IMPACT OF PETROLEUM EFFLUENT FROM JETTY SITES ON PHYSICOCHEMICAL PROPERTIES AND MICROBIAL PROFILE OF AN AGRICULTURAL SOIL

BY

ANI, EBERECHUKWU ADLINE
20184140228

FEDERAL UNIVERSITY OF TECHNOLOGY, OWERRI
SCHOOL OF BIOLOGICAL SCIENCES
DEPARTMENT OF MICROBIOLOGY

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THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF DEGREE OF MASTER OF SCIENCE (MSc.) IN ENVIRONMENTAL MICROBIOLOGY, FEDERAL UNIVERSITY OF TECHNOLOGY, OWERRI

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20184140228

FEDERAL UNIVERSITY OF TECHNOLOGY, OWERRI
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DEPARTMENT OF MICROBIOLOGY

SUPERVISORS: DR. E.C. CHINAKWE.

DR (MRS) N.U. NWOGWUGWU

AUGUST, 2023

CERTIFICATION

This is to certify that this work; **Impact of Petroleum Effluent from Jetty Sites on Physicochemical Properties and Microbial Profile of an Agricultural Soil** was done by ANI EBERECHUKWU ADLINE of registration number 20184140228 in the Department of Microbiology, School of Biological Science, in partial fulfilment of the requirements for the award of Master of Science (M.Sc.) degree in Environmental Microbiology.

----------------------------	14-12-2023
Dr. E.C Chinakwe (Supervisor)	Date
----------------------------	14-12-2023
Dr.(Mrs) N.U Nwogwugwu (Co-Supervisor)	Date
----------------------------	14-12-2023
Prof. I. E Adieze (Head of Department)	Date
----------------------------	14/12/23
Prof. C. S. Alisi (Dean, School of Biological Science)	Date
----------------------------	--------------------
Prof. B.O Esonu (Dean, Postgraduate School)	Date
----------------------------	14-12-2023
Prof. C.J. Ogugbue (External Examiner)	Date

DEDICATION

This project is dedicated to my dearest spouse; words cannot express my heartfelt gratitude for all you have gone through for my sake.

ACKNOWLEDGMENTS

God has my deepest gratitude for guiding me through this task. I would like to express my sincere gratitude to my capable and knowledgeable supervisors, Dr. E.C. Chinakwe for his insightful research counsel, advice, and original research initiatives, and Dr. (Mrs.) N.U. Nwogwugwu for her motherly, wise counsel, and encouragement that made it easier to complete this work. I sincerely thank the Head of Department Prof. I.E. Adieze, my lecturers, and everyone else who worked so hard to provide me with unparalleled knowledge. I value the effort of the staff of Department of Microbiology at the University of Nigeria, Nsukka, for their careful attention to detail and astute advice during the isolation and identification of microorganisms. Finally, I want to express my sincere gratitude to my gorgeous sisters for all their efforts and support. I wish to express my unreserved gratitude to my parents, Mr and Mrs. V. A. Ogbozor for their lovely and fraternal care towards me from time immemorial. To my dearest siblings, closest friends, and my research colleagues; Treash, Chiugo and so many more, thanks for the support and company all through both in the laboratory and our other place of meetings. May you all be richly blessed.

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LIST OF ABBREVIATIONS

$CaCl_2$	Calcium Chloride
$MnCl_2$	Manganesse II chloride
$CoCl_2$	Cobalt (II) Chloride
H_20	Water
$FeCl_2$	Iron II chloride
Mg/ml	Milli gram per milli liter
NH_4S0_4	Ammonium sulphate
FTIR	Fourier Transform Infra-Red
NaH_2PO_4	Di hydrogen sodium phosphate
Na_2HPO_4	Di sodium hydrogen phosphate
$\Omega^{-1\ Cm-1}$	per ohms per centimeters
O_3	Ozone
OH^-	Hydroxyl radical

ABSTRACT

Petroleum effluents from oil producing companies have negatively affected all spheres of live including agricultural soil. In the study, soil samples were with 10%, 50% and 100% of the petroleum effluents from Onne, Nembe and Escravos. Soil physicochemical properties, soil stress marker enzymes, soil bacteria populations, emulsification properties of bacterial isolates and their molecular properties and spectrophotometric assessment of their emulsion were studied using standard methods. Effluents from Onne,Nembe and Escravos polluted soils showed pH of 4.24, 5.87 and 6.3 respectively and pH > as the concentration of the effluent increased from 10-100 %. The control experiment maintained a pH of 7.6 throughout the study period. Soil conductance ($\Omega^{-1}cm^{-1}$) was > in the polluted soils with peak value recorded at 100% pollution of the effluents. P, Mg, K, Ca, and Mg (mg/g) were > as the petroleum effluents % > in the contaminated soils. TPH were consistently > in all the polluted soil. The contents of TOC and TOM correlate with the TPH with Escravos effluents exhibiting the highest levels. Peroxidase, catalase, lipase, and urease in the experimented soil were significantly > except for day 0 of 10% effluent pollution, enzyme activity in the contaminated soil samples were consistently higher than that of the control tests. *Pseudomonas* and *Klebsiella* sp. strains were found to be persistent in the polluted soil, according to analyses of soil organisms. In all the polluted soil, the peak heterotrophic counts (CFU/g) of the organisms were observed on day 0 of 10% effluent pollution, and these counts gradually declined with time. Strains of *Bacillus*, *Pseudomonas, Streptomyces* and *Klebsiella* sp. showed the highest emulsification index at drop collapse and oil spread plate assay. Emulsification studies by crude cultures of the isolates from the contaminated soil showed that *Klebsiella* sp. gave the best emulsification activity with 51.2, 56 and 48.9% emulsification index using coconut oil, kerosene, and crude oil respectively. *Pseudomonas aeruginosa* with accession code of NR_075116.1; *Bacillus substilis* with accession code of MN421487; *Klebsiella aerogene* with accession code of NR_102493.2 and *Streptomyces roseiscleroticus* with accession number of NR_112381. FTIR spectrum showed bands of amide I band is split into three components at 2527.1, 2206.6 cm^{-1} and 1785.4 cm^{-1} while amide II band is observed at 14481.4 cm^{-1}. The CH deformation of the β-glycosidic bond is centered at 853.6 cm^{-1} and 711.9 cm^{-1}. The current study has demonstrated the existence of several recalcitrants in petroleum effluents from these jetty sites on agricultural soils in Nigeria and this gives further credence to the importance of treating petroleum effluents before discharge.

Keywords: physicochemical, petroleum effluents, quality marker enzymes, bacteria population, emulsification index

CHAPTER ONE

INTRODUCTION

1.1Background Information

The main hydrocarbon classes found in oil are the normal alkanes (rapidly degraded), branched alkanes, and cycloalkanes (difficult to identify), isoprenoids (very resistant to biodegradation), the aromatics (fairly identified and much more soluble than other hydrocarbons), and finally the polar ones containing carbon, hydrogen, and oxygen (Chukwu & Adams, 2016) primarily sulfur, oxygen, and/or nitrogen compounds (Callot & Ocamp). Porphyrins and their derivatives are non-hydrocarbon chemicals that can also be found in crude oil (Callot & Ocampo, 2009). Exploration of these mineral composites has left the nation vulnerable in a special way (Nduka & Orisakwe, 2009). Human aggression, marginalization, and major health issues brought on by environmental pollution are among these vulnerabilities (Nweke & Okpokwasili, 2004).

Oil companies and their allies have produced a variety of pollutants over time, primarily in the Niger Delta region of Nigeria, in the form of gaseous emissions, oil spills, effluents, and solid waste (Adieze, Nwabueze, & Onyeze, 2004). These pollutants have contaminated the environment beyond what is sustainable. Increased navigational activity in the Niger Delta's inland and coastal waters is one of the human-made environmental pollutants caused by refined petroleum(Chikere& Chikere, 2012).

Polycyclic Aromatic Hydrocarbon (PAH) concentrations in some Niger Delta sediment were examined by Ezemoye & Ezemoye (2005), who found that these pollutants were present in high amounts in the sediments under study. These petrochemical and refinery facilities produce solid waste and sludge that is made up of organic and inorganic compounds as well as chelated heavy metals. Polycyclic aromatic hydrocarbons (PAHs), phenols, metal derivatives, surface active compounds (surfactants), sulfides, naphthylenic acids, and other chemicals are abundant in the effluents discharged by these petrochemical companies (Valerro, 2010). The incomplete combustion of coal, oil, and other organic molecules results in the production of a collection of around 100 different chemical compounds known as polycyclic aromatic hydrocarbons (PAHs), also known as polynuclear aromatic hydrocarbons (PNAs) and polycyclic organic matter (POM) (2015) Ahmad, Allobaidy, and Ahmad. According to Harvey et al. (2015), they are made up of hydrogen and carbon arranged as two or more fused benzene rings in linear, angular, or cluster patterns, with or without substituted groups linked to one or more rings. When compared to the parent chemical, the newly described substituted PAH occasionally has considerably worse toxicological consequences (Kemsly, 2012).

Polycyclic aromatic hydrocarbons are of particular environmental significance because to their ubiquitous occurrence, significant cytotoxicity, carcinogenic, mutagenic, and endocrine effects (León et al., 2014). The primary source of polycyclic aromatic hydrocarbons in the environment is still incomplete thermal combustion of organic materials, however unintentional oil spills have had a considerable negative influence on the ecosystem. The most dangerous environmental PAHs are those with relatively low molecular weights (128.1–300.36) or from naphthalene to coronene), poor volatility, and low solubility; larger PAHs are more clinically concerning because to their relative immobility.

Metals with comparatively high atomic masses, which show in their atomic weights, are considered heavy metals. Arsenic, beryllium, cadmium, chromium, lead, manganese, mercury, nickel, and selenium are a few examples (Osei, 2008). They participate in bio-geochemical reactions and are moved across compartments by natural processes, the rates of which can occasionally be significantly influenced by human activity (Ajiboye, Yakubu & Adams, 2011). They are persistent in nature and even at extremely low concentrations (1 to 2 micrograms in some circumstances) can harm or kill humans, animals, and plants(Ajiboye, Yakubu & Adams, 2011).

This is because the metals cannot be metabolized by natural means. Heavy metals are primarily produced by oil spills (Osuji & Onajeke, 2004). Heavy metals have the remarkable potential to build up to dangerous levels unnoticed (Wegwu and Akaninwor, 2006). Nephrotoxicity, among other severe health issues, are linked to heavy metals. neurotoxicity and malignancies of various types (Goyer, 1996). Certain bioindicators activities within the soil are mark quality of these persistent recalcitrant, they are constitutive element of high imperativeness in the assessment of soil quality (USDA-NRCS 2009, Markou, Chatzipavlidis & Georgakakis (2016). Biomonitoring is the quantification of bioindication in order to identify trends over time and space. Bioindication is the qualitative indicator of environmental qualities. Due to their significance to the soil system, frequency, ease of collection, and relative simplicity of identification, soil microorganisms and their constitutive enzymes are frequently suggested as bioindicators of soil quality (USDA-NRCS 2009). These biological elements frequently present in healthy soil and have beneficial benefits which largely depend on the following: (1) the abundance and species composition of the organismal strains at a specific location; (2) the behavior of individual soil microorganisms and their constitutive enzymes in contact with a soil substrate (preference/avoidance/activity), and other factors can all be used to determine the soil quality; (3) the body's buildup of chemicals from the environment; (4) the presence of biochemical and cytological stress-biomarkers in organisms (Li, Liu, Yin, L., Xue, Qi, & Li, 2020).

1.2 Statement of the Problem

To fulfill the demands of an exponentially growing population, agriculture and the activities that surround it continue to be the key drivers of our country's productivity and developmental advancements. Agricultural soil fertility continues to be the key to success for all farmers in these regions and the country as a whole, notwithstanding increased farming activity along the southern and eastern coastline. In Nigeria, where population pressure has led to an increase in these practices, agricultural soil fertility quality has received very little attention. Due to their expanding populations, environmental pollution from petroleum and petrochemical derivatives is acknowledged as one of the most important issues in developing nations (Adieze, 2012). All aspects of life, including human health risk and damage, have been significantly impacted by oil pollution health risks to aquatic species, threats to food security, disturbance of lives and livelihoods, and poverty. Polycyclic aromatic hydrocarbons, heavy metals among other toxicant released from oil spills and unprofessional handling impact on the genotoxicity of the ecosystem through impartation with the food chain and other linked abiotic ecological significance. Specific soil fertility indices (physicochemical) and their relationship with these petroleum wastes (even at different concentrations) remain almost unexplainable as arguable data on these are not available.

1.2.1 Scope of the Study

It is impossible to overstate the importance of agricultural sustainability in our developing nation of Nigeria; practices and plenty will only advance if the surrounding soil's integrity is maintained. Environmentalists attempt to highlight the eco-dynamic aspect of the environment through the use of ecological impact assessments and other toxicological research, along with supporting data. There have been a number of non-scientific theories put up regarding how petroleum effluents affect soil fertility, but none of them seem to provide a solution. The current work seeks to close this gap by providing empirical data on soil fertility parameters upon exposures to different concentrations of petroleum effluents.

1.3 Aim of the Study

The present study is aimed at assessment of impact of petroleum effluents from jetty sites in Niger delta on soil physicochemical properties, microbial loading index and stress marker enzyme responses.

1.3.1 Objectives

The following specific objectives will be achieved and they include:

1. Determination of physicochemical properties of the non-polluted agricultural soil
2. Assessment of physicochemical properties of the polluted soil at different concentrations of the petroleum effluents
3. Assay of soil quality marker enzymes such as: Lipase, catalase, peroxidase and urease activity of both control and contaminated soils, respectively.
4. Soil microbial enumerations and identification.

CHAPTER TWO

LITERATURE REVIEW

2.1Petroleum Compound

Petroleum (or crude oil) an engrossed natural resource is a multi-biological liquid composite of hydrocarbons with a little amount of oxygen, nitrogen, and sulfur compounds (Stephen, 2019). Kerogen is a component of petroleum, which is created when big molecules of lipids, oils, and waxes fracture (a variant type crude oil).

Several decades ago, when little marine organisms were common in the seas, this process began. These organisms settled at the sea floor and were eventually covered in layers of clay, silt, and sand (Callot and Ocampo, 2009). Numerous compounds were created as a result of the progressive degradation caused by the combined effects of heat and pressure. Petroleum may move through the soil as it forms because it is a fluid. underground to produce significant, commercially viable amounts of oil (Valero, 2010).

There are two principal sources for processing crude oil:

a. a pool of hydrocarbon
b. a trap for oil.

2.2 Properties of Petroleum

Petroleum has a significantly more stable elemental makeup than solid minerals like coal deposits, which are composed of 83–87% carbon, 11–16% hydrogen, 0% oxygen plus nitrogen, and 4% sulfur make up the composition. According to Lee and Grant (2010), petroleum compounds can be roughly divided into two major classes depending on the catenation of their carbon subunits: aliphatic (paraffin) and aromatic compounds.

2.3Petroleum Refinery

Distillation is the main refinement process stage. Distillation is the process of separating things according to how volatile they are (as shown based on their melting points). As may be seen, this procedure is carried out in a column or distillation tower. Relatively low boiling point evaporatable substances become proportionally richer toward the top of the column. There are chemicals with rather high boiling points near the bottom of the column (ATSDR, 2009).

Even though there are still numerous components in such a liquid, their boiling points are very close to one another. Distillation is a physical procedure used to separate crude oil since different chemical components differbecause neither chemical reactions nor chemical bonds are broken during the distillation process. The sole basis for separation is a physical procedure (boiling) as well (Valero, 2010).

2.3.1 Petrochemicals and Petrochemical Industry

Petroleum waste water (effluents) is a predominant waste and difficult to degrade and managed in any site they are found (contamination). The various refinery layouts allowed for the conversion of crude oil into usable petrochemical products like ethylene and other aromatic olefins (Brooijmans*et al*., 2009).

The hydro-cracker, hydro-cracker flare, hydro-skimming, hydro-skimmer flare, sour water, condensate, condensate flare, and de-salter units all produced petroleum effluent during these processes. According to Ajiboye *et al.* (2011), normal-alkanes (C10-C21), aromatics, and polycyclic hydrocarbons were the main contaminants found in petrochemical effluent.

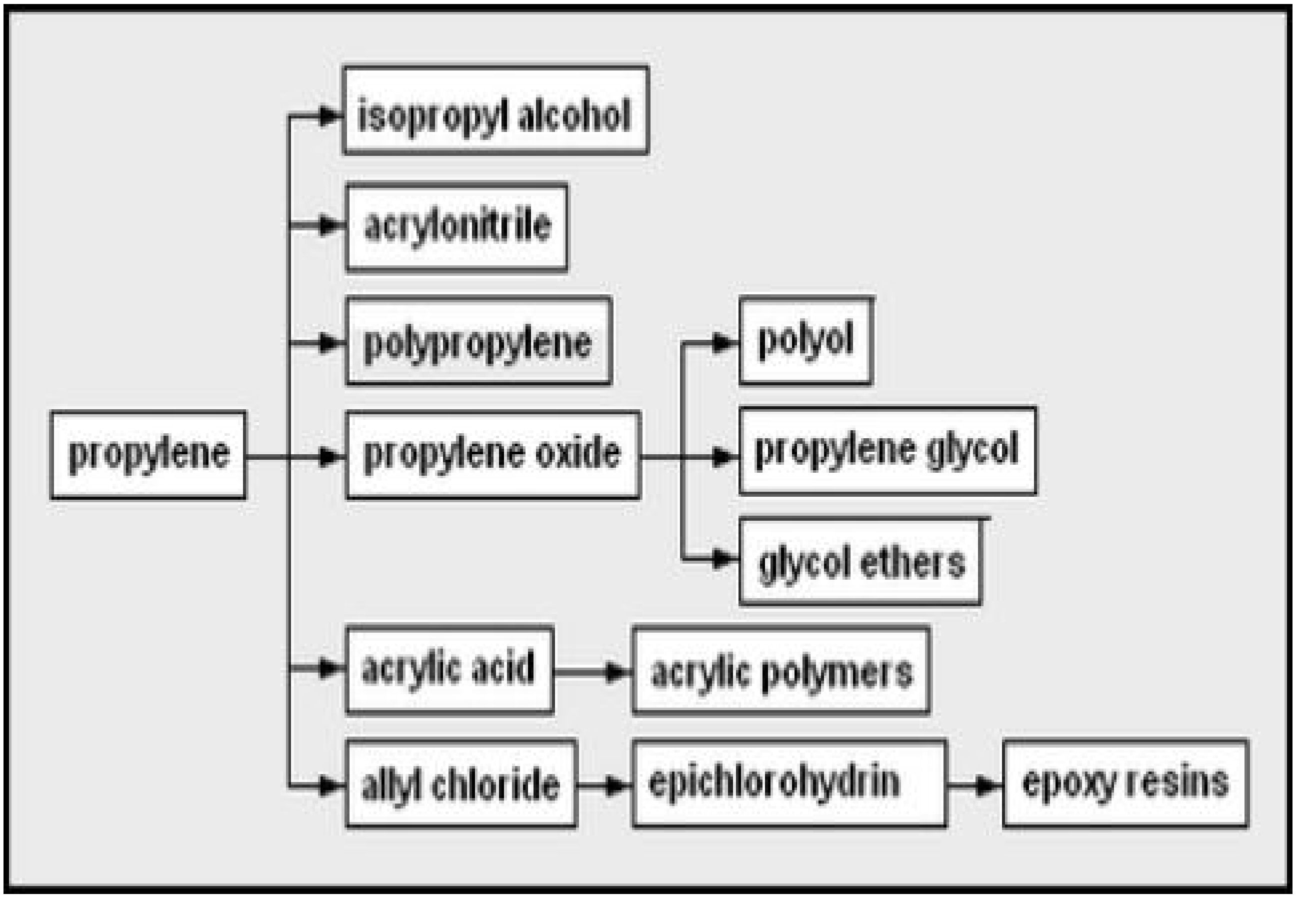

Fig. 2.1 Variant petrochemical products from various refinery processing

Source. (Kutty, Ngatenah, Isa & Malakahmad, 2019).

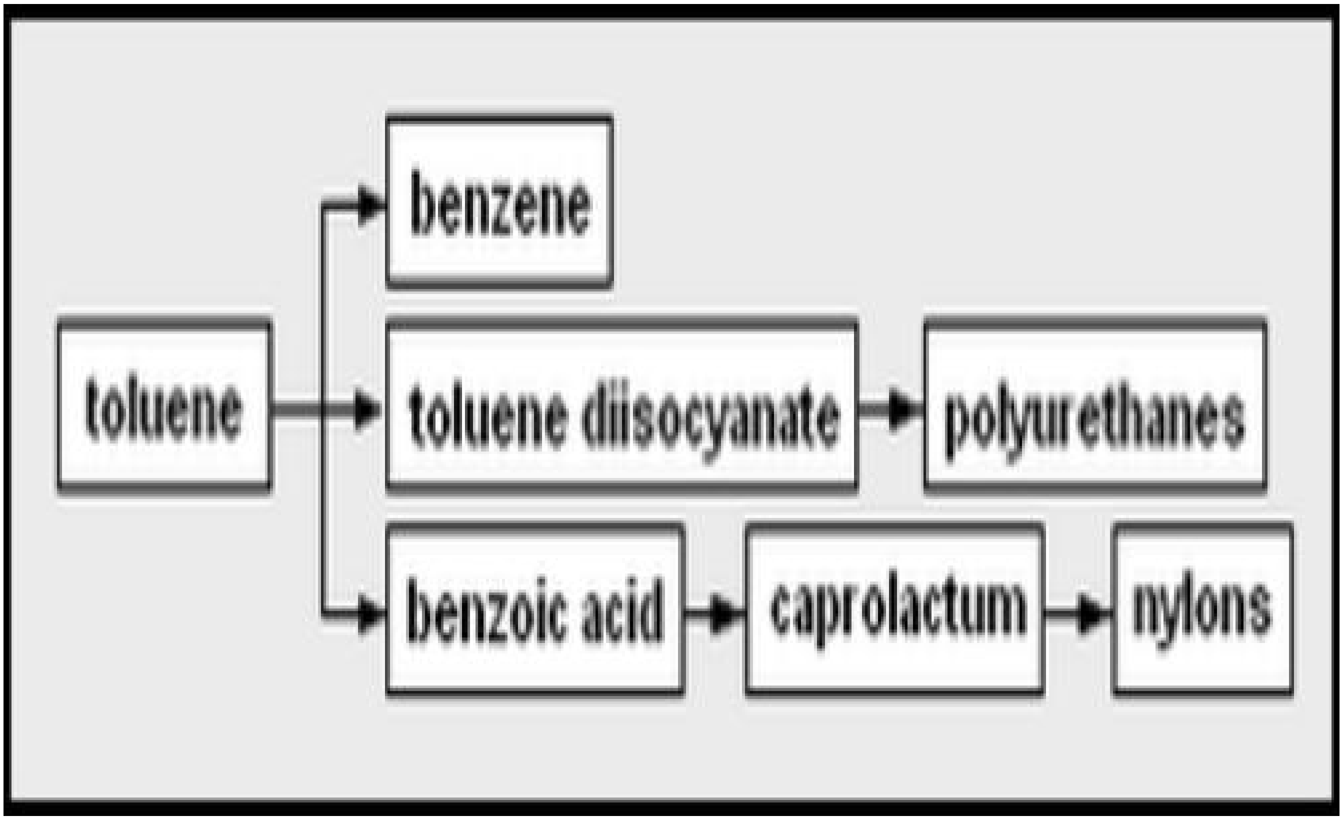

Fig 2.2 Compounds produced from aromatic olefin, Toluene

(Kutty, Ngatenah, Isa & Malakahmad, 2019).

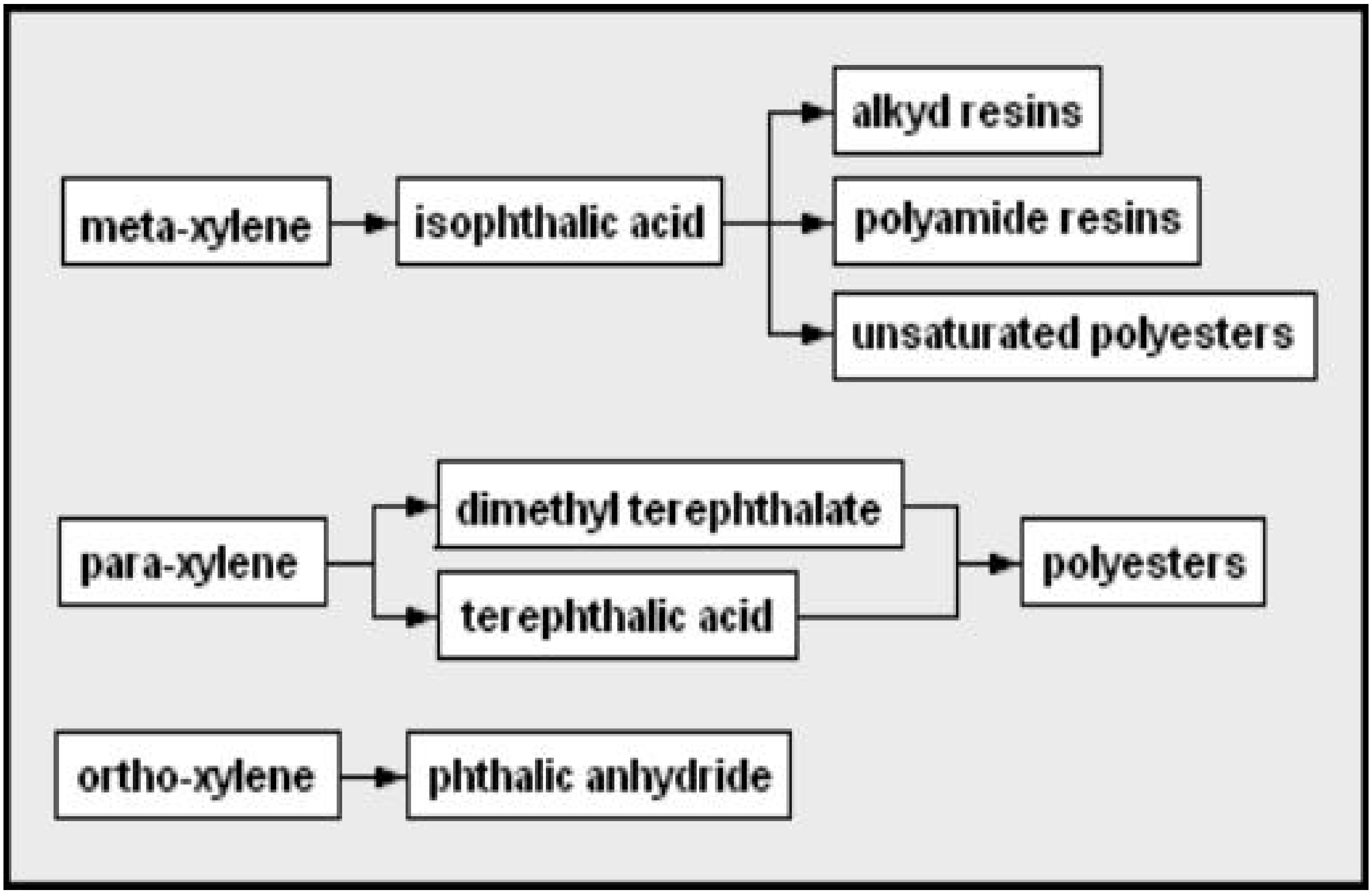

Fig 2.3 Compounds produced from xylene aromatic olefin

(Kutty, Ngatenah, Isa & Malakahmad, 2019).

2.3.2 Petroleum Effluents: Source of Contamination to the Soil.

Petroleum wastewater (effluents) is a predominant waste and difficult to degrade and managed in any site they are found (contamination). The various refinery layouts allowed for the conversion of crude oil into usable petrochemical products like ethylene and other aromatic olefins (Brooijmans*et al*., 2009).

The hydro-cracker, hydro-cracker flare, hydro-skimming, hydro-skimmer flare, sour water, condensate, condensate flare, and de-salter units all produced petroleum effluent during these processes. According to Ajiboye *et al.* (2011), normal-alkanes (C_{10}-C_{21}), aromatics, and polycyclic hydrocarbons were the main contaminants found in petrochemical effluent.

2.3.2.1 Characteristic of Petroleum Wastewater

The physical and chemical features of petroleum wastewater as broadly categorized and enlisted below is authenticated for petroleum industry waste (Stephen, 2019). The principal features Fig. 4's representation of waste water shows that the solids content, color, odor, and temperature of petroleum effluents are some of its physical properties. Whereas the chemical composition of petroleum waste consists of volatile organic carbons, inorganic compounds, and organic chemicals (VOC).

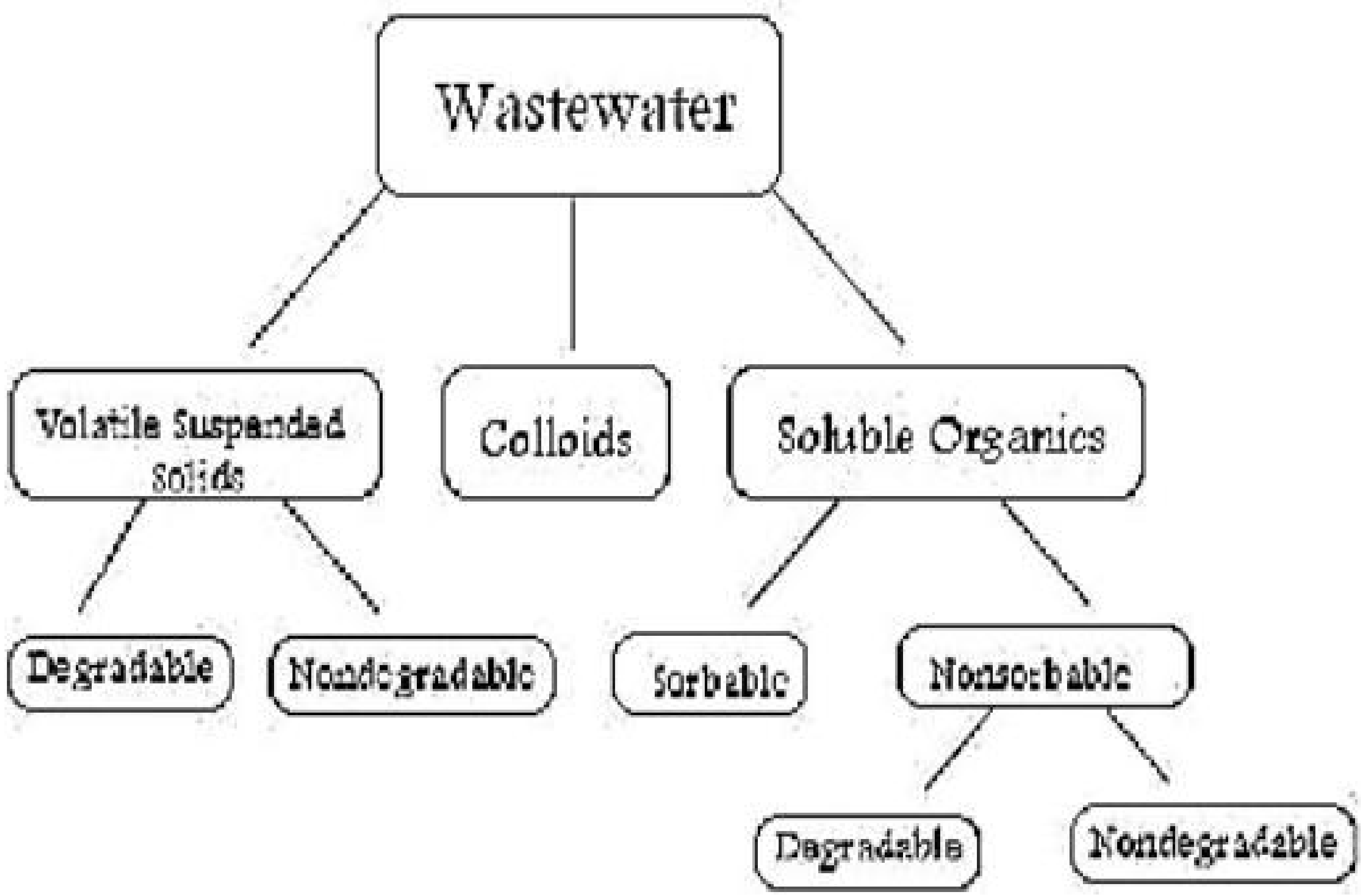

Fig 2.4 Partitioning of organic matter in wastewater (Eckenfelder, 2009)

Fig2.5 Discharge of untreated Surge effluent of Mexico petroleum refinery plant to a river

(Eckenfelder, 2009).

Various fractions of petroleum aliphatic hydrocarbons (up to C10) and well-known aromatic compounds like benzene, toluene, and ethyl-benzene were found to be the major organic pollutants in some refineries' wastewater from the petroleum refining process (Saien et al., 2007). Chang, Holland, Bumpus, Churchwell, and Doerge (2019) demonstrated that the maximum removal for COD and BOD from various sources of petrochemical wastewater by using physical treatment were approximately 1400-1500 mg/l and 25–30 mg/l, respectively. They came to the conclusion that separation and individual treatment for each source was agood alternative to treatment full quantity after mixing various sources. As Table 1 demonstrates, the petroleum effluent is a complex matrix of organic contaminants, with the majority of them contained. Grease and oil could plug drain pipes and cause sticky, corroding, and have terrible scents (Xu & Zhu, 2004), phenolic compounds, which threat the environment due to their extreme toxicity and ability to remain for long periods (Abdelwahab, Baussant, Balk, Liewenborg & Andersen, 2009), and the nitrogen and sulphur components, which are represented in the form of ammonia and hydrogen sulphide (H2S), respectively (Altas & Buyukgungor, 2008). The removal of Naphthenic Acids (NAs) from oilfield wastewater is a significant problem for remediation of large quantities of petrochemical effluents since NAs are a classof compounds in wastewaters from the petroleum industry that are known to induce hazardous consequences (Wang et al., 2015). The proportion of aromatic naphthenic acids (NAs) in total naphthenic acids was reported by Wang et al. in 2015. In a refinery wastewater treatment plant, (NAs) was calculated to be between 2.1 and 8.8%(Murtaza Mulvihill, &Pritchard,2010).

2.4 Ecological Impact of Petroleum Wastewater

Due to the presence of potentially toxic elements (PTEs), which have long-term effects on the environment and human health, the pollution of agricultural soils by wastewater of petroleum origin poses health problems (Amin et al., 2015). PTEs are mostly exposed to humans through the eating of food crops, which might result in a number of health problems in people if the PTE concentration surpassed the safe level (Khan et al., 2015). Utilizing PTE-contaminated plants for an extended period of time might result in the steady accumulation of toxic metals in human kidney and liver tissue, which can disrupt physic-biochemical processes (Amin et al., 2015). In affluent nations, research is being done on how eating PTE-contaminated food or vegetables may affect one's health. However, hardly any is experimented in third world countries (Khan *et al*., 2011).

2.4.1 Impact of Petroleum Waste Water on Soil Physicochemical Properties

Petroleum wastewater and its concomitant pollution within the ecosystem change some physicochemical properties of the polluted biosphere. Several previous studies have shown that the petroleum wastewater significantly changes the soil's physical, chemical, and biological properties through biochemodynamics (Hussain *et al*., 2013), which can, in turn, alter the biogeochemical behavior (mobility and bioavailability) of metals and other nutrients. Therefore, the variation in soil properties as a result of petroleum wastewater can have a significant impact (both positive and negative) on the soil quality and agricultural activities with regards to crop production (Shilpi *et al*., 2018).

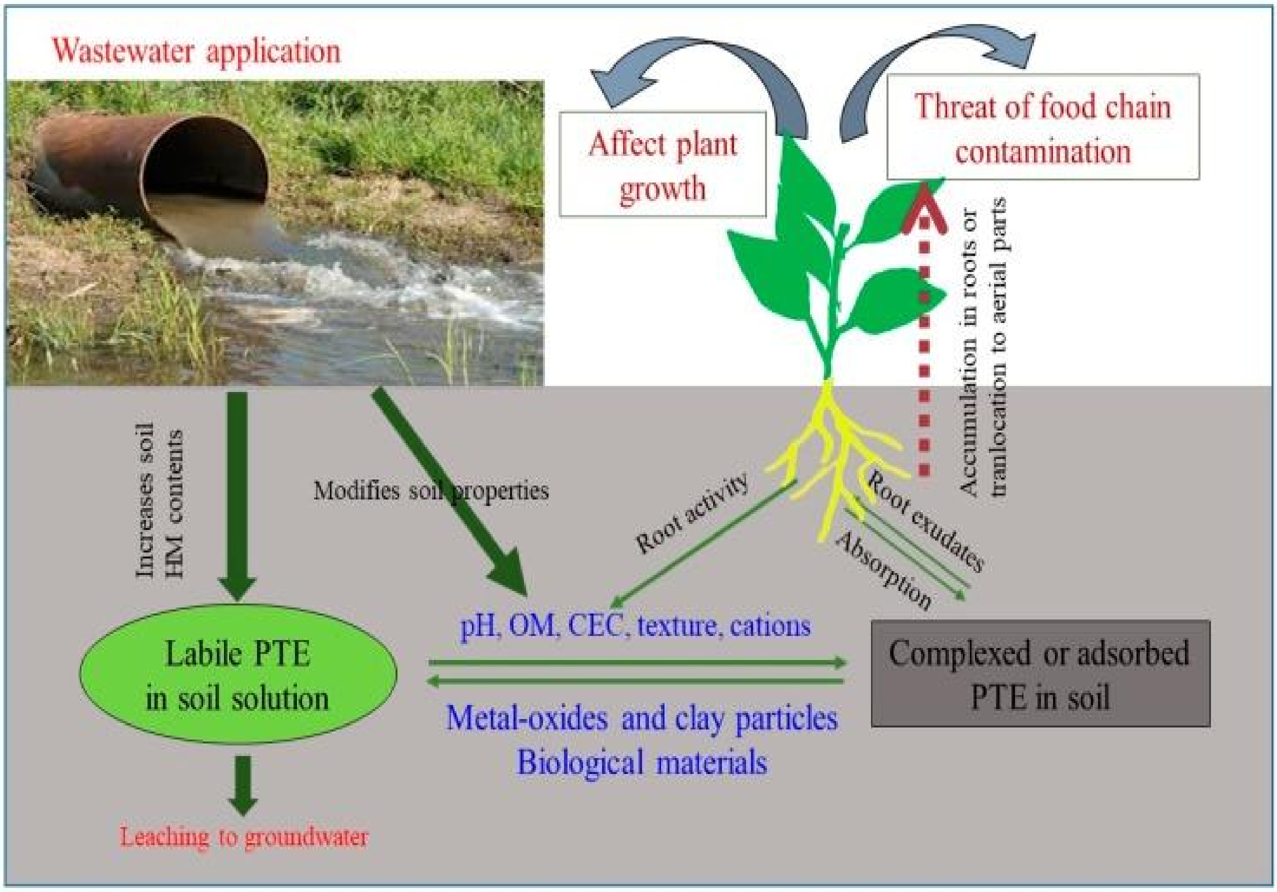

Fig 2.6 Petroleum waste water discharge into the ecosystem and their ecological significances

(Shilpi *et al*., 2018)

2.4.2 The Effect of Petroleum Wastewater on Soil pH

Soil pH which reflects the hydrogen ion concentration of The soil is the primary factor that regulates how metals (ionized ions) are distributed between the soil's solid and solubilized phases. Potentially toxic elements (PTEs) adsorb and desorb in soils, which alters their biogeochemical behavior in the soil-plant system (Farahat et al., 2015). For a number of PTEs, there is a negative relationship between soil pH and the PTEs' bioavailability to plants (Hussain et al., 2013). Due to the improved availability of the majority of nutrients, soils with a pH between 6.0 and 7.5 are ideal for growing most vegetables. The impact of wastewater irrigation on soil pH might vary significantly (Farahat et al.,2015) The amount of acidic compounds in petroleum effluent increases due to the breakdown of organic matter and the development of organic acids in the soils, the pH of the soil has decreased (Farahat et al., 2015). After long-term wastewater contamination from various sources of anthropogenic crude oil operations, the pH of the soil dramatically increased in the majority of studies (Gupta et al., 2012). An increase in soil pH brought on by wastewater application may be due to the sulfate component of the wastewater. In general, how wastewater irrigation impacts soil pH depends on the pH of the wastewater source and the soil's capacity to serve as a pH buffer. In general, changes in the equilibrium of the complex dynamic reactions taking place simultaneously in the soil may cause the pH of the soil to fluctuate after wastewater application. According to earlier research, wastewater irrigation raised the pH of the soil (Zavadil et al., 2009).On the other hand, several research found that the pollution of petroleum effluent caused pH drops. The generation of H+ ions during oxidation reactions is what causes the pH of the soil to fall following wastewater contamination, while the neutralization of the H+ ions by the calcium carbonate in wastewater/sludge is what causes the reduction in soil pH. Because of this, the overall impact of applying petroleum wastewater on soil pH relies on the soil's starting pH, cation and anion ratio, and chemistry of the wastewater (Zavadil *et al*., 2009).

2.4.3 The Effect of Petroleum Wastewater on Soil Dissolved Mineral contents

The majority of the dissolved nutrients and salts in petroleum waste water are inorganic; however, the composition and concentration of these salts vary widely. According to Zavadil et al. (2009), the main components of dissolved salts are anions such sulfate, bicarbonate, and chloride as well as cations like magnesium (Mg2+), sodium (Na+), and calcium (Ca2+). According to Zavadil et al. (2009), soil irrigation with wastewater typically contributes considerable amounts of cations as well as their salts such sulfates, phosphates, bicarbonates, and chlorides. The modification of soil's cation content caused by wastewater application is well documented to have an impact on the metal/nutrient balance between the soil's solid and aqueous phases (Tiwari et al., 2011). However, the impact is dependent on the amount of these cations present in the hydrocarbon effluent. Moreover, the concentration of these cations in the soil also varies with the type of vegetable cultivated because the nutrient uptake and accumulation varies with the vegetable type (Tiwari *et al.,* 2011). In comparison to crops and plants, vegetables are said to be a rich source of nutrients and have a higher capacity to absorb cations (Na, K, Ca, and Ba). According to several researches, wastewater application during irrigation increased the concentrations of Ca, K, and Mg in the soil and in vegetables (Tiwari et al., 2011). Although the consequences of applying soil with a greater K ion content have not been well researched, their long-term application may change the soil's physicochemical characteristics (Becerra-Castro et al., 2015). The soil structure suffers from the excess Na concentration, which also greatly lowers the soil's ability to transfer water (Becerra-Castro et al., 2015). Pollution from petroleum effluent in the soil raises the quantity of Cl in the soil (Keraita *et al*., 2004).

Overall, crop type and species, soil fertility, and nutrient concentrations in wastewater all have a role in how valuable petroleum effluent is as a source of agricultural nutrients. According to Keraita et al. (2004), the efficiency of nutrient usage for wastewater is close to 100%. This is so that plants can easily absorb the nutrients that are present in wastewater because they are frequently contained in dissolved form. Because nutrients are delivered in patches at each pollution, as opposed to synthetic fertilizers, which are often put to crops in two to three splits, the wastewater-induced nutrient supply fits the requirement of crops (Valipour *et al*., 2015).

In terms of agricultural productivity, the impact of petroleum wastewater application on soil nutrient status and nutrient usage efficiency is also documented. The yield of marketable fruit was shown to be higher with wastewater than with groundwater (Chen et al., 2013). Other research revealed that as compared to well-water irrigation, wastewater irrigation considerably boosted the dry and fresh forage yield of crops and vegetables.

2.4.4 The Effect of Petroleum Wastewater on Soil Organic Matter

After pH, soil organic matter (SOM) is the second most crucial factor in determining soil quality. According to Winpenny et al. (2010), SOM controls the biogeochemical behavior (mobility/bioavailability) of metals and nutrients in the soil-plant system. The types/nature of organic matter have a major role in the mechanisms regulating the behavior of metals in soil.

SOM is said to reside in an aqueous media in either a suspended or dissolved state (Rattan et al., 2005). While in the solid phase, Soil Organic Matter can adsorb metals from the aqueous media through chemical adsorptivity when provided in the dissolved form, SOM can also form metal-OM complexes with metals (Winpenny *et al*., 2010). Therefore, SOM has a significant impact on the mobility of PTEs in soils and their availability to plants (increasing or decreasing)

(Winpenny et al., 2010). Additionally serving as a sink for vital nutrients necessary for plant growth is organic matter (OM). Additionally crucial for regulating soil microbial activity is the SOM concentration. Large OM inputs promote microbial development, obstruct soil pores, reduce soil penetration, and encourage anaerobic microbiological growth as a result of aeration issues in the soil (Winpenny *et al*., 2010).

As a result of pollution from petroleum effluent, which has been previously documented (Singh et al., 2010), organic matter has accumulated in the surface soil. The increase in OM content caused by the presence of wastewater with a petroleum origin is thought to be good for the soil. By introducing organic matter into the soil by wastewater application, the soil's structure is enhanced, its capacity for cation exchange is raised, it helps to retain metals by lowering their bioavailability and mobility, and it also provides the soil with nutrients (Singh *et al.,* 2010).

However, wastewater application that results in greater organic matter concentrations might have a negative impact on soil porosity and lead to anaerobic conditions in the root zone (Murtaza et al., 2010). Additionally, if agricultural runoff with a greater OM concentration enters surface water, it may deplete the water's supply of dissolved oxygen, leading to hypoxic conditions and a rise in the mortality of aquatic species (Singh et al., 2010). Due to organic matter obstructing water transmission holes, continuous wastewater irrigation drastically changed theamount of water infiltration into soil (Murtaza et al., 2010).

The agricultural water use and crop yield will be significantly impacted by the decreased soil water efficiency and water retention, as well as the increased surface runoff and altered water infiltration.

2.4.5 Impact of Petroleum Waste Water on Soil Microorganisms

The microbial activity is concentrated at the localized areas on and around the organic leftovers because the soil is a heterogeneous environment in both space and time. As organic and inorganic residues vary, decomposer communities go through succession (El-Nahhal et al., 2013).

According to Vaseghi et al. (2005), the alteration of the soil's physical-chemical properties might have a direct or indirect impact on the microbiological activity of the soil. The use of wastewater for crop irrigation is one of the sources of medically significant bacteria in many poor nations (Murtaza et al., 2010). As a result, even in locations where irrigation water (wastewater) seems to be safe, the qualityof food and hygienic conditions are still crucial.

Petroleum wastewater used as irrigation in soil changed the bacterial population that oxidizes ammonia andThe microbial activity is concentrated at the localized areas on and around the organic leftovers because the soil is a heterogeneous environment in both space and time. As organic and inorganic residues vary, decomposer communities go through succession (El-Nahhal et al., 2013).

According to Vaseghi et al. (2005), the alteration of the soil's physical-chemical properties might have a direct or indirect impact on the microbiological activity of the soil. The use of wastewater for crop irrigation is one of the sources of medically significant bacteria in many poor nations (Murtaza et al., 2010). As a result, even in locations where irrigation water (wastewater) seems to be safe, the quality of food and hygienic conditions are still crucial.

Petroleum wastewater used as the bacterial community that oxidizes ammonia and nitrogen in soil was modified by irrigation. Although waste water and soil have quite distinct qualities from one another, both are home to a variety of bacteria. For instance, in N cycling, the ability of the bacteria to remove soil pollutants (such as PTEs, antibiotics, or pesticides) may help to improve the soil's quality (Vaseghi *et al.,* 2005)

Through the delivery of nutrients and organic materials, it is anticipated that the contamination of soil with petroleum effluent will stimulate various metabolic pathways and species. Accordingly, it is hypothesized that irrigation of wastewater may increase the activity of microorganisms involved in the biochemical balance of elements like N, P, and C. (Khai et al., 2008). However, the quantity and encouragement of soil microbial activity could be detrimental to the soil's physical characteristics.

2.5Petroleum Effluent Compositions and the Environment

2.5.1 Polycyclic aromatic hydrocarbons (PAHs)

Each year, 230,000 tons of PAHs enter aquatic habitats and 43,000 metric tons are released into the atmosphere (Eisler, 1987). Although PAHs are present everywhere in nature as a result of synthesis in terrestrial plants, microbiological synthesis, and volcanic activity, the amounts produced by these natural processes are negligible in comparison to those from anthropogenic sources, such as forest and prairie fires (UNEP, 2011). Examples of anthropogenic activities linked to significant PAH production include coke production in the iron and steel industry, catalytic cracking in the petroleum industry, the production of carbon black, coil tar pitch, and asphalt, the generation of heat and power, controlled refuse incineration, open burning, and emissions from internal combustion engines used in transportation (UNEP, 2012).

Given enough time (millions of years), pyrolysis of organic materials at temperatures as low as 100 to 150 °C can also produce PAHs, as evidenced by the discovery of complex combinations of PAHs covering a wide range of molecular weights in fossil fuels (UNEP, 2011).

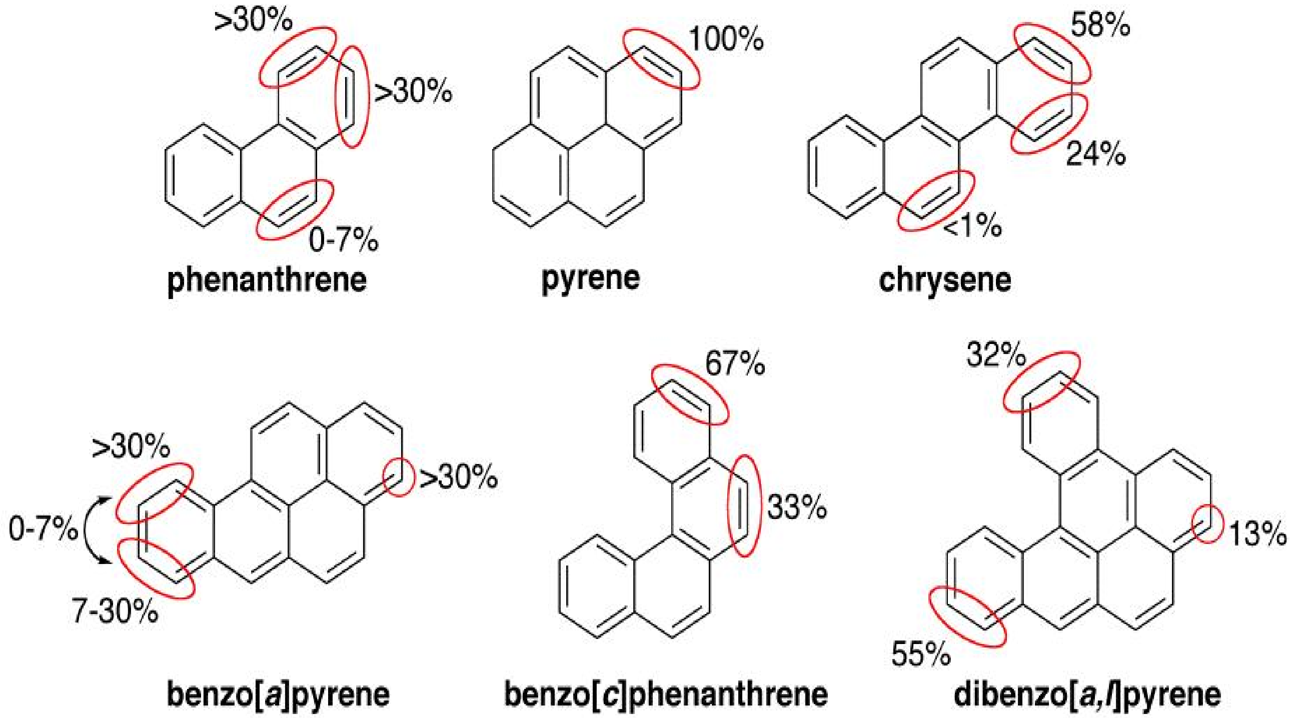

Fig2.7 Structures of some Polycyclic Aromatic Hydrocarbons. (Pampanin and Sydnes, 2013).

TABLE 2.1: Minimum, maximum, and mean PAH content in 48 different Petroleum oils

Crude oil oils	48 Different crude			North Sea	Goliat b
PAH	Minimum mg/kg oil	Maximum mg/kg oil	Mean mg/kg oil	mg/kg oil	mg/kg oil
Naphthalene	1.2	3700	427	1169	1030
Acenaphthene	0	58	11.1	18	12
Acenaphthylene	0	0	0	0	-
Fluorene	1.4	380	70.34	265	75
Anthracene	0	17	4.3	1.5	-
Phenanthrene	0	400	146	238	175
Fluoranthene	0	15	1.98	10	6
Pyrene	0	9.2	-	20	-
Benzo[a]anthracene	0	16	2.88	11	Not analysed
Chrysene	4	120	30.36	26	Not analysed
Benzo[b]fluoranthene	0	14	4.08	4.2	Not analysed
Benzo[k]fluoranthene	0	1.3	0.07	Not detected	Not analysed
Benzo[a]pyrene	0	7.7	1.5	1.3	Not analysed
Dibenz[a,h]anthracene	0	7.7	1.25	Not detected	Not analysed
Benzo[g,h,j]perylene	0	1.7	0.08	1	Not analysed
Indeno[1,2,3-cd]pyrenec	0	1.7	0.08	Not detected	Not analysed

Adapted from Pampanin and Sydnes, 2013

2.5.2Environmental Implications of Polycyclic Aromatic Hydrocarbons

Although the environmental impacts of the majority of non-carcinogenic PAHs are poorly understood, concerns regarding PAHs in the environment stems from their persistence and the knowledge that certain of them are potent mammalian carcinogens; A natural equilibrium between the synthesis and breakdown of PAHs existed before 1900. According to Morris and Calvin (2015), the destruction of PAHs through photodegradation and microbial transformation appeared to balance the generation of PAHs by man-made high temperature pyrolytic reactions, open burning, and microorganism- and volcanic-activity-induced synthesis of the chemicals. The balance has been thrown off to such an extent that PAH generation and introduction into the environment far exceed known PAH removal methods as a result of greater industrial development and increased emphasis on fossil fuels as energy sources (Torrice, 2016).

The atmospheric emission of PAH chemicals will cause them to mix with particulate matter. Particle size, weather, and atmospheric physics all influence how long they stay in the atmosphere and how they move to various areas (Morris and Calvin, 2015). By interacting with ozone and other oxidants, the highly reactive PAHs photo-degrade rapidly in the atmosphere. Degradation times range from a few days to six weeks for PAHs adsorbed onto particles with a diameter of less than one um (in the absence of precipitation) to a few hours to several days for those adsorbed to larger particles (Suess 1976). Morris and Calvin (2015) found that smaller atmospheric particles with PAHs are easily inhaled and may present unique challenges for flying creatures including birds, insects, and bats. Although little is known about their permanence, photooxidation, one of the most significant mechanisms in the elimination of PAHs from the atmosphere, can also yield reaction products that are carcinogenic or mutagenic. The creation of endoperoxides, which eventually go through a sequence of events to generate quinones, is one of the more frequent photo oxidation reactions of PAHs (Edwards 1983). Light intensity, the concentration of gaseous pollutants (03, NOx, SOx), and the chemico-physical properties of the particulates or substrates into which the PAHs are adsorbed are some of the factors that may modify the chemical and photochemical transformation of PAHs in the atmosphere. Depending on these factors, the half-life of benzo(a)pyrene in the atmosphere can range from 10 minutes to 72 days (Valero 2010). PAHs in the atmosphere travel over a pretty long distance. Nevertheless, sites closer to urban centers have considerably greater PAH deposition rates than more rural places (UNEP, 2011), regardless of distances from industrial areas and from natural forest and prairie fires (Edwards 1983).

Many of the PAHs that are discharged into the air eventually find their way to the soil, either directly or by deposition on vegetation. Although some adsorbed PAHs may be washed off by rain, chemically reduced to other compounds, or returned to the soil as the plants decompose, the PAHs may be absorbed or digested by plant leaves before entering the animal food chain (Morris and Calvin, 2015). Vegetation-assimilated PAHs may be moved about, digested, and even photodegraded inside the plant. Assimilation may outpace metabolism and breakdown in some plants living in severely contaminated environments, leading to a buildup in plant tissues (UNEP, 2012). In water, PAHs can either evaporate, diffuse into the water column, mix into sediments at the bottom, concentrate in aquatic biota, or undergo chemical oxidation and biodegradation. In aquatic systems, photooxidation, chemical oxidation, and biological transformation by bacteria and animals are the three most significant degradative mechanisms for PAHs (Neff 1979). Only around 33% of PAHs are found in dissolved form in aquatic environments; the majority are linked to particulate materials (Lee and Grant 1981).

Through photo-oxidation, PAHs dispersed in the water column would likely decay quickly; this process is particularly accelerated at greater concentrations, higher temperatures, higher oxygen levels, and higher sun radiation incidences (UNEP, 2011). The biotransformation and biodegradation of those PAHs by benthic organisms is thought to be the final fate of those PAHs that collect in sediment organism. However, in the absence of irradiation and oxygen, PAHs in aquatic sediments degrade very slowly and may exist permanently in oxygen-poor basins or in anoxic strata (Morris and Calvin (2015).

2.5.3Impact of Polycyclic Aromatic Hydrocarbon from Petroleum Waste Water on Soil Microorganisms

The microbial activity is concentrated at the localized areas on and around the organic leftovers because the soil is a heterogeneous environment in both space and time. As organic and inorganic residues vary, decomposer communities go through succession (El-Nahhal et al., 2013).

The alteration of the soil's physical-chemical properties might have a direct or indirect impact on the microbiological activity of the soil (Vaseghi et al., 2005). The ammonia-oxidizing bacterial community was transformed by some wastewater used as irrigation in soil, and *Nitrosomonas* and *Nitrosospira* species took over (Murtaza et al., 2010). Although soil and PAHs have quite distinct properties from one another, both are home to a large variety of bacterial species.

For instance, the bacteria involved in N cycling have the capacity to remove soil pollutants (such as Pesticides, PTEs, and antibiotics all have the potential to improve soil quality (Vaseghi et al., 2005). Through the delivery of nutrients and organic matter, PAHs in wastewater are anticipated to promote several metabolic pathways and organisms (Khai et al., 2008).

However, the quantity and encouragement of soil microbial activity could be detrimental to the soil's physical characteristics.

2.5.4Impact of Polycyclic Aromatic Hydrocarbon from Petroleum Waste Water Waste Water on Soil Marker Enzyme

Enzyme activity in soils is essential for nutrient cycling and energy conversion. Some enzymes, like urease, are constitutive and created by cells on a regular basis; however, others, like cellulase, are adaptive or triggered and only develop when a susceptible substrate, another initiator, or none of these requirements are present. Mutating bacteria are not always known to behave in this manner (Chilaka et al., 2002).Various developed methods for assessment of soil enzymes activity which include:

(A) Spectrophotometric

(B) Flourimetry methods (Dick, 2011).

Assessment of impact of PAHs in petrochemical waste water indicates either a bloom or attenuation of quality marker enzymes in the soil which in turn signifies an impact in the microbial proliferation and load numbers within the soil (Walker *et al.,* 2008). Some specific quality marker enzymes like Dehydrogenase, peroxidases, lipases increases in activity in the presence of recalcitrants like crude oil, pharmaceuticular effluents and peroxide or other superoxide containing compounds while in the presence of sugar containing effluents, activity of amylases, pollulanase, glucosidases increases. This expresses the mechanism of feedback mechanisms of enzymes in the presence of desired substrates in the soil (Walker *et al.,* 2008).

2.6Environmental Monitoring of Polycyclic Aromatic Hydrocarbons

The risk of PAH contamination for species can be evaluated, and the environmental quality of ecosystems can be categorized, using a variety of environmental monitoring techniques (Pampanin and Sydnes, 2011). The following five methods are reported: Chemical monitoring, which assesses exposure by determining the concentrations of a certain group of substances in compartments of an abiotic environment, (1); (2). monitoring of bioaccumulation: evaluation of exposure through assessment of PAH levels in biota or estimation of the critical dose at a crucial site; (4) Health monitoring: Impact evaluation by assessing the onset of irreversible diseases or tissue damage in organisms; (3) Biological effect monitoring: exposure and effect assessment by identifying the early unfavorable modifications that are partially or completely reversible (biomarkers); (5) Monitoring ecosystems allows for evaluation of the health of an ecosystem through invention of species composition, density and diversity (Francoini et al., 2007; Baumard et al., 1999).

TABLE 2.2: StandardProcedures for Determining of PAHs in Aqueous Samples.

Method Name	Application	Sample preparation	Determination Technique
EPA-610 .	PAHs in municipal and industrial wastewaters	About 1L of water samples is extracted with dichloromethane, the extract is then dried and concentrated to a final volume of less than 10 mL	HPLC or GC (with a packed column)
EPA-8310	Groundwater and wastewater	EPA 3500 series methods (version 3, 2000)	HPLC
EPA-8270D	Aqueous samples	EPA 3500 series methods (version 3, 2000)	GC-MS with use of high resolution capillary columns and deuterated internal standard
ISO 17993:2002	Water quality(determination of15 PAHs)	Water samples collected in brown glass bottles are stabilized by Adding sodium thiosulfate. 1 L of sample is extracted using hexane. The extract is dried with sodium sulfate and then enriched by removal of hexane by rotary evaporation	HPLC and fluorescence detector

Wolska, (2008).

2.7Heavy Metals and Their Environmental Implications

Metals can be mobilized as a direct result of human activity or as a side effect of human activity, as well as through natural weathering processes as erosion and dissolution (Ajiboye et al., 2011). For instance, lead is released from automobile exhaust pipes at a rate that is twice as fast as that at which it is mobilized globally by natural processes, cadmium and zinc oxides are vaporized and released to the air during smelting, and acid mine drainage leaches metals from rocks and soils (Mulvihill and Pritchard, 2010). Since many of their uses tend to disseminate them widely in the environment, making recycling highly challenging, cadmium and lead are particularly toxic contaminants present inmany petrochemical products and their effluents. A large portion of this enters water systems directly or through runoff streams (2011) Ajiboye et al. In aquatic systems, metal pollutants typically persist in soluble or suspension form before either tending to sink to the bottom or being ingested by organisms (Reddy et al., 2007).The biological balance of the recipient habitat and a variety of aquatic creatures may be severely harmed by heavy metal contamination (Vosyliene and Jankaite, 2006; Ashraj, 2005). As one of the primary aquatic creatures in the food chain, fish frequently collect high levels of certain metals (Mansour and Sidky, 2002). Fish are the only animal species present that are unable to escape the negative impacts of these pollutants (Olaifa et al., 2004). Furthermore, it is important to keep in mind that these heavy metals accumulate in a variety of fish species' tissues and organs, where they may then enter the human system through intake of fish that pose a major risk to health.

TABLE2.3: Estimations of Different Concentrations of Heavy Metals Found in Different Petroleum Effluents.

Locations	Mediums	Cd	Cu	Hg	Pb	Zn	Reference
Onne surge Effluents	Water mgl-1 Sediments mgl-1	1.9 42.1	22 94.5	- -	25 108.3	64.5 805	Oyeyiola *et al* (2006)
	Water μgl-1	-	-	-	20.1	90.2	Brummett RE (2003)
Forthare Petrochem	Sediment mgg-1 Fish mgg-1	<1.9 4.67	32.6 07	-	58.1 0.13	101.9 8.8	Machiwa, JF (2003)
Fouarat lake, Morocco	Sediment μgg-1	0.9	75	-	110	400	Bouih, BH. *et al* (2005)
Lakes Awassa, Ziway, Ethiopia	Fish mgkg-1	4.9	797	-	42.3	115.9	Aweke, K. Taddese, W (2004)
Santa Gilla lagoon, Italy	Sediment ppm	-	33	6.28	172	209	Deguetto, S. *et al* (1997)
Marano lagoon, Italy	Sediment μgg-1	-	-	6.6	-	-	Piani, R. *et al* (2005)

Lakshmanan *et al* (2009).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Material

The reagents and chemicals used during the experiment were of analytical grade.

3.1.2 Apparatus / Equipment

Apparatus and equipment used during the experiment were calibrated during each use of the equipment.

3.2 Methods

3.2.1 Soil Samples

Soil samples were collected using the stratified sampling method at different location sited at Soil Science Department Garden, University of Nigeria, Nsukka.

3.2.2 Petroleum Effluent

Petrochemical effluents were collected from the surge pipe flow of a petroleum drilling plant situated within the off coast of Onne, Escravos and Nembe shore-line; the waste water were collected using a clean container at three different locations of the surge reservoir.

3.2.2.1 Contamination of the Soil with the Petroleum Effluents

Soil sample obtained from Soil Science Garden of University of Nigeria, Nsukka was polluted with petroleum effluents from Onne, Escravos and Nembe, respectively at concentrations of 0, 10, 50 and 100 % as described by Vallero (2010). The polluted soils and the control experiment were allowed to acclimatize for 12 hours after the pollution prior to the assessment studies that were carried out on day 0, 7, 14 and 21.

3.2.3 Determination of Soil Physicochemical Properties

The following physicochemical properties of the polluted soil and the control soil sample were determined as described by the journal of ATSDR (2009), they include:

3.2.3.1 Soil pH Test

Ten grams of the soil samples were measured out using the weighing balance; the weighed soil was further dissolved in 40 ml of distilled water and the solution was shaken vigorously for 5 minutes and left standing. The pH value of the solution was determined using the pH meter by dipping the calibrated electrode into the waste water.

3.2.3.2 Soil Conductivity Test

This was carried out using the conductance meter. The conductance meter electrode was dipped in a 5 ml of the soil solution from where the conductivity and resistivity of ions present in the water was deduced.

3.2.3.3 Analyzing the Soil's Macro and Micro Contents

K, Cl, Ca, and Mg ions were among the soil electrolytes present in the mineralsas described in the Journal of ATSDR, 2010.

3.2.3.3.1 Potassium ion (K).

Five mililiter from the dissolved soil solution was measured out as described above and equally equilibrated in 250 ml of deionized water; the mixture was shaken and left standing. 10 ml of the constituted solution was added with a sachet of potassium a gram (1g) and assayed for potassium concentration at wavelength of 610 nm (ATSDR, 2010).

3.2.3.3.2 Chloride ion

Twenty(20) mililiters ml from the dissolved soil solution was measured out; the solution was well shaken and heated for 30 minutes to break up the carbon chains. This was followed by the addition of 1ml of potassium chromate indicator to the solution; the mixture was then titrated against silver nitrate (AgNO3). We can infer the chloride ion concentration in both mixes from the form: A-B X M X 70900Vol. (ml).

Where: B = titre volume of the test sample, where A = titre volume of the blank.

M stands for the molecular weight of the pippeted waste.

3.2.3.3.3 Calcium ion

Fifty mililiter from the dissolved soil solution was pipetted into a 250ml conical flask and dilute to 100 ml with deionized water. 2 ml of 2 M NaOH aq solution and six (6) drops of the Solochrome dark blue solution was added to the solution. The pH Value of the solution was adjusted to 10.0; it is further titrate against the EDTA solution, to a distinct blue endpoint (V ml).

Calcium metal (mg/kg) = Tx 400.5 x 1.05 50 T= titre value. $CaCO_3$ content (mg/Kg) = Vx E($CaCO_3$) x 100/ 50.

3.2.3.3.4 Magnesium ion

Formula: Magnesium (as mg/l) = (T-C) x 0.243 Total hardness and calcium hardness measurements can be used to compute magnesium hardness.

• Where; T stands for total hardness (as $CaCO_3$) and C for calcium hardness (as $CaCO_3$).

3.2.3.3.5 Total Organic Carbon Contents

Total organic carbon contents (TOC) were determined as follows:1 ml from the dissolved soil sample solution was mixed with 10ml (0.5M) of $K_2Cr_20_7$ and 20ml (18 M) of sulphuric acid and vortexed for 30min. the mixture were diluted with 20ml of deionize water. 10ml of phosphoric acid together with 3-4 drops of ferroin indicator was added to the solution and titrate against 50ml of ferrous ammonium sulphate (FAS).

Total organic matter content (TOC) (mg) = Vb - Vs X 16,000 vol. of the sample utilized TOM = 1.23 X TOC

3.2.3.4 Heavy Metal Determination

3.2.3.4.1 Iron (Fe) using Spectrophotometer.

Iron metal from the wastewater was identified using spectrophotometric assay method as described by Morris (1952).

Procedure

Ten milliliter of distilled water and 100 ml of the soil sample solution were combined, agitated for 30 minutes, and then left standing. To the solution prepared, 25 ml volumetric flask was filled with 10 ml of hydroxylamine hydrochloric acid after 10 ml of the solution was pipetted into it. After shaking the mixture for five minutes, 5 ml of sodium acetate, 4 ml of phenanthroline reagent, and 30 ml of deionized water were added. A 1ml aliquot was collected, and it was put into the cuvette. Spectrophotometric assay of iron concentration was taken at wavelength of 510nm.

3.2.3.4.2 Analysis of Heavy Metals from Soil Digestion

In a 250 ml conical flask, 10 mililiters of the dissolved soil solution were digested by adding 30 ml of aqua regia (HNO3, HCl, and HF in a 3:2:1 ratio). It was cooked on a hot plate for an additional 7–12 ml or so. The digest was prepared for atomic absorption spectra (AAS) analysis after being filtered through no. 1 what-man filter paper and volumetrically adjusted to the proper level in a 50ml flask. For the preparations of the standard metals, see appendix 1.

3.2.3.4 Contents of total petroleum hydrocarbons (TPH).

According to the ASTDR Journal of 2009, the total hydrocarbon (TPH) of the polluted soil was calculated.5g of the contaminated soil sample was weighed out and mixed together with 5g of anhydrous sodium sulphate, 25ml of n-Hexane was added to the prepared colloidal sample and mixed thoroughly.

To the standing solution, 25ml of n-Hexane was again added to it, shaken very well. The resulting solution was later partitioned out using the separating funnel. The aqueous layer of the mixture was discarded and the solvent partition was used for spectrophotometric analysis at wavelength of 420nm.

3.2.4 Preparation of Buffer Solutions

3.2.4.1Sodium acetate buffer (stock solution)

Sodium acetate salt (6.804g) was immersed in 1000ml of distillate solution. The pH was adjusted to 5.5 with the conjugate acid.

3.2.4.2 Sodium phosphate buffer (stock solution)

Sodium phosphate salt (7.098g) was immersed in 1000ml of distillate solution. The pH was adjusted with phosphoric acid.

3.2.4.3 Buffer with Tris (stock solution)

By measuring 6.07g of Tris (hydroxmethyl) aminomethane, dissolving some of it in 1000ml of distilled water, and adjusting the pH with hydrochloric acid, Tris buffer (0.1M) was created.

3.2.5 Soil Enzyme Assessment

Soil quality marker enzymes were determined from the contaminated soil with the petrochemical effluent using standard assay protocols, enzyme such as: lipase, catalase, urease and peroxidase were assayed.

3.2.5.1 Lipase

Lipase activity in the soil was determined as described by Okwaye *et al.* (*2*010).

Five (5) grams of the soil was weighed out and dissolved in 10 ml of distilled water, the solution was vortexed for 10 min, it was further filtered using Whatman no1 filter paper. The filtrate was used as crude enzyme solution and 0.5 ml of the crude extract was pippetted into a test tube containing 0.5 ml of prepared p-NPP in an acetate buffer at pH 5.5. The resulting solution was boiled for 30min at 50^{O}C. The reaction was stopped with 0.1 m NaOH. Absorbance was taken in duplicate at wavelength of 410 nm.

3.2.5.2 Catalase

Catalase activity in the soil was determined as described by Haluk, Khawar, Julia, Tim and Ricardo (2012).

Briefly, 5g of the soil was weighed out and dissolved in 10 ml of distilled water, the solution was vortexed for 10 min, it was further filtered using Whatman no1 filter paper. The filtrate was used as crude enzyme solution. To the dissolved soil sample, 0.1 ml of the crude extract was pippetted into a test tube containing 0.2 ml of prepared 30% H_2O_2 in a phosphate buffer at pH 6.5. 0.1 ml of 2,6 DMP was added to the resultant solution and absorbance was taken at wavelength of 400 nm for 60 min.

3.2.5.3 Peroxidase

Peroxidase activity in the soil was determined as described by Eze, Chilaka, and Nwanguma, (2012). From the collected sample, 5g of the soil was weighed out and dissolved in 10 ml of distilled water, the solution was vortexed for 10 min, it was further filtered using Whatman no1 filter paper. The filtrate was used as crude enzyme solution. 0.5 ml of the crude extract was pippetted into a test tube containing 0.3 ml of prepared 30% H_2O_2 in a phosphate buffer at pH 6.0. To the solution, 0.4 ml of o-dianisidine was added to the resultant solution and absorbance was taken at wavelength of 410 nm for 120 min.

3.2.5.4 Urease

This was done as described by Douglas & Bremner (1971).

From the collected sample, 5g of the soil was weighed out and dissolved in 10 ml of distilled water, the solution was vortexed for 10 min, it was further filtered using Whatman no1 filter paper. The filtrate was used as crude enzyme solution. Briefly, 1ml of the crude extract was pippetted into a test tube containing 0.5 ml of 5g urea in a tris-HCl buffer at pH 9.0. The resulting solution was boiled for 1hour at 50^{o}C. To the prepared solution, 0.2 ml of toluene was added to the incubated solution and shaked vigorously. 4ml of KCl was added to the solution to stop the reaction. Absorbance was taken in duplicate at wavelength of 620 nm.

3.2.6 Isolation of microorganisms from contaminated soil

The Ezeonu, Okafor and Ogbonna (2013) approach was used to determine the microbial ecology of the polluted soil. A portion of the soil sample weighing 20g was added to 40ml of distilled water in a clean conical flask and vigorously shaken (stock solution) before being used as the starting point for the isolation process. From the prepared stock solution, ten folds of serial dilutions were carried out, and the 10^{-4} to 10^{-6} dilutions were plated out in a media plate.

3.2.6.1 Preparation of Media and Plate Pouring

Nutrient and Mackonkey media were prepared by dissolving 28g and 13.5 g of the media in 1000ml 0f distilled water respectively. The mixtures were sterilized at of 121^{o}C for 15minutes and then allowed to cool. After cooling for a while, media inoculation was carried out.

Also, differential hydrocarbon utilizing media formulation was prepared according to the manufacturer descriptions. The prepared media was sterilized as described above.

3.2.6.2 Inoculations of Folds of Diluted Soil Solution on the Prepared Plate and Sub Culturing

Using a 1 ml insulin syringe and a glass rod spreader around a bunsen flame, inoculations were carried out on the prepared nutrition, Mackonkey, and the Bush-Nell-Hass medium plates, respectively. Each side of the plate was marked with streaks to indicate the starting point, and once each side was finished, the wire loop was sterilized. But for the Bush-Nell-Haas media sterilized filter paper with discs of petroleum effluents patches were used to cover the inoculated plates. After the inoculation, the inoculated plates were incubated for 3-4 days at 37°C for colonies growth.

3.2.6.3 Preservation of the Bacteria

Non contrasting microbes were kept on the respective media slants as stock cultures in a bijou bottle. The medium was autoclaved at 121°C for 15minutes. Cooling to about 45°C and the bijou bottle were slanted slightly and allowed to gel. The bijou bottles were then incubated at room temperature for 3-4days to check for sterility.

3.2.6.4 Microscopic Features of the Isolated Bacteria

Pure cultures that were three days old were inspected physically and under a microscope. It was also possible to see the color, texture, spores, and growth patterns.

3.2.6.5 Bacteria Identification

Ezeonu *et al.* (2013) described how microscopic slides were created using the three-day-old pure cultures. Gram staining was done on the colonies to separate the Gram positive bacteria from the gram negative bacteria prior to the microscopic analyses of the microbiological isolates. During the Gram staining, each of the pure microbial cells was smeared on a heat fixed glass slide and immediately covered with gentian violet; the set up was covered with glass slit and stand for 15 minutes. The gentian violet was quickly washed off from the mounted microbial cells using 3% acetone and the mounted glass slide was dried using cotton wool and heat fixed by smearing around the bunsen flame.

The mounted slide was counter stained with safranine and allowed to stand for 15 minutes. It was thereafter decolorized using the 3% acetone, washed off with water and dried using cottn wool and heat fixed by smearing around the bursen flame.

A quantity of the culture suspensions and a drop of safranine red were applied to the slide after the Gram staining. It was covered with a cover slip, and an X100 light microscope inspection was conducted on it. By comparing the microbiological characteristics and images of the micrographs to "Atlas of Bacteriology" by Barnett and Hunters (1972), microbial isolates were identified.

3.2.6.6 Biochemical Test

This was done as follows:

2.2.6.6.1 Catalase Test

An aliquote of the overnight bacterial culture was emulsified to a smooth suspension on a clean glass slide and a drop of 3% hydrogen peroxide (H_2O_2) was added to the aliquote using a sterile pastuer pipette (Ezeonu *et al.,* 2013).

3.2.6.6.2 Citrate Utilization Test

Citrate degrading potentials of the organisms were experimented using Simmon's medium. The Simmon's media were prepared according to the manufacturer's instruction. Each of the isolates was streaked on the surface of the prepared citrate slant using a sterile wire loop and was incubated anaerobically and aerobically for 24 hours at 37°C (Mamta *et al*., 2010).

3.2.6.6.3 Indole Test

Indole test detects the presence or absence of tryptophanase which breaks aromatic amino acid, tryptophan into indole, pyruvic acid and ammonia**.** An overnight culture (12 hours) of the test organisms were inoculated each into test tubes containing 5 ml of sterile prepared peptone water and were incubated for 24 h at 37°C anaerobically. After incubation, 0.5 ml of kovac's reagent (3% dimethylaminobenzoaldehyde and 5% HCl) was added to each of the test tubes (Cheesbrough, 2006).

3.2.6.6.4 Methyl red-Voges Proskauer (MRVP) test

Microbial isolates were screened for methyl red-voges proskauer (MRVP) test. This test detects presence or absence of formic hydrogenenlyase. Each of the test organisms was inoculated into test tubes containing 5 ml of glucose phosphate broth (prepared by dissolving 1 g of glucose into 0.1 M of sodium monohydrogen phosphate) and was incubated anaerobically at 37 °C for 48 hours. After incubation, 5 drops of 0.04 % solution of methyl red solution was added into the incubated test tubes (Ezeonu *et* al., 2013).

3.2.6.6.5 Motility and Hydrogen sulphide production

Sulphide indole motility medium (SIM) was used to determine if the isolates were motile and to detect the ability of an organism to reduce sulphur to hydrogen sulphide, Here, a pure culture of the isolates grown overnight (12 hours) were inoculated in test tubes containing 5 ml of prepared sulphide indole motility media by making a single stab to a depth of about ¾ of the media in the test tube and were incubated at 37°C for 24 h aerobically and anaerobically. Motility (diffused growth from the line of inoculation/stab) and hydrogen sulphide production from the inoculum (blackening of the medium) were observed (Mamta *et al*., 2010).

3.2.6.6.6 Sugar Fermentation Test

The sugars tested for fermentation were glucose, lactose, galactose and mannose. One percent of the sugars were prepared according to the detailed specifications of the method of Chessbrough, (2006). Using an overnight (12 hours) culture of the test isolates, the tubes containing 5 ml of the respective sugars with inverted Durham tubes were inoculated and incubated anaerobically at 37°C for 24 hours (Chessbrough, 2006).

3.2.6.7 Heterotrophic Counting and Microbial Standardization

From the growing media plate, Following are total heterotrophic colonies (CFU/g) from nutritional, Mackonkey, and differential media.:

TCFU/ml=

$$microbial\ colonies\ observed\ X\ innoculum\ dilution\ factor\ X\ innoculum\ volume\ pippetted$$

Where TFCU/ml is total coliform unit per volume of the innoculum in mililitres.

Marcfarland standard solution (2% Barium chloride and 5% sulphuric acid aq.) was used in standardization of the bacteria population stored in liquid broth at various incubation days of 0, 7,14 and 21. Absorbance was taken at λ 610 nm as described by Ezeonu *et al.* (2013).

3.2.7 Emulsification Studies of the Isolated Bacteria

Prior to the emulsification assays, the isolates identified was inoculated into 10ml of broth medium each and the incubated at 37^{o}C for 72 h. The culture media was centrifuged at 3000 revolutions per minute (r.p.m.) for 30 minutes. The supernatant was collected and the cells discarded. The supernatant was used for various emulsification tests.

Emulsifying Assays

The following emulsification assays were carried out on the identified isolates as follows:

3.2.7.1 Drop Collapse Assay

The assay was carried out as described by Onugbolu, andAdieze (2016). A drop of the culture supernatant was placed carefully on an oil coated glass slide and observed after one minute. This test was simultaneously carried out on distilled water as control.

3.2.7.2 Oil Spreading Assay

A tin oil layer was created by adding ten microliters of petroleum effluents and diesels to the top of 40 ml of distilled water in a danger dish using a micropipette. As described by Onugbolu, and Adieze (2016), 10 ml of the culture supernatant was gently dropped on the center of the oil layer interface

3.2.7.3 Emulsifying Capacity

Onugbolu, and Adieze (2016) created a biosurfactant with the ability to emulsify. The culture supernatant was mixed with two mililiters (2ml) of petroleum effluents, diesels, and vortexed vigorously at high speed for two minutes. After the mixture had been mixed for 24 hours, the stable emulsion layer's height was measured. The ratio of the height to the emulsion index E24 was used to compute the liquid's overall height and the emulsion layer thickness.

E24 = (H) total X 100% (H) emulsion.

Molecular identification of Isolates

This was carried out as described by Kumar *et al.* (2016). The following processes were carried out during the molecular identifications:

i. **DNA Extraction**

The genomic DNA (gDNA) of the selected bacteria isolates were extracted at the 16 s region using ZR fungal/bacterial DNA miniprep (produced by Zymo Research) using the product as directed by the maker. A ZR Bashing TM Lysis Tube was filled with 750 mL of Lysis Solution and 2 mL of fungus cells broth. This was processed for more than 5 minutes at maximum speed inside a bead equipped with a 2 ml tube holder assembly; The ZR Bashing Bead TM lysis Tube was centrifuged at > 10,000 x g for 1 minute in a microcentrifuge. A Zymo-Spin TM IV Spin Filter (orange top) was used tofilter up to 400 l of the supernatant, and it was centrifuged at 7,000 x g for 1 minute. The filtrate in the Collection Tube from Step 4 was then mixed with 1,200 l of

Fungal/Bacterial DNA Binding Buffer. Following the transfer to a Zymo-Spin TM IIC Column in a Collection Tube, 800 l of the Step 5 mixture was centrifuged at 10,000 x g for 1 minute. After removing the flow from the Collection Tube, the former was repeated. 200 l of DNA Pre-Wash Buffer were added to a brand-new Collection Tube, and the Zymo-Spin TM IIC Column was centrifuged at 10,000 x g for one minute. Additionally, 500 l of Bacterial DNA Wash Buffer was added to the Zymo-Spin TM IIC Column, and the mixture was centrifuged at 10,000 x g for one minute. The Zymo-Spin TM IIC Column was then transferred to a clean 1.5 ml microcentrifuge tube, and 100 l (at least 35 l) of DNA Elution Buffer was added right away to the column matrix. This was centrifuged at 10,000 x g for 30 seconds to elute the DNA.

ii. DNA Amplification

The 16s region of the extracted DNA was then amplified with the following polymerase chain reaction (PCR) techniques. The amplicon size was clarified by comparison with DNA molecular weight marker on agarose gel electrophoresis. The PCR mixture is composed of 2 liters of DNA template, 1 liter each of 10 mM forward and reverse primers, 12.5 liters of Taq 2X Master Mix from New England Biolabs (M0270), and 8.5 liters of nuclease-free water.

iii. Primer Sequences

The primer used has the following sequences:

Forward: 5’ TCC GTA GGT GAA CCT GCG G 3’

Reverse 5’ TCCTCCGCTTATTGACATGS 3’

iv. Cycling Conditions

PCR mix was initially heated at 94˚C for 5mins followed by 36 cycles of denaturation at 94˚C for 30sec. Heating at 94^{0}C was for denaturation leading to separation of the DNA double strand into single strands. The temperature was then reduced to at 54˚C for 30secs to allow annealing to occur. This was followed by an increase in temperature to 72˚C for 45sec for elongation and followed by a final elongation step at 72˚C for 7 minutes. The temperature was then held at 10 ˚C forever.

v. Electrophoresis for DNA and PCR

This was carried out by the procedure below:

1. Agarose weights of 1 g for DNA and 2 g for PCR were measured.

2. In a microwave-safe flask, 100 mL of 1xTAE were combined with the agarose powder.

3. This was microwaved for one to three minutes to completely dissolve the agarose; however, it was important to avoid boiling the solution to the point that some of the buffer evaporated and changed the final concentration of agarose in the gel.

4. The agarose solution was allowed to cool for about five4. The agarose solution was allowed to cool for about five minutes, to a temperature of around 50 °C (about the point at which hands can be comfortably resting on the flask).

5. Ten liters of EZ vision DNA stain were applied. The DNA may be seen when exposed to ultraviolet (UV) light thanks to the DNA-binding property of EZ eyesight.

6. It was the agarosepoured with the well comb in place into a gel tray.

7. To fully solidify, the freshly poured gel was either left at room temperature for 20 to 30 minutes or placed at 4 °C for 10 to 15 minutes..

1. Running an Agarose Gel after Loading Samples

i. Each of the DNA samples or PCR products received loading buffer.

ii. The agarose gel was put into the gel box (electrophoresis apparatus) once it had solidified.

iii. 1xTAE (or TBE) was then added to the gel box until the gel was completely coated.

iv. The first lane of the gel was properly loaded with a v. The samples were then placed into the additional gel wells.

vi. For roughly one and a half hours, the gel was run at 80-150 V.

vii. The gel was carefully taken from the gel box once the power was turned off, the electrodes were unplugged, and the gel.

Viii The DNA fragments and PCR products were seen using UV light in 1. Running an Agarose Gel after Loading Samples

i. Each of the DNA samples or PCR products received loading buffer.

ii. The agarose gel was put into the gel box (electrophoresis apparatus) once it had solidified.

iii. 1xTAE (or TBE) was then added to the gel box until the gel was completely coated.

iv. The first lane of the gel was properly loaded with a molecular weight ladder.

v. The samples were then placed into the additional gel wells.

vi. For roughly one and a half hours, the gel was run at 80-150 V.

vii. The gel was carefully taken from the gel box once the power was turned off, the electrodes were unplugged, and the gel.

The DNA fragments and PCR products were seen using UV light in paragraph viii.

vi DNA Sequencing

Following the manufacturer's instructions, the amplified fragments were sequenced using an Applied Biosystems Genetic Analyzer 3130 xl sequencer and a BigDye terminator v3.1 cycle sequencing kit. All genetic analyses were conducted using MEGA X and Bio-Edit software.

Vii Bioinformatics analysis

The DNA sequence generated was assembled into one complete sequence and the most homologous sequences were determined in comparison to the Genbank using BLASTn (Basic Local Alignment Search Tool for nucleotides) search algorithm. DNA sequence was aligned with reference from NCBI using multiple sequences Alignment. Phylogenetic analysis of the DNA sequence was conducted by neighbour joining using Molecular Evolutionary Genetics Analysis (MEGA) version 7.

3.3 Spectroscopic Analysis

At room temperature, the generated microbial emulsion's Fourier transform infrared (FTIR) spectra were captured using a 400 Perkin Elmer Agilent spectrometer (Perkin-Elmer, Norwalk, CA, USA). Between the wavelengths of 4000 cm-1 and 500 cm-1, spectra were acquired. After being completely dry, samples were crushed into a paste using KBr at a sample-to-KBr ratio of 1:40. Averaging over 32 images, 4 cm-1 resolution spectra were collected.

3.4 Statistical Analysis

Data obtained was expressed as mean replicate of n=3 using the T-student test. Microsoft excel data package used to show significance in the activity of stress marker enzyme activity.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Results

4.1.1 Physicochemical properties of soil

The results showed variations in the physicochemical properties of the soil contaminated with different percentages of petroleum effluents. Soil contaminated with 10% petroleum effluent from Onne, Escravos and Nembe shoreline showed pH of 4.24, 6.3 and 5.87 respectively while the control experiment maintained a pH of 7.6 throughout the experiment. At 50 and 100% of the effluent contaminations, there was no significant difference in the pH of the contaminated soil respectively. Soil conductivity of soils contaminated with effluents from Onne, Escravos and Nembe showed a progressive increase of soil conductivity as effluent concentration increases from 0-100% with effluent from Escravos showing the highest impact on soil conductivity (table 4.1).

Dissolved mineral contents were found in the following order: Cl>Ca>Cu>Fe>Mg>K>PO_3 > Pb while heavy metals of Hg, As and Cd were found at below detectable limit range (BDL) in both the contaminated and non-contaminated soil. Concentration of Cl, Ca, Mg, K, PO_3 and Pb increases as the concentration of the contaminants increases from 0-100 %.

With 10% Onne effluents, the levels of total organic matter (TOM) and total organic carbon (TOC) were 196.73 and 78.64 mg/g, respectively, and they grow as the effluents' concentration rises. Total organic carbons (TOC), as well as total organic matter (TOM) were discovered at 119.04, 146.42; 112.41 and 138.26 mg/g with 10 % effluents from Escravos and Nembe respectively.

Table 4.1 Physicochemical properties of soil sample polluted with different concentrations of petroleum effluent from Onne.

Physiochemical parameters	**Control experiment**	**10% Onne**	**50% Onne**	**100% onne**
pH	7.6	4.24	4.45	4.5
Soil Conductance	610	433	613	673
Chloride ion (mg/g)	433	1151.614	1002.21	1102
Phosphorus (mg/g)	1.78	1.23	2.31	2.01
Magnesium (mg/g)	6.27	11.27	13.05	14.75
Potassium (mg/g)	7.22	11.52	7.68	8.92
Calcium (mg/g)	18.23	19.34	34.55	37.32
Iron(mg/g)	3.52	38.7	39.54	39.54
Cadmium(mg/g)	BDL	0.015	0.024	0.08
Mercury(mg/g)	BDL	BDL	BDL	BDL
Arsenic(mg/g)	BDL	BDL	BDL	BDL
Lead(mg/g)	BDL	1.73	3.3	4.9
Copper(mg/g)	1.08	25.49	30.3	36.1
Total petroleum hydrocarbon (TPH)(mg/g)	0.34	1014.93	1234	1341
Total Organic Carbon (TOC) (mg/g)	10.45	78.64	87.91	90.91
Total Organic Matter (TOM) (mg/g)	12.85	196.73	108.13	111.8

N=2

Table 4.2Physicochemical properties of soil sample polluted with different concentrations of petroleum effluent from Escravos.

Physiochemical parameters	**Control experiment**	**10% Escravos**	**50% Escravos**	**100% Escravos**
pH	7.6	6.3	6.62	6.16
Soil Conductance	610	1033	1134	1219
Chloride ion (mg/g)	433	1031.32	1143.18	1265.09
Phosphorus (mg/g)	1.78	2.28	1.43	0.94
Magnesium (mg/g)	6.27	09.44	16.04	13.28
Potassium (Mg/g)	7.22	14.17	16.28	18.63
Calcium (mg/g)	18.23	34.76	46.72	41.06
Iron(mg/g)	3.52	36.87	27.72	21.39
Cadmium(mg/g)	BDL	0.021	0.042	0.047
Mercury(mg/g)	BDL	BDL	BDL	BDL
Arsenic(mg/g)	BDL	BDL	BDL	BDL
Lead(mg/g)	BDL	7.62	9.23	11.16
Copper(mg/g)	1.08	14.52	29.08	32.24
Total petroleum hydrocarbon (TPH)(mg/g)	0.34	1139.29	1309.06	1342.21
Total Organic Carbon (TOC) (mg/g)	10.45	119.04	127.12	131.43
Total Organic Matter(TOM) (mg/g)	12.85	146.42	156.36	161.65

N=2

Table 4.3 Physicochemical properties of soil sample polluted with petroleum effluents from Nembe surge tank.

Physiochemical parameters	Control experiment	10% Nembe	50% Nembe	100% Nembe
pH	7.6	5.87	5.87	5.87
Soil Conductance	610	965	965	965
Chloride ion (mg/g)	433	1056.45	1121.27	1149.09
Phosphorus (mg/g)	1.78	4.04	8.13	6.29
Magnesium (mg/g)	6.27	10.13	14.28	18.66
Potassium (mg/g)	7.22	08.21	16.05	13.87
Calcium (mg/g)	18.23	10.11	26.24	31.33
Iron(mg/g)	3.52	21.28	18.46	12.09
Cadmium(mg/g)	BDL	BDL	BDL	BDL
Mercury(mg/g)	BDL	BDL	BDL	BDL
Arsenic(mg/g)	BDL	BDL	BDL	BDL
Lead(mg/g)	BDL	1.02	4.38	6.02
Copper(mg/g)	1.08	24.12	20.08	21.23
Total petroleum hydrocarbon (TPH)(mg/g)	0.34	1102.34	1178.29	1214.32
Total Organic Carbon (TOC) (mg/g)	10.45	112.41	127.51	138.44
Total Organic Matter (TOM) (mg/g)	12.85	138.26	156.84	170.28

N=2

4.1.2 Soil Enzyme Analysis

Figures 4.1-13 show the soil enzyme activity at day 0-21 of the incubation of the respective petroleum effluent contamination percentage. Enzyme activity relatively decreases as the contaminant concentrations increases from 0-100 v/w. Lipase activity was seen relatively low in all the soil at low concentrations of the effluents (0-50 v/w). At high concentrations of the effluents, there was a significant increase in the enzyme activity. Peroxidase and catalase activity were significant at low effluent concentrations but was attenuated at high treatment (100%) of the soil with effluent from petroleum hydrocarbon. Peroxidase showed significant activity in all the sampled soils (Escravos, Onne, and Nembe). It was so seen from the result that enzyme activities assayed were relatively high when compared with the control experiment. Escravos and Onne showed marker enzymes with peak activities when compared with enzymes from Nembe.

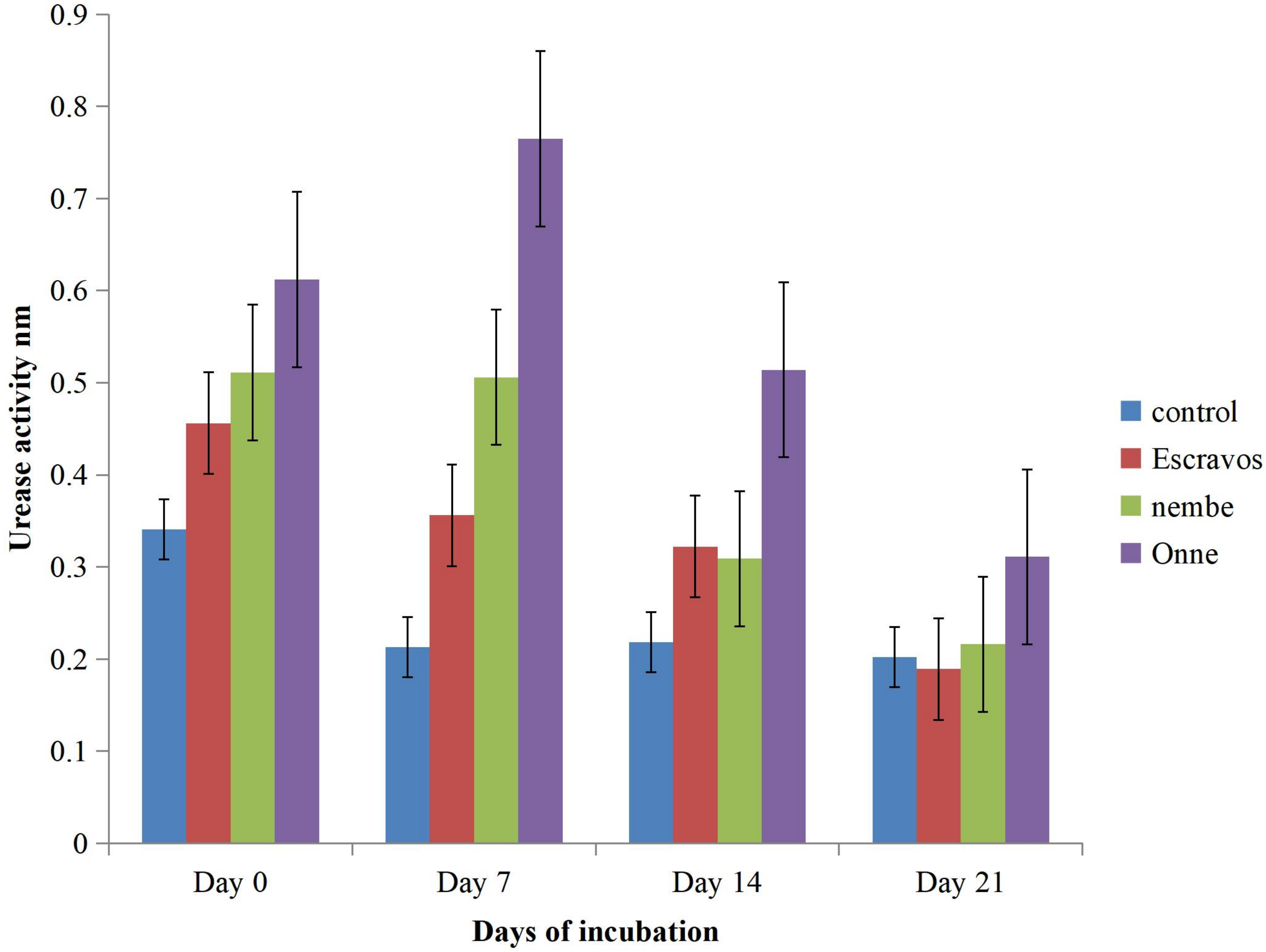

Fig. 4.1 Soil urease activity on different days at 10 % contamination of soil with different effluents.

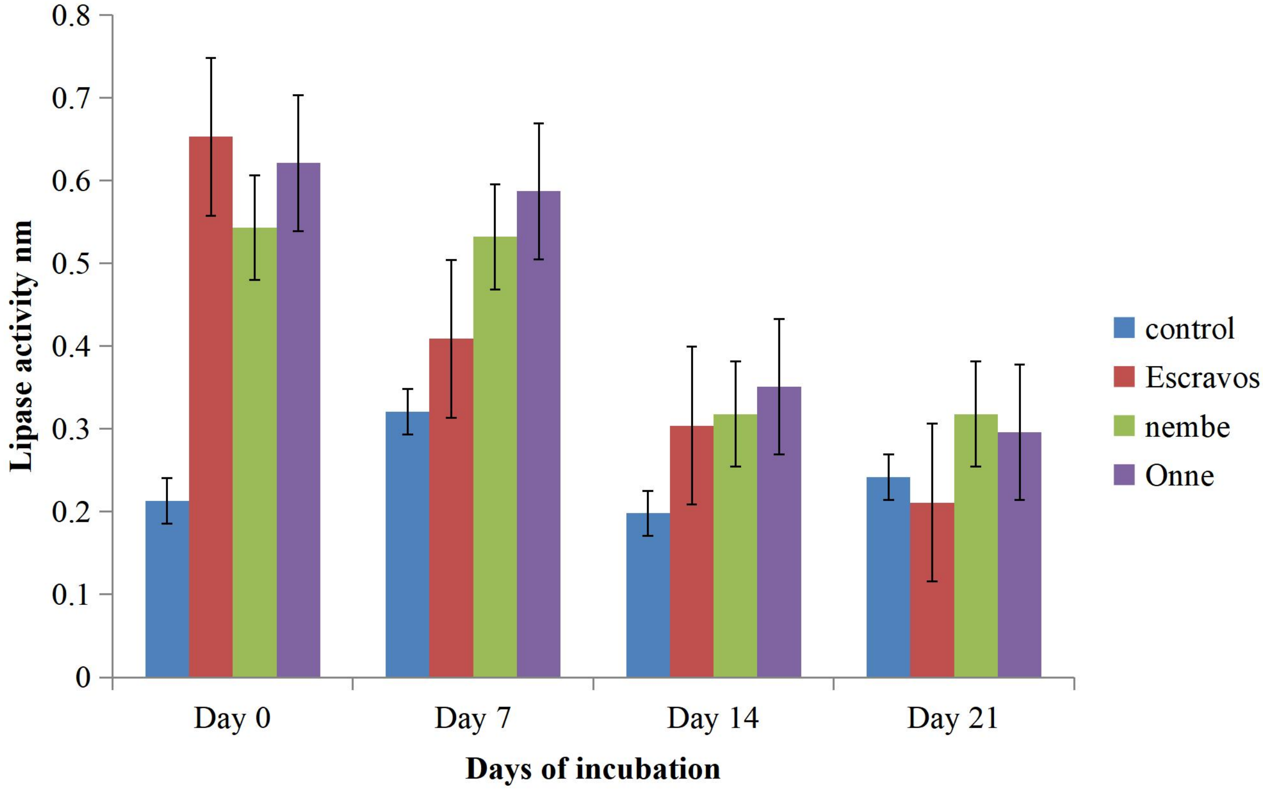

Fig. 4.2 Soil lipase activity on different days at 10 % contamination of the soil with different effluents.

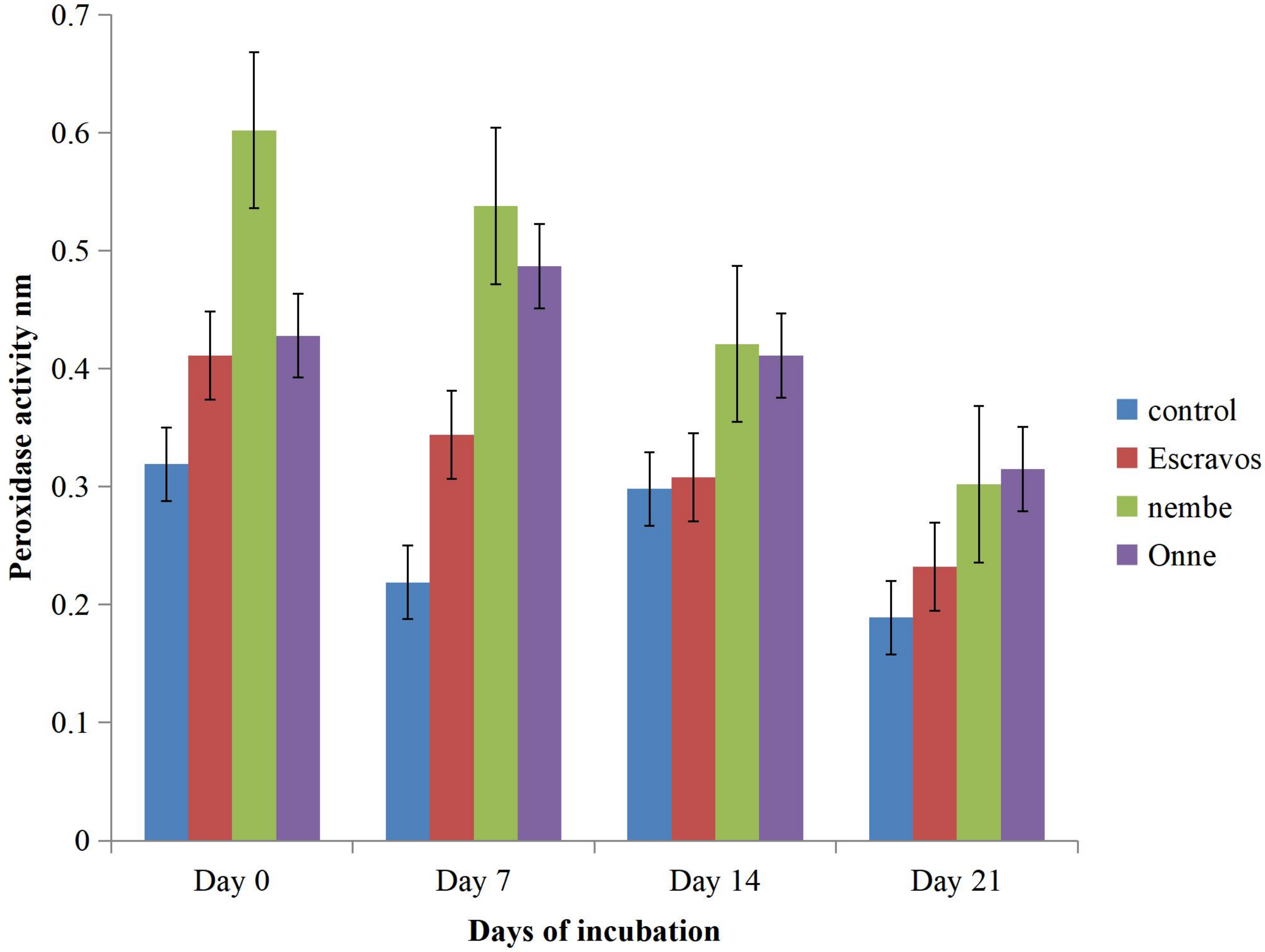

Fig. 4.3 Soil peroxidase activity on different days at 10 % contamination of the soil with different effluents.

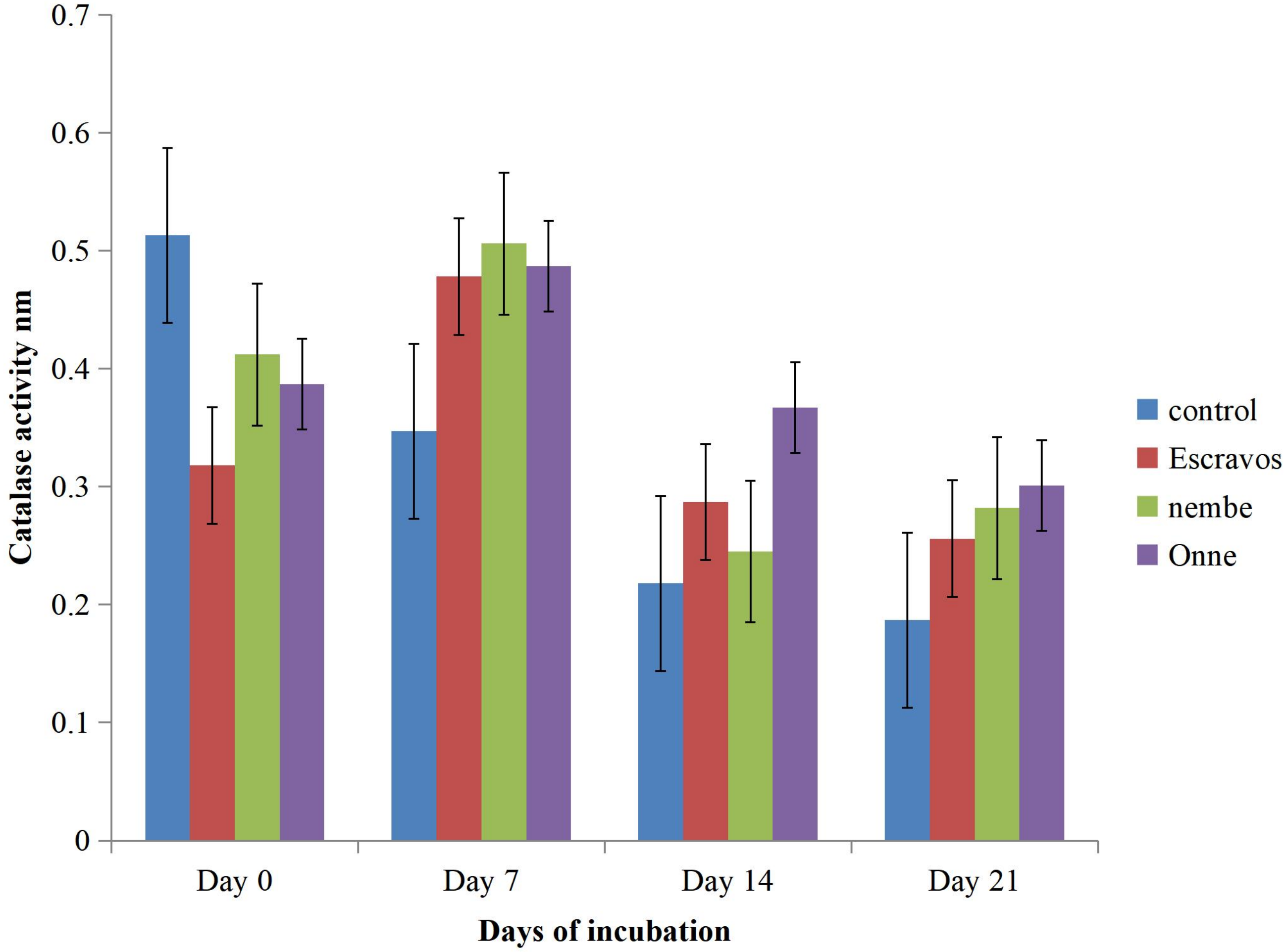

Fig. 4.4 Soil catalase activity on different days at 10% contamination of the soil with different effluents.

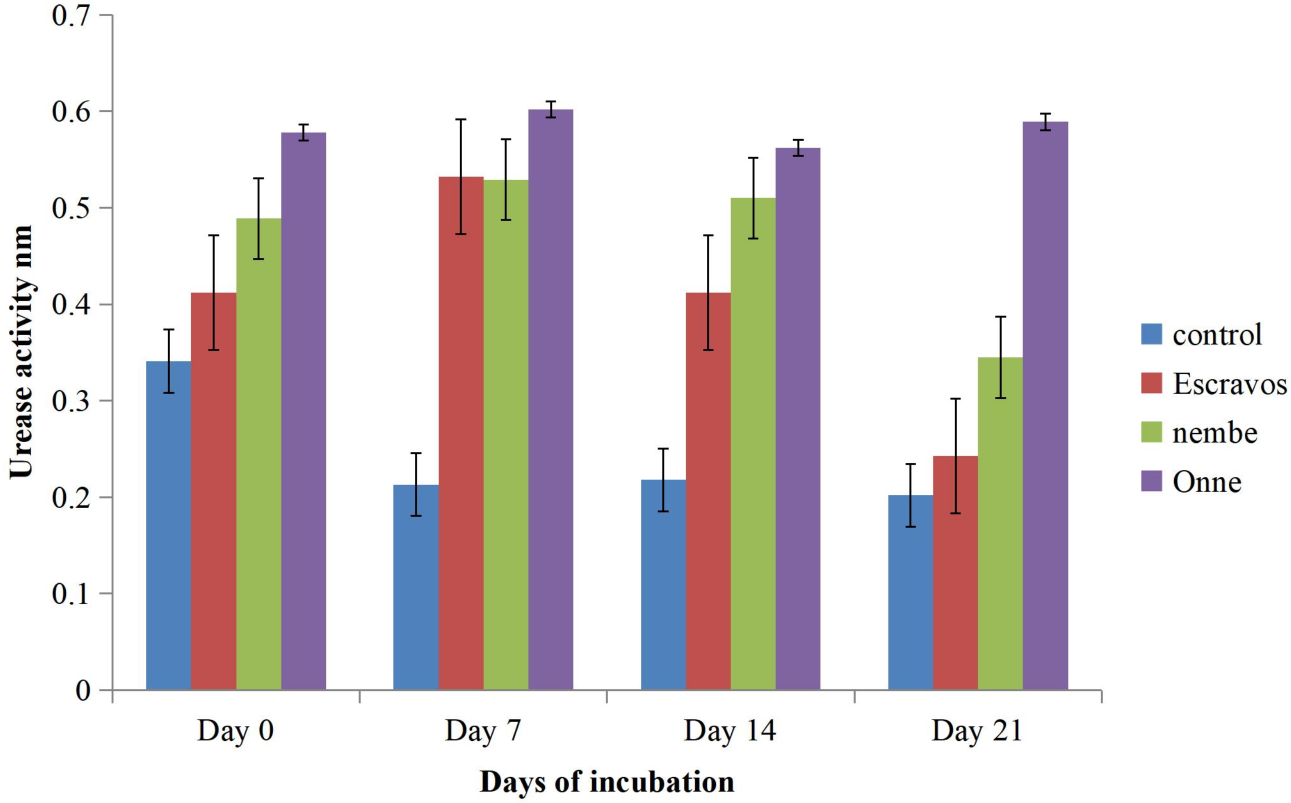

Fig. 4.6 Soil urease activity on different days at 50 % contamination of the soil with different effluents.

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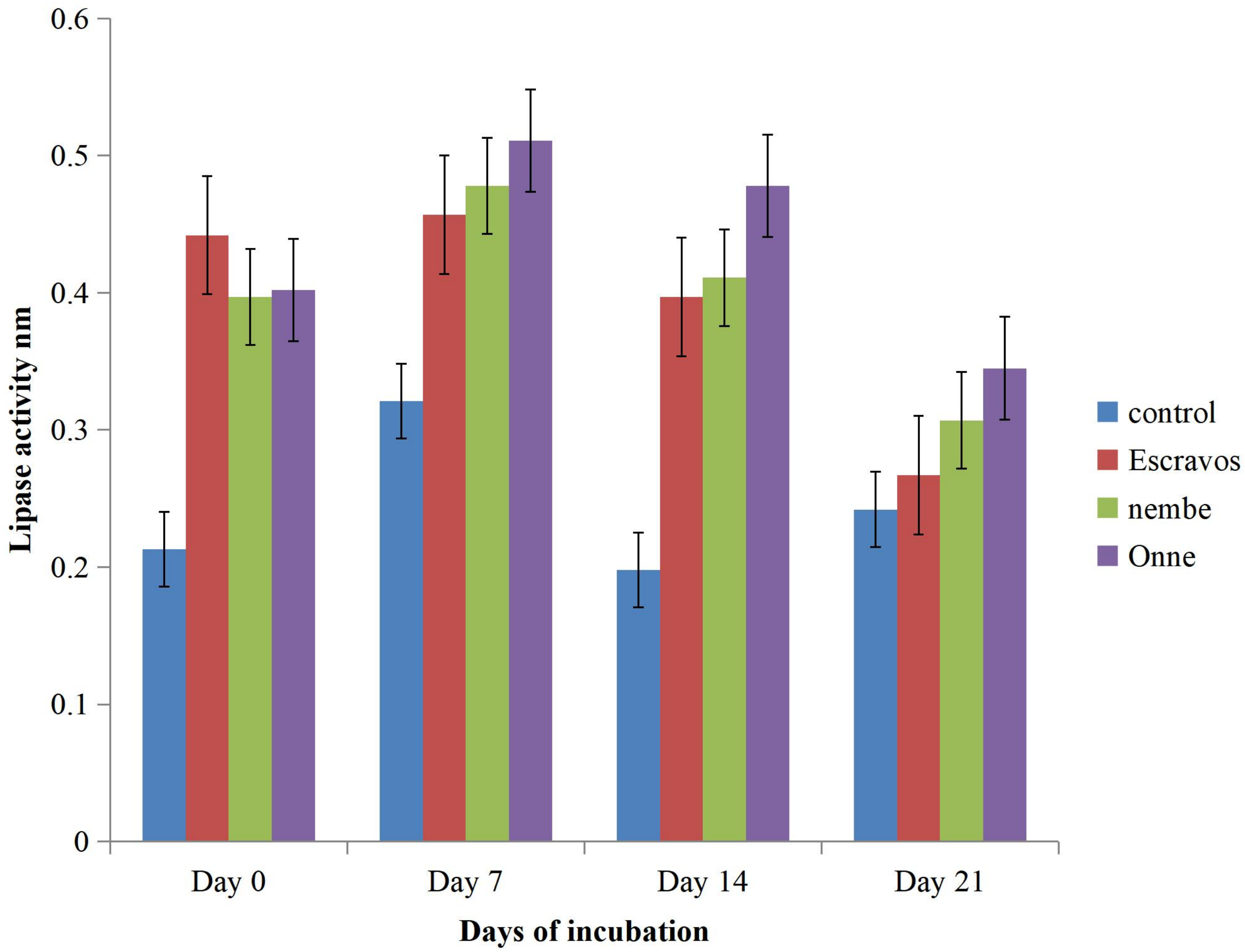

Figure 4.7 Soil lipase activity on different days at 50 % contamination of the soil with different effluents.

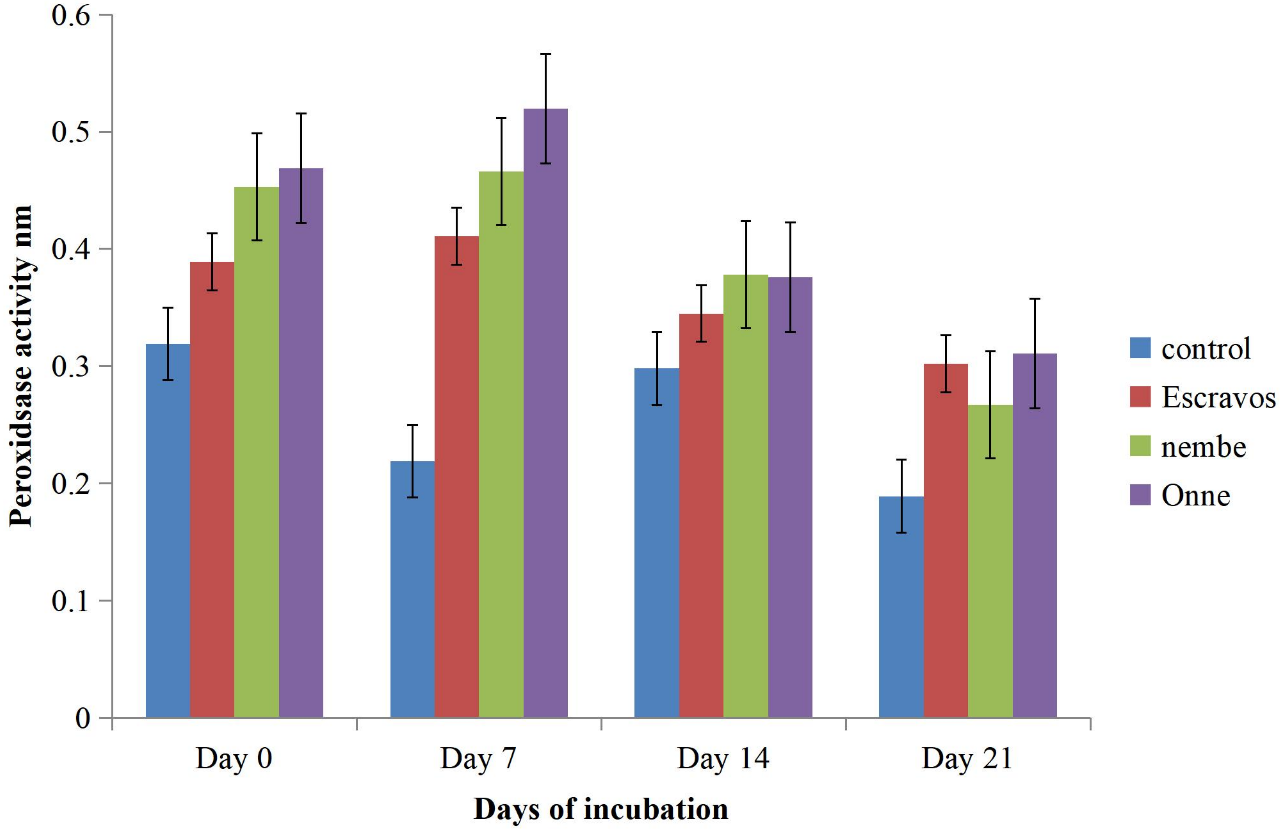

Fig 4.8 Soil peroxidase activity on different days at 50 % contamination of the soil with different effluents.

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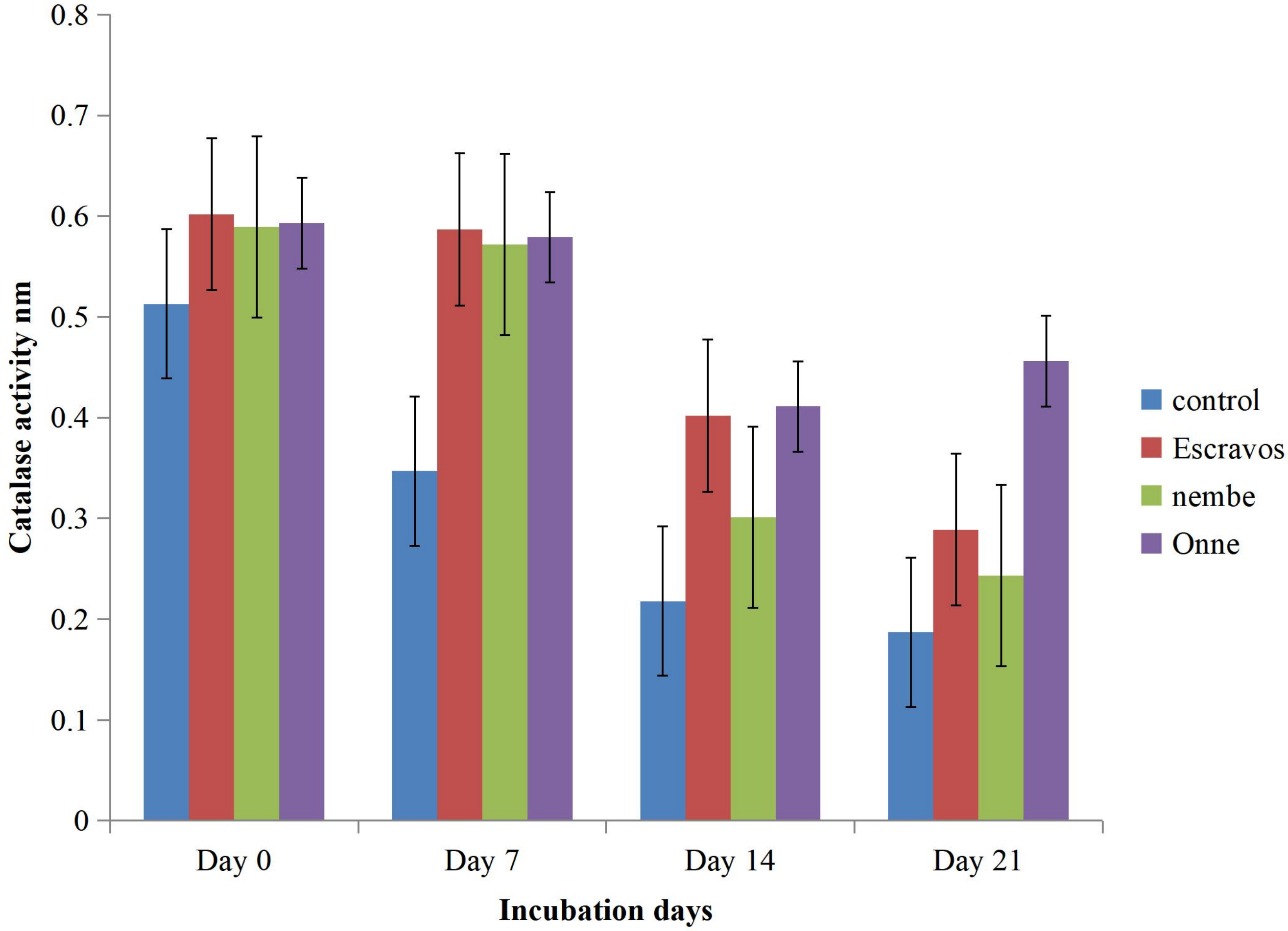

Fig 4.9 Soil catalase activity on different days at 100 % contamination of the soil with different effluents.

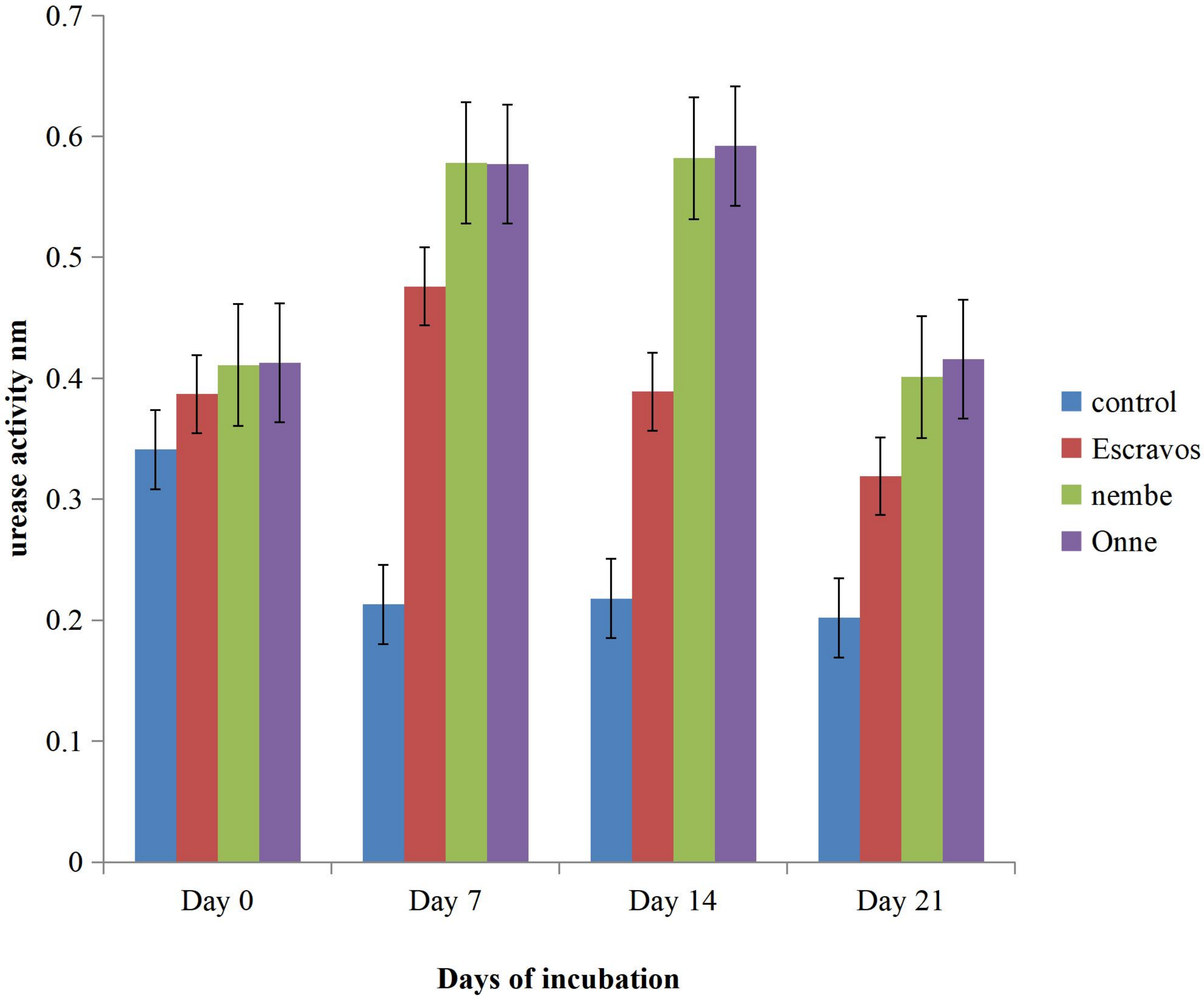

Fig 4.10 Soil urease activity on different days at 100 % contamination of the soil with different effluents.

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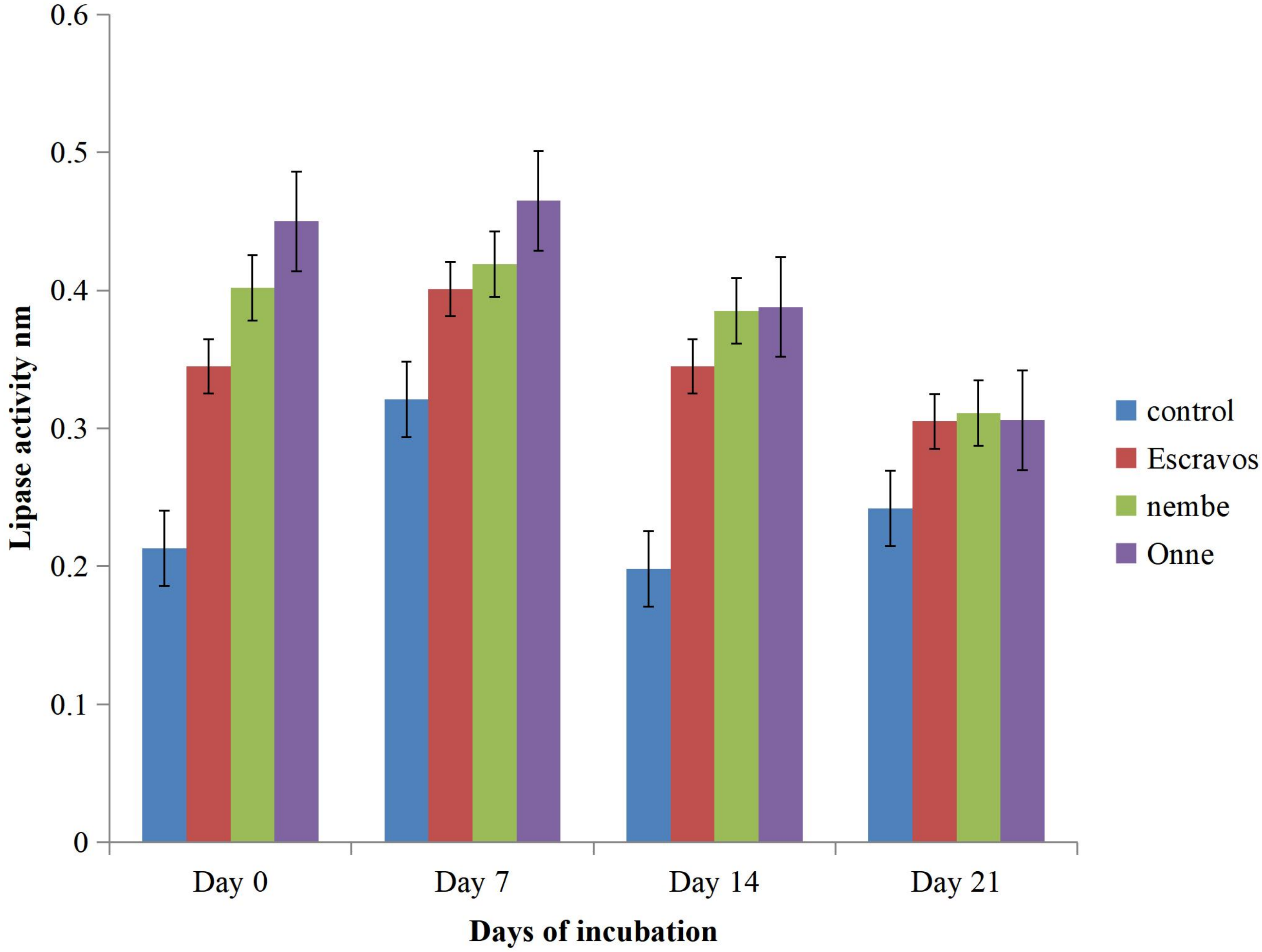

Fig.4.11 Soil lipase activity on different days at 100 % contamination of the soil with different effluents.

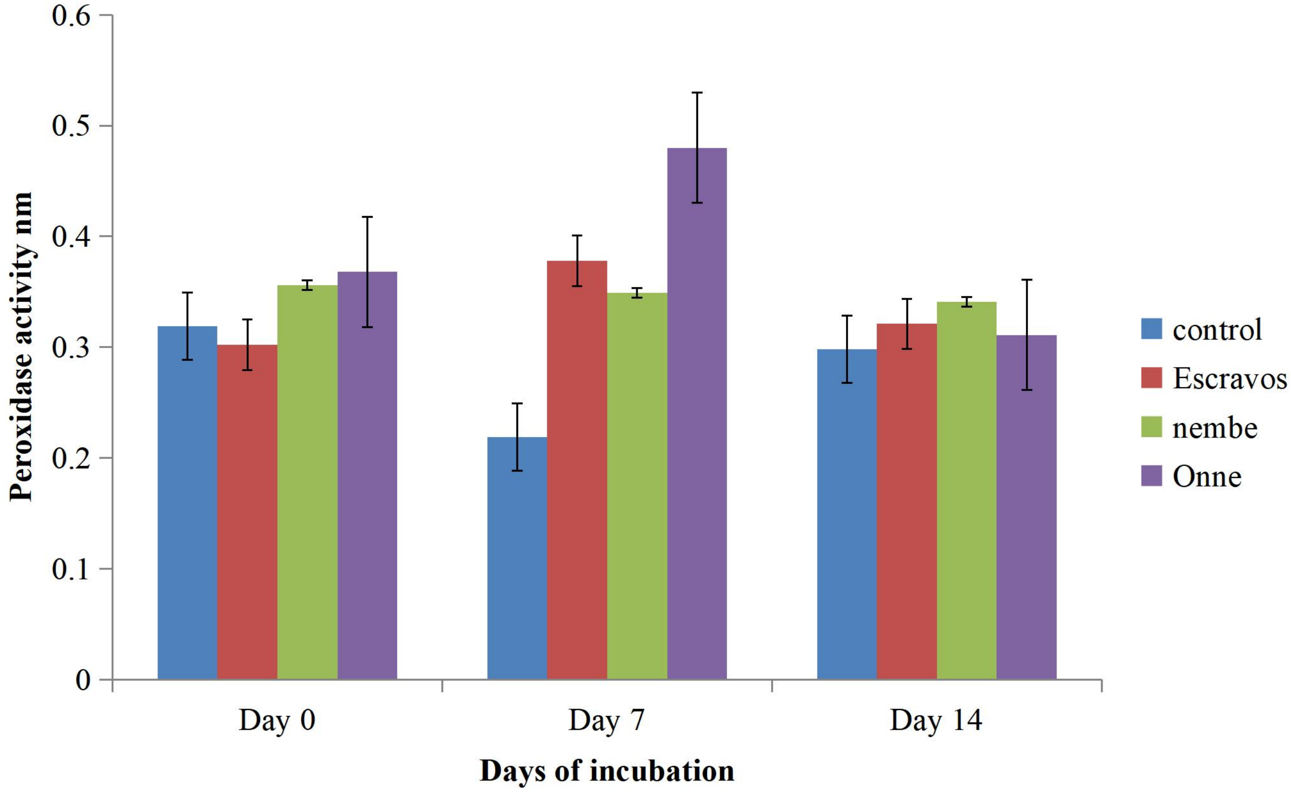

Fig. 4.12 Soil peroxidase activity on different days at 100 % contamination of the soil with different effluents.

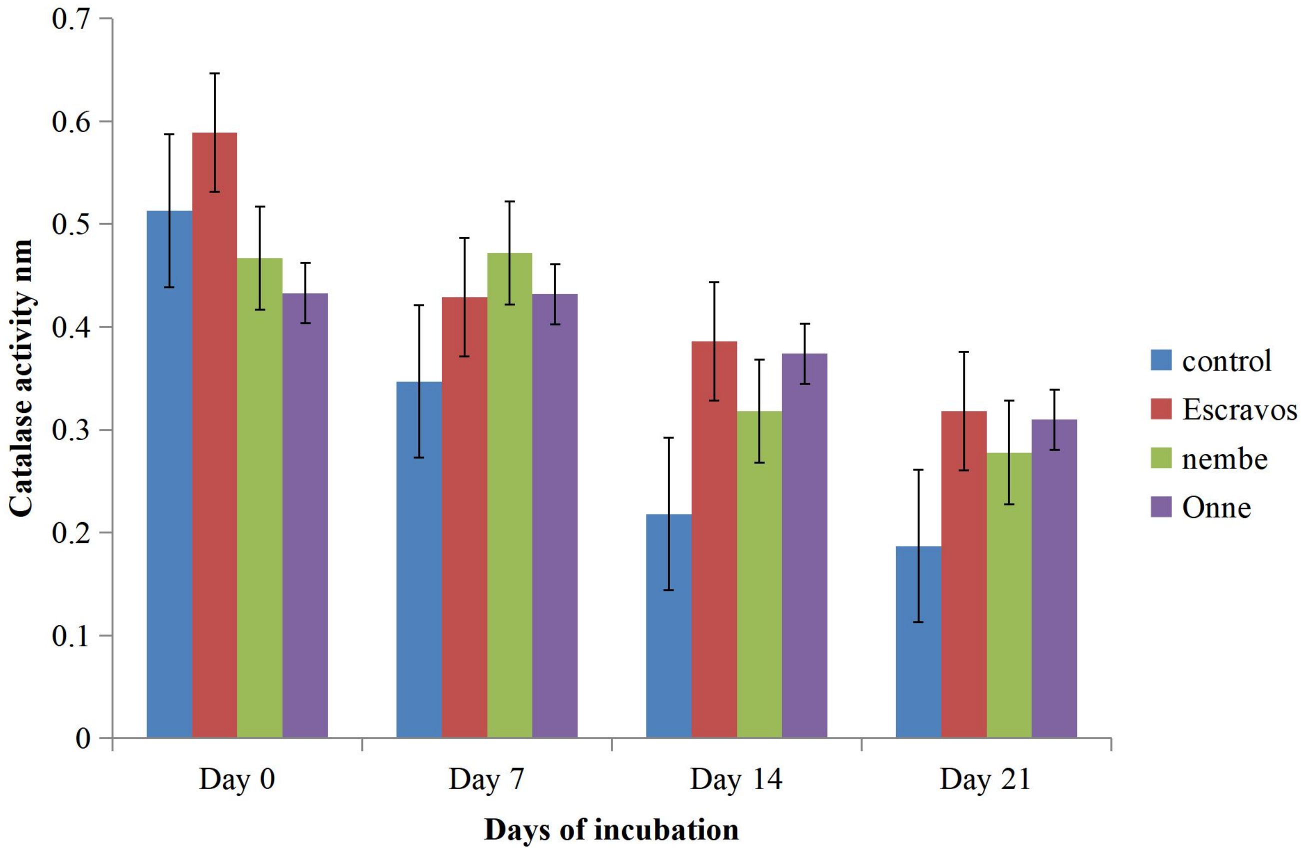

Fig. 4.13 Soil catalase activity on different days at 100 % contamination of the soil with different effluents.

4.1.3 Soil Microbial Populations

The analysis of soil microorganisms in soil samples contaminated with varying amounts of petroleum effluent revealed a significant decline in soil organism as the effluent concentrations increased. The multiplication of the organisms contaminated with petroleum effluent, however, increased noticeably from days 0–7 of incubation but decreased from days 14–21. Soil from Nembe showed relatively low microbial activity (Table 4.4). Total coliform counts (CFU/g) (Table 4.5) showed the same trend as the total viable cells in all the polluted soil with effluents from Nembe, Escravos and Onne respectively but with relatively less number of bacteria population per gram of the soil. Furthermore, bacteria degrading hydrocarbons isolated from the respective contaminated soils with the petroleum effluents showed similar trend of heterotrophic bacteria population (Table 4.6). Table 4.7 showed the bacterial distribution in the different soil samples. Bacillus species were more abundant.

Table 4.4 Distribution of bacteria (CFU/g) at different effluent pollution.

Effluent source	control				10%				50%				100%			
	day 0	day 7	day 14	day 21	day 0	day 7	day 14	day 21	day 0	day 7	day 14	day 21	day 0	day 7	day 14	day 21
Escravos	3.4×10^5	4.2×10^3	3.2×10^3	1.9×10^3	5.1×10^8	4.2×10^6	4.5×10^5	3.2×10^4	7.2×10^7	1.6×10^6	2.7×10^4	0.16×10^3	8.2×10^5	0.3×10^3	6.4×10^2	6.4×10^2
Onne	3.4×10^5	4.2×10^3	3.2×10^3	1.9×10^3	3.3×10^7	5.6×10^6	5.6×10^5	4.2×10^4	4.9×10^7	2.8×10^7	1.5×10^4	0.14×10^3	4.2×10^5	1.7×10^3	7.3×10^2	7.3×10^2
Nembe	3.4×10^5	4.2×10^3	3.2×10^3	1.9×10^3	3.9×10^8	2.9×10^7	3.2×10^6	3.4×10^4	4.8×10^8	4.2×10^6	0.2×10^5	1.15×10^3	5.4×10^5	1.04×10^3	7.6×10^2	7.6×10^2

Table 4.5 Total Coliform Counts (CFU/g) of Bacteria Population

Effluent source	Control				10%				50%				100%			
	day 0	day 7	day 14	day 21	day 0	day 7	day 14	day 21	day 0	day 7	day 14	day 21	day 0	day 7	day 14	day 21
Escravos	2.6×10^3	1.2×10^2	2.7×10^2	0.5×10^2	7.02×10^4	2.30×10^4	2.7×10^3	2.9×10^2	4.5×10^4	1.6×10^6	0.6×10^3	3.5×10^2	9.1×10^3	1.30×10^2	1.3×10^1	1.3×10^1
Onne	2.6×10^3	1.2×10^2	2.7×10^2	0.5×10^2	8.2×10^3	2.39×10^4	1.6×10^4	3.12×10^2	3.5×10^4	2.8×10^7	1.2×10^3	4.12×10^2	5.8×10^3	1.21×10^2	2.4×10^1	2.4×10^1
Nembe	2.6×10^3	1.2×10^2	2.7×10^2	0.5×10^2	3.5×10^4	3.4×10^4	4.1×10^4	3.4×10^3	1.65×10^5	4.2×10^6	0.4×10^4	4.14×10^2	2.21×10^4	1.02×10^2	3.9×10^1	3.9×10^1

Table 4.6 Hydrocarbon Degrading Bacteria Counts (CFU/g)

Effluent source	Control				10%				50%				100%			
	day 0	day 7	day 14	day 21	day 0	day 7	day 14	day 21	day 0	day 7	day 14	day 21	day 0	day 7	day 14	day 21
Escravos	5.8×10^{5}	3.9×10^{4}	4.4×10^{3}	1.9×10^{3}	4.2×10^{4}	6.3×10^{5}	4.5×10^{6}	1.24×10^{7}	6.6×10^{5}	1.65×10^{7}	0.6×10^{3}	2.8×10^{7}	2.1×10^{5}	2.3×10^{6}	1.41×10^{6}	1.2×10^{4}
Onne	5.8×10^{5}	3.9×10^{4}	4.4×10^{3}	1.9×10^{3}	4.9×10^{5}	4.0×10^{5}	1.8×10^{6}	6.2×10^{6}	7.2×10^{5}	2.2×10^{6}	2.1×10^{8}	3.5×10^{8}	7.9×10^{4}	1.4×10^{6}	0.42×10^{7}	1.4×10^{5}
Nembe	5.8×10^{5}	3.9×10^{4}	4.4×10^{3}	1.9×10^{3}	5.2×10^{4}	3.8×10^{4}	1.8×10^{6}	1.39×10^{7}	3.9×10^{6}	7.4×10^{6}	4.3×10^{7}	5.21×10^{7}	1.4×10^{5}	3.5×10^{6}	1.21×10^{6}	2.3×10^{5}

Table 4.7 Bacteria Distribution in the Respective Treated Soil Samples

Effluent source	Control				10				50				100			
	Day 0	Day 7	Day 14	Day 21	Day 0	Day 7	Day 14	Day 21	Day 0	Day 7	Day 14	Day 21	Day 0	Day 7	Day 14	Day 21
Escravos	bacilli	spiral	cocci	Cocci in pairs		spiral	cocci	Cocci in pairs	bacilli	spiral	cocci	Cocci in pairs	cocci	spiral	cocci	Cocci in pairs
Onne	bacill	Cocci in pairs	rods	Large cocci		Cocci in pairs	rods	Large cocci	bacil	Cocci in pairs	rods	Large cocci	cocci	Cocci in pairs	rods	Large cocci
Nembe	bacill	Non sheat spiral	staphylococci	rods		Non sheat spiral	staphylococci	rods	bacil	Non sheat spiral	staphylococci	rods	cocci	Non sheat spiral	staphylococci	rods

4.1.4 Emulsification Studies

Tables 4.8-4.9 show the emulsification properties/potentials of the bacterial isolates using drop collapse, oil spread technique and emulsification index methods.. Strains of *Pseudomonas, Bacillus* and *Klebsiella* showed high potentials of emulsification in the two techniques used. Table 4.9 showed the emulsification index proportion of bacterial isolates in the presence of petroleum effluents and automated gas oil (diesel). Strains of *Pseudomonas, Bacillus* and *Klebsiella* showed high potentials of emulsification in both experimented petroleum fractions.

Table 4.8 Emulsification properties of the bacteria isolates using drop collapse and oil spread plate

Bacterial Isolate	Drop-Collapse	Oil Spreading Technique
Vibro sp.	-	-
E.Coli	+	+
Pseudomonas sp.	++	++
Klebsiella sp.	++	+++
Pseudomonas sp.	+	+
Salmonella sp.	+	+
Bacillus sp.	++	++
Pseudomonas sp.	++	+++
Streptomyce sp.	++	+
Bacillus sp.	++	++
Pseudomonas sp.	+	+

Guidiance: -, negative, + weak, ++ strongly positive

Table 4.9 Emulsification index (%E_{24}) of the bacteria isolates on the effluent and diesel respectively.

Bacteria isolates	Petroleum effluent	Diesel
Vibro sp.	32±0.5	40±0.54
E.coli	43±0.7	46±0.62
Pseudomonas sp.	40±0.56	43±0.56
Klebsiella sp.	43.3± 0.6	51.2±0.74
Pseudomonas sp.	50±0.62	42±0.54
Salmonella sp.	43.8±0.5	40±0.48
Bacillus sp.	50±0.42	51±0.34
Pseudomonas sp.	48.0±0.40	47±0.57
*Streptomyce*sp.	46±0.75	45±0.54
Bacillus sp.	51±0.54	39±0.32
Pseudomonas sp.	53±0.54	42±0.48

N=3

4.1.5 Molecular Studies

Plates 4.1-4 represent the electrophoretic analysis of the amplified bacterial genome. The respective plates showed a basepair (Bp) of approximately 750bp for all the bacterial amplicons.

Figs 4.14-17 show the phylogenetic evolutionary relatedness of the bacteria strains; from the figures sequenced bacteria genomes showed the presence of the following species: *Bacillus substilis, Klebsiella aerogenes, Pseudomonas aeruginosa and Streptomyces rosieclerotus* as the organisms with the highest similarity in the phylogene.

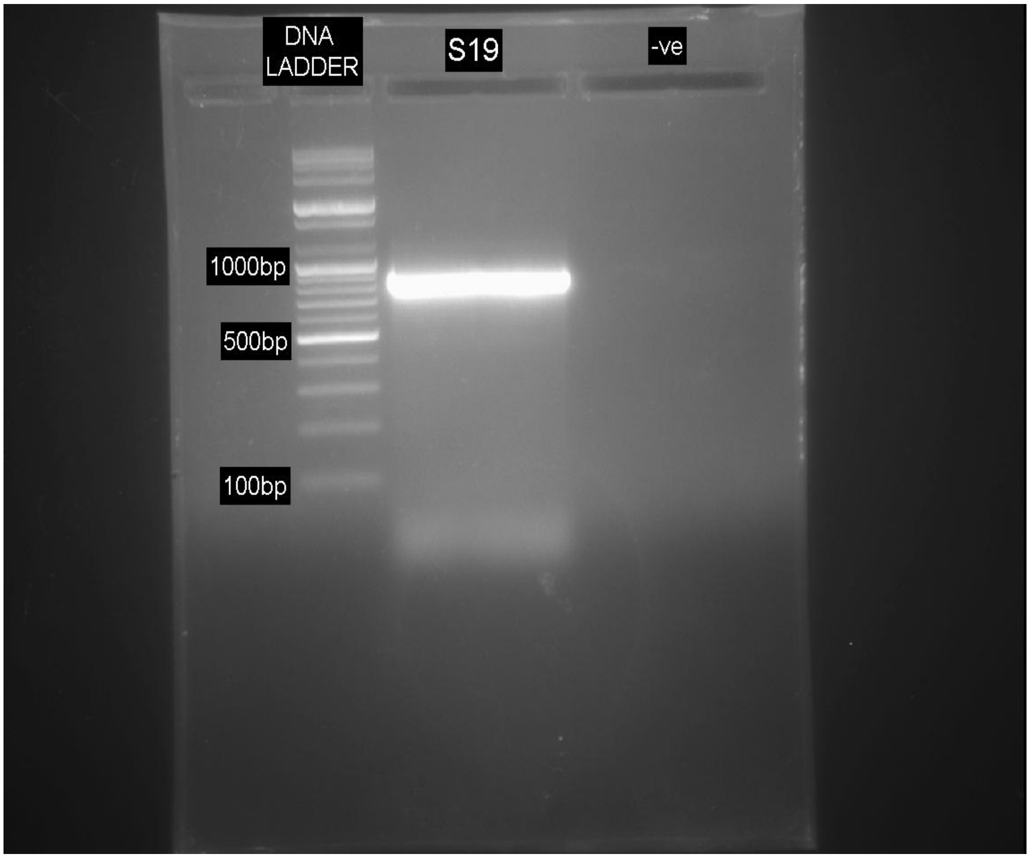

Plate 4.1. Electrophoretogram of the amplified genomic DNA from *Bacillus* sp. viewed on a UV trans-illuminator

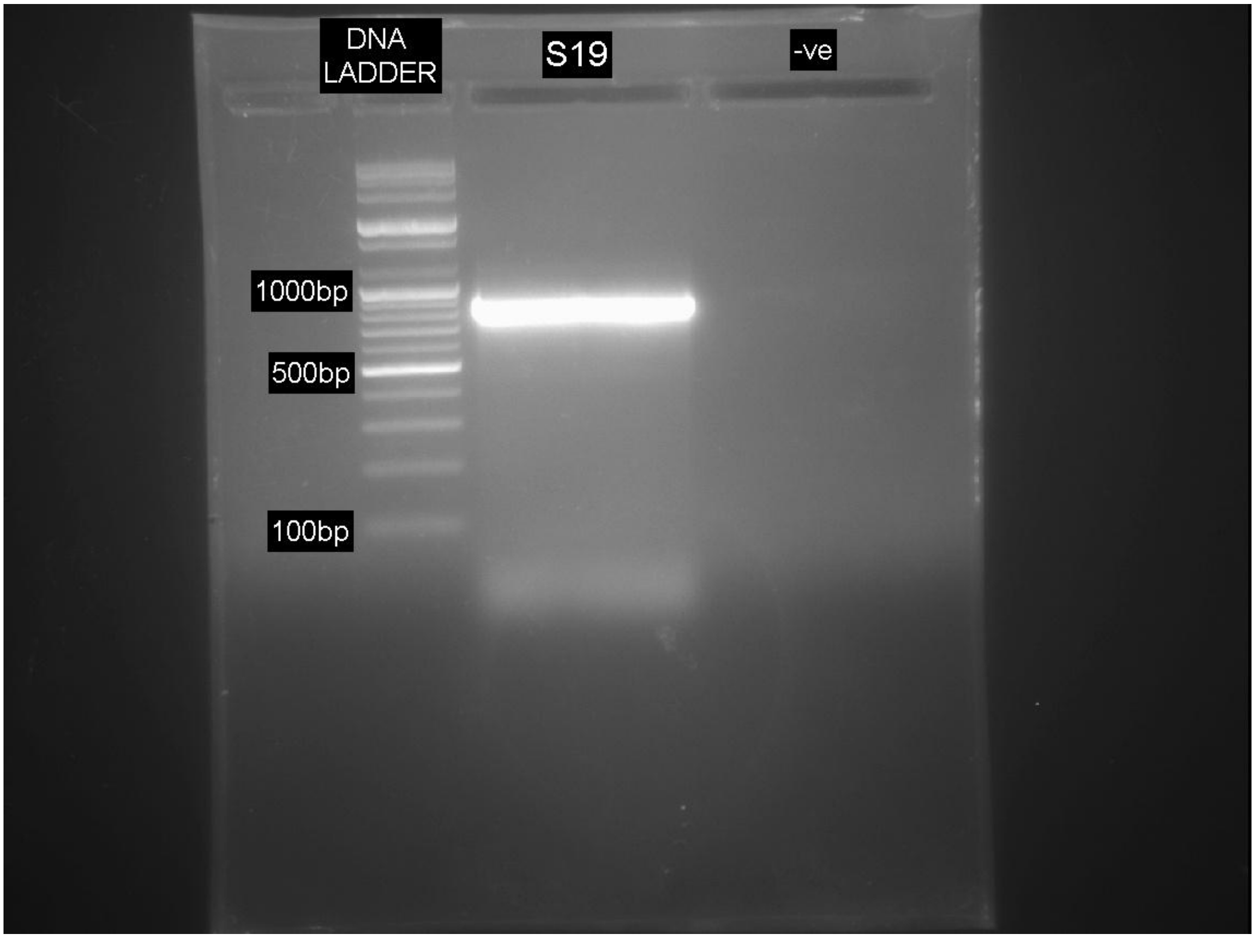

Plate4.2. Electrophoretogram of the amplified genomic DNA from *Pseudomonas* sp. viewed on a UV trans-illuminator.

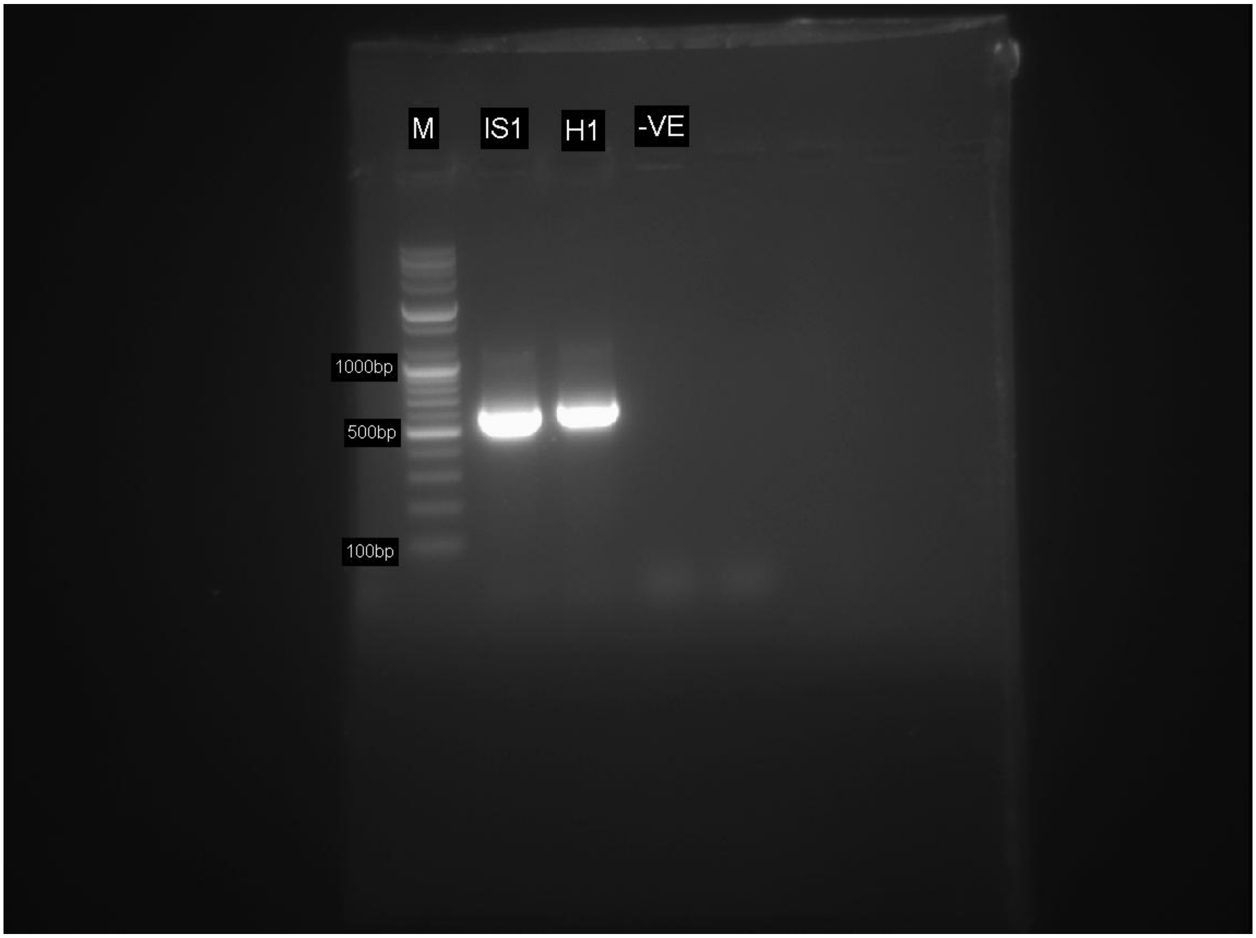

Plate 4.3. Electrophoretogram of the amplified genomic DNA from *Streptomyce* sp. viewed on a UV trans-illuminator

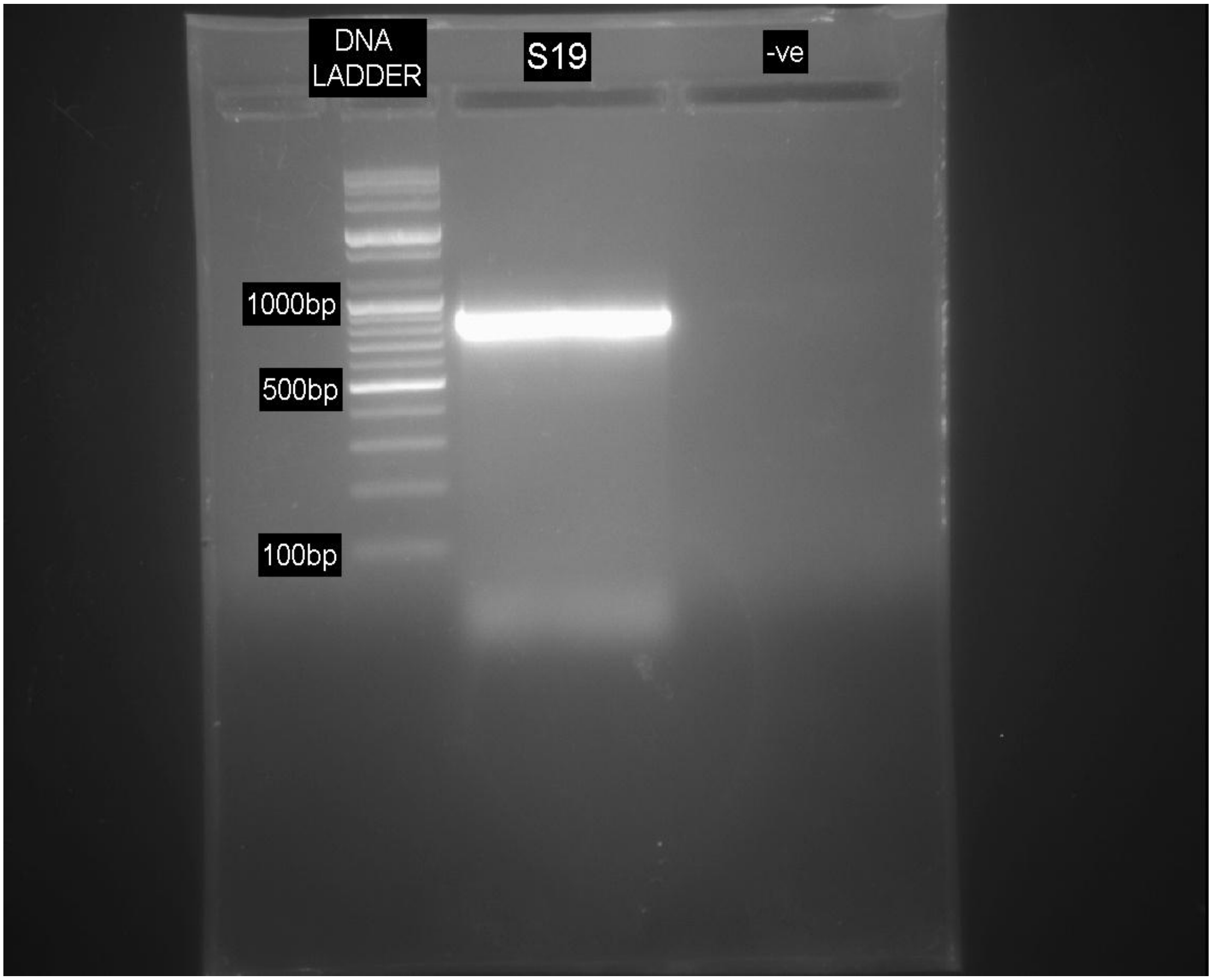

Plate4.4 Electrophoretogram of the amplified genomic DNA from *Klebsiella* sp. viewed on a UV trans-illuminator.

Figure 4.14.Geneomic pattern of strains of *Bacillus substilis* obtained using NCBI BLAST tools

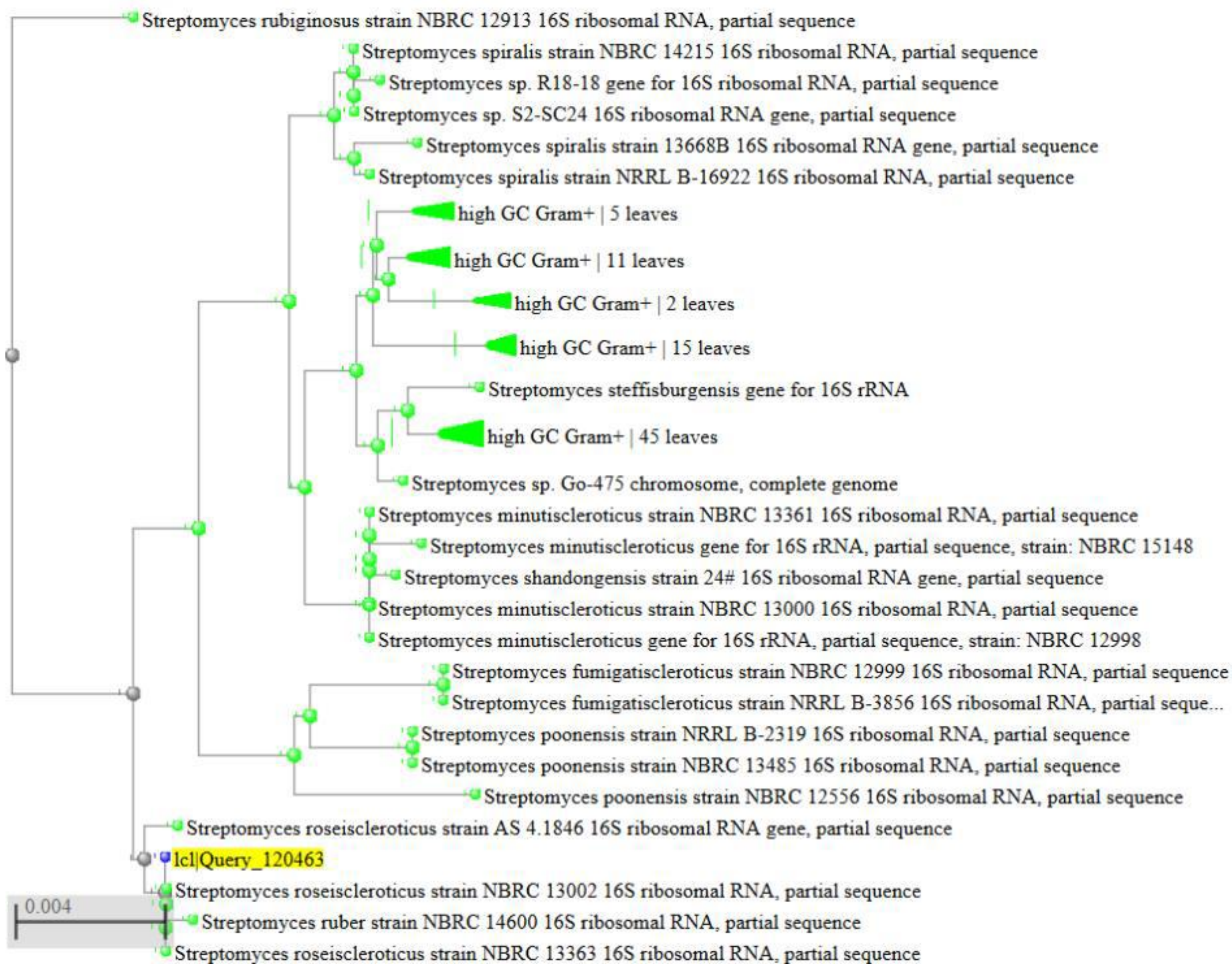

Figure 4.15.Geneomic pattern of strains of *Streptomyce roseisclerotus* obtained using NCBI BLAST tools

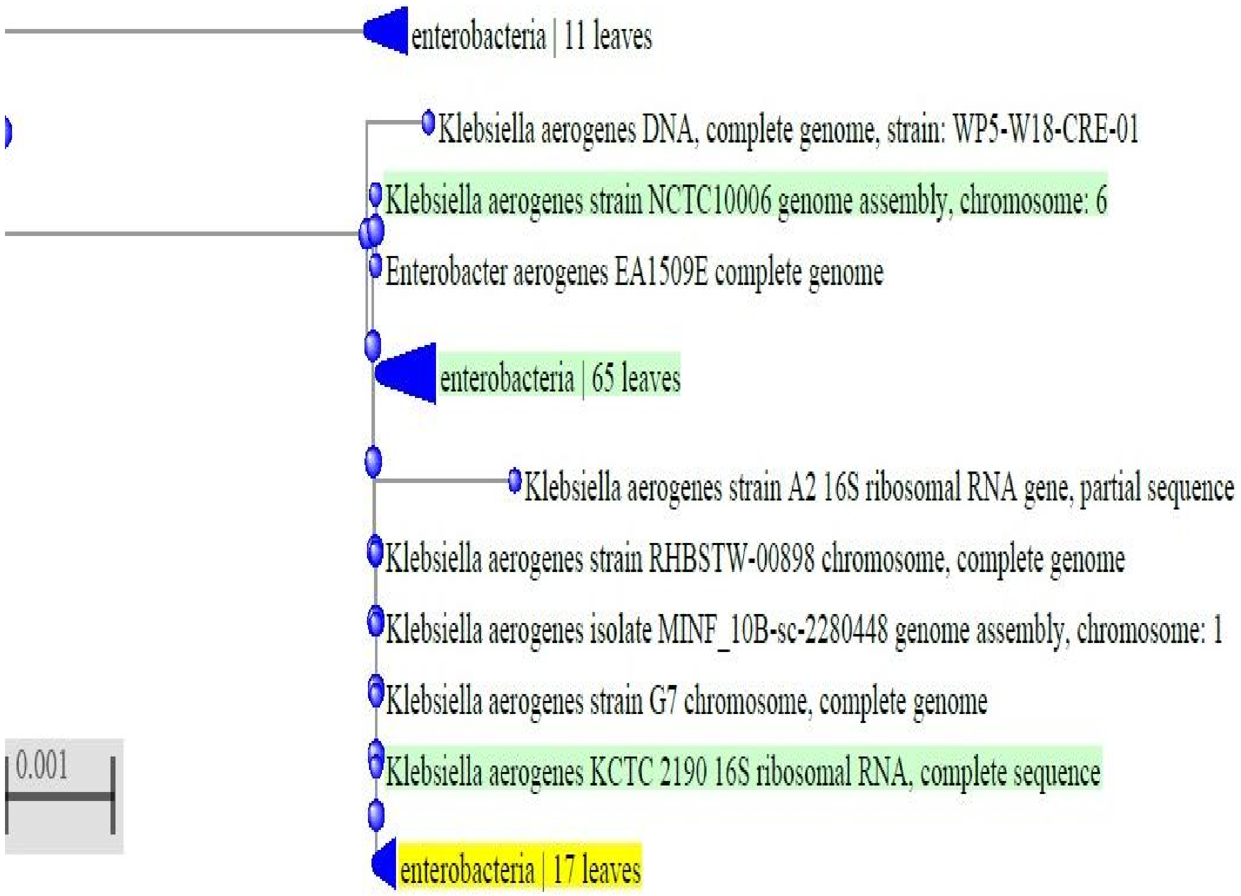

Figure 4.16Geneomic pattern of strains of *Klebsiella aerogene* obtained using NCBI BLAST tools.

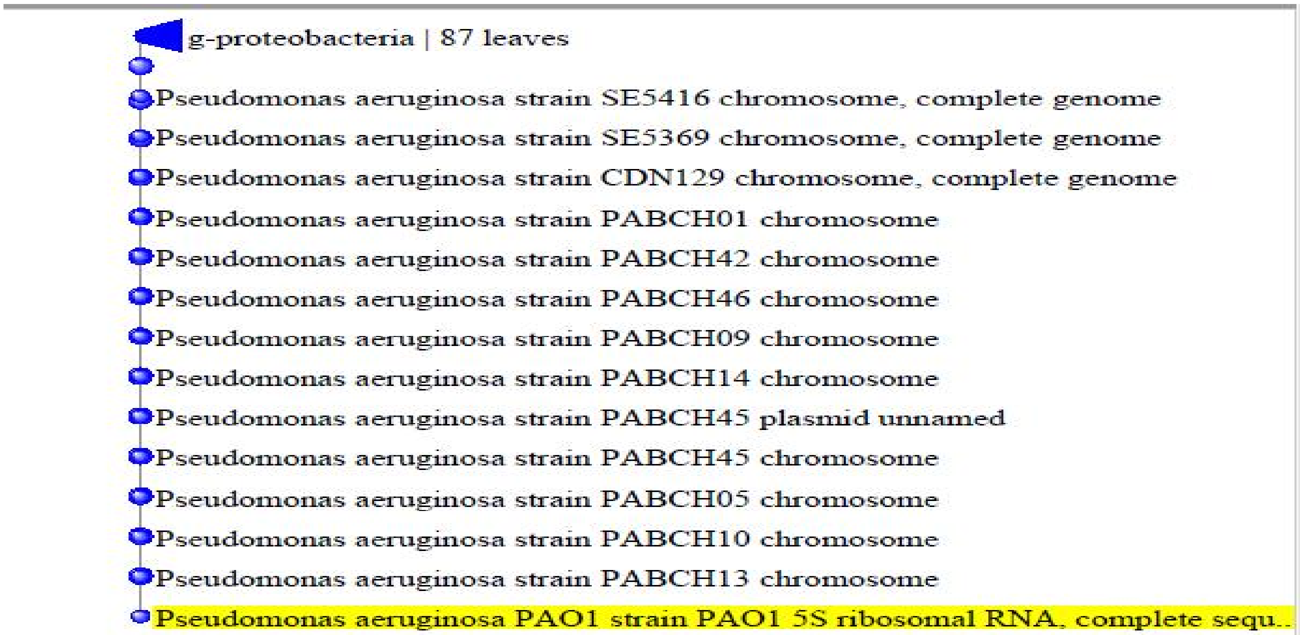

Figure 4.17.Geneomic pattern of *Pseudomonas aeruginosa* obtained using NCBI BLAST tools.

4.1.6 Spectroscopy Studies

FTIR spectroscopy results of soil samples contaminated with Onne, Escravos and Nembe respectively as shown in the figures below. Figures 4.18 and 4.19 showed a characteristic absorption band of phenol, carbon catenation with the families of alkanone and amine. Fig 4.20 describes the attraction sequences of anti-symmetric stretching of the C-O-C bridge, skeletal vibrations involving the C-O stretching are characteristic of its saccharide structure.

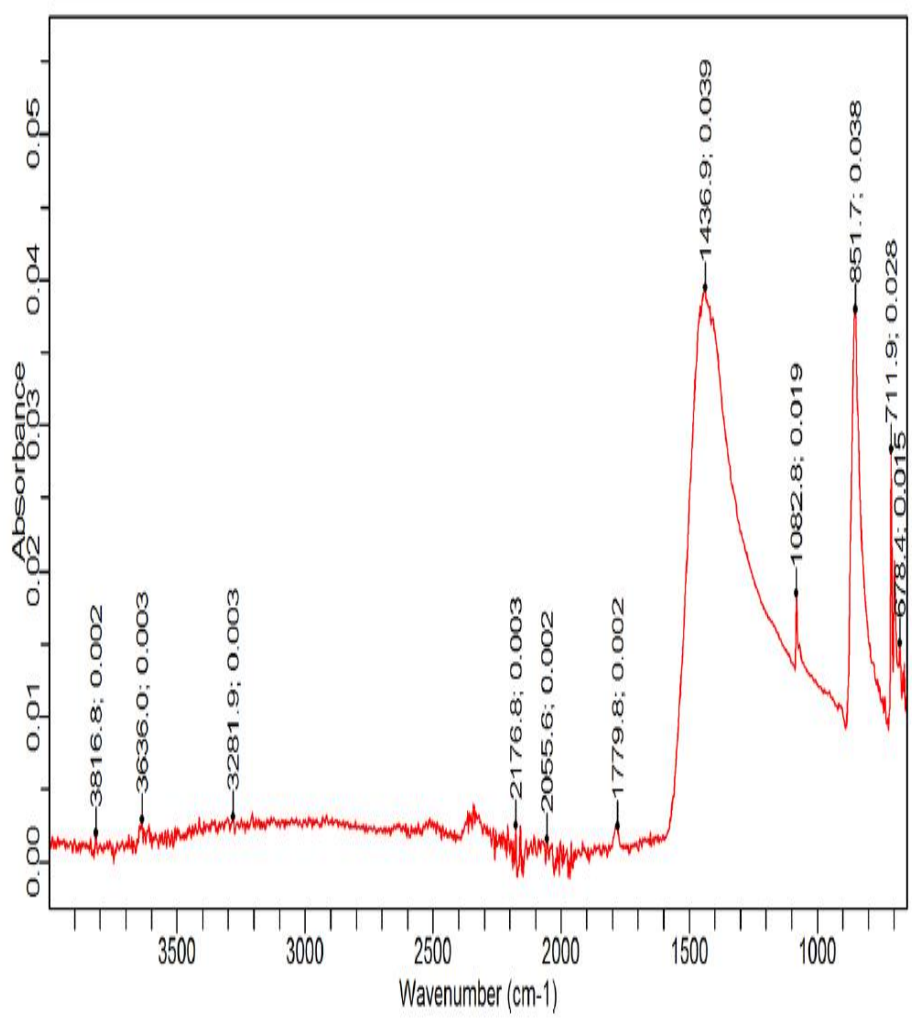

Fig. 4.18 FTIR spectroscopy analysis of microbial emulsion produced from the microbial broth supplemented with petroleum effluents

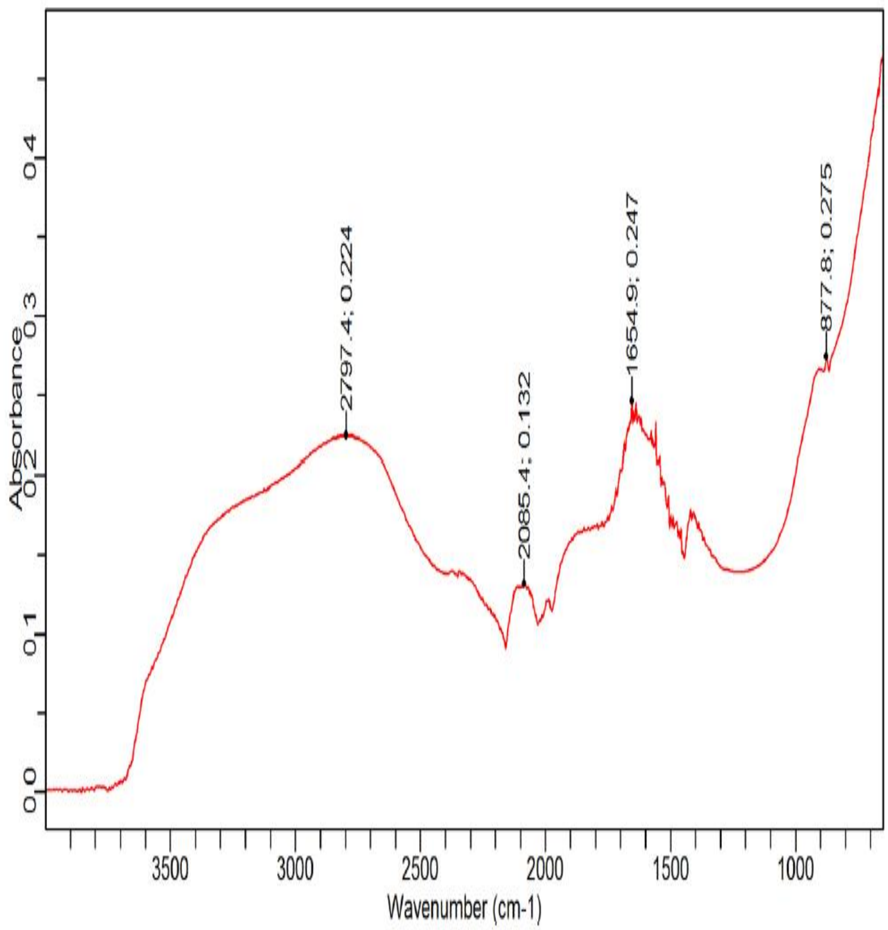

Fig. 4.19 FTIR spectroscopy analysis of microbial emulsion produced from the microbial broth supplemented with coconut oil.

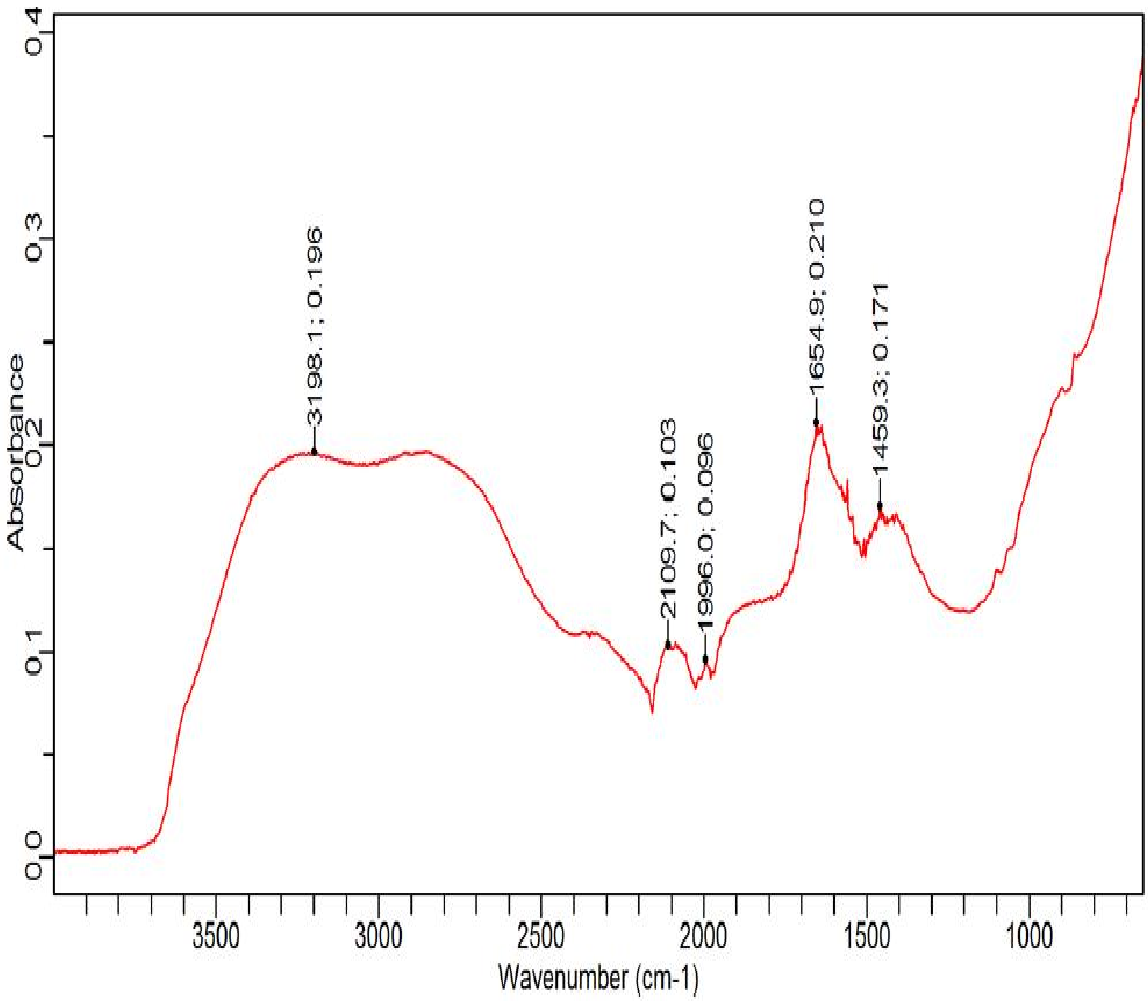

Fig. 4.20 FTIR spectroscopy analysis of microbial emulsion produced from the microbial broth supplemented with kerosene.

4.2 Discussion

According to Allaiges *et al*. (2006), the biggest contamination issue is oil pollution. These oil spills have the potential to seriously harm creatures on land, at sea, and along the shoreline (Pradecpt et al., 2011). The main sources of pollution are service stations, garages, scrap yards, waste treatment facilities, sawmills, and wood impregnation plants (Pradecpt *et al*., 2011). Physical-chemical testing of the soil from the shorelines of Onne, Escravos, and Nembe contaminated with 10% petroleum effluent revealed pH values of 4.24, 6.3, and 5.87, respectively, while the control experiment maintained a pH of 7.6 throughout the experiment. There was no discernible difference in the pH of the polluted soil at 50 and 100% of the effluent contaminations, respectively. This is explained by the type of soil pollutant present, which includes oil and other acidic substances (oleic, benzoic acids) as reported by ASTDR (2009). Soil conductivity of soils contaminated with effluents from Onne, Escravos and Nembe showed a progressive increase of soil conductivity as effluent concentration increased with effluent from Escravos showing the highest impact on soil conductivity.

The following list of dissolved mineral contents was discovered Cl > Ca > Cu > Fe > Mg > K > PO3 > Pb, in that order, while Hg, As, and Cd were found in soil samples from both polluted and uncontaminated areas at concentrations below the detectable limit (BDL). The concentration of Cl, Ca, Mg, K, PO3, and Pb increases when the concentration of pollutants increases from 0 to 100%, with the exception of Fe, which showed a downward trend as the petroleum effluent concentrations increased. Petroleum effluents, in accordance with Vallerro (2010), comprise a variety of minerals that impound in any receiving body and improve its physicochemical properties. The concentration of iron has been steadily decreasing due to the existence of masking chemicals like oxygen, sulfur, and other ligands that compete with iron and decrease its bioavailability. Organic matter in total (TOM) and carbon in all forms (TOC) contents were recorded as 196.73 and 78.64 with 10% Onne effluents, progressing as the concentration of effluents rises. The contents of total organic matter (TOM) and total organic carbon (TOC) were discovered at 119.04, 146.42; 112.41 and 138.26 mg/g with 10 % effluents from Escravos and Nembe respectively. This progresses as the effluents concentration increases. Chikere *et al.* (2006), reported a similar association of ions concentrations in the contaminated Eleme port soil that was discovered in a research at the Eleme petrochemical jetting port site. They showed a greater concentration of the mineral ions in the following order: K, nitrate, magnesium, and chloride ions, 2.28, 1.84, 5.22, and 1789.22 mg/g, respectively.

In their study on soil pollution and Lead (Pb) accumulation, Khalid *et al*. (2017) discovered increased concentrations of heavy metals including Fe, Pb, and Cu in the soil while heavy metals like Hg, As, and Cd were identified in the soil below detectable levels.

The main effects of crude oil spills are caused by total petroleum hydrocarbon, which represents all of the petroleum's poly aliphatic and polycyclic aromatic hydrocarbon (PAH) constituents. The total petroleum content (TPH) of the polluted soil with 10 % effluents from Onne, Escravos and Nembe were 1014.93, 1139.29 and 1102.34 mg/g respectively. The TPH contents increases as the concentration of the effluents increases from 0-100 %. Escravos light implicated with soil contamination showed the highest total petroleum hydrocarbon content with respect to Nembe and Onne contaminated soil each. There is a regressive correlation of the present study to the reference experiment.

Petroleum and its products have been considered as the major source of PAHs that may be absorbed by soil particles due to their high hydrophobicity and thus replace water molecules, reducing the oxygen and water infiltration in the petroleum-polluted soil (Sakshi and Haritash 2019). According to Terytze et al. (1995), PAHs arealso characterized by a strong sorption affinity to soil organic matter. Furthermore, soil geotechnical characteristics may be affected by hydrocarbon contamination, such as permeability, hydraulic conductivity, and compaction, as well as the biological properties (biomass and enzymes) of the soil matrix (Zahermand *et al*. 2020). Petroleum polluted areas are characterized by a lower self-purification capacity that reduces the indigenous microbes involved in soil purification processes (Hreniuc *et al*. 2015).

Enzymes are proteins responsible for various biochemical activities within a biota. They catalyze the significant reduction of energy barrier in every reaction pathways so as to attain the reaction complex within shorter time (Anosike, 2002). Analysis of soil marker enzymes: lipase, manganese peroxidase, and catalase in all the contaminated soil revealed a significant activity of the enzymes in all the contaminated soil at the concentration quotients. Enzyme activity relatively decreases as the contaminant concentrations increases from 0-100 v/w. Lipases are responsible for hydrolysis of fatty acyl esters in a long chain fatty acids to water and alcohol base while the family of peroxidases (peroxidase, manganese peroxidase and catalase) are housekeeping enzymes responsible for dismutation of peroxide and other superoxide to water and release of atmospheric oxygen. Lipases activity was seen relatively low in all the soil at low concentrations of the effluents (0-50 v/w). At high concentrations of the effluents, there was a significant increase in the enzyme activity.

Peroxidase and catalase activity was significant at ambient effluent concentrations but was attenuated at high treatment of the soil with effluent from petroleum hydrocarbon. Peroxidase showed significant activity in all the sampled soils (Escravos, Onne, and Nembe). It was so seen from the result that enzyme activities assayed were relatively high when compared with the control experiment. Escravos and Onne showed marker enzymes with peak activities when compared with enzymes from Nembe. Ezenwelu, Aribodor, Ezeonyejiaku, Okafor, and Oparaji (2022) reported reversibly to the findings of the present study, they stated an optimum activity of lipase produced from strains of *Aspergillus* from a refuse dump site. They went further to state that lipase a catabolically induced with the presence of oil implicated recalcitrant and can only be attenuated with the oily deposits are of composite heavy metals.

Soil organisms are responsible for numerous biogeochemical and metabolic processes in the soil. They serve as important markers of mineral input and outflow in the soil. Analysis of soil microorganisms in soil samples contaminated with varying amounts of petroleum effluent in the experiment revealed a significant decline in soil organism as effluent concentrations rose. However, the multiplication of the organisms in the polluted soil as petroleum effluent increased became noticeably from day 0 to day 7, although it decreased from day 14 to day 21 of incubation. Nembe soil had a low level of microbial activity. Physicochemical components of soil, such as those of soil structures, were reported by Ezeonu *et al.* (2013) and Adieze *et al* (2004). The soil's texture has an impact on the organisms that live there and how they multiply and double. Alrumman *et al*. (2015) had earlier reported that oil contamination in the soil matrix influences soil enzymatic activities and microbial biomass carbon. Also, certain essential soil functions may be lost due to the high toxicity of such persistent aromatic hydrocarbon structures (Khomarbaghi *et al*. 2019).).

Petroleum-contaminated areas suffer tremendously from the drastic impact of the toxicity as well as the concentration induced by PAHs' high molecular weight. Klamerus-Iwan *et al*. (2015)

demonstrated a significant decline of microbial biomass and enzymatic activities (urease and dehydrogenase) in soil polluted by chainsaw oil.

Several researchers have reported the potential of bacteria to degrade low molecular weight PAH structures ($<C_3$) (Xu *et al.* 2021). However, other studies had also revealed the persistence of higher molecular weight hydrocarbons (C_{20}-icosane) after the bacterial degradation of petroleum hydrocarbons. Biodegradation processes can occur aerobically and anaerobically to convert PAHs into CO_2 and H_2O.

The most common bacterial species that have been involved in PAHs degradation processes are; *Alcaligens denitrificans, Acinetobacter calcoaceticus, Pseudomonas putida, Mycobacterium sp., Pseudomonas fluorescens, Corynebacterium renale, Pseudomonas cepacia, Rhodococcus sp., Pseudomonas vesicularis, Moraxella* sp., *Bacillus cereus,* Beijerinckia sp., *Micrococcus* sp., *Pseudomonas paucimobilis*, and *Sphingomonas* sp. (Lu *et al*. 2019; Khomarbaghi *et al*. 2019)

The isolation of high number of certain oil-degrading microorganism from an environment is commonly taken as evidence that those microorganisms are the most active oil degraders of the environment (Atlas and Bartha, 1998). Results from the emulsification assays carried out on the isolated bacteria, (five out the seven (7) isolates) showed higher emulsification potentials during the study. Emulsification assayS carried out which included drop collapse, oil spread plate and emulsification index test on the isolates using petroleum effluent and diesel oil as the sole carbon source showed the following results: Using drop collapse assay as described by Jain *et al.,* (1991), two out of the seven isolates from the contaminated soil scored higher (++) and (+++) in drop collapse assay and also in oil displacement assay using petroleum effluent and diesel oil as sole carbon sources. Culture suspensions on petroleum effluent showed positive correlation when compared to assay outcome with diesel oil but significantly showed a high emulsification than the control experiments without the culture cells.

Drops of cell free culture from *Vibrio* sp. remained intact on glass slide coated with petroleum effluent and diesel after one hour. This isolate is considered non biosurfactant producer. They may have utilized hydrocarbons to produce other metabolites such as bioactive compounds, enzymes etc. The same trend was also seen during the oil spread plate assay on the isolated bacteria. Strains of *Bacillus*, *Klebsiella* and *Pseudomonas* sp. showed the highest in oil displacement. Also as stated afore oil such as petroleum effluent and automated gas oil showed much promising emulsifying feature with the identified organisms. Okpokwasili and Amanchukwu (1996) reports on the biodegradation of crude oil with strains of *Candida* sp. stated that crude oil especially that of Bonny light are complex mixtures of hydrocarbons comprising of PAHs, aliphatic hydrocarbons and centrally total petroleum hydrocarbons (TPH) which will take on a hydrocarbonolistic organism(s) to act on and utilize.

Also *E. coli* showed no positive reaction upon immersion on the oil water interface in all the used oil. This according to Mbachu *et al.* (2016) reported that strains of imperfect bacteria such as the *Actinomycetes* have much compromised ability to produce surface active compounds. Upon assessment on isolates from contaminated water; similar trend of emulsification capacity were obtained as that of the isolates from contaminated soil. Out of the seven bacterial isolates, only three showed greater emulsification potentials on all the three emulsification assays carried out which include drop collapse, oil spread plate and emulsification index assays.

Strains of *Bacillus*, *Pseudomonas, Streptomyces* and *Klebsiella* sp. showed the highest emulsification in drop collapse and oil spread plate assay. Strains of *Salmonella* showed the least among all the isolates in the-afore tested emulsification assays. Emulsification studies by crude cultures of the isolates from the contaminated soil showed that *Klebsiella* sp. showed the best emulsification activity with 48.9% and 51.2, emulsification index respectively using petroleum effluent and diesel oil respectively. This was followed by *Pseudomonas* sp. with, 45.6% and 48% emulsification activity for petroleum effluent and diesel oil as sole carbon source. Strains of *Streptomyces* sp. showed relatively high emulsification index in all the three tested oil. This when compared with the control experiment showed a sharp significant variation with preparations without the cell suspensions that showed emulsification activity of 12%, 16% and 8% indexes with kerosene, coconut oil and crude oil respectively. Vallero (2010) reported that emulsifying organisms (bacteria) are very delicate hydrocarbonolistic organisms that forms close range partition coefficient with hydrocarbons such as petroleum and alikes. They are of high utility in oil cleaning and production of surfactants.

Molecular analysis of the strains with appreciable emulsification index using both culture dependent and independent techniques respectively showed the typical of electrophoretogram of a bacterial strain after DNA extraction from their 16s. base pairs (bp) from the ladder sequence showed the following: 690, 750, 700 and 800 bp when viewed under the trans-illuminance machine. Amplicons taken from the organisms for sequencing showed bands of nucleotide base pairs in accompanying others of their pairing. Phylogenic analysis of the codons showed the following: *Pseudomonas aeruginosa* with accession code of NR_075116.1;

Bacillus substilis with accession code of MN421487; *Klebsiella aerogenes* with accession code of NR_102493.2 and *Streptomyces roseiscleroticus* with accession number of NR_112381, produced the highest emulsions. Fourier transform analysis of the emulsion formed by the organism in the presence of the various oils after 32 scans and resolution of four (4) analyses showed characteristic bands such as: amide I band is split into three components at 2527.1 cm^{-1} , 2206.6 cm^{-1} and 1785.4 cm^{-1} while amide II band is observed at 14481.4 cm^{-1}. The CH deformation of the β-glycosidic bond is centered at 853.6 cm^{-1} and 711.9 cm^{-1}. Antonino *et al*. (2017) reported a double amide splitting of amide I bond at 1680 cm^{-1} and 1630 cm^{-1} while amide bond II was seen at 1535 cm^{-1} and the rest of the saccharide (CH deformation) was at 895 cm^{-1} for biosurfactant produced from dicultures of *Klebsiella* and *Bacillus* sp.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

The present study has presented the risk index of crude oil polluted soil using indices of stress marker enzymes, impacted physicochemical changes and soil organismal diversities. Physicochemical analysis of the petroleum polluted soils in the presence of the control experiment revealed the equivalence of certain hazardous entities in an off threshold numerical. The biomarker enzymes showed that the activities of soil enzymes were affected at different concentrations of the petroleum hydrocarbon concentration. Further studies on proliferation of soil microorganisms showed evidence of heterotrophic counts of organisms in the soil decreasing as the effluent concentration increased and the emulsification potentials of bacterial isolates from the soil showed the presence of emulsifying bacteria within the assayed organisms in the presence of various experimented oils.

5.2 Recommendation

For further understanding of the ecological and toxicological impact assessment studies on petroleum hydrocarbons in the soil, its revealing impact on certain natural markers of terrestrial habitats, interests should be channeled into the following:

1. Life cycle assessment of the petroleum hydrocarbon effluent on the agricultural soil.
2. Assessment of the impact seasonally.
3. Clinical studies of the cultivated plants in the respective area and its assessment on certain biochemical parameters

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APPENDIX

Appendix I

Reagents and equipments from the respective companies were used during the present study and they include: Merck (Germany), British Drug House Chemicals Limited Poole (England), sigma Aldrich, Puroerba, respectively.

Appendix II Bacteria culture plates

Bacteria isolates on nutrient media.

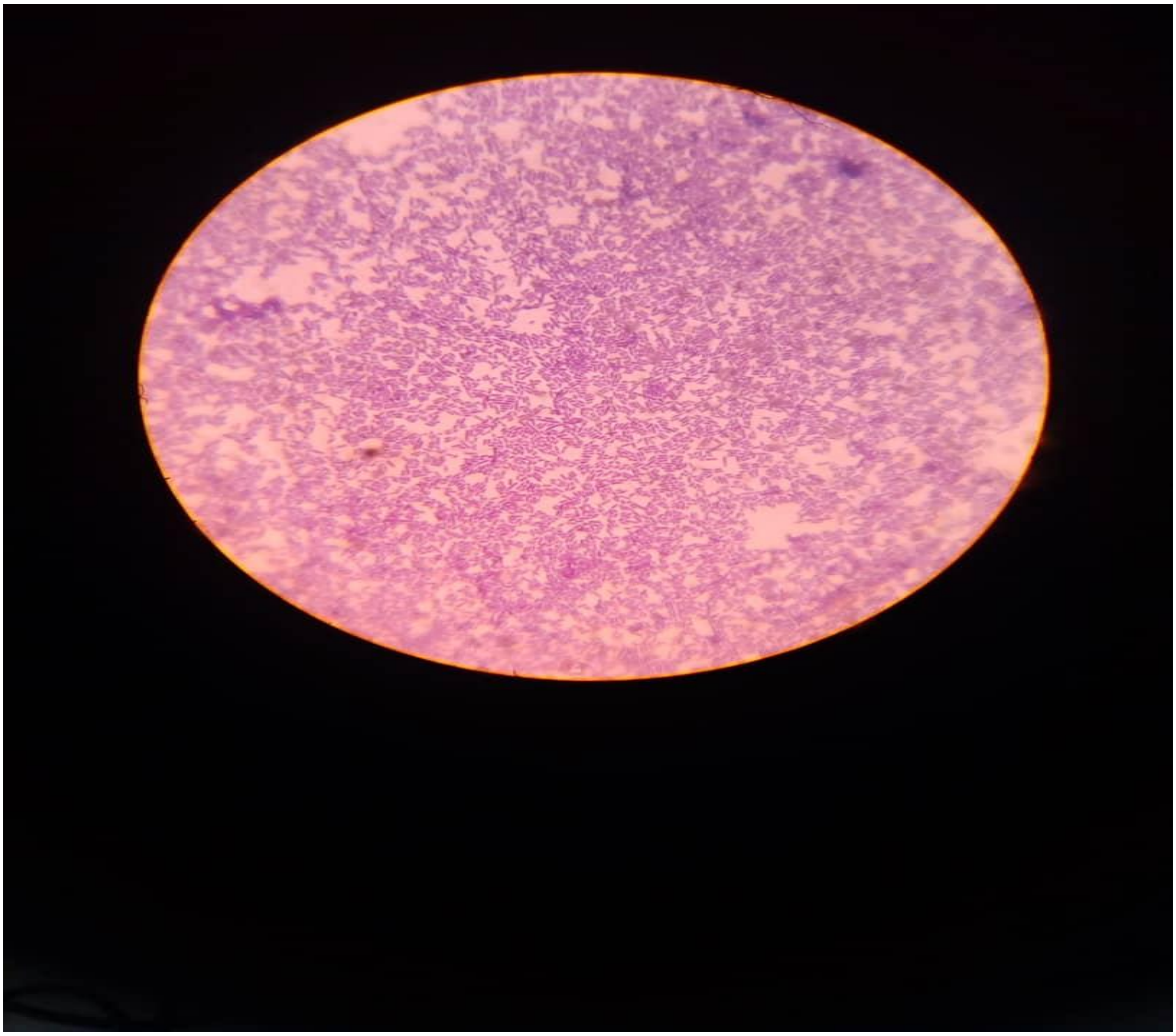


Plate 4.5: micrograph of strains of *Klebsiella* sp under magnification x 100.

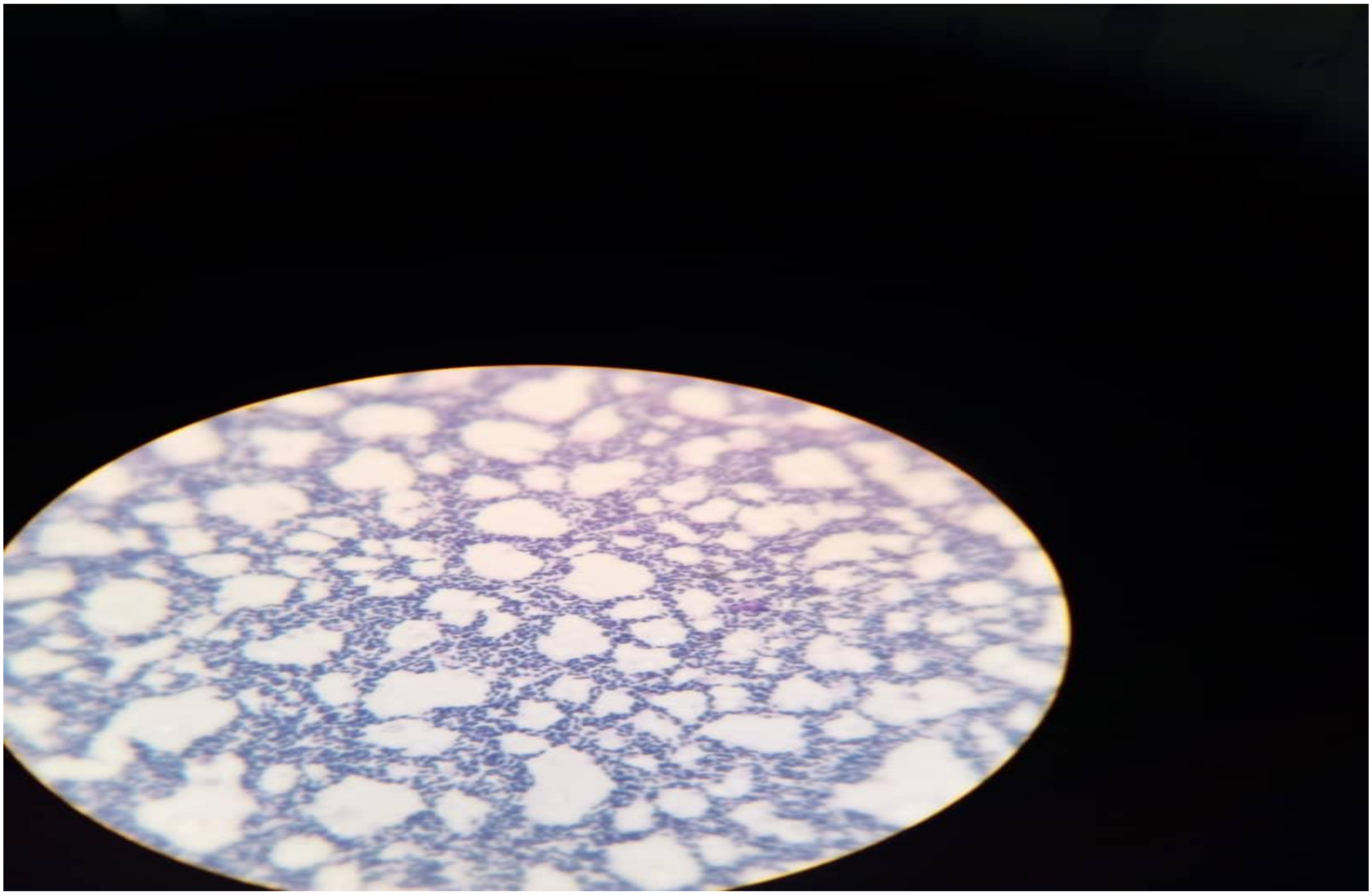


Plate 4.6:Micrograph of strains of *Bacillus* under x100 magnification.

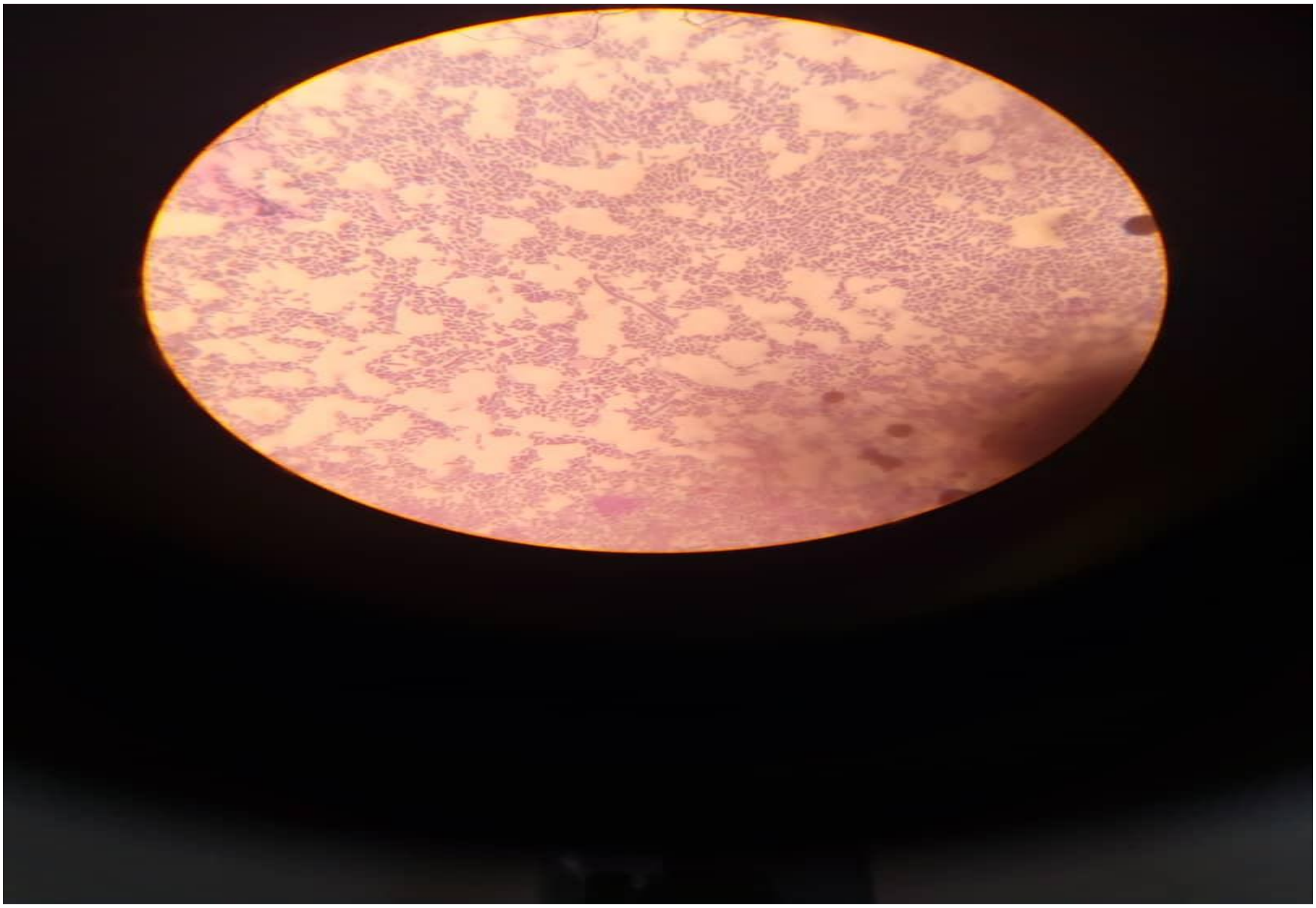
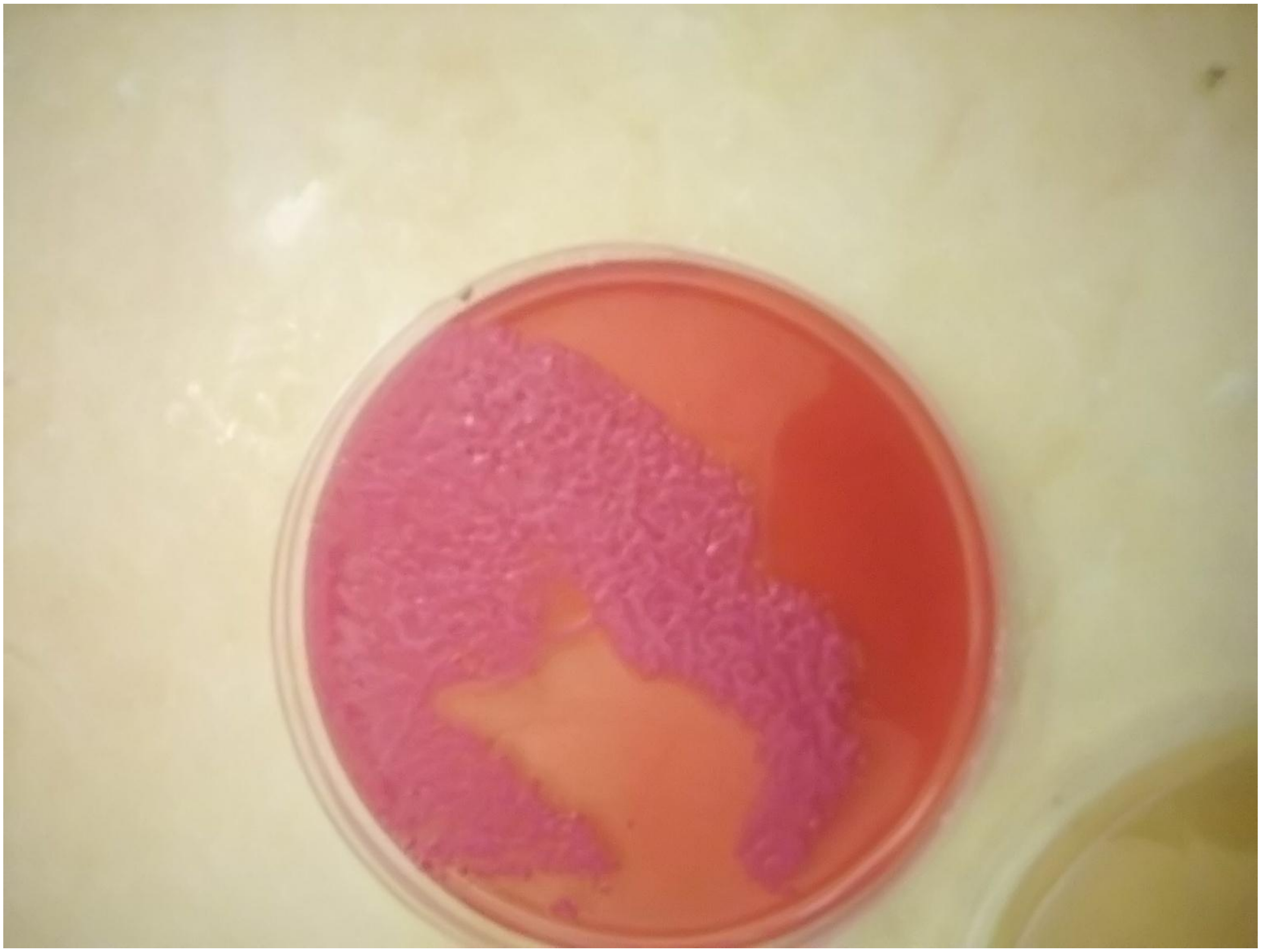
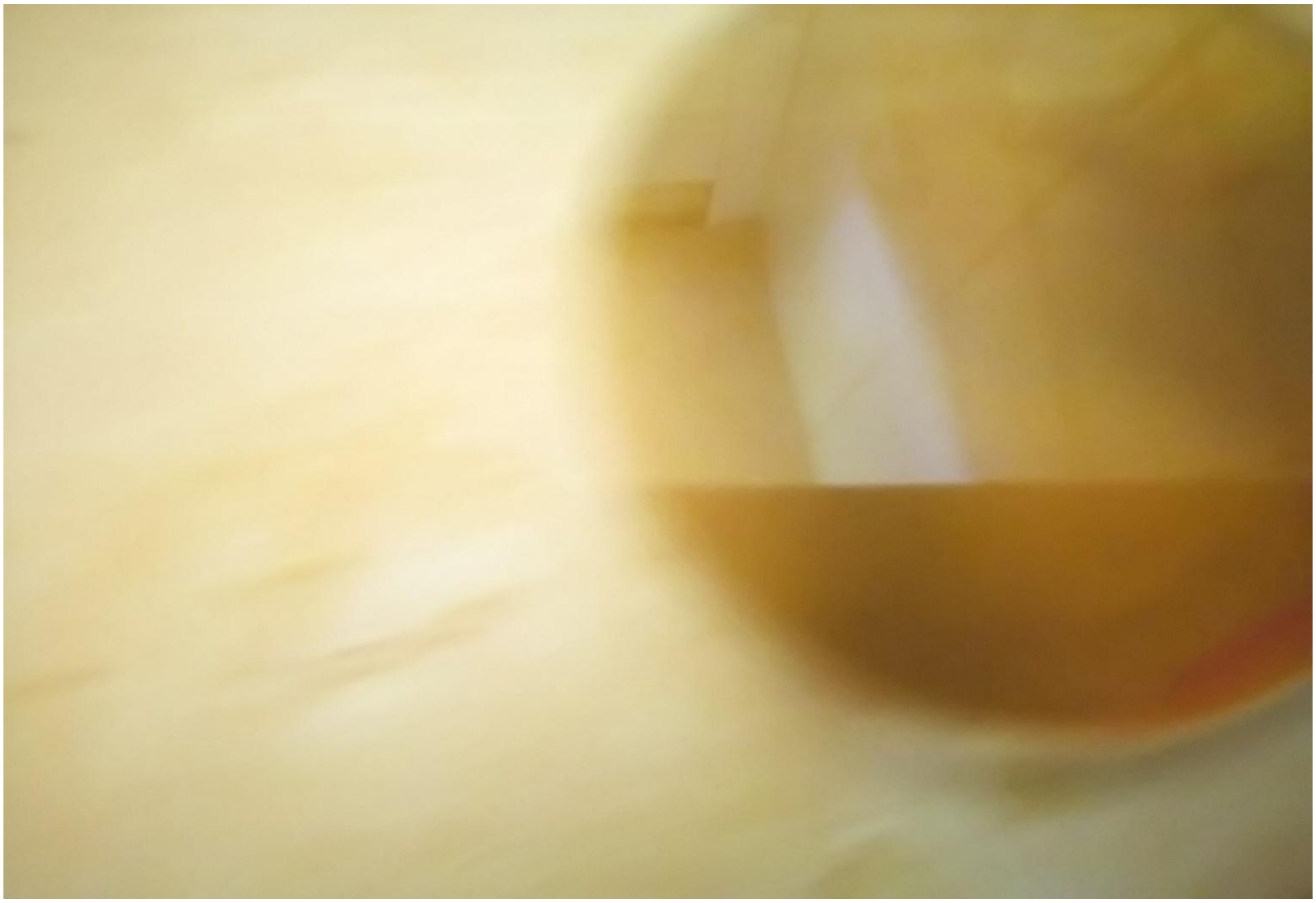


Plate 4.7: Micrograph of strains of Streptomyce under objectives of x 100 magnification.

Plate 4.8: Micrograph of strains of Streptomyce under objectives of x 100 magnification.



Bacteria isolates on Mackonkey media.



Bacteria isolates on Bush-Nell-Hass media.

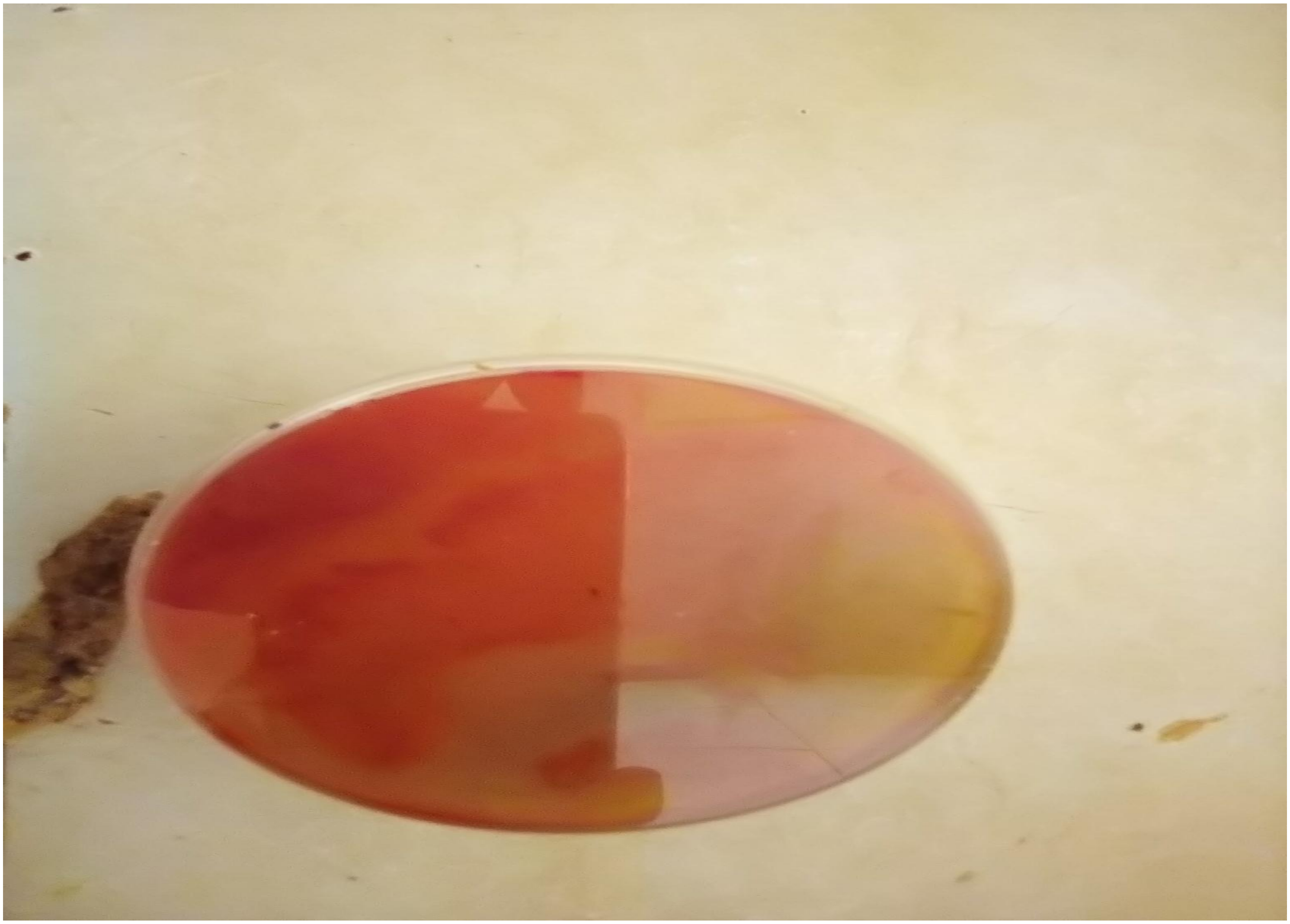


Plate 4.9 strains of the bacteria isolates on Bush-Nell-Hass media