NAMIBIA UNIVERSITY
OF SCIENCE AND TECHNOLOGY

BACTERIA ASSOCIATED WITH PETROLEUM HYDROCARBON WASTES IN KUPFERBERG LANDFILL SITE, WINDHOEK

By

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Thesis presented in fulfilment of the requirements for the degree of Master of Health Sciences, Faculty of Health and Applied Sciences, Namibia University of Science and Technology, Windhoek, Namibia

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8th February 2022

DECLARATION

I, Florencia Petelo hereby declare that the work contained in the thesis entitled “Bacteria associated with petroleum hydrocarbon wastes in Kupferberg landfill site, Windhoek, Namibia” is my own original work and that I have not previously, in its entirety or in part, submitted it at any university or other higher education institution for the award of a degree.

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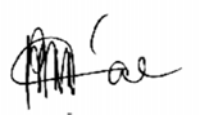
Co-supervisor: 

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ACKNOWLEDGEMENTS

I wish to thank:

1. Firstly, the Almighty Father for the opportunity He has granted me to finish my studies and for all His mercies, endless love, protection and grace. Nothing would be accomplished, if not through Him who strengthens me.
2. Secondly, a huge thank you to my family and friends, for their prayers, unconditional love, support and commitment during the course of my study.
3. Ms Marjory Hlasek for allowing me to use her laboratory throughout the course of my research project and for providing me with the necessary equipment needed.
4. Mr Tuwilika Tobias for his assistance in the biology laboratory.
5. Lastly, my Supervisors, Prof Percy Chimwamurombe and Dr Edosa Omoregie for their time, patience, consideration, help, advice, motivation and mentorship throughout my study.

Dedication

I would like to dedicate this piece of work and give special thanks to my Superhero, friend and lovely father, Dr. Petelo Pedro, for his endless prayers, support and mentorship throughout the course of my education. My dad has been my best cheerleader. I would also like to dedicate this work in the spirit and loving memory of my dearest mother, Mbiavanga Lema.

ABSTRACT

Petroleum compounds are organic contaminants of great interest due to their extensive dispersal, stubbornness, versatile structure and harmful elements that have been generally known to belong to the family of carcinogens and mutagens organic toxins. They contaminate many environments worldwide and enter the global environment through crude oil spillage, fossil fuel combustion as well as natural inputs like natural petroleum seepage. A range of indigenous microbes have the ability of decontaminating, breaking down, transforming and removing these hydrocarbon contaminants from the environment through biodegradation processes. Therefore, the aim of this study was to isolate and identify the bacterial strains present in the soil contaminated waste samples collected from the Kupferberg landfill site in Windhoek and to ascertain their ability to grow efficiently in hydrocarbon based medium. Collected bacterial strains were grown on nutrient agar and were characterised based on their colony features and biochemical reactions using the API 20NE identification database system. Sterile nutrient broth media was inoculated with a loop full of the bacterial isolates supplemented with 1 ml of sterile old diesel engine oil, and the optical density was measured spectrophotometrically on a daily basis. The highest mean bacterial count was found out to be 3.6 x 10^4 CFU/ml in site G, and the lowest mean bacterial count was found out to be 0.9 x 10^4 CFU/ml in site I. The bacterial strains isolated were *Aeromonas hydrophila, Stenotrophomonas maltophilia, Sphingomonas paucimobilis, Pseudomonas luteola, Burkholderia gladioli, Photobacterium damselae, Pseudomonas aeruginosa, Pasteurella spp., Brevundimonas vesicularis, Burkholderia cepacia, Chryseobacterium indologenes* and *Aeromonas salmonicida*, and it was observed that *Sphingomonas paucimobilis* was the predominant isolate in all the samples. Ten selected bacterial strains were subjected to hydrocarbon utilisation/ degradation test, and it was observed that *Pseudomonas aeruginosa* strain with a mean average optical density of 1.738, utilised the hydrocarbon in the medium more efficiently than the other isolates. The study demonstrated that the isolated *Pseudomonas aeruginosa* could be considered as good prospects for the bioremediation of hydrocarbons.

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GLOSSARY

BaP - Benzo (α) pyrene

HUB - Hydrocarbon-utilising bacteria

OD - Optical Density

MAHs - Monocyclic aromatic hydrocarbons

MSM- Mineral salt medium

PAHs - Polycyclic aromatic hydrocarbons

TCA- Tricarboxylic acid cycle

TEAs - Terminal electron acceptor

CHAPTER ONE
INTRODUCTION

1.1. Background of study

A landfill is a site where both hazardous and non-hazardous waste materials are disposed of (Aderemi et al., 2011). Hazardous waste includes petroleum hydrocarbon compounds such as diesel fuels, gasoline, kerosene, oil and gas mixtures. Whereas, non-hazardous compounds found in a landfill site include plastics, glass, cans, paper and office products. Furthermore, a landfill is constructed by using a layering system modelled to cautiously isolate and monitor unwanted substances, leaks and anything else that can damage the environment (Ke et al., 2017). Thus, isolating waste from the air and groundwater is crucial for avoiding contamination. Landfills are an excellent source of energy because when waste materials build up and begins to break down through microbial activity, CO_2 and CH_4 are produced as by-products (Han et al., 2016). These gases are often taken out, filtered and used for energy production (Zeng et al., 2021). However, landfills can have several drawbacks as they are partially responsible for climate change, due to great amounts of CO_2 and CH_4 produced (Zeng et al., 2021). In addition, methane (CH_4) is a greenhouse gas that is more potent than carbon dioxide. Thus, raises implications of climate change and global warming (Ke et al., 2017).

Moreover, microorganisms are found throughout the landfill site and play a leading role in many natural processes (Usman et al., 2017). Many indigenous microbes found naturally in both soil and groundwater habitats within a landfill are capable of degrading both hazardous and non-hazardous waste through a process of biodegradation (Zeng et al., 2021). Therefore, the remediation of pollutants in waste disposal sites is crucial. It helps to reduce hazardous substances where waste is disposed (Hansen et al., 2012). Consequently, waste management procedures are carried out to help ensure that fewer waste materials go to the general waste stream by minimising various forms of pollution.

Nonetheless, bioremediation has become one of the major procedures in the repair of oil-polluted habitats. Therefore, the elimination of polycyclic aromatic hydrocarbons (PAHs) from the contaminated soil environment is more cost-effective than physicochemical treatment as it exhibits potential advantages as in greater safety (Ma et al., 2018; Zharikova et al., 2018), cheaper treatment cost and reduced soil disruption such as clean up mechanisms in treating oily sediments consisting of

degradable substrates as well as specialised indigenous microorganisms (Marchand et al., 2017; Tremblay et al., 2017).

In the past years, immense quantities of both inorganic and organic compounds are released into the environment annually as a result of human activities causing major global environmental pollution (Varjani, 2017; Wu et al., 2020). Petroleum-based products are the world's most widely used and great source of energy and fuel for daily life and industries due to the energy that they produce (Hou et al., 2021; Liu et al., 2021). However, they are exceedingly toxic to all forms of life and present a major concern as they contribute to environmental pollution (Fan et al., 2019). It was evaluated that about 6 million tons of petroleum-based hydrocarbons enters the environment annually (Guo et al., 2016), through accidental leaks or as a result of human activities such as refining, transportation, exploration and production of petroleum products (Das and Chandran, 2011, Li et al., 2017).

Hydrocarbons in the soil lower oxygen tension and increase anaerobiosis which damages plant roots (Varjani and Upasani, 2016; Varjani, 2017). Some effective physical processes such as photo and chemical oxidation as well as leaching are used in the reduction of petroleum compounds in the environment (Al Kharusi et al., 2016). However, microbial degradation is the most preferred and major route of removing petroleum hydrocarbons from petroleum contaminated areas. This method is cost effective compared to physical processes that are often restricted to aquatic environments (Sarkar et al., 2017). However, both physical and chemical processes are of great concern, as they contribute to climate changes (Brown et al., 2017), the greenhouse effect (Wu et al., 2017), due to the natural gases and pesticides used, which increases the development of cancer and respiratory diseases in humans because of the carbonates that they contain (Wongbunk et al., 2020).

In recent years, the application of technology to biodegradation research has provided a broader understanding of the key pathways and the adaptability of microbes to changing environmental conditions. According to Safdari et al., (2018), the ability of microorganisms to utilise and break down petroleum-based contaminants is carried out by a wide diversity of microbial genera. Consistent with Jadhav et al., (2019), several researchers have reported the effect of different environmental factors (such as oxygen, temperature, nutrients, pH and salinity) on microbial degradation of hydrocarbons. Das and Chandran, (2011), stressed that in order for microbes to catalyse the breakdown of hydrocarbon pollutants to less harmful forms also depend on the environment and seasonal ambient

conditions, nature, amount, and composition of the existing microbial community. Thus, both genetic engineering and biotechnology strategies have been developed to help improve degradation rates by existing microorganisms (Safdari et al., 2018).

Thus, microorganisms are used in biodegradation a biological process that has been applied worldwide to control environmental problems such as treating undesirable waste and or pollutants by using active microbes such as bacteria to break down or degrade organic and toxic substances from soil and groundwater medium to less toxic forms or simpler compounds either in the environment or under laboratory conditions (Semple et al., 2001; Sarkar et al., 2017).

1.2. Statement of the problem

Petroleum hydrocarbons consist of a great and diversified group of highly toxic and poisonous inorganic and organic compounds. They are extensively used as fuel worldwide and considered as the main source of energy and materials for various industries (Sarkar et al., 2017). Through human activities such as municipal run-offs, transportation, exploration and illegal disposal of oil waste, petroleum hydrocarbons threaten the environment (Khan et al., 2013; Varjani, 2017).

Furthermore, the composition of petroleum hydrocarbons varies slightly by its source, but the toxic properties are consistent. Chemicals such as polycyclic aromatic hydrocarbons (PAHs) are petroleum-based contaminants that are extremely toxic and potentially harmful mutagens and carcinogens (Ke et al., 2017). In addition, the risk of exposure through inhalation or ingestion is associated with the susceptibility of a compound to evaporate either straight from the soil or from water grounds that have eventually affected subsequent to its release (Adipah, 2019). Contaminated soil negatively affects the health of human beings by breathing mixed chemicals being evaporated from polluted soil (Ja'afaru and Cheng, 2018). Previous long-term studies demonstrated that longer exposures to PAHs could lead to permanent damage, hence depression of the central nervous system as well as both kidney and liver damage can occur (Sajna et al., 2015; Varjani, 2017).

1.3. Aim of research

The main aim of this study was to quantify the culturable bacterial community associated with petroleum hydrocarbon wastes in Kupferberg landfill soils and evaluate their biotechnological potential to degrade petroleum hydrocarbons.

1.4. Objectives of the study

1. To isolate and identify the bacterial strains present in the soil contaminated sample from Kupferberg landfill site.
2. To determine the ability of the selected bacterial strains to grow efficiently in hydrocarbon based medium under laboratory conditions.
3. To describe the evolutionary relationship among the identified bacterial strains from contaminated soil samples in Kupferberg landfill site.

1.5. Research hypothesis

Null hypothesis: There is no significant difference in the growth rate of bacteria grown in petroleum.

Alternative hypothesis: There is a significant difference in the growth rate of bacteria grown in petroleum.

1.6. Significance of the study

Petroleum hydrocarbon contamination causes various hazardous effects on ecosystems and humans. Bioremediation is a promising technology for the remediation of environments polluted with petroleum hydrocarbons, since chemical and physical methods used for the treatment of contaminated sites are not that much efficient and are very costly compared to bioremediation, which is eco-friendly, more efficient and less costly. The significance of this study was to characterize bacteria associated with petroleum hydrocarbon wastes, which could be considered as a key component in the clean-up strategy for the remediation of petroleum hydrocarbons.

This study could also be very useful and beneficial to nature (beneficial to both animal and human health) by maintaining the environment clean, thus providing a much safer and healthier environment for wildlife and by keeping both animal and humans out of danger or being exposed to hazardous

compounds, is a serious demand to promote sustainable development for the Namibian society with low environmental effect.

1.7. Limitations of the study

Due to the pandemic and lockdown associated with COVID-19 restrictions, temporal variation as well as studying the effect of environmental factors associated with the bacterial isolates in degrading petroleum hydrocarbons could not be fully carried out in this study. Sample collection could not be repeated seasonally due to COVID-19 restrictions.

CHAPTER TWO
LITERATURE REVIEW

2.1. Introduction

Bacteria are considered as one of the most vital agents and important petroleum biodegradable microorganisms in the environment. They carry out a vital role in breaking down oil pollutants released in the environment (Bandopadhyay et al., 2018; Varjani and Gnansounou, 2017). In addition, bacteria are ubiquitous because they are found throughout the environment; in the soil, air, water, on the surface of objects and humans both internally and externally (Fosso-Kankeu et al., 2017). Numerous microbes have been utilised in industrial-scale activities to produce fuel and antibiotics, to ferment beverages and food, to treat sewage and many others that prosecute various enzymatic biotransformations (Wu et al., 2017). However, hydrocarbon-utilising bacteria (HUB) are extensively spread in soil habitats and aquatic environments such as seawater. Additionally, their use in biodegradation of petroleum hydrocarbon polluted soils, proves their capability to detoxify organic contaminants. Thus, they have been recognized as a versatile, cost-effective, multi-skilled and environmentally sound treatment (Varjani, 2017).

Petroleum-based hydrocarbons have been associated with the most common pollutants of the soil and groundwater. Although independent organisms can only metabolise a small range of hydrocarbon pollutants (Kuypers et al., 2018). Polycyclic aromatic substrates are a group of hydrophobic chemical substances, which consist of carbon, and hydrogen atoms with two or more merged benzene ringed structures, which are either in linear or cluster arrays (Crampon et al., 2018). In soils, most of these polycyclic aromatic hydrocarbons (PAHs) are considered to be highly toxic, carcinogenic and mutagenic in several cases. They are the major constituent of crude oil and pollute the soil through various routes such as automobile gasoline, burning of fuel, the production of gas and the incineration of waste (Eigenbrod et al., 2009).

2.2. Landfarming bioremediation of petroleum hydrocarbons

Landfarming is a soil bioremediation procedure that triggers mixing of the hydrocarbon-polluted soil. It involves relying on the chemical, physical and biological approaches within the soil for biodegradation (Pipit and Rinanti, 2021). Land farming has been productive in breaking down several hydrocarbon compounds since most oleophilic microorganisms are restricted to the superficial layers of the soil, 15-30 cm deep. (Raffa and Chiampo, 2021). This technique has been employed for many

years due to its efficiency and effectiveness (Yadav et al., 2021). In addition, it is a specialized control method for cleaning up oil-contaminated uppermost soil surfaces either *in situ* or *ex situ* (Paul et al., 2021).

Furthermore, polluted soil is often transported to a treatment site where it is overspread of a prepared soil surface to allow aerobic microbial degradation to occur (Yadav et al., 2021). However, landfarming has many drawbacks which can impact the lives of humans as well as negatively influence soil structure (Paul et al., 2021). For instance, the consequential challenges related to land farming are that it is a very slow biodegradation procedure and has been unsuccessful in degrading high molar mass PAHs (Raffa and Chiampo, 2021). Other challenges include the inhalation of toxic hydrocarbon compounds by humans and the leaching of other hydrocarbon pollutants through the soil to the groundwater region, causing water pollution (Karimi et al., 2021).

In recent years, challenges associated with landfarming have been successfully managed by constructing an impermeable polythene layer which is laid at the bottom of the topsoil to prevent the leachates from sinking to the groundwater region (Paul et al., 2021).

2.3. Chemical composition of persistent environmental petroleum hydrocarbon pollutants.

The rate at which petroleum compounds are degraded, depends mostly on the arrangements and physical structure of the component parts (Abu Laban et al., 2015). Petroleum means “rock oil”, which exists as a dark, thick and sticky liquid (Chiu et al., 2017), consists mainly of carbon and hydrogen atoms with oxygen, nitrogen and sulphur in small portions (Ji et al., 2019). Each element has a special chemical behaviour that enables it to be either efficiently degradable, or difficult to break down or completely indestructible (Patel et al., 2020). Their composition can vary with the oldness of the oilfield, the location and the profundity of the oil rig (Aanderud and Lennon, 2011).

Petroleum crude oil is divided into four groups mainly: saturates, asphaltenes, aromatics and resins. In the configuration of the four main elements of petroleum, the outermost layer of the oil is composed of the saturates, while the innermost part of the oil consists mostly of the asphaltenes due to their large molar mass (Zhu et al., 2019). On the other hand, aromatic compounds such as polycyclic aromatic hydrocarbons and monocyclic aromatic hydrocarbons (MAHs) contain molecules with ringed

hydrocarbons arrangements. Resins consist of light polar oil-surface arrangements (Patel et al., 2020), they can be dissolved in saturates and aromatics. According to Wongbunmak et al., (2020), microbial degradation of crude oil elements takes place in the serial order: MAHs, alkanes, cycloalkanes, PAHs and asphaltenes.

2.3.1. Saturates

Saturated hydrocarbons also known as aliphatic are defined as hydrocarbons that contain single bonds between their carbon atoms (Varjani, 2017). They are considered saturated because each carbon atom is bonded to as many hydrogen atoms as possible. Saturates represent the greatest percentage of petroleum components.

2.3.2. Aromatic

Aromatic compounds have one or various aromatic rings which are commonly replaced with a particular alkyl group (Zhang et al., 2020). MAHs are composed of a single aromatic ring in their structure which include toluene, benzene, xylenes and ethylbenzene. Furthermore, PAHs consist of two or more benzene rings in their structures, they include naphthalene, anthracene and phenanthrene (Hou et al., 2018; Zhu et al., 2019), these are light PAHs and their hydrocarbons consists of two to three benzene ring structures, whereas high molar mass of PAHs consists of more benzene rings in their structures such as pyrene, chrysenes, benzo (a) pyrene and fluoranthene (Margesin et al., 2013; Song et al., 2017).

Polycyclic aromatic compounds are common environmental pollutants that are likely to be mutagenic and carcinogenic (Dumanoglu et al., 2017; Vieira et al., 2018). The Breast Cancer Association stated that dense PAHs such as benzo (a) pyrene have been associated with human breast cancer and it may cause severe damage to the DNA of living beings (Lemieux et al., 2015; Wanapaisan et al., 2018; Wongbunmak et al., 2020). Therefore, this accounts for many researches done on the degradation of heavy PAHs to protect the biodiversity as well as environment from serious long-lasting ecological and medical harm caused by oil leaks (Lee at al., 2018).

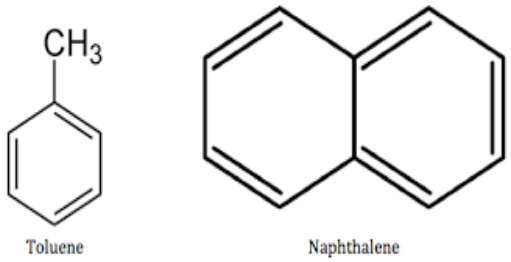

Figure 1: Monocyclic (Toluene) and polycyclic aromatic hydrocarbons (Naphthalene). Source: (Wen et al., 2021)

2.3.3. Asphaltenes

Asphaltenes have been considered as non-volatile constituents of crude oil. They are well known for their existence as diverse organic compounds, consisting primarily of oxygen, carbon, nitrogen as well as sulphur. Asphaltenes are indissoluble in alkanes and soluble in aromatics such as benzene and toluene (Crampon et al., 2018). Asphaltenes are considered as shiny, brittle, black in colour and solid powders, they possess polar fragments with very high molar weight that promotes their high resistance to hydrocarbon biodegradation (Khan et al., 2013).

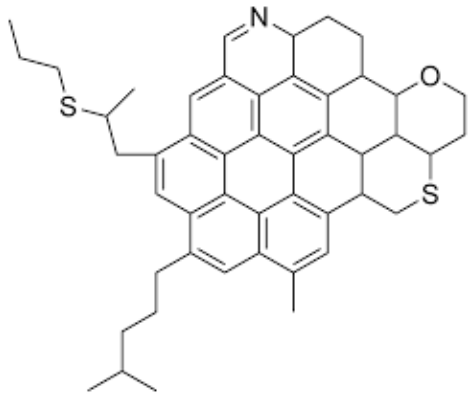

Figure 2: Chemical structure of an asphaltene. Source: (Komeev et al., 2021)

2.3.4. Resins

Resins contain both polar and non-volatile components of crude oil. Their chemical structure resembles that of asphaltenes but with much lower molecular mass instead and greater carbon to hydrogen rate (Zharikova et al., 2018). Resins are highly soluble in alkanes and aromatic solvents except in propane. They are shiny and dark brown to black in colour (Patel et al., 2020).

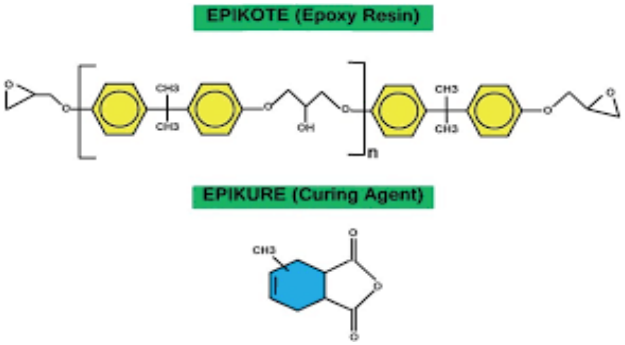

Figure 3: Chemical structure of a resin. Source: (Das et al., 2021).

2.4. Petroleum hydrocarbons as persistent pollutants in the environment

Petroleum also known as crude oil and oil is an organic resource trapped in great amounts in the crust of the earth (Acosta-Santoyo et al., 2019). Petroleum is formed by natural geological processes, when huge amounts of dead bodies such as algae and zooplankton containing organic molecules originating in ancient photosynthesis are hidden beneath limestones and exposed to both extreme pressure and heat under anaerobic decomposition (Lee et al., 2018). Petroleum hydrocarbons are colourless, some are white and/or pale-yellow solids that are insoluble in water, with elevated melting and boiling points and low gas pressure (Zhou et al., 2018).

Polycyclic aromatic hydrocarbons are typically found in the environment, in the soil particularly at several concentrations posing significant environmental harm (Marchand et al., 2017 and Vieira et al., 2018). After these hydrocarbons combine with water, they are likely to sink into the soil, by persisting, thus minimising the condition and production of the soil by making it inappropriate for crop growing and investment (Durodola et al., 2019). PAHs are extremely toxic at low concentrations as they are considered to be mutagenic or carcinogenic to humans and wildlife (Hou et al., 2021).

Petroleum hydrocarbon is a combination of multiple organic matter that differ in their arrangements from one source to another (Wu et al., 2017). Chemicals such as PAHs and benzene are significantly toxic elements of great concern. Their variation in composition affects how they differ from source to source. However, certain characteristics of their potential biological defects will rely on the biome where the oil discharge occurs (Fan et al., 2019).

Aromatic hydrocarbons are organic chemical substrates that consist of one to more ringed arrangements with pi electrons delocalized all the way around them (Nzila, 2018). In contrast, saturates are specified as hydrocarbons with no double bonds. They have been characterised according to their chemical arrangements. However, they also exhibit the greatest percentage of crude oil elements (Fan et al., 2019). Similarly, to the aromatic and saturated portions, asphaltenes and resins are diverse and mainly unknown carbon arrangements with the addition of multiple nitrogen, oxygen and sulphur atoms (Nowak et al., 2018).

Petroleum hydrocarbon pollutants are extremely diverse organic compounds of significant concern due to their harmfulness, broad distribution in various habitats, persistent in the environment, and their complex structures (Brown et al., 2017). Accidental leaks of petroleum contaminants negatively affect the environment and can lead to the development as well as the implementations of remediation approaches for cleaning up contaminated sites. Once petroleum hydrocarbons reach the environment, the immediate damage to the environment can be the result of several causes (Shintani et al., 2019).

2.5. Impact of petroleum hydrocarbons on soil and aquatic ecosystems

The moment petroleum hydrocarbons are discharged into the environment as a result of industrial activities through leaks, the spill moves from the soil to the groundwater, and some of these pollutants are degraded into simpler compounds by present microbes found in the soil (Wu et al., 2017). However, other pollutants may persist in the soil for a lengthy period, while others may evaporate into the atmosphere (Poorsoleiman et al., 2020).

2.5.1. Soil ecosystems

The soil is an irreplaceable and essential natural resource, which maintains an important link between the elements such as air, liquids, bedrock and biota that complete the environment, and these elements collaborate with one another to supply necessary needs like food, fuel, fibre to sustain the microorganism (Li et al., 2019; Patel et al., 2020). Soil polluted by petroleum compounds is a major global drawback that has been attracting awareness over many years (Li et al., 2015). Human practices are one of the serious factors that promote the spill of hydrocarbons through crop production or commercial products, seepage of subterranean storage tanks, and accidental leaks during disposal and transportation (Lee et al., 2018). When petroleum hydrocarbons enter an environment, immediate biological damage emerges by obstructing the supply of water, minerals, and light, thus

negatively influencing soil fertility and plant development (Kronenberg et al., 2017). Moreover, petroleum contamination in soil results in an imbalance in the carbon-nitrogen ratio at the spill site, because petroleum is significantly composed of carbon and hydrogen (Wang et al., 2021). This results in the shortage of nitrogen in oil-soaked soil, thus restraining bacterial growth and the usage of carbon sources (Fan et al., 2019). In addition, the existence of aliphatic substrates also negatively affects soil microbiota and structure, by building oil layers and blocking the exchange of oxygen and nutrients in the deeper layers of the soil (Li et al., 2018). Various indigenous microorganisms in the soil are competent in degrading petroleum pollutants. Petroleum hydrocarbons are broken down by diversified groups of microorganisms, which are suitable for using hydrocarbons as food (Patel et al., 2020).

A variety of bacteria are known to consume entirely on hydrocarbons (Alabresm et al., 2018). Nonetheless, Gurav et al., (2017) reported that *Acinetobacter* sp. was able to use *n*-alkanes of chain length C_{10} -C_{40} as a sole carbon source. Whereas, the degradation of PAHs by *Sphingomonas* was reported by Poorsoleiman et al., (2020). Bacterial genera, namely *Burkholderia*, *Gordonia*, *Aeromonas*, *Mycobacterium* and *Brevibacterium* isolated from a gasoline contaminated soil proved their potential in the degradation of hydrocarbons (Han et al., 2018). Crampon et al., (2018) reported the degradation process of crude oil from an oil-contaminated site in North East India by a mixed consortium. The consortium included *Sphingomonas paucimobilis, Pandoraea pnomenusa, Burkholderia cepacia* and *Pseudomonas luteola* who are known for their complementary degradative abilities, were reported to completely eliminate alkanes, alkyl cycloalkanes and alkyl benzene in 7 days incubation respectively.

2.5.2. Aquatic ecosystems

Oil seepage in aquatic environments, respectively in the open sea results in the production of a heavy constituent known as a floating film, which later ends up sinking in the sediments (Durodola et al., 2019), where it affects animals and plants in marine ecosystem (Jakariya, 2000).

When oil spill occurs in aquatic ecosystems, it moves and spreads with the effect of wind current on the water surface as a slick. Nevertheless, when magnificent amounts of oil in aquatic ecosystems tend to last because of insufficient surface areas accessible for microbial mechanisms (Bandopadhyay et al., 2018), a combination of recuperation, discarding and management of oil is completed afterwards. Conventional approaches such as chemical cleaning of spilled oil by making use of pesticides and biosurfactants, are fundamental techniques for removing spilled oil from aquatic habitats (Tremblay et al., 2017; Marchut-Mikolajczyk et al., 2018).

Khan et al., (2013) reported that *Rhodococcus* sp. and *Pseudomonas aeruginosa* were isolated from groundwater of a crude oil refinery plant. These bacterial isolates and consortia were reported to show differing preferences for nitrogen source and degraded 97-99% of the oil without any significance difference when fortified with the preferred nitrogen source. Moreover, Premnath et al., (2021) investigated the degradation of petroleum hydrocarbon compounds by an oilfield isolated bacterial consortium and it was reported that *Pseudomonas aeruginosa*, *Bacillus subtilis Micrococcus roseus and Corynebacterium* sp were capable of removing 96% of the alkanes, thus enabling them to metabolize both short and long-chain alkanes due to their diverse array of alkane hydroxylase systems (Varjani and Upasani, 2016).

2.6. Effect of petroleum hydrocarbons and other PAHs on human health

PAHs are an important concern with regards to their potential exposure effects on the health of humans (Ke et al., 2017). Toxic hydrocarbons are known as carcinogens and mutagens therefore they pose a major risk to the well-being of living things (Da Silva et al., 2021). The impact of PAHs on human health depends on the way of exposure and length of PAHs as well as the number of PAHs one is exposed to. The major routes of exposure to PAHs are from ingestion, inhalation, eating contaminated food with PAHs, dermal contact, smoking cigarettes or breathing in smoke from fireplaces (Das and Chandran, 2011; Xu et al., 2017). Other factors that affect health have also been reported such as health status (pre-existing underlying health condition) and age. Since petroleum hydrocarbons can remain in the biome for prolonged periods, they can build up in animal and human tissues via dermal exposure. Persistent exposure to high amounts of these toxic compounds can lead to many biological disorders, causing several health effects which include weight loss, liver problems, gastrointestinal deterioration, increased cancer risk, haemolytic anaemia, decreased immunity and reproductive problems (Agudelo-Castañeda et al., 2017). Experimental studies were conducted on long-term health effects and it was demonstrated that longer exposures to PAHs can lead to both kidney and liver damage, lung function abnormalities, breathing problems like asthma and cataract (Sajna et al., 2015; Varjani, 2017; Chen et al., 2019).

Additionally, it has also been revealed that repeated exposure to PAHs by several animals may cause skin inflammation and irritation (Abu Laban et al., 2015; Mueller et al., 2019). However, aliphatic hydrocarbons can also contribute to short-term health problems after exposure by affecting the nervous system leading to severe dizziness and irritability, headaches and memory loss, fatigue, skin injuries as well as rashes (Ellickson et al., 2020).

2.6.1. *Burkholderia* spp.

Burkholderia cepacia is a lactose-non-fermenting organism with poor virulence that is found in soil and water and can survive for longer periods in moist environments. However, *Burkholderia cepacia* is an opportunistic pathogen found that affects organisms that suffer with sickle abnormalities (Mendez et al., 2011) and causes pneumonia in individuals with underlying lung disease such as cystic fibrosis (Jakariya, 2000). In addition, *Burkholderia gladioli* is an aerobic organism that causes disease in both plants and humans. In plants they affect onions and cause whole plant decay, and in humans it causes severe pulmonary infections (Koshlaf and Ball, 2017).

2.6.2. *Aeromonas* spp.

Aeromonas hydrophila is a heterotrophic microbe that is found in warm weather and is very toxic to many organisms. It is resistant to most antibiotics and cold temperatures and is highly pathogenic to fish and amphibians (Coscelli et al., 2017). However, in humans it can cause gastroenteritis, which occurs mostly in young children (Mueller et al., 2019), and other people have compromised immune systems. *Aeromonas salmonicida* have been reported to be etiological agents for furunculosis (Pang et al., 2015) and have been associated with inflammation of the lower intestine, spleen enlargement and muscle lesions in humans and death in freshwater fish populations (Awan et al., 2018).

2.6.3. *Sphingomonas* sp.

Sphingomonas paucimobilis is a non-pathogenic organism that causes a variety of infections which include bacteraemia, urinary tract diseases, meningitis and pneumonia (Guo et al., 2011). Most people that become infected with *Sphingomonas paucimobilis* are individuals who suffer from underlying medical illness.

2.6.4. *Stenotrophomonas* sp.

Stenotrophomonas maltophilia is a multi-drug resistant bacillus that is an opportunistic pathogen. It is an obligate aerobe whose infections have been associated with high morbidity and mortality in severely immunocompromised and debilitated individuals (Arulazhagan et al., 2017).

2.7. Petroleum hydrocarbon-degrading microorganisms

The diversity of microorganisms has been analysed over a broad scale of ecosystems, including aquatic environments, soils, the human body (Fan et al., 2019), hydrocarbon contaminated soil (Koshlaf and Ball, 2017). Petroleum compounds can be broken down by a variety of microorganisms such as bacteria, fungi, yeast and microalgae. However, hydrocarbon-utilising bacteria (HUB) are able to degrade most of the petroleum compounds in the environment, by using it for their cellular growth

and metabolic activities (Bandopadhyay et al., 2018). They include the following species: *Bacillus, Mycobacterium, Acinetobacter, Stenotrophomonas, Pseudomonas* and *Streptococcus* (Das and Chandran, 2011).

Moreover, growth curve measurements derived from optical density (OD) is one of the most typical techniques used in microbiology for determining and monitoring the proliferation of microbes in a given time (Krishnamurthi et al., 2021). Traditionally, the growth rate of microbes is measured at a wavelength of 600 nm using a spectrophotometer (Krishnamurthi et al., 2021). Nonetheless, Meenakshisundaram and Bharathiraja, (2014) did an experiment on microbial degradation of hydrocarbons based on the optical density and they reported that *Pseudomonas putida* and *Bacillus badius* had the greatest degrading abilities. They also revealed that the cells of these microorganisms were able to multiply within the days of the study, based on their OD readings, thus indicating that they were able to degrade and utilize oil for their growth and development. A similar study done by Ebakota et al., (2017) showed that there was an increased plate count and absorbance of all isolates, thus concluding that all isolates were capable of degrading the spent engine oil introduced into the growth medium.

Within the past years, numerous bacteria competent of ecologically favourable degradation properties have been identified and investigated (Pelletier et al., 2004). However, the population of indigenous microbes, that are widespread in the soil and water surfaces, have adjusted to the harsh conditions of the surroundings by growing and utilising the carbon present from the oil pollutants sites as their source of energy (Poorsoleiman et al., 2020).

The ability of a bacteria to break down and to utilise hydrocarbon compounds is displayed by an expansive range of bacterial and fungi species. Based on various published journals, about 25 genera of hydrocarbon-utilising microorganisms have been identified from both soil and damp environments (Shintani et al., 2019). Studies on petroleum degradation have affirmed that various fungi are capable of petroleum hydrocarbon degradation, utilising them as their source of energy and metabolism (Mueller et al., 2019).

Various investigation has made known that a mixed genus of fungi such as those belonging to *Aspergillus spp*., *Penicillium spp*., *Fusarium spp*., *Neosartorya spp*., *Graphim spp.*, *Saccharomyces spp*., *Talaromyces spp*. and *Paecilomyces spp*. (Margesin et al., 2013; Sarkar et al., 2017) are able to metabolise toxic hydrocarbons at different rates. Fungi are considered common soil microorganisms, all these groups have been recited to be existent in hydrocarbon-contaminated habitats and have also been described as hydrocarbon degraders (Bandopadhyay et al., 2018). The role of fungi in the biodegradation process of petroleum products has been extensively studied and species namely, *Penicillium, Geotrichum* and *Cephalosporium* were isolated from a contaminated water medium and were noted to degrade petroleum compounds (Song et al., 2019). On the contrary, Kumari et al., (2018) investigated yeast species namely, *Candida lipolytica, Fusarium* sp. and *Geotrichum* sp. for use of alkanes and it was concluded that degradation of longer chain alkanes such as pentacosane ($C_{25}H_{52}$) occurred at a much slower rate thus resulting in only 15% of degradation by *Fusarium* sp.

2.8. Factors influencing petroleum hydrocarbon degradation

Biodegradation of different classes of petroleum compounds occurs at different rates (Ilori, 2008). Many experiments have indicated that the rate of biodegradation of oil depends not only on the weathering, but also the activity of specific microbes can be influenced by other environmental conditions such as optimum temperature, oxygen, pH, salinity and nutrients available (Da Silva et al., 2021). Moreover, the continuance of petroleum contaminants in the environment depends on the quality and quantity of the petroleum compound mixture and on the properties of the damaged ecosystem (Varjani and Gnansounou, 2017; Varjani, 2017; Guerra et al., 2018). On one hand, petroleum compounds can remain indefinitely in the environment, whereas on the other hand, the same hydrocarbons can be degraded completely within a short period of time (Durodola et al., 2019).

2.8.1. Temperature

Biodegradation of petroleum hydrocarbons can take place over a broad range of conditions. Hydrocarbon-utilising bacteria that have been isolated below 20°C are known to be psychrophilic, mesophilic (15 - 45°C) and thermophilic (above 50°C). Bacteria can adjust to climate alterations in order to sustain enzymatic activity (Al-Mur et al., 2021). Ernakovich and Wallenstein, (2015) reported on microorganisms that can degrade hydrocarbons at below 0°C, whereas Xiong et al., (2015) and Han et al., (2018) stated that microorganisms are capable of metabolising poisonous hydrocarbons at temperatures of 70 - 80°C. Moreover, the majority of microorganisms have efficiency for digesting petroleum toxins (Mendez et al., 2011) at temperatures varying within 20 and 35°C.

Heat influences petroleum hydrocarbon degradation and its effect has been marked on the physical form and chemical composition of hydrocarbons (Paliwal et al., 2012; Fida et al., 2017). Although hydrocarbon degradation occurs at different temperatures, the proportionality at which biodegradation occurs is also influenced by seasonal changes (Schiewer and Horel, 2017). Nevertheless, the rate of biodegradation is typically observed to decrease as the temperature decreases, which is thought to occur due to reduced enzymatic activity (Wang et al., 2021). Figure 4 indicates that the greatest degradation rate usually takes place in the range of 30-40°C in soil habitats, 20-30°C in freshwater ecosystems and 15-20°C in marine environments (Das and Chandran, 2011).

Xiong et al., (2015); Mohanakrishna et al., (2019) and Wang et al., (2021) studied the effects of temperature on the hydrocarbon arrangements of petroleum mixtures. They found that at reduced temperatures, the density of crude oil increases, water solubility increases and the ratio of volatilization of the molar weight is reduced. This implies that the toxic reduced carbon blocks do not evaporate and produce toxicity to the hydrocarbon-utilising microorganisms (Wu et al., 2017) thus delaying the onset of the biodegradation process.

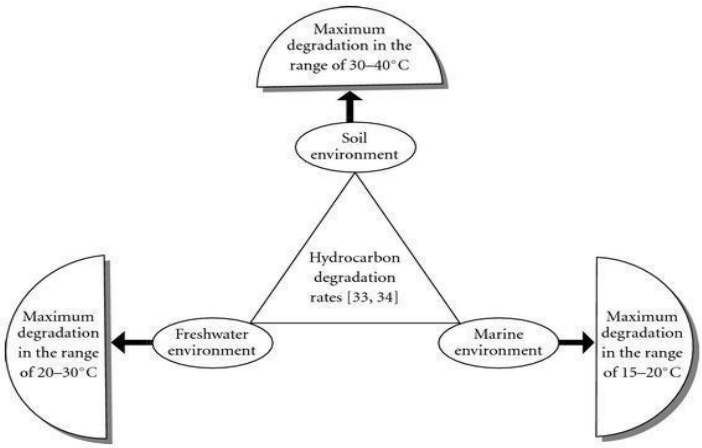

Figure 4: Hydrocarbon degradation rates in soil, freshwater and marine environments. Source: (Das and Chandran, 2011)

2.8.2. Nutrients

Nutrients are extremely essential elements for a productive degradation of environmental toxins, particularly nitrogen, phosphorus and in some cases iron (Varjani et al., 2021). Various investigators (Song et al., 2019; Zhu et al., 2019; Bao et al., 2020; Wang et al., 2020) have outlined that the available amounts of nitrogen and phosphorus in saltwater are seriously restricting bacterial degradation.

Moreover, hydrocarbons containing low concentrations of inorganic nutrients which have been released into aquatic environments, often produce extremely high concentrations of carbon/ phosphorus or carbon/nitrogen, or both, which are unfavourable for microbial growth (Jiang et al., 2019). To the contrary, previous studies (Varjani and Gnansounou, 2017; Wang et al., 2018), have reported an opposite conclusion. However, other work done by (Chen et al., 2017; Zhou et al., 2017; Das et al., 2020) have demonstrated no rise in biodegradation rates, even after fertiliser amendments were used. Furthermore, Jiao et al., (2018) studied the effectiveness of fertilisers for crude oil bioremediation, and it was concluded that the use of organic fertilisers such as poultry manure alone in oil-contaminated soil increased microbial activity in the soil that enhancing an increase in the biodegradability of petroleum hydrocarbons.

Furthermore, Conejo-Saucedo et al., (2019) investigated the degradation of PAHs by *Trichoderma* using pyrene as their carbon source, with an extra addition of 0.02% yeast extract, and 0.1% sucrose, and it was revealed that the pyrene-degrading efficacy and growth were boosted compared with the control after 7- and 14-days incubation period.

2.8.3. pH

Soil pH of 5 to 9 is typically considered as the ideal pH for the elimination of hydrocarbon toxins in both aquatic and terrestrial systems (Margesin et al., 2013). Nonetheless, the pH of the soil may impact the accessibility of nutrients in the environment thus limiting microbial activity in the decaying of petroleum hydrocarbon compounds (Mallick and Dutta, 2008) by affecting bacterial plasma membrane movement and enzymatic reaction as well as biochemical processes (Cheng et al., 2020).

In general, bacteria and fungi are heterotrophic and grow well near neutral to alkaline (6.5 to 8.5) pH, however, fungi are more non-resistant to acidic environments. Wentzel et al., (2019) found that both hydrocarbon-utilising microbes are adequately abundant in the soil, and that adherents of both genera promote the disintegration of petroleum substrates in both water and soil environments (Abu Laban et al., 2015). In previously published research, Chowdhury et al., (2017) observed that about 80% of the mineralization of naphthalene was achieved by hydrocarbon-utilising bacteria at pH of 6.5, whereas only 13% was attributed to fungi for the mineralization of hydrocarbons at a range of pH values (pH 2.5-11).

Liu et al., (2021) and Wu et al., 2018, demonstrated that in oily sludge soils and crude oil Pseudomonas *aeruginosa* had a maximum degradation rate at ranging pH 7.8 and 8 respectively. Overall, Woo et al.,

(2017) concluded that greatest degree of degradation was mainly noted at neutral pH. Nonetheless, microorganisms thriving on hydrocarbons have been identified from historical locations even at pH values of 2 - 3.

2.8.4. Oxygen

Oxygen is a crucial element in the decomposition of petroleum compounds, as it determines whether the reaction pathway is aerobic or anaerobic. Oxygen obtainability in the soil relies on the bacterial usage of oxygen, the genre of soil as well as the presence of elements which can be used by the hydrocarbon-utilising microorganisms to lead to oxygen depletion (Jiang et al., 2019).

Moreover, when oxygen is utilised throughout the degradable process then the routeway is termed aerobic. In anaerobic degradation, oxygen is not used but other organic molecules such as iron, sulphate and or nitrate are involved (Lahiri et al., 2021; Wang et al., 2021). Anaerobic decaying microbes can be designated as strict anaerobes as they can only break down substrates strictly in the absence of oxygen (Wartell et al., 2011; Mansur et al., 2014). Whereas, most of these bacteria are sulphate reducing bacteria. On the other hand, facultative anaerobic bacteria can degrade hydrocarbons either in the presence or absence of oxygen. Facultative bacteria are typical nitrate or iron utilising bacteria. Anaerobic procedures occur in anaerobic sites which can be sediment surfaces but also in profound layers in soil or aquatic sites (Palma et al., 2018).

Nonetheless, aerobic conditions are more beneficial as oxygenases are the principal catalysts required for the decaying of petroleum hydrocarbons to happen (Huang et al., 2021). Numerous researches done, have shown that anaerobic biodegradation of petroleum hydrocarbons by hydrocarbon utilising-bacteria arise only at insignificant rates (Kuypers et al., 2018; Abena et al., 2019), and that its biological implications have been principally measured to be minimal. Although oxygenases only function in the existence of oxygen, decaying rates are greater in aerobic environments as in comparison to those in anaerobic sites (Rajbongshi and Gogoi, 2021).

2.8.4.1 Aerobic degradation

Petroleum hydrocarbon substances are degraded easily and faster under aerobic conditions. Algae, fungi and bacteria are all qualified in breaking down hydrocarbon toxins aerobically (Zhou et al., 2017; Daghio et al., 2018). In aerobic degradation of PAHs, the hydrocarbons with double bonds such as alkanes and alkenes are the only hydrocarbon elements that are efficiently degraded in comparison to aromatic hydrocarbons (Dombrowski et al., 2016; Li et al., 2019). The degradation of diesel, crude oil, *n*-alkanes and PAHs by *Pseudomonas aeruginosa* was investigated by Mohanakrishna et al., (2019), and reported *n*-alkanes degradation via terminal oxidation pathway. Under aerobic conditions, present microbes break down organic materials into carbon dioxide, water and other inorganic

compounds including nitrate and sulphate as by-products (Chen et al., 2017). The aerobic route happens fastest and effectively (Eskandari et al., 2017; Han et al., 2018) since aerobic reactions demand low free energy for initiation and generate additional energy per reaction. However, the rate of decomposition fluctuates, generally relying on plentiful biological parameters, and the degree of decomposition diminishes as complexity in hydrocarbons increases. Igun et al., (2019) suggested that oxygen is the principal commanding element in aerobic ecological decomposition. However, Gurav et al., (2017) and Zhang et al., (2021) reported that the obtainability of oxygen principally counts on its potential to spread-out or locomote towards an environmental area, where present microbes are assumed to achieve the decomposition process the fastest.

Furthermore, the complete degradation of hydrocarbons mainly occurs under aerobic conditions. This process generates various steps as illustrated in Fig. 5. The first step involves the availability of chemicals to microbes with degradation capability (Ji et al., 2019). Secondly, the activation and integration of oxygen is a fundamental reaction generated by oxygenase and peroxidase (Guo et al., 2016). Followed by the peripheral degradation pathways which transforms hydrocarbons into intermediates of the tricarboxylic acid cycle (TCA), and the biosynthesis of cell biomass from the central precursor metabolites i.e. acetyl-CoA, succinate, pyruvate and sugars are needed for several biosynthesis and gluconeogenesis for growth (Ojewumi, 2018).

In addition, *Pseudomonas* sp. have shown to be ubiquitous in oil contaminated soils and soil in general (Liu et al., 2021). This bacterium species has adapted to the hard conditions of the polluted environments and to many different hydrocarbon contaminants (Zharikova et al., 2018), and it is dependable for breaking down the majority of the aromatics in diesel and crude oil, although its efficacy in decaying aromatics hydrocarbons can broaden between bacterial strains (Dvořák et al., 2017).

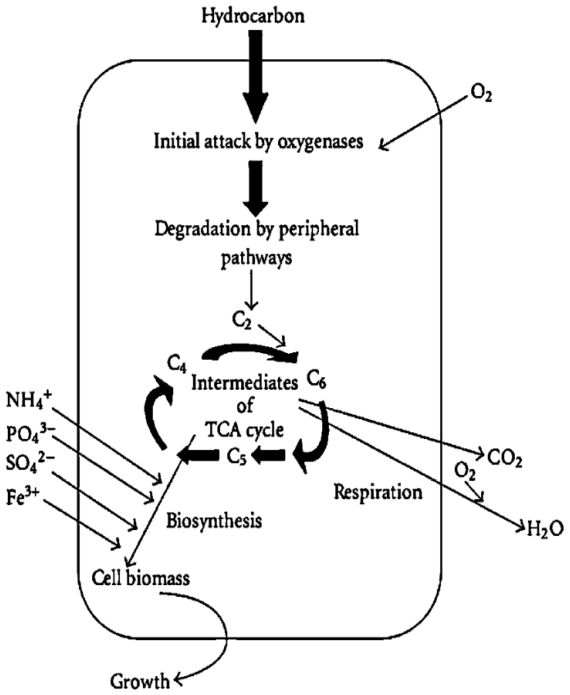

Figure 5: Aerobic degradation of petroleum hydrocarbons by microorganisms. Source: (Das and Chandran, 2011).

2.8.4.2. Anaerobic degradation

PHAs are ubiquitous environmental pollutants of natural and anthropogenic origin. Their high hydrophobicity and low bioavailability increase with molecular masses and influences their stubbornness in the habitat (Ke et al., 2018). The breaking down of hydrocarbons in the presence of oxygen is continually faster as compared to the anoxic method. Naturally, the length of anaerobic decomposition occurs within a prolonged period of time (Zheng et al., 2018). However, alkanes and many other aromatics are broken down anaerobically to produce fatty acids in bacteria (Palma and Costa, 2021). In the shortage of oxygen, oxidised inorganic compounds including nitrate, manganese, iron, sulphate and carbon dioxide act as the terminal electron acceptors (TEAs) (Pavlova et al., 2021).

Most recent studies showed that PAH are also metabolised under anaerobic conditions. Zharikova et al., (2018) and Tran et al., (2021) reveal that degradation of petroleum compounds happens at a much faster rate in the existence of oxygen than in the absence of oxygen, this is because aerobes are able to degrade larger amounts of hydrocarbon compounds than anaerobic microorganisms. However, under anoxic conditions, Aulenta et al., (2021) proved that decomposition of PAHs happens over a long-lasting period whereas, Ahmed et al., (2020) reveals that the degradation of benzene occurred within 120 weeks. In contrast, anaerobic degradation provides an economic and beneficial in situ

biodegradation mechanism (Akob et al., 2017) which is utilised for the purification of sediment affected with petroleum toxins. In contrast, the aerobic bioremediation process is a very effective process (Tucci et al., 2021) by handling hydrocarbon impurity, it is however usually costly.

Anaerobic microbes use terminal electron acceptors (TEA) including nitrate, carbon dioxide, sulphate, oxidized metals (Palma et al., 2018). At polluted sites, microbes use TEA in order to decrease reduction potential in oxygen, nitrate, ferric iron, sulphate and H_2 (Premnath et al., 2021). Particular species of denitrifying or sulphate reducing microbes have been shown to metabolise certain hydrocarbons completely to CO_2 and water (Palma and Costa, 2021).

Furthermore, the anaerobic mechanism for the metabolism of PAHs was reported by Marozava et al., (2019), the pathways of activation involve the methylation via methyltransferases, that is a significant set of enzymes that methylate their components. Secondly, the introduction of succinate synthase, an enzyme that consists of a glycyl radical to fumarate (Oliveira et al., 2020). Lastly the carboxylation via carboxylase, an enzyme that allows the production of new carbon-carbon bonds via inserting HCO_3- or CO_2 to the targeted substances (Kleinsteuber et al., 2012).

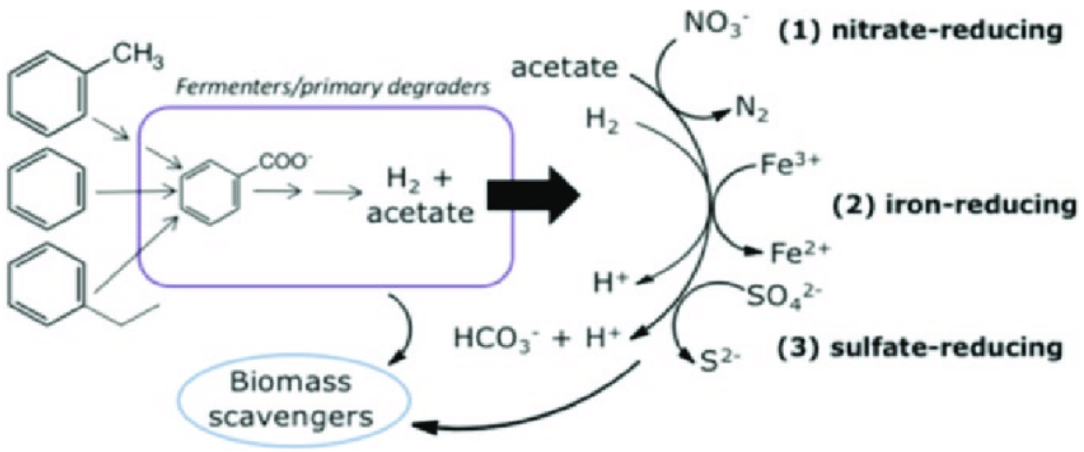

Figure 6: Theoretical model of syntrophic anaerobic biodegradation of alkylbenzenes and benzene (Gieg et al., 2014).

2.8.5. Salinity

Microorganisms necessitating salt for proliferation are considered as halophiles, whereas microorganisms that can grow at higher salt concentrations than those required for growth are referred to as halotolerant. In general, salt concentration is an essential biological component which is involved in the degradation process of organic pollutants (Camacho-Montealegre et al., 2021). Different salt concentrations can have various effects on the degradation of hydrocarbon degradation depending on the type of organisms or microbial population involved as well as the type of environment (Abed et al., 2015). For instance, Jaafarzadeh et al., (2021) reported that the isolate of *Microbacterium paraoxydans B3F* was more sensitive to the existence of salt in the phenanthrene based medium, with no growth at 2 and 2.5% salt, while isolate of *Citrobacter NB2* showed growth in all salinity values. The stress of high osmotic potential from higher salt concentrations tends to inhibit the degradation of hydrocarbons. However, it is necessary to consider that hydrocarbons are less bioavailable in hypersaline environments than in non-saline ones (Tucci et al., 2021). This is because soluble salts reduce hydrophobic organic compounds solubility in water, the higher the salts concentration in aqueous phase, the higher the tendency of organic compounds to be absorbed to the solid matrix (Singh et al., 2021). In addition, the ability of variation in salinity to affect the rate of hydrocarbon degradation appears to be dependent on the natural variation in salinity regime of the sample source (Abena et al., 2019).

Furthermore, Zhang et al., (2021) investigated the effects of salinity on soil microorganisms and microbial activity, and it was shown that the mineralization of petroleum hydrocarbons by hydrocarbon-utilising bacteria was successful at high salt content, this is because higher salt content increased the inhibition of microbial growth leading to a faster degradation of organic pollutants. However, Jamal and Pugazhendi, (2021), reported that the rate of hydrocarbon metabolism decreased increasing salinity in the range of 3.3 to 28.4% and attributed the results to a general reduction in microbial metabolic rates. However, Zheng et al., (2018) and Abu Laban et al., (2015) studies revealed that low concentrations of salt decrease hydrocarbon clastic activity and the optimum degradation results are achieved within an average salinity range.

2.8.6 Microbial community dynamics during petroleum hydrocarbon biodegradation

One of the most complex and diversified groups of bacterial populations thrive in polluted sites with hydrocarbons (Jiao et al., 2018). Hydrocarbons in the environment are biodegraded primarily by a broad range of both bacterial and fungal groups. However, a small fraction of hydrocarbon-utilising microorganisms such as yeast and algae were reported to metabolise a limiting range of hydrocarbon

pollutants like gasoline and naphthalene in soil, and marine environments (Hou et al., 2021). The availability of microorganisms that can catabolise pollutants in the environment is one of the main factors for a successful biodegradation of petroleum hydrocarbons.

Furthermore, a number of published reports have demonstrated that microorganisms use petroleum hydrocarbons as their source of energy in oil-contaminated sites or facilities (Liu et al., 2016; Varjani, 2017; Bacosa et al., 2018). The adaptation of microbial communities to hydrocarbons in these oil-contaminated sites, increases their ability in the biodegradation process.

Indigenous populations of microorganisms which are ubiquitous in soil and groundwater have self-adapted to the hard conditions of the environments, thus they grow by using the carbon present on the pollutants as their source of energy and cellular growth which drives microbial activity.

However, site pollution with hydrocarbons minimises the range and stability of a microbial community (Truskewycz et al., 2019) for several reasons such as: continuous toxicity of the organic compounds, trouble in adjusting to the exceedingly nonpolar conditions, which influences microbial cells to break by disintegrating the cytoplasm membrane lipids and sealing up nutrients and water (Alabresm et al., 2018), by blocking microorganisms from getting necessary building blocks for growth.

In addition, oil pollution events can have strong, immediate negative effects on the indigenous soil microbial community by reducing the diversity and total biomass. Bacterial biofilms in the soil are naturally resistant to disturbances as they are capable of protecting their components through slow diffusion rates and co-metabolism of organic compounds (Thomas et al., 2021).

Furthermore, according to Kronenberg et al., (2017), the presence of microorganisms with the appropriate metabolic capabilities is the most important requirement for oil biodegradation. The bacterial communities which are exposed to hydrocarbons become adapted thus exhibiting selective enrichment and genetic changes (Chi et al., 2018). The adapted microbial communities can respond to the presence of hydrocarbon contaminants within hours and exhibit higher biodegradation rates than communities with no history of hydrocarbon contamination (Ding et al., 2021).

Thus, the capability to isolate large numbers of particular oil degrading microbes from an environment is normally taken as proof that those microbes are highly effective oil degraders of that particular environment (Mitter et al., 2021) and they can be utilised in bioremediation of petroleum contaminated sites. Since petroleum consists of a mixture of compounds and because single microorganisms catalyse only a minimal range of hydrocarbon components (Delgado et al., 2019), thus biodegradation of hydrocarbons demands a mixture of particular bacterial groups functioning to metabolise a greater range of hydrocarbon compounds (Bacosa et al., 2018). The concentration of contaminants in an environment instantly influences the activity of microbes. For instance, when pollutant concentrations are extremely high, contaminants may present toxic effects on the existing bacteria. Oppositely, when contaminant concentrations are low, they may halt the initiation of bacterial degradation enzymes (Thomas et al., 2021).

2.9. Petroleum and Polycyclic aromatic hydrocarbon biodegradation

Petroleum hydrocarbon contamination is immensely harmful to the environment and negatively impacts the life of humans, plants as well as animal ecosystem (Premnath et al., 2021). Petroleum hydrocarbons are highly used globally as a fuel because of its massive demand as a power source, and pollution occurs frequently because of petroleum exploration, production, accidental leaks during transportation and storage (Meruvu, 2021).

Environmental contamination by petroleum hydrocarbons and other PAHs is a serious concern of the modern world (Ma et al., 2018). Biodegradation of petroleum hydrocarbons is a complex mechanism that depends on the physical nature and on the quantity of the hydrocarbons present (Abena et al., 2019). Indigenous microbial populations play a significant role in breaking down hydrocarbon contaminants. Therefore, several microorganisms in polluted ecosystems have the capability to degrade persistent carbon-based contaminants (Hao et al., 2021) and have also adapted to the harsh conditions of the environment as result genomic mutations are caused in subsequent generations preparing them to become hydrocarbon degraders (Abena et al., 2019). Although the fastest degradation process of various organic contaminants takes place in the presence of oxygen, Meruvu, (2021) affirmed that among the microbial flora, bacteria were found to be most active in the degradation of PAHs and that the degradation of petroleum pollutants can be conducted by a certain enzymatic system (Truskewycz et al., 2019).

Different biological factors influencing hydrocarbon degradation have been reported by (Zharikova et al., 2018). However, hydrocarbon degradation by microbial communities depends on the composition of the community and its adaptive response to the presence of hydrocarbons. Biodegradation of petroleum usually requires the cooperation of more than one single species (Bacosa et al., 2018), this is because a consortium composed of many different bacterial species with overall broad enzymatic capacities is required to increase the rate of petroleum biodegradation.

Therefore, the purpose of this research study is to isolate, characterise and identify the different bacteria growing in oil-contaminated sites of Kupferberg landfill dumpsite site. Five previously studied bacterial strains mainly: *Stenotrophomonas sp.*, *Pseudomonas sp*., *Burkholderia sp*., *Aeromonas sp*., and *Sphingomonas sp* (Chaerun et al., 2004; Sarkar et al., 2017; Varjani, 2017; Xu et al., 2017) have been associated with biodegradation of petroleum hydrocarbons and will be used in this study to determine their degradation rates at different time intervals in degrading petroleum hydrocarbons under laboratory conditions. However, several studies have also shown that these selected bacteria have been reported to grow well in nutrient agar and Tryptic Soy Agar and have also adapted to diverse conditions of the environment such as temperature and pH (Nie et al., 2014 and Xu et al., 2017). Moreover, this research intends to provide a better understanding on microbial degradation of petroleum contaminants under different contaminated oil sites in Kupferberg landfill site towards a better understanding in biodegradation challenges and its effect on human health and the environment.

CHAPTER THREE
MATERIALS AND METHODS

3.1. Study area and sample collection

3.1.1. Study area

Windhoek is the capital city of Namibia, which is in Southern Africa. Windhoek's population growth rate is about 4.4%, which contributes to an increase in waste production and pollution, which further threatens the environment.

Samples for this study were obtained from a waste solid management facility in Windhoek, Namibia, namely Kupferberg landfill site (22.63712°S; 17.02672°E) located approximately 11 km southwest of the city. The landfill is divided into sections as per type of wastes present. The landfill consists of only one petroleum contaminated site, where both water and soil waste samples contaminated with petroleum hydrocarbons were collected from.

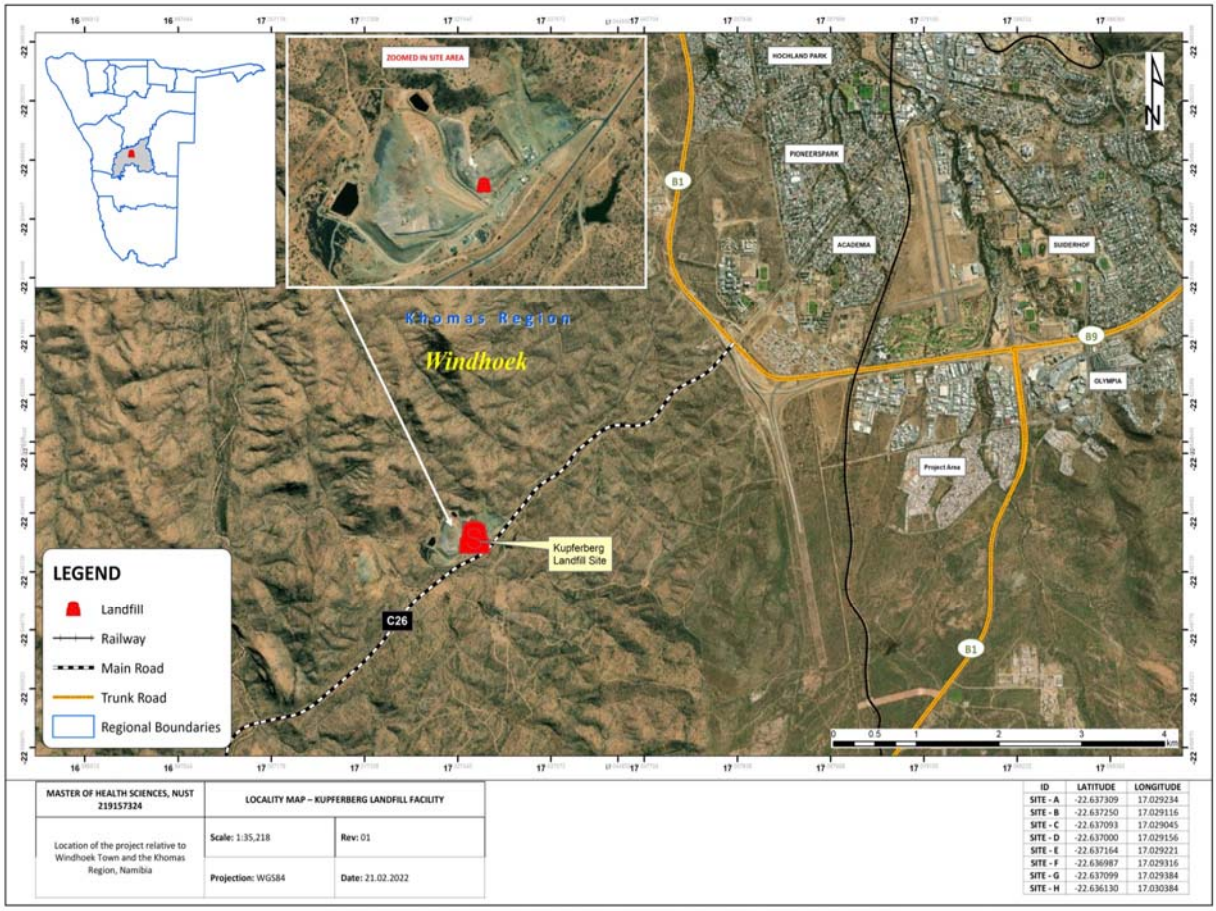

Figure 7: Study area (Kupferberg landfill site).

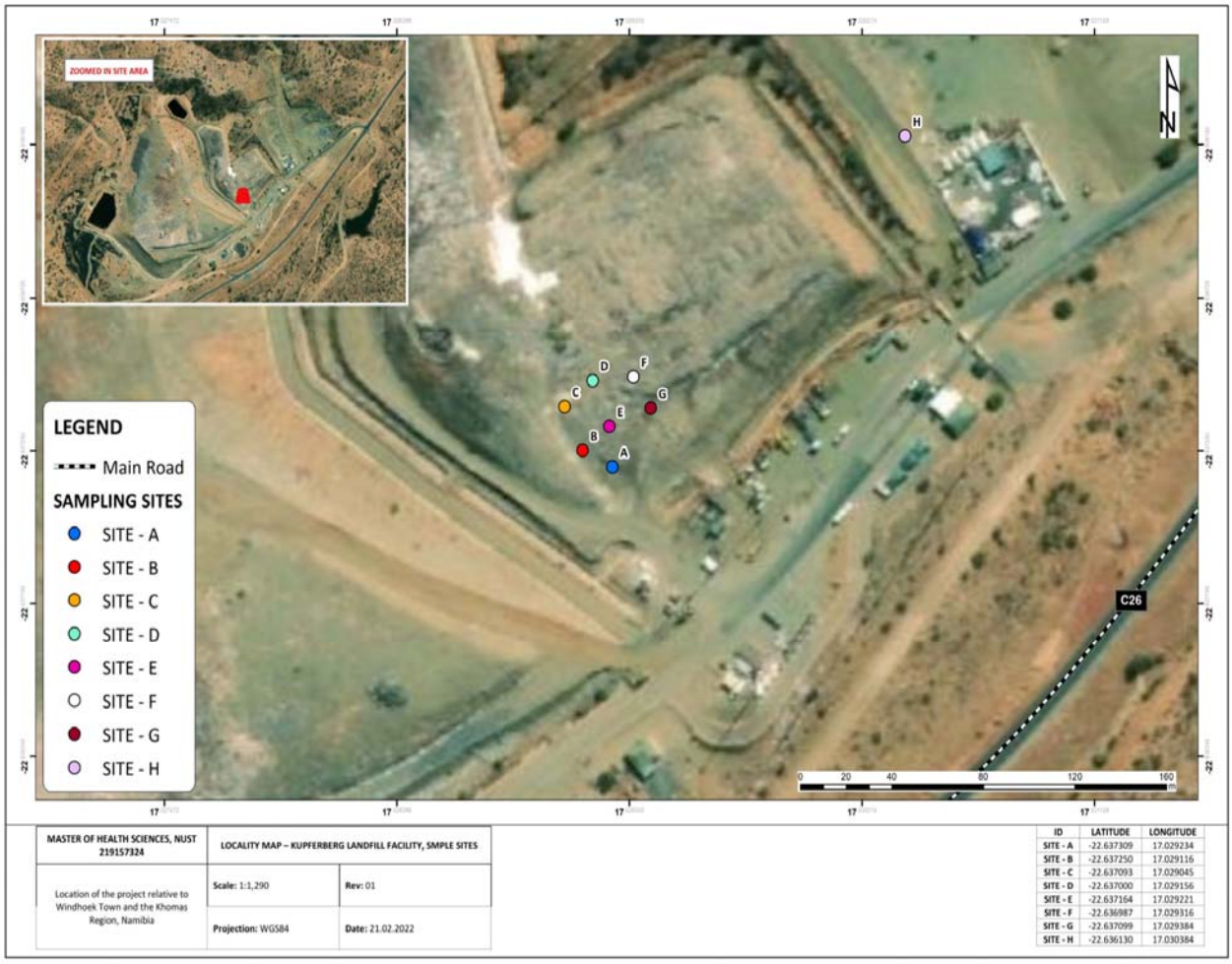

Figure 8: Zoomed area of interest where samples were collected.

3.1.2. Sample collection

Oil-contaminated soil was aseptically collected from Kupferberg landfill site during winter season. A total of twenty (20) samples was collected for this study. Eighteen (18) different contaminated waste samples (soil and combinations of soil and water) were obtained from the topsoil at a depth of 5 cm. The procedure for collection of topsoil samples was repeated for the two (2) other samples, one sample was collected within the landfill site, from an oil free polluted station, about 5-6 metres away from the oil contaminated site and the other was obtained about 11 km outside Kupferberg landfill site from a petroleum free polluted site, which were both used as controls for this study. Sterile plastic bottles, polythene bags, spoons and alcohol were used in collecting the samples. Sampling was not repeated due to COVID-19 restrictions. The samples collected were arranged in a box, transported and stored in the laboratory at 4°C for further analysis.

Figure 9: Oil-contaminated site at Kupferberg landfill site where samples were collected.

3.2. Petroleum hydrocarbon

Petroleum hydrocarbon used in this study was old engine diesel oil from Indongo Toyota, Windhoek, to see the ability of the isolated bacterial strains to grow in petroleum-based medium. Diesel fuel is approximately composed of 75% saturated hydrocarbons, with an average chemical formula of $C_{12}H_{23}$.

3.3. Preparation of culture media

In preparation of the media, 28 g of nutrient agar (NA) was weighed and dissolved in a 1000ml of distilled water, which was later used for culturing. 1000ml of distilled water, measured using a volumetric cylinder, placed in a 1000ml bottle, which was later used for serial dilution. Both the media and distilled water were autoclaved at 121°C for 15minutes.

3.4. Isolation of bacterial strains and microbial count

Serial dilution of each sample was done separately. Six test tubes all containing 9 ml sterilised distilled water. Tenfold serial dilution was made by mixing 1g of soil sample in the first test tube and then transferring 1 ml (1000µL) of the dilution by using a sterilised micropipette tip to the second and so on, a sterilised micropipette tip was used every time before the next transfer (Ebakota et al., 2017). A

new sterile micropipette tip was used every time to dispense 0.1 ml (100µL) from each dilution tube onto nutrient agar plates. A glass spreader, soaked in 70% ethanol, flamed, and cooled, used for spreading on the plates. The plates were incubated at 36.5**°**C for the growth of bacterial colonies and total viable counts for bacteria were determined by enumerating the colony forming units after 24 hours incubation (Ebakota et al., 2017). After incubation, the number of colonies was counted only when 30 - 300 colonies were measurable. The microbial population in the soil was presented as CFU/ml and calculated as:

CFU/ml= (no. of colonies x dilution factor) / volume of culture plate

Pure cultures of bacterial isolates were obtained by sub-culturing on the nutrient agar plates and pure cultures were then transferred to agar slants for further biochemical tests.

3.5. Characterization and Identification of the isolates

The bacterial isolates were characterized by both morphological and biochemical characteristics (Ebakota et al., 2017). The morphological features included elevation, margins, form and colour of bacterial colonies. Biochemical tests were carried out using the standard methodology, based on the manufacturer's (BioMerieux, France) instructions.

The conventional tests are inoculated with a saline bacterial suspension which reconstitutes the media. Therefore, 1-4 colonies of identical morphology were picked up using a sterile inoculating loop from the agar plates and inoculated in the API NaCl 0.85% medium, fresh cultures (18-24 hours) were used (BioMerieux, France) according to the product protocols. The density of the inoculum was adjusted to 0.5 McFarland, otherwise the API 20NE strip test may not function correctly. In particular, a weaker inoculum may lead to false negative results. About 5 ml of distilled water is placed on the tray to create a humid atmosphere. During incubation, metabolism produces colour changes that are either spontaneous or revealed by the addition of reagents. The assimilation tests are inoculated with a minimal medium and the bacteria grow if they are capable of utilising the corresponding substrate. Test results were read after 24 hours and the numerical profile (API codes) were computerized in the API 20 NE information database system Version 7. All the bacterial species that had an identity% ranging from 97-99% similarity was considered accurate with a good homology.

3.6. Hydrocarbon degradation potential of bacterial of isolates

Thirty millilitres of sterile nutrient broth (10 g of peptones, 1 g of beef extract, 2 g of yeast extract, 5 g of NaCl in 1000 ml of distilled water) was prepared and poured into twelve conical flasks, supplemented with 1 ml of sterile old diesel engine oil (Ebakota et al., 2017). The media was inoculated

with a loop full of the bacterial isolates, conical flasks were shaken well to mix all the content and it was then incubated at 37°C. The microbial growth was determined using a UV spectrophotometer at 600 nm. The optical density of the medium was measured to determine the degrading ability of hydrocarbons as the sole source of carbon (Meenakshisundaram and Bharathiraja, 2014).

3.7. Phylogenetic analysis

Phylogenetic tree was used to show a relationship among the ten selected isolates. DNA extraction was not carried out. However, bacterial 16S rRNA gene sequences were obtained from NCBI (www.ncbi.nlm.nih.gov) database using the basic local alignment search tool (BLAST) algorithm. Ten highly homologous sequences of the isolated and identified strains were obtained by blast in results and were downloaded and a phylogenetic tree was constructed to show the resemblance among the selected isolated strains. The phylogenetic tree of the aligned sequences was constructed using the Neighbor-Joining method. The evolutionary analyses were conducted in MEGA version 6.

3.8. Data analysis

Numerical data obtained for the degradative activity from each experimental assay were subjected to two-way ANOVA without replication using Excel Data Analysis Tools. Two-way ANOVA test was used to conclude whether petroleum degradation differs significantly according to the type of bacteria isolated and time. *P* value of less than 0.05 was considered to show statistical significance.

3.9. Research Ethics

Research permit and ethical clearance was obtained as per NUST regulations. Then a collection permit was obtained from the City of Windhoek, Solid Waste Management. Maximising benefits and minimising harm are one of the ethical principles that was used in this research as few samples were used to characterise and identify bacterial strains, of which the study will be of great benefit to Namibia once the project is adopted by any intellectual benefactor e.g. the government for the mass production of these bacterial strains in order to eliminate oil pollution caused by oil spills around Namibia. See **Appendix F and G**.

CHAPTER FOUR
RESULTS

4.1. Microbial count

Total bacterial count of the samples was determined by serial dilution followed by pour plate method using nutrient agar as medium. The number of microorganisms of each sample was calculated as follows: CFU/ml= (no. of colonies x dilution factor) / volume of culture plate, mean average, standard deviation as well as standard error for each contaminated site and the results are given in the figure below. The results in Fig. 10 show that sites G (3.6 x 10^4 CFU/ml), A (3.0 x 10^4 CFU/ml), and F (2.9 x 10^4 CFU/ml) had the highest mean values.

The data in the graph below shows that amongst the different petroleum contaminated sites from Kupferberg landfill site, site G had the highest population count, followed by site A and F comparing to the remaining sampling sites based on the mean average of the microbial count (cfu/ml) from each sample obtained for that particular site. However, some error bars on the graph are overlapping and the others are not overlapping. For the bars that are overlapping, it can be concluded that there was no significant difference in growth (Abdulrasheed et al., 2020), meaning that the bacteria grew faster and better in sites C, D, E, F, G and I compared to the other sites. For the bars that are not overlapping, it means that there is a statistical difference in the growth because the bacteria found in sites A, B, F, G and H grew more or less at the same rate (Abdulrasheed et al., 2020).

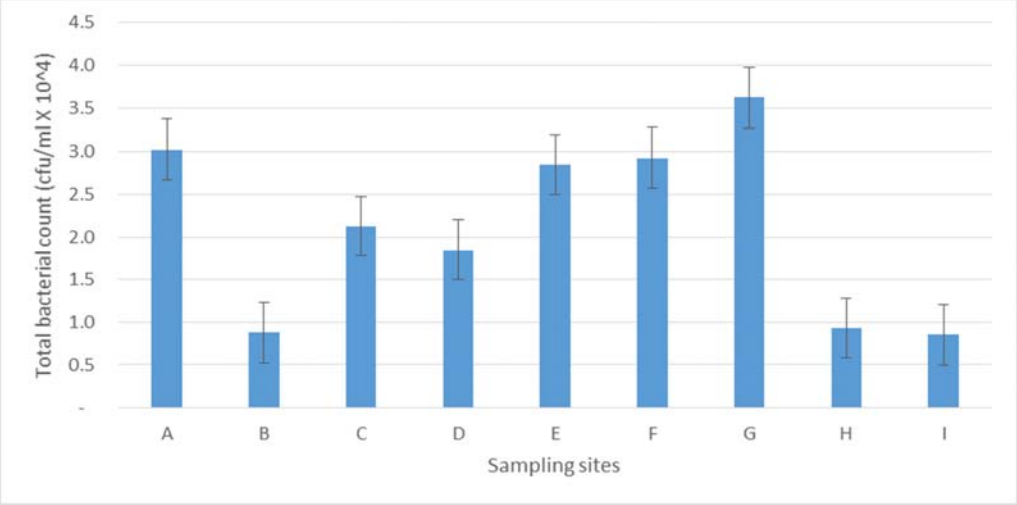

Figure 10: Total bacterial count per sampling sites of soil contaminated with petroleum hydrocarbon wastes from Kupferberg landfill site.

4.2. Morphology identification of the isolates obtained from Kupferberg landfill dumpsite

Colony morphology is one of the first steps in characterising and identifying a bacterial culture. Thus, the bacterial species obtained from Kupferberg landfill site were characterised according to their colony form (shape), colour, elevation and margin as shown in Table 4.2. Pictures of the bacteria isolates grown on nutrient agar medium have been attached in **Appendix A.**

Table 4.1. Morphological features of bacterial isolates from soil contaminated with petroleum hydrocarbon wastes from Kupferberg landfill site.

Sample	Colony morphology			
	Colour	Form	Elevation	Margin
Site A, sample 1	cream	round	convex	entire
Site A, sample 2	white/cream	round	convex	entire
Site A, sample 3	yellow	irregular	raised	undulated
Site B, sample 1	yellow	circular	flat	entire
Site C, sample 1	yellow	irregular	raised	undulated
Site C, sample 2	opaque	circular	raised	entire
Site D, sample 1	opaque	punctiform	flat	undulated
Site D, sample 2	yellow	round	convex	entire
Site E, sample 1	orange	round	raised	entire
Site E, sample 2	yellow	irregular	raised	undulated
Site E, sample 3	opaque/yellow	round	raised	entire
Site F, sample 1	yellow	circular	flat	entire
Site F, sample 2	opaque/white	punctiform	flat	entire
Site F, sample 3	yellow	irregular	flat	lobate
Site G, sample 1	opaque/white	circular	convex	entire
Site G, sample 2	white/cream	round	convex	entire
Site G, sample 3	yellow	irregular	raised	undulated
Site G, sample 4	opaque/white	circular	convex	entire
Site H, sample 1	opaque/yellow	round	raised	entire
Site I, sample 1	white/cream	round	convex	entire

4.3. Biochemical characteristics and identification of bacterial isolates

The identification of bacterial isolates from the polluted contaminated site was obtained from the various biochemical tests done. Whereas, +ve= Positive results and -ve= Negative results. The identification was obtained with a numerical profile from the API 20 NE result paper sheet. The numerical code consists of a 7-digit profile, which was entered on an analytical database software that identified the organism according to the positive results obtained from the different biochemical tests performed on the API 20 NE strip. All the bacterial species that had an identity% ranging from 97-99% similarity was considered accurate with a good homology.

Table 4.2: Biochemical characteristics and identification of bacterial isolates by API 20 NE from soil contaminated with petroleum hydrocarbon wastes from Kupferberg landfill site.

	Biochemical test and bacterial identification					
Sample	Citrate	Indole	Oxidase	Urease	Bacterial specie(s)	Identity %
Site A, sample 1	+ve	-ve	+ve	-ve	*Aeromonas hydrophila*	99.2
Site A, sample 2	+ve	-ve	-ve	-ve	*Stenotrophomonas maltophilia*	98.5
Site A, sample 3	-ve	-ve	-ve	-ve	*Sphingomonas paucimobilis*	99.5
Site B, sample 1	+ve	-ve	-ve	-ve	*Pseudomonas luteola*	99.9
Site C, sample 1	-ve	-ve	-ve	-ve	*Sphingomonas paucimobilis*	98.9
Site C, sample 2	+ve	-ve	+ve	+ve	*Burkholderia gladioli*	98.7
Site D, sample 1	-ve	-ve	+ve	+ve	*Photobacterium damselae*	99.4
Site D, sample 2	-ve	-ve	-ve	-ve	*Chryseobacterium indologenes*	98
Site E, sample 1	-ve	-ve	+ve	+ve	*Brevundimonas vesicularis*	97.5
Site E, sample 2	-ve	-ve	-ve	-ve	*Sphingomonas paucimobilis*	99.1
Site E, sample 3	+ve	-ve	+ve	+ve	*Burkholderia cepacia*	99.9
Site F, sample 1	+ve	-ve	-ve	-ve	*Pseudomonas luteola*	98
Site F, sample 2	-ve	-ve	-ve	-ve	*Pasteurella spp*	98.9
Site F, sample 3	+ve	-ve	+ve	-ve	*Pseudomonas aeruginosa*	98.2
Site G, sample 1	-ve	-ve	-ve	-ve	*Aeromonas salmonicida*	99.6
Site G, sample 2	+ve	-ve	-ve	-ve	*Stenotrophomonas maltophilia*	97.5
Site G, sample 3	-ve	-ve	-ve	-ve	*Sphingomonas paucimobilis*	99.5
Site G, sample 4	-ve	-ve	-ve	-ve	*Aeromonas salmonicida*	99.9
Site H, sample 1	+ve	-ve	+ve	+ve	*Burkholderia cepacia*	98.8
Site I, sample 1	+ve	-ve	-ve	-ve	*Stenotrophomonas maltophilia*	96.8

4.4. Optical density

The optical density for the bacterial isolates was obtained on a daily basis, by measuring the wavelength of 1ml of the inoculum at 600 nm using a spectrophotometer. According to table 4.5, it is well indicated that all bacterial species were able to degrade diesel after 7 days of incubation. The increase in the optical density in different time (day) intervals shows that the bacterial isolates in diesel were able to reproduce and use diesel as their source of carbon and energy.

Table 4.3. Optical Density (OD) at 600 nm of isolated bacterial samples from soil contaminated with petroleum hydrocarbon wastes from Kupferberg landfill site, incubated at 37°C.

Bacteria	Optical density at 600 nm							
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Control (Nutrient broth)	0.003	0.011	0.017	0.019	0.023	0.124	0.488	0.871
Control + Organism	0.066	0.086	0.158	0.245	0.461	0.806	1.461	1.763
Aeromonas hydrophila	0.174	0.262	0.527	0.880	1.537	1.808	1.990	2.009
Stenotrophomonas maltophilia	0.130	0.215	0.480	0.730	1.191	1.210	1.339	1.488
Sphingomonas paucimobilis	0.257	0.491	0.705	1.080	1.552	1.672	1.812	2.070
Pseudomonas luteola	0.126	0.287	0.392	0.858	1.362	1.582	1.956	2.207
Burkholderia gladioli	0.110	0.223	0.348	0.522	0.635	0.825	0.957	1.077
Photobacterium damselae	0.144	0.258	0.296	0.352	0.414	0.593	0.683	0.740
Pseudomonas aeruginosa	0.202	0.861	1.540	1.952	2.150	2.240	2.440	2.521
Brevundimonas vesicularis	0.084	0.174	0.262	0.405	0.640	0.737	0.821	0.896
Burkholderia cepacia	0.107	0.140	0.242	0.636	0.757	0.821	1.025	1.990
Aeromonas salmonicida	0.184	0.290	0.631	0.895	0.932	1.136	1.478	1.732

4.4.1. Optical density (growth rate) determination

The optical density of the sample from culture medium was determined at 600 nm after 7 days incubation using a spectrophotometer. The changes observed in the optical density of the culture medium during the degradation of diesel engine oil by the isolated bacteria can be seen in the graph below. The results show that the incubation time was directly proportional to the optical density, this means that the bacteria in the medium continued to grow as time increased and were still able to utilise and degrade the hydrocarbons present in the diesel oil for their cellular growth and metabolic activities. The degradation process for each incubation day by the selected bacteria is presented individually. See **Appendix E.**

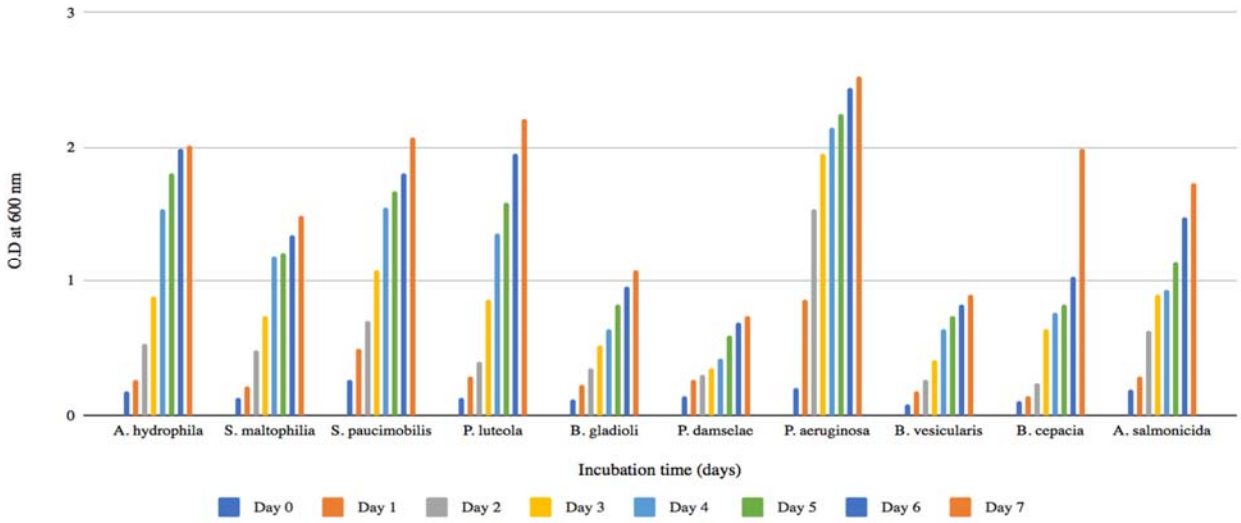

Figure 11: The effect of time (days) on the degradation of diesel by the hydrocarbon-degrading bacteria from soil contaminated with petroleum hydrocarbon wastes from Kupferberg landfill site.

4.6. Data analysis

From the bacterial isolates grown in diesel oil, *Pseudomonas aeruginosa* had the highest mean average of about 1.738, followed by *Sphingomonas paucimobilis* with a mean of 1.204 and lastly *Aeromonas hydrophila* with a mean average of 1.148. The data obtained showed that for rows/optical density (*P*= 0.00 < 0.05) and for columns/ incubation period (*P*= 0.00 < 0.05), therefore we reject the null hypothesis. This means that there was statistical difference within the collected data, and that the bacteria isolated from Kupferberg landfill site, could grow in diesel oil and successfully degrade the hydrocarbons present in the diesel by utilising it as their energy source for cellular metabolism, due to the increase in their OD readings. See **Appendix D.**

4.7. Species isolated and blasted on blastn NCBI

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.66376653 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths (below the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. The analysis involved 10 nucleotide sequences obtained from NCBI. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed

for each sequence pair. There were a total of 1562 positions in the final dataset. Evolutionary analyses were conducted in MEGA version 6 (Tamura et al., 2013).

As seen from the phylogenetic tree (Figure 12) shows that all the species share common ancestry. However, *Brevundimonas vesicularis* and *Sphingomonas paucimobilis* share a common clade. On one hand, *Burkholderia gladioli* and *Burkholderia cepacia* are in a similar clade, although *Stenotrophomonas maltophilia* and *Photobacterium damselae* do not share a clade with any of these species, this means that they are different and have unique sequence information that makes them different from the others. On the other hand, species of *Aeromonas salmonicida* (length of 0.006) and *Aeromonas hydrophila* (length of 0.007) are closely associated to each other in comparison to any other species in this study, because the distance between them are shorter as seen in the tree below.

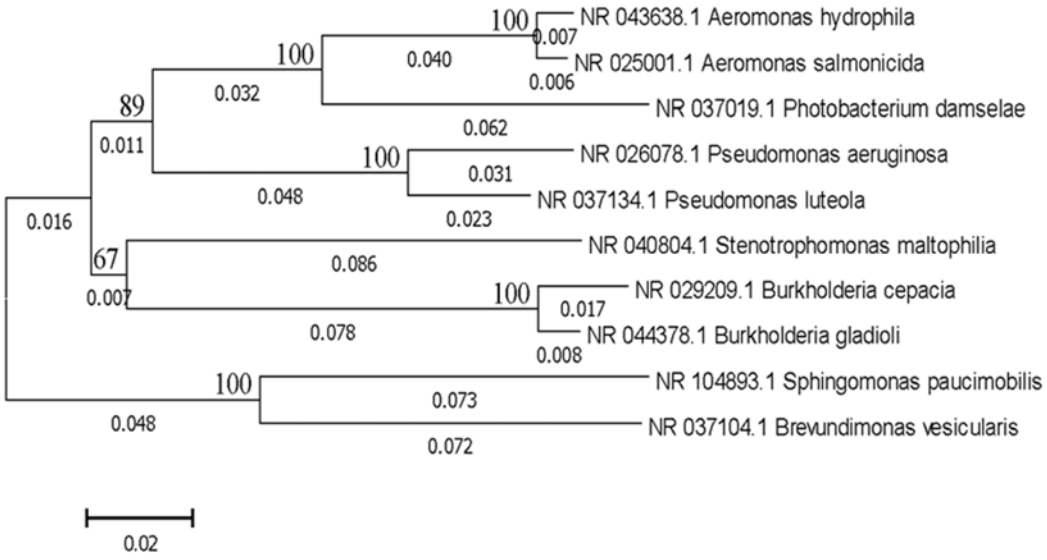

Figure 12: The evolutionary history was inferred using the Neighbor-Joining method. The tree is based on the 16S rRNA sequences (from NCBI) of the bacterial isolates obtained from Kupferberg landfill site.

CHAPTER FIVE
DISCUSSION

Hydrocarbon-degrading bacteria are extremely diverse and widely distributed in nature. They play an important role in the natural surroundings, they promote geological rearrangements of organic chemicals and can break down numerous complex compounds by modifying their degradative enzymatic structure (Wu et al., 2017). However, they are considered as important petroleum biodegradable microorganisms because they present a significant role in the degradation of spilled oil-sludge contaminants in the environment and can efficiently utilise hydrocarbons for their sources of carbon and energy (Sarkar et al., 2017).

In the present study, several soil contaminated samples were collected from an environment highly contaminated with petroleum waste. Thus, native bacterial isolates possess great potential in mineralizing diesel oil in hydrocarbon polluted habitats (Ke et al., 2017). Thus, the considerable efficiency of these indigenous isolates in metabolising petroleum in oil-sludge contaminated sites was confirmed before by many scientists (Djumyom Wafo et al., 2018; Nguemté et al., 2018; Iqbal et al., 2019; Kiamarsi et al., 2020; Li and Ding, 2021). The indigenous bacterial populations studied in this research grew efficiently in nutrient enriched medium with diesel, thus allowing them to utilise petroleum for their cellular growth. All strains isolated in this study were found to be oil degraders which grew in agar plates and liquid media containing diesel oil. These oil degraders were identified using cell morphology, and biochemical tests.

In accordance with several published studies, the most significant hydrocarbon-utilising bacteria present in soil habitats are *Bacillus, Micrococcus, Pseudomonas, Enterococcus, Corynebacterium, Aeromonas, Stenotrophomonas, Rhodococcus, Burkholderia, Flavobacterium* and *Vibrio* (Chaerun et al., 2004; Sarkar et al., 2017; Varjani, 2017; Xu et al., 2017; Wu et al., 2020; Kuyukina et al., 2021; Rahmeh et al., 2021). It is striking that *Bacillus spp*. which are commonly detected by several researchers in various hydrocarbon-contaminated environments (Chang et al., 2016; El-Sheshtawy and Ahmed, 2017; Khanpour-Alikelayeh et al., 2020), but were not encountered during the present study. However, the present findings agree with Wang et al., (2019) who isolated diesel oil degrading bacteria from five different soils in the USA such as: *Sphingomonas paucimobilis, Pseudomonas aeruginosa* and *Aeromonas Salmonicida*. Li et al., (2017), showed in his study that bacterial populations were high in the subsoil samples with 2.93 x 10^6 CFU/g, while the topsoil sample had a mean bacterial count of 2.49 x 10^6 CFU/g collected from a petroleum hydrocarbon-contaminated soil,

thus great amounts of petroleum contents were highly observed. However, the findings in this research, shows that both site G (3.9 x 10^4CFU/ml) and site A (3.0 x 10^4 CFU/ml) had the highest population count in topsoil samples. Similarly, a large population of bacteria was also noted from an oil-spilled environment in the subsoil by Skariyachan et al., (2021) and Bhatt et al., (2021), it was revealed that the native microbial isolates as well as levels of hydrocarbon degraders appeared to be easily affected by environmental exposure to hydrocarbons within their community. However, the difference amongst the CFU values recorded from the different sites in each study done, indicates that the topsoil with the large number of microorganisms could be due to the presence of decayed organic matter (humus), which must have resulted from an increase of oxygen production, where most aerobes thrive (Ebakota et al., 2017). On the other hand, in the case of the subsoil, the smaller number of microbes could have resulted due to a reduction in the oxygen and humus levels, thus a smaller number of microorganisms, especially anaerobes were found in the subsoil (Ebakota et al., 2017).

According to this present study, bacterial identification as well as bacterial count were achieved on various petroleum polluted soil waste samples from Kupferberg landfill site. Whereas, twelve species were identified and it was found that *Sphingomonas paucimobilis* was the predominant isolate in all the samples. The abundance of *Sphingomonas* at the dumpsite revealed that the genus has evolved the ability to acclimatise to the hydrocarbon environment (Semenova et al., 2021). However, it has been proven that not only strains belonging to *Sphingomonas* spp.*,* but overall, *Pseudomonas, Aeromonas, Stenotrophomonas* and *Burkholderia* species could be useful in cleaning up oil spills (Sarkar et al., 2017; Wu et al., 2020; Jiang et al., 2021). A similar investigation performed by Nwankwo and Godson, (2021), showed that from the five genera identified, *Corynebacterium* was the only genus detected in abundance out of all the samples collected from oil polluted sites in Ejama state. Bacteria belonging to this genus are described to be developing a primary role in the degradation of PAHs released in soil environments (Dotaniya et al., 2020), which could justify the abundance of these isolates in different petroleum polluted sites. However, the capability of these bacterial cultures in degrading diesel were analysed individually, respectively at a temperature of 37°C in this study. Adetunji et al., (2021) also tested the capacity of individual isolates as well as the mixed consortium obtained from a polluted soil sample in breaking down hydrocarbons at different pH values and temperatures.

The significance of this research was to investigate the potentiality of different microbial isolates from Kupferberg landfill site in metabolising petroleum such as diesel oil in the presence of oxygen under

laboratory conditions (Ebakota et al., 2017). These microbial isolates were characterised by the API 20 NE identification database programme, which enabled preliminary identification of the isolates as *Burkholderia* genus.

Moreover, the microbial community and composition present in the environment can lead to several changes in the dynamic stability state of the soil (Liu et al., 2016). Twelve bacterial isolates were identified and ten were selected for further research in this study. Since, the selected bacterial isolates have been associated with hydrocarbon degradation in previous studies, thus, the capacity of these selected isolates in degrading hydrocarbons were considered and analysed in the present study to determine their ability to grow in a petroleum based medium, suspended with old engine diesel oil. Based on the outcome of this research, it was observed that the ten microbial strains differed in their capacity to degrade diesel. All isolated bacteria could degrade diesel, however, *Pseudomonas aeruginosa* and *Pseudomonas luteola* showed to have the maximal degradation of petroleum hydrocarbons among all other isolates. Nonetheless, Bekele et al., (2020) carried out a similar experiment, by analysing the biodegradation of diesel by the isolated bacteria from oil polluted soil waste, and it was reported that *Pseudomonas aeruginosa* and *Bacillus subtilis* showed maximum degradation with a higher concentration of diesel. Kalantar et al., (2021) investigated the bacterial strains obtained from oil-sludge contaminated waste and their ability to grow in aromatic hydrocarbons. Kalantar et al., (2021) revealed that the maximum degradation of toluene was shown by *Burkholderia* strains. Furthermore, in this present study all isolated bacterial strains grew successfully on nutrient broth suspended with diesel oil and were able to degrade the hydrocarbons present in diesel, some at faster rate and some at a slower rate. In contrast, Wu et al., (2019) revealed that *Bacillus* strains were unable to grow in hydrocarbon-based media, as it lost the ability to degrade hydrocarbons.

The optical densities in this research indicate the growth dynamics achieved by the various microorganisms in degrading diesel or growing in the presence of hydrocarbons. All the identified bacteria could successfully grow in petroleum enriched medium and it is expected that the hydrocarbon assimilating capabilities in the liquid medium is due to adaptation of isolate due to previous exposure to hydrocarbons (Ebakota et al., 2017). This same procedure was applied and used in numerous researches conducted by (Chaudhary et al., 2019; Sher and Rehman, 2019). However, following a seven-day incubation period experiment done in this research, the identified bacterial isolates had efficiently utilised diesel, although the degradation level among the isolates differs.

According to the ANOVA statistical analysis, *Pseudomonas aeruginosa, Sphingomonas paucimobillis* and *Aeromonas hydrophila* had the highest optical density. A similar study done by Farag et al., (2018) reveals that a linear increase in the optical density at 420 nm was observed for fifteen days, and it was concluded that *Pseudomonas aeruginosa, Sphingomonas paucimobilis* and *Enterococcus faecalis* had the maximum growth in 1% crude oil enriched medium. In another study by (Liu et al., 2016; Chantarasiri, 2021), showed that *Pseudomonas aeruginosa* and *Achromobacter xylosoxidans* had the highest optical densities at 540 nm in a medium enriched with aromatic hydrocarbons. The gradual increase of the optical density of all these isolates at different wavelengths demonstrates that the bacteria that were obtained from an oil-sludge contaminated soil, however, their cells were able to reproduce in the period of incubation days, indicating an increase in microbial activity (Rehman et al., 2021), which lead these isolates to degrade oil and use it for their cellular growth.

Meanwhile, an experiment done by Sher et al., (2020); Jabbar and Hussein, (2021), showed that *Staphylococcus haemolyticus* and *Burkholderia cepacia* had the lowest ability to degrade crude oil. Moreover, a similar research carried out by Peng et al., (2021), demonstrated that *Citrobacter freundii* exhibited lowest engine oil degradation capacity. However, this study shows that *Photobacterium damselae* and *Brevundimonas vesicularis* were able to degrade diesel at a slower rate. Therefore, the low degradation rate of these isolates in certain oil-based mediums could be as a result that certain bacteria are limited, and are only able to eliminate specific hydrocarbons under certain biological conditions (Tariq et al., 2019). The level of utilisation of diesel differs from one bacterium to another due to the differences in their optical density which shows their growth rate in the presence of diesel. This could be resulting in the behaviour of the microbial cells in diesel oil.

In accordance with the optical density, it was deduced that the incubation period was directly proportional to the optical density, this means that as the incubation period increases, the rate at which the identified organisms degrade diesel also increases. Nevertheless, this might have occurred because of the cellular growth during the bacterial exponential phase (Lara-Moreno et al., 2021). However, the isolates with the highest degradation potential were observed and this could have resulted either because of the natural composition of the present hydrocarbon, thus assimilating their capabilities in the degradation process or because the strains have adapted to previous exposure of contaminants (Kumar et al., 2020). Furthermore, the strains identified in this experiment were obtained through the same supplemented medium, thus greatest diesel degraders found in this study

might possibly occupy the same niche as the other low degraders and might still compete for the same organic compounds (Kumar and Fulekar, 2021).

Nevertheless, the capacity of the hydrocarbon-utilising bacteria to mineralize an extensive range of organic compounds are affected by a variety of environmental factors: biotic and abiotic (Palma et al., 2018). In addition, both the physical nature and chemical structure of the microbial community are affected by temperature which influences the rate of the hydrocarbon metabolism by various microorganisms (Okonkwo et al., 2021). However, the density of the oil rises at low temperatures, whilst the evaporation of alkanes decreases, and the water miscibility is reduced, thus retarding and reducing the onset of biodegradation (Han et al., 2018). Moreover, Shakya et al., (2021) and Dhaulaniya et al., (2019) suggested that the degradation ratio typically decreased with dropping temperature, which was believed to result primarily from reduced rates of enzymatic dynamics.

However, this present study showed the maximum biodegradation activity by the isolated bacterial strains at 37°C. Although microbial degradation of hydrocarbons can occur in a wide range of temperatures, the optimal condition for the biodegradation process to take place is between 30-40°C (Wang et al., 2021). Since most enzyme functions are performed at 37°C, enzymes are able to retain its structure at that temperature, allowing it to break down complex molecules efficiently (Ojha et al., 2019). Thus, the degradation of diesel oil by the selected bacterial strains in this present study, was indicated by an increase of OD readings at 37°C, which lead to an increase in enzymatic activity, leading to an accelerated metabolic process, which agrees with the findings of (Ebakota et al., 2017; Krishnamurthi et al., 2021).

Moreover, cold-adapted, psychrophilic microorganisms are extensively distributed in nature and are capable of developing at temperatures around 0°C (Miri et al., 2021). They play a fundamental role in the *in-situ* breakdown of hydrocarbon compounds in cold environments, where surrounding temperatures usually correspond with their proliferation temperature range (Turchetti et al., 2020). In addition, a similar study done by Yi-bin et al., (2014), showed that petroleum hydrocarbon-degrading strains isolated from the Antarctic ocean, based on the analysis of the Raman spectrum of degradation production, showed that *Shewanella* sp and *Planococcus* sp degraded diesel, PAHs and other petroleum hydrocarbons with high efficiency at low temperatures (0-10°C).

Furthermore, at temperatures above 40°C, the rate of hydrocarbon degradation is decreased, and this may contribute to membrane toxicity of hydrocarbons (Mandelli et al., 2017). In addition, greater temperatures lead to protein denaturation by altering the organism's cellular wall and membrane (Mino and Nakagawa, 2018). Nonetheless, a similar study to this research was done by Tao et al., (2017), and they reported on hydrocarbon degradation at 50°C. The study showed the degradative loss at 45°C by a consortium of (*Bacillus cereus, Bacillus firmus, Bacillus lentus* and *Kocuria flava)*. Where the mixed consortium had a weight loss of 8.9 mg of hydrocarbons per 5 ml mineral salt medium (MSM), while *Bacillus subtilis* at 50°C had a weight loss of only 3.8 mg of hydrocarbons alone (about 10% loss), thus indicating that *Bacillus subtilis* is a survival strain in higher temperatures.

CHAPTER SIX
CONCLUSION AND RECOMMENDATIONS

6. 1. Conclusion

This study focused on characterising bacterial isolates obtained from a petroleum polluted waste dumpsite for the degradation of hydrocarbons. The isolated bacterial genera from the oil contaminated soil in Kupferberg landfill dumpsite have been shown to successfully degrade a wide variety of hydrocarbons. Based on the laboratory results, it was observed that the cellular growth of the bacterial isolates due to their optical densities was directly proportional to the diesel oil biodegradation. It is, however, evident from the outcome of this investigation that *Pseudomonas aeruginosa* is an excellent petroleum degrader. Besides being a fast-growing organism, *Pseudomonas aeruginosa* is also known for its ability in degrading a wide variety of oil-sludge contaminants. Therefore, *Pseudomonas aeruginosa* could be considered as a future key component in the clean-up and treatment strategy for the remediation of petroleum compounds.

6.2. Recommendations:

1. To analyse the impact of different biological factors associated with the degradation of petroleum hydrocarbons (different temperatures, pH, nutrients and salinity.
2. Investigate the effect of mixed consortia versus individual bacterial strains from petroleum contaminated sites on degrading abilities.
3. Future research should also address the ability of both mixed consortia and individual strains in degrading different concentrations of petroleum hydrocarbons.
4. Study the use of fungi and algae in degrading petroleum hydrocarbons.
5. Study the impact of seasonal change of the bacterial isolates in degrading hydrocarbons.
6. Investigate the metabolic processes of mixed consortia under anaerobic conditions.

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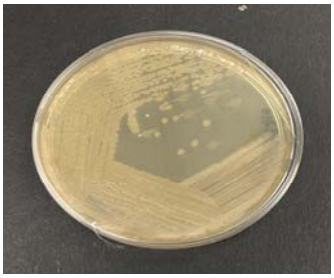
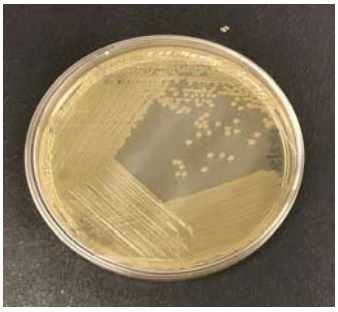
Appendices

Appendix A. Plates of bacterial isolates obtained from soil contaminated with petroleum hydrocarbon wastes from Kupferberg landfill dumpsite.



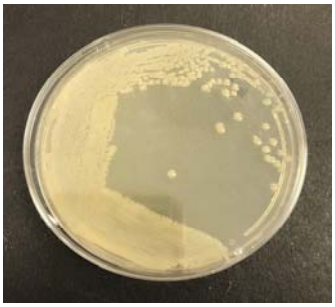
Burkholderia cepacia

Sphingomonas paucimobilis

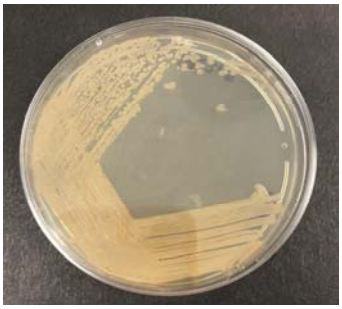


Pseudomonas luteola

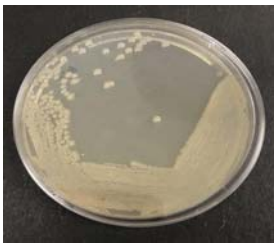
Chryseobacterium indologenes



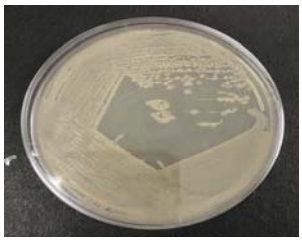
Aeromonas hydrophila



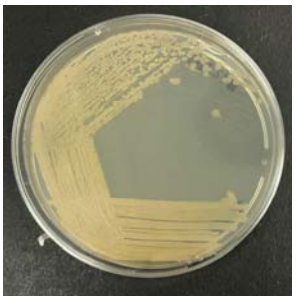
Pseudomonas aeruginosa



Stenotrophomonas maltophilia



Photobacterium damselae



Brevundimonas vesicularis

Burkholderia gladioli

Appendix B. Bacterial count per sampling sites of soil contaminated with petroleum hydrocarbon wastes from Kupferberg landfill dumpsite.

	Site (cfu/ml X 10^4)								
Sample	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**
1	4.0	3.5	3.8	3.2	3.1	3.3	3.2	3.7	3.4
2	4.9	0	4.7	4.2	3.5	3.9	3.9	0	0
3	3.2	0	0	0	4.8	4.5	4.3	0	0
4	-	0	0	0	0	0	3.1	0	0
Mean	3.0	0.9	2.1	1.9	2.9	2.9	3.6	0.9	0.9
SD	0.9	2.0	2.5	2.2	0.9	0.6	0.6	2.1	2.0
SE	0.5	1.2	1.4	1.3	0.5	0.3	0.3	1.2	1.1
n	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0

Appendix C. Statistical analysis on population count.

Statistically, the results show that the *p*-value for the rows = 1.275 > 0.05, which indicates that there was no statistical difference in the bacterial growth, concluding that some bacteria grow better than others. Whereas, the *p*-value for the columns= 0.020 < 0.05, shows that there is statistical difference in the bacterial growth, indicating that bacteria found in some sites grow more or less at the same rate.

Anova: Two-Factor Without Replication

SUMMARY	*Count*	*Sum*	*Average*	*Variance*

SD	9	13.70459	1.522732	0.606447
SE	9	7.912349	0.87915	0.202149
n	9	27	3	0
3.025	3	4.341521	1.447174	1.840755
0.875	3	6.187393	2.062464	0.841584
2.125	3	6.934954	2.311651	0.633291
1.85	3	6.460598	2.153533	0.752335
2.85	3	4.40198	1.467327	1.797096
2.925	3	3.94641	1.31547	2.144308
3.625	3	3.878231	1.292744	2.199887
0.925	3	6.369529	2.123176	0.780405
0.85	3	6.096324	2.032108	0.874694

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	21.28357	2	10.64178	69.63551	1.275308	3.633723
Columns	4.023629	8	0.502954	3.291124	0.020329	2.591096
Error	2.44514	16	0.152821			
Total	27.75234	26				

Appendix D. Statistical analysis on optical density

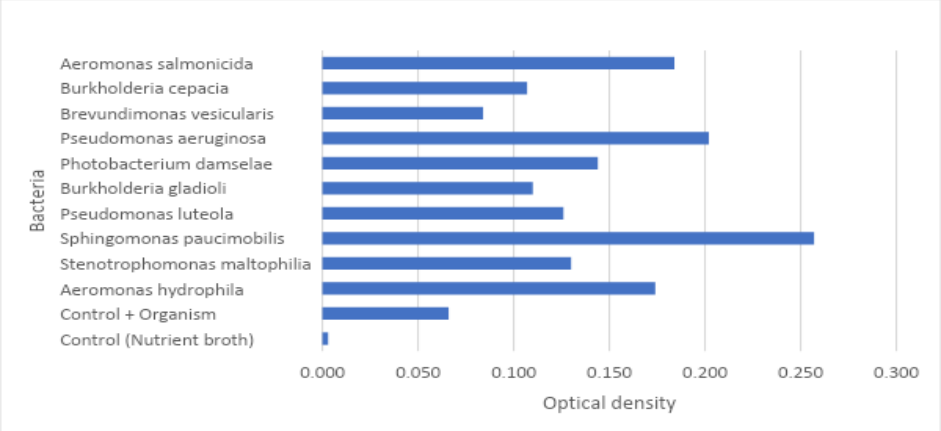
Anova: Two-Factor Without Replication

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
Control (Nutrient broth)	8	1.5560	0.1945	0.1015
Control + Organism	8	5.0460	0.6308	0.4313
Aeromonas hydrophila	8	9.1870	1.1484	0.6040
Stenotrophomonas maltophilia	8	6.7830	0.8479	0.2807
Sphingomonas paucimobilis	8	9.6390	1.2049	0.4470
Pseudomonas luteola	8	8.7700	1.0963	0.6326
Burkholderia gladioli	8	4.6970	0.5871	0.1225
Photobacterium damselae	8	3.4800	0.4350	0.0460
Pseudomonas aeruginosa	8	13.9060	1.7383	0.6773
Brevundimonas vesicularis	8	4.0190	0.5024	0.0972
Burkholderia cepacia	8	5.7180	0.7148	0.3807
Aeromonas salmonicida	8	7.2780	0.9098	0.2913
Day 0	12	1.5870	0.1323	0.0045
Day 1	12	3.2980	0.2748	0.0483
Day 2	12	5.5980	0.4665	0.1526
Day 3	12	8.5740	0.7145	0.2473
Day 4	12	11.6540	0.9712	0.3626
Day 5	12	13.5540	1.1295	0.3586
Day 6	12	16.4500	1.3708	0.3573
Day 7	12	19.3640	1.6137	0.3501

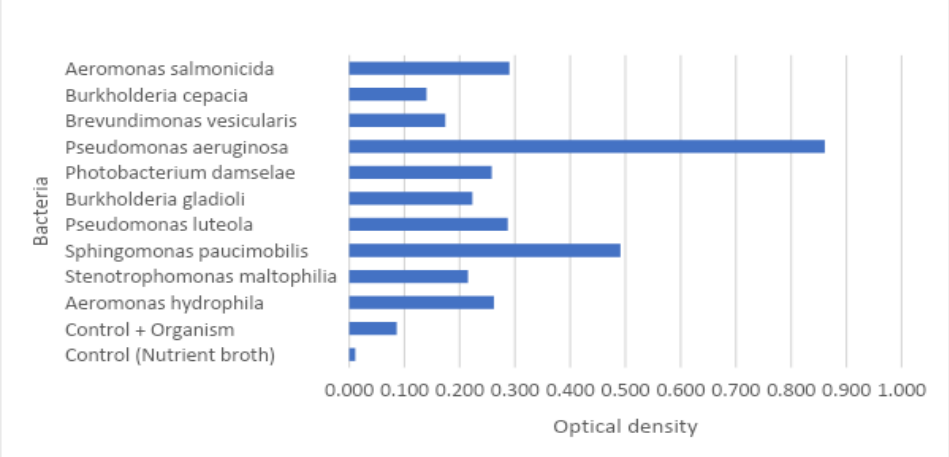
ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Bacteria	15.38694	11	1.399	20.298	0.000	1.915
Days	23.47994	7	3.354	48.674	0.000	2.131
Error	5.30631	77	0.069			
Total	44.1732	95				

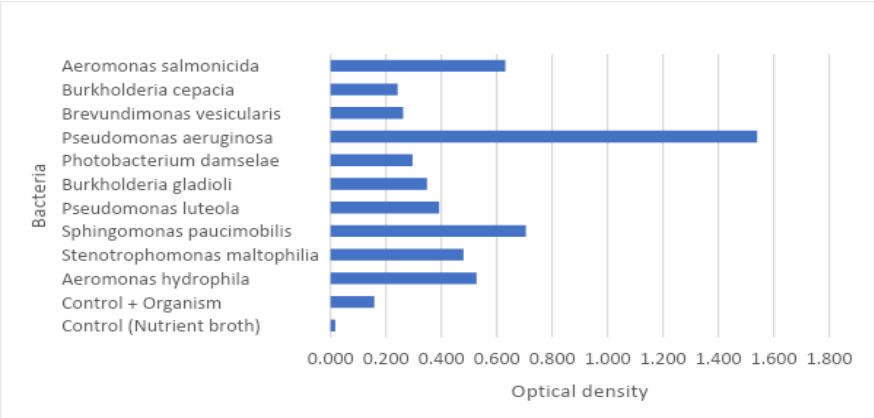
Appendix E. The effect of time (days) on the degradation of diesel by the isolated bacterial strains from soil contaminated with petroleum hydrocarbon wastes from Kupferberg landfill dumpsite, incubated at 37°C, at an optical density of 600 nm.



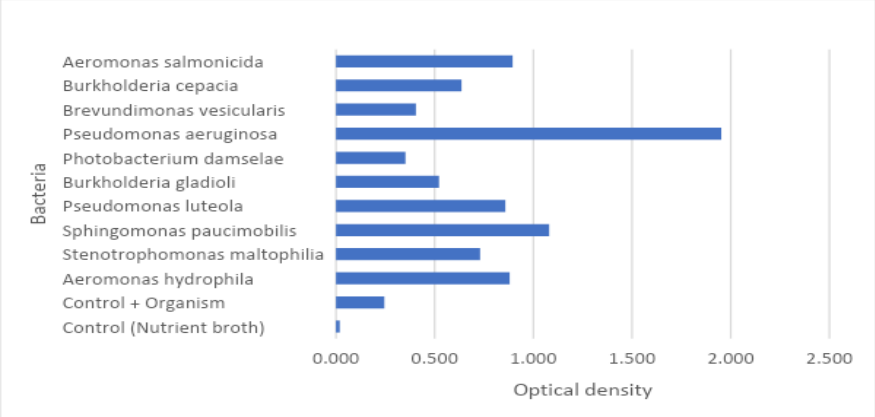
Day 0



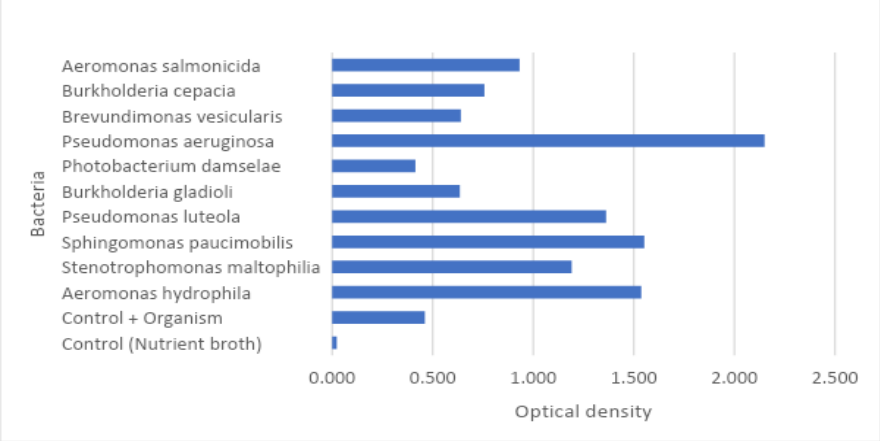
Day 1



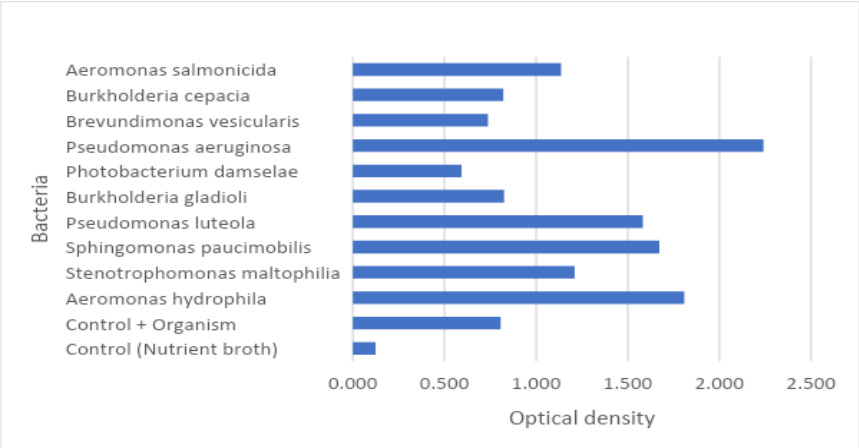
Day 2



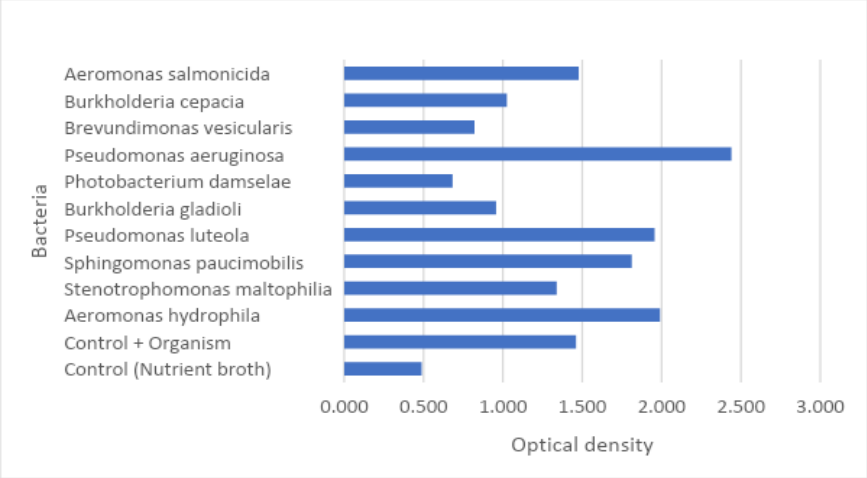
Day 3



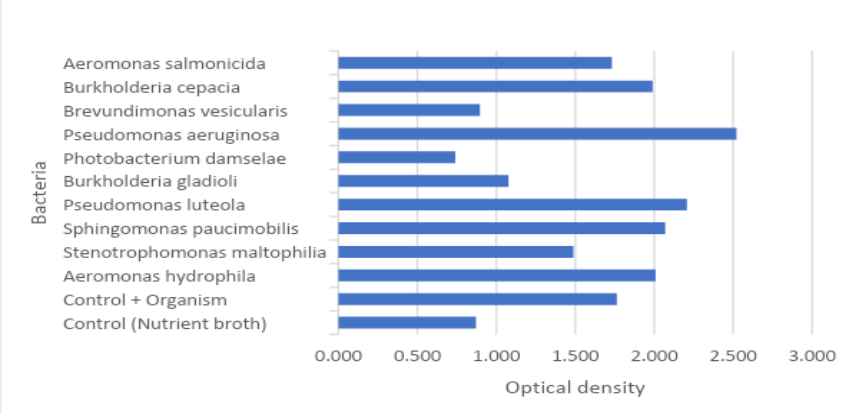
Day 4



Day 5

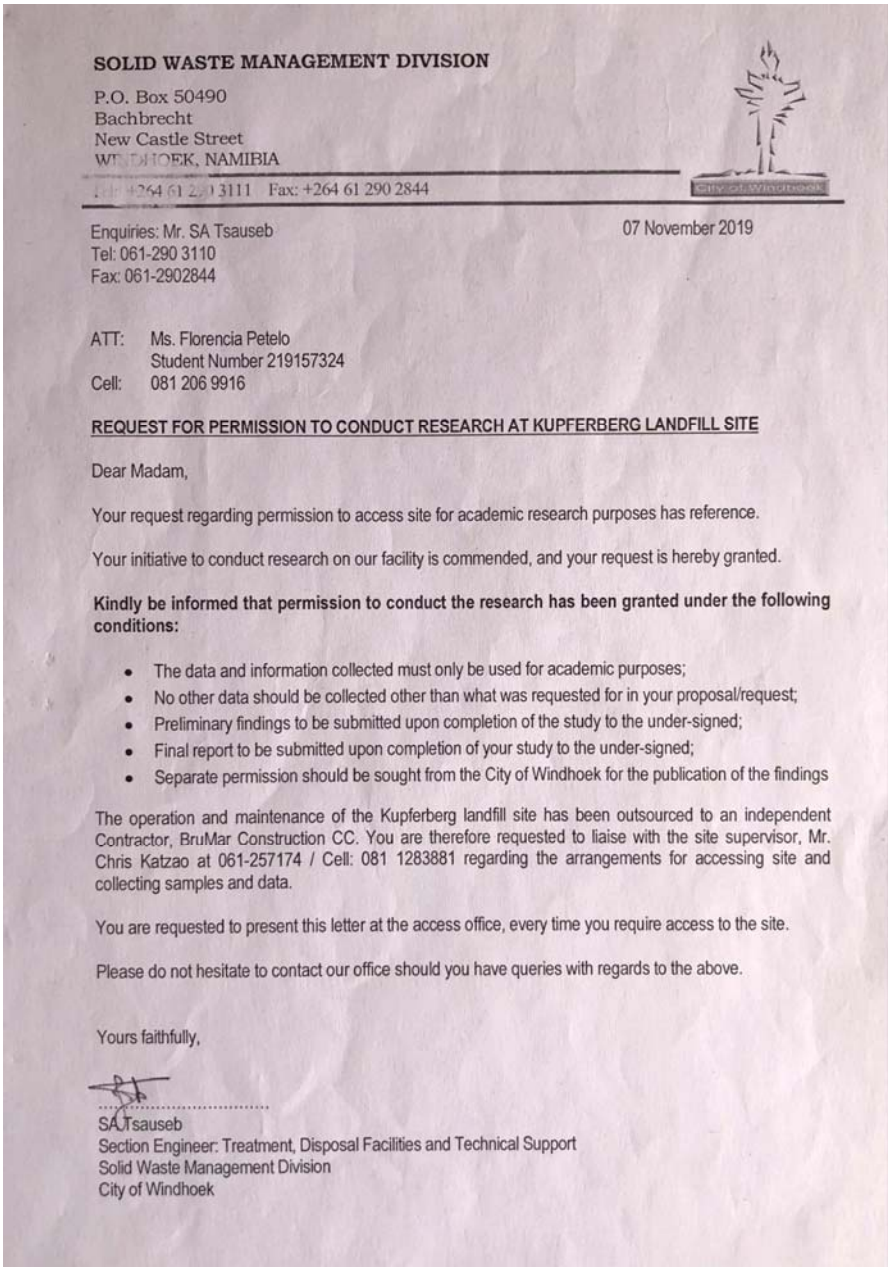

Aeromonas salmonicida
Burkholderia cepacia
Brevundimonas vesicularis
Pseudomonas aeruginosa
Photobacterium damselae
Burkholderia gladioli
Pseudomonas luteola
Sphingomonas paucimobilis
Stenotrophomonas maltophilia
Aeromonas hydrophila
Control + Organism
Control (Nutrient broth)
Bacteria
0.000
0.500
1.000
1.500
2.000
2.500
3.000
Optical density

Day 6


Aeromonas salmonicida
Burkholderia cepacia
Brevundimonas vesicularis
Pseudomonas aeruginosa
Photobacterium damselae
Burkholderia gladioli
Pseudomonas luteola
Sphingomonas paucimobilis
Stenotrophomonas maltophilia
Aeromonas hydrophila
Control + Organism
Control (Nutrient broth)
Bacteria
0.000
0.500
1.000
1.500
2.000
2.500
3.000
Optical density

Day 7

Appendix F. Sample collection (at Kupferberg landfill dumpsite) permit obtained from the City of Windhoek, Solid Waste Management.



SOLID WASTE MANAGEMENT DIVISION

P.O. Box 50490
Bachbrecht
New Castle Street
WINDHOEK, NAMIBIA
Tel: +264 61 290 3111 Fax: +264 61 290 2844

City of Windhoek

Enquiries: Mr. SA Tsauseb
Tel: 061-290 3110
Fax: 061-2902844

07 November 2019

ATT: Ms. Florencia Petelo
Student Number 219157324
Cell: 081 206 9916

REQUEST FOR PERMISSION TO CONDUCT RESEARCH AT KUPFERBERG LANDFILL SITE

Dear Madam,

Your request regarding permission to access site for academic research purposes has reference.

Your initiative to conduct research on our facility is commended, and your request is hereby granted.

Kindly be informed that permission to conduct the research has been granted under the following conditions:

- The data and information collected must only be used for academic purposes;
- No other data should be collected other than what was requested for in your proposal/request;
- Preliminary findings to be submitted upon completion of the study to the under-signed;
- Final report to be submitted upon completion of your study to the under-signed;
- Separate permission should be sought from the City of Windhoek for the publication of the findings

The operation and maintenance of the Kupferberg landfill site has been outsourced to an independent Contractor, BruMar Construction CC. You are therefore requested to liaise with the site supervisor, Mr. Chris Katzao at 061-257174 / Cell: 081 1283881 regarding the arrangements for accessing site and collecting samples and data.

You are requested to present this letter at the access office, every time you require access to the site.

Please do not hesitate to contact our office should you have queries with regards to the above.

Yours faithfully,

.....
SA Tsauseb
Section Engineer: Treatment, Disposal Facilities and Technical Support
Solid Waste Management Division
City of Windhoek

Appendix G. Research permit and ethical clearance obtained as per NUST regulations.

FACULTY OF HEALTH AND APPLIED SCIENCES RESEARCH ETHICS COMMITTEE (FHAS-REC)

DECISION/FEEDBACK ON RESEARCH PROPOSAL ETHICAL CLEARANCE

Dear: Prof/Dr/Mr/Ms/Other	Florencia Silvia Tueevo Petelo
Research Topic:	An investigation of bacterial degradation of petroleum hydrocarbon in Kupferberg landfill dumpsite, Windhoek
Supervisor (if applicable):	Dr Edosa Omoragie
Co-supervisor(s): if applicable	Prof Percy Chimwamurombe
Qualification registered for (if applicable):	MHS

Re: Ethical Screening Application No: FHAS 21/2019

The Faculty of Health and Applied Sciences Research Ethics Screening Committee has reviewed your application for the above-mentioned research project. Based on the recommendation of the expert reviewer, the research as set out in the application is hereby:

(Indicate with an X)

Approved: i.e. may proceed with the project	X
Approved provisionally: i.e. may proceed but subject to compliance with recommendation(s) listed below	
Not approved: Not to proceed with the project until compliance with recommendation listed below and resubmit ethics application for consideration	

It is important to note that as a researcher, you are expected to maintain ethical integrity of your research. You are encouraged to strictly adhere to the research ethics policy of NUST. You should remain within the scope of your research proposal and support evidence as submitted to the FHAS-REC. Should any aspect of your research change from the information as presented, which could have an impact or effect on any research participants/subjects/environment, you are to report this immediately to your supervisor and to the FHAS-REC as applicable in writing. Failure to do so may result in withdrawal of approval. Kindly consult the committee if you need further clarification in this regard.

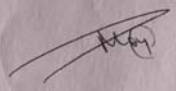
We wish you success in your research endeavour and are of the belief that it will have positive impact on your career as well as the development of NUST and the society in general.

Ethical issues that require compliance/ must be addressed : None

No.	Ethical issues	Comment/recommendation
1.		
2.		
3.		

NB: May attach additional page as required

Sincerely,



Name: Prof Sylvester R Moyo Signature: Date: 25/10/2019
Chairperson: FHAS Ethics Screening Committee.