DECLARATION

Declaration by the candidate

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Wanjohi Lucy Waruguru **SC/PGB/026/09**

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Declaration by the supervisors

This thesis has been submitted for examination with our approval as the University Supervisors.

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DEDICATION

To my dear husband Mr. Francis Mungai and children Steve and Mike

ABSTRACT

Halophilic bacteria have been largely omitted from ecosystem studies of saline Lakes, especially with regard to their importance in decomposition process under aerobic conditions. Plastics are widely used as packaging material due to their light weight, inertness and low cost. The disposal of plastics has become a major environmental challenge because they are not easily degraded. Petroleum oil released into the environment is a world wide problem because it contains aromatic compounds that are toxic to most life forms. Episodic and chronic pollution of the environment by petroleum oil causes major ecological perturbations. Petroleum oil spills affects many species of plants and animals in the environment, as well as humans. This research project was carried out to determine the load and biodiversity of bacteria in five selected sites of Lake Nakuru and to establish their ability to degrade disposable plastics and petroleum oil. Water samples were collected in sterile bottles from five sites of Lake Nakuru namely; Middle Lake, Makalia, Enderit, Njoro and Hippopoint. Serial dilution was carried out and culturing was done using spread plate method on nutrient agar. The cultures were incubated at 35° C for 24 hours. Bacterial load was determined by counting the number of colony forming units. Sub-culturing was done to obtain pure cultures which were then isolated and stocked in agar slants and brain heart infusion media. Identification of bacteria was carried out by observing morphological characteristics, gram staining, biochemical tests, and serotyping using Analytical Profile Index Kits. Degradation of plastics experiment was determined by percentage weight loss of the materials. The experiment had four replicates and was carried out for 90 days. A "greasy spot" test was carried out for seven weeks to determine oil degradation. A total of twenty one isolates were identified; two bacteria were gram positive while nineteen were gram negative. Various bacteria were able to degrade plastics and petroleum oil. They included *Sphingomonas paucimobilis, Streptococcus pyogenes, Tatumela ptyseas, Bacillus anthracoides, Chryseobacterium indologenes, Chryseobacterium meningosepticum, Pseudomonas cepacia, Proteus penneri, Moraxella* sp., *Alcaligen* sp., *Providencia stuarti, and Providencia rettgeri. Yersinia pseudotuberculosis* degraded petroleum oil but was unable to degrade plastics. In this study the bacteria that have not been reported in biodegradation of plastic and oil included *Tatumella ptyseas, Proteus penneri* and *Providencia stuarti.* These bacteria are recommended in bioremediation of oil spills and in the biodegradation of plastics. This will result in broad applicability, low cost and low risk of exposure to hazardous chemicals during clean up.

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LIST OF ABBREVIATIONS/ACRONYMS

AFM	Atomic Force Microscopy
API	Analytical Profile Index
CFU	Colony Forming Units
DMRT	Duncan Multiple Range Test
GC	Gas Chromatography
GDL	Glycosphingolipids
GPS	Geographical Positioning System
HCB	Hydrocarbonoclastic Bacteria
KEMRI	Kenya Medical Research Institute
KWS	Kenya Wildlife Service
M	Molarity
MTRH	Moi Teaching and Refferal Hospital
NCST	National Council for Science and Technology
PAHs	Poly Aromatic Hydrocarbons
PCL	Polycaprolactone
PHA	Polyhydroxy Alkanoates
PHB	Polyhydroxybutyrate
SEM	Scanning Electron Microscopy
TSI	Tripple Sugar Ion

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CHAPTER ONE

INTRODUCTION

1.1 Background information

Bacteria are one of the most abundant and species rich groups of organisms that mediate many critical ecosystem processes (Claire *et al.,* 2003). They inhabit an extra ordinary array of habitats from those that offer ideal conditions for most living organisms to those that are extreme to support most life forms. They inhabit the nutrient rich environments of soils, Lakes, oceans and other organisms but they are also found in extreme environments such as hot springs (Brock, 1978). They also inhabit nearly saturated salt brines (Anton *et al.,* 2000), and acid mine waters at pH of near zero (Baker and Banfield, 2003), deep in Antarctic ice (Price, 2000), and kilometers below the earth's surface (White *et al.,* 1998). The total number of bacteria on earth may be as high as $4 - 6 \times 10^{30}$, a large proportion of these bacteria possibly residing in the oceanic and terestrial subsurfaces (3.5×10^{30}) and ($0.25 - 2.5 \times 10^{30}$) respectively (Whitman *et al.,* 1998). These bacteria contribute to the flow of nutrients and also constitute a significant proportion of nutrients to living biomass (Claire *et al.,* 2003).

It is important to understand patterns of bacterial biodiversity because they mediate many of the environmental processes that sustain life on the earth and their diversity is greatly applied in bioremediation and in search for novel bio-chemicals for use in medicine and industry (Claire *et al*., 2003). Significant theoretical and practical constraints have hindered the quantification of bacterial diversity and have prevented comprehensive studies of bacterial-biodiversity patterns (Brock, 1987). These constraints are; small

proportions of bacterial species that can be cultured, the large number of individuals that may be present at small scale (Klug and Tiedje, 1993) and the difficulty of defining a bacterial species (Goodfellow and O'Donnell, 1993). Challenges in defining a species occurs because the operational definitions of bacterial species are not consistently applied and also due to their genetics (Cohan, 2002).

Scientific interest in extremophilic microorganisms, especially hyperthermophiles, thermoacidophiles, archaebacterial anaerobes, and hyper halophiles has increased (Lowe *et al.,* 1993). One reason for this interest is the need to understand the biochemical mechanisms involved under extreme conditions such as hot springs, saline, acidic, alkaline and arctic. This is important because of possibility of biotechnological use of enzymes and molecules from such organisms (Woese *et al.*, 1990).

The inland Lakes of the world such as the Dead Sea and the Great Salt Lake provide some of the most extreme natural environments for halophilic microorganisms (Williams, 1996). Saline Lakes have significant economic, ecological, biodiversity and cultural value. They are an important source of minerals, water, fish, biochemical products and food stuffs or aquaculture. Many have a high aesthetic value and cultural significance (Hammer, 1986). Such halophilic Lakes are subject to high rates of evaporation because of high temperatures. Halophiles which are found in these halophilic Lakes are used in bioremediation, biodegradation and in oil recovery (Dubey *et al*., 2003).

Bioremediation is any process that uses microorganisms, fungi, green plants or their enzymes to return the environment altered by contaminants to its original conditions (Shristi *et al.,* 2006).

Biodegradation is the degradation caused by biological activity, especially by enzymatic action leading to a significant change in the chemical structure of a material (Yutaka *et al.,* 2009). Waste is grouped into biodegradable and non-biodegradable matter. Biodegradable waste is broken down by microorganisms and become part of the earth and soil, while non-biodegradable waste is not easily broken down by microorganisms, though they finally break down over time (Shristi *et al.,* 2006).

There is considerable research interest in the microbial degradation of plastics waste material since microbes are able to degrade most organic and inorganic materials (Shristi *et al.,* 2006). Plastics are high molecular weight polymers that are at some stage in their existence capable of flow but may also be brought into a non-fluid form in which they have sufficient toughness and strength to be useful in self supporting applications (Brysdon, 2010).

Petroleum oil spills from transportation accidents, pipeline breakages and tank leakages can be considered as the most frequent causes of aromatic hydrocarbon release (Bossert *et al.,* 1984), which are common environmental pollutants with toxic, mutagenic and carcinogenic properties (Mastrangela *et al*., 1997). Bacteria have been reported to play a major role in hydrocarbon degradation by using them to satisfy their cell growth and energy needs (Ghazali *et al.,* 2004). Microbes play a major role in poly aromatic hydrocarbons (PAHs) removal from contaminated environments due to their advantages

such as cost effectiveness and more complete clean up (Pothuluri *et al*., 1994). This research investigated the effective use of some of these bacteria in the biodegradation of plastic and petroleum oil.

1.2 Statement of the problem

Plastics which are non-biodegradable waste have been widely used due to their light weight, inertness and low cost. Their disposal especially those used for packaging have become a major environmental concern due to poor waste management practices. Their accumulation especially in urban areas is a challenge worldwide. The town of Nakuru which is on the northernmost shore of Lake Nakuru is a rapidly growing local centre of industry and agriculture. The town is polluted by polythene bags and disposable cups and bottles. Nakuru Municipal Council tries to collect the garbage but is overwhelmed by the large deposits of these wastes. Some plastics collect water and become the breeding places of mosquitoes worsening the problem. Plastics have also been recently recognized as a major threat to marine life. They sometimes cause blockage in the intestine of fish, birds and marine mammals. They also block drainage systems. It has however been reported that various bacteria species are able to degrade plastics.

There is a high demand of petroleum oil as a source of energy. While trying to meet this demand, accidental and associated petroleum oil spills occurs during the process of exploration, production, transportation and storage. Oil spills occur in Nakuru town garages and are washed by the run off during the rainy season to Lake Nakuru. Oil spills results in ecological risks and long term environmental disturbance. There is therefore

need to identify the bacteria in Lake Nakuru that are able to remediate our environment from plastic and petroleum oil pollutants.

1.3 Justification

The elimination of a wide range of pollutants and wastes from the environment is a requirement to promote a sustainable development of the society with low negative environmental impact. Biological processes play a major role in the removal of contaminants due to catabolic versatility of microorganisms to degrade or convert such compounds (Diaz, 2008).

Interest in the microbial biodegradation of pollutants has intensified in recent years as researchers try to find sustainable ways to clean up contaminated environments (Diaz, 2008). Microorganisms are capable of degrading most of the organic and inorganic materials hence creating research interest in the microbial degradation of plastics waste materials. These microbes may be used in bioremediation (Shristis *et al.*, 2006). Bioremediation and biotransformation methods harness the naturally occurring microbial catabolic diversity to degrade, transform or accumulate a huge range of compounds (Mcleod *et al*., 2008). Microbes with enzymes capable of degrading organic and inorganic materials have been isolated in some of the saline Lakes in Kenya especially Lake Bogoria and Lake Elementaita. Studies on bacterial ecology of Lake Nakuru have not been well covered although a lot of studies on zooplankton ecology in Lake Nakuru have been undertaken by Yasindi *et al.,* (2002) and phytoplankton ecology by Oduor and Schagerl (2007). The waters of Lake Nakuru may be rich in halophilic bacteria which may be

important in bioremediation. There is need to therefore identify novel bacteria from Lake Nakuru with the ability to degrade plastics and petroleum oil.

1.4 Objectives

1.4.1 General objective

The general objective of the study was:

To determine the bacterial load and diversity in selected sites of Lake Nakuru and their ability to degrade plastics and petroleum oil and possible use of identified bacteria for bioremediation.

1.4.2 Specific objective

The specific objectives of this study were;

1. To determine bacterial load in selected sites in the waters of Lake Nakuru.
2. To determine the diversity of bacteria in selected sites in the waters of Lake Nakuru.
3. To determine the ability of the bacteria in selected sites of Lake Nakuru to degrade plastics and petroleum oil.

1.5 Hypothesis

1. There is no significant difference in bacteria densities in different sites in Lake Nakuru
2. There is no bacterial diversity in different sites in Lake Nakuru.
3. Bacteria isolates from Lake Nakuru are incapable of degrading plastics and petroleum oil.

CHAPTER TWO

LITERATURE REVIEW

2.1 Biodiversity and identification of bacteria

Biodiversity boosts ecosystem productivity where each species has an important role to play (Cohan, 2002). An individual ecosystem is composed of populations of organisms interacting within the community and contributing to the cycling of nutrients and flow of energy (Wessels, 1988). Bacterial diversity is thought to be heterogeneously distributed across the earth based on agricultural practice (McCaig *et al.,* 1999), temperature (Ward *et al.,* 1998), nutrient status (Broughton & Gross 2000), salinity (Nubel *et al*., 2000), contamination with pollutants (Muller *et al.,* 2001), predation (Kuske *et al.,* 2002) and other environmental variables. Bacteria are economically important as they are used in industrial microbiology, as biological pest control, in vitamin synthesis and in remediation of the environment (Liese *et al.,* 1999). Despite their importance, practical and theoretical constraints have limited the ability to document patterns of bacterial diversity and to understand the processes that determine these patterns (Kuske *et al.,* 2002).

Bacteria are identified using a number of techniques. The most commonly used techniques are observing morphological characteristics, biochemical tests, and molecular techniques (Altschul *et al.,* 1990). Morphological techniques involve observing the shape and size of the bacteria cell, colony morphology, presence or absence of endospores, flagella and glycocalyx. Based on this technique, the bacteria can be grouped into bacilli, cocci, spirilla, spirochaetes, coccobacillus and vibrio. The bacteria can also be grouped as

micro depending on its size e.g. micrococcus (Science daily, 2010). Biochemical tests involve various tests such as gram stain, oxidase test, triple sugar ion (TSI) test, motility test and analytical profile index (API) tests. API tests involve testing for enteric, non-enteric, streptococcus and staphylococcus bacteria. The API 20NE is a standardized micro method combining eight convectional tests and twelve assimilation tests for the identification of non fastidious gram negative rods not belonging to enterobacteriaceae (Geiss *et al.,* 1985). API 20E is a standardized identification system for enterobacteriaceae and other non-fastidious gram negative rods which uses 21 miniaturized biochemical test and database (Smith *et al.,* 1972). API 20strep is a standardized system combining twenty biochemical tests that offer widespread capabilities. It enables group of species identification of most streptococci and enterococci (Tillotson *et al.,* 1982). There are other characteristics that are important in bacterial identification such as testing for antibiotics resistance, enzymes produced and sugars that are fermented (Geiss *et al.,* 1985).

2.2 Halophiles in Kenya saline Lakes

Saline Lakes are naturally occurring with unique abiotic and biotic conditions. They are mostly located in arid and semi-arid areas. They normally have accumulated salts which are deposited in the Lakes from their catchment areas through inflowing rivers and run-offs. The salts come from the ions dissolved from soluble rocks or from a slow chemical reaction with rocks in the catchment area (Williams, 1996). These Lakes have simple food webs and relatively simple microbiota communities (Humayuon, 2003). Limited studies have been done on spartial and temporal distribution of bacterial diversity in Kenyan

saline Lakes, and on the ascribing aspects of biogeochemical cycles of saline Lakes to these bacteria (Jones *et al.,* 1998). Kilham, (1981) observed that bacterioplanktons biomass in Lake Elementaita and Lake Bogoria were high. The abundant bacteria in Kenya saline Lakes are aerobic gram negative bacteria of *Proteobacteria* lineage, including large numbers of types affiliated to the *Halomonadaceae* family of moderately halophilic bacteria found in terrestrial and marine saline environments. *Proteobacteria* related to *Pseudomonads* and enterics can also be isolated from saline Lakes (Grant, 2006). Other isolates from Kenyan Rift Valley saline Lakes are *Halomonas magadiensis*, from Lake Magadi (Duckworth et al., 2000), *Dietzia natronolimnea* from elementaita, *Cellulomonas bogonensis* (Jones *et al*., 2005) and *Alkalimonas delamenensis* (Ma *et al.*, 2004) from Lake Bogoria.

2.3 Ecology and importance of halophiles

Halophiles are gram-negative, non-spore forming, non-motile bacteria that reproduce by sexual and asexual means (Anton *et al.,* 2000). Halophiles can be classified into slight, moderate and extreme halophiles based on their response to NaCl. The slight halophiles have optimum growth at 2 to 5% NaCl (0.34 to 0.85 M), the moderate halophiles have optimum growth at 5 to 20% NaCl (0.85 to 3.4 M) while the extreme halophiles have optimum growth at 20 to 30% NaCl (3.4 to 5.1 M). Some examples of halophilic bacteria occurring in nature are *Halobacterium salinum, H. halobium, Halofelax mediterranei, Haloarcula sp., Halococcus acetoinfaciens, H. agglomeratus, Natronobacterium gregoryi, and Natronococcus sp.* (Dubey *et al.,* 2003).

Halophiles appear red pigmented due to the presence of carotenoids but sometimes they are colourless. They contain the largest plasmid so far known among all the bacteria (Dubey *et al.,* 2003). These microorganisms use osmotic pressure and chemical substances like sugars, amino acids and alcohols to control the amount of salts inside their cell. The proteins inside the microorganism play the role of making it possible to survive in extreme saline environments (Oren, 2002). Halophiles are coated with a special protein covering which is used to allow only certain levels of salt into the cell. This covering helps to seal in water with the right level of salinity, using the process of diffusion to help it keep the salt at the right level all the time (Sandip *et al*., 2008).

Halophiles are used in biodegradation and bioremediation processes. Williams, (1996) observed that the halobacterial strain EH4 isolated from a salt marsh was found to degrade alkanes and other aromatic compounds in the presence of salt. This bacterium may be used for bioremediation. *Haloferax mediterranei* is a halobacterium which is used to accelerate the production of polyhydroxy alkanoates (PHA) in industries. Halophiles are used in microbially enhanced oil recovery where residue oil in natural oil fields can be extracted by the injection of pressurized water down in a new well (Williams, 1996). Halophiles are also important in the production of protein called bacteriorhodopsin which is used in optical data processing and as a light sensor. Halophiles produce exopolysaccharides which are used as stabilizers, thickeners, gelling agent and as emulsifiers in the pharmaceutical paint, paper, textile and food industry. *Halobacterium halobium* produces a protein which is used as an antigen to detect antibodies against the human *e-myc* oncogene product in the sera of cancer patients suffering from pyrolytic leukaemia cell line (Dubey *et al., 2003*).

2.4 Biodegradation of waste

Biodegradation is a process caused by biological activity which leads to the change of the chemical structure of a material to naturally occurring metabolic products (Yutaka *et al.*, 2009). It can also be defined as degradation caused by biological activity, especially by enzymatic action, leading to a significant change in the chemical structure of a material (Suyama *et al.*, 1998). The European Union deems a material biodegradable if it will break down into water, carbon dioxide and organic matter within six months (Suyama *et al.*, 1998). But despite such definitions, the term "biodegradable" has been applied to a wide range of products even those that might take centuries to decompose, or those that break down into harmful environmental toxins (Tsuchii *et al*., 1980).

Waste which is also known as garbage is defined as unwanted or useless material (Wilson *et al.*, 2006). It is classified into two categories; biodegradable and non-biodegradable waste. Biodegradable waste eventually breaks down to become part of the earth and soil, for example food, while non biodegradable waste will not break down easily for example plastics and glass though eventually they break down over time (Carlsons *et al*., 2006).

2.4.1 Biodegradable plastics

A plastic is a broad name given to different polymers with high molecular weight which can be degraded by various processes (Iwata *et al.*, 1998). Polymers are a broad class of material which are made of repeating units of smaller molecules called monomers. They can be natural for example lignin and chitin or synthetic for example polythene, polybutylene, polypropylene, polyvinyl chloride, nylon and polyesters. They are strong

and durable with a wide range of application in every aspect of life and in industries. They are used as packaging materials, in manufacture of disposable goods, in making plastic containers among others (Tokiwa *et al.,* 2007).

A plastic material is called biodegradable if all its organic compounds undergo a complete biodegradation process (Iwata *et al.,* 1998). It is also said to be biodegradable if the degradation results from the action of naturally occurring microorganisms such as bacteria, fungi and algae and ultimately the material is converted to water, carbon dioxide and/or methane and a new cell biomass (Suyama *et al.*, 1998). According to Yutaka *et al*. (2009) bioplastics or organic plastics are a form of plastics derived from renewable biomass sources such as vegetable oil, corn starch, pea starch, or microbiota, rather than fossil fuel plastics which are derived from petroleum. Most industries use the term bioplastic to mean a plastic produced from a biological source. Both bio- and petroleum-based plastics are technically biodegradable, meaning they can be degraded by microbes under suitable conditions (Tokiwa *et al.*, 1994). However many degrade at such slow rates as to be considered non-biodegradable. The degree of biodegradation varies with temperature, polymer stability, and available oxygen content (Carlsons *et al*., 2006). Consequently, most bioplastics will only degrade in the tightly controlled conditions of industrial composting units. In compost piles or simply in the soil or water, most bioplastics will not degrade, however starch-based bioplastics will (Suyama *et al., 1998*). Biodegradation of plastics by microorganism and enzymes seems to be the most effective process of bioremediation (Carlsons *et al*., 2006). Evaluation of biodegradation of plastics when they are used as substrates for microorganism should be based on chemical

structure and physical properties such as melting point, temperature, crystallinity and storage modules (Mor *et al.,* 2008).

Some conventional plastics such as polythene, poly vinyl chloride are non-biodegradable and their increasing accumulation in the environment has been a threat. Some strategies have been employed to overcome this threat. The first strategy involves production of plastics with high degree of degradability. Bio-plastics have been produced which can be degraded by microorganisms (Tokiwa, 1994). Bio-plastics can be derived from renewable feed-stocks hence reducing emission of green house gasses (Yutaka *et al.,* 2009). Biodegradable plastics have advantages such as increased soil fertility, low accumulation of bulk plastic materials in the environment and reduction of waste management. They can also be recycled to useful metabolites (monomers and oligomers) by microorganisms and enzymes. The second strategy involves degradation of petroleum derived plastics by biological processes for example some aliphatic polyesters such as polycaprolactone (PCL) can be degraded by enzymes and microorganisms (Suyama *et al*., 1998). Thirdly attempts are being made to recycle non biodegradable plastics, for example polystyrene used for making disposable utensils (such as spoons, plates and cups) that can be recycled and used as a filler for other plastics (Tsuchii *et al.,* 1980).

2.4.2 Factors affecting biodegradability of polymers.

Environmental factors have a crucial influence on the polymer to be degraded, on the microbial population and on the activity of the different microorganisms themselves (Gu

et al., 2000). Parameters such as humidity, temperature, pH, salinity, presence or absence of oxygen and the supply of different nutrients have an important effect on the microbial degradation of the polymers. Presence of molecular oxygen is a prerequisite for the degradation of polymers **(**Doi, 1990).

The polymer characteristics such as its mobility, tacticity, chrystallinity, molecular weight, type of fuctional groups and substituents present in its structure and additives added to the polymer play an important role in degradation (Artham and Doble, 2008). The molecular weight determines many physical properties of the polymer. Generally, increase in molecular weight of the polymer decreases its degradability by microorganism (Gu *et al*., 2000). This is because high molecular weight results in a sharp decrease in solubility making them unfavourable for microbial attack. The reason is that bacteria assimilates the substrate through the cellular membrane and then further degrade it by cellular enzymes (Gu *et al.,* 2000). The degree of crystallinity is a crucial factor affecting the biodegradability since enzymes mainly attack the amorphous domain of a polymer. The molecules in the amorphous region are loosely packed and this makes its more susceptible to degradation. The crystalline part of the polymer is more resistant than the amorphous region (Iwata *et al.,* 1998). The type of microorganism also affects the biodegradation of polymer (Artham and Doble, 2008).

2.4.3 Mechanism of biodegradation

Biodegradation of plastics is a heterogeneous process which involves biotic and abiotic processes (Tokiwa, 1994). The major mechanisms involved in the biotic degradation of plastic are; the adherence of the microorganism on the surface of the plastic followed by

colonization of the exposed surface. Due to properties and the size of the polymer molecules, microbes are unable to transport the polymeric material directly into the cells where most biochemical processes take place. They first excrete extracellular enzymes which depolymerize the polymers outside the cells. This yields smaller molecules of short chains, for example oligomers, dimers, and monomers that are able to pass semi-permeable outer bacterial membrane and be utilised as carbon and energy sources (Frazer, 1994). Consequently if the molar mass of the polymers can be sufficiently reduced to generate water soluble intermediates, these are transported into the microorganism and fed into the appropriate metabolic pathways. As a result the end products of these metabolic processes include water, carbon dioxide and methane in case of anaerobic degradation. This degradation process is called mineralization (Barzal *et al.*, 1989).

Microorganisms depolymerize polymers by hydrolysis which is a two step process. First enzymes binds to the polymer substrate then subsequently catalyses a hydrolytic cleavage leading to the scission of the polymer chain bonds and hence decrease in the molecular mass of the polymer (Goldberg, 1995). Two categories of enzymes are actively involved in biological degradation of polymers; extracellular and intracellular depolymerases (Doi, 1990). Certain fungi secrete hydrogen peroxide and a specific enzyme which act slowly initiating degradation on some resistance natural polymers (Brydson, 2010). Microbial exudates other than enzymes can create a micro-environment in which certain polymers become chemically unstable, for example sulfur bacteria produce sulfuric acid from

sulfide or sulfur. This results to cracking or to the chemical disintegraton of the polymer which is referred to as stress corrosion cracking (Brysdon, 2010).

Abiotic processes also act on the polymer either parallel or as first stage. The non-biotic effects include physical forces such as thermal-polymer degradation and oxidation or scission of the polymer chains by irradiation (photo-degradation) and chemical hydrolysis (Lee, 1996). Physical forces involve embrittlement and microfragmentation**.** Embrittlement process begins when a polymer is disposed off as waste into the environment. It is activated primarily by sunlight. The polymer begins fragmenting into small pieces and the molecular weight of the polymer chain is reduced, thus increasing the microbial accessibility, and eventual bio-digestion. Micro-fragmentation is the further fragmentation of the embrittlement stage into smaller fragments, which then begins the bio-digestion. This micro-fragmentation of the polymer chain promotes the growth of microbial colonies to speed up biodegradation. Bio-digestion is the consumption of the micro-fragmented pieces, by the microbial colonies, resulting in the biodegradable polymer being ultimately consumed (Villeti *et al.*, 2002).

2.5 Biodiversity and occurrence of plastic degrading microbes

Biodiversity and occurrence of plastic degrading microbes vary depending on the environment such as soil, sea, compost and activated sludge (Yutaka *et al.*, 2009). A number of aerobic and anaerobic microorganisms that degrade plastics particularly fungi and bacteria have been isolated from various environments (Lee, 1996). *Acidovorax faecalis, Aspergillus fumigatus, Comamonas sp., Pseudomonas lemoignei and*

Variovorax paradoxus are found in soil. *Alcaligenes faecalis and Pseudomonas* have been isolated from activated sludge. *Ilyobacter delafieldii* is present in the anaerobic sludge while *Comamonas testosteroni* has been found in seawater and coastal sediments (Luzier, 1992). According to Kathiresan (2003), microorganisms that degrade plastics were isolated from the mangrove sediments. The microbial species identified for degrading polythene bags were fungi, gram positive and gram negative bacteria. Fungi species identified were; *Aspergillus niger, A. ornatus, A. cremeus, A. flavus, A. candidus, A. ochraceus, A. nidulans,* and *A. glaucus.* Gram positive bacteria were*; Bacillus* sp. *Staphylococcus* sp., *Streptococcus* sp., *Diplococcus* sp., and *Micrococcus* sp. while gram negative bacteria were *Moraxella* sp. and *Pseudomonas* sp. These microbial species were also recorded for degrading plastic except *Bacillus* sp., *Diplococcus* sp., *Aspergillus ornatus, A. cremeus, A. flavus, A. candidus, A. ochraceus,* and *A. nidulans* (Kathiresan, *2003*).

2.6 Standard testing methods for polymer degradation

Various standard methods are used to test the degradation of polymers. One of the methods used is visual observation which involves the evaluation of visible changes in the plastic material. Effects used to describe degradation include roughening of the surface, formation of holes or cracks, de-fragmentation, colour change, or formation of bio-films on the surface (Ikada, 1999). These changes do not prove the presence of a biodegradation process in terms of metabolism, but the parameter of visual changes can be used as a first indication of any microbial attack (Ikada, 1999). Information on the degradation mechanism can be obtained by using equipments like atomic force microscopy (AFM) or scanning electron microscopy (SEM) (Kikkawa *et al.,* 2002).

Determination of residual polymer or measurements of the mass loss of test specimens such as films or test bars is widely applied in degradation tests although no direct prove of biodegradation is obtained. Problems can arise if the material disintegrates excessively or with the incorrect cleaning of the specimen. In the former case, the samples can be placed into small nets to facilitate recovery; this method is used in the full-scale composting procedure. A sieving analysis of the matrix surrounding the plastic samples allows a better quantitative determination of the disintegration characteristics (Witt *et al*., 2001). A change in mechanical properties and molar mass of the specimen is used to test degradation of plastics. This method cannot be proved directly due to metabolism of the polymer material. However, changes in mechanical properties are often used when only minor changes in the mass of the test specimen are observed (Erlandsson *et al.,* 1997).

The consumption of oxygen (respirometric test) or the formation of carbon dioxide (Sturm test) are good indicators for polymer degradation, and are the most often used methods to measure biodegradation in laboratory tests (Hoffmann *et al*., 1997). Under aerobic conditions, microbes use oxygen to oxidize carbon and form carbon dioxide as one of the major metabolic end product (Mueller, 2006). Due to the normally low amount of other carbon sources present in addition to the polymer itself when using synthetic mineral media, only a relatively low background respiration must be identified, and the accuracy of the tests is usually good. Conventional trapping of carbon dioxide in $Ba(OH)_2$ solution, followed by manual titration or infrared and paramagnetic oxygen detectors can also be used to monitor oxygen and carbon dioxide concentrations in the air

stream (Pagga *et al.,* 2001). Carbon dioxide analysis was also adapted for tests in solid matrices such as compost (Pagga, 1998), and this method has now been standardized under the name, controlled composting test (ASTM, 1998; DIN, 1998; ISO 14855, 1998)

Radiolabeling is also used to test degradation of plastics. Materials containing a randomly distributed C_{14} marker can be exposed to selected microbial environments. The amount of carbon dioxide evolved is estimated using a scintillation counter. This method is not subject to interference by biodegradable impurities or additives in the polymer. Biodegradability investigations using this technique for polymeric materials in different microbial environments show a high degree of precision and consistency (Sharabi and Bartha, 1993). However, it has challenges in that labeled materials are expensive, unsafe and not always available and also problems connected to licensing of radioactive waste disposal.

Clear-zone test is a simple semi-quantitative method used to test polymer degradation. It is an agar plate test in which the polymer is dispersed as very fine particles within the synthetic medium agar. This results in the agar having an opaque appearance (Nishida and Tokiwa, 1993). The formation of a clear halo around the colony after inoculation with microorganisms indicates that the organisms are at least able to depolymerize the polymer, which is the first step of biodegradation. This method is used to screen organisms that can degrade polymers (Abou-Zeid, 2001), but it can also be used to obtain semi-quantitative results by analyzing the growth of clear zones (Augusta *et al.,* 1993).

Enzymatic degradation is also used to test polymer degradation. Enzymatic degradation of polymers by hydrolysis is a two step process where the enzyme binds to the polymer substrate then subsequently catalyzes a hydrolytic cleavage. Plastics can be degraded either by the action of intracellular and extracellular depolymerases in plastic degrading bacteria and fungi (Tokiwa and Calabia, 2004).

Controlled composting test involves treatment of solid waste in controlled composting facilities or anaerobic digesters. This is a valuable method for treating and recycling organic waste material (Biological Waste Management Symposium, 1995). Composting of biodegradable plastics is a form of recovery of waste which can cut the increasing need of new land filling sites. Only compostable materials can be recycled through biological treatment, since materials not compatible with composting could decrease the compost quality and impair its commercial value (Tosin *et al*., 1998). The test method is based on the determination of the net carbon dioxide evolution that is, the carbon dioxide evolved from the mixture of polymer compost minus the carbon dioxide evolved from the unamended compost (blank) tested in a different reactor (Bellina *et al.*, 1999).

2.7 Bioremediation

Bioremediation can be defined as any process that uses microorganisms, fungi, green plants or their enzymes to return the environment altered by contaminants to its original condition (Hoff, 1993). Naturally-occurring bioremediation and phytoremediation technologies have been used for example, desalination of agricultural land by phytoextraction. Bioremediation technology using microorganisms was invented by

George M. Robinson in 1960. Bioremediation can be employed in degradation of chlorinated hydrocarbons by bacteria. Another approach is the cleanup of oil spills by the addition of nitrate and/or sulfate fertilizers to facilitate the decomposition of crude oil by indigenous or exogenous bacteria (Pothuluri and Cerniglia, 1994).

Bioremediation technologies can be generally classified as *in situ* or *ex situ. In situ* bioremediation involves treating the contaminated material at the site while *ex situ* involves the removal of the contaminated material to be treated elsewhere. Some examples of bioremediation technologies are bioventing, landfarming, bioreactor, composting, bioaugmentation, rhizofiltration, and biostimulation (Mandri and Lin, 2007).

Not all contaminants, however, are easily treated by bioremediation using microorganisms. For example, heavy metals such as cadmium and lead are not readily absorbed or captured by organisms. Phytoremediation is useful in these circumstances, because natural plants or transgenic plants are able to bio-accumulate these toxins in their above-ground parts, which are then harvested for removal. The heavy metals in the harvested biomass may be further destroyed by incineration or even recycled for industrial use (Sivapriya and Nirmala, 2003).

2.7.1 Organisms used in bioremediation

The organisms best suited for bioremediation are the species that are indigenous to a particular polluted habitat or one that is similar to it. Mixed cultures are superior, in many cases to axenic cultures in the biodegradation of chlorinated aromatic hydrocarbons and

pollutants (Atlas, 1991). Genetically altered organisms tend to be less stable than unaltered native populations (Atlas, 1981). However this is not always the case, and genetically selected microbes have been employed successfully in bioremediation. The genes for many catabolic enzymes involved in the biodegradation are carried on plasmids (Hoff, 1993). Therefore enhancing the number of these plasmids in a microbe selected for bioremediation is advantageous. The microbe selected to remediate a hazardous environment should not only remediate one group of compounds but also be able to survive the toxic effects of others and grow in such unfavourable conditions. Increased tolerance to acid, base, temperature, salinity or heavy metals is essential (Ghazali *et al.*, 2004).

2.7.2 Advantages of bioremediation

Bioremediation has a number of advantages over other processes of remediation, such as broad applicability and low cost (Hoff, 1993). It has low risk of exposure to hazardous chemicals during cleanup. Chemical/physical excavation methods of remediation often require the handling of hazardous material (Hoff, 1993). Polluted sediments in riverbeds, harbours and Lakes should be remediated without disturbing the sediments. Sediments disturbance increases the chances of enlarging the area polluted or transporting the pollutants downstream or to adjacent environments. When bioremediation is successful, there is minimal danger to the environment and the products generated such as carbon dioxide, water and fatty acids are innocuous (Atlas, 1991).

There are a number of advantages of bioremediation, which can be employed in areas that are inaccessible without excavation. For example, hydrocarbon spills or certain chlorinated solvents may contaminate ground water. Introducing the appropriate electron acceptor or electron donor amendment, as appropriate, may significantly reduce contaminant concentrations after a lag time allowing for acclimation. This is typically much less expensive than excavation followed by disposal elsewhere, incineration or other *ex situ* treatment strategies (Ghazali *et al.*, 2004).

2.8 Biodegradation and bioremediation of oil

Aromatic hydrocarbons are common environmental pollutants with toxic, carcinogenic and mutagenic properties (Mastrangela *et al*., 1997). One of the impact associated with oil seepages on land includes loss of soil fertility, water holding capacity, permeability and binding capacity (Bossert *et al*., 1984). The search for effective and efficient methods of oil removal from contaminated sites has intensified (Hoff, 1993). One promising method that has been researched is the biological degradation of oil by bacteria. Bacteria play a major role in hydrocarbon degradation by metabolizing oil which is rich in carbon to satisfy their cell growth and energy needs (Coral *et al*., 2005). Oil biodegradation is a large component of oil weathering and is a natural process whereby bacteria or other microorganisms alter and break down organic molecules into other substances, eventually producing fatty acids and carbon dioxide (Atlas, 1991). Bioremediation is the acceleration of this process through the addition of exogenous microbial populations, through the stimulation of indigenous populations or through the manipulation of the

contaminated media using techniques such as aeration or temperature control (Bosserti *et al.,* 1984).

Marine environments are usually vulnerable to oil contamination since oil spills of coastal regions and the open sea are poorly containable and mitigation is difficult. Despite its toxicity, a considerable fraction of the petroleum oil entering marine system is eliminated by the hydrocarbon-degrading activities of microbial communities, in particular by a group of specialist bacteria called hydrocarbonoclastic bacteria (HCB) (Santos *et al.,* 2006).

The increasing number of marine oil spills requires effective solutions for the environment. Bioremediation techniques are major mechanism for removing the oil residues on the affected shorelines. Among the different techniques to enhance natural biodegradation, seeding of new bacteria and fertilizing the indigenous populations have attracted more interest (Pothuluri, 1994). The application of nutrients such as nitrogen and phosphorous in the form of fertilizers is effective in accelerating the biodegradation process and is also enviromentaly safe (Ghazali *et al.,* 2004).

Many microorganisms possess the enzymatic capability to degrade petroleum hydrocarbons. Some microorganisms degrade alkanes, others aromatics and others both paraffinic and aromatic hydrocarbons. Often the normal alkanes in the range of C_{10} to C_{26} are viewed as the most readily degraded, but low- molecular- weight aromatics, such as benzene, toluene and xylene, which are among the toxic compounds found in

petroleum are also very readily biodegraded by marine microorganisms. More complex structures are more resistant to biodegradation. This means that fewer microorganisms can degrade them and the rate of biodegradation are lower than biodegradation rates of the simpler hydrocarbon structure, that is, the higher the number of methyl branched constituents or condensed aromatic rings, the slower the rate of biodegradation (Atlas, 1991).

Researchers have consistently documented a lag time after oil is spilled before indigenous microorganisms begin to break down the oil molecules (Hoff, 1993). This lag time is related to the initial toxicity of the volatile fractions of the oil, which evaporates in the first few days of a spill. Microbial populations must begin to use the oil and expand their population before measurable degradation takes place, a period usually lasting several days. This fact is important when considering the appropriateness of bioremediation as a quick response technique (Pothuluri, 1994).

2.8.1. Degrading species

Biodegradation of petroleum in the marine environment is carried out largely by diverse bacterial population, including various *Pseudomonas* species (Vila *et al.*, 2010). The hydrocarbon-degrading microorganisms are ubiquitously distributed in the marine environment. Generally in the pristine environments, the hydrocarbons degrading bacteria comprise of less than one percent of the total bacterial population (Atlas, 1991). These bacteria presumably utilize hydrocarbons that are naturally produced by plants, algae and other living organisms. They also utilize other substrates, such as carbohydrates

and proteins. The proportion of hydrocarbon degrading microorganisms increases rapidly when an environment is contaminated with petroleum. In particular, in marine environments contaminated with hydrocarbons, there is an increase in the proportion of bacterial populations with plasmids containing genes for hydrocarbon utilization.

The proportions of hydrocarbon-degrading bacterial populations in hydrocarbon-contaminated marine environments often exceed ten percent of the total bacterial population (Sivapriya and Nirmala, 2003). These bacteria possess diverse metabolic pathways which allow them to utilize most recalcitrant petroleum hydrocarbons. Bacterial degradation of aromatic compounds can be divided into three steps. The first step involves modification and conversion of the many different compounds into a few central aromatic intermediates. This step is referred to as peripheral pathway and involves considerable modification of the ring and perhaps elimination of substituent groups. The second step involves oxidative ring cleavage by dioxygenases, which are responsible for the oxygenolytic ring cleavage of dihydroxylated aromatic compounds. The third step involves further degradation of the non-cyclic, non-aromatic ring-fission products to intermediates of central metabolic pathways. Degradation of *n*-alkanes requires activation of the inert substrates by molecular oxygen with the help of oxygenase. Many strains within one species of bacteria usually exist with some strains being capable of hydrocarbon degradation while others can cause opportunistic infections in humans and animals (Connan, 1984).

Biodegradation of oil in soil is carried out by bacteria which are found in oil contaminated sites, such as vehicle service stations. Hydrocarbon degrading bacteria from

these sites have been reported to utilize aromatic hydrocarbons at wide range of temperature and pH (Swamakaran and Panchanathan, 2011). However, oil is composed only of hydrogen and carbon, and the bacteria need additional nutrients to grow. The inorganic nutrients and oxygen should be provided in order to provide the bacteria with nitrogen and several essential mineral (Hoff, 1993).

The following bacteria families are known to degrade hydrocarbons; Micrococcaceae, Brevibacteriaceae, Dermabacteraceae, Dietziaceae, Cellulomonadaceae, Intrasporangiaceae, Corynebacteriaceae (Brito *et al.,* 2004). Gordoniaceae, Nocardioidaceae, Nocardiacae, (such as chryseobacterium), Bacillaceae, Staphylococcaceae, Sphingomonadaceae, Rhodobacteraceae, Rhodospirillaceae, Brucellaceae (Teralmoto *et al*., 2009). Alcaligenaceae, Comamonadaceae, Burkholderiaceae, Rhodocyclaceae, Geobacteraceae, Desulfobactaceae, Piscirickettsiaceae, Pseudomonadaceae, Alteromonadaceae, Pseudoalteromonadaceae, Pasteurellaceae, Shewanellaceae, Moraxellaceae, Alcanivoracaceae, Oceanospillaceae, Oleiphilaceae, Xanthomonadaceae (Ollivier *et al.,* 2000). Zetaproteobacteriaceae, Flavobacteriaceae, Actinobacteriaceae, Delta-proteobateriaceae, Proteobacteriaceae, Alphaproteobacteriaceae, Betaproteobacteriaceae (Ollivier *et al.,* 2000).

2.8.2 Degradation process

The initial steps in the biodegradation of hydrocarbons by bacteria and fungi involve the oxidation of the substrate by oxygenases, for which molecular oxygen is required. Alkanes are subsequently converted to carboxylic acids that are further biodegraded via

beta-oxidation. Aromatic hydrocarbon rings are generally diols; the rings are then cleaved with the formation of catechols which are subsequently degraded into intermediates of the tri-carboxylic acid cycle (Winter *et al.*, 1969). Fungi and bacteria form intermediates with differing stereochemistry. Fungi, like mammalian enzyme systems, form *trans*-diols, whereas bacteria almost always form *cis*-diols. Many *trans*-diols are potent carcinogens whereas *cis*-diols are biologically inactive. Since bacteria are the main hydrocarbons degraders in the marine environment, the biodegradation of aromatic carbons results in detoxification and does not produce potential carcinogens. The complete biodegradation (mineralization) of hydrocarbons produces the non-toxic end products, carbon dioxide and water, as well as cell biomass which is largely proteins which can be safely assimilated into the food chain (Hoff, 1993).

During oil biodegradation, properties of oil fluid change because different classes of compounds in petroleum have different susceptibilities to biodegradation. The early stages of oil biodegradation are characterized by the loss of n-paraffins (n-alkanes or normal alkanes) followed by loss of acyclic isoprenoids (e.g., norpristane, pristine, phytane, etc.). Compared with these compound groups, other compound classes such as highly branched and cyclic saturated hydrocarbons as well as aromatic compounds are more resistant to biodegradation. As biodegradation process progresses, even the more-resistant compound classes are eventually destroyed (Goodwin *et al.*, 1981). Larter *et al.*, *(*2005) estimated that heavily degraded oils losses about 50% of their mass. The early stages of oil biodegradation that is loss of n-paraffins followed by loss of acyclic isoprenoids can be detected by gas chromatography (GC) analysis of the oil.

2.8.3 Conditions under which biodegradation can occur

Biodegradation of oil by bacteria can occur under both oxic and anoxic conditions by the action of different organisms (Zengler *et al*., 1999). In the subsurface, oil biodegradation occurs primarily under anoxic conditions, mediated by sulfate reducing bacteria in cases where dissolved sulfate is present (Holba *et al.,* 1996), or methanogenic bacteria in cases where dissolved sulfate is low. Subsurface oil biodegradation does not require oxygen, but it requires certain essential nutrients such as nitrogen, phosphorus and potassium (Bennett *et al.*, 1993).

The rate of microbial degradation of hydrocarbon is affected by several biological, physical, and chemical parameters. Biological parameters such as the number of species of microorganisms present and vegetation type affects the rate of oil degradation (Larter *et al.,* 2005). Physical parameters such as physical status of oil spill, temperature, oxygen, nutrients, soil type and characteristics also affects oil degradation rate. Physical status of oil spill is the most important factor that determines the environment the petroleum degraders lives (Teralmoto *et al.*, 2009). Generally, there are two kinds of oil spills: Oil spill in water and oil spill in soil. The difference between them is the degree of spread. In soil, hydrocarbon is absorbed by plant and soil particles, limiting its spreading (Prince, 1993). In marine environment, most of the oil normally spreads, forming a thin slick on the top of water body. A large portion of the oil also forms emulsions or dissolves in the water. Some heavy portions settle at the bottom. The difference in these environmental factors can create a big difference in the petroleum degradation process since they have different oxygen availability level (Hamnrick *et al*., 1980).

The influence of temperature on biodegradation is interactive with other factors, such as the quality of the hydrocarbon mixtures and the composition of the microbial community. Temperature affects the physical status of petroleum, in that, higher temperature will result in higher viscosity, the volatilization of toxic alkanes is reduced, and their water solubility increased, delaying the rate of biodegradations. The other effect is on microbial activity whereby higher temperature usually results in better activity of enzymes and therefore increased rate of metabolism. Biodegradation activity has been found in almost all environments with extreme temperatures. Biodegradation can occur at very low temperatures found in the arctic zone (Mulkinsphillips and Stewart, 1974) and in tropical areas (Teralmoto *et al.*, 2009).

Microorganisms require nitrogen and phosphorus for decomposition of hydrocarbons and incorporation into biomass, therefore the availability of these nutrients are important for bioremediation. The nutrient availability depends heavily on the physical status of oil spill, whether it is in marine or soil, whether it is slick or in dissolved form (Xu and Obbard, 2004). The major degradation pathways for petroleum hydrocarbons involve molecular oxygen, indicating the importance of oxygen to oil degrading microbes (Boufadel *et al*., 2010). However there are anaerobes capable of degradation, but the rate is significantly lower. Maintenance of aerobic conditions is important in the bioremediation since most of the degradation is by aerobic processes (Liang *et al.,* 2011).

Generally the biodegradation rate decreases as salinity and pressure increases (Tyagi *et al.*, 2011). The deep benthic zone in marine ecosystems has been found to be one of those

with least microbial activity, partly due to the higher pressure that limits the microbial activity (Shin and Pardue, 2001). The quality and quantity of the contaminant and the soil characteristics such as particle size distribution also have an effect.

2.8.4 Oil bioremediation techniques

Different bioremediation techniques have been developed that help to accelerate the rates of natural hydrocarbon biodegradation by overcoming the rate limiting factors. The first technique involves stimulation of the indigenous microbial populations through the addition of nutrients or other materials (Goodwin *et al.,* 1981). The other technique involves introducing exogenous microbial population in the contaminated environment. This is known as bio-augmentation. Another technique involves the use of genetically altered bacteria (Goodwin *et al.,* 1981). Most microorganisms considered for seeding are obtained by enrichment cultures from previously contaminated sites. Seed cultures are of little benefit over the naturally occurring microorganisms at a contaminated site for the biodegradation of the bulk of petroleum contaminants (Atlas, 1991).

Environmental modification is another technique used in bioremediation. The contaminated media can be manipulated by aeration or by temperature control. Hydrocarbon biodegradation in marine environment is often limited by abiotic environmental factors such as molecular oxygen, phosphates, ammonium nitrate and organic nitrogen concentration (Later *et al*., 2006). Rates of petroleum biodegradation are negligible in anaerobic sediments because molecular oxygen is required by most microorganisms for the initial step in hydrocarbon metabolism. Oxygen, however is not

limiting in well aerated marine environments. Usually marine waters have very low concentration of nitrogen, phosphorous and various mineral nutrients that are needed for the incorporation into cellular biomass, and the availability of these within the area of hydrocarbon degradation is critical (Larter and Aplin, 2003).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Experimental sites

The study site was Lake Nakuru which is located in the Rift Valley Province, Nakuru County, Kenya, (Figure 1) at latitude 0^{o} 22'S, longitude 36^{o} 05'E and at a height of 1,759 m above sea level (Hammer, 1986). The Lake consists of a shallow pan of water lying on salt impregnated clay which retains coarser polar sediments (Kairu, 1991). The surface area is 40-60 km^2 but it is subject to marked fluctuations as the Lake level is constantly rising and falling (Kairu, 1991). It has an average depth of one meter. However, a mean depth of 2.3 meters was calculated between 1972 and 1973 (Kairu, 1991). The length of the shoreline is 27 km. The water level is unregulated. It has a catchment area of 1,800 km^2 (Vareshi, 1982).

Lake Nakuru is one of the national parks of Kenya known for its diverse bird fauna (495 species), particularly the vast flock of lesser flamingo; *Phoenicopterus minor* (Melack *et al.,* 1974). Other aquatic animal communities within Lake Nakuru include the greater flamingo *(Phonocopterus rubber),* pelican *(Pelicanius onoarotalus),* cichlid *(Oreochromis alcalicus grahami),* copepod *(Lovenula africana)* and midge larva *(Leptochironomous deribae)* (Kairu, 1991). The biota in the Lake consists of phytoplanktons dominated by blue-green algae (Vareshi, 1978). However, the Lake is highly eutrophic owing to the vigorous growth of the planktonic blue-green algae, (*Spirulina platensis),* which supports a large number of algae-grazing lesser flamingos

(Vareshi, 1978). According to Kairu, (1991) the primary producers are almost exclusively the cyanophyte algae, *Arthrospira fusiformis* and *Anabaena spp.*

Laboratory work was conducted in various laboratories; Culturing, determining the bacterial load and degradation experiments were conducted in University of Eldoret microbiology laboratory, while identification was carried out in Moi Teaching and Referral Hospital (MTRH) microbiology laboratory and Kenya Medical Research Institute (KEMRI) Nairobi microbiology laboratory.

3.2 Materials

White disposable plastic cups, clear polythene bags (light gauge), paraffin, soil from Mtwapa, soil from Huruma service station in Eldoret and water samples from Lake Nakuru were used to carry out this study. Inorganic nutrients used were; Ammonium phosphate, Magnesium sulfate, Potassium phosphate and non iodinated Sodium chloride.

3.3 Methods

3.3.1 Sampling

Five previously established locations by Kenya Wildlife Services (KWS) monitoring team, which were also used by Vareshi (1982), were selected (Figure 1) for sampling to determine the bacterial load and diversity of bacteria in Lake Nakuru. The selected sites were geo-referenced using Geographical Positioning System (GPS) to ensure that sampling was conducted accurately from the same sampling site throughout the study period. The sites selected were; the middle of the Lake (Jetty mid) (latitude -0.354781 and longitude 36.093118), Hippo point (latitude -0.319546 and longitude 36.105102) and near the mouth of three rivers feeding the Lake. The site near river Enderit lies at latitude -0.386313 and longitude 36.110497, river Makalia (latitude -0.391499 and longitude

36.083254) and river Njoro (latitude -0.331833 and longitude 36.092667). These sampling points were selected to reflect different catchment areas. Sampling was done from a boat in the five sites on a monthly basis for six months consecutively from December 2010 to May 2011. The samples were collected randomly. At each sampling, three water samples of 500 ml each were collected using sterile plastic bottles at each site by hand dipping the bottles at ten centimeters (10 cm) beneath the water surface. The samples were kept in a cool box under ice at 4°C during transportation. These samples were used for serial dilution and culturing of the bacteria. Rainfall data for the six months was obtained from Kenya meteorological centre, Nakuru (Table 1). This data was obtained in order to investigate whether there was any relationship between the bacterial load of the selected sampling points and the amount of precipitation for each month.

3.3.2 Culturing and counting the colony forming units (CFU)

Serial dilution was carried out by using a sterile pipette and transferring 1ml of the sample water to 9ml of sterile water to make a dilution of up to 10^{6}. 1ml of the diluted sample water was inoculated on sterile Nutrient agar media using spread plate method. The media was sterilized by autoclaving at 121ºC for 15 minutes. The inoculated plates were incubated upside down at 35°C for 24 hours. This was done to prevent condensation droplets from falling onto the surface of the agar. The petri dishes were sealed using adhesive tape to prevent contamination. The number of colonies formed (CFU) was counted per site to determine bacterial load. Sub-culturing was done by streaking method. Single colonies were picked using a sterile wire loop and streaked on sterile media to obtain pure cultures.

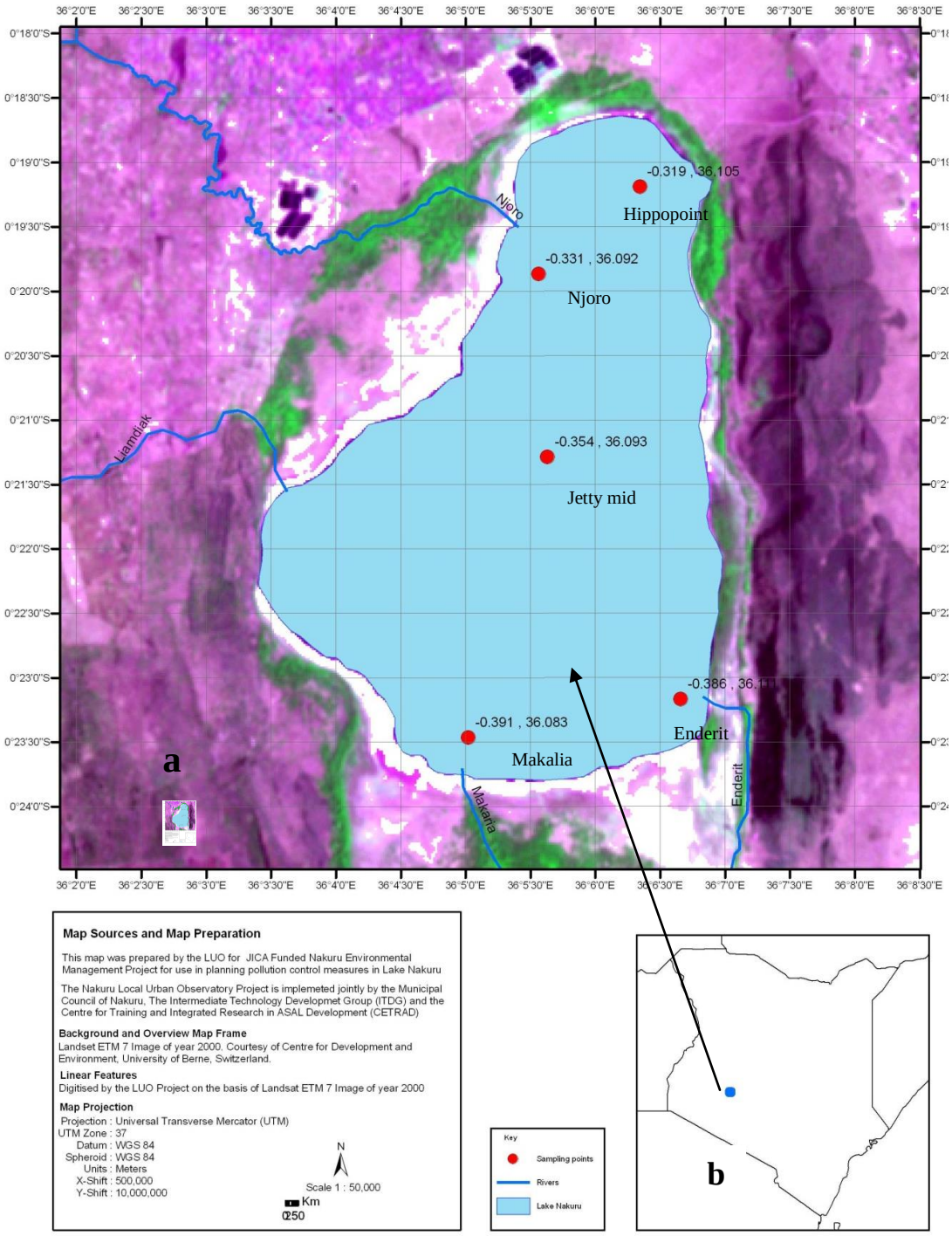

Figure 1: (a) Map of Lake Nakuru showing the sampling points and (b) map of Kenya showing the location of Lake Nakuru.

Note: Map source; Municipal Council of Nakuru

Table 1: Rainfall data during the six months of sampling

Month	Mean Rainfall (mm)
December	13.9
January	1.1
February	0.1
March	104.5
April	58.4
May	111.4

Source: Kenya meteorological centre, Nakuru

3.3.3 Identification of bacteria

The bacteria isolated were identified based on physical characterization and biochemical tests as outlined in Bergey's manual of determinative bacteriology (Holt, 1994). Morphological characteristics such as shape and size were observed under light microscope.

Gram stain which is the most fundamental technique in bacteria identification was conducted. In this procedure, a thin film of each isolate was smeared on the surface of the slide and heated gently over fire to fix it. The smear was first stained with crystal violet, left to stand for a few seconds and then rinsed with a stream of water. It was then treated with a mordant (iodine). The slide smear was then washed with a decolorizing agent (acetone) and counterstained with safrinin. The smear slide was then observed under oil immersion using a light microscope.

Motility test was determined by microscopically observing the bacteria in a wet mount. An inoculum from a freshly prepared culture was used to prepare the wet mount. The inoculum was transferred to a drop of water on a microscope slide, mixed and covered using a cover slip. The slide was observed under light microscope. The bacteria that were observed to swim randomly against the current of water streaming across the slide surface were positive for this test.

Lactose test was conducted in order to determine whether the bacteria fermented this carbohydrate as a carbon source. An inoculum from a pure culture was transferred aseptically to a sterile tube of phenol red lactose broth. The inoculated tube was incubated at 35° C for 24 hours. A positive test indicated colour change from red to yellow.

Hydrogen sulfide gas production test was carried out to determine whether the bacteria were able to reduce sulfur containing compounds to sulfides during metabolism process. An inoculum from a pure culture was transferred aseptically to a sterile triple sugar ion agar slant. The inoculated tube was incubated at 35° C for 24 hours. A black colour in the agar slant media indicated a positive test.

Citrate test was carried out to determine the ability of the bacteria to utilize sodium citrate as the only source of carbon. A sterile wire loop was used to inoculate 3ml of sterile Koser citrate medium with a broth culture of bacteria. The inoculated broth was

incubated at 35° C for three days. A change of colour from green to blue indicated a positive result.

Serotyping using Analytical Profile Index (API) kits was carried out. API kits used were enteric (API 20E) non-enteric (API 20NE) and streptococcus kits (API 20strep.) manufactured by Biomerieux Inc. USA.

3. 3.4 Degradation of plastics disks

Degradation of plastic disks was determined by percentage weight loss of the materials, as described by Kathiresan, (2003). The experiment was set up in four replicates and carried out for a period of 90 days. Disks of 0.6 cm diameter were prepared from clear polythene bags and white disposable plastic cups. Ten mg of each type of disk was put in a conical flask, 150 ml of distilled water and inorganic nutrients composed of 0.01M ammonium phosphate, 0.002M magnesium sulfate, 0.012M potassium phosphate and 0.144 M non-iodinated sodium chloride were added to the conical flasks, sealed using alluminium foil and sterilized by autoclaving at 121° C for 20 minutes. The contents in the conical flasks were inoculated with different bacterial species separately after cooling to 25° C. The conical flasks were covered with parafilm to provide aeration, avoid contamination and evaporation.

The negative control contained ten mg of sterile disks measuring 0.6 cm in diameter, 150 ml of distilled water, and the inorganic nutrients as stated above. A positive control contained 150 ml of distilled water, 5 g of soil from mangrove forest collected at Mtwapa, Kenya, and the inorganic nutrients. The experimental set up was put in a shaker

set at seventy revolutions per minute and at room temperature for 90 days. After 90 days of shaking, the plastic and polythene disks were washed thoroughly using distilled water, shade dried and then weighed for the final weight. From the data collected, the average weight loss caused by each bacterium was computed for both plastic and polythene bags. The degradation was then determined as percentage weight loss and calculated as

$$\text{Degradation} = \frac{\text{Initial weight - Final weight}}{\text{Initial weight}} \times 100$$

3.3.5 Degradation of oil

Oil degradation experiment was done as described by Spring (1994). The experiment was set up in four replicates. Each set up of the main experiment contained 2 ml of paraffin, 150 ml of distilled water, the bacterium being tested and a mixture of inorganic nutrients comprising of 0.011 M ammonium phosphate, 0.002 M magnesium sulfate, 0.012 M potassium phosphate and 0.144 M non-iodinated sodium chloride. These were put in a conical flask which was sealed using parafilm to provide aeration, avoid contamination and evaporation.

The negative control comprised of 2 ml of paraffin, 150 ml distilled water and the inorganic nutrients. The positive control comprised of 2 ml of paraffin, 150 ml of distilled water, inorganic nutrients and 5 grams of soil sample from Huruma service station in Eldoret, Kenya. This soil was collected at a depth of 5 cm and placed in sterile polythene bags. These were put in conical flasks which were covered using parafilm.

Results were recorded from each conical flask on weekly basis for seven weeks. This was done by performing a "greasy spot" test. A brown paper bag was divided into 5.08×5.08 cm squares and labeled as per the conical flasks. A small quantity of liquid just below the surface of oil from each jar was drawn using a dropper. Two drops of this liquid were put onto the center of the square on the brown paper. This procedure was repeated two times for each jar. A few minutes after the water evaporated a greasy spot in each small square was observed. The circumference of each greasy spot was marked using a pencil and its diameter recorded. The initial diameter measurement of the greasy spot formed by the drop of oil was carried out immediately after setting up the degradation experiment. The initial mean diameter of all the set up was 5 cm. Subsequent diameter measurements were carried out on weekly basis for seven weeks.

3.4 Data analysis

Data analysis was done using Minitab^{TM} Version 14.0 for Windows. Data for the microbial load for each site was averaged. One way ANOVA was carried out to test whether there was any significance difference in the microbial load obtained in the five sampling points and at different sampling months. Means were separated using Duncan's multiple range test at 95% level of significance. Data for plastic and polythene disks degradation was averaged and percentage weight loss for each bacterium calculated.

The diameters obtained from the oil degradation experiments were averaged for each bacterium species for each week. One way ANOVA was carried out to determine whether the degradation of oil by each bacteria species was significant. Post hoc test was

carried out to separate the means of the greasy spot formed by each bacterium. Means were separated using Duncan's multiple range test (DMRT) at 95% level of significance.

CHAPTER FOUR

RESULTS

4.1 Bacterial load in selected sampling points at Lake Nakuru

There was a significant difference in the number of bacteria sampled at different months and at different sites (F = 7. 36, P = 0.00). The results in Table 2 show that the month of February had the lowest bacterial load with a mean of 41.07 cfu/ml, while the month of May had the highest mean of 101.20 cfu/ml. Among the sites, Middle Lake had the lowest bacterial load of 50.56 cfu/ml while Njoro had the highest mean of 70.28 cfu/ml (Table 2).

In the month of December, Njoro had the highest bacterial load of 73.67 cfu/ml while Enderit had the lowest mean of 32.67 cfu/ml. In the month of January, Njoro had the highest bacterial load of 54.67 cfu/ml while Middle Lake had the lowest mean of 35.67 cfu/ml. During the month of February, Njoro had the highest bacterial load of 61.00 cfu/ml while Middle Lake had the lowest mean of 21.33 cfu/ml. In the month of March, Makalia had the highest bacterial load of 120.00 cfu/ml while Middle Lake had a mean of 47.00 cfu/ml. Makalia had the highest bacterial load in the month of April 61.67 cfu/ml while Enderit had the lowest mean of 34.33 cfu/ml. Njoro site had the highest bacterial load in the month of May of 117.67 cfu/ml while Makalia had the lowest mean of 78.00 cfu/ml.

The highest bacterial load at Enderit site was in the month of May 105.00 cfu/ml and the lowest mean was in December of 32.67 cfu/ml. At Makalia, the month of March had the highest bacterial load of 120.00 cfu/ml while February had the lowest mean of 28.67

cfu/ml. At Hippopoint, May had the highest bacterial load of 104.00 cfu/ml while February had the lowest of 43.67 cfu/ml. At Middle Lake, the month of May had the highest bacterial load of 101.33 cfu/ml while February had the lowest mean of 21.33 cfu/ml. At Njoro, May had the highest bacterial load of 117.67 cfu/ml while April had the lowest mean 44.33 cfu/ml.

4.2 Diversity and identification of bacteria

The waters of Lake Nakuru were found to have diverse bacteria (Figures 2 to 8). Various types of bacteria grew on nutrient agar. Some formed large colonies while others formed small ones. The colonies formed were of different colours. The colours observed were white, yellow, cream, pink and orange (Figure 2 and 3). Twenty one species were isolated and identified from the waters of Lake Nakuru (Table 3). The bacteria were classified according to their different morphological characteristics. There were seventeen bacilli, one coccus, one coccobacillus, one vibrio and 1 filamentous. There were nineteen Gram negative and two Gram positive bacteria. They were further identified using other biochemical tests and API kits (Table 3). There were twelve enteric and nine non- enteric bacteria (Table 3). Two bacteria were citrate positive while nineteen were citrate negative. *Morganella morganii* and *Erwinia amylovora* produced hydrogen sulphide gas while the rest were negative for this test. Twelve bacteria fermented lactose while nine were non- fermenters. Thirteen bacteria were motile while eight were non- motile (Table 3). The microorganisms isolated and identified from the mangrove soil were *Bacillus* sp., *Micrococcus* sp., *Staphylococcus* sp., and *Streptococcus* sp. while those identified from Huruma service station, Eldoret were *Bacillus* sp., *Pseudomonas* sp., and *Micrococcus* sp.

Table 2: Mean bacteria colony forming units per millilitre sampled from selected sites in Lake Nakuru for six months.

MONTHS	SITES					Mean of means/ month
	Nderit	**Makalia**	**Hippo point**	**Middle Lake**	**Njoro**	
December	$32.67 \pm 11.80^{c(d)}$	$41.00 \pm 18.60^{c(cd)}$	$52.67 \pm 19.40^{b(c)}$	$50.33 \pm 4.33^{b(b)}$	$73.67 \pm 6.69^{a(b)}$	**50.07**
January	$46.67 \pm 13.00^{c(c)}$	$40.67 \pm 17.6^{cd(cd)}$	$53.67 \pm 18.20^{b(c)}$	$35.67 \pm 13.2^{d(cd)}$	$54.67 \pm 12.20^{a(c)}$	**46.27**
February	$50.67 \pm 9.82^{b(b)}$	$28.67 \pm 2.19^{c(d)}$	$43.67 \pm 10.60^{b(d)}$	$21.33 \pm 3.33^{d(d)}$	$61.00 \pm 8.62^{a(c)}$	**41.07**
March	$50.67 \pm 1.76^{c(b)}$	$120.0 \pm 20.20^{a(a)}$	$83.33 \pm 14.50^{b(b)}$	$47.00 \pm 7.37^{c(c)}$	$70.33 \pm 10.10^{ab(bc)}$	**74.27**
April	$34.33 \pm 10.80^{c(cd)}$	$61.67 \pm 21.40^{a(c)}$	$44.33 \pm 17.20^{b(d)}$	$47.67 \pm 6.74^{ab(c)}$	$44.33 \pm 21.40^{b(d)}$	**46.47**
May	$105.00 \pm 8.54^{b(a)}$	$78.00 \pm 14.50^{c(b)}$	$104.0 \pm 10.10^{b(a)}$	$101.33 \pm 3.50^{b(a)}$	$117.67 \pm 15.3^{a(a)}$	**101.20**
Mean of means/site	**53.33**	**61.67**	**63.61**	**50.56**	**70.28**	

Note: Means having the same letter within the rows are not significantly different. Means having the same letter (in brackets) within the columns are not significantly different. Means were separated using DMRT at 95 % level of significance.

Table 3: Bacteria species isolated from Lake Nakuru, classified according to shape, gram staining and biochemical tests.

Bacteria species	**Gram stain**	**Citrate test**	**H_2S production**	**fermentation**	**Motility test**	**Enteric/ non enteric**	**Shape**
Bacillus anthracoides	+	-	-	+	+	Non enteric	Rods
Streptococcus pyogenes	+	-	-	-	-	Non enteric	spherical
Erwinia mallotivora	-	-	-	-	+	Enteric	Rods
Erwinia amylovora	-	-	+	+	+	Enteric	Rods
Sphingomonas paucimobilis	-	-	-	-	+	Non enteric	Rods
Morganella morganii	-	-	+	+	-	Enteric	Rods
Enterobacter or Pantonea agglomerans	-	-	-	+	-	Enteric	Rods
Yersinia pseudotuberculosis	-	-	-	+	-	Enteric	Rods
Chryseobacterium meningosepticum	-	-	-	-	-	Non enteric	Rods
Providencia stuarti	-	+	-	-	+	Enteric	Rods
Vibrio vulnificus	-	-	-	+	+	Non enteric	Comma
Pseudomonas cepacia	-	-	-	-	+	Enteric	Rods
Proteus penneri	-	-	-	-	+	Enteric	Rods
Erwinia nigrifluence	-	-	-	+	-	Enteric	Rods
Agrobacterium radiobacter	-	-	-	+	+	Non enteric	Rods
Providencia rettgeri	-	+	-	+	+	Non enteric	Rods
Alcaligen sp.	-	-	-	+	+	Enteric	Rods
Tatumella ptyseas	-	-	-	+	+	Enteric	Rods
Moraxella sp.	-	-	-	+	+	Non enteric	Spherical and rods
Chryseobacterium indologenes	-	-	-	-	-	Non enteric	Filamentous
Acinetobacter sp.	-	-	-	-	-	Enteric	Rods

Figure 2: Bacteria streaks showing a variation of bacteria growing on a petri

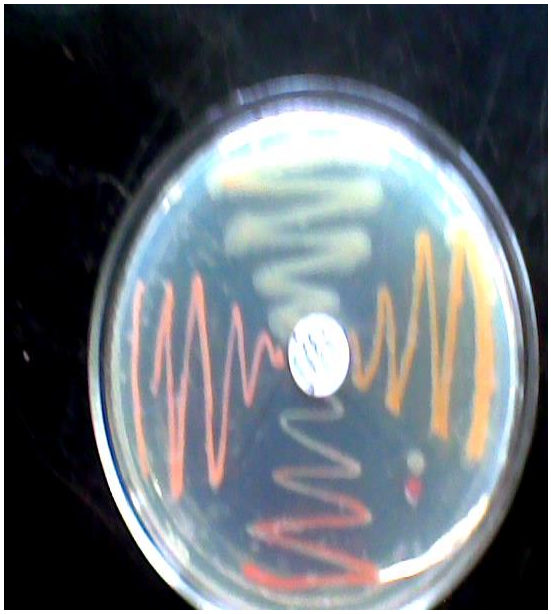

Figure 3: Bacteria streaks showing a variation of bacteria growing on a petri dish

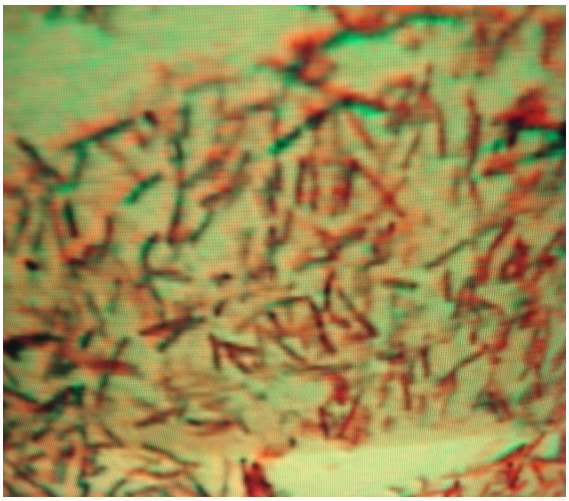

Figure 2: Gram Negative rods isolated from Lake Nakuru

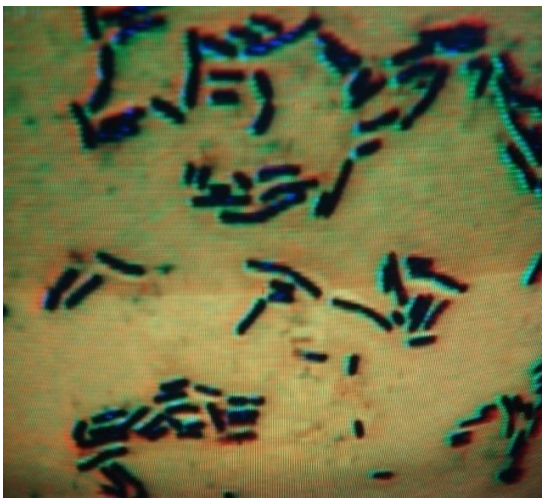

Figure 3: Gram positive rods isolated from Lake Nakuru

(Souce: Author, 2011)

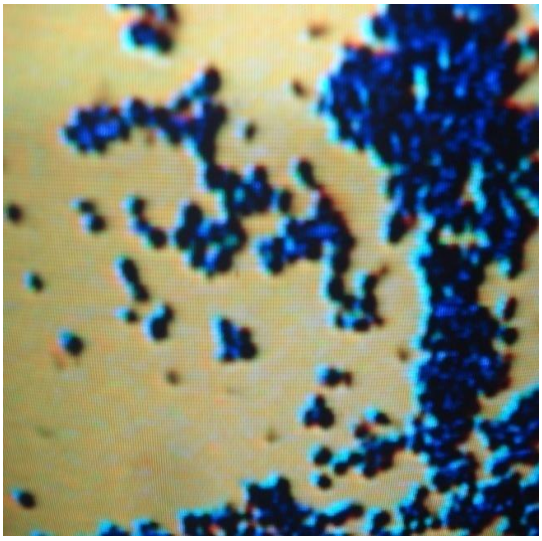

Figure 6: Gram positive coccus isolated from Lake Nakuru

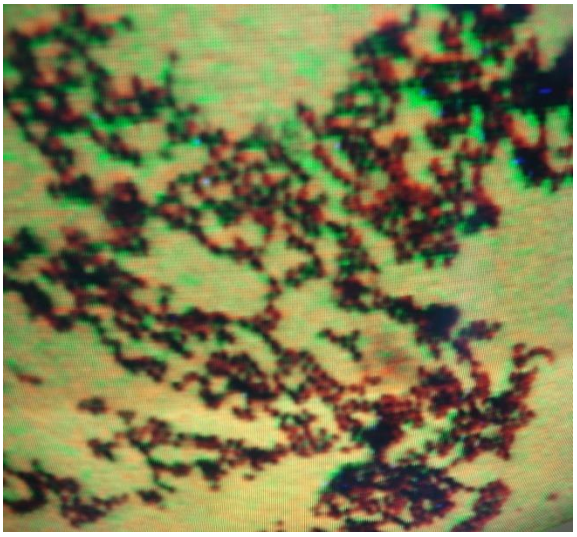

Figure 7: Gram negative coccobacillus isolated from Lake Nakuru

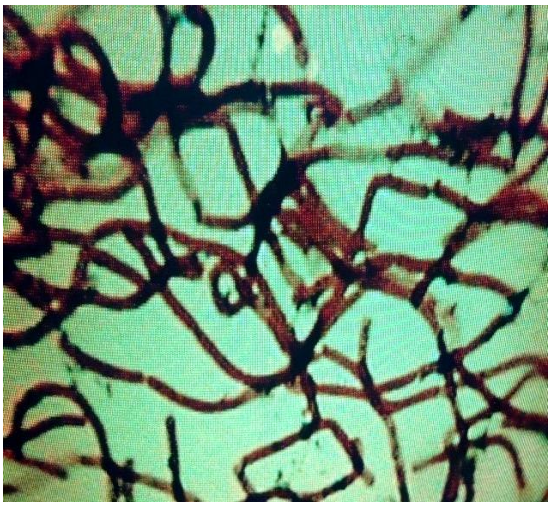

Figure 8: Filamentous, Gram negative bacteria isolated from Lake Nakuru

(Souce: Author, 2011)

4.3 Biodegradation of plastics disks by selected bacteria isolates from Lake Nakuru.

The percentage weight loss of white disposable plastic cups disks that was caused by the bacteria was calculated as the percentage weight loss that was obtained after subtracting the amount of weight lost by the plastic disks in the negative control. Table 4 shows the percentage degradation of plastics that had taken place after ninety days of degradation. The initial weight of plastic that was put in each experimental set up was 10 mg. The bacteria from mangrove soil that were put in the positive control had the highest percentage of plastic degradation with the plastic disks loosing 27.5% of the original weight (Table 4). These bacteria which were isolated from the mangrove soil included *Bacillus* sp., *Micrococcus* sp., *Staphylococcus* sp., and *Streptococcus sp.* The microorganism that caused the highest percentage of plastic degredation from Lake Nakuru was *Sphingomonas paucimobilis* with a percentage of 17.5%. *Erwinia nigrifluence* was also effective in degradation of plastic with weight loss of 12.5% of the original weight of plastics. Other bacteria that were identified as plastic degraders were *Streptococcus pyogenes (11.5%) Tatumella ptyseas (11%) Pseudomonas cepacia*, (9.5), *Chryseobacterium meningosepticum (8%), Erwinia Amylovora (7%) Moraxella sp., (6.5%) Bacillus anthracoides (6%), Providencia rettgeri (5.0%) Proteus penneri (4.50%), Chryseobacterium indologenes (3%), Morganella morganii (2%) Providencia stuarti (1.5%)* and *Alcaligen sp. (0.5%). Providencia stuarti* and *Alcaligen sp.* were slow degraders. *Yersinia pseudotuberculosis* and *Acinetobacter* species were unable to degrade plastics.

Table 4: **Mean weight (mg) and percentage weight loss of plastic disks after treatment with different species of bacteria isolated from Lake Nakuru.**

Name of bacteria species	Mean weight(mg)±SE after 90 days of degradation	Weight loss (mg) after 90 days	Percentage weight loss	percentage weight loss attributed to bacteria
Bacillus anthracoides	5.750 ± 0.0005	4.25	42.5	6.00
Y. pseudotuberculosis	6.350 ± 0.0005	3.65	36.5	0.00
Providencia rettgeri	5.850 ± 0.0005	4.15	41.5	5.00
Positive control	3.600 ± 0.0090	6.40	64.0	27.5
Providencia stuarti	6.200 ± 0.0040	3.80	38.0	1.50
Morganella morganii	6.150 ± 0.0015	3.85	38.5	2.00
S. paucimobilis	4.600 ± 0.0060	5.40	54.0	17.5
Acinetobacter sp.	6.350 ± 0.0015	3.65	36.5	0.00
Moraxella sp.	5.700 ± 0.0030	4.30	43.0	6.50
Erwinia amylovora	5.650 ± 0.0025	4.35	43.5	7.00
Negative control	6.350 ± 0.0005	3.65	36.5	0.00
Alcaligen spp.	6.300 ± 0.0100	3.70	37.0	0.50
Proteus penneri	5.900 ± 0.0090	4.10	41.0	4.50
C. meningosepticum	5.550 ± 0.0005	4.45	44.5	8.00
Pseudomonas cepacia	5.400 ± 0.0020	4.60	46.0	9.50
Erwinia nigrifluens	5.100 ± 0.0000	4.90	49.0	12.5
Tatumella ptyseas	5.250 ± 0.0015	4.75	47.5	11.0
C. indologenes	6.050 ± 0.0035	3.95	39.5	3.00
Streptococcus pyogenes	5.200 ± 0.0010	4.80	48.0	11.5

Note: Weight loss was calculated by subtracting the mean weight from the original weight (10mg) put in the set up. Weight loss attributed to bacteria was calculated by subtracting the weight lost by the negative control from the weight lost in each bacterium set up.

4.4 Biodegradation of Polythene disks by Selected Bacteria Isolates from Lake Nakuru.

The results in Table 4 show that the bacteria from the mangrove soil presented the highest percentage (50.5%) of degradation of polythene. These bacteria were *Bacillus sp., Micrococcus sp., Staphylococcus sp.* and *Streptococcus sp.* Among the bacteria isolated

from Lake Nakuru, *Sphingomonas paucimobilis* presented the highest percentage of polythene degradation (37.5%). *Pseudomonas cepacia* caused 35.5% polythene degradation. *Streptococcus pyogenes* and *Alcaligen sp*. had the same percentage of degradation (27.0%). *Tatumella ptyseas* degraded polythene by 21.5%. *Chryseobacterium meningosepticum* reduced the polythene weight by 19.5 % while *Proteus penneri* reduced the weight of polythene by 18%. Other bacteria from Lake Nakuru that were able to degrade polythene were *Providencia rettgeri (13.5%), Erwinia nigrifluence (11.5%), Providencia stuarti (5.50)* and *Chryseobacterium indologenes* (7.5%). *Erwinia amylovora* and *Acinetobacter sp.* were slow degraders as they had only degraded (1%) of plastics in 90 days. *Yersinia pseudotuberculosis* and *Morganella morganii* were unable to degrade polythene (0%).

Most bacteria were able to degrade polythene at a higher percentage than plastics (Figure 9). *Morganella morganii* degraded plastics though to a smaller degree but was unable to degrade polythene (Figure 9). *Yersinia pseudotuberculosis* was unable to degrade both plastics and polythene. *Sphingomonas paucimobilis, Erwinia nigrifluence, Tatumella ptyseas* and *Streptococcus pyogenes* showed high rates of degradation of both plastics and polythene (Figure 9). *Acinetobacter sp*. was able to degrade polythene but unable to degrade plastics (Figure 9). *Alcaligen sp*. indicated a high percentage of degradation of polythene but low percentage of degradation of plastics (Figure 9).

Table 5: Mean weight (mg) and percentage weight loss of polythene disks after 90 days of treatment with different species of bacteria isolated from Lake Nakuru.

Name of bacteria species	Mean weight(mg)±SE after 90 days of degradation	Weight loss (mg) after 90 days	Percentage weight loss	Percentage weight loss attributed to bacteria
Baccilus anthracoides	8.40 ± 0.0010	1.60	16.0	7.50
Y. pseudotuberculosis	9.15 ± 0.0005	0.85	8.50	0.00
Providencia rettgeri	7.80 ± 0.0110	2.20	22.0	13.50
Positive control	4.10 ± 0.0030	5.90	59.0	50.50
Providencia stuarti	8.60 ± 0.0050	1.40	14.0	5.50
Morganella morganii	9.15 ± 0.0005	0.85	8.50	0.00
S. paucimobilis	5.40 ± 0.0020	4.60	46.0	37.50
Acinetobacter sp.	9.05 ± 0.0005	0.95	9.50	1.00
Moraxella sp.	7.25 ± 0.0105	2.75	27.5	19.00
Erwinia amylovora	9.05 ± 0.0005	0.95	9.50	1.00
Negative control	9.15 ± 0.0005	0.85	8.50	0.00
Alcaligen spp.	6.45 ± 0.0045	3.55	35.5	27.00
Proteus penneri	7.35 ± 0.0005	2.65	26.5	18.00
C. meningosepticum	7.20 ± 0.0010	2.80	28.0	19.50
Pseudomonas cepacia	5.60 ± 0.0010	4.40	44.0	35.50
Erwinia nigrifluens	8.00 ± 0.0040	2.00	20.0	11.50
Tatumella ptyseas	7.00 ± 0.0150	3.00	30.0	21.50
C. indologenes	8.40 ± 0.0100	1.60	16.0	7.50
Streptococcus pyogenes	6.45 ± 0.0045	3.55	35.5	27.00

Note: Weight loss was calculated by subtracting the mean weight from the original weight (10mg) put in the set up. Weight loss attributed to bacteria was calculated by subtracting the weight lost by the negative control from the weight lost in each bacterium set up.

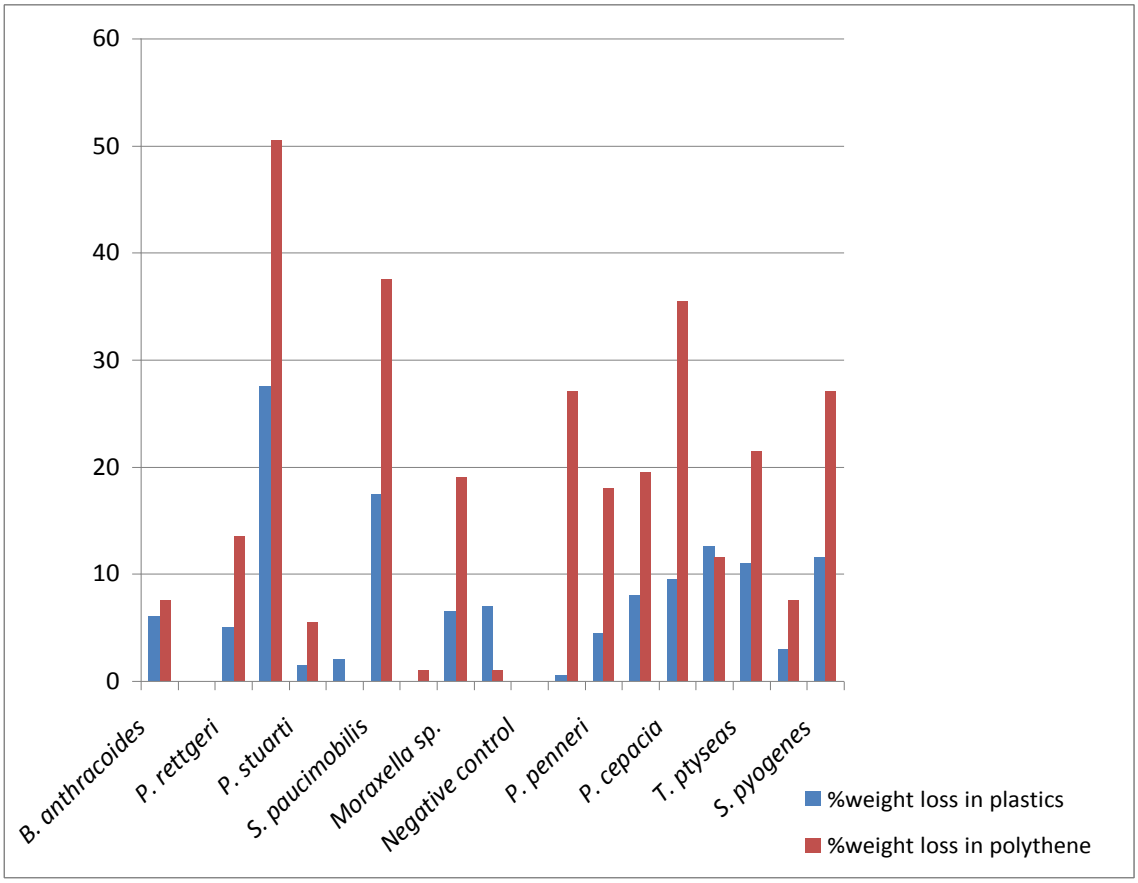

Figure 9: Comparison of the percentage weight loss in plastic and polythene disks by each bacterium species isolated from Lake Nakuru.

(Source Author, 2011)

4.5 Biodegradation of oil

All bacteria species showed a progressive reduction of the amount of oil in each set up (Table 6). The negative control also showed slight reduction of the greasy spot formed.

After one week, *Streptococcus pyogenes* and *chryseobacterium indologenes* caused a higher reduction of the greasy spot diameter than the rest with a diameter of 3.800 cm and 3.225 cm respectively (Table 6). The negative control also showed a slight change in the size of the greasy spot formed as it had a diameter of 4.950cm (Table 6). *Chryseobacterium indologenes* caused the highest change in the size of the greasy spot formed.

Providencia rettgeri, Chryseobacterium indologenes, and *Streptococcus pyogenes* caused a significant reduction in the size of greasy spot diameter after two weeks. *Providencia rettgeri* had a diameter of 2.250cm (Table 6). This was the smallest diameter overall. The bacteria in the positive control that were isolated from the Eldoret service station were also good in oil degradation as they had a diameter of 2.775 cm. These bacteria were *Bacillus sp., Pseudomonas sp*., and *Micrococcus sp.*

After three weeks the greasy spot diameter measurement showed that *Providencia rettgeri* was still the most active in oil degradation followed by *Chryseobacterium indologenes. Tatumella ptyseas* showed the least change in the size of the greasy spot formed (Table 6). All the bacteria showed a progressive decrease in the size of the greasy spot formed.

The greasy spot test taken after four weeks indicated gradual changes on the diameter size. *Providencia rettgeri, Chryseobacterium indologenes and Streptoccus pyogenes* had degraded a large amount of oil. *Providencia rettgeri* indicated the greatest change in the size of the greasy spot formed, as it had a diameter of 0.875. The negative control had a

diameter of 4.725cm of the greasy spot formed. *Chryseobacterium indologenes* had a diameter of 1.575cm (Table 6).

The fifth diameter measurement indicated that *Providencia rettgeri* had the smallest diameter measurement over all. Bacteria from vehicle service station, and *C. indologenes* showed less than one centimeter of the greasy spot formed. All bacteria had a diameter of less than 4 cm except *Chryseobacterium meningosepticum* which had a diameter of 4.050 cm.

The diameter measurements taken after six weeks indicated that P*rovidencia rettgeri, Chryseobacterium indologenes* and the bacteria from vehicle service station had degraded all the oil as there was no greasy spot formed. *Streptococcus pyogenes* and *Morganella morganii* were also good in oil degradation. *Streptococcus pyogenes* had a diameter of 1.425 cm while *Morganella Morganii* had a greasy spot diameter of 0.675. *Bacillus anthracoides*, *Yersinia pseudotuberculosis* and *moraxella sp*. had a diameter of less than 3 cm (Table 6).

At week seven the greasy spot test indicated that *Streptococcus pyogenes and Morganella Morganii* had a greasy spot diameter of less than 1cm. *Bacillus anthracoides* had a greasy spot diameter of 1.5 cm. All the bacteria had a greasy spot diameter of less than 3.5 cm (Table 6).

Table 6: Mean weekly diameter measurements (cm) of the greasy spot formed by the oil drop for each bacterium species.

Bacteria species	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
	1st diameter measurement	2nd diameter measurement	3rd diameter measurement	4th diameter measurement	5th diameter measurement	6th diameter measurement	7th diameter measurement
B. anthracoides	4.325±0.217[b]	4.200±0.178[b]	4.025±0.278[b]	3.125±0.301[b]	2.800±0.329[c]	2.525±0.335[c]	1.500±0.381[c]
Y. pseudotuberculosis	4.925±0.377[a]	4.375±0.450[b]	4.225±0.217[c]	3.725±0.170[b]	3.000±0.342[b]	2.975±0.371[b]	2.350±0.733[c]
P. rettgeri	4.275±0.214[c]	2.250±0.104[b]	1.950±0.220[d]	0.875±0.515[d]	0.325±0.320[d]	0.000±0.000[d]	0.000±0.000[d]
Positive control	4.300±0.173[b]	2.775±0.330[b]	2.625±0.450[d]	2.265±0.450[c]	0.525±0.197[d]	0.000±0.000[d]	0.000±0.000[d]
P. stuarti	4.100±0.070[c]	4.075±0.750[b]	3.975±0.225[b]	3.750±0.166[b]	3.600±0.910[b]	3.525±0.193[b]	3.300±0.212[b]
M. morganni	4.500±0.180[c]	4.150±0.086[b]	3.325±0.250[c]	2.300±0.122[c]	1.850±0.275[c]	0.675±0.312[d]	0.325±0.236[d]
S. paucimobilis,	4.375±0.085[b]	4.175±0.138[b]	3.925±0.286[b]	3.880±0.568[b]	3.775±0.144[b]	3.700±0.070[b]	3.400±0.168[b]
Acinetobacter sp.	4.475±0.206[b]	4.250±0.176[b]	4.000±0.187[b]	3.975±0.085[b]	3.850±0.119[b]	3.800±0.334[b]	3.425±0.189[b]
Moraxella sp.	4.400±0.168[b]	4.200±0.040[b]	3.950±0.064[b]	3.550±0.150[b]	3.475±0.125[b]	2.825±0.350[c]	2.600±0.668[c]
E. amylovora	4.725±0.229[b]	4.475±0.189[b]	4.225±0.125[b]	4.125±0.335[b]	3.825±0.338[b]	3.700±0.248[b]	3.350±0.185[b]
Negative control	4.950±0.189[a]	4.900±0.168[a]	4.850±0.210[a]	4.725±0.210[a]	4.575±0.155[a]	4.275±0.165[a]	4.225±0.165[a]
Alcaligen sp.	4.475±0.301[b]	4.175±0.315[b]	4.025±0.278[b]	3.650±0.132[b]	3.450±0.210[b]	3.350±0.222[b]	2.075±0.687[c]
P. penneri	4.900±0.158[a]	4.650±0.064[b]	4.225±0.217[b]	4.150±0.328[b]	3.925±0.075[b]	3.675±0.222[b]	3.370±0.111[b]
C. meningosepticum	4.800±0.248[ab]	4.325±0.131[b]	4.275±0.214[b]	4.100±0.231[b]	4.050±0.266[b]	3.600±0.286[b]	2.675±0.373[c]
P. cepacia	4.875±0.125[a]	4.300±0.108[b]	4.200±0.265[b]	3.925±0.269[b]	3.625±0.304[b]	3.400±0.300[b]	2.750±0.366[c]
E. nigrifluence	4.300±0.187[b]	4.000±0.212[b]	3.975±0.225[b]	3.775±0.029[b]	3.475±0.502[b]	3.300±0.168[b]	3.200±0.456[b]
T. ptyseas	4.725±0.111[b]	4.600±0.108[b]	4.500±0.108[b]	4.050±0.104[b]	3.800±0.268[b]	3.450±0.104[b]	2.900±0.212[c]
C. indologenes	3.225±0.175[d]	2.575±0.440[d]	2.125±0.568[d]	1.575±0.421[c]	0.950±0.210[d]	0.000±0.000[d]	0.000±0.000[d]
S. pyogenes	3.800±0.141[c]	3.500±0.178[c]	3.425±0.189[c]	2.125±0.111[c]	1.950±0.333[c]	1.425±0.878[c]	0.450±0.287[d]

Note: Means with similar letters within a column are not significantly different at P < 0.05. Means were separated using DMRT test.

Analysis of variance indicated that *Bacillus anthracoides, Yersinia pseudotuberculosis, Providencia rettgeri,* bacteria from vehicle service station, that is, *Bacillus sp., Pseudomonas sp.,* and *Micrococcus sp, Providencia stuarti, Morganella morganii, Sphingomonas paucimobilis, Moraxella sp., Alcaligen sp., Proteus penneri, Chryseobacterium meningosepticum, Pseudomonas cepacia, Tatumella ptyseas, Chryseobacterium indologenes and Streptococcus pyogenes* were significant in oil degradation. *Acinetobacter sp., E. amylovora and E. nigrifluence* were unable to degrade oil. The P value of these bacteria were higher than 0.05, hence not significant. *B. anthracoides, P rettgeri, Bacillus sp., Pseudomonas sp., Micrococcus sp., M. morganii, P. cepacia, T. ptyseas, C. indologenes and S. pyogenes* had a P value of *0.000. P. penneri* had a P value of 0.001, *Alcaligen sp. and Y. pseudotuberculosis* had a P value of 0.002, *Moraxella sp,* had a P value of 0.003, *P. stuarti* had a P value of 0.005, *and S. paucimobilis* had a P value of 0.011.

The post hoc test on the degradation of oil experiment indicated that there was significant difference between means of the bacteria species for the seven weeks. The variation was caused by *Chryseobacterium indologenes, Morganella morganii, Providencia rettgeri,* bacteria in positive control and *Streptococcus pyogenes.* These bacteria had a higher degree of degradation than the rest of the bacteria. The variation was also caused by *Acinetobacter sp.* and *Erwinia amylovora* which were unable to degrade oil.

CHAPTER FIVE

DISCUSSION

5.1 Bacterial load

Lake Nakuru was found to have abundant bacteria which were diverse in all the sampling points. According to the results obtained in this study, there was a significant difference in the number of bacteria (CFU) sampled at different sampling months. The months of March and May had more bacteria (CFU) than the rest of the sampling period. During these two months, the amount of rainfall at Nakuru town and its environs was higher than the rest of the sampling periods. Probably this contributed to the higher bacterial load due to the run offs from the Lake catchment area that ended up in the Lake leading to an increase in the the number of bacteria. The fresh water from run offs tend to lower salinity levels hence abundance of bacteria species (Melack, 1988). Eutrophication may have also lead to an increase in the bacterial load.

The month of February had the lowest bacterial load. During this month, the amount of rainfall was low. According to Livingstone, (2000) biological communities in shallow tropical saline Lakes are susceptible to slight variations in water balance and salinity. Periods of low rainfall leads to increase in salinity hence reduction in abundance of aquatic organisms (Melack, 1988). There were no run offs to the Lake hence no bacteria nor nutrients were added to the Lake from the Lake environs. Probably, decrease in the Lake nutrients lead to the decrease in the bacterial load.

Njoro had the highest bacterial load throughout the sampling period. The high bacterial load at Njoro site was probably due to the catchment area of river Njoro which has various activities including horticultural and industrial activities. Also run offs joining river Njoro comes from various towns including Njoro and Nakuru town. Run offs from these towns probably brought more bacteria into the Lake.

Middle Lake had the lowest bacterial load through out the sampling period. This was probably due to minimal disturbance from animals and runoffs. Rain water is the only water that enters directly at the middle of the Lake. The Lake has inlets and no outlets hence the microorganisms that come into the Lake through the inlets remain in the Lake or die if they are unable to adapt to the Lake's environment. Makalia, Enderit and Hippopoint had more bacteria load than middle Lake. Makalia and Enderit are at the mouths of river Makalia and river Enderit respectively. The catchment areas of these rivers probably had more bacteria which were carried by the run offs to the rivers and finally to the Lake leading to high bacterial load at these sites.

Hippopoint site was the home for hippopotamus, and this could have contributed to the higher bacterial load at this site. The Hippo site is likely to have more bacterial load as there are bacteria that live in the hippos as normal flora and also hippos are likely to transfer bacteria from the neighbouring habitat to the water. Also, the effluents from Nakuru sewage (old sewage plant) are directed into the northern part of the Lake which is near the Hippopoint site. These Effluents probably brings more bacteria in the Lake (Vareshi, 1982).

5.2 Diversity of bacteria in Lake Nakuru

The results from this study show that Lake Nakuru has a rich diversity of bacteria. This may be due to diverse ecological niche of the Lake. Twenty one species of bacteria were identified. They had different morphological and biochemical characteristics. Some were motile while others were non-motile. There were bacillus, coccus, coccobacillus, vibrio and filamentous bacteria. Some produced hydrogen sulphide gas while others did not. Some of the bacteria were inhabitants of the Lake as they are known to be found in various habitats including salty waters for example *Vibrio vulnicus*. Other bacteria were likely to be in the Lake as a result of pollution for example *Streptococcus pyogenes.* Vareshi, (1982) had reported that the ecosystem in the Lake may have changed due to pollution.

Chryseobacterium meningosepticum was among the isolates from Lake Nakuru. This bacterium is an inhabitant of Lake Nakuru as it has been reported to be widely distributed in nature, in fresh and salty water and also in soil by Murrey *et al*., (2007). The presence of *Providencia stuarti* in the Lake may be due to the sewage that is directed to the Lake after treatment or due to the run offs from Nakuru town. This bacterium is commonly found in soil, water, and sewage and is a human pathogen (Lin *et al.,* 2008). Vareshi, (1982) reported that the potential danger of pollution from the effluents of the two sewages in Nakuru town is suspected but it was not clear whether this pollution was responsible for the change of the ecosystem in the Lake.

Erwinia mallotivora is a plant pathogen which causes papaya die back (Garden *et al*., 2004). Its presence in the Lake may be due to the surface run offs from the catchment areas of the rivers feeding the Lake where farming is carried out. *Chryseobacterium indologenes* was the only filamentous bacteria observed. It is found in the soil, plants, food, sweet, salty and potable water (Doiz *et al*., 1999). It is widely distributed in nature and is a rare human pathogen (Hsueh *et al*., 1996). It is an inhabitant of Lake Nakuru since it has been reported to be found in salty water.

Yersinia pseudotuberculosis had bacillus morphology. It is reported to occur widely in various host species, including dogs, cats, horses, cattle, rabbits, deer, rodents, and birds (Bronson, 1972). Its presence in the Lake may be from the wild animals that are present in the Lake environs e.g. primates (baboons) or due to the sewage that is directed to the Lake after treatment. Documented cases of occupational exposure to *Y. pseudotuberculosis* include butchers working in abattoirs slaughtering swine (Iwata *et al.*, 2008). Outbreaks of *Y. pseudotuberculosis* in animal facilities have occasionally been reported, including in primate facilities (Iwata *et al.*, 2008). *Streptococcus pyogenes* was observed to occur as long chains of cocci. Ryan *et al*., (2004) reported that it causes streptococcal infections. Its likely source is the sewage which is disposed in the Lake after treatment or from surface run offs from Nakuru town.

Sphingomonas paucimobilis has been found in various environments, including fresh and seawater, terrestrial habitats, plant root systems and clinical specimens (Nilgiriwala, 2008). Sphingomonas is normally widely spread in the environment hence an inhabitant

of the Lake. Its widespread distribution in the environment is due to its ability to utilize a wide range of organic compounds and to grow and survive under low-nutrient conditions (Nilgiriwala, 2008).

Vibrio vulnificus is an inhabitant of the Lake as it is normally found in marine environment, estuaries, brackish pond or coastal area (Olivier, 2005). Murrey *et al.* (2007) reported that it normally lives in warm seawater and is part of a group of vibrios that are called "halophilic" because they require salt. It is a human pathogen. *Morganella morganii* is an inhabitant of Lake Nakuru. It is also commonly found in the mammals and reptiles that are found in the park (Lipuma 2005). Grierson, (1955) showed that it is commonly found in the environment and in the intestinal tracts of humans, mammals, and reptiles as normal flora. It has two subspecies *M. morganii* and *M. sibonii* (Kilcoyne *et al.,* 2002). *Burkholderia cepacia,* formerly known as *providencia cepacia* is an inhabitant of Lake Nakuru. This bacterium is found in water and soil and can survive for prolonged periods in moist environments (Lipuma, 2005).

Acinetobacter sp. was observed to have bacillus morphology. It is an important soil microorganism because it contributes to the mineralization of aromatic compounds (Gerischer, 2008). It is an inhabitant of the Lake as it is readily found throughout the environment including drinking and surface waters, soil, and sewage and in various types of foods. *Pantoea agglomerans* which was formerly known as *Enterobacter agglomerans* can be isolated from feculent material, plants, and soil where it can be either pathogen or commensal (Becton 2003) hence an inhabitant of Lake Nakuru. *Erwinia amylovora*

causes fireblight to apple and pear trees (Holt *et al.,* 1994). Its presence in the Lake may be due to the surface run offs joining river Njoro coming from areas where horticulture farming is practiced.

In this study, the biochemical test results of *Erwinia nigrifluens* were in agreement with the findings of Wilson, (1987) who also reported that the rods are slightely pointed at the end and can be curved. Its presence in the Lake may be due to run off from cultivated catchments areas of the various rivers feeding the Lake.

Agrobacterium radiobactor is a soil microorganism (Sawada *et al*., 1993). It is used as a biopesticide on nursery stock to prevent crown gall caused by *agrobacterium tumefaciens*. Its presence in the Lake may be due to the run off from river Njoro catchment area where horticultural farming is practiced. *Providencia rettgeri* biochemical test results from the current study were in agreement with the findings of Baron, (1996) who also reported that it can be distinguished from other common *Providencia* species such as *P. alcalifaciens* and *P. stuarti* through biochemical tests such as urease test which is positive (+) and phenylalanine deaminase test.

Bacillus anthracoides were observed to occur in pairs or short chains. Grierson *et al.,* (1955) reported that they are found in animal hair. Their presence in the Lake may be from the wild animals found in the park. *Alcaligenes sp.* is an inhabitant of the Lake. It has been reported by Anderson, (2003) to be found in various locations, including soil and aquatic environments; *Alcaligenes denitrificans* has been known to inhibit the growth

of certain types of algae. *Bordetella pertussis* natural habitat is in the human respiratory mucosa and humans are the only host (Todar, 2005). It is the causative agent of whooping cough. It is used to develop a vaccine for whooping cough (Burnette *et al.*, 1992). Its presence may be due to the sewage that is normally released to the Lake after treatment. *Tatumella ptyseas* and *Moraxella sp.* are inhabitants of the Lake. Most of the bacteria identified are human, animal or plant pathogens but despite this, they are important in degradation and bioremediation.

5.3 Degradation of plastics and polythene

This study revealed that the waters of Lake Nakuru are a good source of bacteria capable of degrading plastics and polythene. There were visible changes in the plastic and polythene materials which indicated that the bacteria had degraded them. The surface was rough and the plastic disc appeared dull in colour. In the control, there was no observable change. Ikada, (1999) reported that parameters of visual changes can be used as a first indication of any microbial attack. Studies that have been carried out by other researchers show that various microorganisms are able to degrade polythene and plastics. According to kathiresan, (2003) *Streptococcus* sp., *Pseudomonas* sp., and *Moraxella* sp. were able to degrade polythene and plastics. *Bacillus sp.* was able to degrade polythene but unable to degrade plastics. In this study these bacteria, including *Bacillus* sp. were able to degrade both plastic and polythene.

Sphingomonas paucimobilis caused the highest percentage weight loss which is attributed to degradation for both plastics (17.5%) and polythene (37.5%). This bacterium can be

used in bioremediation and biodegradation. *S. paucimobilis* occurs in various enviroments hence easy to isolate and culture for remediation purpose. *S. paucimobilis* is metabolically versatile, which means it can utilize a wide range of naturally occurring compounds as well as some types of environmental contaminants. Burd, discovered that *Sphingomonas* can degrade over 40% of the weight of polythene in less than three months (http://www.mnn.com/green-tech/research-innovations). Studies have been held to further explore its metabolic mechanisms for application in biotechnology, in addition to its current utilization in bioremediation and in the food technology. *Sphingomonas paucimobilis* is able to degrade lignin-related biphenyl chemical compounds (Nilgiriwala, 2008). According to Ni'matuzahroh *et al.,* (1999), *Sphingomonads* have been utilised for a wide range of biotechnological applications, from bioremediation of environmental contaminants to production of extracellular polymers such as sphingans e.g. gellan, wellan, and rhamsan which are used extensively in the food and other industries due to their biodegradative and biosynthetic capabilities. One strain, *Sphingomonas* sp. 2MPII, can degrade 2-methylphenanthrene. Apart from being important in bioremediation, it is also clinically important since *Sphingomonas paucimobilis* is known to play a role in human disease, primarily by causing a range of nosocomial non-life-threatening infections that are treated by antibiotic therapy (Nilgiriwala, 2008).

Streptococcus pyogenes was able to degrade both plastics (11.5%) and polythene (27.0%). According to Kathiresan, (2003) *Streptococcus sp.* was able to degrade polythene at 2.19% and 1.07% of plastics per month. It can be used for the elimination of these two pollutants. It is also applied in biotechnology whereby many of its proteins are

known to have unique properties, which have been harnessed to produce a highly specific "superglue" (Zakeri, 2012) and a route to enhance the effectiveness of antibody therapy (Baruah, 2012). *Streptococcus pyogenes* is also clinically important as it is the cause of many important human diseases, ranging from mild superficial skin infections to life-threatening systemic diseases (Ryan, 2004).

Alcaligen sp. was able to degrade polythene at a high percentage (27%) and plastics at low percentage (0.5%). This may be due to the molecular weight of plastic which is higher than that of the polythene. This bacterium was able to degrade polythene at a higher percentage probably due to its low molecular weight. The surface area of the plastic exposed to this bacterium was smaller compared to the surface area of polythene that was exposed to it. The surface area of the material being degraded that is exposed to the bacteria affects the percentage of degradation. The more the surface area exposed to the bacteria, the higher the rate of degradation (Goldberg, 1995). *Alcaligen sp.* is known to be used in remediation of environmental pollutants. According to Anderson (2003), *A. faecalis* converts the most toxic form of arsenic, arsenite (AsO_2^-) to its less dangerous form, arsenate (AsO_4^-). *Alcaligenes* has been used for the industrial production of non standard amino acids. *Alcaligen eutrophus* also produces the biopolymer polyhydroxybutyrate (PHB). Species of *Alcaligenes* generate energy in a number of ways, including arsenite oxidation. This species can be used to clean up environments contaminated with polythene.

Acinetobater sp. was able to degrade polythene (1%) but unable to degrade plastics. This may be attributed to the thickness of plastics that was higher than that of the polythene and also to the surface area of polythene exposed to the bacteria compared to the plastics. This species is metabolically versatile and hence can be exploited in various biotechnological applications including biodegradation and bioremediation (Gutnik, 2008). Gerischer (2008) reported that many of the characteristics of *Acinetobacter* ecology, taxonomy, physiology, and genetics point to the possibility of exploiting its unique features for future applications. *Acinetobacter* strains are often ubiquitous and robust (Gutnik, 2008). Some provide convenient systems for modern molecular genetic manipulation and subsequent product engineering. These characteristics are being exploited in various biotechnological applications including novel lipid and peptide production, enzyme engineering, biosurfacant and biopolymer production and engineering of novel derivatives of these products. It is anticipated that progress in these fields will broaden the range of applications of *Acinetobacter* for modern biotechnology (Gutnik, 2008).

Burkhoderia cepacia formerly, *Providencia cepacia* was able to degrade plastics (9.5%) and polythene (35.5%). This bacterium can be used in the remediation of environment polluted by these wastes. This bacterium is found in water and soil and can survive for prolonged periods in moist enviroments hence easy to isolate and culture for remediation purpose. This bacterium is also clinically important as it is a human pathogen which most often causes pneumonia in immunocompromised individuals (Mahenthiralingam, 2005).

Erwinia nigrifluens and *Tatumella ptyseas* were able to degrade plastics and polythene (12.5%, 11%) and (11.5%, 21.5%) respectively. These bacteria can be used for remediation of plastic and polythene polluted environments. Apart from being important in biodegradation they are also clinically important. *Erwinia nigrifluens* is the causative agent of shallow bark canker of walnut (Wilson *et al.,* 1957), while *Tatumella ptyseas* is a human pathogen, (Berka, 2001)

Moraxella sp. was able to degrade plastics (6.50%) and polythene (19.0%). Kathiresan, (*2003*) reported that *Moraxella sp.* was able to degrade 7.75% of polythene and 8.16% of plastic per month. This bacterium can be utilized in bioremediation of the environment from these pollutants. *Proteus penneri, Chryseobacterium meningosepticum* and *Pseudomonas cepacia* were also good in degradation of both plastic and polythene. Apart from being important in biodegradation, *Chrysebacterium meningosepticum* is also clinically important as it causes opportunistic infections in immunocompromised patients (Murrey *et al.,* 2007). *Pseudomonas cepacia* degraded plastic (9.50%) and polythene (35.50%). Kathiresan, (2003) reported that *Pseudomonas sp*. degraded polythene at 20.54% and plastics at 8.16% per month. It is typically found in water and soil hence easy to isolate and culture for remediation purposes.

Yersinia pseudotuberculosis was unable to degrade both plastics and polythene whereas *Morganella morganii* was unable to degrade polythene. However *Y. pseudotuberculosis* was good at oil degradation. It is also clinically important as it causes infections in humans with tuberculosis-like symptoms.

The bacteria in the positive control were the best in degradation of plastic and polythene. These bacteria were *Bacillus* sp., *Staphylococcus* sp., *Streptococcus* sp and *Miccrococcus* sp. The high percentage of degradation in the positive control was probably due to the combined degradation effect of these bacteria. Combining the bacteria isolated from Lake Nakuru that caused significant degradation in one experimental set up may probably accelerate the degradation process. The degradation caused by each individual bacterium in the positive control was not determined. These bacteria were preserved for further identification upto species level and for further studies in degradation.

The type of microorganism affects the rate of degradation of polymer (Artham and Doble, 2008). Hence there were different rates of degradation of both plastics and polythene depending on the species of the bacteria present. Different bacteria species were able to degrade plastic and polythene at different rates although they had received the same amount and type of nutrients. *Sphingomonas paucimobilis* had the highest percentage of degradation compared to the rest of the bacteria species. This bacterium is known to be metabolically versatile since it can utilize a wide range of compounds as well as pollutants. It was able to metabolise the plastics and polythene at a higher percentage than the rest of the bacteria. Other microorganisms that were good in degradation of both plastic and polythene were *Streptococcus pyogenes, Tatumella ptyseas, Pseudomonas cepacia, Erwinia nigrifluence, Chryseobacterium meningosepticum* and *Moraxella* sp.

The surface area of the plastic and polythene exposed to microorganism affects the rate of degradation. The more the surface area exposed, the higher the rate of degradation

(Goldberg, 1995). The higher rate of degradation by bacteria on the polythene bags may be attributed to the surface area exposed to the bacteria. More polythene disks were put in the experiment set up than the number of plastic disks to achieve the same mass. Hence more surface area of polythene was exposed to the bacteria than that of plastics hence leading to a higher percentage of degradation of polythene than that of plastics.

Generally, increase in molecular weight of the polymer decreases its degradability by microorganisms. High molecular weight results in a decrease in solubility making them unfavourable for microbial attack. This is because bacteria require the substrate to be assimilated through the cellular membrane and then be further degraded by cellular enzymes (Gu *et al.,* 2000). Plastics have a higher molecular weight and are thicker than polythene. In this experiment, this might have contributed to the higher weight loss of the polythene than that of plastic for each bacteria species. The weight loss of polythene was higher than that of plastics for each bacteria species except for *Erwinia spp.* and *Morganella morganii*. *Erwinia spp*. had a higher weight loss of plastics than polythene while *Morganella morganii* was unable to degrade polythene. These bacteria could be having enzymes that are more specific to the degradation of the compounds found in the plastic cups or in the polythene bags.

Polymer degradation is a change in the polymer properties such as tensile strength, colour, shape or molecular weight under the influence of one or more environment factors such as light, chemicals, and in some cases by galvanic action (Faudree, 1991). Degradation is due to the scission of polymer chain via hydrolysis leading to a decrease

in the molecular mass of the polymer. The initial breakdown of a polymer can result from a variety of physical, chemical and biological forces with chemical hydrolysis being the most important. Embrittlement which is one of the physical forces initiating degradation of polymers is activated primarily by sunlight or heat (Goldberg, 1995). These leads to reduction of molecular weight of the polymer and hence increase in the microbial accessibility (Ghazali, 2004).

In this study, initial reduction of molecular weight is attributed to the physical and chemical forces which were activated by heat generated during shaking. These results were in agreement with the findings of Doi, (1990). The moleculer weight reduction may have been as a result of carboxyl group oxidation. Oxidation starts at tertiary carbon atoms because the free radicals formed are more stable and long lasting making them more susceptible to attack by oxygen (Faudree, 1991). Discolouration on the surface may have been as a result of the deposition of carbonates from the salts added. Lapishin *et al.*, (2010) reported that salts cause the discolouration effect on plastic during the process of degradation. Chlorine gas also has an oxidizing effect on polymer. This gas attacks sensitive parts of the chain molecules, oxidizing the chain cleavage. Cracks can be formed by Ozone.

In the plastic experiment, 36.5% weight reductions occurred in the negative control. This may be attributed to physical and chemical forces (Faudree, 1991). In the polythene experiment, 8.5% weight reduction occurred in the negative control. This is attributed to the physical and chemical forces of degradation (Goldberg, 1995).

5.4 Oil degradation

There was a lag time before the process of degradation of oil commenced. The lag time may have allowed for the evaporation of the initial toxicity of the volatile fraction of oils and also increase in bacteria population. This was in agreement with the findings of Pothuluri, (1994) who reported that microbial populations must begin to use the oil and expand their population before measurable degradation takes place, a period usually lasting several days. Application of nitrogen can stimulate the bioremediation of oil contaminated sites (Drozdowicz *et al.,* 2002). Atlas, (1981) reported that in aerobic oil degradation process, the hydrocarbon in oil is converted to carbon dioxide and water by bacteria. The initial step in aerobic biodegradation involves the oxidation of the substrate by oxygenases for which molecular oxygen is required. Hoff, (1993) reported that nitrogen and other essential minerals need to be provided in order to provide the bacteria with the necessary minerals.

Providencia rettgeri presented the highest percentage of degradation of petroleum oil. This bacterium was found to be useful not only in oil degradation but also in the degradation of polythene and plastics. Apart from bioremediation, this bacterium can also be used as a biological pest control agent (Jackson *et al*., 2004).

Alcaligenes sp. was able to degrade the oil significantly. This bacterium can be used in bioremediation of oil contaminated sites. Drozdowicz *et al*., (2002) reported that two bacterial strains which belong to the genera Agrobacterium and Alcaligens exhibited the ability to degrade gasoline aromatic compounds and fix nitrogen at the same time.

Other bacteria that were able to degrade oil were; *Morganella morganii*, *Tatumella ptyseas, Bacillus anthracoides, Chryseobacterium indologenes, Streptococcus pyogenes, Chryseobacterium meningosepticum, Pseudomonas cepacia, Proteus penneri, Alcaligen sp., Yersinia pseudotuberculosis, Moraxella sp., Providencia stuarti, and Sphingomonas paucimobilis.* These bacteria can be used to remediate the environment from oil contaminants. Results for the positive control shows that the bacteria in this soil were able to degrade oil. *Erwinia nigrifluence, Erwinia amylovora and Acinetobactor sp.* were unable to degrade oil. The results for *Acinetobacter sp.* were in contrast with what was reported by Head *et al.,* (2003). According to his experiment, *Acinetobacter sp.* was able to degrade oil.

The following families are known to degrade oil; Chryseobactericeae, Bacillaceae, Staphylococcaceae, Sphingomonadaceae, Alcaligenaceae, Burkholderiaceae, Pseudomonadaceae and Moraxellaceae. From this experiment, species from the above mentioned families were able to degrade oil. Apart from these families, other genera that have not been reported in oil degradation were *Providencia, Tatumella, Streptococcus* and *Yersinia.*

Burkhoderia cepacia formerly known as *Providencia cepacia* was able to degrade oil, polythene and plastics. This bacterium is known to metabolise chlorinated hydrocarbon. These hydrocarbons are commonly found in commercial pesticides and herbicides. This bacterium can be added to sites that are contaminated by these toxins to clean up the

environment. *Burkhoderia cepacia* is one of the most effective bacteria in degrading the chemicals found in house hold herbicide (weed-B-Gone) (Springer, 1992).

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

There were variations in bacterial load within the sampling periods and sites. The month of May had the highest bacterial load while the month of February had the lowest bacterial load. Njoro and Hippo point sites had higher bacterial load than the rest of the sites.

Lake Nakuru has high diversity of bacteria that are of economical and ecological importance. In this study, sixteen genera were isolated and they included; *Proteus* sp., *Tatumella* sp., *Acinetobacter* sp., *Pseudomonas* sp., *Providencia* spp., *Enterobacter* sp., *Morganella* sp., *Erwinnia* spp., *Alcaligen* sp., *Yersinia* sp., *Chrysobacterium* spp., *Agrobacterium* sp., *Vibrio sp., Streptococcus* sp., *Moraxella* sp., *and Bacillus* sp.

There were variations in the ability to degrade plastic cups and polythene bags disks among the bacteria species isolated. Bacteria that were able to degrade plastics cups and polythene bags disks included; *Sphingomonas paucimobilis, Erwinia nigrifluence, Streptococcus pyogenes, Tatumella ptyseas, Pseudomonas cepacia, Providencia stuarti, Chryseobacterium meningosepticum, Erwinia amylovora, Moraxella* sp., *Bacillus anthracoides, Providencia rettgeri, Proteus penneri* and *Chryseobacterium indologenes. Sphingomonas paucimobilis* was the best plastic and polythene degrader.

Bacteria that were able to degrade oil included *Providencia rettgeri, Morganella morganii, Tatumella ptyseas, Bacillus anthracoides, Chryseobacterium indologenes, Streptococcus pyogenes, Chryseobacterium meningosepticum, Pseudomonas cepacia, Proteus penneri, Alcaligene* sp., *Moraxella* sp., *Providencia stuarti* and *Sphingomonas paucimobilis*. *Providencia rettgeri* was the best in oil degradation.

Some bacteria were able to degrade plastic cups, polythene bags and oil. They included *Sphingomonas paucimobilis, Streptococcus pyogenes, Pseudomonas cepacia, Moraxella* sp, *Providencia rettgeri, Chryseobacterium meningosepticum, Chryseobacterium indologenes, Providencia stuarti Tatumella ptyseas, Proteus penneri* and *Alcaligene* sp. Among these bacterial species *Tatumella ptyseas, Proteus penneri* and Providencia *stuarti* are new novel species identified in this study.

6.2 Recommendations

The bacteria in Lake Nakuru are important in biodegradation. Majority of the isolated species can be used in the elimination of a wide range of pollutants such as plastics and petroleum oil.

The mechanism of degradation is not known. The surface of plastic materials had turned from smooth to rough with cracking. This may be due to the compounds secreted extracellularly by the bacteria that may break the complex molecular

structure of plastics. Hence, further study on bacterial enzymes or organic acids that may be involved in degradation of the polythene and plastics will pave way for finding technology for degrading the plastic and polythene materials, which are otherwise hazardous to environment.

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APPENDICES

Appendix I: Bacteria growth in petri dishes.

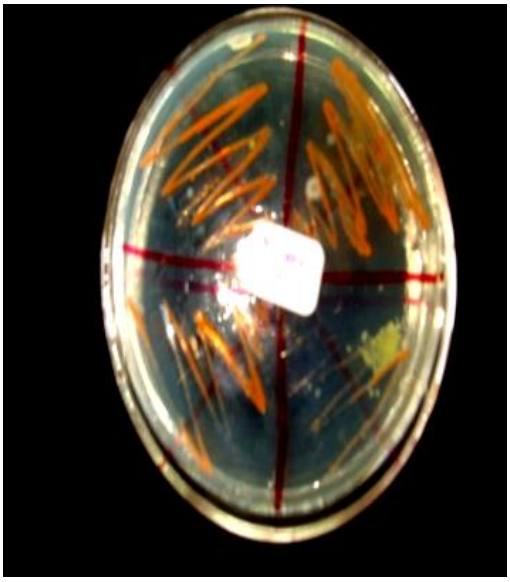


Plate 1: Subcultures of bacteria

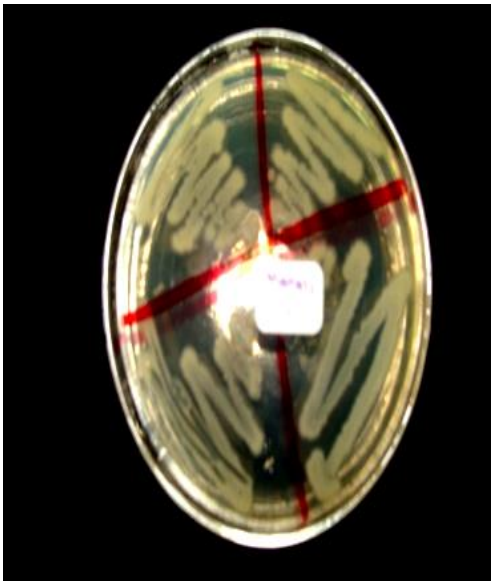


Plate 2: Subcultures of bacteria

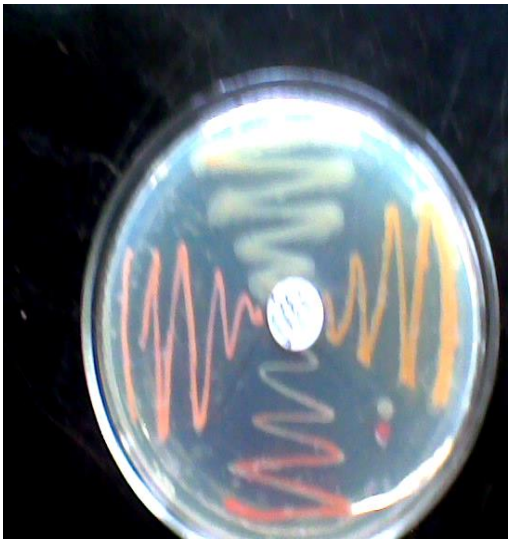
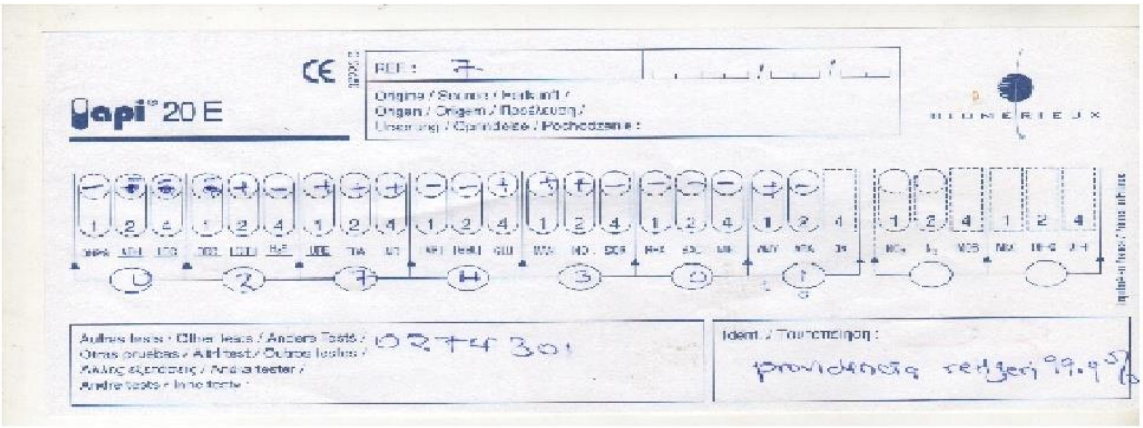


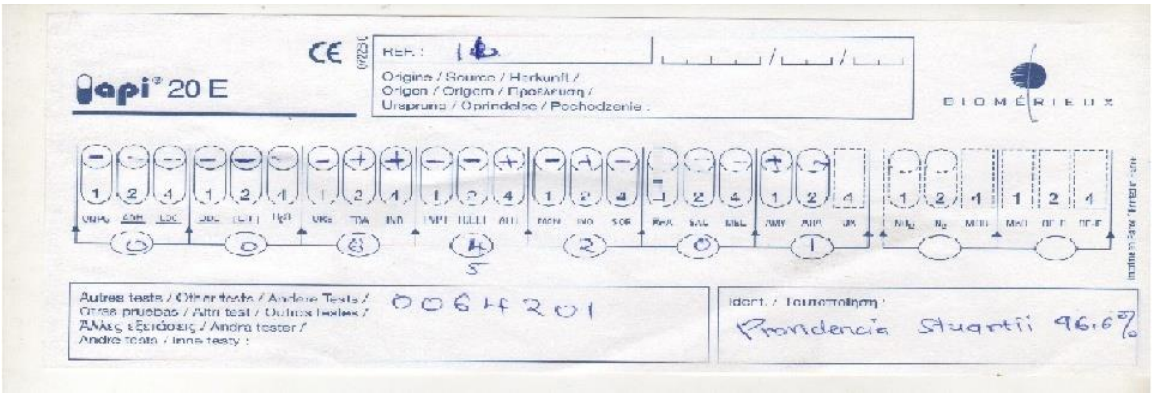
Plate 3: Subcultures of bacteria

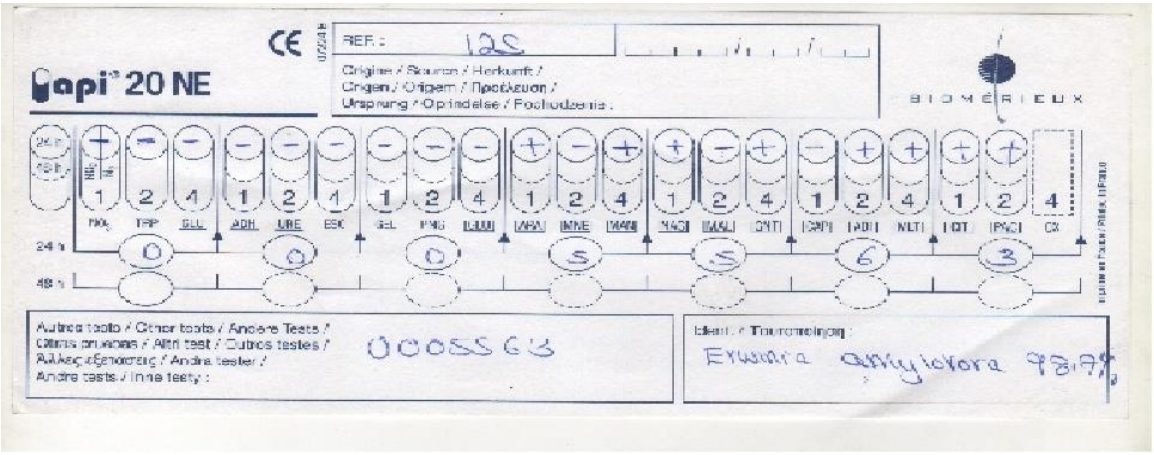
Plate 4: Subcultures of bacteria

(Source: Author, 2011)

Appendix II: Analytical Profile Index (API) scoring sheets







Appendix III: The species isolated and identified using API kits, the book identification number and the level of confidence specified in the book.

Species identified	Book identification no. as per API kits	Level of confidence on identification as specified in the book (in %)
Proteus penneli	0020000	97
Tatumella ptyseas	0000020	99
Acinetobacter spp	0006002	87.4
Pseudomonas cepacia	1006021	92.8
Providenicia stuarti	0261100	97.9
Enterobactor agglomerans	1025132	99.3
Morganella morganii	0374000	95.8
Erwinia amylovora	0002000	98.6
Erwinia nigrifluens	0007173	87.9
Bordetella/Alcaligens spp	0023021	81.6
Providencia rettgeri	1277573	99.9
Erwinia Mallotivora	0002000	98.6
Yersinia pseudotuberculosis	0215120	83.8
Chryseobacterium indologenes	3730000	90
Agrobacterium radiobacter	1730000	99.9
Vibrio vulnificus	7710000	99.9
Chryseobacterium meningosepticum	0730000	99.8
Streptococcus pyogenes	5100000	99.9
Sphingomonas paucimobilis	1001021	90.7
Moraxella sp.	0010000	87.7
Bacillus anthracoides		

Appendix IV: Analysis of variance for each bacteria species. P value Significant at P < 0.05

Bacteria species	F value	P value
B. anthracoides	14.79	0.000
Y. pseudotuberculosis	5.04	0.002
P. rettgeri	42.74	0.000
Positive control	44.75	0.000
P. stuati	4.37	0.005
M. morganni	30.16	0.000
S. paucimobilis,	3.77	0.011
Acinetobacter sp.	1.82	0.170
Moraxella sp.	4.98	0.003
E. amylovora	2.11	0.095
Negative control	2.58	0.050
Alcaligen sp.	5.06	0.002
P. penneri	6.25	0.001
C. meningosepticum	7.57	0.000
P. cepacia	7.07	0.000
E. nigrifluence	1.72	0.166
T. ptyseas	18.04	0.000
C indologenes	14.17	0.000
S. pyogenes	10.15	0.000

Appendix V: Post hoc tests.

Post hoc test for testing the difference in means at different site for the six months

```
                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level      N   Mean  StDev  ------+---------+---------+---------+---
Enderit    18  53.33  28.98    (----------*---------)
Makalia    18  61.67  40.06          (---------*---------)
H. point   18  63.61  32.19           (---------*---------)
M.Lake     18  50.56  27.57  (---------*---------)
Njoro      18  70.28  30.82               (---------*---------)
                          ------+---------+---------+---------+---
                               45        60        75        90

Pooled StDev = 32.22
```

Post hoc test for testing the difference in means at different months of sampling

```
                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level   N    Mean  StDev  ---------+---------+---------+---------+
Jan    15   46.27  23.52    (-----*----)
Feb    15   41.07  18.73  (----*-----)
Mar    15   74.27  32.89               (----*----)
Apr    15   46.47  25.94    (-----*----)
May    15  101.20  21.17                         (----*-----)
Dec    15   50.07  24.48      (----*----)
                          ---------+---------+---------+---------+
                                  50        75       100       125

Pooled StDev = 24.85
```

Post hoc test for the oil degradation.

```
Individual 95% CIs For Mean Based on
                                      Pooled StDev
Level              N    Mean   StDev  ---------+---------+---------+---------+
Acinetobacter sp  28  4.0964  0.4087                            (---*---)
Alcaligen spp     28  3.6000  0.9615                       (---*---)
B. anthracoides   28  3.3607  1.1464                    (---*--)
C. indologenes    28  1.4929  1.3129  (---*---)
C. meningoseptic  28  3.9321  0.8124                          (--*---)
E. amylovora      28  4.2036  0.5507                            (---*---)
E. nigrifluens    28  3.7179  0.6673                        (---*---)
M. morganii       28  2.1857  1.3648         (---*---)
M. virosa         28  3.5714  0.8308                       (---*--)
Negative          28  4.6393  0.4219                                (--*---)
P. cepacia        28  3.8536  0.8076                          (---*--)
P. perineri       28  4.1821  0.5368                             (---*---)
P. rettgeri       28  1.6143  1.7717   (---*---)
P. stuarti        28  3.7500  0.3747                          (---*--)
Positive          28  1.7429  1.6310     (--*---)
S. paucimobilis   28  3.8821  0.4199                           (---*---)
S. pyogenes       28  2.3821  1.3576           (---*---)
T. ptyseas        28  4.0036  0.6899                            (---*---)
Y. pseudotubercu  28  3.4357  1.1618                       (--*---)
                                      ---------+---------+---------+---------+
                                             2.0       3.0       4.0       5.0

Pooled StDev = 0.9998
```

Appendix VI: Research Permit.

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THIS IS TO CERTIFY THAT:

Prof./Dr./Mr./Mrs./Miss....LUCY WARUGURU WANJOHI

of (Address) MOI UNIVERSITY P.O. BOX 1125, ELDORET

has been permitted to conduct research in .....Location,

.....NAKURU.....District,

.....RIFT VALLEY.....Province,

on the topic.Determination of bacterial load and diversity of bacteria in lake Nakuru & their ability to degrade municipal waste.

for a period ending..30TH NOVEMBER,20 11

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Research Permit No. NCST/RRI/12/1/BS/231

Date of issue 07/03/2011

Fee received SHS 1,000

Applicant's Signature

Secretary National Council for Science and Technology

CONDITIONS

1. You must report to the District Commissioner and the District Education Officer of the area before embarking on your research. Failure to do that may lead to the cancellation of your permit
2. Government Officers will not be interviewed with-out prior appointment.
3. No questionnaire will be used unless it has been approved.
4. Excavation, filming and collection of biological specimens are subject to further permission from the relevant Government Ministries.
5. You are required to submit at least two(2)/four(4) bound copies of your final report for Kenyans and non-Kenyans respectively.
6. The Government of Kenya reserves the right to modify the conditions of this permit including its cancellation without notice

GPK6055t3mt10/2009

REPUBLIC OF KENYA

RESEARCH CLEARANCE PERMIT

(CONDITIONS— see back page)