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IKENYI KENNETH CKIKE

TABLE OF CONTENTS

ABSTRACT

A laboratory experiment was carried out to determine the rate of biodegradation of slop oil from a petrochemical complex. The biodegradation of slop oil is accomplished by microbes which show great potential of growth in slop oil and their ability to utilize and degrade slop oil as sole source of carbon and energy was investigated. Acinetobacter sp, Pseudonas sp, Mucor Sp, and Aspergilus sp, were found to be efficient slop oil utilizers.

These microbes were discovered to be efficient biodegraders of slop oil.

Mixed isolates of these microbes gave reasonable degradation rate of 82%. However, individual isolates of these microbes (Acinetobacter sp, Pseudomonas sp, Mucor sp, and Aspergillus sp.) gave percentage degradation rate of 67, 69 and 70 respectively. Microbial isolates of slop oil utilizers have been found to be potential slop oil degraders with maximum degradation rate attained when the isolates are mixed.

CHAPTER ONE

1.0 INTRODUCTION

Petroleum production is the major source of income and support for the Nigerian economy. She generates a lot of money from crude oil. This is because of its demand as a source of energy which has since its discovery been increasing. Petroleum provides about 90% of the export earning and serves as a primary raw material for chemical industries (Petrochemical Industries). The high demand for petroleum / petrochemical products in the form of cooking gas, aviation fuel, gas oil, engine lubricating oil, asphalt and coal tar means increase in production and this eventually result in oil pollution and hydrocarbon contamination, mostly through oil well blowout, tanker accidents, accidental rupture of pipelines and specifically industrial waste oil sludge (slop oil)

The oil pollution is a detrimental problem in our environment in recent times. Biodegradation of waste oil is accomplished by petroleum degrading microbes that are isolated from oil, marine and fresh water sediment and pipelines transporting the oil (Odu, 1978; Pinholt et al, 1979, Tagger et al, 1983, Ijah and Ukpe, 1992). However, the presence of petroleum degraders is not restricted to a particular ecosystem. Any medium with hydrocarbon as a source of carbon and energy can enhance the growth of petroleum degraders (Amadi, 1988).

The environmentally acceptable disposal of slop oil and other waste hydrocarbons pose an important contemporary challenge to the petroleum/ Petrochemical industry. Considering this tendency, a promising and relatively low – cost option recommended is the use of oil as a biological incinerator (Dibble and Bartha, 1979). The rehabilitation of the waste oil polluted areas with concentrated bacterial slurry of high potential petroleum degraders may depend on other environmental parameters such as the average temperature, pH and nutrients (Antai, 1990).

Biodegradation could perhaps be accelerated by addition of nutrients. Methods involving increase of soil microbial degraders' biomass include the

need for limming and adding of mineral fertilizers as a source of nitrogen and phosphorus (Dibble and Bartha, 1979; Atlas, 1981; Ijah and Antai, 1988). This process may however be economically impracticable in a developing country like Nigeria where inorganic fertilizers are expensive and even insufficient for use in agriculture let alone on oil pollution. An unconventional alternative that is cheap is the search for the use of chicken (droppings) manure in stipulating oil biodegradation in the soil; this is suggested recently by Amadi and Antai (1991b). Poultry manure which is not a new farm waste product has long been used as organic fertilizers in agriculture because of its high nitrogen, phosphorus and salt contents (Tiney and Nowakowski, 1959). However, these advantages have not been utilized in the area of slop oil biodegradation. Investigations have also proven that chicken dropping contain a wide range of microbial flora that include known bacterial flora and fungal species implicated in biodegradation of cellulose and hydrocarbons (Amadi and Antai, 1991).

These double functions of chicken manure in both agricultural and petroleum industries could be used in solving some of the environmental problems affecting the slow rate of microbial oil degradation and total rehabilitation of soil for farming after spill. Biodegradation of slop oil is achieved by microbial activities that are beneficial oriented which degrade waste disposed by petroleum/ petrochemical industries.

However, the word petroleum was formed from two Latin words vis petra and oleum which symbolizes rock oil. Petroleum is used synonymously with the terms crude oil and mineral oil, meanwhile, there are many other types of oil not of fossil origin and are quite distinct from petroleum (Clark Jr. and Brown, 1977). According to Atlas and Bartha, (1973), petroleum differs in chemical compositions, viscosity, colour, specific gravity and other physical properties depending on the source. The colour of petroleum varies from light yellow brown to black and the viscosity varies from water-like to almost entirely solid.

Specific gravity and viscosity are the two major parameters for classifying petroleum. Most petroleum specific gravity lies between 0.735

and 0.950 (Atlas and Bartha, 1972). Specificity for instance, Nigeria crude (Bonny light) petroleum has a viscosity of 3.59Cst at 38^{o}C and specific gravity of 0.844 at 16^{o}C while Bonny medium petroleum has a viscosity of 14.1cst and a specific gravity of 0.0907 at 38^{o}C and 15^{o}C respectively (CONCAWE, 1972). The viscosity of petroleum, besides relate to its chemical composition (molecular weight configuration) is a function of temperature; as rises the viscosity decreases and the oil flows more freely.

Petroleum has been accepted as being organic origin, and conditions for the origin and evolution of oil has been established. Petroleum is being formed from partial decomposition of plant and animal remains over a long period of time termed “geologic time”. As at the time of early history the ocean had alternatively covered and receded from land areas, at the same time, animals and plant debris were carried to and deposited in the seas. The abundant animal and plants of the sea have contributed in no small measure to the accumulation of sedimentary deposits; it is believed that this deposits make up the raw materials for the natural production of petroleum (Clark Jr. and Brown, 1977).

However, many theories supporting an inorganic petroleum source have been propounded at different times, most of, or all of these theories conflict in one way or the other with geological proofs. The major source of materials for oil is the plant and animal remains. Some crude oil contain humin and chlorphyl porphyrins, optically active compounds and nitrogen - sulphur derivatives of hydrocarbons (petrov. 1984). These are the convincing evidence of the source of crude oil. Crude oil is a dynamic product of fossils and is a naturally complex mixture of organic compounds having mainly hydrocarbons (Smith et al, 1985).

The history of the origin of crude oil has been further illustrated in recent years. According to Bossert and Bartha(1984), crude oil originated from dead aquatic and organic organisms and materials that their remains gradually deposit at the bottom of the sea or land covered by materials like mud, salt and numerous other sediments over time. The increase in sedimentary pile up resulted in increase in pressure with resultant

transformation of sediment organic materials and aquatic organisms into hard sedimentary rock. During this process of transformation, bacterial activity with resultant heat and pressure change the plant and animals remains into crude oil.

Newly formed oil resulting from the aforementioned process flows to other areas, an oil trap is formed and the sips upwards until it come in contact with a non-porous layer of rock where it then settles. The period of crude oil formation is yet known; however, geologists have over the years given different number of millions of years as the duration.

1.1 JUSTIFICATION

The study is carried out in order to know the rate of biodegradation of slop oil by microbes, also for the determination of microbes that utilizes slope oil as survival medium.

1.2 AIMS AND OBJECTIVES OF THE WORK

This project work is aimed at studying the biodegradation of slop oil from a petrochemical complex. Specific objectives include:

i. To isolate micro organism associated with the utilization of slop oil as their sole carbon and energy source.
ii. To characterize the microbes isolated in (i) above.
iii. To utilize the isolated and characterized isolate either individually or mixed (Mixed pairwise) for the determination of their biodegradation rate of slop oil.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 NATURE AND COMPOSITION OF SLOP SOIL

Slop oil is very complex mixture of substances that cannot be seprated completely by conventional analystical means (CONCAWE, 1981). Slop oil contain smaller amount of oxygen (Atlas and Bartha 1973). Slop oil possess a viscosity of about 3.59cst at 38^{o}C and specific gravity of 0.8444 at 15oC (CONCAWE, 1981). The viscosity of petroleum relates to its chemical composition (molecular weight and configuration) which is a function of temperature, as the temperature increases the viscosity decreases resulting to increased flow of the oil (CONCAWE, 1981).

McGill et al (1981) has calculated the total percentage of hydrogen slop oil to be 76% by weight, but general overview of the percentage composition of the individual component after breaking are not specific.

However, waste oil from petrochemical industry contains at least 240 hydrocarbon components of which 54% are from C3 to C11 and the rest from C12 to C25. Slop oil is a complex mixture of aliphatic, alicyclic and aromatic hydrocarbons co-existing with non hydrocarbon material, although small in amount and collectively designated as resins, Mc Gill et al, (1981).

Table 1.0 – composition of Slop Oil

Component	Percentage by weight
Nickel wppm	5.0
Vanadium wppm	1.1
Conranson	3.6
Asphatane	4.4
Nitrogen wppm	2.0
Hydrocarbon	69.4
Sulphur	0.15

Source: Mc Gill et al (1981)
W.p.p.m – Water part per million.

HYDROCARBON COMPONENT OF SLOP OIL

Petroleum comprises of a variety of hydrocarbons displaying a wide range of physical characteristics as well as an appreciable diversity in molecular structure (Perry, 1979). Hydrocarbons are compounds of hydrogen and carbon; they fall into three classes of alicyclic and aromatic hydrocarbon.

Aliphatic Component of Hydrocarbon

The aliphatic hydrocarbons are open chain compounds, their carbon atoms can be arranged in lines either in straight or branched forms. They could be saturated or unsaturated (Atlas and Bartha 1973). The saturated open chain hydrocarbon are referred to as paraffins or alkaline and have the empirical formula CnH2n+ 2. The alkanes can also exist as gaseous (C1 to C4 atoms) and solids.

The unsaturated hydrocarbons however, consists of those containing one double bound (olefins) one triple bond (acetylenes) two double bonds (diolefins), more than two double or triple bonds or both (alkatrienes), alkadynes and alkadienynes (Atlas and Bartha, 1973).

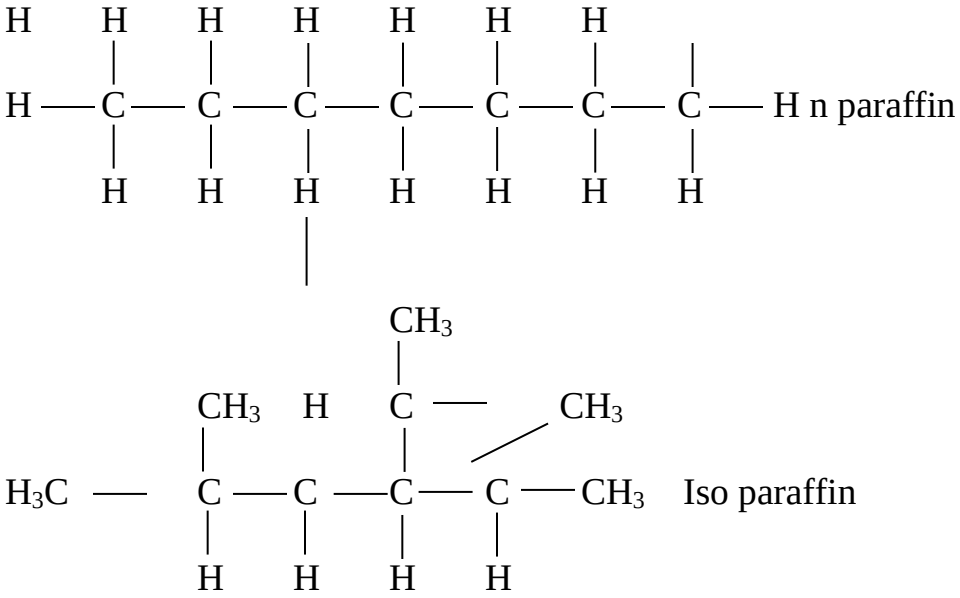


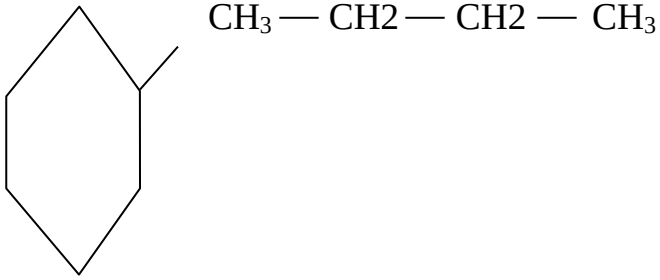
Fig. 1. Examples of aliphatic hydrocarbons

Alicyclic Components (Hydrocarbons)

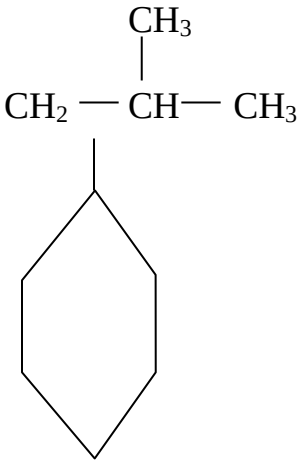
The alicyclic hydrocarbons are made up of carbon atoms arranged in rings. They exist in both saturated and unsaturated forms with the saturated from

dominating. They are highly resistant to microbial degradation. The saturated forms are monocyclic in nature and are referred to as cyclo-alkanes (CnH2n). the unsaturated forms are called cycloalkenes, or cycloefins (CnH2n-2) (Clark and Brown, 1977).

Most of the compound formed here are fairly stable, resist oxidation and are relatively insoluble in strong sulphuric acid (Clark and Brown, 1977).



Alkyl cyclohexane

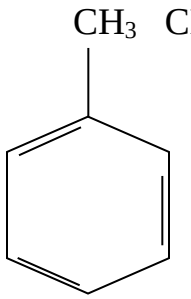


Alkyl cyclopentane

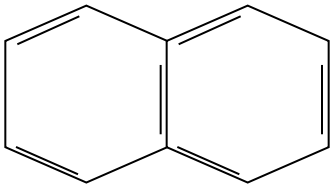
(b) examples of alicyclic hydrocarbons

Aromatic Component (Hydrocarbon)

The aromatic hydrocarbon consists of at least one benzene ring. They normally occur as single or multiple ring compounds with various alkyl substituents. They are all cyclic compounds. The simplest aromatic hydrocarbon is benzene which has 6 carbon atoms ring. The paraffin and naphthalene portion of petroleum are usually higher than the aromatic portion. The aromatics such as boluene and rylene, Polynuclear aromatic components are also considered to be part of aromatic hydrocarbons found in slop oil. (Harrison, 1975).



Alkyl benzene



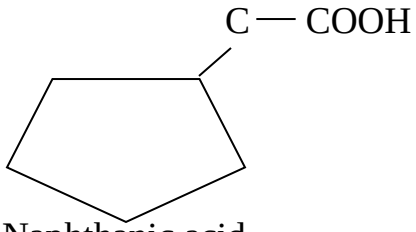
Alkyl Naphthalen

(c) Examples of aromatic hydrocarbons

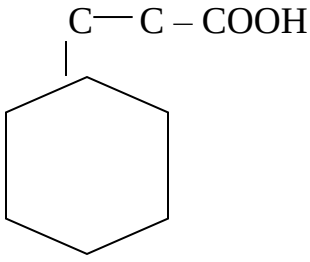
Non hydrocarbon Components of slop oil

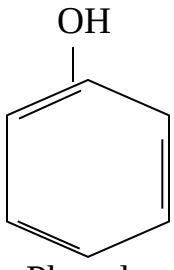
The major non-hydrocarbon components of slop oil are organic compounds containing nitrogen sulphur, oxygen and trace metals.

i. Nitrogen containing compounds includes pyridine, quinolines, pyroles, indoles, bennols etc.
ii. Sulphur containing compounds includes alkylthiols, thiophenes, cyclo-alkylthiols, hydrogen sulphide and elemental sulphur
iii. Oxygen containing compounds includes phenols, carboxylin acid, naphthenic acids, ketones, esters, lactones etc.
iv. Organo metallic compounds which include complexes of organically bound metals such as nickel, vanadium and cobalt.



Naphthanic acid



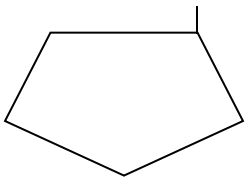


Phenol

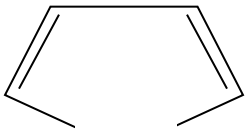
Examples of oxygen containing compounds

On the other hand the following are sulphur containg compounds

CH3 — CH2 — CH2 — CH2 — SH
Butyl mercaptane alkythiol

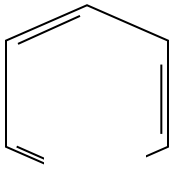


Cyclo alkylthiol

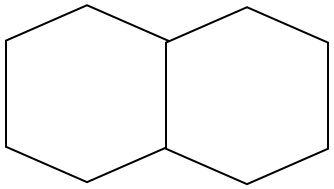


S

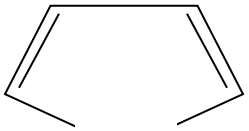
Nitrogen containing compounds includes



N
Pyridine



Quinoline



N
Pyrole

These are all examples of non-hydrocarbon component of petroleum.

2.2 SOURCES OF OIL POLLUTION IN THE ENVIRONMENT

Petroleum hydrocarbon (oil) may be introduced to the environment from a variety of sources thereby causing pollution, both natural anthropogenic, and by an extensive range of mechanism. The total quantity of such compounds entering the environment annually has been estimated in millions of tones (NRC, 1986). This annual estimate gives a rough global picture of the scale of the hazard of oil pollution to the environment. However, they are of much less value in providing a realistic impression of the devastating effect of a major oil spill on a coastline or within a estuary, or of the rather different problems associated with chronic low level discharges (Menzie, 1982). Global estimates also generally include the contribution of natural seeps to the pollution load of the environment (oceans). Natural seeps are certainly outside the United Nations (U.N) definition of marine pollution because they are not man-made; however, there is no doubt that crude oil input s to the seas are important whether their source. They are or should therefore

presumably be included in any general discussion of oil pollution (Reed and Kaplan, 1984; Higging et al, 1978; Gunkel, 1980).

Global estimate are also generally deficient because it fails to take into consideration the recent biological sources of oil (hydrocarbons). These are of great magnitude of about 2.6 X 10^7t a^{-1} (Kornberg, 1981). These estimate are of concern because on occasion a perfectly natural process may be misinterpreted as being a pollution incident, they therefore do not present a pollution threat.

There can be no doubt that whatever the source, petroleum hydrocarbons do present a pollution threat to the oceans, and because the nature and extent of the threat which they give frequently depends on the nature of the source, I shall list and give a brief description of these sources.

a) Off-shore operation

The pollution hazards from offshore operations are generally dependent upon the stage of development or exploitation of the oil field. Such hazards may be divided into the following: (i) hazards arising from drilling operations (ii) those arising from accidents and (iii) hazards arising from routine production (Gunkel, 1980).

The hazards arising from drilling operations results during the process of drilling in which case the drilling mud is not disposed thereby causing the inability of meeting the physical or chemical requirement of the drilling operation with a consequential pollution of the environment, this could as well be enhanced by rig –fouling organisms and additives to the drilling muds. (Komberg, 1981)

On the other hand, hazards arising from accidents results from blow out which is the uncontrolled release of gas and /or oil from a well, also pipeline damage causes pollution which occurs as a result of damage to under-water and underground surface pipelines. This could arise either naturally as a result of scouring sediments by bottom currents, or by marine and terrestrial activities of man like travelling. Finally this note, routine production posses hazards, once the initial drilling stage is completed and production begins a new range of environmental hazards becomes real. These are greatly associated with discharges of oily water which has been utilized either as production water or displacement water.

b) Shipping

On this aspect of oil pollution, the major hazards are caused by tankers shipping methods other than tankers. According to Wardley Smith

(1979) the pollution hazard from crude oil carrying tankers, derives primarily from the impossibility of completely discharging a cargo at the receiving port. The average efficiency of discharge falls in the range of about 99.7% of the total cargo carried. For a large tanker some 1000 tonnes of "clingage" may thus remain in the bank plus an additional quantity which is retained in pipes, pumps etc. The inability of the complete discharge and subsequent residual oils constitute pollution to later (next) cargo, also they constitute what is termed dead weight (Wardley Smith 1979). Shipping, other than tankers, constitutes hazard or pollution. Oily wastes can arise from a wide range of variety of sources which the engine room is the most important. The oil that accumulates in the bilges and machinery spaces is normally a mixture of lubricating and fuel oils. Occasionally, bunker tanks may be used to hold ballast water on dry cargo carriers, thus creating handling problems akin to those encountered with ballast water. There are no international requirements relating to the discharge of contaminated bilge water to the sea; though for ships using bunker tanks for ballast oil – water separators are required. The net result is that considerable quantities of oil may be discharged from ordinary vessels to oceans.

According to NRC (1985), the magnitude of this contribution to oil pollution has recently been estimated to be $3.2 \times 10^5 t\ a^{-1}$ with a minor additional contribution of some $2 \times 10^4 t\ a^{-1}$ from non-tanker accidents.

c) Land-based source

According to Bossert and Bartha (1984), land – based sources of oil pollution normally represents low level, chronic pollution hazards to the environment but may be more disastrous in the terrestrial environment, these may derive from a number of entirely different processes. They include (i) effluent from oil refineries (ii) oil parts and bulk oil handling facilities like petrochemical industries (iii) industrial and municipal waste. All these to a great extent constitute oil pollution of the environment in every different ways.

d) Miscellaneous Source

Here the atmosphere plays role in that when an oil spillage occurs the higher volatile components of the oil diffuses into the atmosphere and cause pollution. According to Duce and Gagosian, (1982), various estimates have been given of the quality of oil reaching the environment, via the atmosphere. Another source here is dumping; the dumping of crude fuel and lubricating oil substances cause pollution of the environment, since they are acted upon and reduced to harmful components by either terrestrial or

aquatic life. According, to NRC (1985), the amount of oil reaching the ocean in this way is estimated at or within the range $0.005 – 0.02 \times 10^6$ $ta^{-1,}$ the higher figure being considered to be the best estimate.

2.3 EFFECT OF OIL POLLUTION IN THE ENVIRONMENT

Effect of oil pollution on marine terrestrial life, occupation and health (environment) are of great importance. The toxicity of oil pollutants depends largely on the physiochemical properties of the type of oil in question and the quality of the environment to be polluted (Adams and Jackson, 1983). As earlier mentioned, oil differ in their composition of hydrocarbon and non hydrocarbon compounds. Oil from petrochemical gas processing and other refinery processes may be composed of polycyclic aromatic hydrocarbons, cyclo alkanes and other hydrocarbons. They may also be composed of mecaptans, cyanide, sulphide, iron oxide, soda and other heavy metals like mercury, lead, cadmium, arsenic, yhalium, nickel, selenium and aluminium. These are all industrial chemical pollutants that can cause hazardous effect in the environment when their concentrations are higher that the accepted standards set by national and international agencies (Borup M.B. et al, 1987).

2.3.1 EFFECT ON MICRO ORGANISMS

Micro organisms (bacteria) can actually thrive on oil polluted environment but their survival and subsequent activities are to some extent affected. The effects of oil pollution in microbial environment are of two folds. According to Baker, (1978), Walker et al (1973) and GESAMP (1977) the presence of oil pollution inhibits the growth of sensitive microbial species. This happens in the short term of pollution. These bacteria are important in maintaining the natural ecological balance of the environment, but affects are largely limited to the immediate vicinity of the pollution. In the longer term, the populations of petroleum degrading microbes develop and may result to overall increase in the microbial biomass of the zone. The effect may be temporary in the case of an isolated spillage or more permanent in areas susceptible to chronic pollution (Mironov, 1971; GESAMP, 1977).

In areas where oil pollution have occurred previously, further introductions of oil may acts as growth stimulating agents to existing petroleum – degrading bacterial (Walker and Colwell, 1975), thus altering the structure of microbial populations (Gunkel et al; 1980; and maki, 1991).

2.3.2 EFFECTS ON FISHES AND BIRDS

Fishes and birds are affected by oil pollution just like microbes are the fact they are vertebrates notwithstanding. In the case of fishes, their high mobility i.e. the result fishes and their powerful ability of detecting contaminants in their environment therefore suggests that they are normally capable of avoiding oil polluted waters. In general harmful effects results when fishes are trapped by behavioural or hydrographic conditions in the vicinity of an oil slick. In this case, severe fouling by oil may clog the gills, but this is a relatively unusual and localized occurrence. Exposure to water soluble toxic components may cause some fish deaths. This effect is however short – lived and again is restricted to the immediate vicinity of the slick. According to Blumer et al, (1970), Blumer and sass, (1972), some oil components (notably the PAHS(Polycyclic aromatic hydrocarbons) might be bio-accumulated and bi-magnified. Lee et al (1991) indicated that depuration of all organs is generally complete after 7 – 14 days provided there is no further exposure of oil. Sub-lethal contamination of fish by oil may cause problems with "tainting". According Hyland and Schneider, (1989), the human palate is quite sensitive to tainting and can frequently detect hydrocarbon contamination in the concentration range of 5-20 ppm. The sub-lethal contamination has been implicated as the cause of increased incidence of diseases of fish (Sindermann, 1979; GESAMP, 1982).According to Komberg, (1981) effect of oil contamination is much more detrimental on fish eggs and other relatively immobile juvenile forms and the adult mobile ones. Also, chronic exposure to low levels of petroleum hydrocarbons may have a pronounced effect on egg hatching rate and larval growth characteristics. This chronic exposure comes more from industrial out-falls, rather than with slicks (Middlebrooks et al, 1987).

On the other, marine birds are the animals most obviously vulnerable to oil pollution. In the wake of each significant oil spill numerous dead birds are seen washed ashore or covered in oil. The death of the bird as a result of oil pollution is at least accomplished in two ways. Oil collapses the circular structure of their feathers. Air pockets vital for the purposes of buoyancy and insulation are destroyed and birds may rapidly drown or if they accidentally reach shore, die of hypothermia. Secondly, oily birds attempts to clean their feathers by preening, and in so doing they ingest oil that causes a degree of poisoning that normally result in death. Oiling to even an extremely minute extent may lead to death of a bird when the effect is synergistically combined with the stresses imposed by severe environmental conditions (Levy, 1980). According to RPI et al,(1987), possessing of ingested oil, the prognosis for a bird, even after cleaning, is not usually good although some species may be more suitable for treatment than others.

2.4 MICRO ORGANISMS ASSOCIATED WITH THE BIODEGRADATION OF PETROLEUM HYDROCARBON.

Microorganisms associated with the degradation of petroleum hydrocarbon abound. These are those organisms which are capable of degrading hydrocarbon. These microbes are ubiquitously distributed in the marine, soil and fresh water habitats. According to Buckley et al, (1984), and Atlas (1981), the relative abundance of microbes vary considerably according to environmental conditions and hydrocarbons pollution history. In terms of their evolution, the occurrence of natural oil seeps over geological time periods has led, not surprising to the evolution of a variety of these hydrocarbon degrading microbes. These microbes are capable of degrading selected fractions of oil (hydrocarbon, usually n – alkanes) in a few days or weeks under suitable temperature range, abundant supply of oxygen, nitrogen and phosphorus (Walker et al, 1975, GESAMP 1977). Is particularly, important to recognize that no single microbial specie is able to degrade all of the constituents of oil. It is therefore in the long term that the effect of oil spill will be determined as a result of a wide interaction between much microbial specie (Austin et al 1976).

These micro organisms being talked about actualize their objective based on the principle of utilizing hydrocarbon as their sole energy and carbon source. These microbes are listed on the table below:

Table 2.0 Petroleum hydrocarbon degrading micro organisms

Fungi	**Bacteria**	**Algae**
Aspergillus sp.	*Pseudomonas sp.*	*Prototheca*
Aureobasidium sp.	*Achromobacter sp.*	*Cryptococus*
Candida sp.	*Acinetobacter sp.*	*Rhodotorula*
Cephalasporium sp	*Actinomyces sp*	*Trichosporon*
Cladosporium sp.	*Aeromonas sp*	*Hensenula*
Cumingghamella sp	*Alcaligens sp*	
Hensenula	*Athrobacter sp*	
Rhodosporidium sp.	*Bacillus sp*	
Rhodotorula sp	*Bacterium sp*	
Saccharomyces sp	*Corynebacterium sp*	
Torelopsis sp	*Flavobacterium sp*	
Trichosporon sp	*Microco ccus sp.*	
	Mycobacterium sp	
	Nocardia sp.	

	Pseudobacterium sp *Sarcina sp.* *Vibrio sp*	

Source Atlas 1981, Venkateswaran et al. 1991.

Although there are many ways of eliminating spilled oil from the environment, biodegradation using micro – organism is a major method (Colwell, 1976, Atlas, 1981). The major organism mediating the degradation of hydrocarbons by plasmids borne genes are well documented (Walker et al, 1975; Singer and Finnety, 1984a).

According to Okpokwasili et al, (1988) bacteria and fungi have similar patterns of hydrocarbon degradation. The evolution of the hydrocarbon mixtures depends on the nature of the microbial community and on the variety of the environmental factors which influences microbial activities among which are physical state of the soil (environment) pH, temperature, oxgen and mineral nutrients.

2.5 BIODEGRADATION OF PETROLEUM HYDROCARBON

Microbial degradation of oil is a process whereby oil is reduced to asphatic or tar residues and plays a major role in weathering process (Atlas, 1981). The biologically and chemically induced changes in composition of a pollution petroleum hydrocarbon mixture are referred to as weathering. The degradation process is as a result of microbial growth. The presence of hydrocarbons cause the inevitable growth of those types of bacteria and fungi which are most efficient in utilizing the petroleum hydrocarbons for carbon and energy source and are able to grow under the environmental conditions created by the addition of oily wastes (Atlas, 1981). The biodegradation process of petroleum hydrocarbon in nature is an intricate interaction between a chemically complex substrate and mixed microbial populations, which are genetically and biochemically adapted for this process (Davis et al, 1967).

Under laboratory conditions it is relatively easy to demonstrate biological degradation of oil. Given a suitable temperature (25 – 37^{o}C) and abundant supplies of nitrogen, phosphorus and oxygen, many bacteria can degrade selected fractions of oil (usually n-alkanes) in a few days or weeks (Walker et al, 1975; GESAMP, 1977). In the ocean for instance, environmental condition are normally for from ideal for the growth of oil degrading bacteria. Low temperatures and lack of enough nutrients are particularly important inhibitors of bacterial attack. Degradation of marine oil spill is therefore very slow, and even potentially

biodegradable compounds may persist for years (Morgan, 1990; Menzie, 1982). From this it could be said that petroleum hydrocarbon biodegradation in the aquatic environment is very gradual process that lingers before degradation is accomplished.

On general perspective, and according to Atlas, (1981), Leahy and Colwell, (1990), biodegradation of petroleum hydrocarbon may be enhanced by supplementation with fixed N, PO_4, O_2 and other nutrient or according to Fedorak and Westlake, (1981). Ismailov (1985), Brown (1987), and Von Wedel et al, (1988) by the direct argumentation with hydrocarbon – degrading micro organisms. When the petroleum hydrocarbon biodegradation is considered individually, n – alkanes are the most widely and readily utilized. Meanwhile, those with chain length between C_{10} and C_{25} are the most suitable as substrates for micro-organisms. Iso-alkanes are less effective as microbial substrate than n – alkanes , particularly if the branching is extensive or involves quaternary carbon atoms. Olefins are more toxic and less readily utilized than the corresponding alkanes. Low molecular weight aromatic hydrocarbons are quite toxic to microbes but are metabolized at low concentrations. Condense polynuclear hydrocarbon are less toxic to micro organisms than are low molecular weight aromatics, they are metabolized on rarely and slowly. Cycloalkanes are highly toxic ans severe, as growth substrate for micro organisms only in isolated, exceptional cases. Some are readily degraded however by the co-metabolic attack of mixed microbial communities.

The biodegradation mechanisms of different classes of petroleum hydrocarbon compounds are quite different but again a few general principles have been established.

i. The degradation of n – alkanes is generally initiated by a mono-terminal attack leading to formation of primary alcohol. Subsequent, reactions lead to the formation of aldehydes and monocarboxylic acids (Atlas, 1981). Subsequent degradation of the carboxylic and proceeds by oxidation under mediation of acetyl co-enzyme A with the subsequent formation of a two – carbon – unit shorter fatty acid and liberation of CO_2 i.e.

$$R-(CH_2)_2-CH_3 \xrightarrow{O_2} R-(CH_2)_2-CH_2OH \xrightarrow{O_2} R-(CH_2)_2-CHO \longrightarrow R-(CH_2)_2-COOH \longrightarrow R-COOH$$

ii. Methyl branching generally increases of hydrocarbons to microbial attack. However, whilst sure highly branched isoprenoid compounds as pristine are relatively stable, they have been shown on occasions to undergo oxidation with the predominant formation of dicarboxylic acids (Mckenna and Kallio, 1971; Pirnik et al, 19734; Pirnik, 1977; Atlas, 1981).

iii. Cyclohexanes are particularly resistant to microbial attack (Atlas, 1981) and complex cycoalkanes are amongst the most persistent components of oil spills in the environment (Atlas et al, 1981). Perry (1979 and 1984) reviewed the microbial metabolism of cyclic hydrocarbon and related compounds.

iv. Aromatic hydrocarbons are degraded by mechanism that involves ring cleavage with the formation of a diacid or dialcohol e.g. is given below in figure 2.0 (Ismailov, 1985).

v. Polynuclear aromatic structures are very resistant to microbial attack. Thus Lee and Ryan (1976) have shown that biodegradation rates for naphthalene are more than 10^3 times higher than those for the benzopyrenes which are as a consequence, very persistent.

vi. The rate of asphaltene degradation is so slow as to be insignificant (Bridie and Blumer et al, 1971). However, their toxicity to marine life is also very low (Atlas, 1981). Studies on the biodegradation reactions of asphaltenes have been somewhat limited by the inability of chemical analytical methodology to tackle these highly complex molecules.

vii. The most degradation resistant fraction of oils will tend to form relatively inert tar balls which, after their density have been increased by absorption or inorganic particulate material, will eventually sink and remain for long periods in the sediment in a largely unaltered state (McKenna et al, 1971).

Table 3.0 Biodegradation rate and toxicity hydrocarbon component of petroleum

Compound	Degradation rate	Toxicity
Linear alkanes		
C_5 - C_9	Rapid at low concentration	Toxic at low concentration
C_{10} – C_{22}	Rapid	Same
C_{23} – C_{44}	Moderate	Same
Branched Chain		
Alkanes	Slow – Moderate	Same
Alkene	Slow – Moderate	Same
Cyclic alkanes	Slow – Moderate	Same
Monoaromatics (B – TEX)	Rapid at low concentration	Toxic at low concentration
Polyaromatics		
Ring	Rapid	Toxic at low concentration
3 – 4 rings	Slow – Moderate	Same
>4 rings	Very slow	Same

Rapid ⟶ days/weeks
Moderate ⟶ weeks/months
Slow ⟶ months/years
Source Middle brooks and Borup (1987).

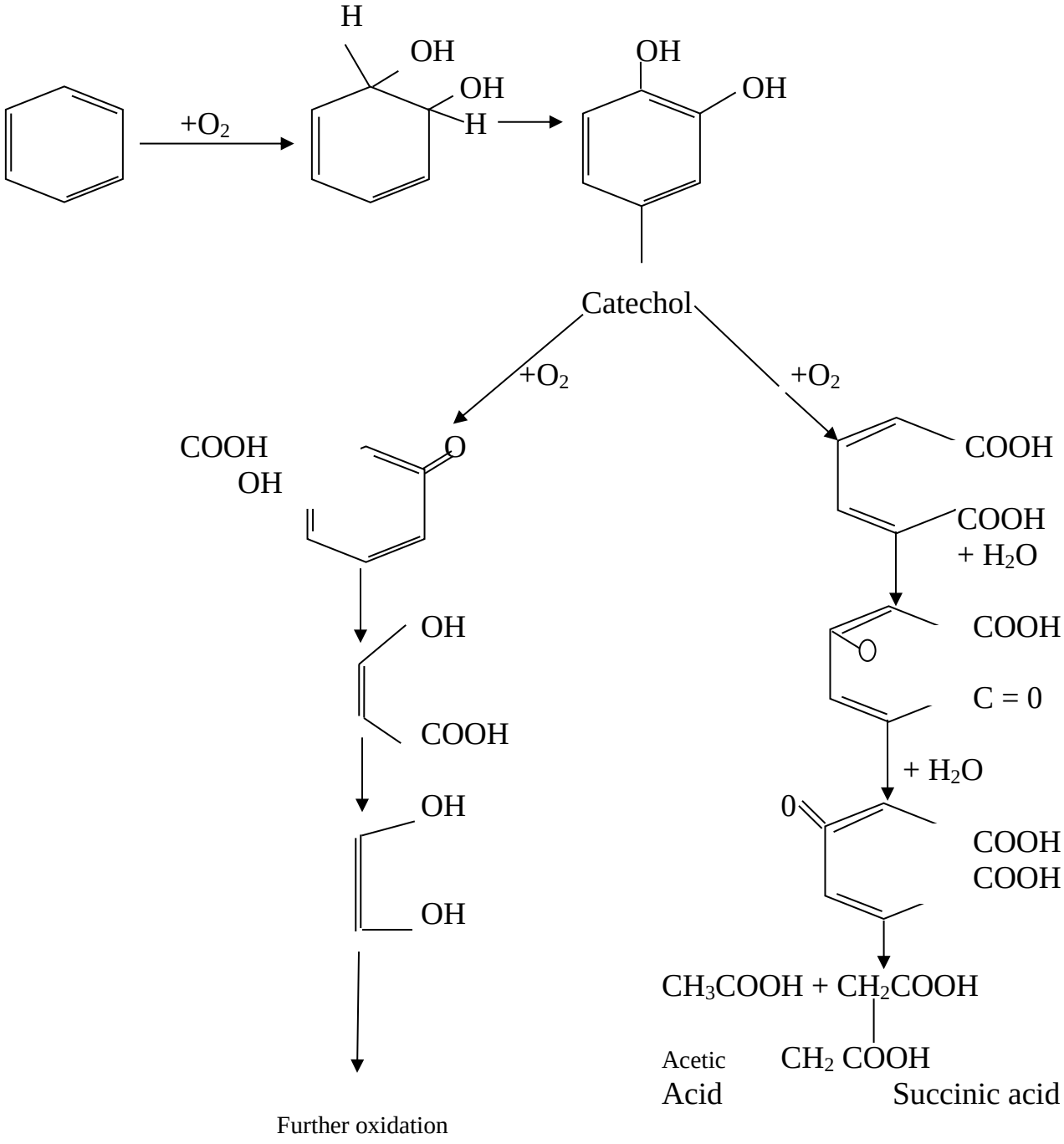


Fig. 2.0 Metabolic pathway for benzene degradation (Ismailov,1985)

Microbial biodegradation of petroleum hydrocarbon is usually initiated by production of bio-surfactants from particular species of bacteria. In reported cases,

micro organisms are found to be “clustered” in great numbers at the oil/water interface of a hydrocarbon layer. These surfactant molecules encapsulate a small quality of oil (an emulsification process), which is then transported across the bacterial cell wall and membrane (Harrison, 1975 and Hess et al, 1990). Once the oil has been internalized, the microbial degradation of hydrocarbon molecules can occur through two main route; straight chain compounds are first oxidized to alcohol, then dehydrogenated to form an aldehyde converted to carboxylic acid, and then transported into acetic acid. In contrast, two hydroxyl groups are added to aromatic molecules through a dioxygenase, in order to saturate a pair of adjacent carbons. Such a transformation then facilitates enzymatic cleavage of the delocalized annulus to yield β – ketoadipate or short- chain acids. Both acetic acids and adipate are then assimilated into the microbial TCA cycle for the production of energy in the form of ATP (Harrison, 1975, and Hess et al, 1990). Figure 3.0 is an illustration of the biodegradation of a petroleum hydrocarbon (n – alkane) extracted from the work of Watkinson and Morgan (1990). The genera of bacter involved in biodegradation of a variety of different classes of petroleum hydrocarbon molecules have been enumerated by Adams and Jackson (1989) and are listed below on table 4.0

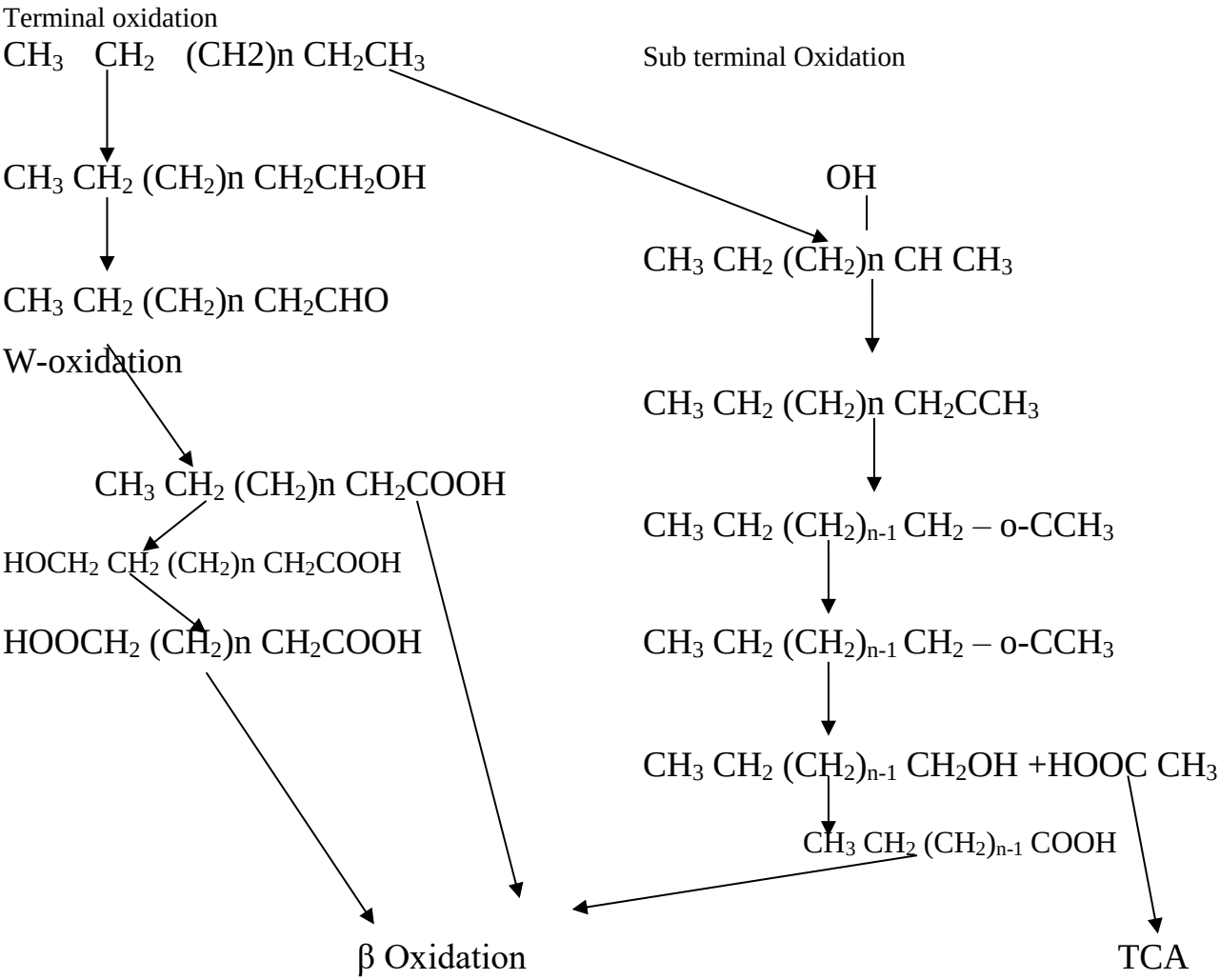


Figure 3.0 Basic metabolic path ways for the degradation of n – alkanes Showing the three main metabolic routes documented for micro organisms.
Source: Watkinson and Morgan, 1990.

Table 4.0 Bacteria that biodegrade variety of different classes of hydrocarbon petroleum

Alkanes	**Cycloalkanes**	**Aromatics**
Pseudomonas sp	*Achromabacter sp*	*Flavobacterium sp*
Acinetobacter sp.	*Norcadia sp*	*Achromobacter sp*
Bacillus sp	*Acetobacter sp*	*Pseudomonas sp*
Arthrobacter sp	*Alcaligens sp*	*Corynebacterium sp*
Mycobacterium sp.	*Pseudomonas sp*	*Nocardia sp*
Brevibacterium sp	*Mycobacterium sp.*	*Aerinibas sp.*

Source: Adams and Jackson (1989)

From table 4.0 and according to Davis (1967) and Ijah et al, (1992), it is evident that Pseudomonas sp are able to degrade all classes of petroleum hydrocarbons and has been noted as one of the best degraders.

Adams and Jackson; (1989) elucidated the fact that the biodegradation process or pathways for petroleum hydrocarbon, are catalyzed by specific enzymes being produced by specific microbes living symbiotically. So, for the complete biodegradation of petroleum hydrocarbon, a consortium of microbes must be present. Shailubhai et al (1985) observed that the composition of petroleum hydrocarbons determines the ease of microbial attack and also investigated and found out that *Rhodotorula rubra* and *Pseudomonas aeroginosa* degraded petroleum hydrocarbon. The order of biodegradation of various fractions decreased from saturated > aromatic > asphatic. Maki (1991) in the research work stated that petroleum hydrocarbon containing polycylic aromatic hydrocarbon can readily be destroyed under optimized conditions. Bossert et al, (1984) showed that microbially mediated mineralization and humification removed petroleum hydrocarbon reasonably and efficiently. Different studies on microbial utilization of petroleum hydrocarbons have established basic knowledge for research workers, to construct oil degrading bacteria clones by natural plasmid transfer and in vitro recombinant DNA technology. Chakrabarty et al (1973) and Brown, (1987), observed that the genetic methodology that involves transfer of natural plasnoids between bacterial, suggests for production of microbial strains for oil spill clean up tasks. Reports by researchers have shown that 50% of the carbon content of hydrocarbon metabolized by bacteria is changed in to biomass, the remaining will be reconverted into CO_2 and water or unattacked compounds. Certain group of bacteria may release low molecular compounds for example acetate and lactate, through incomplete oxidation of hydrocarbons. In fact, literature on this is inexhaustive.

2.6 FACTORS AFFECTING MICROBIAL DEGRADATION OF HYDROCARBON

Numerous factors affect the biodegradation of hydrocarbon, most of which are biotic factors that influence the weathering and subsequent degradation of the hydrocarbons. Dibble, (1978). According to Bartha, (1986), the most important factors that limit petroleum hydrocarbon biodegradation in various environment includes, the nature of the spilled oil, physical state of the oil, weathering water potential, temperature, oxygen, mineral nutrients, pH and the types of micro organisms present. It could therefore be said that apart from some natural tendencies influencing hydrocarbon biodegradation two main generalized factors are involved i.e. the biotic and abiotic factors. I shall discuss these factors briefly

a) **The nature of spilled oil** – In this case, the number of carbon constituting the hydrocarbon plays a role also, inhibitory substances presence in the hydrocarbon oil jointly affect the microbial biodegradation rate. For instance, kerosene that consist almost exclusively of medium chain length alkanes is under suitable conditions 100% biodegradable (Amadu and Antai, 1991). It is also observed that no crude oil is biodegraded quantitatively, and some heavy asphaltic naphthenic crude oils as little as % per gram may be observed within a reasonable time period (Bartha 1986). Some of the non-hydrocarbon components of crude oil are considerably toxic to micro organism and thereby hinder biodegradation.

   The difference in the biodegradation of the medium crude (gulf crude – desalter inlet) and light crude (transniger pipeline crude oil has been investigated (Ijah and Antai, 1988). They observed that by the use of mixed culture of *Pseudomonas* species and *Steptomyces viridosporus* T7A to degrade the two types of crude oil, there was a considerable higher level of biodegradation (47%) of light crude oil while the medium crude oil was lower (32.8%). Amadi and Antai, (1991) have also reported the effect of the boiling points of the hydrocarbons on biodegradation. C_{18} and C_{20} were degraded while C_{16} showed no changes at all by the microbial test species. The physical state of which favours the aggregation, spreading, dispersion, adsorption and weathering (evaporation and photo-oxidation) are also investigated and seen to increase biodegradation (Bartha, 1986; Okpokwasili, 1987).

b) **Temperature** – This has a significant influence on the rate of all biochemical process and of hydrocarbon. Numerous reports have classified

the biodegradation process to take place under conditions that range from thermophilic to psychrophilic (Bartha and Atlas 1977). Bacteria growth on alkanes at temperature up to 70^{o}C was observed by Keuth (1991) and Markowetz (1967). The effect of temperature on biodegradation of hydrocarbon depends solely on the effect of temperature on the microbes. The most suitable temperature range was found to be at 30 – 40^{o} C (Okpokwasiliu, 1997), Schwartze et al (1973). However, very low temperature has been investigated to disrupt, microbial activity, but it has been reported that hydrocarbon Biodegradation can take place at temperature as low as – 1.1^{o}C in the soil and at 5^{o}C in seawater (Atlas and Bartha, 1972). With related example from Nigeria which has two diverse seasons, it is observed that the highest biodegradation rates occurred above 20^{o}C. With maximum rate 36^{o}C (Atlas, 1990).

In general, since temperature affects microbial activity, it then follows that for every temperature rise 10^{o}C, there is a proportional increase in enzymatic activity of these microbes with subsequent increase in biodegradation of hydrocarbon till a peak is reached where increase in temperature affect enzyme activity of the microbe hence reduce microbial degradation of the hydrocarbon.

c) **Oxygen –** Hydrocarbon are reduced organic substrates that can be metabolized only to an oxidative manner. Therefore, the availability of molecular oxygen is considered to be an important limiting factor of hydrocarbon biodegradation by microbes. There has been a lot of controversy on weather oxygen is really needed for hydrocarbon biodegradation or whether anaerobic oxidation with nitrare or sulphate serving as election sinks. This has been demonstrated in the laboratory but knowledge gained so far has shown that from practical point of view this type of biodegradation has a negligible effect on oil pollutants (Davis, 1967; Friede et al, 1972).

d) **Nutrient** – Some beneficial compounds that affect and promote growth and other activities of micro organisms are mineral nutrients. Refined hydrocarbons supply only carbon and energy to degrading micro organisms but, crude petroleum contain little amount of sulphur, nitrogen and heavy metals, while in the natural environments of bacterial, mineral salt, carbondioxide and water are always present (Calominis et al, 1976). The quality of nitrogen and phosphorus, often limits the growth of hydrocarbon utilizers in water or in soils. Laboratory studies and field experimentation

have provided substantial evidence on the relevance of nutrient in crude oil degradation; this is same with hydrocarbon degradation by microbes (Jannasch and Wirsen, 1973, Tagger et al 1983, Antai and Ijah, 1990, Antai, 1991).

e) **Hydrogen ion concentration** – The slightly alkaline pH of seawater is observed to be quite favourable for petroleum hydrocarbon biodegradation, but in acid soil limming to pH 7.8 to 8.0 had a definite stimulating effect (Ward and Brook, 1978). The soil pH determines the type of micro organism participating in petroleum biodegradation.
Report from Amadi and Antai (1991) showed that microbial growth on hydrocarbon varies at different pH levels. Low pH i.e. 4.0 was observed to be more favourable for fungi whoile bacteria degrade maximally at a pH of 10.0. However, the growth rate for all isolates were found to vary within 5% to 33% at pH 10.0 and 33% to 66% at pH 7.0

f) **Moisture –** Similar to all life processes requiring moisture, the microbial degradation of hydrogen may not occur in water absence, since microbes carry out metabolic and enzymatic processes in presence of water. Therefore, the moisture content of an environment of microbes is essential in all activities. It is reported that there is a great difference in the rate of microbial activities of degraders in water saturated areas than in dry environment (Berridge et al, 1968, Bossert and Bartha, 1984). This is also seen in the rapid microbial degradation of hydrocarbon in water than in soil.

g) **Salinity and Pressure** – There is a realization that high hydrostatic pressures in combination with temperature drastically reduce the rate of microbial metabolism (Jannasch and Wiresen, 1973). Stimulated interest in hydrocarbon biodegradation under high pressure conditions is on the increase this day.

Schwartze et al, (1963), on examing the growth and utilization of hydrocarbons at ambient and in situ pressure for deep sea bacteria, found that the rate of hydrocarbons utilization under pressure and ambient temperatures was significantly less than the rates under conditions of ambient temperature and atmospheric pressure. Whereas about 94% hexadecane was utilized within 8 weeks at 1 bar, at 500 bars it took 40 weeks for similar degradation. They included that petroleum would be extremely slow.

Ward and Brook (1978), reported that the rates of metabolism of certain hydrocarbons in hypersaline seas increased as the salinity of the ecosystem

increased. Amadi and Antai (1991), in laboratory studies observed optimal biodegradation with increasing salt concentration of the basal medium.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 STUDY AREA

The study of the biodegradation of slop oil from Eleme Petrochemical complex was carried out in microbiology Laboratory University of Calabar, Cross River State, Nigeria.

3.2 SAMPLE COLLECTION

Slop oil sample used in this study was obtained from Eleme Petrochemical Company Limited Port Harcourt Rivers State, Nigeria. The slop was collected in a clean sterile plastic container at the effluent point of the company.

After collection, the sample oil was placed in a cool box and transported to the laboratory for immediate analysis.

3.3 MEDIA AND REAGENTS

3.3.1 Media Used

The following media were used during the course of this work, nutrient agar, nutrient broth, malt extract agar, mineral salt medium and mineral salt broth.

3.3.2 Reagents Used

The reagents used were grams reagent, oxidase reagent, catalase reagent, lactose phenol cotton blue, etc. The recipe for the preparation of these reagents and media are in appendix column.

3.4 PROCESSING THE SPECIMEN

The specimens were processed using microbiological analysis techniques.

3.4.1 MICROBIOLOGICAL ANALYSIS

3.4.1.1 Enumeration and Isolation of Crude oil degrading bacteria

The method as cited Raymond et al (1977) was used. Crude oil degrading bacteria in the slop oil sample were isolated and enumerated using a surface spread techniques. Serial dilution of the slop oil sample was prepared from 10^{-1} to 10^{-8} and 0.1ml of 10^{-4} dilution was plated onto mineral salt agar containing 58µg/ml of Nystatin (an anti fungal agent) to inhibit fungal growth. Sterile Whatman number 1 filter paper was saturated in 0.5ml of crude oil and placed aseptically onto the lid of the plates and was taped round with masking tape. This was done to provide the crude oil as a sole source of carbon and energy for the growth of the organism on

the surface through vapour pressure phase transfer. The plates were prepared in duplicates, inverted and incubated at 37˚C for 7 days before the enumeration.

3.4.1.2 Enumeration and Isolation of Crude oil Degrading Fungi

The method of Raymond et al, (1977) was as well used. Crude oil degrading fungi in the slop oil sample were isolated and enumerated using a surface spread techniques. Serial dilution of slop oil sample was prepared from 10^{-1} to 10^{-8} and 0.1ml of 10^{-4} dilution was plated onto mineral salt agar containing 15mglml of penicillin and 100mg/ml of streptomycin (an anti bacterial agent) to inhibit bacterial growth. Sterile Whatman No. 1 filter paper saturated with 0.5ml of crude oil and placed aseptically onto the lid of the plate and taped round with masking tape. This again was done to provide the crude oil as a sole source of carbon and energy for the growth of the organisms on the surface through vapour pressure phase transfer. The plate were prepared in duplicates, inverted and incubated at room temperature (28˚C)for 7 days before enumeration.

3.4.1.3 Purification and Maintenance of Microbial Isolates

The bacteria and fungi isolate obtained from mineral salt agar plates were purified by repeated sub culturing (three times). Isolates were subjected to a series of transfer, bacterial isolate into fresh medium (nutrient agar), and fungi into malt extract agar and incubated at 37˚C for 24 hours and at 28˚C for 3 days respectively. Pure colonies of bacteria and fungi were maintained on slope of nutrient agar and malt extract agar slants respectively and stored in a refrigerator at 8˚C until when needed.

3.4.1.4 Biochemical tests and Identification of Microbial Isolates

The biochemical tests and identification of microbial isolates were based on microscopic techniques and biochemical test as described by Cowam &Steel (1974), fungi isolates were characterized using techniques as described by Cruckshank and Co. (1975).

3.4.1.5 Screen test for the utilization of slop oil by microbial isolates

The ability of the microbial isolates to utilize slop oil as a source of carbon and energy was determined according to the method of Okpokwasili and Okarie, (1988), using sterile mineralsalt ajic and supplison, (1972). The medium in 9.9ml medium amounts was contained in test tubes; 0.1ml of slop oil was added to each tube. After capping, all the tubes were sterilized by autoclaving at 121˚C for 15 mins and allowed to cool. Upon cooling, each set of tubes numbered was inoculated with 0.1ml (3.8×10^4 cells) of a 24 hours old nutrient broth grown

bacteria or fungal culture as the case may be. The experiment was repeated but instead unused slop oil was incorporated in the second set of medium. Two control tubes (one containing crude oil and the other and the other slop oil) remained un-inoculated. All the tubes were incubated without shaking at room temperature for 16 days. At 4 days intervals the culture were observed either visually or by colimetric reading with absorbance of 520nM for turbidity (as evidence of microbial growth). The growth density was scored at high (+++); moderate (+ +) and minimal (+) or no growth (-). To confirm the results each microbial culture was compared with the uninoculated control.

3.5 DETERMINATION OF THE BIODEGRADATION RATE SLOP OIL BY MICRO ORGANISMS

3.5.1 Bacterial Isolates

The result of the screen test for utilization of slop oil was used. Isolates that showed maximum turbidity from the screen test result were used.

A 24 hour old nutrient broth culture of these bacterial isolates was made. 9.9ml of mineral salt broth medium was dispensed into sterile test tubes and 0.1ml of the slop oil was transferred into each of the test tubes sterilized by autoclaving at 121˚C for 15 minutes. The test tubes were allowed to cool before inoculation of 1ml of the 24 hours bacterial culture contained 3.8×10^4 cfu/ml into different tubes.

A control experiment with no inoculums was also set up for each isolates and all the tubes were incubated at room temperature for 16 days in a shaking incubator. At the end of the incubation period, the tubes were analysed for each isolates ability to degrade slop oil by extracting the residual oil with n – Hexane and the absorbance, determined using spectro photometric method as decribed by Parsons et al, (1984).

Standard solution of oil n – hexane were prepared in the range of 0.45mg – 4.62mg/ml. the analytical wavelength was selected at 520nM. From the absorption spectrum of the oil in n – hexane, the corresponding curve prepared from this, the curve is shown in appendix section.

Calibration constant β, was determined as the mean of the quotient of standard concentrations to their respective absorbance.

That is $B = \frac{\sum c/A}{n}$

Where C= concentration of standard

A= Absorbance of standard

N= the total number of standard

∑= Summation constant

The concentration was then repeated in relation to a sample volume. In carrying out extraction, separating funnel was clamped to a retort stand funnel filled with known volume of the sample, and known volume of n – hexane was introduced into the sample, the cork was replaced and the separating funnel was shaken vigorously. The built up gas from the funnel was discharged by releasing the tap, shaking was done continuously for 5 to 10 minutes and the funnel cap was opened, clarified and allowed to settle for about 5 to 8 minutes and separation of n – hexane extracted from the water.

Absorbance reading was carried out 520nM with spectrophotometer. A known volume of n – hexane extracted was used for this.

3.5.2 Fungal Isolates

The same procedure as used for bacteria above was utilized except that the fungi were cultured in a 72 – hour malt extract broth before transferring into mineral salt broth. Isolates that showed high turbidity from screen test results were used. Control experiments were set up. All tubes were incubated at 28˚C for 16 days and the degradation rate determined using spectrophotometric method as was described for bacteria, the curve and table are in appendix section.

CHAPTER FOUR

4.0 RESULT

4.1 TOTAL HYDROCARBON UTILIZING BACTERIA AND FUNGI, TOTAL HYDROCARBON AND PERCENTAGE FRACTION OF HYDROCARBON (SATURATED, AROMATIC AND POLAR COMPOUND).

Table 5.0 shows the average of the hydrocarbon utilizing bacteria and fungi count, total hydrocarbon and fraction in slop oil.

The average hydrocarbon utilizing bacteria in the slop sample was 230×10^3 CFU/ml. The average of hydrocarbon utilizing fungi was 175×10^2 CFU/ml. On the other hand the total hydrocarbon in the sample compound was 2832mg/l. The percentage fractions of saturated, aromatic and polar compounds were 64%, 19% and 17% respectively. This table indicates that there are more hydrocarbon utilizing bacteria than fungi in the slop oil sample. It also indicates that out of the total hydrocarbon in the sample 64% is composed of saturated followed by aromatic with 19% and polar compound with the least percentage of 17. The statistical representation of this is shown in Table 8.0 below.

TABLE 6.0 BIOCHEMICAL CHARACTERISTICS AND IDENTIFICATION OF HYDROCARBON UTILIZING BACTERIA IN SLOP OIL.

													CARBOHYDRATE UTILIZATION					
Isolate	**Gram Reaction**	**Rod/Cocci**	**Motility**	**Catalase**	**Oxidase**	**Methyl Red**	**Vogus Proscueus**	**Indole**	**Urease**	**Nitrate Reduction**	**Starch Hydrolysis**	**Gelatin Liquifaction**	**Glucose**	**Sucrose**	**Lactose**	**Mantol**	**Maltose**	**PROBABLE ORGANISM**
B_1	-	**Cocco Baccilli**	+	+	-	-	-	-	+	+	-	-	**A/G**	**A**	-	**A**	**A**	**Acinetobacter Sp.**
B_4	+	**Rod**	+	+	+	+	-	-	+	+	+	-	**A**	**A**	**A**	**A**	**A**	**Bacillus Sp**
B_3	-	**Cocco Baccilli**	+	+	+	-	-	-	+	+	+	+	**A/G**	**A**	**A/G**	**A**	**A**	**Achromobacter Sp.**
B_2	-	**Rod**	+	+	+	-	-	-	+	+	-	+	**A**	-	-	-	-	**Pseudomonas Sp**
B_5	+	**Rod**	+	+	+	-	-	-	-	-	+	-	**A**	**A**	**A**	**A**	-	**Flavobacterium Sp**
B_6	-	**Rod**	+	**ND**	-	-	+	-	-	-	-	-	**A/G**	**A**	**A**	**A/G**	**A/G**	**Serratia Sp**
B_7	+	**Rod**	-	+	+	-	-	-	+	+	+	-	**A**	**A**	**A**	**A**	**A**	**Norcadia Sp**

KEY

A/G = Acid/Gas Production
G = Gas production
A = Acid production
- = Negative
+ = Positive
ND = Not Determine

TABLE 7.0 IDENTIFICATION AND CHARACTERISTICS OF HYDROCARBON UTILIZING FUNGAL ISOLATES FROM OR IN SLOP OI

Isolates	**Colour of Areaial Hyphae**	**Colour of Sunstrate Hyphae**	**Nature of Hyphae**	**Shape and kind of spore**	**Appearance of sporangrophore or conidiophore**	**Probable organism**
F_1	Dark	White	Aseptate	Round sporangium	Long erect non-septate sporangiosphere	Mucor sp.
F_2	Yellow	Brown	Septate Multinucleate	Oval greenish canidia	Long erect and non conidiosphere	Aspergillus sp
F_3	Pink	Dark Brown	Non-Septate	Green Concave shape condition	Simple erect concaveconidiosphere	Fusarium sp
F_4	Green	Brown	Septate Multinucleate branches	Chains of glucose oval brown conidia	Simple long erect conidiosphere	Penicillum sp
F_5	Grey	White	Asiptate	Round sporangium	Long erect non-septate sporangiophore	Rhizopus sp.

Table 5.0 Average hydrocarbon utilizing bacteria and fungal count, total hydrocarbon and fraction of hydrocarbon slop oil

				Fraction of Hydrocarbon %		
Sample	Hydrocarbon utilizing Bacterial x 10^3 CFU/ml	Hydrocarbon utilizing fungal x 10^2 CFU/ml	Total hydrocarbon mg/l	Saturated	Aromatic	Polar compound
Slop oil	230	175	2832	64	19	17

Table 8.0 Statistical Representation of HUB AND HUF

a)

HUB	**COUNT Number (x)**
Acinetobacter sp	245
Bacillus sp	238
Achromobacter sp	217
Pseudomonas	242
Flavobacterium sp	215
Serratia sp	218
Noracadia sp	235
	$\sum x = 1610$

Average HUB =1610/7 =230
= Average HUB in slop oil = 230 x dilution factor x ml plated
= 230 x 10^3 x ml
= 230 x 10^3 CFU/ml

b)

HUB	Count Number (x)
Aspergillus sp	184
Fusarium sp	179
Mucor sp	187
Penicillum sp	164
Rhizopus sp	161

Average HUF = ∑x/HUF Number = 875/5
= Average HUF in slop oil = 175 x dilution factor x ml plated
= 175 x 10^3 x ml
= 175 x 10^3 CFU/ml

However, the table below shows the hydrocarbon utilizing bacteria and fungi in the slop oil.

Table 9.0 Hydrocarbon utilizing bacteria and fungi in slop oil

Hydrocarbon Utilizing Bacteria	Hydrocarbon Utilizing fungi
Achromobacter sp	Aspergillus sp
Acinetobacter sp	Fusarium sp
Bacillus sp	Penicillum sp
Flavobacterium sp	Rhizopus sp
Norcadia sp	Mucor sp
Pseudomonas sp	
Serratia sp	

4.2 BIOCHEMICAL TESTS

In table 6.0 the result of the biochemical tests of bacterial isolates are presented. Based on the results obtained from Table 6.0 and with references to Berggy's Manual of determinative bacteriology, the isolates were determined and identified and were found to belong to the following genera, Acinetobacter sp,

Bacillus sp, Achromobacter sp, Pseudomonas sp, Flavobacterium sp, Serratia sp, and Norcadia sp.

4.3 IDENTIFICATION AND CHARACTERIZATION OF HYDROCARBON UTILIZING FUNGI

Table 7.0 shows the hydrocarbon utilizing fungi. Their identification was based on the colour of their aerial hypae, colour of substrate hypae, nature of hypae, whether septate aseptate and non-septate, the shape and kind of spore, whether round, oval, concave and chains of globules to oval, if it is conidia or sporangium. Based on this, the following fungi were identified, Mucoe sp, Aspergillus sp, Penicillium sp, Rhizopus, and Fusarium sp

4.4 SCREEN TEST FOR THE UTILIZATION OF SLOP OIL BY BACTERIAL AND FUNGAL ISOLATES

Table 10.0 and 11.0 shows the rate of utilization of slop oil as only source of carbon and energy by bacterial and fungal isolates respectively. In consideration of Table 10.0 , it will be observed that two species Acinetobacter sp and Pseudomonas sp, utilize slop oil most when compared with the other isolates. Bacillius sp, and Norcadia sp, moderately utilize slop oil where as species of Serratia, achromobacter and Flavobacterium are least utilizers of slop oil from Eleme Petrochemicals complex.

On the other hand, when fungal isolates were considered based on screen test for utilization of slop oil as only source of carbon and energy, it is observed from Table 11.0 that Mucor sp and Aspergillus sp utilize slop oil maximally where as Fusarium sp, moderately utilize slop oil for growth, finally, Penicillin sp and Rhizopus sp, are the least utilizers of slop oil, hence they grow minimally in slop oil.

4.5 BIODEGRADATION RATE OF SLOP OIL BY MICROBIAL ISOLATES

Table 12.0 shows the assessment of the rate of biodegradation of slop oil from Eleme Petrochemical complex by microbial isolates. The organisms used for the study were selected based on their fast rate of growth on slop oil made available from the screen test as presented in Table 10.0 and 11.0

The organisms were assessed individually and pair wise to ascertain their rate of degradation of slop oil. The result gotten shows that Pseudomonas sp and Aspergillus sp individually degrade slop oil most in 16 days with percentage degradation rate of 69% and 70% respectively.

When these microbes were combined, the percentage rate of degradation increase, this may be attributed to the increased rate of activity of such combination. It is evident in Table 13.0 that the combination of Acinetobacter sp, Pseudomonas sp, Mucor sp and Aspergillus sp gave the highest percentage rate of degradation of 82%; this was followed by the combination of Pseudomonas sp and Mucor sp, which gave the least percentage rate of degradation in combination with 71% rate.

Table 10.0 Screen Test for the Utilization of Slop Oil by Bacterial Isolates

Isolates Tested	**Bacteria Organism**	**Growth in mineral salt medium plus slop oil after 16 days.**
B_1	Acinebacter sp	+ + +
B_2	Bacillus sp	+ +
B_3	Achromobacter sp	+
B_4	Pseudomonas sp	+ + +
B_5	Flavobacterium sp	+
B_6	Sarratia sp	+
B_7	Norcadia sp	+ +

Key
+ + + = Maximum growth
+ + = Moderate growth
+ = Minimal growth

Table 11.0 Screen test for the utilization of slop oil by fungal isolates

Isolates Tested	**Bacteria Organism**	**Growth in mineral salt medium plus slop oil after 16 days.**
F_1	Mucor sp	+ + +
F_2	Aspergillus sp	+ + +
F_3	Fusarium sp	+ +
F_4	Penicillim sp	+
F_5	Rhizopus sp	+

Key
+ + + = Maximum growth
+ + = Moderate
+ = Minimum

Table 12.0 Percentage (%) rate of Biodegradation of slop oil by Bacterial and Fungal isolates

Isolates	Weight of Extract (mg/l)	Weight Loss (Mg/m)	Percentage of Biodegradation
Control	950		
B_1	315	635	67%
B_2	292	658	69%
F_1	320	630	66%
F_2	281	669	70%
B_2 F_1	276	674	71%
B_2 F_2	260	690	73%
B_2 F_1 F_2	210	740	78%
B_1 B_2 F_1 F_2	173	777	82%

Weight Loss = a – b
a = weight of extract (control)
b = weight of respective extracts for B_1 B_2 F_1 ... and B_1 B_2 F_1 F_2
Percentage Rate of degradation (c) = $\frac{a - b}{a}$ X 100

CHAPTER FIVE

5.0 DISCUSSION, SUMMARY AND CONCLUSION

5.1 DISCUSSION

The biodegradation of slop oil was assessed using some microbial isolates individually and in combination. The isolates used were those that showed potential growth in slop oil as presented in the screen tests on tables 11.0 and 12.0. It was observed that the rate of biodegradation of slop oil increased to maximum when these microbial isolates are combined in the biodegradation assay. This indicates that biodegradation rate of slop oil is enhanced by combination of slop oil utilizer. The biodegradation rates are as follows, on individual basis Pseudomonas sp, and Aspergillus sp are highest biodegraders with 69 and 70 percentage respectively. On combination, Acinetobacter sp, Pseudomonas sp, Mucor sp and Aspergillus sp gave the highest percentage degradation rates, whereas Pseudomonas sp and Mucor species combination gave 71% rate of degradation.

These results agree with those stated by authors like Austin at al (1976); Fedorak and Westlake, (1981); Ismailiv, (1985); Brown, (1987); Von Wedel et al, (1988) and Adams and Jackson, (1989). Meanwhile, the different hydrocarbon utilizing bacterial isolates were characterized. On identification, they were grouped into the following genera namely; Acinetobacter, Achromobacter, Norcadia, Flavobacterium, Pseudomonas, Serratia and Bacillus. This is illustrated in Table 7.0. The hydrocarbon utilizing fungal isolates were as well characterized and identified; they belong to the following genera, Mucor, Aspergillus, Penicillum, Rhizopus and Fusarium. The isolation of these microbes from effluent (slop oil) have been previously reported by Kirk et al, (1989); Atlas, (1981); Perry, (1984); Brown (1987); Leahy and Colwell, (1990) and Adams and Jackson (1989).

The screen test to determine the ability of microbial isolates to utilize slop oil as their sole source of carbon and energy showed that , the organism utilized slop oil at various rate (Table 10.0 and Table 11.0). the result of the screen test indicates microbes such as Pseudomonas sp and Acinetobacter sp are bacterial isolates with highest utilization rate. On the other hand, Mucor sp and Aspergillus sp for fungal isolates utilized slop oil most. These findings agree with the work of Kirk et al; (1989); Atlas, (1981); Singer and Finnerty, (1984) and Okpokwasili et al; (1986).

5.2 SUMMARY AND CONCLUSION

From the data obtained from this study, it can be deduced that some microbes are capable of degrading slop oil. These microbes were those that showed great potential in utilizing slop oil as their sole source of carbon and energy.

The maximum percentage of degradation was obtained, when consortia of these potential degraders are utilized for biodegradation analysis. Therefore, it could be concluded that, the combination of slop oil degraders, is a strong tool, for the biodegradation of slop oil from Eleme Petrochemicalls complex. This is substantial and typified by tables 10.0, 11.0 and 12.0.

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APPENDIX 1

I. MEDIA

a) Nutrient Agar

Composition	Weight
Yeast extract	2.0g
Lab. Lemco Powder	1.0g
Peptone	5.0g
Sodium Chloride	5.0g
Agar	15.0g
Distilled water	1000ml

14g of Nutrient agar base was measured using the mettle weighing balance (Mettler P. 185, Mettler instrument A. G. Switzerland) and suspended in 500ml of distilled water and brought to boil to dissolve completely. It was sterilized by autoclaving at 121ºC for 15minutes. This was then cooled to 45ºC before pouring into sterile peri dishes Nutrient agar medium was used for growing heterotropic bacteria and subculturing isolates.

b) Nutrient Agar Broth

Formula	**Weight (g)**
Yeast extract	2.0
Lab. Lemco	1.0
Peptone	5.0
Sodium Chloride	5.0
Distiiled water	1000ml

13g of the medium was dissolve in liters of distilled water, boiled to mix well, dispense into non abstract cotton plugged test tubes and sterilized, by autoclaving at 121ºC for 15 minutes. This is a nutrient medium generating recommended for growing micro organism in broth.

c) MacConkey Agar (Diffco)

Formula	**Weight (g)**
Peptone	20.0
Lactose	1.0
Bile Salt	5.0
Neutral red	0.075
Agar	12.0

Distilled water	1000ml
PH	7.3

26.0g of the medium was weight out and dissolved in 500ml of distilled water by boiling over flame. It was sterilized by autoclaving at 121ºC for 15 minutes and was aseptically poured into sterile petri-dishes and allowed to solidified. Mac Conkey agar medium was used to differentiate lactose fermenting organism from non-lactose fermenting ones in slop oil. It is of general use in the detecting and isolation of members of the enterbacteriacea.

d) Starch Agar

Formula	**Weight (g)**
Soluble starch	5.0
Nutrient Agar	28.0
Distilled water	1000ml

The starch agar medium was prepared by dissolving 20g of nutrient agar in 1 litre of distilled water. 5g of starch was incorporated into the nutrient agar and the mixtures sterilized by autoclaving at 121oC for 15 minutes before dispensing into sterile petri-dishes and allow to solidify for streaking. The unhydrolized starch from a blue colour with iodine. But if the bacteria can hydrolyze starch, there would appear a clear zone and result from and any large activity. Reddish brown zones around the colony indicate partial hydrolysis of starch.

e) Mineral salt medium (Zajic and Supplison, 1972)

Formula	**Weight (g)**
K_2HPO_4	1.8
KH_2PO_4	1.2
NH_4CL	4.0
$MgSO_4$	0.2
NaCl	0.1

The above salts were mixed in 1 litre of distilled water, dispensed into tubes and sterilized by autoclaving at 121ºC for 15 minutes.

f) Malt Extract Agar

Formula	Weight (g)
Peptone	3.0
Malt Extract	17.0
Agar	15.0
Distilled water	1000ml

Dissolve these in one litre of distilled water and sterilize by autoclaving at 121ºC for 15 minutes.

g) Mineral salt medium (Zajic and Supplison, 1972)

Formula	Weight (g)
NH_4Cl	0.5
$K2HPO_4$	0.5
Na_2HPO_4	2.5
NaCL	10.0
Mg_2Cl	2.3
KCl	0.3
Distilled water	1000ml

II. REAGENTS

a) Gram's stain reagents (Pelczer et al., 1977).
Methyl violet
Grams iodine (mordant)
Alcohol (70%)
Safranin (Counterstain)

b) Catalase test reagent (Cruickshank et al 1975)
Hydrogen peroxide (H_2O_2) 3ml
Distilled water 97ml

c) Oxidase test reagent (Cruickshank et al. 1975)
Tetramethyl paraphenylene 1.0g
Diamine dihydrochloride
Distilled water

1g of tetra methylpara Phenylene diamine dihydrochloride was dissolved in 100ml of distilled water to obtain 1% of the reagent.

d) Nitrate reduction test reagents

Nitrate broth

Potassium nitrate (KNO_3)	0.1g
Nutrient broth	0.13g
Distilled water	100ml

Nitrate reagent A

Sulphamilic acid	0.8
Acetic acid (5N)	1000ml

Nitrate B

Alpha Naphthylamine

Acetic acid (5N)

Reagent B was dissolved by gentle heating. Both reagents were stored at 4^{o}C in brown bottles.

e) Indole test (kovac's)

Composition concentration

Para-dimethyl amino benzaldehyde	5g
Amyl acohol	75ml
Conc. Hydrochloride acid	225ml

The aldehyde was dissolved in alcohol in alcohol and the HCL acid was added, stored at 4^{o}C in a brown bottles.

f) MR – Vp test reagents

Peptone	5.0g
Dextrose	5.0g
Phosphate buffer	5.0g
Distilled water	1 liter

g) Urease test

Medium	Weight (g)
Peptone	1.0
Dextrose	1.0
Sodium chloride	5.0
Disodium phosphate	1.2

Potassium dihydrogen phosphate	0.8
Phenol red	0.012
Agar No. 3	15.0
pH	6.8

2.4g of medium was dissolved in 95ml of distilled water, boiled to dissolve and sterilized by autoclaving at 121°C for 15min. 5ml of sterile 40% urea solution and 10% glucose were added, after cooling to 50°C.

h) Citrate utilization test reagent

NaCl	5g
Magnesium sulphate	0.2g
Ammonium dihydrogen phosphate	10.0g
Bromothymol blue	0.008g
Agar	20.0g
Citric acid	2g

The salt and agar were dissolved in a the distilled water and the 2g citric acid was added o the salt solution and pH adjusted to 6.8 with NaCl. The medium was sterilized in autoclave at 121°C for 15minutes.

i) Citrate utilization test reagent

NaCl	5g
Magnesium sulphate	0.2g
Ammonium dihydrogen phosphate	10.0g
Bromothymol blue	0.008g
Agar	20.0g
Citric acid	2g

The salt and agar were dissolved in a the distilled water and the 2g citric acid was added o the salt solution and pH adjusted to 6.8 with NaCl. The medium was sterilized in autoclave at 121°C for 15minutes.

j) Indone production test reagent

Tryptone broth	20g
Sodium chloride	5.0g
Distilled water	1000ml
Kavac's reagent	0.3ml

The component was dissolved in 1000ml of distilled water dispensed into test tubes and sterilized by autoclaving at 121^{o}C for 15minutes.

III. Chemicals and Dilluents

a) Diethyl ether
b) Antifungal drug (Nystatin)
c) Antibacterial drug (Streptomycine)
d) Anti-bacterial drug (Penicillin)
e) Normal (Physiological saline)

Composition	Concentration
NaCl	0.55g
Distilled water	100ml

The salt was discovered in sterile water and dispensed into test tubes foe dilution process.

APPENDIX 2

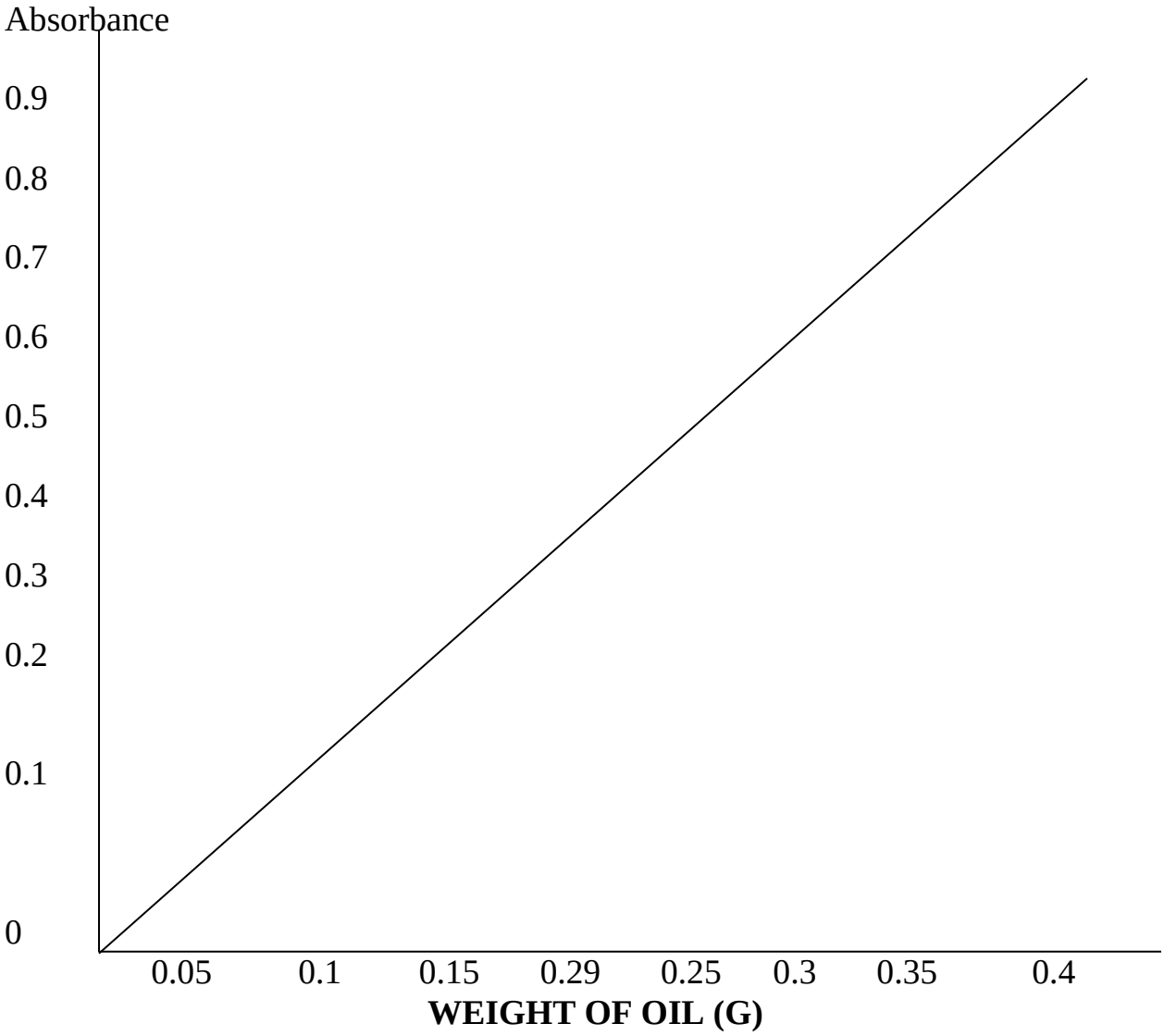
a) Raw Reading for Caliberation of standard and curve using slop oil (bacterial degradation)

Volume of oil (ml)	Equivalent weight (g) x 10^{-1}	Absorbance
0.05	0.30	0.068
0.1	0.59	0.13
0.2	1.18	0.27
0.3	1.77	0.41
0.4	2.36	0.58
0.5	2.94	0.68
0.6	3.54	0.82

b) Raw Reading for Caliberation of standard and curve using slop oil (Fungal degradation)

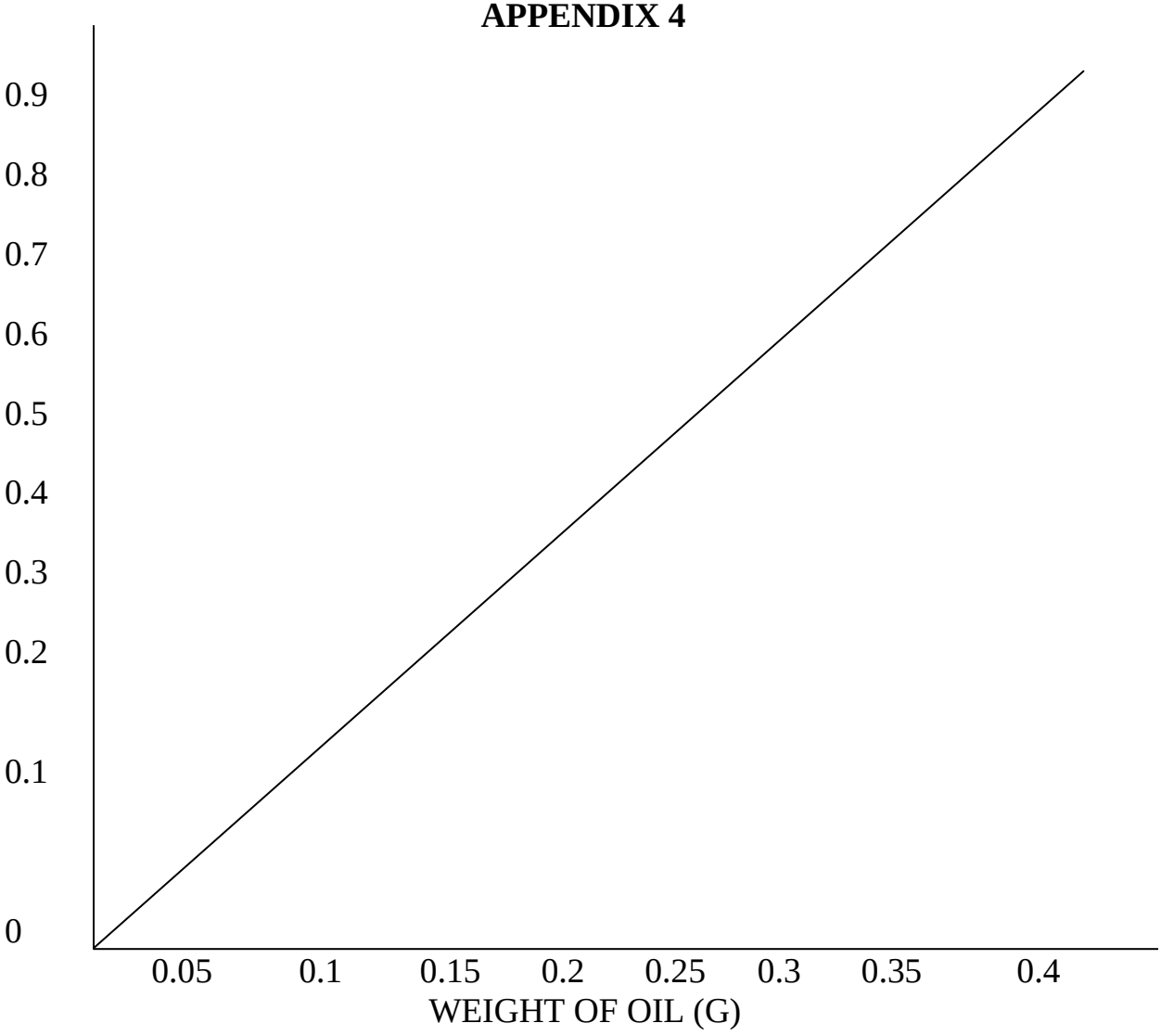
Volume of oil (ml)	Equivalent weight (g) x 10^{-1}	Absorbance
0.05	0.45	0.10
0.1	0.19	0.19
0.2	1.82	0.41
0.3	2.73	0.61
0.4	3.61	0.78
0.5	4.62	1.20

APPENDIX 3



Standard curve of fungal degraded slop oil showing relationship between absorbance and weight oil.

APPENDIX 4



Standard curve of bacterial degraded slop oil showing relationship between absorbance and weight oil.

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ENVIRONMENTAL MANAGEMENT

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