DISTRIBUTION, CHARACTERIZATION AND HYDROCARBON–DEGRADING POTENTIALS OF MICROORGANISMS IN SOME PARTS OF BIDA BASIN, NIGERIA

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ABSTRACT

The distribution and characterization of a suspected petroleum bearing site (SPBS) is generally called microbial prospecting. Microbial surface prospecting is a vital process in the exploration of hydrocarbon suspected site. This study was aimed at assessing the distribution, characterization and hydrocarbon degrading potentials of microorganisms in some parts of Bida Basin, Nigeria. The study assessed the physicochemical and microbiological properties of soil and water from Ahoko, Kogi State, and Patishabakolo, Niger state, Nigeria, to ascertain the potent site for exploration using the presence of microbial anomalies. Standard plate count, ArcGIS, gravimetric and GC-MS methods were used to ascertain the total bacterial and total fungal counts, distribution of specific hydrocarbon–oxidizing microbes and hydrocarbon-degrading potential of isolates respectively. Physicochemical properties showed that pH of the soil samples were within the range of 5.58 – 7.05. Site A had the highest total organic carbon (TOC) (1.18%), conductivity (12.0 μʃ/cm), available Phosphorus (12.06 mg/kg), base saturation (66.03 ± 0.00%) and exchangeable acid (2.28 cmol/kg) compared to other sites in Ahoko. The physicochemical parameters such as pH, TOC, conductivity and total pore space in samples from Patishabakolo vary from 5.30-6.81, 1.75%-8.78%, 0.10μʃ-2.0μʃ/cm and 160.8%-192.3% respectively. The study revealed the presence of counts for methane-oxidizing microbes (MOM) (4.8 x 10^2 CFU/mL, 5.0 x 10^2 CFU/mL), ethane-oxidizing microbes (EOM) (4.6 x 10^2 CFU/mL, 10.0 x 10^2 CFU/mL) propane-oxidizing microbes (POM) (3.6 x 10^2 CFU/mL, 10.0 x 10^2 CFU/mL) and Butane oxidizing microbes (BOM) (2.80 x 10^2 CFU/mL, 6.65 x 10^2 CFU/mL) in water samples from Ahoko, and Patishabakolo respectively. There were significant differences ($p<0.05$) in counts of the various groups of bacteria except for BOM. In general, the EOM predominated in all study sites and areas. The EOM was the most widely distributed across all the study locations and there were correlative relationship ($r = 0.9$) between EOM and BOM. The most frequently encountered bacterial and fungal hydrocarbon-gas utilizers were *Corynebacteria, Acinetobacter, Micrococcus* and *A. niger* and *A. flavus* respectively. Ten crude oil–utilizing organisms which included one bacterium and 9 fungi were amplified using using universal primers. The organisms were identified as, *Enterobacter asburiae* RSo-A, *Aspergillus flavus* A-Feb, *A. tubingensis* D2S3, *Alternaria tenuissima* JYW, *A. flavipes* BH *A. terreus* JYS, *Penicillium pimiteouiense* JS-B, *Aspergillus sublatus* D1S4, *Aspergillus stellatus* Borehole, and *Candida orthopsilosis* JS-A. The rate of crude oil degradation by the identified organisms was determined after 35 days of incubation, using gravimetric analysis method. The results revealed that 68.9 %, 73 %, 68.4 %, 62 %, 72.11 %, 77.8 %, 83.68 %, 68.89 %, 88.05 %, and 85 % degradation were respectively achieved by the organisms. Biodegradation potential of isolates were observed by conversion of high molecular weight (HMW) compounds to low molecular weight (LMW) compound, reduction in the height of peaks and the concentrations of carbon content as well as presence and emergence of intermediates compounds. All the isolates used in this study utilized the crude oil luxuriantly but isolates of *C. orthopsilosis* JS-A and *A. stellatus* borehole were the most effective and efficient degraders, achieved 85 % and 88 % rate of degradation and was able to completely remove 9 and 13 carbon compounds from the

crude oil mixture respectively. Hence, they can be used as clean up agents in oil spills in future if oil exploration begins in these zones.

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ABBREVIATIONS

Symbol	Name
As	Arsenic
BOM	Butane oxidizing microbes
Cd	Cadmium
CO_2	Carbon dioxide

CEC	Cation exchange capacity
Cr	Chromium
Cu	Copper
CMC	Critical micelle concentration
Cubic gcm^{-1}	Cubic gram per centimetre
^{o}C	Degree centigrade
DNA	Deoxyribonucleic acid
EC	Electrical conductivity
EOM	Ethane–oxidizing microbes
GCMS	Gas Chromatography and Mass Spectroscopy
g cm^{-3}	Gram per centimetre cube
gL^{-1}	Grams per litre
Pb	Lead
POM	Propane–oxidizing microbe
LPS	Lipopolysaccaride
MEL	Mannosylerythritol lipids
MOM	Methane–oxidizing microbe

N$_2$	Nitrogen
Hg	Mercury
HOF	Hydrocarbon–oxidizing fungi
HOB	Hydrocarbon–oxidizing bacteria
HOM	Hydrocarbon–oxidizing microbe
µ∫cm^{-1}	microSiemens per centimeter
meq	Milliequivalents
mg kg^{-1}	Milligram per kilogram
nm	Nanometre
NESREA	National Environmental Standards and Regulations Enforcement Agency
Ni	Nickel
N	Nitrogen
ppm	Parts per million
%	Percentage
P	Phosphorus
PAHs	Poly aromatic hydrocarbons
K	Potassium

SPBS   Suspected petroleum bearing sites

TPH   Total petroleum hydrocarbon

TOC   Total organic carbon

USEPA   United State Environmental

                                  Protection Agency

WHO   World Health Organisation

CHAPTER ONE

1.0   INTRODUCTION

1.1 Background to the Study

One of the suspected petroleum bearing sites in Nigeria is in the Bida Basin, which is
located in the North Central Region, Nigeria. Ladipo (1998) reported that the Basins

sedimentary fill consists of post-orogenic molasses and slender unfurled marine residue. It is characterised by means of the absence of carbonates, volcanic, and rocks of tertiary ages (Rahaman *et al*., 2019). The basin has an inverted arch due to fracturing that originates close to Santonian orogenic tendencies in South East Nigeria and the Benue valley (Obaje *et al.,* 2011; Usman, 2019).

Petroleum forms through the setting apart of large debris of waxes, oils and fats that associates with the formation of kerogen (Mansoori *et al*., 2016). This system commenced extraordinary a few years back, whilst little marine lifestyles bureaucracy possessed big quantities of the oceans died, they settled at the sea depths and ended up blanketed in layers of dirt, sediment and sand. The non-stop rot via way of means of the effect of heat and strain pressure delivered approximately the improvement of many additives of oil compounds (Mansoori *et al.*, 2016). Petroleum is a liquid, due to that, it could move via the earth as its forms.

Oil has mostly been recouped via means of oil dull that is achieved after investigations of auxiliary topography (on the repository scale), sedimentary bowl examination, and shop portrayal were finished (Guerriero *et al*., 2012). One of the strategies utilized for hydrocarbon investigation, portrayal and distinguishing proof of microorganisms from hydrocarbon presumed site is regularly known as microbial prospecting and it depends on the way vaporous hydrocarbons through emission and diffusion migrate upward from subsurface petroleum accumulations, and are utilized by a variety of microorganisms present in the sub-soil ecosystem (Etiope, 2015; Hubert and Judd, 2020). The methane, ethane, propane, and butane-oxidizing bacteria exclusively use these gases as carbon source for their metabolic activities and growth. These

microorganisms are mostly found enriched in the shallow soils and can differentiate between hydrocarbon prospective and non-prospective areas (Rasheed *et al.,* 2014).

Biodegradation is characterized as the naturally catalyzed decrease in multifaceted nature of substance mixes (Joutey *et al*., 2013). It is the procedures that make use of microbes to breakdown large compounds into smaller mixes (Marinescu *et al.,* 2009). In biodegradation, living things change the substance through metabolic or enzymatic procedures which depends on development and co-digestion (Fritsche and Hofrichter, 2008). A few microorganisms, including parasites, microscopic organisms and yeasts are engaged with biodegradation process. The reports for algae and protozoa are meagre with respect to their association in biodegradation (Das and Chandran, 2011; Joutey *et al.,* 2013). Biodegradation by natural populations of microorganisms represents one of the primary mechanisms by which petroleum and other hydrocarbon pollutants can be removed from the environment and is much less expensive than different remediation innovations (Ulrici *et al.*, 2000; Abioye *et al.,* 2013). Biodegradation of hydrocarbons begins by the conversion of the alkane chain or polycyclic aromatic hydrocarbon (PAH) into an alcohol. The next step in the process is oxidation, which then converts the compound to an aldehyde and thereafter, into an acid and eventually into water, carbon dioxide, and biomass. In the case of the PAH, fission occurs which leads to mineralisation (Shallu *et al.,* 2014). The process of bioremediation, defined as the use of microorganisms to detoxify or remove pollutants owing to their diverse metabolic capabilities is an evolving method for the removal and degradation of many environmental pollutants including the products of petroleum industry, this technology is believed to be non-invasive and relatively cost-effective (Rasheed *et al.,* 2015).

The success of oil spill bioremediation depends on one's ability to establish and maintain conditions that favour enhanced oil biodegradation rates in the contaminated environment. Numerous factors influence the rate of oil biodegradation. One important requirement is the presence of microorganisms with the appropriate metabolic capabilities. If these microorganisms are present, then optimal rates of growth and hydrocarbon biodegradation can be sustained by ensuring that adequate concentrations of nutrients and oxygen are present and that the pH is between 6 and 9. The physical and chemical characteristics of the oil and oil surface area are also important determinants of bioremediation success (Das and Chandran, 2011).

1.2 Statement of the Research Problem

The Bida and Sokoto Basins are the only Basins in the country yet to produce oil blocks for auctioning by the Federal Government. With the increase in demand for petroleum products and diminishing indigenous production, it has become necessary to search for probable potential zones or areas with the aim of expanding the national exploration and production base and adding to the proven reserves. In addition to the search for other potential area for exploration, the problem of hydrocarbon contamination known to be widespread in oil producing areas should not be overlooked.

One of the major environmental problems today is hydrocarbon contamination resulting from the activities related to the petrochemical industry with accidental release of petroleum products being a particular concern in the environment. Hydrocarbon components have been known to belong to the family of carcinogens and neurotoxic organic pollutants (Das and Chandran, 2011). Currently, accepted disposal methods of incineration or burial in secure landfills can become prohibitively expensive when amounts of contaminants are large. Besides, mechanical and chemical methods

generally used to remove hydrocarbons from contaminated sites have limited effectiveness and can be expensive (Malik and Ahmed, 2012; Abioye *et al.,* 2013). Bioremediation is the promising technology for the treatment of these contaminated sites since it is cost-effective and will lead to complete mineralization. Bioremediation functions basically on biodegradation, which may refer to complete mineralization of organic contaminants into carbon dioxide, water, inorganic compounds, and cell protein or transformation of complex organic contaminants to other simpler organic compounds by biological agents like microorganisms (Malik and Ahmed, 2012).

1.3 Justification for the Study

The Bida Basin is one of the inland Basins in Nigeria that is located in the North Central and extends to the West-Central region of Nigeria. Through geological survey, Bida Basin has been suspected to have the potential for an active petroleum system with the generation, migration and accumulation of hydrocarbons in commercial quantities (Obaje *et al.,* 2011). There is therefore the need for microbiological studies to prove that this Basin indeed can be explored for petroleum. Hence this study was directed to identify the presence and distribution of hydrocarbon degrading microorganisms in the basin. Besides, in oil exploration site there is always pollution, if exploration begins in this basin pollution will be inevitable, for effective remediation of oil polluted site, suitable microbial inoculants as clean up agents in oil spill incidences should be formulated. Suitable microbial inoculants or soil microcosm cannot be efficiently formulated if the microorganisms are not characterized and identified (Tharaka *et al*., 2016), therefore characterization of isolates is a very important aspect in order to develop a successful bioremediation strategy for petroleum oil contaminants. In addition, identifying the degradation capacity of these organisms helps in constructing more competent oil degraders for oil spill remediation.

1.4 Aim and Objectives

The aim of the study was to assess the distribution, characterization and hydrocarbon degrading potentials of microorganisms in some parts of Bida Basin, Nigeria.

The objectives of the study were to:

 i. determine the physicochemical and microbiological properties of soil and water sources in the suspected petroleum bearing sites (SPBS)

 ii. characterize and identify the microbial isolates

 iii. study the distribution of the hydrocarbon utilizing microorganisms in the SPBS.

 iv. determine the petroleum biodegradation potential of the microbial isolates

CHAPTER TWO

2.0   **LITERATURE REVIEW**

2.1 Brief History and Characteristics of Bida Basin

The Nupe Basin, also known as the Mid-Niger Basin, is located in north-central Nigeria and is also known as the Bida Basin. In terms of Paleogeography, Stratigraphy, and Hydrocarbon Resource Potentials, it is one of the freshwater sedimentary basins. It is an intra-cratonic sedimentary basin with two sub-segments extending northwest from Kontagora in the north to an area near Lokoja in the south (Abdullahi, 2016).

The remaining absolute magnetic field esteems more than a few areas of the basin revealed a normal depth to the basement rock of 3.41 km thickness sedimentary reaching 4.70 km in the central and southern parts of the basin (Usman *et al*., 2019). The basin is nearly 350 km long and varies in width from 75 km to 150 km (Rahaman *et al*., 2019).

The sand grain located at the basement of the Northern Bida Bowl is seen regularly around through the Enagi, and Batati and Sakpe arrangements, indicating a stratigraphic progression within the basin. The Lokoja association is found at the base of the Southern Bida Bay, and it is closely observed by the Agbaja and Patti associations. Campania is responsible for all of the construction within the basin. The topography and stratigraphic progressions of mineral deposition within the basin highlight the possibility of developing capability oil structures, which typically include the age, migration, generation, and gathering of crudes in large quantities.

Hydrocarbon leakages have been reported near Petegi/Muregi on the Niger River's shore, as well as at Ahoko in Niger and Kogi State respectively, existed in the Bida Basin. The basin's hydrocarbon resources are mostly gaseous, according to geochemical source rock examinations. The areas around Bida and to the south of Bida, such as Agbaja, Baro, Muregi, Pategi, Abaji, GadaBiyu, and Ahoko, are the most planned for hydrocarbon exploration (Figure 2.1).
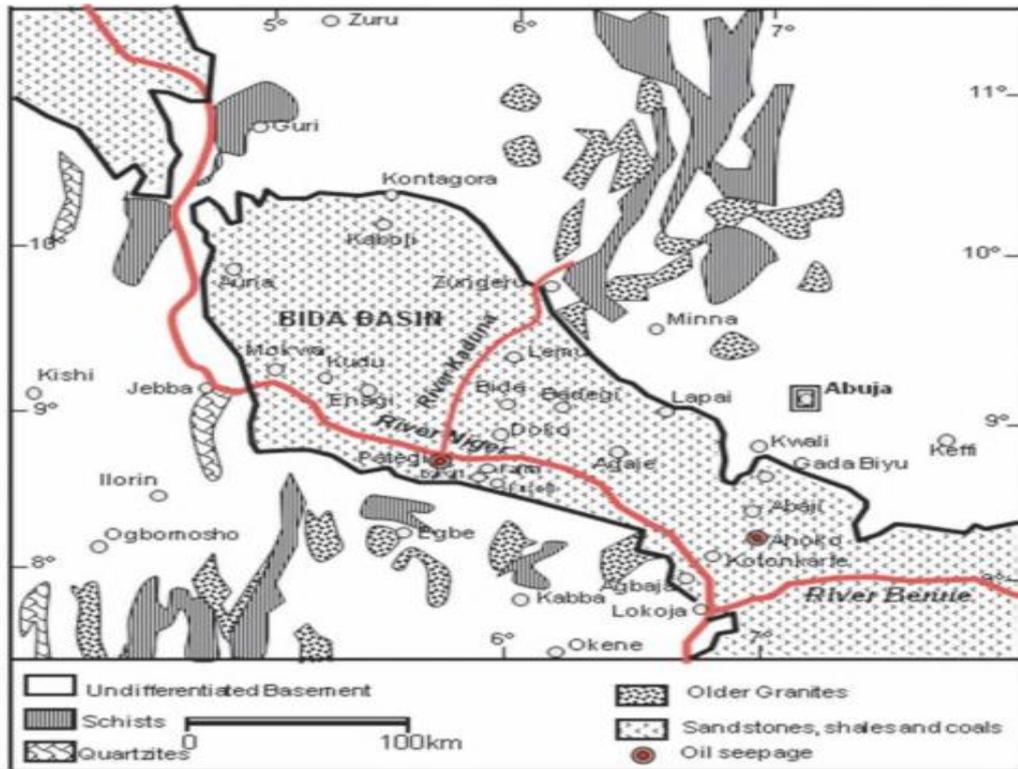
Figure 2.1: Geology and Location of Bida Basin and its environs, showing Ahoko

the study site (Obaje *et al.,* 2011)

The inland basins of Nigeria comprise the Anambra Basin, the Dahomey Basin, the

Lower, Middle and Upper Benue Trough, the Chad Basin, the Bida Basin, and the

Sokoto Basin. However, these inland basins have continued to frustrate the efforts of

many explorers, principally because of the poor knowledge of their geology and the far

distance from existing infrastructure, and for these reasons, many international

companies have turned their focus away from frontier onshore to frontier deep-water

and ultra-deep water offshore. The inland basins of Nigeria constitute one set of a series

of Cretaceous and later rift basins in Central and West Africa whose origin is related to

the opening of the South Atlantic (Figure 2. 2).
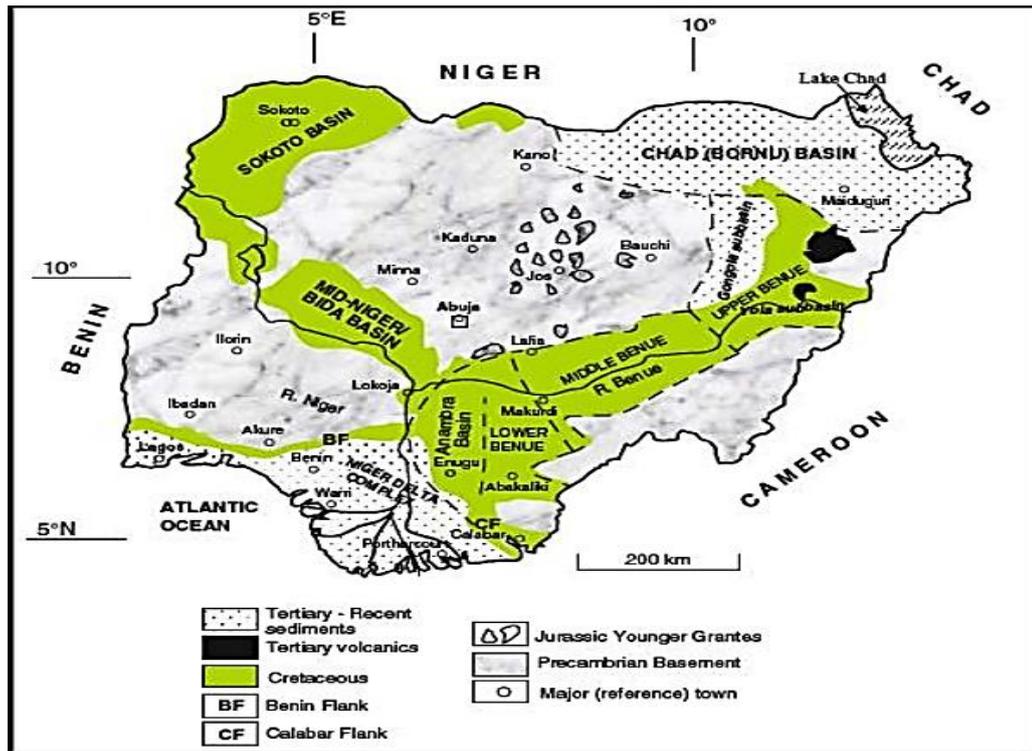
Figure 2.2: Geological map of Nigeria showing the position of Bida Basin

(Obaje *et al.,* 2011)

It is expected that with relentless and reinvigorated geological, geochemical and geophysical studies, particularly with respect to the evaluation of potential petroleum systems, commercial success may also be achieved in the Nigerian sector of Africa's inland basins, even if it may take some time to put all the elements together. It is important to continue with and to step up efforts on the hydrocarbon exploration programmes in Nigeria's other sedimentary basins outside the Niger Delta for more revenue to the country.

2.2 Microorganisms as Indicators in Prospecting for Hydrocarbon Deposits

Bacteria are ubiquitous in distribution and their exceptionally high adaptability to grow on different nutrient sources form the basis of microbial prospecting. Several investigators have used bacteria that degrade hydrocarbons as indicators for finding oil and gas reservoirs (Rasheed *et al.,* 2013; Rasheed *et al.,* 2015; Ning *et al*., 2018;

American Association of Petroleum Geologists (AAPG), 2020). Microbial prospecting of hydrocarbons is based on the detection of anomalous population of hydrocarbon oxidizing bacteria in the surface soils which indicates the presence of subsurface oil and gas accumulation. The technique is based on the seepage of light hydrocarbon gases such as C_1–C_4 from the oil and gas pools to the shallow surface that provide the suitable conditions for the development of highly specialized bacterial population. These bacteria utilize hydrocarbon gases as their only food source and are found enriched in the near surface soils above the hydrocarbon bearing structures (Pareja, 1994; Mohammed *et al.,* 2012; Rasheed *et al.,* 2015). The detection of various groups of methane, ethane, propane or butane oxidizing bacteria, in the surface soils or sediments, helps to evaluate the prospects for hydrocarbon exploration (Rasheed *et al.,* 2012).

In view of the connection between bitumen deposits and microorganisms, microbiological research has been a part of complex geochemical and geological prospecting methods in oil exploration for many years. The oil and gas Institute in United States of America has conducted research using the microbial well survey techniques and a surface method based on the isolation of bacteria which use hydrocarbons as their sole carbon source. The first technique-the microbial well survey technique, is based on the isolation of indicator microbes from oil and gas-bearing zones of cores representing different geographical deposits using specialized microbiological media. Mogilevsky's methodology has been modified, so that its scope has been enlarged and new methods of sample collection and analysis have been developed (Shumacher, 2000). The modified method allows determination of the distribution of particular microbial groups and their level of activity in a geographical profile by observing their hydrocarbon-oxidizing activity. Moreover, it allows an

assessment of the potential areas of interest, and the geo-microbial data confirm the geochemical data on the distribution of an organic substance in materials. The advantage of this method is its high sensitivity, which detects trace amounts of hydrocarbons (Niewiadomski *et al.,* 2000; Yi *et al*., 2016; Ning *et al*., 2018).

The second method-the surface-prospecting method (Shumacher, 2003) is based on the detection of anomalies in microbial distribution in soil samples. It is a method based on the premise that hydrocarbons are generated and/ or trapped in subsurface oil reservoirs (at depth) and migrate upward in varying but detectable quantities. The seepage of hydrocarbon is a long lasting process, but it is recognizable by the presence of analytically detectable (anomalous) concentrations of light hydrocarbons (C_1-C_5) in soils and waters. A higher concentration of these hydrocarbons is often correlated with an increased concentration of hydrocarbon-oxidizing microbes, such as methane-oxidizing bacteria, propane and butane-oxidizing microbes, in the area above hydrocarbon reservoirs (Veena-Prasanna *et al.,* 2013). Hence, the discovery of a surface geochemical anomaly can establish hydrocarbon accumulation in the area. Traps and structures along such pathways should be considered as significantly more promising than those not associated with such anomalies. The results of microbiological analysis of soil samples have been applied in geological studies which concern concentrations of methane and the bacteria that oxidize gaseous hydrocarbons (Ram, 2012; Veena-Prasanna *et al.,* 2013).

2.3 Microbial Targets for Petroleum Seep Prospecting

Surface geochemical petroleum exploration is defined as the search for migrated surface hydrocarbons and their alteration products, including changes in vegetation and microbial populations (Schumacher, 2000; Rasheed *et al.*, 2008b; Hitzman *et al.*, 2009).

Microbes may serve as pathfinders for petroleum exploration. The fact that those microorganisms utilize petroleum compounds as growth substrates makes it possible for them to colonize environments where hydrocarbons occur naturally; they play a profound role on the oxidation of migrating hydrocarbons, and are directly responsible for many surface manifestations of petroleum seepage.

The Microbial Prospection for Oil and Gas (MPOG), developed in Germany by Wanger and Schwarz since 1961 had been used as a stand-alone technique for detecting micro-seepages, is based on the knowledge that oil and gas fields emit a continuous stream of light-hydrocarbon gases towards the Earth's surface (Tucker and Hitzman, 1996; Schumacher, 2000; Wagner *et al.,* 2002; Waheed *et al*., 2018). Specialized microorganisms, such as the hydrocarbon-oxidizing bacteria, depend on light-hydrocarbon gases as their only energy source (Wagner *et al.,* 2002). In terms of investigation aiming at the microbial hydrocarbon prospection, two groups are relevant: gram-positive bacteria, represented mainly by Actinobacteria from CRNM complex (*Corynebacterium, Rhodococcus, Nocardia* and *Mycobacterium*) that uses short-chain hydrocarbons (C_2-C_8) as an energy source, and gram-negative bacteria, mainly the genus *Pseudomonas* that possesses the ALK system responsible for alkane degradation (Shennan, 2006; Kotani *et al.,* 2006).

The Microbial Oil Survey Technique (MOST), developed by Phillips Petroleum Company, is one of the exploration methods and has been available to the petroleum industry since 1985 (Hitzman *et al*., 2009). This methodology is based on the isolation of microorganisms on agar plates containing selective growth medium and subsequent counting of colony-forming units. Microbial anomalies have been proven to be reliable

indicatives of oil and thermogenic gas occurrences in the subsurface, and the method has been widely used throughout the world. Generally, these methods involve microbial activity analysis in samples taken from depths of 0.2 to one meter (Shennan, 2006). MOST uses higher butanol resistance of butane-oxidizing bacteria to detect hydrocarbon micro-seepages. Microbial anomalies have been proved to be reliable indicators of oil and gas in the subsurface (Pareja *et al.,* 1994). Hydrocarbon micro-seepage detection adds value to two dimensional (2-D) and three dimensional (3-D) seismic by identifying those features that are charged with hydrocarbons (Schumacher, 2003; Rasheed *et al*., 2015; Waheed *et al*., 2018).

There is a direct positive relationship between the increased hydrocarbon concentrations and increased hydrocarbon indicating microbial populations. This relationship is easily measurable and distinctly reproducible. Microbial anomalies can also be used for development of field and reservoir characterization studies (Hitzman, 1994; Hitzman *et al*., 1999; Schumacher *et al*., 2003).

Light hydrocarbon gases that migrate upward from buried reservoirs and become adsorbed to near-surface soils and sediments also represent a useful tool for oil and gas prospection, constituting the Sorbed Soil Gas (SSG) technique (Hitzman *et al.,* 2009). Areas of micro seepage are detected by observing the concentration and composition of light hydrocarbons, chiefly methane through butane, extracted from these soils and sediments. These exploration methods can be used in combination with other data, such as geological and geophysical; to reduce exploration costs and increase success rates (Schumacher, 2000). According to Hitzman *et al.* (2009), prospections associated with micro-seepage anomalies are 4-6 times more likely to result in a commercial discovery than prospects with no associated seepage anomaly.

The Microbial Oil Survey Technique (MOST), developed by Philips petroleum company in the 1980s (Hitzman *et al.,* 2009) depends on the relationship between surface microbial anomalies. This kind of approach has proven successful in the discovery of oil and gas reservoirs both on shore and offshore (Turkiewics, 2011).

A second possibility relates to the transport of microbes from oil reservoirs via the upward flow of petroleum fluids to the seabed and into the seawater. Indigenous reservoir microorganisms may become so called 'hitchhiker' cells that are carried from one environment to another, adhering to the surface of rising gas bubbles and/ or oil globules (Leifer and Judd, 2002). In the marine environment these hitchhiker may represent conspicuous indicators of this transport pathway. The hitchhiker cell phenomenon and strategy which was hypothetical have been developed and its development depends on the microbiology of surface hydrocarbon seeps and deep petroleum reservoir (Hubert and Judd, 2020). It is critical that the microbes targeted by either proposed strategy are appropriate for seep prospecting. Careful selection of indicator organisms minimized the possibility of false positive while hunting for petroleum seepage (Tucker and Hitzman, 1996; Hubert and Judd, 2020).

2.4 Microbial Seeding of Oil Spills

Seeding involves the introduction of allochthonous microorganisms into the natural environment for the purpose of increasing the rate or extent, or both, of biodegradation of pollutants (Unimke *et al*., 2018). The rationale for this approach is that the autochthonous microbial populations may not be capable of degrading the wide range of potential substrates present in complex mixtures such as petroleum. The criteria to be met by effective seed organisms include the ability to degrade most petroleum components, genetic stability and viability during storage, high degree of enzymatic

activity and growth in the environment, the ability to compete with indigenous microorganisms, non-pathogenicity, and the inability to produce toxic metabolites (Abioye *et al.*, 2019).

Mixed cultures have been most commonly used as inocula for seeding because of relative ease with which microorganisms with different and complementary hydrocarbon degrading capacities can be isolated. The potential for creating microbial strains able to degrade a variety of different types of hydrocarbon through genetic manipulation has been demonstrated (Leahy and Colwell, 1990). Friello *et al.* (1976) successfully produced a multi plasmid-containing *Pseudomonas* strain capable of oxidising aliphatic, aromatic, terpenic, and polyaromatic hydrocarbons. The use of such strain as an inoculum during seeding would preclude the problems associated with competition between strains in a mixed culture. However, there is considerable controversy surrounding the release of such genetically engineered microorganisms into the environment, and field testing of these organisms must therefore be delayed until the issues of safety, containment, and potential damage are resolved (Leahy and Colwell, 1990). However, recently biosafety measure such as development of biosecurity tools to monitor and detect genetically engineered microbes in the wild (www.sciencedaily.com/releases/2019). Seeding of petroleum-contaminated aquatic environments has been attempted, with mixed results.

2.5 Microbial Degradation of Hydrocarbons

Biodegradation of hydrocarbons is a complex process that depends on the nature and on the amount of the hydrocarbons present. Petroleum hydrocarbons can be divided into four classes: the saturated, the aromatics, the asphaltenes (phenols, fatty acids, ketones, esters, and porphyrins), and the resins (pyridines, quinolines, carbazoles, sulfoxides, and amides) (Prakash *et al.*, 2014). The availability of these compounds determines the

degradation rate because one of the important factors that limit biodegradation of oil pollutants in the environment is their limited availability to microorganisms (Cooney *et al.,* 1995*;* Allamin *et al.,* 2014). Also, hydrocarbons differ in their susceptibility to microbial attack; the susceptibility of hydrocarbons to microbial degradation can be generally ranked as follows: linear alkanes > branched alkanes > small aromatics > cyclic alkanes (Ulrici, 2000; Allamin *et al.,* 2014). Some compounds, such as the high molecular weight polycyclic aromatic hydrocarbons (PAHs), may not be degraded at all (Atlas and Bragg, 2009).

Microbial degradation is the major and ultimate natural mechanism by which one can cleanup the petroleum hydrocarbon pollutants from the environment, the recognition of biodegraded petroleum-derived aromatic hydrocarbons in marine sediments was reviewed by Das and Chandran, (2011), they stated the extensive biodegradation of alkyl aromatics in marine sediments which occurred prior to detectable biodegradation of n-alkane profile of the crude oil and the microorganisms, namely, *Arthrobacter, Burkholderia, Mycobacterium, Pseudomonas, Sphingomonas, and Rhodococcus* were found to be involved for alkylaromatic degradation. Microbial degradation of petroleum hydrocarbons in a polluted tropical stream in Lagos, Nigeria was reported by Adebusoye *et al.* (2007), nine bacterial strains, namely, *Pseudomonas fluorescens, P. aeruginosa, Bacillus subtilis, Bacillus* sp*., Alcaligenes* sp., *Acinetobacter lwoffi, Flavobacterium sp., Micrococcus roseus, and Corynebacterium* sp. were isolated from the polluted stream which could degrade crude oil.

Hydrocarbons in the environment are biodegraded primarily by bacteria, yeast, and fungi. The reported efficiency of biodegradation ranged from 6 % to 82 % for soil fungi, 0.13 % to 50 % for soil bacteria, and 0.003 % to 100 % for marine bacteria. Many

scientists reported that mixed populations with overall broad enzymatic capacities are required to degrade complex mixtures of hydrocarbons such as crude oil in soil, fresh water, and marine environments (Rahman *et al.,* 2003; Yakimov *et al.,* 2007; Brooijmans *et al.,* 2009).

Bacteria are the most active agents in petroleum degradation, and they work as primary degraders of spilled oil. Das and Chandran (2011) listed 25 genera of hydrocarbon degrading bacteria and 25 genera of hydrocarbon degrading fungi which were isolated from marine environment. In earlier days, the extent to which bacteria, yeasts, and filamentous fungi participate in the biodegradation of petroleum hydrocarbons was the subject of limited study, but appeared to be a function of the ecosystem and local environmental conditions (Das and Chandran 2011).

Fungal genera, namely, *Amorphoteca*, *Neosartorya*, *Talaromyces,* and *Graphium* and yeast genera, namely, *Candida, Yarrowia,* and *Pichia* were isolated from petroleum-contaminated soil and proved to be the potential organisms for hydrocarbon degradation (Chaillan *et al.,* 2004). Singh (2006) also reported a group of terrestrial fungi, namely, *Aspergillus*, *Cephalosporium,* and *Pencillium* which were also found to be the potential degraders of crude oil hydrocarbons. The yeast species, namely, *Candida lipolytica, Rhodotorula mucilaginosa, Geotrichum* sp*, and Trichosporon mucoides* isolated from contaminated water were noted to degrade petroleum compounds (Bogusławska and Dąbrowski, 2001).

Though algae and protozoa are the important members of the microbial community in both aquatic and terrestrial ecosystems, reports are scanty regarding their involvement in hydrocarbon biodegradation. An alga, *Prototheca zopfi* which was capable of

utilizing crude oil and a mixed hydrocarbon substrate and exhibited extensive degradation of n-alkanes and isoalkanes as well as aromatic hydrocarbons has been reported. Similarly it has been observed that nine cyanobacteria, five green algae, one red alga, one brown alga, and two diatoms could oxidize naphthalene (Obayori *et al.,* 2009; Das and Chandran, 2011; Unimke *et al*., 2018).

2.6 Factors Influencing Petroleum Hydrocarbon Degradation

A number of limiting factors have been recognized to affect the biodegradation of petroleum hydrocarbons.

2.6.1 Composition and type of the hydrocarbon

The primary significance massive concept to be surveyed is the synthesis and inalienable biodegradability of the petroleum hydrocarbon pollutant. Saturates aromatics, resins, and asphaltene (Shallu *et al*., 2014), n-alkanes of intermediate length (C_{10}-C_{25}) are the most suitable substrates for microorganisms and will be predisposed to be with out problem degradable, whilst the shorter chain compounds are enormously more toxic in petroleum hydrocarbons. Alkanes with long chains (C_{25}-C_{40}) are water-haters, hence difficult to degrade due to their non bioavailability and poor water contact, whereas the cycloalkanes and alkanes of branched-chain degrade very slowly than alkanes. Tars, bitumen, and asphaltic substances, which are highly conpacted fragrant and cycloparaffinic systems, have first-class boiling elements and display the finest resistance to biodegradation. The tempo of microbial take-up and biodegradation of hydrocarbons is reliant upon the dissolvable dissolvability of the hydrocarbons (Maletic *et al.,* 2011).

2.6.2 Effects of temperature

A significant role is played by temperature in the hydrocarbons biodegradation by influencing directly the chemical component of the pollutants, even the body structure and type of microbes. The viscosity consistency of the oil expanded at low temperatures, while the instability of the toxic low molecular weight hydrocarbons decreased, according to research, deferring the onset of biodegradation (Abioye *et al*., 2019). Moreover, Temperature influences the solubility of hydrocarbons (Das and Chandran, 2011). In as much as biodegradation of hydrocarbon can take place at wide range of temperatures, yet, the biodegradation rate slows as the temperature drops. The most famous corruption quotes come from people who have achieved about 40°C in soil conditions, 20 to 30°C in some freshwater conditions, and 20°C in marine conditions (Das and Chandran, 2011). According to Venosa and Zhu (2003), the ambient-room temperature of the weather influenced the movement of microorganisms and the houses of spilled oil. In psychrophilic conditions in calm regions, critical hydrocarbon biodegradation was accounted for. (Pelletier *et al.,* 2004; Delille *et al.,* 2004).

2.6.3 Availability of nutrients

Nutrients are essential for the biodegradation of hydrocarbon pollutants, especially nitrogen, iron and phosphorus. In order to influence biodegradation, a component of these dietary supplements must end up as a restricting element. According to Atlas (2010), as stated by Das and Chandran (2011), after a large spill of oil in freshwater and marine ecosystems, carbons' supply of carbon has been increased greatly, while nitrogen and phosphorus accessibility has become the limiting variable in oil degradation. It has become more articulated in marine environments as a result of low visibility, stages of nitrogen and phosphorous in seawater (Brooijmans *et al.,* 2009).

Freshwater wetlands are frequently thought to be nutrient-poor due to high vitamin demands via plant technique. As a result, vitamin additions have been critical in improving oil pollutant biodegradation. Excessive nutrient concentrations, on the other hand, may limit tha activity of biodegradation. Series of authors have designated the dangerous outcomes of immoderate NPK stages on the biodegradation of hydrocarbons particularly on aromatics (Ijah *et al*., 2008; Abioye *et al*., 2019; Rop *et al*., 2019).

2.6.4 Effects of moisture content

Moisture impacts the price of breakdown of contaminant because it influences the nature and amount of soluble substances which may be to be had even the osmotic stress and pH of terrestrial and aquatic systems. As referenced over, the diploma of water withinside the pore regions of soil furthermore influences the shopping for and promoting of oxygen (Oliveira *et al*., 2020). In saturated situations, oxygen can be quicker than its miles recharged withinside the dirt fume area and the dirt can get anaerobic. This can retard the price of biodegradation and reason number one changes in microbial metabolic interest to occur. Conversely, soil moisture content material fabric ought to be amongst 25 and 85 % of the water maintaining capability, and greater than a few 50 - 80 % is ultimate for biodegradation (Nkwopara *et al*., 2019).

2.6.5   Effects of pH

The acidity or alkalinity of water affects the pH of the soil. Biodegradation can occur at a wide range of pH levels; however, most aquatic and terrestrial systems have a pH range of 6.5 to 8.5, and values from 5.0 to 9.0 are acceptable considered (Chikere and Fenibo, 2018). The rate of dissolution of phosphorus, an important nutrient in ecosystems, is maximal at a pH of 6.5 and decreases at pH values that are neither higher nor lower than the aforementioned (Chikere and Fenibo, 2018; Nkwopara *et al*., 2019).

2. 7 Advantages and Disadvantages of Microbial Prospecting

According to Shumacher (2003), the advantages of the surface-prospecting method are many and include:

i.   Evidence of the presence of hydrocarbon generation and migration

ii. The low cost, ease and rapidity of sample collection and analysis

iii. High sensitivity, which allows detection even ″discrete″ anomaly state on the assumption that the threshold value is estimated properly.

iv. The detection of hydrocarbons in both soils and on the sea floor

v.   The possibility of prospecting before conducting detailed seismic surveys

vi. Having little or no negative environmental impact and having the ability to evaluate areas where seismic surveys are impractical or ineffective due to geological factors

vii. Providing methods applicable to both stratigraphic traps and structural traps, with the ability to locate traps invisible or poorly imaged with seismic data or due to environmental factors.

viii. Reproducibility of results

ix. Providing methods applicable in many different climate conditions and establishing a clear distinction between gas reservoirs and oil bearing structures with gas caps.

x.   The method helps in prioritizing drilling locations (Pareja, 1994)

The success of this method depends on the defining background values adequately. This can fluctuate widely depending on the geographical area. The level of background should be defined individually. Microbial examinations are a valuable supplement to complex geochemical and geological surveys conducted for the exploration of hydrocarbon deposits (Turkiewics, 2011).

However, despite its advantages, it has disadvantages which include:

a. In-ability to predict the reservoir's depth. This is because it is a surface prospecting method and therefore depends solely on the migration of light hydrocarbon gases from gas and oil reservoirs (Rasheed *et al*., 2015).

b. Under-sampling and use of improper sampling techniques causes ambiguity that leads to interpretation failure (Luo *et al*., 2019).

c. Discovery of surface anomaly does not guarantee discovery of commercially significant volumes of hydrocarbon.

2.8   Characterization of Hydrocarbon–Oxidizing Microbes

Hydrocarbon–oxidizing microbes for oil prospection are characterized using cultivation-dependent and cultivation-independent characterization method.

2.8.1: Cultivation-dependent method

This method has to do with the use of suitable culture media for the inoculation of samples. The methodology involves the collection of soil and water samples from the survey area, packing, preservation and storage of samples in pre-sterilized sample bags under aseptic conditions till analysis and isolation and enumeration of hydrocarbon–utilizing bacteria such as methane, ethane, propane, and butane oxidizers on agar plates containing selective growth medium and subsequent counting of colony-forming units (Paula *et al*., 2011, Rasheed *et al*., 2015, Abioye *et al*., 2019). The contour maps for the population density of hydrocarbon oxidizing bacteria are drawn and the data can be integrated with geological, geochemical, and geophysical methods to evaluate the hydrocarbon prospect of an area and to prioritize the drilling locations thereby reducing the drilling risks and achieve higher success in petroleum exploration (Rasheed *et al*., 2015).

2.8.2 Cultivation-independent characterization method (Metagenomics)

Metagenomics is a molecular tool used to analyse DNA acquired from environmental samples, in order to study the community of microorganisms present without obtaining pure culture (Surajit and Ranjan, 2019; Rastogi and Sani, 2020). Culture-independent approaches include analyses of whole genomes or selected genes such as 16S and 18S rRNA (ribosomal RNA) for prokaryotes and eukaryotes, respectively. Broadly, these techniques have been classified into two major categories (Figure 2.3) depending on their capability of revealing the microbial diversity structure and function: (1) partial community analysis approaches and (2) whole community analysis approaches.

There are no specific information on the use of metagenomics in characterization of hydrocarbon gas oxidizing microbes for oil and gas prospecting. However, Stable-isotope probing (SIP), a valuable tool has been used successfully to link the phylogeny and function of targeted microbial groups, to investigate polycyclic aromatic hydrocarbon (PAH)-degrading bacteria under aerobic conditions in sediments from Guaymas Basin with uniformly labeled ^{13}C-phenanthrene (PHE), quantitative PCR primers targeting the 16S rRNA gene of the SIP-identified was emplored in order to expand current knowledge on the diversity of hydrocarbon-degrading microbial communities in Guaymas Basin oil-rich sediments (Gutierrez *et al*., 2015).
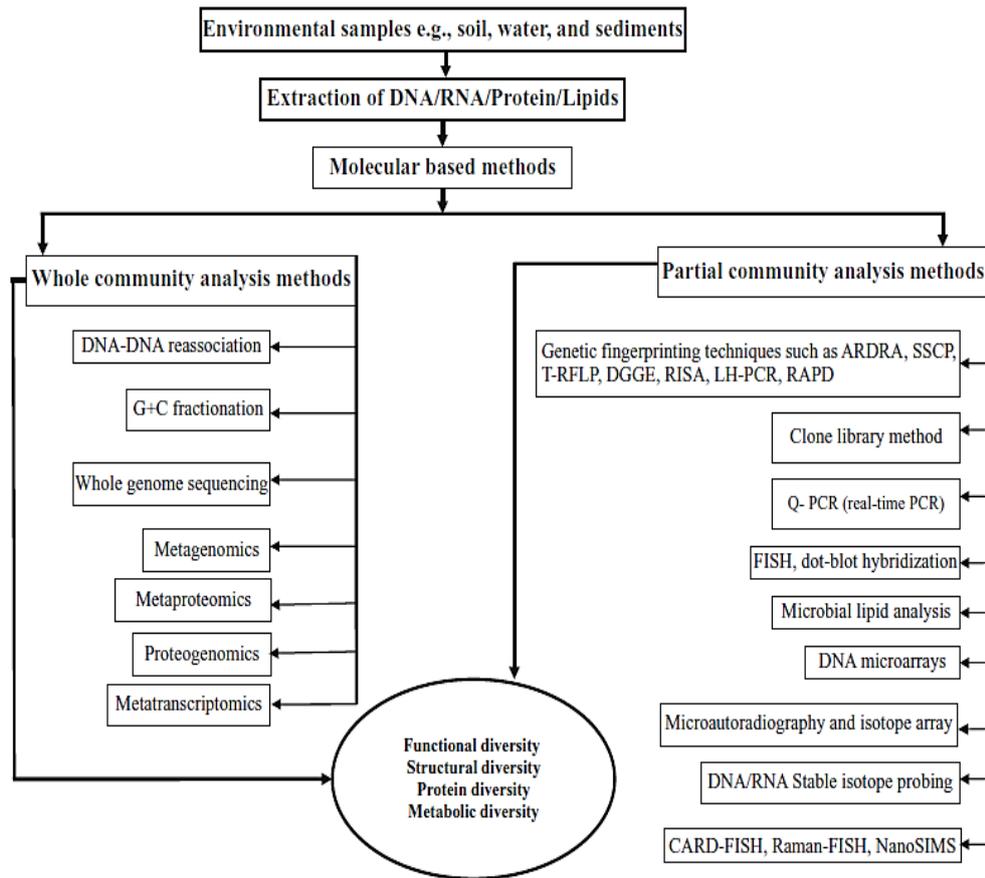
Figure 2.3: Culture-independent molecular toolbox to characterize the structural and functional diversity of microorganisms in the environment (Surajit and Ranjan, 2019; Rastogi and Sani, 2020).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Description and Mapping of the Study Area

Two study areas (Ahoko and Patishabakolo) were taken for the study after recognisance visit to the four major areas (Ahoko, Petegi/Muregi, Enagi and Patitiabakolo) within the Bida basin.

Ahoko (Ebira, Gbagi and Idu) found along Abuja-Lokoja Express Way is a Local Government in Kogi State, North Central Nigeria, with a population of over 15,077 people (National Population Commission of Nigeria (NPCN, 2016). The area is characterized by two climatic seasons: dry season (November – March) and rainy season (April – October). The residents of the area are mostly Ebira, Gwagi and few Fulani, with farming and fishing as their main occupations. The area is dominated by deciduous woodland and rocky hills of open savannah vegetation (Adinoyi-ojo, 2018). It is located at latitude 6° 00″E and longitude 8° 00″N (Figure 3.1).

Patishabakolo is one of the villages in Bida, Niger State, with a population of over 175,840 people (NPCN, 2016), it has mono-modal rainfall pattern ranging from 750 mm to 1000 mm with annual mean precipitation of 875 mm. The mean annual temperature is 30ºC. The vegetation is made up of short grasses that form a matrix for thorny shrubs to grow in. The area's residents are mostly Nupe, with a few Hausa and Fulani. Their main occupations are farming and animal rearing. It is located at longitude 11°56′00″N and latitude 6°54′00″E (Figure 3.2).
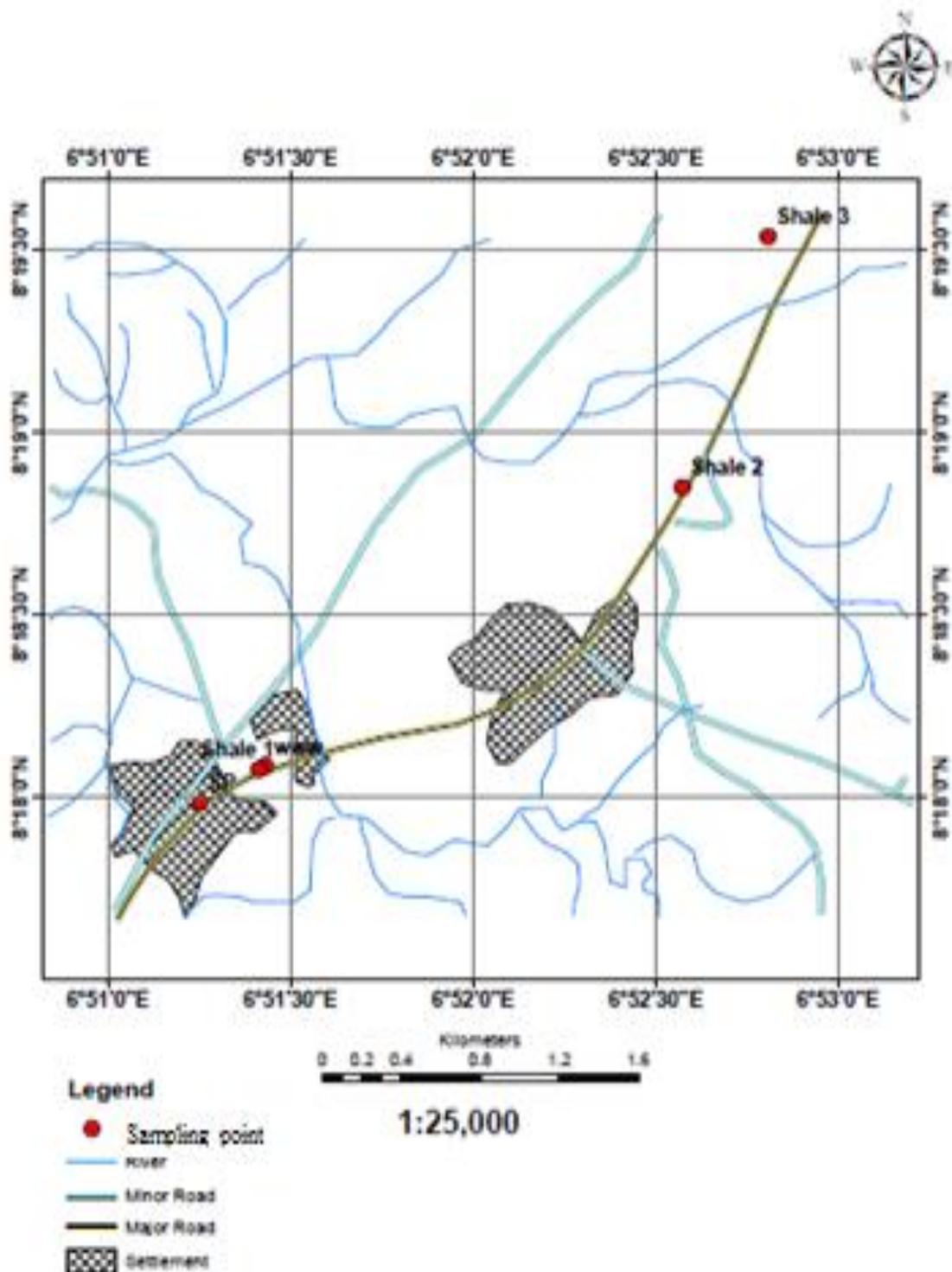
Figure 3.1: Ahoko study site Map, showing points of sample collection
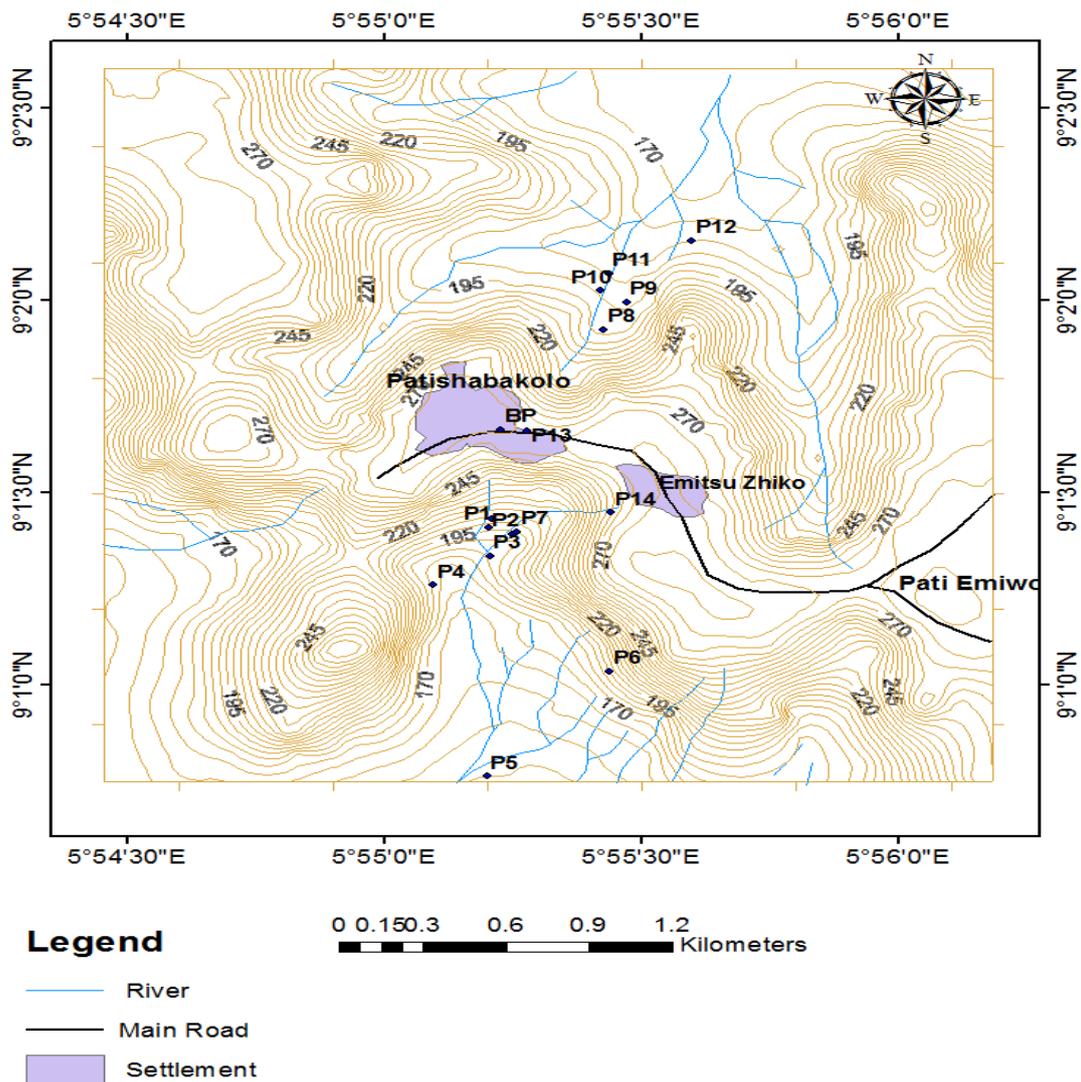
Figure 3.2: Patishabakolo study site Map: showing contour lines, sampling points, settlement

3.2 Experimental Design and Sample Collection

The identified study sites (Ahoko and Patishabakolo) were mapped in a complete randomized design and gridded 1km by 1km interval. Each kilometre was partitioned into 5 points and soil samples were randomly collected in each point and were clustered and bulked into one sample over an area of approximately 4 km^2 within the suspected sites of crude oil. The soil samples were collected using soil core sampler by physical

hammering in a depth of 1m. The soil samples were transferred into a transparent polythene bag; the bags were properly tied (Reyes and Mendoza, 2016).

The water samples (Mini river and Kumi waters) were collected using plastic cans which were properly washed and sterilized with 70 % alcohol, then allowed to air dry. The pre-sterilized dried container was used to collect water halfway between the surface and bottom of the water (Mini river) while the Kumi water was collected by placing the can directly under the oozing point. The plastic cans were filled to the brim, leaving airspace of 2.0cm and the lid was covered immediately after collections. The samples were labelled along with their coordinates (Latitude and Longitude) ascertained using a Global Positioning System (GPS), thereafter they were packed into a bucket containing ice and transported to the Microbiology Laboratory Alex Ekwueme Federal University, Ndufu Alike (AE-FUNAI), Ebonyi State, and Step B Laboratory of the Federal University of Technology Minna, Niger State, Nigeria where samples were analysed.

3.3 Physicochemical Analysis of Soil and Water Samples

The physical and chemical properties of soil and water samples collected from the study areas were analysed using standard methods as described below.

3.3.1 Determination of pH

pH of soil and water samples were determined using pH meter (Model IP67, pH meter, Techmel and Techmel, USA). Twenty gram (20 g) soil sample was mixed with 40 mL distilled water. The suspension was stirred intermittently with a glass rod for 30 minutes and left to stand for one hour. The electrode was immersed into the soil suspension and pH was recorded. The electrode was washed with distilled water before each new reading of the soil was taken. The pH of the water sample was similarly determined by

inserting the electrode of the pH meter into 20 mL of the water samples (Brady and Weil, 1990).

3.3.2 Determination of electrical conductivity

The electrical conductivity (EC) of the water and soil samples was determined by immersing Equiptronics digital electrical conductivity bridge into the water samples and soil suspensions respectively and readings were taken and the EC values were recorded (Chikere *et al.,* 2017).

3.3.3 Determination of Moisture

Moisture content of the soil samples was determined by oven drying method as reported by Das and Bandi (2014). Ten grams (10 g) of soil samples was kept in hot air oven at 105°C for 24 hours. Dry weight of the sample was taken when a constant weight was achieved. The percentage moisture was calculated using Equation 3.1

$$\text{Percentage moisture} = \frac{(W_3 - W_1)}{(W_2 - W_3)} \times 100 \tag{3.1}$$

W_1- Weight of empty beaker

W_2- Weight of empty beaker+ soil before oven drying

W_3- Weight of empty beaker + soil after oven drying

3.3.4   Determination of Total Organic Carbon

The quantity of organic carbon in the soil was estimated using Walkey- Black method as described by Chikere *et al.* (2017). One (1 g) gram of finely ground dry soil sample was passed through 0.5 mm sieve into a 500 mL capacity conical flask. To this, 10 mL of 1N potassium dichromate (KCr_2O_7) and 20 mL concentrated H_2SO_4 was added and the contents was shaken for one minute and allowed to stand for 30 minutes. Then 200

ml of distilled water, 10 mL of phosphoric acid and 1 mL of diphenylamine indicator was added. The solution was titrated against standard ferrous ammonium sulphate till a colour change from blue violet to green was observed. The blank titration was also carried out without soil using Equation 3.2

% Organic carbon in soil=

$$\frac{(Me\ K_2Cr_2O_7 - MeFeSO_4)}{1g\ of\ air-dry\ soil} \times 0.003100 \times (f) \qquad\qquad (3.2)$$

Where:

Me= molarity of solution x cm^3 of solution used; correction factor, f =1.33

% Organc matter in soil = organic carbon x1.729

3.3.5   Determination of Avaliable Nitrogen

The total nitrogen content of the soil was estimated by alkaline permanganate method (Subbaiah and Asija, 1965). Twenty grams (20 g) of the soil sample was weighed and transferred into 1 litre distillation flask followed by the addition of 10 mL of 0.32 % potassium permanganate, 10 mL of 2.5 % sodium hydroxide, and 10 mL of distilled water. This was fitted up in the distillation apparatus, then 25 mL of 0.02 N sulphuric acid was pipetted into a 250 mL beaker, two drops of methyl red indicator was added. The contents of the flask were distilled to collect about 30 mL of the distillate into the known excess of 0.02 N sulphuric acids. The excess of the acid was titrated against 0.02 N potassium hydroxide, a colour change from pink to the light yellow was observed. Then from the volume of 0.02 N sulphuric acid actually consumed by ammonia, the percentage nitrogen present in the given soil sample was extrapolated (Oyem and Oyem, 2013) using Equation 3.3.

$$\text{\% Available Nitrogen} = (A-B) \times (N.\ of\ acid) \times 0.014 \times \frac{100}{wt.of\ soil\ (g)} \qquad (3.3)$$

Where:

Wt. = Weight of soil sample

A= Volume of acid used for treated soil

B= Volume of acid required for blank

N=Normality of Sulphuric acid

3.3.6 Determination of particle size and textural class of the soil

Hydrometer method as described by Oyem and Oyem (2013) was used. Fifty grams of the soil sample was passed through a 2 mm sieve and oven dried. The oven dried sample was transferred into a mixcup; 50 mL of 5% hexametaphosphate was added along with 100 mL of distilled water (Oyem and Oyem, 2013). This was mixed using a stirring rod and allowed to stand for 30 minutes. The suspension was stirred again for 15 minutes with a multimix machine, then transferred from cup to glass cylinder and hydrometer was inserted at a depth of 5 cm to take the initial reading (T_0), thereafter the suspension was allowed to stand for 60 seconds (T_1) and another reading was taken, then allowed to stand for 2 hours (T_2) after which the third reading was taken. The textural class was identified using the textural triangle, the size was calculated using Equation 3.4a –3.4d

$$\text{Mass of sand} = \text{Initial density} - \text{density at time } (T_0) \qquad (3.4)$$

$$\text{\% sand} = \frac{\text{Mass of sand}}{\text{Mass of original sample}} \times 100 \qquad (3.4b)$$

$$\text{\% clay} = \frac{\text{Density at time } (T1)}{\text{Mass of original sample}} \times 100 \qquad (3.4c)$$

$$\text{\% silt} = 100\ \% - (\text{sand} + \text{clay}) \qquad (3.4d)$$

3.3.7 Determination of exchangable cation (Ca^{2+}, K^+, Na^+, Mg^{2+})

Thirty millilitres (30 mL) of 1M NH_4OAc was added into 5 g of soil and 5 mL of water samples, the mixtures were shaken separately for 2 hours in mechanical shaker, then it was centrifuged at 9000g for 10 minutes and clear supernatant solution was decanted into 1000 mL volumetric flask, the process was repeated twice, then the liquid was made up to 1L mark with NH_4OAc solution. Na^+ and K^+ was determined using flame photometer calibrated with Na^+ and K^+ standard (Chikere *et al.*, 2017), while Ca^+ and Mg^+ was titrated using EDTA (American Public Health Association (APHA, 2008).

3.4 Microbiological Analysis of the Soil and Water samples

3.4.1 Enumeration of heteroaerobic bacteria

Freshly prepared Ringer's solution according to Anyanwu *et al.* (2016) was prepared and used to dislodge the microbial load in the samples. This was done by dissolving 10 g of soil sample into 90 mL of the Ringer's solution; the mixtures were shaken for 5 minutes using magnetic stirrer at 30^oC. It was allowed to stand for 30 miuntes, then one millilitre of the stock was taken into 9 mL of the ringer's solution dispensed into test tubes and serial dilutions were made to obtain the dilutions of 10^{-1} to 10^{-10}, 0.05 mL of the dilutions of 10^{-4} to 10^{-10} was inoculated into 18 mL each of freshly prepared sterile molten Nutrient Agar, the mixture was transferred by pour plate method into sterile Petri dishes which were swirled and allowed to solidify at ambient temperature ($26^oC \pm 2^oC$) for 30 minutes on a flat surface. The inoculated culture plates were incubated at 37^oC for 18 hours; after which the colonies that developed were counted using a colony counter (Model TT-02 Colony Counter, Techmel and Techmel, USA). The results were recorded as colony forming units per gram (CFU/g) of soil. Similarly 10 mL of water sample was withdrawn from the original sample and was transferred into 90 mL of

ringer's solution which was serially diluted and 0.05 mL was inoculated into NA, the inoculated culture plates were incubated at 37^oC for 18 hours. The results were recorded as colony forming units per millilitre (CFU/mL) of water samples (Anyanwu *et al.*, 2016).

3.4.2 Enumeration of total fungi

Total fungal count was carried out on Sabourand Dextrose Agar (SDA) (Hi-Media) supplemented with 10 mg/L of streptomycin. Triplicate plates were prepared, inoculated and incubated at 28^oC for 72 hours. The colonies that developed were counted and recorded. This was done for all the samples across the months sampled (February 2017-January 2018). The colony counts were expressed as colony forming units per gram of soil (CFU/g) and colony forming unit per millilitre (CFU/mL) of water (Anyanwu *et al.*, 2016).

3.4.3 Enumeration of Specific Hydrocarbon Utilizers

Specific isolation of microbes associated with hydrocarbon seepage was done using Light carbon gas of C_1-C_4 (methane, ethane, propane, butane). Isolation and enumeration of microorganisms for each sample was carried out by Standard Plate Count (SPC) method as reported by Rasheed *et al.* (2015).

Serially diluted soil suspension was inoculated into sterile molten Mineral Salts Medium (MSM) containing 1.0 g $MgSO_4.7H_20$, 0.7g K_2HPO_4, 0.54 g KH_2PO_4, 0.5 g NH_4Cl, 0.2 g $CaCl_2.2H_2O$, 4.0 mg $FeSO_4.7H_2O$, 0.3 mg H_3BO_4, 0.2 mg $CoCl_2.6H_2O$, 0.1 mg $ZnSO_4.7H_2O$, 0.06 mg $Na_2MoO_4.2H_2O$, 0.03 mg $MnCl_2.4H_2O$, 0.02 mg $NiCl_2.6H_2O$, and 0.01 mg $CuCl_2.2H_2O$ in 1000 mL of distilled water, at pH 7.0. The plates were placed in a desiccator, loaded with potassium hydroxide pellet at the bottom

and closed with greased lid and paraffin. Then it was connected to vacuum pump for 5 minutes to remove air, after vacuum was created, the loaded desiccator was filled with the desired hydrocarbon gas (methane/ethane/propane with 99.99 % purity): For isolation of methane oxidizing bacteria, the desiccator was filled with methane gas. Similarly, for isolation of ethane, propane and butane oxidizing bacteria, the desiccators were filled with ethane/propane/butane gas respectively. The desiccators were then kept in incubators at $35 \pm 2°C$ for 10 days. After incubation, the colonies of methane, ethane, propane and butane oxidizing bacteria were counted using a colony counter (Model TT-02, Techmel amd Techmel, USA) and reported in colony forming units per gram (CFU/g) of soil sample.

3.5 Physiological and Morphological Characterization and Identification of Isolates

3.5.1 Characterization of bacterial isolates

The bacterial isolates including the potential hydrocarbon oxidizers' were characterized based on their gram stain reaction and biochemical tests. Some of the biochemical tests are described below (following the methods of Fawole and Oso, 1988; Harley and Prescott, 2002 and Oyeleke and Manga, 2008). The isolates were identified by comparing their characteristics with those of known taxa using Bergey's Manual of Determinative Bacteriology (George *et al.,* 2004).

(i)   Gram staining

A wire loop was flamed to red hot and allowed to cool; this sterile loop was used to pick culture from a discrete colony. A smear of the discrete colony was made on a clean slide which was allowed to air dry before it was fixed by passing it gently over a flame. The

fixed smear was flooded with crystal violet for 60 seconds after which the stain was drained off and washed over a running tap; then it was flooded with Lugol's iodine for 60 seconds and washed gently using tap water. This was flooded with 95 % alcohol to decolorize for 30 seconds and rinsed with water. The smear was counter stained using safranin for 30 seconds, after which the slide was washed gently with water and was left to air-dry. This was viewed under oil immersion objective lens (x100) of the microscope (Appendix C). Cultures that appeared purple/blue were recorded as Gram-positive bacteria while those that appeared red/pink were recorded as gram-negative bacteria.

(ii)   Catalase test

Two drops of 3% hydrogen peroxide (H_2O_2) was placed on each end of a clean grease free slide and labelled A and B. With the help of a clean glass rod the test organism was transferred to drop A and was observed immediately for gas bubbling (effervescence) while drop B served as control. Results were recorded as negative or positive based on the evolution of gas or bubbles formed.

(iii)   Indole test

Test organisms were grown in 5 mL of 1% peptone water at 37°C for 48 hours. This was followed by the addition of 0.5 mL of Kovac's reagent (prepared by dissolving 5 g P-dimethylamino-benzaldehyde in 75 mL amyl alcohol and 25 mL concentrated HCl, (Appendix B) and shaken gently. Appearance of red colour ring at the reagent layer was recorded as positive result while absence of colour ring indicated negative result.

(iv)   Oxidase test

Three drops of a freshly prepared oxidase reagent (tetraethyl-p-phenylenediamine dihydrochloride) was placed on a piece of filter paper placed on a clean Petri dish, and a sterile wooden stick was used to collect the test organism and smear it on the filter paper. The appearance of a blue-purple colour within ten seconds was recorded as positive result while the absence of blue-purple colour after fifteen seconds was recorded as negative.

(v)   Citrate test

Twenty four point two eight grams (24.28 g) of Simmons citrate agar was weighed and dissolved into 100 mL of distilled water by heating. Then it was dispensed into test tubes. The citrate agar in the test tubes was sterilized by autoclaving at 121°C for 15 minutes, and the tube was placed in an angular position for the agar to gel into slant. The test organisms were streak inoculated into the citrate agar slants and incubated at 37°C for 4 days. Colour change from green to blue was recorded as positive result, while no colour change (green colour retained) indicated a negative result.

(vi)   Carbohydrate utilization test (acid and gas production from carbohydrate)

One hundred millilitres (100 mL) of peptone water was prepared with the addition of two grams (2 g) of the test sugar (sucrose, fructose, D-mannitol, lactose, D-glucose, sorbitol, arabinose, D-mannose) and 0.08 g of phenol red was incorporated as indicator. Five millilitres (5 mL) of the mixture was dispensed into test tubes and sterilized by autoclaving at 121°C for 15 minutes along with an inverted Durham's tube inside the medium and was allowed to cool. Thereafter, the test organisms were inoculated into the sterile medium, a control was set up without the inoculation of the test organism.

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This was incubated at 37°C for 48 hours after which the medium was observed for colour change from red to orange (yellow) indicating acid production while a void in the Durham's tube indicated formation of gas.

(vii)   Triple Sugar Iron Agar test (TSI) (Hydrogen sulphide production)

Triple sugar iron agar slant was prepared and the test isolates were aseptically inoculated (using inoculating wire loop) into the agar by stabbing the agar to the bottom and streaking the surface of the slant. This was incubated along with uninoculated duplicate tubes as controls at 37°C for three days, it was examined daily and the results were recorded with black precipitate indicating hydrogen-sulphide production, the extent of yellow coloration to indicated sucrose, lactose and glucose fermentation while the presence of cracks in the butt pushed from the bottom indicated gas formation.

(viii)   Motility test (Stab culture techniques)

Motility medium was prepared in a test tube using 10 g of peptone, 5 g of agar agar, and 5 g of sodium chloride (NaCl) per liter. This was sterilized by autoclaving at 121°C for 15 minutes. The bacterial isolates were inoculated into the sterile motility medium by stabbing with a sterile needle to a depth of about 2 cm just about the centre of the medium. The tubes were then incubated at 37°C for 18-24 hours. Organisms that grew only along the line of stab as compared to the control was recorded as non-motile whereas those that grew along the line of stab and diffused into the medium away from the line of stabbing causing turbidity (rendering the medium not clear or opaque) was recorded as motile.

(ix)   Hanging drop techniques

A clean grease free cavity slide was placed on the bench with the cavity uppermost, then a clean cover slip was held between two fingers and a drop of molten vaseline was carefully placed on the four edge of the cover slip. Then a drop of 18 hours old bacterial suspension was placed gently at the centre of the cover slip to which vaseline was applied; this was quickly and carefully inverted to the cavity slide, making the drop of the bacterial suspension on the cover slip to suspend in the centre of the slide. The slide along with the cover slip was examined for motility, first by using low power objective (x10) to focus the edge of the drop after which it was carefully turned to high power objective (x40) with reduced illumination. Organisms whose movement was directional, different from zigzag movement was recorded as positive (motile)

(x)   Urease production

Bacterial isolates were inoculated in urea agar slants in bijou bottles and were incubated at 37°C for 24 hours. Bright pink (or red colour) indicated a positive reaction while a negative reaction was indicated by the absence of coloration (i.e. the colour remains pale yellow).

(xi)   Methyl red (MR) and Voges Proskauer (VP) test

The bacterial isolates were inoculated into test tubes containing 2 mL of sterile glucose phosphate peptone water labelled A and B and incubated at 37°C for 48 hours. To test tube A, four drops of methyl red reagent was added using a Pasteur's pipette; this was mixed by shaking gently and was observed for immediate colour change. Positive and negative M-R inference was indicated by bright red rings on the surface of the medium and yellow colour respectively. To tube B, one millilitre of 40 % potassium hydroxide (KOH) and 3 mL of 5 % alcoholic alpha-naphtol was added and shaken properly. This

was allowed to stand for 3 minutes. A pink colour formation in 3 minutes was recorded as positive V-P reaction while no colour change (i.e. remains black) indicated negative reaction.

(xii) Spore staining

Smears of the bacterial isolates were flooded with malachite green. The slides were brought to steaming for 3 minutes (by placing the stained slide over boiling water) and rinsed with tap water. Safranine solution was applied and was left for 30 seconds, after which it was washed, dried and examined under the oil immersion objective. The spores stained green and the remainder of the cell were light red.

(xiii) Hydrolysis of macromolecules (Starch hydrolysis)

Starch agar was prepared by adding 2 g of soluble starch into 100 mL of nutrient agar. This was sterilized by autoclaving at $121^{o}C$ for 15 minutes, and then poured into sterile Petri dishes and allowed to solidify at room temperature. The bacterial isolates were streak inoculated onto the surface of the starch agar and incubated at $37^{o}C$ for 48 hours. After incubation, the plates (both inoculated and uninoculated) were flooded with Gram's iodine and were observed for holo zones around the isolates. The uninoculated plates remained blue-black (negative) while the inoculated plates with clear zone around the colonies of the streaked isolates were recorded as positive.

3.5.2 Identification of fungal isolates

Two drops of seventy per cent (70 %) alcohol was placed on a clean microscope slide, and then a sterilized inoculating needle was used to transfer cultures of filamentous fungi into the drops of alcohol, then two drops of Lactophenol cotton blue dye was added and a coverslip was used to cover the culture. Initial examination was done using a low power objective lens (x10) and then a higher power objective lens (x40) for more detailed examination of spores and other structures. Results obtained were compared with the characteristics of known taxa outlined in established atlas to identify the fungi (Acharya, 2014).

3.6 Molecular Characterization of Specific Hydrocarbon-Utilizing Bacteria and Fungi

3.6.1 Extraction of genomic DNA

The total genomic DNA of isolate were extracted using the universal ZR Fungal/Bacterial DNA kit™ (Zymo Research, USA) following the manufacturer instructions. Briefly 100 mg wet weight of culture were suspended in 200 µL phosphate buffer solution, then introduced into ZR BashingBead TM lysis tube and vortexed at 10,000 rpm for 5 minutes. Thereafter, the ZR BashingBead TM lysis tube was centrifuged at 1000 rpm for 1 minute, 400 µL of the supernatant transferred into a Zymo-SpinTM IV spin filter in a collection tube and centrifuged at 7000 rpm for 1 minute. Exactly 1200 µL of Fungal/Bacterial DNA binding buffer was added to the filtrate and 800 µL of the mixture was centrifuged twice at 10,000 rpm for 1 minute. In a new Zymo-SpinTM IIC column, 200 µl of DNA pre-wash buffer and 500 µL Fungal/Bacterial DNA wash buffer was added and centrifuged at 10, 000 rpm for 1 minute respectively. The column was then transferred into a clean 1.5 mL

microcentrifuge tube, after which 100 µL DNA elution buffer was added directly into the column matrix and centrifuged at 10,000 rpm for 30s to elute the DNA. Ultra-pure DNA was stored at -80°C for further use (Aremu and Babalola, 2015).

3.6.2 PCR amplification

The internal transcribed spacer (ITS) region of the rRNAfrom the extracted DNA of fungi was amplified by PCR with universal primers ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (Aremu and Babalola, 2015). The PCR reaction mixture was performed in a total volume of 50 µL containing 30-50 ng DNA, 100 mM of each primer, 0.05 U/µL *Taq* DNA polymerase, 4mM $MgCl_2$, and 0.4mM of each dNTP. The amplification reaction was done with a C1000 Touch thermal cycler (BioRad, USA). Method of Das *et al.* (2013) with slight modifications were employed for thermal cycling conditions. Initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 1 minute, annealing at 57°C for 1 minute and extension at 72°C for 2 minutes and a final extension at 72°C for 7 minutes. The PCR amplicons were analyzed by electrophoresis.

The 16S rDNA bacterial gene was amplified from genomic DNA obtained from bacterial cultures PCR with universal primer F_1 (5'-AGAGTTTGATCCTGGCTCAG-3') and R5 (5'-ACGGCTACCTTGTTACGACTT-3'). PCR was performed in a total volume of 50 µL containing 30-50 ng DNA, 100mM of each primer, 0.05 U/µL *Taq* DNA polymerase, 4 mM $MgCl_2$, and 0.4 mM of each dNTP. The amplification reaction was also performed with a C1000 Touch thermal cycler (BioRad, USA). The thermal cycling condition used was an initial denaturation at 96°C for 5 minutes, followed by 30 cycles of denaturation at 96°C for 45s, annealing at 56°C for 30s and extension at 72°C

for 2 minutes, followed by a final extension at 72°C for 5 minutes and a holding period at 4°C for infinite time.

3.6.3 Gel electrophoresis

The PCR amplicons were analysed by electrophoresis in 1 % (w/v) agarose gel with EtBr (Ethidium Bromide), 1 kb DNA ladders were loaded in 5 μL volumes, while 7 μL of the sample was loaded with 2 μL of loading dye. The gel was allowed to run for 2 h at 60 V. The gel results were visualized with a ChemiDoc™ MP System (Bio-Rad Laboratories, Hercules, CA, USA) to confirm the expected size of the product. The remaining PCR products were purified using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Germany) (Aremu and Babalola, 2015).

3.6.4 DNA sequencing

The sequencing of the purified PCR products were done at Inqaba Biotechnical Industrial (Pty) Ltd, Pretoria, South Africa with PRISM™ Ready Reaction Dye Terminator Cycle Sequencing Kit using the dideoxy chain termination method and electrophoresed with a model ABI PRISM® 3500XL DNA Sequencer (Applied Biosystems, Foster City, CA, USA) by following manufacturer's instructions.

3.6.5 Sequence analysis

ChromasLite version 2.33 software was used for the analysis of Chromatograms, (sense and antisense) resulting from sequencing reaction for good quality sequence assurance. The resulting chromatograms were edited using BioEdit Sequence Alignment Editor. After this, the resulting consensus 16S rDNA sequences obtained were Blast in the NCBI (www.ncbi.nlm.nih.gov) database with the Basic Alignment Search Tool (BLASTn) for homology in order to identify the probable organism in question (Altschul *et al.*, 1997). These sequences were deposited in the GenBank.

3.6.6 Phylogenetic analysis

The phylogenetic analyses based on the 16S rDNA gene were further used to characterize the organisms in order to establish relationships among them. The partial 16S rDNA sequences obtained were utilized in the search of reference nucleotide sequence available in NCBI GenBank database using BlastN algorithm (Altschul *et al*., 1997). Mafft version 7.0 was employed in the multiple alignment of nucleotide sequences (Katoh and Toh, 2010) while trees were drawn based on character based method (Maximum Likelihood) for comparing set of data against set of models of evolution using MEGA 7 (Kumar *et al*., 2016). Putative chimeric sequences were identified using the Chimera Buster 1.0 software. Manipulation and tree editing were carried out using TreeView (Page, 1996).

3.7 Distribution of Hydrocarbon−Oxidizing Microorganisms (HOM)

The distribution of hydrocarbon oxidizing microbes was achieved from distribution maps that were prepared with Geographical Information System (GIS) to delineate the potential areas with high yields (Veena-Prasanna *et al.,* 2013). The concentration distribution maps of methane, ethane, propane and butane oxidizing microbes enumerated from the soil samples was plotted on geological map of the study area. The higher populations of these hydrocarbon oxidizing microbes was observed and used to delineate the hydrocarbon potential zones (Veena-Prasanna *et al*., 2013).

3.8 Biodegradation Studies

3.8.1 Evaluation of crude oil degradation potential of isolates by gravimetric analysis method

Gravimetric analysis method as reported by Chikere and Fenibo (2018) was used to determine the biodegradation potential of ten identified isolates. The stock culture of all the isolates were resuscitated using Lauria broth for 18 hours, 1 mL of the 18 hours

cultures was standardised to a turbidity equivalent to 0.5 McFarland standards with an absorbance of 0.08 nm at 660 nm using photoelectric colorimeter. Two millilitres (2 mL) of the prepared cultures were inoculated into 100 mL MSM broth supplemented with 1.9 g (2 % w/v) of Transniger pipeline crude oil (originally collected from Shell Petroleum Development Company and supplied by Prof. Chuma Okoro of the Department of Microbiology AE-FUNAI). The flasks were kept on a water bath shaker for 35 days at 27°C. After every 7 days of incubation, dublicate flasks were taken out for gravimetric analysis. Optical Density (O.D) of the oil medium was measured on UV spectrophotometer (model 100vis spectrophotometer Buck Scientific USA) at 620 nm for growth kinetics. To separate the residual Transniger pipeline crude oil from the medium, 5 mL of chloroform was added to the flask and the mixture was separated using separating funnel. Two layers were formed (Appendix G), the upper layer was the oil while lower layer was the broth. The oil from the upper layer was collected in the pre-weighed Petri plate. The percentage of oil degradation was determined using Equation 3.5 (Chikere and Fenibo, (2018).

$$\% \text{ degradation} = \frac{\text{Amount of oil degraded}}{\text{Amount of oil added in the media}} \times 100 \qquad (3.5)$$

Where:

Weight of residual oil = weight of beaker containing extracted oil – weight of empty beaker

Amount of oil degraded = Weight of oil added in media – weight of residual oil.

3.8.2 Evaluation of crude oil degradation potential of isolates using Gas Chromatography-Mass Spectrophotometry (GC-MS)

After the period of 7 days and 35 days, the residual crude oil from each flask was extracted using dichloromethane (5 mL). The extracted crude oil with the solvent was kept open on the laboratory bench for 45 minutes, to allow all solvent to evaporate, then Anhydrous sodium sulphate was added to the extracted residual crude oil to dry off any left over water and reagents, thereafter, 0.5 mL of the extracted residual crude oil in a cryogenic storage vial was taken to Environmental Laboratory of International Energy Services Limited, Portharcourt, Nigeria, for analysis.

Biodegradation potential using GC-MS was analysed according to the method employed by Environmental and Laboratory Services of International Energy Services Limited (**IES-ELS**) (2019). One microliter (1 µL) of the extracted residual crude oil and standards (mixture of aliphatic and aromatic compounds) were transferred into the gas chromatography vial, which was introduced into the gas chromatography by direction injection using helium as the carrier gas. The samples were analysed using gas chromatography (GC-FID) fitted with a split-less injector, a fused silica capillary column (HP:5MS, 30mx0.25mm x 0.25µm). The initial temperature for step 1 was at 40°C for 2mins which was increased at step 2 to 310°C at 16 min at a rate of 10°C/min. Injector and detector temperature were at 270ºC and 320ºC respectively. The components were separated via gas chromatography and detected with FID. Identification of the compound was achieved by comparing a query mass spectrum with reference mass spectra in a library via spectrum matching. The concentrations of the TPH were determined using Equation 3.6 (IES-ELS, 2019).

Cs = (Cc X V X Z) / Vv (3.6)

Where:

Cs = concentration of TPH (ppm)

Cc = concentration from calibration curve in ppm

z   =   dilution factor

v   =   volume of final extract (mL)

Vv   = Volume of sample extracted (mL)

3.9 Statistical Data analysis

Two-way analysis of variance (ANOVA) using SPSS version 20.0 was used to determine the statistical significance difference in mean ($p < 0.05$) of data generated. Data was presented as mean $\pm$ standard error (SE). The graphs were plotted using Sigma Plot version 14. Mafft version 7.0 was employed in the multiple alignment of nucleotide sequence, MEGA 7 software was used for phylogenic relatedness and the trees were drawn based on character based method, the tree editing were carried out using TreeView.

CHAPTER FOUR

4.0   **RESULTS AND DISCUSSION**

4.1 Results

4.1.1 Physical and Chemical Properties of Soil and Water Samples

4.1.1.1 The physical and chemical properties of soil samples obtained from Ahoko study sites

Physicochemical properties of soil in samples collected from Ahoko study sites revealed that samples, despite being from same location, possessed varying properties. pH value ranged from 5.58 to 7.05 showing that the soil was slightly acidic to neutral limit, total organic carbon 0.41 % to 1.18 %, and conductivity 2.60 µ∫/cm to 12.0 µ∫/cm. It was observed that the total organic carbon, conductivity, total pore space, base saturation

and available phosphorus for site A were high (1.18 %, 12 µ∫/cm, 129 %, 66.03 % and 12.06 mg/g respectively) as compared to the other sites (Table 4.11a and 4.1b).This is is an indication that site A has a better soil characteristics for oil bearing site. Calcium and ECEC were high and significantly (p<0.05) different across the sampling sites. Textural class for site A, B and C were Loamy sandy while site C was sandy loamy. However, the components (sand, silt, clay) were all significantly different (p<0.05) across the sites (Table 4.1a and 4.1b).

Table 4.1a: Physicochemical properties of soil in Ahoko

Parameters/ Site	Soil samples			
	Site A	Site B	Site C	Site D
pH	$5.58 \pm 1.00^{bc*}$	5.95 ± 0.05^{bc}	7.05 ± 0.05^{c}	6.15 ± 0.10^{bc}
TOC (%)	1.18 ± 0.00^{ab}	0.75 ± 0.10^{a}	0.90 ± 0.01^{a}	0.41 ± 0.02^{a}
Nitrogen (%)	0.90 ± 1.00^{ab}	0.75 ± 0.05^{a}	0.90 ± 0.01^{a}	0.41 ± 0.00^{a}
Conductivity (µ∫/cm)	12.0 ± 3.50^{cd}	2.60 ± 0.85^{b}	4.10 ± 0.11^{abc}	3.3 ± 0.15^{bc}
SAR	0.13 ± 1.00^{a}	2.55 ± 0.10^{b}	0.14 ± 0.00^{a}	0.12 ± 0.00^{a}
TPS (%)	129.3 ± 0.00^{g}	121.8 ± 1.00^{g}	112.34 ± 0.5^{f}	107.5 ± 3.00^{ef}
Bulk density (g/cm^3)	1.01 ± 0.10^{ab}	1.14 ± 0.01^{ab}	1.34 ± 0.01^{ab}	1.32 ± 0.00^{ab}
Particle density	2.27 ± 0.00^{ab}	2.22 ± 0.00^{ab}	2.30 ± 0.10^{b}	2.39 ± 0.01^{a}
Available P (mg/kg)	12.06 ± 0.00^{cd}	0.71 ± 0.11^{a}	4.84 ± 0.50^{c}	2.65 ± 0.10^{ab}
ECEC (Cmc)	3.71 ± 0.10^{bc}	4.36 ± 0.50^{c}	2.71 ± 0.00^{b}	2.73 ± 0.10^{ab}
Mg^{2++}(cmol/kg)	0.6 ± 0.10^{a}	0.80 ± 0.05^{a}	0.81 ± 0.05^{a}	1.10 ± 0.02^{a}

Key: TOC = Total organic carbon, SAR= Sodium Adsorption Ratio, TPS = Total pore space Values are mean ± standard error of triplicate determinations. *Values with different alphabets are significantly different (p<0.05) between means along the column. Site A, B C and D are alphabetical codes used to represent the points where samples were collected (referring to soil samples).

Table 4.1b: Physicochemical properties of soil in Ahoko

Parameters/ Site	Soil samples			
	Site A	Site B	Site C	Site D
Ca^{2+}(cmol/kg)	$2.20 \pm 0.05^{b*}$	2.80 ± 0.10^{b}	1.70 ± 0.00^{a}	2.40 ± 0.01^{ab}
K^{2++}(cmol/kg)	0.05 ± 0.00^{a}	0.08 ± 0.10^{a}	0.021 ± 0.15^{a}	0.05 ± 0.25^{a}
Na^{2++}(cmol/kg)	0.043 ± 0.00^{a}	3.47 ± 0.01^{b}	0.17 ± 0.10^{a}	0.19 ± 0.11^{a}
Exchangeable acid $^{+}$(cmol/kg)	2.28 ± 0.58^{b}	2.21 ± 0.00^{ab}	0.03 ± 0.00^{a}	2.01 ± 0.01^{ab}
Base saturation (%)	66.03 ± 0.00^{ef}	57.74 ± 0.10^{de}	50.7 ± 0.01^{de}	65.60 ± 0.10^{ef}
ESP	5.59 ± 1.00^{bcd}	45.23 ± 0.00^{e}	5.59 ± 0.00^{bcd}	4.19 ± 0.00^{c}
Sand (%)	84.3 ± 0.10^{f}	86.10 ± 0.12^{g}	65.20 ± 2.00^{ef}	78.9 ± 0.10^{ef}
Silt (%)	8.7 ± 1.00^{bcd}	7.80 ± 0.21^{c}	20.51 ± 0.11^{de}	9.70 ± 0.50^{cd}
Clay (%)	7.0 ± 0.06^{c}	6.10 ± 0.04^{bc}	14.29 ± 0.15^{d}	11.40 ± 0.1^{bcd}
Textural class	Loamy sand	Loamy sand	Sandy Loamy	Loamy sand

Key: Values are mean ± standard error of triplicate determinations. *Values with different alphabets are significantly different (p<0.05) between means along the column. Site A, B C and D are alphabetical codes used to represent the points where samples were collected (referring to soil samples)

The physicochemical properties of water samples collected from Ahoko study sites revealed the pH for the water samples were slightly acidic (6.69) to alkaline (7.80), very

high calcium content (90.0mg/L), low total organic carbon (0.35%), nitrogen (0.01%) and bod 8.6. the water was hard, having 146mg/h of Mg salts (Table 4.2).

Table 4.2: Physicochemical properties of water samples obtained from Ahoko and Patishabakolo

Parameters	Water samples	
	Ahoko (Mini river)	Patishabakolo (Kumi)
pH	7.80 ±0.17	6.69±0.10
Temperature (^{o}C)	27±1.01	25±2.5
Total Organic Carbon (TOC) (%)	0.35±0.0	1.25 ± 0.01
Nitrogen (N_2) (%)	0.01±0.0	0.82 ± 0.00
Conductivity ($\mu\int$/cm)	-	0.1±0.05
Magnesium (Mg) hardness (mg/L)	14.6±0.19	9.0±0.19
Calcium (Ca^{2+}) (mg/L)	90.0±4.7	2.0 ± 0.10
Potassium (K^{2+}) (mg/L)	9.6±0.13	0.8 ± 0.01
Sodium (Na^{2+}) (mg/L)	4.0±0.01	2.21 ± 0.11
Exchangeable Acid (mg/L)	1.15±0.01	-
Chloride (Cl)	56.73±0.99	-
Total alkalinity	60.0±3.5	-
Biochemical Oxygen Demand (BOD)	8.6±0.1	2.83±0.4

Key: (mg/L) = milligrams per litre**;** % = percentage; - = absent; ^{o}C= degree Celsius

4.1.1.2 The physical and chemical properties of soil and water from Patitiabakolo

The physicochemical properties of water (Table 4.2) and soil (Table 4.3) from Patishabakolo revealed that the water had pH of 6.69 and very low Ca, Na and Mg salts (Table 4.2) while the soil is slightly alkaline (7.57) with varying carbon contents from 1.75% to 8.78% (Table 4.3a). The total pore spaces were not significantly different and the soil conductivity across the sampling points was low (0. 10 µ∫/cm to 2.0 µ∫/cm). The textural class for D2S3 and D2S4 are clayey while riverside, borehole and D1S4 are sandy (Table 4.3b).

Table 4.3a: Physicochemical properties of soil in Patishabakolo

Parameters/ Site	Soil Samples				
	Borehole	Riverside	D1S4	D2S3	D2S4
pH	7.57 ±1.00^{d*}	7.09 ±0.0^{cd}	7.09±0.5^{cd}	7.15±0.1^{d}	6.53±1.1^{c}
TOC (%)	2.63±1.00^{b}	8.78 ±1.00^{d}	3.80±0.00^{c}	3.21±0.01^{c}	1.75±0.01
Nitrogen	0.126±1.00^{a}	0.81 ±0.01^{a}	0.154±0.1^{a}	0.11±0.1^{a}	0.10±0.1a
Conductivity (µ∫/cm)	2.0±0.50^{abc}	0.60±0.12^{a}	0. 10±0.1^{a}	1.3±0.13^{ab}	0. 10±0.00a
SAR (%)	0.13±1.00^{a}	2.55 ±0.12^{bc}	0.14±0.0^{a}	0.12±0.01^{a}	0.12±0.00a
TPS (%)	160.8±1.00^{gh}	174.34 ±2.50^{h}	178.5±2.7^{h}	192.3±1.15^{h}	176.9±1.92^{h}
Bulk density (g/cm3)	1.38 ± 0.10^{ab}	1.65 ±0.00^{b}	1.81±0.13^{b}	2.21±0.28^{bc}	1.74±0.11^{ab}
Particle density	2.27± 0.01^{b}	2.22 ±0.33^{b}	2.30±0.01^{b}	2.39±0.23^{b}	2.27±0.12^{b}

Key: TOC= Total organic carbon, SAR= Sodium Adsorption Ratio, TPS =Total pore space, D1S4= Day 1 Sample 4, Borehole sample, D2S3= Day 2 Sample 3, D2S4= Day 2 Sample 4, River side sample

(referring to soil samples), Values are mean ± standard error of triplicate determinations. *Values with different alphabets are significantly different (p<0.05) between means along the column.

Table 4.3b: Physicochemical properties of soil in Patishabakolo

Parameters/ Site	Soil Samples				
	Borehole	Riverside	D1S4	D2S3	D2S4
Available P (mg/kg)	26.83 ± 0.50^f	10.71 ± 1.51^e	9.54 ± 1.8^d	2.65 ± 0.02^{abc}	0.85 ± 0.00^a
ECEC (Cmc)	2.71 ± 0.10^{bc}	7.66 ± 1.01^d	2.71 ± 0.00^{bc}	3.73 ± 0.4^c	2.93 ± 0.1^c
Mg^{2++}(cmol/kg)	0.6 ± 0.10^a	0.90 ± 0.02^{ab}	0.8 ± 0.01^a	1.10 ± 0.00^b	0.70 ± 0.6^a
Ca^{2+}(cmol/kg)	1.80 ± 0.05^{ab}	2.80 ± 0.22^b	1.70 ± 0.7^{ab}	2.40 ± 0.05^b	1.90 ± 1.0^a
K^{2++}(cmol/kg)	0.00 ± 0.00^a	0.48 ± 0.01^a	0.021 ± 0.0^a	0.05 ± 0.01^a	0.16 ± 0.00^a
Na^{2++}(cmol/kg)	0.143 ± 0.00^a	3.47 ± 1.00^b	0.15 ± 1.0^a	0.16 ± 0.7^{ab}	0.14 ± 0.13^a
Exchangeable Acid (cmol/kg)	0.03 ± 0.58^a	0.02 ± 0.00^a	0.035 ± 0.0^a	0.015 ± 0.00^a	0.03 ± 0.1^a
Base saturation (%)	99.03 ± 0.00^{fg}	99.74 ± 6.20^{efg}	98.7 ± 4.9^g	99.60 ± 2.64^{fg}	98.98 ± 2.6^g
ESP	5.59 ± 1.00^{bc}	45.23 ± 0.99^f	5.59 ± 0.06^{bc}	4.19 ± 1.1^c	4.75 ± 0.01^c
Sand (%)	92.8 ± 0.10^{fg}	88.90 ± 1.12^f	81.20 ± 2.5^{ef}	83.1 ± 4.9^f	84.5 ± 1.7^f

Silt (%)	1.10 ± 1.00^a	$6.30 \pm 0.08b^c$	8.20 ± 0.4^d	6.70 ± 1.9^{cd}	6.90 ± 0.23^d
Clay (%)	6.10 ± 0.06^{cd}	4.80 ± 0.11^c	10.60 ± 0.1^e	10.10 ± 0.9^{de}	8.60 ± 0.04^{de}
Textural class	Sandy soil	Sandy Loamy	Sandy loamy	Clayey sandy	Clayey sandy

Key: TOC= Total organic carbon, D1S4= Day 1 Sample 4, Borehole sample, D2S3= Day 2 Sample 3, D2S4= Day 2 Sample 4, River side sample (referring to soil samples), *Values are mean ± standard error of triplicate determinations. Values with different alphabets are significantly different (p<0.05) between means along the column.

4.1.2. Microbiological Qualities of the Soil and Water samples

4.1.2.1 Total aerobic heterotrophic bacteria in soil and water from Ahoko

The counts of bacteria in the soil from Ahoko study area varied with respect to the months of sampling and sites. The counts ranged from 1.17×10^6 CFU/g (March) to 2.67×10^6 CFU/g (April) for site A (SA), 0.95×10^6 CFU/g (August) – 2.88×10^6 CFU/g (April) for site B (SB), 0.55×10^6 CFU/g (August), 2.8×10^6 CFU/g (May) for site C (SC) and 0.38×10^6 CFU/g (February) – 2.02×10^6 CFU/g (October) for site D (SD) (Table 4.4). It was observed that the bacterial counts increased from April to the end of the study in all sampling sites (A-D). There were no significant differences (p>0.05) in counts in March, July and August in all soil samples analysed.

Similarly, for the water samples, high counts of 5.15×10^4 CFU/mL, 5.20×10^4 CFU/mL, and 5.01×10^4 CFU/mL were obtained in rainy season (July, August, and September, respectively), with no significant difference (p>0.05). However, there were low counts in dry season (November and February, 2017) (Table 4.4).

Table 4.4: Bacterial (CFU/g x10^6) counts in Ahoko soil and water samples analysed

Months	Site A	Site B	Soil Samples Site C	Site D	Water (x10^4 CFU/mL)
February (2017)	1.4±0.3^{ab*}	1.05±0.00^a	0.6±0.025^a	0.38±0.10^a	2.82 ±0.1^e
March	1.17±0.05^a	0.99±0.03^a	0.57±0.029^a	0.67±0.11^a	2.89±1.33^e
April	2.67±0.2^e	2.88±0.33^e	1.62±0.075^{ab}	1.44±0.2^{ab}	4.15±1.2^{ef}
May	1.71±0.25^b	0.97±0.06^a	2.8±0.05^e	1.5±0.00^b	4.12±2.01^{ef}
June	1.75±0.38^{bc}	1.03±0.06^a	0.8±0.075^a	0.77±0.025^a	4.10±0.8^{ef}
July	1.18±0.28^a	1.0±0.05^a	0.58±0.1^a	0.95±0.05^a	5.15±2.05^g
August	1.20±0.26^a	0.95±0.00^a	0.55±0.05^a	1.03±0.025^a	5.20± 1.63^g
September	1.63±0.15^a	1.0±0.05^a	1.09±0.00^a	1.5±0.1^{ab}	5.01± 1.21^g
October	2.53±0.1^{de}	2.27±0.24^{abcd}	1.63±0.3^{ab}	2.02±0.3^{bc}	4.27± 1.0^f
November	2.15±0.21^{cd}	2.05±0.1^{bcd}	1.90±0.02^{bc}	1.60±0.09^{ab}	3.61 ±0.7^{ef}
December	2.17±0.14^{cd}	1.52±0.06^{ab}	1.42±0.07^{ab}	1.52±0.1^b	2.95 ±0.01^e
January 2018	1.75±0.05^b	1.32±0.021^a	1.42±0.07^{ab}	1.20±0.19^a	2.75 ±0.25^e

Values are mean ± standard error of triplicate determinations. *Values with different alphabets are significantly different (p<0.05) between means along the column. Site A, B C and D are alphabetical codes used to represent the points where samples were collected (referring to soil samples).

4.1.2.2 Total fungi in soil and water samples from Ahoko

The total number of fungi in the soil samples ranged from 3.16×10^3 CFU/g to 6.60×10^3 Cfu/g; 1.27×10^3 CFU/g – 4.51×10^3 CFU/g; 2.22×10^3 CFU/g – 11.0×10^3 CFU/g; 2.27×10^3 CFU/g – 6.6×10^3 CFU/g for site A, site B, site C and site D respectively.

It was observed that the counts were not consistent following the month and season of the year, December, January and February had the least counts across the study site, while May, June and July had high counts of the fungi (Table 4.5)**.** More so, there was an increase in the number of fungi from 3.05×10^3 CFU/g, 1.86×10^3 CFU/g, 3.71×10^3 CFU/g and 2.87×10^3 CFU/g (March) to 3.51×10^3 CFU/g, 2.19×10^3 CFU/g 4.35×10^3 CFU/g and 3.36×10^3 CFU/g (April) within the sites A, B, C and D respectively, this increase continued consistently till July and a slight decrease was observed in August. this maybe as a result in the break of rainfall popularly known as August break which lead to the decrease in the moisture content of the soil and thereby affect the growth of the fungi (Table 4.5). For the water samples the counts ranged from 1.91×10^3 CFU/mL (December) to 3.51×10^3 CFU/mL (July). The counts increased from March to September after which the counts decreased till the end of the sampling period (January, 2018); the decease being drastic in December. The results were significantly different ($p < 0.05$) between months (Table 4.5).

Table 4.5: Total fungal counts (CFU/g x10^3) in Ahoko soil and water samples analysed

| Months | Soil sample | | | | Water |
	Site A	Site B	Site C	Site D	CFU/mL x10^3
February (2017)	3.16±0.00^b	1.86±0.15^{a*}	3.9±0.17^{bc}	2.98±0.7^{ab}	2.64±0.01^{ab}
March	3.05±0.01^b	1.86±0.00^a	3.71±0.2^{bc}	2.87±0.05^{ab}	3.35±0.50^{bc}
April	3.51±0.00^{bc}	2.19±0.1^{ab}	4.35±0.01^c	3.36±0.02^{bc}	3.30±0.11^{bc}
May	6.6±0.1^{de}	2.34±0.06^b	11.0±4.72^{defg}	6.4±0.01^{de}	3.13±0.05^b
June	6.01±0.00^d	4.14±0.2^c	9.8±5.0^f	6.6±0.70^e	3.03±0.50^b
July	5.45±0.02^{cd}	4.51±0.06^{cd}	9.85±0.04^{fg}	6.6±0.70de	3.51±0.25^{bc}
August	5.01±0.5^{bcd}	4.01±0.70^c	9.19±1.27^f	6.07±0.99^e	3.50±0.02^{bc}
September	5.4±0.02^{cd}	3.47±0.27^{abc}	9.05±2.2^f	5.97±0.15^{de}	3.43±0.40^{abc}
October	6.0±0.7^{abcd}	3.42±0.50^{ab}	9.05±1.0^f	6.16±0.1^{de}	2.67±0.15^{ab}
November	5.4±0.33^{cd}	3.0±0.70^b	5.3±0.7^d	4.57±0.13^{abcd}	2.37±0.50^{ab}
December	4.71±0.06^d	2.5±0.03^b	3.65±0.33^c	3.5±0.25^c	1.91±0.04^{ab}
January (2018)	3.17±0.25^b	1.27±0.33^a	2.22±0.25^{ab}	2.27±0.25^{ab}	2.26±0.50^{ab}

Key: Values are mean ± standard error of mean of triplicate determinations. *Values with different alphabets are significantly different ($p<0.05$) between means along the column. Site A, B C and D are alphabetical codes used to represent the points where samples were collected (referring to soil samples).

4.1.2.3. Specific hydrocarbon-utilizing microorganisms from Ahoko

The enumeration of specific hydrocarbon utilizers from water samples was carried out from February 2017 to January 2018 and it was observed that Methane-oxidizing microbes (MOM) in the sample, decreased from 3.1×10^2 CFU/mL (February) to 3.0×10^2 CFU/mL (March), similar decrease was observed among the other groups of organisms (ethane oxidizing microbes, EOM; propane-oxidizing microbes, POM and butane-oxidizing microbes, BOM). However, among all groups there was an increase in microbial counts in April except for BOM which decreased from 1.2×10^2 CFU/mL to 1.0×10^2 CFU/mL and increased gradually to 3.0×10^2 CFU/mL in October, while POM were not significantly different ($p > 0.05$) within the peak of the rainy season (July – October) (Table 4.6).   Similar results were obtained for EOM with the exception of September where the EOM counts were high. The counts of MOM and EOM were significantly different ($p < 0.05$) in November, December and January as compared to counts of POM and BOM where no significant differences ($P > 0.05$) in counts existed (Table 4.6).

Table 4.6: Counts (x10^2 CFU/mL) of specific hydrocarbon utilizers in water samples from Ahoko

Months	Hydrocarbon gas oxidizers			
	MOM	EOM	POM	BOM
February (2017)	3.1±0.21^{abc*}	2.3±0.12^{bc}	2.0±0.13^b	1.4±0.5^{ab}
March	3.0±1.1^{abc}	2.0±0.00^b	2.0±0.15^b	1.2±0.02^a
April	4.8±1.0^{def}	4.6±0.12^e	3.4±0.24^{bcd}	1.0±0.21^a
May	4.5±0.31^e	4.0±0.00^{ae}	3.5±1.0^{cd}	2.0±0.05^b
June	4.3±0.01^e	4.0±0.5^{de}	3.5±0.01^d	2.5±0.11^{bc}
July	4.2±0.01^e	3.67±0.05^{cd}	3.4±0.4^{cd}	2.6±0.10^c
August	4.2±0.11^e	3.67±0.0^{cd}	3.4±0.2^{cd}	2.6±0.25^c
September	4.0±0.05^{de}	4.0±0.02^e	3.4±0.00^{cd}	2.8±0.00^{bcd}
October	4.0±0.05^{de}	3.67±0.01^{cd}	3.4±0.00^{cd}	2.6±0.02^{ac}
November	3.9±0.05^{cde}	4.0±0.33^e	3.6±0.01^d	3.0±1.1^{bc}
December	3.6±0.01^{cd}	4.6±0.12^{def}	3.0±1.1^{bc}	2.4±0.1^{bc}
January(2018)	3.2±1.1^{bc}	3.67±0.01^{cd}	2.4±1.0^{bc}	2.0±0.01^{bc}

Key: MOM (Methane - oxidizing microbes); EOM (Ethane - oxidizing microbes). POM (Propane - oxidizing microbes); BOM (Butane- oxidizing microbes); Values are Mean of triplicate determinations; *Values with different alphabets are significantly different (p < 0.05) between means within the column. Site A, B C and D are alphabetical codes used to represent the points where samples were collected (referring to soil samples).

The results of methane -oxidizing microbes (MOM) as shown in Figure 4.1 revealed that Site A had the highest counts throughout the study period with no significant difference (P>0.05) in the months sampled from February 2017 to January 2018. It was also observed that the counts of MOM in site C consistently decreased from March

$(0.17 \times 10^2$ CFU/g) to October $(0.03 \times 10^2$ CFU/g) and remainded unchanged at 0.02×10^2 CFU/g from November to January (Figure 4.1).
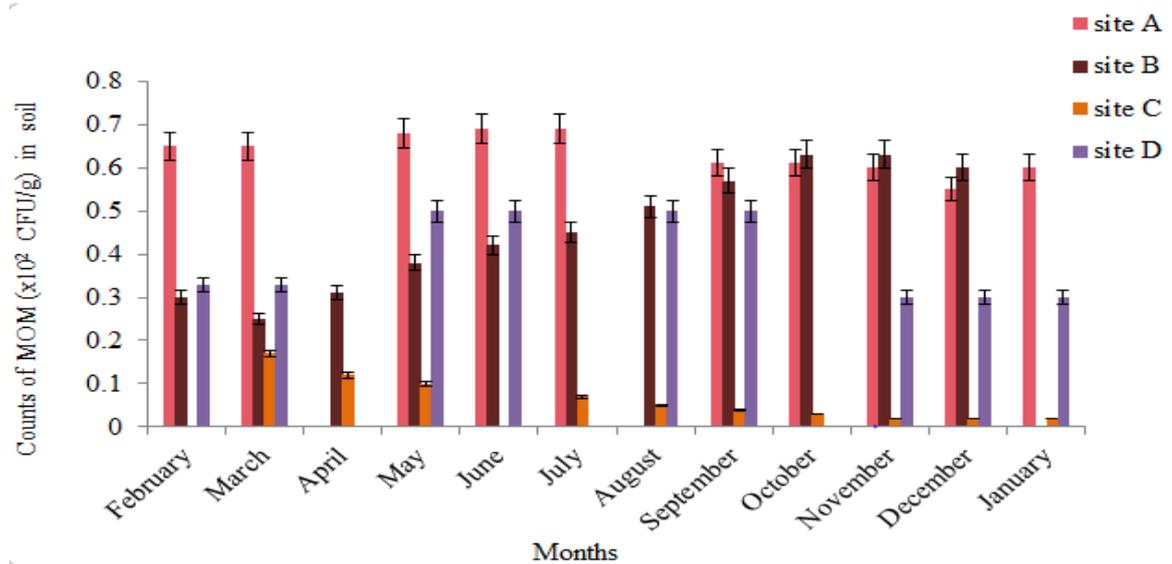
Figure 4.1: Counts of methane-oxidizing microbes (MOM) in soil
Key: Site A, B C and D are alphabetical codes used to represent the points where samples were collected (referring to soil samples).

The results for the ethane-oxidizing microbes ranged from 0.42×10^2 CFU/g to 1.33×10^2 CFU/g for Site A, 0.3×10^2 CFU/g – 0.92×10^2 CFU/g, 0.05×10^2 CFU/g – 0.67×10^2 CFU/g, and $0.00 – 0.8 \times 10^2$ CFU/g for Site B, C and D respectively (Figure 4.2). The results revealed that the seasonal changes within the period of study affected the microbial growth. For site A, the highest microbial count was observed in February $(1.33 \times 10^2$ CFU/g), this was closely followed by 1.2×10^2 CFU/g in March, 1.07×10^2 CFU/g in November and 1.12×10^2 CFU/g in January (Figure 4.2). This implies that the growths were favoured during the dry season rather than wet season as observed in site C, an observation that may be associated with the nature of the soil type.
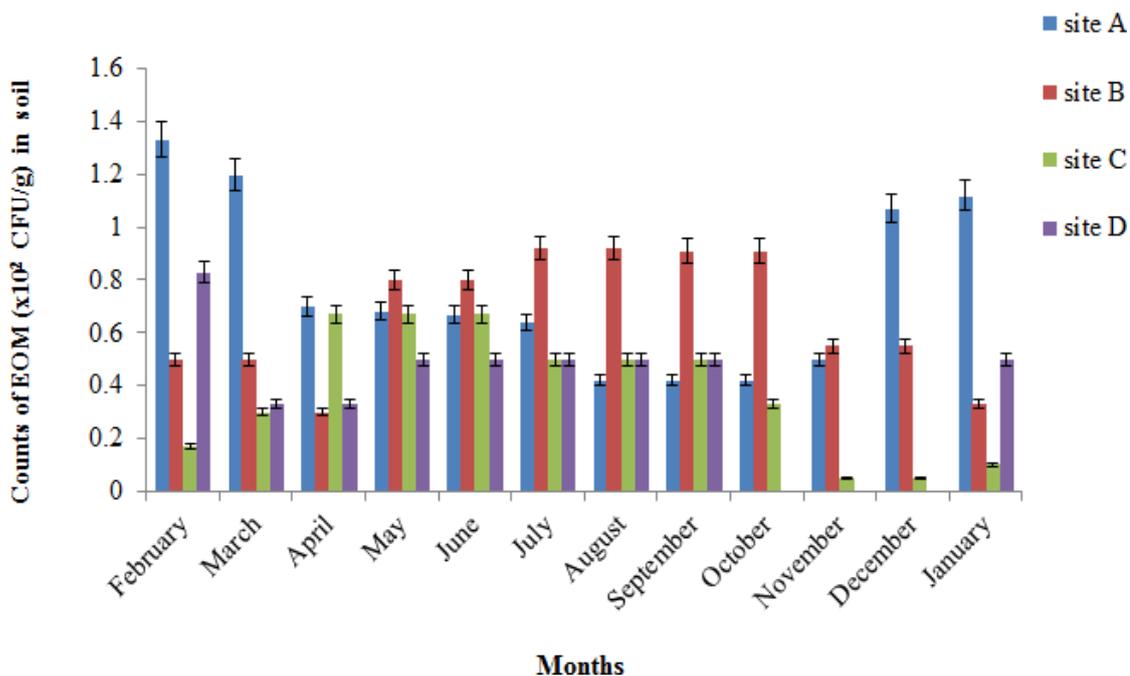
Figure 4.2: Counts of Ethane-oxidizing microbes (EOM) in soil
Key: Site A, B C and D are alphabetical codes used to represent the points where samples were collected
(referring to soil samples).

The counts of propane-oxidizing microbe were carried out from February 2017 to January 2018. It was observed that there were variations in counts with respect to months and seasons of the year (Figure 4.3). The counts ranged from 0.04×10^2 CFU/g to 0.3×10^2 CFU/g for Site A, 0.0×10^2 CFU/g $-$ 0.52×10^2 CFU/g, 0.00×10^2 CFU/g $-$ 1.5710^2 CFU/g, and $0.00 - 0.08 \times 10^2$ CFU/g for Site B, C and D respectively. There were no significant differences ($P>0.05$) between the counts from April to September for site C (Figure 4.3).
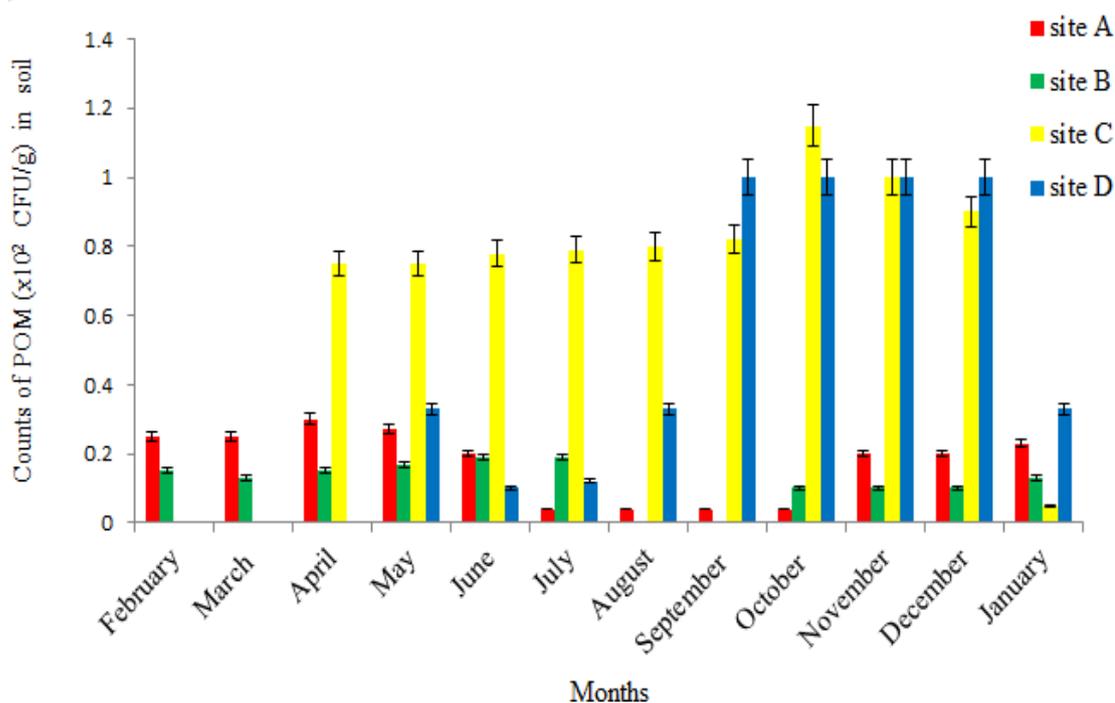
Figure 4.3: Counts of Propane-oxidizing microbes (POM) in soil
Key: Site A, B C and D are alphabetical codes used to represent the points where samples were collected
(referring to samples)

The count of Butane-oxidizing microbe was carried out from February 2017 to January 2018. It was observed that there were sparing number with respect to month and seasons of the year (Table 4.7). The counts ranged from 0.00×10^2 CFU/g to 0.2×10^2 CFU/g for Site A, 0.0×10^2 CFU/g – 0.15×10^2 CFU/g, 0.00×10^2 CFU/g – 1.3210^2 CFU/g, and 0.00 – 0.1×10^2 CFU/g for Site B, C and D respectively. There were no significant differences ($p > 0.05$) between the counts within and across the group. However, it was observed that the growth of BOM was more pronounced from August to December (Table 4.7).

Table 4.7: Counts (10^2 CFU/g) of Butane-oxidizing microbes (BOM) in soil

Months	Samples			
	Site A	Site B	Site C	Site D
February (2017)	0.15 ± 0.00^{ab}	$0.15\pm0.001^{ab*}$	NG	NG
March	$0.1\pm0.001a$	0.13 ± 0.002^{a}	NG	NG
April	NG	NG	NG	NG
May	NG	NG	NG	NG
June	NG	NG	NG	NG
July	NG	NG	NG	NG
August	0.04 ± 0.00^{a}	NG	0.22 ± 0.00^{a}	0.33 ± 0.00^{b}
September	0.04 ± 0.001^{a}	NG	0.32 ± 0.00^{b}	0.1 ± 0.00^{ab}
October	$0.04\pm0.001a$	0.1 ± 0.00^{ab}	NG	0.01 ± 0.00^{a}
November	0.2 ± 0.00^{b}	0.1 ± 0.0011^{ab}	0.01 ± 0.00	0.01 ± 0.00^{a}
December	0.2 ± 0.00^{b}	0.1 ± 0.003^{ab}	0.15 ± 0.01	0.1 ± 0.011^{a}
January (2018)	NG	NG	NG	NG

Key: NG=No growth. Values are Means of triplicate determinations. *Values with different alphabets are significantly different (p< 0.05) between means within the column. Site A, B C and D are alphabetical codes used to represent the points where samples were collected (referring to soil samples).

4.1.2.4. Total aerobic heterotrophic bacteria in soil and water samples from Patishabakolo

The total aerobic heterotrophic bacteria counts ranged from $1.8\text{x}10^6$ CFU/g (January) to $3.51\text{x}10^6$ CFU/g (November), $3.1\text{x}10^6$ CFU/g (March) – $6.7\text{x}10^6$ CFU/g (October), $1.3\text{x}10^6$ CFU/g (January) – $3.50\text{x}10^6$ CFU/g (October), $1.55\text{ x}10^6$ CFU/g (January) – $3.50\text{ x}10^6$ CFU/g (November) and $0.08\text{x}10^6$ CFU/g (January) – $1.0\text{ x}10^6$ CFU/g (August) for Borehole, Riverside, D1S4, D2S3 and D2S4 soil samples respectively. It

was also observed that the riverside samples had more counts of bacteria than other samples, both in the dry and rainy seasons (Table 4.8). Similarly, the counts for water sample ranged from 1.20 $\times 10^6$ CFU/mL (December) to 1.81 $\times 10^6$ CFU/mL (October) (Table 4.8).

Table 4.8: Total aerobic heterotrophic bacterial counts (CFU/g x10^6) in soil and water samples from Patishabakolo

	Sample					
	Borehole	Riverside	D1S4	D2S3	D2S4	Water (kumi)
Months						
February (2017)	1.9±0.21^{a*}	3.9±0.9^d	1.35±0.04^a	1.62±0.10^b	0.00±0.00^a	0.00±0.00^a
March	1.55±0.05^a	3.1±0.41^{cd}	1.4±0.04 ^{a}	2.19±0.00^{bc}	0.00±0.00^a	1.30±0.04^a
April	1.73±0.02^b	3.5±0.01^d	1.7±0.02^b	2.4±0.40^{bc}	0.7±0.02^a	1.38±0.40^a
May	2.12±0.1^c	4.3±0.3^d	2.0±0.1^b	3.09±0.6^{cd}	0.7±0.02^a	1.41±0.01^a
June	2.65±0.1^{ab}	5.3±0.40^e	2.2±0.51^b	1.8±0.21^a	0.8±0.012^{ab}	0.00±0.00^a
July	2.9±0.23^c	5.9±0.25^e	2.53±0.3^c	2.75±0.02^{abc}	0.95±021^{ab}	0.00±0.00^a
August	2.8±0.02^{abc}	5.6±0.07^e	2.50±0.10^c	2.65±0.13^{bc}	1.0±0.00^{ab}	1.73±0.05^b
September	2.7±0.4^{abc}	5.4±0.40^{de}	2.55±0.9^c	2.62±0.13^{bc}	0.5±0.05^a	1.70±0.1^b
October	3.35±0.11^{cd}	6.7±2.72^e	3.50±0.3^d	2.80±0.02^{abc}	0.00±0.00^a	1.81±0.06^{ab}
November	3.51±0.7^{cd}	3.54±0.41^d	3.50±0.15^d	3.50±0.00^b	0.00±0.00^a	1.78±0.15^b
December	1.9±0.21^a	3.7±0.1^d	2.1±0.00^{bc}	2.0±0.00^b	0.00±0.00^a	1.20±0.02^a
January 2018	1.8±0.31^{ab}	5.2±0.01^e	1.3±0.04 ^{a}	1.55±0.05^b	0.08±0.01^a	1.23±0.01^a

Key: D1S4= Day 1 Sample 4, Borehole sample, D2S3= Day 2 Sample 3, D2S4= Day 2 Sample 4, River side sample (referring to soil samples), Values are mean ± standard error of mean of triplicate determinations. *Values with different alphabets are significantly different (p<0.05) between means along the column.

4.1.2.5. Total fungi in Soil and water samples from Patishabakolo

The total fungi (TF) counts ranged from 2.79×10^3 CFU/g (January) to 10.36×10^3 CFU/g (August), 3.0×10^3 CFU/g (February) – 14.20×10^3 CFU/g (August), 1.54×10^3 CFU/g (February) – 4.55×10^3 CFU/g (August), 1.65×10^3 CFU/g (January) – 4.09×10^3 CFU/g (June), and 2.29×10^3 CFU/g (November) – 3.37×10^3 CFU/g (July) for Borehole, Riverside, D1S4, D2S3 and D2S4 samples respectively. It was also observed that the riverside soil samples had more counts of fungi than other samples both in the dry and rainy seasons (Table 4.9). Similarly, the counts for water sample ranged from 0.00×10^3 CFU/mL (June, July) to 2.41×10^3 CFU/mL (September) (Table 4.9).

Table 4.9: Total fungal counts (x10³ CFU/g) in soil and water samples analysed from Patishabakolo

Months	Borehole	Riverside	D1S4	D2S3	D2S4	Water (kumi)
February (2017)	3.43 ± 0.01^{ab}	3.0 ± 0.8^{a}	$1.54\pm0.07^{a*}$	2.79 ± 0.69^{bc}	2.71 ± 0.12^{ab}	1.94 ± 0.5^{a}
March	3.02 ± 0.5^{a}	4.33 ± 0.21^{bc}	2.58 ± 0.13^{b}	3.94 ± 0.19^{d}	3.21 ± 0.02^{c}	2.37 ± 0.01^{ab}
April	3.9 ± 0.01^{cd}	4.90 ± 0.33^{d}	2.75 ± 0.14^{abc}	3.85 ± 1.02^{cd}	3.17 ± 0.65^{c}	2.40 ± 0.4^{ab}
May	4.8 ± 0.24^{d}	$7.23\pm1.15d^{e}$	2.85 ± 0.14^{bc}	3.65 ± 0.18^{cd}	3.03 ± 0.23^{c}	2.21 ± 0.4^{b}
June	6.45 ± 0.32^{ef}	11.0 ± 3.8^{f}	2.17 ± 0.14^{b}	4.09 ± 2.0^{d}	2.99 ± 0.13^{bc}	0.00 ± 0.00^{a}
July	8.24 ± 2.41^{def}	$12.17\pm2.00f^{g}$	3.04 ± 0.51^{c}	4.06 ± 1.11^{d}	3.37 ± 0.27^{cd}	0.00 ± 0.00^{a}
August	10.36 ± 0.52^{efg}	14.20 ± 1.65^{g}	4.55 ± 0.23^{d}	3.96 ± 0.55^{abc}	3.32 ± 0.09^{cd}	2.40 ± 0.13^{bc}
September	7.69 ± 1.3^{e}	10.57 ± 0.53^{efg}	3.90 ± 1.0^{dc}	2.74 ± 0.02^{abc}	2.78 ± 0.14^{abc}	2.41 ± 0.11^{bc}
October	4.71 ± 0.24^{cd}	6.46 ± 2.72^{de}	3.28 ± 0.16^{c}	2.37 ± 0.51^{ab}	2.62 ± 0.13^{ab}	2.09 ± 0.06^{a}
November	3.85 ± 0.19^{cd}	$4.77\pm0\pm.4^{c}$	2.02 ± 0.01^{abc}	1.67 ± 0.08^{a}	2.29 ± 0.11^{bc}	1.84 ± 0.21^{a}
December	5.42 ± 1.7^{cde}	7.43 ± 0.8^{e}	2.99 ± 0.11^{bc}	2.87 ± 0.04^{b}	2.97 ± 0.05^{c}	1.71 ± 0.02^{a}
January (2018)	2.79 ± 0.14^{abc}	4.90 ± 0.8^{b}	1.72 ± 0.09^{ab}	1.65 ± 0.02^{a}	$2.72\pm0,15^{ab}$	1.88 ± 0.2

Key: D1S4= Day 1 Sample 4, Borehole sample, D2S3= Day 2 Sample 3, D2S4= Day 2 Sample 4, River side sample (referring to soil samples), Values are mean ± standard error of mean of triplicate determinations. *Values with different alphabets are significantly different (p<0.05) between means along the column.

4.1.2.6 Specific hydrocarbon−utilizing microorganisms from Patishabakolo

The total counts of methane oxidizing microbes (MOM) in water sample from Patitiabakolo ranged from 3.35×10^2 CFU/mL to 5.0×10^2 CFU/mL, the ethane-oxidizing microbes (EOM) ranged from 5.0×10^2 CFU/mL to 10.0×10^2 CFU/mL, the propane-oxidizing microbes (POM) ranged from 1.67×10^2 CFU/mL to 10.0×10^2 CFU/mL, while the counts for butane-oxidizing microbes (BOM) ranged from 1.67×10^2 CFU/mL (August) to 6.65×10^2 CFU/mL (Table 4.10). However, it was observed that microbial growth was not recorded in 4, 6, and 7 of the 12 months of study for MOM, EOM, POM, and BOM respectively (Table 4.10). The results revealed that there were significant differences ($p < 0.05$) between MOM and EOM.

Table 4.10: Counts (x10^2 CFU/mL) of specific hydrocarbon utilizers in water samples from Patishabakolo

| Months | Hydrocarbon-gas oxidizers | | | |
	MOM	EOM	POM	BOM
February (2017)	0.00±0.00^{a*}	5.00±0.33^c	5.00±0.00^c	0.00±0.00^a
March	0.00±0.00^a	5.00±0.00^c	0.00±0.00^a	0.00±0.00^a
April	3.35±0.57^{ab}	6.65±0.00^d	0.00±0.00^a	3.33±0.33^b
May	5.00±0.00^b	10.0±0.03^e	10.0±0.00^d	0.00±0.00^a
June	5.00±0.00^b	10.0±0.00^e	5.00±0.00^c	6.65±0.05^d
July	5.00±0.00^b	5.00±0.57^c	5.00±0.00^c	5.00±0.00^c
August	3.35±0.57^{ab}	0.00±0.00^a	0.00±0.00^a	1.67±0.01^{ab}
September	0.00±0.00^a	0.00±0.00^a	0.00±0.00^a	0.00±0.00^a
October	0.00±0.00^a	0.00±0.00^a	1.67±0.03^b	5.00±0.00^c
November	3.35±0.57^{ab}	0.00±0.00^a	0.00±0.00^a	0.00±0.00^a
December	0.00±0.00^a	5.00±0.00^c	0.00±0.00^a	5.00±0.00^c
January (2018)	0.00±0.00^a	6.65±0.92^d	0.00±0.00^a	0.00±0.00^a

Key: MOM (Methane-oxidizing microbes); EOM (Ethane oxidizing microbes); POM (Propane-oxidizing microbes); BOM (Butane-oxidizing microbes); Values are Mean of triplicate determinations. *Values with different alphabets are significantly different (p<0.05) between means within the column.

The results of methane oxidizing microbes (MOM) as shown in Figure 4.4 revealed that there were growth only in 4 (October, November, December and January) of the 12 months of study, with November having the highest counts of 8.0 x 10^3 CFU/g for D2S4 samples. The counts observed in the riverside samples were constant (2.0x10^3 CFU/g) and remained unchanged throughout the 7 months of the 12 months sampled and there were no significant differences (p>0.05) within the months.

The counts for borehole samples ranged from 2. 0x10^3 CFU/g – 6. 0x10^3 CFU/g, the high counts (6. 0x10^3 CFU/g) were observed in November and December. It was also observed that the counts of MOM in D2S3 remainded 4.0x10^3 CFU/g from October to January with a slight change (decrease in count) in April (Figure 4.4). In general, MOM growth across the samples were favoured during the dry season and this may be associated with temperature which is a dependent factor to the rate of gas influx and deposition.
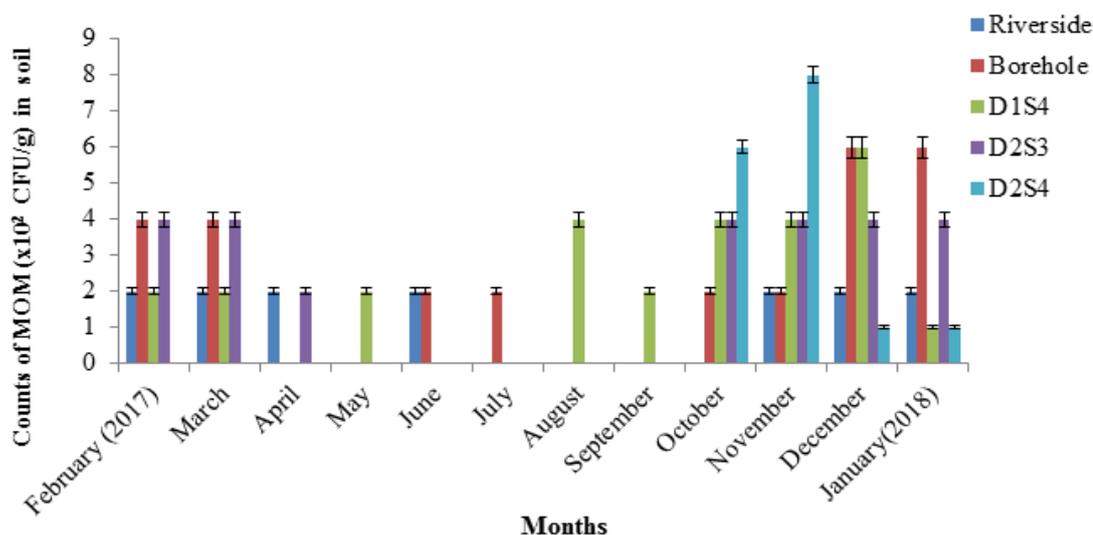
Figure 4.4: Counts of methane-oxidizing microbes (MOM) in soil

Key: D1S4= Day 1 Sample 4, Borehole sample, D2S3= Day 2 Sample 3, D2S4= Day 2 Sample 4, Riverside sample, Values are mean ± standard error of triplicate determination

The total counts of EOM in soil samples from Patishabakolo ranged from 2.0 x10^2 CFU/g (December) to 6.60x10^2 CFU/g (January), 1.3 x10^3 CFU/g –10.00x10^4 CFU/g , 1.3 x10^3 CFU/g – 6.70x10^2 CFU/g, 1.0x10^3 – 6.70x10^2 for Borehole, Riverside, D1S4, and D2S3 samples respectively (Table 4.11). For D2S4 sample, no growth was observed from February to June, although the highest growth was recorded in September (3.33x10^3 CFU/g), this implies that the rates of growth was not consistent and was not dependent on the season of the year. However, there were significant differences ($p < 0.05$) within and between the samples and months of study (Table 4.11).

Table 4.11: Counts of ethane-oxidizing microbes in soil samples from Patishabakolo

	Samples				
Months	Borehole	Riverside	D1S4	D2S3	D2S4
February (2017)	$4.0\text{x}10^3\pm0.00^{b*}$	$6.67\text{x}10^3\pm0.33^{bc}$	0.00 ± 0.00^a	$6.7\text{x}10^2\pm0.33\text{ab}$	0.00 ± 0.00^a
March	$4.0\text{x}10^3\pm1.00^a$	$2.67\text{x}10^3\pm0.33^{ab}$	0.00 ± 0.00^a	$6.7\text{x}10^2\pm0.30^{ab}$	0.00 ± 0.00^a
April	$4.0\text{x}10^3\pm0.58^b$	$4.00\text{ x}10^3\pm0.00^{abc}$	$0.00\pm0.00\text{a}$	$6.7\text{x}10^2\pm0.01$	0.00 ± 0.00^a
May	$4.0\text{x}10^3\pm0.00^b$	2.67 ± 1.00^{ab}	$6.70\text{x}10^2\pm0.30^{ab}$	$2.0\text{x}10^3\pm0.00^{bc}$	0.00 ± 0.00^a
June	$4.0\text{x}10^3\pm1.73^b$	$5.0\text{ x}10^3\pm0.67^{bc}$	$6.70\text{x}10^2\pm0.30^{ab}$	0.00 ± 0.00^a	0.00 ± 0.00^a
July	$2.67\text{ x}10^3\pm0.58^{ab}$	$1.3\text{ x}10^3\pm0.58^a$	$2.0\text{x}10^3\pm0.00^c$	0.00 ± 00^a	$2.0\text{x}10^3\pm0.00^{bc}$
August	0.00 ± 0.00^a	$8.0\text{ x}10^3\pm0.60^c$	$1.3\text{ x}10^3\pm0.29^{bc}$	$1.0\text{x}10^3\pm0.2\text{ab}$	0.00 ± 0.00^a
September	0.00 ± 0.00^a	$2.0\text{ x}10^3\pm0.001^a$	0.00 ± 0.00^a	$6.7\text{x}10^2\pm0.5^{ab}$	$3.33\text{x}10^3\pm0.33^c$
October	0.00 ± 0.00^a	$2.0\text{x}10^3\pm0.00^a$	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a
November	$2.670\text{x}10^3\pm0.50^{ab}$	$4.0\text{x}10^3\pm0.00^{ab}$	0.00 ± 0.00^a	$2.0\text{x}10^3\pm0.16^{bc}$	$2.0\text{x}10^3\pm0.00^{bc}$
December	$2.0\text{x}10^3\pm0.01^{ab}$	$1.34\text{x}10^3\pm0.80^a$	0.00 ± 0.00^a	$3.33\text{x}10^3\pm0.01^c$	$2.0\text{x}10^3\pm0.57^{ab}$
January (2018)	$6.60\text{x}10^2\pm0.00^a$	$10.00\text{x}10^4\pm0.50^c$	$6.70\text{ x}10^3\pm0.58^{ab}$	$2.67\text{x}10^3\pm0.03^c$	$1.3\text{ x}10^3\pm0.03^a$

Key: D1S4= Day 1 Sample 4, Borehole sample, D2S3= Day 2 Sample 3, D2S4= Day 2 Sample 4, River side sample (referring to soil samples), Values are mean ± standard error of triplicate determinations. *Values with different alphabets are significantly different (p<0.05) between means along the column.

The POM results (Table 4.12) it was observed that samples D2S3 and D2S4, had no growth from February to June and from September to January. Growth was however, observed only in July and August and the counts in each month were similar at 3.34 $x10^3$ CFU/g and $2.0x10^3$ CFU/g respectively (Table 4.12). The highest count was observed as 6.60 $x10^2$ CFU/g for Borehole and were constant from June to October; there were significant difference ($p<0.05$) among the samples.

Table 4.12: Counts of propane–oxidizing microbes in soil samples from Patishabakolo

Months	Sample				
	Borehole	Riverside	D1S4	D2S3	D2S4
February(2017)	0.00 ± 0.00^a	$4.0\text{x}10^3\pm0.33^{b*}$	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a
March	0.00 ± 0.00^a	$4.6\text{x}10^3\pm0.56^b$	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a
April	$4.0\text{x}10^3\pm0.00^d$	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a
May	$2.66\text{x}10^3\pm0.60^{cd}$	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a
June	$6.60\text{ x}10^2\pm0.57^{ab}$	0.00 ± 0.00^a	$1.34\text{x}10^3\pm0.06^b$	0.00 ± 0.00^a	0.00 ± 0.00^a
July	$6.60\text{ x}10^2\pm0.58^{ab}$	0.00 ± 0.00^a	$2.0\text{x}10^3\pm0.00^c$	$3.34\text{x}10^3\pm0.90^b$	$2.0\text{x}10^3\pm0.001^b$
August	$6.60\text{ x}10^2\pm0.5^{ab}$	0.00 ± 0.00^a	0.00 ± 0.00^a	$3.34\text{ x}10^3\pm0.33^b$	$2.0\text{x}10^3\pm0.002^b$
September	$6.60\text{ x}10^2\pm0.01^{ab}$	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a
October	$6.60\text{ x}10^2\pm0.60^{ab}$	$4.0\text{x}10^3\pm0.001^b$	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a
November	$2.0\text{x}10^3\pm0.002^b$	$4.0\text{x}10^3\pm0.50^b$	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a
December	$2.0\text{x}10^3\pm0.01^b$	$4.6\text{x}10^3\pm0.88^b$	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a
January 2018	0.00 ± 0.00^a	0.00 ± 0.00	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a

Key: D1S4= Day 1 Sample 4, Borehole sample, D2S3= Day 2 Sample 3, D2S4= Day 2 Sample 4, River side sample (referring to soil samples), Values are mean ± standard error of mean of triplicate determinations. *Values with different alphabets are significantly different ($p<0.05$) between means along the column.

Total counts of BOM in soil samples from Patishabakolo ranged from 0.00 to 2.0×10^3 CFU/g for all the samples (Table 4.13). Many months, particularly April to September had no BOM growth. The results revealed the growth of microbes only in 1, 3, 6, and 2 month of the 12 months of study for D2S4, borehole, riverside, D1S4, and respectively. There was no growth for BOM in D2S3 samples. However, the months with BOM counts had no significant differences ($p > 0.05$) (Table 4.13).

Table 4.13: Counts of butane-oxidizing microbes in soil sample from Patishabakolo

Months	Sample				
	Borehole	Riverside	D1S4	D2S3	D2S4
February (2017)	0.00 ± 0.00^a	$2.0\text{x}10^3\pm0.001^{b*}$	$1.34\text{x}10^3\pm0.06^b$	0.00 ± 0.00^a	0.00 ± 0.00^a
March	0.00 ± 0.00^a	$2.0\text{x}10^3\pm0.001^c$	$2.0\text{x}10^3\pm0.00^c$	0.00 ± 0.00^a	0.00 ± 0.00^a
April	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a
May	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a
June	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a
July	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a
August	0.00 ± 0.00^a	0.04 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a
September	0.00 ± 0.00^a	0.00 ± 0.00	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a
October	0.22 ± 0.00^{ab}	0.1 ± 0.0011^a	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a
November	0.32 ± 0.00^{ab}	0.1 ± 0.003^a	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a
December	$2.0\text{x}10^3\pm0.01^b$	$2.0\text{x}10^3\pm0.001^b$	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a
January(2018)	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a

Key: D1S4= Day 1 Sample 4, Borehole sample, D2S3= Day 2 Sample 3, D2S4= Day 2 Sample 4, River side sample (referring to soil samples), Values are mean ± standard error of mean of triplicate determinations. *Values with different alphabets are significantly different ($p<0.05$) between means along the column.

4.1.3   Distribution and Frequency of Occurrence of Microbes in the Samples

4.1.3.1 Distribution and frequency of occurrence of bacteria and fungi in soil and

water samples

The most frequently isolated bacteria in site A and B were *Micrococcus* sp. with a frequency of occurrence of 5.03 % and 2.79 % respectively, closely followed by *Enterobacter* sp. (4.50 %, 2.23 %) and *Corynebacterium* sp. (3.35 %, 1.68 %), while in site C the most frequently encountered tsolate was *Serratia* sp. (2.23 %), site D and water had *Salmonella* sp.at 3.91 % and 3.35 % respectively (Table 4.14).

Table 4.14: Frequency of occurrence (%) of bacterial isolates in Ahoko soil and water samples analyzed

Bacteria	Soil samples				Water	Total
	Site A	Site B	Site C	Site D		
Pseudomonas alcaligenes	3(1.68)	0(0.0)	2(1.12)	3(1.68)	3(1.68)	11(6.16)
Acinetobacter sp.	2(1.12)	0(0.0)	3(1.68)	5(2.79)	0(0.0)	10(5.59)
Enterococcus sp	4(2.23)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	4(2.23)
Alcaligenes sp.	3(1.68)	2(1.12)	0(0.0)	0(0.0)	0(0.0)	5(2.8)
B. licheniformis	3(1.68)	0(0.0)	0(0.0)	0(0.0)	4(2.23)	7(3.91)
Serratia sp.	2(1.12)	2(1.12)	4(2.23)	4(2.23)	0(0.0)	12(6.7)
Micrococcus sp.	9(5.03)	5(2.79)	3(1.68)	2(1.12)	0(0.0)	19(10.62)
B .coagulans	3(1.68)	5(2.79)	3(1.68)	0(0.0)	2(1.12)	13(7.27)
S. aureus	1(0.56)	2(1.12)	3(1.68)	3(1.68)	0(0.0)	9(5.04)
Corynebacterium sp	6(3.35)	3(1.68)	0(0.0)	2(1.12)	4(2.23)	15(8.38)
Enterobacter sp.	8(4.50)	4(2.23)	0(0.0)	2(1.12)	4(2.23)	18(10.08)
Salmonella	0(0.0)	0(0.0)	7(3.91)	6(3.35)	10(5.59)	23(12.85)
Pseudomonas sp.	3(1.68)	2(1.12)	2(1.12)	3(1.68)	3(1.68)	13(7.28)
Citrobacter freundii	2(1.12)	0(0.0)	0(0.0)	0(0.0)	2(1.12)	4(2.24)
E. coli	0(0.0)	0(0.0)	0(0.0)	2(1.12)	6(3.35)	8(4.47)
Stenotrophomonas	0(0.0)	0(0.0)	0(0.0)	0(0.0)	2(1.12)	2(1.12)
Klebsiella sp.	0(0.0)	0(0.0)	2(1.12)	4(2.23)	0(0.0)	6(3.35)
Total	49(27.40)	25(13.97)	29(16.22)	36(20.12)	40(22.34)	179(100)

Site A, B C and D are alphabetical codes used to represent the points where samples were collected (referring to soil samples). Extrapolated from Appendix F1-F9

The most frequently isolated and widely distributed fungi in both the soil and water samples from Ahoko was *Aspergillus niger* with a frequency of occurrence of 15.49 %, closely followed by *A. flavus* and *Candida* species with 13.78 % and 13.77 % frequency of occurrence respectively (Table 4.15). The results revealed that Site A had *A. flavus* (6.90 %) and *Rhodotorula* species (6.90 %) as the most frequently isolated fungi. It was also observed that *A. fumigatus* was only present in site D and water samples with frequencies of occurrence of 8.62 % and 3.44 % respectively (Table 4.15).

Table 4.15: Frequency of occurrence (%) of fungal isolates in Ahoko soil and water samples analyzed

Fungi	Soil samples				Water	Total
	Site A	Site B	Site C	Site D		
A. fumigatus	0(0.00)	0(0.00)	0(0.00)	5(8.62)	2(3.44)	7(12.06)
Aspergillus niger	3(5.17)	2(3.44)	2(3.44)	1(1.72)	1(1.72)	9(15.49)
Aspergillus flavus	4(6.90)	0(0.00)	2(3.44)	0(0.00)	2(3.44)	8(13.78)
Candida sp.	3(5.17)	1(1.72)	1(1.72)	2(3.44)	1(1.72)	8(13.77)
Rhodotorula sp.	4(6.90)	1(1.72)	0(0.00)	0(0.00)	0(0.00)	5(8.62)
Penicillium sp.	1(1.72)	2(3.44)	1(1.72)	0(0.00)	0(0.00)	4(6.91)
Rhizopus sp.	2(3.44)	0(0.00)	0(0.00)	3(5.17)	0(0.00)	5(8.61)
Alternaria sp	1(1.72)	0(0.00)	0(0.00)	0(0.00)	1(1.72)	2(3.44)
Aspergillus sp	2(3.44)	2(3.44)	0(0.00)	0(0.00)	1(1.72)	5(8.60)
Geotricum sp	2(3.44)	0(0.00)	1(1.72)	2(3.44)	1(1.72)	6(10.32)
Total	22(37.93)	7(12.07)	7(12.07)	13(22.41)	9(15.52)	58(100)

Key: Site A, B C and D are alphabetical codes used to represent the points where samples were collected (referring to soil samples). Extrapolated from Appendix F1-F9

The most frequently encountered and widely distributed bacteria from Patishabakolo sites were *B. coagulans* with total frequency of occurrence of 15.8 %, this was followed by *Corynebacterium* species (12.2 %), *Acinetobacter* and *Pseudomonas* species with 11.4 % frequency of occurrence. The most frequently occurred for D2S3 was *Acinetobacter* sp (8.51 %) and *Corynebacterium* sp (8.51 %) for D2S4 (Table 4.16). The results also revealed that the Riverside samples had tatal frequency of occurrence of 25.4% while D2S4 had the least (12.28 %) frequency of occurrence. It was observed that *Pseudomonas* sp. (16.67 %) occurred most frequently in the water samples while *Stenotrophomonas* was isolated only from the water samples with a frequency of 1.75 % (Table 4.16).

Table 4.16: Frequency of occurrence (%) of bacterial isolates in Patishabakolo soil and water samples analyzed

			Soil samples				
Bacteria	River side	Borehole	D1S4	D2S3	D2S4	Water	Total
Enterobacter sp.	3(2.63)	0(0.0)	2(1.75)	3(2.63)	0(0.00)	1(0.88)	9(7.89)
B. coagulans	5(4.39)	3(2.63)	3(2.63)	2(1.75)	3(2.63)	2(1.75)	18(15.8)
S. aureus	0(0.0)	0(0.0)`	0(0.0)	1(0.88)	0(0.00)	1(0.88)	2(1.75)
Enterococcus sp.	4(8.51)	0(0.0)	0(0.0)	0(0.0)	1(7.14)	1(0.88)	6(5.26)
Alcaligenes sp.	2(1.75)	2(1.75)	2(1.75)	0(0.0)	0(0.00)	0(0.00)	6(5.26)
B. licheniformis	3(2.63)	0(0.0)	1(0.88)	0(0.0)	0(0.00)	2(11.11)	6(5.25)
Acinetobacter sp.	2(1.75)	2(1.75)	3(2.63)	4(8.51)	2(1.75)	0(0.00)	13(11.4)
Micrococcus sp.	1(0.88)	3(2.63)	1(0.88)	2(1.75)	2(1.75)	1(0.88)	10(8.77)
Stenotrophomonas sp.	0(0.0))	0(0.00)	0(0.0)	0(0.0)	0(0.00)	2(1.75)	2(1.75)
Corynebacterium sp.	3(2.63)	3(2.63)	0(0.0)	2(1.75)	4(8.51)	2(1.75)	14(12.2)
Salmonella sp.	2(1.75)	0(0.0)	0(0.0)	2(1.75)	1(0.88)	1(0.88)	6(5.26)
Pseudomonas sp.	3(2.63)	2(1.75)	2(11.7)	3(2.63)	0(0.00)	3(2.63)	13(11.4)
E. coli	0(0.0)	0(0.0)	0(0.0)	2(1.75)	1(0.88)	1(0.88)	4(3.51)
Aeromonas spp.	1(0.88)	0(0.0)	1(0.88)	0(0.00)	0(0.00)	1(0.88)	3(2.63)
Klebsiella sp.	0(0.0)	0(0.0)	2(11.7)	0(0.00)	0(0.00)	0(0.00)	2(1.75)
Total	29(25.4)	15(13.2)	17(14.9)	21(18.42)	14(12.28)	18(15.8)	114(100)

Key: D1S4= Day 1 Sample 4, Borehole sample, D2S3= Day 2 Sample 3, D2S4= Day 2 Sample 4, River side sample (referring to soil samples). Values are mean ± standard error of mean of triplicate determinations. Values with different alphabets are significantly different ($p<0.05$) between means along the column. Extrapolated from Appendix F1-F9.

The results for the frequency of occurrence of fungi in Patishabakolo soil and water samples shown in Table 4.17 revealed the most frequently occurring and widely distributed fungi were *A. niger* with a total frequency of 21.8 %. Riverside, borehole, D1S4, D2S3, D2S4 and water samples had total frequencies of occurrence of 18.2 %, 23.6 %, 18.2 %, 18.2 %, 10.9 % and 10.9 % respectively. It was observed that in the water samples analysed, *A. niger, A. flavus, A. fumigatus* and *Candida* species were absent (0.00 %) while *Penicillium* species were present only in D2S4 and water samples with the same frequency of occurrence of 1.82 % (Table 4.17).

Table 4.17: Frequency of occurrence (%) of fungal isolates in Patishabakolo soil and water samples analyzed

			Soil samples				
Fungi	Riverside	Borehole	D1S4	D2S3	D2S4	Water	Total
Aspergillus sp.	1(1.8)	3(5.5)	2(3.6)	1(1.8)	0 (0.0)	2 (3.6)	9 (16.4)
Aspergillus niger	3(5.5)	4(7.27)	3(5.45)	2(3.6)	2(3.64)	0 (0.0)	14 (21.8)
Aspergillus flavus	2(3.6)	2(3.6)	1(1.8)	3(5.5)	0 (0.0)	0 (0.0)	8 (14.6)
Aspergillus fumigatus	0(0.0)	1(1.82)	1(1.8)	0(0.0)	0 (0.0)	0(0.0)	2 (3.6)
Mucor sp.	2(3.6)	2(3.64)	2 (3.6)	0(0.0)	1 (1.8)	2 (3.6)	9 (16.4)
Penicillum sp.	0(0.0)	0(0.0)	0(0.0)	1(1.82)	0 (0.0)	1 (1.82)	2 (3.6)
Rhizopus sp.	0(0.0)	0(0.0)	1(1.8)	0(0.0)	1(1.82)	1(1.82)	3(5.5)
Candida sp.	2(3.6)	1(1.82)	0(0.0)	3(5.5)	2(3.64)	0(0.0)	8(14.5)
Total	10(18.2)	13(23.6)	10(18.2)	10(18.2)	6(10.92)	6(10.92)	55(100)

Key: D1S4= Day 1 Sample 4, Borehole sample, D2S3= Day 2 Sample 3, D2S4= Day 2 Sample 4, River side sample (referring to soil samples). Values are mean ± standard error of triplicate determinations. Values with different alphabets are significantly different ($p<0.05$) between means along the column. Extrapolated from Appendix F1-F9.

4.1.3.2 Distribution and frequency of occurrence of specific hydrocarbon oxidizing microorganisms in the suspected petroleum bearing sites

A total of 61 hydrocarbon oxidizing bacteria (HOB), which represented 16 different genera were isolated across the groups studied. (3 Methane, 9 Ethane, 3 Propane, 1 Butane-oxidizing bacteria). The most widely distributed and frequently occurring group was ethane oxidizing bacteria (EOB) which included: *Achromobacter* (8.20 %), *Actinobacillus* (4.92 %), *Norcadia* (3.28 %), propane-oxidizing bacteria (POB) comprised*; Mycobacterium* (4.92 %) and *Gordonia* (4.92 %), while the only butane oxidizing bacteria (BOB) identified were *Ochrobacteria* at a frequency of 4.92% (Table 4.18a and 4.18b).

Table 4.18a: Frequency of occurrence (%) of specific hydrocarbon gas–oxidizing bacteria in soil and water samples analyzed

	Soil samples						
	Site A	Site B	Site C	Site D		HC-Gas	
Bacteria					Water		Total
Mycobacterium sp.	3(4.92)	0(0.00)	0(0.00)	0(0.00)	2(3.28)	P	5(8.20)
Actinobacillus sp.	3(4.92)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	E	3(4.92)
Norcadia sp.	0(0.00)	2(3.28)	1(1.64)	0(0.00)	2(3.28)	E	5(8.20)
Enterobacter sp.	1(1.64)	1(1.64)	2(3.28)	0(0.00)	0(0.00)	E	4(6.56)
Sporosarcina sp.	0(0.00)	0(0.00)	0(0.00)	1(1.64)	0(0.00)	M	1(1.64)

Key: HC-Gas= Hydrocarbon gas, M=methane, P=propane, E=ethane, B=butane. Site A, B C and D are alphabetical codes used to represent the points where samples were collected (referring to soil samples). Extrapolated from Appendix F1-F9.

Table 4.18b: Frequency of occurrence (%) of specific hydrocarbon gas–oxidizing bacteria in soil and water samples analyzed

Bacteria	Site A	Site B	Site C	Site D	Water	HC-Gas	Total
			Soil samples				
Gordonia	3(4.92)	0(0.00)	1(1.64)	0(0.00)	2(3.28)	P	6(9.84)
Achromobacter	5(8.20)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	E	5(8.20)
Serratia sp.	1(1.64)	1(1.64)	1(1.64)	2(3.28)	0(0.00)	E	5(8.20)
Methylomonas	2(3.28)	1(1.64)	0(0.00)	0(0.00)	2(3.28)	M	5(8.20)
UNIDENTIFIED	3(4.92)	1(1.64)	0(0.00)	1(1.64)	4(6.56)	P	9(14.76)
Stenotrophomonas	0(0.00)	0(0.00)	2(3.28)	0(0.00)	0(0.00)	E	2(3.28)
Geobacter	0(0.00)	0(0.00)	0(0.00)	0(0.00)	4(6.56)	E	4(4.65)
Aeromonas	0(0.00)	0(0.00)	0(0.00)	0(0.00)	1(1.64)	E	1(1.64)
Methylobaceter	0(0.00)	0(0.00)	0(0.00)	0(0.00)	3(4.92)	M	3(4.92)
Ochrobacteria	0(0.00)	0(0.00)	0(0.00)	0(0.00)	3(4.92)	B	3(4.92)
Arthrobacter sp	0(0.00)	0(0.00)	0(0.00)	0(0.00)	1(1.64)	E	1(1.64)

Key: HC-Gas= Hydrocarbon gas, M=methane, P=propane, E=ethane, B=butane. Site A, B C and D are alphabetical codes used to represent the points where samples were collected (referring to soil samples). Extrapolated from Appendix F1-F9.

Similarly, fifty three (53) hydrocarbon-oxidizing fungi (HOF) were identified and belonged to 11 different genera which comprised 3 Methane, 5 Ethane, 1Propane and 2 Butane, with *Aspergillus* (39.62%) being the most frequently occurring genus (Table 4.19). The most widely distributed and frequently encountered groups were the ethane oxidizing fungi (EOF) which comprised; *Aspergillus, Rhizopus, Alterneria*, and *Candida*; closely followed by Methane oxidizing fungi (MOF): *Penicillium*, *Rhodotorula*, and butane oxidizing fungi, *Pichia* and *Talaromyces*, while *Geotricium* was the only propane oxidizing fungi (POF) identified (Table 4.19).

Table 4.19: Frequency of occurrence (%) of specific hydrocarbon gas-oxidizing fungi in soil and water samples analyzed

Fungi	Soil samples					HC-Gas	Total
	Site A	Site B	Site C	Site D	Water		
Aspergillus sp.	11(20.75)	4(7.55)	2(3.45)	2(3.45)	2(3.45)	E	21(39.62)
Aspergillus niger	4(7.55)	1(1.89)	1(1.89)	1(1.89)	1(1.89)	E	8(15.09)
Geotrichum sp.	2(3.45)	0(0.00)	0(0.00)	0(0.00)	2(3.45)	P	4(7.55)
Penicillium sp	4(7.55)	1(1.89)	2(3.45)	1(25.00)	1(1.89)	M	9(16.98)
Rhizopus sp	1(1.89)	1(1.89)	0(0.00)	0(0.00)	0(0.00)	E	2(3.45)
Pichia sp	1(1.89)	0(0.00)	1(14.28)	0(0.00)	0(0.00)	B	2(3.45)
Rhodotorula sp	1(1.89)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	M	1(1.89)
Talaromyces sp	0(0.00)	0(0.00)	0(0.00)	0(0.00)	1(1.89)	B	1(1.89)
Alterneria sp	0(0.00)	0(0.00)	0(0.00)	0(0.00)	1(1.89)	E	1(1.89)
Aspergillus flavus	1(1.89)	0(0.00)	0(0.00)	0(0.00)	1(1.89)	M	2(3.45)
Candida sp	3(10.71)	0(0.00)	1(14.28)	0(0.00)	0(0.00)	E	4(7.55)
TOTAL	27(50.94)	7(13.21)	7(13.21)	4(7.55)	9(16.98)		53(100)

Key: HC=Hydrocarbon M=methane, P=propane, E=ethane, B=butane gas, Site A, B C and D are alphabetical codes used to represent the points where samples were collected (referring to soil samples).Extrapolated from Appendix F1-F9.

The results of the relative abundance (Figure 4.5) of the hydrocarbon gas–utilizing microbes in soil and water samples analysed revealed that MOB were 4.92 % (Site A), 1.64 (Site B and D); EOB: 9.84 % (Site A), 4.92 % (Site B), 8.20 % (Site C) and 1.64 % (site D) and 16.39 % (water) (Figure 4.5a). Also the MOF were: 3.85 %, 1.92 %, 0.00 % and 0% and 1.92 %; EOF, 40.38 %, 1.92 %, 3.85 %, 5.77 % and 7.69 % for site A, B, C, D and water samples respectively (Figure 4.5b). However there were significant differences in abundance as revealed by the comparative line series within the graph (Figure 4.5), also the results revealed a correlation of 10 % and 100 % between MOB of site A, D, water samples, and among BOB across all the groups of organisms within the study locations respectively, EOB and POB of site A, EOB of site D and water samples are also correlated at 90 %, and 60 % respectively (r = 0.9) (Figure 4.5a). Similarly, there were correlations among MOF of site A, D, water samples and EOF of site B and C at 5 % and 15 % respectively and 100 % BOF across the groups within the samples (Figure 4.5b). These implied that these organisms with correlation above 50 % originated from same source and are related as revealed by the cross plots in the relative abundance graph (Figure 4.5a and Figure 4.5b).
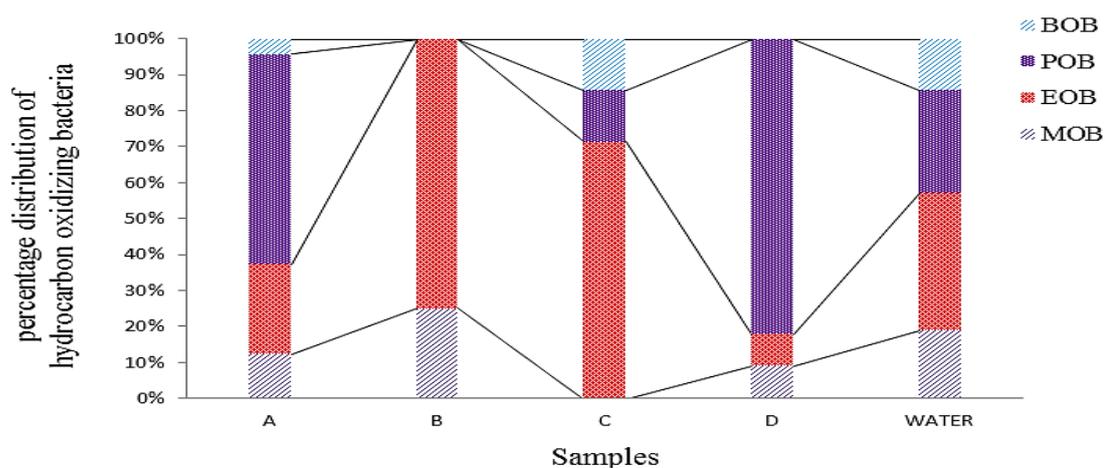
Figure 4.5a: Relative abundance and relationship between hydrocarbon-oxidizing bacteria (HOB) in soil and water samples from Ahoko

Key: Site A, B C and D are alphabetical codes used to represent the points where samples were collected (referring to soil samples). BOB, POB, EOB, and MOB represent Butane, propane, ethane and methane oxidizing bacteria respectively
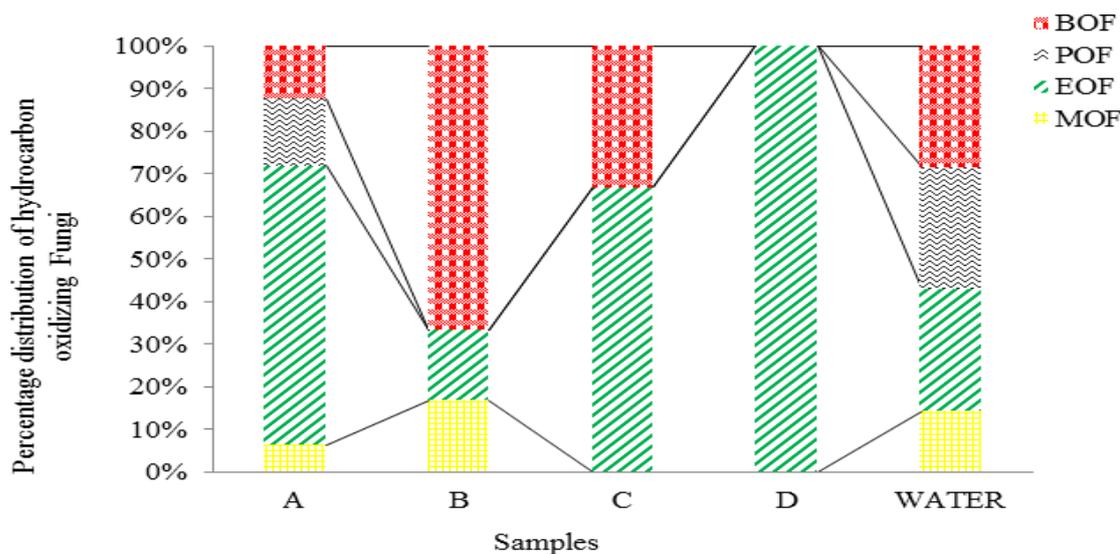
Figure 4.5b: Relative abundance and relationship between hydrocarbon-oxidizing fungi (HOF) in soil and water samples from Ahoko

Key: Site A, B C and D are alphabetical codes used to represent the points where samples were collected (referring to soil samples). BOF, POF, EOF, and MOF represent Butane, propane, ethane and methane oxidizing fungi respectively

The concentration distribution maps (Figure 4. 6) of hydrocarbon oxidizers in the soil and water samples of Ahoko were prepared with the help of Arc Geographical Information System (GIS) to delineate the potential area with high yields. The concentration distribution maps of methane, ethane, propane and butane oxidizers, enumerated from the soil samples, were plotted on the geological map of the study area (Figure 4.6a–d). The higher populations and concentration of these oxidizers were observed in nearby areas of Ahoko Ebira (site A). However, the light gaseous hydrocarbons (C_2, C_3, and C_4) desorbed from the soils samples, as plotted on the geological map of the study area delineate the hydrocarbon potential zones (Figure 4.6a – 4.6d) with site A being more potent.
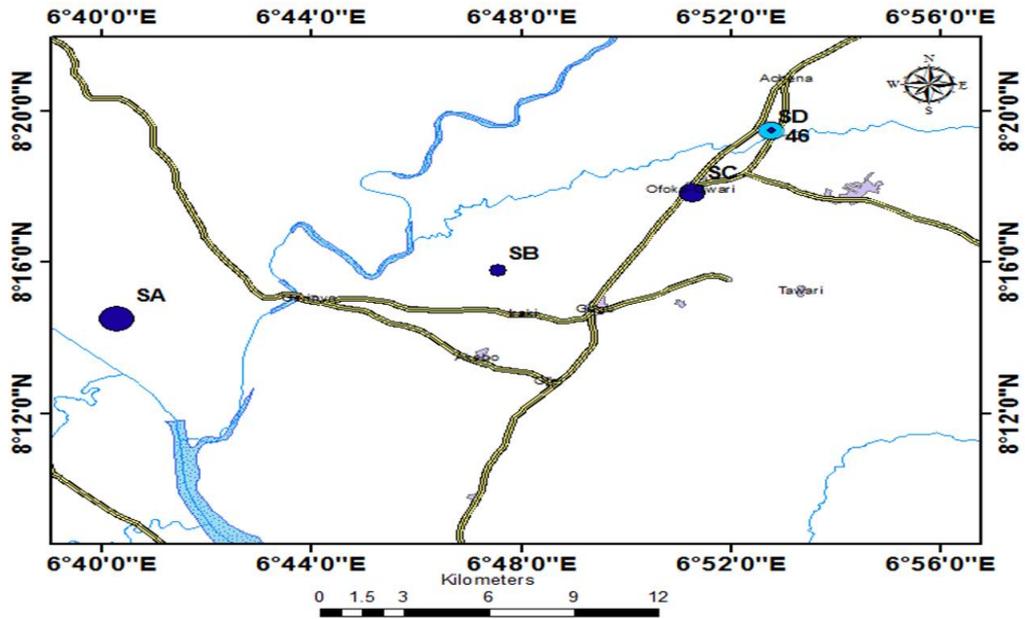
Figure 4.6a: Concentration distribution map of methane-oxidizing microbe (MOM).

Key: SA=site A; SB= site B; SC= site C; SD= site D; Site A, B C and D are alphabetical codes used to represent the points where samples were collected (referring to soil samples).
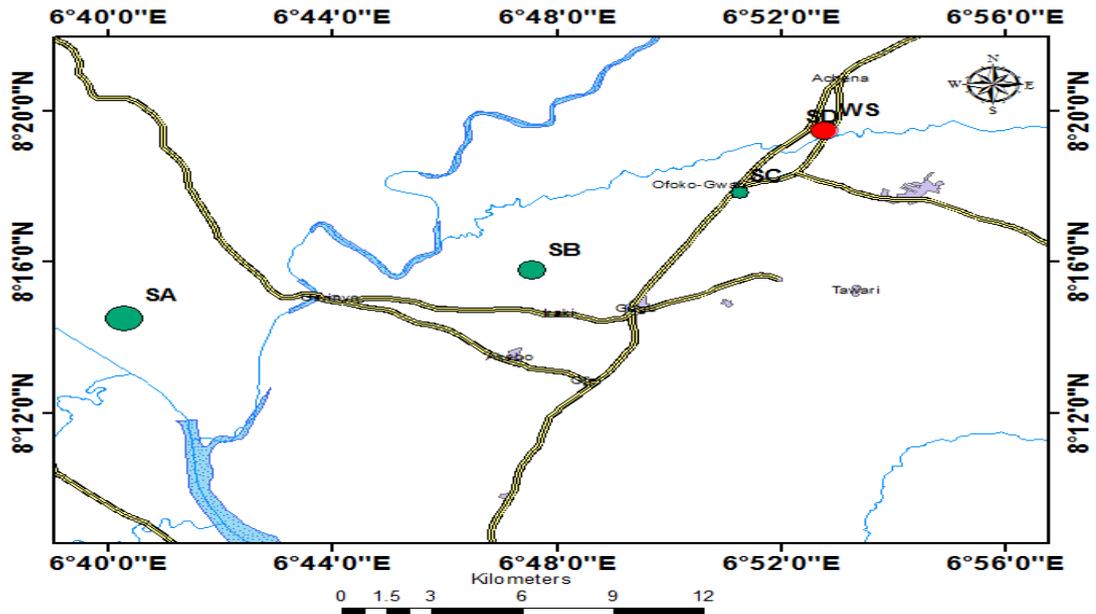
Figure 4.6b: Concentration distribution maps of Ethane-oxidizing microbe (EOM)
Key: SA=site A; SB= site B; SC= site C; SD= site D; Site A, B C and D are alphabetical codes used to represent the points where samples were collected (referring to soil samples).
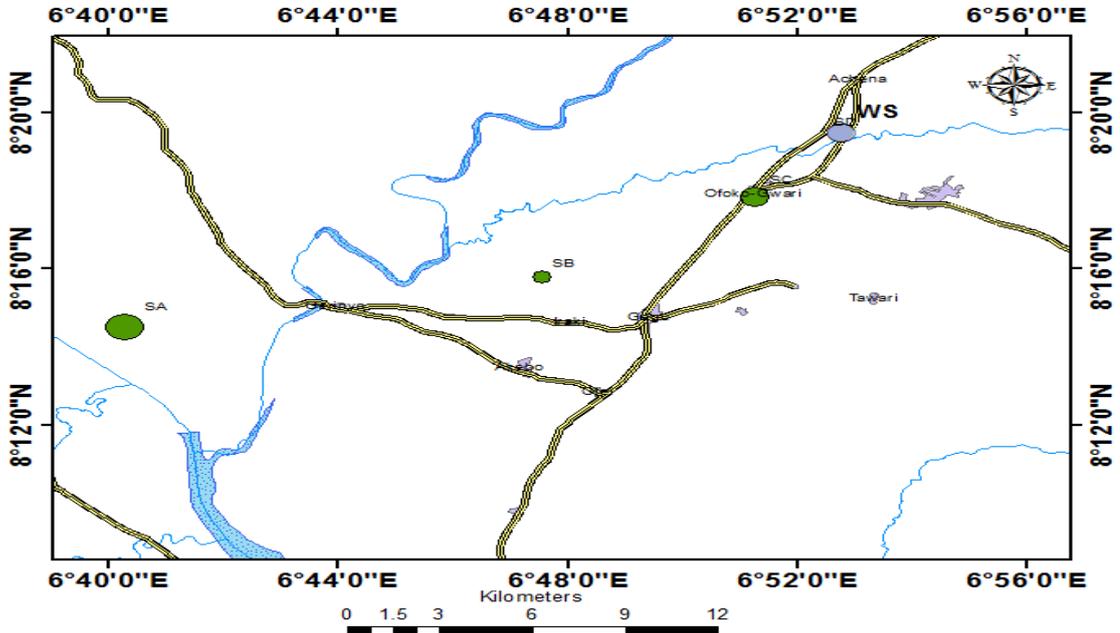
Figure 4.6c: Concentration distribution maps propane-oxidizing microbe (POM)
Key: SA=site A; SB= site B; SC= site C; SD= site D; Site A, B C and D are alphabetical codes used to represent the points where samples were collected (referring to soil samples).
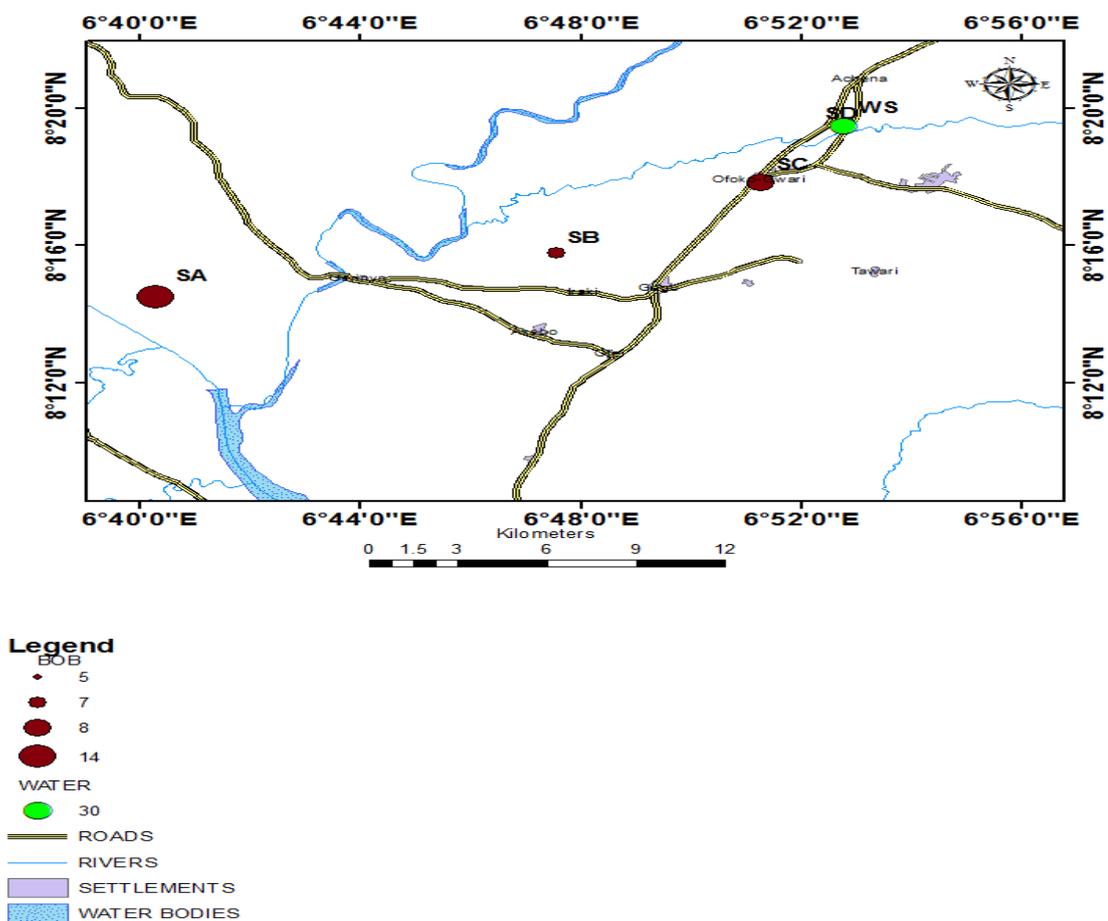
Figure 4.6d: Concentration distribution maps butane-oxidizing microbe (BOM)

Key: SA=site A; SB= site B; SC= site C; SD= site D; Site A, B C and D are alphabetical codes used to represent the points where samples were collected (referring to soil samples).

The result of the percentage distribution and relative abundance of the hydrocarbon gas-utilizing microbes as shown in the graph below (Figure 4.7) compares the percentage that each value contributes to a total across the sampled categories and it is used to emphasize the proportion of each sample. The results of the relative abundance revealed that EOB had 16.67 % abundance, (Borehole), 33.33 % (riverside), 8.33 % (D1S4), 20.83 % (D2S3), 12.5 % (D2S4) and 8.33 % (water); POB: 6.67 %, 11.11 %, 2.22 %, and 2.22 % for Borehole, riverside, D1S4 and water respectively (Figure 4.7). EOF: 23.81 % (Borehole), 28.57 % (riverside), 14.29 % (D1S4), 19.05 % (D2S3), 9.52 % (D2S4) and 4.76 % (water); POF had abundance of 8.89 %, 11.11 %, 2.44 %, 2.44 % and 2.22 % for Borehole, riverside, D1S4 D2S3 and water samples respectively (Figure

4.7). However there were significant differences in abundance as revealed by the comparative line series within the graph (Figure 4.7). More so, the relative abundance using a cross plot had revealed there was 80 %-100 % correlation (r = 0.8) between BOB and BOF across the samples within the locations studied; this implied that organisms originated from same source (Figure 4.7a and 4.7b).
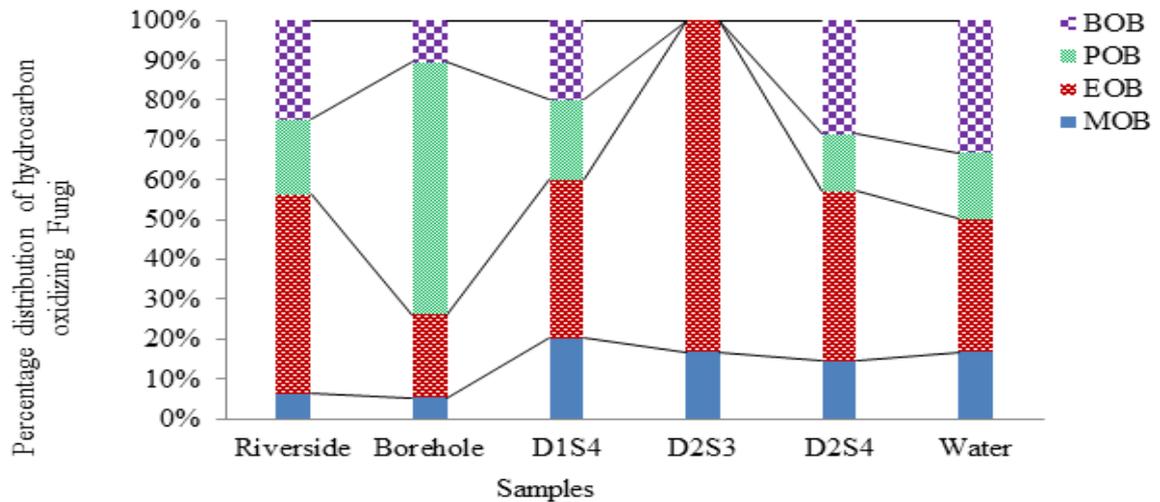
Figure 4:7a Relative Abundance and relationship between hydrocarbon-oxidizing bacteria (HOB) in soil and water samples from Patishabakolo

Key: D1S4= Day 1 Sample 4, Borehole sample, D2S3= Day 2 Sample 3, D2S4= Day 2 Sample 4, River side sample (referring to soil samples). BOB, POB, EOB, MOB= (Butane, Propane Ethane and Methane oxidizing bacteria respectively
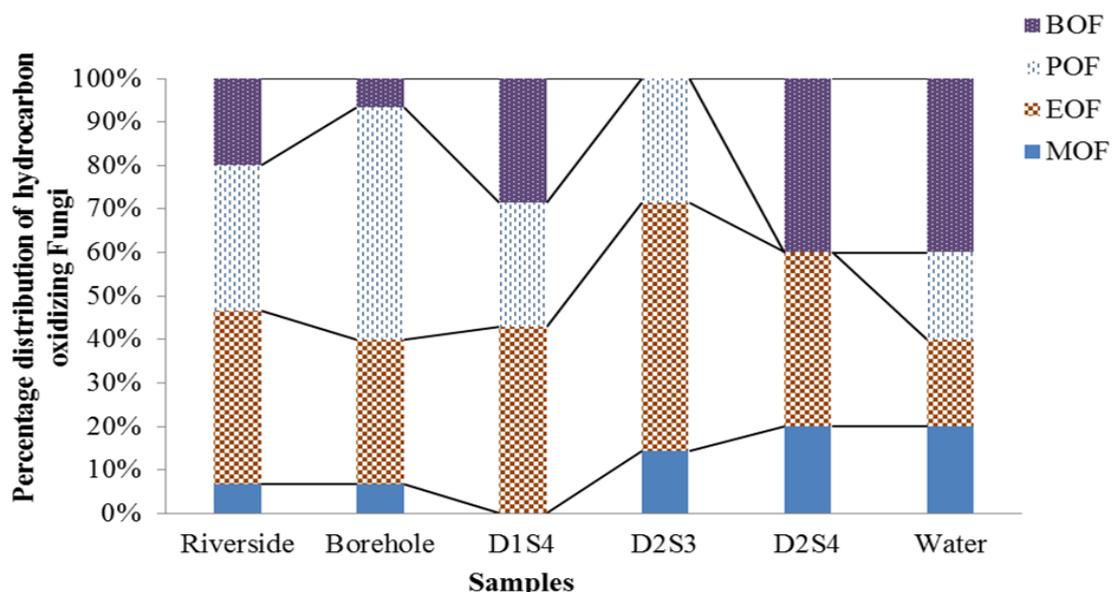
Figure 4:7b Relative Abundance and relationship between hydrocarbon-oxidizing fungi (HOF) in soil and water samples from Patishabakolo

Key: D1S4= Day 1 Sample 4, Borehole sample, D2S3= Day 2 Sample 3, D2S4= Day 2 Sample 4, River side sample (referring to soil samples). BOB, POB, EOB, MOB= Butane, Propane Ethane and Methane oxidizing bacteria respectively

Similarly, the concentration distribution maps of hydrocarbon oxidizers in the soil and water samples of Patishabakolo were prepared with the help of Geographical Information System (GIS) to delineate the potential area with high yields. However, the light gaseous hydrocarbons (C_2, C_3, and C_4) desorbed from the soils samples, as plotted on the geological map of the study area delineate the hydrocarbon potential zones to be riverside and borehole areas, with Borehole area being more potent with higher proportion of light gaseous hydrocarbons (C_2, C_3, and C_4) (Figure 4.8a–c).
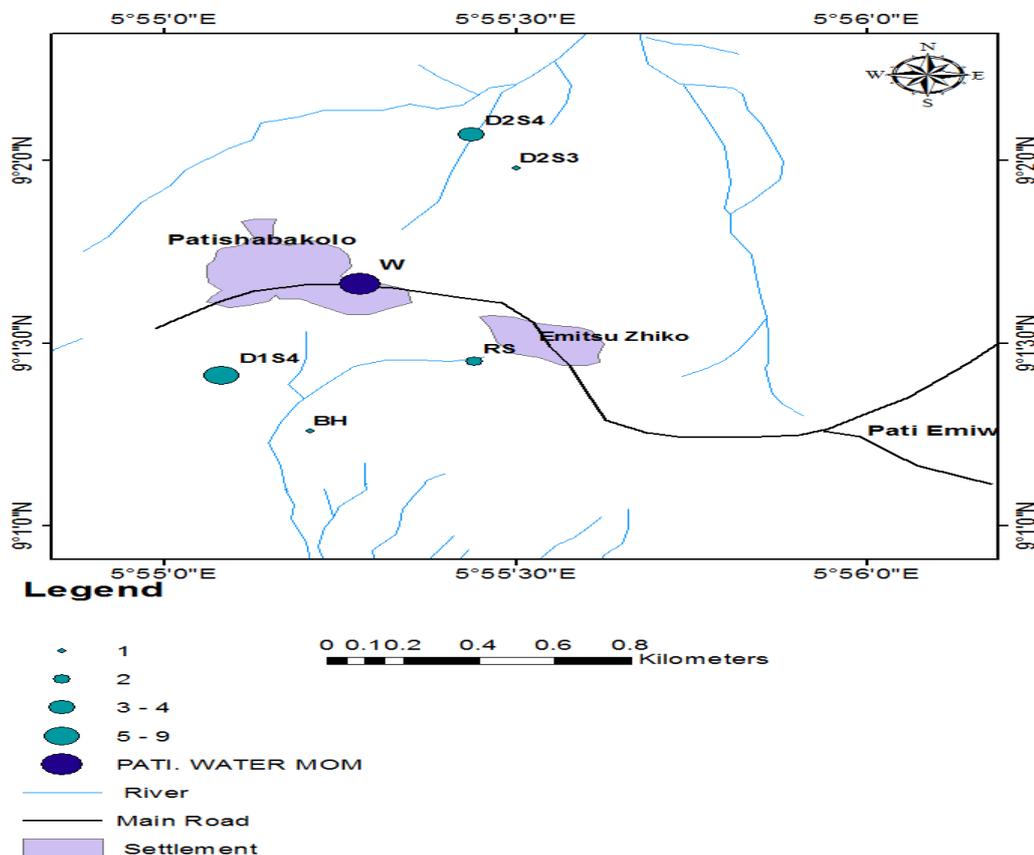
Figure 4.8a: Concentration distribution maps of methane-oxidizing microbe (MOM)

Key: D1S4= Day 1 Sample 4, BH= Borehole sample, D2S3= Day 2 Sample 3, D2S4= Day 2 Sample 4, River side sample (referring to soil samples).
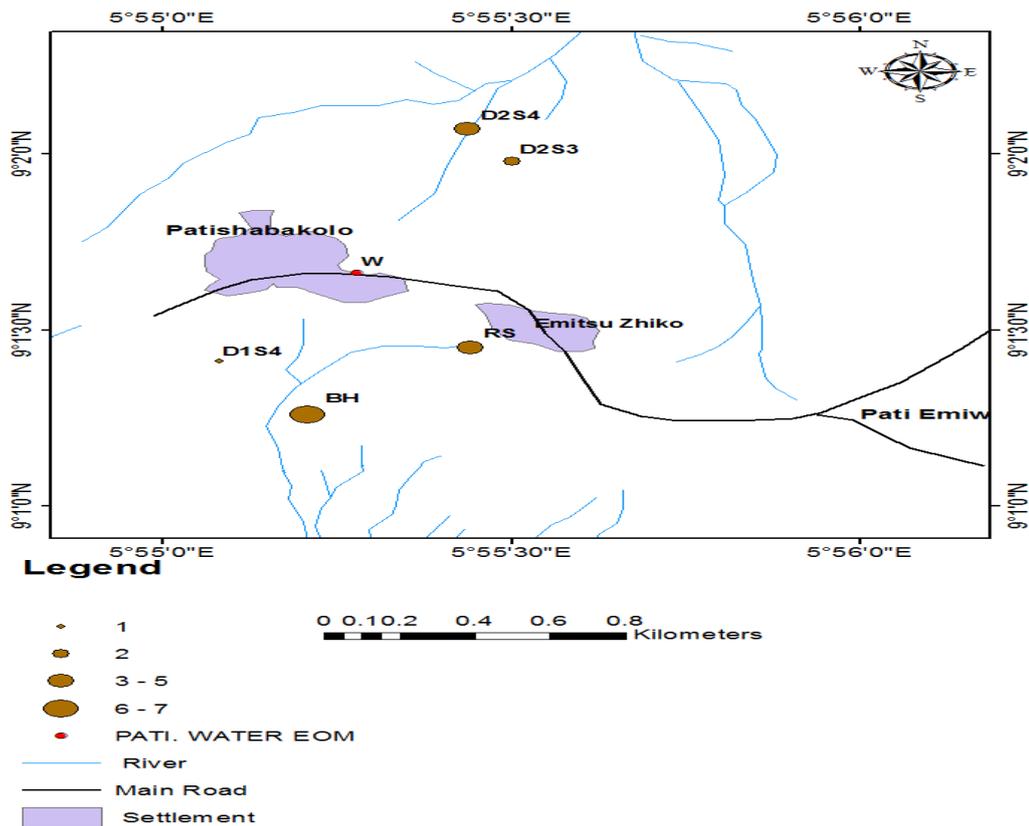
Figure 4.8b: Concentration distribution maps of ethane-oxidizing microbe (EOM)

Key: D1S4= Day 1 Sample 4, BH= Borehole sample, D2S3= Day 2 Sample 3, D2S4= Day 2 Sample 4, River side sample (referring to soil samples).
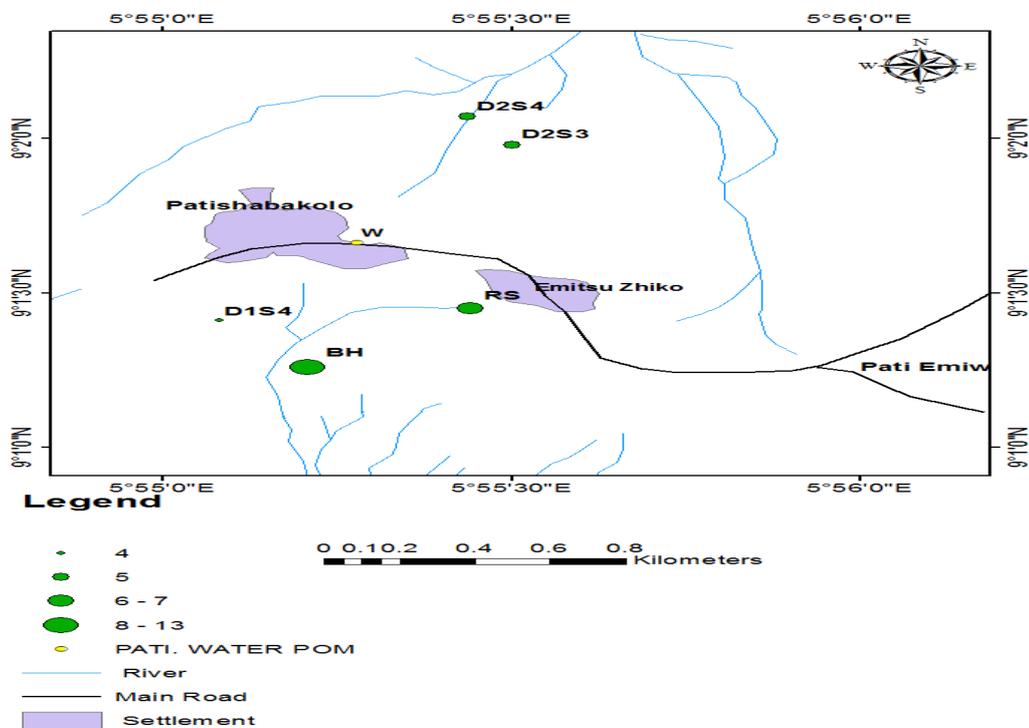
Figure 4.8c: Concentration distribution maps of propane-oxidizing microbe (POM)

Key: D1S4= Day 1 Sample 4, BH= Borehole sample, D2S3= Day 2 Sample 3, D2S4= Day 2 Sample 4, River side sample (referring to soil samples).
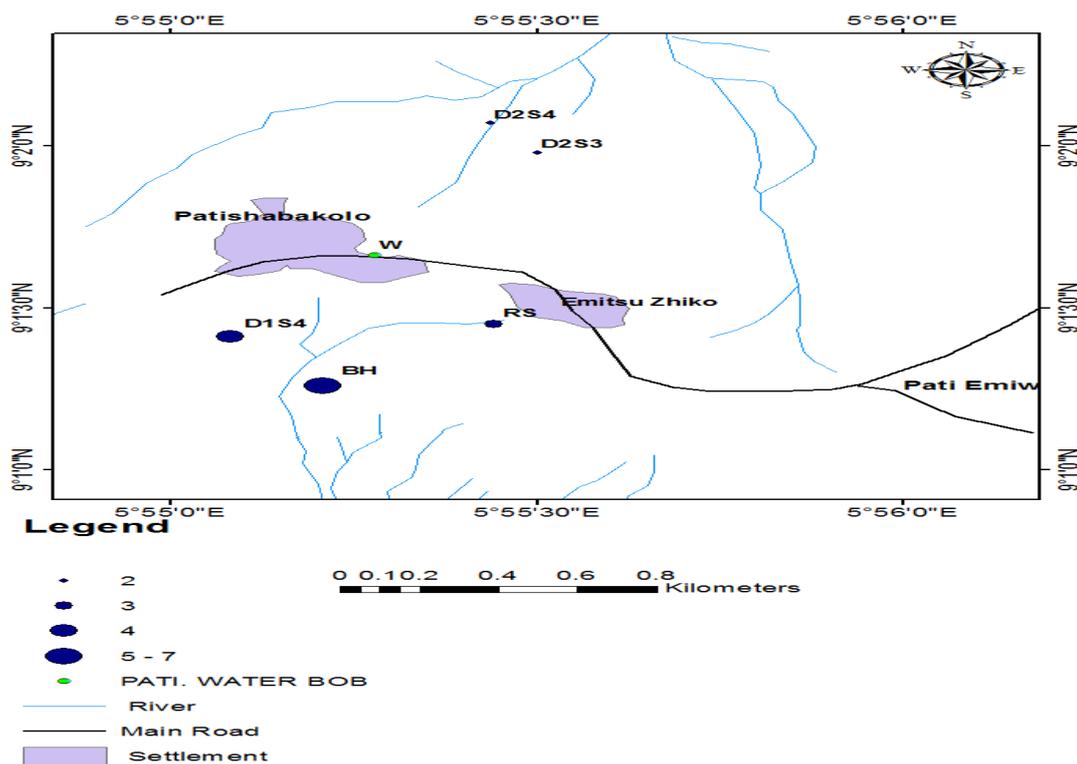
Figure 4.8d: Concentration Distribution maps of Butane oxidizing microbe (BOM)
Key: D1S4= Day 1 Sample 4, Borehole sample, D2S3= Day 2 Sample 3, D2S4= Day 2 Sample 4, River side sample

4.1.4 Molecular Identification of Selected Crude Oil Utilizers

Screening of isolates for crude oil utilization

A total of 61 uncharacterized hydrocarbon gas oxidizing microbes (HOM) were screened for ability to utilize transniger crude oil incorporated into an MSM media for 18hours. Out of the 61, only 30(49.18%) were able to grow in the media, out of the 30, 10 (33.33%) scored as good degraders, 8(26.67%) intermediate and 12(40%) poor degrader based on degree of turbidity and biomass increase evidenced with physical view. Only the 10(33.33 %) isolates scored as good degraders were selected and successfully sequenced, characterized molecularly and subjected to biodegradation study. Unfortunately, only one of the ten isolates was a bacterium (Table 4.21).

4.1.4.1: Electrophoresis analysis

The agarose gel of the amplified bacteria and fungi is shown below (Plate 4.1 and 4.2), revealing the 16S rRNA of 1500 base pairs and ITS of 613 base pair (bp) sequences respectively. The amplicon of the identified bacteria from the Rso-A sample falls within the expected amplicon size (1500 bp) for 16S rRNA gene conserve regions for all bacteria (Plate 4.1). Also, all the amplicons of the identified fungi are within the expected range for the ITS region for all fungi (Plate 4.2). The names and accession numbers of the identified isolates are revealed in Table 4.21 below.
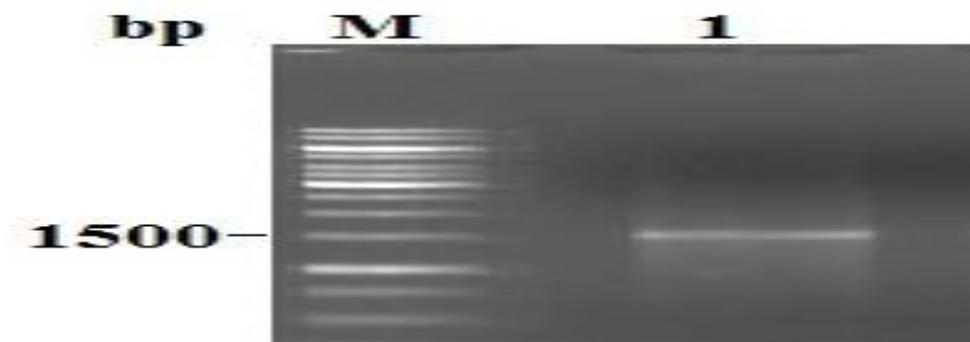
Plate 4.1: Agarose gel of amplified bacteria 16S rRNA sequences of 1500 bp.
Key: M= 1Kb ladder; 1= Rso-A
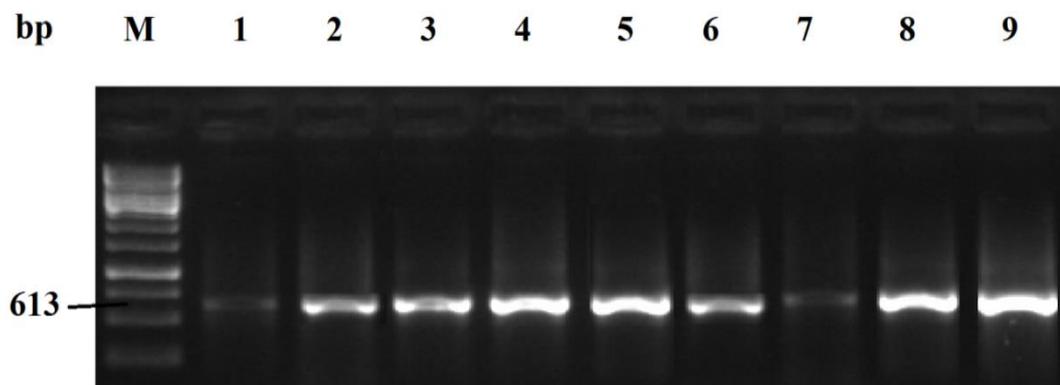
Plate 4.2: Agarose gel of amplified fungi ITS sequences of 613 bp.
Key: M= 1Kb ladder: The numbers represents the samples as follows: 1= A-Feb; 2= D2S3; 3= JYW; 4=BH; 5= JYS; 6= JS-B; 7=D1S4; 8= Borehole; 9= JS-A

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Table 4.21: Identified organisms with the assigned accession numbers from the GenBank

Sequence – ID	Organism	Accession Numbers	Locus (bp)	HC-Gas
Rso-A	*Enterobacter asburiae*	MK551149	799	E
A-Feb	*Aspergillus flavus*	MK551152	569	M
D2S3	*Aspergillus tubingensis*	MK551153	572	E
JYW	*Alternaria tenuissima*	MK551154	230	B
BH	*Aspergillus flavipes*	MK551155	564	E
JYS	*Aspergillus terreus*	MK551156	575	E
JS-B	*Penicillium pimiteouiense*	MK551157	579	M
D1S4	*Aspergillus sublatus*	MK551158	538	E
Borehole	*Aspergillus stellatus*	MK551159	542	P
JS-A	*Candida orthopsilosis*	MK551160	427	E

Key: HC= hydrocarbon, E= ethane, M= methane, P= propane, B= butane

4.1.4.2 Phylogenetic Tree

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analysed. Branches corresponding to partitions reproduced in less than 50 % bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Figure 4.9). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value.
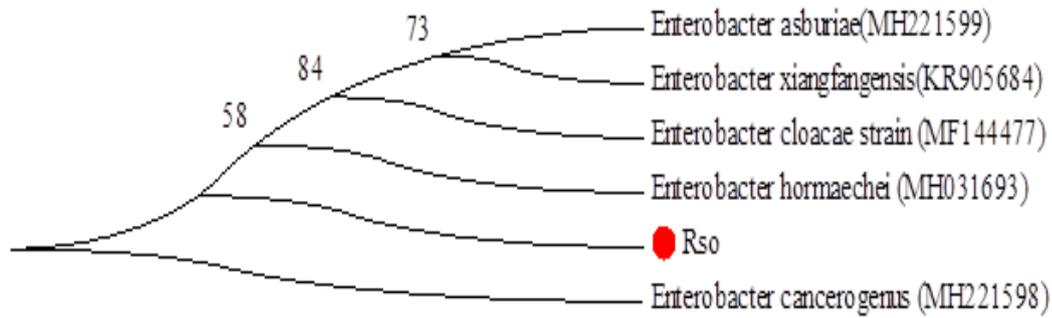
Figure 4.9 Maximum Likelihood phylogenetic tree based on 16S rRNA gene sequence, showing the phylogenetic relationships between identified bacteria and the most closely related strains from the GenBank.

Numbers at the nodes indicate the levels of bootstrap support based on 1000 resampled data sets. Only values greater than 50% are shown. The scale bar indicates 0.2 nucleotide substitution per site
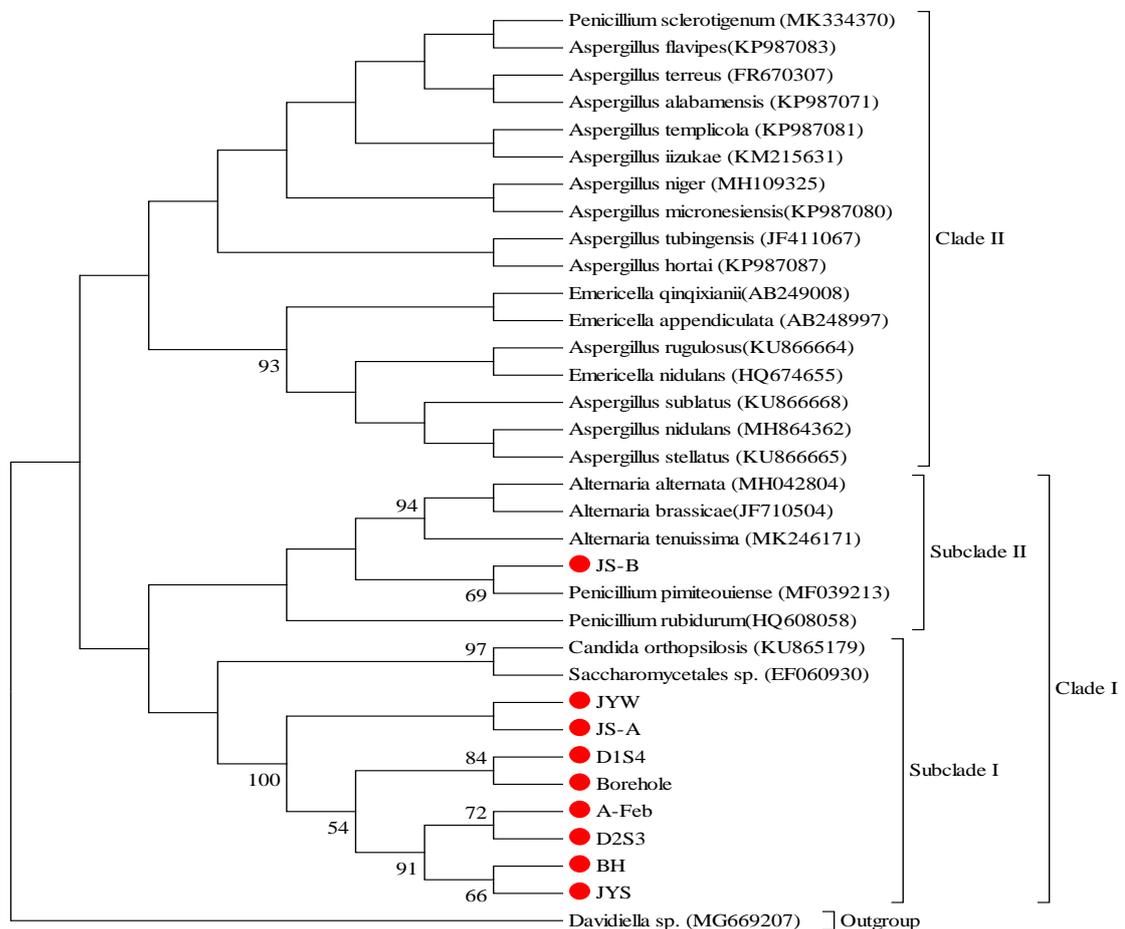
Figure 4.10 Maximum Likelihood phylogenetic tree based on ITS gene sequence, showing the phylogenetic relationships between identified fungi and the most closely related strains from the GenBank.

Numbers at the nodes indicate the levels of bootstrap support based on 1000 resampled data sets. Only values greater than 50% are shown. The scale bar indicates 0.2 nucleotide substitution per site. *Davidiella* sp. is set as the outgroup. Sequences obtained in this study are denoted with a red circle

The phylogenetic analysis of the identified fungi resulted in two main clades (Figure 4.10). The first clade comprises of the *Alternaria* sp., *Penicillium* sp., *Candida* sp., *Saccharomycetales* sp. clustering with the identified fungi from this study. Clade I is subdivided into two: subclade I and subclade II. Subclade I consists of the identified fungi: A-Feb, D2S3, BH, JYS, D1S4, Borehole, JS-A, JYW clustering with the reference strains from the GenBank with less than 50 % having their closest relative to be *Candida orthopsilosis* and *Saccharomycetales* sp. (Figure 4.10). This similarity index is quite low because it falls below the 70% expected borderline for the degree of relatedness according to Wayne *et al*. (1987).

4.1.5 Biodegradation Studies

4.1.5.1   The growth kinetics of the isolates in the medium during the biodegradation process

The rate of growth for all the ten isolates used varied, the growth profile for *Enterobacter asburiae* RSo-A revealed an initial growth of 0.01 nm, which increased to 0.37 nm and 0.47 nm after 14 days and thereafter it decreased to 0.34 nm after 21days.The growth after 28 days increased to 0.55 nm and after 35 days the growth decreased to 0.41 nm.(Figure 4.11); the growth profile for *Candida orthopsilosis* JS-A had a perfect curve with progressive increase in growth from 0.06 (Day 0) to 0.24 nm, 0.26 nm, 0.29 nm, 0.41 nm and 0.51 nm for day 7, 14, 21, 28 and 35 days respectively (Figure 4.12).

The growth rates of *Aspergillus sublatus* D1S4 and *Alternaria tenuissima* JYW were progressive throughout the 35days although *Aspergillus sublatus* D1S4 exhibited a sluggish growth (Figure 4.13). *Aspergillus flavipes* BH and *Aspergillus tubingensis* D2S3 were erratic in growth; for *Aspergillus flavipes* BH a decline in growth was

observed after 21 days while a pronounced decrease was observed after 14 day for *Aspergillus tubingensis* D2S3 (Figure 4.13).

It was observed that the growth of *Aspergillus flavus* A-Feb, *Aspergillus stellatus* Borehole, *Aspergillus terreus* JYS and *Penicillium pimiteouiense* JS-B, increased gradually from day 0 to day 14 and decrease from day 21 with the exception of A-Feb which decrease in growth remained constant and unchanged (0.27 nm) even after 35 days (Figure 4.14). JYS and JS-B had increased and continuous growth rate throughout the time of study, however, there were no significant differences ($p > 0.05$) in the rate of growth between day 21, 28 and 35 for *Aspergillus flavus* A-Feb; other isolates growth rates were significantly different ($p < 0.05$) in all the days of inoculation (Figure 4.14).
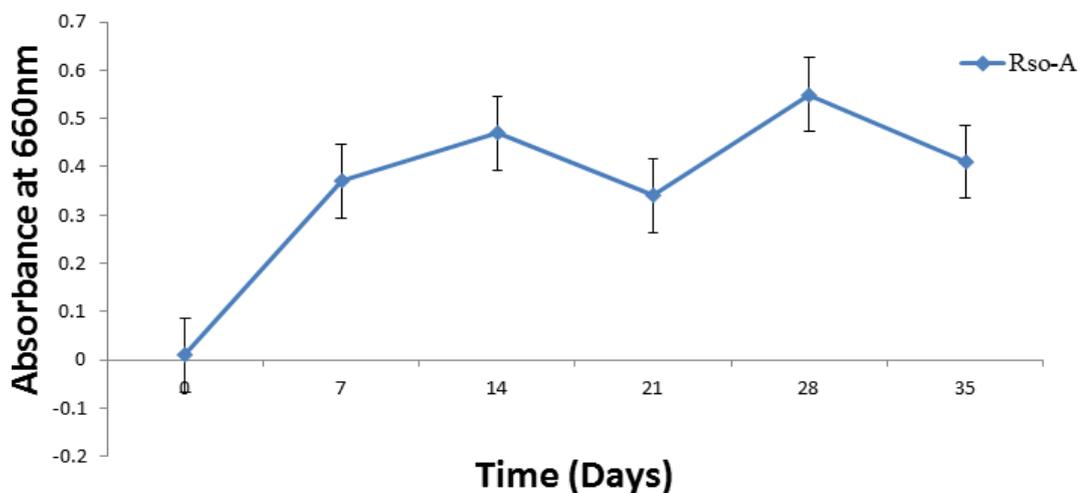
Figure 4.11: Growth of *Enterobacter asburiae* RSo-A in crude oil medium during oil biodegradation.
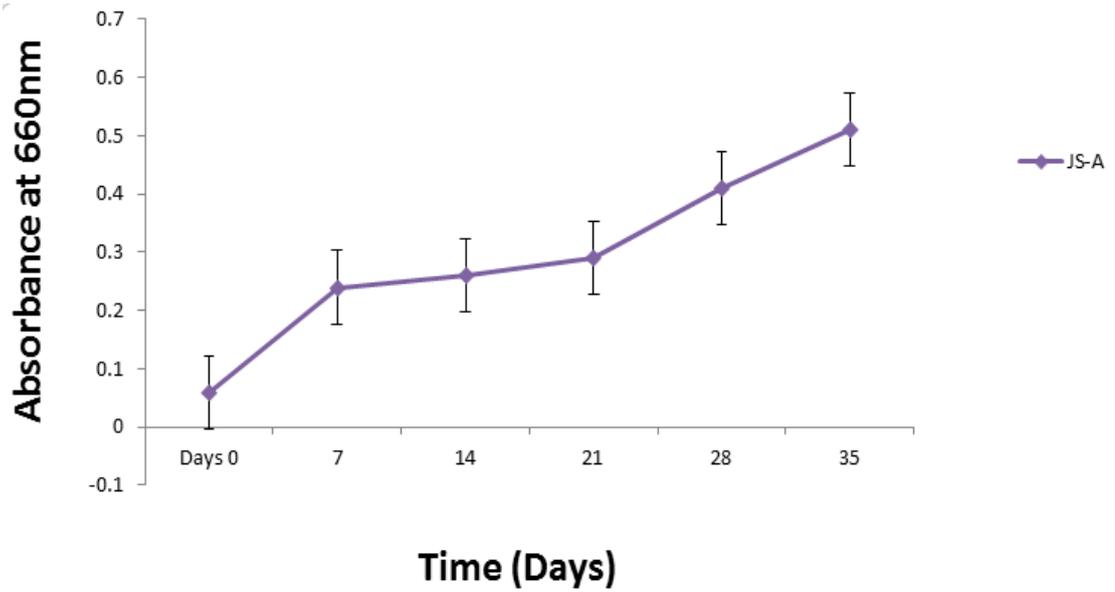
Figure 4.12: Growth of *Candida orthopsilosis* JS-A in crude oil medium during oil biodegradation.
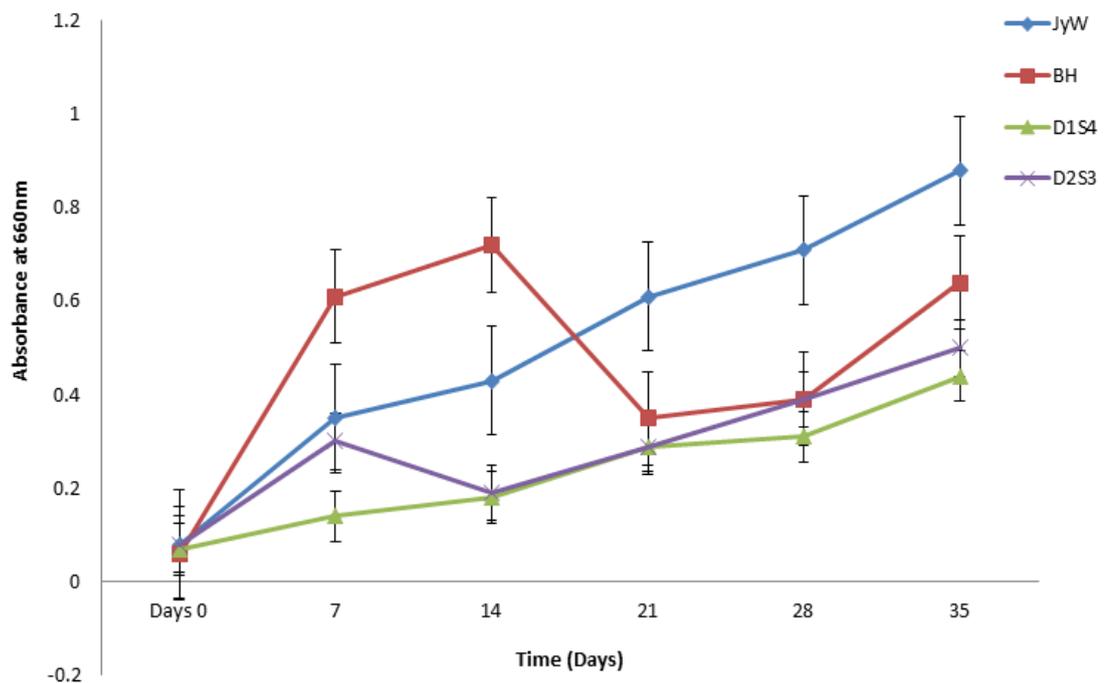
Figure 4.13: Growth of *Alternaria tenuissima*- JYW, *Aspergillus flavipes* BH, *Aspergillus sublatus* D1S4 and *Aspergillus tubingensis* D2S3 in crude oil medium during oil biodegradation
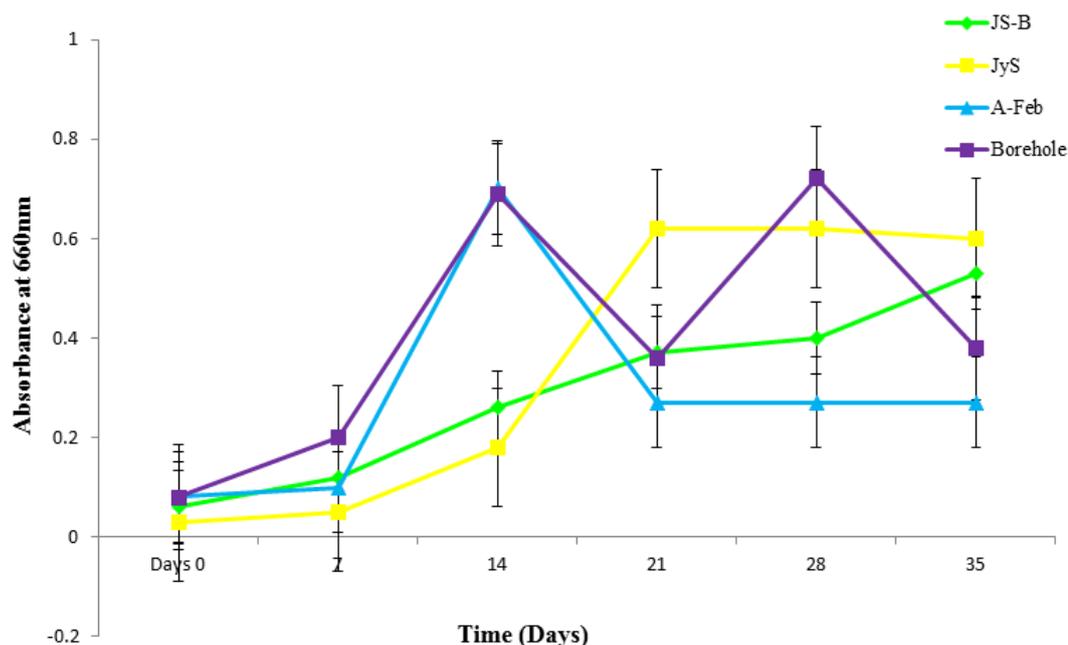
Figure 4.14: Growth of *Penicillium pimiteouiense* JS-B *Aspergillus terreus* JYS, *Aspergillus flavus* A-Feb and *Aspergillus stellatus* Borehole in crude oil medium during oil biodegradation

The growth profile for the mixed isolate revealed that the mixed isolates of had a perfect growth profile, the growth started off from 0.08 nm (day 0) and increased to 0.75 nm (Day 21), it decreased to 0.36 nm and 0.30 nm for day 28 and 35 respectively (Figure *Aspergillus tubingensis* D2S3 and *Aspergillus flavus* A-Feb 4.15a). Isolates of *Aspergillus terreus* JYS+ *Aspergillus sublatus* D1S4 and *Aspergillus tubingensis* D2S3+ *Aspergillus flavipes* BH had an erratic growth; for *Aspergillus terreus* JYS+ *Aspergillus sublatus* D1S4, the growth rate was 0.05 nm at day 0, it remained unchanged till after day 7, then increased to 0.43 nm at day 28 and decreased to 0.36 nm after day 35 while for *Aspergillus tubingensis* D2S3 + *Aspergillus flavipes* BH a pronounced decrease occurred between day 28 and day 35. However, the results were significantly different ($p < 0.05$) in all the days of inoculation (Figure 4.15a). More so, *for Aspergillus flavus* A-Feb + *Aspergillus stellatus* Borehole, decrease in growth was not observed, growth was continuous and progressive throughout the 35 days of incubation while the mixed isolates of *Aspergillus sublatus* D1S4 + *Aspergillus stellatus*

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growth was inconsistent with an observed decrease between day 21 and day 28 (Figure

4.15b).
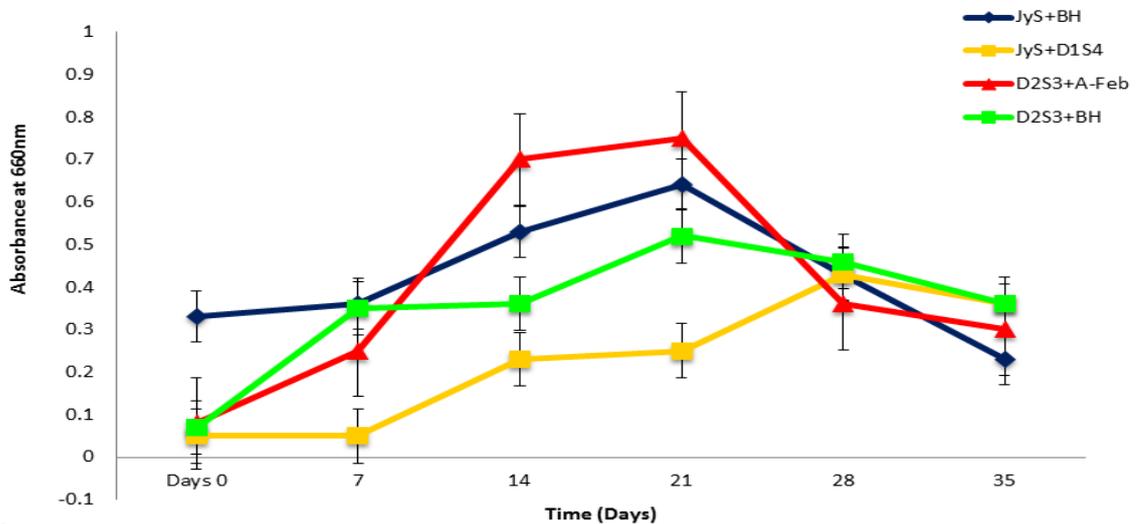
Figure 4.15a: Growth of mixed fungi isolates in crude oil medium during oil

biodegradation.

Key: the codes: (JYS + BH), (JYS + D1S4), (D2S3 + A-Feb) and (D2S3+ BH) represent mixed isolates of *Aspergillus terreus* JYS + *Aspergillus flavipes* BH, *Aspergillus terreus* JYS+ *Aspergillus sublatus* D1S4, *Aspergillus tubingensis* D2S3 + *Aspergillus flavus* A-Feb *and Aspergillus tubingensis* D2S3 + *Aspergillus flavipes* BH
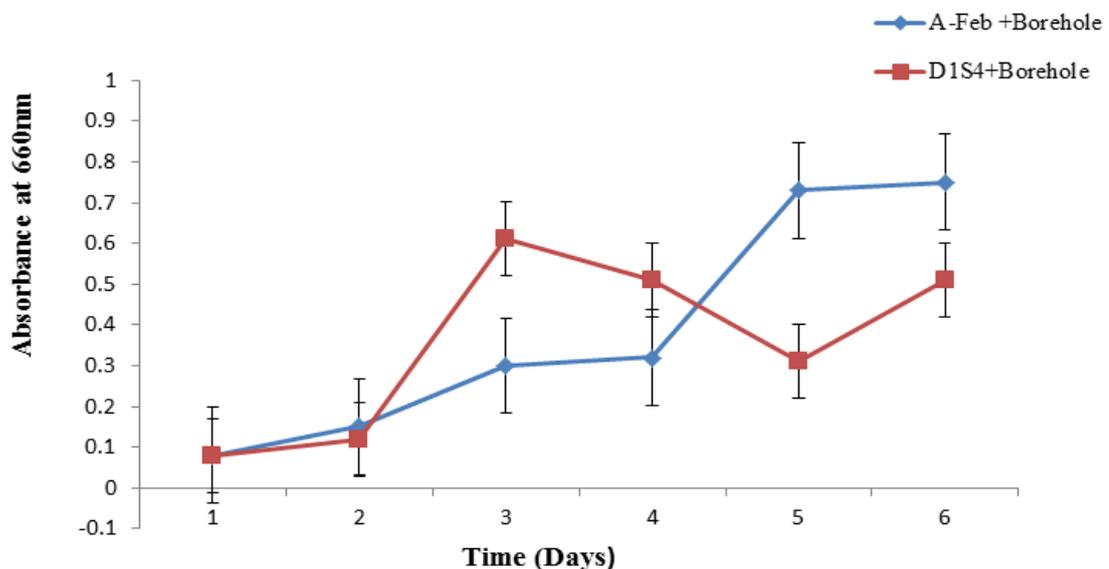
Figure 4.15b: Growth of mixed fungi isolates in crude oil medium during oil biodegradation

Key: the codes: (A-Feb + Borehole) and (D1S4 + Borehole) represent mixed isolates of *Aspergillus flavus* A-Feb + *Aspergillus stellatus*-Borehole and *Aspergillus sublatus* D1S4 + *Aspergillus stellatus* Borehole respectively

4.1.5.2   Percentage degradation (gravimetric analysis method) of crude oil by microbial isolates

The results of biodegradation of petroleum hydrocarbons revealed that *Enterobacter asburiae* RSo-A achieved 68 % degradation, *Aspergillus flavus* A-Feb (73 %), *Aspergillus tubingensis* D2S3 (65.4 %), *Alternaria tenuissima* JYW (62.1 %), *Aspergillus flavipes* BH (72.11 %), *Aspergillus terreus* JYS (77.5 %), *Penicillium pimiteouiense* JS-B (83.68 %), *Aspergillus sublatus* D1S4 (68.89 %), *Aspergillus stellatus* Borehole (88.05 %) and *Candida orthopsilosis* JS-A (85 %) after 35 days (Figure 4.16). The mixed isolates of *Aspergillus terreus* JYS + *Aspergillus sublatus* D1S4 achieved 60 % degradation, *Aspergillus flavipes* BH + *Aspergillus terreus* JYS (64.33 %), *Aspergillus tubingensis* D2S3+ *Aspergillus flavipes* BH (77.5 %). *Aspergillus tubingensis* D2S3+ *Aspergillus flavus* A-Feb (80.01 %), *Aspergillus flavus* A-Feb + *Aspergillus stellatus* Borehole (78.3 %) and *Aspergillus sublatus* D1S4 + *Aspergillus stellatus* Borehole (79.0 %) after 35 days (Figure 4.16). It was observed that the highest rate of degradation was achieved by *Aspergillus stellatus*-Borehole (88.0 %) while the lowest degradation was achieved by *Alternaria tenuissima* JYW (62.1 %). For the mixed isolates the higest and lowest degradation was observed among the consortium of *Aspergillus tubingensis* D2S3+ *Aspergillus flavus* A-Feb and *Aspergillus terreus* JYS + *Aspergillus sublatus* D1S4 with a percentage degradation of 80.01 % and 60 % respectively. However, the rate of degradation among the ten isolates and their consortium were significantly differnent (p<0.05) within the time of study.
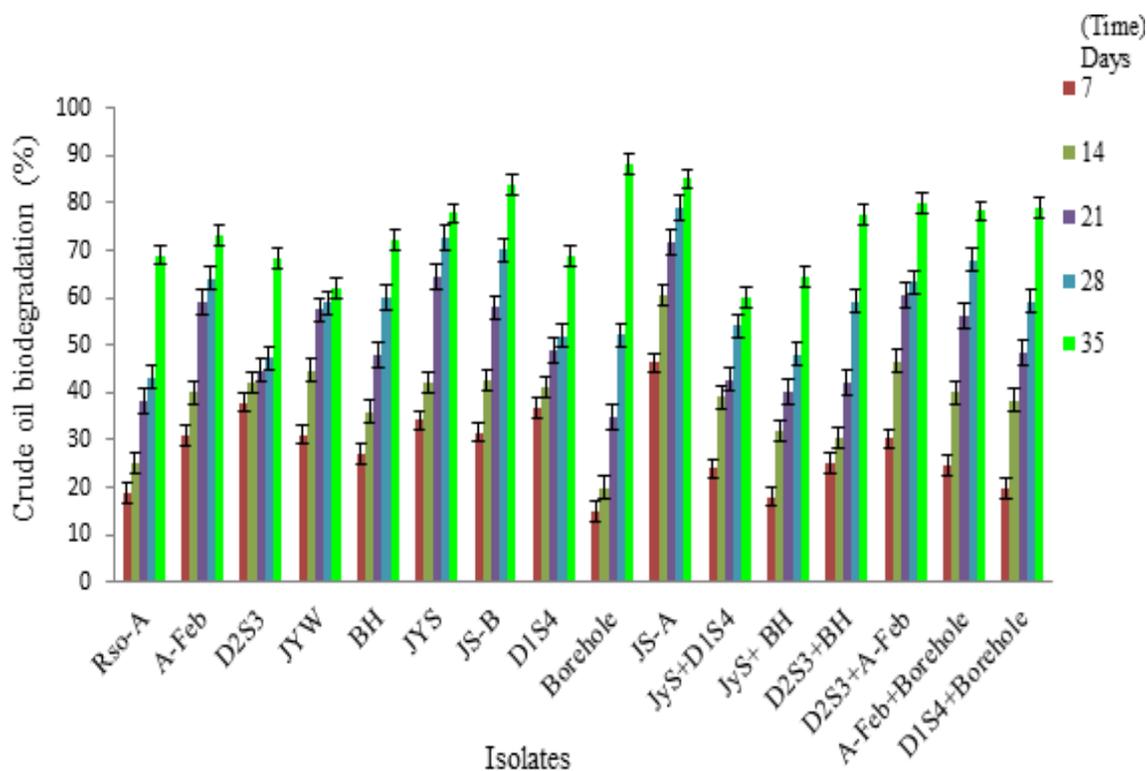
Figure 4.16: Percentage degradation of crude oil by microbial isolates after 35days

Key: Rso-A= *Enterobacter asburiae* RSo-A, *A-Feb= Aspergillus flavus* A-Feb, D2S3 = *Aspergillus tubingensis* D2S3, JYW = *Alternaria tenuissima* JYW, *BH= Aspergillus flavipes* BH, JYS = *Aspergillus terreus* JYS, JS-B = *Penicillium pimiteouiense* JS-B, D1S4 = *Aspergillus sublatus* D1S4, Borehole = *Aspergillus stellatus* Borehole, JS-A = *Candida orthopsilosis* JS-A, JYS+D1S4= *Aspergillus terreus* JyS+ *Aspergillus sublatus,* D1S4, BH+ JyS = *Aspergillus terreus* JYS + *Aspergillus flavipes* BH, D2S3 + BH = *Aspergillus tubingensis* D2S3 + *Aspergillus flavipes* BH, D2S3 + A-Feb = *Aspergillus tubingensis* D2S3 + *Aspergillus flavus* A-Feb, A-Feb + Borehole = *Aspergillus flavus* A-Feb + *Aspergillus stellatus-* Borehole, and D1S4 +Borehole= *Aspergillus sublatus* D1S4 + *Aspergillus stellatus* Borehole

4.1.5.3 Gas Chromatographic-Mass Spectroscopic (GC–MS) profiles of degraded crude oil by microbial isolates

The GC-MS profiles revealed that transniger pipeline crude oil has various hydrocarbon components with varying concentrations of C_8 to C_{38} (Figure 4. 17a and 4.17b).
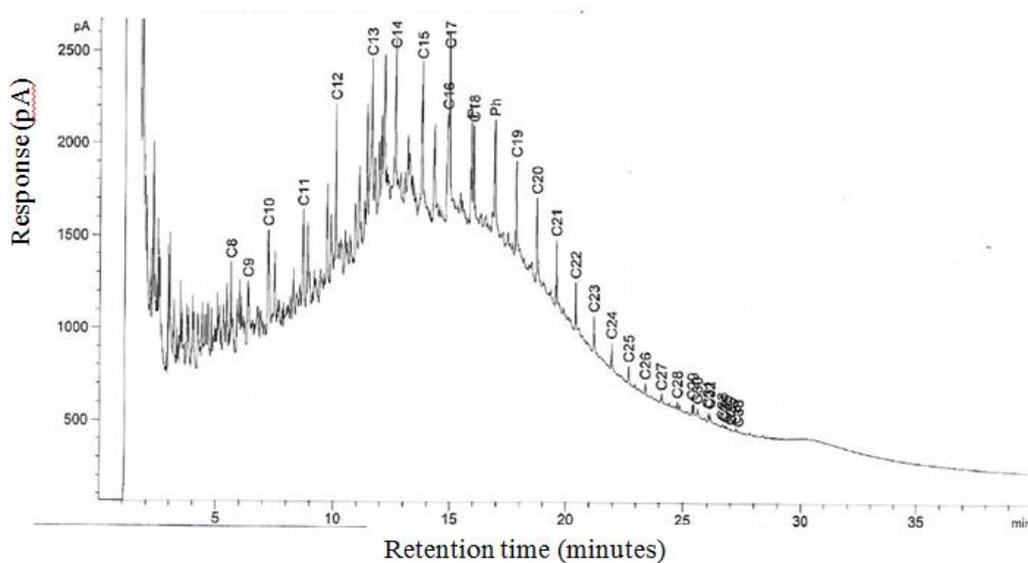
Figure 4.17a: Chromatograms of transniger pipeline crude oil (undegraded)
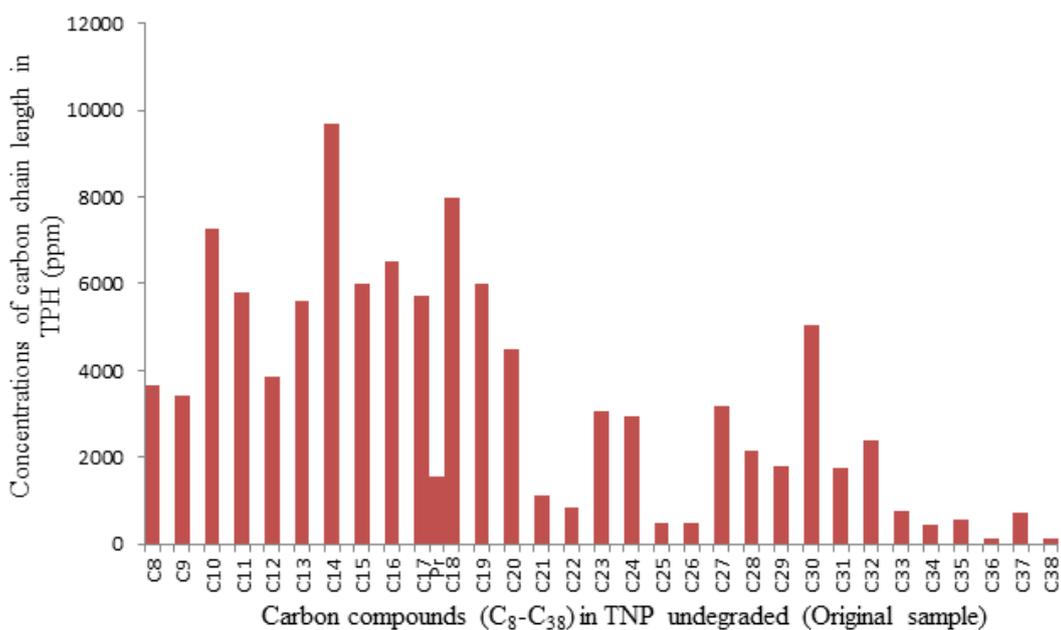
Figure 4.17b: Concentrations of carbon compounds in transniger pipeline original crude oil (undegraded)

The concentrations of the residual total petroleum hydrocarbon present in the transniger pipeline crude oil after 7 days and 35 days inoculation with microorganisms was determined. The GC-MS profile of crude oil used revealed various degree of biodegradation by the organisms. For *Aspergillus flavus* A-Feb, the GC-MS analysis revealed that there were no significant reductions in some compounds present in the oil after 7 days although, three new peaks emerged between C_{15} and C_{16} (Figure 4.18a) as a result of minimal biodegradation and they were: 2, 2, 4, 4-tetramethylnonane, 1-

tetradecanol and 3-ethyl-5, 5-dimethyloctane. However, after 35days some compounds such as n-Dotriacontane (C_{32}) and the newly emerged (2, 2, 4, 4-tetramethylnonane, 1-tetradecanol and 3-ethyl-5, 5-dimethyloctane) were completely biodegraded (0.0 ppm) (Figure 4.18b), while Octacosane (C_{28}), nonacosane (C_{29}) n-Triacontane (C_{30}), n-Hentriacontane (C_{31}), were reduced to 3.6 ppm, 17 ppm, 9 ppm, and 4.0 ppm respectively, others had intermediate biodegradation ranging from 20 ppm to 1000 ppm (Figure 4.19).
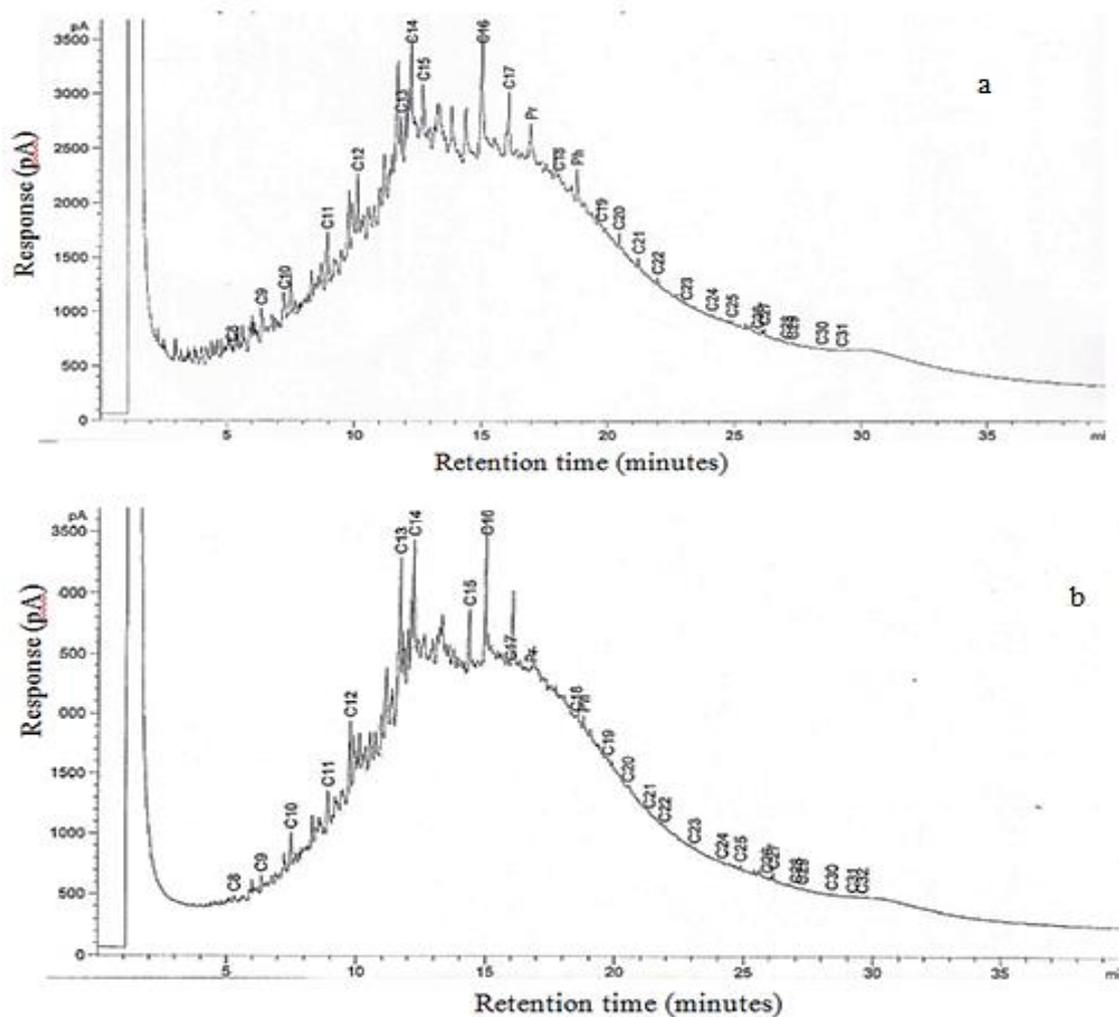
Figure 4.18: Chromatograms of transniger pipeline crude oil degraded by

Aspergillus flavus **A-Feb after (a) 7days and (b) 35 days**
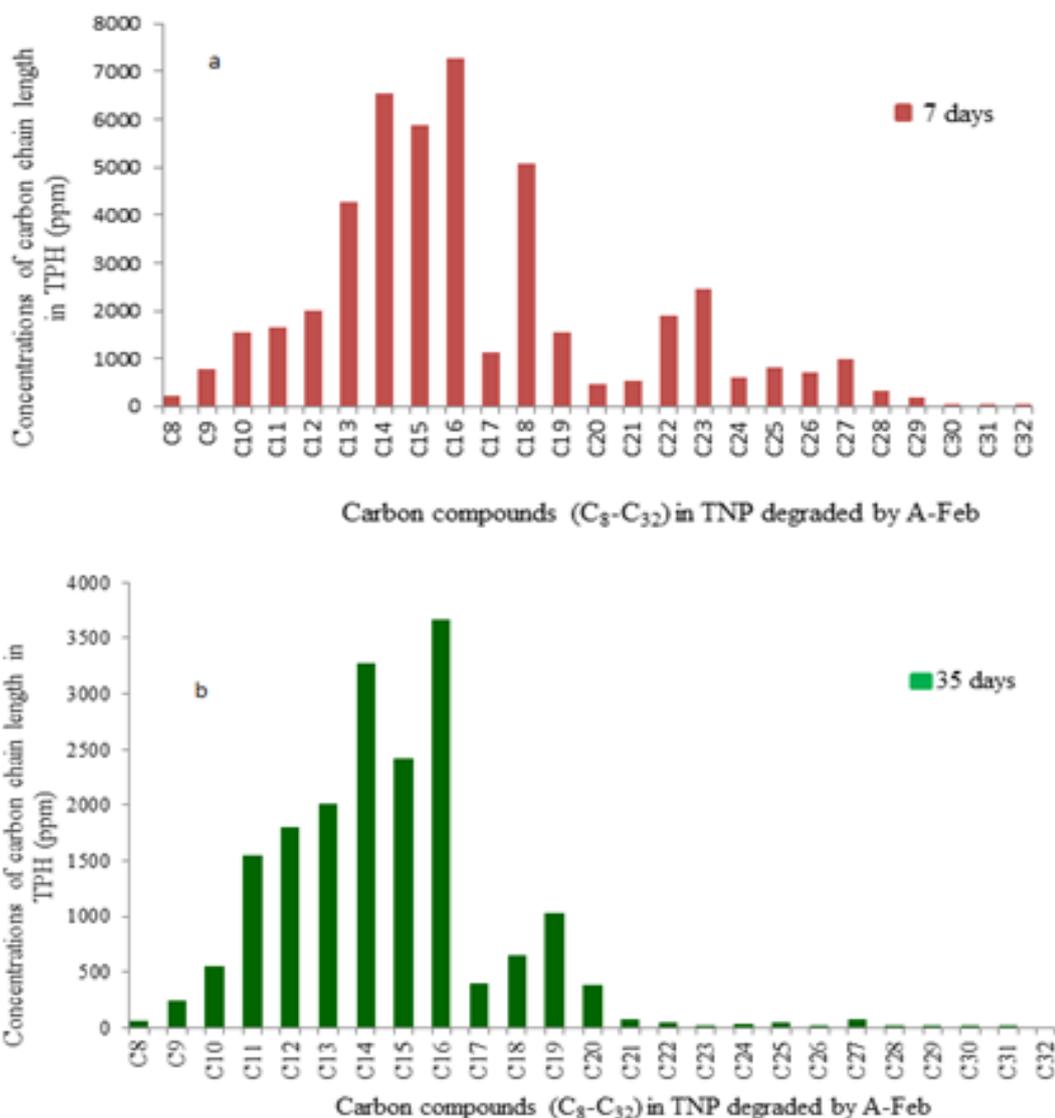
Figure 4.19. Concentrations of carbon compounds in transniger pipeline crude oil degraded by *Aspergillus flavus* A-Feb, after (a) 7 days (b) 35 Days

Key: A-Feb= *Aspergillus flavus* A-Feb

The results of GC-MS analysis revealed that biodegradation took place in the transniger pipeline crude oil treated with *Penicillium pimiteouiense* JS-B after 7 days, this led to the complete removal of Pentadecane C_{15} and emergence of 2, 6, 10, 14-Tetramethylpentadecane (Figure 4.20a). However, after 35 days of incubation more of the oil components (Octane (C_8), (Pentadecane C_{15}) and Octadecane (C_{18}) were completely degraded (0.0ppm) (Figure 4.20b), while, other components such as; n-

Heptadecane (C_{17}), Eisosane (C_{20}), n-Heneicosane (C_{21}) and Docosane (C_{22}) were reduced from 6129 ppm, 4897.6 ppm, 3977.65 ppm, and 5487.6 ppm (Figure 4.21a) to 21.0 ppm 6.0 ppm, 44 ppm and 28 ppm respectively (Figure 4.21b).
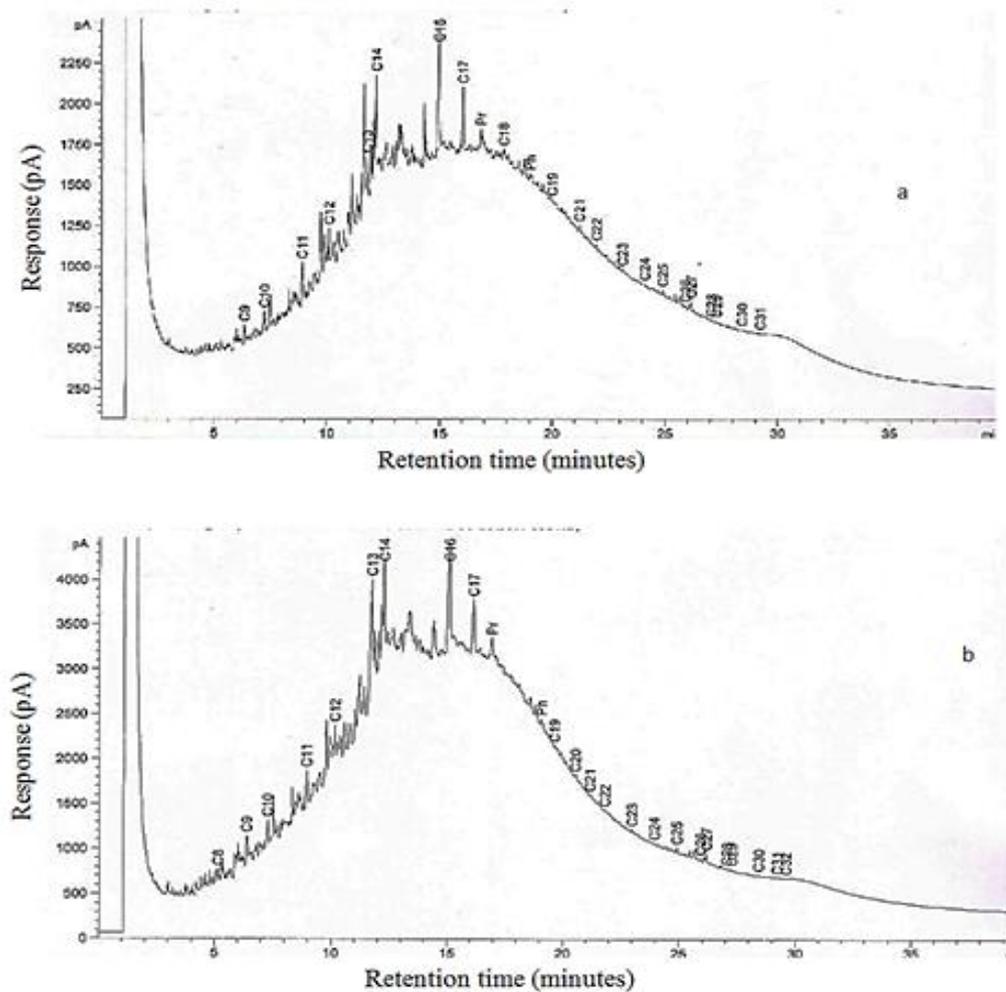
Figure 4.20: Chromatograms of transniger pipeline crude oil degraded by

Penicillium pimiteouiense **JS-B after (a) 7days and (b) 35 days**
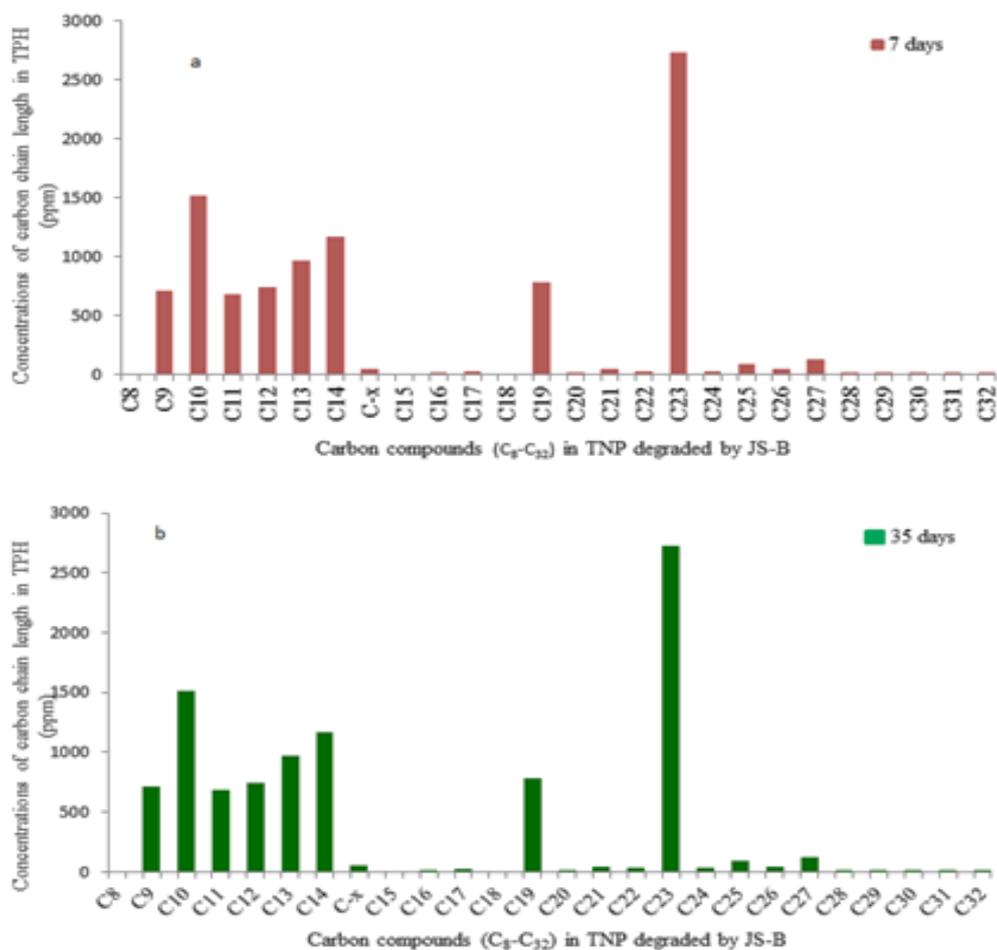
Figure 4.21: Concentrations of carbon compounds in transniger pipeline crude oil degraded by *Penicillium pimiteouiense* JS-B after (a) 7 days (b) 35 days

Key: JS-B = *Penicillium pimiteouiense* JS-B

The GC-MS analysis for the transniger pipeline crude oil degraded by *Enterobacter asburiae* RSo-A revealed that there was no significant reduction in the concentrations of carbon compounds present in the oil after 7 days. However, there was evidence of biodegradation with the emergence of new peaks between C_{15} and C_{16} (Figure 4.22a) these peaks which represent two compounds (3-methylpent-4-pentadecane and 3, 8-dimethylundecane) were completely removed after 35 days (Figure 4.22b). Futhermore, it was observed that the concentration of C_{20} in the oil which initially reduced to 359 ppm from 4481 ppm after 7 days (Figure 4.23a) increased to 2104 ppm after 35 days

(Figure 4.23b), although all the concentrations for the other oil components were minimally reduced with the least being C_8 (6.9 ppm) (Figure 4.23b).
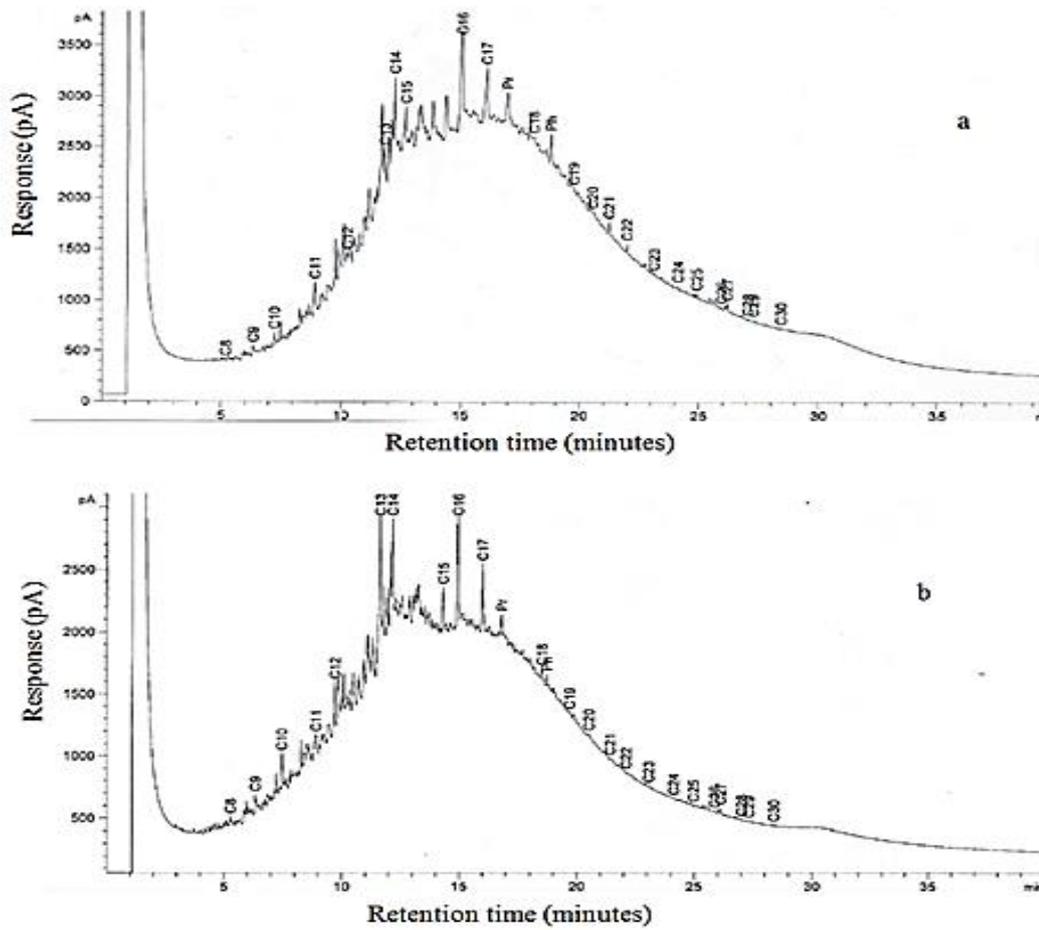
Figure 4. 22: Chromatograms of transniger pipeline crude oil degraded by

Enterobacter asburiae **RSo-A after (a) 7days and (b) 35 days**
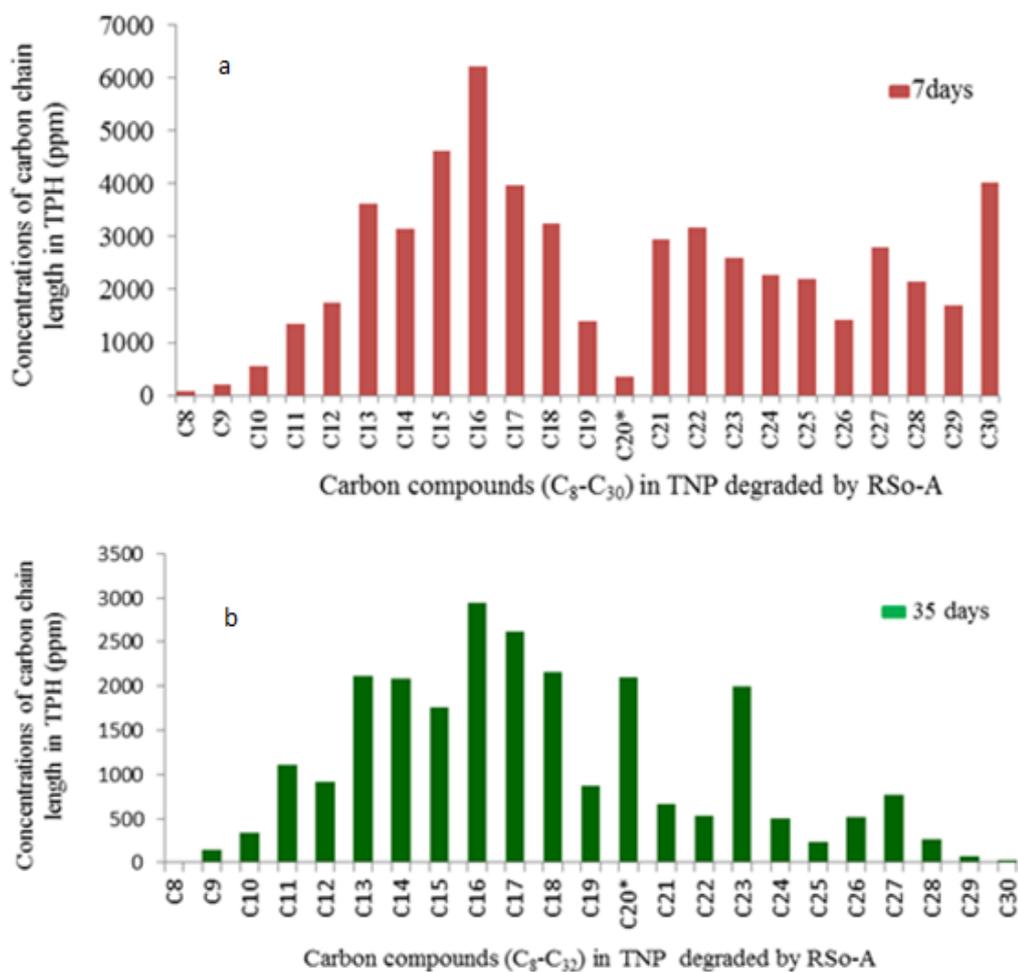
Figure 4.23: Concentrations of carbon compounds in transniger pipeline crude oil degraded by *Enterobacter asburiae* RSo-A after (a) 7 days (b) 35 days

Key: RSo-A = *Enterobacter asburiae* RSo-A

For *Aspergillus tubingensis* D2S3, the GC-MS analysis revealed significant reductions in some compounds present in the oil after 7 days with emergence of three new peaks between C_{15} and C_{16} (Figure 4.24a) as a result of minimal biodegradation and they were: 2, 2, 4, 4-tetramethylnonane, 1-tetradecanol and 3-ethyl-5, 5-dimethyloctane. However, after 35days all the emerged compounds (2, 2, 4, 4-tetramethylnonane, 1-tetradecanol and 3-ethyl-5, 5-dimethyloctane) were completely biodegraded (0.0ppm) and an obvious new peak between C_{14} and C_{15} was observed (Figure 4.24b). Other compounds such as Octane (C_8), n-Triacontane (C_{30}), n-Hentriacontane (C_{31}),, n-

Dotriacontane (C_{32}) were reduced to less detectable quantity of 28.0 ppm, 51.0 ppm, 28.12 ppm, 28.13 ppm respectively (Figure 4.25b).
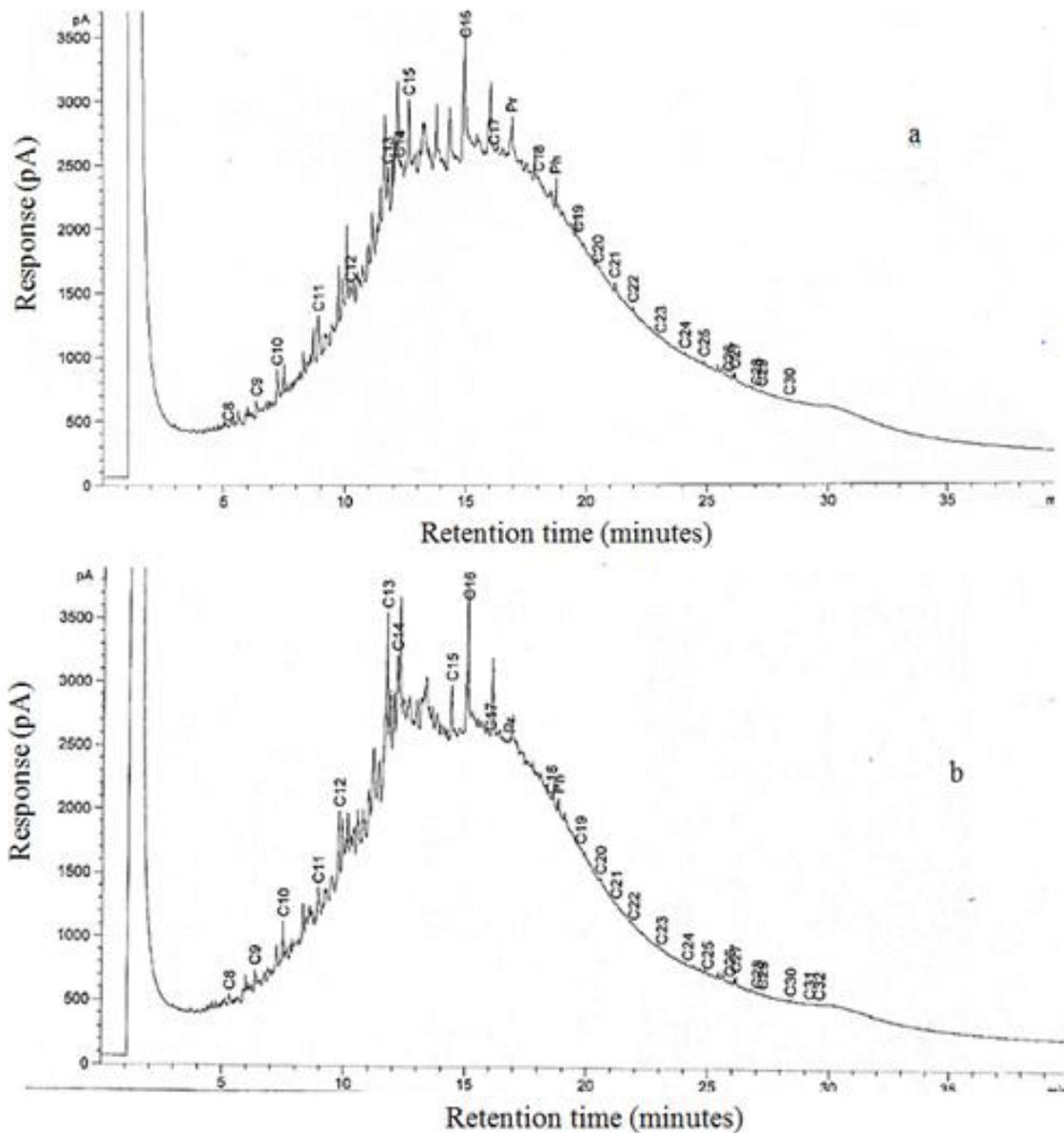
Figure 4. 24: Chromatograms of transniger pipeline crude oil degraded by

Aspergillus tubingensis **D2S3 after (a) 7days and (b) 35 days**

Figure 4.25: Concentrations of carbon compounds in transniger pipeline crude oil

degraded by *Aspergillus tubingensis* D2S3 after (a) 7 days (b) 35 days

Key: D2S3 = *Aspergillus tubingensis* D2S3

The GC-MS profiles revealed that after 7 days inoculation of *Candida orthopsilopsis* JS-A into the petroleum medium, caused compounds like; Tridecane (C_{13}) and Pentadecane (C_{15}) to be completely degraded, while C_{10} (Decane) was resistant to microbial attack with concentration (Figure 4.26a and figure 4.27a). Thirty five days later, more compounds (Undecane (C_{11}), Tetradecane (C_{14}), Hexadecane (C_{16}) , n-Heptadecane (C_{17}), Octadecane (C_{18}) n-Docosane (C_{23}) and n-Tetracosane (C_{24}) were completely degraded and removed from the hydrocarbon mix (Figure 4.26b and Figure

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4. 27b) while Decane (C_{10}), Nonadecane (C_{19}) and Eisosane (C_{20}) were resistant to the microbial attack evidence with no decrease in concentration (Figure 4.27b). No new intermediate compound emerged, meaning that organism was able to assimilate the compounds as energy and carbon source without an intermediate.
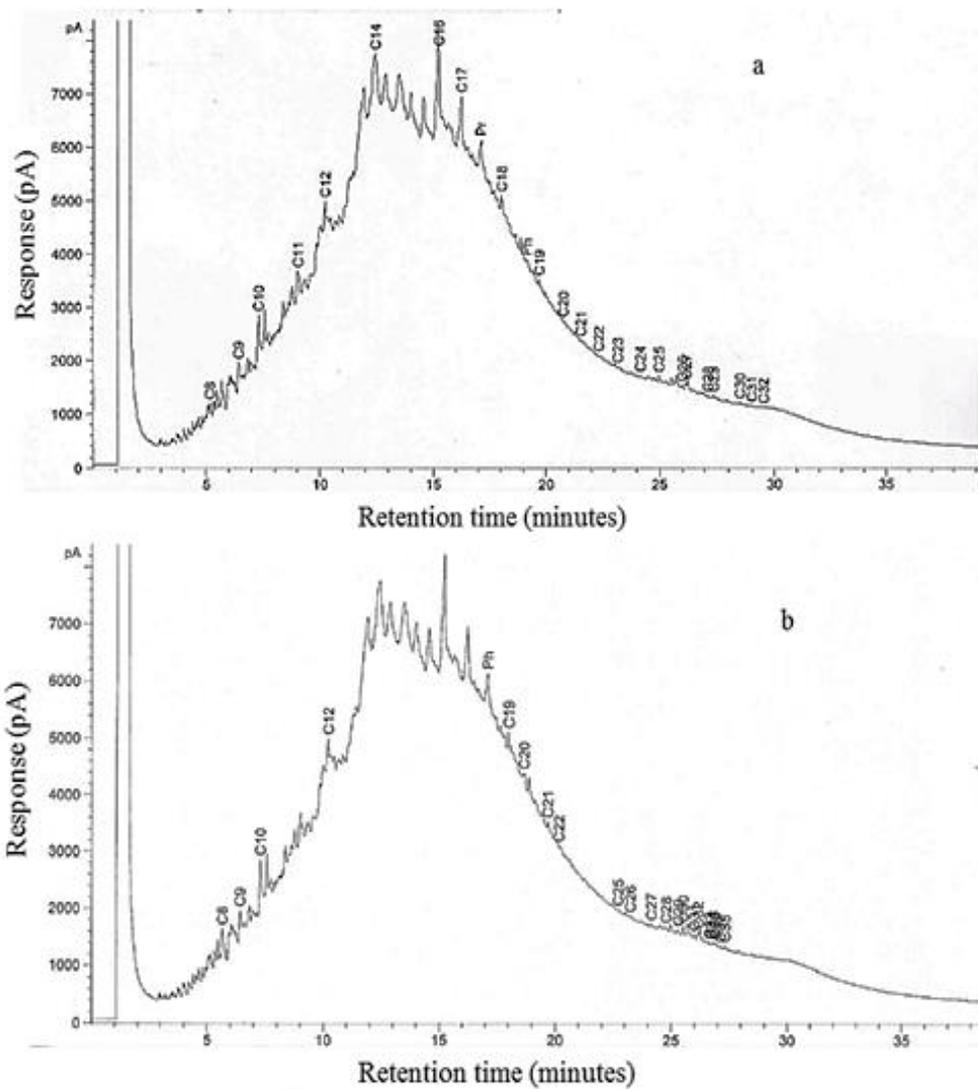
Figure 4.26: Chromatograms of transniger pipeline crude oil degraded by *Candida orthopsilopsis* JS-A after (a) 7 days and (b) 35 days
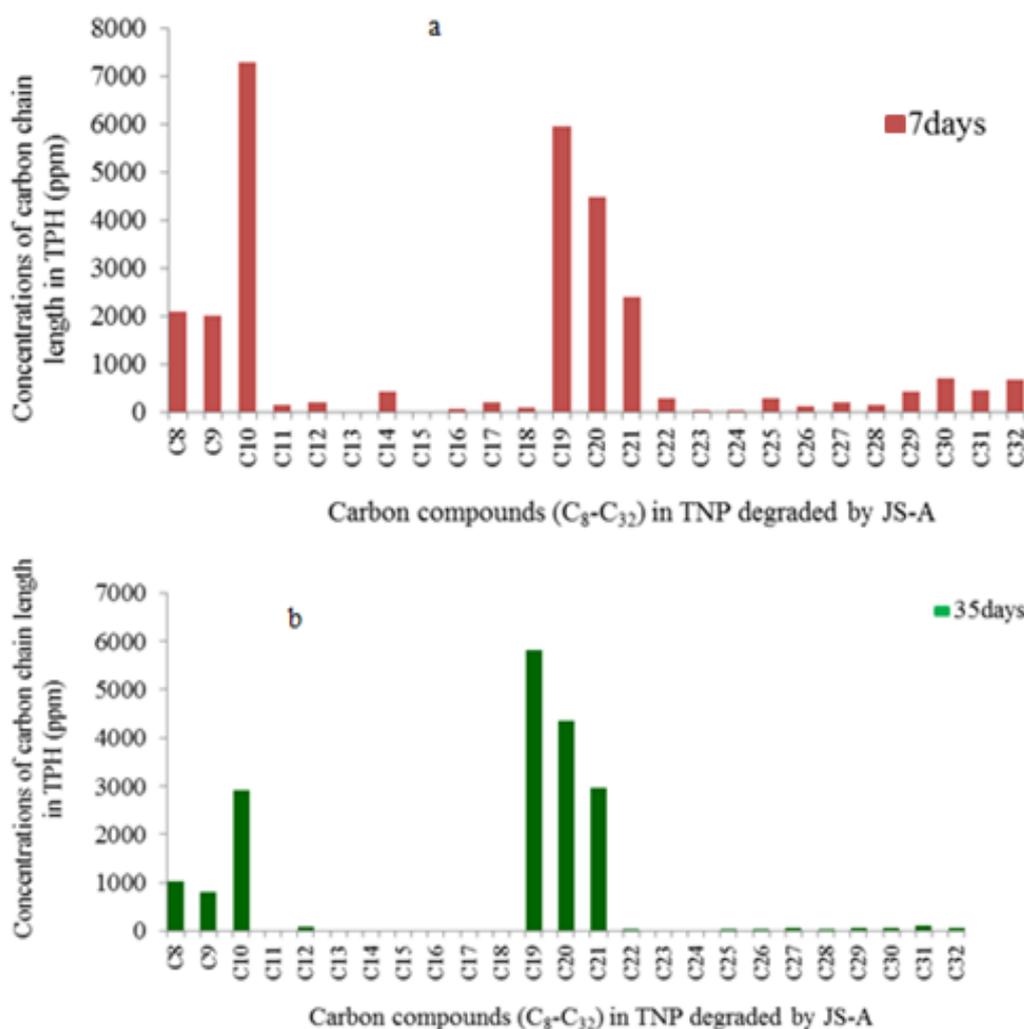
Figure 4.27: Concentrations of carbon compounds in transniger pipeline crude oil degraded by *Candida orthopsilopsis* JS-A, after (a) 7 days (b) 35 days

key: JS-A= *Candida orthopsilopsis* JS-A

The GC-MS profiles of transniger pipeline crude oil degraded by *Aspergillus sublatus-*D1S4 revealed that the organism effectively degraded and reduced the concentrations of the compounds present in the oil after 7 days (Figure 4.28a), although the concentrations of some compounds (Tridecane, Tetradecane, Pentadecane, Hexadecane, n-Heptadecane, Octadecane and n-Docosane) were still above 1000ppm. However, the organism could not achieve 100% removal of the original compound but all the intermediate compounds (1-bromo-4-nonene, (9E)-9-hexadecenol) and 3-ethyl-5, 5-

dimethyl-3-hexanol) between peak C_{15} and C_{16} (Figure 4.28a) were completely degraded (Figure 4. 28b) while the original compounds were reduced greatly (n-Hexacaosane 6.8 ppm) and (n-Octacosane, 10ppm) after 35 days (Figure 4.29b). More so, it was observed that the concentration of C_{27} and C_{30} which were initially reduced to 125.07 ppm and 15.5 ppm after 7 days (Figure 4.29a) increased to 283.2ppm and 24.8ppm respectively after 35 days (Figure 4.29b), this implies that the product of degradation was similar (analogue) to the compound in C_{28} and thereby added to its concentrations.
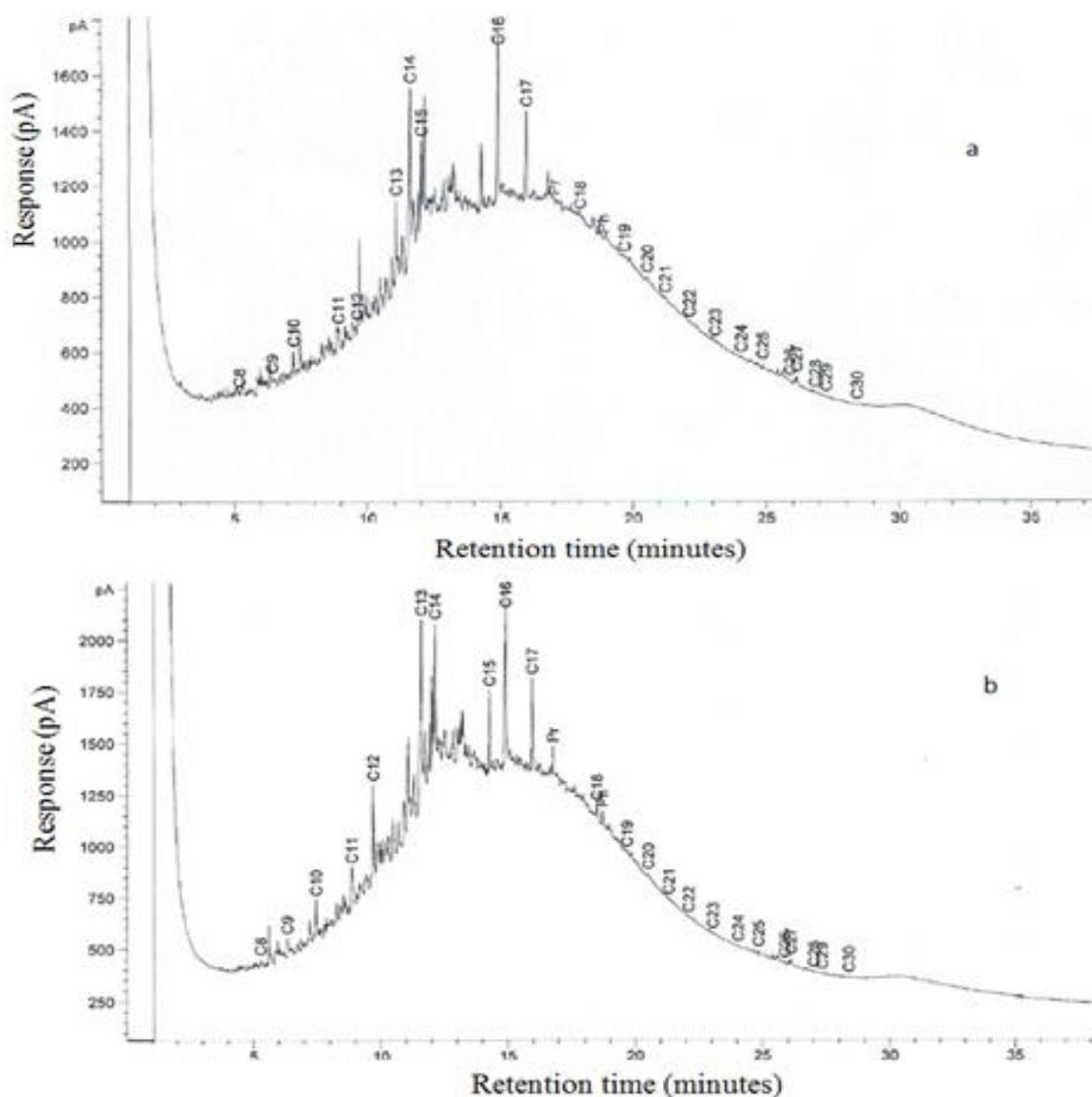
Figure 4.28: Chromatograms of transniger pipeline crude oil degraded by
Aspergillus sublatus-**D1S4 after (a) 7days and (b) 35 days**
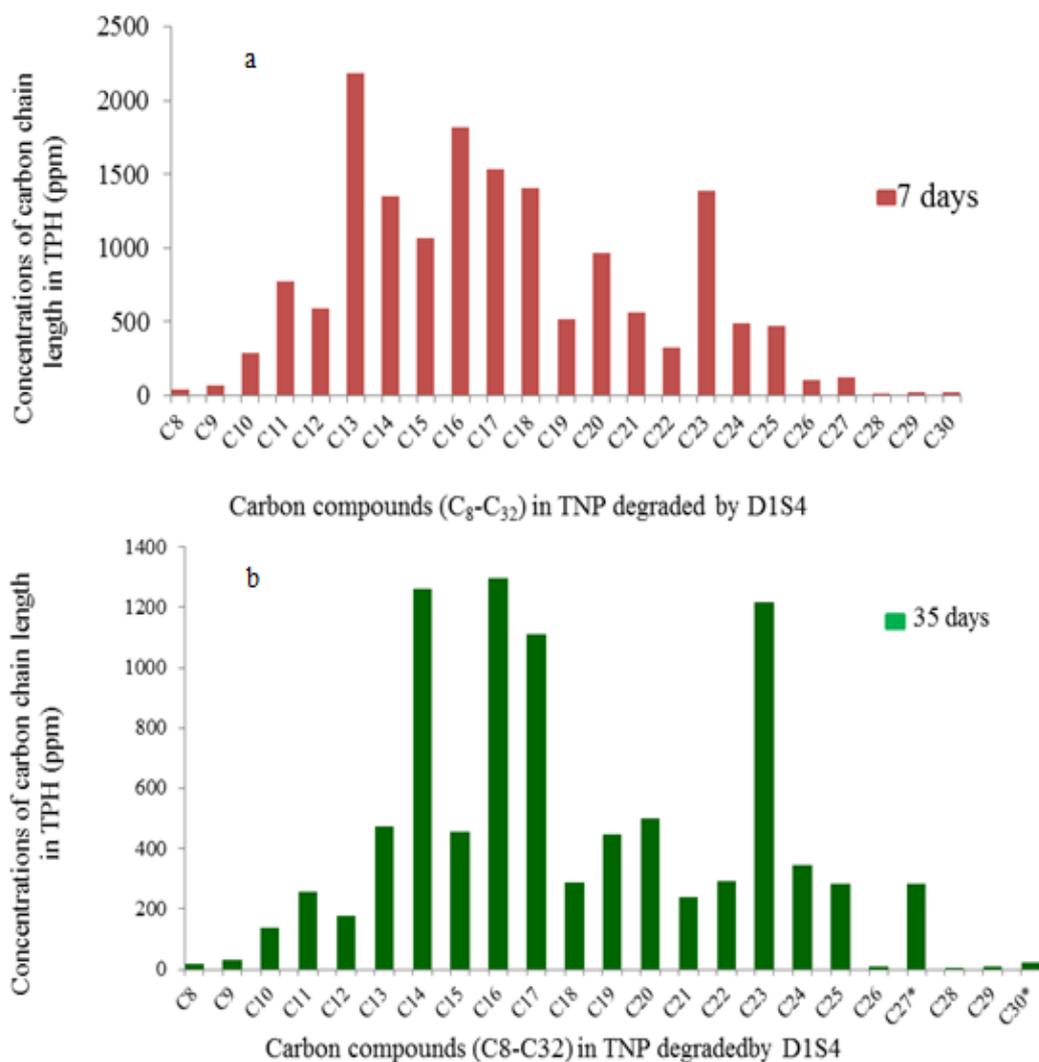
Figure 4.29: Concentrations of carbon compounds in transniger pipeline crude oil

degraded by *Aspergillus sublatus*-D1S4, after (a) 7days (b) 35 days

Key: D1S4= *Aspergillus sublatus*-D1S4

The GC-MS analysis of transniger pipeline crude oil degraded by *Aspergillus terreus*-JYS revealed an emergence of an intermediate compound between peak C_{14} and C_{15} as a result of degradation after 7 days of incubation (Figure 4.30a), this compound remained undegraded after 35 days (Figure 4.30b). However, all the carbon compounds present in the oil were considerably reduced to a concentration less than 500ppm except C_{14}, C_{15} C_{16} and C_{17} which persisted and remained unattacked by the organism after 7 days (Figure 4.31a). The unattacked compounds were eventually degraded and their concentrations were reduced from 3533 ppm, 3592 ppm, 3592.5 ppm, and 4701 ppm

(Figure 4.31a) to 382.6 ppm, 256 ppm, 477 ppm, and 43 ppm (Figure 4.31b) for C_{14}, C_{15} C_{16} and C_{17} respectively. Some compounds (Octane C_8, n-Nonacosane C_{29} and n-Triacontane C_{30}) were degraded completely (100% 0ppm) and removed from the oil, others were minimally degraded while none was resistant to attack by the organism after 35 days (Figure 4. 31b).
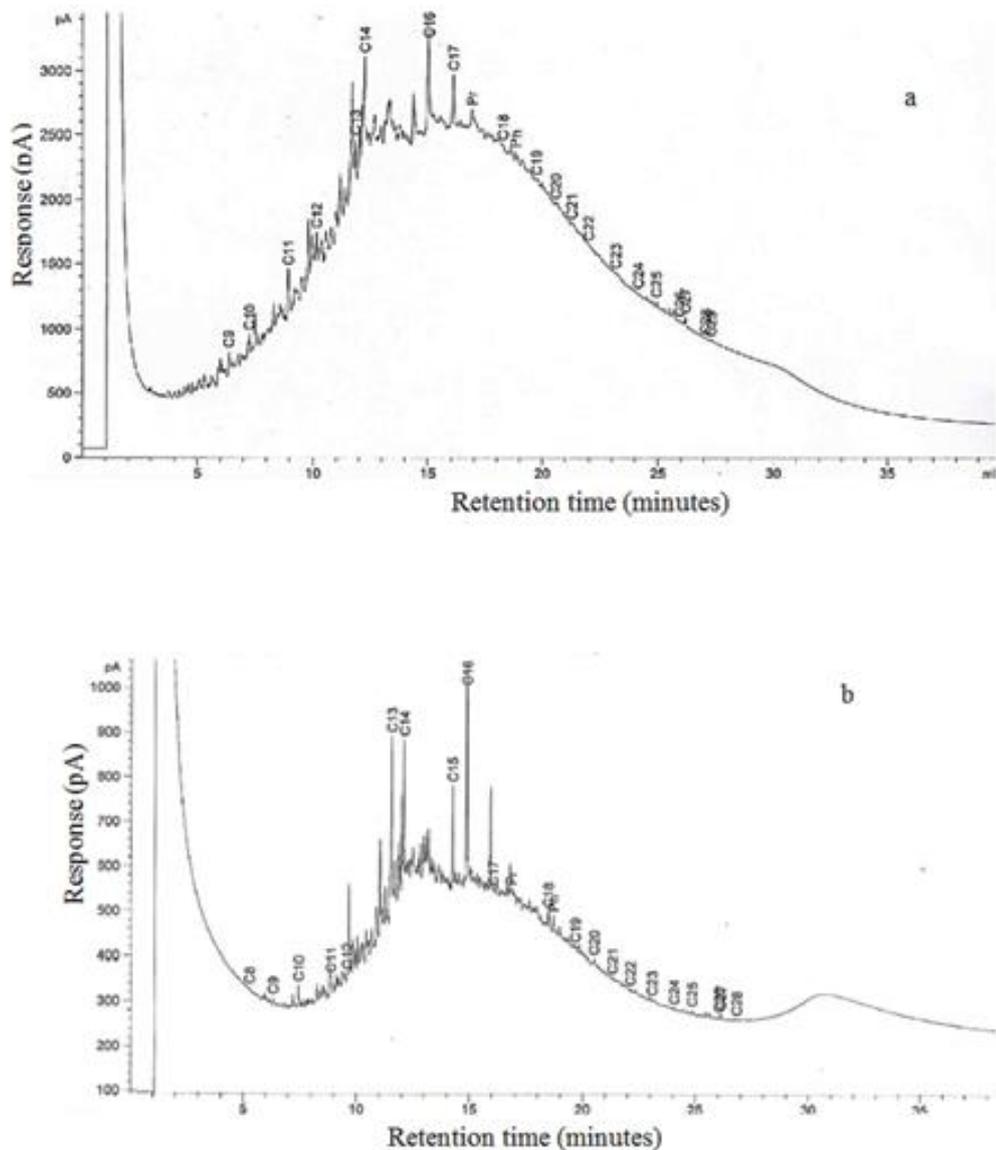
Figure 4.30: Chromatograms of transniger pipeline crude oil degraded by

Aspergillus terreus **JYS after (a) 7days and (b) 35 days**
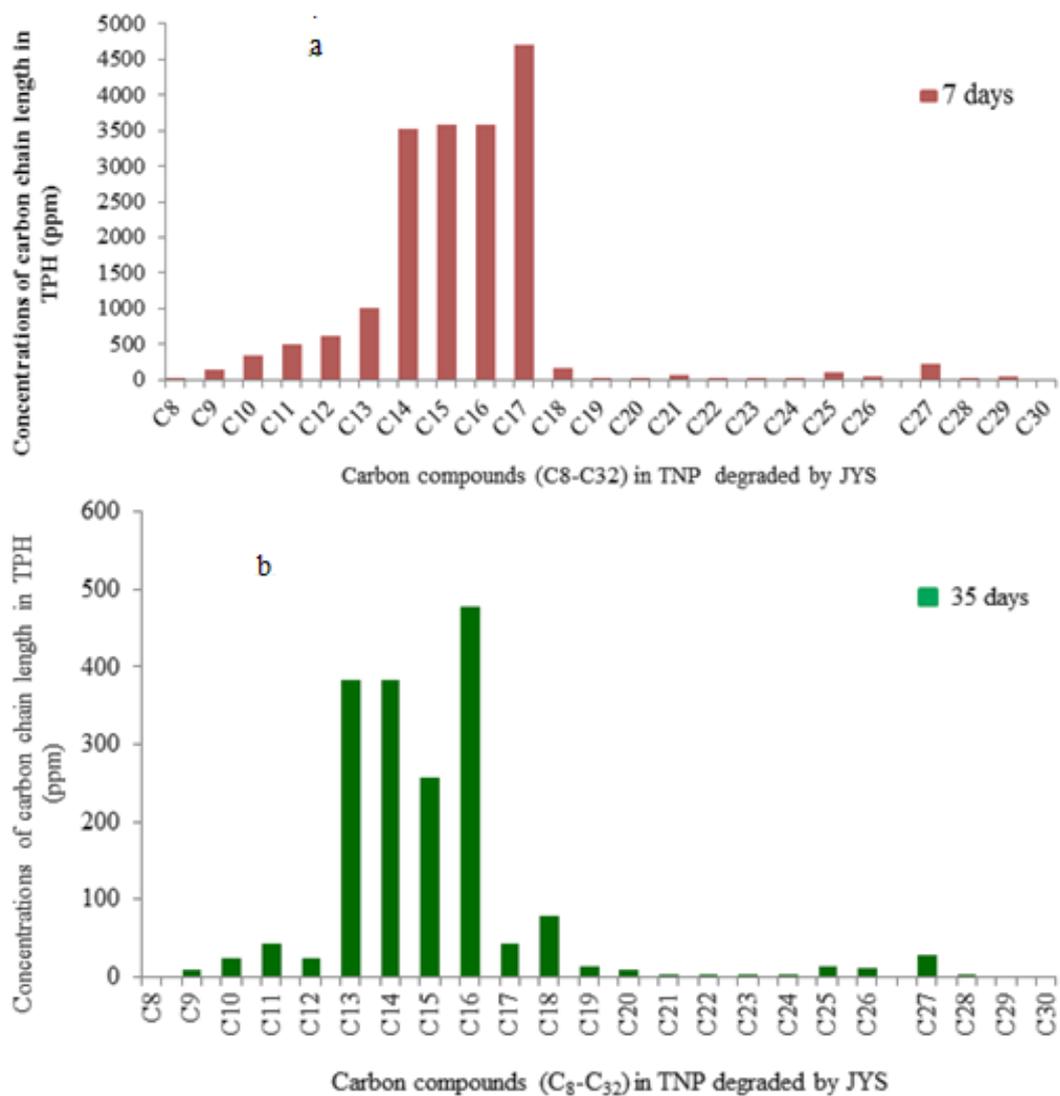
Figure 4.31: Concentrations of carbon compounds in transniger pipeline crude oil

degraded by *Aspergillus terreus* JYS, after (a) 7 days (b) 35 days

Key: JYS= *Aspergillus terreus*-JYS

The GC-MS result of transniger pipeline crude oil treated by *Alternaria tenuissima*-JyW revealed that the concentrations of all the carbon compounds present in the the oil medium were significantly reduced and degraded after 7 days (Figure 4. 32). However, after 35 days more compounds (Pentadecane C_{15}) including the intermediates were degraded completely and removed from the oil by the microbe (*Alternaria tenuissima* JyW) while reduction in concentration of Hexadecane C_{17} was insignificant (Figure 4.33), which implies the compound was resistant to the attack of the organism.
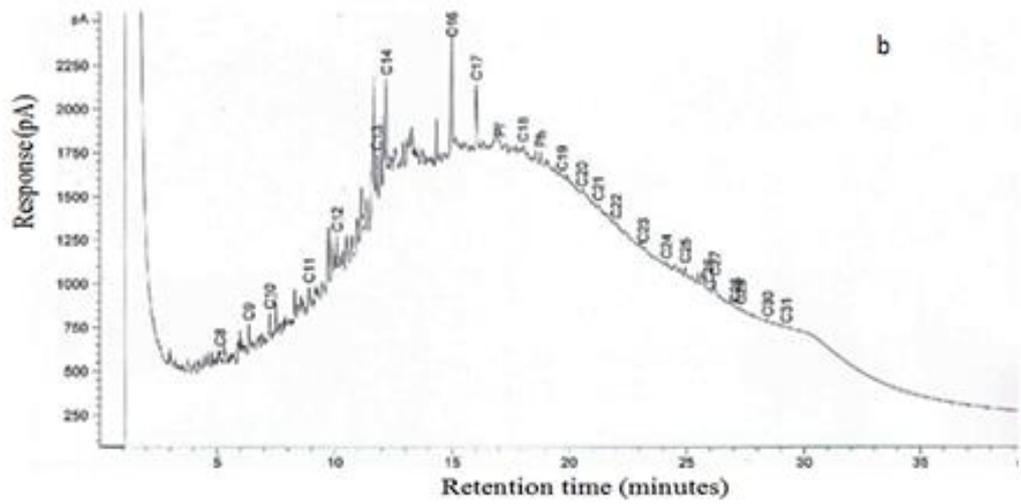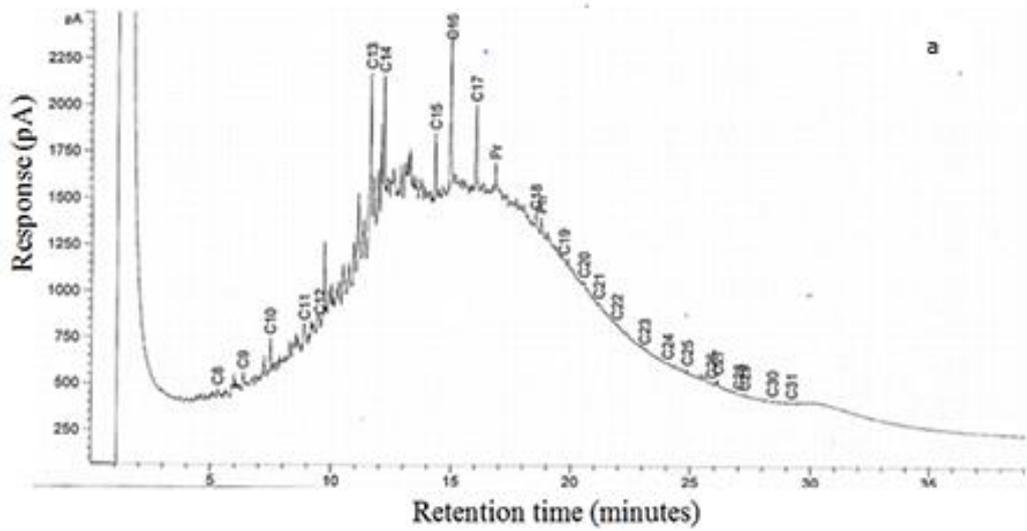
Figure 4.32: Chromatograms of transniger pipeline crude oil degraded by

Alternaria tenuissima **JyW after (a) 7days and (b) 35 days**

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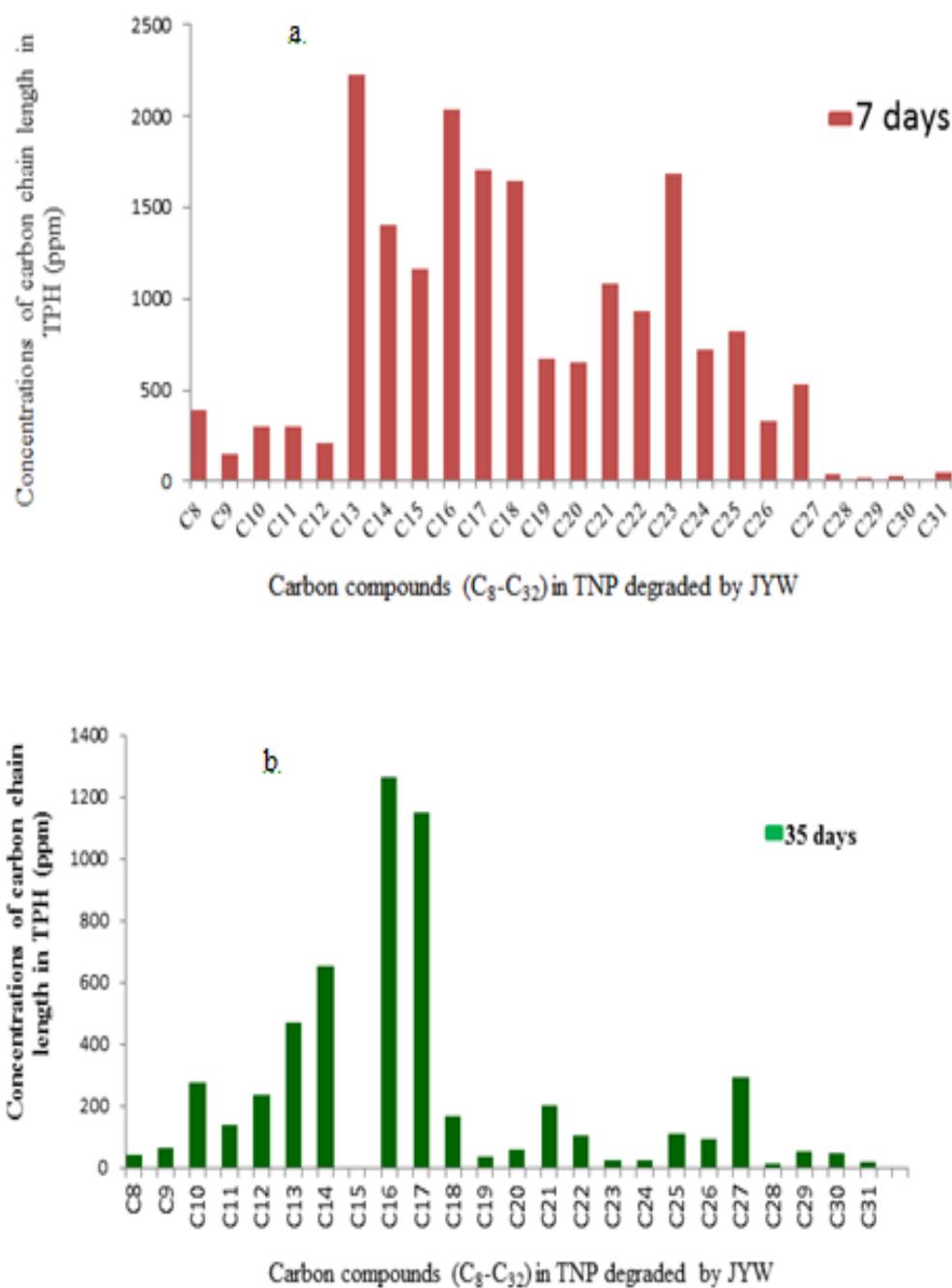
Figure 4.33: Concentrations of carbon compounds in transniger pipeline crude oil degraded by *Alternaria tenuissima* JyW, after (a) 7 days (b) 35 days

Key: JyW= *Alternaria tenuissima*-JyW

The results of GC-MS analysis of transniger pipeline crude oil degraded by *Aspergillus flavipes* BH revealed that there were significant reductions in the concentrations of oil components present in the transniger crude. This led to the biodegradation of certain oil components and emergence of new compounds (four new peaks between C_{15} and C_{16})

(Figure 4.34a) as a result of the biodegradation activities. These include, Pentadec-1-ene, Octane, 2, 6-dimethyldecane, 3, 6-dimethylCyclohexane, and 2, 6, 10, 14-Tetramethylpentadecane after 7 days (Figure 4. 34a). However, after 35days n-Hentriacontane (C_{31}), n-Dotriacontane (C_{32}) and all the newly emerged intermediate compounds were completely degraded (Figure 4. 34b) while concentrations of other compounds were greatly reduced to about 4ppm (C_8) and 877ppm (C_{18}) (Figur 4.35b).
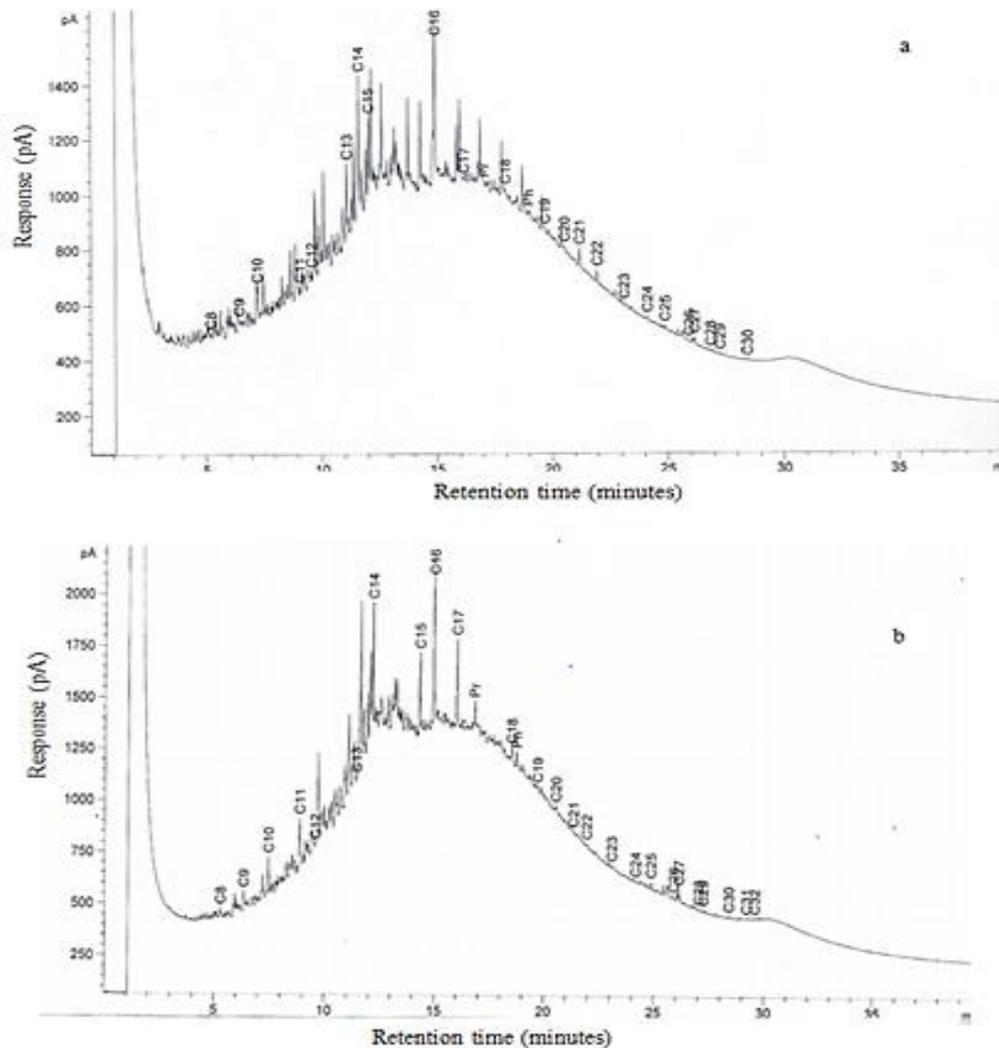
Figure 4.34: Chromatograms of transniger pipeline crude oil degraded by

Aspergillus flavipes **BH, after (a) 7days and (b) 35 days**
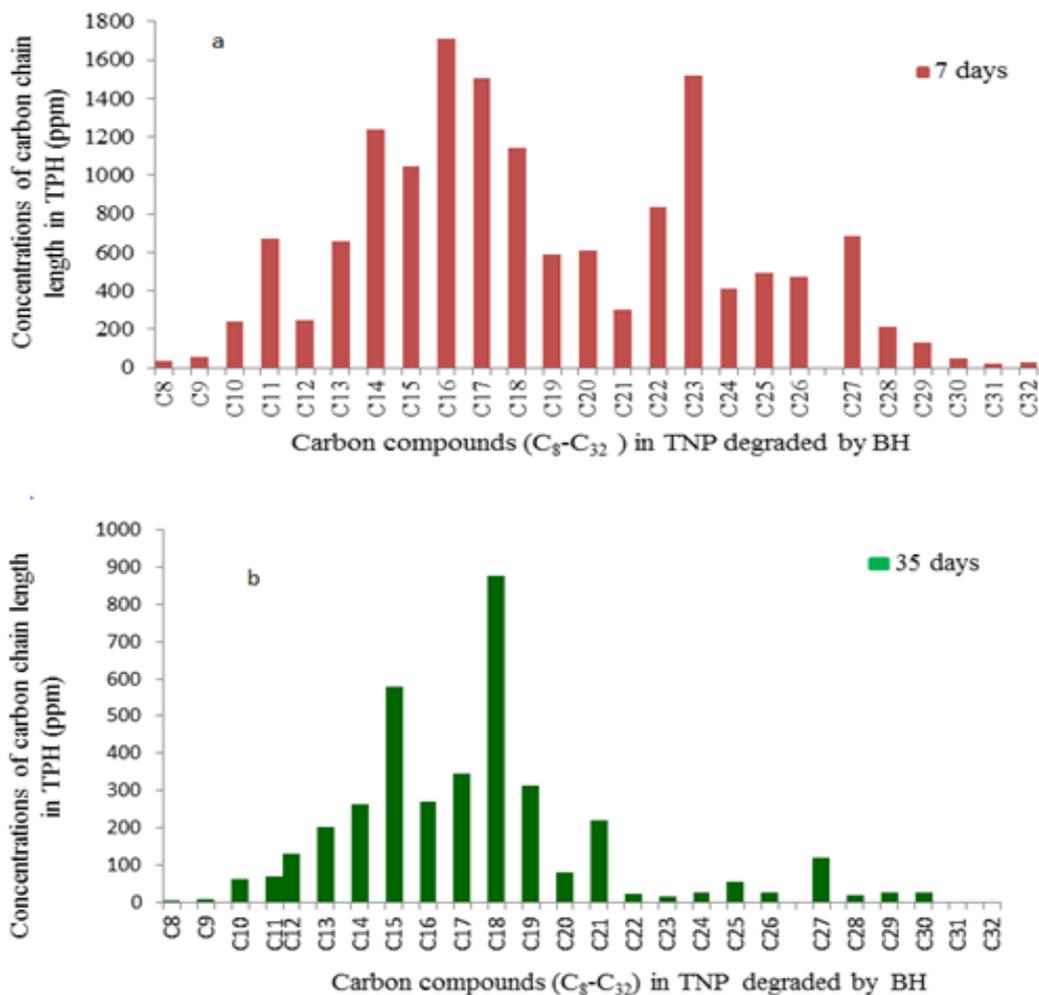
Figure 4. 35: Concentrations of carbon compounds in transniger pipeline crude oil

degraded by *Aspergillus flavipes* BH, after (a) 7 days (b) 35 days.

Key: BH = *Aspergillus flavipes* BH

The result of the GC-MS analysis revealed that *Aspergillus stallatus*-Borehole, effectively degraded and reduced the concentrations of the compounds present in the petroleum hydrocarbon mix after 7 days (Figure 4.36a) although the concentrations of some compounds (Undecane C_{11}, Dodecane C_{12}, Tridecane C_{13}, Tetradecane C_{14}, Hexadecane C_{16}, n-Docosane C_{23}, n-Pentacosane C_{25}, and n-Heptadecane (C_{17}) were still above 1000 ppm. However, after 35 days incubation the organism was able to completely degrade 13 compounds namely (Undecane C_{11}, Tridecane C_{13}, Tetradecane C_{14}, Hexadecane C_{16}, n-Heptadecane C_{17}, Octadecane C_{18}, n-Docosane C_{23}, n-

Tetracosane C_{24}, n-Nonacosane C_{29}, n-Triacontane C_{30}, n-Hentriacontane C_{31}, and n-Dotriacontane C_{32}) (Figure 4.36b). more so, it was observed that there was no emergence of new peak but, the concentration of C_{28} which was initially reduced to 90.3 ppm (Figure 4.37a) increased to 149 ppm after 35 days (Figure 4.37b), this implies that the product of degradation was similar (analogue) to the compound in C_{28} and thereby added to its concentration (Figure 4.37b).
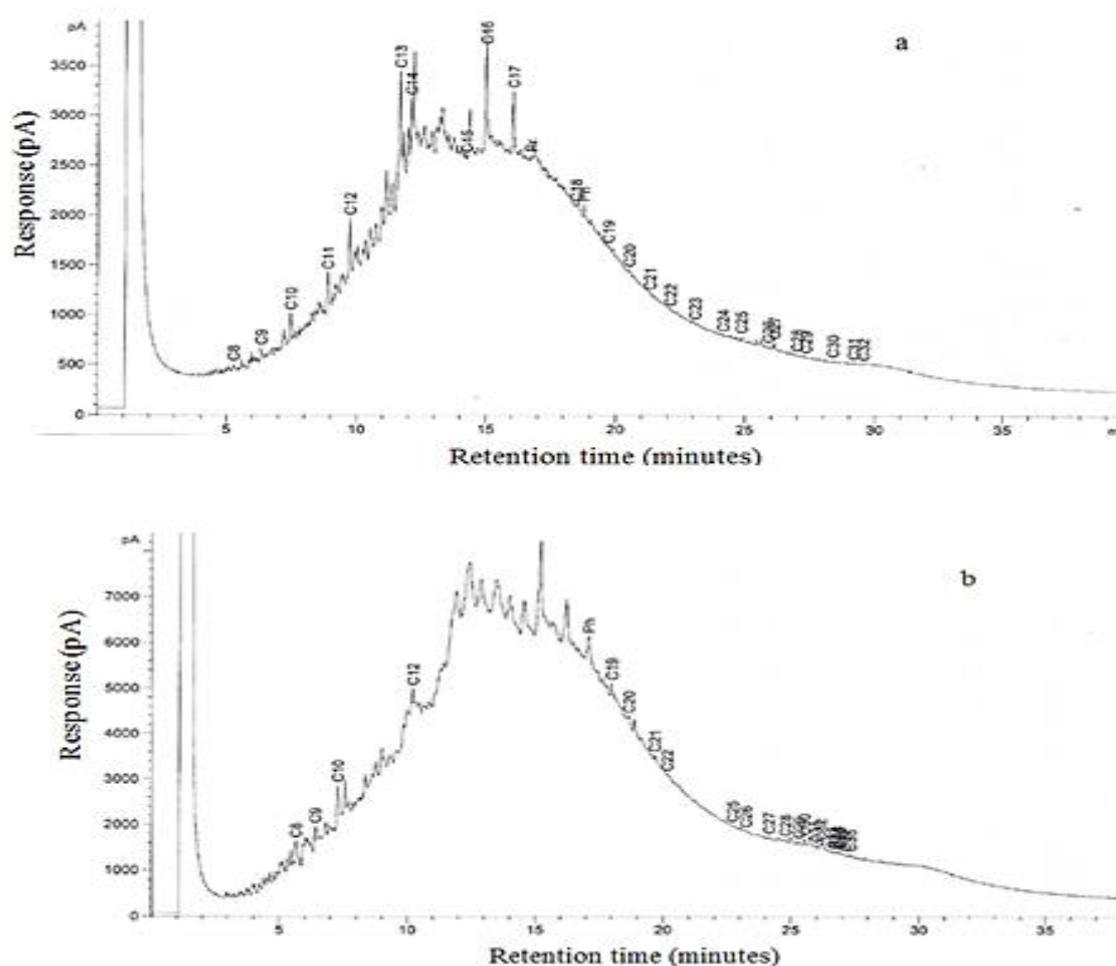
Figure 4.36: Chromatograms of transniger pipeline crude oil degraded by

Aspergillus stallatus-**Borehole after (a) 7days and (b) 35 days**

Key: Borehole = *Aspergillus stallatus*-Borehole
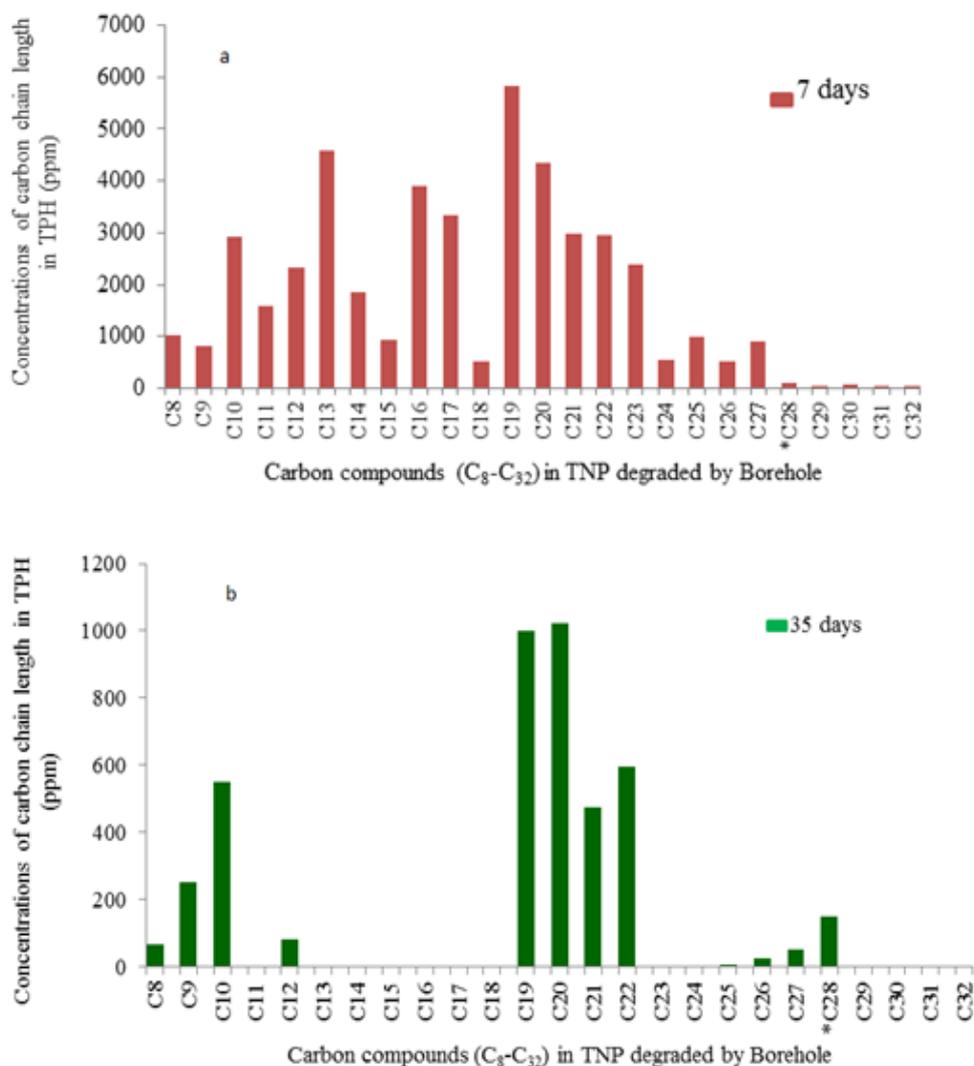
Figure 4.37: Concentrations of carbon compounds in transniger pipeline crude oil degraded by *Aspergillus stallatus*-Borehole after (a) 7 days (b) 35 days

Key: Borehole = *Aspergillus stallatus*-Borehole

The results of the GC-MS analysis of the residual crude oil revealed that certain carbon compound such as C_8 (Octane) was completely degraded after 7 days of inoculation with the mixed microorganisms of *Aspergillus flavus* A-Feb and *Aspergillus flavipes* BH (Figure 4.38a); some components such as C_{23}, C_{24}, C_{26} were reduced to 28 ppm, 72.9 ppm and 41.48 ppm (Figure 4.38b) respectively as compared to the initial quantity of 750 ppm, 557.6 ppm and 505.9 ppm respectively, but C_{12}, C_{14}, C_{15}, C_{16}, C_{17} and C_{18} were resistant with a concentration of 2383 ppm, 5400 ppm, 4200 ppm, 4200 ppm, 4400

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ppm and 2369 ppm respectively (Figure 4. 39a). After 35 days of inoculation, the resistant carbons were broken down to lesser concentrations of 460 ppm(C_{12}), 525 ppm (C_{16}) 981.8 ppm (C_{17}) and 377.4 ppm (C_{18}) (Figure 4.39b).
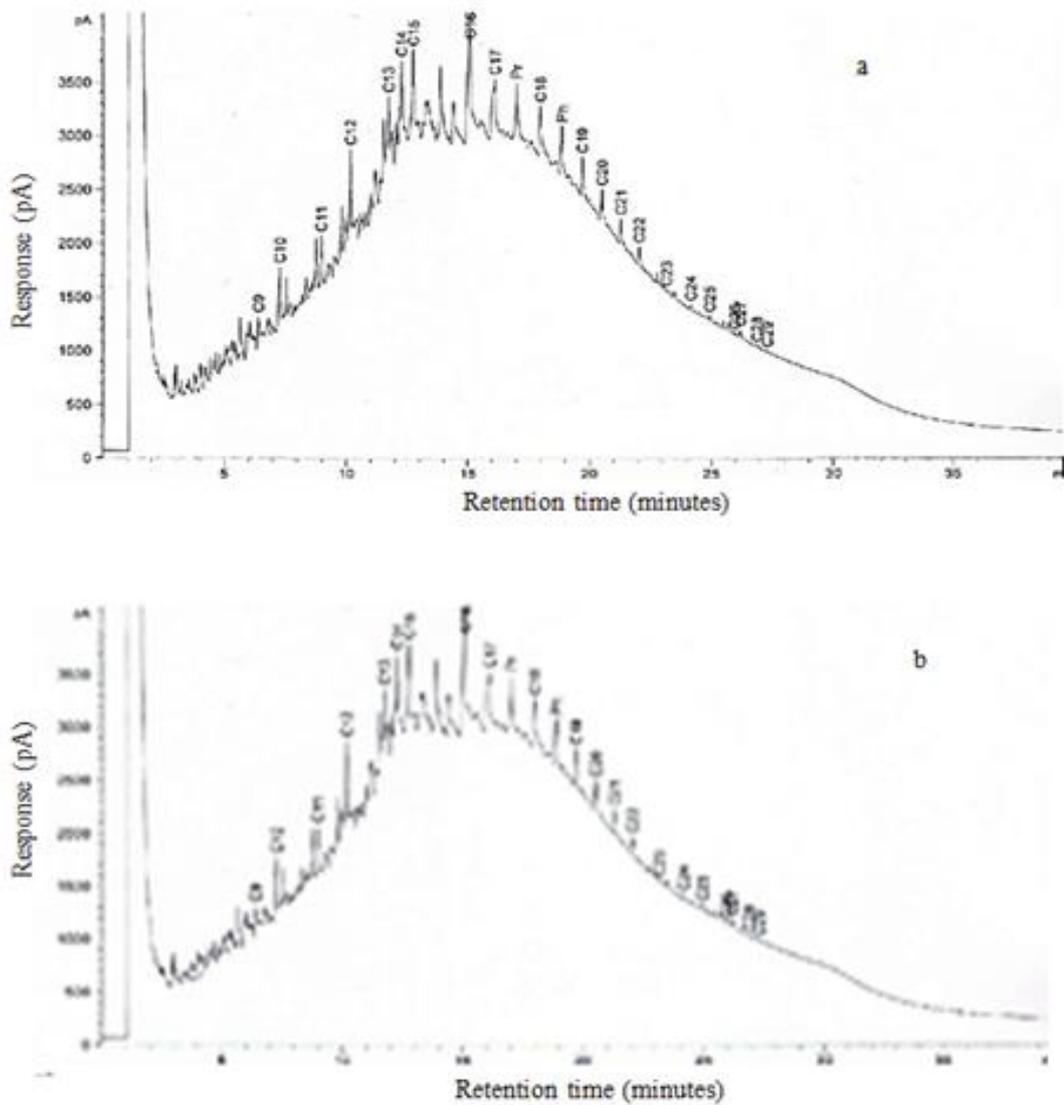
Figure 4.38: Chromatograms of transniger pipeline crude oil degraded by

Aspergillus flavus **A-Feb +** *Aspergillus flavipes* **BH after (a) 7days and (b) 35 days**
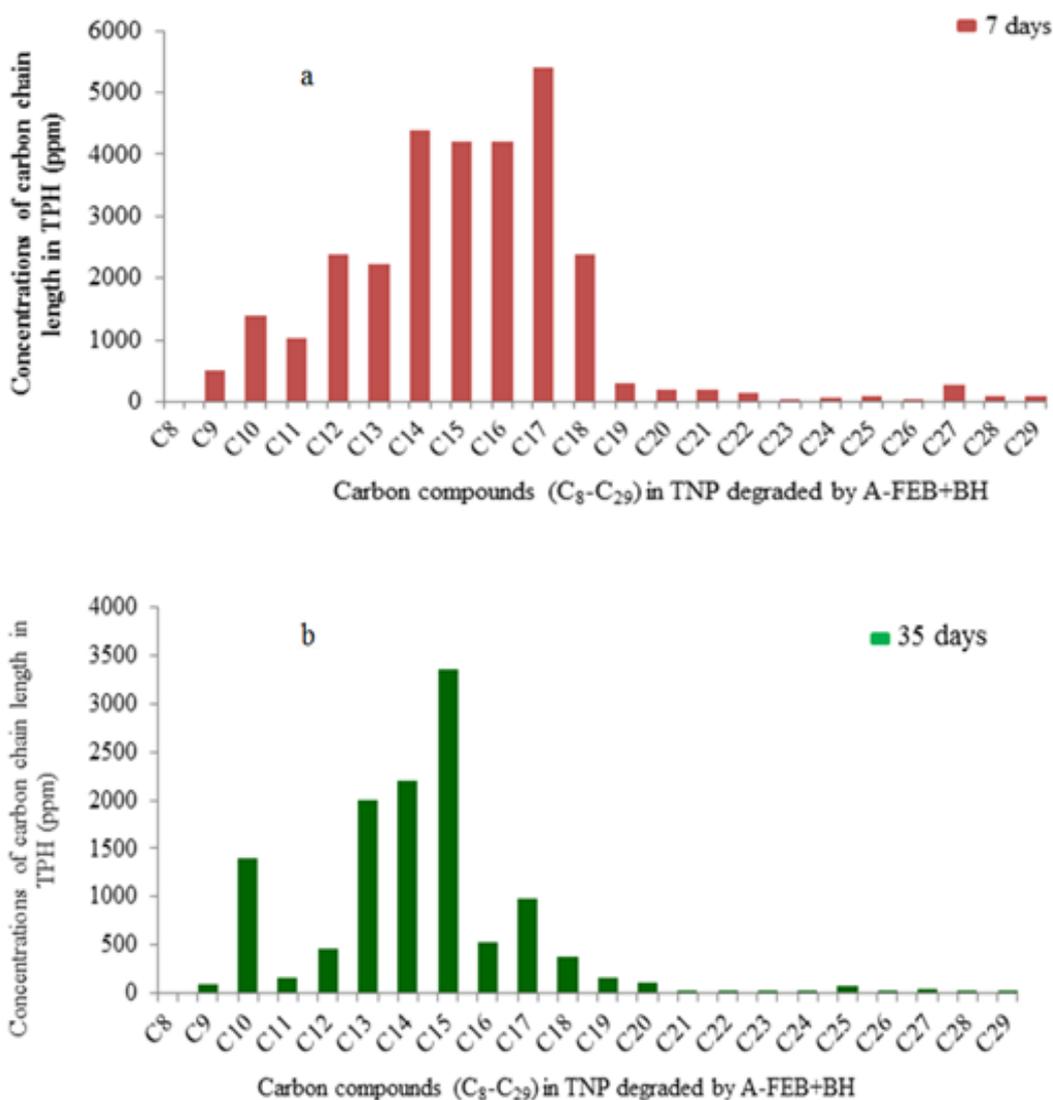
Figure 4.39: Concentrations of carbon compounds in transniger pipeline crude oil degraded by *Aspergillus flavus* A-Feb + *Aspergillus flavipes* BH after (a) 7 days (b) 35 days

Key: A-Feb= *Aspergillus flavus* A-Feb, BH= *Aspergillus flavipes* BH

The result of the GC-MS analysis of transniger pipeline crude oil degraded by mix culture of *Aspergillus terreus* JYS + *Aspergillus flavipes* BH revealed an emergence of four (4) intermediate compounds between peak C_{15} and C_{16} as a result of biodegradation after 7 days (Figure 4.40a), these compounds (4-methylpentanoic acid, methylpropanedioic acid, 2-methylpentadioic acid and 4, 8-dimethylnonanoic acid) were completely degrade (0.00 ppm) and removed from the oil after 35 days (Figure

4.40b). In the same vain, the concentrations of all the carbon compounds present were greatly reduced after 7 days (Figure 4.41a) while the concentrations of C_{12}, C_{31} and C_{32} were completely reduced to 0.00 ppm after 35 days of inoculation with the mix culture (Figure 4.41b) other components (C_{13} and C_{16}) remained unattacked and the reduction in concentrations were not significant (Figure 4.41b).
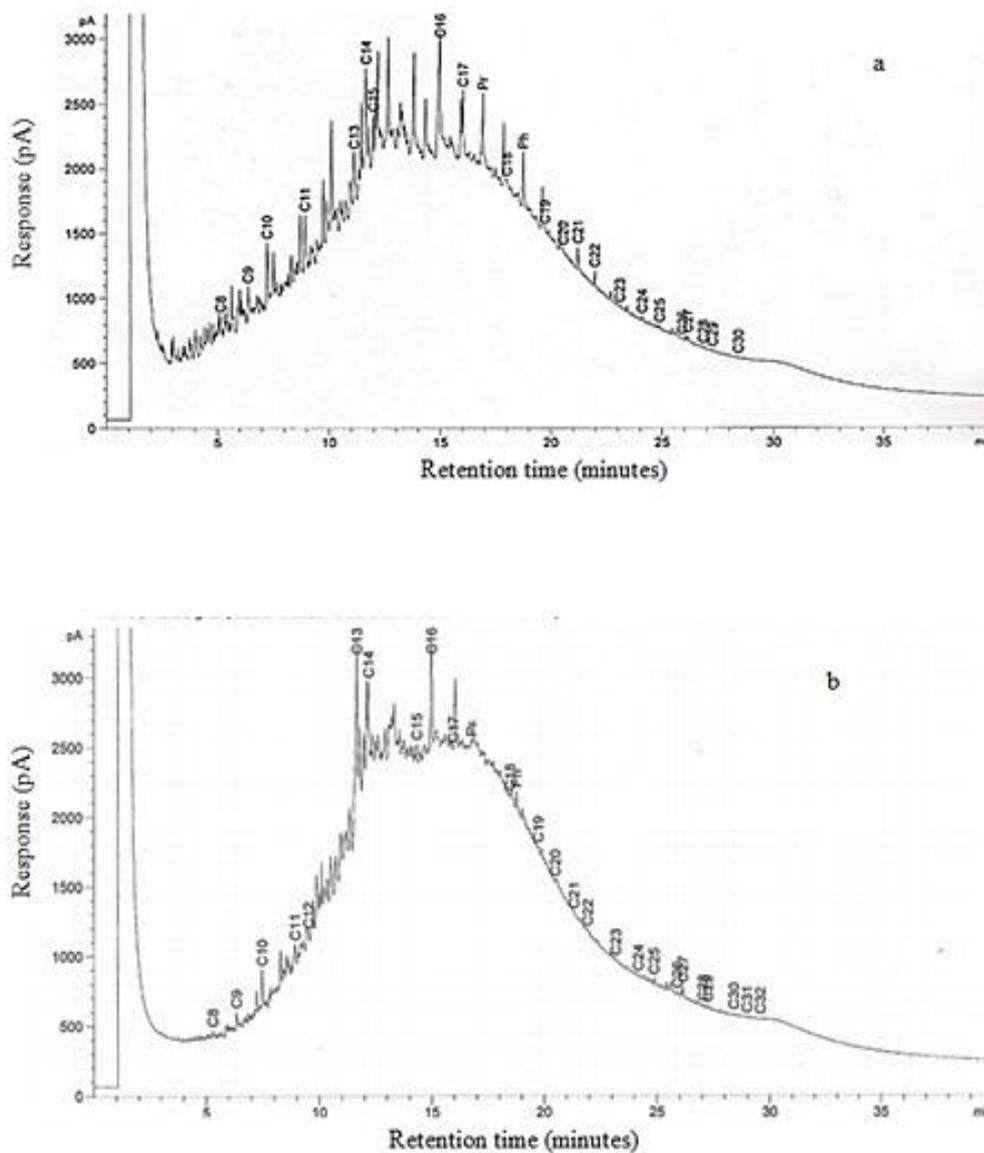
Figure 4.40: Chromatograms of transniger pipeline crude oil degraded by

Aspergillus terreus **JYS +** *Aspergillus flavipes* **BH after (a) 7days and (b) 35 days**
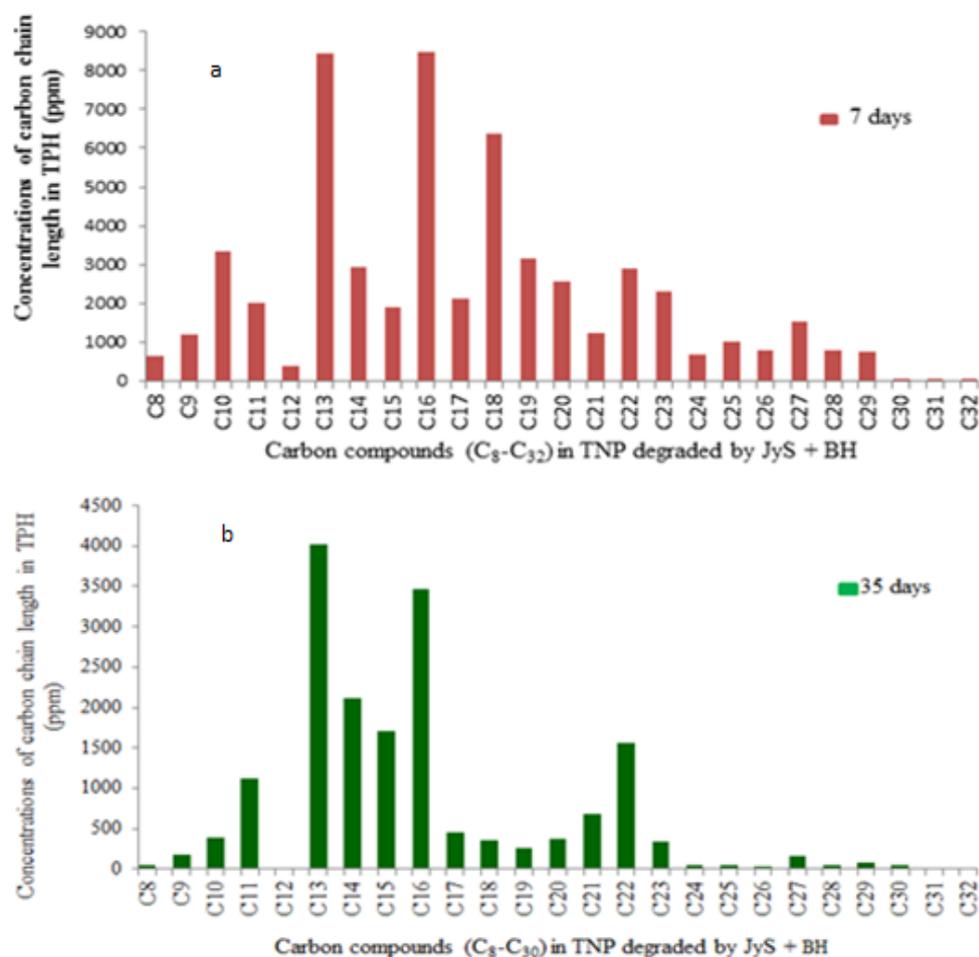
Figure 4. 41: Concentrations of carbon compounds in transniger pipeline crude oil degraded by consortium of *Aspergillus terreus* JYS + *Aspergillus flavipes* BH after (a) 7 days (b) 35 days

Key: JyS +BH = *Aspergillus terreus* JYS + *Aspergillus flavipes* BH

It was observed from the GC-MS profile for *Aspergillus tubingensis* D2S3 + *Aspergillus flavus* A-Feb that biodegradation occurred with evidence in the emergence of three intermediate compounds between peaks C_{15} and C_{16} (Figure 4. 42a), two out of these intermediate compounds were completely degraded and removed from the hydrocarbon mix while the other compound was significantly reduced as revealed by reduction in the length of peak (Figure 4. 42b). The result also revealed that the concentrations of 15 carbon compounds were greatly reduced to a concentration less than 1000 ppm after 7 days (Figure 4. 43a), it was observed that no carbon compound

in the residual transniger pipeline crude oil was completely degraded after 35 days of incubation, however, all the carbon components especially (C_8, C_{28} C_{30} C_{31} C_{32}) were considerably degraded reaching to concentration of 3.9 ppm, 5.7 ppm, 10.1 ppm, 3.11 ppm, 3.39 ppm respectively except C_{18} that remained unattacked by the organisms and the reduction in concentration was not significant (Figure 4. 43b).
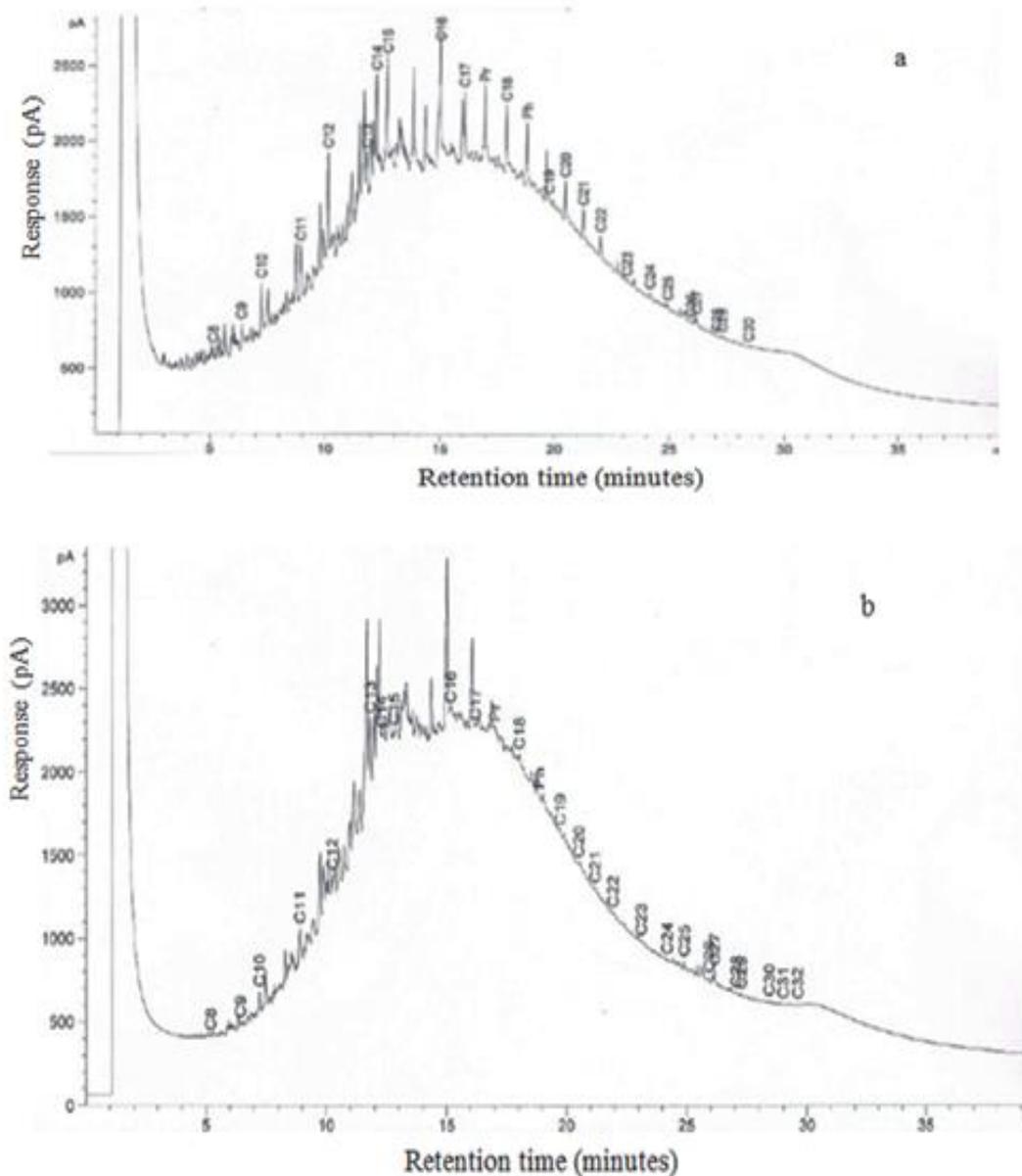
Figure 4.42: Chromatograms of transniger pipeline crude oil degraded by

Aspergillus tubingensis **D2S3 +** *Aspergillus flavus* **A-Feb after (a) 7days and (b) 35**
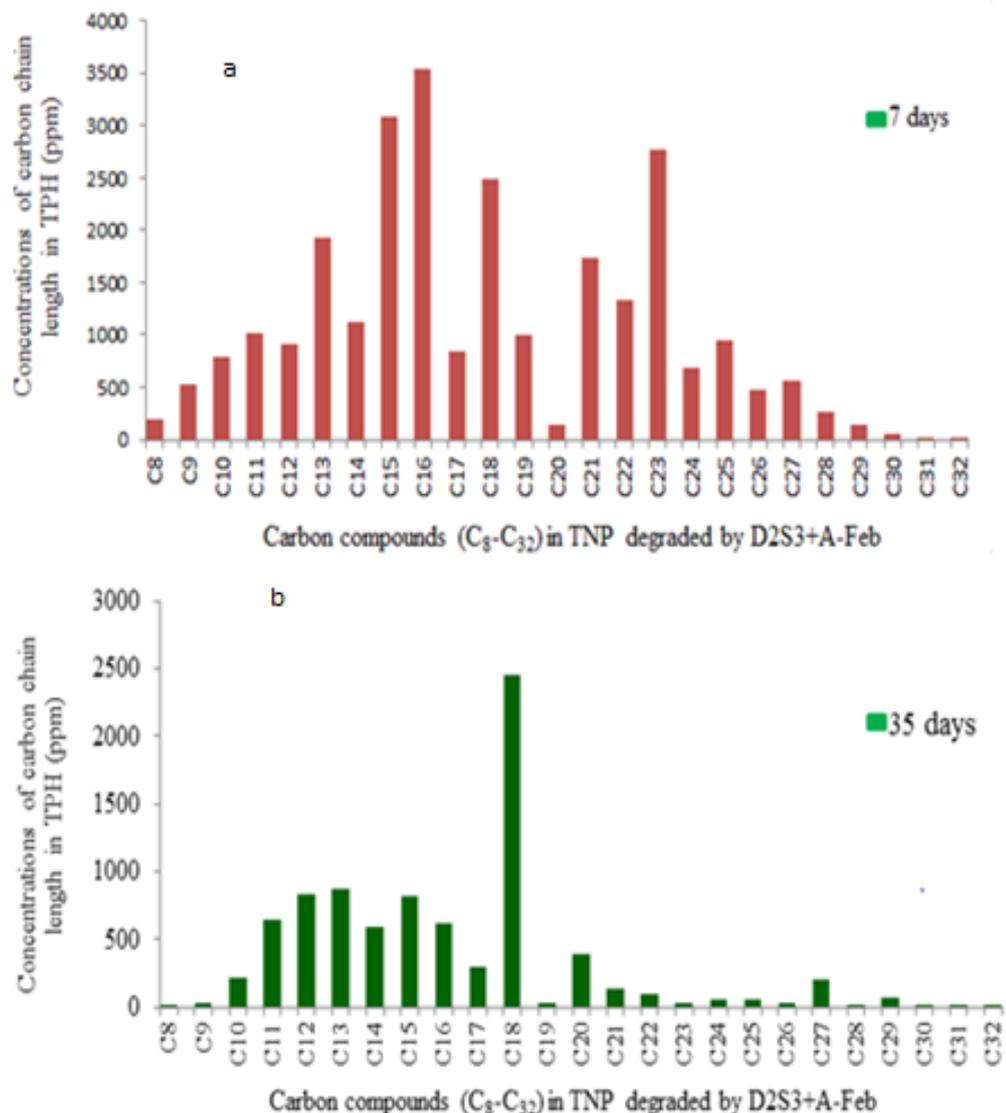
days

Figure 4.43: Concentrations of carbon compounds in transniger pipeline crude oil

degraded by consortium of *Aspergillus tubingensis* D2S3 + *Aspergillus flavus* A-Feb

(a) 7 days (b) 35 days

Key: D2S3+A-Feb = *Aspergillus tubingensis* D2S3 + *Aspergillus flavus* A-Feb

The result of the GC-MS analysis of transniger pipeline crude oil degraded by mix culture of *Aspergillus tubingensis* D2S3 +*Aspergillus flavipes* BH revealed an emergence of an intermediate compound between peak C_{13} and C_{14} as a result of biodegradation after 7 days (Figure 4. 44a), the compound (2, 4-methyldecane) was completely degrade (0.00 ppm) and removed from the oil after 35 days (Figure 4.44b).

Similarly, the concentrations of all the carbon compounds present were greatly reduced after 7 days (Figure 4.45a) while the concentrations of C_{16}, C_{22}, C_{25}, C_{29}, C_{30}, C_{31}, C_{32} were completely reduced to 0.00 ppm after 35 days of inoculation with the mix culture (Figure 4.45b) no components was unattacked and the reduction in concentrations were all significant (Figure 4.45b).
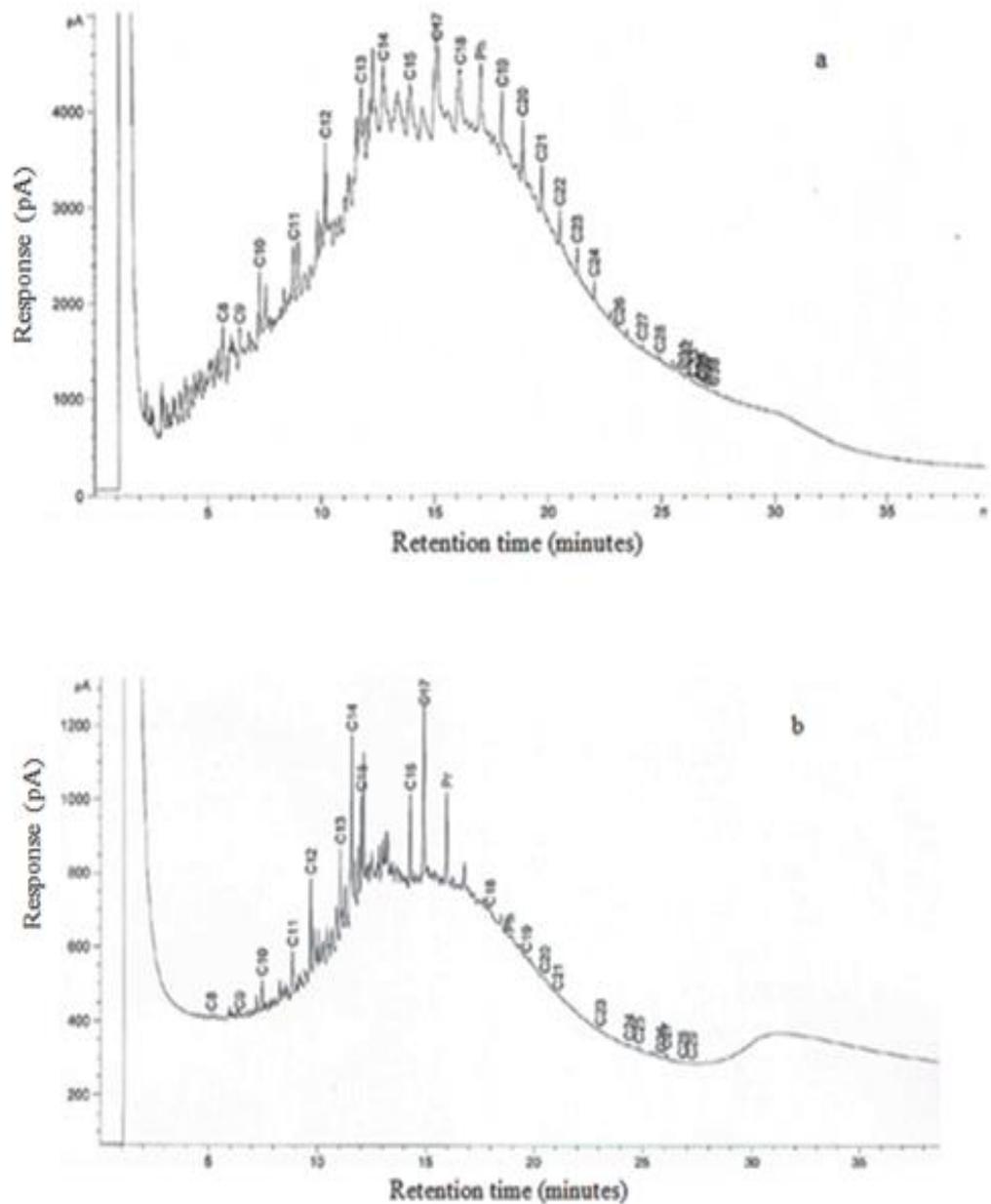
Figure 4.44: Chromatograms of transniger pipeline crude oil degraded by *Aspergillus tubingensis* **D2S3+***Aspergillus flavipes* **BH after (a) 7days and (b) 35 days**
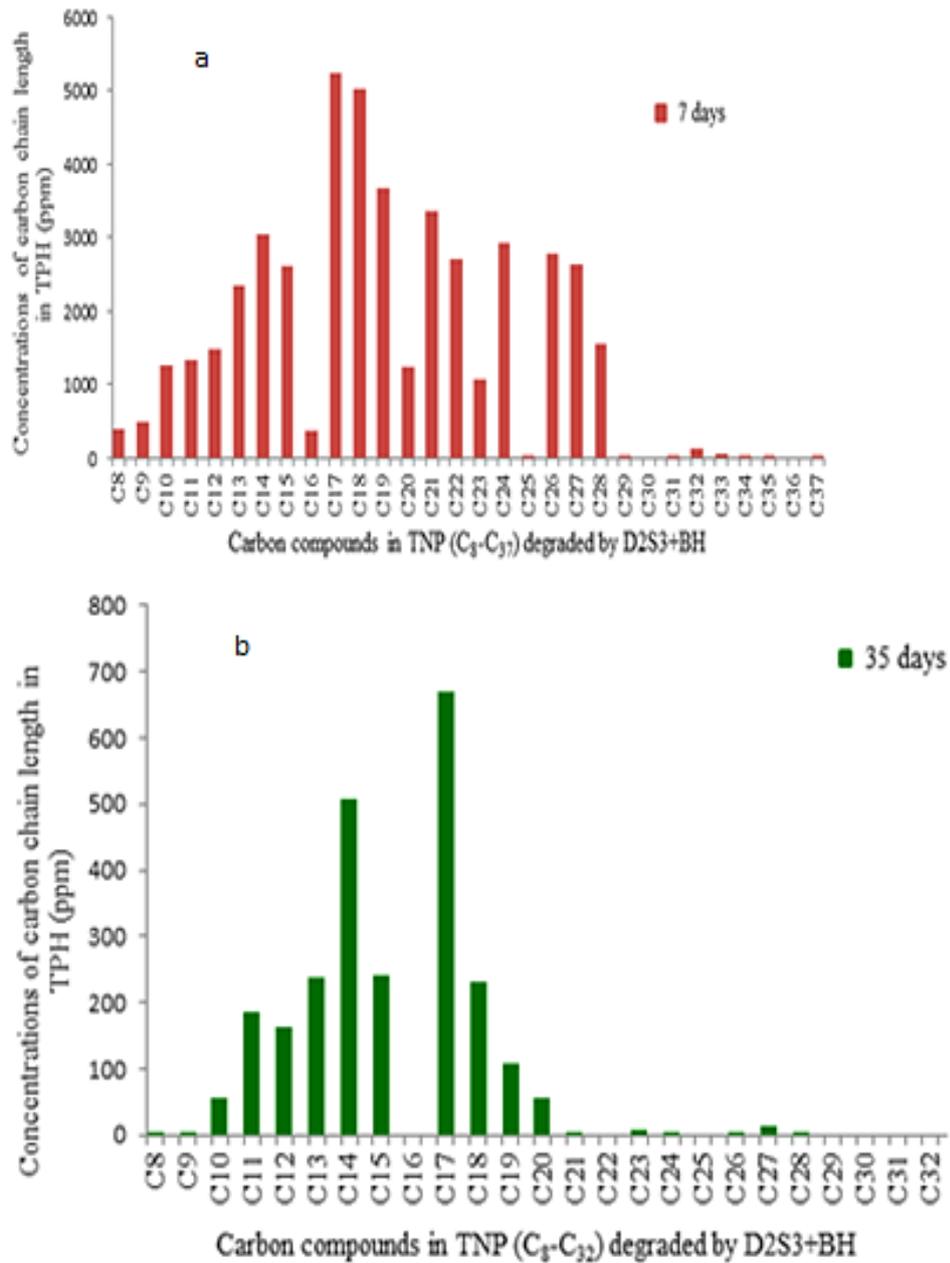
Figure 4.45: Concentrations of carbon compounds in transniger pipeline crude oil degraded by consortium of *Aspergillus tubingensis* D2S3+*Aspergillus flavipes* BH (a) 7 days (b) 35 days
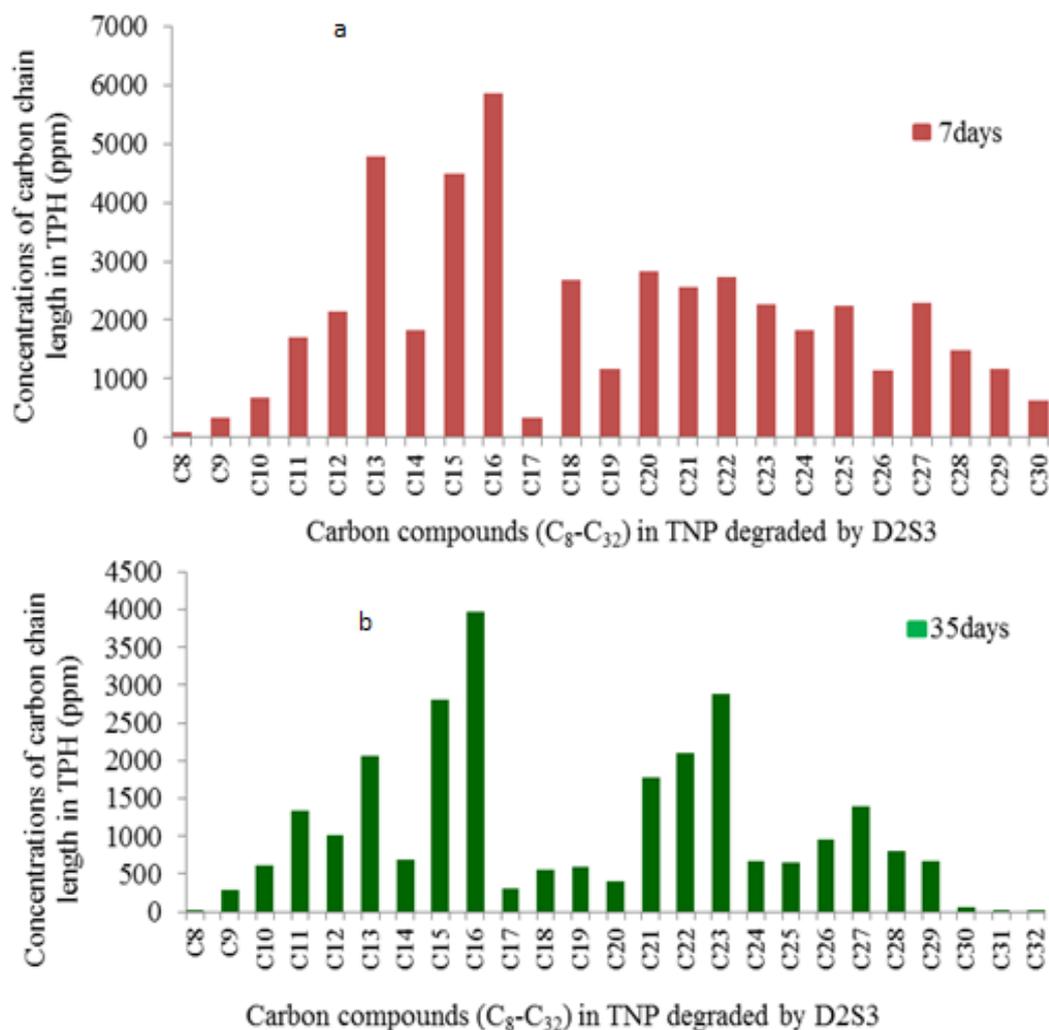
`Key: D2S3+ BH = *Aspergillus tubingensis* D2S3+*Aspergillus flavipes* BH

4.2　Discussion

The physicochemical parameters of soil and water samples collected from the sites were analysed to ascertain the quality of the samples. Some of the parameters analysed were, pH, electrical conductivity (EC), bulk density, N, P, K, and TOC (Table 4.1 to Table 4.3). The results revealed that the EC for soil ranged from 2.60 μ∫/cm to 12.0 μ∫/cm (Table 4.1), 0.1 μ∫/cm to 2.0 μ∫/cm (Table 4.2) and water was 0.1 μ∫/cm (Table 4.3), these are considered low and in contrast to the electrical conductivity of soil and water (21.90 μ∫/cm, 53 μ∫/cm and 68 μ∫/cm) from Niger delta exploration site as recorded by Edwin-Wosu and Nkang, (2019) and Nkwopara $et\ al.$ (2019). Similarly, Chikere $et\ al.$ (2017) and Hussain $et\ al.$ (2019) had reported a high EC value of 3320 and 143.60 μ∫/cm from a crude oil polluted and hydrocarbon spiked soil respectively. The cationic content (Mg^{2+}, Ca^{2+}, K^{2+}, Na^{2++}) of the soil were significantly different ($p<0.05$) with low values (0.6 – 1.10 cmol/kg) (Table 4.1 – Table 4.3) as compared to 7.74 – 193.78 cmol/kg repoted by Nkwopara $et\ al.$ (2019) and Awasthi and Ray (2020). These variations may be as results of differences in study location, quantity of oil in the soil and water, anthropogenic activities which could lead to introduction of pollutants which results in increased in the amount of ions present, as well as the soil type and texture (Oliveira $et\ al.$, 2020; Pedrotti, 2020). The low values in the physicochemical properties is an indication that the sites are not polluted or are not being polluted and hence low quantity of crude are present (Pedrotti, 2020).

The results indicated that all sites (Ahoko and Patishabakolo) harboured hydrocarbon oxidizing bacteria and fungi, although there were variations in counts. The mean total bacterial (TB) and fungal counts (TF) showed that high counts of TB were observed in the April and October (Table 4.4). The high count in April maybe because it's a month of rainfall onset where the temperature of the soil changes to favour the growth of

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organisms (Ijah *et al*., 2008; Allamin *et al*., 2014; Abioye *et al*., 2019). High count of fungi were observed in May, July and October (Table 4.5) this probably due to water content of the soil because in October the soil was wetter than December and February. This is in accordance to the work of Eze and Okpokwasili, (2010), Allamin *et al*. (2014); Abioye *et al*. (2014) and Awasthi and Ray (2020) who stated that seasonal differences with higher microbial counts in wet than in dry season months may be attributed to increased water content of the soil. TB and TF abundance patterns were similar in all the sites A to D with differences in counts; the difference in count of the sites could be due to the difference in sites receiving domestic effluents, grazing activity and agricultural runoff which impart the sites. These findings are similar to the reports of Eze and Okpokwasili, (2010), Allamin *et al*. (2014), and Abioye *et al*. (2014) who reported that variations in microbial counts may be due to the industrial and domestic discharges to the sites.

The results of hydrocarbon oxidizing microbe in water (Table 4. 6) revealed that butane oxidizing microbe (BOM) had it least count of 1.0×10^3 CFU/mL in April while MOM, EOM and POM had their least count of 3.0×10^3 CFU/mL, 2.0×10^3 CFU/mL and 2.0×10^3 CFU/mL respectively, in March. MOM recorded its highest count (4.8×10^2 CFU/mL) in April, EOM had its highest count (4.6×10^2 CFU/mL) in December, and POM had its peak count in November, while BOM recorded a peak count of 3.0×10^2 in October. This implies that the counts of these groups of organisms in water were influenced by the seasons of the year which was tied to temperature that regulated the rate of gas in-flux and oil deposition to the water surface. This is in agreement with Clark *et al.* (2000); Margesin *et al. (*2003); Head *et al.* (2006); Hamamura *et al.* (2006) and Kinnaman *et al.* (2010) who reported a count of 2×10^{10} gyear^{-1} (methane), 1.9×10^9

(ethane), and $1.4\text{x}10^9$ (propane) from an established oil point seep field off shore of Santa Barbara,Califonia, US; reported that the variations due to high dynamic environments is as a result of variable gas influx levels and seasonal changes in deposition.

The growth of Ethane oxiding microbes (EOM) for site A and site C (Figure 4.2) were favoured during the dry seasons of February and January respectively, while high counts of EOM for borehole and riverside (Table 4.11) were observed in January (dry season) and there was significant difference ($p<0.05$) in number of counts. The growth of propane oxidizing microbe (POM) across the sites was also favoured during the dry season (December) except for site B that had its highest count in June (wet season). Similarly, high counts for butane oxidizing microbes (BOM) for all the samples studied (Site A, B, C and D (Table 4.7), borehole, riverside, D1S4, and D2S4 (Table 4.14) were recorded mainly in February, March, November, and December. These variations in microbial number and population is as a result of seasonal changes which regulate the rate of influx of variable gas and hydrocarbon deposit in subsurface for utilization by microbes (Molly *et al.,* 2020).

Recent report by Molly *et al.* (2020) identified novel methane, ethane and propane oxidizing microbes using SIP-DNA probing revealing that, high population of propane and ethane-oxidizing microbes from a hydrocarbon seep environment were obtained at high temperature during the dry than wet season. Hubert and Judd (2020) added that these increase is temperature dependent as it resulted to increase in the rate of in-flux of hydrocarbon to the surface to be utilized by the microbes present. This implies that the EOM for Site A and C, borehole, riversite; POM for site A, C and D, and BOM of all the sites studied were regulated by the rate of influx of gas and hydrocarbon deposition

while the other groups of organisms from site B, D1S4 D2S3 and D2S4 were not associated with hydrocarbon deposition (Hubert and Judd, 2020).

The dominant and most frequently occurring organisms in the two sites were *Micrococcus* sp. (18.7 % and 20.0 %), *Enterobacter* sp. (16.33 %, 16 %), *Corynebacterium* sp. (12.24 %, 12 %), and *Serratia* sp. (13.8 %) (CENM). According to some researchers (Shennan, 2006; Kotani *et al.,* 2006), who conducted investigations aimed at the microbial hydrocarbon prospection, two groups of microbes are relevant: gram-positive bacteria, represented mainly by Actinobacteria from CRNM complex (*Corynebacterium, Rhodococcus, Nocardia* and *Mycobacterium*) that uses short-chain hydrocarbons (C_2-C_8) as energy source, and gram-negative bacteria, mainly the genus *Pseudomonas* (Shennan, 2006; Kotani *et al.,* 2006). Following this statement, it means that, site A (Ahoko area) and borehole samples (Patishabakolo area) of this study are most likely to be the hydrocarbon potent sites, because all the aforementioned CRNM complex except R (*Rhodococcus*) were frequently present in the samples (Table 4.15 and Table 4.17).

The specific hydrocarbon gas-oxidizing microbes (HOM) were identified microscopically and biochemically, they were separated as hydrocarbon oxidizing bacteria (HOB) and hydrocarbon oxidizing fungi (HOF). The results revealed 16 HOB (Table 4.18) and 11 HOF (Table 4.19). Out of the 16, 9 were EOB which belong to the genera: *Enterobacter*, *Achromobacter*, *Serratia*, *Stenotrophomonas, Geobacter, Aeromonas*, and *Arthrobacter*, *Actinobacillus*, *Norcadia*, 3 MOB: *Sporosarcina, Methylomonas, Methylobaceter*, 3 POB, *Mycobacterium, Gordonia,* unidentified and 1 BOB *Ochrobacteria* (Table 4.18). For the 11 HOF, 5 were EOF which included,

Aspergillus spp., *Aspergillus niger* , *Rhizopus, Alterneria, and Candida*, 3 MOF *Penicillium, Rhodotorula, Aspergillus flavus*, 1 POF; *Geotrichum* and 2 BOF; *Pichia* and *Talaromyces* (Table 4.19). Shennan (2006) and Allamin *et al.* (2014) isolated similar fungi isolates mainly *Aspergillus*, *Rhizopus, Candida. Alternaria, Penicillium*, *Pichia, Rhodotorula* and *Geotrichum* species from Kukawa Borno (Nigeria) oil exploration site. Molly *et al.* (2014) stated that less is known about the organisms that oxidize ethane or propane in the environments, but the number of such isolates are primarily represented by high G+C Gram-positive bacteria which include *Nocardia*, *Pseudonocardia*, *Gordonia*, *Mycobacterium*, and *Rhodococcus* with limited *Pseudomonas* species (Shennan, 2006). These organisms have similarly been isolated in the present study.

The presence of MOM is a sparing pointer to a potent site, but the high relative abundance and wide distribution of EOM (which are oxidizers of ethane gas C_2) and POM (oxidizers of propane gas C_3) especially in site A and borehole samples confirmed the potency of these sites for oil exploration (Shennan, 2006; Kotani *et al.,* 2006), however, the production may not be in large commercial quantity because the total counts were less than 10000ppm as affirmed by Rasheed *et al*. (2015).

The cross-plots between C_1, C_2, C_3 and C_4 samples from Ahoko area revealed by line series in the relative distribution and abundance result, displayed accurate correlation (r = 0.9) with a relationship ratio between MOM and MOF of 50 % (Figure 4.5) the results implied that the hydrocarbons might have been generated from a thermogenic source. Similarly, the cross-plots between C_1, C_2, C_3 and C_4 samples from Patishabakolo had a correlation (r = 0.8) (Figure 4.7) with a relationship ratio between MOM, MOF and BOB of 80-100 %. This implies that the hydrocarbons might have been generated from

a biogenic source. These interpretations followed the genetic diagram for correlating fuel wetness, reported by some researchers (Fred *et al*., 2017; Perez-Drago *et al*., 2019; Sowizdzal *et al*., 2020) Who used $C_1/ (C_2 + C_3)$ ratios with the 13C of methane to categorise natural gasoline types as biogenic or thermogenic. They reported that ratios of $C_1/ (C_2 + C_3)$ less than 50 % are usual for thermogenic hydrocarbon gases with values among 25 % and 50 % whereas the ratios of $C_1/ (C_2 + C_3)$ above a thousand with values between 60 % and 85 % are indicative of biogenic origin of hydrocarbon gases.

The distribution map constructed to delinate the hydrocarbon potent zone revealed that site A (Figure 4.6) harboured more of the hydrocarbon oxidizing microbes from Ahoko, while borehole (Figure 4.8) from Patishabakolo area were more populated and widely distributed with EOM and POM. According to Rasheed *et al*. 2018, there is a direct positive relationship between the increased hydrocarbon concentrations and increased hydrocarbon indicating microbial populations. With reference to the above statement it implies that, Site A and Borehole zones of this study are the most propable zones for hydrocarbon exploration.

Ten crude oil utilizing isolates were successfully characterized and sequenced were identified as *E. asburiae* RSo-A*, A. flavus* A-Feb*, A. tubingensis* D2S3*, A. tenuissima* JYW, *A. flavipes* BH *A. terreus* JYS, *P. pimiteouiense* JS-B, *A. sublatus* D1S4 *A. stellatus* Borehole and *C. orthopsilosis* JS-A with accession number from MK551149-MK551160. Of the ten, only one was a bacterium (*Enterobacter asburiae* RSo-A)

The phylogenetic analysis of the identified fungi resulted in two main clades (Plate 4. 2). The first clade comprises the *Alternaria* sp., *Penicillium* sp., *Candida* sp., *Saccharomycetales* sp. clustering with the identified fungi from this study. Clade I is

subdivided into two: subclade I and subclade II. Subclade I consists of he identified fungi: A-Feb, D2S3, BH, JYS, D1S4, Borehole, JS-A, JYW clustering with the reference strains from the GenBank with less than 50 % having their closest relative to be *Candida orthopsilosis* and *Saccharomycetales* sp (Figure 4.10). This similarity index is quite low because it falls below the 70 % expected border-line for the degree of relatedness according to Wayne *et al*. (1987). This 50 % homology percentage shows that they possessed different nucleotide signature with their relative counterpart from the Genbank with little similarities (Aremu and Babalola, 2015). Subclade II is made up of JS-B clustering with 69 % similarities with *Penicillium pimiteouiense* and less than 50 % with the *Alternaria* species. In addition to this, similarities expressed is due to their low similarity value, which result in DNA reassociation values that fall above the 70 % threshold values (Stackebrandt *et al*., 2002). This showed low genetic relatedness that is not quite reliable because they cannot be wiped out overnight according to Konstantinidis and Stackebrandt, (2013) and Nwagu *et al*. (2019). These identified fungi from this study based on their low level of relatedness to the reference strains from the GenBank might be a probably novel isolates, since they have different nucleotide signature (Aremu and Babalola, 2015; Nwagu *et al*., 2019).

Light chain alkanes (C_1-C_7) were probably lost by volatilization during the days of incubation. Comparing the chromatograms of the control and undegraded in Figure 4.16 and Figure 4.17, with those of the degraded (Figure 4. 18 to Figure 4.41), it was observed that the fungi were slow in attacking and degrading the crude oil on the 7th day of incubation but were later able to degrade the oil effectively after 35 days. This may be due to the fact that the fungi are slow growers (Olukunle and Oyegoke, 2016), so it took them longer time to start attacking the crude oil. It was also observed that after 7

days of incubation the organisms did not show any significant (p>0.05) degradation but after 35 days of incubation a significant degradation and concentration reduction was observed.

All the isolates used showed efficient biodegradation potential that increased with increased in chain length, this implies that all the isolates utilized both the shorter and longer chain n-alkane in the crude oil, which is in contrast to the report of Ijah (1998) Farag and Soliman, (2011) and Okwute *et al*. (2017), who observed decrease in degradation ability with increasing chain length. The implication of these variations is that the organisms used had very efficient degradative enzyme systems.

The results of the biodegradation study revealed that the borehole isolate identified as *Aspergillus stellatus*-borehole was the best out of the ten isolates used, it achieved 88% degradation (Figure 4.16) and was able to completely (100%) remove 13 compounds from the crude oil as revealed by the GC-MS analysis (Figure 4. 36). It was closely followed by *Candida orthopsilopsis* JS-A with 85% degradation (Figure 4.16) and was able to completely remove 9 compounds from the crude oil, reducing other compound considerably (Figure 4.27); this implies that these organisms possess active and efficient enzymatic system which led to high ability to consume and break down carbon compounds. This finding is in agreement with the report of Marchand *et al*. (2017) and Mustafa *et al.* (2019); which affirms that organisms with high degradation potential of 80% possess active and efficient enzymatic system which aids their ability to consume and break down carbon compounds efficiently.

Sabah *et al* (2016) reported large number of *Aspergillus* species with *Aspergillus niger* top on the list of crude oil degraders. They reported that fungi thrived more in the oil

exploration site than bacteria and all the fungi had high ability to degrade crude oil. Other investigators (Marchand *et al.,* 2017; Mustafa *et al.,* 2019) concurred with the above statement. The investigators added that fungi had the ability to withstand harsh environmental conditions due to their vast enzymatic capacities and this could be the reason for their prevalence in the environment. This implies that this present study was not far from their findings, because all the organisms with the exception of one used for the oil biodegradation studies were fungi with more of *Aspergillus* species.

CHAPTER FIVE

5.0　　　　CONCLUSION AND RECOMMENDATIONS

5.1　　Conclusion

The physicochemical properties of the studied zones were low as compared to those areas with established oil well, thereby indicating that the zones had not been polluted by crude oil and no oil spillage had occurred, hence a proof of presence of little quantity of oil.

The microbial results indicate the presence of bacterial anomalies for methane, ethane, and propane, and butane oxidizers with the higher population of ethane and propane widely distributed, indicating the zones are prospective for hydrocarbons. However, the presence of propane and butane oxidising microbes which are strong reliable indicators of oil and gas in the subsurface, an indication of micro-seepages, confirms site A of Ahoko zone and Borehole of Patishabakolo zone (which had consistent high counts of ethane, propane and butane oxidizing microbes) as a more potent site for oil exploration, although the quantity of oil may not be of commercial quantity.

Ten crude oil utilizers based on their strong ability to utilize transniger crude oil were selected and subjected to biodegradation for a period of 35 days. Out of the ten, one was a bacterium (*Enterobacter asburiae* Rso-A), one yeast (*Candida orthopsilosis* JS-A) and 8 filamentous mold of which 6 were of the genus *Aspergillus* (*A. flavus* A-Feb; *A. tubingensis* D2S3*; A. flavipes* BH; *A. terreus* JYS; *A. sublatus* D1S4 and *A. stellatus* Borehole), *Alternaria tenuissima* JYW and *Penicillium pimiteouiense* JS-B. The isolates used showed adequate degradation potential that increased with increase in chain length. There was complete degradation and total removal (0.00ppm) of C_{33}, C_{34}, C_{35}, C_{36}, C_{37} and C_{38} from the crude oil mix by these isolates. Also the concentrations of the

recalcitrant and toxic compounds: phytane (ph) and pristane (pr) were greatly reduced. In fact significant differnces were found in the utilization of the carbon compound present in the transniger crude oil among the isolates. Except *A. stellatus* Borehole*, Candida orthopsilosis* JS-A, and *A. flavipes* BH, all the other 7 isolates had C_{16} as a common residual carbon compound that persisted throughout the period of degradation, this implies that C_{16} were more resistant to attack by the organisms.

Intermediates compounds such as: (3-methylpent-4-pentadecane and 3, 8-dimethylundecane); (Pentadec-1-ene, Octane, 2, 6-dimethyldecane, 3, 6-dimethylCyclohexane, and 2, 6, 10, 14-Tetramethylpentadecane); (2, 2, 4, 4-tetramethylnonane, 1-tetradecanol and 3-ethyl-5, 5-dimethyloctane) and (1-bromo-4-nonene, (9E)-9-hexadecenol) and 3-ethyl-5, 5-dimethyl-3-hexanol) that emerged during the process of biodegradation by *Enterobacter asburiae* Rso-A, *A. flavipes* BH, *A. tubingensis* D2S3 and *A. sublatus* D1S4 respectively, were completely degraded (100%) and removed (0.00 ppm) from the oil mixure.

The least performed isolate was *Alternaria tenuissima* JYW with 62.1 % rate of degradation and completely removal of only C_{15} while the most effective and efficient degraders were *A. stellatus* Borehole*, Candida orthopsilosis* JS-A that achieved 88 % and 85 % rate of degradation and was able to completely remove 13 and 9 carnon compounds from the crude oil mixture respectively.

All the ten isolates identified in this study were capable of utilizing the crude oil but the utilization by *A. stellatus* Borehole *and Candida orthopsilosis* JS-A were more effective and efficient than others, hence they can be used as clean up agents in oil spills in future if oil exploration begins in these zones.

5.2   Recommendations

Search for crude oil and gas is a national priority and the economy depends on it. Various types of surveys are being carried out to find the accumulations of hydrocarbons, the most commonly used conventional survey are geological and seismic. Nevertheless most oil companies resort to unconventional methodologies termed as surface prospecting techniques. Surface prospecting reduces the risk and enhances success ratio. Geomicrobial prospecting technique using surface soil and culture media is one methodology which is found highly target focussing and that was what this study employed, as a result:

It is recommended that:

i.   Other method aside culturing Such as Metagenomic, SNIP should be employed in studying the site in order to identify the non-culturable organisms

ii.   Field trial on the isolates should be done to identify their bioremediation capacities in a non-regulated environment

iii. The individual gene responsible for degradation should be isolated and studied

iv.   The isolates should be subjected to biosurfactant production to ascertain if they are potent producers of biosurfactant.

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APPENDIX A

(i) Mineral salt medium MSM (Rasheed *et al.,* 2016)

Dipotassium phosphate (K_2HPO_4)	0.3g
Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$)	0.5g
$MgSO_4$	5g
$NaNO_3$	3g
$KH2PO_4$	1g
Distilled water	1000ml
pH	7.4±0.2

Hydrocarbon gases (methane, ethane, propane, butane)

These were weighed into a flask, and 1000ml of distilled water was added to dissolve the salts, which was then sterilized by autoclaving at 121°C for 15minutes

For 100ml quantity: 0.36g of the composition was weighed and dissolved in 100ml.

(ii) Nutrient agar (NA)

lab-lemco powder	1.0g
Yeast extract	2.0g
Peptone	5.0g
Sodium chloride (NaCl)	5.0g
Agar (No.3 Oxoid)	15g

This was prepared according to the manufacture's direction (28g makes 1liter)

For every 200ml of NA that was prepared 5.6g of NA was weighed and dissolved in 200ml of distilled water, it was then sterilized by autoclaving at 121°C for 15mnutes.

(iii) Muller Hinton broth   (MHB) (Oxoid, CM0405)

Beef dehydrated infusion	300g
Casein hydrolysate	17.5g
Starch	1.5g

| pH | 7.3±0.1 at 25°C |

This was prepared according to the manufacture's direction. 21.0g was weighed into a flask and then dissolved with a1000ml distilled water. It was sterilized by autoclaving at 121°C for 15mnutes.

(iv)   Nutrient broth (Oxoid)

Peptic digests of animal tissue	5.0g
Sodium chloride (NaCl)	5.0g
Beef extract	1.5g
Yeast extract	1.5g
Distilled water	1litre
pH	7.4±0.2 at 25 °C

13g of NB was weighed, dissolved in 1liter, and then sterilized by autoclaving at 121°C for 15mnutes.

(v)   M-R and V-P Media (Glucose-phosphate medium)

Peptone	5g
K_2HPO_4	5g
Glucose	5g
Distilled	100ml
pH	7.5

The components (15g) were introduced into 100ml of distilled water in a conical flask, and then 2ml of the mixture was dispensed into test tubes and was sterilized by autoclaving at 121°C for 15minutes.

(vi)   Sugar Fermentation Test Broth

Phenol red indicator	0.04g
Peptone water (merck)	2g
Distilled water	100ml

The phenol red was dissolved along with the peptone water in 100ml of distilled and was sterilized along with inverted Durham's tubes inside the mixture, at 121°C for 15minute. Then 10ml of filter sterilized sugars (glucose, D-manitol, sucrose, sorbitol etc dissolved in sterile water and heated for 5minutes over a hot burner) were added.

(vii)   Motility Test Media

Peptone water (merck)	2.0g
Sodium chloride	1.5g
Agar agar (oxoid)	2.5g
Distilled water	100ml
pH	7.1±0.2

The composition was weighed and dissolved in 100ml of distilled water; it was dispensed into test tube (5ml) before it was sterilized by autoclaving at 121°C for 15minutes.

(viii)   Simmons Citrate Agar (biomark™)

Ammonium dihydrogen phosphate	1 g
Dipotassium phosphate	1 g
Sodium chloride	5 g
Sodium citrate	2g
Magnesium sulfate	0.2g
Agar	15g
Bromothymol blue.	0.08g
Distilled water	1000 ml

pH   6.9±0.2

   (ix)   Starch Agar

Beef extract   1.5g

Soluble starch   5g

Agar   12g

Distilled water   500ml

pH   7.3±0.2

This was weighed into a flask containing distilled water and dissolved by heating over a

Bunsen burner for 10minutes, before it was sterilized by autoclaving at 121^{oC} for

15minutes.

   (x)   Urea agar base

Yeast extracts   0.1 g

Monopotassium phosphate   0.091 g

Disodium phosphate   0.095 g

Urea   20.0 g

Phenol red   0.01 g

Distilled water (sterile)   1000ml

pH   6.9±0.2

All the composition was weghed and dissolved in distilled water and then sterilized by

autoclaving at 121^{oC} for 15minutes.

xiii. MSM used for biodegradation

KH_2PO_4 1g

K_2HPO_4 1g

NH_4NO_3 1g

$MgSO_4.7H_2O$ 0.2g

$CaCl_2.H_2O$ 0.02g

APPENDIX B

REAGENTS USED

(i)   Acid Ferric Chloride

$FeCl_3.6H_2O$ 12g

Conc. HCl   2.5ml

Distilled H_2O 100ml

(ii)   Benedict Solution

Sodium citrate   17.3g

$NaCO_3$ anhydride   10g

$CuSO_4.5H_2O$ 1.73g

Distilled H_2O 100ml

(iii)   Ehrlich's Reagents

P-dimetylaminobenzaldehyde   1g

Absolute Ethanol   95ml

Conc. HCl   20ml

(iv)   Kovac Reagent

P-dimetylaminobenzaldehyde   5g

Amy alcohol (95%)   75ml

Conc. HCl   25ml

(v)   Crystal violet

Crystal violet powder	0.5g
Distilled water	100ml

(vi) Safranine

Safranin powder	2.5g
95% ethyl alcohol	100ml
Distilled water	900ml

(vii) Methyl red solution

Methyl red	0.04g
Absolute ethanol	40ml
Distilled water	100ml

(viii) Alpha (α)-naphthol solution

5% α-naphthol in absolute ethanol

(ix) Nitrite test reagents

Solution A

0.8% Sulphanilic acid

5N-acetic acid

Solution B

0.6% dimethyl-∝-naphthylamine

5N-acetic acid

1% Zinc dust

(x) Oxidase reagent: 1% tetrametyhl-p-phenylenediamine aqueous solution

APPENDIX C

MICROSCOPIC and MACROSCOPIC VIEW OF SOME OF THE TEST ORGANISMS

C1: *Methylobacterium* sp. (x100)

C2: *Micrococcus* sp. (x100)
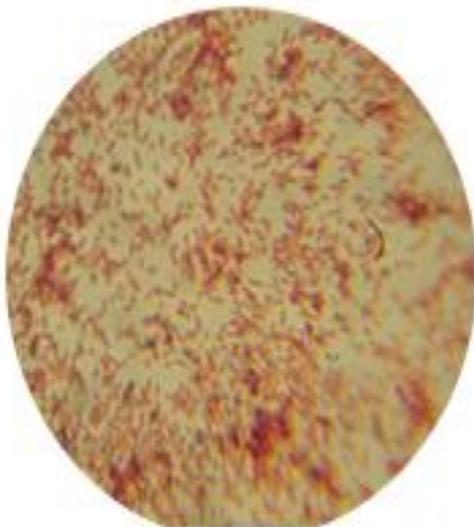
C3: *Acinetobacter* sp. (x100)
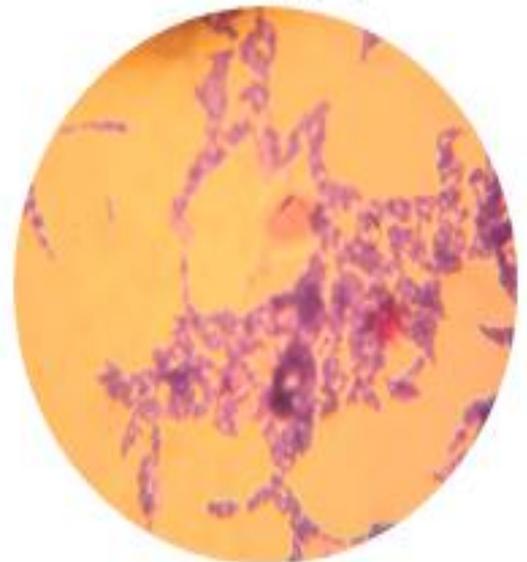
C4: *Bacillus* sp. (x100)

C5: *Alternaria* sp.(x40)

C6: *Aeromonas* sp. (x40)

C7: *Aspergillus oryzae* (x40)
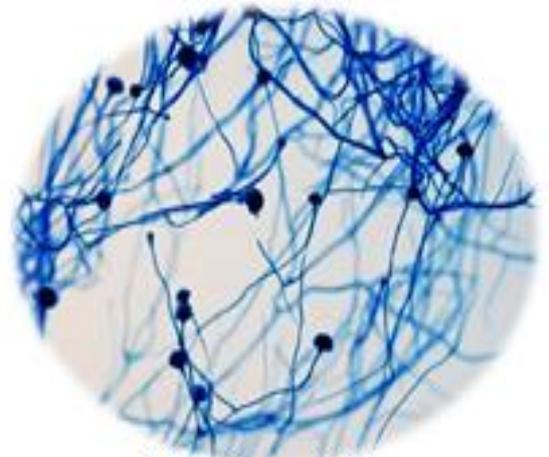
C8: *A. flavus* (x40)
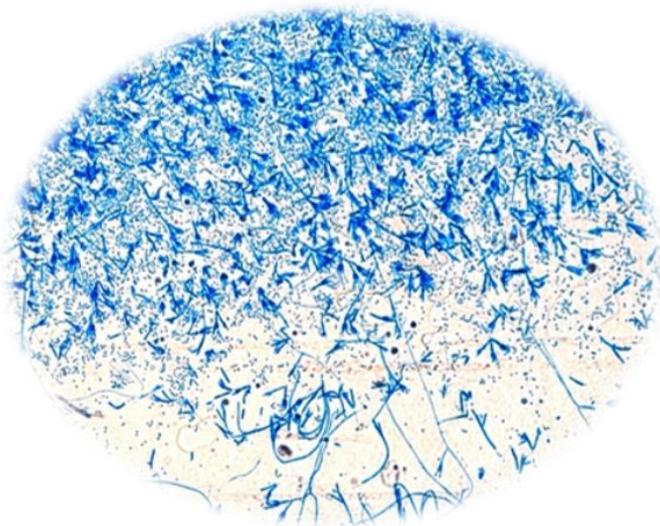
C9: *Penicillium* JS-A (x40)

C10: *A. fumigatus*

C11: *penicillium* sp.

C12: *A. flavus* A-feb

C13: *Penicillium* JS-A.

APPENDIX D

Data obtained from the field and its analysis output

APPENDIX D1: METHANE OXIDIZERS (MOM)

Months	Site A	Site B	SAMPLE Site C	Site D
February (2017)	1.33	0.5	0.17	0.83
March	1.2	0.5	0.3	0.33
April	0.7	0.3	0.67	0.33
May	0.68	0.8	0.67	0.5
June	0.67	0.8	0.67	0.5
July	0.64	0.92	0.5	0.5
August	0.42	0.92	0.5	0.5
September	0.42	0.91	0.5	0.5
October	0.42	0.91	0.33	0
November	0.5	0.55	0.05	0
December	1.07	0.55	0.05	0
January (2018)	1.12	0.33	0.1	0.5

APPENDIX D2: ETHANE OXIDIZERS

Months	Ahoko Soil Colony forming units of Organisms in different sites			
	SA	SB	SC	SD
February	2.33	0.05	0.017	0.083
March	0.05	0.05	0.03	0.033
April	0.033	0.03	0.067	0.033
May	0.08	0.08	0.067	0.05
June	0.067	0.08	0.067	0.05
July	0.04	0.1	0.05	0.05

Months	SA	SB	SC	SD
August	0.042	0.12	0.05	0.05
September	0.067	0.1	0.033	0.05
October	0.067	0.1	0.05	NG
November	0.05	0.05	0.05	NG
December	0.17	0.05	0.05	NG
January	0.12	0.033	0.1	0.05

Values are Means of triplicate determinations
Key: SA=Site A, SB= Site B; SC= Site C; SD=Site D

Appendix D3: Enumeration of Propane oxidizing microbes (POM)@10^2 **CFU/g**

Months	Ahoko Soil Colony forming units of Organisms in different sites			
	SA	SB	SC	SD
February	0.25	0.05	0.05	NG
March	0.25	0.033	NG	NG
April	0.3	0.033	1.0	NG
May	0.27	0.05	1.0	0.033
June	0.2	0.1	1.0	0.01
July	0.04	NG	0.1	NG
August	0.04	NG	1.0	0.033
September	0.04	NG	1.0	1.0
October	0.04	0.01	1.5	1.0
November	0.2	0.01	1.2	1.0
December	2.0	0.01	1.2	1.0
January	0.083	NG	NG	0.033

Values are Means of triplicate determinations
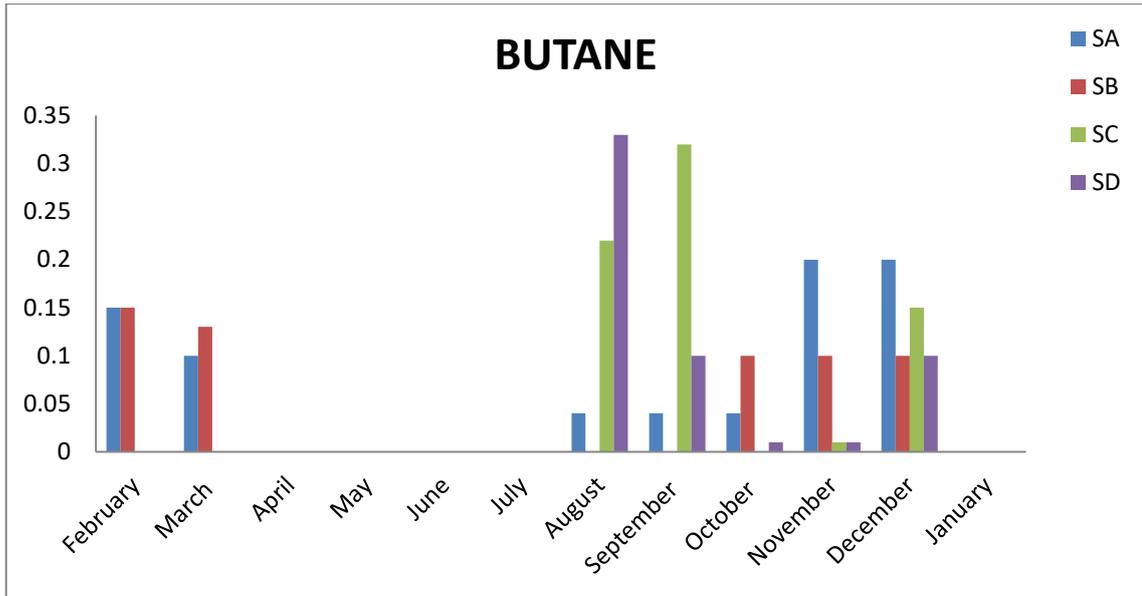
Key: SA=Site A, SB= Site B; SC= Site C; SD=Site D

Figure: Enumeration of Butane oxidizing microbes (BOM)@10^2 **CFU/g**

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
	January	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	February	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	March	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	April	3	1.0000	.00000	.00000	1.0000	1.0000	1.00	1.00
	May	3	1.0000	1.00000	.57735	-1.4841	3.4841	.00	2.00
	June	3	.6667	.57735	.33333	-.7676	2.1009	.00	1.00
WATER	July	3	1.0000	.00000	.00000	1.0000	1.0000	1.00	1.00
	August	3	.6667	.57735	.33333	-.7676	2.1009	.00	1.00
	September	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	October	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	November	3	.6667	.57735	.33333	-.7676	2.1009	.00	1.00
	December	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	36	.4167	.55420	.09237	.2292	.6042	.00	2.00
	January	3	1.3333	.57735	.33333	-.1009	2.7676	1.00	2.00
	February	1	1.0000	.	.	.	.	1.00	1.00
EOM	March	3	1.0000	.00000	.00000	1.0000	1.0000	1.00	1.00
	April	3	1.3333	.57735	.33333	-.1009	2.7676	1.00	2.00
	May	3	2.0000	1.00000	.57735	-.4841	4.4841	1.00	3.00

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		N	Mean	Std. Deviation	Std. Error	Lower Bound	Upper Bound	Minimum	Maximum
	June	3	2.0000	1.00000	.57735	-.4841	4.4841	1.00	3.00
	July	3	1.0000	1.00000	.57735	-1.4841	3.4841	.00	2.00
	August	3	1.0000	.00000	.00000	1.0000	1.0000	1.00	1.00
	September	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	October	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	November	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	December	3	1.0000	.00000	.00000	1.0000	1.0000	1.00	1.00
	Total	34	.9706	.83431	.14308	.6795	1.2617	.00	3.00
	January	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	February	3	1.0000	.00000	.00000	1.0000	1.0000	1.00	1.00
	March	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	April	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	May	3	2.0000	.00000	.00000	2.0000	2.0000	2.00	2.00
	June	3	1.0000	.00000	.00000	1.0000	1.0000	1.00	1.00
POM	July	3	1.0000	.00000	.00000	1.0000	1.0000	1.00	1.00
	August	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	September	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	October	3	.3333	.57735	.33333	-1.1009	1.7676	.00	1.00
	November	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	December	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	36	.4444	.65222	.10870	.2238	.6651	.00	2.00
	January	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	February	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	March	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	April	3	.6667	.57735	.33333	-.7676	2.1009	.00	1.00
	May	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	June	3	1.3333	.57735	.33333	-.1009	2.7676	1.00	2.00
BOM	July	3	1.0000	.00000	.00000	1.0000	1.0000	1.00	1.00
	August	3	.3333	.57735	.33333	-1.1009	1.7676	.00	1.00
	September	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	October	3	1.0000	.00000	.00000	1.0000	1.0000	1.00	1.00
	November	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	December	3	1.0000	.00000	.00000	1.0000	1.0000	1.00	1.00
	Total	36	.4444	.55777	.09296	.2557	.6332	.00	2.00

ANOVA

Patitiabakolo Ethane		Sum of Squares	df	Mean Square	F	Sig.
borehole	Between Groups	25.000	11	2.273	3.409	.006
	Within Groups	16.000	24	.667		
	Total	41.000	35			
riverside	Between Groups	64.750	11	5.886	4.709	.001
	Within Groups	30.000	24	1.250		
	Total	94.750	35			
D1S4	Between Groups	3.556	11	.323	3.580	.004
	Within Groups	2.167	24	.090		
	Total	5.722	35			
D2S3	Between Groups	10.076	11	.916	5.496	.000
	Within Groups	4.000	24	.167		
	Total	14.076	35			
D2S4	Between Groups	10.409	10	1.041	6.245	.000
	Within Groups	3.333	20	.167		
	Total	13.742	30			

ANOVA

PARTITIABAKOLO		Sum of Squares	df	Mean Square	F	Sig.
WATER	Between Groups	6.750	11	.614	3.682	.004
	Within Groups	4.000	24	.167		
	Total	10.750	35			
EOM	Between Groups	15.637	11	1.422	4.265	.002
	Within Groups	7.333	22	.333		
	Total	22.971	33			
POM	Between Groups	14.222	11	1.293	46.545	.000
	Within Groups	.667	24	.028		
	Total	14.889	35			
BOM	Between Groups	8.889	11	.808	9.697	.000
	Within Groups	2.000	24	.083		
	Total	10.889	35			

Appendix D: Statistical analysis output for percentage rate of degradation

Time (DAY) 0	Rso-A	A-Feb	D2S3	JYW	BH	JYS	JS-B	D1S4
	0	0	0	0	0	0	0	0
7	18.95	31	37.9	31.1	27	34.2	31.6	36.8
14	25.09	40	42.1	44.7	36	42	42.6	41.2
21	38.35	59	44.74	57.4	48	64.4	57.9	48.95
28	43.11	64	47.36	59	60	72.6	70	52
35	68.9	73	68.4	62.1	72.11	77.8	83.68	68.89

Time (DAY)	Borehole	JS-A	JyS+D1S4	BH+JyS	D2S3+BH	D2S3 + A-Feb	A-Feb + Borehole	D1S4 + Borehole
0	0	0	0	0	0	0	0	0
7	15	46.31	24	18.07	25.09	30.31	24.71	19.75
14	20	60.52	39	32.01	30.4	46.62	40.02	38.35
21	34.74	71.58	42.8	40	42.05	60.56	56.11	48.35
28	52.09	78.95	54.01	48.13	59.25	63.29	67.86	59.25
35	88.05	85	60	64.33	77.5	80.01	78.3	79

APPENDIX E
SEQUENCE DATA AND RAW SEQUENCE OF THE IDENTIFIED ISOLATES

Appendix E1: The graphic view of the sequence

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            10        20        30        40        50        60        70        80        90
      ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|..
Rso   TCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGAGCGTCAGTCTTTGTCCAGGGGGCCGCCTT

A-Feb AAAGATTGATTTGCGTTCGGCAAGCGCCGGCCGGGCCTACAGAGCGGGTGACAAAGCCCCATACGCTCGAGGATCGGACGCGGTGCCGCCGC

D2S3  ACCTGGAAAAAATGGTTGGAAAACGTCGGCAGGCGCCGGCCAATCCTACAGAGCATGTGACAAAGCCCCATACGCTCGAGGATCGGACGCGG
                  MetValGlyLysArgArgGlnAlaProAlaAsnProThrGluHisValThrLysProHisThrLeuGluAspArgThrArg
JYW   TGCATATCAGGTAGTCCCCAAACCCCCAACCCCCAAGGAGCTTTGAGGGTTCAAATGACGCTCGAACAGGCATGCCCTCCGGAATGCCAAAG
                                                        MetThrLeuGluGlnAlaCysProProGluCysGlnAr
BH    ACCTGAAAAAAAGGATGATCTGCGTGGGCTGGCGCCGGCCGGGCCTACAGAGCGGGTGACAAAGCCCCATACGCTCGAGGACCGGACGCGGT
                MetIleCysValGlyTrpArgArgProGlyLeuGlnSerGlyEndGlnSerProIleArgSerArgThrGlyArgGly
JYS   AAAATAAAAGGTTGCAAAAAATTGCGCCGGCGGGCGCCGGCCGGGCCTGCAGAGCGGAAGACAAAGCCCCATACGCTCGAGGACCGGACGCG

JS-B  TTCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTGAGGGCCCTCTGGGTCCMACCTCCCACCCGTGTTTAACGTACCTTGTTGCTTCGG

D1S4  ACCTGAGAAAAATAAGGTTGGAGACGCCGGCTGGCGCCCGGCCGGCCCTAATCGAGCGGGTGACAAAGCCCCATACGCTCGAGGACCGGACA

Borehole AACCTGAGAAAAATAAGGTTGGAGACGCCGGCTGGCGCCCGGCCGGCCCTAATCGAGCGGGTGACAAAGCCCCATACGCTCGAGGACCGGAC

JS-A  GGAAAAAAAACCTATCCATTAGTTTATACTCCGCCTTTCTTTCAAGCAAACCCAGCGTATCGCTCAACACCAAACCCAAGGGTTTGAGGGAG
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            110       120       130       140       150       160       170       180       190
      ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|..
Rso   GTATTCCTCCAGATCTCTACGCATTTCACCGCTACACCTGGAATTCTACCCCCCTCTACAAGACTCTAGCCTGCCAGTTTCGAATGCAGTTC
                                                                                       MetGlnPhe
A-Feb GGGCCCGTCCCCCCCGGGGAGGGGACGACGACCCAACACACAAGCCGTGCTTGATGGGCAGCAATGACGCTCGGACAGGCATGCCCCCCGGA
                                                                    MetGlySerAsnAspAlaArgThrGlyMetProProGly
D2S3  CTGCCTTTCGGGCCCGTCCCCCCGGAGAGGGGGACGGCGACCCAACACACAAGCCGGGCTTGAGGGCAGCAATGACGCTCGGACAGGCATGC
        CysLeuSerGlyProSerProArgArgGlyGlyArgArgProAsnThrGlnAlaGlyLeuGlyLysSerAsnAspAlaArgThrGlyMetP
JYW   GTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACACTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAG
      CysAlaPheLysAspSerMetIleHisEndIleLeuGlnPheThrLeuLeuIleAlaPheArgCysValLeuHisArgCysGlnAsnGlnGlu
BH    TGCCTTTCGGGCCCGTCCCCCGGGGGGGGACGAGGACCCAATACAAGCCGGGCTTGAGGGCAGTAATGACGCTCGGACAGGCATGCCCCCCGG
        AlaPheArgAlaArgProProGlyGlyThrArgThrGlnTyrLysProGlyLeuArgAlaValMetThrLeuGlyGlnAlaCysProProG
JYS   GCTGCCTTTCGGGCCCGTCCCCCGGAGCCGGGGGACGAGGACCCAACACACAAGCCGGGCTTGAGGGCAGCAATGACGCTCGGACAGGCATG
                                                                         MetThrLeuGlyGlnAlaCy
JS-B  CCGCAAGGCCGCCGGGGGGCATCCGCCCCCGGGTCCGCGCCCGCCGAAGACACCTGTGAACGCTGTCTGAAGATTGCAGTCTGAGCGAAAAG

D1S4  CCGCTGCCTTTCGGGCCCGTCCCCCGGGGGGGACGACGACCCAACACACAAGCCGGGCTTGAGGGCAGCAATGACGCTCGGACAGGCATGCC
                                                                          MetThrLeuGlyGlnAlaCysP
Borehole GCCGCTGCCTTTCGGGCCCGTCCCCCGGGGGGGACGACGACCCAACACACAAGCCGGGCTTGAAGGGCAGCAATGACGCTCGGACAGGCATG
                                                                          MetThrLeuGlyGlnAlaCy
JS-A  CTCAAACAGGCATGCCCTTTGGAATACCAAAGGGCGCAATGTGCGTTCAAAGATTCAATGATTCACGAATATCTGCAATTCAGATTACTTAT
            MetProPheGlyIleProLysGlyAlaMetCysValGlnArgPheAsnAspSerArgIleSerAlaIleGlnIleThrTyr
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            210       220       230       240       250       260       270       280       290
      ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|..
Rso   AGCCCGGGGATTTCACATCCGACTTGACAGACCGCCTGCGTGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTACCG
        AlaArgGlyPheHisIleArgLeuAspArgProProAlaCysAlaLeuArgProValIleProIleAsnAlaCysThrLeuArgIleThrA
A-Feb GGGCGCAATGGGCGTTCAAAGACTCGATGATTCACGGAATTCTGCAATTCACACTAGTTATCGCATTTCGCTGCGTTCTTCATCGATGCCGG
        GlyAlaMetGlyValGlnArgLeuAspAspSerArgAsnSerAlaIleHisThrSerTyrArgIleSerLeuArgSerSerSerMetProG
D2S3  ATACCAGGGGGCGCAATGTGCGTTCAAAGACTCGATGATTCACTGAATTCTGCAATTCACATTAGTTATCGCATTTCGCTGCGTTCTTCATC
        IleProGlyGlyAlaMetCysValGlnArgLeuAspAspSerLeuAsnSerAlaIleHisIleSerTyrArgIleSerLeuArgSerSerSe
JYW   GTGAAAGTTGTAATTATTAATTTGTAACTG
        EndLysLeuEndLeuLeuIleCysAsnXXX
BH    GGGGCGCAATGTGCGTTCAAAGACTCGATGATTCACTGAATTCTGCAATTCACATTAGTTATCGCATTTCGCTGCGTTCTTCATCGATGCCG
        GlyAlaGlnCysAlaPheLysAspSerMetIleHisEndIleLeuGlnPheThrLeuValIleAlaPheArgCysValLeuHisArgCysAr
JYS   AATACCAGGGGGCGCAATGTGCGTTCAAAGACTCGATGATTCACTGAATTCTGCAATTCACATTAGTTATCGCATTTCGCTGCGTTCTTCAT
        GluTyrGlnGlyAlaGlnCysAlaPheLysAspSerMetIleHisEndIleLeuGlnPheThrLeuValIleAlaPheArgCysValLeuHis
JS-B  TTAAAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAA
                              MetLysAsnAlaAlaLysCysAspLysEndCysGluLeuGlnAsnSerValAs
D1S4  TGCCAGGGGGCGCAATGTGCGTTCAAAGACTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCAGTTCGCTGCGTTCTTCATCG
        CysGlnGlyAlaGlnCysAlaPheLysAspSerMetIleHisEndIleLeuGlnPheThrLeuLeuIleAlaValArgCysValLeuHisAr
Borehole AATGCCAGGGGGCGCAATGTGCGTTCAAAGACTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCAGTTCGCTGCGTTCTTCAT
        GluCysGlnGlyAlaGlnCysAlaPheLysAspSerMetIleHisEndIleLeuGlnPheThrLeuLeuIleAlaValArgCysValLeuHis
JS-A  GCTGCGTTCTTCTTCGATGCGAGAACCAAGAAATCCGTTGTTGAAAGTTTTGACTATAAGTCAATCACTTGACTTAAATAAAAGTTTGGTTG
        LeuArgSerSerSerMetArgGluProArgAsnProLeuLeuLysValLeuThrIleSerGlnSerLeuAspLeuAsnLysSerLeuValG
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            310       320       330       340       350       360       370       380       390
      ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|..
Rso   GGCACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAATCGATGAGGTTATTAACCTTATCGCCTTCCTCCCCGCTGAAAGTACTTTAC
        GlyThrGluLeuAlaGlyAlaSerSerAlaGlyAsnValAsnArgEndGlyTyrEndProTyrArgLeuProProArgEndLysTyrPheTh
A-Feb GATCCATTGTTGAAAGTTTTAACTGATTGCGATACAATCAACTCAGACTTCACTAGATCAGACAGAGTTCGTGGTGTCTCCGGCGGGCGCGG
        AspProLeuLeuLysValLeuThrAspCysAspThrIleAsnSerAspPheThrArgSerAspArgValArgGlyValSerGlyGlyArgGl
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Appendix E2: The raw sequence of the identified isolates

>Rso

TCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGC
ACCTGAGCGTCAGTCTTTGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGAT
CTCTACGCATTTCACCGCTACACCTGGAATTCTACCCCCCTCTACAAGACTCTAGCC
TGCCAGTTTCGAATGCAGTTCCCAGGTTGAGCCCGGGGATTTCACATCCGACTTGAC
AGACCGCCTGCGTGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGT
ATTACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAA
TCGATGAGGTTATTAACCTTATCGCCTTCCTCCCCGCTGAAAGTACTTTACAACCCG
AAGGCCTTCTTCATACACGCGGCATGGCTGCATCAGGCTTGCGCCCATTGTGCAAT
ATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGGCT
GGTCATCCTCTCAGACCAGCTAGGGATCGTCGCCTAGGTGAGCCGTTACCCCACCT
ACTAGCTAATCCCATCTGGGCACATCCGATGGCAAGAGGCCCGAAGGTCCCCCTCT
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TCAGGCAGTTTCCCAGACATTACTCACCCGTCCGCCACTCGTCAGCGAAGCAGCAA
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>A-Feb

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>D2S3

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>JYW

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>BH

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>JYS

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>JS-B

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>D1S4

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>Borehole

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>JS-A

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APPENDIX F

MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF ISOLATES FROM SOILS SAMPLED

APPENDIX F1:Morphological and biochemical characteristics of bacteria isolates in Ahoko soil SITE A.

Isolates codes	Cultural characteristics	Gram's reaction	Cell morphology	Catalase	Coagulase	Urease	Motility	Citrate	Starch utilization	Indole	oxidase	MR/VP	Glucose fermentation	H$_2$S	Suspected organism
FSa	Creamy, moist colonies	–	rod	+	-	-	+	+	-	-	+		-	-	*Pseudomonas alcaligenes*
FSa	Non-pigmented mucoid colony with pitted raised surface	–	coccobacilli	+	-	-	+	-	-	-		-/+	+	-	*Acinetobacter* sp.
FSa		+	diplococci	-	-	-	-	- Nsf	ND	- Nr+	-	-/+	+	-	*Enterococcus* sp.
MSa	Flat, opaque gray-white colony	–	Cocco-bacilli	+	_	_		+	-	_	+		+	-	*Alcaligenes* sp.
APSa	Creamy, moist colonies	+	Short rods	+	-		+	+	+	-		+/+	-	-	*B. licheniformis*
APSa	Smooth circular Moderately Raised Creamy colonies		Rod	+	-	-	+	+	ND	-	-	+/-	-	-	*Serratia* sp.
APSa	Circular, smooth and slight yellow colonies	+	Cocci	+	-	+	-	+	+	-	-	+/+	+	-	*Micrococcus* sp.
MySa	Glisten flat colonies	+	Short rod	+	+		+	+	+	-	-	+/+			*B .coagulase*
JSa	Round smooth, whitish colony	+	Cocci in cluster	+	+	-	-	-	-	-	-	-/-	+		*S. aureus*
JSa	Small whitish colony, dry	+	Rod	+	-	-	-	+	-	+	-	+/-	+	+	*Corynebacterium*

Code	Colony characteristics	Gram	Morphology									MR/VP			Organism
	concentrically ringed colony							Nsf							sp
JySa	Colony appers like cauliflower	-	Rod	+	-	+	+		-	-		-/+	+	-	*Enterobacter* sp.
JySa		+	cocci	-											*Enterococcus* sp.
AUSa	Irregular round. Rough cauliflower tye colonies	-	Rod	+	-	+	+		-	-		-/+	+	-	*Enterobacter* sp.
SSa	Cream, Flat, opaque	+		+	-	-	+	+		-	+	-/+	+		*Pseudomonas* sp.
OCSa	Circular, smooth and slight yellow colonies	+	Cocci	+	-	+	-	+	+	-	-	+/+	+	-	*Micrococcus* sp.
NOSa	Small whitish colony, dry concentrically ringed colony	+	Rod	+	-	-	-	+	-	+	-	+/-	+	+	*Corynebacterium* sp
DESa	Slight yellow colonies	+	Cocci in tetrads	+	-	-	-	-	+	-	-	+/+	+	-	*Micrococcus* sp
JANSa	Moist smooth colonies, with shiny surface	-	rod	+	-	+	+	+	-	-	-	+/-	+	+	*Citrobacter freundii*
JANSa	Smooth circular Moderately Raised Creamy colonies	-	Rod	+	-	-	+	+	ND	-	-	+/-	-	-	*Serratia* sp.

Key: + = positive,–means: negative. ND= not determined; Nsf= Non- spore former, Nr+ = Nitrate reduction positive, MR/VP= Methyl red/Vogas Poskture. FSa: Soil collected from site A in the month of February, MSa: Soil collected from site A in the month of March, APSa: Soil collected from site A in the month of April; MySa: Soil collected from site A in the month of May; JSa: Soil collected from site A in the month of June; JySa: Soil collected from site A in the July, AUSa: Soil collected from site A in the month of August: SSa: Soil collected from site A in the month of September OCSa: Soil collected from site A in the month of October, NOSa: Soil collected from site A in the month of November, DESa: Soil collected from site A in the month of December and JANSa: Soil collected from site A in the month of January

APPENDIX F2: Morphological and biochemical characteristics of organisms in water sample from ahoko

Isolates codes	Cultural characteristics	Gram's reaction	Cell morphology	Catalase	Coagulase	Urease	Motility	Citrate	Starch utilization	Indole	Oxidase	MR/VP	Glucose fermentatio	H2S	Suspected organism
FW	Creamy, moist colonies	+	Short rods	+	-		+	+	+	-		+/+	-	-	*B. licheniformis*
FW	Moderately- Raised, Smooth, Opaque colonies	-	Rod	+	-	-	+	-	-	-		+/-	+		*Salmonella spp.*
FW		-	Rod	+			+		-		-		+	-	*E.coli*
FW	Glisten flat colonies	+	Short rod	+	-		+	+		-	-	+/+			*Bacillus coagulase*
MW	Moderately- Raised, Smooth, Opaque colonies	-	Rod	+	-	-	+	-	-	-		+/-	+		*Salmonella spp.*
MW		-	Rod	+			+		-		-		+	-	*E. coli*
MW	Cream, Flat, opaque	-	Rod	+	-	-	+	+	+	-	+	-/-	+	-	*Pseudomonas sp.*
APW		-	Rod	+	-	-	+	+	-	-	-	-/+ Nr+	+	+	*Enterobacter aerogenes*
APW	Creamy, moist colonies	-	Rod	+	-	-	+	+	-	-	+		-	-	*Pseudomonas alcaligenes*
MyW	Moist smooth colonies, with shiny surface	-		+	-	+	+	+	-	-	-	+/-	+	+	*Citrobacter freundii*
MyW	Creamy, moist colonies	+	Short rods	+	-		+	+	+	-		+/+	-	-	*B. licheniformis*
MyW	Small whitish colony, dry concentrically ringed colony	+	Rod	+	-	-	-	+		+	-				*Corynebacterium sp*

Code	Colony morphology	Gram	Shape												Organism
JS-W	Small whitish colony, dry concentrically ringed colony	+	Rod	+	-	-	-	+			+	-				*Corynebacterium* sp
JyW	Button-like watery colony	-	rod	+	-		+	-	-	-	-	-/-	- Nr+	-		*Stenotrophomonas*
AUW	Moist smooth colonies, with shiny surface	-		+	-	+	+	+	-	-	-	+/-	+	+		*Citrobacter* freundii
SSW	Button-like watery colony	-	rod	+	-		+	-	-	-	-	-/-	- Nr+	-		*Stenotrophomonas*
NoW	Moderately, Raised, Entire, Smooth, Opaque colonies	-	Rod	+	-	-	+	-	-	-		+/-	+			*Salmonella spp.*
JANW	Moderately, Raised, Entire, Smooth, Opaque colonies	-	Rod	+	-	-	+	-	-	-		+/-	+			*Salmonella spp.*
JANW	Cream, Flat, opaque	-	Rod	+	-	-	+	+	+	-	+	-/-	+	-		*Pseudomonas sp.*

APPENDIX F3: MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF ISOLATES FROM AHOKO SOIL (Site B)

Isolates codes	Cultural characteristics	Gram's reaction	Cell morphology	Catalase	Coagulase	Urease	Motility	Citrate	Starch utilization	Indole	oxidase	MR/VP	Glucose fermentation	H₂S	Suspected organism	Frequency Occurrence
FSb	Round, smoothcolonies	+ve	Coccus	+	+	+	-	+	-	-	-	-/-	+		*S. aureus*	1
FSb	Small whitish colony, dry concentrically ringed colony	+	Rod	+	-	-	-	+ Nsf	-	+	-	+/-	+	+	*Corynebacterium* sp	1
FSb	Shiny white smooth colonies	-	Rod	+	-	-	+	+	-	-	+	-/-	- Nr-	-	*Alcaligenes faecalis*	1
FSb	Smooth circular Moderately Raised Creamy colonies	-	Rod	+	-	-	+	+	ND	-	-	+/-	-	-	*Serratia* sp.	2
FW	Glisten flat colonies	+ve	Short rod	+	-		+	+		-	-	+/+			*Bacillus coagulase*	2
MSb	Slight yellow colonies	+ve	Cocci in tetrads	+	-	-	-	-							*Micrococcus* sp	1
MSb	Cream, Flat, opaque	-ve	Rod	+	-	-	+	+	+	-	+	-/-	+	-	*Pseudomonas sp.*	1
ASb	Cream, Flat, opaque	-ve	Rod	+	-	+	+	+	+	-	+	-/+	+	-	*Pseudomonas sp.*	1
ASb	Glisten flat colonies	-ve	Rod	+	-	-	+	+	-	-	-	+/-	-	-	*Bacillus coagulase*	1
MySb	Glisten flat colonies	+ve	Short rod	+	-	-	+	+	-	-	-	+/+	-	-	*Bacillus coagulase*	1
JSb		+ve	coccus	+	+	-	-	-	-	-	-	-/-	+			3
JSb	Small whitish colony, dry concentrically ringed colony	+ve	Rod	+	-	-	-	+		+	-				*Corynebacterium* sp	2
JSb	Shiny white smooth colonies	-	Rod	+	-	-	+	+	-	-	+	-/-	- Nr-	-	*Alcaligenes faecalis*	1
JySb	Irregular round. Rough	-	Rod	+		-	+	+		-	-	-/+	+	-	*Enterobacter* sp.	1

Code	Colony morphology	Gram	Shape												Organism	No.
	cauliflower type colonies															
AUSb	Irregular round. Rough cauliflower type colonies	-	Rod	+	-	+	+		-	-	-/+	+	-	*Enterobacter* sp.	1
SSb	Slight yellow colonies	+ve	Cocci in tetrads	+	-	-	-	-	ND	-	-	+/+	+	-	*Micrococcus* sp	2
OCSb	Slight yellow colonies	+ve	Cocci	+	-	+	-	+	ND	-	-	+/+	+	-	*Micrococcus* Sp.	1
NOSb	White to yellow colonies	+ve	Cocci	+	-	+	-	+	ND	-	-	+/+	+	-	*Micrococcus* sp	1
DESb		-	Rod	+	-	-	+	+	-	-	-	-/+	+ Nr+	+	*Enterobacter aerogenes*	2
JANSb	Round, smoothcolonies	+ve	Coccus	+	+	+	-	+	-	-	-	-/-	+		*S. aureus*	1
JANSb	Glisten flat colonies	+ve	Short rod	+	-		+	+		-	-	+/+			*Bacillus coagulase*	1
					Total											25

APPENDICE F4: MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF ISOLATES FROM AHOKO SOIL (SITE C)

Isolates codes	Cultural characteristics	Gram's reaction	Cell morphology	Catalase	Coagulase	Urease	Motility	Citrate	Starch utilization	Indole	oxidase	MR/VP	Glucose fermentation	H2S	Suspected organism	Frequency Occurrence
FSc	Single moist colonies	-	Rod	+	-	+	-	+	+	-	-	-/-	+	-	*Klebsiella*	2
FSc	Moderately- Raised, Smooth, Opaque colonies	-	Rod	+	-	-	+	-	-	-		+/-	+		*Salmonella spp.*	2
FSc	Creamy, moist colonies	-	Rod	+	-	-	+	+	-	-	+		-	-	*Pseudomonas alcaligenes*	2
MSc	White to yellow colonies	+	Cocci	+	-	+	-	+	ND	-	-	+/+	+	-	*Micrococcus* sp	1
MSc	Moderately- Raised, Smooth, Opaque colonies	-	Rod	+	-	-	+	-	-	-		+/-	+		*Salmonella spp.*	1
ASc	Mucoid colony with pitted raised surface	–	Coccobacilli	+		-	-	+	-	-	-	-/+	+	-	*Acinetobacter sp.*	1
ASc	Smooth circular Moderately Raised Creamy colonies	-	Rod	+	-	-	+	+	ND	-	-	+/-	-	-	*Serratia sp.*	1
MySc	Glisten flat colonies	+	Short rod	+	-		+	+		-	-	+/+			*B .coagulase*	1
JSc	Round, smooth colonies	+	Coccus	+	+	+	-	+	-	-	-	-/-	+		*S. aureus*	3
JSc	Moderately- Raised, Smooth, Opaque colonies	-	Rod	+	-	-	+	-	-	-		+/-	+		*Salmonella spp.*	2
JSc	Smooth circular Moderately Raised Creamy colonies	-	Rod	+	-	-	+	+	ND	-	-	+/-	-	-	*Serratia sp.*	1
JySc	Moderately- Raised, Smooth, Opaque colonies	-	Rod	+	-	-	+	-	-	-		+/-	+		*Salmonella spp.*	1
JySc	Non-pigmented mucoid colony with pitted raised surface	–	Coccobacilli	+		-	-	+	-	-	-	-/+	+	-	*Acinetobacter sp.*	1
AUSc	Cream, Flat, opaque	-	Rod	+	-	-	+	+	+	-	+	-/-	+	-	*Pseudomonas sp.*	1

Isolates codes	Cultural characteristics	Gram's reaction	Cell morphology	Catalase	Coagulase	Urease	Motility	Citrate	Starch utilization	Indole	oxidase	MR/VP	Glucose fermentation	H2S	Suspected organism	Frequency Occurrence
SSc	Cream, Flat, opaque	-	Rod	+	-	-	+	+	+	-	+	-/-	+	-	*Pseudomonas sp.*	1
OCSc	White to yellow colonies	+	Cocci	+	-	+	-	+	ND	-	-	+/+	+	-	*Micrococcus* sp	1
NOSc	Smooth circular Moderately Raised Creamy colonies	-	Rod	+	-	-	+	+	ND	-	-	+/-	-	-	*Serratia* sp.	2
DESc	White to yellow colonies	+	Cocci	+	-	+	-	+	ND	-	-	+/+	+	-	*Micrococcus* sp	1
DESc	Non-pigmented mucoid colony with pitted raised surface	–	Coccobacilli	+	-	-	+	-	-	-		-/+	+	-	*Acinetobacter sp.*	1
JANSc	Glisten flat colonies	+	Short rod	+	-		+	+		-	-	+/+			*B .coagulase*	2
JANSc	Cream, Flat, opaque	-	Rod	+	-	-	+	+	+	-	+	-/-	+	-	*Pseudomonas sp.*	1
								Total								29

APPENDICE F5: MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF ISOLATES FROM AHOKO SOIL (SITE D)

Isolates codes	Cultural characteristics	Gram's reaction	Cell morphology	Catalase	Coagulase	Urease	Motility	Citrate	Starch utilization	Indole	oxidase	MR/VP	Glucose fermentation	H2S	Suspected organism	Frequency Occurrence
FSd	Cream, Flat, opaque	-	Rod	+	-	-	+	+	+	-	+	-/-	+	-	*Pseudomonas sp.*	2
FSd	Mucoid colony with pitted raised surface	–	Coccobacilli	+	-	-	+	-	-	-		-/+	+	-	*Acinetobacter sp.*	1
FSd	Small whitish colony, dry concentrically ringed colony	+	Rod	+	-	-	-	+ Nsf	-	+	-	+/-	+	+	*Corynebacterium* sp	1
MSd	White to yellow colonies	+ve	Cocci	+	-	+	-	+	ND	-	-	+/+	+	-	*Micrococcus* sp	1

Code	Colony morphology	Gram	Shape												Organism	No.
MSd		-	Rod	+		+		-		-			+	-	*E. coli*	2
ASd	Mucoid colony with pitted raised surface	–	Coccobacilli	+	-	-	+	-	-	-		-/+	+	-	*Acinetobacter sp.*	2
ASd	Smooth circular Moderately Raised Creamy colonies	-	Rod	+	-	-	+	+	ND	-	-	+/-	-	-	*Serratia sp.*	1
MySd	Single moist colonies	-ve	Rod	+	-	+	-	+	+	-	-	-/-	+	-	*Klebsiella*	2
JSd	Round, smooth colonies	+ve	Coccus	+	+	+	-	+	-	-	-	-/-	+		*S. aureus*	2
JSd	Moderately- Raised, Smooth, Opaque colonies	-	Rod	+	-	-	+	-	-	-		+/-	+		*Salmonella spp.*	2
JSc	Smooth circular Moderately Raised Creamy colonies	-	Rod	+	-	-	+	+	ND	-	-	+/-	-	-	*Serratia sp.*	1
JySd	Moderately- Raised, Smooth, Opaque colonies	-	Rod	+	-	-	+	-	-	-		+/-	+		*Salmonella spp.*	1
JySd	Non-pigmented mucoid colony with pitted raised surface	–	Coccobacilli	+	-	-	+	-	-	-		-/+	+	-	*Acinetobacter sp.*	1
AUSd	Moderately- Raised, Smooth, Opaque colonies	-	Rod	+	-	-	+	-	-	-		+/-	+		*Salmonella spp.*	1
AUSd		-ve	Rod	+	-	+	+	-	-			-/+	+	-	*Enterobacter sp.*	2
SSd	Creamy, moist colonies	-ve	Rod	+	-	-	+	+	-	-	+	-/+	-	-	*Pseudomonas alcaligenes*	2
SSd	Cream, Flat, opaque	-	Rod	+	-	-	+	+	+	-	+	-/-	+	-	*Pseudomonas sp.*	1
OCSd	Small whitish colony, dry concentrically ringed colony	+	Rod	+	-	-	-	+ Nsf	-	+	-	+/-	+	+	*Corynebacterium sp*	1
OCSd	Single moist colonies	-ve	Rod	+	-	+	-	+	+	-	-	-/-	+	-	*Klebsiella*	2
NOSd	Moderately- Raised, Smooth, Opaque colonies	-	Rod	+	-	-	+	-	-	-		+/-	+		*Salmonella spp.*	2
NOSd	Smooth circular Moderately Raised	-	Rod	+	-	-	+	+	ND	-	-	+/-	-	-	*Serratia sp.*	2

Isolates codes	Cultural characteristics	Gram reaction	Cell morphology	Spore form.	Catalase	Coagulase	Urease	Motility	Citrate	Starch utilization	Indole	Oxidase	MR/VP	Glucose fermentation	H2S	Suspected organism	Occurrence
DESd	Creamy colonies Cream, Flat, opaque	-	Rod	+	-	-	+	+	+	-	+		-/-	+	-	*Pseudomonas sp.*	1
DESd	Non-pigmented mucoid colony with pitted raised surface	–	Coccobacilli	+	-	-	+	-	-	-			-/+	+	-	*Acinetobacter sp.*	1
JANSd	Round, smoothcolonies	+ve	Coccus	+	+	+	-	+	-	-	-		-/-	+		*S. aureus*	1
JANSd	White to yellow colonies	+ve	Cocci	+	-	+	-	+	ND	-	-		+/+	+	-	*Micrococcus* sp	1
									Total								36

Appendice F6: Morphological and biochemical characteristics of specific hydrocarbon oxidizers in water from Ahoko

Isolates codes	Cultural characteristics	Gram reaction	Cell morphology	Spore form.	Catalase	Coagulase	Urease	Motility	Citrate	Starch utilization	Indole	Oxidase	MR/VP	Glucose fermentation	H2S	Suspected organism	Occurrence	Gas
Feb-W	White, circular small colonies , slightly raised at the centre	-	Rod (curved)	-	+	-	-	+	- NR+	-	+	+	+/-	+	-	*Geobacter*	1	E
Feb-W	Tiny-spot colonies	+	Short rod	+	+	-	+	+	+	+	-	-	+/+	+	+	UNIDENTIFIED	2	P
Feb-W	Smooth rounded, colored colonies	+	Rod	-	+		-	+	-	+ Nr+	+	+	-/-	+	+	*Arthrobacter sp.*	1	E
Ma-W	Circular, Creamy, mucoid colonies, with smell	-	Rod	-	+	-	-	+	+	+	-	+	-/+	-	-	*Pseudomonas*	1	E
Ma-W	Creamy, dull white wet colonies	+	Rod (mycelium-like)		+	-	-	-	+	-	-	-		-	-	*Mycobacterium*	1	P
Ap-W	Creamy-white, slimy	-	Rod	-	-			+	-	-			-/-	+	-	*Methylobacter*	1	M

Code	Colony	Gram	Shape													ID	No.	Group
	colony																	
AP-W **AP-W**	Non-pigmented colony	-	Rod		+		+		+	-	+					*Ochrobacteria*	3	B
MyW	Tiny-spot colonies	+	Short rod	+	+	-	+	+	+	+	-	-	+/+	+	+	UNIDENTIFIED	1	P
My-W	Slimy COLONY embedded in the media and difficult	-	Rod	+		+	-	ND	-	+	N D	+	-	-		*Methylomonas*	1	M
MyW	Creamy-white, slimy colony	-	Rod	-		+					ND	+	-			*Methylobacter*	2	M
JnW	Tiny-spot colonies	+	Short rod	+	+	-	+	+	+	+	-	-	+/+	+	+	UNIDENTIFIED	1	P
Jy-W	Slimy, rough colony with irregular edges	+	Coccobaccili		+		-									*Gordonia*	2	P
Au-W	Slimy COLONY embedded in the media and difficult	-	Rod	+		+	-	ND	-	+	N D	+	-	-		*Methylomonas*	1	M
Au-W	White, circular small colonies , slightly raised at the centre	-	Rod (curved)	-	+	-	-	+	- NR+	-	+	+	+/-	+	-	*Geobacter*	1	E
Au-W	White, circular small colonies , slightly raised at the centre	-	Rod (curved)	-	+	-	-	+	- NR+	-	+	+	+/-	+	-	*Geobacter*	2	E
Se-W	Smooth rounded, Tan/buff-colored colonies	-	Rod	-	+	-	+		+ Nr+	+	+	-/-	+	+		*Aeromonas sp.*	2	E
De-W	Dull-white colonies, with chalky look	+	Rod	-	+	-	+	-	-	-	-	+	+/+	-	-	*Norcadia*	1	E
Jan-W	Creamy, dull white wet colonies	+	Rod (mycelium-like)		+	-	-	-	+	-	-	-		-	-	*Mycobacterium*	1	P
Jan-W	Dull-white colonies, with chalky look	+	Rod	-	+	-	+	-	-	-	-	+	+/+	-	-	*Norcadia*	1	E
TOTAL																	24	10E,3B,4M,6P

Appendix f7: Morphological and biochemical characteristics of specific hydrocarbon oxidizers in water from Patitiabakolo

Isolates codes	Cultural characteristics	Gram reaction	Cell morphology	Spore	Catalase	Coagulase	Urease	Motility	Citrate	Starch utilization	Indole	Oxidase	MR/VP	Glucose fermented	H2S	Suspected organism	Gas
JanW	Dry flat and broad, irregular edges	-	Rod	+	+	-	-	+	+		-	-	-/+	+	-	*Enterobacter sp.*	E
JanW	Dull-white colonies, with chalky look	+	Rod	-	+	-	+	-	-	-	-	+	+/+	-	-	*Norcadia*	E
FebW	Smooth rounded, Tan/buff-colored colonies	-	Rod	-	+	-	-	+	Nr+	+	+	+	-/-	+	+	*Aeromonas* sp.	E
MaW	Circular, Creamy, mucoid colonies, with smell	-	Rod	-	+	-	-	+	+	+	-	+	-/+	-	-	*Pseudomonas* sp.	E
ApW	Creamy-white, slimy colony	-	Rod	-				+					-/-	+	-	*Methylobacter* sp.	M
APW	Non-pigmented colony	-	Rod		+			+		+	-	+				*Ochrobacteria*	E
APW	White-spot, almost invisible	-	Spiral shape	-	-	-	-	+	+ Nr-	-	-	-	-/-	-	+	*Giebergeria*	B
MyW	Tiny-spot colonies	+	Short rod	+	+	-	+	+	+	+	-	-	+/+	+	+	UNIDENTIFIED	P
MyW	Creamy-tiny colonies	-	Rod/coccoid	-	+	-	+	-	+ Nr+	+	-	+	ND	+	-	*Microbulbifer thermotolerans*	P
MyW	Creamy-white, slimy colony	-	Rod	-				+					ND	+	-	*Methylobacter*	M
JnW	White-spot, almost invisible	-	Spiral shape	-	-	-	-	+	+ Nr-	-	-	-	-/-	-	+	*Giebergeria*	B
AuW	Creamy-white, slimy colony	-	Rod	-				+					-/-	+	-	*Methylobacter*	M
AuW	White, circular small colonies with smooth	-	Rod	-	+	-	-	-	- Nr+	-	+	+	+/-	-	-	*Ramlibacter* sp.	B, P

Isolates codes	Cultural characteristics	Gram reaction	Cell morphology	Spore	Catalase	Coagulase	Urease	Motility	Citrate	Starch utilization	Indole	Oxidase	MR/VP	Glucose fermentation	H2S	Suspected organism	Gas
	edge																
SeW	Smooth rounded, Tan/buff-colored colonies	-	Rod	-	+	-	+		Nr+	+	+	+	-/-	+	+	*Aeromonas sp.*	E
DeW	Mucoid colonies	-	Rod	+	-	+	+			-	-		-/+	+	-	*Enterobacter sp.*	E

F8: Morphological and biochemical characteristics of specific hydrocarbon oxidizers in water from Patitiabakolo

Isolates codes	Cultural characteristics	Gram reaction	Cell morphology	Spore	Catalase	Coagulase	Urease	Motility	Citrate	Starch utilization	Indole	Oxidase	MR/VP	Glucose fermentation	H2S	Suspected organism	Gas
JanW	Dry flat and broad, irregular edges	-	Rod	+	+	-	-	+	+		-	-	-/+	+	-	*Enterobacter sp.*	E
JanW	Dull-white colonies, with chalky look	+	Rod	-	+	-	+	-	-	-	-	+	+/+	-	-	*Norcadia*	E
FebW	Smooth rounded, Tan/buff-colored colonies	-	Rod	-	+		-	+	Nr+	+	+	+	-/-	+	+	*Aeromonas* sp.	E
MaW	Circular, Creamy, mucoid colonies, with smell	-	Rod	-	+	-	-	+	+	+	-	+	-/+	-	-	*Pseudomonas* sp.	E
ApW	Creamy-white, slimy colony	-	Rod	-				+					-/-	+	-	*Methylobacter* sp.	M
APW	Non-pigmented colony	-	Rod		+			+		+	-	+				*Ochrobacteria*	E
APW	White-spot, almost invisible	-	Spiral shape	-	-	-	-	+	+ / Nr-	-	-	-	-/-	-	+	*Giebergeria*	B
MyW	Tiny-spot colonies	+	Short rod	+	+	-	+	+	+	+	-	-	+/+	+	+	UNIDENTIFIED	P

Isolates codes	Cultural characteristics	Grams Reaction	Cell morphology	Catalase	Coagulase	Urease	Motility	Citrate	Starch utilization	Indole	Oxidase	MR/VP	Glucose fermentation	H2S	Suspected organism	Gas
MyW	Creamy-tiny colonies	-	Rod/coccoid	-	+	-	+	-	+ (Nr+)	+	-	ND	+	-	*Microbulbifer thermotolerans*	P
MyW	Creamy-white, slimy colony	-	Rod	-				+				ND	+	-	*Methylobacter*	M
JnW	White-spot, almost invisible	-	Spiral shape	-	-	-	+	+ (Nr-)	-	-	-	-/-	-	+	*Giebergeria*	B
AuW	Creamy-white, slimy colony	-	Rod	-				+				-/-	+	-	*Methylobacter*	M
AuW	White, circular small colonies with smooth edge	-	Rod	-	+	-	-	-	- (Nr+)	-	+	+/-	-	-	*Ramlibacter* sp.	B, P
SeW	Smooth rounded, Tan/buff-colored colonies	-	Rod	-	+	-	+	+ (Nr+)	+	+	+	-/-	+	+	*Aeromonas sp.*	E
DeW	Mucoid colonies	-	Rod	+	-	+	+	-	-			-/+	+	-	*Enterobacter* sp.	E

Appendice F8: Morphological and biochemical characteristics of specific hydrocarbon oxidizers in soil from Ahoko

Isolates codes	Cultural characteristics	Grams Reaction	Cell morphology	Catalase	Coagulase	Urease	Motility	Citrate	Starch utilization	Indole	Oxidase	MR/VP	Glucose fermentation	H2S	Suspected organism	Occurrence	Gas
A-Feb	Creamy, dull white wet colonies	+	Rod (mycelium-like)	+	-	-	-	+	-	-	-	-	-	-	*Mycobacterium*	1	P
A-Feb	Small-tiny colonies, that are sticky	-	Rod	+	-	-	- (nsf)	+ (Nr)	-	-	+	-/+	+	-	*Actinobacillus.*	2	E
Feb-B	Whitishy-dusty,chalk-like	+	Rod shaped	+	-	-	-	-	-	-	-	+/+	-	-	*Norcadia*	1	E

colonies

Code	Colony morphology		Shape											Identification		
Feb-C	Mucoid colonies	-	Rod	+	-	+	+		-	-	-/+	+	-	*Enterobacter sp.*	2	E
Feb-D	Circular Gray to whitish, opaque colony	+	Coccus in pairs	+	-	+	+	-	-	-	+	-/-	-	-	*Sporosarcina sp.*	1	M
MS-A	Slimy, rough colony with irregular edges	+	coccobaccili	+	-	-	-								*Gordonia terrae*	3	P
MS-A	Small-tiny colonies, that are sticky	-	Rod	+	-	-	- nsf	+ Nr		-	+	-/+	+	-	*Actinobacillus.*	1	E
MS-B	Whitishy-dusty,chalk-like colonies	+	Rod shaped	+	-	-	-	-	-	-	-				*Norcadia*	1	E
AS-A	Flat dry colonies with wrong edges	-	Rod	+	+	+	+			+	+	+/+	+		*Achromobacter*	3	P
AS-A		-	Rod	+	-	-	+	+		-	-	+/-	-	-	*Serratia spp.*	1	E
AS-B	Slimy colony embedded in the media and difficult to remove	-	Rod	+		+	-	ND	-	+	ND	+	-		*Methylomonas*	1	M
AS-C	Slimy, rough colony with irregular edges	+	coccobaccili	+			-								*Gordonia*	1	P
AS-D		-	Rod	+	-	-	+	+		-	-	+/-	-	-	*Serratia spp.*	1	E
A-My	Tiny colonies	+	Short rod	+	-		+	+		-	-	+/+			UNIDENTIFIED,	3	P
A-My	Flat dry colonies with wrong edges	-	Rod	+	+	+	+			+	+	+/+	+		*Achromobacter*	2	P
JS-A	Slimy COLONY embedded in the media and difficult to remove	-	Rod	+		+	NR	ND	NR	+	ND	+	-		*Methylomonas*	1	M

Code	Description	Gram	Shape												Organism	No.	Gas
JyS-B	Tiny colonies	+	Short rod	-	-	+	+	+	+	-	-	+/+	+	+	UNIDENTIFIED	1	E
JyW	Dry small tiny white colonies with smooth round edges	Not specific	Short rod	-	-	-	-	-	-	-	-	-/-	-	-	UNIDENTIFIED	2	E
AUS-A	Mucoid colonies	-	Rod	+	-	+	+	-	-			-/+	+	-	*Enterobacter sp.*	1	E
OCS-A	Creamy, dull white wet colonies	+	Rod (mycelium-like)	+	-	-	-	+	-	-	-		-	-	*Mycobacterium*	1	P
OC-C	Button-like colony														*Stenotrophomonas*	2	E
NOS-C	Whitishy-dusty,chalk-like colonies	+	Rod shaped	+	-	-	-	-	-	-	-				*Norcadia*	1	E
NOS-B	Tiny-spot colonies	+	Short rod	+	-	+	+	+	+	-	-	+/+	+	+	UNIDENTIFIED	1	B
NOS-D	Tiny-spot colonies	+	Short rod	+	-	+	+	+	+	-	-	+/+	+	+	UNIDENTIFIED	1	B
A-DES	Creamy, dull white wet colonies	+	Rod (mycelium-like)	+	-	-	-	+	-	-	-		-	-	*Mycobacterium*	1	P
A-JANS	Slimy COLONY embedded in the media and difficult to remove	-	Rod	+		+	-	ND	-	+	ND		+	-	*Methylomonas*	1	M

TOTAL propane SITE A=14P, B=0 C=1P
BUTANE= SITE A=0B, B=1B, C=0B, D=1B
ETHANE: SITE A= 6E, B=3E, C=5E, D=1E
METHANE SITEA=3M. B=1M, C=0, D=1M

37   4M, 16E, 15P, 2B

Appendices F9: Morphological and biochemical characteristics of total Fungal isolates in soils and water studied

Isolates codes	Cultural characteristics	Microscopic characteristics	Suspected organism	Occurrence
FSa	Light yellow-green colony with rough velvet edges	Radiating conidial heads with rough conidiophores	*Aspergillus* flavus	4
FSa	Dusty dark brown to black fluffy colonies	Branced septate-hyphea, with long and smooth conidiophores, unbrachen sponrandiophoress with large round head	*Aspergillus niger*	3
FSa	White mucoid colonies		*Candida*	1
FSa	Slightly red fluffy colonies, whose pigment disappeared after subculturing	Cocci in shape with budding cells	*Rhodotorula*	2
FSb	Dusty black fluffy colonies	Branced septate-hyphea, with long and smooth conidiophores, unbrachen sponrandiophoress with large round head	*Aspergillus niger*	2
FSb	Slightly red fluffy colonies, whose pigment disappeared after sub-culturing	Cocci in shape with budding cells	*Rhodotorula*	1
FSc	White dry, powdery, suede-like colonies	Presence of septate hyphae branching spores (arthroconidia) in chains	*Geotrichum*	1
FSd	White to grey velvet colonies with black velvet in the middle	rough conidiophore, with biseriate phialides, with round vesicles and radiating head	*Aspergillus fumigatus*	2
MSa	White mucoid colonies		*Candida*	1
MSd	White dry, powdery, suede-like colonies	Presence of septate hyphae branching spores (arthroconidia) in chains	*Geotrichum*	1
ASa	White mucoid colonies		*Candida*	1

ASb	White mucoid colonies		*Candida*	1
ASa	Dusty grey	Long slender septate conidiophore	*Aspergillus*. sp.	2
ASc	White to grey velvet colonies with black velvet in the middle	rough conidiophore, with biseriate phialides, with round vesicles and radiating head	*Aspergillus flavus*	2
ASb	Initially White colonies, that turned dark-brown after 5days		*Aspergillus* sp.	2
ASd	White to grey velvet colonies with black velvet in the middle	rough conidiophore, with biseriate phialides, with round vesicles and radiating head	*Aspergillus fumigatus*	2
MySa	Slightly red fluffy colonies, whose pigment disappeared after sub-culturing	Cocci in shape with budding cells	*Rhodotorula*	1
MySc	Dark brown to black fluffy colonies	Branced septate-hyphea, with long and smooth conidiophores, unbrachen sponrandiophoress with large round head	*Aspergillus* niger	2
JSa	Dark brown to black fluffy colonies	Branced septate-hyphea, with long and smooth conidiophores, unbrachen sponrandiophoress with large round head	*Aspergillus* niger	1
JSd	White cotton-like and fluffy mass		*Rhizopus*	3
JySb	Dark green and powdery colonies	Smooth walled conidiophores Chain of single celled	*Penicillium*	2
JySc			*Candida*	1
JySd	White velvet colonies with black velvet in the middle	rough conidiophore, with biseriate phialides, with round vesicles and radiating head	*Aspergillus fumigatus*	1

AUSa	White dry, powdery, suede-like colonies	Presence of septate hyphae branching spores (arthroconidia) in chains	*Geotrichum*	1
AUSc	White dry, powdery, suede-like colonies	Presence of septate hyphae branching spores (arthroconidia) in chains	*Geotrichum*	1
SSc	Dark green and powdery colonies	Smooth walled conidiophores Chain of single celled	*Penicillium*	1
OCSa	Slightly red fluffy colonies, whose pigment disappeared after subculturing	Cocci in shape with budding cells	*Rhodotorula*	1
OCSd	White mucoid colonies		*Candida*	2
NOSa	Dark green and powdery colonies	Smooth walled conidiophores Chain of single celled	*Penicillium*	1
DESa	White cotton-like and fluffy mass		*Rhizopus*	2
JANSa			*Alterneria*	1
JANSa	White dry, powdery, suede-like colonies	Presence of septate hyphae branching spores (arthroconidia) in chains	*Geotrichum*	1

Appendices F10: Morphological and biochemical characteristics of specific hydrocarbon oxidizing Fungi in soils and water studied

Isolates codes	Cultural characteristics	Microscopic characteristics	Glucose	Lactose	Suspected organism	Occurrence	Gas
A-Feb	White velvet colonies	Rough conidiophores with swollen vesicles and colourless septate hypae	-	+	*Aspergillus* spp.	2	E
A-Feb	Light green and powery colony	Long slender septate conidiophore	+	+	*Aspergilus flavus*	1	M
A-Feb	White mucoid colonies		+	+	*Candida,*	2	E
A-Feb	Colonies are creamy, flat, moist and smooth,	Presence of pseudohyphea with clusters of small blastosphore	+	+	*Pichia*	2	B
FEB-D	White to creamy velvet colonies	Rough conidiophores with swollen vesicles and septate hypae	-	-	*Aspergillus* spp.	2	E
Feb-B	Smooth white		-	-	*Talaromyces*	1	M
Feb-W	Colonies are creamy, flat, moist and smooth,	Presence of pseudohyphea with clusters of small blastosphore	+	+	*Pichia*	2	B
MS-A	Initially White colonies, that turned dark-brown after 5days		+	-	*Aspergillus spp*	2	E
MS-B	White cotton-like and fluffy mass	Sporangiosphores in group from node directly above the rhizods.	+	-	*Rhizopus*	1	B

MS-C	Dark brown to black fluffy colonies	Branced septate-hyphea, with long and smooth conidiophores, unbrachen sponrandiophoress with large round head			*Aspergillus* niger	2	E
AS-A	Initially White colonies, that turned dark-brown after 5days	Branched septate-hyphea, with long and smooth conidiophores, unbranched sponrandiophore with large round head	-	+	*Aspergillus* niger	2	E
AS-A	Coral moist-soft colonies, whose pigment disappeared after sub-culturing	Cocci in shape with budding cells	-	+	*Rhodotorula*	1	E
AS-D	White cotton-like and fluffy mass	Sporangiosphores in group from node directly above the rhizods.	+	-	*Rhizopus*	1	B
AW			-	-	*Talaromyces*	1	M
My-A	White velvet colonies	Rough conidiophores with swollen vesicles and colourless septate hypae	-	+	*Aspergillus* spp.	2	E
My-A	Gray-greenish	Conidiophores with phialide bearing the	-	-	*Penicillium*	1	E
My-C	Colonies are creamy, flat, moist and smooth,	Presence of pseudohyphea with clusters of small blastosphore	+	+	*Pichia*	1	B

Code	Macroscopic	Microscopic			Organism		
My-W	Flat white suede-like colonies	Presence of septate hyphae breaching spores (arthroconidia) in chains	-	-	*Geotrichum spp.*	2	P
JS-A	Creamy pasty colonies		+	+	*Candida sp.*	1	P
JS-A	Flat white suede-like colonies	Presence of septate hyphae breaching spores (arthroconidia) in chains	-	-	*Geotrichum spp.*	2	P
JS-B	Greenish to black, white mycelia at the margin, white droplet, yellow golden in the media.	Conidiophores with phialide bearing the conidia in chains	-	-	*Penicillium*	1	M
JS-C	Colonies are creamy, flat, moist and smooth,	Presence of pseudohyphea with clusters of small blastosphore	+	+	*Pichia*	2	B
JyS-A	White velvet colonies	Rough conidiophores with swollen vesicles and colourless septate hypae	+	-	*Aspergillus*	2	E
JyW			-	-	*Alterneria*	1	E
A-AUS	White dry colonies with carpet grass/matt apprearance	Presence of septate hyphae breaching spores (arthroconidia) in chain	-	-	*Geotrichum candidum*	2	P
AUW	Initially White colonies, that turned dark-brown after 5days	Branched septate-hyphea, with long and smooth conidiophores, unbranched sponrandiophore with large round head	-	+	*Aspergillus* niger	1	E
SS-A	black colonies with white margin around, having irregular radial form	Branched conidiophore with chains of conidia looking like brush	-	-	*Penicillium spp*	1	E
A-OCS	Initially White colonies, that turned	Rough conidiophores	-	+	*Aspergillus niger*	1	E

	dark-brown after 5days	with swollen vesicles and colourless septate hypae					
OCW	White velvet colonies	Rough conidiophores with swollen vesicles and colourless septate hypae	-	+	*Aspergillus* spp.	2	E
A-NOS	Greenish to black colonies with white margin around, having irregular radial form	Smooth walled conidiophores with conidia in chains	-	-	*Penicillium*	1	E
A-DES	White cotton-like and fluffy mass	Sporangiosphores in group from node directly above the rhizods.	+	-	*Rhizopus*	1	B
A-JANS	Colonies are creamy, flat, moist and smooth	Presence of pseudohyphea with clusters of small blastosphore	+	+	*Pichia*	1	B
JAN-D	Greenish to black colonies with white margin around, having irregular radial form	Smooth walled conidiophores with conidia in chains	-	-	*Penicillium*	1	M
A-JANS	Coral moist-soft colonies, whose pigment disappeared after sub-culturing	Cocci in shape with budding cells	-	+	*Rhodotorula*	1	E
	ETHANE=SITE A=21E, , B=1E C=2E, D=3E, W=4E METHANE: SITE A=2M, B-1M C=0M, D=0 W=1M BUTANE: SITE A=4B, B=4B C=1, D=0 W=2 PROPANE: SITE A=5P, W=2P					53	31E, 11B,4M 7P

Appendices F11: Biodegradation ability of hydrocarbon gas utilizing microbes on transniger pipeline crude oil

S/No.	Isolates codes	Biodegradation ability	Gas	scores
1	A-Feb	+	E	Poor degraders
2	A-Feb	+++	M	Good degraders
3	Borehole	+++	P	Good degraders
4	Borehole 2 (BH)	+++	E	Good degraders
5	FEB-P	+	E	Poor degrader
6	D2S4	+	E	Poor degrader
7	Riverside	+	P	Poor degrader
8	MS-A	++	E	Intermediate
9	MS-B	+	B	Poor degrader
10	D2S3	+++	E	Good degrader
11	AS-A	++	E	Intermediate
12	AS-P	+	E	Poor degrader
13	AS-D	+	B	Poor degrader
14	AW	+	M	Poor degrader
15	My-A	++	E	Intermediate
16	My-P	+	E	Poor degrader
17	My-C	++	B	Intermediate
18	My-W	++	P	Intermediate
19	JS-A	+++	P	Good degraders
20	JW	+	P	Poor degraders
21	JS-B	+++	M	Good degraders

22	JS-C	+	B	Poor degraders
23	JYS	+++	E	Good degraders
24	JYW	+++	B	Good degraders
25	Riverside 2 (RSo)	+++	E	Good degraders
26	D1S4	+++	E	Good degraders
27	SS-A	+	E	Poor degraders
28	A-OCS	++	E	Intermediate
29	OCW	++	E	Poor degraders
30	A-NOS	++	E	Intermediate

G1: Extraction of residual crude oil after microbial degradation in a liquid medium (broth