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BACTERIAL COMMUNITY DYNAMICS DURING THE BIOREMEDIATION OF DIESEL CONTAMINATED SOIL USING BREWERY SPENT GRAIN

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A thesis submitted in partial fulfilment of the requirements of the University of Sunderland for the degree of Doctor of Philosophy

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DECLARATION

I wish to say that no component of the work referred to in this report has been submitted in support of any application for another qualification for this or any other institutions of learning
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ABSTRACT

Brewery spent grain (BSG) has been shown to be nutrient rich and support the growth of microbes. It has thus been exploited in studies for the bioremediation of hydrocarbon contaminated soils. However, previous studies using BSG, have tended to investigate contaminant reduction and microbial growth. The associated bacterial community dynamics, metabolites, catabolic genes, and any changes over time have not been investigated. This knowledge could proffer a more informed rate enhancing strategy to optimise the remediation process.

The study reported here has undertaken research to evaluate the bioremediation potential of BSG in diesel contaminated soil in terms of bacterial population dynamics, bacterial metabolic potential in key hydrocarbon degradation pathways, presence and abundance of degradation genes and shifts in bacterial populations towards known hydrocarbon degraders. It has also evaluated the breakdown of the diesel and attempted to correlate this with changes in bacterial populations.

The results show that BSG accelerates the biodegradation of diesel contamination in soils. Bacterial counts increased significantly in soil samples with BSG added, from log 3.9 up to log 5.6. The alkB gene for alkane degradation and catA and xylE genes for aromatic hydrocarbon degradation, were significantly higher in the microbial community of soils treated with BSG; implying that BSG supported the proliferation of both aromatic and aliphatic hydrocarbon degrading microbes possessing these genes.

Also, the metagenomic evaluation of bioremediation over time, adopted in this study, revealed shifts in bacterial populations in favour of Flavobacterium, Pseudomonas and Acinetobacter species and the latter two were shown to be intrinsic populations of the BSG control treatment. Of these species, Acinetobacter spp. were associated with the xylE gene while Pseudomonas putida was associated with both the catA and alkB genes; thus enhancing both aliphatic and aromatic hydrocarbon biodegradation. The BSG control treatment was also shown to harbour five other known hydrocarbon degrading bacterial species.
The findings of this study revealed the bacterial community dynamics in response to hydrocarbon contamination and provides insight to the association of *Pseudomonas putida* with enhanced biodegradation in temperate soil. Promoting the growth of this bacteria, and harnessing its catabolic potential, coupled with the addition of BSG could be one method of optimising the rate and extent of bioremediation in hydrocarbon contaminated soils.
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Chapter 1

INTRODUCTION

1.1 Introduction

Industrialisation has not been realised without costs to environment and health. The reason being that energy, which is the driving force of industrialisation, is obtained mainly from petroleum hydrocarbons which have become one of the major environmental pollutants of global concern (Fernández et al., 2011; Gao et al., 2014); with diesel being the most commonly found in the environment (Fernando et al., 2017). Petroleum hydrocarbons are not only an important energy resource for daily life but are also raw materials for a number of products such as plastics, paints and cosmetics (Cohen, 2002; Shahsavari et al., 2013).

As such, in the UK alone, an average of 999,000 barrels of oil was produced daily in 2017. This includes crude oil and natural gas liquids (URL1). Petroleum hydrocarbons have become ubiquitous being the most widespread contaminant in the environment worldwide; polluting air, water and soil (Margesin et al., 2003). They are introduced to the environment mainly by the spillage of petroleum products such as diesel and crude oil (Yanto et al., 2013) during their exploration, refining and transportation (Molina-Barahona et al., 2004; Agamuthu et al., 2013). Examples are the Exxon Valdex oil spill in 1989 which affected the Alaskan shoreline of Prince William Sound (Boopathy, 2000) and the Deepwater Horizon oil spill of 2010 in the Gulf of Mexico (Adams et al., 2015).

Petroleum hydrocarbons are also introduced to the environment from the combustion of petroleum products (Mao et al., 2012). Soil, which acts as an ultimate repository of contaminants, accumulates contamination from the air and water through precipitation and sedimentation (Zhang et al., 2006) and can retain polycyclic aromatic hydrocarbons, for several years causing health and environmental impacts.
1.2 Risks of petroleum hydrocarbon contaminated land

Environmental contaminants, such as petrogenic hydrocarbons, present a hazard because they can cause serious environmental and health problems (Zhang et al., 2012) due to their potential toxicity, carcinogenicity and mutagenicity to living organisms (Peng et al., 2010; Shepherd et al., 2011; Shahsavari et al., 2013). Chronic exposure of humans to hydrocarbon contaminated soils can be carcinogenic (Balachandran et al., 2012). Among environmental media however, soil contamination has been reported to be the most threatening; affecting the soil microbiota (Agamuthu et al., 2013). Health risks arise from direct contact with contaminated soils and vapours from the hydrocarbons.

Another major concern is that they can affect the food chain and accumulate up the trophic ladder (Semple et al., 2001). Petroleum hydrocarbon contamination from soil can get washed by run off storm water into water bodies such as rivers and seas resulting in its uptake and accumulation in tissues of fish and sea animals, which when ingested by man further up the food chain, can cause mutations or death (Das and Chandran, 2011). It can also contaminate water supplies when hydrocarbons seep into ground water.

In developing countries, like Nigeria however, petroleum hydrocarbon pollution impacts on the economy and livelihood of affected communities due to the resultant loss of fertility in agricultural soils (Okoh, 2006; Bayode et al., 2011) resulting in disputes between communities and oil exploration companies (URL 2). Hydrocarbon contaminated soil, which is widespread in the UK and globally, stemming from industrial and manufacturing processes, has become one of the major threats to sustainability and several challenges have been encountered in the redevelopment of such land due to the high toxicity effects associated with the removal of contaminants on living organisms and humans (Meckenstock and Mouttaki, 2011). These concerns drive the need for remediation.
1.3 Risk assessment of petroleum hydrocarbon contaminated land

The release of petroleum hydrocarbons into the soil environment poses a potential risk and it is essential to understand its toxicology to responsibly manage the risk it poses to human health, water resources, ecosystems, other environmental receptors and property (EA, 2003). In order to manage this risk and develop a structured risk assessment framework, it is also essential to understand the impact of exposure of petroleum hydrocarbons to each of these receptors. However, toxicological evaluation is difficult since petroleum hydrocarbons are a complex mixture containing hundreds of individual compounds and it is not practicable to analyse for individual compounds when present in complex mixtures. Thus, a practical approach is required to protect human health (EA, 2003).

As such, contaminated land management in the UK is risk based and it is driven by legislation. The two predominant legislations for contaminated land in the UK are the Environmental Protection Act 1990 Part 2A, also called contaminated land regime, which evaluates risk posed by historic contamination, and The Town and Country Planning Acts. Both legislations, however, are aimed at the identification and remediation of land that poses significant risk to health and environment to make it suitable for use. The risk associated with contaminated land, however, is dependent on the presence of a receptor or pathway (DEFRA, 2012; EA, 2003).

Thus, to determine risk, well established three tiers of risk assessments have to be conducted (GOV.UK, 2016) based on the source of the contaminant, its pathway and receptor (URL 3). A generic quantitative risk assessment is usually preceded by a preliminary risk assessment and followed by a detailed quantitative risk assessment which would inform decisions on how to manage the contamination (GOV.UK, 2016; URL 3). Aspects considered during the risk assessment would be the mobility of the contaminant to such receptors as water table or surface water, concentration of pollutants and bioavailability of the contaminant (Kuppasamy et al., 2017).
The approach adopted in the UK for petroleum hydrocarbons, present in the environment, is a focus on components that pose high risk; based on toxicology and their environmental behaviour and fate (EA, 2003). Risk based approaches require total petroleum hydrocarbons (TPH), to be divided into fractions based on physiochemical and toxicological properties. Fractionation is in terms of aliphatic and aromatic compounds and also according to their equivalent carbon number (EC) (Pinedo et al., 2012).

However, approaches for the risk evaluation of petroleum hydrocarbon fractions differ among countries and groups. The Total Petroleum Hydrocarbons Criteria Working Group (TPHCWG) established in the USA to develop information for soil cleanup and protect human health at contaminated sites, grouped petroleum hydrocarbons into six aliphatic and seven aromatic fractions based on EC number and transport or mobility (EA, 2003).

The Environmental Protection Department of Hong Kong, however, assigned threshold concentrations to three groups of target aliphatic petroleum hydrocarbons (C6 – C8, C9 – C16, and C17 – C35) in line with risk-based remediation goals (RBRG); such that, exposure to soil contaminants at the threshold concentration is considered to have minimal risk. Above the RBRG threshold however, clean up or remediation would be required (EPD, 2007).

Polyaromatic hydrocarbons (PAHs) were grouped as semi volatile chemicals into individual RBRG target compounds such as anthracene, benzo(a)pyrene, pyrene and benzo(a)anthracene (EPD, 2007). The threshold concentrations for these aromatic compounds are much lower than the aliphatic hydrocarbons because of the high risk they pose due to their chemical and toxicological properties (URL 4). The risk assessed by the RBRG process includes exposure concentrations and toxicity. Also included is the identification of exposure pathways and receptors (EPD, 2007).

Nevertheless, the U.S. Environmental Protection Agency (US, EPA) has listed many PAHs such as naphthalene, phenanthrene and BTEX (benzene, toluene, ethylbenzene and xylene) compounds as priority pollutants for health
risk assessments because of their inherent toxicity (Khan et al., 2018). Also, along with other health and environmental agencies, they have classified benzene as the only aromatic hydrocarbon known to be a human carcinogen and therefore, the primary focus of many petroleum hydrocarbon risk assessments. However, the potential exposure to benzene at a petroleum contaminated site depends on the concentrations of benzene in the soil, water, and air, and the frequency and duration of expected human contact with the contaminated media.

In Nigeria, The Environmental Guidelines and Standards of the Petroleum Industry in Nigeria (EGASPIN) guides the control of petroleum hydrocarbon pollution in the environment while The National Oil Spill Detection and Response Agency (NOSDRA) has the responsibility of detection, monitoring and remediation of petroleum hydrocarbon spills (Ogbonnaya et al., 2017).

1.4 Risk based management and remediation of contaminated land

Following the risk assessment, risk management strategies would be adopted to break the source-pathway-receptor linkage (URL 3). Thus, if risk from soil to a receptor, such as the aquatic ecosystem is eminent from contaminated land, a remediation technique preventing destruction to the ecosystem and subsequent health hazards would be decided upon to break the contaminant-pathway-receptor linkage (URL 3; Kuppusamy et al., 2017). The development of efficient and cost effective technologies targeted at decontaminating soil is therefore imperative and has become a global challenge (Molina-Barahona, 2004; Shahsavari et al., 2013).

1.4.1 Physiochemical remediation

Physiochemical remediation techniques for decontaminating contaminated land include soil washing, solvent extraction, incineration and removal to landfill (Semple et al., 2001). Among these, however, incineration and landfilling are accepted disposal methods to avert the hazards of pollution
(Okoh, 2006). However, they are expensive, labour intensive and have safety concerns (Shahsavari et al., 2013) since they lead to an incomplete decomposition of contaminants (Gao et al., 2014). Also, with the waste management directive to reduce waste to landfill, more sustainable approaches such as waste recycling and remediation is required (DEFRA, 2007). Thus, the decontamination of soil using natural mechanisms is preferred.

1.4.2 Natural attenuation

This comprises the natural and unassisted physical, chemical and biological processes by which hydrocarbon contamination in soil is removed under favourable conditions. During natural attenuation, contaminants can be transformed to less harmful forms or immobilised to reduce their threat to the environment and risk to living things including humans. It results in a reduction of mass, mobility, toxicity, volume or concentration. When it is brought about by biological means such as bacteria or fungi, it is referred to as bioattenuation or intrinsic remediation. Natural attenuation includes dispersion, sorption, volatilisation and degradation (Bjerg et al., 2003; Brown et al., 2017).

Degradation, however, is the most interesting option since it completely mineralises the contaminants into carbon dioxide and water which are safe to the environment (Bjerg et al., 2003). Degradation can be either abiotic or biotic. In abiotic degradation such as hydrolysis and photolysis, contaminants react with water or light respectively to form new compounds while in biotic degradation, the contaminants are metabolised by living organisms such as bacteria, fungi and plants.

1.4.3 Biodegradation

Biotic degradation is also known as biodegradation. Thus, biodegradation is a form of natural attenuation. Biodegradation of hydrocarbons is essential for the cleanup of pollution in soils, to reduce the risk to environmental and human health. It is considered the ultimate natural mechanism and preferred
treatment option for hydrocarbon contaminated soils because of its complete mineralisation of hydrocarbons into innocuous, highly oxidised products (Jain and Bajpai, 2012). However, biodegradation is usually slow and depending on the toxicity of the contaminant, may not be effective. Bioremediation techniques are therefore used to monitor and enhance biodegradation under controlled conditions.

1.4.4 Bioremediation

Bioremediation, which is monitored biodegradation, involves the adoption of enhancement techniques to improve upon the biodegradation process. It is therefore more advantageous because it improves the rate and extent of biodegradation. Soil, however, is the most expensive media to decontaminate (Agamuthu et al., 2013). Thus, soil bioremediation strategies effective on a large scale to improve sustainability and enhance better health for society are being investigated (Rodrigues et al., 2009). Organic amendments in the form of crop residues like corn and sugar cane (Barahona et al., 2004) and plant residues like pea straw (Shahsavari et al., 2013) have been used to stimulate bioremediation.

Brewery spent grain (BSG), which is a food by product with high nutritional content is readily available in many countries (Thomas and Rahman, 2006) and has also been used for bioremediation. Its effluents have also been used for diesel bioremediation, indicating strong bioremediation potential (Agarry and Latinwo, 2015). However, its use has been mostly in tropical climates (Agamuthu et al., 2010; Abioye et al., 2012) and microbial metabolism and activity, which determine biodegradation rate, are temperature dependent. Thus, biodegradation rate tends to increase with increasing temperature; allowing for higher biodegradation rates in tropical climates (Adams et al., 2015).

Nevertheless, since effective biodegradation has been carried out in Arctic soils (Whyte et al., 2002; Okere et al., 2012; Yergeau et al., 2012; Okere et al., 2017), biodegradation in temperate climates is also feasible. However,
knowledge of the microbial populations and functional genes that are effective in the biodegradation of different climates is essential to harness and promote bioremediation (Yergeau et al., 2012). This study will explore the use of BSG in the bioremediation of diesel contamination in a temperate soil.

1.5 Background to the Research

Bacteria, in particular, have been found to be very instrumental in the bioremediation of hydrocarbon contaminated soil (Chikere et al., 2009). This is because they can degrade pollutants using their enzymatic machinery and utilise the associated metabolites via the tricarboxylic acid (TCA) cycle for energy and growth (Kulshreshtha, 2012). Following the contamination of soil, bacterial strains that are resistant to the toxicity of the pollutant can biodegrade the chemicals; thereby, detoxifying the soil and making nutrients available for other quiescent populations to grow and increase their numbers. This leads to changes in the bacterial community within the soil; resulting in increased population diversity and activity (Zucchi et al, 2003).

Thus, the microbial community composition varies with time; with those most suited to the metabolic breakdown products becoming dominant as bioremediation progresses (Reddy et al., 2011). It is important to note also, that the distribution of microorganisms influence the efficiency of bioremediation (Maila, 2005). Knowledge of bacterial populations present over time, therefore, would mean that the bioremediation process could be tailored to introduce bacteria most suited to the metabolites at different times during the bioremediation process (Hamamura et al., 2006). This could improve upon the rate and extent of bioremediation since the availability of microorganisms with appropriate metabolic capabilities is essential for enhanced bioremediation (Jain and Bajpai, 2012).

So far, bioremediation has been considered advantageous from an economic point of view and not time efficiency. Currently, the process is slow particularly in temperate and Arctic climates and is difficult to perform over large expanses of land (Thapa et al, 2012). Strategies to promote the efficiency on a large
scale are being sought and it is the intention of this study, to explore the bacterial community composition of diesel contaminated soils and their dominant catabolic genes over time, and to review how the nutritional content and resident microflora of BSG can enhance bioremediation in a temperate climate.

1.6 Aims and Objectives

The aim of this research was to evaluate the impact of BSG on diesel bioremediation in soil and answer a series of three research questions; thus, enabling recommendations for the improvement of the bioremediation process to be made:

- How does BSG influence diesel biodegradation in soil?

- What are the pathways and metabolites associated with the biodegradation process and how does it fit to the elucidated hydrocarbon breakdown pathways?

- What bacterial populations are associated with the biodegradation process; how do they change over time with and without BSG and does the process result in an increased population of known hydrocarbon degrading bacteria and catabolic genes?

This was achieved through the following objectives:

- Reviewing the literature to determine the elucidated hydrocarbon degradation pathways and associated enzymes and genes.

- Laboratory scale investigations into the bioremediation of diesel contaminated soil with and without BSG, over time, to determine the following:
The extent and rate of diesel degradation by determining the percentage reduction in peak heights of TPH, which is the total extractable hydrocarbon in any environmental sample, and concentration of aliphatic TPH fractions during the process of bioremediation, using Gas Chromatography (GC).

The colony forming units (CFUs) for enumerating the bacterial population during the bioremediation process and also, the ability of the bacteria to break down substrates in the key metabolic pathways for hydrocarbon biodegradation using Gas Chromatography Mass Spectrometry (GC-MS).

The presence of catabolic genes encoding key degradation enzymes associated with hydrocarbon breakdown and their changes in abundance, over time, during the bioremediation process, using real time PCR (qPCR).

The succession of the bacterial community based on shifts in bacterial populations over time, during the process of bioremediation, and identifying species abundance with and without BSG.

1.7 Structure of the Thesis

The outline of the chapters is summarised to provide an overview of this study as follows:

Chapter 1 is an introduction of the research and includes the background and structure of the thesis.

Chapter 2 discusses the scientific literature relevant to the research in an attempt to highlight the already known facts about bioremediation including the various remediation methods and gaps in the literature that would be addressed in this study.
Chapter 3 describes the development and evaluation of the methods used in this study and includes a series of laboratory scale experiments. It covers a range of techniques such as soil preparation, microbiological methods, development of sampling protocol, spiking protocol, universal primers evaluation, DNA extraction kit for BSG, metagenomic analysis including real time quantitative PCR and next generation sequencing, chemical analysis and the statistical analysis used in the study.

Chapter 4 summarises the investigation into the use of BSG to stimulate the bioremediation process. Results on the percentage TPH reduction, concentration of aliphatic TPH (C10–C28) fractions and CFUs during the laboratory scale bioremediation experiments on diesel breakdown over time with and without BSG are presented to answer the research question ‘how does brewery spent grain (BSG) influence diesel breakdown in soil?’

Chapter 5 details the analysis of compounds and metabolites present during the bioremediation process, over time, with and without BSG and how they fit into the key metabolic pathways for diesel biodegradation.

Chapter 6 profiles the metagenomic analysis including the changes in gene copy numbers of known catabolic genes for hydrocarbon degradation and the succession of the microbial community based on shifts in bacterial species composition and abundance; over time, during the bioremediation process, with and without BSG. The results are evaluated and analysed to determine the impact BSG has on bacterial populations.

Chapter 7 provides a general discussion of the results of the research and how the research questions are answered based on the findings.

Chapter 8 concludes the thesis by discussing the benefits of using BSG to remediate diesel contaminated soil and recommendations for future work.
Chapter Two

LITERATURE REVIEW

2.1 Introduction

Contaminated land is a widespread and acknowledged environmental concern resulting from poor industrial practices and incidents. In the UK alone, it is estimated that about 300,000 hectares of land could be contaminated as a result of industrial activity (EA, 2009). Petroleum hydrocarbons, however, are one of the most common contaminants (Jiang et al., 2016) and diesel, one of the most commonly found hydrocarbons in the environment (Fernando et al., 2017). It is also the most dominant hydrocarbon contaminant in the UK (Stroud et al., 2007). Hydrocarbon pollution in soil not only causes changes in the physiochemical properties of soil, but also changes in the composition and dynamics of the soil microbial community (Hamamura et al., 2008; Tang et al., 2012).

To avert these negative effects caused by hydrocarbon contamination, it is essential to develop strategies for inexpensive but effective decontamination (Skipper, 1999; Bento et al., 2005). Bioremediation strategies are usually based on field trials and laboratory studies, aimed at improving upon the biodegradation process (Abioye et al., 2009). However, studies have focussed on the feasibility of cleaning up contaminated sites (Molina-Barahona et al., 2004; Liu et al., 2011; Dussan and Numpaque, 2012; Sharma et al., 2014) with only a little attention given to the microbial communities involved in the clean-up process, and their ecology, in response to the perturbation (Bento et al., 2005; Kulshreshtha, 2012; Yergeau et al., 2012).

This is essential because the microbial community composition of the contaminated site and the populations of degrading bacteria in it, are key to achieving enhanced biodegradation. Also of importance is the concentration of the contaminant and the environmental conditions such as temperature and pH (Semple et al., 2001; Molina-Barahona et al., 2004). Populations exposed to hydrocarbons become adapted and exhibit genetic changes that selectively enrich them to exhibit preference for hydrocarbons (Leahy and Colwell, 1990).
Thus, bacterial species isolated from contaminated sites, such as *Pseudomonas* spp., *Sphingomonas* spp., and *Flavobacterium* spp., have been shown to have the ability to metabolise complex aromatic hydrocarbons such as naphthalene and pyrene in soil (Zhang *et al.*, 2006). Nevertheless, hydrocarbonoclastic microbes have been found to exist in non contaminated environments where they consist of less than 1% of the total microbial community. The reason for this could be because petroleum hydrocarbons, also known as petrogenic hydrocarbons, are naturally occurring organic compounds; originating from rocks formed from plant and animal remains over millions of years (Kingston, 2002).

In contaminated environments however, hydrocarbonoclastic microbes can account for up to 10% of the total microbial community (Atlas, 1991) and exposed communities exhibit higher degradation rates than pristine communities with no history of hydrocarbon contamination (Leahy and Colwell, 1990; Adams *et al.*, 2015). Thus, a knowledge of the microbial communities and their biodegradation capability is essential to determine the extent to which contaminants are degraded and the bioremediation techniques to be adopted to achieve enhanced biodegradation (Bento *et al.*, 2005).

Though the process of bioremediation has been achieved by bioaugmenting with bacterial consortium, indigenous microorganisms have the advantage of being better suited and adaptable to the prevailing conditions of the particular contaminated site to be remediated. Thus, the biostimulation of indigenous catabolically active microbes in contaminated soils, by the addition of inorganic amendments such as fertilisers enriched with nitrogen and phosphorus, or the addition of organic amendments which supply nutrients (Chikere *et al.*, 2012; Silva-Castro *et al.*, 2013; Wu *et al.*, 2016), has proved successful in many remediation studies in enhancing intrinsic soil microbial activity (Margesin and Schinner, 2001; Chikere *et al.*, 2012; Shahsavari *et al.*, 2013).

Also, biostimulation balances the carbon, nitrogen and phosphorus (C:N:P) ratio which is required to provide adequate nutrition for the optimal growth of a catabolically active soil bacterial population (Okoh, 2006) to the optimal 100:10:1 (Wu *et al.*, 2016). This is necessary because the introduction of
hydrocarbon pollutants into soil causes an imbalance of nutrients; greatly increasing the carbon content and limiting other essential nutrients such as nitrogen and phosphorus; thus, creating a toxic environment for the indigenous microbial population (Shahsavari et al., 2013).

However, there is still a gap in knowledge regarding the microbial community composition and abundance during diesel bioremediation over time in temperate regions and their associated catabolic genes. This information is necessary to design bioremediation strategies that would target specific microorganisms associated with high degradation rates. Such targeted and tailored approach could lead to a more rapid and enhanced bioremediation and could be a useful indicator for soils with high hydrocarbon remediation potential (Yergeau, 2012). Knowledge of diesel composition, however, is necessary to understand its degradation by microorganisms.

2.2 Composition and degradability of petroleum diesel

Petroleum diesel, also called diesel fuel, is produced from the fractional distillation of crude oil between 200°C (392°F) and 350°C (662°F) at atmospheric pressure, resulting in a mixture of carbon chains (Collins, 2007). It comprises of about 10 – 20% volatile fraction which acts as a precursor of tropospheric ozone formation when released into the atmosphere; thus, increasing ozone pollution. The less volatile 80 – 90% aliphatic fraction, however, causes significant deterioration to soil physical properties (Serrano et al., 2006). Most low molecular weight hydrocarbons are volatile while the high molecular weight hydrocarbons remain in the soil and become biodegraded (Chikere et al., 2011).

It is composed of aliphatic and aromatic hydrocarbons. The aliphatic hydrocarbons consist of either saturated or unsaturated, straight, branched or cyclic carbon and hydrogen chains. The saturated aliphatics are called alkanes while alkenes and alkynes are unsaturated. Alkanes have single C-C bond structures, alkenes have double C=C bonds and alkynes have triple C≡C bonds (Stroud et al., 2007).
Aromatic hydrocarbons, on the other hand, are composed of carbon and hydrogen chains containing benzene as part of their structure. They are characterised by having a benzene ring and are named based on the number of benzene rings; the simplest of which is the monoaromatic benzene. The simplest polyaromatic hydrocarbon, having two benzene rings is naphthalene (Chikere et al., 2012). Diesel fuels contain a mixture of hydrocarbons of C10 to C28 hydrocarbons and the susceptibility of diesel to degradation is based on molecular weight; usually with the lightest compounds degrading first in the order: linear alkanes, branched alkanes, cyclic alkanes, simple aromatics and poly aromatics with some high molecular weight poly aromatics remaining recalcitrant (Das and Chandran, 2011).

The low molecular weight alkanes such as C1 – C4 are gases at ambient temperatures and in addition to the volatile aromatic hydrocarbon fraction, are susceptible to volatilisation (Serrano et al., 2008). The high molecular weight alkanes with higher carbon atoms are more persistent in the soil, binding to soil particles and removed only through biodegradation by bacteria such as *Pseudomonas* spp. which has been reported to have the ability to emulsify and degrade hydrocarbons (Dussan and Numpaque, 2012). Others get sequestered in the soil and persist; especially in clayey soils (Ogbonnaya et al., 2017).

In their study, Serrano et al. (2006) observed that fifty days after a diesel spill, volatilisation was the main cause of disappearance of the volatile aromatic fractions, which include the BTEX compounds; benzene, toluene, ethylbenzene and xylene. However, three months after the spill, the soil had still not attained its biological activity indicating that the less volatile aliphatic components of diesel were still retained in the soil. Jiang et al. (2016) also observed that when petroleum products are released into the environment, they are immediately subject to a variety of abiotic weathering processes including evaporation, dissolution, dispersion, adsorption on suspended materials or photooxidation. This was followed, in their study, by biotic or microbial degradation (biodegradation) in which soil microorganisms used up aliphatic hydrocarbons as sources of carbon and energy (Serrano et al., 2008).
For this reason, criteria other than hydrocarbon disappearance such as an increase in the number of hydrocarbon degrading bacteria, as the bioremediation progresses, provides evidence of biodegradation. Also, the evolution of carbon dioxide, which is a by product of metabolism, can be measured as an evidence of biodegradation by using $^{14}$C-labelled substrate respirometry; such that, the labelled $^{14}$CO$_2$ produced as a result of microbial degradation, is detected and measured (Stroud et al., 2007b).

Sterile controls are also used in bioremediation to demonstrate the biotic activity of the biodegradation process. Thus, to achieve soil sterilisation, autoclaving soil at 121°C has been employed (Ghazali et al., 2004; Liu et al., 2015). Other sterilisation methods explored in soil bioremediation studies include the use of gamma radiation (Berns et al., 2008); although there are risks of side effects (Harell et al, 2018) with this method. Bactericides such as sodium azide have also been used; nevertheless, autoclaving is still the most commonly used sterilisation method for bioremediation (Abioye et al., 2012). Nonetheless, for biodegradation to occur, the hydrocarbon has to be available to the microorganisms.

2.3 Fate and behaviour of petroleum hydrocarbons in soil

Following the spill of hydrocarbons into soil, there is a sequestering into the soil matrix. The fate of these hydrocarbons include volatilisation to air, biodegradation, binding to soil particles and leaching into ground water (Semple et al., 2001). Although hydrocarbons may be subject to a number of physical chemical and biological processes in soil, microbial degradation is the major reason for their loss in the soil (Stroud et al., 2007). Thus, biodegradation by microbes is the key process by which hydrocarbons are removed in soil. However, knowledge of the fate and behaviour of organic pollutants in soil is important to predict how successful bioremediation will be (Stroud et al., 2007).
2.3.1 Interactions between hydrocarbons and soil

The time contaminants spend in contact with soil is termed ageing and the longer that time, the decrease in compound availability. Ageing results in the movement of contaminants from accessible to less or non accessible soil compartments; thus, reducing bioavailability of the contaminants. (Semple et al., 2001). Factors that influence contaminant interaction with soil includes hydrophobicity, pollutant volatility and affinity for organic matter (Stroud et al., 2007).

Also, the concentration of the contaminant influences bioavailability in that higher concentrations enhance sorption to soil. Another factor that significantly influences bioremediation is the rate at which contaminants transfer to the aqueous phase which contains the microorganisms. Thus, the interaction between the hydrocarbons and the aqueous phase is very important for the success of bioremediation (Guarino et al., 2017).

Soil is composed of organic and inorganic matter which are separated by pores containing either water or air. Soil organic matter is important to the fate and behaviour of organic compounds because adsorption to either the soft and rubbery or hard and glassy phases are irreversible (Stroud et al., 2007; van Elsas et al., 2007; Chikere et al., 2011). Humus, the organic component of soil, is composed of microorganisms and decomposed plant materials while the inorganic component of soil is composed of rock broken down into sand, silt and clay depending on particle size.

As hydrocarbons diffuse within soil, they can partition into soil organic matter and get concealed by adsorbing to organic matter, mineral fractions or the three-dimensional structure of the soil. The octanol/water partition coefficient ($K_{ow}$) is used to describe the extent to which chemicals partition into organic matter (Stroud et al., 2007). This will affect the bioavailability and bioaccessibility of the hydrocarbon and limit its biodegradation (Stroud et al., 2007). The availability of hydrocarbons in soil is dependent on microbe-contaminant interactions and degradation studies have shown that some microbes are extremely versatile in catabolising organic environmental pollutants.
This catabolic potential can be harnessed to improve biodegradation. However, the success of any bioremediation strategy depends on contaminant bioavailability and degradability (Semple et al., 2001). An assessment of the bioavailability of contaminants in soil is also an important factor to consider regarding the risk posed by the contaminant and the achievement of successful bioremediation (Semple et al., 2006).

2.3.2 Hydrocarbon bioavailability and bioaccessibility to soil microbes

A bioavailable compound is one which is freely available to cross an organism’s membrane from the medium in which the organism resides at a given point in time; while a bioaccessible compound is one which is available to cross an organism’s membrane from the environment in which it resides if the organism has access to it. However, it may be physically removed from it or only bioavailable after a period of time (Semple et al., 2004; Stroud et al., 2007; Chikere et al., 2011; Ogbonnaya et al., 2017). Thus, sequestered hydrocarbons, though bioavailable, may not be bioaccessible to the soil microbes.

Sorption is reversible and does not limit biodegradation and earthworms have been shown to retard the binding of organic contaminants to soil and releasing previously soil bound contaminants; making them available for degradation (Hickman and Reid, 2008). Thus, hydrocarbons which were sorbed and not available at a particular time, may become bioavailable after being desorbed (Stroud et al., 2007).

The assessment of bioavailability is important in understanding the risk posed by hydrocarbon contaminants and $^{14}$C labelled substrates have been used to understand bioavailability. This is achieved by measuring the amount of $^{14}$CO$_2$ produced after metabolism, which will be equivalent to the bioavailability of the contaminant in soil at a given time (Semple et al., 2006). The understanding of bioavailability and bioaccessibility is therefore essential in determining strategies for bioremediation and is prerequisite to the success of any bioremediation process (Chikere et al., 2011, Ogbonnaya et al, 2017).
Figure 2.1 is a diagrammatic representation of how hydrocarbon compounds may be sequestered within the soil showing the possible interactions between soil matrices and aliphatic hydrocarbons (adapted from Stroud et al., 2007).

In Figure 2.1 with arrows labelled 1 to 5, hydrocarbon compound labelled 1 is bioavailable and can be bioaccessible if any microbes come into contact with it, 2 and 5 are not bioavailable and so cannot be bioaccessible since they are irreversibly adsorbed into organic matter, 3 is both bioavailable (can be desorbed) and bioaccessible, while 4 is bioavailable but sequestered; thus, not bioaccessible. Thus, strategies to enhance the bioremediation rates of hydrocarbon contaminated soils may be improved by the addition of biosurfactants to improve availability of hydrocarbons to microbes (Guarino et al., 2017).
2.3.3 Biosurfactants and the bacterial uptake of hydrocarbons

Aliphatic hydrocarbons such as hexadecane are very hydrophobic and immiscible in water and can partition extensively into the solid phase; getting sorbed to mineral particles making them less bioaccessible to microbes for biodegradation (Stroud et al., 2007). However, the production of surface active agents or biosurfactants by bacteria, reduces the surface tension of hydrocarbons and enhances the ability of bacteria to adhere to the hydrocarbon surface leading to increased bioavailability and subsequent biodegradation of the hydrocarbon (Hassanshahian et al., 2014).

Biosurfactants are amphiphilic compounds having both hydrophobic and hydrophilic regions which can interact with hydrocarbons and make them more water soluble (Karanth et al., 1999). It thus, facilitates the direct contact of hydrocarbons with soil microbes which is prerequisite for biodegradation and promotes cell motility on surfaces (van Hamme et al., 2003). As the production of biosurfactants enhances biodegradation, many hydrocarbon degrading bacteria are known to produce biosurfactants which emulsify hydrocarbons and enhance their adhesion and detachment to surfaces and the hydrocarbons.

A wide variety of microbes produce biosurfactants; an example of which are Pseudomonads. They are the best bacteria known to produce biosurfactants. P. aeruginosa and P. putida produce glycolipid biosurfactants which increase the hydrocarbon surface area making it available to be utilised by encapsulation into the microbial cell. Acinetobacter species also produce biosurfactants which gives them a hydrophobic exterior and allows contact with hydrocarbons during degradation (Stroud et al., 2007).

Also, Pseudomonas spp. produce biosurfactants known as rhamnolipids which emulsify hydrocarbon molecules into microdroplets; making it easier for adsorption by bacteria (Hua and Wang, 2014). The biosurfactant, rhamnolipid, was used by Liu et al. (2011) in their bioremediation experiment to enhance the bioavailability of petroleum oil to microorganisms by increasing its solubility. However, increase in temperature also increases the solubility of hydrocarbons and enhances their biodegradation rates. Thus, summer months
will be more favourable for bioremediation than winter months (Hemalatha and Veeramanikandan 2011).

Hydrocarbon biodegradation enzymes are intracellular; thus, after soil microbes adhere to hydrocarbons or access them in the dissolved aqueous phase, the hydrocarbons need to be transported across the cell membrane into their cell for biodegradation. Biodegradation enzymes (oxygenases) then introduce molecular oxygen into the hydrocarbons to begin the breakdown process (Hua and Wang, 2014). The uptake of hydrocarbons by microbes is another factor that could limit bioremediation (Stroud et al., 2007).

2.4 Diesel degradation pathway

The efficiency of the biodegradation process is associated with the genetic potential of the microbes which enables them to digest the substrates in the degradation pathway. The microorganisms most associated with the biodegradation of hydrocarbons include bacteria and fungi. However, bacteria have been shown to be more versatile than fungi and play a greater role in hydrocarbon biodegradation (Chikere et al., 2011); functioning as the primary biodegraders of spilled hydrocarbon in the environment (Das and Chandran, 2011).

The ability of microbes to degrade petroleum hydrocarbons, especially aromatic hydrocarbons such as naphthalene in soil, is associated with their possession of enzymes such as catechol oxygenases that break down hydrocarbons so the microbes can utilise them as sole substrates for carbon and energy (Zhang et al, 2006). Bacterial enzymes responsible for the metabolism of some aromatic hydrocarbons have been identified and quantified to aid and improve bioremediation strategies and it is essential to assess the metabolic pathways and enzymes involved in hydrocarbon biodegradation (Kim et al., 2009). Benzene is an example of an aromatic hydrocarbon contained in diesel and its degradation pathway has been established (Kasahara et al., 2012).
Different microbes utilise different pathways for the degradation of different aromatic hydrocarbons. However, most bacteria that breakdown aromatic hydrocarbons aerobically have been reported to use similar metabolic pathways. The main principles of aerobic degradation of hydrocarbons by microorganisms as illustrated by Das and Chandran, (2011) is shown in Figure 2.2.

Figure 2.2: Main principles of the aerobic degradation of hydrocarbons by microorganisms (Das and Chandran, 2011).

Following the oxidative catabolism of hydrocarbons, the tricarboxylic acid (TCA) metabolites formed in the peripheral pathway get metabolised via the β-oxidation pathway to the TCA cycle for energy and biomass. The diesel degradation pathway is a combination of the breakdown pathways of its constituents and can be summarised into two groups of aliphatic and aromatic hydrocarbons.
2.4.1 Aerobic degradation of aliphatic hydrocarbons (alkanes)

The aerobic degradation of alkanes is catalysed mostly by alkane monoxygenases. It is an intracellular oxidative process in which molecular oxygen is incorporated into the methyl group of the hydrocarbon in three possible ways. The methyl group to which oxygen is incorporated determines the degradation pathway utilised and could be terminal, subterminal or diterminal. Oxidation of the terminal methyl group results in the formation of primary alcohols which are further oxidised to aldehydes and fatty acids and metabolised via the tricarboxylic acid (TCA) cycle for the release of energy and biomass (Chikere et al., 2011; Varjani, 2017) and is illustrated in Figure 2.3.

In diterminal oxidation, the methyl groups at both terminals of the hydrocarbon are oxidised to form diols, dialdehydes and dicarboxylic acids before going through the β-oxidation pathway to the TCA cycle for energy and biomass release. In the subterminal oxidation however, an intermediate methyl group is oxidised forming different compounds such as secondary alcohols, ketones and esters (Varjani, 2017) and is illustrated in Figure 2.3.
Figure 2.3: Degradation pathways of alkanes (adapted from van Elsas et al., 2007; Varjani, 2017)
2.4.2 Aerobic degradation of aromatic hydrocarbons

For the aromatic hydrocarbons, there is an upper degradation pathway in which benzene is catalysed via different intermediates to benzene-1, 2-diol (catechol). Dioxygenases and monooxygenases catalyse the reaction via the cyclohexa-3, 5-diene-1, 2-diol (cis-dihydrodiol) and 7-oxabicyclo hepta-2, 4-diene (arene oxide) intermediates respectively (Das and Chandran, 2011; Fuchs et al., 2011). This is illustrated in Figure 2.4.

![Benzene dioxygenase and benzene monoxygenase](image)

*Figure 2.4: Upper degradation pathway of aromatic hydrocarbons catalysed by benzene dioxygenase and benzene monoxygenase respectively (Das and Chandran, 2011).*

Benzene-1, 2-diol (catechol) is then then degraded via the *ortho* or *meta*-cleavage pathway depending on the position where the benzene ring is cleaved. For the *ortho*-cleavage pathway, the benzene ring is cleaved between two carbon atoms with hydroxyl groups by catechol-1, 2-dioxygenase while for the *meta*-cleavage pathway the ring is cleaved adjacent two carbon atoms with or without hydroxyl groups by catechol-2, 3-dioxygenase (van Elsas et al., 2007; Chikere et al., 2011; Fuchs et al., 2011; Olajire and Essien, 2014; Varjani and Upasani, 2017). This is illustrated in Figure 2.5. Benzene is the simplest aromatic hydrocarbon having a single cyclic ring while naphthalene is the simplest polycyclic aromatic hydrocarbon having two cyclic rings (Chikere et al., 2011).
Figure 2.5: Degradation pathways of aromatic hydrocarbons (adapted from van Elsas et al., 2007; Olajire and Essien, 2014).
2.5 Bioremediation as a risk mitigation strategy for contaminated soil

Bioremediation, which is monitored biodegradation, is aimed at enhancing contaminant biodegradation. It is an exploitation of the metabolic capabilities of microorganisms (Guarino et al., 2017), during biodegradation, to remove hydrocarbons from the environment (Mesarch et al., 2000) in order to mitigate their toxic and mutagenic effects and restore soil property (Thapa et al., 2012). It is an efficient, economic and eco-friendly option and is accepted as a valuable and safe alternative treatment to physiochemical remediation such as landfill and incineration (Molina-Barahona et al., 2004; Tang et al., 2010; Zhang et al., 2011), which results in an incomplete decomposition of contaminants (Gao et al., 2014).

It entails the complete transformation of environmental pollutants by biological systems such as micro-organisms and plants into completely mineralised innocuous products (Dua et al., 2002; Kuiper et al., 2004; Fernández et al., 2012). It is therefore considered the ultimate natural mechanism for clean-up of hydrocarbon contaminated soils (Jain and Bajpai, 2012). Also, since biodegradation by microbes is the key process by which hydrocarbons in soil are removed (Stroud et al., 2007), it has gained acceptance globally as the preferred treatment for the decontamination of hydrocarbon contaminated soils (Van Hamme et al., 2003; Chikere et al., 2011).

It also gained popularity when it was used to treat the Alaskan shoreline of Prince William Sound following the Exxon Valdez oil spill in 1989 (Boopathy, 2000). Biodegradation is achieved by the metabolic activity of microorganisms which possess adequate enzymes that can break down the contaminants and their corresponding metabolites (Maletic et al., 2013). They then assimilate the breakdown products into their biomass (Kulshreshtha, 2012); thus, utilising it as a sole source of carbon for energy and growth (Thassitou and Arvanitoyannis 2001; Mahiuddin et al., 2012; Adetutu et al., 2015).

Lack of appropriate enzymes will therefore limit the degradation rate (Thapa et al., 2012); and although fungi have the potential for degrading organic compounds due to their symbiotic association with plants, bacteria are more important colonisers in oil contaminated soil. This could be attributed to the
fact that bacteria have high survival capabilities by adapting easily to the presence of contaminants through mutations, horizontal gene transfer and forming new catabolic pathways. However, fungi and bacteria can have a mutualistic relationship in which the presence of fungi supports bacterial growth (Kauppi, 2011).

Nevertheless, since biodegradation relies only on the autochthonous bacterial populations present, the process is slow and needs enhancement to support large contaminant concentrations especially due to the associated nutritional imbalance and resultant toxicity to soil and its intrinsic microbial population. Thus, bioremediation strategies, involving the enhancement of contaminant biodegradation, under controlled conditions are required to improve upon the process (Shahsavari et al., 2013; Cycon´ et al., 2009; Adams et al., 2015).

However, since the efficiency of the biodegradation process is associated with the genetic potential of the degrading microorganisms which breakdown the contaminants and metabolites in the degradation pathway, it is essential to explore the enzymatic activities and genes encoding key enzymes (Foght, 2008) involved in the established degradation pathways of microorganisms as they utilise hydrocarbon substrates especially with the elucidation of new pathways to improve the biodegradation process.

Also, to improve biodegradation efficiency, bioremediation strategies such as phytoremediation (Shahsavari et al., 2013), bioaugmentation and or biostimulation (Cycon´ et al., 2009; Adams et al, 2015) are employed. Such strategies are usually determined by the evaluation of contaminant biodegradation in field trials and laboratory studies (Abioye et al., 2009).

2.6 Types of bioremediation

Three types of bioremediation strategies have been used for the decontamination of petroleum hydrocarbon contaminated soil.
2.6.1 Phytoremediation

This involves the use of plants and their rhizosphere microorganisms to degrade contaminants. However, the associated toxicity of the pollutants to plants often limits this technology (Shahsavari et al., 2013).

2.6.2 Bioaugmentation

This is the introduction of microorganisms with adequate degradation potential or an enriched microbial consortium into the polluted soil (Barathi and Vasudevan, 2001; Ruberto et al., 2003; Lebkowskaa et al., 2011; Taccari et al., 2012; Wu et al., 2013) to start the degradation process; thus, detoxifying and the soil and enabling the otherwise stressed indigenous microbes to regain their biological activity and grow (Chikere et al., 2009; Das and Chandran, 2011; Okoh, 2006). However, the challenge is ensuring that the introduced allochthonous microbes can thrive and survive the new harsh environment (Baek et al., 2009). It is possible that allochthonous microbes may not adapt readily to the new soil environmental condition (Kauppi et al., 2011) thus making them ineffective (Kulshreshtha, 2012; Mrozik and Zofia, 2010).

2.6.3 Biostimulation

This is the introduction of nutrients by the addition of inorganic or organic amendments and oxygen into soil to make up for the deficiency caused by the contaminants and enhance autochthonous bacteria to thrive, proliferate and metabolise the pollutants (Yu et al., 2005; Kauppi et al., 2011; Sayara et al., 2011; Taccari et al., 2012; Abed et al., 2014; Adams et al., 2015). This establishes shifts in microbial population in favour of hydrocarbon degraders (Molina-Barahona, 2004; Shahsavari et al., 2013).

Inorganic amendments include nitrogen, potassium and phosphorous fertilisers while organic amendments include plant residues (Abioye et al., 2012; Dadrasnia and Agamuthu, 2013; Shahsavari et al., 2013; Hamzah et al., 2014), animal dung (Orji et al., 2012), bird droppings (Chikere et al., 2012) and sewage sludge (Agarry and Latinwo, 2015). Biostimulation has also been
applied successfully in studies (Abioye et al., 2012; Dadrasnia and Agamuthu, 2013; Chikere et al., 2012; Hamzah et al., 2014).

The advantage of stimulating autochthonous bacteria is that being native makes them stable and adapted to the environment in which they are. They have also developed effective mechanisms to regulate their cellular functions in response to changes in their environment (Kulshreshtha, 2012). In biostimulation, the supplementation of nutrients supports the growth and metabolism of autochthonous microbes and increases their activity. They can then effectively degrade any pollutants present in their environment (Kulshreshtha, 2012).

However, bioaugmentation can be applied together with biostimulation (Bento et al., 2005). In their study, Adetutu et al. (2015) treated soils contaminated with oil tank bottom sludge using both biostimulation and bioaugmentation to achieve TPH levels suitable for landfill disposal and observed that intrinsic microbial populations in the sludge were better suited at degrading it than those from allochthonous sources.

2.7 Bioremediation Techniques

Bioremediation techniques could be *in situ* or *ex situ*. The former, involves the treatment of contaminated soil in the contaminated site while the later involves the excavation of contaminated soil from contaminated sites to other sites for treatment. The technique adopted is based on various factors including cost of treatment, depth of pollution, type of pollutant, geology and geographic location of polluted sites (Azubuike et al., 2016).

2.7.1 *In situ* Bioremediation

*In situ* bioremediation is usually more desirable because of its low disturbance to the contaminated soil and the avoidance of excavation and transport; which in turn reduces cost. Moisture, nutrients, oxygen, heat and pH are controlled to enhance biodegradation (Sutar and Das, 2012) and includes:
**Bioventing**, which is the most common *in situ* treatment and involves the supply of oxygen and or nutrients to contaminated soil by pumping them through injection wells to enhance biodegradation by indigenous bacteria. It is used for the bioremediation of hydrocarbons in soils from leaking underground tanks (Azubuike *et al.*, 2016).

**Biosparging**, which is another *in situ* treatment, involves the injection of air under pressure below the water table to increase ground water oxygen and enhance biodegradation by indigenous bacteria.

For *in situ* bioremediation, however, it is important that oxygen, and or nutrients, are distributed evenly within the soil. Fine textured clay has a low permeability and limits the permeability of air and nutrients throughout soil. Clayey soils also retain water because of their small pores and high surface area. Water thus drains slowly from them preventing oxygen reaching the soil microbes (Azubuike *et al.*, 2016).

It is thus, not suitable for all types of soil; especially, clayey soils. However, it is suitable for well drained medium or coarse textured soils. Also, it is difficult to achieve complete biodegradation and control natural conditions for biodegradation such as temperature. Thus, adequate environmental conditions are the limiting factor for these techniques.

### 2.7.2 Ex situ Bioremediation

*Ex situ* techniques involve the supply of oxygen and nutrients to excavated soil through the continuous mixing or tilling of slurries in a prepared bed or reactor (Xu and Lu, 2010) examples include:

**Land farming**, which is a simple technique in which contaminated soil is spread over a prepared bed and tilled periodically until pollutants are biodegraded. Indigenous microbial activity is stimulated by allowing good aeration and monitoring nutrient availability to facilitate the aerobic degradation of contaminants. It has the potential of reducing monitoring and maintenance costs as well as clean up liabilities and so is used as a disposal alternative (Sutar and Das, 2012).
Composting and biopiles, in which organic amendments like manure in the form of municipal and green waste or agricultural waste such as bark chips, wheat bran or saw dust is mixed into the excavated contaminated soil to enhance aeration and microbial activity. Nutrients and bacterial consortiums can also be added to accelerate the bioremediation rate. The same technique is involved in biopile and composting except that in biopile, the soil is stored in aerated piles (Xu and Lu, 2010).

Bioreactors, in which contaminated soil is mixed with water and nutrients to form a slurry which is agitated to stimulate microbial action in a bioreactor. The bioreactor is a containment apparatus used to create a three phase (solid, liquid and gas) mixing condition that helps to increase the bioremediation rate of soil bound and water soluble pollutants. Thus, the rate and extent of biodegradation using this method is greater than in situ conditions. This technique is also used for contaminated sludge and water (Sutar and Das, 2012) and is suitable for clay soils (U.S.EPA, 2006).

2.8 Factors that affect soil bioremediation

For bioremediation to be applied efficiently and effectively, adequate knowledge of the contamination site and the conditions that affect the microbial degradation of contaminants is essential (Sabate et al., 2004). The most important factors that affect the bioremediation of hydrocarbon contamination includes the chemical structure of the hydrocarbons, the presence of viable microorganisms with appropriate enzymatic capabilities to degrade the hydrocarbon and optimal environmental conditions in which microorganisms can thrive (Bundy et al., 2002; Stroud et al., 2007).

The environmental factors that limit the rate of bioremediation include temperature, moisture content, nutrient availability, pH, concentration of contaminants, soil characteristics and presence or absence of oxygen (Adams, 2006). However, bioremediation can occur in both aerobic (Wiegel and Wu, 2000) and anaerobic environments (Komancova et al., 2003).
2.8.1 Temperature

The optimum temperature for hydrocarbon degradation is 20°C – 30°C and biodegradation tends to increase with increasing temperature such that bioremediation in temperate climates and climates with extremely low temperatures occurs very slowly (Olajire and Essien, 2014). The UK is associated with having low temperatures in winter which may limit the rate of bioremediation. However, since biodegradation studies have been successfully carried out in colder Arctic climates with resultant mineralisation of pollutants (Okere et al., 2017), biodegradation can also be successfully carried out in temperate climates by applying appropriate bioremediation strategies (Yergeau et al., 2012).

2.8.2 Moisture content

Microorganisms require water in their environmental matrix for optimum growth and proliferation. It also aids in transporting nutrients to the microorganisms during nutrient uptake and regulates soil temperature. Without adequate moisture, microbes will dry up and die since they require up to 12% to 25% of moisture for survival (Adams et al., 2015).

2.8.3 Oxygen

Microorganisms require oxygen for aerobic respiration. However, since they can degrade hydrocarbons both aerobically and anaerobically, in the absence of oxygen, anaerobic respiration can occur in the presence of electron acceptors such as nitrates, oxides and sulphates (ICSS, 2006; Adams et al., 2015). Nevertheless, aerobic respiration is known to have a higher biodegradation rate (Thapa et al., 2012).

2.8.4 Nutrient Availability

Micro-organisms like all living things, require nutrition for growth and metabolism. The most important nutrients they require are carbon, nitrogen, phosphorus and potassium (Okoh, 2006). However, they need a greater amount of carbon than the other nutrients since they utilise it for energy and biomass. The optimum concentration of these elements required for bacterial
growth is a carbon, nitrogen and phosphorous (C:N:P) ratio of 100:10:1 (Wu et al., 2016). However there seems to be a high variation in the C:N:P ratio of fungi based on taxonomic groups and environmental conditions. In their study, Zhang and Elser, (2017) observed a median fungal C:N:P ratio of 250:16:1 from four fungal phyla.

2.8.5 Soil properties and pH

Most microbes have an optimum pH for growth of 6 to 8 (ICSS, 2006; Adams et al., 2015). Properties of soil such as type is very important for microbial activities. The proportion of clay to sand and silt would determine how permeable the soil would be to moisture and air which are vital for microbial growth. It will also determine the availability of the pollutant to the microorganisms (Paul and Clark, 1998).

2.8.6 Availability of contaminant

For any remediation strategy to succeed, the contaminant must be available and accessible to the microorganisms and this is termed bioaccessibility and bioavailability (Kulshreshtha, 2012). The availability of hydrocarbons in soil has been explained in detail in Section 2.3.

2.9 Monitoring bioremediation by TPH measurement

Measurement of TPH (total petroleum hydrocarbons) is the commonly used parameter for quantifying environmental contamination originating from petroleum hydrocarbons. The determination of TPH in soil samples is based on extraction with solvents such as n-hexane and acetone followed by gas chromatograph analysis. It is dependent on the analytical method used for its determination and measurement and different analytical methods are recommended for the different ranges of hydrocarbon components of crude oil such as oil and grease, diesel range organics and gasoline range organics. Since it is a measured gross quantity without identification of its constituents, the TPH value represents a mixture of compounds (URL 4).
For diesel range organics, the EPA Method 8015 which is a solvent extraction, followed by gas chromatography with flame ionisation detection, is the required analytical method for use. For volatile fractions in gasoline, purge and trap gas chromatography is used with a flame ionising detector. TPHs are thus, defined as the measurable amount of petroleum-based hydrocarbon in an environmental media and is dependent on the analysis of the medium in which it is found (URL 4).

It is used to ascertain the severity of contamination and the progress of remediation effort based on concentration (Bacosa et al, 2012). However, it is not a direct indicator of risk posed by contamination to humans or to the environment (EA, 2003) and does not take into consideration the site or source of contamination. Thus, activities such as pipeline explosion, vandalism and illegal refining in countries like Nigeria, which have significant levels of specific contaminants of carcinogenic concern, would be underestimated by TPH measurements (Ogbonnaya et al., 2017) since emphasis is paid to concentration of contamination without considering the risk (Ogbonnaya et al., 2017).

For this reason, polyaromatic hydrocarbons and other chemicals of critical concern which are usually low in concentration, though hazardous to health are considered low in TPHs. Similarly, studies have shown that a TPH of ≤10,000 mg kg\(^{-1}\) of crude oil in soil did not adversely affect plants and posed no risk of leaching to ground water since crude oil is heavy and has a low leaching potential. However, for gasoline leaks at underground storage sites, a TPH as low as ≤100 mg kg\(^{-1}\) in soil is recommended to prevent leaching (URL 5). The quantitation of TPHs in hydrocarbon contaminated soil is thus, the first tier of analysis conducted prior to remediation and is followed by a risk based assessment (Okparanma et al., 2013).

The limitations of TPH measurement, apart from being concentration based; and not suitable for hazard investigation, is that, it can be measured in high levels in materials that are not derived from petroleum and pose no risk to human health such as grass (14,000 mg kg\(^{-1}\)), dried oak leaves (18,000 mg
kg$^{-1}$), and petroleum jelly (749,000 mg kg$^{-1}$) (API, 2000). Nevertheless, it is still used for environmental assessment and not health risk assessment (URL 4).

2.10. Monitoring bioremediation by metagenomic analysis

The broad enzymatic capabilities of microbial populations enabling the breaking down and utilisation of hydrocarbons can affect the extent and rate of biodegradation (Ghazali et al., 2004). Such populations would possess degradation genes that encode and regulate the catabolic enzymes responsible for the degradation of hydrocarbons in different hydrocarbon degradation routes or pathways (Hemalatha and Veeramanikandan 2011). Genes are genetic material usually stored in the chromosomes of microorganisms and sometimes in extra chromosomal material called plasmids. Hydrocarbon degradation genes, however, are frequently located on plasmids.

The presence of degradation genes determine the bioremediation potential of any given community (Tancsics et al., 2008) and serve as markers for monitoring and detecting hydrocarbon degrading enzymes and thus, the identification and assessment of hydrocarbon utilising microbes in the environment (Mesarch et al., 2000; Kasuga et al., 2007). As such, the relationship between environmental pollution and shifts in microbial communities have been established; showing that populations which survive environmental perturbations due to their enhanced physiological and substrate utilisation capabilities, become dominant in the community structure (Chikere et al., 2011).

Metagenomics is useful in detecting unculturable bacteria. This is important because less than 1% of environmental isolates are not culturable (Chikere et al., 2011). Zucchi et al. (2003) in their study, noted that although bioremediation is usually accompanied by an increase in bacterial diversity and gene abundance, the increase in bacterial diversity and gene abundance observed did not result in an accompanying increase in cultivable bacterial count; suggesting that uncultivable bacteria have an important contribution to soil bioremediation.
Metagenomic analysis and quantification of environmental catabolic genes is achieved using polymerase chain reaction (PCR) to replicate the gene of interest in an environmental sample into millions of copies to facilitate its detection and identification. It has been used in a study by Marco (2011) to detect microbial community shifts from Proteobacteria to Firmicutes following long term chromium soil pollution. It has also been employed to characterise population dynamics in contaminated environments using targeted quantitative real time polymerase chain reaction (qPCR) (Lee et al., 2010).

Baek et al. (2009), in their bioremediation study of an oil contaminated soil using the bacterium Norcardia sp H17-1, evaluated changes in the microbial community structure in response to hydrocarbon degradation, using targeted primers in real time polymerase chain reaction (qPCR), to quantify the 16S rDNA gene and two catabolic genes involved in hydrocarbon degradation. The two catabolic genes studied were the alkane monoxygenase (alkB) gene and the catechol-2, 3-dioxygenase (xylE) gene.

Also, the presence and abundance of meta-cleavage genes (xylE) in microbial communities from jet fuel-contaminated soil undergoing bioremediation were revealed using metagenomics (Brennerova et al., 2009; Shah et al., 2011). However, these technologies have not been applied to studies on the bioremediation of diesel using food waste such as BSG in temperate climates.

Nevertheless, the microbial degradation of diesel requires several different genes and pathways. Different genera of bacteria are known for their oil degradation potential and possess different degradation enzymes for hydrocarbon metabolism in a variety of known hydrocarbon degradation pathways and include:

2.10.1. Alkane monoxygenase gene (alkB)

This gene, possessed by alkane degrading organisms, encode alkane monooxygenases, which are one of the key enzymes in the mineralisation of alkanes and catalyse the hydroxylation of the terminal or subterminal methyl group of alkanes. This is usually the first step in the degradation of aliphatic hydrocarbons. Alkane monooxygenase genes (alkB) from *Pseudomonas*
*Pseudomonas putida* are responsible for the degradation of C5 – C12 alkanes (Malkawi *et al.*, 2009).

The alkane monooxygenase enzyme is responsible for converting alkanes in diesel to acetyl Co-A which passes through the TCA cycle to produce energy and biomass for growth in bacterial cells with respiratory products of CO₂ and H₂O. Dussan and Numpaque (2012) in their study, confirmed the presence and expression of the alkane monooxygenase gene from two *Pseudomonas* spp., *P. stutzeri* and *P. aeruginosa*, which were involved in a diesel bioremediation efficiency of 95%; thus, suggesting the consideration of these species for use in bioremediation.

Hydrocarbonoclastic bacteria, possessing this gene, are able to remove hydrocarbon pollutants from the environment (Wang *et al.*, 2013). The OCT plasmid in *P. putida* GX Po1 encodes all of the genes required for the assimilation of C3 – C13 alkanes (van Beilen *et al.*, 2005). Alkane degrading organisms are ubiquitous in the environment; thus, *alkB* genes can be used as molecular markers in the detection of oil degrading organisms using primers that can detect the genes in the environment (Wang *et al.*, 2010; Yang *et al.*, 2015).

Alkane degrading bacterial communities on Sub-Antarctic Macquarie Island comprised of *Pseudomonas* and *Acinetobacter* (Powell *et al.*, 2010). *AlkB* gene clone libraries from contaminated soils also consisted of *Acinetobacter* (Perez de Mora *et al.*, 2011). Also, Wallish *et al.* (2014) reported that the *alkB* gene sequences detected in soil with heavy hydrocarbon contamination were mainly related to *Pseudomonas, Bacillus* and *Acinetobacter* species.

### 2.10.2 Catechol-2, 3-dioxygenase gene (*xylE*)

This gene is recognised as a useful marker in evaluating the bioremediation of hydrocarbons in soil as it catalyses the cleavage of the catechol aromatic ring of aromatic hydrocarbons (Zucchi *et al.*, 2003). Catechol-2, 3-dioxygenase gene (*xylE*) from *Pseudomonas putida*, is responsible for the degradation of xylene and toluene.
Several bacteria produce catechol-2, 3-dioxygenase when soil is contaminated with hydrocarbons or hydrocarbon based compounds such as benzene, toluene or xylene and it is one of the key biochemical pathways through which the contaminants are degraded. As a result of its dominance and importance, it can be used to monitor the clean-up of contaminated sites. Catechol-2, 3-dioxygenase has been detected in *Acinetobacter* and *Pseudomonas* species isolated from hydrocarbon contaminated sites (Mendez *et al.*, 2010; Fuentes *et al.*, 2014).

Most aerobic aromatic hydrocarbon biodegradation pathways converge through catechol-like intermediates that are typically cleaved by *ortho* or *meta*-cleavage dioxygenases. *Meta*-cleavage dioxygenases, or catechol-2, 3-dioxygenases are believed to be more capable than *ortho*-cleavage dioxygenases or catechol-1, 2-dioxygenases, in degrading alkyl-substituted aromatics such as xylenes. Catechol-2, 3-dioxygenase genes also have a well characterized phylogeny that allows for the systematic design of dioxygenase-specific primers (Mesarch *et al.*, 2000).

### 2.10.3 Catechol-1, 2-dioxygenase gene (*catA*)

Catechol-1, 2-dioxygenase is a key enzyme in the degradation of monoaromatic hydrocarbons through the *ortho*-cleavage pathway. *Rhodococcus* species are known to possess the catechol-1, 2-dioxygenase gene and are usually isolated from hydrocarbon contaminated environment (Andreoni *et al.*, 2004; Abraham *et al.*, 2005).

The presence of the *catA* gene has been monitored in the literature by measuring the activity of catechol-1, 2-dioxygenase (Margesin *et al.*, 2013). However, there is not much literature on the determination of its gene copy number based on metagenomic analysis. However, *Pseudomonas putida* is known to metabolise aromatic hydrocarbons via the *ortho*-cleavage pathway catalysed by catechol-1, 2-dioxygenase (Harwood and Parales, 1996; Nelson *et al.*, 2002).
2.11 16S Ribosomal gene (16S rDNA) as a molecular marker for bacterial identification during bioremediation

Studies on the bioremediation of petroleum hydrocarbons have investigated the bacterial population dynamics during biodegradation using the 16S ribosomal deoxyribonucleic acid (16S rDNA) gene as a molecular marker (Chikere et al., 2012). This is because it encodes the 16S ribosomal ribonucleic acid (16S rRNA) which is present in all bacteria. It is therefore, also referred to as the 16S rRNA gene. It is highly conserved between species and contains nine hypervariable regions (V1 – V9) which exhibit considerable sequence diversity among different bacteria and is flanked by conserved regions which allow for species specific sequences within a given hypervariable region (Chakravorty et al., 2007).

The gene sequences are conserved enough to allow for the design of PCR primers which target different taxonomic groups and also has enough variability to allow for the phenotypic comparison of microbial communities (Malik et al., 2008). Molecular techniques involve the amplification of the 16S rDNA, from extracted DNA, using universal primers which targets and anneals to the conserved region of the gene. The resultant PCR products are then sequenced to identify the bacterial species present (Sergeant et al., 2012).

16S rDNA gene sequences provide more objective and reliable classification of microbes than phenotyping. Thus, PCR amplification, cloning and sequencing have been used in determining gene sequences from various environments such as soil and water (Kim et al., 2011). High sequence conservation enables targeting a broad taxonomic group while the interspersed hypervariable regions, which are the regions where variations in amino acid sequences occur, allow discrimination at low levels (Hartmann et al., 2010).

Being the most conserved or least variable DNA in cells, portions of the 16S rDNA sequence from distantly related organisms are remarkably similar and can be precisely aligned enabling true differences to be measured. Conserved regions are the same for all bacteria while the variable regions contain specific sequences unique to individual bacteria (URL 6). The comparison of 16S rDNA
sequences can thus show evolutionary relatedness among organisms and has been used to estimate rates of species divergence among bacteria (URL 7) and for evaluating phylogenetic relationships among organisms (URL 6).

Though no single region can differentiate among all bacteria, the V2 to V4 regions were found most suitable for distinguishing bacterial species to genus level (Chakravorty et al., 2007). The highly conserved regions to which it is flanked makes it easy to design PCR primers that will work across a variety of organisms and the large size of its variable region helps to make PCR reliable.

The Ribosomal Data Project (RDP) obtains bacterial rRNA sequences from the International Nucleotide Sequence Database Gen Bank monthly and provides aligned and annotated rRNA gene sequences for research (Cole et al., 2005). The 100bp region downstream of the forward primer 515F and 100bp upstream of the reverse primer 806R when compared to RDP sequences were found to accurately estimate species richness.

### 2.12 Biostimulation monitoring and evaluation studies

Both inorganic and organic nutrients have been applied in bioremediation studies. Silva – Castro et al. (2013) used an NPK fertiliser in the bioremediation of diesel contaminated soil and achieved a 58% TPH reduction. Also, Chikere et al. (2012) achieved an 87% TPH reduction of crude oil contaminated soil using NPK fertiliser. In the investigation of Gallego et al. (2001) to find the best way to remediate diesel contaminated soil in the laboratory, they observed that biostimulation with nitrogen and phosphorus inorganic nutrients, produced the highest bioremediation rate of above 90% after 45 days with an associated presence of *Acinetobacter* sp. degrading most of the hydrocarbons compared to natural attenuation (intrinsic bioremediation) and control sterilised soils.

However, the use of inorganic nutrients and fertilisers is expensive and in order to minimise costs of remediation, organic nutrients have been applied in bioremediation studies. The use of organic nutrients has an added environmental advantage in that wastes that would have been sent to landfill
are being reused and utilised. Shahsavari *et al.* (2013) used crop residues to achieve an 83% TPH reduction in hydrocarbon contaminated soil mixed with pea straw as compared to the unamended control in which 57% TPH degradation was observed. Tea leaf, soy cake, and potato skins have been used in the bioremediation of a 5% (w/w) diesel contaminated soil and 75%, 88% and 81% TPH reductions obtained respectively after 84 days compared to the control of 35% (Dadrasnia and Agamuthu, 2013).

Table 2.1 presents a list of biostimulation studies in nutrient amendments were utilised to achieve high TPH degradation.
Table 2.1: Some published studies investigating the impact of nutrient amendments on the removal of hydrocarbon contaminants from soil

<table>
<thead>
<tr>
<th>S/NO</th>
<th>AUTHOR</th>
<th>STUMULANTS</th>
<th>% REDUCTION</th>
<th>CONTAMINANT</th>
<th>CONT. CONC</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Dadrasnia and Agamuthu, 2013</td>
<td>Tea leaf Soy cake Potato skin Control</td>
<td>75 88 81 35</td>
<td>Diesel</td>
<td>5% (w/w)</td>
<td>84 days</td>
</tr>
<tr>
<td>2</td>
<td>Shahsavari et al., 2013</td>
<td>2.5% pea straw 2.5% wheat straw 2.5% hay Control</td>
<td>83</td>
<td>60% Diesel + 40% engine oil</td>
<td>1% (w/w)</td>
<td>90 days</td>
</tr>
<tr>
<td>3</td>
<td>Agamuthu et al., 2013</td>
<td>10% sewage sludge 10% cow dung Control</td>
<td>82 94 56</td>
<td>used lubricant oil</td>
<td>10% (w/w)</td>
<td>98 days</td>
</tr>
<tr>
<td>4</td>
<td>Abioye et al, 2012</td>
<td>10% BSG Banana skin (BS) Spent mushroom compoat (SMC)</td>
<td>92</td>
<td>lubricating oil</td>
<td>5% (w/w)</td>
<td>84 days</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10% BSG Banana skin (BS) Spent mushroom compoat (SMC)</td>
<td>55</td>
<td>lubricating oil</td>
<td>15% (w/w)</td>
<td>84 days</td>
</tr>
<tr>
<td>5</td>
<td>Agarry S and Latinwo, G, L., 2015</td>
<td>BSG Effluent bioventing Natural bioattenuation</td>
<td>79 62 40</td>
<td>Diesel</td>
<td>10% (w/w)</td>
<td>28 days</td>
</tr>
<tr>
<td>6</td>
<td>Guarino et al., 2017</td>
<td>Natural attenuation Land farming (biostimulation with nutrients containing NPK mixtures) Land farming (biostimulation) + bioaugmentation with indigenous 10^9 cfu g⁻¹ bacteria after 60 days</td>
<td>57 70 86</td>
<td>TPH in soil from old refinery site</td>
<td>Field trial</td>
<td>90 days</td>
</tr>
<tr>
<td>7</td>
<td>Ross et al., 2010</td>
<td>Fresh sewage (FS) Composted sewage (CS) Unamended soil (US)</td>
<td>46 27 22</td>
<td>Aged hydrocarbon contaminated soil</td>
<td>Field trial</td>
<td>8 months</td>
</tr>
</tbody>
</table>
Table 2.1 represents studies in which BSG and its effluents have been successfully used to achieve reduced TPH in both diesel and lubricating oil contaminated soils (Abioye et al., 2012; Agarry and Latinwo, 2015). Other studies have achieved reduced TPH in diesel contaminated soil using crop residues (Shahsavari et al., 2013; Dadrasnia and Agamuthu, 2013; Chikere et al., 2012; Ross et al., 2010).

2.13 Brewery Spent Grain

Also called brewers spent grains (BSG), it is the main by-product of the brewing process and comprises about 85% of the total waste of the brewery Industry (Lynch et al., 2016). It is obtained mostly from barley after the barley malt is crushed, liquefied and the liquor, also called wort, is separated from the solid residue (Forssell, 2008). Most of the carbohydrate or sugar content is also removed during the brewery process (Khidzir et al., 2010).

About three million tonnes of BSG are produced annually in the UK alone and with the need to diversify landfill waste and promote green environmental technology, it has become inevitable to recycle BSG and convert it to a raw material by finding alternative uses for it. The high nutritional content of BSG is the factor that is being exploited in its reuse (Faulds et al., 2009).

BSG has a high dry mass protein and fibre content of 20% and 75% respectively (Mussatto et al., 2006) and contains polyphenols which are recognised antioxidants, carbohydrates; made up of starch and non-starch polysaccharides, protein, lignin and ash (Faulds et al., 2009). Being a cellulosic waste, when mixed with soil, it reduces soil bulk density and increases aeration. The lignin component increases soil humic acid concentration; encouraging growth of microbes and the cellulose and hemicellulose serve as a source of nutrition (Shahsavari et al., 2013).

It also contains mineral elements such as phosphorus and potassium; though in concentrations lower than 0.5% (Mussatto et al., 2006). For this reason, it has been used as components of animal feed. Its use as baking flour has also been studied (Santos et al., 2003). Alternative uses such as its use in paper
manufacture, incorporation into brick making and its application in biotechnological processes such as the cultivation of mushrooms and enzyme production have also been explored (Mussatto et al., 2006).

Similarly, its high moisture content and fermentable sugar makes it a potentially valuable resource as wet BSG promotes microbial activity and fresh samples of BSG have been shown to have a resident microflora of thermophilic aerobic bacteria which are susceptible to rapid growth (Robertson et al., 2010). Also to note, is that BSG has been used for the cultivation of microbial species such as *Actinobacter*, *Lactobacillus* and *Penicillin* and has been recommended as suitable to support the growth of micro-organisms (Aliyu et al., 2011). The inclusion of BSG to enhance bioremediation in diesel contaminated soil has been explored in previous studies at the University of Sunderland and include the work by Oruru, (2014) which investigated the sustainability of this approach.

Table 2.2 describes the composition of BSG from various studies; adapted from Khidzir et al. (2010) and Lynch et al. (2016).
Table 2.2: Composition of brewery spent grain (BSG) from various studies, adapted from Khidzir et al. (2010) and Lynch et al. (2016)

<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>Cellulose</td>
<td></td>
<td>25.4</td>
<td>21.9</td>
<td>16.8</td>
<td>12</td>
<td>40.2</td>
<td>26.0</td>
<td>21.7</td>
</tr>
<tr>
<td>Hemicellulose</td>
<td></td>
<td>21.8</td>
<td>29.6</td>
<td>28.4</td>
<td>40</td>
<td>-</td>
<td>22.2</td>
<td>19.2</td>
</tr>
<tr>
<td>Lignin</td>
<td></td>
<td>11.9</td>
<td>21.7</td>
<td>27.8</td>
<td>11.5</td>
<td>56.7</td>
<td>n.d.</td>
<td>19.4</td>
</tr>
<tr>
<td>Proteins</td>
<td></td>
<td>24</td>
<td>24.6</td>
<td>15.2</td>
<td>14.2</td>
<td>6.4</td>
<td>22.1</td>
<td>24.7</td>
</tr>
<tr>
<td>Ashes</td>
<td></td>
<td>2.4</td>
<td>1.2</td>
<td>4.6</td>
<td>3.3</td>
<td>2.3</td>
<td>1.1</td>
<td>4.2</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2.7</td>
<td>0.3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Moisture contents (%)</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>73</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lipid</td>
<td></td>
<td>10.6</td>
<td>-</td>
<td>-</td>
<td>13</td>
<td>2.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total carbon (%)</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>35.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total nitrogen (%)</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.025</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The variations in the value range of BSG characteristics in respect to inter-brewing, are a result of the different formulation of grains used; which is dependent on the required flavour (Khidzir et al., 2010), its brewing regime, grain varieties and time of harvesting. However, within brewery sampling has been shown to be quite homogeneous (Lynch et al., 2016).

From Table 2.2, the major constituents of BSG are fibre, in the form of hemicellulose and cellulose, protein and lignin. Fibre constitutes about half of its dry weight composition while protein constitutes up to 30% of its composition. Its high fibre and protein content makes it suitable for both food and non-food applications (Lynch et al., 2016).
Chapter 3

MATERIALS AND METHODS

3.1 Introduction

This chapter describes the methods used in this study and includes a series of laboratory scale experiments to evaluate and validate them.

The methods presented here covers a range of techniques such as soil preparation, experimental design and maintenance, development of spiking and sampling protocol, microbiological methods, universal primers evaluation, DNA extraction for brewery spent grain (BSG), Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR, chemical analysis, molecular analysis such as DNA extraction, real time polymerase chain reaction (qPCR), next generation sequencing and the statistical analysis used in the study.

Soil preparation describes how the soil was prepared from collection up till use. The experimental design and maintenance describe the treatments used in the study and how they were maintained including incubation temperature, aeration and moisture maintenance. The development of spiking and sampling procedures include the experiments that were conducted to evaluate the methods that would be used to spike diesel into the samples and to collect the samples for analysis.

The microbial methods describe the types of bacteriological agar used for the laboratory cultivation of bacteria from the treatments and the characterisation of the bacteria. The universal primers (515F and 1391R), used in this study to amplify the 16S rRNA gene, were evaluated against known bacterial sequences to confirm its suitability. The method for the extraction of DNA from brewery spent grain was developed and evaluated as the literature does not yet mention this procedure. The chemical analysis describes the analytical methods such as the extraction of hydrocarbon from the soil samples and the gas chromatography (GC) and gas chromatography mass spectrometry (GC-MS) methods used to evaluate the total petroleum hydrocarbons (TPHs) present in the samples.
The section on metagenomic analysis describes the direct extraction of genomic DNA for real time polymerase chain reaction (qPCR) and next generation sequencing. qPCR was used to determine the presence of hydrocarbon degrading genes in the samples and the gene copy numbers over time during the bioremediation process while next generation sequencing was used for bacterial community profiling to determine the bacterial populations present in the samples, over time, during the process.

The statistical analysis describes the statistical tests used in the study and the reasons for their choice.

3.2 Materials

3.2.1 Soil

The soil sample used in this study was a sandy loam and was a mixture of 8% clay, 50% sand and 42% silt. It was from a pristine agricultural organic farmland in county Durham, used to grow vegetables, with no known exposure to contamination. Samples were collected from a depth of 10 to 30 cm, after the removal of surface litter. It was air dried and sieved as detailed in Section 3.3.1 and samples were sent for chemical analysis (Anglian Soil Analysis Ltd, UK). The characteristics of the soil is shown in Table 3.1.

Table 3.1: Physical and chemical characteristic of the soil used in this study

<table>
<thead>
<tr>
<th>Soil property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil texture</td>
<td>Sandy/Loam</td>
</tr>
<tr>
<td>pH</td>
<td>6.80</td>
</tr>
<tr>
<td>Moisture (%)</td>
<td>26.04</td>
</tr>
<tr>
<td>Organic matter (%)</td>
<td>12.0</td>
</tr>
<tr>
<td>Sand (%)</td>
<td>50.44</td>
</tr>
<tr>
<td>Silt (%)</td>
<td>41.84</td>
</tr>
<tr>
<td>Clay (%)</td>
<td>7.72</td>
</tr>
<tr>
<td>Total organic carbon (%)</td>
<td>6.96</td>
</tr>
<tr>
<td>Potassium (mg kg⁻¹)</td>
<td>15.4</td>
</tr>
<tr>
<td>Magnesium (mg kg⁻¹)</td>
<td>21.1</td>
</tr>
<tr>
<td>Phosphate (mg kg⁻¹)</td>
<td>17.5</td>
</tr>
<tr>
<td>Nitrate (mg kg⁻¹)</td>
<td>3.5</td>
</tr>
</tbody>
</table>
### 3.2.2 Brewery Spent Grain

The spent grain used in this study was obtained from Darwin Brewery at Sunderland Enterprise Park and the malt used in the brewing process was the recipe for a standard pale ale malt obtained from Fawcett and Sons maltsters, Castlefield, UK. The BSG was stored for four days at 4°C in the laboratory at the University of Sunderland prior to use and had a moisture content of 75% and pH of 5.3. Its inherent bacterial profile was determined using metagenomic analysis (Chapter 6, Figure 6.5). Detailed characteristics of BSG from the literature is shown in Section 2.13 of Chapter 2.

### 3.2.3 Chemicals

Diesel for the experiment (10 litres) was obtained from a commercial Texaco filing station in Sunderland and stored in a closed container at room temperature. The same batch was used throughout the study.

The Acetone and Hexane used were of analytical grade, obtained from Sigma Aldrich, UK and stored in cupboards in the laboratory at room temperature.

### 3.2.4 Microbiological media for bacterial cultivation

Three different growth media were used in this study namely R2A, malt and oil agar. The R2A agar and malt agar were obtained from Sigma Aldrich, UK while the oil agar was formulated by incorporating agar into a minimal salt medium with diesel (oil) as the only carbon source. The formulation for the different media used in this study is shown in Section 3.5.2.

### 3.2.5 Reagent kits used in this study

All laboratory reagent kits used were for research only and include the DNeasy Blood & Tissue Kit (Qiagen, UK) for bacterial genomic DNA extraction, E.Z.N.A.® Soil DNA Kit (Promega, UK) for extraction of DNA from treatments and QIAquick Gel Extraction Kit (Qiagen, UK) for extraction of DNA from gels following PCR.
3.2.6 Primers and Master Mix for qPCR

The universal primers targeting the 16S rDNA gene, primers targeting the *alkB*, *catA* and *xylE* catabolic genes and the KAPA SYBR® FAST Universal qPCR Master Mix were obtained from Sigma Aldrich, UK and stored in the freezer at -20°C.

3.3 Soil preparation and experimental design

3.3.1 Soil collection and diesel spiking

The soil was air dried in a shaded area away from direct sunlight in the greenhouse at 15°C ± 3°C. After breaking large aggregate particles into smaller soil particles (Viji and Rajesh 2012), it was sieved using a 4 mm mesh sieve to remove debris, stones and large particles (Shahsavari *et al.*, 2013).

The pH of the soil was determined by a 1: 2.5 soil: distilled water ratio (Emmett *et al.*, 2010) to be 6.8. For treatments requiring diesel, 20 ml diesel was spiked into 2000 g soil in triplicate to achieve a 1% (v/w) contamination by adding the diesel into 25% of the soil and mixed thoroughly with a stainless steel spoon before adding in the remaining 75% of soil to achieve a good homogeneity (Fernández *et al.*, 2011) as described in Section 3.8.1.

3.3.2 Soil treatments

There were five treatments for the experiment as follows:
1. Soil + Diesel (natural attenuation)
2. Soil + Diesel + BSG (amendment)
3. Soil alone (control)
4. Sterile Soil + Diesel (abiotic control)
5. Sterile Soil + Diesel + Sterile BSG (abiotic control)

Soil sterilisation, for treatments requiring sterile soil, was achieved by autoclaving at 121°C for one hour for three alternate days (Molina-Barahona *et al.*, 2004; Emam *et al.*, 2014) prior to diesel spiking. This was necessary to
inhibit soil microbial activity and destroy microbes that would have developed from spores. This same procedure was used to sterilise brewery spent grain for treatments requiring sterilised grain.

For each treatment, 2000 g soil was put into 5 litre pots in triplicate. 1 % diesel (v/w) and 10 % brewery spent grain (w/w) were added to required treatments. All treatments were covered with gortex cloth and kept in the green house at a temperature of 15°C ± 3°C. Table 3.2 below is a representation of the treatments and their composition.

### Table 3.2: Composition of the treatments with and without BSG, their sterile counterparts and soil control, used in this study

<table>
<thead>
<tr>
<th>S/N</th>
<th>Treatment</th>
<th>Grams of soil</th>
<th>% of diesel</th>
<th>Grams of BSG</th>
<th>Number of replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Soil alone control (S)</td>
<td>2000</td>
<td>-</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>Soil and diesel (S+D)</td>
<td>2000</td>
<td>1.0</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>Sterile soil and diesel (SS+D)</td>
<td>2000</td>
<td>1.0</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>Soil, diesel and BSG (S+D+G)</td>
<td>2000</td>
<td>1.0</td>
<td>200</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>Sterile soil, diesel and sterile grain (SS+D+SG)</td>
<td>2000</td>
<td>1.0</td>
<td>200</td>
<td>3</td>
</tr>
</tbody>
</table>

#### 3.3.3 Aeration and Moisture Content

The treatments were mixed twice a week using a sterile spatula to provide sufficient air and oxygen and the moisture content of the treatments were maintained by the weekly addition of 5 % (v/w) sterile distilled water. Thus, 100 ml sterile distilled water was added to each treatment pot weekly.
3.3.4 Soil Sampling

Following the results of the evaluation for a sampling protocol, and in accordance with the literature, samples were taken from 5 points in each treatment pot and bulked together into composite samples for each sampling time (Bento et al., 2005). Thus, 5 g samples were obtained from each of 5 points and bulked together to a 25 g composite sample per treatment pot. Two sub samples of 10 g each were measured out for chemical analysis (TPH determination) and microbiological analysis (CFU) respectively while 1 g was used for DNA extraction for metagenomic analysis to determine catabolic gene copy numbers and population composition changes among treatments over time. Sampling was carried out on days 0, 2, 5, 7, 12, 14, 21, and 28.

3.4 Analytical Methods

3.4.1 Diesel extraction

For each replicate sample, 10 g of soil was collected for TPH analysis. The soil was weighed into a bottle and 20 ml of hexane added to it. The bottle was then placed on a shaker at 100 rpm for 24 hours at room temperature. After 24 hours, the samples were filtered by vacuum funnel and 1 ml aliquots of the extract were pipetted into 1.5 ml gas chromatography (GC) vials in triplicate and sealed for gas chromatography (GC) and gas chromatography mass spectrometry (GCMS) analysis respectively.

3.4.2 Gas Chromatography (GC)

The GC analysis was carried out using an Agilent Technologies 7890A GC equipped with a flame ionization detector (FID). An Agilent 19091J-413E HP-5 (30 m x 0.32 mm x 0.25 µm) capillary column capillary (Agilent Technologies, UK) was used with helium as the carrier gas at a flow rate of 2 ml min⁻¹, hydrogen gas at a flow of 30 ml min⁻¹ and air at a flow of 300 ml min⁻¹. The temperature program used was a modified version of Bento et al. (2005).
The initial temperature was 50°C with isothermal operation for 5 min, followed by heating to 270°C at a constant rate of 10°C min⁻¹ and a final 5 min isothermal operation. Samples for each sampling time were analysed on the same run since the GC could run up to a hundred samples. Triplicate of the standard were run concurrently with treatment samples for every run to confirm accuracy of the results and resolve any discrepancies.

3.4.3 Determination of percentage TPH reduction and concentration of aliphatic TPH (C₁₀ – C₂₈) fractions

Following gas chromatography, the percentage reduction in total petroleum hydrocarbon (TPH) was calculated using the formula from Bento et al. (2005) as: \[ \% \text{ TPH reduction} = \frac{\text{TPH of control} - \text{TPH treatment}}{\text{TPH control}} \times 100. \]

Day 0 TPH was used as control for each treatment. The concentration of aliphatic TPH (C₁₀ – C₂₈) fractions was calculated by comparing the total peak areas for each fraction in the treatments to the total peak areas for the same fraction in the TPH standard Mix 1 from Sigma Aldrich (UK) and quantified.

3.4.4 Determination of the biodegradation rate of aliphatic TPH (C₁₀ – C₂₈) fractions, in the treatments, using bioremediation kinetics.

The concentration of the TPH (C₁₀ – C₂₈) fractions was fitted to a first order reaction, based on kinetic modelling, to determine the biodegradation rate of the aliphatic TPH (C₁₀ – C₂₈) fractions in the treatments with and without BSG. The order of the reaction in the treatments was determined by plotting the logarithm of TPH concentration versus time using a scatter diagram. The resultant straight line showed that the concentration data fitted to the first order kinetic model. The slope of the line was divided by time to determine the biodegradation rate constant (k day⁻¹) (Sakar et al., 2005; Agarry and Jimoda, 2013).
The biodegradation rate was determined using the formula from Baek, et al. (2004): \( \frac{C}{C_0} = e^{-kt} \), which is same as: \( C = C_0 e^{-kt} \), (Abioye et al., 2012), where C is the concentration of the TPH fractions (mg kg\(^{-1}\)) at time t, \( C_0 \) is the initial concentration of the TPH fractions (mg kg\(^{-1}\)), t is time (day \(^{-1}\)) and k is the biodegradation rate constant (day\(^{-1}\)).

### 3.4.5 Gas Chromatography Mass Spectrometer (GC-MS)

GCMS was analysed using an Agilent GC-MS 7890A/5975C series with an Agilent 19091S-433E HP-5MS (30 m x 0.32 mm x 0.25 µm) capillary column (Agilent Technologies, UK). Helium was the carrier gas with a flow velocity of 1 ml min\(^{-1}\) and 1 µl of sample was injected into the column in a splitless mode. The analytical conditions were an initial temperature of 50°C, with isothermal operation for 1 min followed by heating to 120°C at a constant rate of 20°C min\(^{-1}\) and a final heating to 310°C at a constant rate of 4°C min\(^{-1}\) (Xu and Lu, 2010) with a 5 min isothermal operation. The column was directly connected to an electron ionisation mass spectrometer with an electron energy of 70 eV producing ions that are characterised according to mass-to-charge ratio and relative abundance.

### 3.5 Microbiological Methods

#### 3.5.1 Determination of colony forming units (CFUs) for the enumeration of heterotrophic and hydrocarbon degrading bacteria

Composite samples of 10 g from each treatment pot were added into lidded bottles containing 100 ml of 0.2% by volume sterilised sodium pyrophosphate and mixed on a shaker at 150 rpm (revolutions per minute) for 30 minutes at 20°C. Thereafter, 1 ml of the soil / sodium pyrophosphate solution from each bottle was transferred into 9ml sterile saline in test tubes and a 10-fold serial dilution was carried out from \(10^{-1}\) to \(10^{-6}\).
Following the dilutions, 0.1 ml (100 µl) of each serially diluted solution was plated on R2A agar, for the enumeration of heterotrophic bacteria, and oil agar, for the enumeration of hydrocarbon degrading bacteria, and incubated at 30°C for 24 hours and 25°C for 7 days, respectively.

3.5.2 Formulation of microbiological media used in this study

R2A agar was formulated by Reasoner and Geldreich for enumerating heterotrophic microbes in potable water (Reasoner and Geldreich, 1985; Margesin and Schinner, 2001) but has found use in soil since it is nutritionally rich and able to stimulate the growth of many bacteria (Pepper, 2014). The formulation for R2A agar is shown in Table 3.3 and the experiment carried out to justify the use of R2A agar for this study is as shown in Section 3.8.4.

Oil agar, on the other hand, was formulated by incorporating agar into a minimal salt medium and adding 4% sodium dodecyl sulphate after autoclaving. The hydrocarbon was made available to the bacteria through vapour transfer by impregnating strips of filter paper with 60 ml diesel and placing on the lids of the petri dishes containing sterilised minimal media. It served as the only carbon source for the bacteria; thus, allowing for the selective isolation of hydrocarbon degrading bacteria since only organisms able to metabolise the hydrocarbon (oil) will grow on the media (Margesin and Schinner, 2001). The formulation for oil agar is as shown in Table 3.4 below.
Table 3.3: Composition and formulation of R2A agar (URL 8) used for the laboratory cultivation of heterotrophic bacteria from treatments

<table>
<thead>
<tr>
<th>R2A Agar Composition</th>
<th>Grams</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast Extract</td>
<td>0.5</td>
</tr>
<tr>
<td>Proteose Peptone No. 3</td>
<td>0.5</td>
</tr>
<tr>
<td>Casamino Acids</td>
<td>0.5</td>
</tr>
<tr>
<td>Dextrose</td>
<td>0.5</td>
</tr>
<tr>
<td>Soluble Starch</td>
<td>0.5</td>
</tr>
<tr>
<td>Sodium Pyruvate</td>
<td>0.3</td>
</tr>
<tr>
<td>Dipotassium phosphate</td>
<td>0.3</td>
</tr>
<tr>
<td>Magnesium Sulphate</td>
<td>0.05</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0</td>
</tr>
</tbody>
</table>

Table 3.4: Composition and formulation of oil agar used for the laboratory cultivation of heterotrophic bacteria from treatments

<table>
<thead>
<tr>
<th>Oil Agar Composition</th>
<th>Grams</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferric Ammonium Citrate</td>
<td>0.01</td>
</tr>
<tr>
<td>Calcium Citrate</td>
<td>0.05</td>
</tr>
<tr>
<td>Magnesium Sulphate Heptahydrate</td>
<td>0.20</td>
</tr>
<tr>
<td>Ammonium Sulphate</td>
<td>1.0</td>
</tr>
<tr>
<td>Potassium Dihydrogen Phosphate</td>
<td>1.0</td>
</tr>
<tr>
<td>Sodium Dihydrogen Phosphate Dodecahydrate</td>
<td>7.0</td>
</tr>
<tr>
<td>Agar No. 2</td>
<td>15.0</td>
</tr>
<tr>
<td>Trace Elements</td>
<td>1.0 ml</td>
</tr>
</tbody>
</table>
3.6 Molecular Analysis

The analysis described here utilised bacterial genomic DNA extracted from individual isolated bacteria and total community DNA extracted directly from soil samples. The individual bacterial genomic DNA was amplified using PCR and sequenced to identify the individual bacterial species while the total community genomic DNA was amplified and used for:

i) The detection and quantification of catabolic genes present in the samples using qPCR and

ii) The profiling of bacterial species composition and abundance, and any community changes in the treatments over time, using next generation sequencing.

3.6.1 DNA (deoxyribonucleic acid) extraction

3.6.1a DNA extraction from bacterial isolates

Genomic DNA from bacteria isolated from agar plate cultures, were extracted using the Qiagen DNeasy blood and tissue DNA extraction kit (Qiagen, UK), following the manufacturer’s protocol, in order to identify the bacterial species. Extracted DNA were checked for purity based on absorbance at 260 nm and 280 nm, using a Nanodrop (ND-2000, Thermo Fisher Scientific, UK), to ensure a ratio of 1.8 to 2.0.

3.6.1b Community DNA extraction from soil treatments

Total community genomic DNA was extracted from 1 g sample each, of all treatments, over time, using the EZNZA soil DNA kit (Omega Bio-Tek, Inc., Norcross, GA, USA) according to the manufacturer’s instructions (Dineen et al., 2010) to determine the copy numbers of catabolic genes and microbial species composition and abundance over time. Extracted DNA were checked for purity based on absorbance at 260 nm and 280 nm, using a Nanodrop (ND-2000, Thermo Fisher Scientific, UK), to ensure a ratio of 1.8 to 2.0.
3.6.2 Identification of bacterial isolates

Genomic DNA described in Section 3.6.1a above were analysed for the identification of individual bacterial species.

3.6.2a Polymerase chain reaction (PCR)

The eubacterial gene in the DNA was amplified by polymerase chain reaction (PCR) for characterisation using the Bio Rad thermocycler (Bio Rad, UK). The primers used were the 515F (5’-GTGCCAGCMGCGGCTAA-3’) and 1391R (5’-GACGGGCGGTGWGTRCA-3’) (Turner et al., 1999). These primers amplify the V4 region of the 16S rDNA gene of bacteria. The amplification reaction was performed in a total volume of 25 µl for each sample containing 8 µl PCR water, 1.25 µl each of forward and reverse primers totalling 2.5 µl, 12.5 µl of Immomix enzyme (BIOLINE Reagents Ltd, UK) and 2 µl of DNA. The amplification program was performed with initial denaturation step at 95ºC for 10 min; followed by 40 cycles of 1 min denaturation step at 91ºC, 45 s annealing step at 55ºC and 30 s elongation step at 72ºC; and a final extension step at 72ºC for 10 min.

Experiments to validate the use of these primers are as shown in Section 3.8.5.

3.6.2b Gel electrophoresis, sequencing and identification of bacteria

The PCR products were run on a 2% (w/v) Agarose gel using gel red staining in a 1 x TAE buffer (20 mM tris-HCl, 10 mM acetate, 0.5 mM Na2EDTA) at room temperature on a constant voltage of 70 V for 1 hour. Bands were visualised using a Bio-Rad Chemi Doc™ MP Imaging System (Bio-Rad Laboratories Ltd. Hertfordshire, UK). Prominent bands were excised under UV radiation, extracted using the QI Aquick Gel Extraction Kit (QIAGEN, USA) and sent to Source Bioscience Limited, UK for sequencing. Sequences were identified using the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI) (Pasumarthi et al., 2013) by matching with the GenBank sequence of strains having >98%16S rDNA gene sequence similarity and assigned the same phylotype (Zhang et al., 2012).
3.6.3 Detection and quantification of hydrocarbon catabolic genes

Total community genomic DNA, described in Section 3.6.1b above was analysed for each treatment over time to determine the presence and copy numbers of three catabolic genes namely: the \textit{alkB} gene that encodes the catabolism of aliphatic hydrocarbons, the \textit{catA} gene that encodes the catechol-1, 2-dioxygenase enzyme involved in aromatic hydrocarbon breakdown and the \textit{xylE} gene that encodes the catechol-2, 3-dioxygenase enzyme also involved in aromatic hydrocarbon breakdown.

3.6.3a Real time polymerase chain reaction (qPCR)

Quantitative real time PCR (qPCR) for the amplification of catabolic genes were performed using 1:10 dilutions of microbial community DNA extracted from bioremediation treatments (Shahsavari et al., 2017) with and without BSG overtime. Dilutions were done using sterile nuclease free water. Three hydrocarbon catabolic genes; \textit{alkB} (Powel \textit{et al.}, 2006), \textit{catA} and \textit{xylE} (Shahsavari \textit{et al.}, 2016) were assayed for; using a Rotor Gene Q thermocycler (Qiagen, UK) and the 2x Kapa Sybr Fast qPCR Master Mix Universal kit (Sigma Aldrich, UK). All samples and standards were run in triplicates. Negative controls were included in all amplifications.

Absolute quantification for each gene in the different samples over time was performed by comparison with standard callibration curves (Phillips \textit{et al.}, 2012) generated by assaying ten fold serial dilutions of 6 orders of magnitude ranging from $10^{-2}$ to $10^{-7}$ of known concentrations of cleaned PCR products for each gene. In addition, melt curve analysis was performed at the end of each reaction to check for purity of the amplified product by detecting the production of nonspecific PCR products such as primer dimers (Wang \textit{et al.}, 2010).

PCR products of the gene of interest were verified to be of correct band size by running them on a 2\% (w/v) gel electrophoresis using gel red staining. Bands were visualised using a Bio-Rad Chemi Doc™ MP Imaging System (Bio-Rad Laboratories Ltd. Hertfordshire, UK), excised under UV radiation and extracted using the QI Aquick Gel Extraction Kit (QIAGEN, USA).
Gene copy number was calculated using the formula: number of copies = (ng/ul DNA x 6.022 x10\(^{23}\)) / (PCR product length in base pairs x 1 x 10\(^9\) x 650) (Staroscik, 2004) where Avogadro's number of 6.022\(x10^{23}\) is the number of molecules/mole DNA and 650 Daltons the average weight of a base pair. The gene copy numbers are expressed as log10 of gene copy numbers g\(^{-1}\) soil.

Details of primers targeting specific catabolic genes including their sequences and annealing temperatures are shown in Table 3.4 below while the primer concentrations used and the master mix cocktail is shown in Table 3.5 below.

**Table 3.5: Details of the primers used for the detection and quantification of target hydrocarbon catabolic genes**

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Primer Name</th>
<th>Annealing Temperature °C</th>
<th>Sequence (5' – 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkane monoxygenase</td>
<td><em>AlkB</em></td>
<td>50</td>
<td>F: AAC TAC ATC GAG CAC TAC GG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: TGA AGA TGT GGT TGC TGT TCC</td>
</tr>
<tr>
<td>Catechol-1,2-dioxygenase</td>
<td><em>CatA</em></td>
<td>58</td>
<td>F: ACVCCVCGHACCATYGAAGG</td>
</tr>
<tr>
<td>(C13O)</td>
<td></td>
<td></td>
<td>R: CGSGTNGCAWANGCAAGT</td>
</tr>
<tr>
<td>Catechol-2,3-dioxygenase</td>
<td><em>XylE</em></td>
<td>58</td>
<td>F: AAGAGGGCATGGGGGCGACCGGTTCGATCA</td>
</tr>
<tr>
<td>(C23O)</td>
<td></td>
<td></td>
<td>R: CCAGCAAAACACCTCGTTGGTGGTC</td>
</tr>
</tbody>
</table>
Table 3.6: Concentrations of the components of the PCR reaction mixture used to amplify the detected catabolic genes for quantification

<table>
<thead>
<tr>
<th>Cocktail’s Components + DNA Template</th>
<th>20 µl rxn</th>
<th>Final conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X KAPA SYBR® FAST qPCR Master Mix Universal</td>
<td>10 µl</td>
<td>1X</td>
</tr>
<tr>
<td>Forward Primer (10 pM)</td>
<td>0.4 µl</td>
<td>200 nM</td>
</tr>
<tr>
<td>Reverse Primer (10 pM)</td>
<td>0.4 µl</td>
<td>200 nM</td>
</tr>
<tr>
<td>PCR-grade water</td>
<td>8.2 µl</td>
<td>N/A</td>
</tr>
<tr>
<td>Template DNA</td>
<td>1.0 µl</td>
<td></td>
</tr>
</tbody>
</table>

The cycling programme for the alkB gene was an initial denaturation step at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing at 50°C for 30 seconds, extension at 72°C for 30 seconds and a primer dimer removal and signal acquisition at 80°C for 10 seconds.

The cycling programme for both catA and xylE genes were the same since they have the same annealing temperature and comprised of an initial denaturation step at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing at 58°C for 30 seconds, extension at 72°C for 30 seconds and a primer dimer removal and signal acquisition at 80°C for 10 seconds.

3.6.4 Next generation sequencing

The 16S rRNA gene V4 variable region PCR primers 515 - 806 (Caporaso et al., 2011) were used in a single-step 30 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C for 3 minutes, followed by 28 cycles (5 cycle used on PCR products) of 94°C for 30
seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed.

Sequencing was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on an Ion Torrent PGM following the manufacturer’s guidelines. Sequence data were processed using a proprietary analysis pipeline (MR DNA, Shallowater, TX, USA). In summary, sequences were depleted of barcodes and primers, then sequences <150bp removed, sequences with ambiguous base calls and with homopolymer runs exceeding 6bp were also removed.

Sequences were denoised, OTUs generated and chimeras removed. Operational taxonomic units (OTUs) were defined by clustering at 3% divergence (97% similarity). Final OTUs were taxonomically classified using BLASTn against a curated database derived from GreenGenes, RDPII and NCBI (www.ncbi.nlm.nih.gov, DeSantis et al 2006, http://rdp.cme.msu.edu).

3. 7 Statistical Analysis

Means, standard deviations and standard error were calculated using Microsoft Excel 2013.

The IBM SPSS statistics version 24 statistical tests were used in this study to determine significant differences among treatments in respect to TPH reduction, bacterial count and catabolic gene copy numbers. All data were tested for normality using the SPSS Shapiro-Wilk test and significance was determined as p < 0.05.

The SPSS parametric analysis of variance (ANOVA) and the least significant difference (LSD) post hoc test were used to determine significant differences among treatment means of more than two independent variables while the independent T test (parametric) was used to determine significant differences between two independent variables. Significance was determined as p < 0.05.
3.8 Evaluation of Methods

3.8.1. Evaluation of diesel spiking methodology

A pilot experiment was conducted to compare the effect of three methods of spiking diesel into soil on the homogenous distribution of diesel in soil and the resultant microbial growth.

Laboratory studies on the fate of hydrocarbon contaminants in soil require the organic compounds to be spiked into the soil and acetone has been evaluated as a good diluent for diesel, helping its distribution in soil samples and resulting in high recovery rates (Oruru, 2014) thus it has been used severally in studies for hydrocarbon spiking (Brinch et al., 2002; Sawada et al., 2004; Smídová et al., 2012).

However, Brinch et al., (2002) noted that adding organic solvents such as acetone to soil samples severely affected the indigenous bacteria and proposed only 25% of soil sample to be added to acetone, followed by evaporation of the solvent before mixing with the remaining 75% of soil. Doick et al. (2003) on the other hand, have spiked hydrocarbons into 25% of soil and mixed into the remaining 75% of soil without any diluent and obtained good recovery rates and microbial count. Thus, several studies have followed this procedure (Liu et al., 2015).

This evaluation is therefore to investigate the effect of three diesel spiking procedures, based on the literature (Brinch et al., 2002; Doick et al., 2003), on the distribution and recovery of diesel from soil, and associated bacterial CFUs.

The three treatments for spiking diesel into soil that were investigated are as follows:

Treatment A: 250g soil was mixed with 1% diesel and 1% acetone and left overnight. The remaining 750 g soil was then added gradually and mixed using stainless steel spoon (Brinch et al., 2002).

Treatment B: 1 kg soil was mixed with 1% diesel and 1% acetone and left overnight (Brinch et al., 2002).
Treatment C: 250 g soil was mixed with 1% diesel alone and left overnight. The remaining 750 g soil was then added gradually and mixed using a stainless steel spoon (Doick et al., 2003).

Each of the three treatment pots were prepared in triplicates, with a total of 9 treatment pots. Each treatment was evaluated to determine which spiking procedure had a greater homogeneity of diesel distribution and supported the most bacterial growth to determine which diesel spiking method would be adopted for this study. Also to be determined was the sampling methodology to be used in this study.

3.8.2 Evaluation of sampling methodology

This was conducted to assess the effect of bulking samples, from the three treatments in 3.8.1, into composites.

A. Five individual samples were taken from each tray so there were 15 samples from the three trays representing one treatment and a total of 45 samples for all nine trays representing the three treatments.

B. Five samples were also taken per tray and bulked to form one composite sample from which three subsamples were taken to represent one treatment tray. This was repeated for all nine treatment trays resulting in a total of 27 composite samples for all treatment trays.

These samples were analysed for TPH recovery using GC. Culturable aerobic heterotrophic bacteria were quantified by plate count on nutrient agar (Chemlal et al., 2012) and R2A agar (Margessin and Schinner, 2001) and incubated at T = 30°C and 25°C respectively for optimum growth. Colonies were counted after 24 to 48 hours.
Statistical Analysis

All data are presented as means ± SD. Means and standard deviations were calculated using Microsoft Excel 2013. All data were tested for normality using the SPSS Shapiro-Wilk test after which the statistical significance of the data was evaluated by the SPSS one way analysis of variance (ANOVA). A p value of less than 0.05 was considered statistically significant (Aceves-Diez et al., 2015).

Results

Table 3.7 below shows the results of TPH recovery for the three spiking treatments in Section 3.8.1 based on the two sampling methods in Section 3.8.2.
Table 3.7: Mean peak heights of recovered TPH in diesel spiked soil, using three different spiking procedures, to determine diesel distribution and recovery from soil

Mean peak heights of recovered TPH

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Sampling method 1 (n = 3)</th>
<th>Sampling method 2 (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Treatment pots</td>
<td>Mean peak height (TPH C10 – C28)</td>
</tr>
<tr>
<td>A</td>
<td>1% (v/w) diesel + acetone in 25% soil, mixed later in 75% soil</td>
<td>31.4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>35.8</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>33.4</td>
</tr>
<tr>
<td>B</td>
<td>1% (v/w) diesel + acetone in 100% soil at once</td>
<td>19.5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>25.0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>36.1</td>
</tr>
<tr>
<td>C</td>
<td>1% (v/w) diesel only in 25% soil and mix in 75% later</td>
<td>39.6</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>31.5</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>23.0</td>
</tr>
</tbody>
</table>

Same letters are not statistically different among treatments in each sampling method by the Tukey test (p < 0.005).
From table 3.7 above, the analysis of variance (ANOVA) in TPH recovery between the group means of the three different treatments using sampling method 1 was $P = 0.526 > 0.05$. This indicates that there is no statistically significant difference in recovered TPH between the three treatments using sampling method 1.

However, the total mean TPH recovery, though not statistically significant, is highest for treatment A in which diesel and acetone were added to 25% soil before mixing in the remaining 75% of soil and had the lowest variability between samples as shown by the standard deviation in Table 3.7. The recovered TPH was lowest for treatment B in which diesel and acetone were added to 100% of the soil at.

Also, from table 3.7 above, the analysis of variance (ANOVA) in TPH recovery between the group means of the three different treatments using sampling method 2 was $P = 0.937 > 0.05$. This indicates that there is no statistically significant difference in recovered TPH between the three treatments using sampling method 2.

However, though the total mean TPH recovery is not statistically significant, it is highest for treatment C in which diesel only was added to 25% soil before mixing in the remaining 75% of soil and lowest for treatment B in which diesel and acetone were added to 100% soil at once.

**Conclusion**

The TPH recovery from the three treatments did not vary significantly in both sampling methods. This indicates a considerably good distribution and homogeneity of diesel in the soil after spiking. However, because this study is interested in microbial populations present in contaminated soil, the potential of the treatments to support bacteria was also evaluated. Nevertheless, only method 2 was used in investigating the bacterial count in the treatments since there was no significant difference in TPH recovery between both methods and secondly, the literature supports the use of composite samples for TPH recovery in contaminated soils (Bento *et al.*, 2005; Chikere *et al.*, 2009, Shahsavari *et al.*, 2013).
3.8.3 Evaluation of CFUs for different spiking and sampling methodologies in treatments

Soil samples from each of three treatments in Section 3.8.1 based on the sampling method two in Section 3.8.2 were used for plate count on R2A agar and nutrient agar following the plate count method in Section 3.5.1 to evaluate the effect of treatments on bacterial count and identify which bacteriological medium best supports the bacterial growth.

**Results**

Table 3.8 below shows the results of CFUs for the enumeration of heterotrophic bacteria for the three spiking treatments in Section 3.8.1 based on the two sampling methods in Section 3.8.2.
Table 3.8: Comparison of colony forming units among three different diesel spiked soil treatments, using different growth media, to determine choice of media for the enumeration of heterotrophic bacteria

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Treatment pots</th>
<th>Log10 CFU g⁻¹ soil (n =3)</th>
<th>Mean ± SD</th>
<th>Log10 CFU g⁻¹ soil (n =3)</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1% (v/w) diesel</td>
<td>1</td>
<td>4.7</td>
<td>4.6 ± 0.11</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>+ acetone in 25% soil</td>
<td>2</td>
<td>4.7</td>
<td>4.7</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td>mixed later in 75% soil</td>
<td>3</td>
<td>4.5</td>
<td>4.4</td>
<td>4.4</td>
</tr>
<tr>
<td>B</td>
<td>1% (v/w) diesel +</td>
<td>1</td>
<td>4.1</td>
<td>4.2 ± 0.12</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>acetone in 100% soil at once</td>
<td>2</td>
<td>4.3</td>
<td>4.2</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>mixed later in 75% soil at once</td>
<td>3</td>
<td>4.1</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>C</td>
<td>1% (v/w) diesel only</td>
<td>1</td>
<td>4.9</td>
<td>5.1 ± 0.28</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>in 25% soil and mix in 75% later</td>
<td>2</td>
<td>5.3</td>
<td>5.3</td>
<td>5.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>4.8</td>
<td>4.6</td>
<td></td>
</tr>
</tbody>
</table>

Same letters are not statistically different among treatments in each sampling method by the Tukey test (p < 0.005)
From Table 3.8 above, the analysis of variance (ANOVA) in bacterial count between the group means of the three different treatments on nutrient agar was $P = 0.028 < 0.05$ while that for R2A agar was $P = 0.013 < 0.05$. This indicates that there is a statistically significant difference in bacterial counts between the three treatments on both media with treatment C, in which diesel alone was added to 25% soil prior to the mixing in of the remaining 75%, yielding the highest bacterial count.

This was confirmed by the post hoc tests which revealed that there is no significant difference in the heterotrophic bacterial count between treatments A and C but the bacterial count between treatments B and C are significantly different from each other.

As seen earlier, treatment C yielded the highest TPH recovery in method 2 and the highest bacterial count in both nutrient agar and R2A agar. Thus, treatment C was adopted for use as the spiking protocol while method 2 was adopted for sampling in this study.

**3.8.4 Evaluation of media for heterotrophic bacterial enumeration in this study**

To further evaluate which media would support bacterial growth the most in this study, three types of bacteriological growth agar namely nutrient, R2A and malt agar were used to evaluate microbial growth in treatments.

**Method**

Another pilot experiment was conducted to compare the effect of the three methods of spiking diesel into soil, as described in Section 3.8.1, on microbial growth. Sampling was carried out using the method in Section 3.8.2b on one day treatments and 7 day treatments.

**Results**

Figures 3.1 and 3.2 below are graphs showing the bacterial counts from the treatments in the three different media after 24 hours and seven days respectively. The SPSS one way analysis of variance (ANOVA) test was used to determine the significant differences in the data.
Figure 3.1: CFUs for the enumeration of heterotrophic bacteria after 24hrs of incubation on nutrient agar (NA), Reasoners 2A agar (R2A) and malt agar (MA)

From the one day bacterial count results in Figure 3.1 above, the analysis of variance (ANOVA) test indicates that there is a significant difference in group means of CFUs between the different media ($P = 0.031 < 0.05$). The post hoc test reveals that the CFUs from the R2A media is significantly different from that of malt agar ($P = 0.028 < 0.05$).

However, there is no significant difference in bacterial counts among treatments ($P = 0.969 > 0.05$).
Figure 3.2: CFUs for the enumeration of heterotrophic bacteria after 7 days of incubation on nutrient agar (NA), Reasoners 2A agar (R2A) and malt agar (MA)

From the seven day bacterial count results in Figure 3.2 above, the analysis of variance (ANOVA) test also indicates that there is a significant difference in group means between the CFUs in different media (P = 0.002 < 0.05). The post hoc test reveals that the bacterial count from the R2A media is significantly different from that of nutrient agar (P = 0.027 < 0.05) and malt agar (P = 0.002 < 0.05). However, there is no significant difference in bacterial counts between nutrient agar and malt agar. There is also no significant difference in bacterial counts among treatments (P = 0.919 > 0.05).

The results indicate that there is no significant effect of sample type on bacterial count for overnight and seven day old cultures. However, there is significant effect of media on bacterial count for both overnight and seven day old cultures particularly in R2A Agar.

R2A Agar, though developed by Reasoner and Geldrich for bacterial counts from potable water, is known to be nutritionally rich and able to support the growth of various bacteria (URL 9) and will be used in this study. The formulation of the media is as shown in 3.5.2.
3.8.5 Evaluation of Universal PCR primers targeting the 16S rRNA gene

The 515F and 1391R universal primers, which target the V4 variable region of the 16S rRNA gene have been successfully used to amplify bacterial, archaeal and eukaryotic genes (Wright et al, 2013; Robertson et al, 2009).

To justify the use of these primers for the amplification and subsequent identification of bacterial species in this study, a polymerase chain reaction (PCR) was performed by amplifying the 16S rRNA loci of bacterial DNA isolated from an initial bioremediation experiment of 1% diesel contaminated soil using brewery spent grain and plated on both oil and R2A agar.

The bacterial DNA was amplified using a domain specific universal forward primer 515F (5'- GTGCCAGCMGCGGCGGTAA -3’) and a universal reverse primer 1391R (5'-GACGGCGGTGCGGTGCA-3’).

The amplification reaction was performed in a total volume of 25 µl for each sample containing 8 µl PCR water, 1.25 µl each of forward and reverse primers totalling 2.5 µl, 12.5 µl of Immomix enzyme (BIOLINE Reagents Ltd, UK) and 2 µl of DNA. The amplification program was performed with initial denaturation step at 95°C for 10 min; followed by 40 cycles of 1 min denaturation step at 91°C, 45 s annealing step at 55°C and 30 s elongation step at 72°C; and a final extension step at 72°C for 10 min.

The sequences obtained from the PCR products were used to identify the bacterial species by comparing them with known bacterial sequences using the Basic Local Alignment Search Tool (BLAST) algorithm. Table 3.9 presents the sequences obtained following PCR of bacterial isolates and the bacterial species identified.
In another experiment, DNA was extracted in triplicates from pure cultures of *Salmonella*, *E. coli* and *Pseudomonas* ssp., using the DNeasy blood and tissue kit (Qiagen, UK). The eubacterial universal primers 515F and 1391R were used for amplification and the PCR products were run on a gel as

<table>
<thead>
<tr>
<th>S/N</th>
<th>AGAR PLATE USED</th>
<th>SEQUENCE</th>
<th>SPECIES (sp.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Oil 1</td>
<td>ACTAGCGATTCTCCAGCCTTGGCAGATGGTGACCTGCTGGCGACTCAGTGCAAGGTTTATGGGATTAGCTCCACCTCGCGGCTTGGCAACCCTTTGTACCGACCATTGTAGCACGTGTGTAGCCCTGGCCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCCTTAGAGTGCCCACCCGAGGTGCTGGTAACTAAGGACAAGGGTTGCGCTCGTTACGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTCTGAGTTCCCGAAGGCACCAATCCATCTCTGGAAAGTTCTCAGCATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCGACTTATCGCGTTAGCTGCGCCACTAAGATCTCAAGGATCCCAACGGCTAGTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTCAGTGTCAGTATCAGTCCAGGTGGTCGCCTTCGCCACTGGTGTTCCTTCCTATATCTACGCATTTCACCGCTACACAGGAAATTCCACCACCCTCTACCGTACTCTAGCTCAGTAGTTTGGATGCAGTTCCCAGGTTGAGCCCGGGGATTTCACATCCAACCTTGCTGAACCACCTACGCGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTTCGTATTACCGCGGCTGGCTGGCA</td>
<td><em>Pseudomonas</em> ssp.</td>
</tr>
<tr>
<td>2</td>
<td>Oil 2</td>
<td>CCAAAAGAGGAGGTCGTCGGCCGCACATCTGACATCTTGGATCTCGGACTTCCTGGCGACTGGTTCCGGCGGGATGCCGCTGCGCAGTCTGCAAGGTTTATGGGATTAGCTCCACCTCGCGGCTTGGCAACCCTTTGTACCGACCATTGTAGCACGTGTGTAGCCCTGGCCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCCTTAGAGTGCCCACCCGAGGTGCTGGTAACTAAGGACAAGGGTTGCGCTCGTTACGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTCTGAGTTCCCGAAGGCACCAATCCATCTCTGGAAAGTTCTCAGCATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCGACTTATCGCGTTAGCTGCGCCACTAAGATCTCAAGGATCCCAACGGCTAGTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTCAGTGTCAGTATCAGTCCAGGTGGTCGCCTTCGCCACTGGTGTTCCTTCCTATATCTACGCATTTCACCGCTACACAGGAAATTCCACCACCCTCTACCGTACTCTAGCTCAGTAGTTTGGATGCAGTTCCCAGGTTGAGCCCGGGGATTTCACATCCAACCTTGCTGAACCACCTACGCGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTTCGTATTACCGCGGCTGGCTGGCA</td>
<td><em>Pseudomonas</em> ssp.</td>
</tr>
<tr>
<td>3</td>
<td>R2A 1</td>
<td>TGATCGCGATTCTAGCGATTCCGCTTCCGCAGTCGAGTTGCAGACTGCGACCCCAGAACGATTTGTGGGATTGGCTTAACCTCGCGGTTTCGCTGCCCTTTGTTCTGTCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCCTTAGAGTGCCCACCCGAGGTGCTGGTAACTAAGGACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACTCTGCCCCCGAAGGGGAACGTCCTATCTCTAGGATTGTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACTAAAGGGGCGGAAACCCCCTAACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGCCTCAGCGTCAGTTACAGACCAGAGAGTCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGCATTTCACCGCTACACGTGGAAATTCCACTCCTCCTCTTCTGCACTCAAGTTCCCCAGTTTCCAATGACCCCTCCCCCGGTTGAGCCGGGGGGCTTTCACTACAGACTTTAAGAAACCGCCCTGCGAAGCCCTTTTACGCCCAAATAAATTCCGGGACAACGCTTAGACCTAATTTACCAGCGGCGGCTGCGGGCCACAA</td>
<td><em>Bacillus</em> ssp.</td>
</tr>
<tr>
<td>4</td>
<td>R2A 2</td>
<td>GCCTCCCAAGAAGGACGCTCGGCTTGATTCACCCGGCGAGCTAGTACCTGATCCCGGATGATCTGCCTTGGGCGCGGCCGTACCCCGGATGTCGTCGTCGTTGGGCTTGGGCTTGGGCTTGGGCTTCCGGTGGGCGGTTACCCCAACTACCACTACGAGCTGACGAGCGACTGACGAGAGGAGGCCCTGCGGCTGGGCTTGGGCTTGGGCTTGGGCTTCCGGTGGGCGGTTACCCCAACTACCACTACGAGCTGACGAGCGACTGACGAGAGGAGGCCCTGCGGCTGGGCTTGGGCTTGGGCTTGGGCTTCCGGTGGGCGGTTACCCCAACTACCACTACGAGCTGACGAGCGACTGACGAGAGGAGGCCCTGCGGCTGGGCTTGGGCTTGGGCTTGGGCTTCCGGTGGGCGGTTACCCCAACTACCACTACGAGCTGACGAGCGACTGACGAGAGGAGGCCCTGCGGCTGGGCTTGGGCTTGGGCTTGGGCTTCCGGTGGGCGGTTACCCCAACTACCACTACGAGCTGACGAGCGACTGACGAGAGGAGGCCCTGCGGCTGGGCTTGGGCTTGGGCTTGGGCTTCCGGTGGGCGGTTACCCCAACTAC</td>
<td><em>Bacillus</em> ssp.</td>
</tr>
</tbody>
</table>
described in Section 3.6.2b. Excised bands were extracted and sequenced for identification to verify if the results would match the original pure cultures. Figures 3.3 and 3.4 below shows the gel bands as seen under UV radiation in the gel imager.

Agarose gel electrophoresis of PCR amplification products for pure cultures of *Salmonella* and *E. coli* using universal eubacteria primers 515F and 1391R. First lane shows an external 50bp hyperladder to determine size of bands. Lane 2 is the same 50 bp hyperladder in the gel, lanes 2 to 7 are pure cultures of *Salmonella* while lanes 8 to 13 are pure cultures of *E. coli*. Single bands were detected for each sample showing specificity of primer pairs.

*Figure 3.3: Agarose gel electrophoresis of PCR products, using universal primers 515F and 1391R, showing single DNA bands of the 16S rDNA eubacterial gene for six samples each of *Salmonella* and *E. coli*, showing specificity of the primers.*

Agarose gel electrophoresis of PCR amplification products for pure cultures of *Pseudomonas spp.* using universal eubacteria primers 515F and 1391R. First lane shows an external 50bp hyperladder to determine size of bands. Lane 2 is the same 50 bp hyperladder in the gel, lanes 2 to 7 are blank while lanes 8 to 13 are pure cultures of *Pseudomonas spp.* Single bands were detected for each sample showing specificity of primer pairs.

*Figure 3.4: Agarose gel electrophoresis of PCR products, using universal primers, showing single DNA bands of the 16S rDNA eubacterial gene for six samples of *Pseudomonas spp.*, showing specificity of the primers.*
The sequences were identified using the Basic Local Alignment Search Tool (BLAST) and corresponded to the original cultures. Table 3.9 below shows the corresponding sequences.

Table 3.10 Sequences obtained for the identification of known bacterial species, following PCR amplification, to confirm suitability of primers

<table>
<thead>
<tr>
<th>Sample 1 Salmonella enterica</th>
<th>Sample 2 E.coli</th>
<th>Sample 3 Pseudomas aeruginosa</th>
</tr>
</thead>
<tbody>
<tr>
<td>NNNNNNNNNNNNNATCGGATTACTGGGGCTAAGCGGCACCGCGGCGGTCTGTCAAGTGGATGTAATCTCCGCGGCTCAACCTCGGGATGGAATTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAAACCTGGGAACTGCATTCGAAACTGGCAGGCTTGAGTCTTGTAGAGGGGTTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGTATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTTGGGATCCTTGAGATCTTAGTGGCGCAGCTAACGCGATAAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGCTGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCAGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTGTCCTTAGTTACCAGCACCTCGGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGTCGGTACAAAGGGTTGCCAAGCCGCGAGGTGGAGCATTAATCCCATAAAACCGATCGTANTCCGGATCGCANTCTGCAACTCGACTGCGTGAAGTCNNAATCGCTAGTAATCGTGAATCAAGATGTCACGGTGAATACNTTCCCGGGCCTTGCACTCACCCGCCCGNNNN</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

These results show that the primers targeted the 16S rDNA gene and can be used for the amplification and subsequent identification of bacterial genera.
3.8.6 Use of Enterobacteria repetitive stain (ERIC) polymerase chain reaction (PCR) for preliminary microbial profiling

ERIC sequences are 127 bp palindromes, reading the same in either direction. They have been used to analyse a broad range of species belonging to the group Enterobacteriaceae and Vibrionaceae (Wilson and Sharp 2006). They have also been used widely for the differenciation and identification of *E.coli* and other bacterial strains (Lang *et al.*, 2013).

ERIC PCR fingerprints have been used to discriminate between species (Laciar *et al.*, 2006) hence its consideration in this study to be used as a preliminary screening tool to profile changes in trend of the microbial community during bioremediation represented by changes in intensity and band size of amplified DNA fragments.

It has been used to differentiate the DNA from various bacteria, bacteriophages, vertebrates, invertebrates, fungi and plants (Gillings and Holley, 1997). It has also been used to detect phenol degrading bacterial strains in an activated sludge microbial community (Wang *et al.*, 2009).

To justify the use of these primers for the amplification and fingerprinting of bacterial species present in bioremediation treatments, a microcosm soil bioremediation experiment was set up in triplicate in which 250 g soil was mixed with 1% diesel (v/w) alone and left overnight. The remaining 750 g soil was then added gradually and mixed using a stainless steel spoon. The treatments were as follows:

1. Soil alone control (S)
2. Soil + Grain alone (SG)
3. Soil + Grain + Diesel (SGD)

Sampling was carried out daily using the method in Section 3.8.2b. Total community genomic DNA was extracted from samples using the using the E.Z.N.A soil DNA kit (Omega Bio-Tek, Inc., Norcross, GA, USA) according to the manufacturer’s instructions (Dineen *et al.*, 2010). This was also followed by DNA measurement and gene amplification using Enterobacterial Repetitive Intergenic Consensus (ERIC) primers (Alessandro *et al.*, 2011).
The daily profiles of DNA fragments in the treatments were assessed to determine changes in trend to inform sampling times. A total volume of 25 µl for each sample containing 8 µl water, 1.25 µl each of forward primer ERIC 1 (5’ ATGTAAGCTCCTGGGGATTCA 3’) and reverse primer ERIC 2 (5’ AAGTAAGTGACTGGGGTGAGC 3’) totalling 2.5 µl, 12.5 µl of Immomix enzyme (BIOLINE Reagents Ltd, UK) and 2 µl of DNA template was used for the amplification reaction.

The amplification program was performed with initial denaturation step at 90°C for 7 min; followed by 30 cycles of 30 s denaturation step at 90°C, 1 min annealing step at 58°C and 8 min elongation step at 65°C; and a final extension step at 68°C for 16 min. The products were then run concurrently with a 50 base pair (50 bp) Bioline hyperladder on a 2% agarose gel, to view bands.

The hyperladder is a set of regularly spaced DNA with known sizes, also called standards, and serves as a molecular weight marker to determine the approximate size of DNA fragments since the molecular weight of DNA is inversely proportional to the migration rate through a gel. Thus, lower molecular weight fragments will migrate faster down the gel. The 50bp hyperladder, which was used, can detect double stranded DNA fragments from 50 base pairs to 2000 base pairs.

Figure 3.5 shows the DNA bands in treatments on days 0, 1 and 12 including the control samples, following gel electrophoresis.
ERIC PCR products from different treatments over time to profile community changes evidenced by variations in band intensity and numbers depicting changes in abundance of bacterial strains over time. Lane 1: 50bp hyperladder, Lane 2: blank, Lanes 3 – 4: duplicate day 0 soil + grain + diesel samples, lanes 5 – 6: duplicate day 1 soil + grain samples, lanes 7 – 8: duplicate day 12 soil + grain samples, Lanes 9 – 10: duplicate day 12 soil + grain + diesel samples, lane 11: blank, lanes 12 – 13: duplicate no template control.

Figure 3.5: Agarose gel electrophoresis of PCR products amplified using ERIC primers for preliminary differentiation and detection of community changes in treatments soil + grain (SG) and soil + grain + diesel (SGD) based on band prominence and abundance.

The results, as seen in Figure 3.5, showed an increase in microbial communities abundance, represented by increased intensity in band sizes around day 12. Also, an increase in prominent band numbers was observed on day 12 indicating increased microbial diversity.

ERIC profiles thus confirm that the communities are changing with signs of definite changes occurring on day 12 so sampling will be carried out to include day 12. This is just a preliminary investigation as the community profiling for this study will be evaluated using next generation sequencing.
3.8.7 Evaluation of primers used in qPCR to evaluate catabolic genes.

The three primer pairs targeting the alkB, catA and xylE catabolic genes in contaminated soil were evaluated using total community DNA to confirm their target sequences and ensure the right genes were amplified based on the expected product size in base pairs and their sequences. The expected product sizes for each of the genes are as follows: alkB should be approximately 100bp (Powell et al., 2006), catA approximately 470bp (Sei et al., 1999, Shahsavari et al., 2016) and xylE approximately 380bp (Azhari et al., 2010, Shahsavari et al., 2016).

Real time polymerase chain reactions (qPCR) were run according to the method described in Section 3.6.3a and the product sizes for each gene was determined and excised to confirm sequences. These PCR products were used as positive control for the standard curve. The melt curves were viewed to detect the amplification of any unspecific products and primer dimers and the standard curves were checked to ensure linearity and PCR efficiency. A standard curve with r2 >0.95 was considered good.

PCR efficiency was determined by the slope of the standard curve and a slope of -3.322 indicates that the the amount of PCR product is doubled in each cycle resulting in a PCR efficiency of 1 or 100%. Thus, a PCR efficiency between 0.9 –1.1 was considered good.

3.8.7a AlkB gene detection

Figure 3.6 shows the gel image of the alkB gene following qPCR to determine product size.
The 50 base pair lader shows the \textit{alkB} gene at approximately 100 bp; which corresponds to the expected product size. \textit{AlkB} gene primers were used in qPCR amplification to detect the gene from soil bioremediation samples. Gel bands were excised, extracted and used as positive controls for standard curve and quantification of the gene. Lane 1 is the ladder, lane 2 is blank and lanes 3 to 11 are PCR products of the \textit{alkB} gene from treatments.

\textit{Figure 3.6: Agarose gel electrophoresis of the \textit{alkB} qPCR product showing the size of the 100bp gene fragment}

When compared to the ladder in lane 1 of the gel image, the gene was about 100 bp which corresponded to the expected product size. However, it was excised and sequenced to further confirm its similarity to the \textit{alkB} gene. When compared to the gene bank data using CHROMAS, there was an 83% similarity to the uncultured bacterion clone SL18 alkane monoxygenase (\textit{alkB}) gene.partial cds using CHROMAS.

The purified product was then used for a standard curve and Figure 3.7 below shows the melt curve of the standards following the qPCR assay while Figure 3.8 shows the standard curve.

\textit{Figure 3.7: Melt curve of the \textit{alkB} amplicons during qPCR all having the same peak and no primer dimers}
The melt curve shows just one peak corresponding to about 87 degrees with no primer dimers indicating the purity and specificity of the PCR product.

*Figure 3.8: Standard curve generated from the alkB gene for copy number quantification*

The standard curve for the alk gene in Figure 3.8 shows a PCR efficiency of 1.0, slope of -3.37 and $R^2$ of 1.0 which is acceptable.

**3.8.7b CatA gene detection**

Figures 3.9 below shows the gel image of the catA gene following qPCR to determine product size.
The 50 base pair lader shows the catA gene at approximately 470 bp; which corresponds to the expected product size. CatA gene primers were used in qPCR amplification to detect the gene in the soil bioremediation treatments. Gel bands were excised, extracted and used as positive controls for standard curve and quantification of the gene in treatments. Lane 1 is the hyperladder and lanes 2 to 7 are PCR products of the catA gene from treatments.

*Figure 3.9: Agarose gel electrophoresis of the catA qPCR product showing the size of the 470 bp gene fragment*

When compared to the ladder in lane 1 of the gel image, the gene was about 470 bp and corresponded to the expected product size. However, it was excised and sequenced to further confirm its similarity to the catA gene.

When compared to the gene bank data using BLAST, there was a 90% similarity to the Pseudomonas sp. 6978 clone catechol 1, 2 dioxygenase (catA) partial cds gene and also a 60% similarity to the uncultured bacterium C120 gene for catechol 1, 2 dioxygenase partial cds clone.

The purified product was then used for a standard curve and Figure 3.10 below shows the melt curve of the standards following the qPCR assay while Figure 3.11 shows the standard curve.
**Figure 3.10:** Melt curve of the catA amplicons during qPCR all having the same peak and no primer dimers

The melt curve shows just one peak corresponding to about 90 degrees with no primer dimers indicating the purity and specificity of the PCR product.

<table>
<thead>
<tr>
<th>Standard Curve (1)</th>
<th>conc = 10^{(-0.297*CT + 10.750)}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard Curve (2)</td>
<td>CT = -3.369*log(conc) + 36.213</td>
</tr>
<tr>
<td>Reaction efficiency (*)</td>
<td>0.98082 (* = 10^{(-1/m) - 1})</td>
</tr>
<tr>
<td>M</td>
<td>-3.36877</td>
</tr>
<tr>
<td>B</td>
<td>36.21304</td>
</tr>
<tr>
<td>R Value</td>
<td>0.99969</td>
</tr>
<tr>
<td>R^2 Value</td>
<td>0.99938</td>
</tr>
</tbody>
</table>

**Figure 3.11:** Standard curve generated from the catA gene for copy number quantification

The standard curve for the alk gene in Figure 3.8 shows a PCR efficiency of 1.0, slope of -3.37 and R^2 of 1.0 which is acceptable.
3.8.7c XylE gene detection

Figures 3.12 below show the gel image of the xylE gene following qPCR to determine product size.

The 50 base pair ladder shows the xylE gene at approximately 380 bp; which corresponds to the expected product size. XylE gene primers were used in qPCR amplification to detect the gene from soil bioremediation treatments. Gel bands were excised, extracted and used as positive controls for standard curve and quantification of the gene in treatments. Lane 1 is the hyperladder, lane 2 is blank and lanes 3 to 14 are the xylE PCR products from samples.

FIGURE 3.12: Agarose gel electrophoresis of the xylE qPCR product showing the size of the 380bp gene fragment.

When compared to the ladder in lane 1 of the gel image, the gene was about 380 bp which corresponds to the expected product size. However, it was excised and sequenced to further confirm its similarity to the catA gene.

When compared to the gene bank data using BLAST, there was an 85% similarity to the uncultured bacterium C230 gene for catechol 2, 3 dioxygenase partial cds clone R2-375-91b with accession number AB195034.1 and an 80% similarity to the uncultured bacterium C230 gene for catechol 2, 3 dioxygenase partial cds clone R2-375-2c with accession number AB197265.1.02.
The purified product was then used for a standard curve and Figure 3.13 below shows the melt curve of the standards following the qPCR assay while Figure 3.14 shows the standard curve.

**Figure 3.13:** Melt curve for the xylE amplicons during qPCR all having the same peak and no primer dimers

The melt curve shows just one peak corresponding to about 86 degrees with no primer dimers indicating the purity and specificity of the PCR product.
Figure 3.14: Standard curve generated from the xylE gene for copy number quantification

The standard curve for the alk gene in Figure 3.14 shows a PCR efficiency of 1.0, slope of -3.39 and $R^2$ of 1.0 which is acceptable.
Chapter Four

THE USE OF BREWERY SPENT GRAIN TO STIMULATE BIODEGRADATION

4.1 Introduction

The aim of this chapter is to investigate the bioremediation potential of brewery spent grain on a laboratory spiked diesel contaminated soil. Sterile control treatments were included in this experiment to confirm the biotic loss of hydrocarbons. The bioremediation potential of brewery spent grain on diesel contaminated soil in this experiment, was evaluated based on the percentage reduction in total petroleum hydrocarbons (TPH), percentage reduction in aliphatic TPH fractions and culture based enumeration of heterotrophic and hydrocarbon utilising bacterial population. The next sections cover a review of diesel contamination and evaluation in soil, use of brewery spent grain (BSG) in bioremediation, a brief description of the methods used, and the results obtained following this experiment.

4.2 Diesel contamination and evaluation in soil

Diesel fuel is a complex hydrocarbon mixture which is toxic to plants and soil microbes. Diesel range hydrocarbons comprise of C10 – C28 compounds and it comprises of about 80 – 90% aliphatic hydrocarbons (alkanes) and 10 – 20% aromatic hydrocarbons (Serrano et al., 2009). The volatile aromatic hydrocarbon fractions, when released into the atmosphere, act as precursors for ozone formation while the aliphatic component is responsible for the significant deterioration in soil properties and also initiates microbial population changes in favour of hydrocarbonoclastic bacteria. (Serrano et al., 2006).

Thus, the evaluation of microbial populations is essential to ascertain and monitor bioremediation and the presence of an adequate and active population of hydrocarbon degraders is key to the process (Wolicka et al., 2009). Accordingly, the first steps to bioremediation monitoring requires using
chemical analysis for pollutant evaluation and culture dependent enumeration of the associated microbial population (Atlas and Philip, 2005).

Despite the bias that only a limited bacterial species are culturable in the laboratory (Malik et al., 2008), culture techniques can reveal the presence of important hydrocarbon degrading microbes and establish their propensity to degrade pollutants (Atlas and Phillip, 2005). Culture dependent techniques have been successfully exploited in the assessment and isolation of microbial populations during hydrocarbon bioaugmentation and biostimulation studies (Margesin et al., 2003; Bento et al., 2005; Ros et al., 2010; Chikere et al., 2011; Dadrasnia and Agamuthu, 2013; Shahsavari et al., 2013).

In biostimulation, organic and inorganic nutrients can be added to the soil. Organic nutrients are added by amendment with organic waste or food by products while inorganic amendments are used to provide inorganic nutrients (Agamuthu et al., 2013). Although the use of inorganic amendments, such as nitrogen and NPK (nitrogen, phosphorus and potassium) fertilisers have been effectively demonstrated (Margesin et al., 2007; Chikere et al., 2012), organic waste has proved a cheaper and more environmentally friendly alternative that would reuse biowaste (Agary and Latinwo, 2015). Also, organic amendments are a source of microbial biomass and enzymes. Thus, organic waste and plant residues have been used in recent times for bioremediation (Shahsavari et al., 2013; Dadrasnia and Agamuthu, 2013).

4.3 Use of brewery spent grain (BSG) in bioremediation

Brewery spent grain is one such biowaste that has the potential of both biostimulation and bioaugmentation (Robertson et al., 2010). It has been demonstrated by Abioye et al. (2012) that BSG is effective in bioremediation. During their investigation using a Malaysian soil contaminated with a 5% (w/w) and 15% (w/w) used lubricating oil respectively, under laboratory conditions, biodegradation of 92% and 55% were achieved in 84 days after amending soil with 10% BSG.
Brewery waste effluents have also been evaluated in the bioremediation of a 10% (w/w) diesel contaminated soil, using bioreactors, and a 79% degradation was achieved after 28 days (Agarry and Latinwo, 2015). However, much of the literature on the use of brewery spent grain in bioremediation is in tropical climates (Abioye et al., 2012; Agarry and Latinwo, 2015; Ogugbue et al., 2017) and there is need to evaluate the potential of waste food by products such as brewery spent grain on the bioremediation of diesel contaminated soil in temperate climates.

The sustainability of using BSG for bioremediation has been evaluated in an earlier study at the University of Sunderland. Its use is further investigated in this study to determine its catabolic potential and associated microbial population. The aim of this study is therefore to investigate the bioremediation potential of brewery spent grain on a 1% (w/w) diesel laboratory contaminated soil, kept under natural atmospheric conditions, to simulate in situ biodegradation.

4.4 Methods

The bioremediation potential of brewery spent grain on diesel contaminated soil, in this experiment, was achieved by evaluating the percentage reduction in TPH, which is a measure of the total extractable petroleum hydrocarbons from a given environmental sample (URL 4), in treatments with and without brewery spent grain. It also assessed the heterotrophic and hydrocarbon degrading bacterial populations in the treatments during the biodegradation.

The treatments, composition of the microcosms and all methods used in this experiment are as stated in Section 3.3 of the Methods chapter. The biotic treatment S+G+D is the biostimulation treatment because of the addition of BSG while the biotic treatment S+D, without BSG, is the natural attenuation treatment.
4.4.1 Soil Sampling

Treatments were covered with a gortex cloth and incubated in the green house with temperature fluctuations of 15°C +/- 3°C. Composite samples for each treatment pot were obtained by collecting 5 g of soil from four corners and the centre of each pot, and mixing together, for analysis (Chagas Spinelli et al., 2012) on days 0, 2, 5, 7, 12, 14, 21, and 28.

4.4.2 Analytical and Microbial analysis

From each treatment, 10 g triplicate of composite samples were used for GC to evaluate TPH reduction, concentration of aliphatic hydrocarbon (C10 – C28) fractions, and the rate of biodegradation of the aliphatic hydrocarbon fractions in the treatments, as detailed in Section 3.4 of the Methods Chapter. For the colony forming unit (CFU) analysis to enumerate bacterial population, 10 g composite samples were used for soil suspensions and plated on R2A agar and oil agar plates respectively, as detailed in Section 3.5 of the Methods Chapter.

4.5 Results

4.5.1 Percentage TPH Reduction

Results of the percentage TPH reduction in treatments with and without BSG and their abiotic counterparts are shown in Figure 4.1 below. The soil alone treatment which served as control contained no hydrocarbons and so was not included in the TPH analysis.
Figure 4.1. Percentage reduction of TPH in treatments during bioremediation in soil samples with BSG (S+D+G), without BSG (S+D), their sterile counterparts with BSG (SS+D+SG) and without BSG (SS+D).

The reduction in TPH for the different treatments illustrated in Fig 4.1 were calculated using the initial TPH for day 0 as baseline and measured against subsequent reduction in TPH over time. The formula used is as stated in Section 3.4.3 of the Methods Chapter.

As shown in the two biotic treatments in Figure 4.1, a 92% reduction in TPH was observed after one week in the natural attenuation treatment (S+D) and a 96% reduction in the biostimulated treatment (S+D+G). The highest TPH reduction of 99% was observed in the biotic treatment with BSG on sampling day 28.

In addition, a rapid reduction in TPH of 78% and 84% was observed as early as day two in the natural attenuation treatment (S+D) and the biostimulated
treatment (S+D+G) respectively. However, the marked difference in TPH reduction between the abiotic controls and the biotic treatments from days 2 to 12 reveal that although the hydrocarbon appears to be disappearing fast, it is not totally as a result of evaporation or volatilization but rather due to biotic causes (Serrano et al., 2008).

However, a proliferation of bacteria was observed in the sterile controls which eventually had more bacterial growth than the biotic treatments and so could not serve as counterpart abiotic controls for comparison with the biotic treatments. Therefore, although the TPH reduction and bacterial count of the abiotic controls are reported, all comparison and statistical tests between treatments with and without brewery spent grain exclude the abiotic controls.

Increased bacterial population of sterilised soil has been reported in the literature (Mahmood et al., 2014) to support higher and more virulent microbial species (Ludwig and Henry, 1943). This is likely because other competitive species may have been destroyed and those present have developed and achieved greater resistance (Ibekwe et al., 2011).

4.5.2 Statistical analysis of percentage TPH reduction

An independent samples t-test of significance was used to determine significant differences in TPH total peak heights between the treatments with and without BSG. Significance was determined as $p < 0.05$.

No significant difference was observed in TPH total peak heights between the two abiotic treatments with and without BSG at the start of the experiment on day 0 ($P = 0.10 > 0.05$). This reveals that the diesel was homogeneously mixed in the treatments. Thereafter, clear and significant statistical differences in TPH total peak heights were evident between the treatments as from day 2 up till the end of the experiment.

This reveals that although both treatments with and without BSG exhibit a rapid reduction in TPH on day 2 which is due to biotic causes, the treatment with BSG still exhibits a significantly higher TPH reduction than the natural
attenuation treatment without BSG and contributes more to the degradation process. The table showing the t test results is shown in Appendix 1.

The percentage TPH loss in both treatments with and without BSG on day 2 were 77% and 84% respectively and by day 5 were 89% and 93%. The levels of TPH reduction of these two samples after day 7 was over 90% suggesting that majority of the degradation was complete by this time. This is similar to the results obtained by Agarry and Latinwo (2015) who, in their study, observed a high rate of 79% TPH reduction in a 10% (w/w) diesel contaminated soil, after 28 days, using brewery spent effluent.

The results indicate that the natural attenuation treatment without BSG may have an autochthonous hydrocarbon degrading microflora which is responsible for the rapid TPH reduction observed. The heterotrophic and hydrocarbon degrading bacterial population in both treatments were enumerated following an evaluation of the reduction in concentration and extent of removal of individual TPH fractions during the bioremediation experiment.

4.5.3 Reduction in concentration and removal extent of aliphatic TPH (C10 – C28) fractions, in treatments, over time

The percentage TPH reduction for the treatments with and without BSG, as indicated in the statistical results in Appendix 1, were significantly different from day 2. However, concentrations of the TPH aliphatic (C10 – C28) fractions for treatments with and without BSG, were further evaluated to determine their removal extent and which of these fractions contributed most to TPH reduction. The results would also indicate the aliphatic hydrocarbon reduction potential of BSG.

The concentration of aliphatic TPH (C10 – C28) fractions were quantified using the TPH standard Mix 1 (Sigma Aldrich, UK) by comparing the total peak areas for each fraction (analyte) in the standard to the corresponding total peak areas for the same fraction in the samples.
Table 4.1 shows the description of the analytes in the standard.

**Table 4.1:** Description of the aliphatic TPH (C10 – C28) analytes in the TPH Mix 1 standard (Sigma Aldrich, UK).

<table>
<thead>
<tr>
<th>S/N</th>
<th>Analyte names</th>
<th>No. of carbon atoms in analyte</th>
<th>Analytical Concentration (ug/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Decane</td>
<td>C10</td>
<td>1876</td>
</tr>
<tr>
<td>2</td>
<td>Docosane</td>
<td>C12</td>
<td>1898</td>
</tr>
<tr>
<td>3</td>
<td>Dodecane</td>
<td>C14</td>
<td>1878</td>
</tr>
<tr>
<td>4</td>
<td>Eicosane</td>
<td>C16</td>
<td>1902</td>
</tr>
<tr>
<td>5</td>
<td>Hexacosane</td>
<td>C18</td>
<td>1903</td>
</tr>
<tr>
<td>6</td>
<td>Hexadecane</td>
<td>C20</td>
<td>1886</td>
</tr>
<tr>
<td>7</td>
<td>Octacosane</td>
<td>C22</td>
<td>1918</td>
</tr>
<tr>
<td>8</td>
<td>Octadecane</td>
<td>C24</td>
<td>1900</td>
</tr>
<tr>
<td>9</td>
<td>Tetracosane</td>
<td>C26</td>
<td>1902</td>
</tr>
<tr>
<td>10</td>
<td>Tetradecane</td>
<td>C28</td>
<td>1884</td>
</tr>
</tbody>
</table>

The concentration of the aliphatic TPH (C10 – C28) fractions in the treatments with and without BSG, for each sampling time during the degradation, are represented in Figure 4.2 below.
Figure 4.2: Extent of removal of aliphatic TPH (C10 - C28) fractions, with and without BSG, over time, during diesel biodegradation.
The results from Figure 4.2 show that 100% of the C10 fraction was lost by day 12 in both treatments. However, this is the only hydrocarbon fraction that was completely removed in the unamended treatment without BSG (S+D). In the amended treatment with BSG (S+D+G), there was a complete loss of the C10, C12 and C28 fractions by the end of the experiment. Although the initial concentration of C12 was much higher than that of C28 in both treatments by day 0, C12 was the second fastest removed fraction, having been removed by 98% in treatment S+D and 100% in treatment S+D+G by day 21. Thus, the low molecular weight fractions were more readily removed than the higher molecular weight fractions (Karamalidis et al., 2010).

However, C28 was the third most removed fraction despite having a high molecular weight, resulting in its being less easily degraded and having a slower degradation rate (Karamalidis et al., 2010). It had a 90% removal in treatment S+D and a 100% removal in treatment S+D+G by day 21. This could, however, be a result of its very low concentration in the treatments which may have enabled a fast loss despite its slower degradation.

The C14 to C26 fractions, however, were persistent up to sampling day 21 although their concentrations reduced over time. In this study, the C10 and C12 fractions, which had the lowest molecular weight, were dissipated fastest during the remediation experiment and by day 2, all the fractions achieved considerable reduction. Thus, the rapid reduction in TPH observed in this study was contributed to by all the hydrocarbon fractions.

4.5.4 Statistical Analysis of aliphatic TPH (C10 – C28) fractions

The Mann Whitney U independent samples non parametric test was used to determine if there were any significances in the differences shown in Figure 4.2 between the TPH C10 – C28 fractions in treatments soil and diesel (S+D) and soil, diesel and grain (S+D+G) for each sampling time. Significance was determined as \( p < 0.05 \). The \( p \)-values of significance for the test results are as shown in Appendix 2.
The Mann Whitney U test results indicate that there was no statistically significant difference in concentrations of TPH C10 – C28 fractions between the treatments with and without BSG at the start of the experiment on day 0 (p = 0.09 > 0.05). However, statistically significant differences were observed as from day 2 (p= 0.02 > 0.05) up till the end of the experiment except on day 5, (p=0.11 >0.05). The statistical difference for days 12 and 21 were both p < 0.001.

4.5.5 Rate of biodegradation of aliphatic TPH (C10 – C28) fractions in treatments

First order kinetics was used to determine the rate of biodegradation of the aliphatic TPH (C10 – C28) fractions in the treatments by fitting the data for the TPH concentrations to the first order kinetics model: \[ C = C_0 e^{-kt} \] (Abioye et al., 2012) as detailed in Section 3.4.4 of the Methods Chapter, where \( C \) is the concentration of the TPH fractions (mg kg\(^{-1}\)) at time \( t \), \( C_0 \) is the initial concentration of the TPH fractions (mg kg\(^{-1}\)), \( t \) is time (day\(^{-1}\)) and \( k \) is the biodegradation rate constant (day\(^{-1}\)).

The biodegradation reaction rate constant \( k \) (day\(^{-1}\)) for the unamended treatment without BSG was 0.0590 day\(^{-1}\) while that of the biostimulated treatment with BSG was 0.1021 day\(^{-1}\). This result showed that the aliphatic hydrocarbons in the treatment with BSG were degraded faster than the unamended treatment without BSG. Also, the first order linear model \( R^2 \), which is the correlation coefficient of the data were 0.8699 and 0.9585 for the treatments without BSG and with BSG respectively.

The closer the correlation coefficient to 1, the greater the correlation of the data; thus, there was greater correlation of aliphatic hydrocarbon concentrations in the amended treatment than the unamended. The percentage degradation observed in the aliphatic TPH (C10 – C28) fractions was similar to that of the total TPH reduction shown in Section 4.5; having a 77% and 87% removal of aliphatic hydrocarbons, during the initial rapid degradation phase, in the treatments without BSG and with BSG respectively.
4.5.6 Bioremediation Efficiency (%)

The Bioremediation Efficiency (BE %) of the biostimulated treatment with brewery spent grain was calculated to determine its efficiency during the bioremediation experiment in comparison with the natural attenuation treatment without brewery spent grain.

The calculation was done following the example of Dussan and Numpaque, (2012) as follows: BE (%) = 100 – (As × 100 / Aac) where As = total area of peaks in the biostimulated sample with BSG (SGD) and Aac = total area of peaks in the natural attenuation sample without BSG (SD). The BE (%) of the experiment was calculated over time and the results represented in Table 4.2 below.

Table 4.2: Percentage Biodegradation efficiency between the treatments with and without BSG, over time during diesel bioremediation

<table>
<thead>
<tr>
<th>DAY</th>
<th>BE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>13.4</td>
</tr>
<tr>
<td>2</td>
<td>38.1</td>
</tr>
<tr>
<td>5</td>
<td>39.7</td>
</tr>
<tr>
<td>7</td>
<td>52.6</td>
</tr>
<tr>
<td>12</td>
<td>63.8</td>
</tr>
<tr>
<td>14</td>
<td>67.2</td>
</tr>
<tr>
<td>21</td>
<td>78.3</td>
</tr>
<tr>
<td>28</td>
<td>82.6</td>
</tr>
</tbody>
</table>

The results in Table 4.2 reveal that the treatment with BSG was more efficient in the bioremediation process from the start of the experiment and had a higher bioremediation potential of 13.4% compared to the natural attenuation treatment. Nevertheless, this increased bioremediation potential was not
statistically significant as indicated in the statistical test for difference in TPH total peak heights between treatments with and without BSG in Appendix 1 until day 2 when there was a 38.1 % increase in bioremediation potential.

However, the bioremediation potential of the amended treatment with BSG continued to increase throughout the experiment, with an increase of 82.6% by day 28, as compared to the natural attenuation treatment without BSG. This increase in bioremediation efficiency of the treatment with BSG may be due to its efficiency in degrading the more resistant hydrocarbons at the later stage of bioremediation when the degradation is no longer rapid but still ongoing (Ros et al., 2010).

4.5.7 CFUs for the enumeration of heterotrophic bacteria in treatments

Following microbial culture by the plate count method using R2A agar, the CFUs for the enumeration of heterotrophic bacteria in the treatments at each sampling time were determined. The results are expressed as log10 CFU g⁻¹ soil as shown in Figure 4.3.

Figure 4.3: Colony forming units (CFUs) for the enumeration of heterotrophic bacteria, in treatments with and without BSG, including their sterile counterparts, during diesel bioremediation.
The results in Fig 4.3 show variations in the bacterial population between the different treatment types. No growth was recorded on either of the abiotic sterilised treatments at day 0 although these samples did record growth from day 2 indicating a rapid regrowth and proliferation of bacteria. Interestingly, these samples showed higher microbial counts in subsequent days incubation than samples from all other treatments.

The absence of bacterial growth on day 0 indicates that the sterile controls served as a good abiotic control revealing that bioremediation was responsible for the observed rapid reduction in TPH at the beginning of the experiment. However, as stated in Section 4.5.1, the literature supports increased proliferation of bacterial population soon after sterilisation in autoclaved soil as compared to the unautoclaved soil (Ludwig and Henry, 1943; Mahmood et al., 2014).

### 4.5.8 Statistical analysis

An analysis of variance (ANOVA) and post hoc (LSD) test was used to determine possible significant differences in the CFUs among the treatment types. The sterile abiotic controls were excluded in this analysis because of the regrowth of bacteria observed in them. Significance was determined as $p < 0.05$.

The results, as detailed in Appendix 3, reveal that there were significant differences in the bacterial populations between the treatments with and without BSG from the start of the experiment up till the end. This difference is likely because the treatment amended with BSG supports some microflora that are not present in the soil and diesel treatment (Robertson et al., 2010). Similarly, the soil alone control treatment had significantly different heterotrophic CFUs from the amended treatment with BSG all through the experiment. However, CFUs in the soil only treatment did not differ from that of the unamended soil and diesel treatment at the start of the experiment but thereafter differed all through the experiment.
Analysis of the results indicate that the control soil samples without diesel or BSG remained constant throughout the incubation period. CFUs in biotic samples with only diesel added increased from day 2 and peaked at day 7 after which they continually decreased. However, CFUs in the biotic samples with diesel and BSG added, increased from day 2 and peaked at day 12 before gradually decreasing. Nevertheless, the heterotrophic bacterial CFU in the amended treatment with BSG remained significantly higher (p<0.01).

4.5.9 CFUs for enumeration of hydrocarbon utilising bacteria in treatments

Following microbial culture by the plate count method using oil agar, the CFUs for the enumeration of hydrocarbon utilising bacteria in the treatments at each sampling time were determined. The results are expressed as log10 CFU g⁻¹ soil as shown in Figure 4.4.

Figure 4.4: Colony forming units (CFUs) for the enumeration of hydrocarbon utilising bacteria, in treatments with and without BSG, including their sterile counterparts, during diesel bioremediation.
4.5.10 Statistical analysis

The results in Fig 4.4 show variations in the bacterial population between the different treatments especially the BSG amended treatment which shows much higher hydrocarbon utilising CFUs than all the other treatments. There was no bacterial growth from the sterile treatments on day 0 and no microbial growth from the soil only treatment on days 0 to 5. After this time, microbial growth that slowly declined was observed in the soil alone treatment. The CFUs observed in the soil only control reveal that the soil has got indigenous hydrocarbon degraders which began utilising diesel as their only carbon source after a lag phase of about one week.

An analysis of variance (ANOVA) and post hoc (LSD) tests were used to determine significant differences in CFUs among the treatment types. Significance was determined as $p < 0.05$. The detailed post hoc test results, shown in Appendix 4, show no LSD values on days 0 to 5 because only two variables; hydrocarbon utilising bacterial CFUs with and without BSG were assessed. On these days, the ANOVA significance values ($p=0.00<0.05$), reveal that there is a significant difference in hydrocarbon utilising CFUs between the two treatments with and without BSG.

However, since no growth was observed from the soil alone sample on days 0 to 5, an independent t-test was used to further confirm the significant differences in hydrocarbon degrading CFUs between the treatments with and without BSG (Appendix 5). Significance was determined as $p < 0.05$ and the test results revealed that there was a significant difference in CFUs between the two treatments ($p< 0.005$).

The CFUs in the treatment without BSG remained about the same through this period with only a 6% increase on day 2 and 12% increase on day 12 as compared to its CFU at the start of the experiment. Thereafter, the CFUs remained about the same until the end of the experiment. For the treatment with SDG however, there was a rapid increase in CFUs of 34% on day 2 as compared to the level at the start of the experiment.
Levels of hydrocarbon degrading microorganisms were clearly and significantly higher in biotic samples with diesel and BSG and a 37% increase was observed on day 2 as compared to the treatment without BSG. The CFUs remained high with a 43% increase as compared to the treatment without BSG on day 7. Thereafter, a gradual decrease in CFUs was observed until the end of the experiment by which time there was still a 12% increase in counts as compared to the unamended treatment.

The abiotic controls, though having no measurable CFUs at the start of the experiment, were observed to have a rapid increase in microbial growth by day 2. The levels of hydrocarbon degrading microorganisms increased to match those in the unamended soil and diesel only treatment by day 21.

This experiment determined the hydrocarbon degrading population changes numerically. However, the species composition is unknown, and it is difficult to determine if the population changes are based on variations in species diversity and or abundance. Species diversity refers to the different species that make up the community while species abundance refers to the number of a particular specie in the population.

The use of metagenomics explored to determine the composition and abundance of microbial species in this experiment will be discussed in Chapter 6 of the thesis.

**4.5.11 Relationship between TPH concentration and bacterial CFUs**

The TPH C10 – C28 concentration, not for the individual fractions this time, but for the entire sample, based on the mean peak areas of all the replicates, was quantified over time for the biotic treatments with and without BSG. The TPH Mix 1 aliphatic hydrocarbon standard from Sigma Aldrich (UK) was used for the quantification.

The TPH concentration for each of the biotic treatments, (S+D and S+D+G), on the different sampling days were compared to the corresponding hydrocarbon degrading CFUs to verify the relationship between the
hydrocarbon degrading bacterial population and hydrocarbon concentration following the example of Suja et al. (2014) in Figure 4.5.

Figure 4.5: Relationship between aliphatic TPH concentration and hydrocarbon degrading CFU

Fig. 4.5 above shows the relationship between the hydrocarbon utilising CFUs and the TPH concentration of the biotic treatments with and without BSG (S+D and S+D+G) over time. The treatment with BSG, (S+D+G) is seen to have higher hydrocarbon degrading bacterial CFUs than treatment S+D, which does not have BSG. The hydrocarbon utilising bacterial CFUs increased rapidly from the start of the experiment up till day 2 while the TPH concentration decreased rapidly from the start of the experiment up till day 2 showing a negative correlation between the two variables. Thereafter, there was a more gradual increase in bacterial CFUs and decrease in TPH concentration up till day 12.
Thus, the bacterial population in both treatments peaked at day 12, having CFUs of 3.6 log$_{10}$ g$^{-1}$ and 4.8 log$_{10}$ g$^{-1}$ for treatments S+D and S+D+G respectively. They started to decline thereafter possibly due to a depletion of the carbon source since the TPH concentration had already reduced significantly to 611 mg kg$^{-1}$ in treatment S+D+G and 1764 mg kg$^{-1}$ in treatment S+D as compared to their concentrations at the start of the experiment.

After day 12 however, the bacterial CFUs in the treatment S+D remained constant while that in treatment S+D+G decreased rapidly though it was still 20% higher than treatment S+D by the end of the experiment. The result shows a negative correlation up till day 12 for both treatments. Although the hydrocarbon degrading bacterial growth in treatment S+D+G rapidly declined after the peak on day 12, its bacterial CFUs remained significantly higher than treatment S+D throughout the experiment.

A Pearson correlation between the TPH concentration and hydrocarbon degrading CFUs showed a negative correlation in both treatments indicating that as TPH concentration reduced, there was a corresponding increase in hydrocarbon degrading CFUs. However, there was a stronger association for the treatment amended with BSG (R = -0.926) than the unamended treatment without grain (R = -0.858). This result is similar to that of Suja et al. (2014) in which the petroleum hydrocarbon degradation correlated negatively with bacterial CFUs in the presence of a microbial consortium until the peak of microbial growth on days 14 and 28 in which time TPH reduction was significantly reduced.

The rate of biodegradation of the total aliphatic TPH (C10 – C28) fractions between the treatments with and without BSG, during the initial rapid and later slow degradation phases, were calculated using the equation in Section 4.5.5. The results show biodegradation rates of 0.7416 (S+D) and 1.0139 (S+D+G) for the rapid phase between days 0 and 2, followed by rates of 0.4152 (S+D) and 0.4858 (S+D+G) between days 2 and 5, 0.1921 (S+D) and 0.2803 (S+D+G) between days 5 and 12 and finally 0.1143 (S+D) and 0.1838 (S+D+G) between days 12 and 21.
As observed in Figure 4.5, the biodegradation rates were highest at the onset of the biodegradation for both treatments; but higher still, in the treatment with BSG. The biodegradation rate for the amended treatment with BSG remained higher than the unamended treatment over time, throughout the bioremediation process. However, the difference in rate of biodegradation between the two treatments was lowest between days 2 and 5.

Nevertheless, although the biodegradation rate for the unamended treatment during the rapid phase was lower than that of the amended treatment, it was still considerably high. The reason for this may be because the soil may have a catabolically active microflora that readily degrades hydrocarbons allowing for a high biodegradation rate, close to that of the amended treatment. Also, since the contamination of diesel in the soil is 1% (v/w), the pollutant concentration may not have been toxic to soil microbes; thus, allowing them to retain their catabolic potential. The intrinsic bacterial population of the soil will be evaluated and discussed in Chapter 6 of this thesis.

### 4.6 Discussion

The purpose of this section is to discuss the results obtained from the laboratory bioremediation experiments during which the percentage TPH reduction and the concentration of TPH aliphatic C10 – C28 fractions were evaluated. Also, the associated growth profile of both heterogeneous and hydrocarbon degrading bacterial populations, during the bioremediation process, were enumerated. These evaluations were carried out based on their usefulness for assessing and monitoring the decontamination of diesel in soil.

#### 4.6.1 Total Petroleum Hydrocarbon (TPH) reduction and bacterial CFUs

Microorganisms are the driving agents for TPH loss during bioremediation since they metabolise both aromatic and aliphatic hydrocarbons and utilise them for energy and biomass. For this reason, the TPH reduction in this study will be discussed in relation to bacterial population. Also, since hydrocarbon
utilising bacteria, rather than heterotrophic bacteria, are responsible for the breakdown and utilisation of hydrocarbons, the bacterial population discussed here will be the hydrocarbon degraders only. Although fungi are known to possess extracellular enzymes capable of degrading hydrocarbons, this study focussed on bacterial degradation.

### 4.6.1a TPH and bacterial population changes over time

The TPH reduction pattern observed in this study were of two phases; an initial rapid TPH loss followed by a gradual loss in TPH over time. This pattern was also observed in the study of Ros et al. (2010). However, since the experiment of Ros et al. (2010) involved an 8 month field degradation of aged hydrocarbon contaminated semi-arid soil, the duration of each phase occurred over a longer period.Nevertheless, the initial rapid phase is likely to be the period when the most labile hydrocarbons were degraded and the second slower phase, the period when the more resistant hydrocarbons were degraded (Ros et al., 2010).

Also, the rapid loss of hydrocarbons during the initial degradation stage could be attributed to the presence of mostly straight chain hydrocarbons which are easy to degrade. This may be the case in this study because, as revealed during the reduction in concentration and removal extent of the aliphatic hydrocarbon fractions, which were all alkanes, all of the fractions were degraded by more than half during the initial rapid phase. This pattern of degradation has also been observed in other studies including those of Bento et al. (2005) and Chikere et al. (2012) in which high rates of hydrocarbon degradation were observed in the first two weeks of incubation followed by slower rates of degradation.

Another factor that could be responsible for the rapid degradation, especially in both treatments with and without BSG is the presence of autochthonous hydrocarbon degraders in the soil from the start of the experiment, suggesting the presence of an active bacterial community that do not need to spend time adapting to the pollutant but can readily degrade it (Shahsavari et al, 2013).
However, the soil is pristine and was obtained from a known uncontaminated site and thus not expected to have hydrocarbon degrading microbes since it has not been exposed to petroleum hydrocarbons. Nevertheless, both aliphatic and aromatic hydrocarbons have been found in low concentrations in pristine soils likely originating from seepage from natural deposits and biosynthesis by plants and microbes (Atlas and Phillip 2005) confirming that hydrocarbon degrading microbes are ubiquitous (Margesin et al., 2013). Also, petrogenic hydrocarbons, being natural, are abundant even in pristine soils (Kingston, 2002).

Also, the microbial community in pristine soils have been observed to be well adapted to contaminants enabling a rapid degradation response following the introduction of contamination (Schwarz et al., 2018). Similarly, following culture dependent and molecular methods, alkane degradation bacteria and genes have been detected in pristine Arctic and Antarctic soils (Whyte et al., 2002) and also in pristine Alpine soils (Margesin et al., 2003). In their study, Okere et al. (2011) observed that mineralisation of 14C-phenantrrene occurred in an Antarctic soil with no history of pollution.

However, mineralisation increased with temperature and the highest extent of mineralisation was observed at 22°C. Nevertheless, cold adapted psychrotrophs (cold tolerant bacteria which have optimum temperatures of above 15°C) and psychrophiles (cold loving bacteria which have optimum temperatures of below 15°C) are widely distributed in nature and psychrotrophic bacteria are known to adapt rapidly and multiply following contamination in cold environments and thrive better than psychrophiles. A number of psychrotrophic hydrocarbon degraders of the genera Rhodococcus, Acinobacter, Pseudomonas and Sphingomonas have been isolated from contaminated Antarctic soils (Aislabie et al., 2006; Okere et al., 2012).

Thus, the very rapid degradation observed in this study right after the start of the experiment is not uncommon since adapted microbial communities have high populations of hydrocarbon degraders and can respond to the presence of hydrocarbons within hours (Atlas and Bertha, 1998; van Elsa et al., 2007). Adaptation, which arises from the exposure of microbes to hydrocarbons,
results in genetic changes in the microbial communities that allows for a selective enrichment and increase in hydrocarbon degrading populations (Quatrini et al., 2008).

Also, the rate of organic contaminants differs between classes of chemicals with aliphatic hydrocarbons degrading faster than aromatics. In their study, Stroud et al. (2007) observed a relatively short lag time and an immediate rapid degradation of hexadecane indicating that catabolism was inherent in the soil microbial community. This is similar to the degradation results obtained in this study.

4.6.1b TPH and bacterial population changes with and without BSG

The rapid degradation of hydrocarbons observed at the onset of the remediation process, though occurring in both treatments with and without BSG, was significantly higher in the treatment with BSG (P = 0.01). There was also a corresponding rapid increase in the hydrocarbon degrading bacterial population in the treatment with BSG while the TPH was degrading as opposed to the gradual increase in the unamended treatment. Thus, though both treatments may have supported autochthonous populations of hydrocarbon degraders, the hydrocarbon degrading population in the treatment with BSG proliferated quite rapidly and was significantly more abundant (P = <0.001) than that of the unamended treatment. The treatment with BSG, therefore, contributed more to the initial rapid loss of TPH.

Also, the negative correlation observed between TPH reduction and bacterial growth was higher for the amended treatment (R = -0.926) than the unamended treatment (R = -0.858); indicating that the greater bacterial population in the amended treatment with BSG resulted in a greater reduction in TPH. This result is not unexpected since hydrocarbon degrading bacteria are known to be directly responsible for metabolising the pollutant and thus reducing its concentration. Suja et al. (2014) and Adetutu et al. (2015) in their study, observed increased TPH reduction as hydrocarbon degrading population increased.
This rapid increase in bacterial population in the amended treatment may be the result of additional nutrients provided by BSG which may have stimulated the proliferation of autochthonous microbial populations (Agamuthu et al., 2013) as the experiment progressed since it is known that BSG is very rich in nutrients containing a lot of moisture and polysaccharides (Thomas and Rahman, 2006). The BSG used in this study comprised of barley and the typical barley BSG composition has been described to be 20 – 25% hemicellulose, 12 – 25% cellulose, 19 – 30% protein, 12 – 28 % lignin, 10% lipid and 2 – 5% ash (Lynch et al., 2016).

Bacteria can utilise the polysaccharide in cellulose and hemicellulose as sources of nutrition and the amino acids in protein can be directly incorporated as building blocks into bacterial cells or catalysed by the bacteria. Lignin, on the other hand, is useful for increasing humic acid concentration which promotes bacterial growth and BSG, when mixed with soil, promotes aeration (Shahsavari et al., 2013).

Also, BSG is known to have its own resident microflora (Roberston et al., 2010) some of which may be hydrocarbon degraders. These bacteria which are introduced along with their natural substrate, unlike in bioaugmentation, may thrive and enhance the remediation process since they are still autochthonous in the new environment, being in the BSG and may still be stimulated by the nutrients from BSG.

The introduction of BSG resulted in a significantly higher reduction in TPH in treatment SDG as compared to treatment SD as from day 2. This result is anticipated because no difference in TPH is expected between the treatments at the start of the experiment, so they can be comparable. However, since the degradation was very rapid and much of the hydrocarbons were removed by day 2, it is expected that the enhanced treatment should have as significantly higher hydrocarbon loss.

Also, the amended treatment with brewery spent grain had higher biodegradation rates and supported the disappearance of the C10, C12 and C28 fractions (Sections 4.5.3). It also had a significantly higher reduction in the extent of fractions C14 to C26 than the unamended hydrocarbon contaminated
soil. The reduction of TPH fractions in the amended treatment was also significantly greater than the unamended after the start of the experiment until the end of the experiment except on day 5 when the difference, though higher, was not statistically significant. The unamended contaminated soil however, only supported the disappearance of the C10 fraction as from day 12 of the bioremediation treatment. This result also supports the enhancement potential of brewery spent grain for bioremediation.

The result of this experiment is unlike that of Bento et al. (2005) in which natural attenuation was more effective than biostimulation in soil samples from a diesel contaminated field in Hong Kong, China. Nevertheless it is similar to other studies such as the study of Gallego et al. (2001) in which biostimulation with inorganic nitrogen and phosphorus resulted in the highest biodegradation rate of 90% after 45 days and the study of Shahsavari et al. (2013) in which an amendment with pea straw yielded a TPH reduction of 83% after 90 days as compared to the natural attenuation control which yielded a TPH reduction of 57%.

4.6.2. Potential of brewery spent grain amendment in the bioremediation of hydrocarbon contaminated soil

The increase in the hydrocarbon degrading CFUs following biostimulation with BSG is indicative of an enhancement in biodegradation and the bioremediation potential (Margesin et al., 2003; Shahsavari et al., 2013) of BSG. The amendment with BSG was able to significantly increase the microbial population of both heterotrophs and hydrocarbonoclastic bacteria all through the experiment with the later surpassing its non-amended treatment with up to a 35% increase after the start of the experiment up till day 12 when the bacterial population peaked.

Similarly increases in bioremediation potential have been observed in a number of studies utilising different organic and inorganic nutrients to enhance bioremediation (Abioye et al., 2012; Dadrasnia and Agamuthu, 2013; Silva-Castro et al., 2013; Hamzah et al., 2014). In this investigation of the
bioremediation enhancement potential of BSG, two prerequisites, as suggested by Bento et al., (2005) should to be met to establish the enhancement potential of BSG and inform its use for practical applications.

The first criterion, which is a confirmation of bioremediation has been met by the use of sterile controls. The second, which is its ability to surpass the natural attenuation in contaminant reduction, has also been met by the significantly lower concentrations of hydrocarbon fractions and the reduced percentage TPH observed in the amended treatment with BSG, as compared to the unamended contaminated soil treatment. This is also indicative of the enhanced bioremediation potential contributed by BSG. Thus both criteria have been met in this study.

Previous studies involving the use of brewery spent grain include the study of Abioye et al. (2012) in which 10% BSG was used to stimulate the bioremediation of a 5% and 10% laboratory contaminated used lubricating oil for 84 days. A 92% degradation and 55% degradation were observed respectively, for each contaminant concentration. However, in this study in which 10% BSG was used for the bioremediation of a 1% diesel contaminated soil, a 96% degradation was observed.

This is not unlikely because when mixed in soil, BSG being a lignocellulosic waste, improves soil quality; increasing soil aeration, humic acid concentration and providing nutrients for soil fertility thus accelerating bioremediation (Shahsavari et al., 2013). In addition to stimulating the bioremediation process with its nutritional content, BSG may also augment the process with its microflora of hydrocarbon degrading microbes.

However, concentration of pollutants negatively correlates with microbial activity such that higher pollutant concentrations will result in lower microbial activity (Adams et al., 2015; Abioye et al., 2012). Nevertheless, this may not mean that BSG is not able to stimulate a higher percentage degradation but the treatment with the higher concentration may require more time for complete degradation (Okoh, 2006).
Also, as compared to the 92% degradation in a tropical climate after 84 days in the study of Abioye et al. (2012), a degradation of 96% was achieved in this study after 28 days. Several factors could be responsible for this including the thick consistency of lubricating oil as compared to diesel and also that the soil composition used in this treatment was a sandy loam, which has more aeration than the clayey soil used by Abioye et al. (2012). Although it may be difficult to directly compare both results because of the completely different systems such as mix of TPH and temperature, they both show the potential of BSG in bioremediation.

BSG effluents have also been used in a previous study for the bioremediation of a 10% laboratory diesel contaminated soil in a tropical climate and a 79% degradation was observed after 28 days as compared to a 40% degradation observed in the unstimulated attenuation treatment (Agarry and Latinwo, 2015). Compared to the results of Abioye et al., (2012), this result may mean that degradation does not just depend on the concentration of the contaminant but also on the type of pollutant (Okoh, 2006).

The result of Agarry and Latinwo (2015) is comparable to that in this study where a 96% degradation was achieved in a 1% diesel contaminated soil in 28 days. This is because although the contaminant concentration was 10% and expected to have a much lower degradation rate, the degradation was in a tropical climate with higher temperatures that promote bioremediation. Thus, the use of BSG to augment bioremediation is consistent in increasing hydrocarbon degradation rate.

4.7 Conclusion

The results obtained from both microbial culture and the analytical methods for hydrocarbon reduction reveals that BSG supports the growth of both heterotrophic and hydrocarbon utilising bacteria thus yielding higher CFUs in the amended treatments than the unamended treatments. Statistical analysis used in this study also reveals that the use of BSG efficiently and significantly improved the bioremediation of diesel in this experiment.
Also, the amendment with BSG resulted in a greater bioremediation potential and reduction of hydrocarbon content. It supported a greater extent and higher rate of TPH biodegradation than the unamended treatment, throughout the process of bioremediation. However, the degradation rate was highest during the onset of degradation when the contaminant concentration was highest. Since bacteria are responsible for bioremediation and an active population of hydrocarbon degrading bacteria are essential for bioremediation to occur (Wolicka et al., 2009), the results are in line with the literature and confirmative of bioremediation.

BSG is known to contain a population of resident microflora (Robertson et al., 2010). Therefore, in addition to the high nutritional content of brewery spent grain which supports the bacterial growth of indigenous bacteria in the treatments, it is also likely to have its own autochthonous hydrocarbon degraders that augment the bioremediation process. Thus, BSG may contribute to both biostimulate and bioaugment the hydrocarbon contaminated soil.

Thus, this study has established that BSG does improve the breakdown of diesel in soil and also demonstrates that the pristine soil evaluated harbours autochthonous hydrocarbon degraders that can be biostimulated to enhance the bioremediation process by the addition of nutrients, aeration and moisture which may have been effectively supplemented by BSG. The use of BSG is thus inexpensive, simple, efficient, transforms pollutants to simple innocuous substances and is a means of reusing waste.

To further confirm the use of BSG to enhance diesel bioremediation in soil, the use of metagenomic analysis was employed in this study to profile the bacterial community composition of the various treatments and to establish the presence of known hydrocarbon degrading bacteria in BSG as stated in the literature (Robertson et al., 2010). This will be discussed in Chapter 6.

The next chapter, however, will evaluate the breakdown substrates present at different stages of the bioremediation process and determine how they fit into the known metabolic pathway for diesel remediation, to further establish the remediation potential of brewery spent grain.
Chapter Five

PATHWAYS AND METABOLITES OF DIESEL BIODEGRADATION

5.1 Introduction

The aim of this chapter is to further establish the bioremediation potential of BSG for the remediation of diesel contamination. This was achieved by identifying the compounds present in the treatments with and without BSG, at all stages, during the bioremediation process using gas chromatography mass spectrometry (GC-MS). Secondly, the elucidated metabolic pathways of both aliphatic and aromatic hydrocarbons were assessed to determine the associated catabolic products. These were compared with the degradation compounds identified in the treatments to determine where they fit in the pathway.

5.2 GC-MS and its applications in hydrocarbon biodegradation

GC-MS is useful in environmental monitoring for the detection and identification of organic pollutants in water and soil (Chauhan et al., 2014). Studies have utilised this technique in bioremediation to monitor the extent of degradation by determining the hydrocarbon compounds present during the process (Serrano et al., 2008; Sharma et al., 2009; Xu and Lu, 2010).

Yanto and Tachibana (2013) used GC-MS to successfully identify fifteen metabolites of aliphatic hydrocarbon degradation during their study on the bioremediation of petroleum hydrocarbons by Pestalotiopsis sp. NG007. Similarly, GC-MS was used to identify the metabolites produced during the degradation of polyaromatic hydrocarbons (PAHs) using a previously identified and isolated bacterium Cronobacter sakazakii MM045 (KT933253) (Umar et al., 2017).

The authors identified the organic acids pyruvic acid, acetic acid and lactic acid at the end of the degradation process of phenanthrene and pyrene suggesting the complete degradation of the PAHs by the bacterium.
5.3 Metabolic pathways for petroleum hydrocarbon degradation

The first step in the aerobic degradation of both aliphatic and aromatic hydrocarbons involves the introduction of molecular oxygen to the hydrocarbon and is catalysed by hydrocarbon oxygenases. For aromatic hydrocarbons however, the introduction of molecular oxygen to the benzene ring hydroxylates it into metabolites such as catechol or its derivatives that are substrates for ring cleavage enzymes (Otuka et al., 1998, Chikere et al., 2012, Varjani and Upasani, 2017).

The enzymes associated with the breakdown of hydrocarbons include:

- Monoxygenases which introduce one atom of oxygen into the hydrocarbon.
- Dioxygenases which introduce one molecule of oxygen into the hydrocarbon.
- Dehydrogenases which remove hydrogen atoms from the hydrocarbon.

The primary step in the aerobic degradation of alkanes is catalysed by alkane monoxygenase and involves the oxidation of a methyl group by the introduction of molecular oxygen to form an alcohol. The alcohol formed would be primary or secondary depending on the position of the methyl group oxidised by the enzyme. A primary alcohol is formed during terminal oxidation when the enzyme oxidises the terminal methyl group while a secondary alcohol is formed during subterminal oxidation when the enzyme oxidises an intermediate methyl group (Chikere et al., 2011; Varjani, 2017).

Figure 5.1 is a diagrammatic representation of the key metabolic breakdown products of aliphatic hydrocarbons represented in Figure 2.3 of Chapter 2 and adapted from van Elsas et al. (2007) and Varjani, (2017).
As seen in Figure 5.1 above, alkanes can be oxidised during catabolism to primary or secondary alcohols depending on the position of the methyl group initially attacked by the oxygenase enzyme.

Primary alcohols are further oxidised to aldehydes and fatty acids while secondary alcohols are further oxidised to ketones and esters.

Figure 5.2 is a diagrammatic representation of the key metabolic breakdown products in the breakdown pathway of aromatic hydrocarbons represented in Figure 2.5 of Chapter 2 adapted from van Elsas et al. (2007), Olajire and Essien (2014).
Figure 5.2: Key metabolites of aromatic hydrocarbon degradation pathways adapted from van Elsas et al. (2007), Olajire and Essien (2014).

Figure 5.2 above summarises the breakdown pathways for aromatic hydrocarbons. Aromatics are initially oxidised to catechol, also known as benzene-1, 2-diol. Following this, the benzene ring is cleaved in either of two pathways: firstly, via the ortho-cleavage pathway between carbons 1 and 2 and is catalysed by catechol-1, 2-dioxygenase enzymes to produce ketones and esters as metabolites.
Secondly, via the *meta*-cleavage pathway between carbons 2 and 3 and is catalysed by catechol-2, 3-dioxygenase enzymes to produce aldehydes and esters as metabolites (Chikere *et al.*, 2011; Olajire and Essien, 2014; Varjani and Upasani, 2017).

The distinguishing factor between both pathways is therefore the presence of either ketones or aldehydes. Thus, both aliphatic and aromatic hydrocarbons have aldehydes as the as the key distinguishing metabolites for the terminal oxidation pathway (aliphatics) and the *meta*-oxidation pathways (aromatics) respectively. Ketones on the other hand, are the key distinguishing metabolites for the subterminal oxidation pathway (aliphatics) and the *ortho*-oxidation pathways (aromatics) respectively.

The rest of the chapter will discuss the methods utilised in this study to investigate the compounds associated with the degradation of a 1% diesel contaminated soil with and without the addition of BSG.

### 5.4 Methods

The same bioremediation experiment carried out to investigate the bioremediation potential of BSG on laboratory contaminated diesel soil, discussed in Chapter 4 and fully explained in Chapter 3, is discussed here. GC-MS was used to identify the metabolites associated with diesel degradation all through the experiment and an Agilent Mass Profiler Professional software (Agilent Technologies, UK) was used to analyse the MS data by comparing the statistical differences of metabolites, based on their abundance ratios, between treatments with and without BSG.

#### 5.4.1 Soil preparation and experimental design

The methods for diesel spiking, soil treatment composition and soil sampling used in this experiment are as stated in Section 3.3 of the Methods Chapter. The triplicate biological treatments were covered with a gortex cloth and incubated in the green house with temperature fluctuations of 15°C ± 3°C.
Composite samples obtained by mixing 5 g of soil from the four corners and centre of each pot were analysed on days 0, 2, 5, 7, 12, 14, 21 and 28.

5.4.2 Analytical Methods

5.4.2.1 GC-MS analysis to identify compounds present in treatments

The methods for diesel extraction from the different treatments and chemical analysis by GC-MS are as stated in Section 3.4 of the Methods Chapter. At each sampling time, 10 g composite soil samples were used for diesel extraction and three technical replicates from each of the extracts were analysed using GC-MS, to identify the compounds present in the samples.

GC-MS analysis was performed using an Agilent GC-MS 7890A/5975C series (Agilent Technologies UK). The HP-5MS Agilent column with a 30 m length, (0.32 mm inner diameter and 0.25 film) was used. The injector temperature was set to 280°C and helium was used as the carrier gas at a constant flow rate of 0.5 ml min⁻¹. Each sample (1 μl) was injected in a splitless mode. The GC oven was programmed for a starting temperature of 50°C, held isothermal for one minute, increased at a rate of 20°C min⁻¹ to 120°C, then increased at a rate of 4°C min⁻¹ to 310°C, (Xu and Lu, 2010) and held isothermal for a further 5 minutes. The spectrometer was operated in electron-impact (EI) mode and the ionization energy was 70 eV.

5.4.2.2 Mass Profiler Professional software analysis to compare abundance of compounds, between treatments, over time

Following the GC-MS analysis to identify compounds present in the treatments over time, the Agilent G3835AA MassHunter Mass Profiler Professional Software (Agilent Technologies, UK) was used to analyse the MS data (identified compounds) and differentiate their abundance in the treatments with and without BSG, for each sampling time, to allow for comparison. To achieve this, the MS data (identified compounds) for each treatment and sampling time were inputted into the mass profiler. Thus, the abundance of each compound
in the treatments, over time, were determined and significantly different compounds between the treatments overall, were also determined.

5.5 Results

5.5.1 Compounds present in treatments with and without BSG over time

The compounds identified by the GC-MS in the different samples over time, were assessed to determine the presence of compounds that are metabolites in the elucidated breakdown pathway of diesel, as shown in the literature (Varjani et al., 2017). Compounds were differentiated based on their functional groups.

Table 5.1 is a representation of the compounds present in the different treatments with and without brewery spent grain over time based on functional group.
Table 5.1: Hydrocarbon degradation compounds present in treatments, over time during bioremediation, including diesel control

<table>
<thead>
<tr>
<th>HYDROCARBON TYPE</th>
<th>FUNCTIONAL GROUP</th>
<th>TYPE OF COMPOUND</th>
<th>TREATMENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>DAY 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SOIL AND DIESEL</td>
</tr>
<tr>
<td>ALIPHATIC HYDROCARBONS</td>
<td>C-H</td>
<td>STRAIGHT CHAINED</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td>−OH</td>
<td>ALCOHOLS</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td>−H−C=O</td>
<td>ALDEHYDES</td>
<td>x</td>
</tr>
<tr>
<td></td>
<td>−R−C=O</td>
<td>KETONES</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td>−OH−C=O</td>
<td>CARBOXYLIC ACIDS</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td>−O−C=O</td>
<td>FATTY ACID ESTER</td>
<td>✓</td>
</tr>
<tr>
<td>AROMATIC HYDROCARBONS</td>
<td></td>
<td>Benzene ringed</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td>−OH</td>
<td>CATECHOL (Phenol)</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td>−H−C=O</td>
<td>ALDEHYDES</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td>−R−C=O</td>
<td>KETONES</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td>−OH−C=O</td>
<td>CARBOXYLIC ACIDS</td>
<td>✓</td>
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<tr>
<td></td>
<td>−O−C=O</td>
<td>FATTY ACID ESTER</td>
<td>✓</td>
</tr>
</tbody>
</table>
The results from Table 5.1 show that for aliphatic hydrocarbon degradation, there were no aldehydes present all through the bioremediation process. Since aldehydes are an intermediate product of terminal oxidation as seen in Figure 5.1, the result indicates that the aliphatic hydrocarbons in diesel may have been broken down by the enzymatic oxidation of intermediate or subterminal methyl groups via the subterminal oxidation pathway into secondary alcohols, ketones and esters (Chikere et al., 2011). Ketones, which are intermediary products of the subterminal oxidation pathway, as shown in Figure 5.1, were observed in both treatments SD and SDG all through the degradation process thus further implying that the aliphatic hydrocarbons may have been degraded via the subterminal oxidation pathway.

Nevertheless, aliphatic carboxylic acids, which are another intermediate product of the terminal oxidation pathway were observed at the start of the process in both treatments but quickly disappeared in the treatment with BSG after the start of the experiment while it persisted in the treatment without BSG until after day 5. Although aliphatic carboxylic acids are produced by the enzymatic oxidation of aldehydes in the terminal oxidation pathway of aliphatic hydrocarbons (Varjani, 2017), and aldehydes were not found in any of the treatments all through the bioremediation, they may have been quickly oxidised to aliphatic fatty acids before being detected.

However, since carboxylic acids are oxidised via the $\beta$-oxidation pathway into the TCA cycle via acetyl CoA as shown in Figure 5.1, the disappearance of carboxylic acids in the treatment with S+D+G implies that the oxidation of the carboxylic acids to the TCA cycle occurred just after the start of the experiment in the treatment with BSG but persisted until after day 5 in the treatment without BSG. This is noteworthy and will be discussed later because most of the hydrocarbons in this study were degraded by day 2. For aromatic hydrocarbon degradation however, catechol which is the intermediary breakdown product as shown in Figure 5.2, is present in both treatments for each sampling time from day 0 up till day 21. This indicates that both treatments may have been able to metabolise aromatic hydrocarbons.
Also from Figure 5.2, aromatic ketones and aldehydes are the distinguishing compounds between the ortho-cleavage pathway catalysed by catechol-1, 2-dioxygenase and the meta-cleavage pathway catalysed by catechol-2, 3-dioxygenase, respectively (Fuchs et al., 2011). From Table 5.1 however, aromatic ketones were observed in both treatments with and without BSG all through the bioremediation indicating the activity of catechol-1, 2-dioxygenase. Aromatic aldehydes, however, were only present at the start of the experiment on days 0 and 5 in treatment S+D but present on days 5, 12 and 21 in treatment S+D+G.

Having identified the compounds present in the treatments with and without BSG over time based on their functional groups as shown in Table 5.1, the breakdown pattern of the individual GC-MS identified compounds were further evaluated by inputing the data into an Agilent MasHunter Mass Profiler software for analysis to determine the abundance of individual compounds, in the treatments with and without BSG, over time. Significantly different compounds between the two groups were also determined based on abundance. The functional groups of these compounds, when compared, matched that of the metabolites of the diesel degradation pathway as shown in Figures 5.1 and 5.2.

5.5.2 Abundance of compounds in treatments over time

Since diesel is a mixture of compounds and several compounds were detected, only the compounds which were shown to be significantly different between treatments soil and diesel (S+D) and soil, diesel and grain (S+D+G) using the Agilent MassHunter Mass Profiler software (Agilent Technologies, UK) are listed in Table 5.2 for comparison between the treatments. The abundance of each of these metabolites, over time, in the two treatments were profiled to determine their breakdown pattern in the treatments.

Also shown in Table 5.2 are the chemical characteristics of the metabolites such as chemical formula, molecular weight, type of compound and molecular structure.
Table 5.2: Significantly different compounds between treatments, with and without BSG, during diesel bioremediation.

<table>
<thead>
<tr>
<th>S/N</th>
<th>Name of metabolite</th>
<th>Chemical Formular</th>
<th>Molecular Weight g/mol</th>
<th>Type of Compound</th>
<th>Molecular Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1,2,4,5-tetramethylbenzene</td>
<td>C_{10}H_{14}</td>
<td>134.22</td>
<td>Aromatic HC</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1,6,7-trimethylnaphthalene</td>
<td>C_{13}H_{14}</td>
<td>170.25</td>
<td>Aromatic HC</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1-anthracen-9-yl-N-phenylmethanimine</td>
<td>C_{21}H_{15}N</td>
<td>281.35</td>
<td>Aromatic HC</td>
<td></td>
</tr>
</tbody>
</table>

**Aromatic Hydrocarbons**

| 4   | Eicosane                                               | C_{20}H_{42}      | 282.55                 | Alkane             |                     |
| 5   | 5-propyltridecane                                      | C_{16}H_{34}      | 226.44                 | Alkane             |                     |
| 6   | n-pentadecanol                                         | C_{15}H_{32}O     | 228.41                 | Alcohol            |                     |
| 7   | 2-ethyl-2-methyltridecan-1-ol                          | C_{16}H_{34}O     | 242.44                 | Alcohol            |                     |
| 8   | 1-dodecoxydodecane                                     | C_{24}H_{50}O     | 354.65                 | Methyl Ketone      |                     |
| 9   | Hexadecyl 2,2,2-trichloroacetate                       | C_{18}H_{33}Cl_{3}O_{2} | 387.81               | Ester              |                     |
| 10  | Methyl-octadeca-9,12-dienoate                          | C_{19}H_{34}O_{2}  | 294.47                 | Ester              |                     |
| 11  | Tridecyl octanoate                                     | C_{21}H_{42}O_{2}  | 326.57                 | Ester              |                     |
| 12  | Butyl tetradecane-6-sulfonate                          | C_{18}H_{38}O_{3}S | 334.56                 | Ester              |                     |
| 13  | Hexadecan-4-yl 2-methoxyacetate                        | C_{19}H_{38}O_{3}  | 314.51                 | Ester              |                     |
The results in Table 5.2 do not show any aromatic breakdown products. However, the compounds identified by GC-MS in the different samples over time indicate the presence of catechol, aromatic ketones and aldehydes as represented in Table 5.1. Nevertheless, since the compounds presented were based on significant differences in abundance between the treatments, the products of aromatic hydrocarbon metabolism may not have been significantly different.

The results in Table 5.2 also do not show any aliphatic carboxylic acids which were present in Table 5.1. However, both Tables 5.1 and 5.2 do not show aliphatic aldehydes. Thus, there is consistency in both results in that compounds which were not present in Table 5.1 are also not present in Table 5.2. However, some compounds present in Table 5.1 are not seen in Table 5.2 especially the aromatic aldehydes which show a major difference between treatments in Table 5.1. A reason for this may be the fact that some compounds may be transitory. Thus, some significant metabolites between the treatments may have been missed.

Nevertheless, the aliphatic metabolites listed in Table 5.2 are representative of alcohols, ketones and esters which are all metabolites of aliphatic subterminal oxidation and will be used for determining the breakdown profile of each compound between the treatments with and without BSG. The next section will graph the abundance and breakdown profile of the compounds listed in Table 5.2 (Figures 5.3 to 5.7) to determine the catabolic potential of the treatments. Figure 5.3 shows the breakdown pattern of the aromatic hydrocarbons listed in Table 5.2.
Figure 5.3: Breakdown pattern of aromatic hydrocarbon compounds which were significantly different between treatments over time during diesel bioremediation.

Figure 5.3 shows that the aromatic hydrocarbons were completely metabolised by day 2 of the experiment. It also shows that they were metabolised more in the treatment with BSG. This supports the observation in chapter 4 where there was a rapid reduction in percentage TPH by day 2.
and most of the degradation was accomplished. Thus, aromatic degradation may have contributed greatly to this early TPH loss.

Figure 5.4 shows the breakdown pattern of the aliphatic hydrocarbons listed in Table 5.2.

![Figure 5.4: Breakdown pattern of alkane compounds which were significantly different between treatments over time during diesel bioremediation.](image)

Figure 5.4 shows two different breakdown patterns for alkanes. Eicosane is a straight chained alkane and although having a molecular weight of 283 and 20 carbon atoms as shown in Table 5.1, is completely transformed by day 2 while 5-propyltridecane with a molecular weight of 220 and 16 carbon atoms persists all through the experiment in the treatment without BSG and up till day 14 in the treatment with BSG.
This agrees with the literature because although lower weight molecules are expected to breakdown more quickly than higher molecular weight molecules, single straight chained alkanes are more readily degraded than branched alkanes (Chandra et al., 2013, Varjani and Upasani, 2017). In this example also, the treatment with BSG seems to support a faster transformation of alkanes.

Figure 5.5 shows the breakdown pattern of the alcohols listed in Table 5.2.

Figure 5.5: Breakdown pattern of alcohols which were significantly different between treatments over time during diesel bioremediation.
As shown in Figure 5.5, there are also two degradation patterns for alcohols. Here, n-pentadecanol is present from the start of the bioremediation process and is rapidly and completely metabolised during the early phase of the experiment. However, it is metabolised faster in the treatment with BSG. It is an alcohol and therefore a by-product of metabolism indicating the activity of biodegradation very early in the process.

2-ethyl-2-methyltridecan-1-ol however, became abundant in the treatments later during the bioremediation. However, it was both synthesised and metabolised faster in the treatment with BSG.

Figure 5.6 shows the breakdown pattern of the ketone and esters listed in Table 5.2.
Figure 5.6: Breakdown pattern of ketone and esters which were significantly different between treatments, over time, during diesel bioremediation.
All three metabolites shown in Figure 5.6 are synthesised fastest in the treatment with BSG and degraded fastest in the same treatment.

However, the ester, methyl-octadeca-9,12-dienoate, is both synthesised and metabolised during the early degradation phase while 1-dodecoxydodecane (ketone) and tridecyl octanoate (ester) are synthesised later during the bioremediation process and persist all through the experiment in the treatment without BSG (S+D) but gets metabolised by day 21 in treatment SDG.

Figure 5.7 shows the breakdown pattern of other esters listed in Table 5.7.
Figure 5.7: Breakdown pattern of esters which were significantly different between treatments over time during diesel bioremediation.

The breakdown pattern of all three esters shown in Figure 5.7 are similar in that they are synthesised later during the biodegradation process and persist all through the experiment in the treatment without BSG (S+D) but gets metabolised by day 21 in treatment S+D+G.
5.6 Discussion

Metabolite identification and quantifying, which is the essence of metabolomics, is essential to better understand the biochemical and biological mechanisms in living systems. This is so because the extremely diverse metabolites in living organisms are a result of chemical transformations catalysed mostly by enzymes in response to nutrition and environmental factors (Vinaixa et al., 2016).

A combination of Mass Spectrometry (MS) based identification of metabolites and MassHunter Mass Profiler Professional (MPP) software based analysis of differences in metabolite abundance between treatment groups with and without BSG, were used in this study to understand the metabolomics of diesel bioremediation using BSG. Following the identification of the metabolites by GC-MS, the difference in abundance of these metabolites between the treatments, over time, were used to determine their breakdown patterns.

The purpose of this section is to discuss the results obtained from the compounds identified in the different treatments, over time during the experiment, to determine the presence of known hydrocarbon metabolites and thus confirm the activity of biodegradation. Also, the results obtained by comparing the difference in abundance and breakdown pattern of identifier metabolites in treatments with and without BSG, would be discussed to ascertain the potential of BSG in enhancing the bioremediation of diesel contaminated soil.

5.6.1 Metabolites and likely pathway for diesel bioremediation using BSG

The metabolites for aliphatic hydrocarbons identified in this study include alcohols, ketones, carboxylic acids and esters. These metabolites were identified in both treatments with and without brewery spent grain. Interestingly, these are the same as the signature metabolites identified for aliphatic hydrocarbon degradation in the elucidated hydrocarbon degradation
pathways (Chikere et al., 2011; Fuchs 2011; Varjani and Upasani 2017). This provides evidence that the aliphatic hydrocarbons in diesel were actively degraded by both treatments from the start of the experiment up till the end.

From the elucidated pathways, the breakdown sequence of aliphatic hydrocarbon degradation metabolites via the terminal oxidation pathways is from: primary alcohols → aldehydes → carboxylic acids while that via the subterminal oxidation pathway is from secondary alcohols → ketones → esters (Varjani, 2017). However, aliphatic aldehydes were not identified in either of the treatments indicating that the aliphatic hydrocarbons in the treatments may have been degraded only via the subterminal pathway.

Previous studies have revealed that the presence of metabolites of degradation can be reliable indicators of biodegradation (Agrawal and Gieg, 2013; Bento et al., 2015). However, it is also argued that the disadvantage of using the presence of signature metabolites to determine biodegradation is that sometimes the metabolites may be produced in quantities below analytical detection or their appearance, transitory (Agrawal and Gieg, 2013; Bento et al., 2015).

Thus, aliphatic hydrocarbons may also have been degraded via the terminal oxidation pathway in this study. This is likely because carboxylic acids were identified in the treatments and the degradation pathway reveals that carboxylic acids are oxidised from aldehydes. Thus, the proposed pathway for aliphatic hydrocarbon degradation in this study conforms to the elucidated pathway for the degradation in the literature as illustrated in Figure 5.1 as comprising of both terminal and subterminal oxidation.

The signature aromatic metabolites of concern for the treatments with and without BSG, were aromatic ketones and aromatic aldehydes. These metabolites distinguish between the ortho degradation pathway and the meta-degradation pathway with the aromatic ketones belonging to the ortho-degradation pathway catalysed by catechol-1,2-dioxygenase and the aromatic aldehydes belonging to the meta-degradation pathway catalysed by catechol-2, 3-dioxygenase.
The results obtained indicate that both treatments had aromatic ketones all through the experiment while the treatment with BSG had more aromatic aldehydes over time than the treatment without BSG. This indicates that although the aromatic hydrocarbons in both treatments were degraded via both the *ortho* and *meta*-oxidation pathways, the treatment with BSG may have sustained the degradation via the *meta*-oxidation pathway catalysed by catechol-2, 3-dioxygenase for longer, through the process, unlike the treatment without BSG.

Thus, the aromatic hydrocarbon degradation in this study is consistent with the literature (Chikere *et al.*, 2011; Fuchs *et al.*, 2011; Yanto and Tachibana, 2013; Varjani and Upasani, 2017) and was via both the *ortho* and *meta*-degradation pathways. However, the treatment with BSG may be more efficient at the *meta*-cleavage pathway since it had aldehyde metabolites as from day 5 up till the end of the experiment whereas the treatment without BSG only had aldehydes at the start of the experiment up till day 5.

Degradation metabolites during biodegradation, and likely degradation pathways for individual hydrocarbon substrates, by specific bacterial strains, has been investigated in some studies (Zhang *et al.*, 2012; Mallick *et al.*, 2007; Ghosal *et al.*, 2016). Zhang *et al.* (2012) in their investigation of naphthalene degradation by a novel thermophilic bacterium, *Geobacillus* sp., detected and identified a new metabolite, trans-o-hydroxybenzylidene-pyruvate.

Similarly, Mallick *et al.* (2007) determined the degradation pathway of a single PAH, phenanthrene, using *Staphylococcus* sp. strain PN/Y and observed that the degradation was via both the *ortho* and *meta*-cleavage pathways with phenanthrene-1,2-diol as the initial metabolite. Also, Ghosal *et al.* (2010) reported that phenanthrene was completely degraded by the Gram-negative *Ochrobactrum* sp. strain PWTJD firstly to 2-2-hydroxy-1-naphthoic acid, salicylic acid and catechol in the upper pathway. Catechol was further degraded by catechol-2, 3-dioxygenase in the lower pathway to 2-hydroxymuconic semialdehyde, and TCA cycle intermediates.
However, several studies on the bioremediation of diesel, being a complex mixture of hydrocarbons, have evaluated TPH removal and the associated CFUs and not the biodegradation metabolites (Ghazali et al., 2004; Molina-Barahona et al., 2004; Bento et al., 2005; Sprocati et al., 2012; Dadrasnia and Agamuthu, 2013; Agarry and Latimwo, 2015). Similarly, although studies like that of Serrano et al. (2009) determined the presence of aliphatic hydrocarbons from C11 to C27 during the biodegradation of a diesel contaminated soil using GC-MS and Sharma et al. (2009) utilised GC-MS to determine the hydrocarbon fractions that were degraded during the bioaugmentation of a diesel contaminated soil, metabolites were not identified, over time, to determine the likely degradation pathway.

This study, however, has investigated the metabolites associated with diesel contaminated soil during bioremediation with BSG, and the degradation was carried out by intrinsic soil bacterial populations, and the bacterial populations that may be supported by BSG instead of a single bacterium. Since diesel is a complex mixture of both aromatic and aliphatic hydrocarbons, several metabolites were associated with its biodegradation; thus, the metabolites were assessed based on functional groups rather than individual compounds and the degradation pathways were also established based on the functional groups of metabolites.

The presence of signature metabolites of particular functional groups, in this study, belonging to the elucidated pathways for diesel biodegradation, have confirmed the occurrence of biodegradation though it does not evaluate the abundance of the metabolites in each treatment.

5.6.2 Abundance and breakdown pattern of metabolites in treatments

The presence of metabolites, of particular functional groups, known to be in the breakdown pathway for diesel biodegradation in this study, such as alcohols, fatty acids, esters and ketones (Chikere et al., 2011) confirms that the microbial community present during the remediation were actively involved in the breakdown process (Yanto and Tachibana, 2013) and were contributory to the removal of diesel all through the bioremediation process.
Subsequently, the abundance and degradation pattern of significantly different metabolites between the treatments with and without BSG was used to determine the effect of the amended treatment on the biodegradation process. Any differences observed in favour of the amended treatment would be the result of an improved efficiency of the treatment in stimulating the biodegradation process.

In this study, simple aromatics disappeared after the first two days of the biodegradation just as did linear alkanes and alcohols such as Eicosane and n-pentadecanol. However, branched alkanes and alcohols such as 5-propyltridecane and 2-ethyl-2-methyltridecan-1-ol persisted through the biodegradation process. This is not unexpected because although alkanes are readily degraded, methyl branching increases the resistance of alkanes to microbial attack; making branched alkanes less degradable than linear alkanes (Varjani and Upasani, 2017).

Some esters such as methyl-octadeca-9,12-dienoate, on the other hand, appeared at the beginning of the remediation process and quickly disappeared while others appeared later in the process and persisted through the biodegradation. However, the esters appeared more abundantly and disappeared fastest in the treatment amended with BSG but persisted in the control natural attenuation treatment till the end of the experiment.

In summary, the treatment with BSG had a significantly reduced abundance of metabolites most times, throughout the biodegradation incubation implying that this amended treatment is more efficient in the catabolism of diesel than the unamended control treatment. This improvement is expected because soil hydrocarbon pollution causes an imbalance of the mineral nutrients particularly nitrogen and phosphorus which if deficient, may become a limiting factor to microbial degradation (Shahi et al., 2016). The addition of nutrients will therefore improve the survival of indigenous microbes responsible for the catabolism of the contaminant (Das and Chandran, 2011) and BSG, which was used in this study to stimulate bioremediation, has been shown to be nutrient rich (Aliyu et al., 2011).
The supply of nutrients to contaminated soil undergoing remediation by the addition of fertiliser, nutrients, or the use of organic sources and food by products has been evaluated in previous studies and shown to improve the remediation process as compared to the natural attenuation control (Shahsavari et al., 2013). BSG is also known to support the growth of microbes (Aliyu et al., 2011) as well as providing inocula of its own (Robertson et al., 2010). The intrinsic bacterial population in BSG will be explored and discussed further in Chapter 6.

5.7 Conclusion

The analysis of metabolites in biological samples such as those associated with the biodegradation of hydrocarbons by microbes is vital to deduce the metabolic activity of these microbes. This information could be used to determine the rate limiting step in the transformation process and extrapolate novel biological knowledge (Tsugawa et al., 2011). In this study, the introduction of BSG to diesel contaminated soil encouraged faster metabolism in terms of the synthesis and appearance of metabolic substrates and the catabolism and disappearance of metabolic products.

The amended treatment was observed to support the activity of catechol-2, 3-dioxygenase much more than the control treatment, thus enhancing its bioremediation potential. The monitoring of bioremediation over time in this study, using GC-MS, has enabled the confirmation of an enhanced bioremediation by the addition of BSG based on the presence and abundance of hydrocarbon degradation metabolites.

Thus, this chapter provides evidence that the amendment of diesel contaminated soil with BSG enhances the oxidative breakdown of aliphatic hydrocarbons via the terminal and subterminal pathways and the aromatic hydrocarbons via the ortho and meta-oxidation pathways. The next chapter will discuss the presence and abundance of genes coding for the enzymes seen in this chapter and the microbial communities associated with these genes.
Chapter Six

MICROBIAL GENES AND SUCCESSION DURING BIOREMEDIATION

6.1 Introduction

The aim of this chapter is to determine and evaluate the presence of catabolic genes and bacterial populations during the bioremediation of diesel contaminated soil, with and without BSG, and relate them to the breakdown compounds and metabolic pathways discussed in previous chapters of this study. It will determine the microbial community succession in response to diesel contamination and any accompanying shifts in the microbial community composition. The genes evaluated will be related to the metabolic pathways to yet confirm the bioremediation potential of brewery spent grain since genes encode the degradation enzymes that catalyse the reactions in the metabolic pathways.

To achieve this, the bacterial community composition over time, during the biodegradation incubations, was investigated by metagenomic analysis targeting the 16S rDNA. This was done using next generation sequencing. Also, the presence and abundance of known hydrocarbon catabolic genes harboured by the degrading bacterial community was evaluated using quantitative real time polymerase chain reaction (qPCR). The biodegradation potential of BSG was determined by comparing gene copy numbers in both treatments with and without BSG over time, during the incubation.

The sections in this Chapter will cover a brief summary of the bacterial populations during petrogenic hydrocarbon degradation and the bacterial catabolic genes for hydrocarbon biodegradation. It will then present and discuss results of the investigation described above and the conclusion.

6.2 Bacterial catabolic genes for hydrocarbon bioremediation

Monitoring the presence and abundance of hydrocarbon degradation genes serves as a very useful biomarker for estimating the bioremediation potential of contaminated sites (Mesarch et al., 2000; Zucchi et al., 2003; Zang et al.,
Thus, three bacterial catabolic genes encoding hydrocarbon degradation enzymes were evaluated. They include the alkane degradation gene (*alkB*) which is very important and key in the aerobic transformation of aliphatics (Rojo, 2009; Wang *et al*., 2010; Powell *et al*., 2006; Shahsavari *et al*., 2013). This gene encodes the alkane monooxygenase enzyme that hydrolyses aliphatic hydrocarbons to their corresponding primary or secondary alcohols depending on the position of the methyl group being oxidised (Varjani, 2017).

Oxidation of the terminal methyl group results in a primary alcohol while the oxidation of an intermediate methyl group results in a secondary alcohol. These are further metabolised to their corresponding aldehydes and fatty acids or ketones and esters, via the β-oxidation pathway into Acetyl CoA and into the TCA cycle (Varjani, 2017).

The other catabolic genes evaluated were the aromatic hydrocarbon degradation genes *catA* and *xylE* that encode the catechol-1, 2-dioxygenase and catechol-2, 3-dioxygenase enzymes, respectively, and are associated with the cleavage of the aromatic ring which is a key step in the elucidated pathways for aromatic hydrocarbon degradation (Olajire and Essien, 2014).

Catechol-1, 2-dioxygenase cleaves the ring between the first and second carbon atoms via the ortho-cleavage pathway producing ketones, esters and acetyl CoA metabolites before entering the TCA cycle. Catechol-2, 3-dioxygenase cleaves the ring between the second and third carbon atoms via the meta cleavage pathway producing aldehydes, esters and acetaldehyde metabolites before entering the TCA cycle (Varjani and Upasani, 2017).

### 6.3 Bacterial populations associated with hydrocarbon degradation

Microbial populations are the agents of biodegradation and play a vital role in the decontamination of toxic contaminants in hydrocarbon contaminated soil (Sheppard *et al*., 2011, Shahsavari *et al*., 2013). As such, an assessment and understanding of the microbial community dynamics during bioremediation is
essential in monitoring the bioremediation of petrogenic hydrocarbon contamination (Shahsavari et al., 2013) in order to understand the response and adaptation of microbes to pollution (Fuentes et al., 2014).

To achieve this, metagenomic techniques, which have become a very useful application in bioremediation; to characterise and better understand microbial communities, in different environments at a genomic level, were used (Adetutu et al., 2012, Zhang et al., 2012; Devarapalli and Kumavath, 2015). Metagenomic techniques are based on the molecular analysis of microbial communities using the 16S rDNA gene, which is present in all eubacteria and serves as a molecular gene marker, for identification (Shah et al., 2011) of microorganisms and functional genes (Yergeau et al., 2012).

Studies have shown that bacterial species associated with the degradation of petroleum hydrocarbons include Acinetobacter, Arthrobacter, Flavobacterium, Nocardia, Pseudomonas, and Vibrio (Varjani, 2017; Chandra et al., 2013). Also, bacterial genera known to degrade aliphatics, mono and polyaromatics include Pseudomonas sp. and Bacillus sp. while Acinetobacter sp. is known to degrade aliphatics and monoaromatics (Varjani, 2017). Among the different species associated with bioremediation, Pseudomonas species have been seen in various studies to be versatile in the biodegradation of hydrocarbons especially those in diesel fuel (Dussán and Numpaque, 2012).

However, knowledge of microbial species specific to diesel remediation using food waste such as brewery spent grain in temperate countries is lacking. This study will investigate to determine the response of the populations to the contamination and the potential of BSG in enhancing the bioremediation of the hydrocarbons present in diesel in a temperate climate.

6.4 Methods

Samples were taken from treatments in the same biodegradation experiment detailed in Chapter 3 and discussed in the previous chapters, to investigate the bioremediation potential of BSG on laboratory contaminated diesel soil. This was achieved by quantifying and comparing the gene copy numbers of
catabolic genes present in the natural attenuation and biostimulated treatments over time. The bacterial community profile of the treatments over time were also investigated to determine and compare any community shifts in favour of known hydrocarbon degraders.

6.4.1 Soil preparation and experimental design

The methods for diesel spiking, soil treatment composition and soil sampling used in this experiment are as stated in Section 3.3 of the Methods Chapter. The triplicate biological treatments were covered with a gortex cloth and incubated in the greenhouse with temperature fluctuations of 15°C +/- 3°C. Samples were taken from the natural attenuation and biostimulation treatments on days 0, 5, 12, and 21 for evaluation.

6.4.2 Metagenomic analysis

Deoxyribonucleic acid (DNA) was extracted from the soil and diesel natural attenuation treatment (S+D) and the biostimulated soil, diesel and grain treatment (S+D+G) over time using the E.Z.N.A soil DNA kit (Omega Bio-Tek, Inc., Norcross, GA, USA) according to the manufacturer’s instructions (Dineen et al., 2010). 1 g composite samples of soil from each treatment was obtained per sampling time for the genomic DNA extraction.

Extracted DNA were checked for purity based on absorbance at 260nm and 280nm, using a Nanodrop (Nanodrop technologies, USA) to ensure a ratio between 1.8 to 2.0 and used for the absolute quantification of catabolic genes by quantitative real time polymerase chain reaction (qPCR) assays and community profiling based on next generation sequencing.

6.4.2.1 Quantification of diesel catabolic genes

Quantitative PCR (qPCR) was performed using microbial community DNA extracted from bioremediation treatments with and without brewery spent grain
overtime. Three hydrocarbon catabolic genes: \textit{alkB} (Powel \textit{et al.}, 2006), \textit{catA} and \textit{xylE} (Shahsavari \textit{et al.}, 2016), were assayed on a Rotor Gene Q thermocycler (Qiagen, UK) using the 2x Kapa Sybr® Fast qPCR Master Mix Universal kit (Sigma Aldrich, UK). All samples and standards were run in triplicates. Negative controls were included in all amplifications. The detailed methods are as stated in Section 3.6 of the Methods Chapter.

Absolute quantification for each gene in the different samples over time were performed by comparison with standard calibration curves (Phillips \textit{et al.}, 2012) generated by assaying ten fold serial dilutions of 6 orders of magnitude ranging from $10^{-2}$ to $10^{-7}$ of known concentrations of cleaned PCR products for each gene. The standard curves were linear ($r^2 > 0.95$). In addition, melt curve analysis was performed at the end of each reaction to check for purity of the amplified product by detecting the production of nonspecific PCR products such as primer dimers (Wang \textit{et al.}, 2010).

Gene copy number was calculated using the formular: $\text{number of copies} = \frac{(\text{ng/ul DNA} \times 6.022 \times 10^{23})}{(\text{PCR product length in base pairs} \times 1 \times 10^9 \times 650)}$ (Staroscik, 2004) where Avogadro's number of $6.022 \times 10^{23}$ is the number of molecules/mole DNA and 650 Daltons as the average weight of a base pair.

Details of the primers, primer concentrations, master mix cocktail, annealing temperatures, sequences and amplification conditions of the qPCR assays are as detailed in Section 3.6.3 of the Methods Chapter.

Preliminary evaluations of the qPCR assays were carried out to confirm the suitability of the primers in amplifying the target catabolic genes by confirming the product size of the amplified genes and identifying their sequences by comparing them to the database using BLAST (basic local alignment search tool). Details are shown in Section 3.8.7 of the Methods Chapter.

6.4.2.2 Bacterial community profiling using next generation sequencing

The detailed method is as stated in Section 3.6.4 of the Methods Chapter. The 16S rDNA gene V4 variable region PCR primers 515 – 806 (Caporaso \textit{et al.},
2011) were used in a single-step 30 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C for 3 minutes, followed by 28 cycles (5 cycle used on PCR products) of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed.

Sequencing was performed at MR DNA, Shallowater, TX, USA on an Ion Torrent PGM following the manufacturer’s guidelines. Sequence data were processed using a proprietary analysis pipeline (MR DNA, Shallowater, TX, USA). In summary, sequences were depleted of barcodes and primers, then sequences <150bp removed, sequences with ambiguous base calls and with homopolymer runs exceeding 6bp were also removed.

Sequences were denoised, OTUs generated and chimeras removed. Operational taxonomic units (OTUs) were defined by clustering at 3% divergence (97% similarity). Final OTUs were taxonomically classified using BLASTn against a curated database derived from GreenGenes, RDPII and NCBI (www.ncbi.nlm.nih.gov, DeSantis et al., 2006, http://rdp.cme.msu.edu).

6.5 Results

The melt curves and standard curves generated during the qPCR assays are shown in in Section 3.8.7 of the Methods Chapter.

6.5.1 Quantification of hydrocarbon catabolic genes

The gene copy numbers of the three degradation genes, encoding hydrocarbon degradation enzymes of interest in this study, were evaluated by quantitative real time PCR (qPCR) and are presented below. The abundance of these genes, reflects the abundance of the bacterial populations that possess the genes; and can produce the requisite catabolic enzymes to degrade the respective aliphatic or aromatic hydrocarbons. Thus, an increase in gene copy number of any particular gene means an increase in the population of bacteria harbouring that gene.
Figure 6.1 shows the copy numbers of the \textit{alkB} gene over time. The \textit{alkB} gene encodes the alkane monoxygenase enzyme utilised in aliphatic hydrocarbon degradation.

\textit{Figure 6.1: Gene copy numbers of the \textit{alkB} gene in treatments, with and without BSG, over time, during the bioremediation of diesel contaminated soil.}

From Figure 6.1, a difference in \textit{alkB} gene copy number was observed all through the experiment between the treatments with and without BSG with a very distinct difference as from day 12. However, statistical analysis performed on the data using the independent t-test revealed that there was no significant difference in the gene copy numbers between treatments SD and SDG on day 0 (P= 0.29). Nevertheless, there was a significant difference in the the gene copy numbers between the treatments on all other days: day 5 (P = 0.02), day 12 (P < 0.00) and day 21 (P = 0.04).

The levels of gene copy number decreased in both treatments at day 5. However the decrease observed in the treatment with BSG was small compared to that without BSG. Both levels of gene copy number rose again in later days of incubation; at day 12, for the treatment with BSG and at day 21, for the treatment without BSG. The \textit{alkB} genes in treatment SD from the start
to the end of the experiment ranged from $4.57 \log_{10} \text{g}^{-1}$ to $5.61 \log_{10} \text{g}^{-1}$ representing a 22.76% increase while that in treatment SDG ranged from $5.65 \log_{10} \text{g}^{-1}$ to $7.93 \log_{10} \text{g}^{-1}$ representing a 40.6% increase.

The result obtained in this study is similar to that of Shahsavari et al. (2013) in which alkB gene copy numbers in the soil microbial community increased following the addition of plant residues to a 1% diesel and engine oil (60% diesel/40% engine oil) contaminated soil.

Figure 6.2 shows the copy numbers of the catA gene over time. The catA gene encodes the catechol-1, 2-dioxygenase enzyme utilised in ortho-oxidation pathway of aromatic hydrocarbon degradation.

Figure 6.2: Gene copy numbers of the catA gene in treatments, with and without BSG, over time, during the bioremediation of diesel contaminated soil.
From Figure 6.2, a difference in \textit{catA} gene copy number was also observed all through the experiment between the treatments with and without BSG with a distinct difference as from day 12. As with the \textit{alkB} gene, the levels of gene copy number in both treatments decreased at day 5. However, unlike the \textit{alkB} gene, this decrease was small in both treatments. Nevertheless, levels of gene copy number in both treatments rose again in later days of incubation at day 12 for the treatment with brewery spent grain and at day 21 for the treatment without BSG.

Statistical analysis performed on the data using the independent t-test revealed that there was a significant difference in the gene copy numbers between treatments SD and SDG from day 0 and all through the experiment with p values for the different days as follows: day 0 (P = 0.01), day 5 (P = 0.02), day 12 (P < 0.00) and day 21 (P < 0.00). The \textit{catA} genes in treatment S+D from the start to the end of the experiment ranged from 6.59 log10 g\(^{-1}\) to 5.72 log10 g\(^{-1}\) representing a 13.2 \% decrease while that in treatment S+D+G ranged from 7.10 log10 g\(^{-1}\) to 7.43 log10 g\(^{-1}\) representing a 4.65\% increase. The gene copy numbers in both treatments decreased by day 5 and the treatment with BSG increased again by day 12 while the treatment without BSG increased later by day 21.

Figure 6.3 shows the copy numbers of the \textit{xylE} gene over time. The \textit{xylE} gene encodes the catechol-2, 3-dioxygenase enzyme utilised in \textit{meta}-oxidation pathway of aromatic hydrocarbon degradation.
From Figure 6.3, a difference in \textit{xylE} gene copy number was observed as from day 5 up till the end of the experiment between the treatments with and without BSG with a distinct difference as from day 12. Statistical analysis performed on the data using the independent t-test revealed that there was no significant difference in the gene copy numbers between treatments SD and SDG on day 0 (P= 0.36). Nevertheless, there was a significant difference in the gene copy numbers between the treatments on all other days: day 5 (P = 0.03), day 12 (P < 0.00) and day 21 (P < 0.00). The \textit{xylE} genes in treatment S+D from the start to the end of the experiment ranged from 4.84 log\textsubscript{10} g\textsuperscript{-1} to 4.93 log\textsubscript{10} g\textsuperscript{-1} representing a 1.86 % increase while that in treatment S+D+G ranged from 4.89 log\textsubscript{10} g\textsuperscript{-1} to 5.64% log\textsubscript{10} g\textsuperscript{-1} representing a 15.34% increase.

The results of the qPCR indicate that the \textit{catA} genes were the most abundant in the both treatments at the start of the experiment and its abundance in the treatment with BSG was significantly higher than in the unamended treatment without BSG from the start of the experiment. However, it minimally decreased over time in treatment SD up to 1.86% by day 21 of the experiment. By this
time, its abundance in both treatments was comparable to that of the \textit{alkB} genes in both treatments and slightly more than the \textit{xyIE} genes in the treatments. Since the presence of the genes represents the likely presence of its encoded enzyme, these results will be related to the results of the breakdown profile of both aliphatic and aromatic hydrocarbons in chapter five and discussed at the end of this chapter. However, the presence of the gene does not necessarily mean the enzyme will be expressed but an indication that it can be expressed (Wang \textit{et al.}, 2013; Ghosal \textit{et al.}, 2016).

6.5.2 Bacterial community composition during diesel biodegradation

The results of the investigation to monitor bacterial community changes, during the transformation of aliphatic and aromatic hydrocarbons in diesel contaminated soil, during bioremediation, are presented below. Aliquots from the same community genomic DNA samples with and without BSG, used for qPCR, were used for this analysis for comparability with each other and with the control soil alone and grain alone samples.

The most dominant twelve bacterial genera in terms of relative abundance in the different treatments were analysed and their relative abundance and dynamics shown. Figure 6.4 shows a profile of the relative abundance of these bacterial populations that make up the community, in the treatments with and without BSG and their sterile counterparts including the soil alone control.

As mentioned in Chapter 4, a proliferation of bacterial growth was observed in the sterile treatments after day 2 of the studies so they could no longer serve as controls. Nevertheless, they were useful and effective in determining that the rapid loss of hydrocarbons during the initial stage of the experiment was due to biotic factors. Their consideration in this chapter is to determine their sterility at the onset of the experiment.

Figure 6.4 is a profile of the relative abundance of bacterial populations in the community.
Figure 6.4: Bacterial community profile showing relative abundance of bacterial populations in treatments during diesel bioremediation over time.
From the results seen in Figure 6.4, the treatments with BSG supported the highest microbial populations. This was consistent with the results of the heterotrophic and hydrocarbon utilising bacterial results obtained from culture based methods in Section 4.5 of Chapter 4.

The microbial population on day 0 was similar for all treatments except the sterile controls. The results here reveal that the sterile controls had a very low microbial population from the start of the experiment. This may explain the reason for the growth proliferation observed in the sterile controls during the degradation process. It appears that autoclaving alone did not effectively eliminate the soil bacteria. Thus, at the start of the experiment, the two sterile controls with and without BSG had similar bacterial populations while the remaining three treatments: the treatments with and without BSG and the soil alone control also has similar bacterial populations.

Having observed the bacterial community profile of the treatments based on the relative abundance of bacterial populations present in it, the percentage abundance of bacterial populations in the treatments with and without BSG were evaluated for comparison and determination of bacterial succession.

Figure 6.5 below shows the percentage abundance of bacterial populations in the treatments with and without BSG including the soil alone and grain alone controls for comparison.
Figure 6.5: Bacterial community profile showing percentage abundance of bacterial populations, present in treatments, during diesel bioremediation over time.
From Figure 6.5, it is interesting to note that the twelve most abundant bacterial populations in the treatments, following the amendment of diesel contaminated soil with BSG in this experiment, are known hydrocarbon degraders. This supports the results from studies that the presence of contamination results in a shift in bacterial succession in favour of species that can degrade the contaminant; thus making them more abundant (Cappello et al., 2007; Liu et al., 2011).

From the results in Figure 6.5, it is observed that the percentage abundance of *Flavobacterium succinicans* in the soil alone control treatment increased in percentage abundance as from day 0 from about 7% up to 20% on day 12 after which it reduced to 11% on day 21. The genera *Flavobacterium* are known hydrocarbon degraders (Zhang et al., 2006; Shahsavari et al., 2013; Varjani, 2017) and this may be the reason behind the observation of minimal hydrocarbon utilising bacterial CFUs in this treatment from day 5, as seen in Section 4.5.9 of Chapter 4. Apart from this percentage increase, the percentage composition of other bacterial genera in this treatment showed no significant shifts over time.

In the grain alone treatment however, there was an increase in the percentage abundance of *Arthrobacter* sp. from 2% at the start of the experiment to 15% by day 21. Unlike the soil alone treatment in which the percentage abundance of *Flavobacterium succinicans* peaked on day 12 and reduced thereafter, the *Arthrobacter* sp., which is also a known hydrocarbon degrader, continued to increase throughout the experiment in the grain.

This increase in percentage abundance of *Arthrobacter* sp. in the grain alone control treatment resulted in slight decreases in the abundance of the *Pseudomonas* sp., *Acinetobacter piperi* and *Sphingomonas* species over time. Apart from this, like the soil alone treatment, the percentage composition of other bacterial populations in this treatment showed no significant shifts over time and *Acinetobacter johnsonii* was the most abundant species in the treatment all through the experiment.

In the unamended soil and diesel only treatment, a rapid shift in bacterial community composition was observed after day 0 that introduced three
Acinetobacter species into the treatment at the expense of Acidobacterium spp. on day 5. The introduced species and their abundances are Acinetobacter sp. 45%, Acinetobacter johnsonii 12%, and Acinetobacter piperi 5%. The reduction in abundance of Acidobacterium spp. following contamination is interesting to note because it is ubiquitous and found especially in soils but is not a known hydrocarbon degrader (Naether et al., 2012).

No further shifts in community composition were observed until the end of the experiment. However, there were changes in abundance of species after day 5 in which the three Acinetobacter species gradually reduced until the end of the experiment while both Flavobacterium species increased. Acinetobacter sp. reduced from 45% to 41% and finally to 32% on days 5, 12 and 21, respectively, and remained the most abundant genera in this treatment all through the experiment.

Acinetobacter johnsonii reduced from 12% to 8% and 5% on days 5, 12 and 21 while Acinetobacter piperi reduced from 5% to 3% and 2.5% on days 5, 12 and 21. Flavobacterium sp., on the other hand, increased from 3% to 5% and finally 9%, Flavobacterium succinicans increased from 2% to 3% and then 5% while Pseudomonas sp. increased from 5% to 8% and finally 10%.

In the amended treatment, the addition of BSG to the contaminated soil at the start of the experiment on day 0 may have contributed to the increased percentage abundance of both the Arthrobacter sp. and Pseudomonas putida observed in this treatment in Figure 6.5 as compared to the unamended soil and diesel, and soil alone treatments.

However, after day 0, a rapid shift in microbial community composition was observed in this amended treatment in favour of Flavobacterium succinicans, Flavobacterium sp. and Pseudomonas putida making them the most abundant species in the treatment while almost eliminating Acidobacterium spp. and greatly reducing Arthrobacter spp. The amended treatment with BSG, was thereafter able to sustain these three species as the most abundant all through the experiment. Acinetobacter piperi was also introduced. As with the unamended treatment, the reduction in the abundance of Acidobacterium spp. following contamination was also observed in this treatment. Similarly, in their
biodegradation study of a crude oil contaminated soil, Wu et al. (2017) observed that among the 10 predominant bacteria, Actinobacteria was the most predominant phylum with a relative abundance of 63% in the bioaugmentation treatment while the abundance of Acidobacteria reduced.

A second shift in microbial community composition was observed after day 5 in which Acinetobacter sp. was introduced and the abundance of two other species of Acinetobacter increased. Acinetobacter johnsonii increased from 2% to 8% and 9% on days 5, 12 and 21 while Acinetobacter piperi increased from 4% to 9% and 8% on days 5, 12 and 21 at the expense of Pseudomonas putida which reduced in abundance from 28% to 14% and 12% on days 5, 12 and 21. However, Flavobacterium succinicans, Pseudomonas putida and Flavobacterium sp. remained the most abundant species by the end of the experiment.

6.6 Discussion

Monitoring the abundance of hydrocarbon degrading genes is necessary to understand the catabolic potential of the degrading microbial community and in this case, soil bacterial community. Such knowledge could then be harnessed to help increase the efficiency of the bioremediation process; since understanding the behaviour of hydrocarbonoclastic microbial populations is prerequisite to a successful bioremediation implementation process (Baek et al., 2009).

Metagenomic methods and real time PCR have been effectively and successfully used in monitoring target microbial populations and bacterial catabolic genes involved in the degradation of diesel hydrocarbon contaminants (Baek et al., 2009; Yergeau et al., 2009; Bacosa et al., 2012). These methods have been used in this study to evaluate the presence of three catabolic genes; alkB, catA and xylE during the bioremediation of diesel contaminated soil and also assess the bacterial community successions which occurred during the incubation.
The purpose of this section, therefore, is to discuss the results obtained from these laboratory experiments regarding their usefulness in determining the catabolic potential of BSG to enhance diesel bioremediation in soil.

6.6.1 Hydrocarbon catabolic genes during bioremediation

Among the three genes evaluated to determine the catabolic potential of BSG, in the remediation of diesel contaminated soil in this study, the \textit{alkB} gene had the highest percentage increase from the start of the experiment till the end especially in the treatment with BSG. The reason for this may be because aliphatic hydrocarbons were present in the highest concentrations. However, \textit{catA} was the only gene with a significantly higher abundance in the amended treatment with BSG, from the start of the experiment.

Considering that there was a rapid biodegradation of hydrocarbons from the start of the experiment, catechol-1, 2-dioxygenase, which encodes the \textit{catA} gene that metabolises aromatic hydrocarbons through the \textit{ortho}-cleavage pathway with ketone intermediates as seen in Figure 5.2 of Chapter 5, may have been actively involved in the degradation process at the onset of the biodegradation and may have contributed to the statistically significant reduction in TPH of the treatment with spent grain as compared to the treatment without spent grain.

Also, \textit{Pseudomonas putida}, which was consistently successfully cultivated in the laboratory from the treatments during the biodegradation experiment was also seen in abundance at the start of the experiment in the treatment amended with BSG as shown in the bacterial community profile in Figure 6.5. \textit{Pseudomonas putida} is known to have catabolic genes for both aliphatic and aromatic hydrocarbons (Marcus, 2003) and has metabolic pathways that metabolise aromatic hydrocarbons via catechol through the \textit{ortho}-cleavage pathway catalysed by catechol-1, 2-dioxygenase (Harwood and Parales, 1996; Nelson \textit{et al.}, 2002).

Thus, \textit{Pseudomonas putida} may have been actively involved in the metabolism of aromatics catalysed by catechol-1, 2-dioxygenase to produce
ketones at the start of the experiment. Also, the breakdown pattern of aromatic hydrocarbons shown in Figure 5.3 of Chapter 5 reveals that aromatic hydrocarbons were mostly degraded within the first two days of the experiment. In accordance with this, the gene copy numbers of the catA gene reduced after day 5 of the bioremediation and the abundance of Pseudomonas putida especially in the amended treatment with BSG also reduced after day 5 as seen in the bacterial population profile.

Nevertheless, catechol-2, 3-dioxygenases have been observed to be more effective in the bioremediation of aromatic hydrocarbons than the catechol-1, 2-dioxygenases (Mesarch et al., 2000; Chikere et al., 2012). Thus, catechol-2, 3-dioxygenase may also have been involved in the initial rapid transformation of aromatic hydrocarbons in this study.

The alkB genes increased in copies later in the biodegradation process which appears to be the time when they were most active. However, the breakdown profile for significantly different alkanes between treatments indicates that alkanes were metabolised from the start of the remediation treatment and reduced significantly more in the treatment with BSG. Nevertheless, alkanes persisted up till the end of the experiment and the labile, easier to degrade alkanes are usually degraded during the initial biodegradation phase leaving the higher molecular weight alkanes which are less degradable (Ros et al., 2010), which may require more enzymatic catalysis for biodegradation.

In this study, the gene copy numbers of all three catabolic genes were significantly more abundant in the amended treatment with BSG as from day 5 up till the end of the experiment. Similarly, in the study of Baek et al. (2009) in which a 3% Arabian crude oil contaminated soil was augmented with Norcadia sp. H17-1, there were more alkB gene copies in the augmented treatment at the end of the 30 day study than in the nontreated soils.

Also, similar to the results in this study, Margesin et al. (2003) reported higher levels of alkB and xylE genes from Pseudomonas sp. in polluted soils than pristine. AlkB genes were significantly higher in treatments amended with plant residues such as pea straw and hay than the unamended contaminated soil in
the bioremediation of a 1% diesel oil and engine oil contaminated soil by Shahsavari et al., (2013).

Although there is a concern that encoding genes may be present without their corresponding enzymes being expressed, Yergeau et al. (2012), in their study of diesel contaminated soil undergoing biopile remediation and amended with monoammonium phosphate (NH₄PO₄), observed the same results for the alkB gene copy number abundance as the expression of the gene. Both abundance and expression results were highest after one month of treatment and significantly lower after one year.

Since the presence of these genes can be monitored as indicators for catabolic potential (Baek et al., 2009) and the amendment with BSG significantly increased the abundance of all three genes in consideration, BSG may be said to have the potential to significantly increase the bioremediation potential of diesel contaminated soil.

6.6.2 Microbial community shifts during hydrocarbon bioremediation

The results from the metagenomic profiling of the microbial community for the soil alone and grain alone control treatments show, as expected, that there were no shifts in the population (Figure 6.4). Also, the microbial population in the soil alone control was relatively constant, as was expected, making it a good control for confirming the reliability of the results.

However, the presence of a low microbial population in the sterile controls suggest that not all of the autochthonous bacterial population were eliminated. Thus, the increased population of the treatments may have arisen from a proliferation of the autochthonous soil bacteria, having overcome the stress of the sterilisation procedure (Mahmood et al., 2014).

The introduction of diesel in the unamended soil and diesel treatment led to a major shift in the microbial community in favour of Acinetobacter sp. having an abundance of up to 45% of the entire population and greatly reducing the abundance of Acidobacterium spp.
Acinetobacter sp. is a known hydrocarbon degrader that has been identified in hydrocarbon contaminated soils and the xylE gene encoding catechol-2, 3-dioxygenase that catalyses the degradation of aromatic hydrocarbons has been detected in Acinetobacter sp. and Pseudomonas sp. (Mendez et al., 2010; Fuentes et al., 2014). Acinetobacter sp. was identified in treatment S+D right after the start of the experiment.

Thus, it is not surprising that aromatic aldehydes were identified in this treatment at the start of the experiment at day 0. Acinetobacter sp. may have been involved in the breakdown of aromatics at the start of the experiment in this treatment. Although Acinetobacter sp. persisted in this unamended treatment without grain until the end of the experiment, its abundance was reduced. However, most of the aromatic hydrocarbons detected in this study were degraded early in the experiment and may be the reason for the 29% reduction in the abundance of Acinetobacter sp. by day 21.

As Acinetobacter sp. reduced in the treatment without BSG, Pseudomonas sp. gradually increased. Pseudomonas species are known to be versatile and effective in the degradation of both aliphatic and aromatic hydrocarbons including those in polar soils (Yergeau et al., 2012). It is not surprising then that the abundance of Pseudomonas sp. continually increased after day 5 since the aliphatic hydrocarbons in this study persisted beyond the aromatics as seen in Chapter 5. This result thus supports results obtained in Chapter 5.

The amendment with BSG enriched the autochthonous bacteria such that from the onset of the bioremediation at day 0, Pseudomonas putida and Arthrobacter spp. were more abundant in the amended treatment than other treatments. A shift in the soil microbial community resulted in the increased abundance of Pseudomonas putida, Flavobacterium succinicans and Flavobacterium sp. These are all known hydrocarbon degraders and remained the most abundant species throughout the experiment. Nevertheless, no single microbial specie is capable of completely degrading contaminants rather a synergistic activity of the microbial community is required.

However, the microbial community population in the amended treatment with BSG significantly degraded more hydrocarbons by day 2 of the experiment.
than the unamended treatment. It appears then that the dominance of *Pseudomonas* species and *Flavobacterium* species in the amended treatment with BSG may have had a greater hydrocarbon bioremediation potential than the dominance of *Acinetobacter* sp. in the unamended treatment without BSG.

A second shift in bacterial community in the amended treatment with BSG, as from day 5 introduced *Acinetobacter piperi* at which point aldehydes appeared in the treatment. However, by day 12, *Acinetobacter* sp. increased in abundance alongside *Acinetobacter piperi* as seen in Figure 6.5. From this point, only this treatment contained aromatic aldehydes.

Thus *Acinetobacter* sp. may have been responsible for the sustained metabolism of aromatics in the amended treatment with BSG, as seen in Table 5.1 of Chapter 5, since it is known to possess *xylE* genes which are catalysed by catechol-2, 3-dioxygenase (Mendez *et al*., 2010; Fuentes *et al*., 2014). Catechol-2, 3-dioxygenase has been shown to be very effective in the degradation of aromatic hydrocarbons (Mesarch *et al*., 2000) as opposed to the catechol-1, 2-dioxygenase.

### 6.7 Conclusion

The abundance trend of the *alkB* genes tends to reflect the trend of alkane abundance in Chapter 5. Thus, gene copy numbers positively correlated with bioremediation of the hydrocarbons (Wang *et al*., 2010; Phillips *et al*., 2012). In addition, there was a significant increase in gene copy numbers resulting from biostimulation with brewery spent grain. However, it is known that the presence of a gene does not necessarily reflect the synthesis of the encoded enzyme and a further investigation requiring the use of reverse transcription real time quantitative polymerase chain reaction (RT-qPCR) to quantify the m-RNA and gene expression may be required (Wang *et al*., 2010).

The metagenomic analysis using next generation sequencing in this study, provided a culture independent approach to elucidate the bacterial population dynamics during the biodegradation of diesel contaminated soil using BSG and showed changes in the abundance of autochthonous species in favour of
known hydrocarbon degraders; resulting in an enrichment of autochthonous hydrocarbon degraders and shifts in microbial populations supporting aerobic hydrocarbon degraders.

In addition, the microbial community analysis of the grain alone control treatment showed that the grain supports its own microflora; most of which are known hydrocarbon degraders. This supports the reasoning that the supplementation with BSG is not just a biostimulation treatment but also bioaugmentation.

These results suggest that BSG is an effective treatment to enhance the bioremediation of hydrocarbon contaminated soils.
Chapter Seven

DISCUSSION

7.1 Introduction

The impacts of diesel pollution extend beyond environmental degradation to health risks posed on all forms of life (Margesin and Schinner, 2001; Souza et al., 2014; Varjani, 2017) due to the toxicity, mutagenicity and carcinogenicity of its constituent hydrocarbons (Shahsavari et al., 2013). Diesel fuel, being a mixture of chemicals, remains the most common petrogenic hydrocarbon contaminants found in the environment because of spillage during transport and storage thus contaminating soil and water (Yergeau et al., 2012; Tellechea et al., 2017).

Soil is one of the most important natural resources required to sustain life on the earth (Gao et al., 2013). It is necessary to provide nutrients for plant growth without which neither animals nor man will survive, since plants are the only autotrophs in nature; and provide a medium for the recycling of organic elements in the nutrient cycles (Bezdicek et al., 1996). Thus, soil contamination would mean a disruption to its functions (Mikkonen et al., 2011; Gao et al., 2013) and a mismanagement of the soil resource can lead to drought, malnutrition and poverty (Bezdicek et al., 1996).

However, the majority of soil functions are carried out by soil microbes; which also play a significant role in biogeochemical cycles, a mentioned previously. There is, therefore, a growing concern regarding the impact of soil contamination on the soil microbial diversity and their population (Saadoun et al., 2008). For this reason, environmentally safe, efficient and cost effective remediation methods are being sought to mitigate the environmental impacts of diesel contamination as compared to physiochemical methods like landfilling and incineration which are unsafe and costly (Shahsavari et al., 2013).

Phytoremediation, which is the use of plants and their rhizosphere organisms to biodegrade contaminants, has been found useful. However, the toxicity of pollutants to plants limit its application (Shahsavari et al., 2013). Bacteria, on the other hand, have been found to be versatile in the breakdown of
hydrocarbons; thus bioremediation, in which bacteria with adequate catabolic enzymes break down pollutants resulting in complete mineralisation, is the preferred method for remediating contaminated soil since it is safe and cost efficient (Varjani et al., 2017).

To this end, studies have been conducted to investigate the efficiency of different types of bioremediation techniques such as biostimulation and bioaugmentation on soils contaminated with diesel (Bento et al., 2005). The application of non living plant biomass and plant residues such as pea straw, wheat straw and hay, have been used in biostimulation studies (Barathi and Vasudevan, 2003; Zhang et al., 2008; Hultgren et al., 2009).

In one study, Hultgren et al. (2009) did not observe any conclusive improvement in bioremediation after wheat straw was added to a creosote contaminated soil. However, other studies have used plant and crop residues in biostimulation and observed improved bioremediation rates as compared to natural attenuation rates in hydrocarbon contaminated soils (Shahsavari et al., 2013, Dadrasnia and Agamuthu, 2013, Molina-Barahona et al., 2013).

To anticipate effects, laboratory experiments are required to assess the enhancement potential of specific crop residues or biostimulants for specific hydrocarbon contaminants in specific climatic conditions to establish the scientific credibility of a particular remediation process (Atlas and Bartha, 1972; Suja et al., 2014).

BSG was considered for use as a biostimulant in this study because in addition to providing nutrients for autochthonous bacteria (Thomas and Rahman, 2006) during the remediation process, it is known to have its own resident microflora (Robertson et al., 2010) and so may serve for both biostimulation and bioaugmentation.

Also, bioremediation with BSG and effluents have been effective in diesel contaminated soil in tropical climates (Abioye et al, 2012; Agarry and Latinwo, 2015). Hence in this study, the bioremediation potential of BSG was assessed for its suitability in the biodegradation of diesel contaminated soil in temperate climates and an overview of the main findings are discussed.
7.2 Does brewery spent grain influence diesel breakdown in soil?

This is the first research question that was answered by the experiments in Chapter 4 of this thesis. Following the 28 day bioremediation experiment of a 1% (v/w) diesel contaminated soil kept at a temperature of 15 ± 3°C to reflect normal environmental conditions, a TPH reduction of 96% was observed in the treatment with BSG (S+D+G) while a 92% reduction was observed in that without BSG (S+D). Although the final values are close, statistical analysis revealed a significant difference in TPH reduction between the two treatments.

The results of the reduction in concentration and removal extent of TPH aliphatic fractions also showed a significant difference between the treatments as from day 2; with the treatment with BSG having greater reduction in concentration. Also, while only one fraction, C10, was completely degraded in treatment S+D, three fractions, C10, C12 and C28 were degraded in the treatment with BSG. Higher biodegradation rates were also observed in the amended treatment with BSG, all through the bioremediation process, as compared to the unamended treatment. The hydrocarbons were thus removed quicker in this treatment.

The population of both heterotrophic and hydrocarbon utilising bacteria showed a rapid increase from the start of the bioremediation process when the hydrocarbon reduction was highest and peaked after day 12. However, the bacterial population in the treatment with BSG were significantly higher than that in the treatment without BSG. These results answer the question that stimulating the bioremediation process with BSG resulted in an enhancement of the process. The reason for this is likely because the polysaccharides from the cellulose and hemicellulose in BSG can stimulate the growth and activity of hydrocarbon degrading soil microflora (Zhang et al., 2008).

Like other plant and crop residues, BSG can improve soil properties such as aeration, nutrition and moisture (Thomas and Rahman, 2006) and thus improve conditions for degrading bacteria. Also, BSG has its own associated microbial community that may contribute to the degradation of hydrocarbons (Shahsavari et al., 2013).
However, the breakdown of hydrocarbons is made possible by bacteria that have an adequate metabolic capability (Venosa et al., 2002; Adams et al., 2015) by possessing specific degradation enzymes that can digest the hydrocarbons through degradation pathways. Since the breakdown pathways for hydrocarbon degradation are already elucidated, it is necessary to understand the pathway for diesel breakdown by BSG. To achieve this, the breakdown metabolites present during the bioremediation process were identified by GC-MS and analysed. The results obtained and the deduced diesel remediation pathway in this study are discussed.

7.3 Metabolites and Pathways of diesel bioremediation using BSG

As the bioremediation of organic pollutants involve a sequence of metabolic reactions catalysed by enzymes (Peixoto et al., 2011; Varjani, 2017) and aliphatic hydrocarbons can be degraded via various metabolic pathways such as terminal, subterminal and β-oxidation pathways for aliphatic hydrocarbons and ortho and meta-cleavage pathways for aromatic hydrocarbons (Varjani and Upasani, 2017) as shown in Figures 5.1 and 5.2 of Chapter 5, the metabolites identified were used to determine the oxidative pathways utilised in the breakdown of diesel with and without BSG.

The presence of aliphatic ketones and esters all through the remediation process in both treatments with without BSG may mean that the aliphatic hydrocarbons in diesel were degraded mainly via the subterminal oxidative pathway. However, although aliphatic aldehydes were not detected, the presence of aliphatic fatty acids (carboxylic acids) suggests that aldehydes may have been oxidised very rapidly, especially in the treatment with BSG, to carboxylic acids which may have been oxidised via the β-oxidation pathway to the tricarboxylic acid (TCA) cycle.

Thus, the aliphatic hydrocarbons may have been oxidised via both the terminal and subterminal pathways with the treatment amended with BSG being more efficient in the breakdown process. The results also revealed that the soil may
have intrinsic hydrocarbon degraders since both treatments seemed to rapidly degrade hydrocarbons from the start of the experiment.

The presence of aromatic ketones all through the experiment indicated that the degradation was mostly via the ortho-oxidation pathway catalysed by catechol-1, 2-dioxygenase (Fuchs et al, 2011). Aromatic aldehydes which are the key differentiating metabolites of the ortho-cleavage pathway catalysed by catechol-2, 3-dioxygenase (Varjini and Upasani, 2007) were only present at the start in the treatment without BSG and later in the treatment with BSG which supported its presence throughout the experiment.

This result suggests that the indigenous hydrocarbon degrading microbes did play a significant role in the bioremediation process (Varjani, 2017) but may have been stimulated by the nutrients in BSG (Thomas and Rahman, 2006), thus enabling the treatment with BSG to be more efficient in supporting the activity of both catechol-1, 2-dioxygenase and catechol-2, 3-dioxygenase. Thus, degrading the aromatic hydrocarbons in diesel via both the ortho and meta-cleavage pathways. This may also account for the initial rapid loss of hydrocarbons at the start of the experiment.

The work presented here, thus shows that stimulation with BSG appears to have enhanced the biodegradation by being more efficient in the known degradation pathways for hydrocarbon degradation especially the meta-oxidation pathway for aromatic hydrocarbon degradation catalysed by catechol-2, 3-dioxygenase. Interestingly, the gene that encodes this enzyme is located on plasmids (Van der Meer et al., 1992; Varjani, 2017) which are capable of horizontal gene transfer enabling them to replicate these genes and adapt quickly to the hydrocarbons (de la Cruz and Davies, 2000).

The presence of the catabolic genes encoding these enzymes confirms the potential of the bacterial population that possess them to synthesise them (Olajire and Essien, 2014) and since their presence can be monitored as useful biomarkers for estimating the bioremediation potential of contaminated soils (Mesarch et al., 2000; Margesin et al., 2003; Zucchi et al., 2003; Zhang et al., 2006; Ma, 2006; Yang et al., 2015), the abundance of the alkane monooxygenase gene (alkB), the catechol-1, 2-dioxygenase gene (catA), the
catechol-2, 3-dioxygenase gene (xylE) and the bacterial population that harbours them were assessed and will be discussed in the next section.

7.4 Metagenomic analysis of catabolic genes and bacterial populations during bioremediation

The metagenomic analysis in combination with the analytical GC-MS evaluation used in this study has been useful in elucidating the dynamics of bacterial communities during diesel bioremediation. The results obtained also tally with the literature in that as shown in Figure 6.2 of Chapter 6, the catA gene was the only gene having a significantly higher copy number from the start of the experiment on day 0 in the treatment with BSG.

The results from the percentage TPH reduction in Figure 4.1 of Chapter 4 and the concentration of hydrocarbon fractions in Figure 4.2 of Chapter 4 reveals that TPH and aliphatic hydrocarbons were rapidly degraded from the start of the experiment and was significantly more degraded in the treatment with BSG by day 2. The breakdown profile of aromatic compounds as shown in Figure 5.3 also showed that aromatic hydrocarbons were degraded during the rapid degradation phase. These results all agree and are consistent.

*Pseudomonas putida* which is known to metabolise aliphatic and aromatic hydrocarbons via the ortho-cleavage pathway catalysed by the catA gene (Marcus A, 2003; Harwood and Parales, 1996; Nelson *et al.*, 2002) was present in the treatment amended with BSG from the start of the experiment on day 0 and increased up till day 5 before declining as seen in Figure 6.5 of Chapter 6. In line with this, both the catA gene copy numbers and the abundance of *P. putida* declined in the treatments after day 5.

However, *Pseudomonas putida* was also observed in the treatment without BSG, in which hydrocarbons were also reduced during the initial rapid remediation, Nevertheless, the abundance of *P. putida* was greater in the amended treatment than in the unamended treatment. The reason for this may be the enhancement by brewery spent grain which had an intrinsic population of *P. putida* as also seen in Figure 6.5. These results indicate that *P. putida* is likely to be associated with the rapid degradation of hydrocarbons in this study.
All three catabolic genes; the alkane monooxygenase gene (alkB), the catechol-1, 2-dioxygenase gene (catA) and the catechol-2, 3-dioxygenase gene (xylE) were present in both treatments with and without BSG but the gene copy numbers in the treatment with BSG continually increased after the start of the bioremediation experiment and remained much higher and significantly greater in abundance than in the treatment without BSG.

Also, *Acinetobacter* species which were observed in the treatments without BSG, early during the experiment on day 5, began to decline. Following the decline of *Acinetobacter* sp., aldehydes were no longer present in this treatment. However, *Acinetobacter* appeared in the treatment with spent grains as from day 5 resulting in a corresponding appearance of aldehydes in this treatment as shown in Figure 5.1. This result agrees with the knowledge that *Acinetobacter* sp. are known to possess the *xylE* genes that catalyse the degradation of aromatic hydrocarbons through the *meta*-cleavage pathway with aldehydes as intermediate products.

*Acinetobacter* sp. is also intrinsic in BSG. Thus *Pseudomonas* sp. and *Acinetobacter* sp. may be actively responsible for the enhanced bioremediation potential of BSG. However, the community of bacteria work synergistically to degrade pollutants (Chikere *et al.*, 2011) so a single species may not be solely associated with the enhancement in diesel bioremediation resulting from the stimulation with brewery spent grain.
Chapter Eight

CONCLUSION

8.1 Introduction

This study demonstrated that the addition of BSG to diesel contaminated soil enhanced biodegradation under laboratory conditions in a temperate climate. Thus, waste such as BSG, that could have been added to landfill can be converted to a purposeful use; not just reducing landfill waste and recycling the waste, but also for the bioremediation of diesel contaminated soil which is a very environmentally viable option (Margesin, 2003). The need for bioremediation of contaminated soil is also made obvious by this work in that for as low in concentration as a 1% diesel contaminated soil, natural attenuation did not completely eliminate the contaminants in one month despite the very rapid degradation rate observed at the start of the experiment.

The metabolites present at different times during the bioremediation process indicate that the breakdown of diesel was mostly via the subterminal oxidation pathway for the aliphatic hydrocarbon content of diesel and via the ortho-oxidation pathway for the aromatic hydrocarbon content of diesel. However, the addition of BSG enhanced the metabolism via these two pathways while also sustaining metabolism via the meta-cleavage pathway for aromatic hydrocarbon degradation and speeding up the metabolism of carboxylic acids via the $\beta$-oxidation pathway for aliphatic hydrocarbons. The meta-cleavage pathway, however, is known to be more efficient in the breakdown process of hydrocarbons (Mesarch et al., 2000; Varjani and Upasani, 2017).

The addition of BSG also increased the gene copy numbers of all three catabolic genes analysed in this study namely: $alkB$ for the metabolism of aliphatic hydrocarbons, $catA$ and $xylE$ for the metabolism of aromatic hydrocarbons. Also, the metagenomic analysis of the bacterial community over time enabled the determination of bacterial populations that were most abundant during the biodegradation process and the community dynamics of the bacterial population in the treatment with BSG, responsible for the enhanced bioremediation observed in this study.
Acinetobacter species and Pseudomonas species were the most abundant bacterial populations responsible for the breakdown of hydrocarbons in this study. As such, the use of genetic engineering can be exploited to increase the populations of such autochthonous hydrocarbon degraders to further improve the bioremediation process.

Summarily, the addition of BSG in bioremediation is cheap, sustainable and introduces a lot of benefits for bioremediation enhancement such as aeration, nutrition and the introduction of its own microflora and the use of next generation sequencing to characterise bacterial species composition and abundance at various stages during the bioremediation of diesel contaminated soil has increased the knowledge and understanding of community dynamics.

The biostimulation of diesel contaminated soil in this study was enhanced by the addition of BSG. This was evident by the following reasons:

- The percentage total petroleum hydrocarbon content of diesel in soil was significantly reduced in the treated sample as compared to the untreated sample.

- There was a greater extent and rate of biodegradation in the treated sample; having a biodegradation rate constant (k day⁻¹) of 0.1021 as compared to 0.059 in the untreated sample.

- There was an increased population of hydrocarbon degraders in the treated sample as compared to that of the untreated sample.

Key findings of this study:

- The addition of BSG resulted in a sustained degradation of the aromatic hydrocarbons in diesel via the meta-oxidation pathway.

- The addition of BSG enhanced the aliphatic hydrocarbon metabolism of carboxylic acids via the β-oxidation pathway making it happen quicker.
The *alkB*, *catA* and *xylE* catabolic genes were increased significantly above the natural attenuation.

*Acinetobacter* species and *Pseudomonas* species were most abundant during the biodegradation and key to the enhanced bioremediation of BSG.

*Acinetobacter* species and *Pseudomonas* species were also intrinsic bacterial populations in BSG.

### 8.2 Recommendations for future work

Pilot scale field study is essential to demonstrate the efficiency of this technique *in situ* prior to large scale field application. Also, since the use of lignocellulose inoculum carriers such as wheat straw and corn cobs have been used successfully to significantly degrade polyaromatic hydrocarbons (García-Sánchez *et al.*, 2018), and BSG is rich in cellulose, it would be useful to investigate the role of fungi in the degradation of diesel using BSG.

Other laboratory scale investigations that can be carried out include:

- Determination of gene expression as opposed to the detection and quantification of gene copy numbers evaluated in this study.

- In addition to autoclaving, during laboratory experiments, bactericides such as sodium azide can be added to the sterile samples to keep them sterile.

- Investigation on the role of plasmids and horizontal gene transfer in the *meta*-cleavage pathway catabolised by *xylE* during bioremediation.

- Metagenomic profiling of fungal species during the bioremediation of diesel using brewery spent grain.
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Kim, S., Kweon, O., Cerniglia, C. E. (2009) Proteomic applications to elucidate bacterial aromatic hydrocarbon metabolic pathways *Current Opinion in Microbiology*12, 301–309


Mao, J., Yongming L, Ying T, Zhengao L (2012) Bioremediation of polycyclic aromatic hydrocarbon-contaminated soil by a bacterial consortium and associated microbial community changes. *Int Biodeter & Biodegr.* 70, 141-147


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Electronic resources (internet)

**URL 1** (2018) The Statistics Portal. Oil production in the United Kingdom (UK) from 2003 to 2017 (in 1,000 barrels daily)


**URL 3** Land Contamination Management in UK

**URL 4** Overview of Total petroleum hydrocarbons

**URL 5** EPA Underground Storage Tanks

**URL 6** 16S Ribosomal RNA
https://www.sciencedirect.com/topics/neuroscience/16s-ribosomal-rna (Accessed on 31/01/19)
URL 7 16S rDNA Sequence Analysis - the BIOTECH Project
biotech.bio5.org/sites/default/files/pdf/16s%20rDNA%20SeqAnal.pdf
http://www.google.co.uk/url?sa=t&rct=j&q=&esrc=s&source=web&cd=4&cad=rja&uact=8&ved=0CEEQFjAD&url=http%3A%2F%2Fbiotech.bio5.org%2Fsites%2Fdefault%2Ffiles%2Fpdf%2F16s%2520rDNA%2520SeqAnal.pdf&ei=HGuWVYTkLMOysQH8w66ACg&usg=AFQjCNGOABLLQJ7qIa6clDgTBv18H8rKA&bvm=bv.96952980,d.ZGU
(Accessed on 10/01/2019)

URL 8 Sigma Aldrich Product information for the composition of R2A agar
(Accessed on 10/01/19)
https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma-Aldrich/Datasheet/1/17209dat.pdf (Accessed on 26/01/19)

URL 9 R2A agar (Difco™ & BBL™ Manual, 2nd Edition)
(Accessed on 26/01/19)
APPENDIX

Appendix 1

Appendix 1: Independent samples t-test results for difference in TPH total peak heights between treatments with and without BSG from day 0 to 28.

<table>
<thead>
<tr>
<th>TREATMENTS</th>
<th>Soil + Diesel</th>
</tr>
</thead>
<tbody>
<tr>
<td>S+D+BSG</td>
<td>Day 0</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
</tr>
</tbody>
</table>

Where S+D+BSG is soil + diesel + brewery spent grain
Appendix 2

Appendix 2: Mann Whitney U test results for significant differences in concentrations of TPH (C10 – C28) fractions in contaminated soil samples with and without BSG

<table>
<thead>
<tr>
<th>TREATMENTS</th>
<th>Soil + Diesel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
</tr>
<tr>
<td>S+D+BSG</td>
<td>0.09</td>
</tr>
</tbody>
</table>

Where S + D + BSG is soil + diesel + brewery spent grain
Appendix 3: One-way ANOVA test results for significant differences in colony forming units (CFUS) enumerating heterotrophic bacteria among treatments with and without BSG, and the soil control on all sampling days

<table>
<thead>
<tr>
<th>TREATMENTS</th>
<th>Soil + Diesel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
</tr>
<tr>
<td>S+D+BSG</td>
<td>0.02</td>
</tr>
<tr>
<td>Soil control</td>
<td>0.24</td>
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</table>

Soil + Diesel + Brewery Spent Grain

<table>
<thead>
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<th>TREATMENTS</th>
<th>Soil + Diesel</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
</tr>
<tr>
<td>Soil control</td>
<td>P&lt;0.001</td>
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</tbody>
</table>

ANOVA P values

<table>
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<tr>
<th>TREATMENTS</th>
<th>Soil + Diesel</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
</tr>
</tbody>
</table>

Where S+D+BSG is soil + diesel + brewery spent grain
Appendix 4: One-way ANOVA test results for significant differences in colony forming units (CFU) enumerating hydrocarbon degraders among treatments with and without BSG, and the soil control on all sampling days.

<table>
<thead>
<tr>
<th>TREATMENTS</th>
<th>Soil + Diesel</th>
<th>Soil + Diesel + Brewery Spent Grain</th>
<th>ANOVA P values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 2</td>
<td>Day 5</td>
</tr>
<tr>
<td>S+D+BSG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soil control</td>
<td></td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
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<tr>
<td>Soil control</td>
<td></td>
<td>P&lt;0.001</td>
<td>0.01</td>
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</tbody>
</table>

Where S+D+BSG is soil + diesel + brewery spent grain.

There are no post hoc (LSD) values for days 0 to 5 because only 2 variables were evaluated since there were no values for soil control as hydrocarbon degrading bacteria did not grow in the soil control sample on those days. A separate t-test was run for the two variables.
Appendix 5

Appendix 5: Independent samples t-tests CFU results for enumerating hydrocarbon degrading bacteria between treatments with and without BSG from day 0 to 5.

<table>
<thead>
<tr>
<th>TREATMENTS</th>
<th>Soil + Diesel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
</tr>
<tr>
<td>S+D+BSG</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Where S + D + BSG is soil + diesel + brewery spent grain

The results shown in Appendix 5 are a confirmation of the one way ANOVA results in Appendix 4.