Characterisation and bioremediation of hydrocarbon contaminated soils: A case of Murowa Diamonds mine

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Characterisation and the Potential of Bioremediation of Hydrocarbon Contaminated soils: A Case of Murowa Diamonds

I declare that the above dissertation/thesis is my own work and that all the sources that I have used or quoted have been indicated and acknowledged by means of complete references.

SIGNATURE DATE

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Abstract

Microbial biodegradation methods of hydrocarbon contaminated soils that can occur through biodegradation, bio augmentation, bio stimulation, and phytoremediation, have gained significant interest in recent years when compared to the conventional methods.

The study was designed to explore the influence of petroleum hydrocarbon contamination on physicochemical and microbial characteristics of soils as well as determining the possibility of hydrocarbon biodegradation using biostimulation. The process involved soil characterisation and modification of nitrogen and phosphorus content to stimulate naturally adapting microorganisms. Characterisation process determined that hydrocarbon contamination of Murowa soils introduced hydrophobicity to the naturally wettable sandy loam soils. Naturally adapting microbial species capable of degrading hydrocarbons identified using Direct microscopy, Gram and Melzer's iodine staining included *Pseudomonas, Bacillus, Serratia marcescens, Flavobacterium, Micrococcus, Streptomyces Staphylococcus, Penicillium* and yeasts. The N: P nutrient ratio and moisture levels were identified as potential limiting factors and hence experiments focused on manipulation of N: P nutrients to stimulate the identified hydrocarbon degrading organisms (bio stimulation). Hydrocarbons were identified by solvent extraction using hexane and gas chromatography. These included decane, undecane, hexadecanal, 2-ethylcridine, octadecane and 1-iodo.

Soils weighing 10kgs with hydrocarbons levels of about 265mg/kg were subjected to eight (8) treatments with seven (7) different combinations of N (6000-12000mgN): P (600-3000mgP) concentration ranges including the control. Nitrogen The moisture was adjusted and tilling for aeration was done on a weekly basis. Changes in Total Petroleum Hydrocarbon (TPH), C: N: P ratio, microbial mass and pH were evaluated over 111 consecutive days. The optimum N:P ratio was the determined to 2:1 molar ratio in form of 6000mgN:3000mgP.

TPH concentration was reduced by 73% from the initial concentration within the first 74 days. Beyond 74 days there were no significant changes in the TPH concentration and this was attributed to the presence of more complex insoluble hydrocarbons which needed more time and an additional bio surfactant to complete mineralization.

The conclusion was that a combination of natural attenuation and biostimulation methods can be used to bioremediate Murowa hydrocarbon contaminated soils using the 2:1 molar ratio of what.

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1 CHAPTER 1: INTRODUCTION

1.1 Background and motivation

Soil pollution due to petrochemicals is a serious problem that has attracted considerable human attention in the past two decades because of the long term effects on terrestrial, aquatic ecosystems and groundwater quality (Hollinger *et al.* 1997; Prakash *et al.* 2014). Increased incidents of petrochemical spills occur due to industrial discharges, disposal of wastewater, transportation, maintenance of vehicles and mining operations (Vidali, 2001; Okieimen and Okieimen, 2004; Wang *et al.* 2010; Sharma *et al.* 2014).

Petrochemicals are complex mixtures which contain simple linear or saturated hydrocarbons, branched hydrocarbons also known as unsaturated hydrocarbons as they contain double or triple bonds, cyclic alkanes, alkenes and arenes (aromatic) compounds. The compounds have low water solubility, a high capacity to bond themselves (a process known as catenation) and very stable aromatic rings (Van Hamme *et al.* 2003; Yemashova *et al.* 2007; Mbandinga *et al.* 2011; Olajire and Essien, 2014). They comprise numerous harmful compounds which include well known solvents like benzene, toluene, xylene, and compounds such as ethylene dibromide (EDB) and organic lead that are added to the hydrocarbons during manufacturing processes. These compounds have effects such as cancer, modifying the genetic information (DNA) in humans and animals as well as damaging the foetus during animals and human pregnancy (Bumpus, 1989; Clemente *et al.* 2001; Cerniglia *et al.* 2001; Olajire *et al.* 2007; Olajire *et al.* 2008; Cao *et al.* 2009; Haritash and Kaushik, 2009; Pathak *et al.* 2010). These characteristics make them deemed as major pollutants that pose risky effects on humans and living organisms in the environment (Refaat *et al.* 2008).

The hydrocarbon pollutants take long periods of time to degrade under natural environmental conditions. Soil properties such as the particle size, large surface area and the ability to bind the pollutant or hydrocarbon, water repellency and structure make the soils very difficult to clean to restore them to natural state reference. The hydrocarbon compounds bind to soil components and they are difficult to remove or degrade thereby leading to soil and ground water pollution (Barathi and Vasudevan, 2001; Chadran and Das, 2011).

Petrochemical contamination causes air, soil and water pollution which results in damage of ecosystem by accumulation in plants and animals tissues (Sharma *et al.* 2014; Dao *et al.* 2014; Camus *et al.* 2015; Shahi *et al.* 2016). Hydrocarbon contamination in soils is accompanied by depletion in both nutrients and oxygen levels in the soils (Amadi, 1990;

Atlas, 1991; Atlas and Bartha, 1993; Bossert and Bartha, 1994). Significant incidents sited in literature include the oil tanker Exxon Valdez oil spill incident of 1989 which occurred in Prince William Sound and the Gulf of Alaska where immediate effects included death of many seabirds, sea and river otters, harbor seals and bald eagles etc. as a result of asphyxiation due to sudden drop of oxygen (Atlas and Bartha, 1998; Brady and Weil, 2002 and Atlas and Hazen, 2011). The deep oil spill horizon also referred to as the BP oil disaster which occurred in 2010 in the Gulf of Mexico is another case where such impacts have been recorded and are still being felt years after the incident occurred (Atlas and Hazen 2011).

Conventional remediation methods for spills often involve excavation and burial of contaminated soil in hazardous waste landfills, physical and chemical approaches such as dispersants, skimmers and incineration (Nies and Mesarch, 1996, Chien *et al*. 2010). These methods have limitations as they involve transfer of pollutants from one environmental compartment to another which actually create significant risks in the excavation, handling and transportation of hazardous materials. Apart from transfer of risk, the conventional remediation methods are also limited to physical and chemical approaches such as dispersant, booms, skimmers and *in situ* incineration which are expensive and their by-products would possibly result in secondary contamination of soil and water which again would need further post treatment (Hamzah *et al.* 2013). Landfilling methods are actually expensive in that they fill up quickly; prompting the construction of new ones which is generally expensive especially in areas where there is large population. The cap and contain methods are interim solutions since the contaminate would remain onsite, requiring monitoring and maintenance of the landfills and isolation barriers long into the future which is very costly and rendered high liabilities to companies

To overcome the limitations associated with containment methods, bioremediation emerged as a highly promising secondary treatment option for oil since its first application after the 1989 Exxon Valdez spill (Bragg *et al.* 1994). Bioremediation describes various technologies and practices that make use of natural biological processes to clean up pollution (Sharma *et al.* 2014). It makes use of primarily indigenous living microorganisms, to degrade contaminants into less toxic forms (Medina-Bellver, 2005; Mandri and Lin, 2007). The technology is based on activation of microbial degradation of pollutants in contaminated sites by optimising environmental factors such as nutrient concentrations, water content, pH, oxygen supply, availability of contaminants to microorganisms and temperature (Wolf *et al.* 1988; Arndt *et al.* 1990; DECHEMA 1992; Alef *et al.* 1993; McDonald and Rittmann, 1993; Muller *et al.* 1993; Skladany and Metting, 1993).

Bioremediation technologies are generally classified as *in situ* or *ex situ*. *In situ* bioremediation involves treating the contaminated material at the site whereas *ex situ* involves the removal of the contaminated material to be treated elsewhere (Sharma *et al.* 2014). Types of bioremediation technologies for soils contaminated with petrochemicals include phytoremediation, land-farming, bio augmentation, and bio stimulation.

Land farming is a bioremediation treatment process which is performed in the upper soil zone or in biotreatment cells. Contaminated soils, sludge, or sediments are incorporated into the soil surface and periodically turned over or tilled to aerate the mixture. Additives such as fertilizers and manure are applied into the land farm to increase the rate of bioremediation (Cookson, 1995; Chukwuma *et al.* 2012; Adams *et al*. 2015).

Bioaugmentation is defined as the addition of known biodegrading microbial strains to contaminated soil or water to supplement the existing microbial population (Tanee and Kinako, 2008, Das and Chadran 2010; Adams *et al*. 2015). Bioaugmentation principles involve studying the indigenous varieties present in the location to determine if biostimulation is conceivable. If the diverse indigenous species do not have the metabolic ability to perform the remediation process, exogenous varieties are introduced.

On the other hand biostimulation involves the modification of the environment to stimulate growth of existing bacteria capable of bioremediation. Growth limiting factors such as nutrients (phosphorus, nitrogen or carbon) are added to enhance growth of microbial population (Das and Chadran 2010, Sharma *et al*. 2014, Adams *et al*. 2015). The primary advantage of biostimulation is that biodegradation will be undertaken by native microorganisms that are well-suited to the sub-surface environment, and are well distributed within the sub-surface (Vidali 2001; Adams, 2015). The primary disadvantage is that the delivery of additives in a manner that permits the additives to be readily available to subsurface microorganisms is based on the local geology of the sub-surface. Tight and impermeable sub-surface lithology i.e. tight clays or other fine-grained material make it difficult to spread additives across the affected area. Fractures in the subsurface generate preferential pathways in the sub-surface which additives follow preferentially, preventing even distribution of additives (Vidali, 2001).

In comparison with other remediation techniques such as physical, and chemical treatments, bioremediation has proved to be ecological (natural), cost-effective and most favourable technique. Its most important advantages are that: it is a natural process, resultant products are harmless i.e. carbon dioxide and water and the decontaminated soil can be recultivated/reused (Leahy and Colwell, 1990; Rosenberg and Ron, 1990, Balba *et al*. 1998;

Bouchez- Naitali *et al*. 1999; Nweke and Okpokwasili, 2004; Kaplan and Kitts, 2004; Quatrini *et al*. 2008; Prakash *et al*. 2014).

According to Boopathy and Manning (1999), bioremediation has numerous applications, which include the cleanup of groundwater, soil, lagoons, and sludge and process waste streams. Conditions for bioremediation are optimised by soil water conditioning, aeration, temperature, pH, and nutrient addition. Use of inorganic fertiliser is said to stimulate indigenous microbial population or activity in soil contaminated with petroleum hydrocarbons (Cunningham and Philp, 2000; Hamzah *et al.* 2014).

Many scientific reviews have covered various factors that influence the rate of hydrocarbon degradation (Zobell, 1946; Atlas, 1981; Atlas, 1984; Foght and Westlake, 1987 Leahy and Colwell, 1990; Atlas and Bartha, 1992; Chukwuma, 2012; Wang *et al.* 2010). The presence of microorganisms with appropriate metabolic capabilities, optimal rates for growth of microbial community, adequate nutrients, oxygen, pH range between 6 and 9 and physical and chemical properties of oil and contaminated soil were found to be crucial for the success of hydrocarbon degradation.

Further studies need to be done to investigate the hydrocarbons susceptibility to microbial attack and it has been proven to differ by type of hydrocarbon type. Susceptibility of hydrocarbons to microbial attack is generally ranked as follows;

Straight chain alkanes>branched alkanes>small aromatics>cyclic alkanes (Perry, 1984; Ulrici, 2000). According to Atlas and Bragg, (2009), high molecular weight polycyclic aromatic hydrocarbons (PAH) may not be degraded at all.

Other studies conducted have shown that hydrocarbons in the environment are primarily degraded by bacteria, fungi and yeast. Efficiencies reported ranged from 6% - 82% for soil fungi (Jones *et al*. 1970; Pinholt *et al*. 1979), 0.13 – 50% for soil bacteria (Jones *et al*. 1970; Pinholt *et al*. 1979) and 0.003 – 100% for marine bacteria (Mulkins *et al*. 1974; Halloway *et al*. 1980).

There are certain genera of bacteria, fungi and yeast that have been isolated and well known for effective biodegradation of hydrocarbons and these include *Pseudomonas, Mycobacterium, Arthrobacter, Burkholderia, Sphingomonas, Rhodococcus* just to mention a few. In the tropical climates, e.g. in Lagos Nigeria, nine bacterial strains from the above mentioned genera were isolated from a stream polluted with hydrocarbons (Adebusoye *et al*. 2007). Fungal genera capable of degrading hydrocarbons effectively included *Amorphoteca, Neosartorya, Talaromyces* and *Graphicum* whereas for yeast genera, *Candida, Yarrowia and Pichia.* (Chaillan *et al*. 2004).

Studies are still in progress to identify opportunities for improvement and to further understand the mechanism of biodegradation as it depends on indigenous microorganisms to transform or mineralise the organic contaminants (Wang *et al*. 2010).

In a research study to investigate natural attenuation and the role of indigenous soil bacteria to biodegrade diesel contaminates soil and water in a boreal environment in Finland, Kauppi (2011) showed that bio-stimulation enhanced bioremediation and that indigenous diesel degrading bacteria are present in boreal environments, and hence microbial inocula are not always required. It was further demonstrated that in terrestrial environment experiments, the combination of addition of slowly released nitrogen and aeration advanced hydrocarbon degradation. Previous contamination of soil proved to give bacterial community potential for rapid adaptation and efficient degradation of the same contaminant (Kauppi, 2011).

Whilst a number of microbial strains have been identified and isolated, factors limiting degradation are also known which include pH, temperature, and oxygen nutrient supply. Wang *et al*. (2010) argued that there are several approaches to improving efficiency of hydrocarbon biodegradation and bioremediation of oil pollutants. The common approach was the supply of solid oxygen and adjusting carbon, nitrogen and phosphorus ratio to optimise microbial processes which was found to improve the effectiveness and efficacy of bioremediation of crude oil pollutants.

With respect to performances of various indigenous bacterial strains, the genus Pseudomonas, particularly *Pseudomonas putida F1* is one of the most researched hydrocarbon degrading bacterial strains. The rate of naphthalene bioremediation using *Pseudomonas putida* was found to be as high as 61mg/L/hr (Yu *et al*. 2006). Other studies that were conducted in Japan (Wongsa *et al*., 2004) revealed that strains of *Pseudomonas aeruginosa WatG* and *Serratia marcescens HokM*, could degrade 90 – 95% of total concentration of diesel oil in soil and kerosene within 2 – 3 weeks and petroleum hydrocarbon (TPH) could be degraded by 72% in four weeks (Moriya and Horikoshi, 1993). According to Christova *et al*. (2003), a strain of *Bacillus subtilis* was found to be good at degrading hydrocarbons with degradability of 98% n-hexadecane and 75% naphthalene.

After identification of individual strains, some scientists concurred that biodegradation of hydrocarbons could be enhanced if a combination of bacteria is used than individual strains. In a research conducted by Satiskumar *et al*. (2008), a combination of four bacterial strains was used and they achieved a degradation maximum of 77% for crude oil. The degradation by the individual strains were as following; 69% by *Pseudomonas sp. BPSI* - 8, 64% by

Bacillus sp., 45% by *Pseudomonas sp. HPS2 – 5*, and 41% by *Gorynebacterium sp. BPS2*-6 at 1% crude oil concentration.

Biodegradation time and efficiency used in the studies conducted in the field, microcosms or batch experiments varied from one researcher to another. Atlas and Bartha (1971) observed that degradation by bacteria can start in 2 – 4 day period and reach its maximum within two weeks basing on continuous monitoring of CO_2 evolution. In another study related to time and efficiency conducted by Berwick, (1984), 98% of carbon tetrachloride extractable oil was degraded over 83 days and degradation percent was in the following order: aromatics>saturates>heterocyclics>asphalts though the degradation rate for all of these was above 94%.

Abiotic factors (environmental conditions) such as temperature, pH, oxygen supply, and nutrient balance were proved to play a crucial role in the effectiveness of biodegradation. According to Sathishkumar *et al.* (2008), a temperature of 35^0C and pH 7 were found to be optimum for maximum degradation of crude oil when using a consortium of *Pseudomonas* strains. A 79% removal of PAH was achieved in 60 days with the addition of nutrients while 30% with indigenous microflora alone (Mittal and Singh, 2009). Evidence in improvement of hydrocarbon degradation by aeration or oxygen supply was observed (Soli and Bens 1971, Atlas 1991, Miethe *et al.* 1993, Perissutti *et al.* 2003; Wang *et al*. 2011).

All these previous studies were indicative of an improvement of hydrocarbon degradation efficiency and effectiveness, whether in cases where indigenous microbial strains were isolated and a consortium was used or where oxygen/aeration was applied to adjust nutrient balance and to optimize C: N: P ratio under optimum environmental conditions such as temperature and pH. Oxygen balances out nutrients through processes such as nitrification and denitrification where ammonium nitrogen, nitrite or nitrate nitrogen react with oxygen reducing the concentration of at the same time react with organic compounds releasing carbon dioxide gas. This means that if oxygen levels are low such processes are impaired resulting in high nitrate or nitrogen values in the soil. Oxygen supply therefore requires to be supplied in form of a slow release source such as solid peroxygen e.g. CaO_2 or MgO_2 (Wang *et al.* 2011).

All this substantial literature on biodegradation of hydrocarbons show that most studies were conducted in boreal, temperate, tropical and arctic regions pointing to the fact that such studies are limited in the tropical savanna regions of Zimbabwe particularly in diamond

mining areas and research on bioremediation of hydrocarbon contaminated soils is still limited in Zimbabwe.

It is also noteworthy that no indigenous bacteria, fungi or yeast genera like *Pseudomonas* species have been identified for biodegradation of oil contaminated soils in Zimbabwe. Research regarding manipulation of soil nutrients, physical and chemical properties to enhance bioremediation of oil contaminated soils is also still limited. Accordingly, information on the impacts of oils on soil physical and chemical properties in this case particularly is yet to be understood.

The mining activities at Murowa Diamonds are highly mechanised with heavy mobile equipment for activities such as loading and hauling, drilling and blasting which range from excavators, front end loaders up to articulated dump trucks. There is also a variety of light vehicles and buses for site transportation services. The machinery consumes hydrocarbon oils and diesel fuel for day to day operations. Maintenance of heavy mobile equipment is done at a site workshop and is cleaned at a wash down facility. During the operations incidental spills and leaks from stationary vehicles are experienced and as such the soils are excavated and stock piled at the hazardous waste area in a concrete bund wall. Furthermore during cleaning or maintenance oils flow with muddy water and collect into a sump. Water and oil proceed to an oil trap but the sludge that remains in the sump will be contaminated with oil. The sludge is dried at the bay and is sent to the hazardous waste area for stock piling.

1.2 Problem statement

Since 2004, Murowa Diamonds has been excavating oil contaminated soil and storing it in a concrete lined area designated for hazardous waste. Though the waste storage area is concrete lined and bunded, the soil has been accumulating there for over a decade now and hence needs to be treated to return it to its natural state and use. The storage area is filling up and shortly there will be need to find some other area for disposal. Storage is only a temporary or a stop gap measure since the contamination will be at the location, and would require monitoring to prevent pollution of the surrounding areas which may attract cleaning or corrective actions cost with potential for litigation if not managed well.

Hydrocarbons are known to have persistent impacts on the soil. In the case of Murowa Diamonds the degree of contamination by diesel and oils has not been assessed and hence the impacts of Murowa activities have not been investigated. No remediation methods have been identified for proper treatment and cleaning of the Murowa contaminated soils. Rio Zim (RZ) Murowa Holdings which owns Murowa has got generic guidelines on remediation of

hydrocarbon contaminated soils which have not been tested and applied for Murowa area. Murowa also has an environmental standard on management of hazardous material and contamination control of which requirements are not fully met. Zimbabwean regulations i.e. statutory instruments 10 and 12 of 2007 under the Environment Management Act of 2002 require the polluter to ameliorate any pollution or contamination as a result of his or her activities. Murowa in its closure plan committed itself to exit the mine area restored and in a habitable state for future land use as part of its environmental stewardship and biodiversity policies and hence the need for amelioration.

1.3 Justification

The study sought to investigate the effects of petrochemical hydrocarbons on Murowa soils, possibly identifying microbial genera that are available for biodegradation of hydrocarbon and determine factors that can possibly limit biodegradation process which could include oxygen, nutrient availability and their ratios. This is envisaged to assist in understanding the magnitude of the problem and identification of possible effective conditions that are appropriate for bioremediation of Murowa soils which will in turn assist in soil quality restoration. If these are implemented all the legal and other requirements that the business has to comply to are met.

In terms of scientific research, the research sought to improve, validate and substantiate opportunities for improvement of hydrocarbon degradation efficiency and its effectiveness using present indigenous microbial strains, by optimising oxygen/aeration, temperature and nutrients. Bioremediation techniques that make use of indigenous microorganisms are cost effective which supports the company's environmental stewardship standards and requirements as well as the green industry movement.

The study sought to determine the most effective combination of N: P nutrient ratio for treating hydrocarbon contaminated soil from Murowa activities that could increase the biodegradation of hydrocarbons and allow for prediction of the rates for clean-up. The study also sought to determine the optimum environmental conditions favourable for enhanced growth of hydrocarbon degrading microorganisms.

The study outcomes were expected to assist in setting up of an ex-situ soil remediation site which would be used as the final disposal area for contaminated soils for Murowa Diamonds mine. Furthermore, the study was expected to substantiate literature on biodegradation of hydrocarbon contaminated soils in the semi-arid tropical savannas of Zimbabwe, particularly

the diamond mining area. The study was anticipated to assist to add onto research information on petrochemical pollution remediation studies.

1.4 Specific research questions

The research sought to address the questions as follows;

1. What is the composition and impact of hydrocarbon contamination on soil physical and chemical properties at Murowa Diamonds mine?
2. Which indigenous microorganisms' genera have adapted to contaminated soil conditions?
3. How does soil N: P and moisture affect hydrocarbon degradation at Murowa Diamonds mine?

1.5 Aim of the Study

The study aimed at evaluating the potential for bioremediation of hydrocarbon contaminated soils at Murowa Diamonds mine in Zimbabwe. The study also anticipated to investigate the impact of hydrocarbons contamination on Murowa soils, as well as identifying indigenous microorganisms' genera that can degrade hydrocarbons and determining the effect of N: P ratio on biodegradation of hydrocarbons.

1.6 Objectives

The purpose of the study was to;

1. Determine the composition of the petroleum hydrocarbon in soil and possible impacts on physical and chemical properties of the soil
2. Determine the various hydrocarbon biodegrading microorganisms' genera present in contaminated soils.
3. Investigate the effect of different levels of C: N: P ratios on degradation of hydrocarbons under laboratory (controlled conditions).

1.7 Research approach

It was quantitative research approach where uncontaminated and contaminated soil samples were physically and chemically characterized. Characterisation exercise tested possible nutrient manipulations that could activate biostimulation to initiate bioremediation. The study used a combination of laboratory and field pilot experiments to address the knowledge gap. Data was statistically analysed to determine the effect of nutrient manipulation on hydrocarbon degradation.

1.8 Description of study site

Murowa Diamonds Mine is located in the Murowa ward of Zvishavane district, adjacent to the Runde River, off the Rutenga rail-road link. Murowa GPS Position is 20^0 South, 30^0 East. The mine has been in operation since July 2004 and is located in the south-western region of Zimbabwe. Zvishavane is the nearest town, which is approximately 60km from the mine site by road. **Fig. 1** shows the location of Murowa Diamonds in Zimbabwe.

The Murowa Diamonds kimberlites lie between two main structural features. Immediately to the west, running at around 330 degrees is a dyke of doleritic composition, which forms part of the Sebanga Poort Dyke System. To the east, is a shear zone which can be traced across the granites up to Tokwe block and south to Buchwa Greenstone.

Figure 1- Map of Zimbabwe showing location of Murowa Diamonds extracted from the Famous diamonds mines chart – www.etoolsage.comDiamonds are found on the Cambrian aged Kimberlite hosted by granite greenstone terrain of the Archean Zimbabwe Craton. Situated 220km from Venetia and 70km from River Ranch within the Limpopo mobile belt that separates the Zimabwean and Kaapvaal cratons (Smith *et al*. 2009)

The annual average rainfall recorded at the mine site (580.2mm) is slightly higher than the long-term average observed within the region (558.0mm). There are distinct wet and dry seasons at the mine site which also characterise the region four (4) and five (5) of the

Zimbabwe farming and rainfall regions. The annual total evaporation at the mine is 1,741mm which is lower than the long-term annual average evaporation of the region (1,911mm). The dominant trade wind is from the south easterly direction. Temperatures at the mine site are slightly higher due to the difference in altitude, namely 225m lower than Zvishavane. Maximum temperatures are above 28^{o}C in the period September to January, with June and July experiencing the lowest temperatures 8.1 and 9.6^{o}C respectively at the mine site (Murowa Diamonds project baseline EIA, 2001).

1.8.1 Soils

Murowa soils are moderately acidic to slightly alkaline and are low in humus (organic matter). The area experiences temperatures ranging from about 8 - 40^{o}C which are optimal for biodegradation. However, since the soils are acidic to slightly alkaline and low in humus the factors that could possibly limit biodegradation are microbial communities, oxygen and nutrient supply.

1.8.2 Hydrocarbon pollutant composition

There are various oils used as lubricants, fuels, adhesives and organic detergents which are used at site for heavy mobile equipment maintenance. The oils include 80W 90,15W40 gear oil, Super 700 engine hydraulic oil, SAE30 hydraulic oil, ATF hydraulic oil, 15W40 engine oil. Lubricants include grease (GP), multipurpose grease, hyspin 46/88 and synthetic waterproof EP grease. Detergents used are degreaser R and various hand cleaners. Diesel is the only fuel for all the vehicles at site. Murowa contaminated soils are likely to contain any hydrocarbon present in the substances which may include benzene, cyclohexane, ethylbenzene, decane, undecane, naphthalene, dodecane, 1,7 iso phenol, 6 methoxybicyclo 2, ethanol, tetradecane, hexadecane, nonacosane, PAHs, polyalphaolefins (PAOs), poly internal olefins, esters, polyalkylene glycol, butylene, phosphate esters which are attributed to the substances used at site.

2 CHAPTER 2: LITERATURE REVIEW

2.0 Introduction

This chapter basically reviewed the effects of petroleum hydrocarbons contamination and pollution on soil properties and how the pollution can bioremediated using available bioremediation technologies focusing as well on factors influencing bioremediation.

2.1 Soil contamination by hydrocarbons

If a hydrocarbon spill on soil occurs, oil is absorbed by plants and soil particles and hence its spread is limited unlike in water where it spread very fast. Individual petroleum hydrocarbons behave differently. Some may be very mobile e.g. BTEX compounds and some like PAHs bind strongly to the soil particles very close to the point source. It has been reported that some remain entrapped in the organic phase which means that they will be immobilized and as a result they will not move out of their percolated location or point (Chukwuma *et al.* 2012;). Hydrocarbons have been said to be persistent in the environment and that some of them may take greater than seven years to decompose (Plice 1948; Rowell, 1977; McElroy, 1989; Lafortune *et al.* 2009; Chukwuma *et al*. 2012). Hamnrick *et al.* (1980) determined that petroleum hydrocarbons persist longer in sediments where there is limited oxygen than in aerated surface oil layers. The other component is believed to be bio - accumulated in soil biota i.e. organism and plants (Lafortune *et al.* 2009).

Hydrocarbons in soils can be transformed by hydrocarbon degrading microorganisms under aerobic or anaerobic conditions (Leahy and Cowell, 1990; Leahy *et al.* 2002). Under aerobic conditions, hydrocarbons are oxidized by bacteria using oxygenases. Some bacteria have multiple pathways for oxygenases for dissimilation of the compounds. The multiple oxygenases accommodates further expansion of the substrate range available for co-oxidisation

In anaerobic conditions the bacteria degrade hydrocarbons through reduction. The anaerobic bacteria has been investigated to occur where manganese (Mn^{2t} and iron (Fe^{2t}) II ions are the terminal electron acceptors in sediments or anoxic zones (Atlas, 1991; Essaid *et al.* 1995). Anaerobic degradation was first demonstrated by sulphate reducing bacteria, and since then sulphate, nitrate and chlorate reducing bacteria have been grown anaerobically with the saturated hydrocarbons. The bacteria will be utilising the saturated hydrocarbons as the sole carbon and energy sources (Savage *et al*. 2010; Callaghan *et al.* 2009; Gieg *et al.* 2008 and Jones *et al.* 2008).

2.2 Effects of hydrocarbon contamination on soil properties

Oil concentration above 3% is known to adversely impact soil properties, soil life including the plant community (Baker, 1976; Amadi *et al.* 1993; Osuji *et al.* 2005). Effects of contamination can occur in many dimensions and they are both positive and negative. Some of the effects include limited aeration, water and nutrient availability, as well as improvement on the nutrient content though it is realized after a longtime.

Petroleum contamination in soils or sediments limits oxygen and water availability which in turn affect the availability of nutrients in the form of nitrogen and phosphorus (Njoku 2009; Wang *et al.* 2013).

Effect of hydrocarbon contamination on nutrients is dependent on proportionality of the quantity of oil spillage and the concentration of manganese and ferrous elements. If the levels are high they can become very toxic and as a result plant growth is adversely impacted due to alteration of the nutrients status of the soil (Chokwuma, 2012).

A study conducted by Osuji and Nwoye, (2007), confirmed that petroleum hydrocarbons impact fertility of the soil which means that the ability of soil to support growth of plants on sustained basis under certain climatic conditions and appropriate properties of the land is compromised. They reported that soil pH increased up to about 8.5 and levels of both carbonates and bicarbonates increased which hampered plant growth. Petrochemical contamination alter soil fertility as they reduce bioavailability of organic matter to plants when they are adsorbed to the organic matter, in addition as they filter down the soils they leach nutrients resulting in soil pH changes, reduced cation exchange capacity, salinization and water logging due to the water repellent effect (Chokwuma, 2012).When soils are contaminated they become water repellent or hydrophobic (Takawira *et al.* 2014) which means than the microorganisms in the soil can no longer access adequate moisture required for their activities.

On a positive note, it has been reported that the long lasting effect of hydrocarbons in soils is that it results in nutrient supply (McGill, 1976; Chokwuma, 2012). It was found out that hydrocarbon contaminated fields remained barren for some time estimated at greater than seven years and then become richer in nutrients than uncontaminated areas (Plice, 1948; Rowell, 1977). This was found to happen because when hydrocarbons are broken down they are converted to soil organic matter thereby improving the nutrient content of the soil (Chokwuma, 2012).

Studies to assess the impact of hydrocarbon on soil properties and the extent of pollution were conducted. These considered determination of the concentration of hydrocarbons and

comparing them with data from uncontaminated sites or regulatory acceptable pollution limits (Osuji and Nwoye, 2007). Other reviews done determined factors that can be considered for impact assessment on soil fertility properties which included macro nutrients N, P, K, soil pH and conductivity, moisture content, Total petroleum hydrocarbon (TPH), Total organic carbon (TOC) and the concentration can then be compared with guidelines. For example, the Nigerian government developed environmental guidelines and standards for petroleum industry in Nigeria which are used as reference point. These guidelines and standards specify acceptable levels of concentration of pollutants or nutrients (e.g. acceptable NPK levels are 15000, 200 and 10000 respectively which support growth) to assist in determining the level of impact.

2.1 Factors influencing biodegradation of hydrocarbons in soil

A number of reviews have been conducted to determine factors that influence the rate of hydrocarbon degradation in soils (Zobell, 1946; Atlas, 1981; Atlas, 1984; Foght and Westlake, 1987; Leahy and Colwell, 1990; Atlas and Bartha, 1992; Sathishkumar *et al.* 2008; Wang *et al.* 2011; Kothari, 2013; Sihag *et al.* 2014). Factors include physical, chemical or biological environmental processes such as weathering, sorption, evaporation or volatisation, leaching and photo oxidation (Haritash and Kaushik, 2009). Volatilisation has been found to contribute about 15-60% loss of fuel hydrocarbons including semi volatile 4- and 5-ring PAHs into the atmosphere (Huesemann, 1995; Hawthorne and Grabanski, 2000; Salanitro, 2001; Mphekgo *et al.* 2004; Haritash and Kaushik, 2009; Sharma *et al*. 2014).

Other factors that were found to be crucial for successful hydrocarbon degradation include availability of microorganisms with suitable metabolic capabilities, optimal rates for growth of microbial community, adequate nutrients, oxygen, pH range of 6 - 9 and physical and chemical characteristics of the petroleum hydrocarbons and contaminated soil. According to Vidali, (2001) optimum environmental conditions affecting hydrocarbon degradation and microbial activity during bioremediation are shown in **Table 1.**

Table 1 - Environmental conditions affecting hydrocarbon degradation in soils Source: (Vidali, 2001; Agothos and Reineke, 2002)

Parameters	Microbial activity requirements	Optimum value for oil degradation
Moisture content	25-28% of water holding capacity	30-90%
Soil pH ranges	5.5 - 8.8	6.5 - 8.0
Oxygen availability or concentration	Aerobic, minimum air filled pore space of 10%	10-40%
Nutrient Content	N &P for microbial growth	C:N:P = 100:10.1
Temperature	15-45 ^{0}C	20-30^{0}C
Concentration of Contaminant	Not too toxic	Hydrocarbon 5-10% of dry weight of soil
Heavy metals concentration	Total content 2000ppm	700ppm
Soil type	Low clay or silt content	-

2.1.1 Temperature

The effect of temperature on biodegradation is influenced by factors such as the composition or chemical structure of the hydrocarbon and the microbial types present. Temperature has an effect on physical or chemical structure of petroleum hydrocarbon, metabolic processes as well as the microbial community composition (Crawford and Zhou, 1995; Walworth and Reynolds, 1995). High temperature cause higher viscosity and reduce volatisation of toxic alkanes. Water solubility increases resulting in biodegradation rates decreasing and degradation taking long. In terms of microbial processes, elevated temperatures usually enhance enzyme activity and resulting in improved rate of metabolism. Low temperatures decrease the rates at which volatilization and enzyme processes occur.

The biodegradation rate of hydrocarbons has been found to increase from psychrophilic to mesophilic temperatures (Rike *et al.* 2009). Optimum temperatures have been found in the range 25^{o}C - 40^{o}C (Van Hamme *et al.* 2003). Some species like *Bacillus sp.* can grow in petroleum hydrocarbons with temperature ranging between 45-70^{o}C (Klug and Markovertz, 1971; Sorkhoh *et al.* 1993). Biodegradation activities even occur in most environments with extreme temperatures, for example, in the Arctic zone where temperatures are around 5^{o}C (Mulkins and Stewart, 1974) and also in the tropical areas (Teralmoto *et al.* 2009).

Temperature can indirectly impact other environmental factors that may have direct effects to biodegradation like moisture or water availability. An example of such a scenario was experienced at the Prudhoe Bay Marine ice ecosystems in Arctic where crude oil was degraded extremely slowly due to limited availability of liquid water regardless of low temperatures (Atlas, 1978).

2.1.2 Nutrients

Nitrogen and Phosphorus nutrients are required by microorganisms for decomposition of hydrocarbons and their availability is important for bioremediation. Addition of these essential nutrients (biostimulation) has proven to be effective (Hollender *et al.* 2003; Semple *et al.* 2006; Walworth, 2007). The availability of nutrient largely depends on the physical condition of the spill whether it is in water or on soil or in dissolved form or slick. Research has shown that nutrient amendments can significantly enhance petroleum hydrocarbon degradation both in water and soil (Xu and Obbard, 2003; Liang *et al.* 2011; Tyagi *et al.* 2011). Low concentration of nutrients conditions have been found to limit nitrogen and phosphorus availability in soil (Leahy and Colwell, 1990; Leys *et al*. 2004)

In theory, according to Rosenburg and Ron, (1996), it has been estimated that 150mg Nitrogen and 30mg Phosphorus are utilised to convert a gram of hydrocarbon to cell material. Based on this, optimal C: N: P mole-ratio recommended for enhancing removal of hydrocarbon is 100.10.1 (Malina, 1999). Malina (2007) noted that soil physicochemical properties tend to affect the effectiveness of nutrient sources since the soil environment is complex and heterogeneous. Zawierucha *et al*. (2008) compared various nitrogen and phosphorus nutrient sources with different C: N: P ratios to improve biodegradation of hydrocarbons in soil and found out that the maximum improved rate of biodegradation (2-26 times higher than intrinsic biodegradation rates) was 100:10:5. Ubochi *et al.* (2006) studied the feasibility of bio stimulation alternatives in hydrocarbon contaminated soils with different NPK concentrations and established that 60g of NPK fertiliser was the best biostimulation option.

Nutrient addition is done to augment deficient inorganic nutrients levels depending on hydrocarbon concentration. The N: P addition however, was found contradictory as it affected both negative and positive kinetic aspects of hydrocarbons degradation e.g. lag time and the extent of biodegradation (Alexander 1994; Baker and Herson, 1994; Carmicael and Pfaender 1997; Johnson *et al*. 1974; Johnson and Scow 1990; Manila and Alexander 1991; Morgan and Warkison 1992; Ward *et al*. 1999). Some researchers have however, experienced inconsistent results after addition of nutrients which indicated that the degradation process was not commensurate with the averaged optimal nutrient of 100:10:1(Leys *et al.* 2004).

2.1.3 Oxygen

According to Cernigilia, (1984), Atlas, (1995) and Boufadel *et al.* (2010), major hydrocarbon degradation pathways involve oxygenases and molecular oxygen which indicates that oxygen is important for the microbial hydrocarbon degraders. Though there are microorganisms capable of degrading hydrocarbons under anaerobic conditions, oxygen availability can still be a limiting factor in biodegradation process. For example, oxygen can severely limit hydrocarbon biodegradation in areas like basins or sediments that are anoxic, stratified lakes hypolimnion, and benthic sediments (Boufadel *et al.* 2010). Oil degrading bacteria consume very high levels of oxygen even in water where it is readily available. Slow oxygen diffusion rates and its limited solubility in soil may limit oxygen availability. Oxygen diffusion in soil is affected if soils are water-logged or flooded. Addition of bulking agents and tilling at intervals have been used to improve oxygen diffusion and increase soil porosity which would then allow an increase in oxygen consumption rate due to improved bacteria digestion

(Devinny and Islander, 1989). According to Atlas (1991), maintenance of aerobic condition is important in bioremediation as most processes are aerobic.

2.1.4 Salinity and Pressure

These factors are mostly confined to salt-water saline lakes or deep seas where high levels of hydrostatic pressure are existent. Generally, there is an inverse relationship between rate of biodegradation and salinity i.e. when salinity increase biodegradation decreases. For example, in marine ecosystems it was found out that in the benthic zone experienced the least microbial activities partly due to high pressure that limits microbial processes (Ward and Brock, 1978; Shiaris, 1989; Shin and Pardie, 2001). High salt concentrations in soils have been found to be inhibitory or lethal to microorganisms because they have been found to have an effect on the osmotic balance of microorganisms and their enzyme activities (Chukwuma *et al*. 2012). Soils with high electrical conductivity resulted in retarded biodegradation rates. An EC of 40mmhos has been found to cease microbial activities according to McMillen, (1994; 1995). The methodology for bioremediation of salt hydrocarbon contaminated soils is the same as for ordinary soils except that they require additional treatments (Chukwuma *et al*, 2012).

2.1.5 Microbial communities composition

A wide range of microbes have showed ability to degrade petroleum hydrocarbons (Atlas, 1981; Olivier *et al.* 2000; Van Hamme *et al.* 2003; de Brito *et al.* 2004; Sylvia *et al.* 2005; Santos *et al.* 2006; Teralmoto *et al.* 2009). Among these bacteria and fungi appear to be predominant degraders in hydrocarbon contaminated ecosystems. Contamination along with other physical and chemical properties influence microbial species composition (Saul *et al.* 2005; Grant *et al*. 2007). Competition within and between species affects the microbial community composition. Microbial community composition is also influenced by the soil pore size which provides surfaces for colonization. Pores larger than 2μm have less bacterial biomass (Johnsen *et al.* 2005) which means that where there are large soil pore sizes there are fewer microbes.

2.1.5.1 Bacteria

Over 100 microorganisms' genera with ability to degrade recalcitrant compounds in petroleum hydrocarbon have been isolated to date. **Appendix 1** shows some identified hydrocarbon degrading genera. These include but not limited to the following; *Alpha, Beta*

and Gammaproteeobacteria, Gram positives, Flexibacter – Cytophaga- bacteriods (Teralmoto *et al.* 2009).

Species like *Pseudomonas, Mycobacterium, Haemophilis, Rhodococcus, Paenibacillus* and *Ralstonia* have been extensively studied for their bioremediation capability. Carbonnoclastic bacterium, *Alcanivorax borkumensis* was first to be sequenced (Santos *et al.* 2006) to appreciate its rare metabolic ability in hydrocarbon degradation. The bacterium utilises hydrocarbons as its major source of carbon and energy. It is capable of breaking down alkanes as well as spreading a bio surfactant that aids more oil to be bioavailable for other microbes.

2.1.5.2 Archea

These are common in hydrocarbon polluted environments such as water reservoirs e.g. (lakes), crude oil underground storage activities, and acquifers. These range from A*rchaeoglobus fulgidus, Pyrococcus lithotrophicus,* and*, Thermococcus celer* (Olivier *et al.* 2000; de Brito *et al.* 2004).

With continual research, there is more probing into mechanisms and traits of oil degraders and better utilisation of and efficiency of the degraders is expected (Vila *et al.* 2010).

2.1.5.3 Fungi

Yeast and filamentous fungi have the capability to degrade oil hydrocarbons. Yeast and fungi species that degrade hydrocarbons include but are not limited to C*andida, Rhodotorula, Saccharomyces, Sporobolomyces* and *Trichosporon* (Atlas, 1981; Liu *et al.* 2009).

2.1.6 Microbial community dynamics

The overall soil microbial community structure can be altered by petroleum hydrocarbon contamination according to Cerniglia *et al.* (1980). Populations of petroleum degraders typically establish below 1% of the total microbial communities but when oil pollutants are present their population increase. Taxa of hydrocarbon degrading microorganisms was found to have a tendency of becoming dominant in environments that are contaminated with oils because of natural selection that occurs as a result of the pressure exerted by the contaminants. This may typically range from 10% or even up to 100% of the community (Tyagi *et al.* 2011).

2.1.7 Microbial degradation processes

Aerobic microbial biodegradation processes - occur in the presence of oxygen and the by-products of complete degradation are carbon dioxide and water. It has been proven that the ease of degradation is influenced by size and structure of the hydrocarbon compound.

The hydrocarbon compounds are converted to carbon dioxide and water if the degradation occurs in the presence of oxygen. The initial step of aerobic biodegradation involves the oxidation of substrate by oxygenases which require molecular oxygen (Wentzel *et al.* 2007).Oxygen availability in aerobic processes can possibly be a limiting factor in soils, sediments and aquifers and is also dependent on the type of soil i.e. if it is well aerated or well mixed with water (Kothari *et al.* 2004; Haritash and Kaushik, 2009; Chukwuma *et al.* 2012).

Anaerobic microbial degradation processes- exist and have been investigated to occur where manganese (Mn^{2t} and iron (Fe^{2t}) II ions are the terminal electron acceptors in sediments or anoxic zones (Atlas, 1991; Essaid *et al.* 1995). Anaerobic degradation was first demonstrated by sulphate reducing bacteria, and since then sulphate, nitrate and chlorate reducing bacteria have been grown anaerobically with the saturated hydrocarbons. The bacteria will be utilising the saturated hydrocarbons as the sole carbon and energy sources (Savage *et al*. 2010; Callaghan *et al.* 2009; Gieg *et al.* 2008 and Jones *et al.*2008).

2.1.8 Soil properties

Soil pore size influences biodegradation. Soil pores provide surface area for microbial colonies. Microbes are fewer in soils with large pore sizes than in soils with small pores. Predation reduces bacterial biomass especially in pores that are larger than 2µm (Johnsen *et al.* 2005).

According to Jangid *et al.* (2008), soil type defines the environment for the microbes. Different types of soils vary in terms of organic matter, particle size, pH, water holding capacity, available oxygen, nutrient content, redox potential and these factors control physical and chemical degradation (Margesin and Schinner, 2001).

2.1.9 Concentration of hydrocarbon contaminant

Concentration of petroleum hydrocarbon also determines the extent of breakdown of hydrocarbon from soils. High concentration can inhibit growth of microorganisms and concentration inhibition depends on the type of compound (Ijah and Antai, 2003). High degradation rates were observed in soils contaminated with 10%-20% hydrocarbon than those with 30-40% in a study done over a period of 12 months (Ijah and Antai, 2003).

Biodegradation has also been observed to depend on structure and physicochemical properties of the contaminant. Chemical properties include the presence and expression of appropriate degrading genes by the indigenous microbial community which is referred to as genetic potential (Aelion *et al.* 1989; Leahy and Colwell, 1990), bioavailability (Simoni *et al.* 2001), contaminant structure (Pitter and Chudoba, 1990) as well as toxicity (Rutgers *et al.* 1998). High concentration levels of hydrocarbon contamination can be toxic to microorganisms and hence biodegradation is possible when the concentration of the contamination is below threshold of toxicity (Yang *et al.* 2009), and in turn low concentration of contaminant may not provide enough carbon for efficient degradation (Boopathy, 2000).

2.2 Techniques for removal of hydrocarbons from contaminated soils

Several treatment technologies have been proposed for treating petroleum contaminated soils. Two basic processes are *in situ* and *ex situ* and they make of use of different technologies such as thermal and biological treatment, chemical extraction, soil washing and aerated accumulation techniques (De Pagter and Whiddon 1994; Ratliff, 1994; Nueumann-Hensel *et al.* 1999; Galvez *et al.* 2001; Yrum *et al.* 2004; Childs *et al.* 2004). There is no universal method that was been documented for the removal of hydrocarbons from contaminated sites.

In situ processes of decontamination are conducted in the subsoil either by biological means such as degradation by microorganisms, or a combination of chemical and physical processes like incineration, air sparging, and soil air suction extraction or through combinations. This technique is suitable and more effective on sandy soils than in soils containing clay. This technique can be applied with the help of spreading units in case of contamination at the surface and requires availability of sufficient amount of oxygen.

Ex situ processes involve excavation of contaminated soil to an off site remediation facility. It is applicable if the amount of contaminated soil is small or if contamination occurred at the surface in residential areas or industrial sites where *in situ* treatment is not possible. The advantage of *ex situ* process is more effective when compared to *in situ* because of controllability of many factors such as moisture, temperature, salinity, pH etc. Various *ex situ* methods range from steam stripping and combustion, extraction to biological methods. **Table 2** summarizes bioremediation strategies that are generally applied to hydrocarbon pollution remediation according to Vidali (2001).

Table 2 - Summary of bioremediation strategies applied in pollutant remediation

Technology	Examples	Benefits	Limitations	Factors to consider
In situ	*In situ* bioremediation can be done • Biosparging microorganisms • Bioventing in the presence of metals • Bioaugmentation • Controlling environmental conditions	• Most cost efficient • Non-invasive • Relatively passive • Makes use of Natural processes • Treats soil and water	• Environmental constraints • Extended treatment time • Monitoring challenges • Maybe influenced by environmental conditions such as Biodegradability of pollutants, Solubility of chemicals, Geological condition, Pollutants distribution	Biodegradative abilities of indigenous
Ex situ	• Landfarming • Composting • Biopiles • Biostimulation	• Cost efficient • Low cost • Conducted on site	• Space requirements • Extended treatment time • Need to monitor abiotic factors • Loss • Mass movement challenge • Bioavailability limitation	Same as above
Bioreactors	• Slurry reactors • Aqueous reactors	• Rapid breakdown kinetic • Balanced or Optimized environmental factors • Enhances mass movement • Effective use of surfactants • and inoculants	• Soil excavation required • Relatively high cost capital • Relatively high operating cost	• Bioaugmentation • Amendments toxicity • Contaminants concentrations/level/Toxicity

The bioremediation technique evaluated for Murowa Diamonds mine contaminated soils was *ex situ* biostimulation considering the small volumes of hydrocarbon contaminated soils and that the soils are in containment. Key factors anticipated to influence the ex situ biostimulation process include soil fertility, total hydrocarbon content, total organic content and possibly the availability of microorganisms and oxygen. This was mainly attributed to the region in which Murowa Diamonds is in and the environmental conditions of the region.

2.3 Recommended nutrient ratios for biostimulation

Theoretically, according to Rosenburg and Ron 1996, approximately 150mg of Nitrogen and 30mg Phosphorus are utilised in the conversion of 1g of hydrocarbon to cell material. Based on this, the optimal C: N: P mole-ratio recommended for enhancing hydrocarbon removal is 100.10.1(Malina, 1999). Malina, 2007 noted that the effectiveness of nutrient sources tend to be affected by the soil physicochemical properties since the soil environment is complex and heterogeneous. Zawierucha *et al*. (2008) compared N: P various sources of nutrients with different C: N: P ratios for enhancing biodegradation in soil and found out that the highest enhanced biodegradation rates (2-26 times higher than intrinsic biodegradation rates) were 100:10:5. Ubochi *et al.* (2006) examined the potential of bio stimulation options in oil contaminated soils with different contents of NPK in soil and found out that 60g of NPK fertiliser was the best treatment option.

3 CHAPTER 3: RESEARCH METHODOLOGY

3.1 Introduction

Reviews conducted demonstrated that key issues for successful bioremediation of hydrocarbon contaminated soils are to determine the soil type, soil properties (such as pore size, nutrients (C: N: P ratios), organic matter, soil pH, redox potential), concentration of hydrocarbon and monitoring environmental conditions such temperature and moisture content. Identification of the appropriate technology for removal of hydrocarbons from the soil given the scenario is also crucial. The approach used was initially to characterize Murowa soils focusing on factors which included physicochemical and microbial properties followed by setting up a controlled experiment which involved manipulation of nutrient concentration and moisture availability. Old aged soils that were stockpiled as far back as 2004 and freshly stockpiled hydrocarbon contaminated soils were used. The bioremediation techniques applied were the *ex situ* natural attenuation and bio stimulation.

3.2 Sampling protocol

The old aged stockpiled contaminated soils were profiled according to years in which there were contaminated. Stockpiling since the mine started was done as follows 2004 – 2007, 2008-2011 and 2012 to date (2014). **Fig. 2.0** shows picture of the old stockpile area.

Figure 2 - Picture of different aged hydrocarbon contaminated soils stockpiles. The stockpiles were profiled according to dumping periods since the mine operations started i.e. from to 2004 to date**.**

3.2.1 Sampling for old contaminated soils

The objective and purpose of sampling was to collect representative samples from old aged contaminated soils from a depth of 0-30cm for physical, chemical and microbial characterisation. Six quadrants which were 1m x 1m x 1m x 1m i.e. $1m^2$ were marked out. The quadrants were marked out following existing heaps from the dumping pattern that was followed from each age of soil. Where there were more than 6 heaps judgemental sampling was done where by quadrants were marked from heaps with bare surfaces and there were obvious signs of hydrocarbons on the surface. Bulk samples were collected from the centre of each quadrant using a shovel from the 0-15 cm and 15-30cm depths. Three composite samples were made from the six quadrants from each depth respectively. These were bagged in 1kg zip polythene bags and labelled. **Fig 3.0** shows an illustration of the sampling pattern. The squares represent the 1mx1mx1mx1m quadrants and the black dots are the actual points where samples were collected.

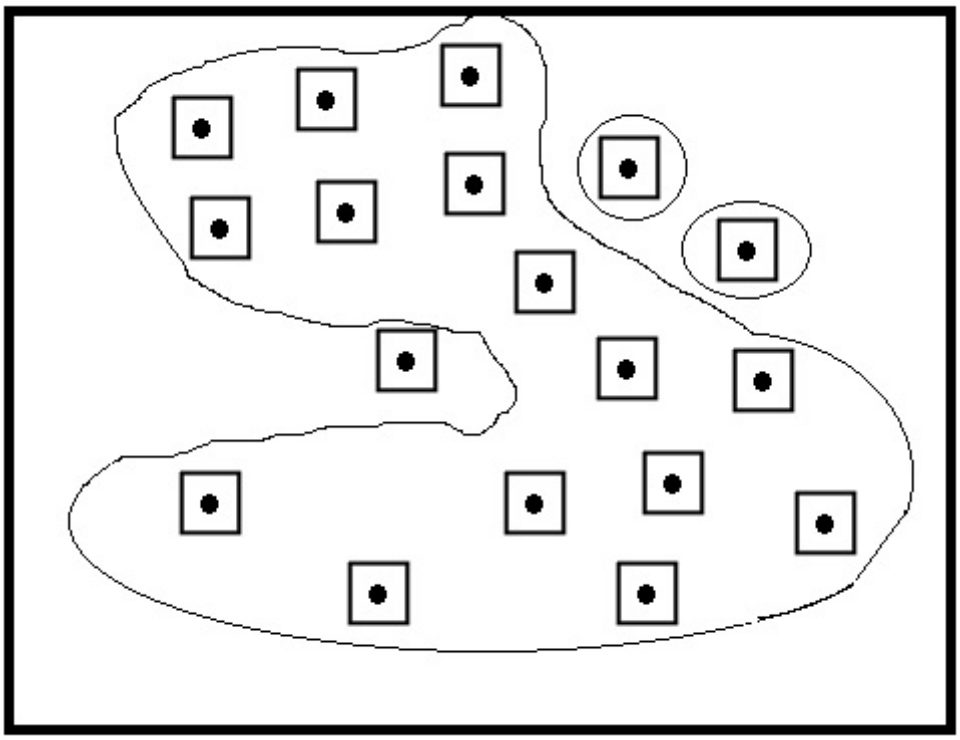

Figure 3 - Illustration of the systematic sampling pattern conducted. Squares represent 1m x 1m x 1m quadrants marked out and the black dots represent the actual sample collection point.

3.2.2 Sampling for bulk density and soil moisture retention parameters

From the same quadrants adjacent to the bulk sampling positions, three (3) undisturbed samples were collected using 7cm diameter and 5cm height metal cores from 0-15cm and 15-30cm profiles for determination of soil bulk density, soil moisture retention and repellency (Blake and Hartge, 1986). **Table 3** shows the number of soil samples collected for moisture retention and soil bulk density.

Table 3 -Samples collected for moisture retention and bulk density determination

Sample code	Age (years)	Depth units (cm)
M1	2004 -2007	0-15
M2		
M3		
M4	2004-2007	15-30
M5		
M6		
M7	2008-2011	0-15
M8		
M9		
M10	2008-2011	15-30
M11		
M12		
M13	2012 - 2014	0-15
M14		
M15		
M16	2012-2014	15-30
M17		
M18		
M19	Freshly contaminated 3 months accumulation	
M20	BWB A	
M21	BWB B	
M22	Uncontaminated	0-15cm A
M23		0-15cm B
M24		15-30cm

3.2.3 Soil sampling for freshly contaminated soils

The freshly contaminated soils were collected from the two washbays at the maintenance workshop referred to as the small wash bay and the big wash bay. **Figs 4** and **5** show the small and the big wash bay respectively.

Figure 4 -Small wash bay showing recently deposited oil contaminated soils where systematic grid sampling method was applied.

Figure 5 -Big washbay showing recently deposited oil contaminated soils where systematic sampling applied.A depth of 30cm could not be attained hence sampling was conducted up to a depth of 10cm.

Systematic grid soil sampling was applied for the first replicates that were collected from the Tarcon Small Wash Bay (TSWB) as the soils were contained in a regular shape as shown in **Fig. 6** illustrating sampling pattern for the small wash bay.

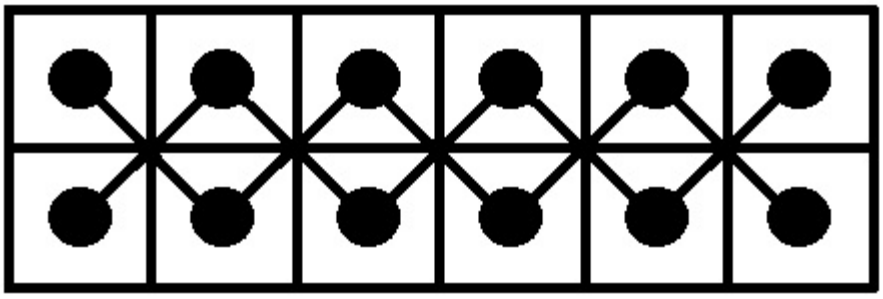

Figure 6 -Illustration of systematic grid soil sampling pattern conducted for the Small wash bay.

Soils collected were mixed to make one composite sample that was named TSWB. Replicates 2 (R2) and 3 (R3) were sampled from Tarcon Big Wash Bay which was named BWB as shown on **Fig. 7**.

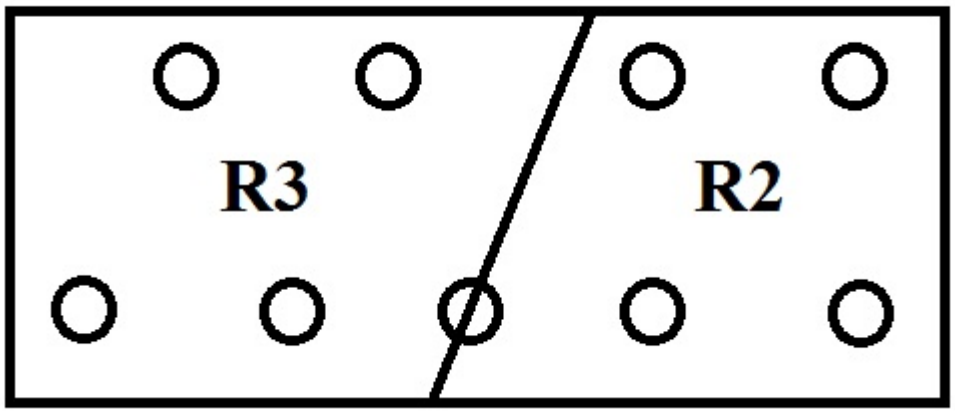

Figure 7 - Illustration of sampling pattern from the Big Wash Bay

Since the soil in the bunker was about 30cm in depth, 5 samples were collected by depth as follows;

Replicate 2 – BWB 0-15cmA, 16-30cm A, 0-15cm B, 0-15cm C and 16-30cm C. These were mixed to form one composite sample which was named BWB 1.

Replicate 3 composite sample was formed from BWB, D, E, F and G which was then named BWB 2.

3.2.4 Soil sampling for uncontaminated site

A total of six bulk composite samples were collected using systematic grid sampling from a 25m^2 grid at 0-15cm and 16-30cm depths respectively from an undisturbed area outside the mining operations area where there was no contamination. These were labelled as follows; UCS 0-15 cm A, B, C, UCS 15-30cm A, B, and C. **Fig. 8** illustrates the sampling pattern.

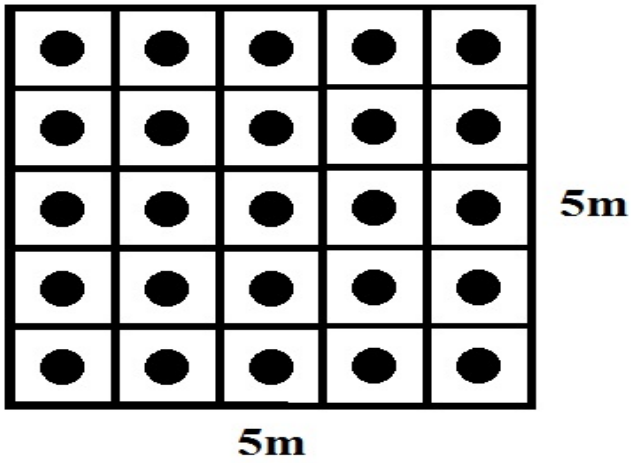

Figure 8- Illustration of systematic grid sampling conducted on an uncontaminated control site

Table 4 summarises the number of bulk, composite and core samples collected from old contaminated stockpiles, uncontaminated site and the recently contaminated from the wash bay.

Table 4 - Total number of samples collected for soil characterisation

Age	Old age soils	No. of Samples	No. of composite samples	No. of Core samples
2004 - 2007	0-15cm 15-30cm	6 6	3 3	3
2008-2011	0-15cm 15-30cm	6 6	3 3	3
2012 to date	0-15cm 15-30cm	6 6	3 3	3
Freshly contaminated samples were collected from a 3 months accumulation at the washbay.	N/A	9 from each monthly accumulation to make 3 composites	3	3
Uncontaminated virgin soils		9 from an area that is 25m^2 as the washbay and the old contaminated area to make 3 composite samples	3	3

Total No. of samples			**24**	**15**

3.3 Sample transportation

The samples were refrigerated soon after sampling to prevent any changes in microbial populations and activities. These were then flown to the laboratory which is about 450km away from the mine on the following day in a cooler box. All the samples were treated identically to ensure comparability of results.

3.4 Experimental Design for Pot experiment

The third and fourth objectives of the study were to investigate the effect of different level of C: N: P ratios on degradation of hydrocarbons at pot experiment and in the field. The pot experiment set up was based on the fact that Murowa soils have low nutrients hence required additional N and P to improve biodegradation efficiency.

Soil characterisation determined that Total Nitrogen average in the soils was approximately 0.06% which translates to about 600mg/kg. Phosphorus concentration average was about 18mg/kg which was a marginal concentration regarding P nutrient levels.

Based on the facts highlighted on the recommended nutrient ratios for biostimulation sited in the literature review, a complete randomized block design pot experiment was set up at the Murowa Mine Weather station. The experiment's focus was on the C: N: P nutrient ratio and moisture as the limiting factors maintaining a ratio of 100:10.1(Thomas *et al*. 1992; Lie beg and Cutright, 1999; Vidali, 2001). The sources of N and P were 34.5% N ammonium nitrate fertiliser and 19.3% P_2O_5 Single Super Phosphate Min 12% S. Moisture was added in form of water to achieve 20% water holding capacity and to ensure proper mixing with contaminants (Akpoveta *et al*. 2011). Watering with 1.6 litres of water on a weekly basis was done across all treatments based on the fact that moisture was already a limiting factor since Murowa fell within a dry region 4-5 of the Zimbabwean farming regions. The samples were mixed/tilled once weekly for aeration (Ayotamuno *et al.* 2006).

3.5 Site preparation and procedure

A site area of $12m^2$ (4m length X 3m width) was cleared and levelled using a total station. The area was then divided into 3 blocks which were $4m^2$. Each block was then subdivided into 8 compartments of 1m X 50cm width for housing each pot with each treatment.

Weights for each clay pot were determined and these were inscripted on all the pots. A bulk sample from the **2012 to date** stockpile of about 300kgs in weight was collected, fragmented and mixed thoroughly to form one uniform sample. From the uniform composite soil sample

10kgs subsamples were collected and filled the 24 earthen ware pots. Diameter of clay pots was 0.3m and 0.3m depth. Pots were labeled with each treatment assigned three (3) replicates. Application rates for Ammonium Nitrate (AN) and Single Super Phosphate (SSP) fertilisers were calculated as per **section 3.5.1.** Both fertilisers were applied and thoroughly mixed with the soil to attain N: P concentrations in the ratios detailed in **Table 5**.

Table 5 - Experimental Treatment ratios

Treatment	Soil sample	Replicates	Nitrogen	Phosphorus	Molar Ratio	Comment
1. Control	2012 to date contaminated sample	3	600mgN	18mgP	33.1	Average Baseline concentration of N:P
2. P only	2012 to date contaminated sample	3	6000mgN	600mgP	10.1	Increased P concentration to attain molar ratio10:1. Added 0.154kg SSS fertiliser containing 4.2g P
3. P only	2012 to date contaminated sample	3	6000mgN	1200mgP	10.5	Increased P concentration to attain molar ratio of 10:5. Added 0.3735 kgs of SSS fertiliser
4.Both N:P	2012 to date contaminated sample	3	9000mgN	900mgP	10.1	Increased both N: P concentration to 10:1 molar ratio. 13.044 g AN and 0.262kg SSS fertiliser added.
5.P only	2012 to date contaminated sample	3	6000mgN	3000mgP	2.1	Increased P concentration to attain ratio 2:1. Added 1kg of SSS fertiliser
6.N:P	2012 to date contaminated sample	3	12000mgN	2400mgP	10.5	17.3913 g AN fertiliser and 0.811 kg SSS added
7.	2012 to date contaminated sample	3	7gN	0.54gP		1ton/ha application rate
8.	2012 to date contaminated sample	3	14gN	1.08gP		2 ton/ha application rate

3.5.1 Calculations for fertiliser application

Phosphorus (http://www.esf.edu/for/briggs/FOR345/Fertilizer%20Worksheet.pdf **)**

Composition of P source was a single superphosphate fertiliser 19.3% P_2O_5 Min 12% S. The calculation of P

Atomic weight of P =31 and O=16

Mass of P_2 =2X31 =62g **Mass O_2** =5X16 =80g Total weight for P_2O_5 = 142g

Proportion of P in P2O5 is [62/142] =0.437 translating to 43.7%

Therefore by simple proportion 19.3% constituted 27.38215g P

Nitrogen

34.5% N in 50kg of AN = 17.25kg

Nitrogen was applied at a rate of 1ton/Ha and 2ton/Ha at two weekly intervals (Chorom and Sharifi, 2010). Calculations for the pots were done as follows;

1. Application of 1ton/Ha would require 345Kg N (10000m^2)
   Surface area of pot =πr^2 = (22/7)*0.15*0.15 = 0.070714 m^2
   Amount of N for pot = (0.070714 m^2/10000m^2)* 345Kg =0.00244 kg N
   Amount of AN fertiliser required was (0.00244 kg N/17.25Kg)*50 = 0.007071KgN which translated to 7.071429g N for 1pot.
2. For application rate of 2ton/Ha it was 7.071429g multiplied by 2 which is 14.14286g N for 1 pot.

To attain 10:1 Molar Ratio of N: P

1 mol N = 14g: 1mol P=31g

N:P 10:1

140g N: 31g P

Therefore a pot required (2.439643g/140g)*31g=0.540207g P.

Amount of fertiliser 19.3% P_2O_5 required = (0.54g/10000g)* 27.38215 = 0.001479g of P_2O_5 fertiliser.

3.5.2 Determination of water to attain full saturation

Porosity of porous materials = (1- bulk density/2.65) x100

Bulk density = mass/volume for the packed pot

i.e. pack 3-4 pots the way the researcher intended to finally pack them. Determined mass (M), determined volume occupied by the mass of soil (V) then BD = M/V

Porosity was 40% which meant that for a soil occupying a volume of 10 litres, 4 litres was the pore space. Therefore applying 4 litres to the soil all pore spaces will be occupied with water and the soil is saturated. To determine 20% water holding capacity (20/50*4litres of water) which concluded to 1.6 litres of water (Wu *et al.* 2016).

3.5.3 Pots arrangement

Randomization process was done using a coin head representing placing direction South to North and tail North to South. The pots were arranged from North to south as shown in the **Table 6** and **Fig 9**.

Table 6 - Experimental Treatment blocks arrangement

Block 1	Block 2	Block 3
6000mgN:600mgP	9000mgN:900mgP	7gN:0.54gP
14gN:1.08mgP	6000mgN:3000mgP	600mgN:18mgP
12000mgN:2400mgP	14gN:1.08mgP	14gN:1.08mgP
600mgN:18mgP	6000mgN:1200mgP	6000mgN:1200mgP
6000mgN:1200mgP	7gN:0.54gP	9000mgN:900mgP
9000mgN:900mgP	600mgN:18mgP	6000mgN:3000mgP
7gN:0.54gP	6000mgN:600mgP	12000mgN:2400mgP
6000mgN:3000mgP	12000mgN:2400mgP	6000mgN:600mgP

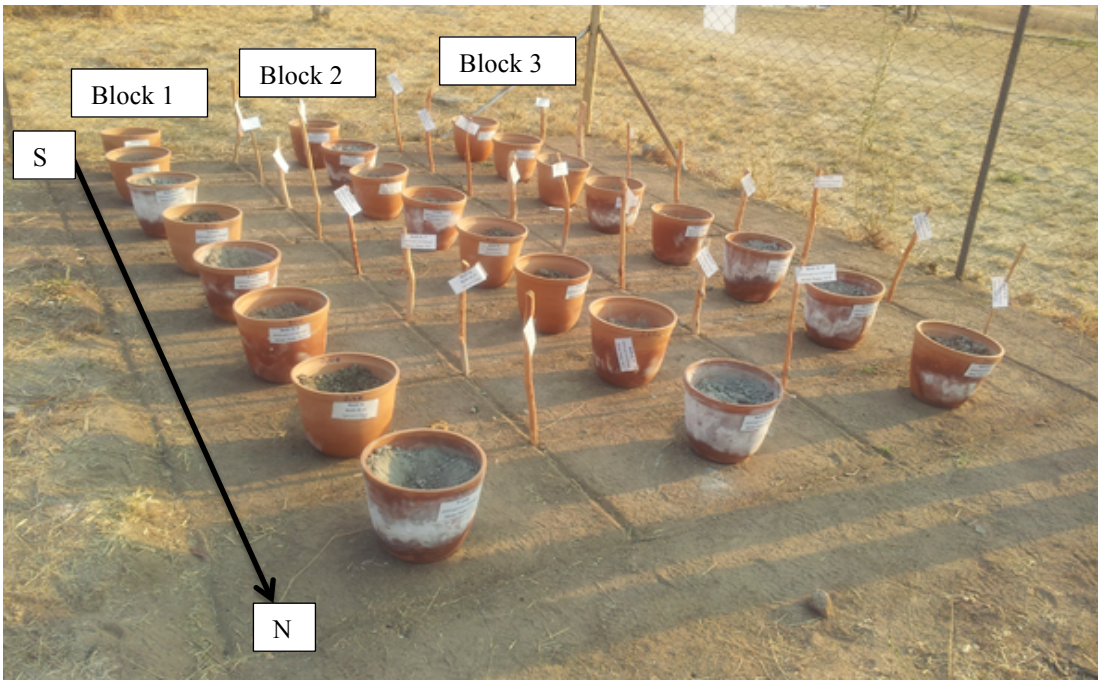

Figure 9 - Pots arrangements within respective blocks

Pots were watered 1.6 litres each to attain a water holding capacity of 20% and tilled once per week for aeration. Samples were collected at **10days, 17days, 24days, 32days, 45days, 74 days, 88 days and 111 days.** The samples were mixed thoroughly for representation. The samples were taken out on a weekly basis to analyse for change in concentration of total hydrocarbon concentration (Chorom *et al*., 2010), pH, and total nitrogen, phosphorus and % carbon. Temperature and other weather conditions were monitored to account for any changes in the experiment results.

3.6 Laboratory Methods and Materials for soil analyses

3.6.1 Soil sample preparation

Soil samples for chemical analysis were air dried for 48hrs in the shed to minimise mineralization and pulverized to pass through a 2mm sieve except for a sub soil sample taken for organic carbon determination which was pulverized to pass through a 0.5mm (500µm) sieve (Okalebo *et al.* 1993).

3.6.2 Determination of physical properties

Particle size analysis, total porosity, soil bulk density as well as water repellency were measured to characterize/determine physical properties of contaminated and uncontaminated soils.

3.6.2.1 Particle size analysis

Particle size analysis involved pre-treatment steps such as removal of organic matter, carbonates and sesquioxides to eliminate cementing agents. Analysis was done by wet sieving and sedimentation technique (Gee and Bauder, 1986) according to their size and density to determine the sand, clay and silt particle ratio/composition.

3.6.2.2 Water repellency

Water repellency was determined using Water Drop Penetration Test (WDPT) (Letey, 1969; Watson and Letey, 1970; King, 1989). The procedure was basically measuring the time it will take for water drop to penetrate or disappear on a soil oven dried overnight. The test was performed in leveled petri dishes and the time taken for each drop was recorded in minutes.

3.6.3 Determination of soil chemical properties

Chemical properties that can potentially influence biodegradation rates of petroleum were determined and these included pH, Nutrient content (Total N, total P, PK, C), Electrical conductivity, total carbon content, Total Petroleum Hydrocarbon (TPH) concentration, cation exchange capacity and the total exchangeable bases (Ca, Mg, K and Na).

3.6.3.1 Determination of soil pH

Both the water and the calcium chloride (0.01M $CaCl_2$) methods were used to determine soil pH for both contaminated and uncontaminated soils. The water method refers to the potentiometric determination of Hydrogen ions in a soil suspension in water (Esterfen *et al.* 2013). The calcium chloride of 0.01M concentration method entailed the hydrogen ions concentration measurement in a soil salt solution using a pH meter with a built in glass electrode and reference electrode (Esterfen *et al.* 2013).

3.6.3.2 Determination of soil Nutrients (C: N: P)

Carbon determination was conducted using the Walkley-Black method (Jackson, 1958). The method involved oxidation of organic matter by potassium dichromate ($K_2Cr_2O_7$- sulphuric acid mixture followed by back titration of the excessive dichromate by ferrous ammonium sulphate (Fe $(NH_4)_2(SO_4)_2$*$6H_2O$).

Determination of total nitrogen was done by Kjeldahl procedure. The method involved three successive phases which included the digestion of organic material to convert nitrogen into HNO_3, distillation of the released NH_3 into an absorbing medium and volumetric analysis of the NH_3 formed during the digestion process. Digestion was carried out by heating the

sample with concentrated sulphuric acid in the presence of selenium as the catalyst and potassium sulphate to raise the digestion temperature. Hydrogen peroxide was then added as an oxidant and the solution cooled. Distillation was done with excess sodium hydroxide base to determine ammonia content of the digest and absorption of evolved ammonia (NH_3) was done in boric acid. The alkaline bicarbonate method of Olsen *et al.* (1954) was used to determine total phosphorus. Available P was extracted by a sodium bicarbonate solution of pH 8.5 for 30 minutes.

3.6.3.3 Determination of total petroleum hydrocarbon concentration

Total petroleum hydrocarbons (TPH) is a commonly used gross parameter for quantifying environmental contamination as a result of various PHC products such as fuels, oils, lubricants, waxes, and others. Gravimetric Methodologies used were US EPA Method 3550 and 1664. The samples were sieved through a 4mm sieve. 5.0 grams of the samples were weighed in triplicates. The samples were transferred to a 50ml glass centrifuge tube to which 25ml of chloroform was poured and the tube was tightly closed by the lid. Extraction was done for 1 hour using the ultrasound bath at a constant temperature of 40^0C. After the extraction, the samples were centrifuged at 3000 rpm for 10 minutes. Each of the extract was transferred into an Erlenmeyer flask and the chloroform was evaporated off at 65^0C and dried to a constant weight. The amount of TPH's was then gravimetrically determined.

3.6.4 Determination of biodegrading microorganisms genera

Microbial characterisation parameters determined included microbial genera identification, Total Microbial Mass, Total Microbial Nitrogen, Total Microbial Carbon and soil respiration.

3.6.4.1 Microbial Genera identification

Soil samples were dissolved in Ringer solution of a concentration of 10g/100ml and serial dilutions subjected to serial dilution from $10^0 - 10^{-9}$. The dilutions were then plated on nutrient agar, and potato dextrose agar for yeasts and molds. The plates for bacteria were incubated for 48 hours at 37^0C while the plates for fungi were incubated for 14 days at 25^0C. Pure cultures were isolated from individual colonies. Direct microscopy and Gram's staining were performed for bacteria.

As for fungi, direct microscopy and staining using Melzer's iodine, Lacto phenol cotton blue and Malachite green were performed. Colony morphology and biochemical tests were performed for identification.

3.6.4.2 Total Microbial Mass, Carbon and Nitrogen

Soil samples were incubated for seven (7) days at field capacity (this refers to amount of moisture remaining in soil after water has drained following a wetting or irrigation event) to resuscitate the microbes before fumigation. Total Microbial biomass, Total microbial nitrogen and total microbial biomass were determined using Chloroform fumigation, fumigation- extraction methods respectively (Vance *et al.* 1987; Sparling and West, 1988). Extraction was done from a 5g sample using 25ml of 0.5M K_2SO_4/2MKCL. The solution was passed through a Whatman filter number one (1) and the clear solution was collected for analysis. Aliquots of 2ml from each of the fumigated and non-fumigated samples. These were treated with 0.5mls of Sodium tricitrate (0.4M) and subsequently 2 ml (millilitres) of reagent mixture was added to every sample and standard. The samples were left for 30 minutes in a water bath (boiling). The samples were cooled using running tap water and 5mls of 50% alcohol were added and the samples were left for colour development. The coloured samples were then passed through a spectrophotometer to read the microbial carbon and nitrogen as referred to by Amato *et al.* (1988) as well as Schinner *et al.* (1996).

3.6.4.3 Soil respiration

Soil respiration was analyzed using CO_2 Flux method as described by Schlesinger, and Andrews (2000). The procedure involved measurement of carbon dioxide gas ($CO_{2)}$ that evolved from a known quantity soil in an airtight chamber and the carbon dioxide gas was absorbed in soda lime solution for a period of 12 hours. As soda lime solution absorbed the gas, the mass of soda increased and the increase in mass was used to estimate the respiration per day or per hour.

3.6.5 Identification of hydrocarbon types

Eleven samples were collected at 5 weeks from the pot experiment including the control pot and were analysed to identify the hydrocarbon types present in the contaminated soils. The hydrocarbons identification test was necessitated by the TPH concentration trend from analysis completed in the first four weeks which exhibited an increase in TPH concentration which was not expected hence the analysis to investigate which hydrocarbons behaved as such. Hydrocarbon types were determined by the solvent extraction using hexane and trailed by a quantitative analysis using Gas chromatography mass spectrometer (GC Model-7980 A and MS model-5975 C) at the Scientific Institute for Research and development Centre (SIRDC) FTBE laboratory. The conditions were: column type- 5% Phenyl methyl siloxane

Column Flow – 0.7 ml/min, Injector Temp- 250^{0}C, Column Oven Temp program – 50^{o}C – 1 min and ramp at 20 ^{o}C per minute to 310MS temp 230 and Mass analyser temp (quadrupole) - 150

3.7 Statistical methods

3.7.1 Soil characterisation data

Soil characterisation data was tested for normality using the Q-Q plots in SPSS version 16 to validate the use of ANOVA. **Appendix 2** and **4** show Q-Q plots and ANOVA for characterisation data respectively. Data which was not normal i.e. for Ca, Mg, Na, and K was transformed and subjected to one- sample Kolmogorov-Smirnov test **(Appendix 3)**. General liner model univariate ANOVA was conducted to determine the individual and interactive effect of soil age, and depth **(Appendix 4).**

3.7.2 Pot experiment data

Data was tested for normality using both GenStat and SPSS software as specified on **appendices 7, 8, 9** and **10**. One way ANOVA was conducted and multiple comparisons of the means were done for all the parameters using the Least Square Differences to establish variations weekly by treatment. Univariate analysis was also conducted to determine the interactive effect of time and treatment**.**

4 CHAPTER 4: RESULTS

Study results are presented in the order: physical, chemical and microbiology data respectively. The letters above the bar graphs define significance levels. In cases where the letters are the same it means the samples are similar the difference is insignificant and where the letters are different, samples would be significantly different.

4.1 Physical properties of Murowa uncontaminated and hydrocarbon contaminated soils

4.1.1 Soil texture

The average sand content across the contaminated sites was 79.74%, Clay 9.3% and Silt 10.89%. The soils were therefore classified as Sandy loams (SaL) according to the Zimbabwean guide to interpretation of soil results which contain more than 20% silt + clay more than 50% sand.

Mean range for sand particles content in the soils was 74%-85%. There was no significant difference in sand particle content in the 2004-2007 stockpile and the fresh contaminated soils 81.3 and 82.2% respectively. This was also the same for the 2012-todate and the uncontaminated sites which had statistically similar sand particles proportion (74 and 84%) whereas the 2008-2011 sand particle content was 71% which was significantly different from the rest at ($p<0.05$) .

Clay content in soil samples for periods 2004-2007, 2012 to date and freshly contaminated was statistically similar (8.5, 9.1 and 7.8%) which was the same for the 2008-2011, 2012-to date and the uncontaminated soils (10.3, 9.1 and 10.3 % respectively) also showed no significant difference at ($p<0.05$).Silt content across the sites was higher in 2012-to date sample at 15.9% where as in 2004-2007, 2008-2011 and freshly contaminated were statistically similar i.e. (10.2, 12.6 and 10%) ($p<0.05$). The 2012-to date and the uncontaminated had significantly different silt content 15.9 and 5.3%. This was expected to differ as the soils were mined or excavated from different levels of the pits and or areas of the mine depending on the contamination would have occurred and also due to the fact that Murowa area has some heterogeneous soil patches. Results are shown in **figs 10, 11** and **12** which show percentage sand, clay and silt particles of Murowa soils.

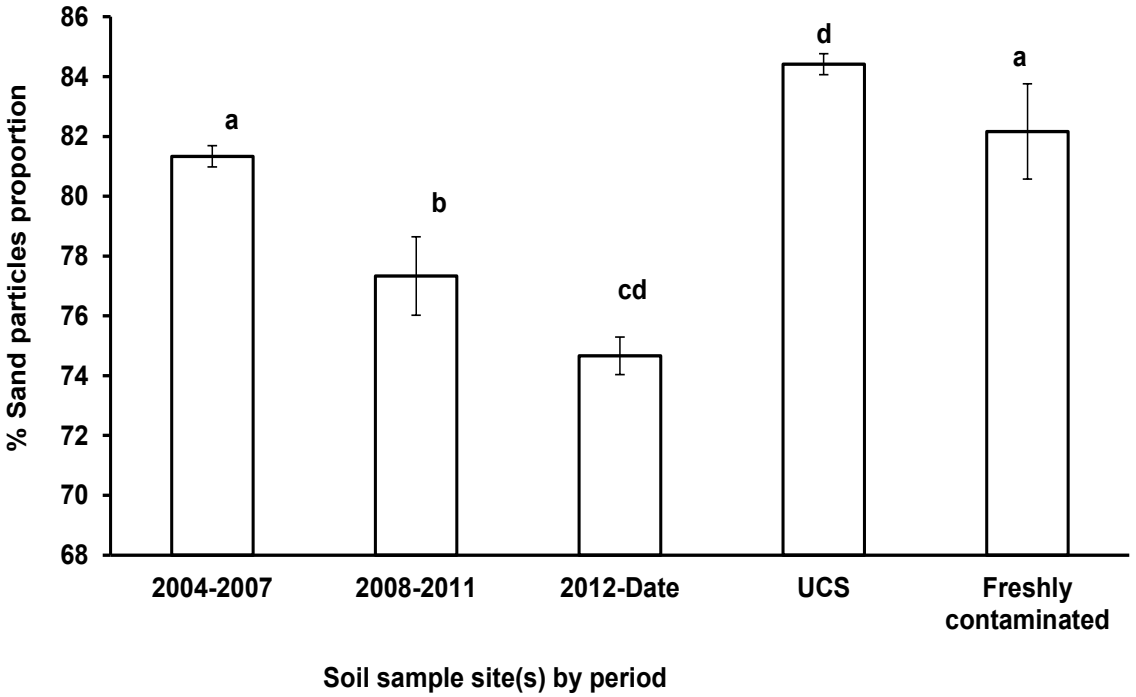

Figure 10 - Percentage sand particles proportion in soil sample sites by dumping period. Particle size analysis was determined by wet sieving and sedimentation. Data is expressed as mean % sand particles on the different sites. Vertical bars show the SE of the mean % of the sand particles. Means with different letters are significantly different (ANOVA $p<0.05$).

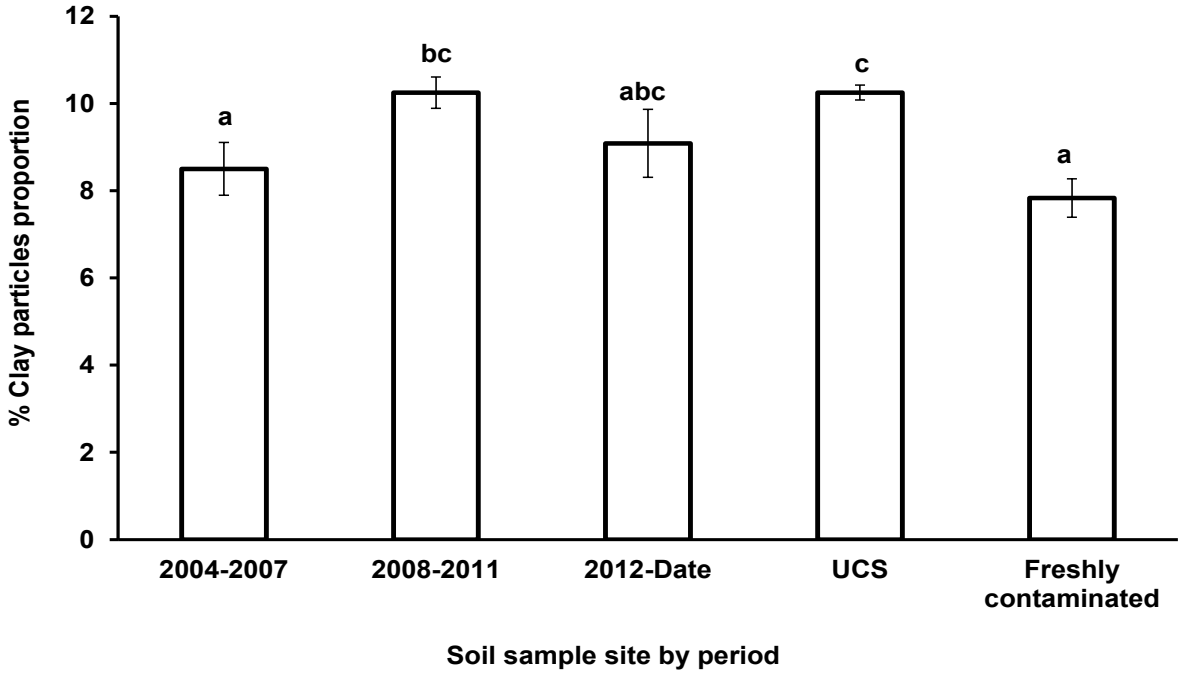

Figure 11 - Percentage clay particles proportion in soil sample sites by dumping period. Particle size analysis was determined by wet sieving and sedimentation. Data is expressed as mean % clay particles on the different sites. Vertical bars show the SE of the mean % of the clay particles. Means with different letters are significantly different (ANOVA $p<0.05$).

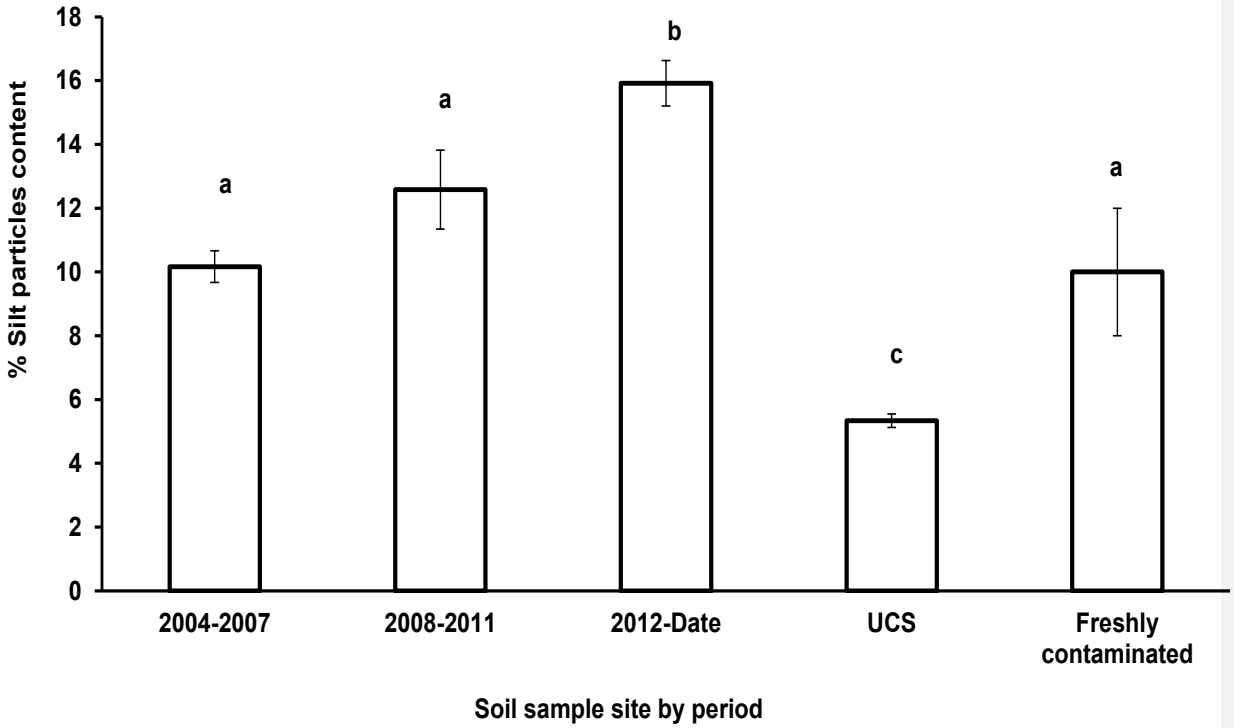

Figure 12 - Comparison of percentage silt content across sites. Particle size analysis was determined by wet sieving and sedimentation.Data is expressed as mean % silt particles on the different sites. Vertical bars show the SE of the mean % of the silt particles. Means with different letters are significantly different (ANOVA $p<0.05$).

4.1.2 Water Repellency

The seven replicates for determining soil water repellency that were done reflected a similar trend across all the sites. The water repellency level was high in soils on site 2012-todate >2008-2011>2004-2007>freshly contaminated>UCS. Statistically 2004-2007, UCS and freshly contaminated soils were the same (8.5, 0.03, 0.03 minutes). The old aged soils had low water repellency than the recently dumped soils i.e. water repellency decreased with age or period it was dumped which was the same case as the TPH concentration or levels. This was exhibited by 2012 to date that had the highest water repellency and TPH concentration which followed suite on the other years. This reflected a correlation between water repellency and the TPH concentration. As the TPH level increased the water repellency increased.

According to (Dekker and Jungerious, 1990; Dekker and Ritsoma, 1994), uncontaminated and the freshly contaminated soil samples are categorised as hydrophilic/wettable if water repellency is less than 5 sec, slightly repellent (5-60s), strongly repellent (60-600s), severely repellent (600-3600s) and extremely repellent (more than 3600s). Murowa contaminated soils are therefore classified as follows 2004-2007 – **mean 297.49sec** strongly repellent, 2008-2011 – **mean 1509sec** severely water repellent, 2012 to date – **mean 2571 sec** severely

repellent, uncontaminated soils (UCS) and the freshly contaminated – **mean 1.8 sec** hydrophilic or wettable. **Figs 13 and 14** give a comparison of water repellency.

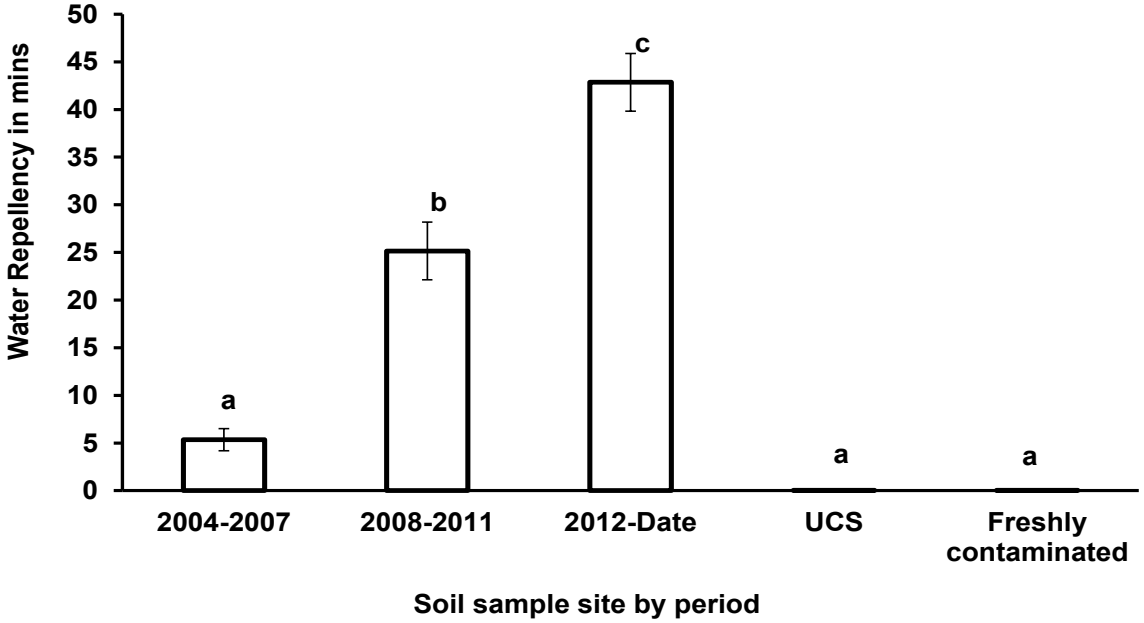

Figure 13 - Comparison of mean water Repellency in minutes across sites from Replicate 1 soil samples which was determined using the Water Drop Penetration Test (WDPT).Data is expressed as mean water repellency on the different sites. Vertical bars show the SE of the mean repellency. Means with different letters are significantly different (ANOVA $p<0.05$).

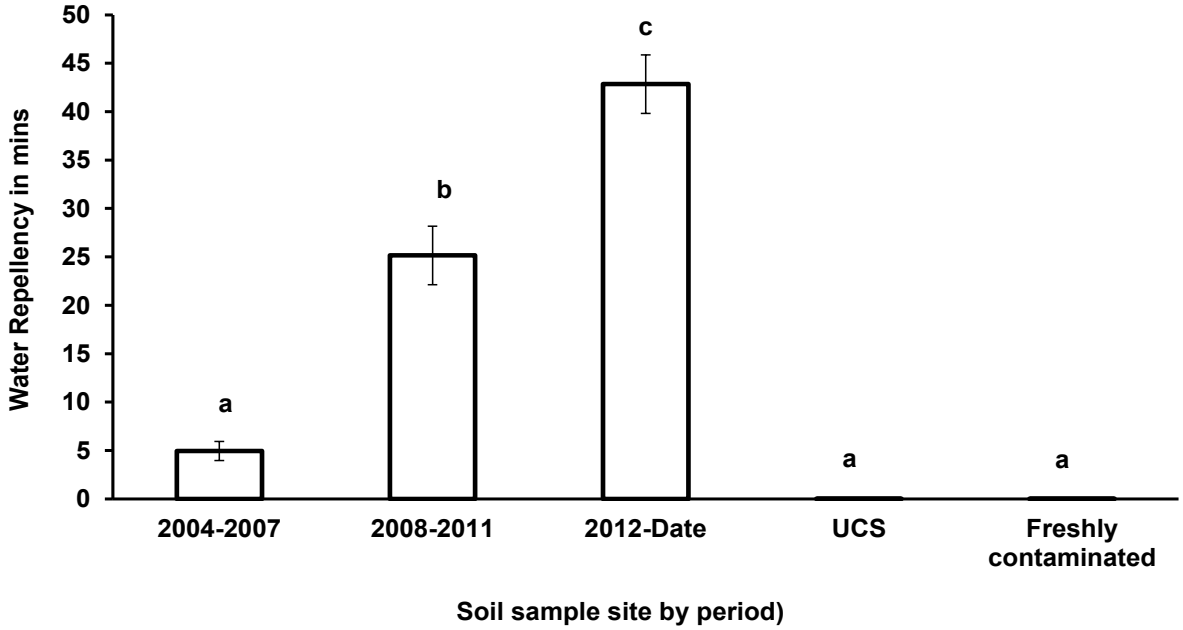

Figure 14 - Comparison of Water Repellency in mins across sites from Replicate 2soil samples which was determined using the Water Drop Penetration Test (WDPT).Data is expressed as mean water repellency on the different sites. Vertical bars show the SE of the mean repellency. Means with different letters are significantly different (ANOVA $p<0.05$).

4.2 Soil chemical properties

4.2.1 Total Petroleum Hydrocarbon

The soils samples from the 2008 -2011 and 2012-to date sites had significantly ($p<0.05$) higher TPH than 2004-2007 site, UCS and the freshly contaminated soils **(figs 15** and **16)**. The soils from site 2008-2011 and 2012 to date showed no significant difference though 2012 had the highest TPH mean 265.7 mg/l when compared with 192.3 mg/l).

Hydrocarbons were detected in UCS (reference site) both 0-15cm and 15-30cm depths which was not expected. This could be attributed to biogenic hydrocarbons which are also referred to as fingerprinting hydrocarbons meaning background hydrocarbons that occur naturally in uncontaminated sites. According to Wang *et al.* (2012) this could lead to overestimation of petroleum hydrocarbon levels in some instances they have been found exceeding regulatory levels e.g. 300ug $g^{(-1)}$ for coarse soils and 1300 ug $g^{(-1)}$ for fine soils.

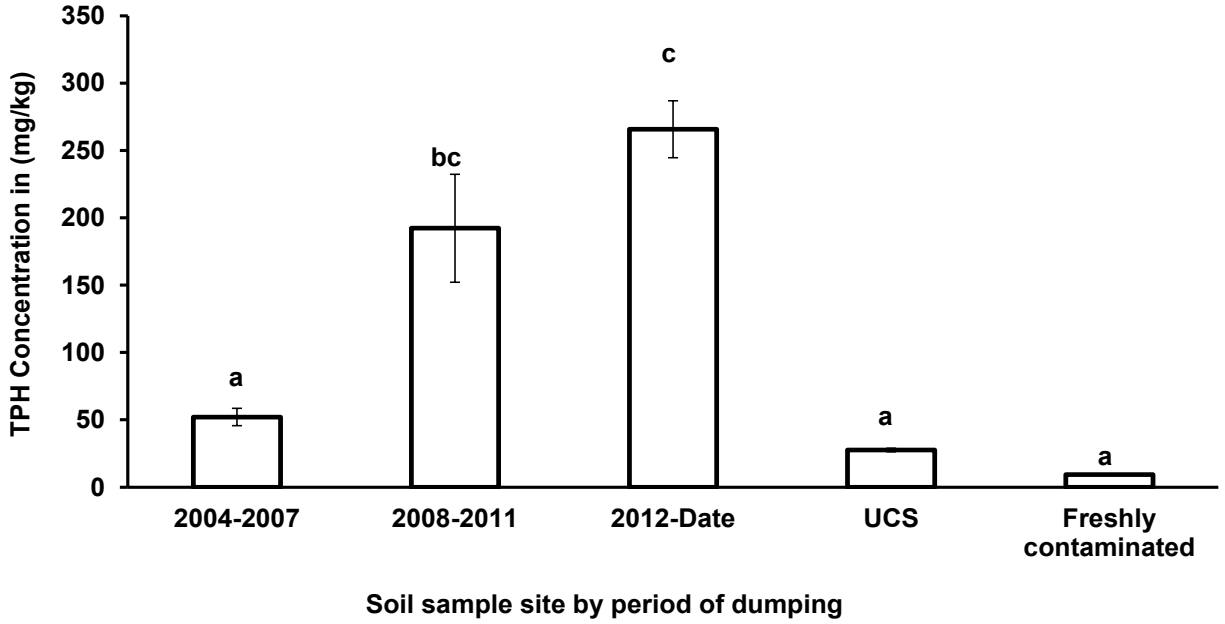

Figure 15 - Comparison of mean TPH concentration by the periodic dumping sites. TPH was determined by gravimetric methods USEPA3350 and 1664.The concentration is expressed as mean TPH mg/kg on the different sites. Vertical bars show the SE of the mean of TPH concentration. Means with different letters are significantly different (ANOVA $p<0.05$).

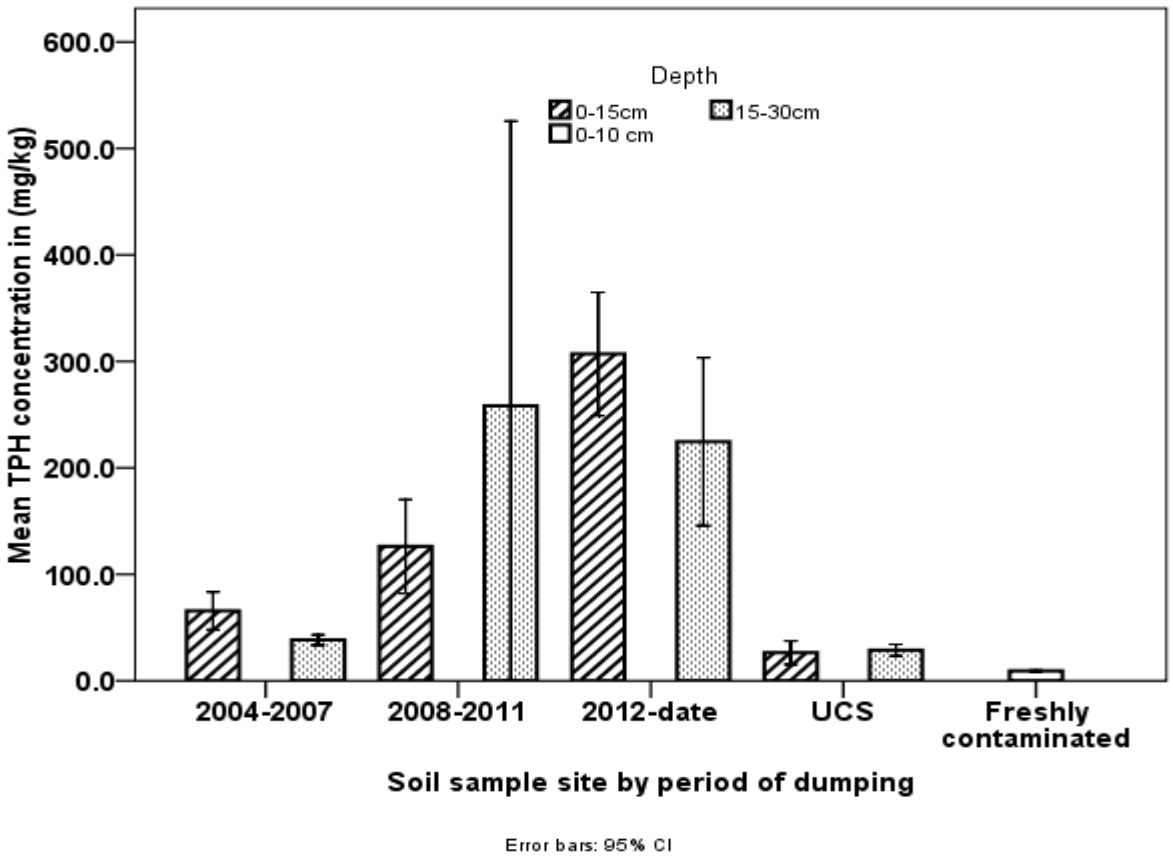

Figure 16 - Comparison of mean TPH concentration interactive effect of period of dumping and depth of soil across sites. The concentration is expressed as mean TPH mg/kg on the different sites. Vertical bars show the SE of the mean TPH concentration (Univariate ANOVA $p<0.05$).

4.2.2 Relationship between water repellency and TPH concentration

The old aged soils had low water repellency than the recently dumped soils i.e. it decreased with age or period it was dumped which was the same case as the TPH concentration or levels i.e. 2012 to date had the highest water repellency and TPH concentration and followed suite on the other years which reflects a correlation between water repellency and the TPH concentration. The higher the TPH level the higher the water repellency.

The plot of water repellency and hydrocarbon concentration demonstrated a strong correlation between TPH and water repellency (r^2=0.99). **Fig. 17** Illustrates and validates the strong correlation between the TPH and water repellency. The higher the TPH concentration the higher the water repellency was.

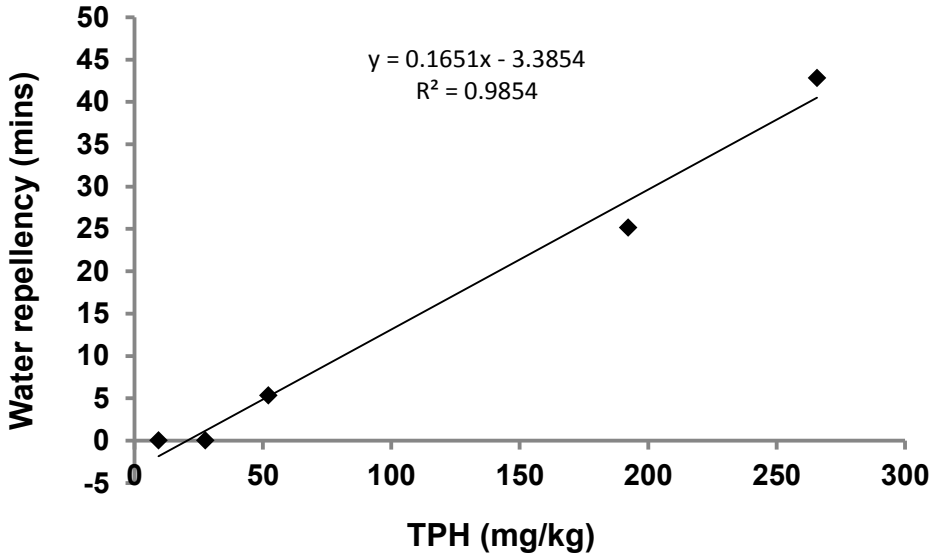

Figure 17 - Correlation between water repellency and mean TPH concentration.

4.2.3 Soil pH

Soil pH was measured to ascertain pH at all soil depths in order to determine whether pH could be a limiting factor for biodegradation to occur and adjust the levels accordingly. pH levels for 2008-2011 and 2012 to date soils were statistically similar (comparable) i.e., pH levels of 8.5 and 8.6 respectively whereas 2004-2007, UCS and the freshly contaminated were significantly different ($p<0.05$) pH levels of 7.6 and 9.4 respectively **(Figs 18** and **19)**. pH mean ranges 8.067-9.367 which according to the Zimbabwean soils are classified as strongly alkaline. pH range suitable for microbial activity is 5.5-8.8 and for oil degradation is 6.5-8.0 (Wang *et al.* 2012; San Martin, 2011). The pH ranges for Murowa soils therefore would not require any pH adjustment as it falls within the range required for biodegradation processes.

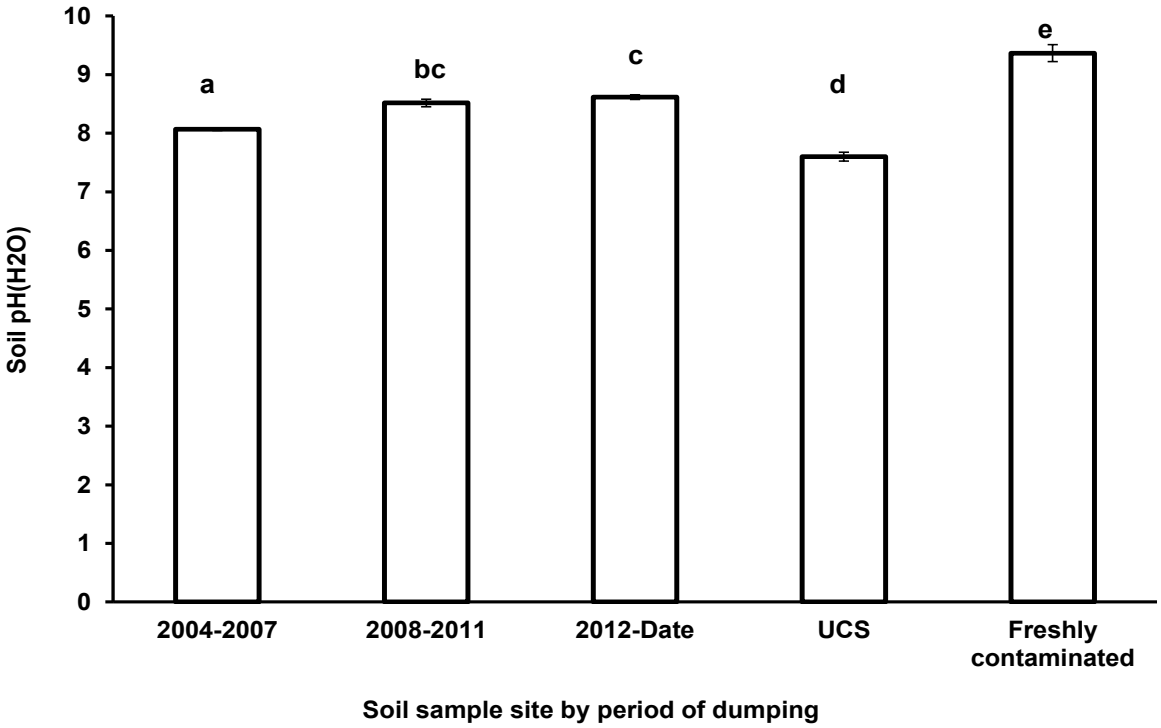

Figure 18 - Comparison of pH level by dumpsite and period. Soil pH was determined using both the water method and the 0.01M CaCl2.The data is expressed as mean pH levels for the different sites. Vertical bars show the SE of the mean soil pH for the different sites. Means with different letters are significantly different (ANOVA $p<0.05$).

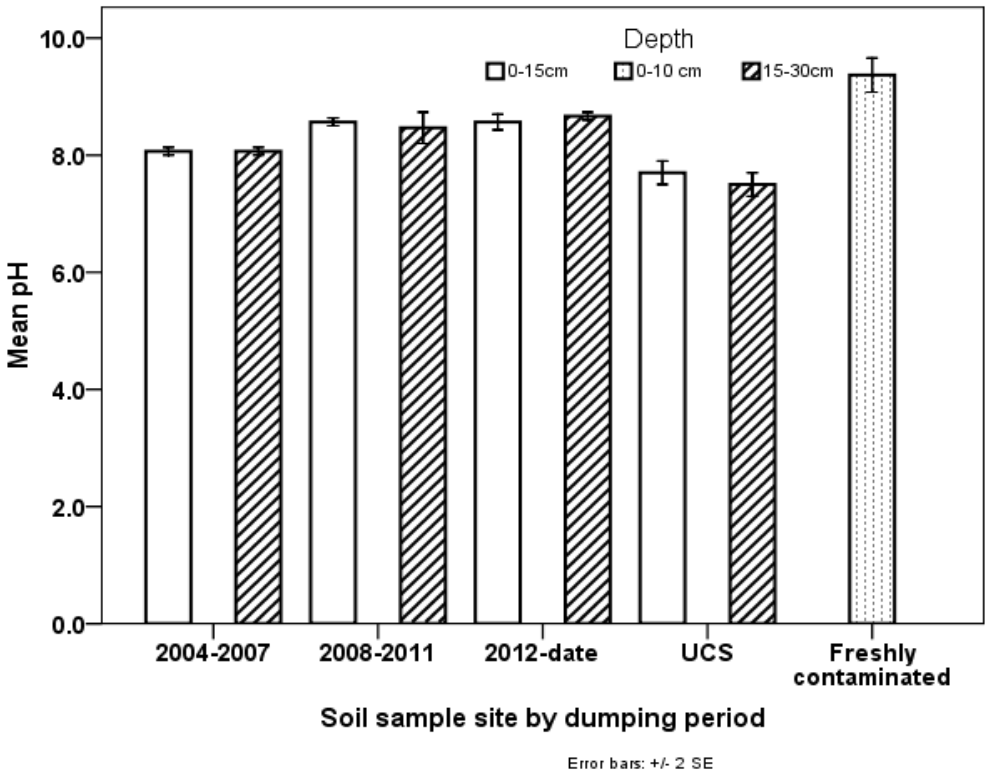

Figure 19 - Comparison of interactive effect pH levels between period of dumping and depth of soil across sites. The data is expressed as mean pH and the vertical bars show the SE of the pH mean (Univariate ANOVA $p<0.05$).

4.2.4 Nutrients

4.2.4.1 Soil Carbon

Mean percentage carbon range was 0.40 -2.4%. Freshly contaminated soil had the highest carbon percentage followed by 2012 to date sample. Statistically, carbon percentage in 2008-2011, 2012 to date and the freshly contaminated soils were more or less the same (1.998 and 2.27% respectively. In the 2004-2007 and UCS carbon percentage was significantly different ($p<0.05$) at 1.08 and 0.40 % respectively.

Carbon concentration was significantly higher in contaminated soils than in UCS which may be attributed to hydrocarbon contamination. The 2004-2007 soils which are the oldest had significantly lower carbon than the younger soils. Natural attenuation can be assumed to have been happening. **Fig. 20** shows comparison of C percentage by period**.**

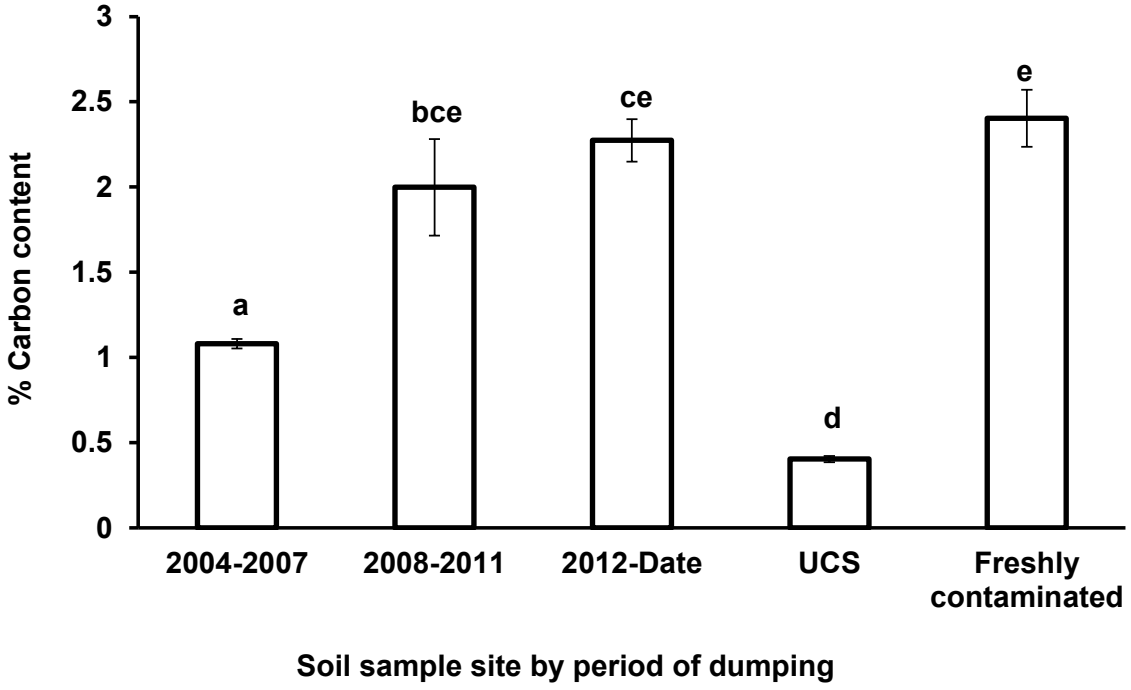

Figure 20 - Comparison of mean carbon % across sites by dumping period. Carbon % was determined by the Walkley-Black method using potassium dichromate as the oxidant. Vertical bars show the SE of the mean % carbon for the different sites. Means with different letters are significantly different (ANOVA $p<0.05$).

4.2.4.2 Total soil Nitrogen

Total nitrogen content of a soil reflects its organic matter content. Nitrogen percentage ranged from 0.04-0.09% (400mg/kg – 900mg/kg). The percentage range falls within the characteristics of upland soils of medium and low rainfall areas of Zimbabwe according to the guide to meaning of soil analysis of Zimbabwe. **Table 7** details typical nitrogen in

Zimbabwean soils. The 2004-2007, 2008-2011 and the UCS have more or less the same Total nitrogen % whereas 2012 to date and the freshly contaminated are significantly different at ($p<0.05$).

The 2012–to date had significantly high nitrogen percentage than other sites while freshly contaminated soils had the least percentage. Oldest soils and the UCS were statistically similar while higher than freshly contaminated. The least mean average nitrogen concentration was 0.0433% and highest being 0.0817% respectively. **Figs. 21** and **22** show comparison of nitrogen percentage over time and depth.

Table 7 - Typical total Nitrogen ranges in Upland soils of medium and low rainfall in Zimbabwe source (Department of Soil Research, 2015).

Texture	Total Nitrogen(%)	Total Nitrogen(mg/kg)
Sands	0.02- 0.05	200- 500
Sand loams	0.04- 0.07	400-700
Sandy clay loam	0.06- 0.10	600- 1000
Clays	0.10- 0.15	100- 1500

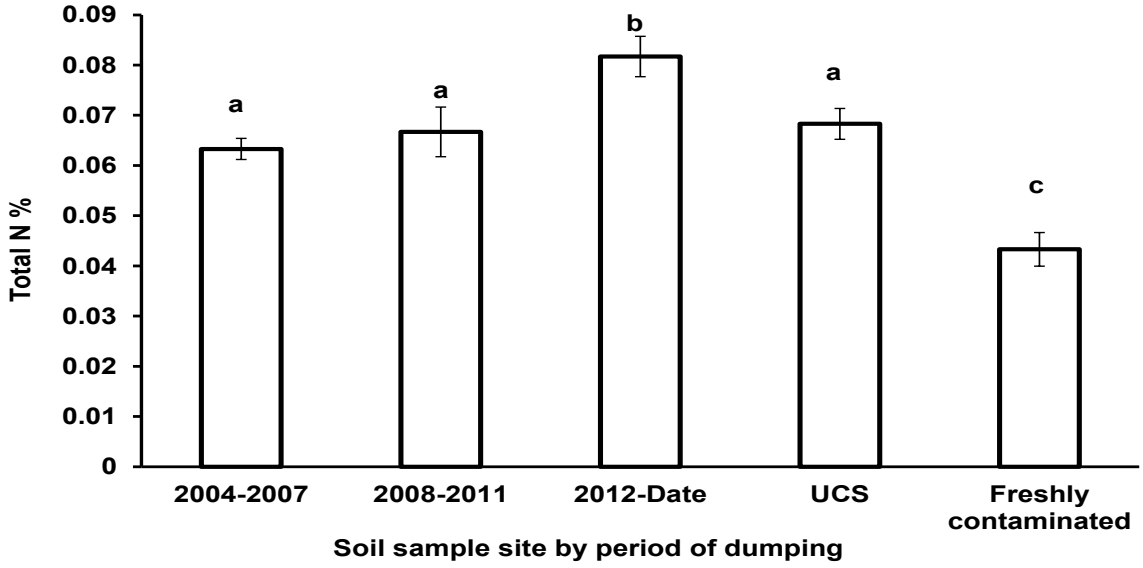

Figure 21 - Comparison of mean % Total N percentage by soil age. Total N % was determined by the Kjeldahl procedure. Vertical bars show the SE of the mean % total nitrogen for the different sites. Means with different letters are significantly different (ANOVA $p<0.05$).

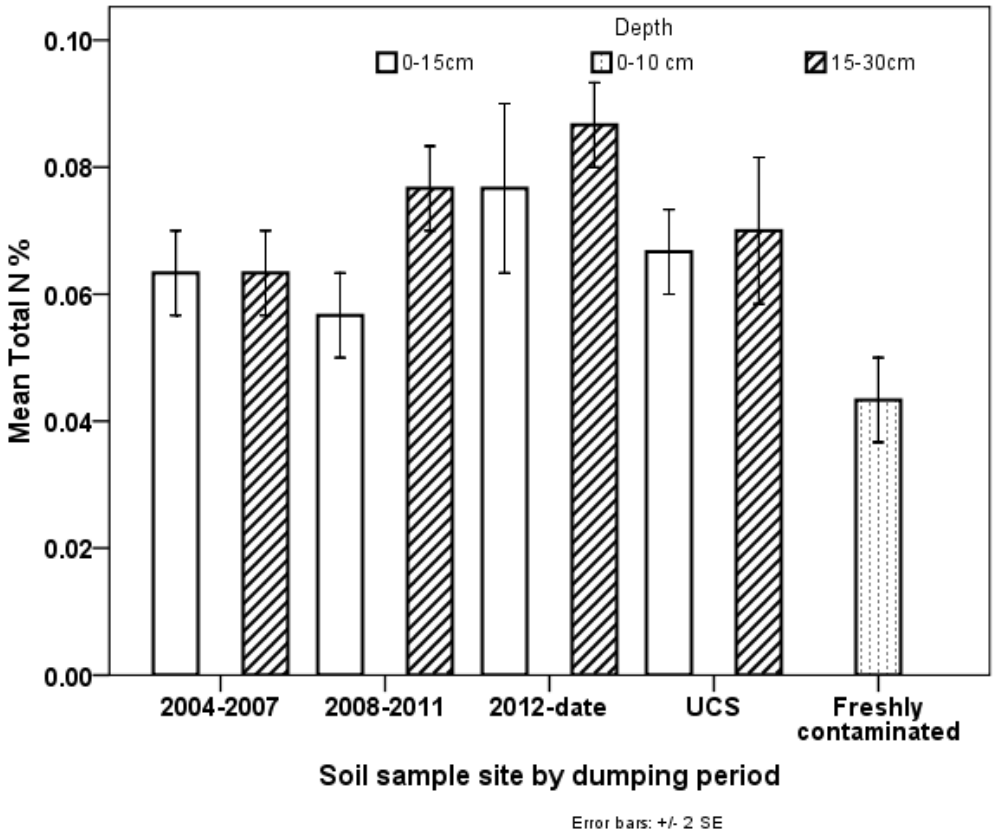

Figure 22 - Comparison of Total nitrogen % variation across the dumping site at 0-15cm and 15-30 cm depths. It shows an interactive effect of depth on the nitrogen levels across sites. The data is expressed as mean % total nitrogen and the vertical bars show the SE of the total nitrogen mean (Univariate ANOVA $p<0.05$).

4.2.4.3 Phosphorus

The 2004-2007, 2012 –to date and the freshly contaminated were statistically the same ($p<0.05$) while 2008-2011 and UCS were significantly lower and different from the rest. Mean phosphorus ranges across all sites were 6.35 – 29.76mg/kg. The results reflect that phosphorus levels were not influenced by age of contaminated. High phosphate levels in the freshly contaminated soils could be attributed to detergents used for washing heavy mobile equipment at the washbays and these would be an added advantage for the microbes that digest hydrocarbons. **Fig. 23** illustrates phosphorus concentration variations overtime.

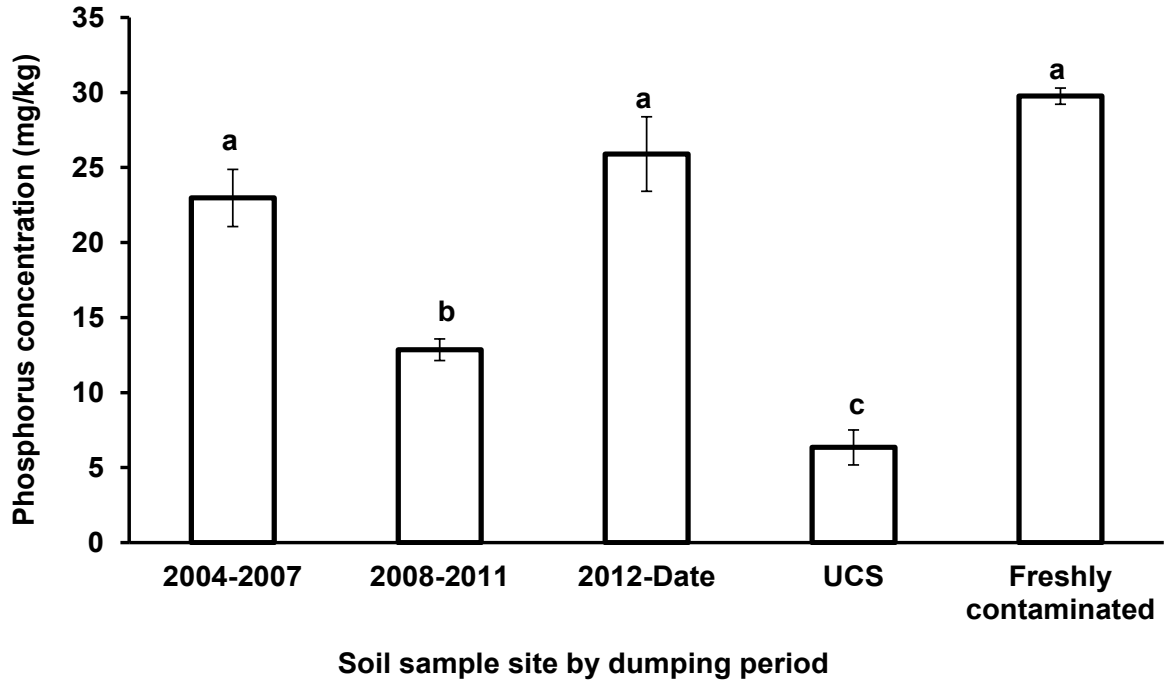

Figure 23 - Comparison of phosphorus concentration by soil age. Phosphorus concentration was determined by the Olsen alkaline bicarbonate method. Vertical bars show the SE of the mean phosphorus concentration in mg/kg for the different sites. Means with different letters are significantly different (ANOVA $p<0.05$).

4.2.4.1 Carbon:Nitrogen:Phosphorus (C:N:P) Ratio

The average Carbon, Nitrogen and Phosphorus ranges were 4000 -24000mg/kg, 400 - 900mg/kg and 6.35 – 29.76mg/kg respectively. This translates to 629.9:62.99:1 as the minimum C:N:P ratio and 806.45:30.24:1 and the maximum C:N:P ratio. The ratios required to be manipulated to achieve recommended C: N: P ratios of 100:10:1 and 100:10:5 accordingly. The ratio of C: N: P is one of the most critical factors that influence biodegradation of hydrocarbons in soils and hence would require accurate calculations for fruitful results.

4.2.4.2 Electrical Conductivity

Electrical conductivity of 1 part of soil in 5 parts of distilled water was determined. EC is a measure of the ability of a suspension to conduct an electric charge and is mostly dependent on the quantity of dissolved salts. Its measurements provide an index of soluble salt content or its salinity. Electrical conductivity mean ranges were 259-893.67uS/cm. Freshly contaminated soil had the highest conductivity followed by 2012-to date. The 2012-date and freshly contaminated samples were not significantly different, 2004-2007, 2008-2011 and UCS had similar EC levels. Conductivity deteriorated with age of contamination of the

contaminated soils i.e. the younger it is the higher the conductivity. **Fig.24** shows mean electrical conductivity across sites.

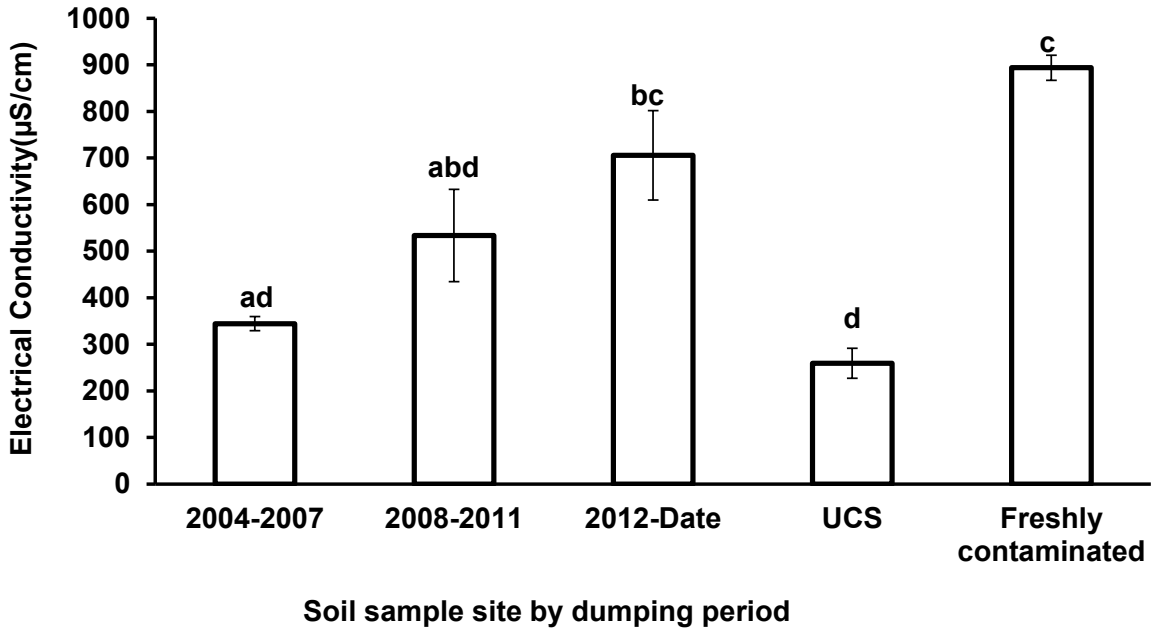

Figure 24- Mean electrical conductivity (EC) across soil sample sites by age. Vertical bars show the SE of the mean EC for the different sites. Means with different letters are significantly different (ANOVA $p<0.05$).

4.2.4.3 Exchangeable cations /CEC

Exchangeable elements include Ca, Mg, K and Na. Average means for cations were Ca- 8.54, Mg – 3.30, K- 0.62 and Na- 0.88.

CEC refers to the number of cations per dry weight that a soil is capable of holding at a certain pH. In this project only the most naturally abundant ones were measured i.e. Ca, Mg, K and Na. CEC was determined by summing up the concentrations of 5 naturally most abundant cations (Ca, Mg, K, Na /Al in acidic soils). Average CEC me/% for the uncontaminated and contaminated soils were 6.4 and 15.6. CEC decreased with age, the older the soil the lower the CEC. **Figs 29** and **30** illustrate comparison of CEC overtime and depth respectively.

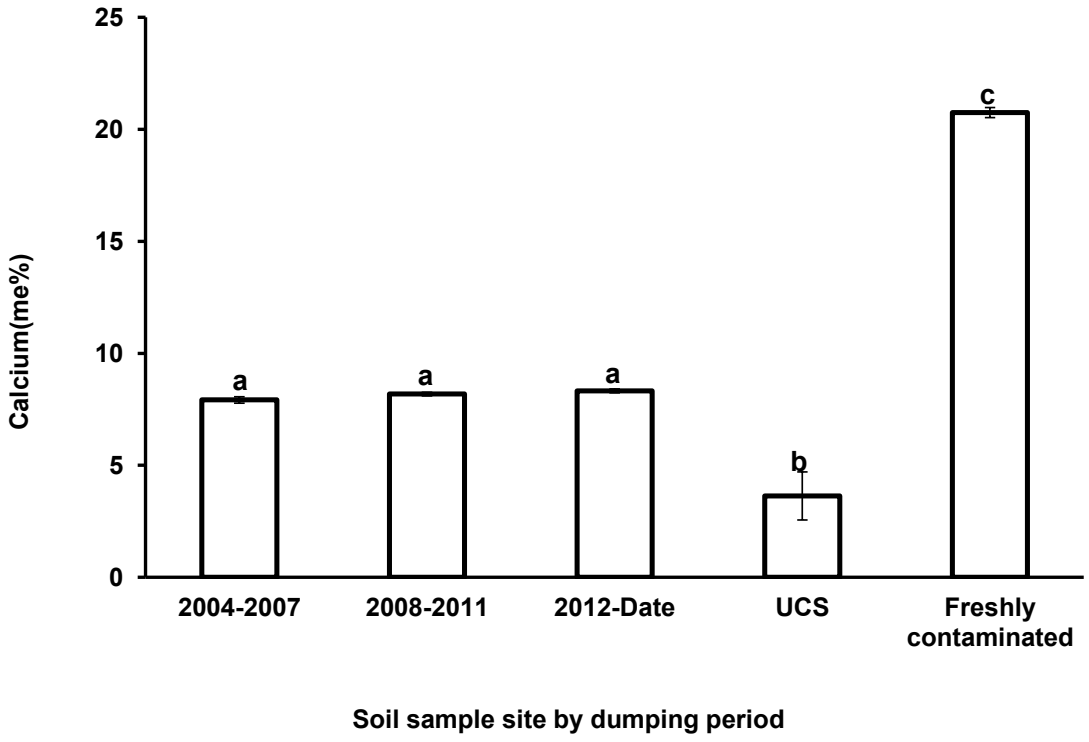

Figure 25 - Comparison of calcium in various soil ages. Calcium levels were determined to assess the micronutrients levels in the soils across the various sites. Vertical bars show the SE of the mean calcium for the different sites. Means with different letters are significantly different (ANOVA $p<0.05$).

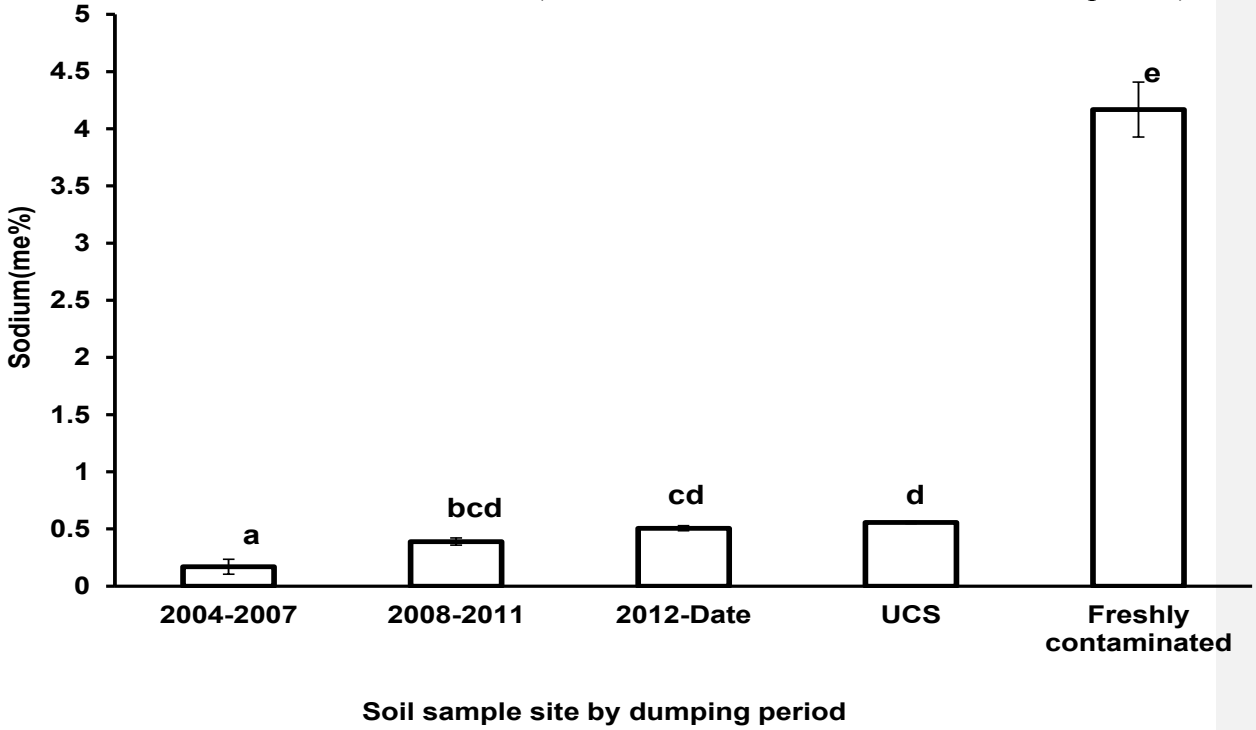

Figure 26 - Comparison of % Sodium by site. Sodium levels were determined to assess the micronutrients levels in the soils across the various sites. Vertical bars show the SE of the mean sodium % for the different sites. Means with different letters are significantly different (ANOVA $p<0.05$).

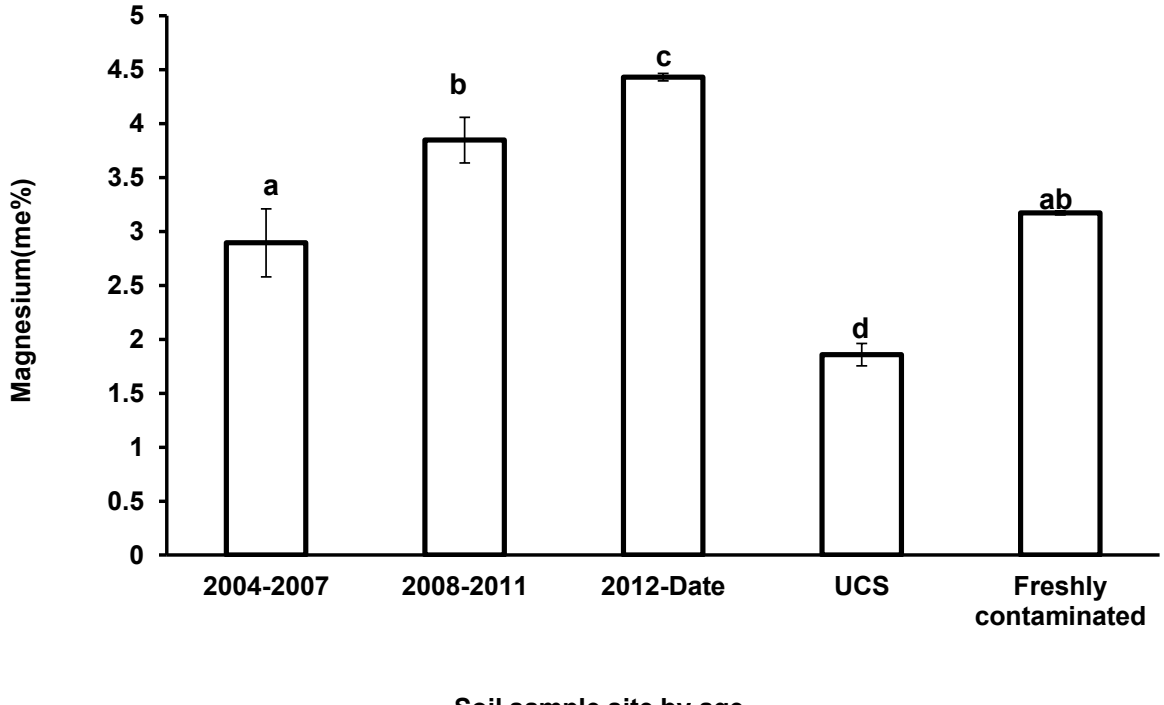

Figure 27 - Comparison of magnesium availability across the study sites. magnesium levels were determined to assess the micronutrients levels in the soils across the various sites. Vertical bars show the SE of the mean magnesium % for the different sites. Means with different letters are significantly different (ANOVA $p<0.05$).

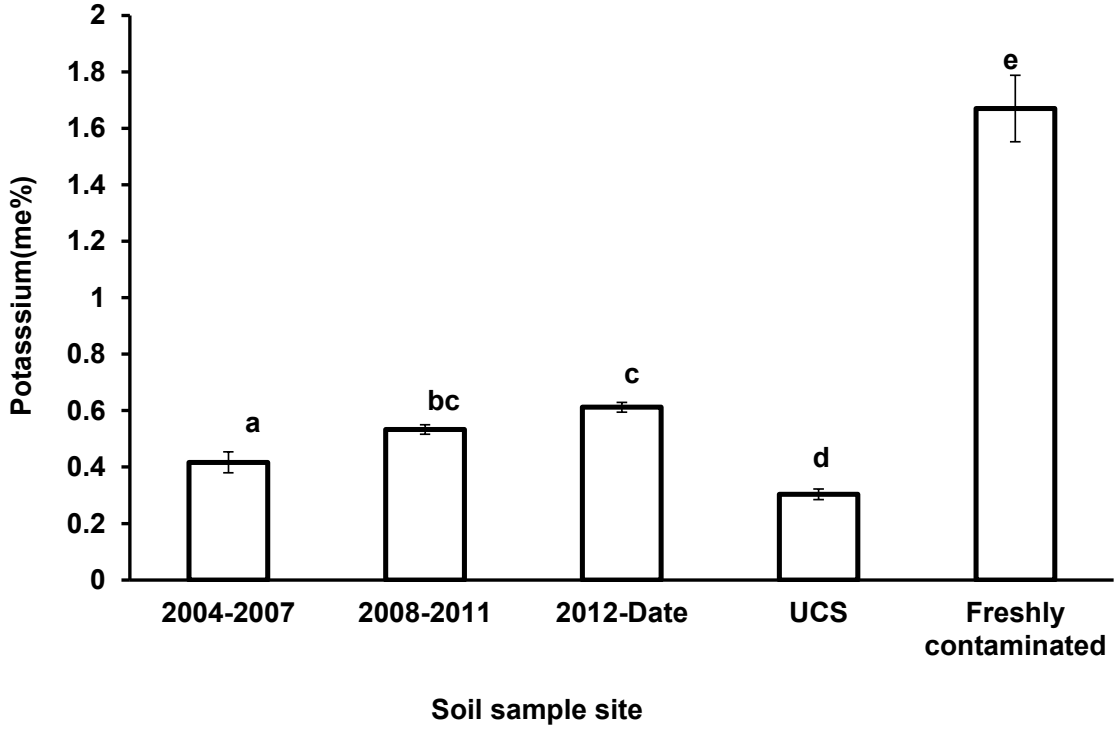

Figure 28 - Comparison of Potassium availability across sites. Potassium levels were determined to assess the micronutrients levels in the soils across the various sites. Vertical bars show the SE of the mean potassium % for the different sites. Means with different letters are significantly different (ANOVA $p<0.05$).

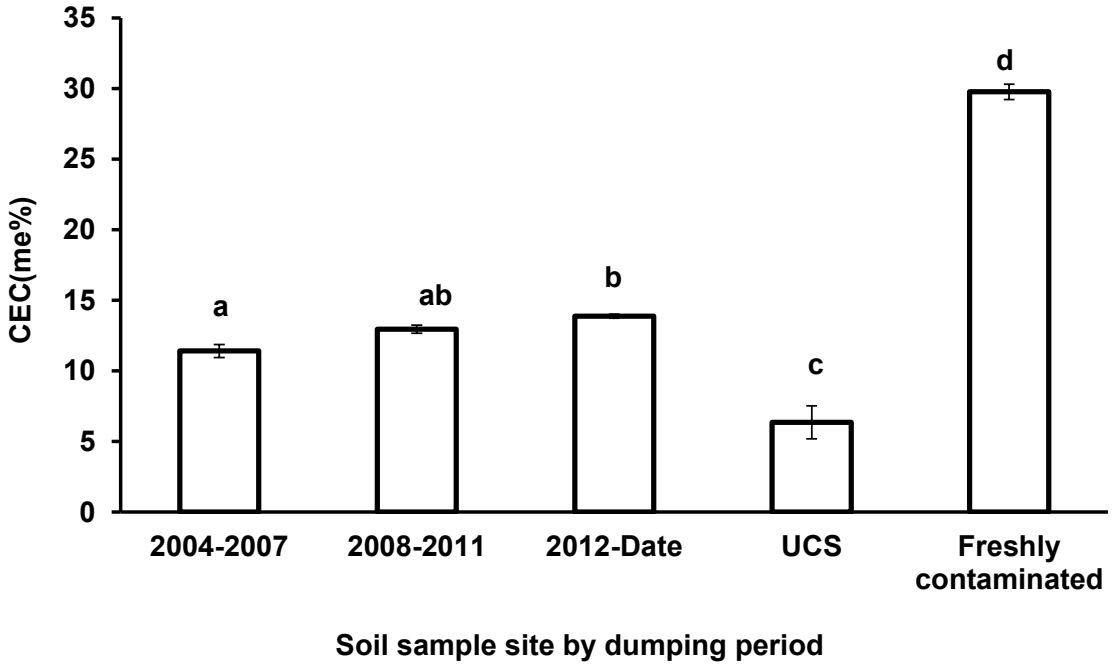

Figure 29 - Comparison of CEC percentage by site. Three replicates were used per site and mean CEC values are presented as me%. Vertical bars show the SE of the mean CEC me% for the different sites. Means with different letters are significantly different (ANOVA $p<0.05$).

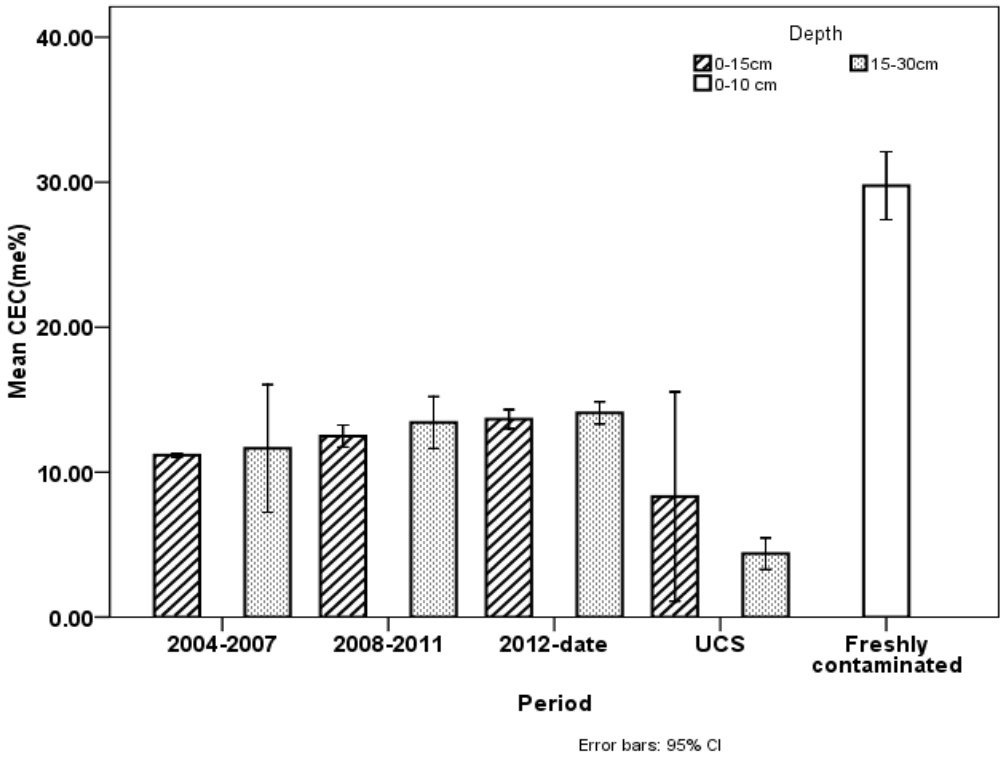

Figure 30 - Illustration of interactive effect of dumping period and depth on CEC. The CEC was determined so as to understand the cation ion exchange capacity of the different soils. Vertical bars show the SE of the mean CEC me% for the different sites (Univariate of ANOVA $p<0.05$).

4.3 Microbiological properties

Results for identified microbial genera, soil respiration, microbial biomass, microbial nitrogen and Carbon determined across the uncontaminated and contaminated sites by depth are presented in this section.

4.3.1 Identification of microbial genera

A total of 42 microbial genera were identified across the sites. **Table 8** details the identified genera. Only 9 out of the 42 identified genera have been verified in literature of their capability to naturally degrade the hydrocarbon is soils. These included one of the predominantly used ***Pseudomonas species, Bacillus, Serratia. marcescens, Flavobacterium, Micrococcus, Streptomyces, Staphylococcus, Penicillium*** and ***the yeasts (***Brinda *et al*. 2013; Bodour *et al.* 2003; Mrozik *et al.* 2003; Cerniglia, 1992). If these are stimulated with nutrients in organic or inorganic fertilizers form they are the species that could be responsible for microbial degradation.

Table 8 - Identified Microbial Genera

Microbial Genera identification	**2004-2007**		**2008-2011**		**2012 to date**		**UCS**	**BWB/ TSWB**	
Freshly contaminated									
	0-15cm	15-30cm	0-15cm	15-30cm	0-15cm	15-30cm	0-15cm	15-30cm	1
	2								
Fusarium	+++	+++	+++	+++	+++	+++	+++	+++	+
	+	+							
Aspergillus	+++	+++	+++	+++	+++	+++	++	-++	+
	+	+							
Penicillium	+++	+++	+++	+++	+++	-++	+-	---	-
	-	-							
Slime molds	+++	+++	+++	+++	+++	+++	++	+++	+
	+	+							
Streptomyces	+++	+++	+++	+++	+++	+++	-+	+++	+
	+	+							
Alternaria	+++	+++	+--	+++	-++	+++	+-	---	-
	+	-							
Bacillus, Mucor Proteus	+++	+++	+++?--+		+++	+++	+++	?+	+++	+
	+	+							
Lactobacillus	-+-	+++	--+		+++	+++	+++	++	+++	+
	+	+							
Pseudomonas	+++	+++	+-+		+++	+++	+++	++	+++	-
	-	-							
Clostridium	---	+++	---		+++	-++	+++	--	---	-
	-?	-							
Rhizobium	---	+++	---		+++	-++	+++	--	+++	+
	+	+							
Rhizoctonia	+++	+++	+++		+++	++	+++	+-	+++	+
	+	+							
Nitrobacteria	--+	+++	---		+++	---	+++	--	+++	+
	+	+							
Nitrosomonas	--+	+++	---		+++	---	+++	--	---	-
	-	-							
Flavobacterium	---	+++	++-		+++	---	-++	--	---	-
	-	-							

Algae	+++	+++	+?+	+++	+++	+++	+-?	+++	+
Filamentous -Bacteria	---	+++	---	+++	-++	+++	--	---	-
Ciliates	+++	+++	+++	+++	+++	+++	++	+++	+
Flagellates	--+	+++	+++	+++	+++	+++	--	---	-
Nematodes	---	+++	---	+++	-++	+++	-+	+++	+
S.marcescens	+++	---	+++	--	+--	---	+-	-++	+
Acetic acid bacteria	-+-	---	--+	--+	---	---	+-	+--	-
Yeasts	+++	---	+++	---	+--	---	++	+++	+
Sordaria	+++	---	+++	---	+--	---	+-	---	-
Cladosporous	--+	---	++-	---	-++	---	++	-++	+
Rhizopus	---	---	-+-	---	-++	---	++	+++	+
Micrococcus	---		---	---	--+	+-+	--	---	-
Trichoderma	+++		+++	---	+--	---	--	---	-
Mucor	+++		+++	---	+++	---	--	---	-
Phoma	+++		+++	---	+++	---	+-	-+-	-
Green algae	+++		+++	---	+--	--+	++	---	+
Coliforms	+++		+++	---	++-	---	--	---	-
Streptococcus	+-+		+++	--+	+--	---	++	+++	+
Protozoa	-+-			---	---	---	--	---	-
Penicillin				---	+--	++	+++	+	+
E.Coli						++	+++	+	+
Staphylococcus								+	-
P.fluorescence								+	+
Collatrotricum									+
Clostridia									+
Absidia									

Scale – the three symbols i.e. **+++, +-+, ---, +--** represent 3 replicates analysed for the microbial genera. The +, -, and? Signs mean that the particular genera were present, absent and result not conclusive in a sample respectively.

The comparison of Total Microbial biomass, Nitrogen, and Carbon percentage across sites and depth is illustrated from **Figs. 31 to 36** respectively. The Total microbial biomass, Nitrogen and Carbon percentages followed the same trend across all sites by depth. There were no significant differences in percentages for the three parameters in the 2004-2007, 2012 to date and the freshly contaminated samples. The parameters were statistically similar and the same applied to depth as well at $p<0.05$. The soil sample site 2008-2011 and the uncontaminated soil sample were significantly different. The Total Microbial Respiration parameters varied across all sites. **Figs. 37** and **38** illustrate the variations.

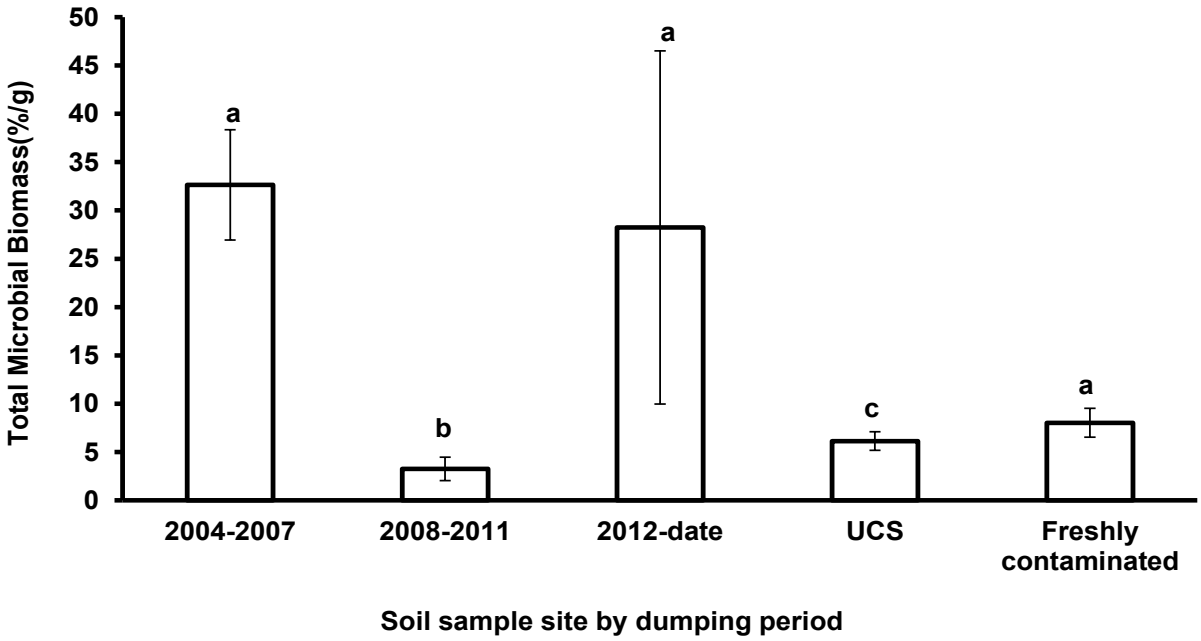

Figure 31 - Comparison of Total microbial biomass across sites. Total microbial biomass was determined using the chloroform fumigation extraction method after seven days of incubation. The biomass was were determined to assess the microbial activities inorder to determine potential biodegradation of hydrocarbons in soils across the various sites. Vertical bars show the SE of the mean microbial biomass %/g for the different sites. Means with different letters are significantly different (ANOVA $p<0.05$).

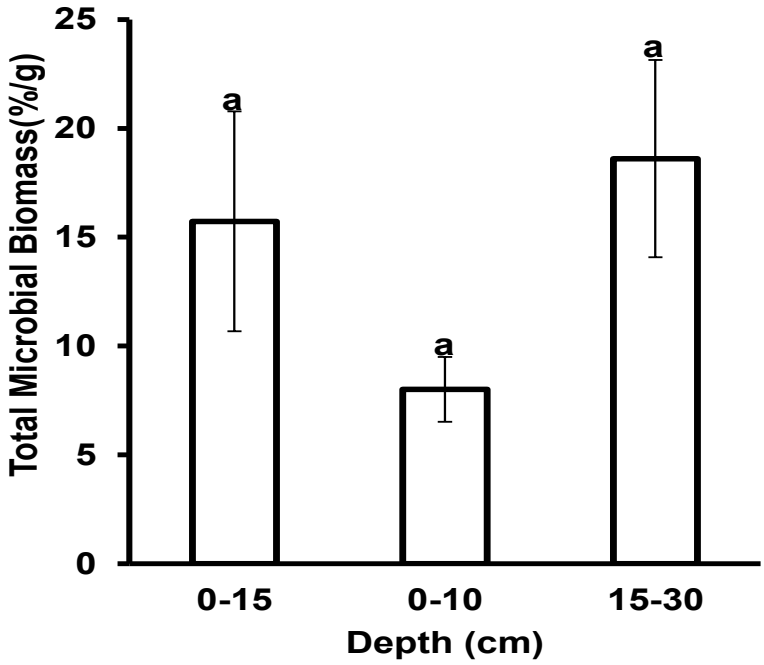

Figure 32- Comparison of total microbial biomass in three composite samples representing depths 0-15, 15-30 (old aged) and 0-10cm (freshly contaminated).

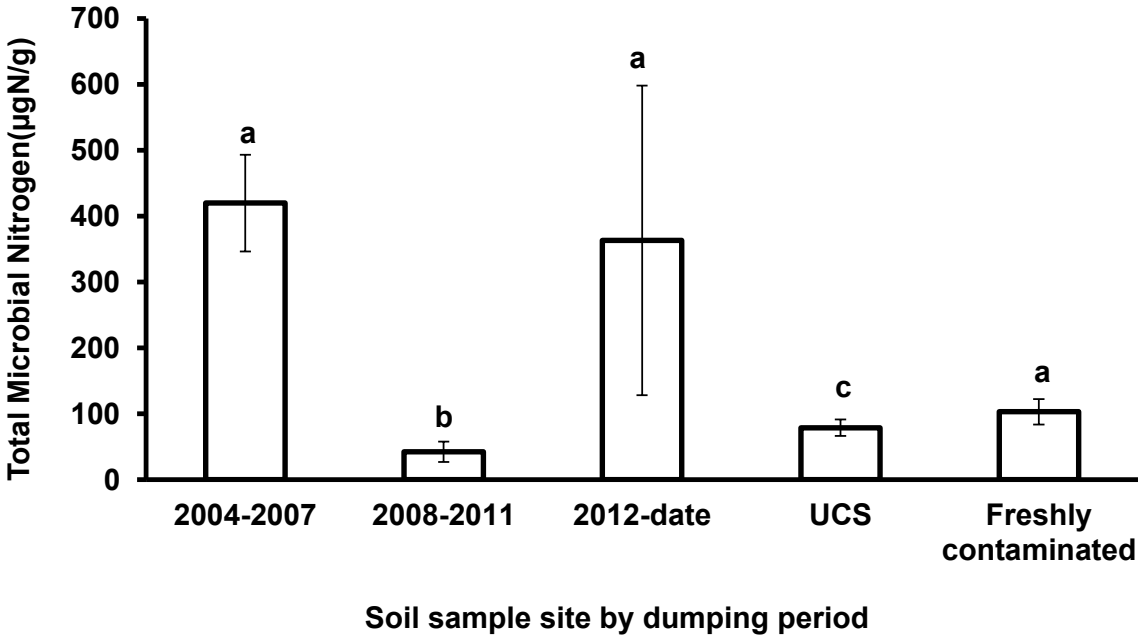

Figure 33 - Comparison of mean microbial nitrogen availability across sites. Total microbial nitrogen was determined by reading through a spectrometer after chloroform fumigation extraction method which was preceded by seven days of incubation. The nitrogen was estimated to aasess nitrogen levels available for microbial activities in soils across the various sites. Vertical bars show the SE of the mean microbial nitrogen %/g for the different sites. Means with different letters are significantly different (ANOVA $p<0.05$).

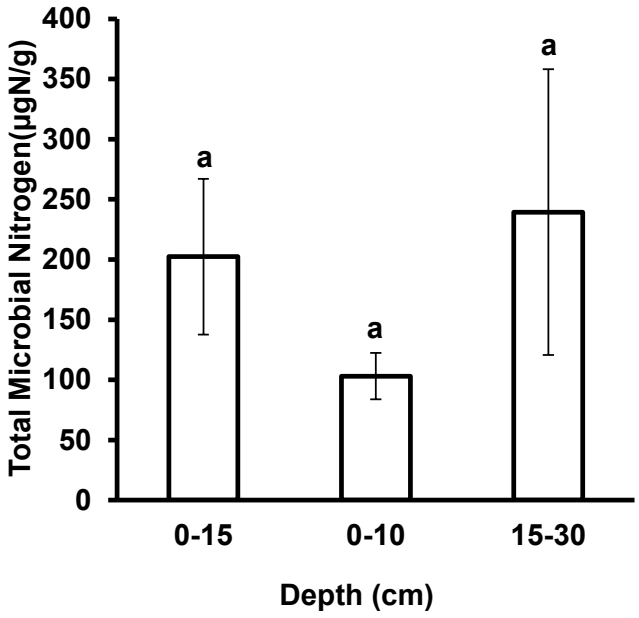

Figure 34 - Comparison of mean microbial nitrogen in three composite samples representing depths 0-15, 15-30 (old aged) and 0-10cm (freshly contaminated).

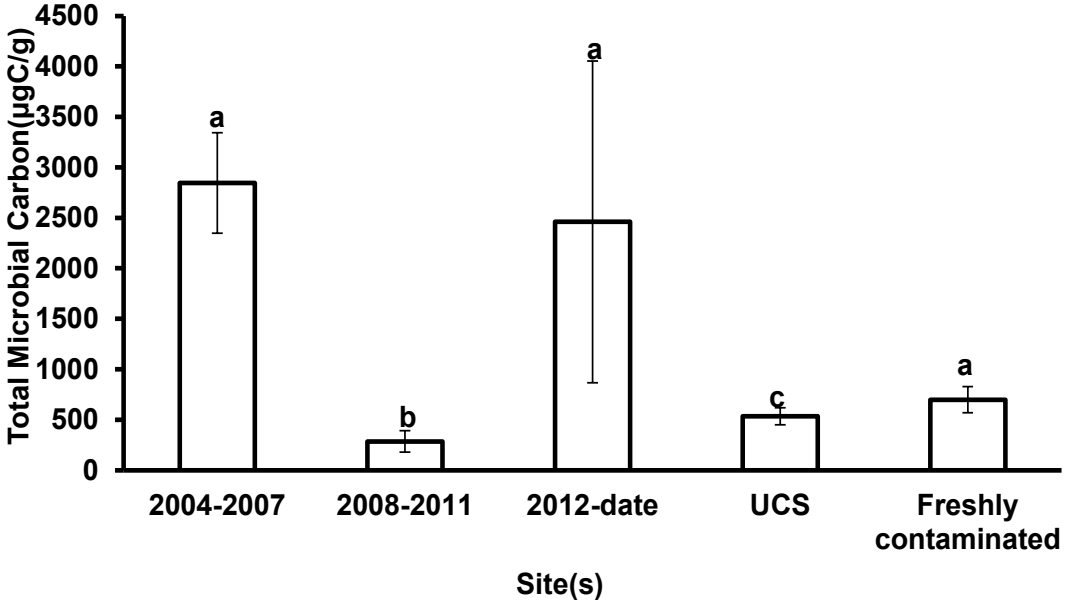

Figure 35 - Comparison of mean microbial carbon availability across sites. Total microbial carbon was determined by reading through a spectrometer after chloroform fumigation extraction method which was preceded by seven days of incubation. The carbon levels were estimated to assess carbon availability for microbial activities in soils across the various sites. Vertical bars show the SE of the mean microbial carbon %/g for the different sites. Means with different letters are significantly different (ANOVA $p<0.05$).

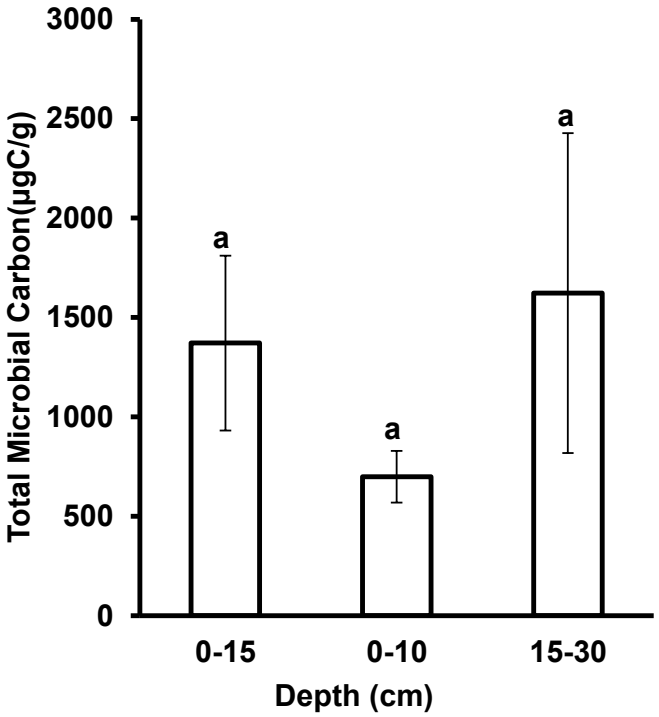

Figure 36 - Comparison of mean microbial carbon in three composite samples representing depths 0-15, 15-30 (old aged) and 0-10cm (freshly contaminated).

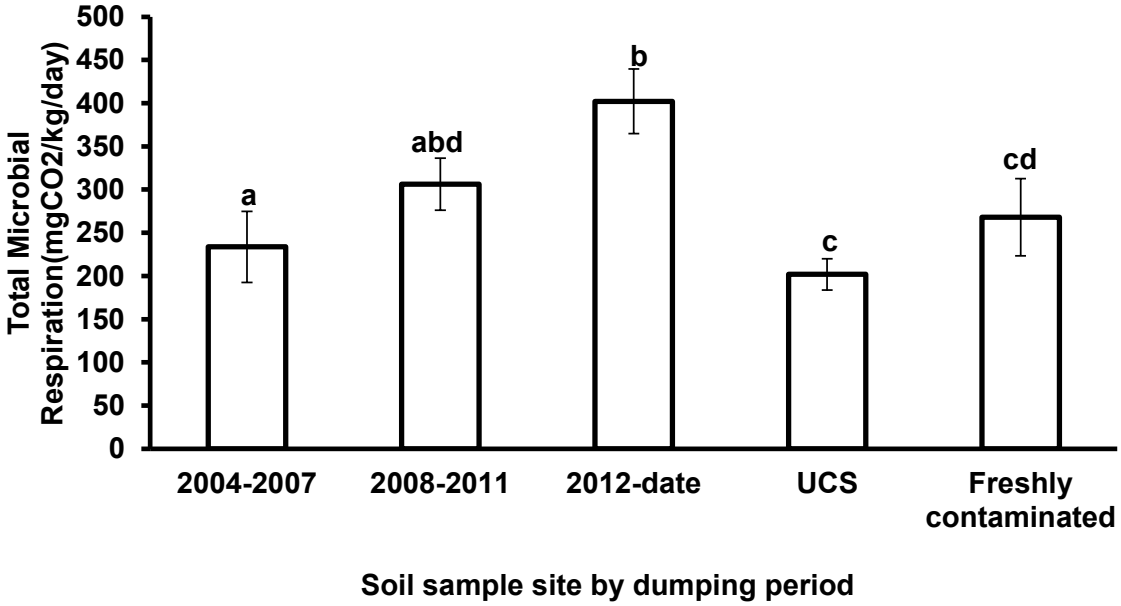

Figure 37 - Comparison of Total Microbial Respiration across sites. Total microbial respiration was estimated using the CO_2 flux method where CO_2 was absorbed by soda lime water. The respiration was estimated to assess the rate of degradation of hydrocarbons under natural conditions soils across the various sites. Vertical bars show the SE of the mean microbial respiration in mgCO2/kg/day for the different sites. Means with different letters are significantly different (ANOVA $p<0.05$).

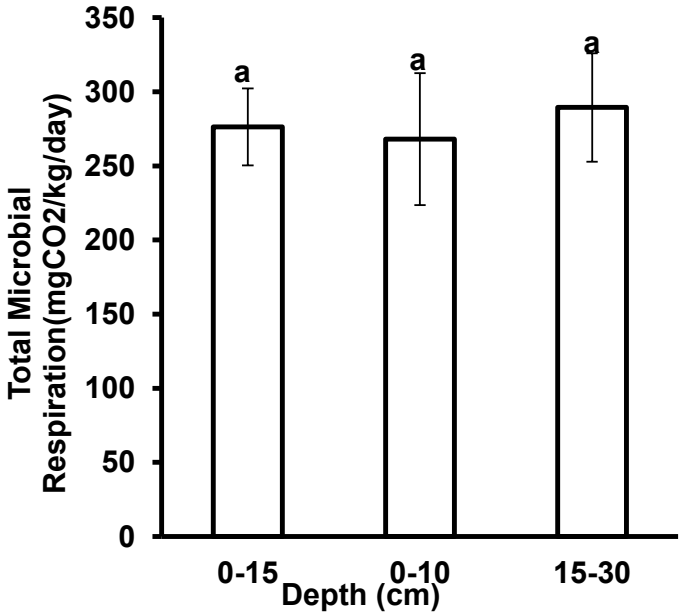

Figure 38 - Comparison of mean Total Microbial Respiration microbial carbon in three composite samples representing depths 0-15, 15-30 (old aged) and 0-10cm (freshly contaminated).

4.4 Murowa Soils characterisation summary

Murowa soils were classified as Sandy loam. Water repellency deteriorated with age of soil, i.e. the recently polluted the higher the repellency. This was also the case for TPH concentration which deteriorated with age. Average pH range for the soils was 8-9.4 which means soils are strongly alkaline and subsequently, the pH would not need any adjustment as it falls within the suitable range required for microbial degradation of hydrocarbons. In terms of the nutrient ratios, C: N: P range, minimum was 629:62.9:1 and maximum was 806:30.3:1 and the nutrient ratios as a result required manipulation to attain the ratios of 100:10:1 and 100:10:5.

As for the microbial properties, out of the 42 genera identified, only nine have been verified of their capability to naturally degrade hydrocarbons. There was insignificant variation in terms of microbial nutrients and respiration activities across sites and soil depth.

Based on the results from the characterisation process, a decision to use the 2012 to date sample for the pot experiment was made. This was so because it contained the highest concentration of hydrocarbon when compared to other soil ages and hence was selected as the sample for the pot experiment. The TPH concentrations in other soil ages were considered very low such that no meaningful trends could be determined overtime. ***Table 9*** shows consolidated physicochemical and microbial characterisation results for the 2012 to date soil sample.

Table 9 - Physicochemical results summary for 2012 to date sample

Parameter	2012 to date sample
Sand	*74.67*
Silt	*15.92*
Clay	*9.08*
Water repellency	*2571*
TPH	*265.733*
pH	*8.62*
Carbon	*2.27%*
Nitrogen	*0.08%*
Phosphorus	*25.90mg/kg*
C:N:P ratio	*22.733: 817: 25.9017 Vs**(100:10:1)***
EC	*705.67*
Ca	*8.32*
Mg	*4.43*
K	*0.61*
Na	*0.51*
CEC	*13.87*
Microbial species capable of degrading TPH	***Pseudomonas, Bacillus, S. Marcescens, Flavobacterium, micrococcus, Streptomyces, staphylococcus, Penicillium and yeasts***
Total Microbial Mass	*28.235*
Total Microbial Nitrogen	*363.22*
Total Microbial carbon	*2460.6*
Respiration	*402.17*

4.5 Pot experiment results

The controlled pot experiment was run for 111days in 2015 from August to November which was essentially during the summer season. The experiment involved monitoring the effect of nutrient amendments on total petroleum hydrocarbon concentration and degradation in hydrocarbon contaminated soils. Changes C: N: P concentration, pH, total microbial mass were also observed during the same period. **Figs. 39** through to **46** summarise the TPH, C: N: P concentration, pH and microbial mass variations over the stated period.

4.5.1 Total Petroleum Hydrocarbon

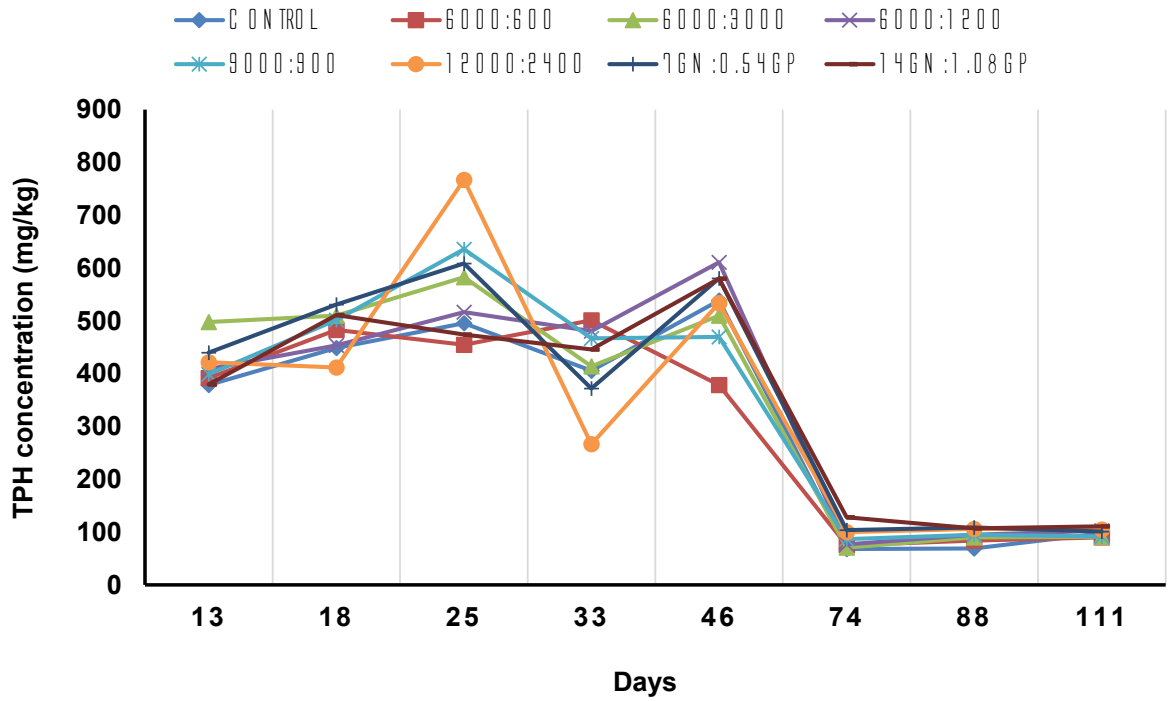

Figure 39 - Influence of nutrient treatments on hydrocarbon degradation and the behaviour of hydrocarbon in natural site (control) over 111days period. Data is expressed as mean TPH concentration in mg/kg.

The TPH results for days 0- 74 show that there was an average decrease of about 77%. At 74 days, the graphs show some convergence in the concentration of hydrocarbons across all treatments which might mean that degradation occurred until it reached comparable values as of the control. **Fig. 39** shows the influence of C: N: P treatment on TPH degradation over time. Generally for all treatments from day zero to day 25 (the first 3 weeks) the TPH concentration increased from the mean initial concentration of TPH was 265.733 mg/kg to average mean of about 527.125 mg/kg, then from day 25 to day 33 there was a sharp decline, then from day 33 to day 46 there was a spike increase and thereafter from day 46 to day 74 there was a steady decrease. It is during this period (days 46-74) where the highest TPH concentration reduction was recorded usually probably due to the fact that hydrocarbons would have been broken into less complicated hydrocarbon compounds which were more available and easier to degrade by microorganisms. **Table 10** highlights percentage decreases for days 0 - 74, 46 - 74 and 0 - 111. The graph shows that within the first 74 days there were significant changes between successive measures over time which might lead one to concluding that degradation was effectively done from day zero to day 74.

Statistically the results show that there was a significant influence of treatment on degradation of TPH (F 8.641; p 0.000) **Appendix 10.** Comparisons of all treatments with the

control show that there were significant differences between control and treatments 6000:1200; 9000:900; and 7gN:0.54gP. There were no significant difference between the control and treatments 6000:600; 6000:3000; 12000:2400 and 14gN:1.08gP

Treatment 6000:3000 yielded the highest degradation of about 82% from the initial concentration and 86% from day zero to day 74 **(Table 10).**

Table 10 - Percentage decreases in TPH from 0-74days, 46-74days and 0-111days

Treatment	0-74 days	46-74days	0-111days
Control	74%	87%	74%
6000:600	80%	80%	77%
6000:3000	**86%**	**86%**	**82%**
6000:1200	81%	87%	75%
9000:900	78%	81%	77%
12000:2400	76%	81%	75%
7gN:0.54gP	76%	82%	77%
14gN:1.08gP	66%	78%	71%

Table 11 - Identified Hydrocarbons at five (5) weeks

Sample reference	Treatment	Hydrocarbon present
121	Control Replicate 1	Decane, undecane, hexadecanal, 2 ethylcridine and octadecane, 1-iodo
123	Control Replicate 3	Decane, undecane, dodecane
124	6000mgN:600mgP replicate 1	Decane, undecane, dodecane
134	9000mgN:900mgP	Decane, undecane, dodecane
136	12000mgN:2400mgP Replicate 1	Decane, undecane, dodecane
142	14gN:1.08gP	Decane, undecane, dodecane
144	14gN:1.08gP	Decane and undecane

Time also played a significant influence on biodegradation (F 14.729; p 0.000) as shown in **Fig. 39**. ANOVA shows that there was a significant interactive effect of treatment and time on biodegradation of hydrocarbons (F 3.003; p 0.000). Weekly comparison reflected that there were significant differences in TPH concentration changes every week except between

day 13 and 33. From day 74 to day 111 there were no significant changes. This means that the period in with which the degradation process occurs is critical. **Fig. 40** illustrates the influence of time and treatment on TPH concentration.

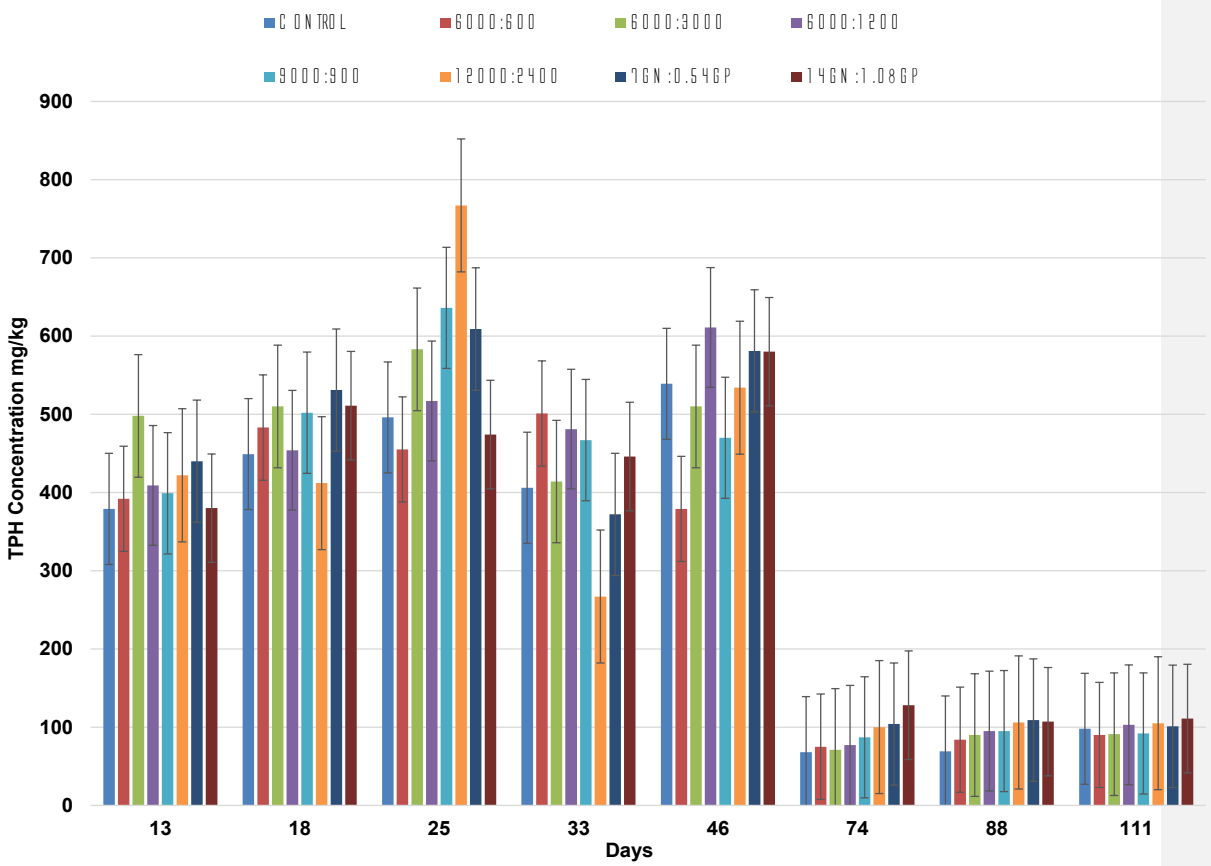

Figure 40 - Interactive effect of nutrient treatment and time on hydrocarbon degradation over a period of 111 days. TPH was determined by gravimetric methods USEPA3350 and 1664. The concentration is expressed as mean TPH mg/kg on the different sites). Vertical bars show the SE of the mean of TPH concentration. Anova (F 3.003; p 0.000 at $p<0.05$).

4.5.2 Total Carbon availability in Murowa soils during treatment

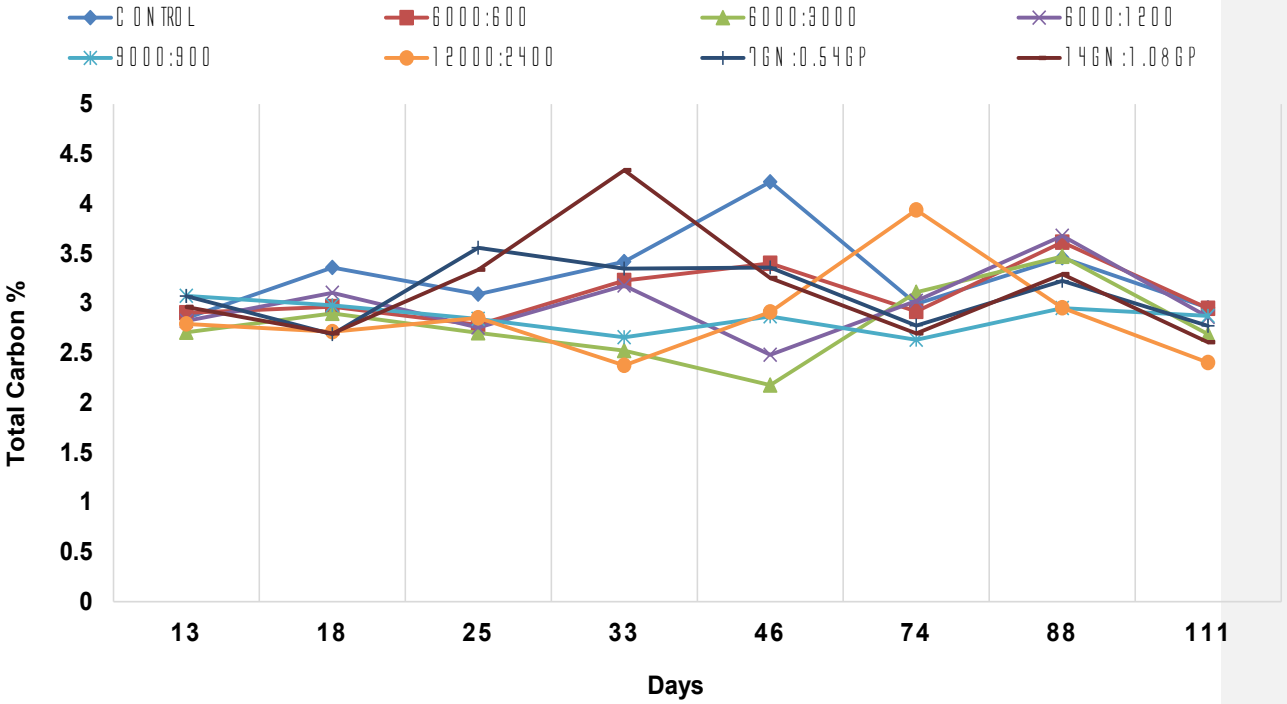

Figure 41 - Mean Total Carbon % variation in the various N:P fertiliser treated soil and in the natural site (control) during over a period of 111 days. Carbon % was determined by the Walkley-Black method using potassium dichromate as the oxidant.

The ratio of C: N: P is critical in biodegradation of hydrocarbons in soils. Carbon availability and levels was one of the elements that were monitored together with TPH over the 111 days. The initial percentage Carbon mean range was 0.40 -2.4% **(Fig. 20)**. The results show that application of fertiliser had minimal influence on Carbon % as the levels slightly increased to range 2.78 – 4.2% probably due to the fact that the microbes could have been utilizing the carbon liberated from the hydrocarbon degradation rather the carbon in the soil.

Carbon levels were not significantly different at P<005, at 13 days they were more or less the same with any other days except at 33 days and 88 days which were significantly different. Sharp peaks of Carbon levels were experienced at 33days and 88 days.

Effect of treatment on Carbon levels was minimal as well. Comparison of carbon levels in all the treatments and the control sample show that the control levels were not significantly different except for treatment 6000:3000 and 9000:900. **Appendix 10** details the comparison of effect of treatment on carbon levels.

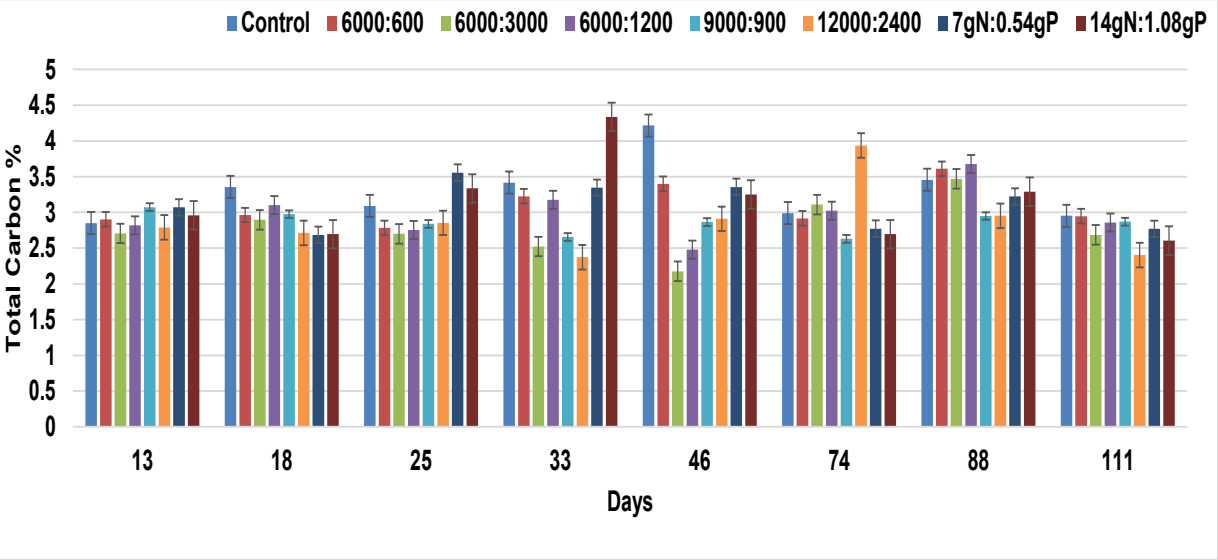

Figure 42 - Interactive effect of nutrient treatment and time on Carbon composition in the treatments and the natural site over a 111 days period. Carbon % was determined by the Walkley-Black method using potassium dichromate as the oxidant. Vertical bars show the SE of the mean % carbon for the different sites on a weekly basis. (ANOVA $p<0.05$).

4.5.3 Total Nitrogen percentage in Murowa Soils during treatment

Percentage nitrogen concentration initially ranged from 0.04 – 0.09% (400-900mg/kg). Addition of nitrogen and phosphate fertilizers resulted in a slight increase in nitrogen concentration which was witnessed from day 0 - 46 though the increment was statistically not significant. After day 46 there was a significant drop in total nitrogen until day 74. From day 74 onwards, a gradual increase was witnessed for most of the treatments except treatments 7gN:0.54g P and 12000:2400 which recorded sharp increases after 88 days. Comparison of the control (Total N decrease range was 0.3 – 0.13%) and each treatment showed that the difference in the levels of nitrogen in 6000:600; 6000:3000; 6000:1200; and 14gN:1.08gP (Total N decrease range was 0.3 – 0.12%) were insignificant while significant difference were noted between 9000:900; 12000:2400 and 7gN:0.54gP in which the increase in total N ranged between 0.3-0.9%. **Fig. 43** demonstrates the effect of nitrogen fertiliser on nitrogen concentration.

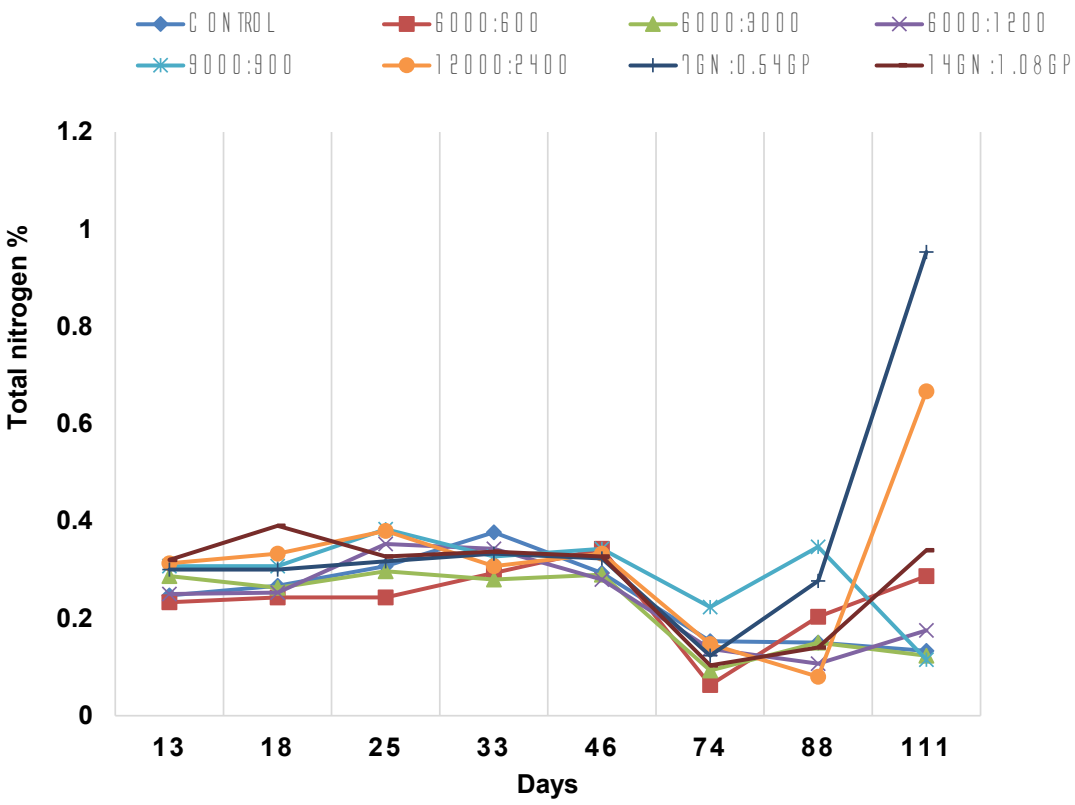

Figure 43 - Illustrates effect of nitrogen fertiliser on nitrogen concentration in treated soils as well as in the natural site over a period of 111days.

4.5.4 Phosphorus concentration

Initial mean phosphorus ranged from 6.35-29.76mg/kg. Addition of nitrogen and phosphorus fertilisers influenced the phosphate concentration in the soils positively. Phosphorus significantly increased to levels above 700mg/kg at 13days. Significant differences in that respect were noted at 18, 33, 46 and 74days, the concentration levels fluctuated downward and upward at 18 and 25 days and downward until day 74 and started appreciating at 88 and 111days. Comparison of the control sample by the various treatments reflects that the control sample was significantly lower in phosphorus concentration from treatments 6000:3000; 6000:1200; 9000:900 and 12000:2400 and statistically similar to 6000:600; 7gN: 0.54gP and 14gN:1.08gP **(Fig. 44)**.

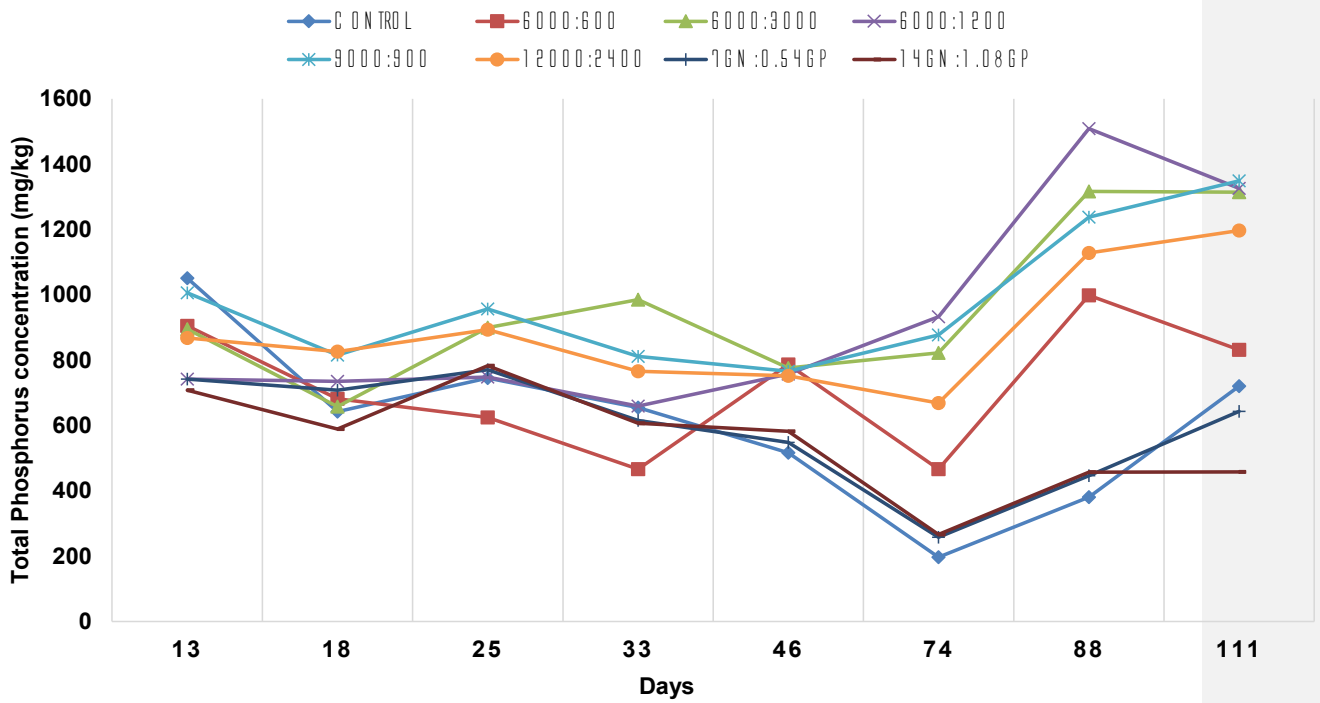

Figure 44 - Total phosphorus concentration variations in nitrogen and phosphorus fertilisers treated soils and untreated soil (control site) over a period of 111 days.

4.5.5 Total Microbial biomass

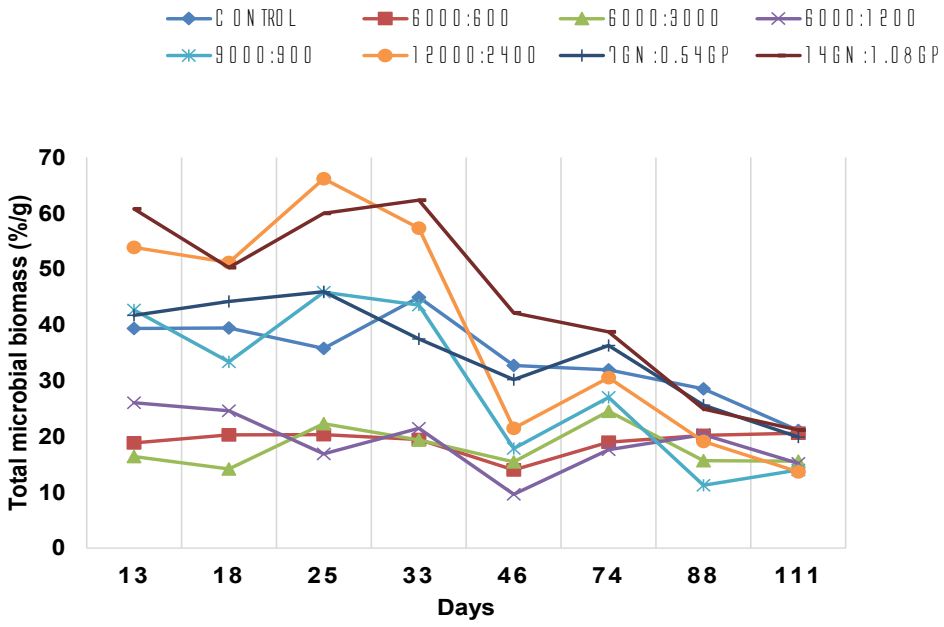

Figure 45 - Microbial biomass response over 111 days following stimulation by different nitrogen and phosphorus treatments inform of inorganic fertiliser.

Microbial biomass percentage per gram before treatment ranged from 25-30%. Generally after addition of the fertilisers the microbial biomass increased to about 40% and above except in two treatments 6000:600 and 6000:3000 which were below 20%. Treatment 12000:2400 had the highest microbial biomass percentage which ranged between 55% and 69% and the least being 6000:3000 which was below 20%. The percentage microbial biomass from 13 to 33days was statistically the same. Significant changes were noted between 33 and 46 days where there were slight to sharp declines for the various treatments. From day 46 to 74 days there was a steady increase in biomass percentage which then declined at 88 and 111days.

Sharp declines between day 33 and day 46 followed by a significant increase which corresponded with a steady decrease in Nitrogen and phosphorus concentration during the same period meaning the microbes utilised the nitrogen and phosphorus. The sharp decline between day 33 and 46 could be attributed to significant temperature changes that prevailed during that period which were above 30^0C averaging 34^0C which could have been unfavourable for the microbes especially in treatments with N concentration greater than 6000mgN. The decrease in the biomass almost converged with the low levels experienced in the treatments that had 6000mgN which could mean that only those microbes that could survive under those nutrient and temperature conditions survived. However, there is an opportunity to further investigate and isolate the microbes which multiplied significantly in high nitrogen content and those that remained active beyond 46days.

The microbial biomass levels in the control sample remained constant throughout the period while the other treatments almost converged at 46days onwards,

4.5.6 Soil pH

Initial pH mean ranges were 8.067-9.367 for the samples that were used for pot experiment. Weekly comparison show that there were significant differences ($p<0.05$) at each stage except at 13, 33 and 46 days which were similar as well as 74, 88 and 111 days **(Fig. 46)**. Treatments also influenced pH to a certain extent. In treatment **6000:600**, pH level lowered from strongly alkaline to alkaline (pH – 7.01), **6000:1200**, **6000:3000**, **9000:900** and **12000:2400** became neutral (pH range 6.2 – 6.7), 7gN:0.54P and 14gN:1.08P remained strongly alkaline (pH – 8.01) while the control did not vary much (pH-8.10) by remained strongly alkaline as well (Zimbabwean guide to the meaning of soil analysis). The pH ranges fall within the pH ranges suitable for microbial activity at 5.5-8.8 and for oil degradation 6.5-8.0.

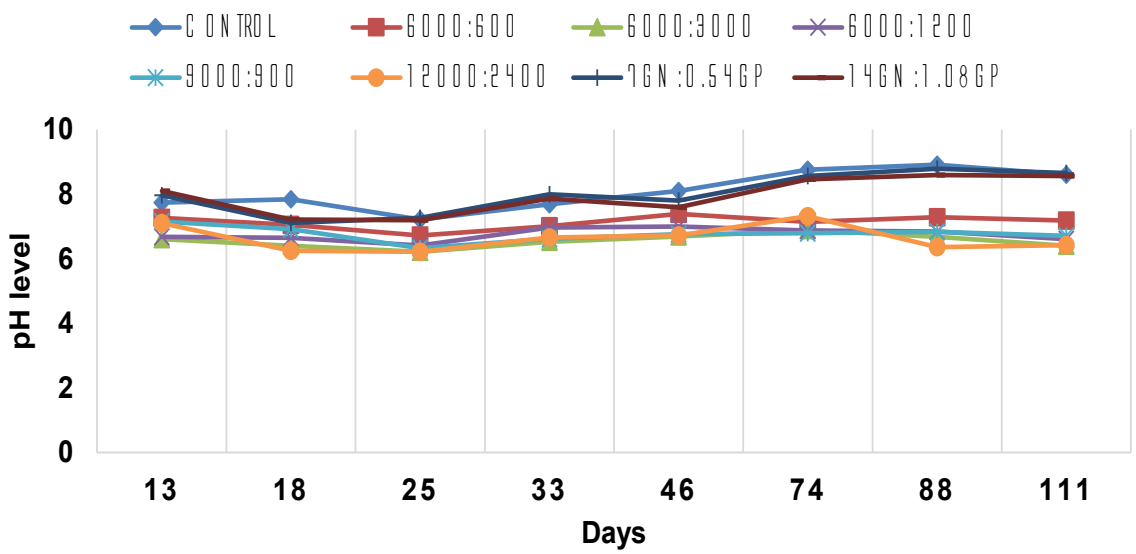

Figure 46 - Soil pH response following addition of N: P fertiliser in the seven different treatments and the control sample over 111 days during degradation hydrocarbon contaminated soils.

5 CHAPTER 5: DISCUSSION

This section deliberates on the findings from an investigation done to determine if there were any significant impacts to Murowa Diamonds' soil physical, chemical and microbial properties as a result of hydrocarbon contamination. The study also explored if there were microorganisms that could potentially degrade hydrocarbons. Understanding the chemical, physical and microbial properties is critical for bioremediation of Murowa hydrocarbon contaminated soils (Takawira *et al.* 2014).

5.1.1 Physicochemical and microbial properties of hydrocarbon contaminated soils

Key findings from soil characterisation were that Murowa soils are strongly alkaline, sandy loam with average sand content 79.74%, Clay 9.3% and Silt 10.89%, low NPK nutrient levels ranges (400-900mg/kg nitrogen, phosphorus 6.35-29.76mg/kg and K 0.3033 - 1.67%) **(Figs. 10-14)**, wettable with 1.8sec water repellency when not contaminated and strongly repellent when contaminated. Water repellency deteriorated with age of soil, i.e. the recently polluted the higher the repellency. This was also the case for TPH concentration which deteriorated with age. Average pH range for the soils was 8-9.4 which means soils are strongly alkaline and subsequently, the pH would not need any adjustment as it fell within the suitable range required for microbial degradation of hydrocarbons. The TPH levels in uncontaminated soils were below 50mg/kg when contaminated which averaged 265.7mg/kg **(Fig. 15)**. In terms of the nutrient ratios, C: N: P range, minimum was 629:62.9:1 and maximum was 806:30.3:1 and the nutrient ratios as a result required manipulation to attain the ratios of 100:10:1 and 100:10:5.

As for the microbial properties, out of the 42 genera partially identified **(Table 8)**, only nine have been verified against literature of their capability to naturally degrade hydrocarbons which included ***Pseudomonas, Bacillus, S. Marcescens, Flavobacterium, Micrococcus, Streptomyces, Staphylococcus, Penicillium,*** **and the yeast families**. While these are the known microbes genera from the consulted literature, the other identified genera could participate in biodegradation. Further to this, the traditional microbial methods are most related to what is known and documented which means that there could be other unknown genera which has not yet been discovered that could contribute to degradation as well. There were insignificant variations in terms of microbial nutrients and respiration activities across sites and soil depth.

The natural site (UCS) contained some biogenic hydrocarbons though they were found to be wettable/ hydrophilic **(Fig. 15)**. However, following the hydrocarbon contamination, all the soils became water repellent (hydrophobic), evidently confirming that hydrocarbon contamination induced water repellency to Murowa soils. This is in line with studies that were conducted by Takawira *et al.* 2014; Acenengui *et al*. 2007; Doerr *et al.* 2000; Hoffman *et al.* 2000; Scott, 2000) which confirmed that hydrocarbons had capacity to induce water repellency due to the presence of long-chain aliphatic and aromatic compounds in petroleum hydrocarbons that are hydrophobic.

A significant difference was noted on the different aged sites that most parameters concentrations or levels decreased with age. The parameters that showed a downward decrease with age included TPH, water repellency, pH and Carbon. The concentration or levels of these parameters were highest in the 2012 to date which is the site that had least age. Phosphorus did not show any defined trend which explains why its levels remained constant during the pot experiment. Nitrogen levels in old aged soils were the same with the uncontaminated site which may mean that the old aged soils almost resembling the reference site.

The study also presents the first evidence that the sandy loam soils associated with the study area are inherently wettable though the uncontaminated soil samples contained some biogenic hydrocarbons.

5.1.2 Nutrients

5.1.2.1 Carbon

Contaminated soils carbon percentage results indicated that carbon levels deteriorated with age as the older soils had the least carbon levels **(Fig. 20)**. This could be possibly due to natural attenuation that could be assumed to have been happening. In this experiment, it can be concluded that the hydrocarbons were not toxic because there is substantive information that there hydrocarbon carbons were reduced. If the hydrocarbon concentration levels were high it could have been toxic to microorganisms and hence biodegradation was not going to be possible. Murowa levels can be concluded that there are below the threshold of toxicity of 300mg/kg (Yang *et al.* 2009). The carbon concentration was adequate, had it been low concentration of contaminant it would provide enough carbon for efficient degradation (Boopathy, 2000).

Carbon concentration was significantly higher in contaminated soils than in UCS which may be attributed to hydrocarbon contamination. This was expected as hydrocarbon contamination

increases Total Organic Carbon in soil (Ekundayo and Obuekwe, 2000). In addition, the difference could have been due to fact that mining activities could have had different effect in terms of input of TPH concentration as these could have been different with period.

5.1.2.2 Nitrogen and Phosphorus

Percentage nitrogen in all soil ages initially ranged from 0.04 – 0.09% (400-900mg/kg). Addition of nitrogen and phosphate fertilisers resulted in a slight increase in nitrogen concentration which was witnessed from day 0 - 46 though the increment was statistically not significant. The insignificant changes in nitrogen concentration after addition of fertilisers until day 46 could be explained by microbes' utilization rate of N perhaps similar to the rate of N replenishment from the inorganic fertiliser. Also it could be due to the fact that if the bulk of N was is in the form of ammonium (NH_4^+), it could be retained on the soil particles because of the positive charge which was attracted to the negative charge on the soil micelle. This can also be further explained by the fact that when fertiliser is added it reacts with the soil minerals and organic matter and become part of the soil reserve. In relation with the microbes, it is possible that the microbes can assimilate nitrogen within them for survival and use it later during the biodegradation.

After day 46 there was a significant drop in total nitrogen until day 74. From day 74 onwards, a gradual increase was witnessed for most of the treatments except treatments 7gN:0.54gP and 12000:2400 which recorded sharp increases after 88 days. The significant drop is attributed to the increase in microbial mass and the highest degradation in TPH witnessed during the same period which means that N was consumed by the microbes when they were degrading the hydrocarbons.

Addition of nitrogen and phosphorus fertilisers positively influenced phosphate concentration in the soils. However, the fluctuations might mean that only a small concentration was required from 0 – 46days and it could have been supplied in excess. A significant drop was witnessed in the month 46-74 days which can also be attributed to the increase in microbial mass and the significant decrease in TPH during the same period. It was during this period when degradation occurred effectively and the microbes utilizing both phosphorus and nitrogen. The N: P ratio that yielded best results was the 6000mgN:3000mgP which achieved 86% TPH reduction.

The increases in both phosphorus and nitrogen from day 74 onwards could be attributed to the decrease in microbial mass experienced during that same period meaning that when the microbes die the nutrients that they would have assimilated become part of the soil or organic

matter. This is line with what McGill, (1976); Rowell, (1977) and Chukwuma, (2012) found out that the long last effect of hydrocarbons in soils eventually result in nutrient supply. This was attributed to the fact that when hydrocarbons degrade there are decomposed and converted to soil organic matter which helps to improve the content of nutrients,

At 46days mean average nitrogen concentration in all soil treatments was estimated at 0.32% and at 74 days it was around about 0.13% meaning that 0.19% was used within that month when degradation occurred effectively. Similarly, mean average phosphorus concentration used during that same period was estimated at 266mg/kgP in all treatments. Treatments which recorded an increase in phosphorus during that same period, the average mean increase was about 110mg/kg. The increase in phosphorus can be attributed to the fact that Engine or motor oils metals contain phosphorus in form of phosphate esters and which when mineralisation has occurred some of the phosphorus is released adding up to the soil phosphorus concentration.

5.1.3 Microbial Biomass

Prior to addition of nutrients, N: P, the total microbial mass was estimated at 27%/g and after two weeks (13days) from addition of nutrients it remained constant in treatments with 6000mgN until day 33. There were insignificant changes to microbial biomass percentage in this first month which was congruent with insignificant changes in nitrogen and phosphorus concentration from day zero to day 46. An increase in total microbial mass or a decrease in nitrogen could not be expected as the microbes were not utilizing the nitrogen meaning there was no change in demand for nitrogen or vice versa. However, between day 33 and day 46 there were sharp declines followed by a significant increase which corresponded with a steady decrease in nitrogen and phosphorus concentration during the same period meaning the microbes utilised the nitrogen and phosphorus. The sharp decline between day 33 and 46 could be attributed to significant temperature changes that prevailed during that period which were above 30^0C averaging 34^0C which could have been unfavourable for the microbes especially in treatments with N concentration greater than 6000mgN. The decrease in the biomass almost converged with the low levels experienced in the treatments that had 6000mgN which could mean that only those microbes that could survive under those nutrient and temperature conditions survived. However, there is an opportunity to further investigate and isolate the microbes which multiplied significantly in high nitrogen content and those that remained active beyond 46days.

The total microbial mass did not change significantly in all the treatments that had nitrogen concentration of 6000mg and the treatment 6000:3000. However, these were the treatments that yielded effective hydrocarbon degradation when compared with those that had higher nitrogen concentration. This also possibly suggests that the 6000mgN concentration was suitable for maintaining a consortium of many different bacterial species that were required to efficiently degrade the hydrocarbon (Wu *et al.* 2013). The inconsistent result possibly indicates that 6000mgN concentration does not or is too low to negatively impact the microbes suggesting that nitrogen level concentration is not a limiting factor to the microbes naturally adapting in the Murowa hydrocarbon contaminated soils.

In relation to pH, the treatments with 6000mgN had pH ranging from 6.2-6.7 to which could suggest the pH levels were suitable for the microbes as it did not make any significant impact to the microbial mass significantly. The soil pH had since however, reduced from the original 8.6 falling within the range 6.2-6.7 following the N: P ratio amendments.

5.1.3.1 Hydrocarbons occurrence and degradation

The uncontaminated soil sites contained biogenic hydrocarbons also referred to as fingerprinting hydrocarbons meaning background hydrocarbons that occur naturally in uncontaminated sites. The biogenic hydrocarbon levels could contribute about 10% to the actual concentration of the hydrocarbon contamination. This was calculated from the average hydrocarbon levels determined in uncontaminated site against the contaminated sites hydrocarbon levels. This is line with discoveries made by Wang *et al.* 2012 where it was determined that the biogenic hydrocarbons could lead to overestimation of petroleum hydrocarbon levels and in some instances they have been found exceeding regulatory levels e.g. 300ug/g for coarse soils and 1300 ug/g for fine soils.

The characterisation process determined the TPH levels in mg/kg as follows; 2012 to date **(265.8)**>2008-2011**(192.3**)>2004-2007**(52.1)**>UCS **(27.58**)>freshly contaminated **(9.4)**. The trend reflected that hydrocarbon concentration deteriorated with age which could possibly indicate/suggest that natural attenuation has been happening since 2004 (**Fig. 15**). This was also confirmed by the pot experiment control sample which showed significant reduction in TPH. However, there could be a possibility that the differences in TPH concentration across sites could be due to the fact that mining activities could have had different effect in terms of input of TPH as these could have been different with period. Naturally present microorganism have hydrocarbon degradative capabilities and the presence of naturally adapting microorganisms as confirmed here and occurrence of processes like volatilisation of

other hydrocarbons e.g. C_{10}-C_{14} can be attributed to the significant disappearance of the hydrocarbons in open environment (Thomassin-lacroix *et al.* 2002, Sanscartier *et al.* 2009). In addition, odour encountered during excavation of soil could support that volatilisation increased by increasing the soil pore space available for diffusion of volatile PHCs while inhibiting biodegradation (Dragun, 1998).

TPH degradation occurred significantly with 86% being recorded as the highest decrease at (P 0.05) within the first 74 days. **Table 9** shows weekly TPH concentration reduction percentages. In the first 3-4 weeks the TPH concentration increased from the initial concentration. This was unexpected and as a result some further investigations were done through literature review and laboratory analysis to determine the type of hydrocarbon in the samples to assist in describing the behaviour of the trend. **Table 10** shows results of hydrocarbon types identified.

The increase in TPH concentration can be explained by the mechanism or pathways in which the alkane hydrocarbons are degraded. The initial attack by oxygenases results in what is referred to as peripheral pathways of degradation. This will be followed by terminal (main pathway) and sub terminal oxidation (sub pathway). As this occurs the long chain hydrocarbon is broken into smaller compounds exposing more C-H bonds which will be encapsulated within the structure. Sanscartier *et al.* 2009 determined a TPH increase from a reduction of 87% to an increase of 187% and Sabate *et al*. 2004 also witnessed apparent increases of TPH levels in contaminated soils by weathered heavy hydrocarbon.

From day 46 to 74 a steady decrease in TPH concentration was witnessed which signifies that it was during this period when mineralisation significantly occurred. According to the expected breakdown pathway it will be at the point when the hydrocarbons have been broken to single C-H bonds when there are saturated (n-alkanes) or light weight alkanes. The concentration of TPH reduced significantly until day 74, this could be attributed to the nature and composition of the hydrocarbons which were largely alkanes C_7-C_{12}, C_{18} which can be easily broken down when compared to cyclic aromatics, PAHs and alkenes (Pitter and Chudoba, 1990; Hamamura, 2006; Das 2010; Chikwuma, 2012; Wang *et al.* 2013 Zampoli, 2014).

From day 74 onwards the retarded biodegradation could be attributed to the fact that simple hydrocarbons had been degraded and there was now the dominance of the large and more complex hydrocarbons which are difficult to degrade due to their size, insolubility and hydrophobicity. These could be alkanes in the range of C20- C40 insoluble PAHs, alkenes and cyclic aromatic or alkanes which are insoluble and hydrophobic. These substances could

be complex organic compounds found in engine oil and hyspin known as heavy polycyclic aromatic, polyalphaolefins (PAO) and polyinternalolefins. Complex PAHS have been found to be not readily available to the microbes as the water is repelled, limiting microbial attachment to the hydrocarbons (Maier, 2000). Another possible dimension to explain the retarded biodegradation could be the fact that the attack of hydrocarbons by microorganisms results in either full degradation (mineralization) or in a partial degradation process producing metabolites which may be used by other members of the biocenosis or which remain in the soils (Agathos and Reineke, 2002). In addition, the microbial biomass graph indicated there was a decrease in the biomass from day 74 onwards which could also have resulted in a decline in the hydrocarbon degradation rate.

TPH identified in the sample included decane, undecane, hexadecanal, 2-ethylcridine, octadecane, I-iodo and dodecane. Decane also known as Decyl hydride is an alkane with formula $C_{10}H_{22}$ which falls within the C_7-C_{12} class (Chem ID, 2002; Lewis, 2002, Sigma – Aldrich, 2002). The substance is a component of engine fuels (Covender, 1994) which can also be found in solvents and cleaning agents (Lewis, 2002; Verseheuren, 2001, Wolkoff *et al.* 1998). This could be attributed to Murowa activities since maintenance work involves use of engine fuels. However, decane has also been found naturally trapped among sedimentary rocks (BIRTH, 2002; Chevron, 2002, NETLAB, 1997). In another study conducted in 1986, hydrocarbon characterisation of soils containing spilled hydrocarbon (>3years) revealed the presence of n-alkanes, including decane. The level of concentration of the n-alkanes decreased in contaminated soils because of biological transformation and volatisation (Saterbak *et al.* 1998) which maybe the case for the Murowa soils. Decane has also been found as a growth substrate for yeasts (Verscheuren, 2001) and to be readily hydroxylated to decanol by *Pseudomonas* which could suggest the two genera as active contributors to the biodegradation process.

Undecane is also another aliphatic saturated hydrocarbon that was present which is an alkane hydrocarbon with chemical formula $C_{11}H_{24}$. It is also found in lubricant additives and greases.

Hexadecanal is a synthetic, solid, fatty alcohol and nonionic surfactant also known as cetyl acid which is used as an additive in fuels manufacture and processing aid specific to petroleum production. It is also used in lubricants and greases and cleaning products as foam stabilisers in detergents. This can be broken down by volatilisation from moist surfaces.

Octadecane $C_{18}C_{38}$ and Dodecane $C_{12}H_{26}$ were also identified. These are solvents in standardised hydrocarbon manufacture of paraffin manufacture which when released in soils are not expected to move. Volatilisation from soil surfaces expected.

Hydrocarbon degradation follows a hierarchy which starts with the saturated molecules followed by aromatic, then resins and asphaltenes (Song *et al.* 1990, Whyte *et al*. 1997 and Leon *et al.* 1998, Haritash and Kaushik, 2009; Chukwuma, 2012). Normal alkanes have been found more prone to oxidation followed by iso-alkanes which are highly branched structures with quaternary carbon and seldom degraded whereas isomers, cyclic and long -chain degrade slowly. Murowa activities mainly contain diesel and engine oil which primarily contain aliphatic compounds C_{14}-C_{24} and C_{14}-C_{22} respectively. Aliphatic compounds degrade more rapidly than aromatics (Ramirez *et al*. 2008, Zrafi-Nouira *et al.* 2009).

The trend demonstrates that it takes about one and half months to reduce the light n-alkanes and 74 days to completely mineralize these alkanes. From day 74 onwards, the remaining hydrocarbons are long chain hydrocarbons, PAOs alluded to earlier. This was expected as some reported studies have indicated that hydrocarbon degradation leaves between 10% and 30% of the initial soil pollution in soil after bioremediation techniques have been applied. In addition, it has been found that complete hydrocarbon degradation cannot occur due to their size and hydrophobic properties well as accumulation of recalcitrant components which reduces their bioavailability to microorganisms (Chukwuma, 2012).

6 CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

The study of the chemical and microbial characterisation of Murowa hydrocarbon contaminated soils revealed that the soils are sandy loam, strongly alkaline with low NPK levels and C: N: P ratio of 634:107:1 for uncontaminated soils and 877:32:1 for the contaminated 2012 to date sample which had the highest hydrocarbon contamination, naturally wettable and severely water repellent when contaminated.

Pseudomonas, Bacillus, S. Marcescens, Flavobacterium, Micrococcus, Streptomyces, Staphycoccus, Penicillum and **yeast** genera were the naturally adapting microorganisms identified with capability to degrade hydrocarbons. Though these are known petroleum hydrocarbon degraders it cannot be concluded that there were the only microorganisms solely responsible for the degradation as it very possible that there could be other microbes that actually contributed to the degradation process but have not been isolated as yet.

Manipulation of nitrogen and phosphorus nutrients presented conflicting results. Nitrogen and phosphorus concentration in treatments with 6000mgN did not change significantly yet they exhibited effective hydrocarbon degradation suggesting the concentration could not fit the averaged optimal nutrient ratio for the degradation process hypothetically in excess.

The treatments with nitrogen concentration greater than 6000mgN yielded significant microbial growth as witnessed by the significant increase in the total microbial mass though they did not yield degradation as high as the 6000mgN. Effective degradation was experienced during the second month between 46 days and 74days.

The 6000mgN:3000mgP yielded the highest biodegradation as it recorded the highest TPH reduction of 86% over 74days and overall throughout the 111 days it reduced by 82%. In comparison with the control sample, the 6000mgN:3000mgP yielded a degradation rate than was 8% higher than the control sample which was 74%.

It can be concluded that Murowa hydrocarbon contaminated soils can be bioremediated using a combination of natural attenuation and biostimulation methods. The recommended ratio being 6000mg N: 3000mg P (2:1 molar ratio) which yielded the highest degradation. Significant biodegradation in fertiliser treated pots as shown by the TPH trend was recorded within the first 74 days. However, there is still an opportunity to study the metagenome of the contaminated area and isolate the most active microorganism that could be responsible for biodegradation during that period and the given conditions. In addition, tests could be performed to identify if the current isolated microorganisms were true hydrocarbon degraders

or if there were heterotrophic organisms that use other organic compounds as their carbon and energy source.

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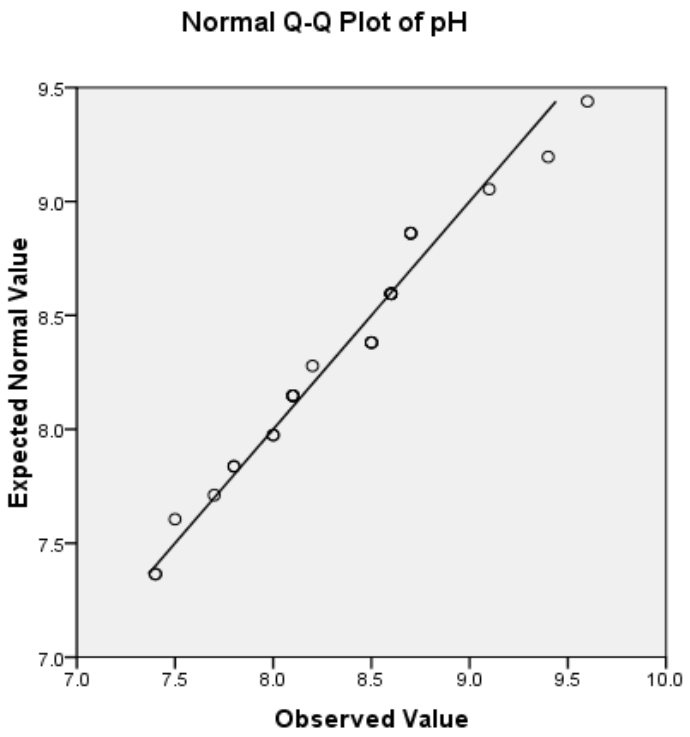
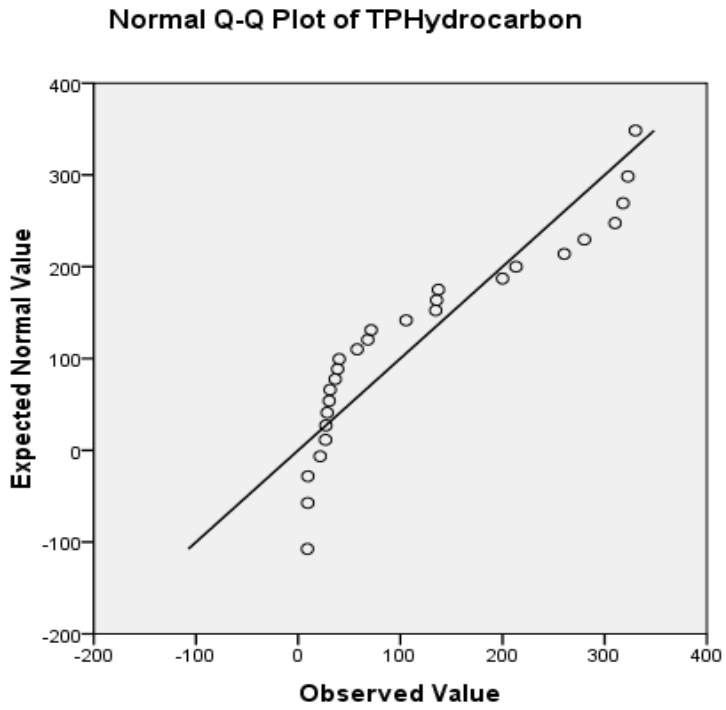
Appendix 1 -List of confirmed hydrocarbon degrading organisms from previous studies

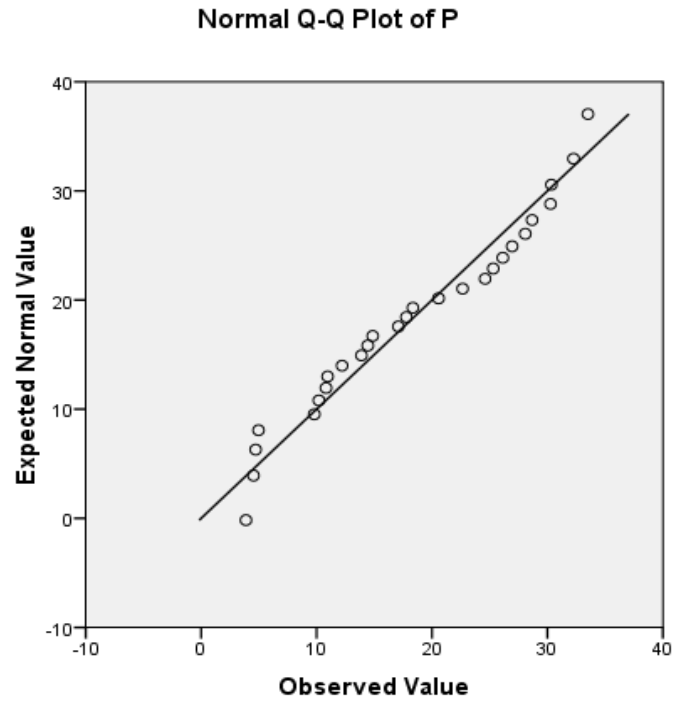
Micro organisms		
Yokonella spp	*Actinobacteria*	*Geobacillus*
Alcaligenes spp	*Micrococcaceae*	*Staphylococcaceae*
Roseomonas spp	*Arthrobacter*	*Staphylococcus*
Stenotrophomonas	*Micrococcus*	*Proteobacteria*
Acinetobacter	*Brevibacteriaceae*	*Alphaproteobacteria .*
Flavobacter	*Brevibacterium*	*Sphingomonadaceae*
Corynebacterium	*Dermabacteraceae*	*Rhodobacteraceae*
Streptococcus	*Brachybacterium*	*Rhodospirillaceae*
Providencia	*Dietziaceae*	*Brucellaceae*
Sphingobacterium	*Dietzia*	*Betaproteobacteria .*
Capno cytophagia	*Cellulomonadaceae*	*Alcaligenaceae*
Moraxella	*Cellulomonas*	*Achromobacrer*
Bacillus	*Intrasporangiaceae*	*Alcaligenes*
Geobacillus	*Janibacter*	*Comamonadaceae*
Staphylococcaceae	*Terrabacter*	*Acidovorax*
Staphylococcus	*Corynebacteriaceae*	*Polaromonas*
Proteobacteria	*Mycobacterium*	*Burkholderiaceae*
Alphaproteobacteria .	*Corynebacterium*	*Burkholderia*
Sphingomonadaceae	*Gordoniaceae*	*Ralstonia*
Rhodobacteraceae	*Gordonia*	*Rhodocyclaceae*
Rhodospirillaceae	*Nocardioidaceae*	*Azoarcus*
Brucellaceae	*Nocardioides*	*Thauera*
Betaproteobacteria .	*Rhodococcus*	*Delta-proteobacteria*
Alcaligenaceae	*Nocardiaceae*	*Geobacteraceae*

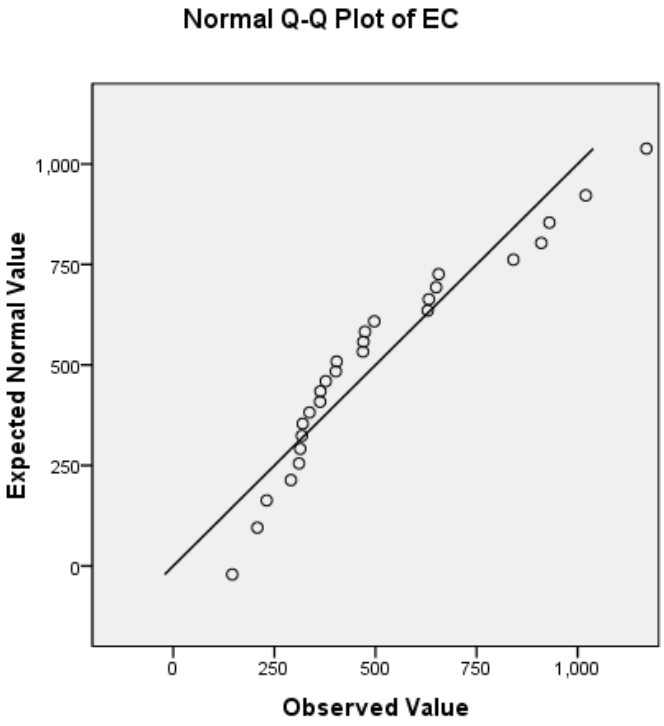
Achromobacrer *Alcaligenes* *Comamonadaceae* *Acidovorax* *Polaromonas* *Burkholderiaceae* *Burkholderia* *Ralstonia* *Rhodocyclaceae* *Azoarcus* *Thauera* *Delta-proteobacteria* *Geobacteraceae* *Geobacter* *Desulfobacteraceae* *Epsilon-proteobacteria* *Gamma-proteobacteria* *Piscirickettsiaceae* *Cycloclasticus* *Pseudomonadaceae* *Pseudomonas* *Alteromonadaceae* *Marinobacter* *Pseudoalteromonadaceae* *Pseudoalteromonas*	*Cyanobacteria).* *Bacteroidetes/ Chlorobi group* *Flavobacteria* *Chryseobacterium* *Achromobacter Flavobacterium* *Yeosuana* *Thermaceae* *Thermus* *Thermotogae* *Firmicutes* *Bacillaceae* *Bacillus* *Shewanellaceae* *Shewanella* *Moraxellaceae* *Acinetobacter* *Moraxella* *Halomonadaceae* *Halomonas* *Alcanivoracaceae* *Alcanivorax* *Oceanospirillaceae* *Oleiphilaceae* *Oleiphilus* *Xanthomonadaceae*	*Geobacter* *Desulfobacteraceae* *Epsilon-proteobacteria* *Gamma-proteobacteria* *Piscirickettsiaceae* *Cycloclasticus* *Pseudomonadaceae* *Pseudomonas* *Alteromonadaceae* *Marinobacter* *Pseudoalteromonadaceae* *PseudoalteromonasStenotrophomonas).* *Xanthomonas Arenimonas* *Zetaproteobacteria* *Pasteurellaceae* *Pasteurella* *Rhodanobacter*

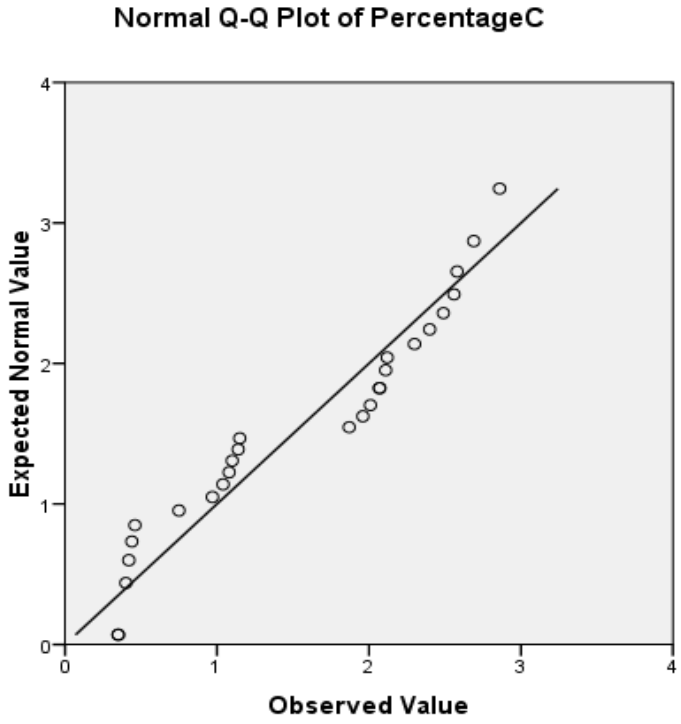
Appendix 2 -Q-Q plots Characterisation result showing data normality test conducted

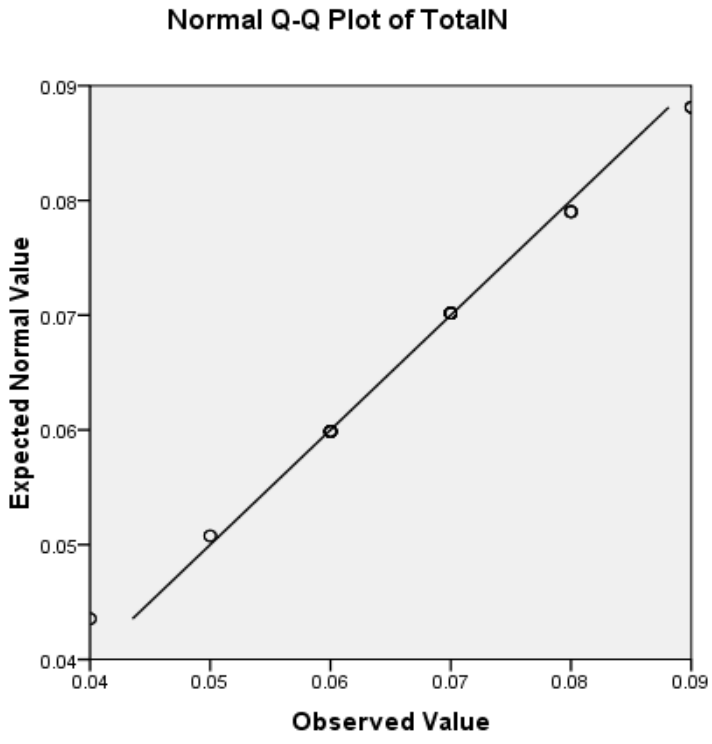


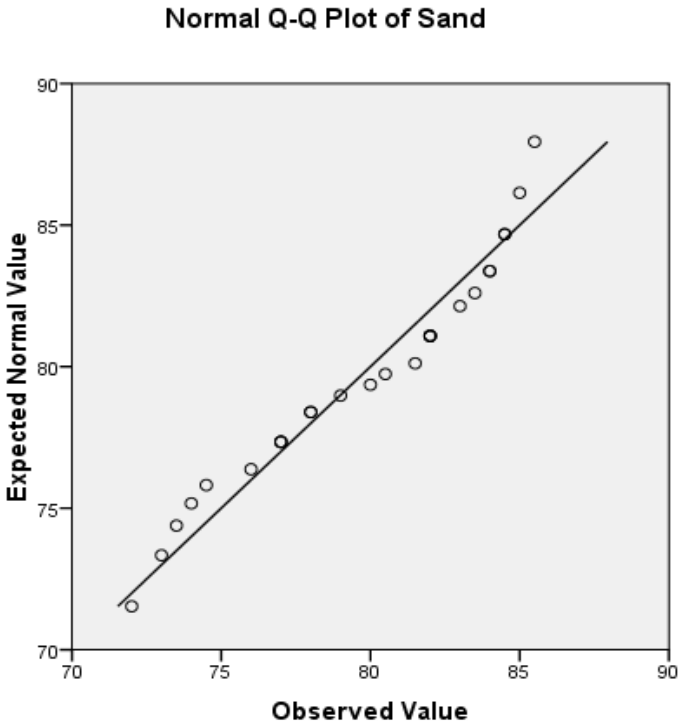


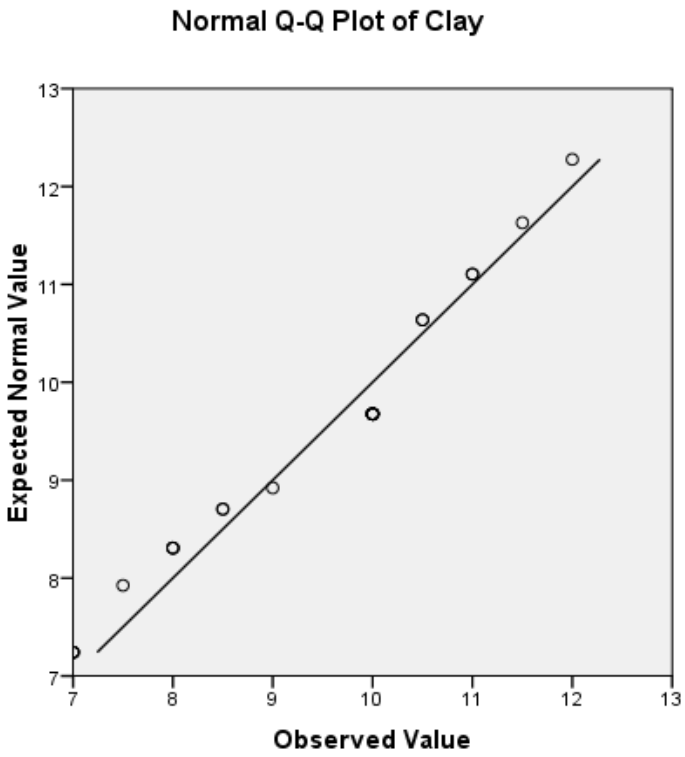

Normal Q-Q Plot of P
Expected Normal Value
Observed Value
40
30
20
10
0
-10
-10
0
10
20
30
40

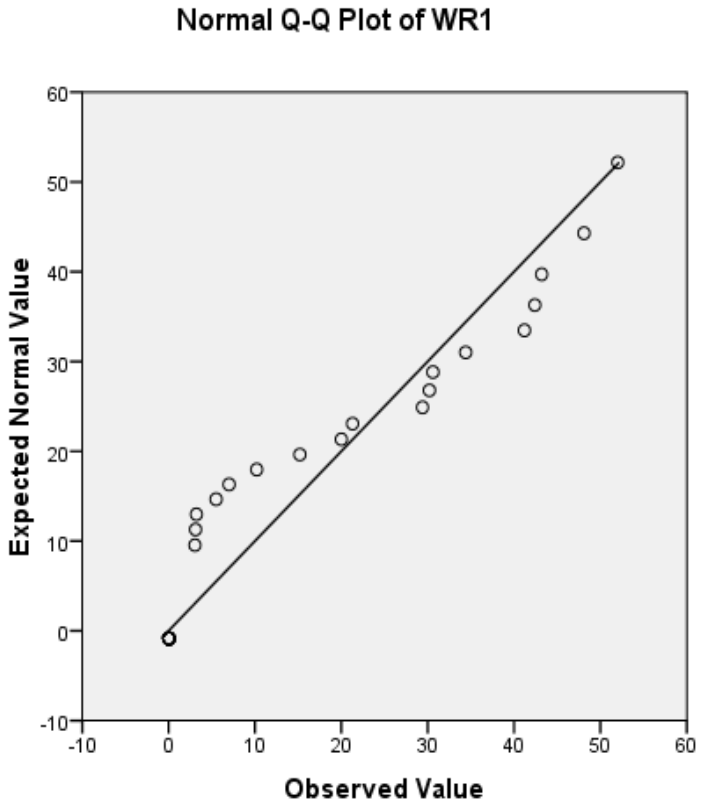

Normal Q-Q Plot of EC
Expected Normal Value
Observed Value
1,000
750
500
250
0
0
250
500
750
1,000

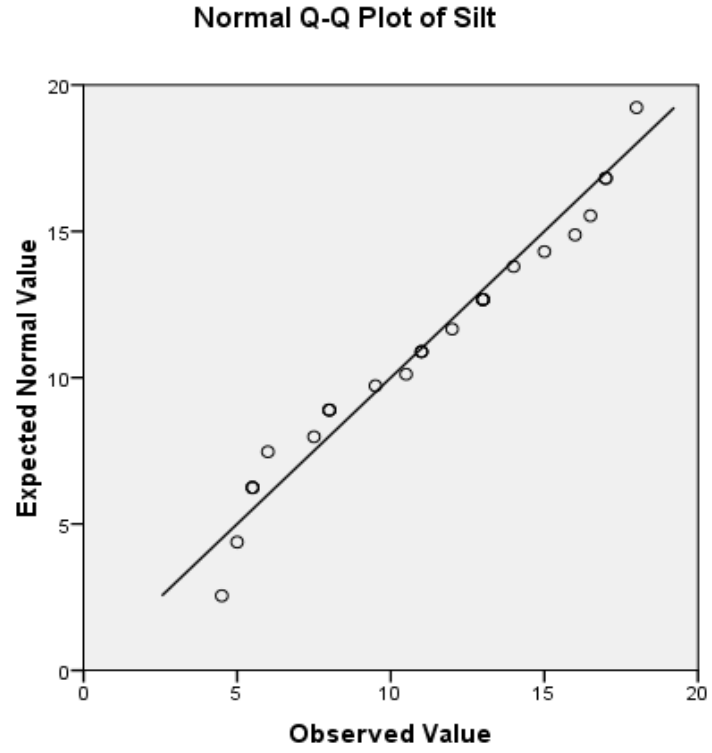

Normal Q-Q Plot of PercentageC
Expected Normal Value
Observed Value
0
1
2
3
4


Normal Q-Q Plot of TotalN
Expected Normal Value
Observed Value
0.04
0.05
0.06
0.07
0.08
0.09


Normal Q-Q Plot of Sand
Expected Normal Value
Observed Value
90
85
80
75
70
70
75
80
85
90


Normal Q-Q Plot of Clay
Expected Normal Value
Observed Value
13
12
11
10
9
8
7
7
8
9
10
11
12
13


Normal Q-Q Plot of WR1
Expected Normal Value
Observed Value
60
50
40
30
20
10
0
-10
-10
0
10
20
30
40
50
60


Normal Q-Q Plot of Silt
Expected Normal Value
Observed Value
20
15
10
5
0
0
5
10
15
20

Appendix 3 - One-Sample Kolmogorov-Smirnov Test for Ca, Mg, Na, K, CEC and Water repellency

		Ca_Tran	Mg_Tran	K_Trans	Na_Trans	CEC_Trans	WR1_Trans	WR2_Trans	WR4_Trans	WR5_Trans	WR7_Trans
N		27	27	27	27	27	27	27	27	27	27
Normal Parameters[a]	Mean	.8572	.4880	-.2862	-.3432	1.0700	.3123	.2979	.3223	.3114	.3060
	Std. Deviation	.28192	.15146	.22100	.44678	.21953	1.36901	1.36087	1.37557	1.36543	1.37054
Most Extreme Differences	Absolute	.320	.165	.216	.295	.231	.243	.243	.243	.244	.242
	Positive	.277	.129	.216	.295	.231	.243	.243	.243	.244	.242
	Negative	-.320	-.165	-.093	-.183	-.211	-.217	-.221	-.219	-.229	-.188
Kolmogorov-Smirnov Z		1.661	.857	1.122	1.531	1.201	1.264	1.262	1.265	1.267	1.259
Asymp. Sig. (2-tailed)		.008	.454	.162	.018	.112	.082	.083	.082	.081	.084
Exact Sig. (2-tailed)		.006	.410	.139	.014	.095	.068	.069	.068	.067	.070
Point Probability		.000	.000	.000	.000	.000	.000	.000	.000	.000	.000

a. Test distribution is Normal.

Appendix 4 -ANOVA for the characterisation results						
		Sum of Squares	df	Mean Square	F	Sig.
TPHydrocarbon	Between Groups	145642.386	2	72821.193	17.192	.000
	Within Groups	59301.956	14	4235.854		
	Total	204944.342	16			
pH	Between Groups	.988	2	.494	36.463	.000
	Within Groups	.190	14	.014		
	Total	1.178	16			
Ca	Between Groups	.428	2	.214	2.843	.092
	Within Groups	1.053	14	.075		
	Total	1.480	16			
Mg	Between Groups	6.740	2	3.370	10.811	.001
	Within Groups	4.364	14	.312		
	Total	11.103	16			
K	Between Groups	.103	2	.052	12.561	.001
	Within Groups	.058	14	.004		
	Total	.161	16			
Na	Between Groups	.313	2	.156	12.834	.001
	Within Groups	.171	14	.012		
	Total	.484	16			
CEC	Between Groups	17.047	2	8.523	12.264	.001
	Within Groups	9.730	14	.695		
	Total	26.777	16			
P	Between Groups	465.703	2	232.851	12.446	.001
	Within Groups	261.916	14	18.708		
	Total	727.619	16			
EC	Between Groups	388522.892	2	194261.446	4.771	.026
	Within Groups	570062.167	14	40718.726		
	Total	958585.059	16			
PercentageC	Between Groups	4.649	2	2.324	11.447	.001
	Within Groups	2.843	14	.203		
	Total	7.492	16			
TotalN	Between Groups	.001	2	.000	4.573	.030
	Within Groups	.001	14	.000		
	Total	.002	16			
WR1	Between Groups	3520.677	2	1760.338	50.432	.000
	Within Groups	488.675	14	34.905		
	Total	4009.351	16			
WR2	Between Groups	3519.192	2	1759.596	17.321	.000
	Within Groups	1422.224	14	101.587		
	Total	4941.415	16			
WR3	Between Groups	3087.020	2	1543.510	20.350	.000
	Within Groups	1061.851	14	75.847		
	Total	4148.871	16			

WR4	Between Groups	3761.789	2	1880.895	24.687	.000
	Within Groups	1066.655	14	76.190		
	Total	4828.445	16			
WR5	Between Groups	3257.017	2	1628.508	31.205	.000
	Within Groups	730.626	14	52.188		
	Total	3987.642	16			
WR6	Between Groups	3208.491	2	1604.246	44.997	.000
	Within Groups	499.135	14	35.652		
	Total	3707.626	16			
WR7	Between Groups	4103.773	2	2051.886	18.951	.000
	Within Groups	1515.829	14	108.273		
	Total	5619.601	16			
Sand	Between Groups	117.369	2	58.684	12.569	.001
	Within Groups	65.367	14	4.669		
	Total	182.735	16			
Clay	Between Groups	9.984	2	4.992	2.179	.150
	Within Groups	32.075	14	2.291		
	Total	42.059	16			
Silt	Between Groups	86.629	2	43.314	8.873	.003
	Within Groups	68.342	14	4.882		
	Total	154.971	16			

Appendix 5 -Multiple Mean comparison for characterisation data

Dependent Variable		(I) Period	(J) Period	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
TPHydrocarbon	LSD	2004	2008	-140.1833*	37.5759	.002	-220.776	-59.591
			2012	-226.8133*	39.4100	.000	-311.339	-142.287
		2008	2004	140.1833*	37.5759	.002	59.591	220.776
			2012	-86.6300*	39.4100	.045	-171.156	-2.104
		2012	2004	226.8133*	39.4100	.000	142.287	311.339
			2008	86.6300*	39.4100	.045	2.104	171.156
	Bonferroni	2004	2008	-140.1833*	37.5759	.007	-242.306	-38.061
			2012	-226.8133*	39.4100	.000	-333.920	-119.707
		2008	2004	140.1833*	37.5759	.007	38.061	242.306
			2012	-86.6300	39.4100	.136	-193.737	20.477
		2012	2004	226.8133*	39.4100	.000	119.707	333.920
			2008	86.6300	39.4100	.136	-20.477	193.737
pH	LSD	2004	2008	-.4500*	.0672	.000	-.594	-.306
			2012	-.5533*	.0705	.000	-.704	-.402
		2008	2004	.4500*	.0672	.000	.306	.594
			2012	-.1033	.0705	.165	-.254	.048
		2012	2004	.5533*	.0705	.000	.402	.704
			2008	.1033	.0705	.165	-.048	.254
	Bonferroni	2004	2008	-.4500*	.0672	.000	-.633	-.267
			2012	-.5533*	.0705	.000	-.745	-.362
		2008	2004	.4500*	.0672	.000	.267	.633
			2012	-.1033	.0705	.494	-.295	.088
		2012	2004	.5533*	.0705	.000	.362	.745
			2008	.1033	.0705	.494	-.088	.295
Ca	LSD	2004	2008	-.26000	.15832	.123	-.5996	.0796
			2012	-.38233*	.16604	.037	-.7385	-.0262
		2008	2004	.26000	.15832	.123	-.0796	.5996
			2012	-.12233	.16604	.473	-.4785	.2338
		2012	2004	.38233*	.16604	.037	.0262	.7385
			2008	.12233	.16604	.473	-.2338	.4785
	Bonferroni	2004	2008	-.26000	.15832	.368	-.6903	.1703

			2012	-.38233	.16604	.111	-.8336	.0689
		2008	2004	.26000	.15832	.368	-.1703	.6903
			2012	-.12233	.16604	1.000	-.5736	.3289
		2012	2004	.38233	.16604	.111	-.0689	.8336
			2008	.12233	.16604	1.000	-.3289	.5736
Mg	LSD	2004	2008	-.95333*	.32233	.010	-1.6447	-.2620
			2012	-1.54300*	.33806	.000	-2.2681	-.8179
		2008	2004	.95333*	.32233	.010	.2620	1.6447
			2012	-.58967	.33806	.103	-1.3147	.1354
		2012	2004	1.54300*	.33806	.000	.8179	2.2681
			2008	.58967	.33806	.103	-.1354	1.3147
	Bonferroni	2004	2008	-.95333*	.32233	.031	-1.8294	-.0773
			2012	-1.54300*	.33806	.001	-2.4618	-.6242
		2008	2004	.95333*	.32233	.031	.0773	1.8294
			2012	-.58967	.33806	.309	-1.5084	.3291
		2012	2004	1.54300*	.33806	.001	.6242	2.4618
			2008	.58967	.33806	.309	-.3291	1.5084
K	LSD	2004	2008	-.11667*	.03702	.007	-.1961	-.0373
			2012	-.19133*	.03882	.000	-.2746	-.1081
		2008	2004	.11667*	.03702	.007	.0373	.1961
			2012	-.07467	.03882	.075	-.1579	.0086
		2012	2004	.19133*	.03882	.000	.1081	.2746
			2008	.07467	.03882	.075	-.0086	.1579
	Bonferroni	2004	2008	-.11667*	.03702	.021	-.2173	-.0161
			2012	-.19133*	.03882	.001	-.2968	-.0858
		2008	2004	.11667*	.03702	.021	.0161	.2173
			2012	-.07467	.03882	.225	-.1802	.0308
		2012	2004	.19133*	.03882	.001	.0858	.2968
			2008	.07467	.03882	.225	-.0308	.1802
Na	LSD	2004	2008	-.22000*	.06375	.004	-.3567	-.0833
			2012	-.32800*	.06686	.000	-.4714	-.1846
		2008	2004	.22000*	.06375	.004	.0833	.3567
			2012	-.10800	.06686	.129	-.2514	.0354
		2012	2004	.32800*	.06686	.000	.1846	.4714

			2008	.10800	.06686	.129	-.0354	.2514
	Bonferroni	2004	2008	-.22000*	.06375	.012	-.3933	-.0467
			2012	-.32800*	.06686	.001	-.5097	-.1463
		2008	2004	.22000*	.06375	.012	.0467	.3933
			2012	-.10800	.06686	.386	-.2897	.0737
		2012	2004	.32800*	.06686	.001	.1463	.5097
			2008	.10800	.06686	.386	-.0737	.2897
CEC	LSD	2004	2008	-1.55000*	.48132	.006	-2.5823	-.5177
			2012	-2.44467*	.50481	.000	-3.5274	-1.3620
		2008	2004	1.55000*	.48132	.006	.5177	2.5823
			2012	-.89467	.50481	.098	-1.9774	.1880
		2012	2004	2.44467*	.50481	.000	1.3620	3.5274
			2008	.89467	.50481	.098	-.1880	1.9774
	Bonferroni	2004	2008	-1.55000*	.48132	.018	-2.8581	-.2419
			2012	-2.44467*	.50481	.001	-3.8166	-1.0727
		2008	2004	1.55000*	.48132	.018	.2419	2.8581
			2012	-.89467	.50481	.294	-2.2666	.4773
		2012	2004	2.44467*	.50481	.001	1.0727	3.8166
			2008	.89467	.50481	.294	-.4773	2.2666
P	LSD	2004	2008	10.11000*	2.49722	.001	4.7540	15.4660
			2012	-1.65833	2.61911	.537	-7.2758	3.9591
		2008	2004	-10.11000*	2.49722	.001	-15.4660	-4.7540
			2012	-11.76833*	2.61911	.001	-17.3858	-6.1509
		2012	2004	1.65833	2.61911	.537	-3.9591	7.2758
			2008	11.76833*	2.61911	.001	6.1509	17.3858
	Bonferroni	2004	2008	10.11000*	2.49722	.004	3.3232	16.8968
			2012	-1.65833	2.61911	1.000	-8.7764	5.4598
		2008	2004	-10.11000*	2.49722	.004	-16.8968	-3.3232
			2012	-11.76833*	2.61911	.002	-18.8864	-4.6502
		2012	2004	1.65833	2.61911	1.000	-5.4598	8.7764
			2008	11.76833*	2.61911	.002	4.6502	18.8864
EC	LSD	2004	2008	-189.16667	1.16503E2	.127	-439.0404	60.7070
			2012	-376.83333*	1.22189E2	.008	-638.9031	-114.7636
		2008	2004	189.16667	1.16503E2	.127	-60.7070	439.0404

			2012	-187.66667	1.22189E2	.147	-449.7364	74.4031
		2012	2004	376.83333*	1.22189E2	.008	114.7636	638.9031
			2008	187.66667	1.22189E2	.147	-74.4031	449.7364
	Bonferroni	2004	2008	-189.16667	1.16503E2	.380	-505.7928	127.4595
			2012	-376.83333*	1.22189E2	.024	-708.9137	-44.7530
		2008	2004	189.16667	1.16503E2	.380	-127.4595	505.7928
			2012	-187.66667	1.22189E2	.441	-519.7470	144.4137
		2012	2004	376.83333*	1.22189E2	.024	44.7530	708.9137
			2008	187.66667	1.22189E2	.441	-144.4137	519.7470
PercentageC	LSD	2004	2008	-.91833*	.26017	.003	-1.4764	-.3603
			2012	-1.23400*	.27287	.000	-1.8193	-.6487
		2008	2004	.91833*	.26017	.003	.3603	1.4764
			2012	-.31567	.27287	.267	-.9009	.2696
		2012	2004	1.23400*	.27287	.000	.6487	1.8193
			2008	.31567	.27287	.267	-.2696	.9009
	Bonferroni	2004	2008	-.91833*	.26017	.010	-1.6254	-.2112
			2012	-1.23400*	.27287	.001	-1.9756	-.4924
		2008	2004	.91833*	.26017	.010	.2112	1.6254
			2012	-.31567	.27287	.800	-1.0573	.4259
		2012	2004	1.23400*	.27287	.001	.4924	1.9756
			2008	.31567	.27287	.800	-.4259	1.0573
TotalN	LSD	2004	2008	-.00333	.00549	.554	-.0151	.0084
			2012	-.01667*	.00576	.012	-.0290	-.0043
		2008	2004	.00333	.00549	.554	-.0084	.0151
			2012	-.01333*	.00576	.036	-.0257	-.0010
		2012	2004	.01667*	.00576	.012	.0043	.0290
			2008	.01333*	.00576	.036	.0010	.0257
	Bonferroni	2004	2008	-.00333	.00549	1.000	-.0183	.0116
			2012	-.01667*	.00576	.035	-.0323	-.0010
		2008	2004	.00333	.00549	1.000	-.0116	.0183
			2012	-.01333	.00576	.109	-.0290	.0023
		2012	2004	.01667*	.00576	.035	.0010	.0323
			2008	.01333	.00576	.109	-.0023	.0290
WR1	LSD	2004	2008	-19.80333*	3.41103	.000	-27.1193	-12.4874

			2012	-35.67333*	3.57752	.000	-43.3463	-28.0003
		2008	2004	19.80333*	3.41103	.000	12.4874	27.1193
			2012	-15.87000*	3.57752	.001	-23.5430	-8.1970
		2012	2004	35.67333*	3.57752	.000	28.0003	43.3463
			2008	15.87000*	3.57752	.001	8.1970	23.5430
	Bonferroni	2004	2008	-19.80333*	3.41103	.000	-29.0737	-10.5330
			2012	-35.67333*	3.57752	.000	-45.3961	-25.9505
		2008	2004	19.80333*	3.41103	.000	10.5330	29.0737
			2012	-15.87000*	3.57752	.002	-25.5928	-6.1472
		2012	2004	35.67333*	3.57752	.000	25.9505	45.3961
			2008	15.87000*	3.57752	.002	6.1472	25.5928
WR2	LSD	2004	2008	-20.07500*	5.81915	.004	-32.5558	-7.5942
			2012	-35.62167*	6.10317	.000	-48.7117	-22.5317
		2008	2004	20.07500*	5.81915	.004	7.5942	32.5558
			2012	-15.54667*	6.10317	.023	-28.6367	-2.4567
		2012	2004	35.62167*	6.10317	.000	22.5317	48.7117
			2008	15.54667*	6.10317	.023	2.4567	28.6367
	Bonferroni	2004	2008	-20.07500*	5.81915	.012	-35.8900	-4.2600
			2012	-35.62167*	6.10317	.000	-52.2086	-19.0347
		2008	2004	20.07500*	5.81915	.012	4.2600	35.8900
			2012	-15.54667	6.10317	.070	-32.1336	1.0403
		2012	2004	35.62167*	6.10317	.000	19.0347	52.2086
			2008	15.54667	6.10317	.070	-1.0403	32.1336
WR3	LSD	2004	2008	-22.62000*	5.02814	.001	-33.4043	-11.8357
			2012	-32.28000*	5.27356	.000	-43.5907	-20.9693
		2008	2004	22.62000*	5.02814	.001	11.8357	33.4043
			2012	-9.66000	5.27356	.088	-20.9707	1.6507
		2012	2004	32.28000*	5.27356	.000	20.9693	43.5907
			2008	9.66000	5.27356	.088	-1.6507	20.9707
	Bonferroni	2004	2008	-22.62000*	5.02814	.002	-36.2852	-8.9548
			2012	-32.28000*	5.27356	.000	-46.6122	-17.9478
		2008	2004	22.62000*	5.02814	.002	8.9548	36.2852
			2012	-9.66000	5.27356	.265	-23.9922	4.6722
		2012	2004	32.28000*	5.27356	.000	17.9478	46.6122

			2008	9.66000	5.27356	.265	-4.6722	23.9922
WR4	LSD	2004	2008	-22.61333*	5.03950	.001	-33.4220	-11.8047
			2012	-36.43000*	5.28547	.000	-47.7662	-25.0938
		2008	2004	22.61333*	5.03950	.001	11.8047	33.4220
			2012	-13.81667*	5.28547	.020	-25.1529	-2.4805
		2012	2004	36.43000*	5.28547	.000	25.0938	47.7662
			2008	13.81667*	5.28547	.020	2.4805	25.1529
	Bonferroni	2004	2008	-22.61333*	5.03950	.002	-36.3095	-8.9172
			2012	-36.43000*	5.28547	.000	-50.7946	-22.0654
		2008	2004	22.61333*	5.03950	.002	8.9172	36.3095
			2012	-13.81667	5.28547	.061	-28.1813	.5480
		2012	2004	36.43000*	5.28547	.000	22.0654	50.7946
			2008	13.81667	5.28547	.061	-.5480	28.1813
WR5	LSD	2004	2008	-20.47500*	4.17083	.000	-29.4205	-11.5295
			2012	-34.03833*	4.37441	.000	-43.4205	-24.6562
		2008	2004	20.47500*	4.17083	.000	11.5295	29.4205
			2012	-13.56333*	4.37441	.008	-22.9455	-4.1812
		2012	2004	34.03833*	4.37441	.000	24.6562	43.4205
			2008	13.56333*	4.37441	.008	4.1812	22.9455
	Bonferroni	2004	2008	-20.47500*	4.17083	.001	-31.8103	-9.1397
			2012	-34.03833*	4.37441	.000	-45.9269	-22.1498
		2008	2004	20.47500*	4.17083	.001	9.1397	31.8103
			2012	-13.56333*	4.37441	.023	-25.4519	-1.6748
		2012	2004	34.03833*	4.37441	.000	22.1498	45.9269
			2008	13.56333*	4.37441	.023	1.6748	25.4519
WR6	LSD	2004	2008	-18.05667*	3.44734	.000	-25.4505	-10.6629
			2012	-34.16667*	3.61560	.000	-41.9214	-26.4120
		2008	2004	18.05667*	3.44734	.000	10.6629	25.4505
			2012	-16.11000*	3.61560	.001	-23.8647	-8.3553
		2012	2004	34.16667*	3.61560	.000	26.4120	41.9214
			2008	16.11000*	3.61560	.001	8.3553	23.8647
	Bonferroni	2004	2008	-18.05667*	3.44734	.000	-27.4257	-8.6876
			2012	-34.16667*	3.61560	.000	-43.9930	-24.3403
		2008	2004	18.05667*	3.44734	.000	8.6876	27.4257

			2012	-16.11000*	3.61560	.002	-25.9363	-6.2837
		2012	2004	34.16667*	3.61560	.000	24.3403	43.9930
			2008	16.11000*	3.61560	.002	6.2837	25.9363
WR7	LSD	2004	2008	-22.42833*	6.00759	.002	-35.3133	-9.5433
			2012	-38.32800*	6.30082	.000	-51.8419	-24.8141
		2008	2004	22.42833*	6.00759	.002	9.5433	35.3133
			2012	-15.89967*	6.30082	.024	-29.4136	-2.3858
		2012	2004	38.32800*	6.30082	.000	24.8141	51.8419
			2008	15.89967*	6.30082	.024	2.3858	29.4136
	Bonferroni	2004	2008	-22.42833*	6.00759	.007	-38.7555	-6.1012
			2012	-38.32800*	6.30082	.000	-55.4521	-21.2039
		2008	2004	22.42833*	6.00759	.007	6.1012	38.7555
			2012	-15.89967	6.30082	.073	-33.0237	1.2244
		2012	2004	38.32800*	6.30082	.000	21.2039	55.4521
			2008	15.89967	6.30082	.073	-1.2244	33.0237
Sand	LSD	2004	2008	4.00000*	1.24754	.006	1.3243	6.6757
			2012	6.43333*	1.30843	.000	3.6270	9.2396
		2008	2004	-4.00000*	1.24754	.006	-6.6757	-1.3243
			2012	2.43333	1.30843	.084	-.3730	5.2396
		2012	2004	-6.43333*	1.30843	.000	-9.2396	-3.6270
			2008	-2.43333	1.30843	.084	-5.2396	.3730
	Bonferroni	2004	2008	4.00000*	1.24754	.019	.6095	7.3905
			2012	6.43333*	1.30843	.001	2.8773	9.9893
		2008	2004	-4.00000*	1.24754	.019	-7.3905	-.6095
			2012	2.43333	1.30843	.252	-1.1227	5.9893
		2012	2004	-6.43333*	1.30843	.001	-9.9893	-2.8773
			2008	-2.43333	1.30843	.252	-5.9893	1.1227
Clay	LSD	2004	2008	-1.75000	.87389	.065	-3.6243	.1243
			2012	-.40000	.91655	.669	-2.3658	1.5658
		2008	2004	1.75000	.87389	.065	-.1243	3.6243
			2012	1.35000	.91655	.163	-.6158	3.3158
		2012	2004	.40000	.91655	.669	-1.5658	2.3658
			2008	-1.35000	.91655	.163	-3.3158	.6158
	Bonferroni	2004	2008	-1.75000	.87389	.195	-4.1250	.6250

			2012	-.40000	.91655	1.000	-2.8910	2.0910
		2008	2004	1.75000	.87389	.195	-.6250	4.1250
			2012	1.35000	.91655	.489	-1.1410	3.8410
		2012	2004	.40000	.91655	1.000	-2.0910	2.8910
			2008	-1.35000	.91655	.489	-3.8410	1.1410
Silt	LSD	2004	2008	-2.41667	1.27561	.079	-5.1526	.3192
			2012	-5.63333*	1.33787	.001	-8.5028	-2.7639
		2008	2004	2.41667	1.27561	.079	-.3192	5.1526
			2012	-3.21667*	1.33787	.031	-6.0861	-.3472
		2012	2004	5.63333*	1.33787	.001	2.7639	8.5028
			2008	3.21667*	1.33787	.031	.3472	6.0861
	Bonferroni	2004	2008	-2.41667	1.27561	.237	-5.8835	1.0501
			2012	-5.63333*	1.33787	.003	-9.2693	-1.9973
		2008	2004	2.41667	1.27561	.237	-1.0501	5.8835
			2012	-3.21667	1.33787	.092	-6.8527	.4193
		2012	2004	5.63333*	1.33787	.003	1.9973	9.2693
			2008	3.21667	1.33787	.092	-.4193	6.8527

*. The mean difference is significant at the 0.05 level.

Appendix 6 - Microbial Biomass mean comparison

	(I) Period	(J) Period	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
LSD	1	2	29.3800*	13.43077	.041	1.2691	57.4909
		3	4.4083	13.43077	.746	-23.7026	32.5193
		4	26.5162	12.94222	.055	-.5722	53.6046
		5	24.6300	16.44927	.151	-9.7987	59.0587
	2	1	-29.3800*	13.43077	.041	-57.4909	-1.2691
		3	-24.9717	13.43077	.079	-53.0826	3.1393
		4	-2.8638	12.94222	.827	-29.9522	24.2246
		5	-4.7500	16.44927	.776	-39.1787	29.6787
	3	1	-4.4083	13.43077	.746	-32.5193	23.7026
		2	24.9717	13.43077	.079	-3.1393	53.0826
		4	22.1079	12.94222	.104	-4.9805	49.1962
		5	20.2217	16.44927	.234	-14.2071	54.6504
	4	1	-26.5162	12.94222	.055	-53.6046	.5722
		2	2.8638	12.94222	.827	-24.2246	29.9522
		3	-22.1079	12.94222	.104	-49.1962	4.9805
		5	-1.8862	16.05284	.908	-35.4852	31.7128
	5	1	-24.6300	16.44927	.151	-59.0587	9.7987
		2	4.7500	16.44927	.776	-29.6787	39.1787
		3	-20.2217	16.44927	.234	-54.6504	14.2071
		4	1.8862	16.05284	.908	-31.7128	35.4852
Bonferroni	1	2	29.3800	13.43077	.414	-13.2456	72.0056
		3	4.4083	13.43077	1.000	-38.2172	47.0339
		4	26.5162	12.94222	.545	-14.5588	67.5912
		5	24.6300	16.44927	1.000	-27.5755	76.8355
	2	1	-29.3800	13.43077	.414	-72.0056	13.2456
		3	-24.9717	13.43077	.785	-67.5972	17.6539
		4	-2.8638	12.94222	1.000	-43.9388	38.2112
		5	-4.7500	16.44927	1.000	-56.9555	47.4555
	3	1	-4.4083	13.43077	1.000	-47.0339	38.2172
		2	24.9717	13.43077	.785	-17.6539	67.5972

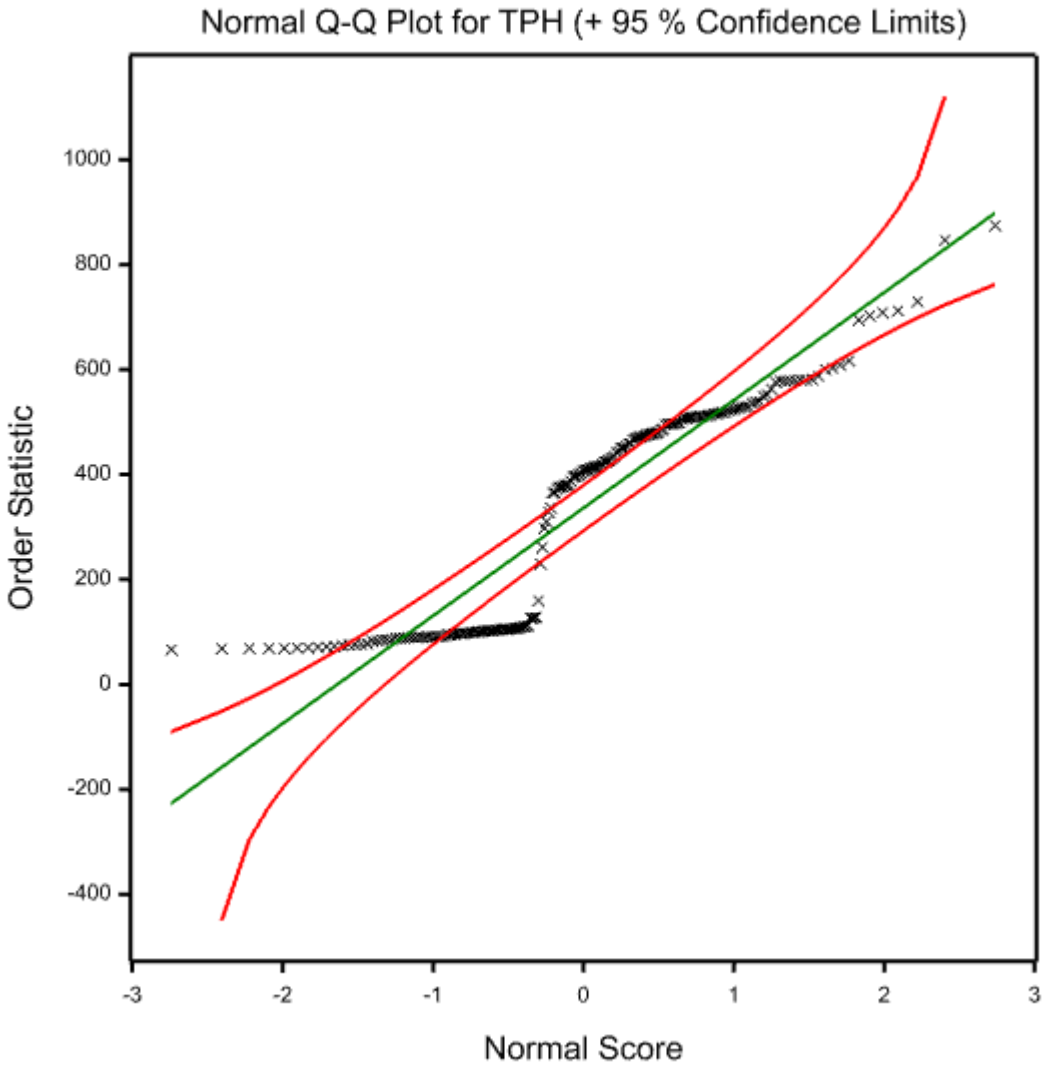
		4	22.1079	12.94222	1.000	-18.9672	63.1829
		5	20.2217	16.44927	1.000	-31.9838	72.4271
	4	1	-26.5162	12.94222	.545	-67.5912	14.5588
		2	2.8638	12.94222	1.000	-38.2112	43.9388
		3	-22.1079	12.94222	1.000	-63.1829	18.9672
		5	-1.8862	16.05284	1.000	-52.8335	49.0611
	5	1	-24.6300	16.44927	1.000	-76.8355	27.5755
		2	4.7500	16.44927	1.000	-47.4555	56.9555
		3	-20.2217	16.44927	1.000	-72.4271	31.9838
		4	1.8862	16.05284	1.000	-49.0611	52.8335

Based on observed means.

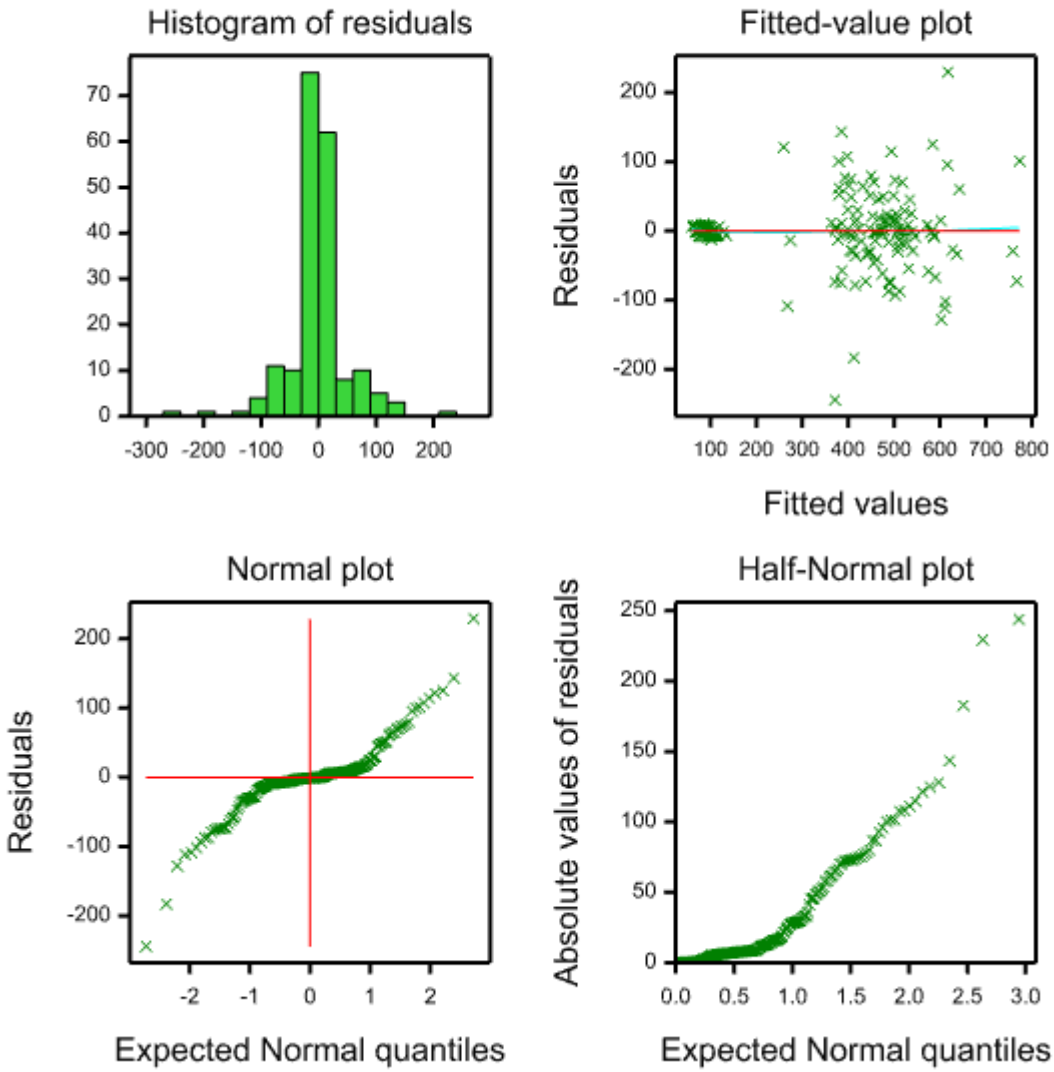
The error term is Mean Square(Error) = 541.157.

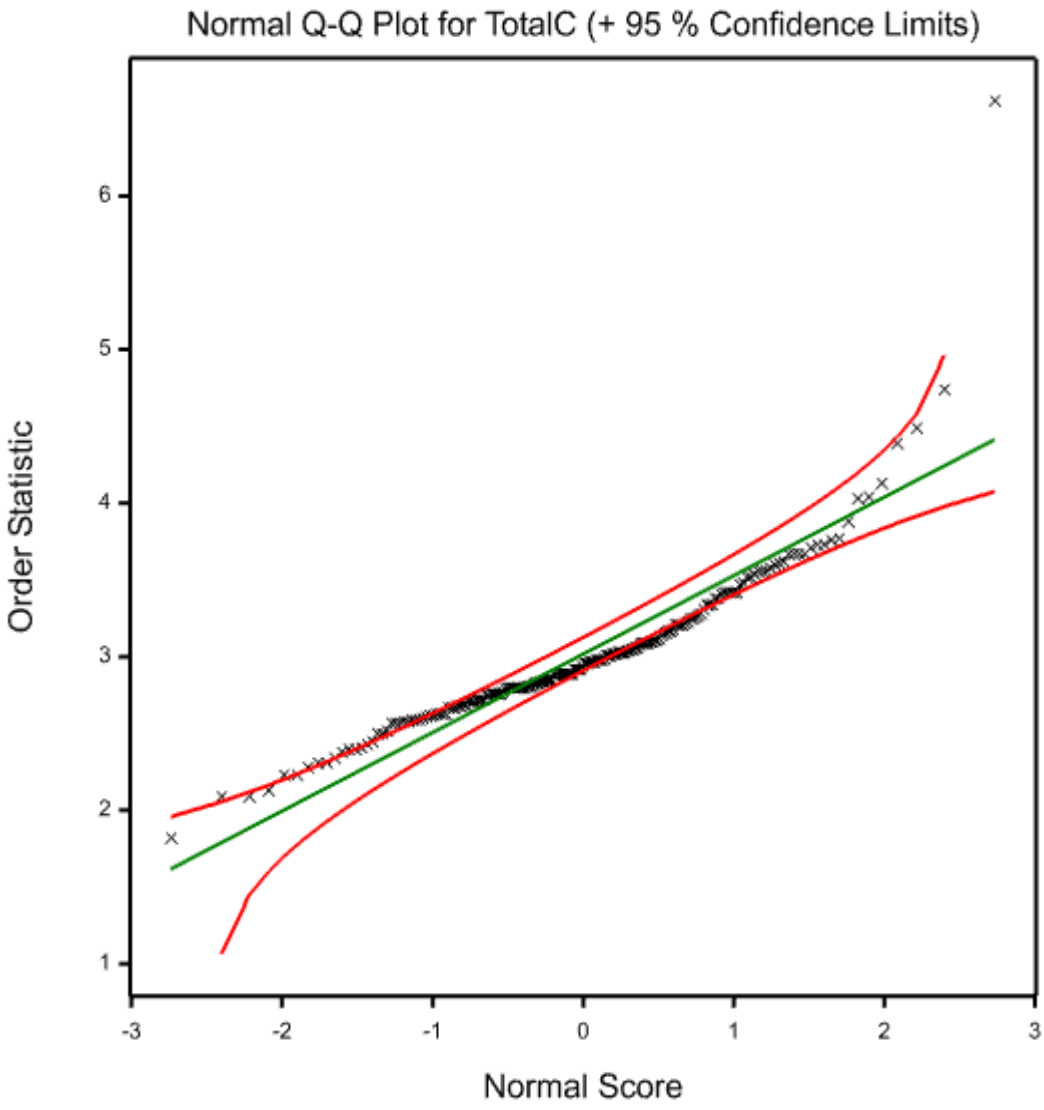
*. The mean difference is significant at the .05 level.

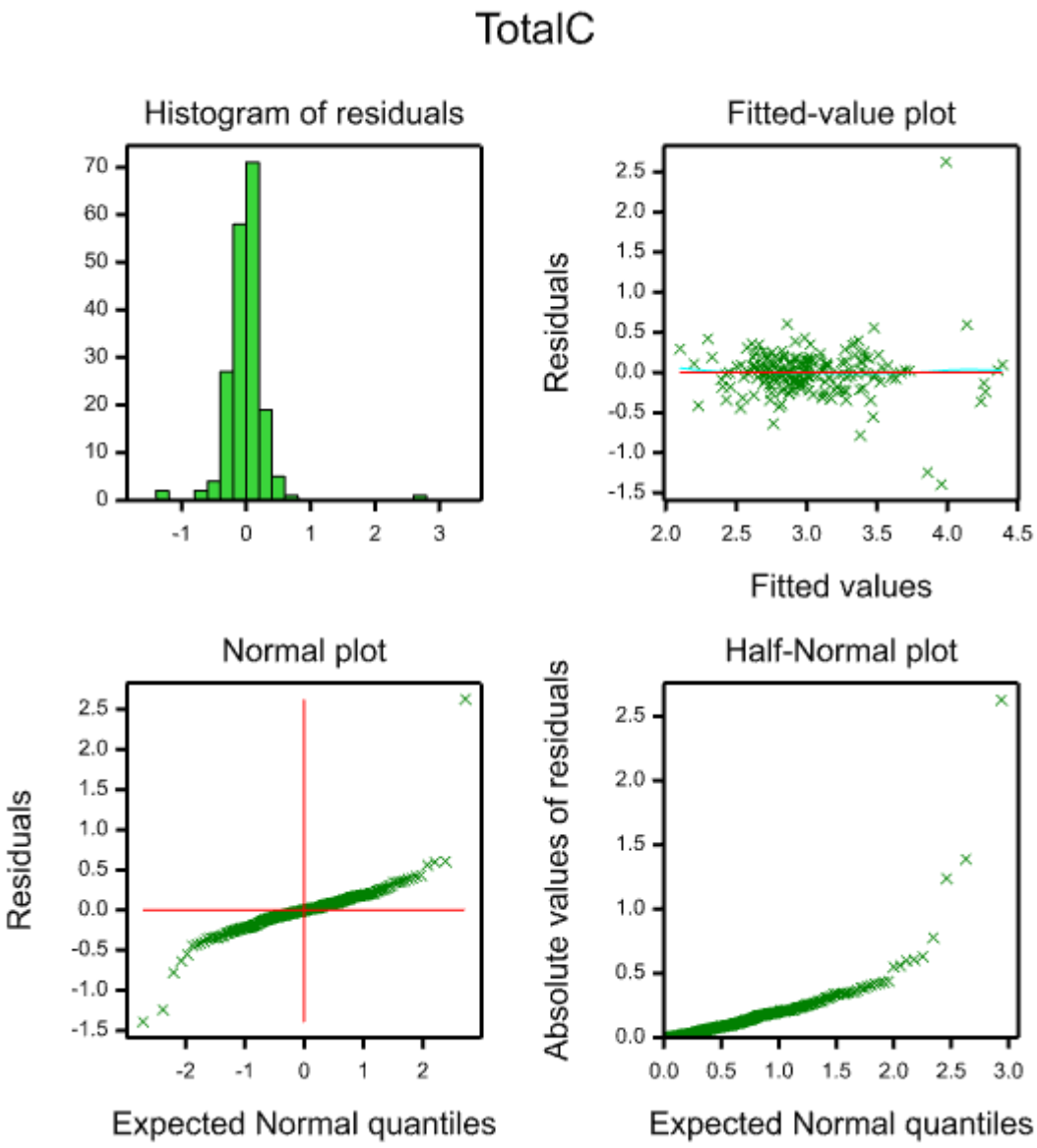
Appendix 7 -Test for Normality- Pot Experiment statistical analysis

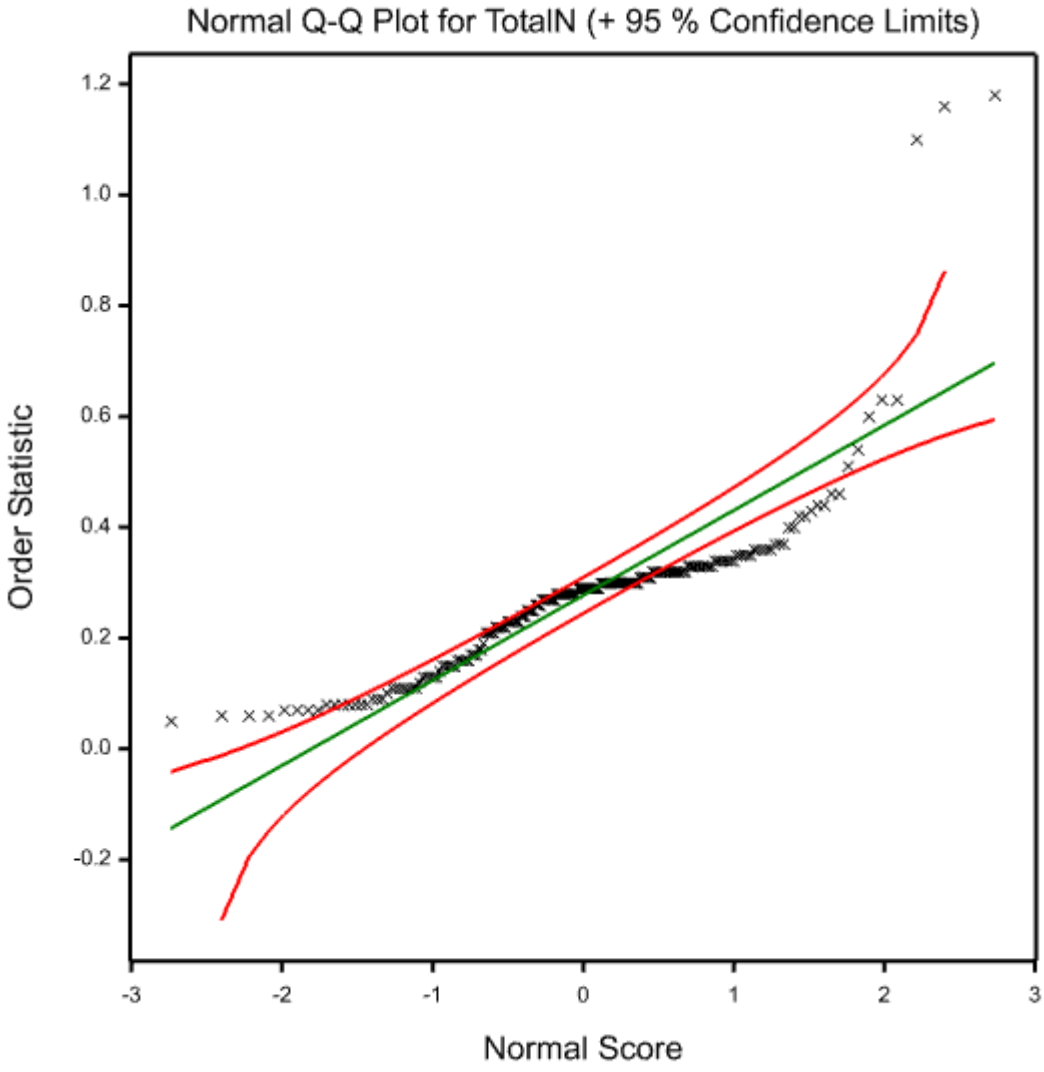


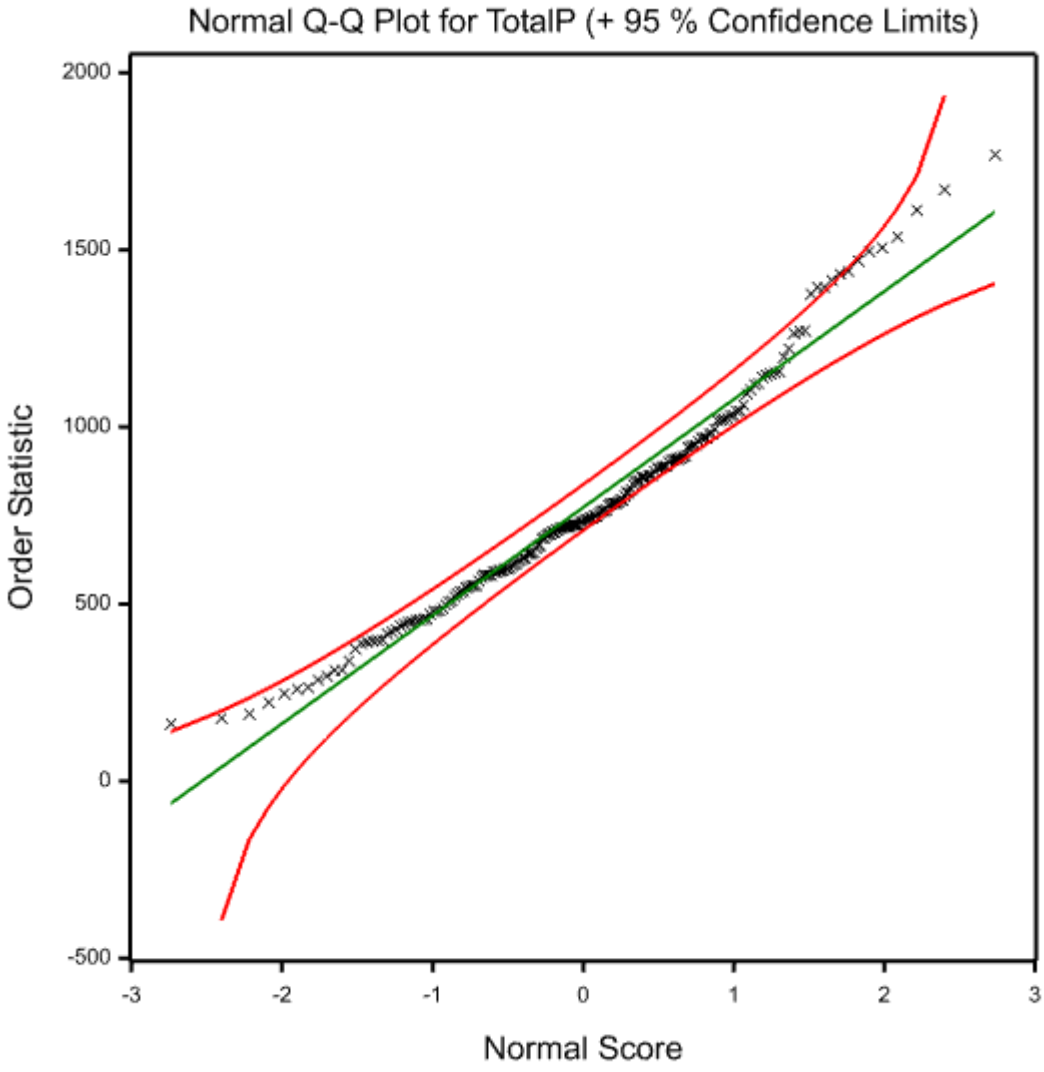
TPH

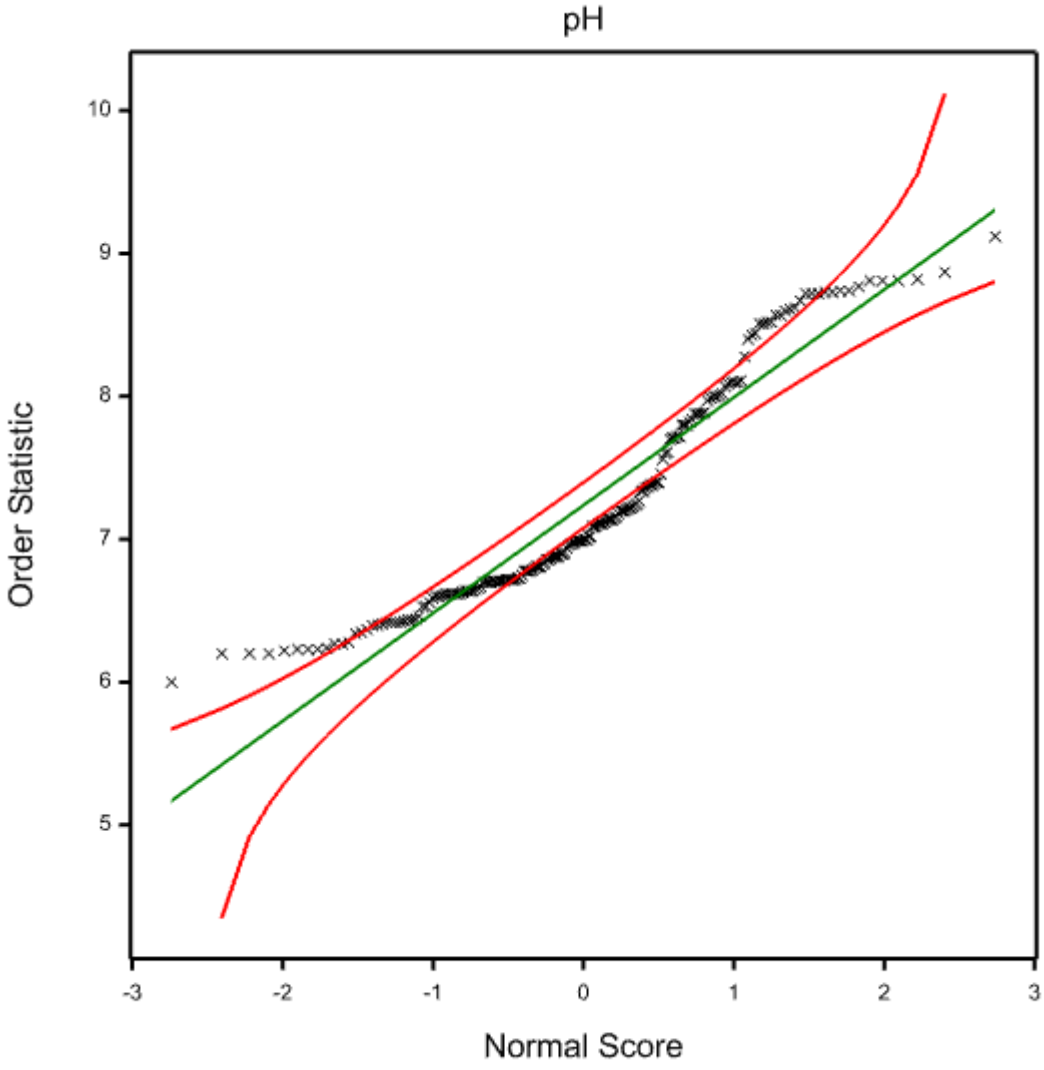

Histogram of residuals
Fitted-value plot
Residuals
Fitted values
Normal plot
Residuals
Expected Normal quantiles
Half-Normal plot
Absolute values of residuals
Expected Normal quantiles

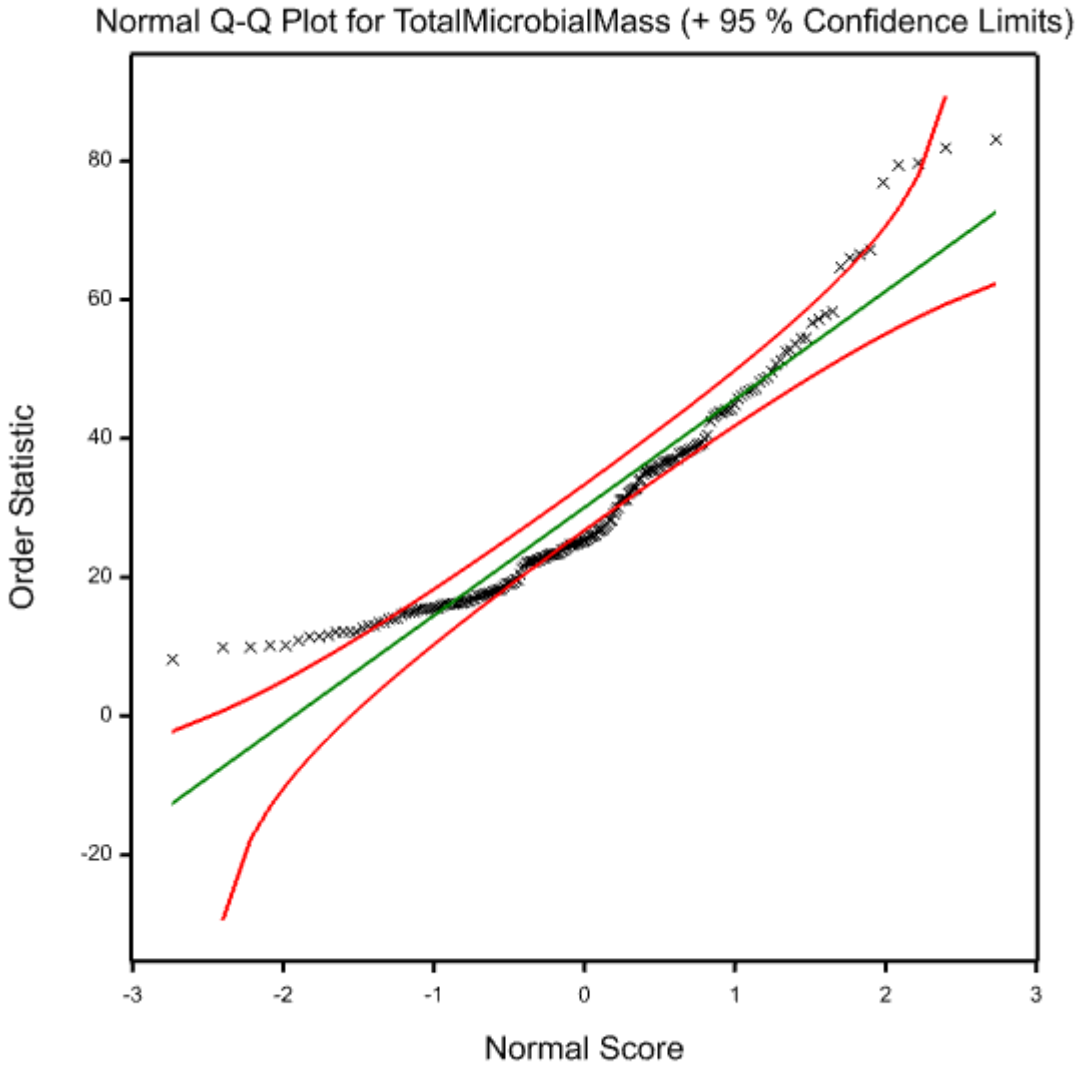

Normal Q-Q Plot for TotalC (+ 95 % Confidence Limits)
Order Statistic
Normal Score
1
2
3
4
5
6
-3
-2
-1
0
1
2
3


TotalC
Histogram of residuals
Fitted-value plot
Residuals
Fitted values
Normal plot
Residuals
Expected Normal quantiles
Half-Normal plot
Absolute values of residuals
Expected Normal quantiles


Normal Q-Q Plot for TotalN (+ 95 % Confidence Limits)
Order Statistic
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-3
-2
-1
0
1
2
3
Normal Score


Normal Q-Q Plot for TotalP (+ 95 % Confidence Limits)
Order Statistic
Normal Score
2000
1500
1000
500
0
-500
-3
-2
-1
0
1
2
3


pH
Order Statistic
Normal Score
10
9
8
7
6
5
-3
-2
-1
0
1
2
3


Normal Q-Q Plot for TotalMicrobialMass (+ 95 % Confidence Limits)
Order Statistic
80
60
40
20
0
-20
-3
-2
-1
0
1
2
3
Normal Score

Appendix 8 -Mean and Standard Error summary – pot experiment

TPH

Treatment	13 days		18 days		25days		33 Days		46 Days		74 Days		88 Days		111 Days	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	379.	35.496	449.	35.496	496.	35.496	406.	35.496	539.	35.496	68	35.496	69	35.496	98	35.496
6000:600	392.	35.496	483.	35.496	455.	35.496	501.	35.496	379.	35.496	75	35.496	84	35.496	90	35.496
6000:3000	498.	35.496	510.	35.496	583.	35.496	414	35.496	510.	35.496	71	35.496	90.	35.496	91.	35.496
6000:1200	409	35.496	454	35.496	517	35.496	481	35.496	611	35.496	77	35.496	95.	35.496	103.	35.496
9000:900	399	35.496	502	35.496	636	35.496	467	35.496	470	35.496	87	35.496	95	35.496	92	35.496
12000:2400	422.	35.496	412.	35.496	767.	35.496	267	35.496	534	35.496	100	35.496	106.	35.496	105.	35.496
7gN:0.54gP	440	35.496	531	35.496	609	35.496	372	35.496	581	35.496	104	35.496	109	35.496	101	35.496
14gN:1.08gP	380	35.496	511	35.496	474	35.496	446	35.496	580	35.496	128	35.496	107	35.496	111	35.496

TOTAL C

Treatment	13 days		18 days		25days		33 Days		46 Days		74 Days		88 Days		111 Days	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	2.85	0.227	3.356	0.227	3.09	0.227	3.416	0.227	4.216	0.227	2.99	0.227	3.456	0.227	2.953	0.227
6000:600	2.903	0.227	2.963	0.227	2.783	0.227	3.226	0.227	3.4	0.227	2.916	0.227	3.61	0.227	2.946	0.227
6000:3000	2.706	0.227	2.896	0.227	2.7	0.227	2.523	0.227	2.176	0.227	3.11	0.227	3.47	0.227	2.686	0.227
6000:1200	2.82	0.227	3.103	0.227	2.753	0.227	3.176	0.227	2.48	0.227	3.023	0.227	3.676	0.227	2.859	0.278
9000:900	3.073	0.227	2.976	0.227	2.84	0.227	2.656	0.227	2.866	0.227	2.63	0.227	2.95	0.227	2.87	0.278
12000:2400	2.79	0.227	2.713	0.227	2.853	0.227	2.373	0.227	2.91	0.227	3.936	0.227	2.953	0.227	2.403	0.227
7gN:0.54gP	3.07	0.227	2.686	0.227	3.556	0.227	3.346	0.227	3.356	0.227	2.773	0.227	3.223	0.227	2.77	0.227
14gN:1.08gP	2.96	0.227	2.696	0.227	3.336	0.227	4.336	0.227	3.25	0.227	2.696	0.227	3.29	0.227	2.606	0.227

TOTAL N

Treatment	13 days		18 days		25days		33 Days		46 Days		74 Days		88 Days		111 Days	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	0.247	0.057	0.267	0.057	0.307	0.057	0.377	0.057	0.293	0.057	0.153	0.057	0.15	0.057	0.133	0.057
6000:600	0.233	0.057	0.243	0.057	0.243	0.057	0.293	0.057	0.343	0.057	0.063	0.057	0.203	0.057	0.287	0.057
6000:3000	0.287	0.057	0.263	0.057	0.297	0.057	0.28	0.057	0.29	0.057	0.093	0.057	0.15	0.057	0.123	0.057
6000:1200	0.25	0.057	0.253	0.057	0.353	0.057	0.343	0.057	0.28	0.057	0.137	0.057	0.107	0.057	0.175	0.057
9000:900	0.307	0.057	0.307	0.057	0.383	0.057	0.327	0.057	0.343	0.057	0.223	0.057	0.347	0.057	0.115	0.057
12000:2400	0.313	0.057	0.333	0.057	0.38	0.057	0.307	0.057	0.333	0.057	0.147	0.057	0.08	0.057	0.667	0.057
7gN:0.54gP	0.3	0.057	0.3	0.057	0.317	0.057	0.333	0.057	0.323	0.057	0.123	0.057	0.277	0.057	0.953	0.057
14gN:1.08gP	0.32	0.057	0.39	0.057	0.327	0.057	0.337	0.057	0.327	0.057	0.103	0.057	0.14	0.057	0.34	0.057

TOTAL P

Treatment	13 days		18 days		25days		33 Days		46 Days		74 Days		88 Days		111 Days	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	1.05E+03	114.245	642.667	114.245	745.333	114.245	655	114.245	516.667	114.245	197.027	114.245	380.867	114.245	720.26	114.245
6000:600	905.333	114.245	681.667	114.245	624.333	114.245	466.333	114.245	786.667	114.245	466.29	114.245	998.123	114.245	831.663	114.245
6000:3000	894.667	114.245	658	114.245	899.333	114.245	985.333	114.245	775.667	114.245	822.137	114.245	1.32E+03	114.245	1.31E+03	114.245
6000:1200	742	114.245	735.333	114.245	748.333	114.245	659.667	114.245	757.667	114.245	932.96	114.245	1.51E+03	114.245	1.33E+03	139.922
9000:900	1.01E+03	114.245	815.667	114.245	956.667	114.245	811.667	114.245	766.333	114.245	877.547	114.245	1.24E+03	114.245	1.35E+03	139.922
12000:2400	867.667	114.245	826	114.245	893.333	114.245	766.333	114.245	751.667	114.245	668.313	114.245	1.13E+03	114.245	1.20E+03	114.245
7gN:0.54gP	742.333	114.245	708.333	114.245	770.667	114.245	615.333	114.245	548.333	114.245	257.92	114.245	446.09	114.245	643.493	114.245
14gN:1.08gP	708	114.245	589.333	114.245	782.667	114.245	607	114.245	582.333	114.245	266.577	114.245	457.347	114.245	457.633	114.245

TOTAL MICROBIAL MASS

Treatment	13 days		18 days		25days		33 Days		46 Days		74 Days		88 Days		111 Days	
	Mean	**SE**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**
Control	39.367	4.393	39.467	4.393	35.767	4.393	44.967	4.393	32.733	4.393	31.933	4.393	28.527	4.393	21.063	4.393
6000:600	18.867	4.393	20.3	4.393	20.333	4.393	19.467	4.393	14	4.393	18.967	4.393	20.22	4.393	20.6	4.393
6000:3000	16.4	4.393	14.2	4.393	22.3	4.393	19.367	4.393	15.433	4.393	24.533	4.393	15.673	4.393	15.593	4.393
6000:1200	26.067	4.393	24.6	4.393	16.9	4.393	21.467	4.393	9.667	4.393	17.633	4.393	20.35	4.393	15.21	5.38
9000:900	42.7	4.393	33.4	4.393	45.867	4.393	43.533	4.393	17.867	4.393	27	4.393	11.247	4.393	14.03	5.38
12000:2400	53.9	4.393	51.167	4.393	66.2	4.393	57.333	4.393	21.467	4.393	30.567	4.393	19.103	4.393	13.64	4.393
7gN:0.54gP	41.733	4.393	44.2	4.393	45.967	4.393	37.5	4.393	30.2	4.393	36.3	4.393	25.633	4.393	19.887	4.393
14gN:1.08gP	60.8	5.38	50.267	5.38	60	5.38	62.367	5.38	42.167	5.38	38.767	5.38	24.92	5.38	21.173	5.38

pH

Treatment	13 days		18 days		25days		33 Days		46 Days		74 Days		88 Days		111 Days	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	7.74	0.113	7.837	0.113	7.213	0.113	7.683	0.113	8.093	0.113	8.76	0.113	8.907	0.113	8.6	0.113
6000:600	7.26	0.113	7.047	0.113	6.72	0.113	7.003	0.113	7.39	0.113	7.137	0.113	7.287	0.113	7.18	0.113
6000:3000	6.607	0.113	6.4	0.113	6.21	0.113	6.523	0.113	6.683	0.113	6.887	0.113	6.677	0.113	6.397	0.113
6000:1200	6.68	0.113	6.643	0.113	6.41	0.113	6.96	0.113	7	0.113	6.87	0.113	6.84	0.113	6.605	0.113
9000:900	7.15	0.113	6.913	0.113	6.323	0.113	6.62	0.113	6.757	0.113	6.787	0.113	6.827	0.113	6.71	0.113
12000:2400	7.1	0.113	6.247	0.113	6.217	0.113	6.653	0.113	6.72	0.113	7.317	0.113	6.353	0.113	6.417	0.113
7gN:0.54gP	7.96	0.113	7.11	0.113	7.267	0.113	7.997	0.113	7.8	0.113	8.56	0.113	8.787	0.113	8.65	0.113
14gN:1.08gP	8.097	0.139	7.213	0.139	7.193	0.139	7.863	0.139	7.59	0.139	8.46	0.139	8.593	0.139	8.563	0.139

Appendix 9 -Multiple comparison of significance differences over time

LSDDependent Variable	(I) Week	(J) Week	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
TPH	13 Days	18 Days	-66.7083*	17.88825	.000	-102.1087	-31.3080
		25 Days	-152.1667*	17.88825	.000	-187.5670	-116.7663
		33 Days	-4.5000	17.88825	.802	-39.9003	30.9003
		46 Days	-110.4583*	17.88825	.000	-145.8587	-75.0580
		74 Days	326.2300*	17.88825	.000	290.8297	361.6303
		88 Days	320.3357*	17.88825	.000	284.9354	355.7360
		111 Days	316.0577*	18.29029	.000	279.8618	352.2537
	18 Days	13 Days	66.7083*	17.88825	.000	31.3080	102.1087
		25 Days	-85.4583*	17.88825	.000	-120.8587	-50.0580
		33 Days	62.2083*	17.88825	.001	26.8080	97.6087
		46 Days	-43.7500*	17.88825	.016	-79.1503	-8.3497
		74 Days	392.9383*	17.88825	.000	357.5380	428.3387
		88 Days	387.0440*	17.88825	.000	351.6437	422.4444
		111 Days	382.7661*	18.29029	.000	346.5701	418.9620
	25 Days	13 Days	152.1667*	17.88825	.000	116.7663	187.5670
		18 Days	85.4583*	17.88825	.000	50.0580	120.8587
		33 Days	147.6667*	17.88825	.000	112.2663	183.0670
		46 Days	41.7083*	17.88825	.021	6.3080	77.1087
		74 Days	478.3967*	17.88825	.000	442.9963	513.7970
		88 Days	472.5024*	17.88825	.000	437.1020	507.9027
		111 Days	468.2244*	18.29029	.000	432.0285	504.4203
	33 Days	13 Days	4.5000	17.88825	.802	-30.9003	39.9003
		18 Days	-62.2083*	17.88825	.001	-97.6087	-26.8080
		25 Days	-147.6667*	17.88825	.000	-183.0670	-112.2663
		46 Days	-105.9583*	17.88825	.000	-141.3587	-70.5580
		74 Days	330.7300*	17.88825	.000	295.3297	366.1303
		88 Days	324.8357*	17.88825	.000	289.4354	360.2360
		111 Days	320.5577*	18.29029	.000	284.3618	356.7537
	46 Days	13 Days	110.4583*	17.88825	.000	75.0580	145.8587
		18 Days	43.7500*	17.88825	.016	8.3497	79.1503

		25 Days	-41.7083*	17.88825	.021	-77.1087	-6.3080
		33 Days	105.9583*	17.88825	.000	70.5580	141.3587
		74 Days	436.6883*	17.88825	.000	401.2880	472.0887
		88 Days	430.7940*	17.88825	.000	395.3937	466.1944
		111 Days	426.5161*	18.29029	.000	390.3201	462.7120
	74 Days	13 Days	-326.2300*	17.88825	.000	-361.6303	-290.8297
		18 Days	-392.9383*	17.88825	.000	-428.3387	-357.5380
		25 Days	-478.3967*	17.88825	.000	-513.7970	-442.9963
		33 Days	-330.7300*	17.88825	.000	-366.1303	-295.3297
		46 Days	-436.6883*	17.88825	.000	-472.0887	-401.2880
		88 Days	-5.8943	17.88825	.742	-41.2946	29.5060
		111 Days	-10.1723	18.29029	.579	-46.3682	26.0237
	88 Days	13 Days	-320.3357*	17.88825	.000	-355.7360	-284.9354
		18 Days	-387.0440*	17.88825	.000	-422.4444	-351.6437
		25 Days	-472.5024*	17.88825	.000	-507.9027	-437.1020
		33 Days	-324.8357*	17.88825	.000	-360.2360	-289.4354
		46 Days	-430.7940*	17.88825	.000	-466.1944	-395.3937
		74 Days	5.8943	17.88825	.742	-29.5060	41.2946
		111 Days	-4.2780	18.29029	.815	-40.4739	31.9180
	111 Days	13 Days	-316.0577*	18.29029	.000	-352.2537	-279.8618
		18 Days	-382.7661*	18.29029	.000	-418.9620	-346.5701
		25 Days	-468.2244*	18.29029	.000	-504.4203	-432.0285
		33 Days	-320.5577*	18.29029	.000	-356.7537	-284.3618
		46 Days	-426.5161*	18.29029	.000	-462.7120	-390.3201
		74 Days	10.1723	18.29029	.579	-26.0237	46.3682
		88 Days	4.2780	18.29029	.815	-31.9180	40.4739
pH	13 Days	18 Days	.3979*	.05657	.000	.2860	.5099
		25 Days	.6300*	.05657	.000	.5181	.7419
		33 Days	.1612*	.05657	.005	.0493	.2732
		46 Days	.0700	.05657	.218	-.0419	.1819
		74 Days	-.2729*	.05657	.000	-.3849	-.1610
		88 Days	-.2096*	.05657	.000	-.3215	-.0976
		111 Days	-.1327*	.05784	.023	-.2471	-.0182

18 Days	13 Days	-.3979*	.05657	.000	-.5099	-.2860
	25 Days	.2321*	.05657	.000	.1201	.3440
	33 Days	-.2367*	.05657	.000	-.3486	-.1247
	46 Days	-.3279*	.05657	.000	-.4399	-.2160
	74 Days	-.6708*	.05657	.000	-.7828	-.5589
	88 Days	-.6075*	.05657	.000	-.7194	-.4956
	111 Days	-.5306*	.05784	.000	-.6450	-.4161
25 Days	13 Days	-.6300*	.05657	.000	-.7419	-.5181
	18 Days	-.2321*	.05657	.000	-.3440	-.1201
	33 Days	-.4688*	.05657	.000	-.5807	-.3568
	46 Days	-.5600*	.05657	.000	-.6719	-.4481
	74 Days	-.9029*	.05657	.000	-1.0149	-.7910
	88 Days	-.8396*	.05657	.000	-.9515	-.7276
	111 Days	-.7627*	.05784	.000	-.8771	-.6482
33 Days	13 Days	-.1612*	.05657	.005	-.2732	-.0493
	18 Days	.2367*	.05657	.000	.1247	.3486
	25 Days	.4688*	.05657	.000	.3568	.5807
	46 Days	-.0912	.05657	.109	-.2032	.0207
	74 Days	-.4342*	.05657	.000	-.5461	-.3222
	88 Days	-.3708*	.05657	.000	-.4828	-.2589
	111 Days	-.2939*	.05784	.000	-.4084	-.1794
46 Days	13 Days	-.0700	.05657	.218	-.1819	.0419
	18 Days	.3279*	.05657	.000	.2160	.4399
	25 Days	.5600*	.05657	.000	.4481	.6719
	33 Days	.0912	.05657	.109	-.0207	.2032
	74 Days	-.3429*	.05657	.000	-.4549	-.2310
	88 Days	-.2796*	.05657	.000	-.3915	-.1676
	111 Days	-.2027*	.05784	.001	-.3171	-.0882
74 Days	13 Days	.2729*	.05657	.000	.1610	.3849
	18 Days	.6708*	.05657	.000	.5589	.7828
	25 Days	.9029*	.05657	.000	.7910	1.0149
	33 Days	.4342*	.05657	.000	.3222	.5461
	46 Days	.3429*	.05657	.000	.2310	.4549
	88 Days	.0633	.05657	.265	-.0486	.1753

		111 Days	.1403*	.05784	.017	.0258	.2547
	88 Days	13 Days	.2096*	.05657	.000	.0976	.3215
		18 Days	.6075*	.05657	.000	.4956	.7194
		25 Days	.8396*	.05657	.000	.7276	.9515
		33 Days	.3708*	.05657	.000	.2589	.4828
		46 Days	.2796*	.05657	.000	.1676	.3915
		74 Days	-.0633	.05657	.265	-.1753	.0486
		111 Days	.0769	.05784	.186	-.0375	.1914
	111 Days	13 Days	.1327*	.05784	.023	.0182	.2471
		18 Days	.5306*	.05784	.000	.4161	.6450
		25 Days	.7627*	.05784	.000	.6482	.8771
		33 Days	.2939*	.05784	.000	.1794	.4084
		46 Days	.2027*	.05784	.001	.0882	.3171
		74 Days	-.1403*	.05784	.017	-.2547	-.0258
		88 Days	-.0769	.05784	.186	-.1914	.0375
TotalC	13 Days	18 Days	-.0275	.11356	.809	-.2522	.1972
		25 Days	-.0925	.11356	.417	-.3172	.1322
		33 Days	-.2354*	.11356	.040	-.4601	-.0107
		46 Days	-.1854	.11356	.105	-.4101	.0393
		74 Days	-.1129	.11356	.322	-.3376	.1118
		88 Days	-.4321*	.11356	.000	-.6568	-.2074
		111 Days	.1439	.11611	.217	-.0858	.3737
	18 Days	13 Days	.0275	.11356	.809	-.1972	.2522
		25 Days	-.0650	.11356	.568	-.2897	.1597
		33 Days	-.2079	.11356	.069	-.4326	.0168
		46 Days	-.1579	.11356	.167	-.3826	.0668
		74 Days	-.0854	.11356	.453	-.3101	.1393
		88 Days	-.4046*	.11356	.001	-.6293	-.1799
		111 Days	.1714	.11611	.142	-.0583	.4012
	25 Days	13 Days	.0925	.11356	.417	-.1322	.3172
		18 Days	.0650	.11356	.568	-.1597	.2897
		33 Days	-.1429	.11356	.211	-.3676	.0818
		46 Days	-.0929	.11356	.415	-.3176	.1318
		74 Days	-.0204	.11356	.858	-.2451	.2043

	88 Days	-.3396*	.11356	.003	-.5643	-.1149
	111 Days	.2364*	.11611	.044	.0067	.4662
33 Days	13 Days	.2354*	.11356	.040	.0107	.4601
	18 Days	.2079	.11356	.069	-.0168	.4326
	25 Days	.1429	.11356	.211	-.0818	.3676
	46 Days	.0500	.11356	.660	-.1747	.2747
	74 Days	.1225	.11356	.283	-.1022	.3472
	88 Days	-.1967	.11356	.086	-.4214	.0281
	111 Days	.3794*	.11611	.001	.1496	.6091
46 Days	13 Days	.1854	.11356	.105	-.0393	.4101
	18 Days	.1579	.11356	.167	-.0668	.3826
	25 Days	.0929	.11356	.415	-.1318	.3176
	33 Days	-.0500	.11356	.660	-.2747	.1747
	74 Days	.0725	.11356	.524	-.1522	.2972
	88 Days	-.2467*	.11356	.032	-.4714	-.0219
	111 Days	.3294*	.11611	.005	.0996	.5591
74 Days	13 Days	.1129	.11356	.322	-.1118	.3376
	18 Days	.0854	.11356	.453	-.1393	.3101
	25 Days	.0204	.11356	.858	-.2043	.2451
	33 Days	-.1225	.11356	.283	-.3472	.1022
	46 Days	-.0725	.11356	.524	-.2972	.1522
	88 Days	-.3192*	.11356	.006	-.5439	-.0944
	111 Days	.2569*	.11611	.029	.0271	.4866
88 Days	13 Days	.4321*	.11356	.000	.2074	.6568
	18 Days	.4046*	.11356	.001	.1799	.6293
	25 Days	.3396*	.11356	.003	.1149	.5643
	33 Days	.1967	.11356	.086	-.0281	.4214
	46 Days	.2467*	.11356	.032	.0219	.4714
	74 Days	.3192*	.11356	.006	.0944	.5439
	111 Days	.5760*	.11611	.000	.3462	.8058
111 Days	13 Days	-.1439	.11611	.217	-.3737	.0858
	18 Days	-.1714	.11611	.142	-.4012	.0583
	25 Days	-.2364*	.11611	.044	-.4662	-.0067
	33 Days	-.3794*	.11611	.001	-.6091	-.1496

		46 Days	-.3294*	.11611	.005	-.5591	-.0996
		74 Days	-.2569*	.11611	.029	-.4866	-.0271
		88 Days	-.5760*	.11611	.000	-.8058	-.3462
TotalN	13 Days	18 Days	-.0125	.02852	.662	-.0689	.0439
		25 Days	-.0438	.02852	.128	-.1002	.0127
		33 Days	-.0425	.02852	.139	-.0989	.0139
		46 Days	-.0346	.02852	.228	-.0910	.0219
		74 Days	.1517*	.02852	.000	.0952	.2081
		88 Days	.1004*	.02852	.001	.0440	.1569
		111 Days	-.0856*	.02916	.004	-.1433	-.0279
	18 Days	13 Days	.0125	.02852	.662	-.0439	.0689
		25 Days	-.0312	.02852	.275	-.0877	.0252
		33 Days	-.0300	.02852	.295	-.0864	.0264
		46 Days	-.0221	.02852	.440	-.0785	.0344
		74 Days	.1642*	.02852	.000	.1077	.2206
		88 Days	.1129*	.02852	.000	.0565	.1694
		111 Days	-.0731*	.02916	.013	-.1308	-.0154
	25 Days	13 Days	.0438	.02852	.128	-.0127	.1002
		18 Days	.0312	.02852	.275	-.0252	.0877
		33 Days	.0012	.02852	.965	-.0552	.0577
		46 Days	.0092	.02852	.748	-.0473	.0656
		74 Days	.1954*	.02852	.000	.1390	.2519
		88 Days	.1442*	.02852	.000	.0877	.2006
		111 Days	-.0419	.02916	.153	-.0996	.0158
	33 Days	13 Days	.0425	.02852	.139	-.0139	.0989
		18 Days	.0300	.02852	.295	-.0264	.0864
		25 Days	-.0012	.02852	.965	-.0577	.0552
		46 Days	.0079	.02852	.782	-.0485	.0644
		74 Days	.1942*	.02852	.000	.1377	.2506
		88 Days	.1429*	.02852	.000	.0865	.1994
		111 Days	-.0431	.02916	.141	-.1008	.0146
	46 Days	13 Days	.0346	.02852	.228	-.0219	.0910
		18 Days	.0221	.02852	.440	-.0344	.0785
		25 Days	-.0092	.02852	.748	-.0656	.0473

		33 Days	-.0079	.02852	.782	-.0644	.0485
		74 Days	.1862*	.02852	.000	.1298	.2427
		88 Days	.1350*	.02852	.000	.0786	.1914
		111 Days	-.0511	.02916	.082	-.1088	.0066
	74 Days	13 Days	-.1517*	.02852	.000	-.2081	-.0952
		18 Days	-.1642*	.02852	.000	-.2206	-.1077
		25 Days	-.1954*	.02852	.000	-.2519	-.1390
		33 Days	-.1942*	.02852	.000	-.2506	-.1377
		46 Days	-.1862*	.02852	.000	-.2427	-.1298
		88 Days	-.0513	.02852	.075	-.1077	.0052
		111 Days	-.2373*	.02916	.000	-.2950	-.1796
	88 Days	13 Days	-.1004*	.02852	.001	-.1569	-.0440
		18 Days	-.1129*	.02852	.000	-.1694	-.0565
		25 Days	-.1442*	.02852	.000	-.2006	-.0877
		33 Days	-.1429*	.02852	.000	-.1994	-.0865
		46 Days	-.1350*	.02852	.000	-.1914	-.0786
		74 Days	.0513	.02852	.075	-.0052	.1077
		111 Days	-.1861*	.02916	.000	-.2438	-.1284
	111 Days	13 Days	.0856*	.02916	.004	.0279	.1433
		18 Days	.0731*	.02916	.013	.0154	.1308
		25 Days	.0419	.02916	.153	-.0158	.0996
		33 Days	.0431	.02916	.141	-.0146	.1008
		46 Days	.0511	.02916	.082	-.0066	.1088
		74 Days	.2373*	.02916	.000	.1796	.2950
		88 Days	.1861*	.02916	.000	.1284	.2438
TotalP	13 Days	18 Days	157.5833*	57.12275	.007	44.5391	270.6276
		25 Days	62.1250	57.12275	.279	-50.9192	175.1692
		33 Days	168.8750*	57.12275	.004	55.8308	281.9192
		46 Days	179.0417*	57.12275	.002	65.9974	292.0859
		74 Days	303.6121*	57.12275	.000	190.5678	416.6563
		88 Days	-69.6142	57.12275	.225	-182.6584	43.4301
		111 Days	-82.6367	58.40656	.160	-198.2215	32.9482
	18 Days	13 Days	-157.5833*	57.12275	.007	-270.6276	-44.5391
		25 Days	-95.4583	57.12275	.097	-208.5026	17.5859

		33 Days	11.2917	57.12275	.844	-101.7526	124.3359
		46 Days	21.4583	57.12275	.708	-91.5859	134.5026
		74 Days	146.0288*	57.12275	.012	32.9845	259.0730
		88 Days	-227.1975*	57.12275	.000	-340.2417	-114.1533
		111 Days	-240.2200*	58.40656	.000	-355.8049	-124.6351
	25 Days	13 Days	-62.1250	57.12275	.279	-175.1692	50.9192
		18 Days	95.4583	57.12275	.097	-17.5859	208.5026
		33 Days	106.7500	57.12275	.064	-6.2942	219.7942
		46 Days	116.9167*	57.12275	.043	3.8724	229.9609
		74 Days	241.4871*	57.12275	.000	128.4428	354.5313
		88 Days	-131.7392*	57.12275	.023	-244.7834	-18.6949
		111 Days	-144.7617*	58.40656	.015	-260.3465	-29.1768
	33 Days	13 Days	-168.8750*	57.12275	.004	-281.9192	-55.8308
		18 Days	-11.2917	57.12275	.844	-124.3359	101.7526
		25 Days	-106.7500	57.12275	.064	-219.7942	6.2942
		46 Days	10.1667	57.12275	.859	-102.8776	123.2109
		74 Days	134.7371*	57.12275	.020	21.6928	247.7813
		88 Days	-238.4892*	57.12275	.000	-351.5334	-125.4449
		111 Days	-251.5117*	58.40656	.000	-367.0965	-135.9268
	46 Days	13 Days	-179.0417*	57.12275	.002	-292.0859	-65.9974
		18 Days	-21.4583	57.12275	.708	-134.5026	91.5859
		25 Days	-116.9167*	57.12275	.043	-229.9609	-3.8724
		33 Days	-10.1667	57.12275	.859	-123.2109	102.8776
		74 Days	124.5704*	57.12275	.031	11.5262	237.6147
		88 Days	-248.6558*	57.12275	.000	-361.7001	-135.6116
		111 Days	-261.6783*	58.40656	.000	-377.2632	-146.0935
	74 Days	13 Days	-303.6121*	57.12275	.000	-416.6563	-190.5678
		18 Days	-146.0288*	57.12275	.012	-259.0730	-32.9845
		25 Days	-241.4871*	57.12275	.000	-354.5313	-128.4428
		33 Days	-134.7371*	57.12275	.020	-247.7813	-21.6928
		46 Days	-124.5704*	57.12275	.031	-237.6147	-11.5262
		88 Days	-373.2262*	57.12275	.000	-486.2705	-260.1820
		111 Days	-386.2488*	58.40656	.000	-501.8336	-270.6639
	88 Days	13 Days	69.6142	57.12275	.225	-43.4301	182.6584

		18 Days	227.1975*	57.12275	.000	114.1533	340.2417
		25 Days	131.7392*	57.12275	.023	18.6949	244.7834
		33 Days	238.4892*	57.12275	.000	125.4449	351.5334
		46 Days	248.6558*	57.12275	.000	135.6116	361.7001
		74 Days	373.2262*	57.12275	.000	260.1820	486.2705
		111 Days	-13.0225	58.40656	.824	-128.6074	102.5624
	111 Days	13 Days	82.6367	58.40656	.160	-32.9482	198.2215
		18 Days	240.2200*	58.40656	.000	124.6351	355.8049
		25 Days	144.7617*	58.40656	.015	29.1768	260.3465
		33 Days	251.5117*	58.40656	.000	135.9268	367.0965
		46 Days	261.6783*	58.40656	.000	146.0935	377.2632
		74 Days	386.2488*	58.40656	.000	270.6639	501.8336
		88 Days	13.0225	58.40656	.824	-102.5624	128.6074
TotalMicrobialMass	13 Days	18 Days	2.77917	2.196461	.208	-1.56757	7.12590
		25 Days	-1.68750	2.196461	.444	-6.03423	2.65923
		33 Days	-.77083	2.196461	.726	-5.11757	3.57590
		46 Days	14.53750*	2.196461	.000	10.19077	18.88423
		74 Days	9.26667*	2.196461	.000	4.91993	13.61340
		88 Days	16.77000*	2.196461	.000	12.42327	21.11673
		111 Days	19.55417*	2.245826	.000	15.10974	23.99859
	18 Days	13 Days	-2.77917	2.196461	.208	-7.12590	1.56757
		25 Days	-4.46667*	2.196461	.044	-8.81340	-.11993
		33 Days	-3.55000	2.196461	.109	-7.89673	.79673
		46 Days	11.75833*	2.196461	.000	7.41160	16.10507
		74 Days	6.48750*	2.196461	.004	2.14077	10.83423
		88 Days	13.99083*	2.196461	.000	9.64410	18.33757
		111 Days	16.77500*	2.245826	.000	12.33058	21.21942
	25 Days	13 Days	1.68750	2.196461	.444	-2.65923	6.03423
		18 Days	4.46667*	2.196461	.044	.11993	8.81340
		33 Days	.91667	2.196461	.677	-3.43007	5.26340
		46 Days	16.22500*	2.196461	.000	11.87827	20.57173
		74 Days	10.95417*	2.196461	.000	6.60743	15.30090
		88 Days	18.45750*	2.196461	.000	14.11077	22.80423
		111 Days	21.24167*	2.245826	.000	16.79724	25.68609

33 Days	13 Days	.77083	2.196461	.726	-3.57590	5.11757
	18 Days	3.55000	2.196461	.109	-.79673	7.89673
	25 Days	-.91667	2.196461	.677	-5.26340	3.43007
	46 Days	15.30833*	2.196461	.000	10.96160	19.65507
	74 Days	10.03750*	2.196461	.000	5.69077	14.38423
	88 Days	17.54083*	2.196461	.000	13.19410	21.88757
	111 Days	20.32500*	2.245826	.000	15.88058	24.76942
46 Days	13 Days	-14.53750*	2.196461	.000	-18.88423	-10.19077
	18 Days	-11.75833*	2.196461	.000	-16.10507	-7.41160
	25 Days	-16.22500*	2.196461	.000	-20.57173	-11.87827
	33 Days	-15.30833*	2.196461	.000	-19.65507	-10.96160
	74 Days	-5.27083*	2.196461	.018	-9.61757	-.92410
	88 Days	2.23250	2.196461	.311	-2.11423	6.57923
	111 Days	5.01667*	2.245826	.027	.57224	9.46109
74 Days	13 Days	-9.26667*	2.196461	.000	-13.61340	-4.91993
	18 Days	-6.48750*	2.196461	.004	-10.83423	-2.14077
	25 Days	-10.95417*	2.196461	.000	-15.30090	-6.60743
	33 Days	-10.03750*	2.196461	.000	-14.38423	-5.69077
	46 Days	5.27083*	2.196461	.018	.92410	9.61757
	88 Days	7.50333*	2.196461	.001	3.15660	11.85007
	111 Days	10.28750*	2.245826	.000	5.84308	14.73192
88 Days	13 Days	-16.77000*	2.196461	.000	-21.11673	-12.42327
	18 Days	-13.99083*	2.196461	.000	-18.33757	-9.64410
	25 Days	-18.45750*	2.196461	.000	-22.80423	-14.11077
	33 Days	-17.54083*	2.196461	.000	-21.88757	-13.19410
	46 Days	-2.23250	2.196461	.311	-6.57923	2.11423
	74 Days	-7.50333*	2.196461	.001	-11.85007	-3.15660
	111 Days	2.78417	2.245826	.217	-1.66026	7.22859
111 Days	13 Days	-19.55417*	2.245826	.000	-23.99859	-15.10974
	18 Days	-16.77500*	2.245826	.000	-21.21942	-12.33058
	25 Days	-21.24167*	2.245826	.000	-25.68609	-16.79724
	33 Days	-20.32500*	2.245826	.000	-24.76942	-15.88058
	46 Days	-5.01667*	2.245826	.027	-9.46109	-.57224
	74 Days	-10.28750*	2.245826	.000	-14.73192	-5.84308

	88 Days	-2.78417	2.245826	.217	-7.22859	1.66026

Based on observed means.

The error term is Mean Square(Error) = 57.893.

*. The mean difference is significant at the .05 level.

Appendix 10 - Multiple Comparisons of significance difference by treatment

Dependent Variable	Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
TPH	6000mgN:600P	6000mgN:3000P	-38.4828*	17.88825	.033	-73.8831	-3.0825
		6000mgN:1200	-46.2166*	18.08165	.012	-81.9996	-10.4335
		Control	-5.4707	17.88825	.760	-40.8710	29.9296
		9000mgN:900P	-46.8053*	18.08165	.011	-82.5883	-11.0222
		12000mgN:2400P	-31.5311	17.88825	.080	-66.9315	3.8692
		7gN:0.54gP	-48.2503*	17.88825	.008	-83.6506	-12.8500
		14gN:1.08P	-34.6028	17.88825	.055	-70.0031	.7975
	6000mgN:3000P	6000mgN:600P	38.4828*	17.88825	.033	3.0825	73.8831
		6000mgN:1200	-7.7338	18.08165	.670	-43.5169	28.0492
		Control	33.0121	17.88825	.067	-2.3882	68.4124
		9000mgN:900P	-8.3225	18.08165	.646	-44.1055	27.4605
		12000mgN:2400P	6.9517	17.88825	.698	-28.4487	42.3520
		7gN:0.54gP	-9.7675	17.88825	.586	-45.1678	25.6328
		14gN:1.08P	3.8800	17.88825	.829	-31.5203	39.2803
	6000mgN:1200	6000mgN:600P	46.2166*	18.08165	.012	10.4335	81.9996
		6000mgN:3000P	7.7338	18.08165	.670	-28.0492	43.5169
		Control	40.7459*	18.08165	.026	4.9628	76.5289
		9000mgN:900P	-.5887	18.27299	.974	-36.7504	35.5730
		12000mgN:2400P	14.6855	18.08165	.418	-21.0976	50.4685
		7gN:0.54gP	-2.0337	18.08165	.911	-37.8167	33.7494
		14gN:1.08P	11.6138	18.08165	.522	-24.1692	47.3969
	Control	6000mgN:600P	5.4707	17.88825	.760	-29.9296	40.8710
		6000mgN:3000P	-33.0121	17.88825	.067	-68.4124	2.3882
		6000mgN:1200	-40.7459*	18.08165	.026	-76.5289	-4.9628
		9000mgN:900P	-41.3346*	18.08165	.024	-77.1176	-5.5515
		12000mgN:2400P	-26.0604	17.88825	.148	-61.4607	9.3399
		7gN:0.54gP	-42.7796*	17.88825	.018	-78.1799	-7.3793
		14gN:1.08P	-29.1321	17.88825	.106	-64.5324	6.2682
	9000mgN:900P	6000mgN:600P	46.8053*	18.08165	.011	11.0222	82.5883

		6000mgN:3000P	8.3225	18.08165	.646	-27.4605	44.1055
		6000mgN:1200	.5887	18.27299	.974	-35.5730	36.7504
		Control	41.3346*	18.08165	.024	5.5515	77.1176
		12000mgN:2400P	15.2742	18.08165	.400	-20.5089	51.0572
		7gN:0.54gP	-1.4450	18.08165	.936	-37.2280	34.3380
		14gN:1.08P	12.2025	18.08165	.501	-23.5805	47.9855
	12000mgN:2400P	6000mgN:600P	31.5311	17.88825	.080	-3.8692	66.9315
		6000mgN:3000P	-6.9517	17.88825	.698	-42.3520	28.4487
		6000mgN:1200	-14.6855	18.08165	.418	-50.4685	21.0976
		Control	26.0604	17.88825	.148	-9.3399	61.4607
		9000mgN:900P	-15.2742	18.08165	.400	-51.0572	20.5089
		7gN:0.54gP	-16.7192	17.88825	.352	-52.1195	18.6812
		14gN:1.08P	-3.0717	17.88825	.864	-38.4720	32.3287
	7gN:0.54gP	6000mgN:600P	48.2503*	17.88825	.008	12.8500	83.6506
		6000mgN:3000P	9.7675	17.88825	.586	-25.6328	45.1678
		6000mgN:1200	2.0337	18.08165	.911	-33.7494	37.8167
		Control	42.7796*	17.88825	.018	7.3793	78.1799
		9000mgN:900P	1.4450	18.08165	.936	-34.3380	37.2280
		12000mgN:2400P	16.7192	17.88825	.352	-18.6812	52.1195
		14gN:1.08P	13.6475	17.88825	.447	-21.7528	49.0478
	14gN:1.08P	6000mgN:600P	34.6028	17.88825	.055	-.7975	70.0031
		6000mgN:3000P	-3.8800	17.88825	.829	-39.2803	31.5203
		6000mgN:1200	-11.6138	18.08165	.522	-47.3969	24.1692
		Control	29.1321	17.88825	.106	-6.2682	64.5324
		9000mgN:900P	-12.2025	18.08165	.501	-47.9855	23.5805
		12000mgN:2400P	3.0717	17.88825	.864	-32.3287	38.4720
		7gN:0.54gP	-13.6475	17.88825	.447	-49.0478	21.7528
pH	6000mgN:600P	6000mgN:3000P	.5800*	.05657	.000	.4681	.6919
		6000mgN:1200	.3705*	.05718	.000	.2574	.4837
		Control	-.9763*	.05657	.000	-1.0882	-.8643
		9000mgN:900P	.3649*	.05718	.000	.2517	.4780
		12000mgN:2400P	.5000*	.05657	.000	.3881	.6119
		7gN:0.54gP	-.8883*	.05657	.000	-1.0003	-.7764

	14gN:1.08P	-.8187*	.05657	.000	-.9307	-.7068
6000mgN:3000P	6000mgN:600P	-.5800*	.05657	.000	-.6919	-.4681
	6000mgN:1200	-.2095*	.05718	.000	-.3226	-.0963
	Control	-1.5563*	.05657	.000	-1.6682	-1.4443
	9000mgN:900P	-.2151*	.05718	.000	-.3283	-.1020
	12000mgN:2400P	-.0800	.05657	.160	-.1919	.0319
	7gN:0.54gP	-1.4683*	.05657	.000	-1.5803	-1.3564
	14gN:1.08P	-1.3987*	.05657	.000	-1.5107	-1.2868
6000mgN:1200	6000mgN:600P	-.3705*	.05718	.000	-.4837	-.2574
	6000mgN:3000P	.2095*	.05718	.000	.0963	.3226
	Control	-1.3468*	.05718	.000	-1.4599	-1.2336
	9000mgN:900P	-.0057	.05778	.922	-.1200	.1087
	12000mgN:2400P	.1295*	.05718	.025	.0163	.2426
	7gN:0.54gP	-1.2589*	.05718	.000	-1.3720	-1.1457
	14gN:1.08P	-1.1893*	.05718	.000	-1.3024	-1.0761
Control	6000mgN:600P	.9763*	.05657	.000	.8643	1.0882
	6000mgN:3000P	1.5563*	.05657	.000	1.4443	1.6682
	6000mgN:1200	1.3468*	.05718	.000	1.2336	1.4599
	9000mgN:900P	1.3411*	.05718	.000	1.2280	1.4543
	12000mgN:2400P	1.4763*	.05657	.000	1.3643	1.5882
	7gN:0.54gP	.0879	.05657	.123	-.0240	.1999
	14gN:1.08P	.1575*	.05657	.006	.0456	.2694
9000mgN:900P	6000mgN:600P	-.3649*	.05718	.000	-.4780	-.2517
	6000mgN:3000P	.2151*	.05718	.000	.1020	.3283
	6000mgN:1200	.0057	.05778	.922	-.1087	.1200
	Control	-1.3411*	.05718	.000	-1.4543	-1.2280
	12000mgN:2400P	.1351*	.05718	.020	.0220	.2483
	7gN:0.54gP	-1.2532*	.05718	.000	-1.3664	-1.1401
	14gN:1.08P	-1.1836*	.05718	.000	-1.2968	-1.0705
12000mgN:2400P	6000mgN:600P	-.5000*	.05657	.000	-.6119	-.3881
	6000mgN:3000P	.0800	.05657	.160	-.0319	.1919
	6000mgN:1200	-.1295*	.05718	.025	-.2426	-.0163
	Control	-1.4763*	.05657	.000	-1.5882	-1.3643
	9000mgN:900P	-.1351*	.05718	.020	-.2483	-.0220

		7gN:0.54gP	-1.3883*	.05657	.000	-1.5003	-1.2764
		14gN:1.08P	-1.3187*	.05657	.000	-1.4307	-1.2068
	7gN:0.54gP	6000mgN:600P	.8883*	.05657	.000	.7764	1.0003
		6000mgN:3000P	1.4683*	.05657	.000	1.3564	1.5803
		6000mgN:1200	1.2589*	.05718	.000	1.1457	1.3720
		Control	-.0879	.05657	.123	-.1999	.0240
		9000mgN:900P	1.2532*	.05718	.000	1.1401	1.3664
		12000mgN:2400P	1.3883*	.05657	.000	1.2764	1.5003
		14gN:1.08P	.0696	.05657	.221	-.0424	.1815
	14gN:1.08P	6000mgN:600P	.8187*	.05657	.000	.7068	.9307
		6000mgN:3000P	1.3987*	.05657	.000	1.2868	1.5107
		6000mgN:1200	1.1893*	.05718	.000	1.0761	1.3024
		Control	-.1575*	.05657	.006	-.2694	-.0456
		9000mgN:900P	1.1836*	.05718	.000	1.0705	1.2968
		12000mgN:2400P	1.3187*	.05657	.000	1.2068	1.4307
		7gN:0.54gP	-.0696	.05657	.221	-.1815	.0424
TotalC	6000mgN:600P	6000mgN:3000P	.3100*	.11356	.007	.0853	.5347
		6000mgN:1200	.1016	.11478	.378	-.1256	.3287
		Control	-.1975	.11356	.084	-.4222	.0272
		9000mgN:900P	.2364*	.11478	.042	.0092	.4635
		12000mgN:2400P	.2271*	.11356	.048	.0024	.4518
		7gN:0.54gP	-.0042	.11356	.971	-.2289	.2206
		14gN:1.08P	-.0529	.11356	.642	-.2776	.1718
	6000mgN:3000P	6000mgN:600P	-.3100*	.11356	.007	-.5347	-.0853
		6000mgN:1200	-.2084	.11478	.072	-.4356	.0187
		Control	-.5075*	.11356	.000	-.7322	-.2828
		9000mgN:900P	-.0736	.11478	.522	-.3008	.1535
		12000mgN:2400P	-.0829	.11356	.467	-.3076	.1418
		7gN:0.54gP	-.3142*	.11356	.007	-.5389	-.0894
		14gN:1.08P	-.3629*	.11356	.002	-.5876	-.1382
	6000mgN:1200	6000mgN:600P	-.1016	.11478	.378	-.3287	.1256
		6000mgN:3000P	.2084	.11478	.072	-.0187	.4356
		Control	-.2991*	.11478	.010	-.5262	-.0719
		9000mgN:900P	.1348	.11600	.247	-.0948	.3643

	12000mgN:2400P	.1255	.11478	.276	-.1016	.3527
	7gN:0.54gP	-.1057	.11478	.359	-.3329	.1214
	14gN:1.08P	-.1545	.11478	.181	-.3816	.0727
Control	6000mgN:600P	.1975	.11356	.084	-.0272	.4222
	6000mgN:3000P	.5075*	.11356	.000	.2828	.7322
	6000mgN:1200	.2991*	.11478	.010	.0719	.5262
	9000mgN:900P	.4339*	.11478	.000	.2067	.6610
	12000mgN:2400P	.4246*	.11356	.000	.1999	.6493
	7gN:0.54gP	.1933	.11356	.091	-.0314	.4181
	14gN:1.08P	.1446	.11356	.205	-.0801	.3693
9000mgN:900P	6000mgN:600P	-.2364*	.11478	.042	-.4635	-.0092
	6000mgN:3000P	.0736	.11478	.522	-.1535	.3008
	6000mgN:1200	-.1348	.11600	.247	-.3643	.0948
	Control	-.4339*	.11478	.000	-.6610	-.2067
	12000mgN:2400P	-.0093	.11478	.936	-.2364	.2179
	7gN:0.54gP	-.2405*	.11478	.038	-.4677	-.0134
	14gN:1.08P	-.2893*	.11478	.013	-.5164	-.0621
12000mgN:2400P	6000mgN:600P	-.2271*	.11356	.048	-.4518	-.0024
	6000mgN:3000P	.0829	.11356	.467	-.1418	.3076
	6000mgN:1200	-.1255	.11478	.276	-.3527	.1016
	Control	-.4246*	.11356	.000	-.6493	-.1999
	9000mgN:900P	.0093	.11478	.936	-.2179	.2364
	7gN:0.54gP	-.2313*	.11356	.044	-.4560	-.0065
	14gN:1.08P	-.2800*	.11356	.015	-.5047	-.0553
7gN:0.54gP	6000mgN:600P	.0042	.11356	.971	-.2206	.2289
	6000mgN:3000P	.3142*	.11356	.007	.0894	.5389
	6000mgN:1200	.1057	.11478	.359	-.1214	.3329
	Control	-.1933	.11356	.091	-.4181	.0314
	9000mgN:900P	.2405*	.11478	.038	.0134	.4677
	12000mgN:2400P	.2313*	.11356	.044	.0065	.4560
	14gN:1.08P	-.0487	.11356	.668	-.2735	.1760
14gN:1.08P	6000mgN:600P	.0529	.11356	.642	-.1718	.2776
	6000mgN:3000P	.3629*	.11356	.002	.1382	.5876
	6000mgN:1200	.1545	.11478	.181	-.0727	.3816

		Control	-.1446	.11356	.205	-.3693	.0801
		9000mgN:900P	.2893*	.11478	.013	.0621	.5164
		12000mgN:2400P	.2800*	.11356	.015	.0553	.5047
		7gN:0.54gP	.0487	.11356	.668	-.1760	.2735
TotalN	6000mgN:600P	6000mgN:3000P	.0158	.02852	.580	-.0406	.0723
		6000mgN:1200	-.0012	.02883	.965	-.0583	.0558
		Control	-.0021	.02852	.942	-.0585	.0544
		9000mgN:900P	-.0630*	.02883	.031	-.1200	-.0059
		12000mgN:2400P	-.0812*	.02852	.005	-.1377	-.0248
		7gN:0.54gP	-.1271*	.02852	.000	-.1835	-.0706
		14gN:1.08P	-.0467	.02852	.104	-.1031	.0098
	6000mgN:3000P	6000mgN:600P	-.0158	.02852	.580	-.0723	.0406
		6000mgN:1200	-.0171	.02883	.554	-.0741	.0400
		Control	-.0179	.02852	.531	-.0744	.0385
		9000mgN:900P	-.0788*	.02883	.007	-.1359	-.0218
		12000mgN:2400P	-.0971*	.02852	.001	-.1535	-.0406
		7gN:0.54gP	-.1429*	.02852	.000	-.1994	-.0865
		14gN:1.08P	-.0625*	.02852	.030	-.1189	-.0061
	6000mgN:1200	6000mgN:600P	.0012	.02883	.965	-.0558	.0583
		6000mgN:3000P	.0171	.02883	.554	-.0400	.0741
		Control	-.0008	.02883	.977	-.0579	.0562
		9000mgN:900P	-.0617*	.02913	.036	-.1194	-.0041
		12000mgN:2400P	-.0800*	.02883	.006	-.1370	-.0230
		7gN:0.54gP	-.1258*	.02883	.000	-.1829	-.0688
		14gN:1.08P	-.0454	.02883	.118	-.1025	.0116
	Control	6000mgN:600P	.0021	.02852	.942	-.0544	.0585
		6000mgN:3000P	.0179	.02852	.531	-.0385	.0744
		6000mgN:1200	.0008	.02883	.977	-.0562	.0579
		9000mgN:900P	-.0609*	.02883	.037	-.1180	-.0039
		12000mgN:2400P	-.0792*	.02852	.006	-.1356	-.0227
		7gN:0.54gP	-.1250*	.02852	.000	-.1814	-.0686
		14gN:1.08P	-.0446	.02852	.120	-.1010	.0119
	9000mgN:900P	6000mgN:600P	.0630*	.02883	.031	.0059	.1200
		6000mgN:3000P	.0788*	.02883	.007	.0218	.1359

		6000mgN:1200	.0617*	.02913	.036	.0041	.1194
		Control	.0609*	.02883	.037	.0039	.1180
		12000mgN:2400P	-.0183	.02883	.528	-.0753	.0388
		7gN:0.54gP	-.0641*	.02883	.028	-.1211	-.0070
		14gN:1.08P	.0163	.02883	.572	-.0407	.0734
	12000mgN:2400P	6000mgN:600P	.0812*	.02852	.005	.0248	.1377
		6000mgN:3000P	.0971*	.02852	.001	.0406	.1535
		6000mgN:1200	.0800*	.02883	.006	.0230	.1370
		Control	.0792*	.02852	.006	.0227	.1356
		9000mgN:900P	.0183	.02883	.528	-.0388	.0753
		7gN:0.54gP	-.0458	.02852	.111	-.1023	.0106
		14gN:1.08P	.0346	.02852	.228	-.0219	.0910
	7gN:0.54gP	6000mgN:600P	.1271*	.02852	.000	.0706	.1835
		6000mgN:3000P	.1429*	.02852	.000	.0865	.1994
		6000mgN:1200	.1258*	.02883	.000	.0688	.1829
		Control	.1250*	.02852	.000	.0686	.1814
		9000mgN:900P	.0641*	.02883	.028	.0070	.1211
		12000mgN:2400P	.0458	.02852	.111	-.0106	.1023
		14gN:1.08P	.0804*	.02852	.006	.0240	.1369
	14gN:1.08P	6000mgN:600P	.0467	.02852	.104	-.0098	.1031
		6000mgN:3000P	.0625*	.02852	.030	.0061	.1189
		6000mgN:1200	.0454	.02883	.118	-.0116	.1025
		Control	.0446	.02852	.120	-.0119	.1010
		9000mgN:900P	-.0163	.02883	.572	-.0734	.0407
		12000mgN:2400P	-.0346	.02852	.228	-.0910	.0219
		7gN:0.54gP	-.0804*	.02852	.006	-.1369	-.0240
TotalP	6000mgN:600P	6000mgN:3000P	-238.3217*	57.12275	.000	-351.3659	-125.2774
		6000mgN:1200	-188.8966*	57.74031	.001	-303.1629	-74.6302
		Control	106.4071	57.12275	.065	-6.6372	219.4513
		9000mgN:900P	-241.4044*	57.74031	.000	-355.6708	-127.1380
		12000mgN:2400P	-167.2212*	57.12275	.004	-280.2655	-54.1770
		7gN:0.54gP	128.4883*	57.12275	.026	15.4441	241.5326
		14gN:1.08P	163.6900*	57.12275	.005	50.6458	276.7342
	6000mgN:3000P	6000mgN:600P	238.3217*	57.12275	.000	125.2774	351.3659

		6000mgN:1200	49.4251	57.74031	.394	-64.8413	163.6915
		Control	344.7288*	57.12275	.000	231.6845	457.7730
		9000mgN:900P	-3.0827	57.74031	.958	-117.3491	111.1836
		12000mgN:2400P	71.1004	57.12275	.216	-41.9438	184.1447
		7gN:0.54gP	366.8100*	57.12275	.000	253.7658	479.8542
		14gN:1.08P	402.0117*	57.12275	.000	288.9674	515.0559
	6000mgN:1200	6000mgN:600P	188.8966*	57.74031	.001	74.6302	303.1629
		6000mgN:3000P	-49.4251	57.74031	.394	-163.6915	64.8413
		Control	295.3037*	57.74031	.000	181.0373	409.5700
		9000mgN:900P	-52.5078	58.35133	.370	-167.9834	62.9677
		12000mgN:2400P	21.6753	57.74031	.708	-92.5910	135.9417
		7gN:0.54gP	317.3849*	57.74031	.000	203.1185	431.6513
		14gN:1.08P	352.5866*	57.74031	.000	238.3202	466.8529
	Control	6000mgN:600P	-106.4071	57.12275	.065	-219.4513	6.6372
		6000mgN:3000P	-344.7288*	57.12275	.000	-457.7730	-231.6845
		6000mgN:1200	-295.3037*	57.74031	.000	-409.5700	-181.0373
		9000mgN:900P	-347.8115*	57.74031	.000	-462.0779	-233.5451
		12000mgN:2400P	-273.6283*	57.12275	.000	-386.6726	-160.5841
		7gN:0.54gP	22.0813	57.12275	.700	-90.9630	135.1255
		14gN:1.08P	57.2829	57.12275	.318	-55.7613	170.3272
	9000mgN:900P	6000mgN:600P	241.4044*	57.74031	.000	127.1380	355.6708
		6000mgN:3000P	3.0827	57.74031	.958	-111.1836	117.3491
		6000mgN:1200	52.5078	58.35133	.370	-62.9677	167.9834
		Control	347.8115*	57.74031	.000	233.5451	462.0779
		12000mgN:2400P	74.1832	57.74031	.201	-40.0832	188.4495
		7gN:0.54gP	369.8927*	57.74031	.000	255.6264	484.1591
		14gN:1.08P	405.0944*	57.74031	.000	290.8280	519.3608
	12000mgN:2400P	6000mgN:600P	167.2212*	57.12275	.004	54.1770	280.2655
		6000mgN:3000P	-71.1004	57.12275	.216	-184.1447	41.9438
		6000mgN:1200	-21.6753	57.74031	.708	-135.9417	92.5910
		Control	273.6283*	57.12275	.000	160.5841	386.6726
		9000mgN:900P	-74.1832	57.74031	.201	-188.4495	40.0832
		7gN:0.54gP	295.7096*	57.12275	.000	182.6653	408.7538
		14gN:1.08P	330.9112*	57.12275	.000	217.8670	443.9555

	7gN:0.54gP	6000mgN:600P	-128.4883*	57.12275	.026	-241.5326	-15.4441
		6000mgN:3000P	-366.8100*	57.12275	.000	-479.8542	-253.7658
		6000mgN:1200	-317.3849*	57.74031	.000	-431.6513	-203.1185
		Control	-22.0813	57.12275	.700	-135.1255	90.9630
		9000mgN:900P	-369.8927*	57.74031	.000	-484.1591	-255.6264
		12000mgN:2400P	-295.7096*	57.12275	.000	-408.7538	-182.6653
		14gN:1.08P	35.2017	57.12275	.539	-77.8426	148.2459
	14gN:1.08P	6000mgN:600P	-163.6900*	57.12275	.005	-276.7342	-50.6458
		6000mgN:3000P	-402.0117*	57.12275	.000	-515.0559	-288.9674
		6000mgN:1200	-352.5866*	57.74031	.000	-466.8529	-238.3202
		Control	-57.2829	57.12275	.318	-170.3272	55.7613
		9000mgN:900P	-405.0944*	57.74031	.000	-519.3608	-290.8280
		12000mgN:2400P	-330.9112*	57.12275	.000	-443.9555	-217.8670
		7gN:0.54gP	-35.2017	57.12275	.539	-148.2459	77.8426
TotalMicrobial Mass	6000mgN:600P	6000mgN:3000P	1.15667	2.196461	.599	-3.19007	5.50340
		6000mgN:1200	-.05670	2.220207	.980	-4.45043	4.33702
		Control	-15.13375*	2.196461	.000	-19.48048	-10.78702
		9000mgN:900P	-11.03192*	2.220207	.000	-15.42565	-6.63820
		12000mgN:2400P	-20.07792*	2.196461	.000	-24.42465	-15.73118
		7gN:0.54gP	-16.08333*	2.196461	.000	-20.43007	-11.73660
		14gN:1.08P	-25.96333*	2.196461	.000	-30.31007	-21.61660
	6000mgN:3000P	6000mgN:600P	-1.15667	2.196461	.599	-5.50340	3.19007
		6000mgN:1200	-1.21337	2.220207	.586	-5.60709	3.18036
		Control	-16.29042*	2.196461	.000	-20.63715	-11.94368
		9000mgN:900P	-12.18859*	2.220207	.000	-16.58231	-7.79486
		12000mgN:2400P	-21.23458*	2.196461	.000	-25.58132	-16.88785
		7gN:0.54gP	-17.24000*	2.196461	.000	-21.58673	-12.89327
		14gN:1.08P	-27.12000*	2.196461	.000	-31.46673	-22.77327
	6000mgN:1200	6000mgN:600P	.05670	2.220207	.980	-4.33702	4.45043
		6000mgN:3000P	1.21337	2.220207	.586	-3.18036	5.60709
		Control	-15.07705*	2.220207	.000	-19.47077	-10.68332
		9000mgN:900P	-10.97522*	2.243702	.000	-15.41544	-6.53500
		12000mgN:2400P	-20.02121*	2.220207	.000	-24.41494	-15.62749
		7gN:0.54gP	-16.02663*	2.220207	.000	-20.42036	-11.63291

	14gN:1.08P	-25.90663*	2.220207	.000	-30.30036	-21.51291
Control	6000mgN:600P	15.13375*	2.196461	.000	10.78702	19.48048
	6000mgN:3000P	16.29042*	2.196461	.000	11.94368	20.63715
	6000mgN:1200	15.07705*	2.220207	.000	10.68332	19.47077
	9000mgN:900P	4.10183	2.220207	.067	-.29190	8.49555
	12000mgN:2400P	-4.94417*	2.196461	.026	-9.29090	-.59743
	7gN:0.54gP	-.94958	2.196461	.666	-5.29632	3.39715
	14gN:1.08P	-10.82958*	2.196461	.000	-15.17632	-6.48285
9000mgN:900P	6000mgN:600P	11.03192*	2.220207	.000	6.63820	15.42565
	6000mgN:3000P	12.18859*	2.220207	.000	7.79486	16.58231
	6000mgN:1200	10.97522*	2.243702	.000	6.53500	15.41544
	Control	-4.10183	2.220207	.067	-8.49555	.29190
	12000mgN:2400P	-9.04600*	2.220207	.000	-13.43972	-4.65227
	7gN:0.54gP	-5.05141*	2.220207	.025	-9.44514	-.65769
	14gN:1.08P	-14.93141*	2.220207	.000	-19.32514	-10.53769
12000mgN:2400P	6000mgN:600P	20.07792*	2.196461	.000	15.73118	24.42465
	6000mgN:3000P	21.23458*	2.196461	.000	16.88785	25.58132
	6000mgN:1200	20.02121*	2.220207	.000	15.62749	24.41494
	Control	4.94417*	2.196461	.026	.59743	9.29090
	9000mgN:900P	9.04600*	2.220207	.000	4.65227	13.43972
	7gN:0.54gP	3.99458	2.196461	.071	-.35215	8.34132
	14gN:1.08P	-5.88542*	2.196461	.008	-10.23215	-1.53868
7gN:0.54gP	6000mgN:600P	16.08333*	2.196461	.000	11.73660	20.43007
	6000mgN:3000P	17.24000*	2.196461	.000	12.89327	21.58673
	6000mgN:1200	16.02663*	2.220207	.000	11.63291	20.42036
	Control	.94958	2.196461	.666	-3.39715	5.29632
	9000mgN:900P	5.05141*	2.220207	.025	.65769	9.44514
	12000mgN:2400P	-3.99458	2.196461	.071	-8.34132	.35215
	14gN:1.08P	-9.88000*	2.196461	.000	-14.22673	-5.53327
14gN:1.08P	6000mgN:600P	25.96333*	2.196461	.000	21.61660	30.31007
	6000mgN:3000P	27.12000*	2.196461	.000	22.77327	31.46673
	6000mgN:1200	25.90663*	2.220207	.000	21.51291	30.30036
	Control	10.82958*	2.196461	.000	6.48285	15.17632
	9000mgN:900P	14.93141*	2.220207	.000	10.53769	19.32514

	12000mgN:2400P	5.88542*	2.196461	.008	1.53868	10.23215
	7gN:0.54gP	9.88000*	2.196461	.000	5.53327	14.22673

Based on observed means.
The error term is Mean Square(Error) = 57.893.

*. The mean difference is significant at the .05 level.