DURBAN UNIVERSITY OF TECHNOLOGY

BIOREMEDIATION OF ACID MINE DRAINAGE AND CRUDE OIL CONTAMINATED SOILS

by

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ABSTRACT

Pollution is one of the greatest ills plaguing the existence of the ecosystem which could lead to the annihilation of terrestrial and aquatic habitat if not remedied. Acid mine drainage (AMD) and crude oil are among the major land and water pollutants cause by industrial and human activities. The constant exploration, mining, and processing of mineral resources and prevalent use of petroleum products for economic purposes have contributed to contamination of soil and proximate water bodies which results in environmental degradation; thus, remediation becomes necessary. The treatment of AMD contaminated soils using the conventional methods has some room for improvement to meet the remediation purpose. Bioremediation technology provides a sustainable and eco-friendly approach to the treatment of contaminants. This study aims to evaluate the performance of different potential bioremediation techniques and conduct a comparative analysis of these methods for the treatment of AMD and crude oil-contaminated soils. The treatment approach for both pollutants comprises of soils separately contaminated with AMD and crude oil before the application of bioremediation techniques. For the biostimulation study, contaminated soils were amended with varying ratios of the brewery or municipal wastewaters (BWW and MWW), while the bioventing (BVT) treatment involved wastewater amendment and supply of atmospheric air from the vadose zone at 3L/min at 30 minutes intervals every 48 hours. The bacteria strain *Pseudomonas aeruginosa* ATCC 15442 used for the study which was inoculated at 5%(w/w) was cultured in two different media for respective treatments and wastewater was amended as an extra energy source for bioaugmentation (BAU) study while Bioattenuation (BAT) which received no amendment was used as a control treatment for the study. The treatments were conducted in plastic bioreactors under mesophilic conditions for 28 days and samples were collected from each treatment system on weekly basis to analyse for sulfate, heavy metals, and total petroleum hydrocarbon (TPH) reduction. The result of the study showed that the amendment of contaminated soils with wastewater increased alkalinity in the system which enhanced microbial activities for effective remediation which recorded 52.43 and 51.23% average TPH and metal removal efficiency for the BSTc treatment. Also, the combined application of bioremediation techniques was more effective than single application as the introduction of oxygen into the treatment system with wastewater amendment increased the TPH and metal removal efficiency by an average of 12.98

and 13.17% respectively but efforts to enhance sulfate removal by air-injection (BVTa) proved abortive with 17.20 and 14.67% removal efficiencies less than BSTa and BAUa respectively as sulfate-reducing bacteria thrive in an anaerobic environment. However, *P. aeruginosa* ATCC 15442 adopts the sorption process in the reduction of hydrocarbon and metal toxicity with 42.02 and 41.81% average removal efficiencies respectively and the amendment extra nutrient (wastewater) increased the removal efficiency of these pollutants by 25.24 and 16.23% respectively. The results of the study inferred that wastewater (BWW and MWW), air-injection and *P. aeruginosa* ATCC 15442 showed great potentials in the degradation and removal of TPH, metals and sulfate contaminants, hence, can serve as a viable strategy for the remediation of AMD and crude oil polluted soils while improving waste management and amelioration of pollution aftermath faced by communities involved in mining and oil production and/or processing. There is a need for optimization to ensure effective remediation while further study is required to validate large scale application.

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CHAPTER ONE

1.0 Introduction

The emerging increase in world economic activities, evolution and advancement of modern technology, industrialization, lifestyles, and a host of other factors have played active roles in waste generation, contributing invariably to environmental pollution. These processes are related to production, manufacturing, exploration, mining, transportation, and other activities of the heavy industries. These activities which pollute the environment have caused a lot of damages to the ecosystem ranging from air contamination, water pollution, and land/soil degradation to the death of habitats and destruction of aquatic and terrestrial inhabitants.

Pollution is the presence, discharge, or release of toxic, unwanted, or undesirable substances, chemicals or toxic materials in a quantity that will cause discomfort to the inhabitants and contaminate the aquatic, terrestrial and atmospheric environment. Soil pollution is a form of land degradation caused by simultaneous variations or alteration in the natural soil environment attributed to the presence of synthetic or foreign bodies, chemicals, or substances in the soil surface and subsurface. These soil degradations are caused by human activities, improper waste disposal, mining activities and industrial activities. Among the land pollution and contamination, Acid Mine Drainage (AMD) and crude oil are the major forms of land and water contaminants that have contributed immensely to the deterioration of soil structure, affecting the soil fertility, proximate water bodies, and gradual annihilation of the ecosystem. This poses a serious challenge to the accessibility of potable water, land use and development worldwide.

Crude oil contamination is attributed to series of operations such as oil exploration, production and processing activities, drilling problems such as rig blow-out, vandalisation of pipelines, and transportation of petroleum products which usually result in the accidental ejection of crude oil or spillage of petroleum products on the surface or subsurface. South Africa which is involved in crude oil processing operations is prone to crude oil spillage. However, some other countries like Nigeria, Algeria and Saudi Arabia whose economy is primarily based on the petroleum industry implies continuous exploration and other petroleum operations to ensure and maintain

approximately 80% of their foreign exchange earnings (World Bank cited in Global Edge 2019). These countries whose budgetary revenue is dependent on crude oil as a major export face similar environmental challenges. The prevalence of oil-related processes in these countries invariably denotes the great possibility of crude oil pollution. These crude oil processing countries like South Africa involved in crude oil processing activities with six crude oil processing plants that account to large processing capacity to meet the daily need for petroleum products consumption constantly faced with the problem of environmental pollution (air, water, and land) caused by crude oil (US Energy Information Administration 2015). This results to pollution of arable land, truncation of the source of livelihood and decline in the standard of living. The increasing demand for petroleum products for daily activities has led to increased exploration, processing, and supply of these products to meet demand and satisfaction of human want. Hence, the need to devise a means to effectively manage eventualities in cases of crude oil pollution is paramount.

Acid Mine Drainage (AMD) can be attributed to excavations and mining activities. This is the waste product of every mining operation. AMD is generated when mine water (sulfide minerals) from old, abandoned, or active mine sites are exposed to oxygen. This triggers pyrite oxidation and the formation of a yellow-orange liquid known as ferrous hydroxide (AMD). This is prevalent mostly in mineral mining regions (coal, gold, platinum, etc). The AMD contaminant, when produced, flows from the mining locations through the land to the nearby water bodies, contaminating the soil, rivers or dams and limiting the availability of potable water for domestic purposes and arable agricultural lands for food production.

The mining sector has remained the major source of economic growth to some countries like South Africa with the largest reserves of coal, diamond, gold, manganese, platinum group metals (PGM) among other minerals (Ferrero *et al.* 2012), which contributes to the annual gross domestic product (GDP) growth and economic stability. South Africa is among the leaders in the world mining industries, contributing immensely to the mineral reserve/deposits and production in the world. In terms of GDP value, South Africa has the fifth world largest mining sector and mineral wealth reserve of estimated worth 22.3 trillion ($2.5 trillion) (Brand South Africa 2012). The country's economy is built extensively on the mining sector (gold and

diamond), which attributes to more than one-third of its exports, hence, mining of minerals has also boosted foreign direct investment, job creation (employment), socio-economic growth and development (Mineral Council South Africa 2018a).

Following the widespread mining operation around the country, AMD has been described as a prolific threat to South African's environment and mining industry due to the disastrous aftermath caused by this contaminant. This is due to increased mining operations mostly in mining regions like Gauteng province (gold mine) with (eight) deepest goldmine in the world (Mineral Council South Africa 2018b) and Mpumalanga province (coal mine) which accounts to 83% of coal production (Mpumalanga Provincial Government 2010). The continuous mining operation in these provinces among other provinces accounts for the constant production of mine wastes (AMD) which has affected the standard of living of inhabitants within the province.

Acid mine drainage has reached a crisis point as most mining companies allow the AMD water to flow through the soil into the streams, dams, and rivers, resulting to contamination of aquatic bodies and soil degradation, polluting arable lands, affecting agriculture and access to quality water, leading to the destruction of life in most mining areas especially Gauteng and Mpumalanga provinces (Idowu *et al.* 2010). These provinces contribute to the high value and potential of arable land for agricultural purposes that promote food production in South Africa. The annual loss of these arable land to soil degradation is a threat to increase food production and supply required to satisfy the increasing population as South Africa will have to produce 50% more food by 2050 to feed the population growth (Department of Agriculture 2018; World Wide Fund for Nature WWF 2019). The availability of these agricultural arable land had declined by almost 4% over the past 23 years, attributed to increasing land pollution, mining/industrial activities, and extension of municipal boundaries (Bureau for Food and Agricultural Policy 2012; Department of Agriculture 2014). The incessant loss of agricultural land will result in low food production for the increasing population. If not mitigated, it will lead to food scarcity, decrease the export rate *(as maize and other crop production declines)* and increase importation of some agricultural products to compliment the available, driving up food prices (inflation) and living cost which will harm the economy (Bureau for Food and Agricultural Policy 2012; Strydom and Struweg 2016.).

Having acknowledged the contributions of exploration and mining of these mineral resources to the country's socio-economic growth and development, generation of waste/effluents, pollution and contamination of the environment remain inevitable and remediation is indispensable.

1.2 Background of Study

The increased industrialization, globalization, and development have contributed immensely to economic growth, provision of basic human needs which makes life and task easier, but these activities do not come without its negative effect as it has continued to generate an immeasurable amount of pollution which contaminate the natural environment worldwide. Pollution is a plague threatening the existence of the ecosystem and if not properly addressed can gradually lead to the destruction of the terrestrial and aquatic habitats. These can be attributed to the activities of heavy industries such as mining operations, exploration, processing, and movement of crude oil and petroleum products (Romero-Baena, González and Galán 2018; Affandi and Ishak 2019).

It is indisputable that most industrial activities, (mining and exploration of mineral resources) contribute to the economic growth, development and sustainability of most countries like South Africa involved in the mining of mineral resources, which account for their major export earnings. Also, the country is involved in the processing of crude oil and the utilization of petroleum products. These activities have brought more wealth to individuals and government but at the price of extensive ecological damage because of little or no strategy to effectively manage the direct and indirect effect of mine waste or crude oil spillage. As mining and crude oil processing operations are critical to development, hence, devising means to mitigate these contaminants and their aftermath is necessary.

Crude oil and AMD contaminant tend to pollute the natural soil environment (Fig. 1-1 & 1-3), causing variation in soil structural setting, reducing the organic content resulting in a loss in soil nutrients and low productivity due to infertility (Alaribe Frank 2016; Olufemi, Andrew and Akpejeluh 2020). The presence of these pollutants in the soil reduces the availability of arable agricultural land for crop production which if not addressed might lead to low production, food

shortage/insecurity and reduce exportation due to reduction in cash crop production which will invariably harm the country's economy. Also, these contaminants (AMD) most times decant from the source (Fig. 1-2) and flows through the soil into the water bodies, contaminating proximate rivers, dams, and lakes which attributes to the reduction in oxygen supply in the water environment, leading to the destruction of the aquatic bodies and even death of aquatic animals. These contaminants in the aquatic environment make the water unfit for human consumption and other domestic activities. These attributes to the reduction in the availability of potable water as experienced in many places in South Africa as a result of crude oil and AMD contamination.

The widespread of this pollution, following the immense economic contributions, has prompted the development of several strategies and technologies required for the mitigation and/or remediation, to enhance the reclamation of contaminated sites. These methods that have been adopted so far for the treatment of AMD/crude oil contaminated sites include a physical or conventional method, chemical method, thermal method, encapsulation, soil vapour extraction (SVE). The use of these methods has been considered viable over the past years for the treatment and reclamation of contaminated sites, although some are not sufficient (Koul and Taak 2018).

Figure 1-1. AMD in West Rand (Gold Mine)

Figure 1-2. Decant of AMD from 18 Winze — a shaft in the Western Basin respectively (Coetzee 2013)

Figure 1-3. Crude oil-polluted sites *(Source; Environmental Pollution Centre 2018)*

1.2.1 Treatment of soil contaminants

Various treatment methods have been adopted previously for the reclamation of polluted soils. The ***Physical or conventional method*** involves the digging-up of the polluted soil and taking it to a dumpsite as a form of disposal. This method is tedious, requires enough equipment, manpower, and transportation of the hazardous material from one place to another might be risky and non-eco-friendly. This approach transferred the contaminated soil from one place to another, without the application of any further treatment to the soil. Hence, the problem is temporarily solved by making the site available for use while the soil remains untreated, thereby resulting in pollution of the new landfill site.

The C***hemical method*** employs selected chemicals for the reduction of contaminant concentration in the soil and makes it less toxic, harmless to the water bodies, soil, and human beings. The application of the chemical method of soil remediation is limited by some factors which include soil texture, type of contaminant and metals present, organic matter, and concentration of the chemical selected for the remediation process. However, harmful effects and concentration of the chemical materials (like metallic oxides) are to be considered during the pilot and large-scale applications. Some chemical remediation methods include chemical fixation, chemical oxidation, chemical leaching, and electric kinetic remediation (Koul and Taak 2018).

The ***Thermal method*** involves the use of heat/heat injection into the contaminated soil to facilitate the removal of wastes or constituents of the contaminants with low boiling point by converting them into vapour, which can be collected and treated in a gas treatment facility. Sequel to the broad application of this method on contaminated site, the high energy requirement can be detrimental to the soil properties which might complicate the whole remediation process (Vidonish *et al.* 2016). The lack of universally accepted application procedure/technology for thermal remediation, due to insufficient work to improve environmental sustainability, suitability, and applicability of this remediation method results in varying considerations for specific contaminants, site, and soil type. Hence, the choice of application is solely a factor of the contaminated soil category, heat requirement of the soil, soil features and nature of the area (Vidonish *et al.* 2016).

The ***Encapsulation method*** of soil remediation requires filtration of contaminants from the soil. The most common type of encapsulation is mixing the contaminated soil with lime, cement, and concrete, to prevent other soils from direct contact with the polluted soil. With this method of soil remediation, restoration of soil organic matters is not guaranteed as the additives might cause further deterioration of soil properties and organic content even though the removal of the contaminant is achieved. Encapsulation method of soil remediation proves to be potent in the reduction of the concentration of heavy metals constituents of the contaminants present in the soil, by precipitation and pH increase (Rojas, Consoli and Heineck 2009). This method is considered if the soil will not be used for future purposes especially for cultivation and

agricultural purposes since a reduction in the soil fertility and organic content is prevalent after the application of this remediation method. These methods are less reliable and sustainable for the remediation of polluted soil due to the high cost of operation, risk of recontamination of site, inability to ensure restoration of soil fertility and the non-eco-friendly nature of these approaches.

1.2.2 Bioremediation Methods

The inability of the previous treatment methods of contaminated soils to effectively remediate the soil and reduce the concentration of pollutants without posing environmental problems prompted the emergence and development of bioremediation technology. Bioremediation is an approach that offers the use of several biologically induced methods in the remediation of polluted soils. Bioremediation utilizes the activities of microorganisms to disintegrate biodegradable pollutants in the presence of favourable site parameters for effective biodegradation and performance (Adams *et al.* 2015). Bioremediation provides green, safe and reliable technology for pollutant clean-up, removal, de-pollution, remediation and reclamation of contaminated sites by microbial activities. This technique has proven to be the most sustainable measure for the remediation of polluted soils and water to restore its original state (Yuniati 2018). In this study, remediation is defined as the act of gradual degradation, reduction, conversion or/and metamorphosis of pollutants into less harmless innocuous material by stimulating the rate of microbial activities.

In the course of biodegradation, the contaminants are disintegrated into smaller molecules by pollutant degrading microorganisms (Adams, Niyomugabo and Sylvester 2017). This mechanism of bioremediation involves the limitation of transfer or movement of contaminants from the contaminated area to other areas within the soil or water environment. Restriction of the mobility of pollutants enhances concentration within a specific area, to improve the bioavailability of contaminants to the pollutant degrading microbes, to promote the biodegradation efficiency of the contaminant without posing an environmental risk. This process ensures the feasibility and reliability of the bioremediation approach. Bioremediation offers a sustainable, reliable, low cost and eco-friendly approach for the treatment of pollutants (e.g. AMD and crude oil) within terrestrial and aquatic habitats through the application of

indigenous microorganisms or microbial consortium for the catalytic process (Yuniati 2018). There are different bioremediation approaches used in the treatment of polluted soils and water. These include bioattenuation, biostimulation, bioaugmentation, bioventing and phytoremediation technology.

Bioattenuation or Natural Attenuation is a bioremediation method where degradation occurs due to natural processes without the addition of substrates or stimulants. With this method, the indigenous contaminant degrading microorganisms are allowed to degrade the pollutants in a controlled environment and prevent spread to the uncontaminated site or zone. The monitored or controlled bioremediation as an in-situ treatment method is becoming more prevalent because of its cost-effective and easy application nature but the major setback with this approach remains the slow rate of biodegradation which results to poor efficiency.

Biostimulation is a bioremediation technique where stimulants such as nutrients and/or substrates (nitrogen, phosphorus, carbon and other electron acceptors) are supplemented or introduced in treated or raw form to the polluted environment to speed up the degradation activity. In this approach, organic or inorganic nutrients are used to stimulate the natural soil environment, to support and energize the indigenous microorganism for optimum performance. The use of this method involves modification of the contaminated site using substrates to alter the existing environmental conditions, by providing a conducive atmosphere for the growth and activities of microorganisms which increases the rate of biodegradation (Adams, Niyomugabo and Sylvester 2017). The nutrients act as soil conditioners that facilitate and improves the soil pH, increases the bioavailability of contaminants to the degrading bacteria, promote aeration, permeability, and dehydrogenases (Liu *et al.* 2018).

Bioaugmentation entails the introduction of a genetically modified strain or consortium (mixed culture) of microorganisms into the contaminated environment to support, augment, enhance and improve the activities of the indigenous microorganisms for effective biodegradation. This approach is considered when the rate of biodegradation is slow due to either insufficient or low number of native microbes present in the contaminated site. For the successful application of this approach, selection of strain or consortium (such as oil-degrading or sulfate-reducing or heavy metal resistant bacteria) to be introduced to augment the existing organisms should be

dependent on some factors like the ability of the strain to maintain stability genetically, compete for nutrient with the indigenous microorganism, tolerate high concentration of contaminants, exist in a hostile/unfavourable environment and speed up the rate of biodegradation (Atlas 1977; Goldstein et al. 1985 cited in Adams *et al.* 2015).

Bioventing is the bioremediation process of ventilating the in-situ contaminated environment by injecting air or oxygen through the subsurface zone to promote dehydrogenase of indigenous microorganisms and enhance aerobic biodegradation. Bioventing aerates the vadose zone by supplying enough air/oxygen using a low flow rate to the unsaturated region for microbial activities. Air or oxygen can directly or indirectly supply into the vadose zone to enhance aerobic processes, through sparging groundwater with air or oxygen or adding hydrogen peroxide to pumped or reinjected groundwater. The use of pure oxygen has proven to be more effective in the bioventing process due to its ability to supply 75000kg of water into the vadose to enhance mineralization of 1kg of TPH present in the contaminated soil. This is more efficient than the use of air sparged (injection of pressurized air) into the contaminated groundwater, which delivers approximately 8 – 9mg/l of dissolved oxygen and also, the use of hydrogen peroxide (H_2O_2) as oxygen (O_2) source delivered 1200kg of water at 500mg/l, thus indicating a lower concentration of oxygen (Hinchee, Dowey and Beard, 1989 cited in Hinchee and Arthur 1991). It is also due to the drawback of using hydrogen peroxide for the bioventing process because the low penetration potential restricts the supply of oxygen to the vadose zone for microbial activities (Hoeppel, Hinchee and Arthur 1991)

Phytoremediation is a method of bioremediation that employs selected plant types to eliminate, convert, maintain and/or kill pollutants in soil and groundwater. There are several distinct types of pathways for phytoremediation such as Rhizosphere biodegradation, Phyto-stabilization, photo-degradation, and Phyto-accumulation etc. Phytoremediation operates by using plants to immobilize or remove toxins from a polluted environment. The plants also convert toxic substances into less harmful substances. Some plants disintegrate organic pollutants by releasing toxin degrading enzymes into the soil or by removing and degrading soil pollutants within their tissues. In other situations, plants promote degradation by supplying the soil microorganisms with nutrients that do the job. Metals and metalloid pollutants cannot be broken

down but by modifying their valence and storing them in roots or leaf tissues, plants can make them less toxic (Beans 2017)

1.2.3 Factors affecting the bioremediation application and performance

The outcome and rate of biodegradation of these bioremediation methods differ between *in-situ* and *ex-situ* bioremediation and factors affecting the application of these methods. These factors that influence the application and performance of bioremediation treatment includes**.**

i. *Temperature* - The highest degradation rate is generally visible within the temperature range of 30 – 40^{o}C. Also, high temperature increases the solubility of pollutant (in the case of crude oil contaminant) and makes contaminants bioavailable for degrading bacteria while low temperature delays the biodegradation process. But extremely high temperatures are disastrous for bioremediation as it's unfavourable for dehydrogenases and could lead to the death of microorganisms.

ii. *Soil pH* is another factor which could be sensitive for the application of bioremediation since the activities of microorganism are a factor of the toxicity of the contaminated environment; moderate pH is favourable for the process. However, the removal of most metallic pollutants depends on pH (Silva *et al.* 2009; Mathiyazhagan and Natarajan 2011).

iii. *Aerobic/anaerobic condition*: the contaminated environment must be aerated, or oxygen supply should be ensured, but some microorganisms (sulfate-reducing bacteria, SRB) operate favourably anaerobically. Thus, great care should be taken to identify the bacteria present and condition for operation.

iv. *Availability of nutrient or energy source*: a constant supply of nutrients rich in N and P as carbon or energy source to stimulate and enhance activities of the microorganisms should be ensured for optimum biodegradation rate and performance (Boopathy 2000).

v. *Nature of contaminant*: the type of pollutant (AMD or Crude oil) involved is a determining factor for the rate of biodegradation. The rate of biodegradation varies according to the type of contaminant. Identifying the type of contaminant provides a platform for the deployment of the bioremediation method best suited for the remediation of such contaminants. The biodegradability of hydrocarbon showed that lower hydrocarbons are easily degradable. Compounds like high molecular wt. polyaromatics (PAHs) may be very difficult to biodegrade

(Yuniati 2018). Also, non-biodegradable contaminants (metals) may pose a challenge to the application of bioremediation.

vi. *Bioavailability of contaminant*: availability of contaminants is an essential factor that ensures the feasibility of bioremediation. Contaminants and substrates must be available for contaminant degrading bacteria for microbial metabolism which is a prerequisite for biodegradation (Boopathy 2000).

vii. *Nature of the contaminated site*: the site condition is crucial for bioremediation applications. These involve soil type, soil properties (physical and chemical) and indigenous microbial population present and other intrinsic site features. These factors determine the extent of biodegradation of the contaminant.

viii. *Microbial communities*: the indigenous microbial community, coupled with the augmentation of a bacteria strain or consortium is more effective than a single strain or indigenous microbial community alone. According to Yuniati (2018), the selection of bacteria strain or consortium must be based on traits with different abilities that will be advantageous to the selected site properties for effective performance.

ix. *Concentration*: the concentration of the contaminant is a determining factor for the rate of biodegradation. The high concentration of the contaminant is detrimental to the microorganisms as it slows microbial metabolism and reduces the rate of biodegradation. Also, at a very low concentration, there is a possibility of non-bioavailability of contaminants to the contaminant degrading bacteria which drastically reduces the biodegradation efficiency. Since most microorganisms are sensitive to high concentration which most times affects metabolism and can lead to the death of the microorganism present, thus, selection of apt microorganism is an important factor to consider which depends on the concentration of the contaminant on the site to be treated (Alexander 1999; Boopathy 2000).

x. *Inhibitors*: the presence of possible inhibitors tends to hinder the bioremediation process, which will help to determine whether to apply the bioremediation technology to the contaminated site.

xi. *Seasonality and Plant type*: Bioremediation method especially, phytoremediation progress can be affected by seasonal variations, which is dependent on the location of the contaminated site. Its effectiveness will also be truncated by other climate factors. Also, the

availability of contaminant tolerant/resistant plant species is essential for the success of this bioremediation technique (Beans 2017).

1.3 Research Problem

The high rate of soil pollution poses a serious threat to the environment, and if not mitigated could result in soil degradation destruction of the ecosystem.

1.4 Research Aim and Objectives

This study aims to assess the performance of bioremediation methods for the treatment of polluted soils.

Research Objectives:

- To investigate the effects of amending contaminated soil with industrial and municipal waste effluent.
- To evaluate the application of potential bioremediation methods for the remediation of AMD and crude oil contaminated soils,
    - Biostimulation using Industrial and Municipal wastewaters
    - Bioventing using atmospheric air.
    - Bioaugmentation using *Pseudomonas aeruginosa* ATCC 15442.
- To conduct a comparative study of the potential bioremediation methods

1.5 Significance and Novelty of study

The need to ensure a clean, healthy, and pollution-free environment necessitated constant research to devise a sustainable, reliable, and low-cost approach for the remediation of contaminated soils. This study considered ways of economically achieving a bioremediation treatment of AMD and crude oil (petroleum) polluted soils using low-cost materials. These two soil contaminants (AMD and Crude oil) were considered because they are among the most prevalent soil contaminants in the world (Cocarta, Stoian and Karademir 2017). Having acknowledged the fact that most studies on AMD focused on the treatment of mine (AMD)

water which accounts to limited work on AMD contaminated soils established the novelty of this present study together with the investigation of potential bioremediation techniques to fill a gap in the literature (as elucidated in ***section 2.8***). This study evaluates the application of wastewaters (specifically South African brewery and municipal wastewater), air-injection and specific bacteria strain (*Pseudomonas aeruginosa* ATCC 15442) for crude oil and AMD contaminated soil remediation. These contaminants have contributed immensely to the deterioration of soil structure and organic content; thus, revitalization becomes imperative. These among other factors under-listed forms the basis and motivation for this research work.

The treatment of AMD and crude oil contaminated soils previously using the conventional and physio-chemical method have not rendered the required remediation as most methods are expensive, non-eco-friendly and often result in further recontamination of the site. The inability of these methods to achieve the remediation objectives necessitated this study which adopts a modified bioremediation technique, a more viable approach which entails the use of biological means to tactically ensure the reclamation of the contaminated site, where the contaminant will be successfully removed while restoring the soil organic content at low cost with little/no risk to the environment and without causing subsequent degradation to the soil. This study is poised to evaluate potential biological methods for the remediation of mine waste (AMD) and crude oil contaminated soil to provide an efficient approach that can be effectively applied for the treatment of AMD and crude oil-polluted soil in polluted areas.

The bioremediation of AMD and crude oil polluted soils is an effective way to mitigate water scarcity as it will prevent the flow of mine water and crude oil into the proximate dams, streams, and rivers. The remediation of contaminated soil will ensure food security as this process will enhance the reclamation and restoration of contaminated soil to provide more arable land for agricultural purposes to meet enough food production requirements for the increasing population. However, with the increasing production, exportation will be feasible as economic growth remains inevitable. This bioremediation study adopts the use of biodegradable wastes for the treatment of AMD contaminated soil while enhancing waste management, thereby preventing the ecosystem from the menace of pollution and total annihilation to ensure a safe and healthy environment for ecological balance.

1.6. Structure of the Thesis

The overall structure of this thesis is composed of five themed chapters with different sections embedded in each chapter. The first chapter covers the introduction, background, and objectives of this study. It goes further to explain the justification of his study as established in the knowledge gap statement and its contributions to the body of knowledge.

Chapter two begins by laying out the theoretical dimension of the research and review of relevant literature. It started with the review of the effects of AMD and crude oil contaminated soils which established that these contaminants posed a problem to the ecosystem that required urgent attention and subsequently focused on the review of biostimulation, bioventing and bioaugmentation methods of bioremediation and its different applications for the treatment of AMD and crude oil-contaminated soils. After extensive review, a gap in the literature was established which the present study seeks to address.

The third chapter is concerned with the methodology used to simultaneously achieve the objective of this study. This chapter outlined the materials used for this study, the experimental set up for each treatment, which involved the applications of biostimulation, bioventing and bioaugmentation methods for the treatment of polluted soils, the conditions at which the experiment was conducted, the technique and instruments used for sample preparation and analyses. Information provided in this chapter together with the appendices will be sufficient to facilitate the reproducibility of this research work.

Chapter four entails results presentation and discussion of all the treatments conducted. It vividly discussed the reasons for the GCMS, XRF and SEM results obtained and the mechanism responsible for the trend evident in the presented results, in conformity with relevant literature and standards. In response to the purpose of this study, a section of this chapter was dedicated to the comparative analysis of these bioremediation techniques for the treatment of AMD and crude oil-contaminated soils.

The final chapter presents the conclusions after thorough investigation and key recommendations that will set the basis for further study.

CHAPTER TWO

Literature Review

The standard of life on planet earth is a factor of the quality of the environment. Invariably, good health is inextricably linked to a clean and pollution-free ecosystem. Pollution is attributed to human or industrial activities which include but are not limited to soil pollution which has presently increased drastically and threatens the existence of life and the sustenance of the ecosystem.

Soil pollution and contamination are a form of land degradation caused by variations in the natural soil environment attributed to the presence of synthetic or foreign bodies, chemicals, or substances. The degradation may be anthropogenic (by humans): improper waste disposal, mining activities, and activities of other heavy industries or geogenic (by nature: volcano) in origin. Among the land pollution and contamination, acid mine drainage and crude oil spillage are the major forms of pollutants that have contributed immensely to the deterioration of soil structure, affecting soil fertility, plant germination, and growth, proximate water bodies, leading to gradual annihilation of the terrestrial inhabitant.

2.1 Effects of contaminants on human, environment/soil and plant growth

2.1.1 Effects of contaminants on human

Human exposure to contaminants can occur through direct ingestion of pollutants or by dermal absorption by the skin. Indirect exposure to contaminants can also occur by ingestion of fish from crude oil or AMD-contaminated water supplies or by food crops (e.g. fruits and vegetables) contaminated with crude oil or AMD-contaminated food items exposed animals. AMD enhance heavy metal deposition in fertile soil surrounding the mining fields. Hence food crop ingestion of heavy metals is a significant health hazard to local populations (McLaughlin, Parker and Clarke 1999; Adriano 2001; Pruvot *et al.* 2006).

McBride (2003) observed that metal absorption from polluted soil through plant roots, and the accumulation of pollutants from the atmosphere on plant surfaces, may be detrimental to human health.

Studies have found that trace metal accumulation in edible sections of plants is unhealthy for humans and other living things. Concentrations of arsenic were recorded frequently in soils and plants affected by mining activities. Concentrations of copper (Cu), zinc (Virginie *et al.*), arsenic (As) and aluminium (Al) has been reported to be more on plant leaves than roots for plants around the mining areas. (Wild 1974; de Koe 1994). Both animals and humans can be harmed by eating these plants.

Each contaminated environment is unique in terms of its capacity and coverage. However, the type and quantity of crude oil or AMD would differ from site to site and the related risk and feasibility of preventive alternatives. Depending on this, the mechanisms of exposure and possible effects of pollutants on human health will also greatly differ. There has also been little research to evaluate which pathway to human contact to contamination will be prevalent or have a significant negative impact (Akcil and Koldas 2006).

2.1.2 Effect of crude oil on soil and plant growth

Crude oil is a complex liquid comprising of thousands of hydrocarbon components and heavy metals. It is precarious to evaluate the exact level of toxicity of complex mixtures when deposited in the soil especially when there's little or insufficient information to prior addition or deposit of such mixture in the soil (Baek *et al.* 2004). The existence of crude oil contaminants in the soil changes soil characteristics. Soil pH and TPH content increase with an increase in the amount of hydrocarbon in the soil (Ogboghodo *et al.* 2004b) which causes altercation in the native soil environment, while N & P content decreases with a corresponding increase in the soil toxicity resulting to decline and lost in the availability of essential nutrients required for plant growth (Odu 1972 cited in Ekundayo, Emede and Osayande 2001). The high concentration of the crude oil contaminant contributes to the deterioration of soil structure and fertility which renders the soil impotent for agricultural purposes and reduces the availability of arable lands.

In a study to access the impact of crude oil on the growth, germination and grain yield of maize, *Zea mays* observed reduction by 95% probability in comparison with the control experiment as reported by Ekundayo, Emede and Osayande (2001). The study recorded that oil contamination had a negative impact on maize growth parameters (plant height, leaf area, stem girth, ear length) that were investigated. The prompt germination of unpolluted samples, when compared to the polluted sample, justified the negative impact of oil contaminants in the soil and plant growth. The delay in germination was due to the absorption of oil by seeds and its penetration in the embryo (Baek *et al.* 2004). Odu (1978) observed that the penetration of soil by crude oil spillage causes a visible nutrient deficiency in plants, insufficient aeration, a reduction in the level of available nutrients and a significant rise in the toxicity of elements such as Mn and Fe (Amadi, Dickson and Moate 1993 cited in Ekundayo, Emede and Osayande 2001) where the insufficiency of nutrients corresponds to low water intake by plants (Terge 1984) which accounts to the poor performance of crops planted in the oil-polluted environment. Similarly, the effect of oil in the germination of *Zea mays* was investigated (Udo and Fayemi 1975) with seedlings planted in the soil of different concentration (0 to 10.6% w/w) of crude oil for six weeks recorded that the growth, germination, and yield drastically decreases as the concentration of crude oil increases. At 4.2% (w/w) concentration, average reduction was reported at 50% and 92% for germination and yield respectively. The poor growth of *Zay mays* was attributed to the suffocation of the microbial and enzymatic activities of the plant due to lack of air caused by exhaustion of oxygen by increased microbial activities.

The study by Abosede (2013) to evaluate the impact of crude oil contamination on some soil characteristics using samples from contaminated site and control (unpolluted) at three different soil depths, recorded that crude oil has no significant effect on particle soil sizes (silt and clay) at different depths, however, the depth of sand particle were higher at 0.5cm depth than 10-15cm by 43.35%. The study reported that crude oil has no significant effect at different depths, but can be noted that the presence of the pollutant increases the bulk density while reducing the total porosity of the pore spaces attributed to clogging or blockage of pores spaces with crude oil, limiting drastically air and water circulation within the natural soil environment (kayode, oyededi and olowoyo 2010 cited in Abosede 2013). It can be suggested according to Abosede (2013) that physical soil properties like saturated hydraulic conductivity, macro-porosity and

total porosity and bulk density can be affected since these properties are controlled by pore spaces present in the soil.

Sequel to the study by Baek *et al.* (2004) to ascertain the extent of inhibition of oil contaminant on the growth of corn (*Zea Mays*) and Red bean (*Phaseolus Nipponese*) using 300g of soil artificially contaminated with varying degrees (0, 1, 3, 5, 10% w/w) of crude oil and 5 seeds each of corn and red bean was planted in darkness at 23^0C for 14 days, showed that uncontaminated soil has the highest shoot and root length which decreases in this progression: 0 > 1 > 3 > 5 > 10% (w/w). Moreover, unlike the *Zea mays,* corn finds it difficult to germinate in 5% oil-polluted soil which suggest its increased susceptibility than a red bean, as little as 1% concentration of oil reduced the root development of corn by 52% against 28% recorded for red bean while shoot development declined by 28.70% and 10.90% for corn and red bean respectively which is in contrast with the unpolluted sample without root and shoot decrease. The discrepancies in crude oil tolerance recorded by these seedlings may be attributed to the individual systematic accumulation of oil compound, availability of nutrients and cell wall structural discrepancies exhibited by these seedlings (Albert 1995 cited Baek *et al.* 2004).

However, Agbogidi, Eruotor and Akparobi (2007) reported a significant decline in the plant height, stem and leaf diameter on the 10.4ml concentration of crude oil unlike the 5.2ml concentration with little appreciable growth. Maize varieties died in 42ml crude oil concentration after 2 to 42 hours. Also, *Zea mays* var. F27 (corn) was reported (Amakiri and Onofeghara 1983) to be destroyed in the presence of crude oil at 31ml. To elucidate the above, Ogboghodo *et al.* (2004a) noted that plant height, survival rate, performance rate, and dry matter yield reduced with an increase in oil pollution. In contrast, (Agbogidi, Eruotor and Akparobi 2007) inferred that little or small amount of mineral oil in soil has little effect (not too harmful) to plant growth but may be beneficial depending on the oil concentration and variety of the plant.

Conclusively, plants respond differently to different pollutants and toxicity due to their genetic modification and response of plant systems as modified by environmental influences and sub-lethal effect can be linked to the presence of metallic ions and trace metals in crude oil polluted soil (Ekundayo, Emede and Osayande 2001).

2.1.3 Effects of heavy metals on soil and plant growth

Soil contaminant, Acid Mine Drainage contains different variations of metals which were dependent on the kind and composition of the mine site. These metals concentration also varies as the AMD water moves from the central basin (source) to the environment and toxicity tends to decrease as the AMD water flows away from the source, causing deterioration of inhabitant and reducing the quality and standard of living. AMD tends to accumulate gradually in the soil with time (days, months, and years), from low to medium and high concentration which is a factor of AMD source. Severe soil pollution by AMD is a function of time as the AMD water constantly flows through the soil. Some of these heavy metals are required by plants in small quantities as high concentration poses a great challenge to plant growth (Aller *et al.* 1990) and study have shown the effect of toxicity of some heavy metal concentration on the terrestrial, aquatic environment and plant growth. Some of these heavy metals that constitute AMD include Iron, Zinc, Manganese, Copper, Aluminium, and Cobalt.

a. **Iron (Fe)**

Iron is a vital mineral needed for the biological redox system in plants (Rout 2015). It is a vital component of enzymatic activities required for physiological and biochemical activities in plants. This acts as an augmentative component of the main enzymes that partake in the plant hormone synthesis and numerous movement of electrons reaction (Kerkeb and Connolly 2006). Plants are subject to different concentrations of Fe in the soil due to Fe mobility and other environmental and/or ecological factors (Abdel-Kader 2007). Thus, deficiency or excess Fe in the soil are suggested to trigger oxidative stress which results in nutritional imbalance and physiological disorder among other adverse/negative effects on the plant (Becker and Asch 2005).

According to Wheeler, Al-Farraj and Cook (1985), iron accumulation in *E. hirsutisms* grown in a Fe-rich environment attributed to the toxicity of iron in the plant. High concentration (approximately $20mgL^{-1}$) of iron in the soil is toxic to plant as it inhibits the development of plant root through the production of superoxide which is iron-induced (Rout 2015) and negatively influence plant distribution in the wetland (Tanaka et al 1996 cited in Rout 2015;

Snowden and Wheeler 1993). Effect of Fe on aquatic plants was investigated by Batty and Younger (2003), recorded that Fe concentration of 1mg/L retards the growth of some aquatic plants (*phragmites Australis*). The result of the study reported that the concentration of Fe may not possibly explain the growth retardation of the plant. Similarly, it was suggested that low concentration of Fe 0.5 – 5.0mg/l resulted in membrane damage of some plants (hydrilla) (Sinha et al 1999 cited in Cubitto *et al.* 2004; Ivshina *et al.* 2015; Rout 2015; Lim, Lau and Poh 2016; Mahjoubi *et al.* 2018; Moradi, M. Smits and O. Sharp 2018). To further buttress the adverse effect of high iron content in the soil, the study by Rout (2015) where the effect of different concentration of Fe on different varieties of upland and low land was investigated showed varying responses by different variations with negative symptoms such as slow germination rate, shoot and root tolerant indices and leaf bronzing symptoms at 40nMFe. In the same vein, Snowden and Wheeler (1993) inferred that high concentration of Fe in soil shows numerous visible effects with some plants species; stunted growth, decrease in leaf size, green leaf colour deepening, shoots wilting, and other symptoms.

b. **Zinc (Virginie *et al.*) and Manganese (Mn)**

Zinc is a trace metal required for plant metabolism. It helps the plant to produce chlorophyll. Plant leaves tend to discolour (chlorosis) when the soil is low in Zn as plant growth is stunted (Van Baker and Brooks 1989 cited in Soltangheisi *et al.* 2014). Manganese is among the nine vital nutrients required by the plants. It facilitates different processes such as the production of chloroplast, photosynthesis, nitrogen metabolism and synthesis of some enzymes (Millaleo *et al.* 2010). Soltangheisi *et al.* (2014) investigated the effects of Zn and Mn on growth, uptake, response and chlorophyll content of sweet corn (*Zea mays var. Saccharate*) using different concentrations of Zn and Mn (introduced as $ZnSO_4.7H_2O$ and $MnSO_4.H_20$) at 0.0, 0.1, 1.0 and 10.0mg/L. The result of the 28 days study showed that Mn and Zn concentration in roots and shoots increased with increasing Mn and Zn concentrations in the nutrient solution. Zn concentration in both root and shoot enhanced with increased Mn concentration level unlike in the case of Mn concentration which showed no relationship with the increasing level of Zn. The combination of Zn and Mn in the ratio of 0:1 recorded the highest yield while 10:0 showed the lowest dry yield of young corn. De Magalhães *et al.* (2004) recorded that *synechocystis aquatilis*

f. aquatilis (cyanophyceae) growing at high Zn concentration (2.20 to 3.30mg/L) showed decreased growth rate and final yield of about 50 – 60%, relative to cells germinated in the presence of 0.2mg/L Zn. Khudsar, Iqbal and Sairam (2004) reported that Zn concentration of 100 – 400ug/g (0.1 – 0.4mg/g) causes an appreciable reduction in the root and shoot growth parameters at several levels of growth of Artemisia annual plant. Growth inhibition and chlorosis were observed with a high concentration of Zn of approximately 0.5 – 5g/kg in the soil; also root and shoot elongation was also retarded with high Zn concentration (Kopponen et al, 2001; Veer and Lafa 1989 cited in Soltangheisi *et al.* 2014). The study by Malik *et al.* (2011) to investigate the effect of different concentrations of Zn (with a total of 20ppm Zn) using $ZnSO_4.H_2O$ (0, 200, 300 400ppm) on the growth and yield of Red Amaranth (Amaranthus spp.) and Rice (Oryza sativa, variety BR49). The result of the 90 days study indicated that the effect of high concentration of Zn increases with increases in Zn with significant effects on the growth, root/shoot length and yield of rice and red amaranth. In Red amaranth, there's no visible discrepancy between control, 300, and 400ppm treatment in case of the root length but 200ppm showed different behaviour. This is in contrast with rice, where 200ppm and control experiment showed similar root length while others (300 and 400ppm treatment) differ significantly. It can be suggested that Zn toxicity varies according to plant varieties, exposure duration, stress, and nutrient components in growth medium (Hafeez, Khanif and Saleem 2013). Total Mn concentration as other metals varies in the soil, fluctuating from 20 to 10,000mg/kg and a high concentration of Mn is a limiting factor to plant growth and development resulting in poor performance (Horst 1988; Millaleo *et al.* 2010). Clarkson (1988) noted that toxicity of Mn concentration ranges from 30 – 50mg/kg as defects and deficiency symptoms of Mn are visible especially at the developmental stage of the plant.

c. **Copper (Cu)**

Copper is another trace element needed for plant growth. Copper activates enzymatic activities in plants, required for photosynthesis, respiration, and plant metabolism (Sommer 1931; Rehm and Schmitt 1997; Perales-Vela *et al.* 2007). The increase in the concentration of this metal can be detrimental to plant growth and metabolism. Nagati *et al.* (2015) investigated the influence of heavy metals (Cu & Cd) on seed germination and plant growth (using grass pea plant) which

showed that Cu and Cd concentration above 5ppm reduces the plant shoot and root length with photo-toxicity, Cu recorded 54.28% - 86.85% and 28.57 – 100% for shoot and root reduction respectively and Cd: 28.57 – 100% and 14.28 - 100% for the shoot and root reduction respectively as the concentration increases from 5ppm to 80ppm. The study of 10 days also recorded that plants have the capacity of resisting heavy metals as they tend to absorb metals considerably within a limited range required for plant metabolism (Nagati *et al.* 2015). This correlates with the study by An *et al.* (2004) where the combined effect of Cu, Cd, and Pb on *Cucumis Sativas* showed an increased reduction in root and shoot of the plant as the metal concentration increases. Similarly, the study by Xiong, Liu and Geng (2006) recorded that high concentration of Cu was toxic to the growth of cultivar Xiayangbai of Chinese cabbage (B. pekinensis rupr.), with significant adverse effects ranging from a reduction in plant biomass, root length and leaf number, to a reduction in nitrogen metabolism which attributed to a decline in nitrate reductase (NR) activity in the root and leaves and decrease in chlorophyll content (Xiong, Liu and Geng 2006). The concentration of Cu in an uncontaminated soil in plant biomass was suggested to be 12mg/kg and values above this range will possibly result to toxicity which is detrimental to plant growth and development (Balsberg Pahlsson, 1989; Fernandes and Henriques, 1991; Kabata-Pendias and Pendias, 2001 cited in Xiong, Liu and Geng 2006).

d. **Aluminium (Al)**

The effect of high Al concentration in the soil can limit plant growth and metabolism. The threshold of Al toxicity in cowpea was evident at 0.1uM and growth inhibition was visible at Al concentration greater than 40uM (Taylor, Blarney and Edwards 1998). It also contributes to the altercations which obstruct uptake, transport, and metabolism of different plant substrates (Mossor-Pietraszewska 2001). Also, the adverse effect of Al was reported by Lee (1971) in the study to investigate the effect of Al on potatoes growth and mineral requirements. The result recorded that increase in Al concentration (5 – 10ppm) in the soil lead to the decrease/inhibition of absorption of plant essential nutrients required for metabolism (Mossor-Pietraszewska 2001).

e. **Cobalt (Co)**

The toxic impact of Co on plants varies according to species and varieties as some plants (species) are more sensitive to a specific Co concentration than others, this attributes to different responses of different plants when exposed to Co due to the level of tolerance (Palit, Sharma and Talukder 1994). The high concentration of Co inhibits RNA synthesis and decreases the population of DNA and RNA by endo and exonucleases activity modification. The toxicity effect of Co ranges from leaf fall and premature leaf closure, inhibition of greening, reduction in shoot weight, to disruption of the transport mechanism (Palit, Sharma and Talukder 1994). The study by Aziz, Gad and Badran (2007) to investigate the effect of different concentration (0, 20, 40mg/kg soil) of Co and Ni on the growth, flower yield of roselle calyces recorded that low concentration of Co (up to 20mg/kg soil) triggered an appreciable increase in plant height, no of branches and fruit per plant and also dry weights of roselle calyces which is contrary to the response with high concentration (40mg/kg and above) of Co. These observation are similar to the study by Atta-Alyet al 1991 cited in Aziz, Gad and Badran (2007) here low level of Co caused a decreased in catalyse and enzymatic activities, contrary to high Co levels.

Conclusively, the negative effects of contaminants showed variation in accordance with nature and toxic level in the soil. It has been shown that the presence of these contaminants, heavy metals, and/or crude oil poses a challenge to the environment, soil, and plant growth. Soil pollution attribute to a decrease in the availability of arable lands by the reduction in soil fertility which results in poor plant growth, development, and yield. To reclaim these contaminated soils, these pollutants in the soil need to be remediated in a manner that will remove the toxic substance while preserving and revitalizing the innate organic materials present in the soil. Hence, this prompted the need for a promising approach for an effective remediation purpose.

2.2 Remediation of contaminated soils

The treatment of polluted soils has been done previously by removal and disposal of contaminated material, physiochemical method (Ejechi and Ozochi 2015), in-situ isolation, encapsulation method, soil vapour extraction (SVE), thermal and chemical method of decontamination. The application of the conventional techniques in the remediation of polluted

soil has not rendered the required outcome in the removal of the pollutant from the soil and recovery of nutrients. The objectives remain to tactically ensure the reclamation of the site, where the contaminant will be successfully removed with minimal risk to the environment and without causing subsequent degradation to the soil. The inability of these methods to meet up with the objective is attributed to some limiting factors like nature of the contaminant exists in the site, the soil type, target concentration required after the decontamination process, the volume of material to be treated, site size and accessibility, the possibility of recontamination of the site (chemical and thermal method), cost-effectiveness, the ability of the method to adequately regain/restore the soil depleted nutrients, the quantity of compost or supplement, environmental tolerance or friendliness of the decontamination materials or chemicals or the approach (Adekunle *et al.* 2017b). `

The inability of these methods to render a satisfactory remediation process provided a platform for more study on this global issue in a bid to develop a more viable, eco-friendly, and low-cost approach for the reclamation of the polluted site, restoring the lost nutrients without causing further degradation to the soil structure. However, the molecular study provides the platform to enhance bio-activities (Sutar, Mane and Ghosh 2012). These biological methods know as bioremediation involves the application of microbes to degrade contaminated soil under controlled environmental parameters to a non-toxic/harmless state or reduced below initial concentration to a tolerable limit. The bioremediation technology is dependent on natural microbe's ability to carry out the mineralization of organic compounds, resulting in ultimate formation of CO_2, H_2O, and biomass (Mariano *et al.* 2007; Adekunle *et al.* 2017b). The optimization of the bioremediation approach is a multisystem technique comprising of many factors that involve potential hydrogen (pH) value, microbial availability, pollution and dehydrogenase, temperature, oxygen supply (or anaerobic) for microbial growth, electron acceptor and nutrients (Atagana, Haynes and Wallis 2003).

2.3 Bioremediation methods for the treatment of crude oil contaminated soils - *Review*

In recent times, petroleum hydrocarbon has become one of the major pollutants of the ecosystem due to its widespread usage and spill (Aislabie, Jordan and Barker 2008; Abioye 2011). Bioremediation technique has successfully been applied for the treatment of soil contaminated with organic contaminants (Sutton *et al.* 2013; Garbisu *et al.* 2017). This technique is recently receiving unlimited attention sequel to the cost-effective and eco-friendly nature. The objective of any soil treatment method is not only to adequately remove the contaminant but also to revive the organic content of the soil (Sutton *et al.* 2013; Garbisu *et al.* 2017).

2.3.1 Bioattenuation and biostimulation treatment of crude oil contaminated soils

Bioattenuation is the biodegradation approach that is dependent on the natural environmental factors to revive and sustain the growth of the microbial communities to enhance natural biodegradation of pollutants with human intervention aside from monitoring the degradation rate (Garbisu *et al.* 2017). Biostimulation entails the adjustment of environmental parameters using organic and inorganic supplements to boost or stimulate the activities of microorganisms in the soil to improve the biodegradation rate and pollutant removal.

Liu *et al.* (2018) investigated the biostimulation of contaminated soil using aged refuse (domestic waste) from landfills. The experiment conducted in 1L plastic tubes of three experiments with each triplicated comprises of bioreactors containing contaminated soil and sterilized aged refuse (SAR); aged refuse (AR) treatment and the controlled treatment at varying loading ratios. The result recorded that AR gave the highest biodegradation efficiency of 89.83% (i.e., a reduction from 47.28.mg/g initial concentration to 2.46mg/g), an increase in the pH was observed from 6.35 to 7.67 and increased the soil organic matter content from 6.1% to 9.5%. These dilute the total petroleum hydrocarbon (TPH) by half, decreasing the soil eco-toxicity (Abbasian *et al.* 2016). The SAR and controlled experiment offered 74.64% and 22.40% representing reduction from an initial concentration of 47.28mg/g to 6.13mg/g and 36.69mg/g respectively. The result showed the potency of organic substrate in the reduction of TPH in the polluted soil. Also, the reduction efficiency of domestic waste was investigated by

Gallego *et al.* (2001) on diesel contaminated soil using domestic sludge from wastewater plants through the investigation of potential in-situ technique by the study of microbial biodegradation. The result reported 90% biodegradation efficiency after 45 days using inorganic nitrogen (N) & phosphorus (P) while the addition of sludge increased the biodegradation efficiency to an appreciable extent. The microbial study indicated the presence of contaminant degrader *Acinetobacter sp*. degrading most of the contaminant of approximately 40000l of diesel fuel released.

Ling and Isa (2006) also recorded high degradation efficiency in the evaluation of the efficacy of composting and sewage sludge for the remediation of refinery oil sludge and the optimum ratio of polluted soil to sewage sludge amendment to facilitate the degradation of refinery sludge was determined. The result showed that optimum oil and grease efficiency was achieved after 9-week study period at 65% degradation efficiency under low temperature for an optimum ratio of 1:0.5 (v/v; soil to sewage sludge ratio) but the treatment failed to remove most of the recalcitrant (heterocyclic) components. Similar removal percentage was reported by (Chorom, Hosseini and Motamedi 2010) where the use of sewage sludge accounts for the degradation efficiency of 45% to 60% of oil in contamination soil for 5 to 10 weeks study period. It reduced hydrocarbon classes $C_{17} – C_{21}$, $C_{22} – C_{25}$, $C_{26} – C_{29}$, $C_{30} – C_{36}$ at 37%, 22%, 69%, and 62% reduction efficiency accordingly within 10 weeks study period. It can be inferred that the organic nutrient amendment (supplementation) can improve, enhanced, and reinforce the microbial activities to foster biodegradation efficiency (Chijioke-Osuji, Ibegbulam-Njoku and Belford 2014a; Obiakalaije, Makinde and Amakoromo 2015).

However, a study by Obiakalaije, Makinde and Amakoromo (2015) to authenticate the efficacy of organic animal waste supplement (goat manure, poultry dropping, and cow dung waste) for the remediation of crude oil polluted soil reported that organic substrate was potent for the decrease in TPH concentration in the polluted soil with biodegradation rate of 70.7% to 87.1% from the three amendments with the highest efficiency shown by goat manure (87.1%), followed by poultry dropping (78.6%) and then cow dung (70.7%) as against the natural attenuation which gave 32.1% after 28 days period. The study recorded an increase in total microbial (heterotrophic bacteria) count in all samples treated with animal waste with the

highest population observed in the sample amended with goat manure. The existence of indigenous microorganisms in animal waste accounts for the significant increase in the total heterotrophic and hydrocarbon utilizing counts.

Similarly, organic and inorganic compounds were used in the study by Ofoegbu, Momoh and Nwaogazie (2014) to determine the rate of biodegradation using these supplements for 40 days. The experiment was performed at varying loading ratios of organic and inorganic fertilizers singly and in combination with a ratio of 100 or 50:50 respectively added to the crude oil polluted soil at 2%, 4%, and 6%(w/w). The result showed that the combination of cow dung (organic) and inorganic fertilizer gave the highest degree of biodegradation efficiency of 84.62% for 2% crude oil contaminated soil followed by NPK, cow dung, (CD and PKHA) with PKHA having the least removal efficiency. The study also reveals that that rate of TPH removal is a factor of the volume of contaminants or the degree of contamination, thus, the higher the contaminant the slower the rate of biodegradation and vice versa and also the significant difference in biodegradation rate of the organic and inorganic stimulants was elucidated (Ofoegbu, Momoh and Nwaogazie 2014).

Also, the study by Chijioke-Osuji, Ibegbulam-Njoku and Belford (2014b) showed similar results of 88% biodegradation efficiency with the combination of poultry manure (organic) and inorganic fertilizer (NPK 15:15:15) after 112 incubation period which elucidates the discrepancy between soil amended with supplement and natural attenuation. The dependence of biodegradation rate on the degree of contamination was reaffirmed by Naowasarn and Leungprasert (2016a) in the remediation of oil-contaminated soil using chicken manure (organic supplement) as the result of 5% contaminated soil treatment gave the highest TPH reduction efficiency of >60% more than the 10% and 20% treatment at equal addition of organic supplement after 42days study period. It can be inferred that a high concentration of contaminant contributes to the decrease in the biodegradation efficiency of hydrocarbons (Kuyukina, Krivoruchko and Ivshina 2018). In contrast, the study by Ani *et al.* (2018) and Obiakalaije, Makinde and Amakoromo (2015) reported that low concentration of contaminant showed a significant decline in the rate of biodegradation due to the limitation of the bioavailable target contaminant for the microbial intake which indicated that the available

contaminants are practically insufficient for the biodegradation activities. Invariably, the optimum concentration of the contaminant is required as the TPH removal efficiency depends on the availability of the nutrients for microorganism utilization (Obiakalaije, Makinde and Amakoromo 2015).

Imafidon and Ogirigbo (2018b), Amhakhian and Faleke (2014) and Omokaro (2006) also showed the efficacy of cow dung (organic) to stimulate soil microbial activities which are facilitated by the amendment of extra nutrients (organic or inorganic) to increase the biodegradation efficiency of organic pollutant. This can be attributed to the ability of the organic substrate to establish a stable environment for microorganisms to thrive and enhance TPH removal in the polluted soil (Amhakhian and Faleke 2014; Kuyukina, Krivoruchko and Ivshina 2018). Imafidon and Ogirigbo (2018b) illustrated the above stated with the addition of nutrient agar to cow dung and NPK which increased the TPH removal efficiency by 10% (from 61.93 to 72.13%) and that of NPK by 3% (from 52.85 to 55.50%) in 10 weeks. This practically falls within the biodegradation efficiency recorded by Isitekhale *et al.* (2013). Invariably, Ramsay *et al.* (2000) noted that the amendment of organic fertilizer will have an appreciable impact on the growth of hydro carbonic degrader microbes in the soil which enhances bioremediation efficiency.

According to the study by Margesin and Schinner (1997a) inorganic fertilizer decreased the initial concentration of crude oil contamination from 4000mgkg^{-1} soil matter to 380 - 400mgkg^{-1} after 155days of incubation in the treatment of oil-polluted soil. Sequel to the result of the study, approximately, 30% of the TPH was eliminated by an abiotic process while 60 – 65% was attributed to microbial degradation. The addition of supplements can significantly improve the biotic and abiotic conditions of the soil polluted with petroleum hydrocarbon (Lin *et al* 2018).

Ani *et al.* (2018) analysed the optimization process of the organic substrate, goat dung as a potential co-additive in the remediation of crude oil-polluted soil. The study reported that the introduction of goat dung increased the biodegradation efficiency (70 – 75% of 130g/l initial concentration) and the pH. The addition of organic substrate tends to enhance dehydrogenase activities which promote biodegradation efficiency and increases the pH (Amhakhian and

Faleke 2014; Adekunle *et al.* 2017a). As the neutral pH of 7 recorded the highest removal efficiency, Ani *et al.* (2018) noted that an optimal pH of 7 provides a favourable condition for the growth of microorganisms which will, in turn, enhance the remediation of process. It has been reported that extreme pH values are harmful to microbial growth and activities and reduce their ability to comfortably degrade target contaminants (Leahy and Colwell 1990). It showed that organic substrate, goat dung is an effective co-substrate and viable approach for the treatment of oil-polluted soil having contributed to little or no risk, to 70-75% reduction efficiency of TPH concentration in the contaminated sample after 56 days period (Ani *et al.* 2018).

Adekunle *et al.* (2017a) investigated the degradation of hydrocarbon contaminated soil using sheep waste and goat waste compost as an organic stimulant. The result of the study recorded that biodegradation can be influenced by soil type, the period of application, and quantity of compost or supplement (Yuniati 2018). The influence of these factors was experienced in the remediation of TPH polluted soil using the same organic substrate, cow dung as investigated by Amhakhian and Faleke (2014) and Imafidon and Ogirigbo (2018b) recorded biodegradation efficiency of 89-98% after 28 days and 61.93% after 70 days period respectively. The discrepancies in the removal efficiencies recorded by different researchers justified the fact that the TPH removal efficiency is dependent on certain conditions (Adekunle *et al.* 2017b). There was also a decrease in the acidity of the polluted soil supplemented with sheep and goat composting indicated that organic substrate can increase pH of soil polluted by petroleum hydrocarbon (PHC) at low cost and without visible risk of recontamination of the sample by the substrate (Nwogu, Azubuike and Ogugbue 2015; Adekunle *et al.* 2017b; Ani *et al.* 2018).

In the study to authenticate the potency of slurry phase biological method and land farming for the remediation of crude oil polluted soil by Kuyukina, Krivoruchko and Ivshina (2018) reported that slurry phase bio-treatment was able to achieve a biodegradation efficiency of 88% after 2 months of the study period and application of land farming as showed 300 – 600ppm/day reduction in oil concentration. Similarly, Einawawy and Salba (1996) reported that land farming was successful in the treatment of TPH contaminant with 80% contaminant removal. The manpower requirement of this approach is enormous as the 120m farming plot under

investigation took 15 months to achieve an appreciable biodegradation efficiency (Einawawy and Salba 1996). The land farming technology has been widely used its cost-effectiveness, but the physical, chemical and biological components of this method may pose serious damage to the entire remediation process if not properly managed. The dominant contaminant removal technique involved in land framing is the volatilization of low weight volatile compound in the course of the early stage of the treatment process, leaching and remaining recalcitrant hydrocarbon can pose serious health and environmental challenge to the rehabilitation workers when designing the land farming technology on the contaminated site. Also, the large expanse of land, flexibility, manpower, and effectiveness of the approach at high TPH concentration (> 50,000ppm) are among the drawback of this approach. (Maila and Cloete 2004).

Adekunle (2011) studied the remediation of soil polluted with Nigeria petroleum products using composted municipal waste and the growth of maize as a risk assessment on soil quality and remediation evidence. The result of the research recorded that the treatment of petroleum-contaminated soil increased pH and electrical conductivity and decreased the TPH at 40-75.8% efficiency with toxicity reduction from 100% to 16.2% after 21 days of treatment. Maize growth was observed in the composted remediated soil which signified the evidence of soil restoration for agricultural purposes. Seed germination in the soil that received no treatment suggests that a certain degree of crude oil contamination supports plant growth. Kuhn *et al.* (1998) supported this with the treatment of Kuwait crude oil contaminated soil where the germination and growth of tomato (*Lycoperisucum esculantum*) was feasible at 0% - 0.36% crude oil contamination with toxicity and growth inhibition recorded at ≥0.48%. However, the Nigerian crude oil used by Adekunle 2011 supports maize seed germination up to 5% contamination. It can be inferred that oil phytotoxicity can vary with location, type of crude oil, specie, or type of plant and/or climatic conditions (Adekunle 2011).

2.3.2 Bioaugmentation of crude oil contaminated soils

Bioaugmentation is the process of introducing isolated bacterial strain or microbial consortium or genetically engineered bacteria with defined catabolic attributes to accelerate the dehydrogenase activities, increase biodegradation efficiency to produce the expected outcome (ESchauer-Gimenez *et al.* 2010; Onuoha *et al.* 2011). Bioaugmentation is favourable in polluted

soil that has possibly undergone bioremediation but still pose an environmental risk since indigenous microorganisms failed to accomplish the biodegradation of contaminant during the process. Hence, this bioremediation is posed to improve the rate of pollutant removal through the injection of biodegradable bacteria consortium or strain of microorganisms. The type of microorganism to be used in the decontamination process is dependent on the type of contaminant, physiological, and microbial metabolic activities to comfortably degrade contaminants. Since there is no single microorganism that can degrade all the contaminants present in a contaminated sample, researchers have studied the ability of most microbes to remove contaminants and most studies have focused on bacteria and fungi.

Olukunle and Oyegoke (2016) investigated the bioaugmentation of crude oil using fungi isolated from cow dung polluted soil. The study identified 16 fungi from the contaminated soil which possesses contaminant degradation ability with *Trichoderma viridae (66.2% TPH reduction efficiency), Aspergillus flavus and Varicosporium elodeae (40% TPH reduction rate)* species demonstrating the best degradation ability in 15 days study period. The study by (Kristanti *et al.* 2011) on the bioaugmentation of crude oil using fungi (white-rot fungi *Polyporus sp.* S133) and the effect of three nutrients (glucose, polypeptide, and wood meal) were evaluated. The outcome of the treatment reported that appreciable TPH removal efficiency of 93% was feasible with the addition of 10% kapok. In the same vein white-rot fungus (basidiomycetes fungal isolate *Armillaria* sp. F022) was applied by (Hadibarata and Kristanti 2013) in biodegradation and metabolite conversion of pyrene. The result of the treatment reported pyrene concentration, <19% after 30 days of incubation as the high removal efficiency correlated with the degree of depletion of carbon source (glucose) used for the treatment. These demonstrated the ability of white-rot fungi to remediate crude oil contaminated if properly developed (Kristanti *et al.* 2011).

Similarly, on mycoremediation, Winquist *et al.* (2014) investigated the removal efficiency of recalcitrant PAHs using fungi inoculum; strain *Phanerochaete velutina* which showed a significant reduction of in TPH concentration most especially PAHs (at 96% of 4 - ring PAHs and 39% of 5 -& 6 – ring PAHs) removed from the polluted sample as against the inoculated treatment stimulated with green waste (biostimulation only) which recorded 55% reduction of

4 – ring and only 7% of 5 & 6 – ring PAHs in 3 months treatment period for the laboratory scale experiment. The field-scale study observed an almost similar biodegradation efficiency for the *P. velutina* inoculated and inoculated treatment recorded 96% of 16 PAHs degraded in 3 months. This indicated that bioaugmentation using fungi has the capacity of biodegrading TPH from contaminated soil and restoring the organic content of the soil, although most of the study on fungi remediation ability are still at the laboratory stage. (Isikhuemhen, Anoliefo and Oghale 2003; Kristanti *et al.* 2011; Winquist *et al.* 2014).

The study by Benyahia and Embaby (2016) to investigate the relative potency of bioaugmentation (BA) and biostimulation (BS) noted that crude oil contaminated soil requires a combined application of BS and BA for effective remediation of contaminated soil. The result of the study showed that bio-piling amended with BS and BA offered 77% TPH removal efficiency after 156 days study period. The single treatment with bioaugmentation gave 55% removal efficiency and biostimulation with indigenous microbes recorded 23% as against bioattenuation with a 4% biodegradation efficiency. The study reported that the availability of biodegradable contaminants and nutrients can affect the rate of biodegradation (Benyahia and Embaby 2016).

Ghaly, Yusran and Dave (2013) evaluated the efficacy of nutrient amendment and augmentation of mycobacteria species for the treatment of PAHs, pyrene polluted soil. An increase in the number of cells: 40, 70, 59, and 132 for control, biostimulation, bioaugmentation, and combined biostimulation & bioaugmentation respectively was observed. It recorded that the experiment which was conducted within the temperature range of 20 - 40C and moisture content of 40 - 60%, within the optimum moisture for PAHs degradation (Wilson and Jones 1993) observed the highest PAHs and pyrene reduction efficiency of 84% with the combined process of biostimulation and bioaugmentation, followed by bioaugmentation (57.86%), biostimulation (50%) and the least was recorded in natural attenuation with 37% pyrene removal efficiency after the 14 days study period. The outcome of the study by Abdulsalam *et al.* (2011) was in contrast with Ghaly, Yusran and Dave (2013) experiment where biostimulation using inorganic fertilizer and dihydrogen orthophosphate recorded 75% higher than bioaugmentation (with bacteria consortium) which gave 66% while bioattenuation offered 50% removal efficiency

after 10 weeks study period in a study conducted in an aerobic fixed bioreactor to compare the bioremediation efficiencies of soil polluted with spent motor oil. Similarly, in the study by Mao *et al.* (2012) to investigate the performance of bacteria consortium for the treatment of PAH contaminated soil reported that the addition of 20% bacteria consortium gave (35.8%) high PAH removal efficiency than the 10% treatment (20.2%) after 90 days of incubation. An increase in bacteria consortium was reported at the early stage of the remediation process and decreases gradually as the treatment progress and the low removal efficiency due to high concentration of PAH in the sample according to Mao *et al.* (2012).

Sugiura *et al.* (1996) studied the correlation between biodegradability and physicochemical properties of petroleum, which expatiates chemical species of petroleum that are recalcitrant to biodegradation using bacteria consortium. In the study, a microbial bacteria consortium SM8 isolated from sediment and sub-cultured in crude oil medium and *Acinetobacter* isolated from the Pacific Ocean was used for the experiment for the treatment of four crude oil samples from different locations. The result showed that both strains of bacteria degraded oil samples at different rates with *Acinetobacter sp. T4* recording 19 – 34% and SM8 consortium 12 – 20% biodegradation efficiencies after 28 days of treatment. Although *Acinetobacter sp.* T4 recorded an appreciable biodegradation efficiency than SM8 sp., it was reported that SM8 consortium recorded significant degradation of naphthalene, phenanthrene, fluorene, dibenzothiophene and their structural analogue than *Acinetobacter sp.* T4 which was more effective on alkyl chains, but polycyclic aromatics were recalcitrant to this bacteria strain (SM8 sp.).

2.3.2.1 Combination of Biostimulation and Bioaugmentation

In a study by Qiao *et al.* (2013) to evaluate the effectiveness of different amendments using NPK, fertilizers, humic substances, organic industrial waste (NOVOGRO) and yeast-bacteria consortium for enhancing the treatment of PAH from polluted soil (up-to 6% hydrocarbon)0 recorded that biostimulation, (the mixture of NPK, HS, and NOVOGro) showed the greatest efficiency of TPH removal in 90 days study period. Also, the addition of exogenous oil-degrading bacteria had a minimal effect on biodegradation efficiency of contaminants, but the introduction of external yeast bacteria consortium (bioaugmentation) showed a significant increase in removal efficiency of more recalcitrant PAH while bioattenuation offers the least

result. This proved that combination biostimulation (organic and inorganic supplement) and bioaugmentation (use of microorganism consortium) are more efficient in the treatment of crude oil polluted soil; the removal of recalcitrant (PAH) hydrocarbons and decrease in toxicity of contaminated soil, since the augmentation with yeast bacteria facilitated the removal of more recalcitrant hydrocarbon. The removal of PAH by yeast bacteria consortium is due to the synergistic impact of bacteria and fungi that exists in the sample which indicated that that the introduction of external microorganism is more efficient that the indigenous organisms in the removal of PAHs citing salient features of microbial consortium as reasons for its potency in the treatment (Qiao *et al.* 2013).

Assessment of biodegradation ability of bacteria isolated from oil-contaminated soil with the animal waste amendment was investigated by Urhibo and Ejechi (2017). The result of the study showed that the TPH removal by bacteria in the animal waste amendment was more than that of strain from soil contaminated with petroleum and the greatest TPH biodegradation efficiency was recorded with poultry waste, strain *P. Vulgaris* (96.6 – 97.3% as against 80.4 – 95.9%) after 6 weeks of treatment. The high degradation efficiency of the sample with bacteria isolated from animal waste amended with animal waste can be attributed to the ability of the animal waste supplement to act as an energy source for the bacteria which enhanced the biodegradation efficiency unlike the strain without carbon source (Jamil and Clark 2013). The use of bacteria for the remediation of contaminated soil is affected by carbon source and environmental factors which limits its potency and metabolic activities resulting in low biodegradation efficiency. Accordingly, while some bacteria are sensitive to PHC, exposure to PHC may adversely affect their potency and activities, others can utilize the cytotoxic intermediate metabolite and flourish (Jamil and Clarke 2013; Xu *et al.* 2018). Thus, cleaning crude oil contaminated soil with bacteria strain will be tedious without energy source to improve the process performance.

The study by Fan, Xie and Qin (2014) reaffirmed the potency of the combined systems of biostimulation and bioaugmentation with yeast which recorded TPH reduction of 83%. Concentration effect contributes to contaminant bioremediation as (low concentration) 0.5% (v/v) gave 96% more than (high concentration 5% (v/v) with 42% biodegradation efficiency. Similarly, records indicated that biostimulation with a stimulant (sludge) and hydrocarbon

sulfate degrading bacteria accelerated the rate of biodegradation of TPH and recalcitrant PAH (Roy *et al.* 2018). Also, an investigation by Suja *et al.* (2014b) on the effect of native microbial bioaugmentation and biostimulation in the bioremediation of TPH polluted soil based on pilot and field study showed that the combined approach is efficient in reinforcing the growth microbial community and dehydrogenase for the treatment of TPH contaminated site as 97% biodegradation efficiency was recorded after 70 days.

In the assessment of bioaugmentation and biostimulation efficiencies for petroleum contaminants, (Mohajeri *et al.* 2017) where bacteria consortium (*Acinetobacter, Alcaligenes, Bacillus, and Pseudomonas*) and nutrient supplement. The result of the process indicated that bioaugmentation has the highest efficiencies of degradation with 73.89, 73.76 and 58.31% respectively for 3g, 30g and 60g oil concentration/kg respectively whereas biostimulation boosted 52.11, 58.36 and 43.02% and natural attenuation with 15.33, 15.48 and 13.01 for 3g, 30g and 60g oil concentration/kg respectively after 90 days incubation period. The result inferred that oil concentration of more than 30g/kg is not appropriate for bioremediation to avoid the increase in toxicity which contributes to the inhibition of the process. The concentration of oil among other environmental factors is a crucial function of bioremediation of oil contamination since the efficacy of bioaugmentation is a factor of effective attachment, retention and metabolic population in the bioreactor system which should be equivalent to the oil concentration for optimum performance (Mohajeri *et al.* 2017).

2.3.3 Bioventing treatment of crude oil contaminated soils

This is a bioremediation approach that cost-effectively removes light and middle hydrocarbon distillates from the unsaturated zone through the combination of soil venting and improved bioremediation. The removal ability of bioventing is by direct air injection through the vadose zone to revive, revitalize, resuscitate and promote aerobic respiration within the contaminated soil environment which enhances biodegradation of more volatile hydrocarbons (Hoeppel, Hinchee and Arthur 1991). Bioventing system is structured to enhance sufficient oxygen supply to ventilate the vadose zone and activate oxic condition in the contaminated site, usually operated at low flow rates with designs and configuration different from soil vapour extraction (SVE) (Frutos *et al.* 2010).

The bioventing treatment of contaminated soil involves the controlled supply of air or oxygen directly or indirectly into the subsurface unsaturated zone of the contaminated site to resuscitate aerobic processes (Dupont 1993). The bioventing of contaminated soil has been studied; the early study focuses on the use of water (as an oxygen source) to deliver oxygen for ventilation of the subsurface zone which is less effective when the penetration rate (low) is compared to gas (O_2). This also attributes to the drawback of the use of hydrogen peroxide for bioventing process, since the low penetration power limits the availability of oxygen to the vadose zone for microbial activities (Hoeppel, Hinchee and Arthur 1991). Similarly, an investigation by Huling, Bledsoe and White (1990) to ascertain the efficiency of H_2O_2 for bioventing revealed that the high concentration of H_2O_2 up to 100mg/l may have an inhibitory effect on the biodegradation rate of contaminated sample/site, also, the toxicity and stability of H_2O_2 depends on some type of contaminants, site and other environmental factors (Hinchee, Downey and Aggarwal 1991).

Mao *et al.* (2009) evaluated the treatment of crude oil polluted soil by bioventing and composting technology application where inorganic fertilizer was used as a stimulant for the bioventing in three varying ratios; 8:2, 7:3, 5:5 for contaminated soil to organic fertilizer (dry weight). The result showed that 45% of TPH was removed from the soil in 40 days period from an initial concentration of $7.0x10^4$mg/kg. The highest reduction efficiency of 45% was observed with the 7:3 treatment, which attributes to the high concentration of the contaminant. Volatilization removal was less than 0.1 which suggested that degradation was most active due to the bioremediation process. Similarly, Lee and Swindoll (1993), conducted a laboratory experiment on the feasibility of bioventing applications for the treatment of hydrocarbon (light and heavy). The study carried out using three treatments was operated for bioventing, organic nutrient, and moisture; bioventing without organic nutrient and moisture, and the controlled experiment. The result recorded that after 90 days of treatment at an operating temperature of 22^oC, bioventing was the most effective TPH removal from the contaminated soil. Bioventing with organic matter and moisture boosted 98%; bioventing without organic matter and moisture gave 83% and the controlled experiment showed the least biodegradation efficiency of 29%. Bioventing with nutrient was effective in the removal of BTEX and recalcitrant PAHs (96%); heavy hydrocarbon (75%). The result showed that bioventing is apt for the treatment of hydrocarbon ranging from light to medium (gasoline and diesel) to heavy hydrocarbons (such

as fuel oils and other volatile, non-volatile HC and PAHs); which inferred that nutrient amendment increased the removal efficiency of bioventing (Lee and Swindoll 1993; Frutos *et al.* 2010).

This was further justified in a study by Møller *et al.* (1996) to evaluate the bioventing of diesel oil-polluted soil using supplements and comparison of removal efficiencies based on actual oil concentration and respirometric data. Bioventing was supplemented with nutrients (Nitrogen and Phosphorus added as a mixture of $NaNO_3$, KNO_3 and $NaHPO_4$ dissolved in water to give C: N: P ratio of 120:10:1 based on concentration content of oil) and inoculated with oil-degrading bacteria (isolated from an enrichment culture of bacteria from diesel contaminated soil). Similarly, bioventing treatment of phenanthrene-polluted soil using optimum conditions of mineralization: humidity: 60% and different C/N/P ratio of 100:20:1 respectively was effective as it enhanced the removal efficiency after 7 months of study. The result indicated that nutrients and inoculation increased the rate of bioremediation of contaminated soil (Lee and Swindoll 1993; Møller *et al.* 1996; Frutos *et al.* 2010) and respirometry test has no appreciable impact on the removal of diesel oil, thus, removal of contaminant was done by bioremediation (Møller *et al.* 1996). The ability to achieve an enhanced bioventing biodegradation with nutrient addition was in contrast with the investigation by Dupont, Doucette and Hinchee (1991) where the amendment nutrient (moisture) was insignificant for the increase rate of biodegradation of fuel contaminated soil. However, Bulman, Newland and Wester (1993) demonstrated that nutrients amendment to bioventing rendered an appreciable increase in the rate of degradation of TPH which suggested a further study on the nature and type of individual nutrient required for each contaminant for the bioventing process to foster biodegradation rate since some additives (nutrients) can reduce or hinder biodegradation or constitute to the increase in toxicity of the sample after the treatment (Frutos *et al.* 2010).

Eslami and Joodat (2018), studied the bioremediation of oil and heavy metals polluted soil using bioventing – bio-sparging and phytoextraction (plant assisted bioremediation) techniques. The result of the study showed that a combined process of venting and bio-sparging rendered the highest efficiency of biodegradation, reducing 60% of the contaminant in 40 days period while the phytoextraction technique was effective in reducing heavy metal contaminants up to 50%

after 50 days of study. The process indicated that air-injection nourishment to the system improved the degradation rate by providing optimum soil medium for the remediation process. In bioventing and bio-sparging, the removal efficiency of ethylbenzene was more than that of pyrene, attributed to the discrepancies in their molecular structures (Eslami and Joodat 2018). Ethylbenzene, which is of BTEX family possesses one cyclic hydrocarbon, different from pyrene (of PAHs group) with multiple cyclic hydrocarbons elements – which made biodegradation of pyrene slow and more difficult than BTEX. Also, the less complexity of the BTEX family attributes to the self-bioremediation of contaminants containing ethylbenzene, unlike pyrene which falls under the recalcitrant PAHs. (Norris et al 1994 cited on Rathfelder, Lang and Abriola 1995; Eslami and Joodat 2018).

Lee *et al.* (2006) monitored the bioremediation of diesel fuel in the bioventing process using in-situ respiration rate. The experiment comprised of 5kg of soil contaminated with 8000mg/kg of petroleum hydrocarbon was conducted in a column, with variation in flow rate; where one received continuous venting and the other column received venting for 6 hours/6 hours resting during the 5 months study period. The result indicated that there is no apparent variation in the biodegradation efficiencies between the two columns with varying flow rates when measured with an online measuring system of respiration rate. The result supports the study by Thomé *et al.* (2014) where there's no significant difference in the biodegradation rate at varying air flow rates and airflow intervals at 2, 4 and 6l/m (corresponding to 0.36, 0.82 and 1.4kpa) at 1-hour flow every 24, 36 and 48 hours for 15, 30 and 60 days respectively. From the result, the highest biodegradation rate was recorded at 85% for bioventing and 64% for natural attenuation. It suggested that bioventing will be more economical if the lowest flow rate (2l/m) and highest flow interval will be considered for the bioventing of contaminated soils while increasing bioventing flow interval and rate is not justifiable due to high operation cost (Thomé *et al.* 2014). Volatilization was not considered in the process because the contaminant (diesel) has fewer volatile components and considering low operating temperature, and low flow intensities, these components have negligible effects (Fingas 2004; Mao *et al.* 2009).

However, the study by Frutos *et al.* (2010) reported the ability of the bioventing technique to significantly remove 93% of phenanthrene (PAHs) from 1026mg/kg initial concentration to

74mg/kg in 7 months period. The Ecotoxicity test indicated that the residual toxicity (which pose an ecological challenge) obtained at the end of treatment was basically due to C/N used for the optimization of the system and not low phenanthrene concentration, which suggested that type of nutrient amendment should be considered in the bioremediation process (Bulman, Newland and Wester 1993). Also, the treatment showed a decrease in degradation efficiencies with a corresponding increase in treatment period where the highest rate of biodegradation was recorded between months 1 – 3 while gradual decline commenced from month 4 with a significant decrease in biodegradation efficiency from month 5 till the 7^{th} month of the study period. It can be inferred according to Martineez Garcia (2004 cited on Frutos *et al.* (2010) that the removal efficiency is a function of time as it tends to increase or decrease significantly with time.

The application of bioventing and bio-trickling filter technologies for soil remediation was investigated by Magalhães, Jorge and Castro (2009). In the study, the soil was artificially polluted with aromatic hydrocarbon (toluene) of 100mg/dm^3 and 500mg/dm^3 homogeneously to attain the desired soil contamination and the mineral medium was added to the soil for moisture regulation at 10%. Microbial inoculum culture of 10cm^3 was added to the two bioreactors for bioventing and combined bioventing and bio-trickling after 6 days. The result should that bioventing and combined bioventing and bio-trickling gave the same rate of biodegradation of 99% removal of toluene (at an initial concentration ranging from 2 to 14mg/g soil) after 20 days study period with toluene attributed removal to the combined effort of biodegradation and volatilization as against 80% removal efficiency recorded for the untreated sample. Sequel to the volatile nature of the toluene, volatilization was able to eliminate some volatile components of the contaminant unlike the case of non-volatile diesel contaminants as earlier reported (Magalhães, Jorge and Castro 2009). Also, the combination of the two processes rendered a significant removal efficiency of 99% toluene removal from the soil. The process which was not carried out with high organic load to determine the extent of biodegradation rate when bioventing is compared to biotrickling posed a limitation to the study. However, Chou and Wu (1999) reported that the treatment of toluene using the combination of BVT and BF showed a higher rate of biodegradation of 90% in 121 days at a high organic load of 30g/h

which inferred that the combination of these techniques can be effective for the remediation of toluene from a polluted site.

Agarry and Latinwo (2015) investigated the application of bioventing and wastewater for the remediation of diesel polluted soil in a microcosm system containing 1kg soil spiked with 10% (w/w) crude oil to achieve desired contamination and monitored for 28 days. The result observed that a combination of brewery waste effluent supplement and bioventing technique gave the highest TPH degradation rate of 91.5%; bioaugmentation and biostimulation with brewery waste effluent recorded 78.7% removal efficiency and 61.7% for bioventing. The natural attenuation gave the lowest rate (≤40%) of diesel removal since the treatment received no amendment or supplementation. Also, the increase in total hydrocarbon-degrading bacteria (THDB) count throughout the treatment period in all the systems was observed with the highest bacteria growth visible with the combined bioventing and brewery waste effluent approach. It was also reported by Thome *et al.* 2014 combined biostimulation and bioventing was more effective than biostimulation or bioventing used alone. Brewery waste tends to increase the nutrient level and microbial density in the soil thus acting as bioaugmentation and biostimulation agent. A similar trend was recorded by Muskus Morales, Santoyo Muñoz and Plata Quintero (2013) where organic components (animal waste) acted as bioaugmentation and biostimulation agent to facilitate the remediation process. The use of bioventing and biostimulation/bioaugmentation was suggested to be an environmentally sustainable approach for the remediation of the natural ecosystem (Thomé *et al.* 2014; Agarry and Latinwo 2015).

Table 2-1. Bioremediation of crude oil contaminated Soils using different approaches.

Bioremediation Methods	**Nutrient Amendment**	**Contaminant**	**Degree of Contamination/ Initial Concentration**	**Process Duration/ Study Period**	**Maximum Removal Efficiency**	**References**
BAT	No amendment	Crude oil	47.28mg/g	98 days	22.40%; 32.1%	(Liu *et al.* 2018)
BAT	No amendment	Spent motor oil	9830mg/kg – 14439mg/kg	10 weeks	50%	(Abdulsalam *et al.* 2011)
BAT	No amendment	Petroleum	3g/kg, 30g/kg and 60g/kg oil conc./kg of soil	90 days	13.01 – 15.33%	(Mohajeri *et al.* 2017)
BST	Aged Refuse	Petroleum	47.28mg/g	98 days	74.64 – 89.83%	(Liu *et al.* 2018)
BST	Slurry + land farming	Crude oil	20454ppm /ha	2 months	88%	(Kuyukina, Krivoruchko and Ivshina 2018)
BST	Land farming	Crude oil		15 Months	80%	(Einawawy and Salba 1996)
BST	Sewage Sludge	Crude oil	5kg/l	9 weeks	45 – 65.6%	(Ling and Isa 2006)
BST	Sewage sludge	Crude oil	1000mg/kg	10 weeks	45% - 60%	(Chorom, Hosseini and Motamedi 2010)
BST	Organic waste; (goat manure, poultry droppings, and cow dung)	Crude oil	53966.60 mg/kg	28 days	70.7 – 87.1%	(Obiakalaije, Makinde and Amakoromo 2015)
BST	Composted Municipal waste	Petroleum	Diesel fuel: 16000±83 mg/kg	15 days	40 – 75.8%	Adekunle 2011

			Spent engine oil: 18333±97 mg/kg; Crude oil: 23000±10 1mg/kg			
BST	Poultry manure	Crude oil	3666mg/g	157 days	96.01%	Chikere et al, 2009 (Chikere *et al.* 2012)
BST	Organic fertilizer	Crude oil	4000mg/kg	155 days	90.01 - 92%	(Margesin and Schinner 1997b)
BST	Organic (cow dung, (CD); palm kernel husk ash, (PKHA) and inorganic fertilizer (NPK)	Crude oil	Varying degrees 2%, 4% and 6% to 1000g soil	40 days	84.62% - (CD + NPK); 76.80% - NPK	(Ofoegbu, Momoh and Nwaogazie 2014)
BST	Organic waste (refuse)	Crude oil	42mg/g	96 days	44 – 87%	(Al-Kindi and Abed 2016)
BST	Organic (poultry droppings, and goat dung) and inorganic fertilizer (NPK) and sawdust	Crude oil	20l of crude oil per 3kg soil	112 days	60.7% - 88% - poultry manure 60.7%	(Chijioke-Osuji, Ibegbulam-Njoku and Belford 2014)
BST	Plant and animal organic and NPK	Crude oil	200g crude oil per 1kg soil	8 weeks	89 – 96.89%	(Mbah and Obahiagbon 2017)
BST	Organic (poultry manure)	Crude oil	300mg/kg	12 weeks	76.42 – 86.97%	(Isitekhale *et al.* 2013)

	and inorganic (NPK) fertilizer					
BST	Chicken manure and	Crude oil	28.8mg/kg to 70.27mg/kg	42 days	>60%	(Naowasarn and Leungprasert 2016b)
BST	Sheep waste and goat waste compost	Petroleum hydrocarbon	NA	28 days	pH reduced from 6.63 to 8.22	(Adekunle *et al.* 2017b)
BST	Cow dung	Crude oil		28 days	89 – 98%	(Amhakhian and Faleke 2014)
BST	Goat dung	Crude oil	130g/l	56 days	70 – 76%	(Ani *et al.* 2018)
BST	Animal Waste (poultry manure, piggery manure, goat manure and chemical fertilizer)	PHC mixture (Kerosene, diesel and gasoline mixture)	1kg soil with 10%(w/w) PHC	4 weeks	Poultry manure: 73%; Piggery manure: 63%; Goat manure: 50%; NPK: 39%	(Agarry, Owabor and Yusuf 2010)
BST	*Capra aegagrum hircus*; Goat manure	Crude oil	50ml/kg	14 days	62.08%	(Nwogu, Azubuike and Ogugbue 2015)
BAU	*Acinetobacter Baumanni S30 Pjes*	Crude oil	89.3g/kg	90 days	40.56%	Mishra, Darma and Lal 2004
BAU	Indigenous microbes, and free cells *of P. aeruginosa*	Petroleum	8025mg/kg; 17780mg/kg	191 days	n – alkane – 94%; Aliphatic – 89% respectively	(Karamalidis *et al.* 2010)

BAU	*Acinetobacter sp. T4* and SM8 consortium	Crude oil	5000mg/L	28 days	19 – 34% and 12 – 20%	(Sugiura *et al.* 1996)
BAU	*P. aeruginosa*	Crude oil	5000ppm	7 days	30 % for resin and aromatics	(Venkateswaran and Harayama 1995)
BAU	*Pseudomonas spp. TMU2-5, Bacillus licheniformis Tmu1-1, B. Lentus TMU5-2, Bacillus cereus TMU8-2 and Bacillus firmus TMU6-2.*	Asphaltene	-	60 days	48% for Mixed culture and 46% for *Pseudomonas sp.*	(Tavassoli *et al.* 2012)
BAU	*Bacillus toyonensis BCT-7112*	Asphaltene	5g/L	50 days	64.8% and 60% at 25^{o}C and 45^{o}C respectively	(Honarmand Kashi, Tabatabaee and Arbab Soleimani 2018)
BAU	a mixed culture comprising *P. aeruginosa, Bacillus, Brevibacillus, Staphylococcus and Corynebacterium*	Asphaltene	5g/L	13 days	46%	(Pineda-Flores *et al.* 2004)
BAU	by mixed consortium containing	Crude oil	1% - 10% crude oil	20 days	78% for mixed consortium and	(Rahman *et al.* 2002)

	Pseudomonas sp.DS10-129, Bacillus sp. DS6-8b, Micrococcus sp. GS2-22, Corynebacterium sp. GS5-66, Flavobacterium sp. DS5-73		concentration		66%, 59%, 49%, 43%, and 41% for single strain respectively,	
BAU	*P. Aeruginosa DQ8*	Diesel oil	2% *vlv*	5 weeks	53.3±2.1%, 66.3±5.3%, and 46.6±3.4% for aromatic, nonhydrocarbons and asphaltenes respectively	(Zhang *et al.* 2011)
BAU	*Pseudomonas sp. BZ-3*	PAH	50mg/L	28 days	>45% PAH with two rings for non-aromatics, 36% of PAH in case of PHE and Anthanthrene	(Lin *et al.* 2014)
BAU	*B. Subtilis* DM-04 strain and *P. Aeruginosa*	Petroleum	2%(v/v) petroleum (84g/kg)	120 days	75% for *P. Aeruginosa* and *B. Subtilis* with 53.6%	(Das and Mukherjee 2007)

	N and NM strains					
BAU	Bacteria consortium	PAH	936.1mcg/kg	90 days	3-ring PAH: 18.7-35.2%; 4-ring PAH: 21.8-33.2%; 5-ring PAH: 17.3 – 40.5%	Mao *et al.* (2012)
BAU	Bacteria consortium	Crude oil	3, 30 and 60 g	90 days	73.89, 73.76 and 58.31% respectively	Mohajeri et al 2016
BST & BAU	Fungi inoculum (strain phanerochaete) and green waste	PAHs	3500mg/kg	3 months	55% (BST); 96% (BAU)	Erika, et al, 2013
BST & BAU	Commercial NPK Fertilizer, Humic Substances (HS), Organic industrial waste (NOGRO) and yeast bacteria consortium	Polyaromatic hydrocarbon (PAHs)	63.0g TPH/kg Soil	90 days	46 – 64%	(Qiao *et al.* 2013)
BST & BAU	Yeast and nutrient amendment	TPH	16300mg/kg	180 days	83% - 96%	(Fan, Xie and Qin 2014)
BST & BAU	Microbial inoculation and	Crude oil	277.5g Crude oil	156 days	56 - 77%	(Benyahia and Embaby 2016)

	nutrient supplement		per 1850g soil			
BST & BAU	Nutrient supplement and mycobacterium	Pyrene (PAHs)	700mg pyrene per 1kg soil	2 weeks	84.29% (BST + BAU); 57.86% - BAU; 50% - BST	(Ghaly, Yusran and Dave 2013)
BST & BAU	Inorganic fertilizer and dihydrogen orthophosphate and consortium of bacteria	Spent motor oil	9830mg/kg – 14439mg/kg	10 weeks	75% (BST); and 66% (BAU)	(Abdulsalam *et al.* 2011)
BST and BAU	Bacteria consortium and nutrient supplement	Petroleum	3g/kg, 30g/kg and 60g/kg oil conc./kg of soil	90 days	43.02 – 58.36% (BST) 58.31% - 73.89%; (BAU)	(Mohajeri *et al.* 2017)
BST & BAU	Microbial consortium and nutrient	Crude oil	3000bbl/acre	70 days	97%	(Suja *et al.* 2014a)
BVT & composting technology	Organic fertilizer as a stimulant	Crude oil	$7.0X10^4$mg/kg	40 days	45%	(Mao *et al.* 2009)
BVT	Organic nutrient and moisture O_2	Hydrocarbon (light and heavy - PAHs)	4900ppm TPH	70 days	62% - 98%	(Lee and Swindoll 1993)
BVT	Nutrient addition + oil degrading bacteria and O_2	Diesel oil	2000mg/kg	112 days	96%	(Møller *et al.* 1996)
BVT & biosparging and	Air	Crude oil and heavy metal	NA	40 – 50 days	60% of c/o (BV and BS); 50%	(Eslami and Joodat 2018)

phytoextraction					of metals (phytoextraction)	
BVT & BST & BAU	Brewery waste (BW)	Diesel oil	1kg soil with 10% (w/w) diesel oil	28 days	BVT + BW: 91.5%; BST + BAU: 78.7%; BVT: 61.7%	(Agarry and Latinwo 2015)
BVT	Oxygen	Diesel oil	8000mg/kg	5 months	Increase TPH removal	(Lee *et al.* 2006)
BVT	Oxygen	Crude oil	40g/kg	120 days	85%	(Thomé *et al.* 2014)
BVT	Oxygen	Phenanthrene	1000mg/kg	7 months	93%	Javier et al, 2010 (Frutos *et al.* 2010)
BVT & bio-trickling filter technology	Microbial inoculum	Crude oil	14mg/g	121 days	90 - 99%	(Magalhães, Jorge and Castro 2009)

BST - Biostimulation; BAU – Bioaugmentation; BVT – Bioventing; BAT: Bioattenuation

2.4 Acid Mine drainage

Acid Mine Drainage (AMD) contamination is the seepage or flow of contaminated water from old, abandoned, or active mining pit/sites/areas into the environment contaminating the soil, water bodies, and contributing to the degradation of the ecosystem. AMD is formed when sulfide-bearing minerals, pyrites (iron disulfide, FeS_2) found in a coal mine and gold reef (Witwatersrand, Gauteng Province, South Africa) is exposed to oxygen and water. The process is known as pyrite oxidation characterized by the formation of sulfuric acid and dissolved iron. AMD consists of high sulfate content, low pH, heavy metals, and the presence of radioactive elements (Ur, Ra) depending on the type of site.

2.4.1 Overview of AMD formation

i. *Stage one*: The first or initial reaction in the pyrite weathering is characterized by oxidation of pyrite by oxygen (O_2). In this reaction, sulfur is oxidized to sulfate, and ferrous iron is formed (Eqn. 2-1).

$2FeS_2 + 7O_2 + 2H_2O \rightarrow 2Fe^{2+} + 4SO_4^{2+} + 4H^+$ Equation 2-1

ii. *Stage two:* The second stage of pyrite disintegration, showed the transformation of ferrous iron to ferric iron. The conversion to ferric iron from ferrous iron utilizes one molecule of acidity (Eqn. 2-2). The reaction which is facilitated by bacteria present during the weathering process is pH sensitive and tends to be faster at pH values of approximately 5 and slow at pH range between 2 – 3 (acidic medium) without the presence of bacteria.

$4Fe^{2+} + O_2 + 4H^+ \rightarrow 4Fe^{3+} + 2H_2O$ Equation 2-2

iii. *Stage three:* This stage involves the hydrolysis of ferric iron. The by-product of this process is 3moles of acidity. The precipitation and formation of ferric hydroxide (Eqn. 2-3) are pH-dependent which forms at the pH value of 3.5 and above, and little or no precipitate is formed at pH below 3.5.

$4Fe^{3+} + 12H_2O \rightarrow 4Fe(OH)_3^- + 12H^+$ Equation 2-3

Where the Iron (III) hydroxide or ferric hydroxide (yellow boy) is the Acid Mine Drainage (AMD).

iv. *Stage four*: The oxidation of additional iron disulfide (pyrite) by ferric iron (Eqn. 2-4) forms the basis of the fourth stage. This reaction is a self-generating and recurring part of the overall process which occurs very fast and continuous until either pyrite or ferric iron is depleted. In this reaction iron acts as the oxidizing agent and not oxygen.

$FeS_2 + 14Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+} + 2SO_4^{2-} + 16H^+$ Equation 2-4

The overall summary of the reaction for the formation of AMD through pyrite weathering is given below in Eqn. 2-5;

$4FeS_2 + 15O_2 + 14H_2O \rightarrow 4Fe(OH)_3^- + 8H_2SO_4$ Equation 2-5

2.4.2 Treatment of Acid Mine Drainage

The application of traditional method involves the application of limestone and other alkaline agents for the treatment of AMD through neutralization, precipitation of metals and metal hydroxides have not effectively achieved the desired result metal removal from the polluted soil or water and/or solve the problem of sulfate pollution but has contributed to the increase in the pollution of the environment pollution (and possibly recontamination of the sample) through the formation of sludge (Bwapwa, Jaiyeola and Chetty 2017a). The inefficacy of conventional methods prompted the use and advancement of a biological approach for the remediation of AMD polluted sites. Various studied has focused on the application of organic and/or inorganic materials (biostimulation) and the use of microorganism – Sulfate-reducing bacteria, algae, and fungi (bioaugmentation) for the reduction of heavy metals and sulfate concentration.

2.5 Sulfate Reducing Bacteria *(An Overview)*

The application of Sulfate Reducing Bacteria (SRB) technology has proven to be a viable approach for sulfate and metals removal at low pH from the polluted sites. Sheoran et al (2010 cited on Jamil and Clarke (2013) reported that SRB can be efficient in the remediation of AMD with no power but the use of SRB during the cold weather (low temperate regions or winter season) will limit the performance of AMD.

2.5.1 Chemistry of SRB for Sulfate and metal reduction

SRB utilizes organic carbon for the removal of sulfate while synthesizing hydrogen sulfide and alkalinity (Eqn. 2-6) (Jamil and Clarke 2013) for metal precipitation.

$$2H^{+} + 2CH_2O + SO_4^{2-} \rightarrow H_2S + 2H_2CO_3^{-} \qquad \text{Equation 2-6}$$

The reaction above illustrates the reduction of sulfate by SRB using CH_2O as an energy source which results in precipitation of metal in the AMD with a subsequent increase in the pH and alkalinity of the sample. During the treatment process, biogenic hydrogen sulfide (H_2S) reacts with the metallic ions (Me^{2+}) present in the AMD sample to produce metal sulfide (Eqn. 2-7) while the hydroxide ion (HCO_3^{-}) combines with the protons present in the AMD to neutralize the acid water as shown below (Eqn. 2-8);

$Me^{2+} + HS^{-} \rightarrow MeS + H^{+}$ Equation 2-7

$HCO_3^{-} + H^{+} \rightarrow CO_2 + H_2O$ Equation 2-8

At the end of the reaction, it's expected that there will be an appreciable increase in the pH and alkalinity of the sample with an effective reduction in the composition and concentration of sulfate and metals (Jamil and Clarke 2013)

2.5.2 Composition and relationship between SRB and microbial community

The inability of SRB to singly decompose/oxidize complex organic chemicals increases its dependence on the microbial community for the breakdown of these complex sugar (like the case of cellulose; glucose as carbon source) into simpler sugar for SRB consumption and utilization. These microbial supports include sulfate reducers, methanogens, acetogens (Neculita, Zagury and Bussière 2007b). Due to the limitation of fermentation products, the inevitability nature of SRB to compete with the microbial community (methanogens and acetogens) for available carbon source is a function of excess sulfate in the AMD (Jamil and Clarke 2013).

2.5.3 Operational pH for SRB

SRB grows and operates favourably at a pH of 6.8 - 7.2, but a pH of <5 is not suitable for SRB activities and sulfate reduction (Lu *et al.* 2011). However, a pH of 5 – 6 will be optimum to produce hydrogen sulfide and precipitation of metals. Jong and Parry reported that SRB at reduced pH from 6.0 to 4.0 achieved a reduction rate of 553 – 1053molm3/day. Also, SRB has been found to survive in ethanol feed at a low pH of 2.5 but was less effective in the production of alkalinity below pH 3 in AMD concentration (Tsukamoto, Killion and Miller 2004). Invariably a slightly higher pH will ensure effective sulfate and metal removal through metal precipitation and effective degradation of carbon source.

2.5.4 Carbon source for SRB Dehydrogenase

The Insufficient nature of carbon source in AMD required for SRB processes contributes to the urgent need for supplementation with an external/extra carbon source for optimal SRB growth and activities (Kolmert, Wikström and Hallberg 2000). Since sulfate reduction is an energy-

demanding activity, the selection of the right carbon source for the SRB is tantamount to efficiency, cost-effectiveness, and economic viability. However, organic carbon attribute is an indispensable factor that must be considered in the selection of carbon source as it determines the efficacy, eco-friendly nature and promotes the activities of SRB (Gibert *et al.* 2002).

2.5.5 Direct and Indirect supplementation of energy source for SRB

Organic carbon substrate can be supplied to the SRB community either directly or indirectly to augment the existing bacteria. Sulfate-reducing bacteria prefer simple organic substrate as an energy source that can be amended directly or indirectly. Organic source such as sugar, organic acids (lactic, acetic) and alcohol are direct organic carbon source since they can be supplied to the SRB for consumption without decomposition while indirect substrate requires further disintegration (breakdown) before it can be adequately utilized by SRB (Examples include paper, waste, wood, organic compost, and food production by-products). In long term consideration, the indirect substrate method will be suitable (Sheoran, Sheoran and Choudhary 2010) since the mine site is mostly located away from the urban settlement than direct substrate which tends to be easily consumed by SRB even though it makes the carbon source readily available for easy usage (Tsukamoto, Killion and Miller 2004). To achieve a favourable environment for microbial activities using direct carbon source, there a need for constant addition of the organic substrate which will result in high operational cost and maintenance.

2.5.6 Hydraulic Residence Time (HRT)

Hydraulic residence time also known as hydraulic retention time is the measure of average time or duration that a given compound remains in a bioreactor or storage unit. It is denoted by HRT = V/Θ; where v is the total volume of the bioreactor and Θ is the amount of compound (feed or substrate) inside the bioreactor (ml/day) and HRT (ml/days or ml/hours). HRT for sulfate metals to precipitate is approximately 3 – 5 days when using direct organic substrate but indirect carbon source allows for double (of direct source) timing, i.e. 7 – 10 days for the activities of microorganism for metal precipitation in the AMD (Chang, Shin and Kim 2000).

2.5.7 Operational and storage temperature for SRB

Most SRB are known to be mesophilic with storage and operating temperature range of 20 – 45C (68 – 113F), but thermophilic which relatively thrive in temperatures between 45 – 122C and others (psychrophilic and hyper-thermophilic) have been identified. Research showed that SRB can comfortably tolerate a wide range temperature range of -5 to 75C including mesophilic and thermophilic strains (Tang, Baskaran and Nemati 2009). Aside from temperature effect, the operability and functionality is still a function of some indigenous microbial proponents (such as methanogens which are sensitive to low temperature but operate favourably in mesophilic condition for optimal growth) to degrade the substrate into simpler form for SRB consumption (Jamil and Clarke 2013). This suggested that biogenic H_2S production is bound to be seasonal due to temperature variation, where cold temperatures inhibit SRB growth and performance.

2.5.8 Inhibitory effect for SRB

The toxicity of heavy metals such as Zn, Fe, Cu, and Mg are may inhibit the growth and performance of SRB. The lethal concentration range for heavy metal ions to SRB are 2 -50mg Cu/l, 13 – 40mg Zn/l, 75 – 125mg Pb/l, 4 – 54mg Cd/l, 10 – 20mg/l, 60mg Cr/l, 74mg Hg/l. (Utgikar *et al.* 2002; Jamil and Clarke 2013). However, this range of concentration is tantamount to variation depending on SRB strain used for the study. The batch study showed that a high concentration of heavy metals inhibits SRB growth and performance (reduction in sulfate removal ability), and can ultimately lead to the death of SRB (Cabrera *et al.* 2006). The study by Cabrera *et al.* (2006) indicated that the mixed culture of *Desulfovibrio Sp.* tend to withstand higher concentration of heavy metals than single strain (*Desulfovibrio Vulgaris*) and where among the population used for the treatment of AMD, but laboratory test showed that heavy metals (Cu, Cd, and Ni) were toxic to SRB at 20mg/l while Zn, Cr, Pb were less toxic at 25, 60 and 75mg/l respectively (Hao *et al.* 1994)

2.6 Bioremediation of Acid Mine Drainage (AMD)

2.6.1 Biostimulation and Bioaugmentation treatment of AMD

In a study by Freitas, André Homrich Schneider and Schwartzbold (2011) using algae strains for the adsorption of heavy metals in AMD showed the algal biomass ability to accumulate significant concentration of heavy metals from the AMD mostly Fe which constitute 6.3% of the biomass. The site under investigation recorded a similar rate of metal removal/adsorption in descending order of magnitude: Fe > Al > Ca > Mg > Zn > Mn > Cu which are among the range of heavy metals removed by algae as reported by Das et al, 2009. The study also reported the presence of acidophilic species of algae with *Microspora* being the dominant specie among *Eunotia, Euglena, Mougeotia,* and *Frustulia,* can survive in AMD environment with pH ranging from 2.9 to 4.1.

Similarly, the study by Chekroun and Baghour (2013) indicated that individual species of algae showed to be 'hyper-accumulators' and 'hyper-absorbents' with significant selectivity for different metals. The use of algae strains in the treatment of AMD is viable because of its sustainability and tend to remove heavy metals using absorbent and adsorbent mechanism but algae's inability to withstand high concentration of heavy metals and the challenge of yet to identify the algae strain with a high rate of heavy metal intake and adsorption (as not all algae can effectively treat AMD) are drawbacks to the use of algae strain as it limits the treatment process, thereby reducing the sulfate and heavy metals removal efficiency, as most treatment approach using algae is still at the laboratory stage (Bwapwa, Jaiyeola and Chetty 2017a). Seasonality of algae also plays a limiting role to the use of algae treatment strategy, since algae are autotrophic, it requires sufficient light (as an energy source) for photosynthetic process and production of adequate biomass for the absorption and adsorption of heavy metals in AMD (Elbaz-Poulichet *et al.* 2000; Bwapwa, Jaiyeola and Chetty 2017a).

The use of aquatic plants (phytoremediation) are low cost, environmentally friendly and can produce biomass easily for the treatment of AMD contaminated site but its poor tolerance to high toxicity and seasonality are limitations to the use of this treatment approach (Rai 2008; Bwapwa, Jaiyeola and Chetty 2017a). To achieve optimal remediation, this treatment method

requires constant harvest to remove dead plants from the site to avoid posing environmental challenge by recontamination of the site through the release of absorbed materials back to the environment/site by the dead plants, thus making the process too cumbersome, tedious and time-consuming (Rai 2008). However, Sharma, Singh and Manchanda (2015) suggested that phytoremediation will preferably work well as a secondary or tertiary treatment approach for the reclamation of AMD contaminated site.

McCullough and Lund (2011) studied the remediation of acidic and metalliferous Drainage (AMD) through organic amendments by municipal sewage and green waste. The result of the treatment conducted for 145 days revealed that green waste and sewage rendered an optimal treatment with an appreciable pH increase from 2.5 to 5.5 after the study period. This combined application of organic substrate was more effective when compared to the individual application of green waste or sewage. Similarly, (Reuter 1997) reported that the use of the organic substrate, sewage sludge from municipal wastewater was effective for the reclamation of surface mine soil. The ability of an organic substrate to remedy the soil can be attributed to the presence of trace metal, toxic organics, and pathogens, such as bacteria and viruses. It suggested the pre-treatment of sewage wastewater to remove elements of toxics and trace metals (Hg, Ni, Pb, Cr, and Cd) not required for plant growth (Reuter 1997). It can be inferred that the addition and use of organic substrate is a cost-effective substrate for AMD treatment and reclamation of contaminated soil but the availability in large quantity might be a challenge for large scale applications (McCullough and Lund 2011; Reuter 2019). Sewage sludge, according to (Linden *et al.* 1995) contains vital plant nutrients, primarily N & P of approximately, 1-6% total N and 0.1-2% P. Considering the treatment method, sludge also contains ~30% organic matter. The organic matter in sludge is an indispensable constitute to its success as an organic supplement for the restoration of the soil organic content and reclamation of AMD contaminated soil (Linden *et al.* 1995). Sewage sludge served as a viable organic amendment due to its appreciable composition.

The efficacy of using indigenous mixed bacteria culture obtained from mine water and single sulfate-reducing bacteria, SRB species (*Desulfovibrio desulfuricans*) for the remediation of AMD was investigated by (Hwang and Jho 2018). The artificial AMD was prepared with some

portion spiked with Cu and the other with Cu/Zn and placed in a 50ml serum bottle using lactate as a carbon source. The result recorded that the rate of heavy metal and sulfate reduction was higher with *D. desulfurican* which (with Zn spiked) gave 61% heavy metal and sulfate reduction in 48 hours while control without Zn spiked gave approximately 35% sulfate removal. The indigenous mixed microorganism offers a slow efficiency of degradation with 64% sulfate removal over a longer period of 240h – 360h. This significant removal of sulfate at 240h – 360h was noted during the rapid of zinc removal. As the zinc was completely removed, sulfate reduction drastically declined, and no sulfate removal was noticed afterwards due to the unavailability of zinc to form sulfide. This was attributed to (Yue et al, 2015) the extracellular polymeric substances (EPS) release by *D. desulfurican*, which tend to bind heavy metals. It can be inferred that the removal of zinc in the study was possibly the sorption to EPS produced by *D. desulfurican* as bio-sorption and precipitation may be considered the zinc removal approach in this study.

Also, the reduction in zinc and carbon source (lactate) showed a remarkable similarity where the decrease in lactate is directly proportional to the decrease in zinc. Thus, a constant supply of direct carbon sources will increase the microbial population and activities and hence increase the sulfate reduction rate and vice versa (Jamil and Clarke 2013). The zinc spiked showed that the availability of Zn enhances the reduction of sulfate by the formation of metal sulfide, this can be linked to the ability of SRB to utilize sulfate as an electron acceptor and organic substrate (lactate) as an electron donor. However, SRB *(D. desulfurican strain)* tends to be more tolerable to a high concentration of AMD than the indigenous microorganism since SRB reduction capacity reduced from 47% to 20% after 720h while indigenous microorganism exhibits reduction from 36% to 6% within the same timeline during the study. Also, the study on microbial treatment of AMD, Barnes (1998) reported that SRB species *(Desulfovibrio desulfuricans and Clostridium desulfuricans)* possess an appreciably level of tolerance to a high concentration of AMD and were effective for the reduction of sulfate and treatment of AMD.

Similarly, a study by Costa and Duarte (2005) to test the viability organic/inorganic substrate, sludge, and inoculum, SRB (lactate as an energy source) for the remediation of AMD in a semi-continuous process recorded a satisfactory outcome. The result showed that the AMD column

containing solid sewage + sludge + acidic soil and SRB was able to remove 80% sulfate concentration in the AMD and other heavy metals after 170 days; the second column with solid sewage + sludge + acidic soil (for SRB inoculation) and AMD rendered 80% to 90% sulfate reduction and column three containing solid sewage + sludge and AMD gave the highest sulfate reduction of 90% because of the absence of acidic soil which increases the sulfate reduction in the treatment as noticed in columns one and two. Reduction in removal efficiency of sulfate and Fe was observed between 100 – 150 days attributed to the depletion of available energy sources since the biosulfidogenic process facilitated the reduction of sulfate to sulfide and precipitation of metal sulfide by SRB is dependent hinges on the presence of available carbon source to enhance dehydrogenase. Thus, the unavailability of energy source reduces the production of bio-hydrogen sulfide resulting to a decline in biodegradation efficiency (Castro et al., 1999; McConchie, 2003 cited in Costa and Duarte 2005; Jamil and Clarke 2013). The column two treatment was amended with a carbon source (lactate) recorded a significant revitalization in the removal efficiency of sulfate and heavy metals, which signifies the indispensable nature (of continuous addition of appropriate quantity) of direct carbon source for the bioremediation of AMD contaminant using SRB but the increase in pH of the sample may lead to a decline in SRB efficiency with a subsequent reduction in sulfate removal efficiency and can lead to poisoning of the column (Jamil and Clarke 2013).

Doshi (2006) recorded that SRB exhibit seasonal performance with significant sulfate reduction in fall than in spring, thus, low temperature may reduce SRB activities. This was observed in the study to remedy the site (using SRB) that cannot be practically treated with other technologies. The result of the 3-year study period showed that the site treated with an indirect substrate, pulp waste (i.e. pulp amended cell) yielded higher sulfate reduction efficiencies of 65% to 70% (5000mg/l) per year, Fe: 80 – 99.5% and Zn: 99% than the site treated with wood chip amended cell which had a sulfate reduction rate of 500mg/l per year. It suggested that pulp waste is more active as a carbon source for SRB than the wood chip for the removal of sulfate and biodegradation process. Although the indirect substrate used as an energy source for the study is viable for a large scale treatment, the additional time required for the substrate to degrade and bioavailable for SRB consumption could have been reduced if direct carbon source

was used, which is easily available but requires constant addition due to its easy depletion nature (Doshi 2006; Jamil and Clarke 2013).

The remediation of AMD according to Martins *et al.* (2011a) with SRB using a two-way stage bioremediation process; a calcite tailing column and an anaerobic up-flow packed bed reactor with lactate as SRB carbon source. The study conducted for 243 days for a continuous system with 15 days HRT reported that anaerobic bioreactor gave a high reduction efficiency of 99% through biosulfidogenic process than the calcite tailing column. The black ppt. formed on top of the column after 3 days of anaerobic inoculation was suggested to be preceded by growth and activities of SRB in the anaerobic treatment which attributed to a decrease in Redox potentials and enhanced sulfate and heavy metal removal. The use of calcite tailing act as a neutralizing and buffer material to facilitate the biodegradation process according to Martins *et al.* (2011a).

The effectiveness and potency of cow dung, molasses, and sewage sludge as an indirect energy source for SRB in the remediation of AMD were demonstrated by Zdyb, 1999 cited in van den Berg *et al.* (2016). In a bid to provide a suitable anaerobic atmosphere for the SRB growth and activities, anaerobically digested sludge was constructed. The result of the study recorded that sewage sludge and Kikuyu grass recorded the highest sulfate reduction efficiency with 128.5mg/l and 138.25mg/l respectively. The increased performance of Kikuyu was linked to the extra lactic acid carbon source supplemented to aid the breakdown of cellulose to small sugar and cellulose into smaller components (Jamil and Clarke 2013) to enhance the bioavailability of substrate for SRB utilization. Similarly, the use of South African grasses as an energy source for SRB for the treatment of AMD by Ramla and Sheridan (2015) gave 80% sulfate and 99% Fe removal efficiency from 6000mg/l and 2000mg/l of sulfate and Fe initial concentrations respectively with pH increased from 3.0 to 8.0 (optimal) after 70day treatment period.

Greben *et al.* (2009) employed the use of grass cellulose as a carbon source for the biomass of rumen fluid microorganism for the treatment of rich mine effluents. High sulfate removal (78-86%) was also recorded with the removal of some metals like Fe in the process. The study reported that the use of grass cutting can be a productive and cost-effective carbon source for

AMD remediation (Greben *et al.* 2009). Column and batch studies showed that cellulose hydrolysis inhibits H_2S production by SRB (required for the precipitation and removal of heavy metals) when liable carbon source is absent (Logan *et al.* 2005). In the same vein, Dill (2001) recorded an appreciable sulfate reduction of 97.8 and 99% with Kikuyu and hay as an indirect carbon source for AMD remediation respectively. Sequel the cellulose hydrolysis as stated above, an indirect substrate (Kikuyu and hay grass, South African grasses, grass cuttings) which contain a large percentage of cellulose can cause interference/distortion of SRB activity with cellulolytic bacteria, occurring at long treatment period, since cellulolytic bacteria are active at pH > 6, extra buffering may be needed to utilize this substrate to prevent the occurrence of cellulose hydrolysis (Logan *et al.* 2005).

Vadapalli *et al.* (2008) studied the neutralization of AMD using fly ash and the strength development of resulting solid residues. The study recorded that the addition of fly ash amendment (collected from local power stations) triggered the increase in pH with a corresponding decrease in the electrical conductivity (EC) value of the sample. The reaction was attributed to the hydrolysis and precipitation of AMD constituents; Al and Fe and subsequent absorption of precipitate upon ash particles (Logan *et al.* 2005). The reductions of sulfate (at 90% reduction rate) and heavy metals were feasible upon the FA/AMD contact in the sample. The main principle that coordinated the treatment of AMD using FA were precipitation, co-precipitation, and adsorption at the surface of the FA particles and precipitating metals. Also, the potency of FA does not require pre-treatment before application according to Vadapalli *et al.* (2008).

Also, Zdyb (1999) studied the efficacy of different carbon sources (lactic acid, butyric acid, and acetic acid) for SRB in AMD treatment. The experiment was conducted in four anaerobic bioreactors with 1000ml/l synthetic AMD mixed with SRB and carbon sources and the controlled experiment received only the SRB inoculum without carbon source. The result of the 4 weeks study recorded that lactic and butyric acid were more effective for sulfate removal. The performance of acetic was below the other two since acetic is known to be a sub-optimal carbon source, and varieties of SRB cannot directly synthesize acetic, thus requires a further breakdown to ensure bioavailability. 90% sulfate removal efficiency was achieved by lactic acid in day 11

while it took butyric acid 15 days to attain the 90% sulfate reduction efficiency and the lowest reduction efficiency was recorded by the controlled experiment which can be attributed to lack of energy source. It can be inferred that lactic acid is an excellent energy source for the growth and dehydrogenase of SRB in the treatment of AMD (Zehnder 1988; Qatibi, Bories and Garcia 1990). It can be inferred that lactate is the energetic carbon source for SRB growth and metabolism (Luptakova and Macingova 2012). Hence, supplementation of SRB with nutrient (energy or carbon source) will be effective for sulfate reduction and treatment of AMD (Chang, Shin and Kim 2000).

The use of municipal waste as an energy source has been studied to show its efficacy in the acid mine drainage (AMD) treatment. Bioremediation of AMD coupled with domestic wastewater remediation was studied by Sanchez-Andrea, Triana and Sanz (2012). In the study, domestic wastewater was used as a low-cost carbon source for the treatment of AMD contaminants. Three reactors with different microbial support and domestic wastewater/AMD loading ratio of 1:10 (v/v), amended with an extra energy source. A higher biodegradation efficiency of 85% was recorded for sulfate and heavy metal reduction as a result of additional carbon source; with COD – 88%, sulfate > 75%, Fe > 85% and other dissolved metals > 99% (except for Mn) through co-precipitation of metals from AMD (Costa and Duarte 2005), with fermentative and reducing bacteria – *Desulfovibrio spp, Desulfosporosinus, Clostridium spp.* noted as the most abundant species present during the treatment (van den Berg *et al.* 2016). Also, Martins *et al.* (2011b) and van den Berg *et al.* (2016) noted similar bacteria (*Desulfovibrio spp, Clostridium spp, Desulfitobacterium sp. and members of Bacteroides order*) as active participants in the remediation of AMD using up-flow anaerobic packed bed system (UAPB). High metal (Zn, Cu, & Fe) and sulfate removal were recorded with 72 – 99% and >72% efficiency respectively with pH increase from 2.5 to 7.1 after 183 days incubation period according to Martins *et al.* (2011a).

Similarly, in the justification of the efficacy of remediation of AMD using co-treatment with municipal wastewater, Strosnider *et al.* (2013) reported appreciable removal efficiencies of 99.8, 87.8, 97.7, 99.8, 13.9, 87.9 and 73.45% for Al, As, Cd, Fe, Mn, Pb, and Zn from the initial concentration of 46, 0.25, 2.0, 290, 55, 1.2, and 390mg/l respectively with pH increase from 2.6 to 6.79 after the 135 days treatment period. The removal of heavy metals in the process was

linked to precipitation and co-precipitation as metal sulfides. Cd, Cu, Fe, Pb, Hg, Ni and Zn are among the metals that precipitated as metal sulfides and Mn and some of these metals may also be removed by co-precipitation with metal sulfide (Logan *et al.* 2005). The study recorded the unchanged nature of Mn throughout the system until the pH increased (>6) which resulted in oxidation and hydrolysis with Mn reduction of 13.6%. The low reduction efficiency of the Mn which is sensitive to high toxicity was also reported by Hughes and Gray (2013) that a high concentration of AMD limits the removal efficiency of Mn and other metals. Similarly, Mn concentration remains unchanged in the treatment in the Kaldnes stage but 20.6% of reduction of dissolved Mn was observed with treatment using limestone. However, the formation of Manganese carbonate ($MnCO_3$) was suggested as the removal mechanism of Mn in AMD treatment using carbonate (Waybrant, Blowes and Ptacek 1998; Bamforth *et al.* 2006). It can be inferred that AMD and MWW co-treatment is an efficient, cost-effective approach for wastewater management and AMD treatment (Strosnider, Winfrey and Nairn 2011; Hughes and Gray 2013). However, recent research showed that Mn and Zn can be efficiently removed were the acidity is not limited to a particular range value; as tertiary treatment with alkali and carbonate will be apt to achieve the required removal efficiency of these metals after co-treatment of AMD (Edenborn and Brickett 2002).

The low removal efficiency recorded by different treatment approaches prompted the study by Silva et al. (2012) in the remediation of high-manganese AMD with limestone and sodium carbonate. The result indicated that manganese can be effectively removed through metal carbonate precipitation (Waybrant, Blowes and Ptacek 1998; Bamforth *et al.* 2006) as manganese carbonate was detected in net alkaline mine water. The study reported that pH and carbonate concentration as factors that can affect the removal of Mn from mine water. The Mn removal efficiency was 99% after the 2 weeks study period achievable with carbonate ions provided the pH within the alkaline range (preferably, above 8.5) (Silva *et al.* 2012).

In contrast to low Mn & Zn removal, the study by Hughes and Gray (2013) recorded high removal efficiency in the co-treatment of AMD with MWW using three treatment processes viz; addition of synthetic raw (untreated) AMD (containing a mixture of Al, Cu, Fe, Mn, Pb, Zn and SO_4 at a range of concentration and pH) to the activated sludge aeration tank; pre-treatment

of AMD before addition to the aeration tank by mixing with activated sludge; pre-treatment of AMD in combination with MWW. The result of the 40-day study recorded that thee continuous AMD loading into the system did not affect COD reduction, TOC removal, and BOD. Average COD removal was 87-93% in the processes. It also reported similar heavy metal removal efficiency for laboratory-scale plug flow treatment and sequencing batch reactor system in the course of AMD and wastewater co-treatment with 52-84%, 47-61%, 74-86% and 100% removal efficiency for dissolved heavy metals Al, Cu, Fe, and Pb respectively except for Mn and Zn whose removal was linked to acidity (pH < 10) but removal from circum-neutral pH was 93-95% for Mn and 58-90% for Zn. Similarly, the study by Deng and Lin (2013) reported high Mn removal efficiency among other metals using two-stage remediation of AMD and Municipal wastewater (MWW) which involved the batch mixing of the two wastes in pH of 6.2 – 7.9 and COD/Sulfate concentration of 0.05 – 5.4 to condition the mixture solution followed by anaerobic biological treatment. The result noted that anaerobic reactor was appropriate for AMD treatment (Zdyb 1999) and electronic microscopy suggested adsorption and co-precipitation of heavy metals (Costa and Duarte 2005) as the removal approach of this mechanism with varying biodegradation efficiencies; Fe > 99%, Al ~ 100%, Mn 75 – 100%, Ca 52 – 81%, Mg 13 – 76% and Na 56 – 76%. The removal of Mn and Zn is poor with activated sludge together with Cu, Cr, Pb and Cd (Oliver and Cosgrove 1974 cited in Hughes and Gray 2013 Chang *et al.* 2007) which can be attributed (for Mn) to the uncatalyzed oxidation of soluble Mn(II) to insoluble Mn(IV) which is sensitive to high acidic concentration and tend to occur at pH > 9, giving Mn a solubility over a wide range of pH (Stumm and Morgan 1970; Brezonik 1994), also, hydroxide precipitation of zinc is feasible at pH > 8 and intolerable of low pH concentration (Katsou, Malamis and Loizidou 2011). This suggested that use of co-treatment of AMD and MWW showed a promising result for the reduction of Zn & Mn to enhance treatment of AMD and municipal wastewater and enhance waste management.

The use of domestic wastewater sludge as an energy source for bacteria inoculum in the anaerobic treatment of AMD and identification of bacteria community present in AMD was studied by van den Berg *et al.* (2016). The study conducted with and without biofilm entailed a homogenous combination of synthetic wastewater and AMD in the ratio of 1:1 and observed for 30 days at a temperature of 25C in a dimly lit environment. The preliminary study by van

den Berg *et al.* (2016), reported that 1:1 (AMD:SDWW) was the optimum ratio for the mixture of AMD and SDWW for the sulfate and COD removal to enhance AMD treatment purpose. The result indicated that the transparent bioreactor in a dimly-lit environment encouraged the growth of microbial community (Hurse and Keller 2004) and removal of sulfate with *Chlorobium Spp* dominating the microbial population in the bio-environ among other species *(Magneto spirillum spp and Ornithobacterium spp*) which also have appreciable population. The study which observed the sulfate removal efficiency showed that treatment with and without biofilm gave approximately similar result with 96% and 58% corresponding to 905.33mg-SO_4/L and 757mg-COD/L respectively while treatment without biofilm gave 96% (674.3mg-SO_4/L) and 60.8% (827mg-COD/L) removal efficiency after 26 days period. Low sulfate reduction was recorded when the incubation temperature was lowered to 17^{o}C and 19^{o}C from the 25^{o}C room temperature. Thus, a lower temperature is not optimum for the growth of sulfate-reducing microbes for sulfate and COD removal (van den Berg *et al.* 2016). The use of domestic wastewater is effective for AMD treatment and can facilitate sulfate and heavy metals removal, thereby limiting the waste of extra resource for the remediation purpose (Davison *et al.* 1989; Strosnider, Winfrey and Nairn 2011; Hughes and Gray 2013).

The study by Davison *et al.* (1989) on the efficacy of domestic sewage sludge in the reclamation and treatment of abandoned sand quarry filled with acid water of pH 3 from pyrite oxidation was treated with calcium hydroxide to neutralize the water to pH 8 and sewage sludge to prevent the further inflow of acid. The treatment recorded appreciable capacity of sewage facilitated sulfate reduction and removal of heavy trace metals through neutralization within the two-year study period. The production of acid was restored after 2 years, due to the depletion of organic substrate (sewage sludge) and the shallow depth covered by the treatment.

Bai *et al.* (2013) evaluated the remediation of AMD using a mixed culture of SRB (from activated sludge of wastewater) and lactate as an organic carbon source. The activities of SRB were noticed through the black precipitate visible on the top of the sample in the bioreactor. The outcome of the 35 days processes recorded sulfate reduction from 20800 mg/l to 8200 mg/l (approximately, 61% removal efficiency) and heavy metals: Cu, Fe, Mn also recorded removal efficiencies of 99%, 86%, and 53% respectively as the pH increases from 2.75 to 6.20 during

the study period. However, Bai *et al.* (2013) suggested that increased removal efficiency of heavy metals can be increased with longer HRT. Similarly, the potency of the mixed culture of SRB was authenticated in the remediation of AMD was investigated by (Luptakov*a* and Kusnierova 2005). The experiment was conducted using genera *Desulfovibrio* and *desulfotomaculum* (mixed SRB culture) from different sources, (wastewater collection tank (SRB-VSZ) and (SRB-GJ) potable mineral water) with a model solution containing Cu prepared from copper sulfate with the pH adjusted using sulfuric acid. The result of the experiment showed 98-99% removal of copper from the liquid phase after 5-6days of the treatment. An increase in activities of the SRB was prevalent as black – brown precipitate was formed on the top layer of the sample and subsequently, a rotten egg odor of H_2S was perceived as sample was taken from liquid phase in the bioreactor which was not observed in the abiotic controlled experiment. Sulfate was reduced from 1.82mg/l to 0.89/l with pH increased from 7.6 to 8.9. Sulfate reduction also reduces acidity, thereby raising the pH which enhances the precipitation and removal of metals from the sample (Gadd 2004). It can be suggested that mixed SRB culture collected from different sources are equivalent and can effectively reduce sulfate and heavy to facilitate AMD treatment (Luptakov*a* and Kusnierova 2005). The *Desulfovibrio* SRB species are exceptional from other bacteria because of the presence of *desulfoviridin*, they oxidize their energy source as acetate, thus excrete as the end product and uses organic acids or alcohols (lactate, pyruvate or hydrogen) as the electron donor for sulfate removal. Their ability to grow easily in sulfate lactate medium in the absence of oxygen makes them viable for the anaerobic treatment of AMD with recent research reporting the existence of many species with the *Desulfovibrio* specie (Heidelberg *et al.* 2004).

Table 2-2. Bioremediation of AMD using different approaches.

BIOREMEDIATION METHODS	ADDITIVES/ CARBON SOURCE	CONTAMINANT	DURATION	SULFATE AND/OR HEAVY METAL REMOVAL EFFICIENCIES	INFERENCE	REFERENCE
BST	Municipal sewage and green waste	AMD	145 days	Sulfate 75% and heavy metals: 45 – 85%	pH increase and effective removal of sulfate	(McCullough and Lund 2011)
BST	Sewage sludge from Municipal wastewater	AMD	2 – 3 years	Enhanced sulfate removal	Pre-treatment of sewage sludge to reduce trace metals not needed by the soil for plant growth is necessary before application as sewage was a viable organic amendment	(Reuter 2019)
BST & BAU	Acidic soil, sludge, and sewage sludge and lactose	AMD	170 days	80 – 90% sulfate	Combined treatment using Sludge and sewage was efficient	(Costa and Duarte 2005)
BST & BAU	SRB with pulp waste and wood chips	AMD	3 years	Sulfate: 65 – 70% (5000mg/l per year); Fe: 80 – 99.5%; Zn: 99%	Pulp waste substrate is more effective	(Doshi 2006)
BST	Calcite tailing	AMD	243 days	Sulfate: 99%	Precipitation of heavy metals observed	(Martins *et al.* 2011a)

BST	Municipal wastewater (MWW)	AMD		Fe > 95; Al ~ 100%; Mn: 75-100%; Ca: 52-80%; Mg: 13-76%; and Na: 56-76% Sulfate: >80%	Indicate great potential as an alternative mechanism for the remediation of two wastes (AMD & MWW)	(Deng and Lin 2013)
BST & BAU	Mixed culture of SRB from the wastewater collection tank and potable mineral water	AMD	6days	98 – 99% Cu	Mixed SRB culture is viable for AMD treatment. The sulfate-reducing ability of SRB from industrial and natural sources are equivalent	(Luptakova and Kusnierova 2005)
BST	Domestic wastewater	AMD		COD: 88%; sulfate > 75%; Fe > 85% `; other dissolved (metals except Mn) > 99%	Sulfate-reducing bacteria were noted to have facilitated sulfate removal and metal precipitation	(Sanchez-Andrea, Triana and Sanz 2012)
BST	Cow manure, molasses (Kikuyu), and sewage sludge	AMD		Sulfate: 125mg/l/d – 135.25mg/l/d	Highest sulfate removal efficiency recorded Sewage sludge and Kikuyu grass	Zdyb, 1999; Dill et al, 2001
BST & BAU	lactic acid, butyric acid, and acetic acid	AMD	11 – 15 days	90% sulfate	Lactic acid was most efficient while acetic gave the least removal efficiency	Zdyb 1999
BST	SRB and South African grass as a carbon source	AMD	70 days	Sulfate: 80% and Fe: 99% removal	Increased pH from 3.0 t0 8.0	(Ramla and Sheridan 2015)

				efficiency from 6000mg/l and 2000mg/l initial conc. respectively		
BST	Limestone and Sodium Carbonate	High Mn mine water	2 weeks	Mn: 99.9%	Carbonate concentration and pH controls the Mn removal efficiency as the treatment was more effective	(Silva *et al.* 2012)
BST	Activated sludge and MWW	AMD	40 days	Cu, Al & Fe; 90 – 100%; Zn: 65 - 100%; Mn: 60 – 75%	MWW was more effective in heavy metal removal than activated sludge	(Hughes and Gray 2013)
BST	MWW	AMD	135 days	Al: 99.8%; As: 87.8%; Cd: 97.7%; Fe: 99.8%; Mn: 13%; Pb: 87.9%; Zn: 73.4%	Low removal efficiency of Mn can be attributed to high acidic concentration	(Strosnider, Winfrey and Nairn 2011)
BST	Synthetic domestic wastewater	AMD	90 days	Sulfate: 96% (674.3mg/l - 905.33mg/l) ; COD: 58 – 60.8% (757mg/l – 827mg/l)	The approach is viable for the co-treatment of domestic waste and AMD with chlorobium spp. dominating the microbial population	(van den Berg *et al.* 2016)
BAU	*P. Aeruginosa* AT18	Heavy metals	72 hours	Cu (86.95%); Cr (99.6%); Zn (87.7%);	Metal removal a factor of pH of the treatment system	(Silva *et al.* 2009)

				Mn (21.69%)		
BAU	*P. Aeruginosa* BS2	Heavy metals	3 days	86 – 96% for Cu, Pb, Cr, Cd, and Ni	The result showed that rhamnolipid biosurfactant from *P. Aeruginosa* was able to respond positively to contaminated soil by effective reduction of metal concentration	(Juwarkar *et al.* 2008)
BAU	*P. Aeruginosa* KUCd1	Heavy metals	96 hours	76 - 89% Cd removal	*P. Aeruginosa* KUCd1 showed survival in wastewater with or without supplementation with extra nutrient	(Sinha and Mukherjee 2009)
BAU	*P. Aeruginosa*	Heavy metals	24 - 72 hours	79.1% at 48hrs, 52.4% at 72hrs for Cu and Zn respectively and 61% at 24hrs for Fe; 41.6% at 72hrs for Cr	*P. aeruginosa* reaction to different concentration of heavy metal with varying intake levels with relative to time and change in biomass	(Awasthi *et al.* 2015)
BAU	*P. aeruginosa*	Metal (Al)	24hrs	80%, 97%, 70%, and 90% of Al at different eluents	The different recovery rate of Al was recorded with different eluents and rate increases with an increase in biomass and	(Tuzen and Soylak 2008)

					absorption of Al were pH-dependent	
BAU	*Pseudomonas putida* (CZ1)	Metals		87.2% of Cu and 99.8% of Zn	Based on heavy metal toxicity, Cu toxicity was higher than that of Zn.	(Chen *et al.* 2006)
BAU	*P. Aeruginosa* and *B. Subtilis*	Salts (Sulfates and Nitrates)	15 days	95%, 88%, 96%, 83%, 97.3%, 61.3%, 62.8% and 61.2% for TS, SS, DS, COD, BOD, Nitrate, Sulfate, and Phosphates respectively	The removal efficiency recorded showed that the application of immobilized mixed culture was effective	(Ajao, Adebayo and Yakubu 2011)
BAU`	SRB, *Desulfomicrobium* sp., *Pseudomonas aeruginosa*, and *Sulfurospirillum* sp., *Sulfurovum* sp. and *Paracoccus denitrificans*	Sulfate and Nitrate	10 days	80% and >99% sulfate and nitrate reduction respectively	At high concentration of sulfate and nitrate, heterotrophic denitrifier (*P. Aeruginosa)* outcompete autotrophic denitrifiers to reduce SRB activities, hence heterotrophic denitrifiers utilize sulfate and nitrates as electron acceptors	
BST & BAU	Rumen fluid microorganism and Grass cellulose as a carbon source	AMD	4 weeks	78 – 86% sulfate	Fermentation product of cut grass can be used as an energy source for sulfate removal	(Greben *et al.* 2009)

BAU	SRB and lactate as carbon source	AMD	35 days	Sulfate: 61% (from 20800mg/l to 8200mg/l); Fe: 86% (from 545mg/l to 75mg/l); Cu: 99%; Mn: 53%	The pH increases from 2.75 to 6.20 showed gradual detoxification of the sample as sulfate and heavy metals are removed	(Bai *et al.* 2013)
BST & BAU	SRB + Calcium tailing and wine waste composting	AMD	226 days	Sulfate: >90%; Fe: 61-91%; Cu & Zn: 97%	Calcite tailing can contribute to the improved sulfate and metal removal efficiency	(Costa *et al.* 2009)
BAU	SRB	AMD	183 days	Sulfate: >72%; Fe, Zn & Cu: 72 – 99%; Mo & Co: 35-45%	Bacteria such as *Desulfovibrio spp, Clostridium spp, Desulfitobacterium sp. and members of bacteroidales order* were identified as active AMD degrading bacteria	(Martins *et al.* (2011b)
BST	Emulsified soybean oil (edible oil substrate) as an energy source	AMD	300 day	75% Sulfate; Al, Cu, and Zn: 99%	Emulsified oil substrate is a promising substrate as it does not pose an inhibitory effect to the microbes	(Lindow and Borden 2005)
BAU	Sludge inoculum and ethanol as carbon source	AMD	130 – 150 days	75% sulfate and COD	The maximum removal of sulfate and COD can be achieved by the reduction in sulfate load; pH increased from 6.8 to 7.0	(Rodriguez *et al.* 2016)

BST - Biostimulation; BAU – Bioaugmentation; BVT – Bioventing; BAT: Bioattenuation

2.7 *Pseudomonas aeruginosa* as a bioremediation tool

The genus *Pseudomonas* is summarily described in terms of phenotypic and genomic features of its member species and can utilize of varieties substrates (organic and inorganic), survive different environmental conditions and may grow in simple media, and their nutritional flexibility enables them to survive in contaminated environs which may be toxic to other bacteria as found in *Pseudomonas* studies. These characteristics suggest *Pseudomonas a*s a viable agent for bioremediation purpose (Moore *et al.* 2006).

To improve the rate of biodegradation of hazardous organic compounds in the terrestrial and aquatic environment, microorganism application has proven to be effective in reducing toxic material concentration so far. However, the study revealed that microorganisms that exhibit or show chemotaxis towards the environment tend to perform better than non-chemotaxis organisms that attribute different degrading microbes to variation in the biodegradation efficiencies. It has been recorded that oil-degrading microorganisms like E, Colin Salmonella, *Pseudomonas Aeruginosa, P.Putida, Bacillus Cereus, Myxococcus sp., Rhizobium and Azopirillium sp.* have some appreciable chemotaxis behaviour which contributes to their performance in the bioremediation treatment (Samanta, Singh and Jain 2002).

P. Aeruginosa is a gram-negative bacterium, gammaproteobacterial, aerobic, rod, and family Pseudomonadaceae that can withstand heavy metals such as copper, cadmium, chromium, nickel (Awasthi *et al.* 2015). The deposition of metals in soil in high concentration is undesirable for plant growth and development due to its non-biodegradable nature. This has made bioremediation of heavy metals a difficult process. Some bacteria have special morphology and can absorb/accumulate metals on their cells (Nagashetti *et al.* 2013). Because of its abundance, availability on earth, cost-effective and eco-friendly nature, the microbe is suitable for remediating metal-polluted soils. Some of these bacteria have been used in the bioremediation process to treat heavy metals and many of these bacteria that have proved to be active in the treatment for bioremediation include the organisms *Pseudomonas, Bacillus, Escherichia, Micrococcus, and Streptomyces*. They develop in the presence of heavy metals by rendering metal binding (Srivastava *et al.* 2018) with functional groups and metal chelating agents present on the cell wall.

Chemotaxis is a systemic, complex mechanical process whereby bacterial cells detect significant (low or high) concentration changes and respond behaviourally to the change, then adjust to tolerate or adapt to the new change in chemical stimulus concentration. Reaction to this situation differs depending on microorganisms where the chemotaxis may be reacted positively when the microorganism moves in the direction or absorbs the compound or moves away or repelled by the compound when it responds negatively to the circumstance. With the above, the response to chemotaxis includes attractant or repellent concentration gradients. Some organisms like *Pseudomonas sp.* are chemotaxis, which accounts for their effectiveness in the bioremediation process (Samanta and Jain 1999).

2.7.1 Biodegradation of Hydrocarbons using *P. aeruginosa.*

Glycolipids are biosurfactants of low molecular weight, to which hydrocarbons bind to long-chain aliphatic acids or lipopeptides. Glycolipids such as rhamnolipids, sophorolipids, trehalose lipids are disaccharides that have long-chain fatty acid acylated. Among the glycolipids known are the most versatile and studied rhamnolipids developed by the species *Pseudomonas* – consisting of two moles of rhamnose and two moles of ß-hydroxy-decanoic acid (Lang and Willbrandt 1999 cited in Ron and Rosenberg 2002). Lang and Willbrandt (1999) noted that rhamnolipids would individually reduce the water surface tension from $72mNm^{-1}$ to 25 – $30mNm^{-1}$ at a concentration of 10 – 200mg / L^{-1}.

2.7.1.1 Biosurfactant Production

Biosurfactants are synthesized during the growing time when they enter the stationary growth stage. Biosurfactant emulsifier production has caused cell proliferation and represents the stationary stage of growth (Brint and Ohman 1995). Since these species may use crude oil as an energy source and at the same time degrade selected fractions of hydrocarbons and become hungry once hydrocarbon range has been depleted (Bezza and Chirwa 2015).

In respect to orientation, the cell surface of the microbial cell may be more hydrophobic if the biosurfactant is cell-bound. This is evident in Pseudomonas aeruginosa, where the hydrophobic aspect of the cell surface is greatly enhanced by the existence of cell-bound rhamnolipids – in

contrast to Acinetobacter, where the cell surface is decreased by the existence of cell-bound emulsifiers (Rosenberg, Gottlieb and Rosenberg 1983). It can be inferred that microbes can use the biosurfactant they generate to curb their cell surfaces, characteristics, connect or remove themselves from the surface according to their requirements.

Rosenberg, Gottlieb and Rosenberg (1983) proved this in their analysis where bacteria degrading oil *A. Calcoacticus RAG-1,* which uses n-alkenes as a carbon source for growth and metabolic activity, suffered malnutrition as hydrocarbon options were reduced while oil droplets are still abundant in aromatics and cyclic paraffin. The famine prompted the production of emulsan mini capsule. Such emulsifiers, emulsion releases starving cells from the depleted oil droplet of n-alkanes by forming polymeric films around the depleted droplet. The depleted oil droplet is classified as empty (exempt from n-alkanes) energy source as the cell is desorbed. The emancipation of the cells from the depleted oil droplets motivates them to search for new oil droplets or nutrients. Oil droplet now has a hydrophilic outer surface after depletion which makes it difficult for the bacterium to bind or bond to any used droplets. Bacteria detachment from depleted oil droplets by emulsifiers increases dehydrogenase and enhances bacteria's free movement in a bid to mobilize the necessary fraction of hydrocarbon for an energy source. Therefore, this cycle improves the productivity of biodegradation and facilitates the bioremediation of polluted areas with hydrocarbons (Ron and Rosenberg 2002).

2.7.1.2 The use of Biosurfactant in Bioremediation of Contaminated Soils

There are two methods or mechanisms adopted by biosurfactant developed by *Pseudomonas aeruginosa* in the treatment of soils polluted by hydrocarbons (Ron and Rosenberg 2002), which includes increasing hydrophobic water surface area-insoluble substrate and enhancing the bioavailability of hydrophobic compounds

i. Increased hydrophobic water surface area-insoluble substrates.

The rate of growth of oil reducing hydrocarbon bacteria may be influenced by the interface surface area that exists between water and oil. The limitation of the surface area can result in arithmetic, rather than exponential biomass production. Emulsification is a cell density-

dependent occurrence, i.e. increased cell numbers increase extracellular products. The concentration of cells in an oil open system like the hydrocarbon polluted environment of water was never sufficient to solubilize the oil. Any solubilized oil is dispersed in water which attributes to its bioavailability to the emulsifying producing strains and the indigenous competing microorganisms. Emulsifiers do not actively participate in the biodegradation process, rather they produce an enabling environment for the degradation of hydrocarbon by producing macroscopic emulsion in the bulk liquid (Ron and Rosenberg 2002)

ii. increased bioavailability of hydrophobic compounds

Bioavailability of hydrocarbon fractions, particularly PAH, depends on solubility, as low solubility tends to reduce the availability of these fractions of hydrocarbons to degrading bacteria. These pose a grave challenge to the successful degradation of hydrocarbons. The poor solubility (which increases surface sorption) of high molecular hydrocarbons is due to their recalcitrant existence-resulting in substrates being restricted to degrading bacteria. This is because bonding organic molecules to surfaces prevent biodegradation. Biosurfactant increase growth rate on the attached substrate by desorbing them from the surface or by enhancing their apparent water solubility (Ron and Rosenberg 2002; Ganesh and Lin 2009; Lutsinge 2018)

Biosurfactant's stability, eco-friendly, and selectivity nature accounts for its effectiveness as the chemical and synthetic surfactant, by increasing the bioavailability of the hydrophobic compound for the hydrocarbon bioremediation process. In mobilizing insoluble molecules and ensuring their availability for bioremediation, surfactants that can conveniently reduce the interfacial are effective. Biosurfactant (emulsifiers) may serve as a substratum (additive) to facilitate the process of biodegradation when produced by microbes. It is possible to introduce bacteria that are capable of overproducing bioemulsifiers which can diffuse in the soil or transfer to bacteria in close contact while participating in biodegradation ((Ron and Rosenberg 2002).

Bioaugmentation (use of microorganisms) can be affected by temperature. Temperature influences the rate of degradation of crude oil in the soil. At low temperatures, oil viscosity tends to increase, and the degradation of alkane decreases significantly as water solubility decreases. With increased temperature, between 30-40^{0}C and optimally within 30^{0}C at pH 7.5,

crude oil is less viscous, dehydrogenases increase as degradation of crude oil is facilitated. At the temperature above 40^0C, the toxicity of crude oil may be experienced which adversely affects microbial activities and reduces the biodegradation rate (Banat 1995; Rahman *et al.* 2002). Since *Pseudomonas Aeruginosa* is mesophilic, thus, tends to adapt and perform optimally at temperature 30^0C (Suguira et al 1997; Rahman *et al.* 2002). However, a high concentration of crude oil is toxic to microbial growth and can lead to the death of microorganisms resulting in distortion of the bioremediation process. Hence, crude oil reduction is inversely proportional to the concentration (Rahman *et al.* 2002), invariably, at a lower concentration of crude oil, *Pseudomonas Aeruginosa* will be more active as a result of a high rate of metabolism. The effect of concentration was evident in the study by Rahman *et al.* (2002) which reported a decrease in biodegradation efficiencies, 70%, 67%, 63%, 52% as the oil concentration increases from 2.5%, 5%, 7.5% to 10% respectively.

The study by Karamalidis *et al.* (2010) on the treatment of petroleum polluted soils involving stimulation of indigenous microorganisms and combined stimulation & inoculation with *Pseudomonas aeruginosa strain* showed varying degrees in hydrocarbon concentration in different treatments. The treatment of 191 days recorded for the indigenous cells; 94% of n-alkane degradation from 8025mg/kg to 481mg/kg at t=191d; total aliphatic fractions decreased by 89% on similar treatment period (from 17780mg/kg at t = 0d to 1951mg/kg at t = 191d). Also, combined stimulation and *Pseudomonas aeruginosa* inoculation showed a reduction in recalcitrant hydrocarbon fraction with 20% and 70% PAH biodegradation efficiency (after 35 and 150 days respectively) from the initial concentration of 58mg/kg to 17mg/kg at the end of the treatment. Degradation of n-alkanes by stimulated indigenous microbes recorded increased in reduction efficiency with corresponding to an increase in time to attain 82 – 98% for n-C_{12} to n-C_{27} after 107 days and >82% for n-C_{28} to n-C_{34} after 191d. The overall biodegradation efficiency according to Karamalidis *et al.* (2010) was observed to be 73.3%. It reported that bioremediation with free cells or encapsulated *Pseudomonas aeruginosa* has little effect on the treatment performance, but It will be noted that introduction of *Pseudomonas aeruginosa* inoculum effectively reduces the treatment time and facilitate the removal of recalcitrant hydrocarbons (Karamalidis *et al.* 2010)

Microbial biodegradation of resins fractionated from Arabian light crude (Venkateswaran and Harayama 1995) using *P. aeruginosa* isolated from emulsified mixed population recorded 30% and 30% of resin and aromatics respectively from 5000ppm concentration of crude oil after 7 days of treatment. It reported an increased *P. aeruginosa* growth rate while degrading fractions of hydrocarbons. Similarly, the study by Mukherjee *et al.* (2010b) further buttresses the ability of *P. aeruginosa* to grow in oil-polluted soils as earlier reported by Venkateswaran and Harayama (1995), while using selected fractions of hydrocarbons as a carbon source.

The degradation of hydrocarbon was investigated (Mukherjee *et al.* 2010a) using bacteria strains isolated from an oil field and cultured in a mineral media and hydrocarbon enrichment environment containing apt proportions of benzene, toluene, hexadecane, tributyrin, and glucose as an energy source for bacteria growth. The study observed, through bacteria identification that *P. aeruginosa* was the most versatile and popular bacteria strain in the isolate among other bacteria; *Acinetobacter spp. Flavobacterium multivorum* and *Flexibacter condensin* identified. The study reported the ability of *P. aeruginosa* strain PTZ-5 from the oil field to effectively utilize various fractions of hydrocarbons: hexadecane, benzene, and toluene as an energy source for growth while facilitating the remediation process of the hydrocarbons. The result reported the ability of *Acinetobacter calccoaceticus* ADPT to grow on hexadecane and not in alkane which suggests the inability of *Acinetobacter sp.* to perform in some hydrocarbon components (Sugiura *et al.* 1997). In contrast to the performance of *Acinetobacter calccoaceticus* in the treatment, *P. aeruginosa* (PTZ-5) was tolerant of various hydrocarbon concentrations and showed great potential in the removal of hydrocarbons (Mukherjee *et al.* 2010b).

Tavassoli *et al.* (2012) evaluated the degradation of asphaltene using microorganisms isolated from crude oil samples. Among the isolated and identified strains based on their morphological and biochemical characteristics is *Pseudomonas spp. TMU2-5, Bacillus licheniformis Tmu1-1, B. Lentus TMU5-2, Bacillus cereus TMU8-2, and Bacillusfirmus TMU6-2.* Biodegradation of asphaltene was highest with the mixed culture which recorded 48% removal efficiency when compared to pure cultures; *Pseudomonas spp.* with a 46% removal efficiency, degrading mostly branched alkanes, phenol, naphthalene, and acetone (Sugiura *et al.* 1997; Mukherjee *et al.*

2010a) and *Bacillus spp*. was effective in the degradation of benzene and PAH. It can be suggested that *P. aeruginosa* and *Bacillus spp.* can be effective in the clean-up of polluted sites by constant biodegradation of HC. (Mukherjee *et al.* 2010b). Similarly, the asphaltene degradation was investigated by Pineda-Flores *et al.* (2004) using of mixed culture of bacteria consortium with and without *P. aeruginosa* was investigated for the potency of each consortium. Biodegradation of asphaltene with a mixed culture (without *P. aeruginosa*) comprising of *Bacillus, Brevibacillus, Staphylococcus, and Corynebacterium* which uses asphaltene as energy source recorded of 46% after utilizing 8% of asphaltene in 13d with initial HC concentration of 5 g/l, at 25 ^{o}C for 60 days treatment period. However, the performance of 4 strains of bacteria containing *Pseudomonas, Citrobacter, Enterobacter, Staphylococcus and Lysinibacillus* recorded asphaltene removal efficiency of 11 – 51% both in shaking and static condition at 40 ^{o}C for 60 days study period (Lavania *et al.* 2012; Honarmand K, Tabatabaee and Arbab S. 2018). Sequel to the mixed culture comparison, it can be inferred that the later, mixed culture containing *Pseudomonas* showed a slight improvement with regards to biodegradation efficiency under the same timeline. However, Honarmand K, Tabatabaee and Arbab S. (2018) recorded a higher asphaltene degradation rate more than Tavassoli *et al.* (2012) and Pineda-Flores *et al.* (2004), in the biodegradation of heavier fractions of crude oil using some bacteria strain, *Bacillus toyonensis BCT-7112*. The result of the study recorded asphaltene's reduction efficiencies of 64.85% and 60% at 25^{o}C and 45^{o}C respectively. The variation in the degradation efficiencies of different treatments may be attributed to concentration, operating temperature, and bioavailability of asphaltene for oil-degrading bacteria (Banat 1995; Sugiura *et al.* 1997).

The study by Rahman *et al.* (2002) to investigate the efficacy of crude oil remediation by mixed consortium containing *Micrococcus sp. GS2-22, Corynebacterium sp. GS5-66, Flavobacterium sp. DS5-73, Bacillus sp. DS6-8b and Pseudomonas sp.DS10-129* isolated from oil-polluted soil samples observed a decrease in biodegradation of crude oil as the concentration of oil increases. The result of the experiment recorded the highest removal efficiency of 78% with mixed consortium after 20 days period of incubation. However, for the single strain, *Pseudomonas sp. DS10-129* showed the highest degradation efficiency of 66% followed by *Bacillus sp. DS6-8b, Micrococcus sp. GS2-22, Corynebacterium sp. GS5-66, Flavobacterium sp. DS5-73* with 59%, 49%, 43%, and 41% respectively, as *Flavobacterium sp. DS5-73* recorded the lowest reduction

efficiency. The appreciable biodegradation efficiencies recorded by these treatments are attributed to the reduction of the lag period required for the microorganisms to respond by the application of mixed or single cultured strain(s) of microorganism(s) (Rahman *et al.* 2002).

Zhang *et al.* (2011) investigated the degradation of n-alkanes and PAH in petroleum using *P. Aeruginosa DQ8* isolated from oil-polluted soil, cultivated in modified Basal Salt medium (BSM) for 5 weeks. The study showed that *P. aeruginosa DQ8* used oil as the energy source for its growth and degraded about 83±1.0% of 2%(v/v) diesel oil which includes C_{12}-C_{25} n-alkanes and other fractions with a total degradation of alkanes length greater than C_{20} and degradation efficiencies of 53.3±2.1%, 66.3±5.3%, and 46.6±3.4% for aromatic, nonhydrocarbons and asphaltenes, respectively. Similarly, Richard and Vogel (1999) recorded a diesel oil reduction efficiency of 90% after 90 days of treatment using sub-cultured bacteria consortium. Also, the study by Lin et al 2014 was in line with the prevailing trend, where effective degradation of phenanthrene (PHE) was attributed to P*seudomonas sp. BZ-3* isolated from crude oil-polluted soil, cultured in a mineral medium, and inoculated to different concentrations of PHE (500 mg, 1000 mg, 4000 mg) supplemented at 50 mg/l. It was observed that *Pseudomonas sp. BZ-3* utilized the PHE as an energy source (Ma, Xu and Jia 2013) which is evident in the appreciable biodegradation efficiency of 75% of PHE (of 50 mg/L initial concentration) after 28 days treatment. Further analysis of the result revealed that *Pseudomonas sp. BZ-3* degraded >45% PAH with two rings for non-aromatics, 36% of PAH in case of PHE, and Anthanthrene were recalcitrant to degradation as only 18% was degraded, PAH with 4 rings recorded 26% degradation efficiency in the case of pyrene. These are also agreed with results obtained by (Yuan *et al.* 2001; Ma, Xu and Jia 2013) – which indicates that *Pseudomonas sp. BZ-3* possibly possesses an effective enzyme to foster the removal of PAHs.

The study by Varjani and Upasani (2017) on the influence of activity parameters on the degradation of crude oil by *P. aeruginosa* NCIM-5514 noted that *P. aeruginosa* was feasible in remediation and enhancing commercial application on the surface and subsurface degradation of hydrocarbon in the polluted soils. The study considered the effect of environmental and nutritional conditions such as agitation, temperature, pH, NaCl concentration, petroleum, and non-petroleum energy source and its concentration, Nitrogen, and inoculum ratio on the growth

of *P. aeruginosa NCIM-5514.* The result of the study showed that optimum growth of *P. Aeruginosa NCIM-5514* was observed at 1%(w/v) glucose at 180rpm with a temperature of 37^0C and pH 7.2, with 1%(w/v) inoculum for 4 days using crude oil and glycerol as an energy source. However, optimization of environmental parameters for growth of *P. aeruginosa* affects the biodegradation ability and efficiency of the hydrocarbons by microbes (Jagadevan and Mukherji 2004) as good biodegradation can be feasible by adjusting some conditions while optimizing physical and chemical factors such as growth media, carbon source for effective biodegradation. The temperature and physicochemical nature of the contaminants affects the growth of *P. aeruginosa* (Varjani and Upasani 2017).

In the biodegradation process, oxygen acts as a substrate in oxygenase catalysed reaction and doubles as an electron acceptor in oxic metabolism. Hydrocarbon is a good carbon source for *P. aeruginosa* growth, Priya and Usharani (2009) signify that the type and C/N concentration used in the media culture is essential for the growth, biomass formation and biodegradation of hydrocarbon by *P. aeruginosa* (Jagadevan and Mukherji 2004). Results showed the mesophilic, aerobic crude oil utilizer and halotolerant, nature of *P. aeruginosa* (Varjani and Upasani 2017).

The study by Das and Mukherjee (2007) to evaluate the biodegradability of *Bacillus Substilis* and *P. aeruginosa* strain isolated from petroleum oil-polluted soil showed that *P. aeruginosa* was effective than *Bacillus Substilis* after 120 days of the experiment. Moreover, the two strains showed a significant decrease in the concentration of crude oil in the soil as compared to control treatment. The study observed extensive growth and biosurfactant synthesis by exogenic microbes in oil-contaminated soils. The isolated bacteria were supplemented with 2%(v/v) petroleum and incubated at 45^0C and pH 7.0 for *P. Aeruginosa N and NM* strains (Das and Mukherjee 2005) or at 55^0C temperature and pH 8.0 for *B. Subtilis* DM-04 strain (Mukherjee and Das 2005) with 200rpm agitation. Conclusively from the study, *P. aeruginosa* showed a higher degradation efficiency of 75% against *B. Subtilis* with 53.6% representing reduction from 84g/kg initial concentration to 21g/kg and 39g/kg respectively after 120 days of treatment. Also, a high level of crude oil degradation exhibited by *P. aeruginosa* in the study (Das and Mukherjee 2007) due to significant breakdown and utilization of petroleum as a carbon source which invariably enhanced the growth of *P. Aeruginosa* as compared to *B.Substilis*. In contrast,

Jackson and Pardue (1999) and Hesnawi and Mogadami (2013) inferred that addition or introduction of nutrients into the treatment has minimal impact on the removal efficiency of the crude oil. However, the study by Shin *et al.* (2001) and Atlas (1995) concluded that microbial or organic amendment is essential since the indigenous microbial community is ineffective for optimum degradation of complex and recalcitrant hydrocarbons while Chaineau *et al.* (2005) advocate for adequate nutrient addition with moderation) for a better TPH removal.

P. aeruginosa tends to decrease the surface tension of culture which suggests that strain might produce biosurfactants (Zhang *et al.* 2011),. The production of surfactant may also contribute to the distribution and effective degradation of crude oil which promotes degradation of TPH with/out the addition of extra nutrients (Thavasi, Jayalakshmi and Banat 2011). Unlike other strains of bacteria, *P. Aeruginosa* can effectively degrade (n-alkanes and PAHs) (Hasanuzzaman *et al.* 2007) and different fractions of hydrocarbon which suggests its effectiveness for TPH degradation (Rahman *et al.* 2002). Besides, (Zhang *et al.* 2011) noted that *P. Aeruginosa* can use diesel oil and crude oil as a substantive energy source for growth while degrading the same effectively. Thus, the ability of *P. Aeruginosa* to degrade major components of crude oil inferred that it can be applied for the remediation of a vast group of petroleum fractions and remediation of crude oil contaminated soils.

2.7.2 Bioremediation of Heavy Metals using *P. aeruginosa.*

According to Abdelbary, Elgamal and Farrag (2019) study, *P. aeruginosa* strains were successful in the heavy metal uptake and removal at polluted sites. Microbes used in the remediation of heavy metal emissions using various biotechnological processes. These methods include biosorption, bioaccumulation, bio-mineralization, phytostabilization, biostimulation, rhizoremediation, mycoremediation, cyano-remediation, and gene remediation and these bacteria adopt several mechanisms for growth in a heavy metal polluted environment. Some bacteria such as *Arthrobacter sp., Pseudomonas sp. Corynebacterium sp., Alcaligenes sp. Bacillus Azotobacterium sp., Flavobacterium sp., Mycobacterium sp. Rhodococcus sp. and Methanogens* have been deployed for the remediation of heavy metals contaminants (Abdelbary, Elgamal and Farrag 2019). *Pseudomonas aeruginosa* is tolerant of heavy metals and resistant to a toxic concentration of most metals, dyes, and antibiotics (De and Ramaiah

2007; Oyetibo *et al.* 2010; Yamina, Tahar and Marie Laure 2012; Wales and Davies 2015). Studies have reported the effectiveness of *P. Aeruginosa* in metal uptake through metal-chelating by producing siderophores which improve bioavailability (Sar *et al.* 1999; Joo, Hassan and Oh 2010; Kőnig-Péter *et al.* 2014; Kőnig-Péter, Kilar and Pernyeszi 2019) and also, synthesize biosurfactants to enhance the solubility of hydrophobic substances and metal mobility (Ron and Rosenberg 2002; Ganesh and Lin 2009; Lutsinge 2018).

The study by Maitra (2016) noted that bacteria strain *P. Aeruginosa* exhibited a great potential in metal tolerance as it grows in the heavy metal concentrated environment while reducing the concentration of the heavy metals while compared to other strains. Invitro assessment (Maitra 2016) recorded that *P. Aeruginosa* isolates tolerated highest concentration of Cu, Zn, Co, Fe, Mn, Mo than other bacteria like *Rhizobium, Bradyhizobium* which are sensitive to Cu, Zn, and Co only. In toxic environments, heavy metals tolerant bacteria prefer to develop antioxidant enzymes to tolerate and adapt to the strenuous nature of the heavy metal-polluted environment. Heavy metal tolerant is based on the level of toxicity and tolerant mechanism adopted by individual bacteria, according to (Ropek and Para 2003; Ezzouhri *et al.* 2009). Such bacteria have a special survival mechanism and tolerance to concentrations of heavy metal in the water.

Biosorption of heavy metals has been proven effective for metal removal from industrial wastewater and natural H_2O (Volesky and Holan 1995; Volesky and May-Phillips 1995; Sar *et al.* 1999; Volesky 2003; Gavrilescu 2004; Alluri *et al.* 2007; Dhir and Kumar 2010; Fu and Wang 2011; Abbas *et al.* 2016). The application of organic waste accounts to the low-cost alternative of metal reduction in comparison with conventional and other physicochemical techniques for remediation purposes, but widely criticized due to its ineffectiveness and/or expensive nature (Volesky and Holan 1995; Volesky 2003). The use of biologically modified methods such as biosorption/bioaccumulation for metal removal have alternatively in comparison with conventional methods, rendered a breakthrough in the remediation of metal contaminants (Brady 1992; Kapoor and Viraraghavan 1995; Mishra and Malik 2012; Olguín and Sánchez-Galván 2012).

Biosorption and bioaccumulation are two obvious pathways for bacterial and fungal removal of heavy metals from the atmosphere (Kumar *et al.* 2014). The metal uptake/deletion cycle

followed by microorganisms is either by active mechanics (bioaccumulation) or through passive processes (biosorption) as suggested by (Zhou and Kiff 1991); (Fourest and Roux 1992; Hussein *et al.* 2004). Biosorption is the application of biomass heavy metals extraction from a polluted site and bioaccumulation requires the use by micro-organisms of active and passive metal ingestion to resist the toxic impact of heavy metals (Brady 1992). The method of biosorption is the process of converting heavy metals employing live or dead biomass to decontaminate the metals or reduce concentration. Studies have shown that metal uptake occurred more prevalently through the mechanism of biosorption because bioaccumulation, which is an active uptake process, involves the introduction of a substrate and increased BOD and/or COD in the system to improve the metal removal process, effectively constant conservation of the active, stable, and sensitive microbial population needed for bioaccumulation is also rigorous due to sub-lethal effect of metal and unfavourable abiotic conditions (Brown and Lester 1982; Silva *et al.* 2009). The critical consideration factor in applying biological methods in extracting heavy metals is the use of low-cost organic substrates, the cost of biomass immobilization, and the reusability of biomass (Volesky 2003).

Most microorganisms will bioaccumulate, which is a passive physicochemical process based on adsorption, complexation of the ion exchange, and/or micro precipitation (Volesky and Holan 1995). Biomass ' ability to absorb metals relies on the cell wall's composition that consists of polysaccharides, proteins, lipids, functional groups such as carboxylate, hydroxyl, phosphate, and amino groups that can effectively bind metals. The adsorption of heavy metals on the outer cell surface of microorganisms is essentially a protection mechanism adopted by microbes to prevent the toxicity and penetration of heavy metals through the cell walls by creating a polymeric coating that sheds the metal from penetration (Scott and Karanjkar 1992). According to Kuyucak and Volesky (1988), metal removal via metal sorption by microbes depends on several factors such as metals' bioavailability to microbes, treatment system temperature, an organic component needed for complexation, presence of other ions that may have high affinity to the organism to enhance competition, system pH, cell metabolic product that may cause metal ppt. formation, pollutant concentration, acceptor electrons, soil composition, and water movement (Brar *et al.* 2006; Mani and Kumar 2014) while factors such as cation exchange capability, the content of clay minerals, metal oxide, and organic matter limit metal availability

in the soil (Brar *et al.* 2006; Mani and Kumar 2014). Treatment of heavy metal is typically accomplished by removing the metal ion from the substructure to minimize the risk associated with exposure to the contaminant.

The study by Silva *et al.* (2009) in the removal of heavy metals from polluted sites using *P. aeruginosa* AT18 recorded the effectiveness of bioremediation of heavy metals using *P. aeruginosa* isolated from petroleum-polluted soil and maintained in nutrient agar slant, stored in ambient temperature, 30^0C. Heavy metal solution of Cr_2O_3 (60 mg/L), $CU(NO_3)_2$ (50 mg/L), $MnSO_4$ (50 mg/L) and, $ZnSO_4$ (80 mg/L) were inoculated with cell suspension (10ml) for 72 hours in rotary shaker at 150rpm. Metal removal was a factor of pH of the treatment system as maximum removal Cr (99.6%) was recorded at pH 7.72, an increase in sorption of Cu (95.0%) occurred at lower pH 6.25 which is the optimum adsorption pH for Cu. Zn removal was effective at pH 7.0 with 87.7% and 21.69% for Mn at pH 7.0 removal efficiency after 72 hours of treatment. The study recorded that the growth of *P. Aeruginosa* was more visible in Zn followed by Cu, Cr, and Mn while adsorbing the metals. However, considering the heavy metals investigated, Mn recorded high toxicity, poor *P. aeruginosa growth,* and low removal due to the possession of the highest atomic mass when compared to other metals (Chen *et al.* 2000).

Silva *et al.* (2009) noted that heavy metals, Zn, Cr, Cu, and Mn were removed as a result of the precipitation cycle and competition may also contribute in the removal of metals beyond sorption and precipitation mechanisms. The rivalry to increase the pH is due to the complexing of the functional group at *P. aeruginosa* cell walls and an increased concentration of alkali ions. Metal removal at pH 5.5–7 is encouraged, and pH much greater than 7.0 reduces metal removal. *P. aeruginosa* AT18's maximum sorption power, at pH values below 6.0, was small (Puranik and Paknikar 1999). Sequel to the performance recorded by the *P. aeruginosa* in heavy metal removal, Silva *et al.* (2009) noted that *P. Aeruginosa* resistance to high metal concentration, suitability for metal removal, and the design of a treatment system using *P. aeruginosa* as a viable alternative approach for remediation purpose.

Similarly, Nagashetti *et al.* (2013), evaluated the ability of *P. aeruginosa* to remove metals by biosorption process. *P. Aeruginosa* used for the treatment was collected from a health centre and cultivated in LB broth media. Heavy metals (Cu, Zn, and Cr) solutions were simulated for

each heavy metal treatment. The result of the study revealed significant absorption of metals by *P. Aeruginosa* as concentration increases from 250 to 100ppm. The adsorption of metals in different concentration are as follows this sequence in decreasing order of adsorption; for 250ppm Cr > Cu > Zn; 500ppm – Cu > Cr > Zn while 1000ppm - Zn > Cr > Cu. It can be inferred that Cr, Cu, and Zn were more favourable at 250, 500, and 1000ppm respectively. This suggested the potency of *P. Aeruginosa* in the reduction of metals and can be exploited for effective removal of Cu, Zn, and Cr with Zn recording the highest absorption at 1000ppm, Cu at 500ppm and Cr at 250ppm. The metal removal trend Cr > Cu > Zn recorded by Nagashetti *et al.* (2013) at 250ppm agreed with Silva *et al.* (2009) and Juwarkar *et al.* (2008) with Cd > Cr > Cu > Pb > Ni. Similarly, Nagashetti *et al.* (2013) investigated the metal tolerance ability of *Pseudomonas sp.* with a wide range of hexavalent Cr, Cu, and Zn concentrations of 250, 500, and 1000ppm. Removal of these metals by *Pseudomonas sp.* was recorded after 72 hours of incubation which suggests the ability of *Pseudomonas sp.* To absorb and survive in varying concentrations of heavy metals (Nagashetti *et al.* 2013; CHESTER *et al.* 2014).

In contrast to *P. aeruginosa* metal tolerance trend recorded by Silva *et al.* (2009), (Zn > Cu > Cr > Mn), Mathiyazhagan and Natarajan (2011) observed a different trend: Zn > Mn > Cu > Cr > Hg while Cr > Co > Cu > Cd > Ni > Zn > Mn. The variation in metal tolerance and removal efficiency recorded can be attributed to the pH and temperature which contribute to metal biosorption process and activities of microbes in the soil. Mathiyazhagan and Natarajan (2011) observed an increase in pH from 6.57 to 9.71 and 3.9 to 6.75 for *T. Ferroxidase* and *P. Aeruginosa* respectively during the treatment with temperature ranging from 25^{o}C to 60^{o}C (with 38^{o}C recorded as optimum temperature for biosorption). Among the two bacteria strains, *T. Ferroxidase* and *P. Aeruginosa*, *P. Aeruginosa* showed effective metal absorption more than *T. Ferroxidase* due to high metal tolerance exhibited by *P. Aeruginosa,* hence, can serve as a bioremediation tool for contaminated soils.

The study by Juwarkar *et al.* (2008) to access the potentiality of rhamnolipid biosurfactants produced by *P. Aeruginosa* strain BS2 for the removal of multi-metal polluted soils. The production of rhamnolipid biosurfactant was carried out using *P. Aeruginosa* BS2 isolated from sludge at a temperature of 37^{o}C agitation 400rpm, an incubation period of 96 hours with aeration

at 28LPM and ½ VVP (Volume per air per volume liquid). The foam was removed by foam fractionation method after 96 hours of incubation with the field of biosurfactant using distillery found to be 0.91g/l. The di-rhamnolipid biosurfactant was inoculated to the metal contaminated soils at o.1% and metals spiked with distilled water was used a control treatment. The high sorption of heavy metal recorded in the treatment ranges from 86 – 96% which is due to competitive metal sorption in the soil which arises from the use of the multi-metal solution for the simulation of metal contaminated soils. This appreciable metal reduction efficiency is similar to Silva et al 2009. Metal removal from the soil is a function of time where increased removal was recorded as treatment time increases. Pb and Cu recorded reduction by 797 and 423ppm of 900 and 480ppm initial concentration. While Cr, Cd, and Ni were reduced by 865, 396, and 686ppm from 940, 430, and 880ppm initial concentration respectively after 36 hours of treatment time.

The low removal of metal recorded in the control treatment may be attributed to unavailability of metals in the soil and also due to the long ageing presence of metals which attributes to the strong bound of heavy metals to the soil which limits the availability of pollutants for remediation (Boopathy 2000). The presence of di-rhamnolipids promotes metal mobilization (Cd > Cr > Pb > Cu > Ni) which is dependent on the stability of required metals with di-rhamnolipids during the treatment. The result showed that rhamnolipid biosurfactant from *P. aeruginosa* was able to respond positively to contaminated soil by effective reduction of metal concentration (Juwarkar *et al.* 2008).

The study by Sinha and Mukherjee (2009) reported that the order of toxicity as follows Cr > Co > Cu > Cd > Ni > Zn > Mn. This was deduced using *P. aeruginosa* KUCd1 strain isolated according to Sinha and Mukherjee 2008. Results recorded 75% Cd removal after 96 hours during the stationary phase. It was observed during the active growth phase that Cd removal is linked to the cell, the increased removal of Cd was found to be unrelated to cell increment which is attributed to adsorption by dead cells as reported by (Kurek, Czaban and Bollag 1982; Vig *et al.* 2003). Sinha and Mukherjee (2009) observed that bioremediation from polluted sites is dependent on microbes tolerance and the availability of nutrients under such conditions. There is a decrease in cell number as time progresses, which affirmed that survival is also a factor of

nutrient availability. Survival rate was increased in wastewater treatment where there is an alternative nutrient source for the *P. Aeruginosa* which facilitated the metal removal efficiency at 89% after 96 hours while media treatment recorded 75% and nutrient-deficient media remove less than 20% Cd from the wastewater. The bacteria strain *P. Aeruginosa* KUCd1 showed survival in wastewater with or without supplementation with extra nutrient and also, the possibility of utilization in metal contaminated agricultural lands to enhance removal, although removal of some metals from metal-polluted sites requires further study due to the recalcitrant nature of some heavy metals (Philip, Iyengar and Venkobachar 2000; Sinha and Mukherjee 2008; Sinha and Mukherjee 2009)

The unavailability of Fe which is a *significant nutrient for the Pseudomonas aeruginosa bacterium* prompted the secretion of siderophore (*iron carriers* - high-affinity iron-chelating compounds) which transport iron across cell membranes. *P. aeruginosa produces siderophores (Peek et al. 2012) for binding and transporting iron. Siderophores are essential for the acquisition of iron by certain pathogenic bacteria.* These siderophores are released by *P. Aeruginosa (Sinha and Mukherjee 2008)* to scavenge available Fe from the mineral phase in the treatment system by the gradual formation of soluble Fe^{3+} complexes for active transport mechanism uptake (Miethke and Marahiel 2007; Hider and Kong 2010). Siderophore synthesized by a microorganism can chelate (Nair, Juwarkar and Singh 2007) and detoxify other metal ions aside Fe, such as Cu, Al, Pb, Mn, Zn, Cd, Cr, Ur among others (Carrillo-Castañeda *et al.* 2002; Del Olmo, Caramelo and SanJose 2003). Such complexes minimize the toxicity of heavy metals and thus siderophore processing can contribute to the bioremediation of toxic metals.

O'Brien, Hodgson and Buckling (2014) studied the effect of toxic metals on siderophore production using *P. Aeruginosa* strain PA01 cultured in kings medium B (KB) with the addition of either Cu or $FeSO_4$ where the later was used as a control for the effect of sulfate and Cu^{2+}. The study observed that high toxicity of copper sulfate (6.17mM), reduced the population growth of *P. aeruginosa* by 63.5% when compared to Fe treatment of the same concentration while treatment with Cu (624uM) reduced the growth population by 29.25%. The toxicity of heavy metal concentration may affect the production of siderophore which depends on iron

availability in the system (O'Brien, Hodgson and Buckling 2014). It is believed that selection for production and utilization of siderophore in a contaminated environment may increase to reduce Fe availability. It was suggested that the selection of siderophore for detoxification may be significant than for iron scavenging in some context which may depend on the association of heavy metals with an excess of bioavailable Fe. Low concentration of Fe increases Fe solubility, reduces siderophore production but increase the bioavailability of other toxic metals to enhance removal (D'Onofrio *et al.* 2010). Recent studies have noted that siderophores are also capable of binding other heavy metals (such as Cu and Zn), but siderophore chelation in this situation greatly decreases bacterial metal uptake. Such complexes minimize the toxicity of heavy metals and thus siderophore processing can contribute to the bioremediation of toxic metals. O'Brien, Hodgson and Buckling (2014) inferred that success and effectiveness of siderophore application for the decontamination of contaminated sites.

According to the study by Awasthi *et al.* (2015), a significant metal uptake was observed after 24 hours of inoculation of *P. aeruginosa* isolated from the contaminated soil sample. The study reported that Cu was significantly removed from all concentrations with the highest removal efficiency of 79% on increasing metal concentration from 5% to 15%. Fe uptake and absorption were evident at all levels with a maximum at 61% in 10% concentration and 52.4% in 15% for Zn with Cr showing the lowest efficiency of absorption of 41.6%. Awasthi *et al.* (2015) observed that high toxicity of Cr on *P. aeruginosa* was observed at 5% concentration after 72 hours of treatment. However, the high removal efficiencies for Cu and Zn can be attributed to the indispensable nature of Cu and Zn as essential nutrients required for cell survival; hence, efficient adsorption is of these metals is evident. The study showed that the effect of Cu concentration in *P. aeruginosa* was negligible as the organism could be effectively applied for metal remediation.

The findings of Awasthi *et al.* (2015) recorded the growth of *P. aeruginosa* from 24 – 72 hrs as growth decline after 72 hours which indicate the bacteria response to different concentrations of heavy metal with varying intake levels in relation to time and change in biomass. These metal removal efficiencies recorded Awasthi *et al.* (2015) agreed with results of other researchers (Kabata-Pendias and Pendias 1999; Kolembkiewicz 1999; Hussein *et al.* 2004) which inferred

that Cr maximum affinity to *P. aeruginosa* was as a result of its low biodegradation efficiency. This suggested that *P. aeruginosa* serves as a viable alternative for metals (Zn, Cu, Fe, and Cr) removal from industrial effluents.

Biosorption of Al was studied by Tuzen and Soylak (2008) using The *P. aeruginosa* cultured in medium broth (prepared by mixing LB broth in 200ml distilled water and sterilized for 20mins). The starter culture was first prepared in a solid stock medium and inoculated into 10ml liquid nutrient medium, incubated at 30^{o}C for 24 hours. The initially prepared 200ml was inoculated with 2ml of started culture and incubated in vails at pH 7.3 at 30^{o}C in continuous shaking. The bacteria cell was harvested and separated at stationary phases (OD: 4.0 – 4.6 at 600nm), after 24 hours using centrifugation at 7000rpm for 15mins. Before inoculation into the treatment medium, 40 – 50ml of the solution containing 0.2ug of Al(III) was added to 10ml buffer solution and *P. aeruginosa* loaded on chromosorb 106 columns preconditioned through passing buffer solution at a flow rate of 5mL^{-1}. Results showed that absorption of Al was pH-dependent as recovery was more evident at a pH range of 6 -7 and pH below 6 or above 9 recorded a decline in Al recovery. This can be due to the positive changes on the surface of biomass which contribute to insignificant recoveries of Al at pH lower than 6 and formation of Al hydroxide ppt which possibly occurred at optimal working conditions leading to the unquantified recovery of Al at pH higher than 8 (Tuzen and Soylak 2008). Tuzen and Soylak (2008) recorded varying recovery efficiencies of Al using *P. aeruginosa* in different eluents as 80%, 97%, 70%, and 90% for 0.5mlL^{-1} HCl, 1mlL^{-1} HCl, 0.5mlL^{-1} HNO_3 and 1mlL^{-1} HNO_3 respectively. The recovery efficiency of Al increased with an increase in the number of microorganisms. The study showed that *P. aeruginosa* exhibits good chemical stability and reusability potentials for optimum result.

2.7.3 Sulfate reduction by *P. aeruginosa*

The bacteria strain, *Pseudomonas spp.* grow in organic or inorganic sulfur (Ismail *et al.* 2014), utilizing the same as a sole surface source in the presence of glucose as a carbon source. *Pseudomonas* synthesize varying degrees of biosurfactants based on the utilized sulfur sources. Studies have shown that under pilot-scale, *P. Aeruginosa*, *P. Stutzeri, Desulfovibrio Vulgaris,*

Desulfovibrio Desulfurican were able to remove a reasonable amount of undesirable salts such as sulfate and nitrate from contaminated sites (Chen *et al.* 2008).

Ajao, Adebayo and Yakubu (2011) investigated the removal efficiency of textile industrial effluent (containing sulfates, nitrate, phosphate, COD, and BOD) using the isolated mixed immobilized culture of *P. aeruginosa* and *B.Subtilis* to remove sulfates and nitrates from polluted water. The mixed culture of *P. aeruginosa* and *B. Subtilis* were cultured in agar solution and incubated for a 24hours. 2litres of effluents was supplemented with Basal Medium and autoclaved at 121^{o}C for 15 mins, followed by 30g of each immobilized bacterium was added and progress monitored for bioremediation process which comprises of 1000ml of sterilized effluents dispensed into 5litres bioreactor with air sparger aseptically and 30g each of immobilized bacteria cells added to each treatment and monitored for 15 days. Ajao, Adebayo and Yakubu (2011) recorded 62.8 % and 61.3% removal efficiency for sulfate and nitrate respectively after 15 days treatment period. The removal efficiency recorded that the application of immobilized mixed culture of *P. aeruginosa* and *B. Subtilis* for the treatment of contaminated effluent and this can ensure maintenance and reusability of strain over a period time. The result of the study also recorded appreciable removal of other pollutant constituents of textile industrial effluents with removal efficiencies of 95%, 88%, 96%, 83%, 97.3%, 61.3%, 62.8% and 61.2% for TS, SS, DS, COD, BOD, Nitrate, Sulfate, and Phosphates respectively after 15 days treatment period. Also, some selected heavy metals (like Cu) were biosorphoned and effectively removed after 15 days. The result showed a good correlation with the study by Rajamohan and Karthikeyan (2004) that reported an effective reduction of COD and other constituents of effluent. These results suggest the efficacy of immobilized mixed culture of *P. aeruginosa* and *B. Subtilis* for the reduction of sulfate and other effluent components and can ensure the reusability of application.

Chen *et al.* (2008) observed similar salt reduction ability of *P. Aeruginosa,* heterotrophic denitrifiers to effectively reduce salts present in wastewater is partly dependent on the interaction with SRB present in the wastewater to ensure a high rate of sulfate and nitrate removal. The study recorded 80% and >99% sulfate and nitrate reduction to elemental sulfur and nitrogen gas respectively after 10 days of treatment. The study showed that microbial

community present in the sludge bed reactor used for the treatment comprises of *P. Aeruginosa* and *sulfurospirillium sp* as heterotrophic; SRB (*Desulfomicrobium sp.*) as autotrophic and *Sulfurovum sp.* and *Paracoccus denitrifican* as denitrifiers. It is evident that at high concentrations of sulfate and nitrate, heterotrophic denitrifiers (*P. aeruginosa)* outcompete autotrophic denitrifiers to reduce SRB activities, hence heterotrophic denitrifiers utilize sulfate and nitrates as electron acceptors (Hubert and Voordouw 2007; Kodama and Watanabe 2007).

2.8 Heavy Metal Speciation in Soil

Speciation is the chemical and physical attribute of an element which can be defined in terms of oxidation state, stoichiometry, coordination (number and type of ligands) and physical condition and interaction with other phases. Metal speciation involves the chemical nature of the soil solution metal, either as a free ion or complexed to a ligand, during the gaseous stage and dispersed in solid phases throughout the soil. These characteristics regulate the chemical activity of components, whether in surface (soil) or the aquatic and contribute immensely to toxicity determination. The chemical and physical aspects defining metal speciation in soil regulate its interaction, reactivity and fate in soil, and certain cases, toxicity, including its solubility, and uptake. In environmental soil chemistry, the quantitative speciation and variation of metals over time are necessary for the study of metals concentrations in the soil. Although species have formed for thousands and millions of years in the presence of metals in the natural world, it is only after industrialization that large concentrations of metals have consistently been added globally to soil ecosystems. The need to classify and quantify those species in soils that pose the greatest potential threat to organisms is highlighted by this increased exposure of organisms to metals (Roberts, Nachtegaal and Sparks 2005; Reeder, Schoonen and Lanzirotti 2006).

2.8.1 Metal species and reaction in soils

In terms of metal species in soils, sorption is the general category of reactions considered to be the most critical. Sorption is a general term, which involves several different processes, the general metal ion elimination from solution and eventual interaction with solid soil fraction of a metal ion from a solution. The opposite of this method is called desorption or the removal and

addition of metal from a solid material into the solution. Metal sorption reactions in soils largely determine their mobility, fate and bioavailability and, thus, are important when trying to understand the spectrum of metals. The removal of metals from soil solutions by inorganic and organic phases is a mechanism by which radioactive metals can be sequestered, potentially alleviating adverse environmental effects. Multiple mechanisms can lead to the simultaneous removal of a metal ion from the solution. One way to illustrate the connection between soil metal sorption and metal speciation is to consider sorption as a reaction involving the metal ion and speciation as the result of this reaction; however, it is important to note that just as the speciation of the metal varies with time, sorption is also a dynamic process (Roberts, Nachtegaal and Sparks 2005; Reeder, Schoonen and Lanzirotti 2006). At any moment, the speciation of metal is simply a snapshot and it can change as the sorption mechanisms changes. In addition to being a significant factor in the determination of metal speciation, the presence of crystalline and amorphous inorganic phases and organic material is crucial in the sorption and speciation of metals. The solid fraction of the soil is an accumulation of non-living, living, and initially living material able to react with metal ions.

2.8.2 Exchangeable metal ions

Both inorganic and organic soil solids have permanent charging sites that are often negatively charged, depending on pH. Positively charged metal cations that encounter these sites may form an electrostatic bond of low energy, often referred to as cation exchange. In soil science, the term "cation exchange" is used to characterize the substitution of one adsorbed cation, which can be easily exchanged by another (Neal and Sposito 1989). The metal cation in the soil solution, which changes with one on the surface (or Ca^{2+}, Na^{+}, etc.), forms an external sphere complex. Organic matter is, in fact, crucial to metal speciation in soils with its variable and often high cation exchange capacity. This is realized if it is considered that the cation exchange capacity (CEC) of organic matter increased incrementally from 36 cmol kg^{-1} at pH 2.5 to 215 cmol kg-J at pH 8.0 or 45% of the total soil CEC in a study of 60 Wisconsin soils (Helling, Chesters and Corey 1964). Generally, when the concentration of the monovalent cation is low, multivalent cations effectively displaces monovalent cations from clay exchange sites. Studies on Na^{+}-Me^{2+} exchange reactions on Na^{+}-saturated montmorillonite have shown that Na^{+}-Me^{2+}

is pH-independent below pH 6 (Inskeep and Baham 1983). However, if the monovalent cation concentration (e.g., high ionic strength) is high, competition for exchange sites can lead to the formation of adsorption complexes between the ion and the surface of the metal. Ionic strength dependence is one of the characteristics of ion exchange-outer sphere complexation and is often used as a macroscopic evaluation to determine whether this sorption mechanism is functional.

The ion exchange process and the formation of an outer sphere complex on clay soil can be represented in equation 2.9 below (McBride 1994):

$Me^{n+} + nNa^{+}$ - clay $\leftrightarrow$ Me^{n+}- clay + nNa^{+} Equation 2-9

Where Me^{n+} is the metal cation with valency *n*

2.8.3 Metal mobility Processes

The vital processes that regulate metal mobility in the soil are dissolution/precipitation, complexation with ligands, sorption/desorption by solid, biotransformation, uptake by soil and aquatic biota and reduction-oxidation (redox) (Brown Jr and Parks 2001; Warren and Haack 2001; Sparks 2003)

i. Dissolution and precipitation involving metal species are subject to both thermodynamic and kinetic control. However, many significant environmental phases lack thermodynamic stability records, including many amorphous phases. Besides, some of the phases regulating metal solubility in nature are solid solutions and adequate solution models are available for relatively few environmentally applicable phases. Due to several potential kinetic conditions, it is necessary to note that supersaturation does not ensure that precipitation occurs. For example, inhibitors, often sorbed metals, can impose kinetic restrictions that limit precipitation (and dissolution) (Roberts, Nachtegaal and Sparks 2005; Reeder, Schoonen and Lanzirotti 2006).

ii. Metal solubility is also usually linked to aqueous phase complexion. Metals with a high affinity to either organic or inorganic ligands can exhibit significantly increased solubility through complex formation. The high affinity of UO_2^{2+} to dissolved CO_3^{2-} is an environmentally significant example. In the absence of dissolved CO_3^{2-}, the total solubility of UO_2^{2+} mediated by mineral schoepite ($UO_3.2H_2O$) at neutral pH is 3.4 μM. With 1 mM of total

carbon dioxide dissolved in the solution, the total solubility of UO_2^{2+} is tenfold higher (56 μM) due to carbonate complexation (Roberts, Nachtegaal and Sparks 2005). Aqueous complexation can also affect the absorption of metals by aquatic and soil organisms. For example, dissolved Cu2+ and Ni2+ are readily absorbed by certain aquatic phytoplankton, whereas absorption is extremely limited when these same metals are highly complexed with organic ligands or natural organic matter. Although several studies of aquatic species have shown associations between the metal absorption and the fraction of the metal occurring as a free ion (i.e. the free ion activity model) (Morel 1983), very little is known about chemical form responsible for the metal taken up (Sunda and Huntsman 1998). Solubility and absorption behaviour, in turn, affect metal mobility in the environment (soil), thereby limiting organisms and human's exposure pathways.

iii. Sorption processes associated with the transfer of dissolved metals to solid surfaces are some of the most critical controls on dissolved metal concentrations in systems containing solid phases (Brown Jr and Parks 2001; Sparks 2003). Adsorption (i.e., surface ion accumulation), surface precipitation (formation of a distinct surface phase), and co-precipitation can be regarded because of sorption (incorporation of ions into a phase, commonly as it precipitates). In general, sorption is best used to extract a dissolved metal from a solution when the metal remains at a low concentration and the solid surface available is high. However, sorption depends also on the presence of complexing ligands or competing species, including pH, ionic strength, and other solvent properties. Also, significant considerations are the characteristics of the solid-liquid interface, including surface load (Davis *et al.* 1990) and the control of the superficial site (Reeder 1996; Elzinga and Reeder 2002).

iv. Biotransformation typically includes processes for reducing/oxidizing (redox). Since there are often significant variations in solubility between different metal oxidation states, the reduction or oxidation of bacteria can be highly effective in regulating metal concentrations in environmental solutions. It has been shown that bacteria present in soil systems reduce dissolved U(VI) to U(IV), resulting in uraninite formation (Lovley *et al.* 1991; Fredrickson *et al.* 2000). This process will effectively immobilize uranium in the subsurface. However, under oxidizing conditions, uraninite can be re-oxidized, resulting in U(VI) remobilization. Without biological mediation, **Redox processes** can also occur, but kinetics are usually sluggish. Important

electron donors/acceptors include organic matter and compounds containing Fe, Mn and sulfur in surface environments. Many redox processes occur at solid surfaces and are related to sorption processes. Metal adsorption on surfaces can alter their electronic structure and facilitate redox reactions that are inhibited when the metal is dissolved. For example, it has been shown that compared to the homogenous reaction between these two metal species, the reduction of Cr(VI) co-adsorbed with Fe(II) onto a Fe(III)-oxide mineral substrate is significantly faster. The same phenomenon has been observed for reactions between Fe(II) and uranyl (Liger, Charlet and Van Cappellen 1999).

Many physical, geochemical, and biological processes influence metals behaviour in surface environments. Much emphasis was put on processes which mobilize or immobilize metals because mobility generally makes metal exposure easier. A dissolved metal can be transported by fluid flow, which eventually enters the supply of water or being used within a food chain. In comparison, a metal that precipitates as a coating on mineral grain into the soil or aquifer is immobilized by coating on mineral grain, which can essentially be removed from exposure unless it has subsequently been remobilized. The bioaccessibility of geomaterials in certain cases is equated with the phenomenon of dissolved metal. Metals that are associated with small mobile particles can have greater exposure potential. Colloids and airborne particles, for example, are mobile and all have been shown to have metals connected to them (Roberts, Nachtegaal and Sparks 2005; Reeder, Schoonen and Lanzirotti 2006).

2.9 Eh and pH - *the pH Diagram*

Soil pH is a key factor that influences the metals speciation, distribution, the redox process, and removal from the soil. Since AMD is mostly caused by iron sulfide oxidation (FeS_2, often known as pyrite), Iron, which is the predominant metal in AMD, is more soluble in low pH soils, which is mostly present in combination with other metals. Higher quantities of ferrous ions can therefore be formed in more acidic soils under redox condition (Kirk 2004). On the other side, iron is more readily oxidized when the soil pH is high, and the solubility of Fe(II) is thus lower. A pe-pH (pourbaix or potential/pH) diagram shows the influence of soil pH and Eh on Fe transformation in soil, in which Fe and other metals speciation in the soil can be theoretically predicted (Kirk 2004).

Eh is a measure of the redox (oxidation-reduction) status of the solution or its solution. Eh is the most crucial aspect in regulating the distribution between Fe(III) and Fe(II) and other metals. This redox potential parameter ranges from 600 to – 300 mV in soil (Lindsay 1979). Redox reactions between Fe(III) and Fe(II) typically occur when soil Eh decreased to about 200 mV (Liesack, Schnell and Revsbech 2000), but this barrier Eh can differ depends on the soil properties (Kirk 2004).

In many soils, both Fe and Mn oxides are normal mineral constituents and are important substrates for many macronutrients and micronutrients to be retained in the soil. In calcareous soils, plant availability of both Fe and Mn is significantly reduced due to the extremely low solubility of oxides of Fe and Mn oxides and carbonates of Mn. Manganese and iron minerals present in redox cycling between oxy and anoxic system (Nealson and Saffarini 1994). Reduced iron and manganese are oxidized within oxic conditions by microbes, precipitated as oxides and oxyhydroxides in the anoxic sediment, where they are re-mobilized by anaerobic iron and manganese reducers (Huang and Zhang 2020). Mobile Fe(II) and Mn(II) may then be diffused into the oxic zone or precipitated as carbonates. In addition to carbonates, other poorly soluble reduced compounds can precipitate and dissociates (Fig. 2-1a & b).

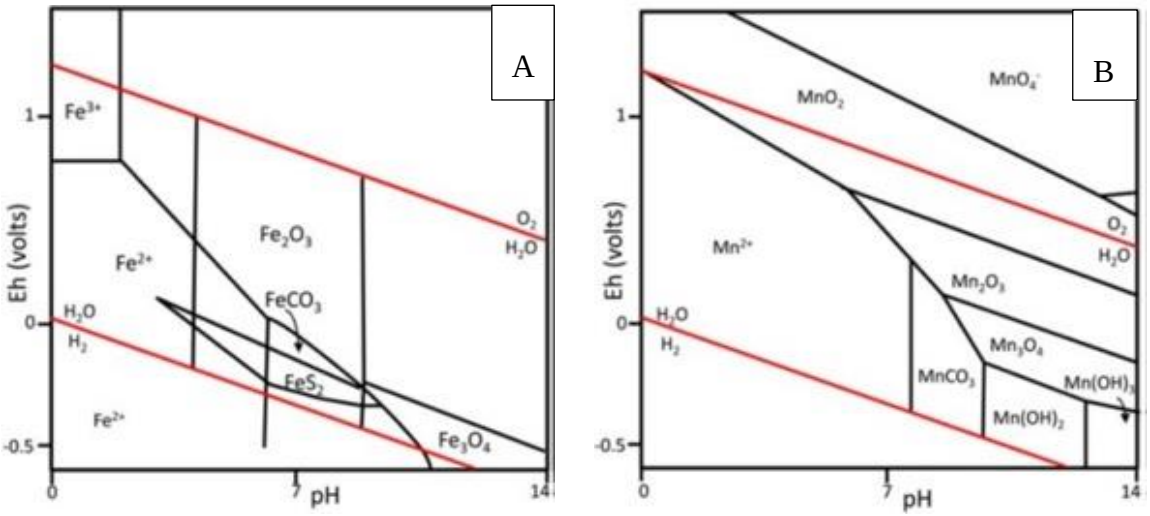


Figure 2-1 a & b. Eh-pH diagram for compounds of Fe(III)-Fe(II) and Mn(IV)-Mn(III)-Mn(II) respectively.

Probably, manganese and iron Carbonates precipitate at pH around/above 7 and conditions are reduced. (Adapted from Glasby and Schulz (1999) with modifications).

In present-day soil ecosystems, fungi, in specifically ascomycetes and basidiomycetes, are prevalent oxidizers of manganese, and abiogenic manganese oxidation occurs primarily at high pH levels (above pH 8). Enzymes including manganese peroxidase are essential for the degradation of lignin where Mn(II) to Mn is oxidized by hydrogen peroxide (III) (Myers and Nealson 1988; Bartlett and James 1993; Huang and Zhang 2020). In general, Mn(IV) and Fe(III) are high redox potential electron acceptors, allowing organisms to obtain more energy from anaerobic oxidation of carbon compounds than with sulfates. The redox cycle of manganese (and iron) also defines the alteration of their solubility (Huang and Zhang 2020). The oxidized products MnO_2, FeOOH, $FeOH_2$ and Fe_2O_3 precipitate and settle in the anoxic sediment where they are reduced within anoxic conditions. Fe(II) and Mn(II) have higher solubility but can form precipitates as carbonates or sulfides for Fe to enhance removal (Nealson and Saffarini 1994). Since the potential of the Mn(IV)/Mn(II) redox lies between the nitrate potentials reduction and iron reduction, Mn(IV) is a viable electron acceptor of an anaerobic microbial electron transport chain. Aside anaerobic sulphate reducers, several Mn(IV) (and also Fe(III) reducers also use other electron acceptors of high redox potential, including oxygen (Myers and Nealson 1988; Huang and Zhang 2020). The rate of oxidation is influenced by several factors, including pH, O_2 content, light, Mn and Fe-oxide surfaces, bacteria, and other cations and anions in the solution. The rate of Mn oxidation and precipitation has been found to increase with the concentration of dissolved Mn and with the abundance of Mn-oxide and hydroxide surfaces under abiotic conditions at a given pH (Hem 1972, 1978).

2.10 Knowledge Gap Statement

Having reviewed relevant works of literature, it is evident that appreciable work has been done on the utilization of bioremediation methods for the treatment of crude oil and/or AMD contaminated sites. The problem associated with these pollutants poses a serious threat to the availability of arable land, food and water security, economic growth and improved standard of living. Hence, the drive to invest in cost-effective, sustainable, and viable approach for the remediation and reclamation of contaminated sites is of great interest.

Most studies focused on the application of bioremediation methods on mine water remediation while the bioremediation of AMD contaminated soils has not been thoroughly investigated. Also, the novelty of the present study is to bridge the below-stated gaps in the literature which have not been extensively investigated:

- The evaluation of the performance of brewery and municipal wastewater for the remediation of AMD and crude oil-polluted soils.
- The investigation of combined application of air-injection and wastewaters (brewery and municipal) for the remediation of AMD and crude oil polluted soils
- The application and evaluation of bacteria strain, *Pseudomonas aeruginosa* ATCC 15442 potentials for contaminant remediation.
- The evaluation of brewery and municipal wastewater amendment as an extra carbon source for *Pseudomonas aeruginosa* ATCC 15442 in AMD and crude oil bioremediation.

CHAPTER THREE

3.0 Materials and Method

3.1 Materials

i. *Soil Samples*

Soil samples used for this study were collected from three different locations at 2-3 cm depth in Durban, South Africa. The soil samples were mixed thoroughly, air-dried, and subsequently passed through a set of sieves between 2 mm and 0.002 mm mesh sizes (Table 3-1). Soil samples for the bioremediation treatment were preserved at ambient temperature in a polyethylene bag for future use.

ii. *Crude oil and AMD*

Crude oil sample used for this study is a light crude oil, collected from a local petrochemical Company in Durban. The crude oil has not undergone any distillation or other conversion processes. Light crude oil is liquid petroleum that flows freely at room temperature. It has low viscosity, low specific gravity and high API gravity (between 32^o (865 kg/m^3) and 42^o (816 kg/m^3) API) due to presence of significant proportion of light hydrocarbon fractions (NYMEX 1995). AMD water was simulated from metal sulfates.

iii. *Brewery Waste (BWW) and Municipal Waste (MWW)*

Brewery wastewater was obtained from South African Brewery (SAB) while Municipal (domestic) wastewater effluent was collected from Isipingo wastewater works. Samples were stored in high-density polyethylene (HDPE) containers, kept in the refrigerator for further use. The wastewaters were characterized by carbon-oxygen demand (COD) and presented in Table 3-2.

iv. *Pseudomonas aeruginosa* ATCC 15442

Pure culture of *P. aeruginosa* ATCC 15442 used for this study was supplied in kwikstik form by Anatech Microbial Laboratories, South Africa.

Table 3-1. Physicochemical properties of Soil

Properties	Parameter/value
Soil pH	7.33
Sand (2 – 0.02 mm) (%)	79.32
Silt (0.02 – 0.002 mm) (%)	14.71
Clay (<0.002 mm) (%)	5.97
Texture Class	Sandy
Moisture Content	10.6 $\pm$ 0.3
Soil Profile	Domestic Subsurface (1-3 cm depth)
Location	Durban, South Africa
Initial soil Contaminant (AMD/Crude oil)	Nil

Table 3-2**.** BWW and MWW Properties

Wastewater/ Composition	**BW**	**MW**	**Deionised water**
COD (mg/L)	750	704	
pH	8.2	7.9	7.1

3.2 Methods

3.2.1 Preparation of crude oil contaminated soil

An appropriate amount of soil sample (1 kg) was spiked with 50 g of crude oil, stirred continuously to achieve a homogenous mixture of the two components to practically achieve artificial contamination. 5 % (*w/w*) was adopted to obtain severe contamination of soil sample as a concentration above 3% has been reported to be increasingly detrimental and deteriorate soil organic content, rendering the soil impotent for plant growth (Baker 1976; Osuji, Egbuson and Ojinnaka 2005). The contaminated soil was kept for 2 days to allow for ageing and to mimic a real contaminated soil scenario after which different bioremediation treatment was applied.

3.2.2 Biostimulation (BSTc) study of crude oil contaminated soil

The experimental set-up for the biostimulation of crude oil polluted soils consists of six plastic bioreactors (Fig. 3-1b) for the soil treatment. Five out the six bioreactors for biostimulation (BSTc) containing 1 kg of contaminated soil each were amended with brewery waste (BWW) and municipal waste (MWW) of varying ratios while the remaining bioreactor designated as bioattenuation (BATc) was used for control experiment with no amendment (Table 3-3).

3.2.3 Bioventing (BVTc) study of crude oil contaminated soil

Crude oil contaminated soil was prepared according to *section **3.3.1*** above. Bioventing study for crude oil contaminated soil treatment consists of five bioreactors designated as BVTc containing 1 kg of contaminated soil. Three bioreactors were amended with/out wastewater (BWW and MWW) and atmospheric air was supplied to these bioreactors while one bioreactor was not amended with wastewaters but ventilated. The bioreactor for bioattenuation (BATc) which was neither supplemented with wastewaters nor ventilated served as control treatment as shown in Table 3-3. Atmospheric air was supplied to the bioreactors through the vadose unsaturated zone using an air compressor pump to allow for adequate air circulation around the bioreactors (Fig. 3-1a). Air was supplied at 3 L/min for 30 mins every 48 hours since bioventing will be more economical if the lowest flow rate and highest flow interval will be considered for

the bioventing of contaminated soils while increase flow rate and the interval is not justifiable due to high operation cost (Thomé *et al.* 2014).

3.2.4 Bioaugmentation (BAUc) study of crude oil-contaminated soil

3.2.4.1 Microbial culture and inoculum preparation

The strain of *P. aeruginosa* ATCC 15442 used in the present study was preserved at 2^oC – 8^oC before the culturing process. The starter culture for *P. aeruginosa* ATCC 15442 strain was prepared in solid slant using 10g of MacConkey agar medium dissolved in 200ml deionized water in 300ml Erlenmeyer flask. The flask was covered with cotton wool and aluminium foil to prevent loss and ensure airtight. The medium was autoclaved at 120^oC for 90mins to sterilize and remove impurities that will trigger the possible growth of undesirable / non-targeted microbes, subsequently transferred to a petri dish and cooled for 30mins in the laminar airflow chamber to avoid contamination. *P. aeruginosa* ATCC 15442 was transferred to slant media and incubated for 48 hours at 37^oC. Bacteria cells were activated according to Varjani and Upasani (2017) by transferring culture from the nutrient agar slant to Bushnell-Hass (BH) broth ($MgSO_4$ – 0.200 g/l, $CaCl_2$ – 0.020 g/l. KH_2PO_4 – 1.000 g/l, K_2HPO_4 – 1.000 g/l, NH_4NO_3 – 1.000 g/l, $FeCl_2$ – 0.050 g/l, pH at 25^oC – 7.1) amended with 1% v/v crude oil and incubated at 37 ± 1^oC, in continuous agitation at 180rpm for 24 hours. The inoculum was prepared in 200ml BH medium by inoculating activated culture broth at 4%, v/v of OD -1.0 at $AU_{600,}$ and incubated at 37 ± 1^oC, 180rpm for 24 hours.

3.2.4.2 Experimental set-up for the bioaugmentation of crude oil contaminated soil using *P. aeruginosa* ATCC 15442

The bioaugmentation treatment was carried out in four bioreactors (BAUc) (Fig. 3-1c) containing 1kg crude oil-contaminated soils each prepared according to *section 3.3.1*. 100ml of BH broth was added to three bioreactors followed by inoculation of enriched culture (OD: 1.0 at AU_{600}) at 5% (w/w), while two bioreactors were amended with wastewaters afterwards. Bioreactors that were neither inoculated nor supplemented with wastewater effluents was used as the control treatment (BATc) as shown in Table 3-3.

All bioreactor treatments were conducted in fours (quadruple) at ambient temperature condition (22 ± 3^{o}C) and monitored for 28 days. Samples were taken from each bioreactor every 7 days for residue TPH extraction and analysis.

Table 3-3. Composition of Bioremediation of crude oil contaminated soils

Bioreactors	**BWW (mg/kg^{-1}) Soil**	**MWW (mg/kg^{-1}) Soil**	**Air (O_2)**	**P. A ATCC 15442 (5% w/w Inoculum)**	**Loading ratio (BWW:MWW)**
BSTc-1	100	0	-	-	4:0
BSTc-2	75	25	-	-	3:1
BSTc-3	50	50	-	-	2:2
BSTc-4	25	75	-	-	1:3
BSTc-5	0	100	-	-	0:4
BVTc-1	*0*	*0*	√	-	*0:0*
BVTc-2	*100*	*0*	√	-	*4:0*
BVTc-3	*0*	*100*	√	-	*0:4*
BVTc-4	*50*	*50*	√	-	*2:2*
BAUc-1	0	0	-	√	0:0
BAUc-2	100	0	-	√	4:0
BAUc-3	0	100	-	√	0:4
BATc (Control)	0	0	-	-	0:0

BSTc: *Biostimulation,* BVTc: *Bioventing,* BAUc: *Bioaugmentation,* BATc: *Bioattenuation* BWW: *Brewery wastewater,* MWW: *Municipal wastewater,* P.A: *P. aeruginosa ATCC 15442*

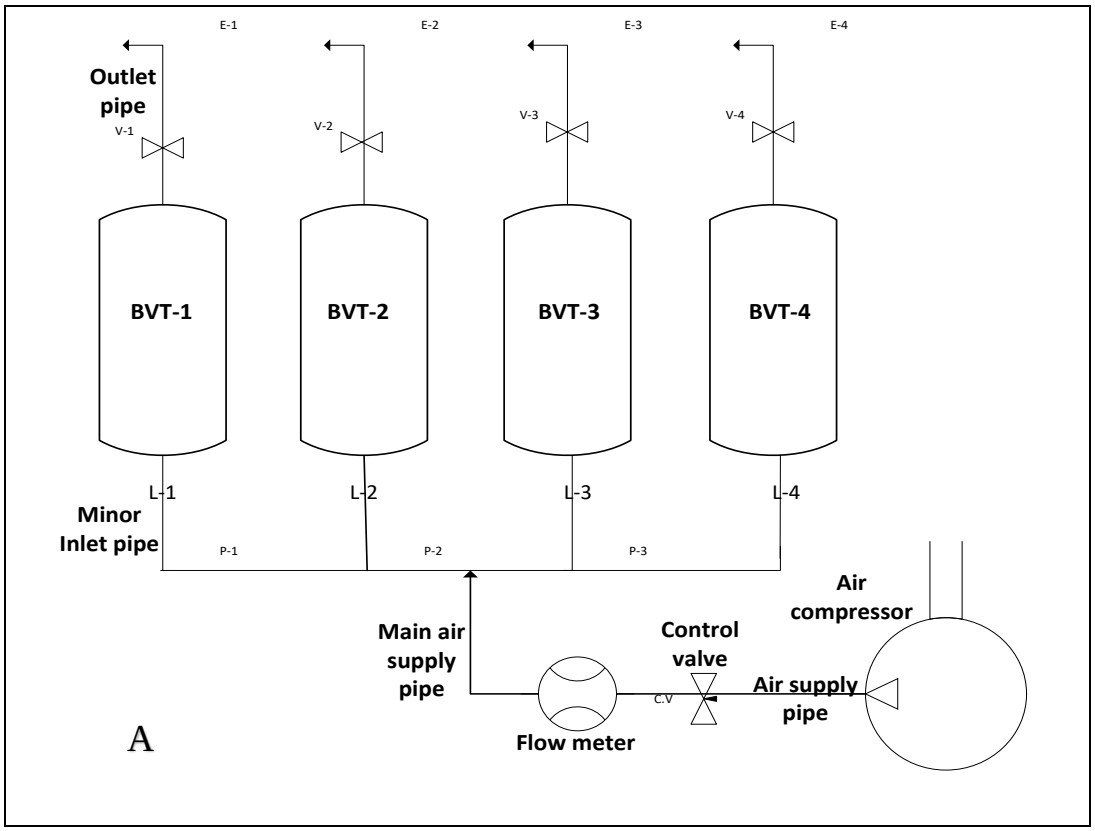


Figure 3-1a. Schematic Diagram for the Bioventing (BVT) Treatment System

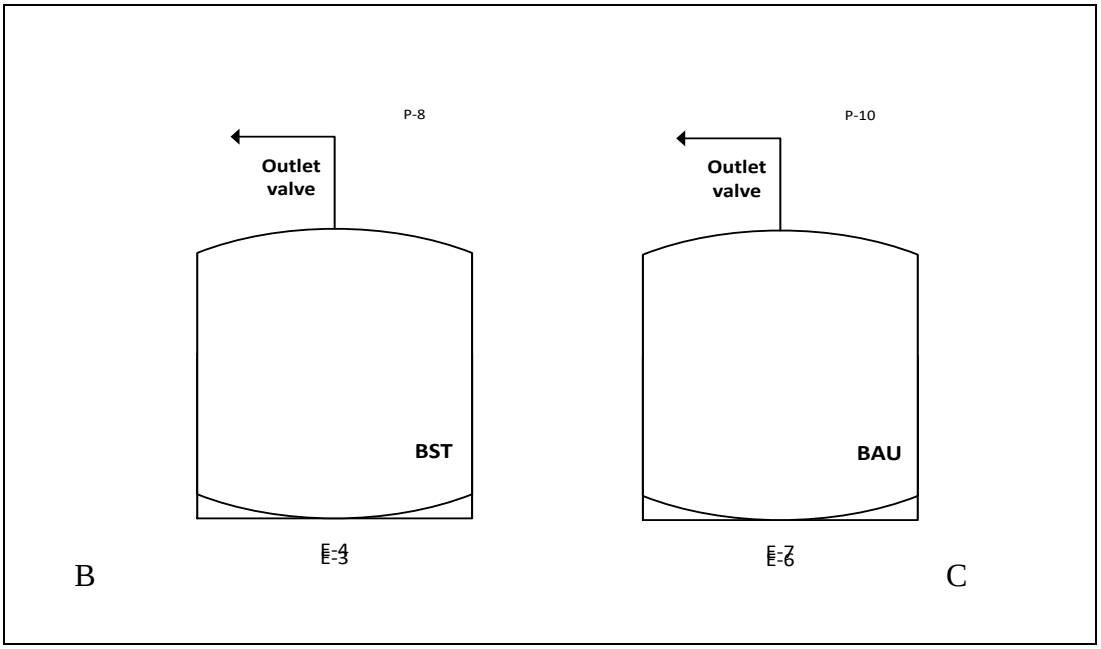


Figure 3-2b & c. Schematic Diagram for the Biostimulation (BST) and Bioaugmentation (BAU) Treatment System respectively

3.3 Bioremediation study of AMD contaminated soil

3.3.1 Simulation of mine water and preparation of AMD contaminated soil

AMD was simulated with metal compositions close to that obtained in central Witwatersrand (Humphries, McCarthy and Pillay 2017): consisting of 25.70 $ZnSO_4.H_2O$; 10.70 $CuSO_4.5H_2O$, 200.01 $FeSO_4.7H_2O$, 20.00 $MnSO_4.H_2O$, 50.50 $Al_2SO_4.18H_2O$ (mg/kg). The required metal sulfates were dissolved in the appropriate quantity of deionized water, and the pH adjusted to 2.7 using sulfuric acid to achieve the required AMD acidic medium (Table 3-4). The mixture was stirred at 200rpm for 60 minutes to ensure a homogeneous solution of the metal sulfate. For the AMD contaminated soil, the simulated mine water was mixed with the soil sample and the contaminated soil was agitated at 180 rpm for even distribution and mixture of AMD solution in the soil sample. The contaminated sample was dried and kept unperturbed for 48hours before the exposure to bioremediation treatments.

3.3.2 Biostimulation (BSTa) study of AMD contaminated soil

Experiments were conducted in six plastic bioreactors designated as BSTa containing 1kg of contaminated soil sample each. Five bioreactors were amended with brewery waste (BWW) and municipal waste (MWW) at varying compositions. The remaining bioreactor for Bioattenuation (BATa) treatment that received no amendment served as a control treatment as shown in Table 3-5.

3.3.3 Bioventing (BVTa) study of AMD contaminated soil

The bioventing treatment of AMD contaminated soils consist of five bioreactors labelled as BVTc, containing 1kg of contaminated soils, with three bioreactors amended with wastewaters (BWW and/or MWW each) and were supplied with atmospheric air at 3 L/min for 30 min every 48 hours using air compressor pump, through the vadose unsaturated zone to allow for ventilation of bioreactors as shown in Fig. 3-1. One bioreactor was ventilated only while the remaining bioreactor which neither received nutrient amendment nor atmospheric air served as a control treatment (BATa). Details as presented in Table 3-5.

3.3.4 Bioaugmentation (BAUa) study of AMD contaminated soil

3.3.4.1 Microbial culture and inoculum preparation

The starter culture for the bacteria was prepared according to ***Section 3.3.4.1*** above. The liquid medium was prepared with Luria-bertani (LB) broth in 300ml Erlenmeyer flask by dissolving peptone (10g/L), NaCl (5g/L), and Yeast (2g/L) in distilled water. The LB solution was autoclaved at 120^{o}C for 90 mins and cooled for 30 mins. Starter culture was transferred to the LB nutrient medium in the laminar airflow chamber, incubated at 37±1^{o}C, with continuous shaking at 180rpm for 48 hours. Bacteria cells used for this study were immobilized according to Philip, Iyengar and Venkobachar (2000) after 48 hours of incubation (optical density 1.0 at 600 nm), cells were harvested and separated from media using centrifugation at 4000 rpm for 30 min at 4 ^{o}C. The supernatants were decanted, and immobilized cells recovered as sediment. The isolated biomass was rinsed with distilled water. The inoculum was prepared in a 200 ml LB medium using the immobilized cells.

3.3.4.2 Experimental set-up for the bioaugmentation of AMD contaminated soil using *P. aeruginosa* ATCC 15442

The bioaugmentation treatment was carried out in four plastic bioreactors (BAU_a) containing 1kg AMD contaminated soils each. 100ml of LB broth was added to three bioreactors and immobilized cells (5% *w/w*, OD: 1.0 at AU_{600}) were inoculated. Two bioreactors received BWW or MWW afterwards. One bioreactor was inoculated, and the control treatment (BATa) was neither inoculated with *P. aeruginosa* nor supplemented with wastewater effluents (Table 3-5).

All AMD treatments were conducted in fours (quadruple) at ambient temperature and monitored for 28 days. Samples were collected from each bioreactor every week and analyzed for heavy metal and sulfate concentration.

Table 3-4. AMD Composition

Metals / Sulfate	Concentration **(mg/kg)**
Fe^{2+}	40.120
Cu^{2+}	2.722
Zn^{2+}	9.363
Al^{3+}	4.091
Mn^{2+}	6.502
SO_4^{2-}	789 – 798
Strength of Solution / Solution Conc. (g/L)	0.314

Table 3-5. Biostimulation treatment of AMD contaminated soil

Bioreactors	**BWW (mg/kg^{-1}) Soil**	**MWW (mg/kg^{-1}) Soil**	**Air (O_2)**	**P. A ATCC 15442 (5% w/w Inoculum)**	**Loading ratio (BWW:MWW)**
BSTa-1	100	0	-	-	4:0
BSTa-2	75	25	-	-	3:1
BSTa-3	50	50	-	-	2:2
BSTa-4	25	75	-	-	1:3
BSTa-5	0	100	-	-	0:4
BVTa-1	*0*	*0*	√	-	*0:0*
BVTa-2	*100*	*0*	√	-	*4:0*
BVTa-3	*0*	*100*	√	-	*0:4*
BVTa-4	*50*	*50*	√	-	*2:2*
BAUa-1	0	0	-	√	0:0
BAUa-2	100	0	-	√	4:0
BAUa-3	0	100	-	√	0:4
BATa (Control)	0	0	-	-	0:0

BSTa: *Biostimulation,* BVTa: *Bioventing,* BAUa: *Bioaugmentation,* BATa: *Bioattenuation* BWW: *Brewery wastewater,* MWW: *Municipal wastewater,* P.A: *P. aeruginosa ATCC 15442*

3.4 Analytical Procedure and Instruments

COD was determined through standard method using COD reactor (Hach) and Spectrophotometry (DR 3900) (APHA 1995) while electronic pH meter was used to determine the pH of samples. Bacteria medium were autoclaved using Vertical Type Steam Sterilizer HL-341. Optical cell density was determined with Thermo Scientific GENESYS™ 150 UV-Visible Spectrophotometer and Eppendorf 5810R Refrigerated Centrifuge w/A-4-81 Rotor was used for cell immobilization. Soil pH was determined according to (Peech *et al.* 1947): 20g of homogenized soil sample was mixed with 20ml of distilled water (1:1 *w/v*) in 50ml beaker and the suspension agitated for 60minutes.

3.4.1 Soil sample preparation and extraction of crude oil for TPH analysis

Mechanical shaking was applied for crude oil extraction from soil samples as it is considered an effective and rapid soil solvent extraction procedure. Before the extraction, samples were dried in the oven at 35^{o}C for 24 hours and pulverized using mortar and pestle to reduce the grain size and sieved using 63 µm standard sieve size to ensure homogeneity of grain sizes. 5 g of homogenized soil was weighed into 250ml glass jar, dichloromethane (DCM) and acetone were added in the ratio of 2:1 (20 and 10 mL respectively), the glass jar was covered with aluminium foil to prevent loss of solvent and shaken vigorously on a mechanical shaker for 90 minutes at 200 rpm to allow DCM and acetone to effectively extract oil from the soil sample. The solution was filtered using Whatman filter paper and syringe filter to remove all impurities. The filtrate (extract) was transferred into a 50 ml volumetric flask and made up to a known volume of 30ml. Literature studies revealed that DCM and acetone proved the most suitable solvent for oil extraction from soil samples because of its consistency, efficiency, and ability of not interfering with hydrocarbon fractions like Benzene, Toluene, and Xylene (BTEX), & C5-C9 (Okop and Ekpo 2012). Total petroleum hydrocarbons were identified and quantified using Gas Chromatography-Mass Spectrometry, Shimadzu GCMS-QP2010 SE.

3.4.2 Heavy Metals Analyses

Soil samples were dried in an oven at 35^{o}C to prevent degradation and loss of metals. 5g of dried soil sample was homogenized using mortar and pestle to obtain a fine texture which was later allowed to pass through 63µm sieve to remove rocks, pebbles, and sticks particles. Subsequently, scanning electron microscopy - Energy-dispersive X-ray spectroscopy (SEM-EDS) and X-ray fluorescence analysis using Wirsam XRF were carried out on the soil samples for qualitative and quantitative analysis of heavy metals in the soil respectively.

3.4.3 Sulfate Analysis

For the sulfate analysis, aqueous extraction was used which entails the addition of 150mL of deionized water to 15g of the homogenized sample, shaking at 300rpm for 30mins, followed by filtration. The filtrate was mixed with reagents and SO_4^{2-} ions were precipitated with $BaCl_2$ in acidic medium and colour was detected at 420nm using a Gallery plus discrete analyser.

CHAPTER FOUR

4.0 Results and Discussion

4.1 Bioremediation study of crude oil contaminated soils

4.1.1 Biostimulation of crude oil contaminated soils (BSTc)

4.1.1.1 BSTc Results

Biostimulation results showed that the amendment of contaminated soil with wastewater (BWW and MWW) facilitated the biodegradation of crude oil as the TPH concentration decreased from week 1 to week 2 (Fig. 4-1). It is evident from the result that TPH percentage removal was relatively fast within the first 2 weeks of the treatment in all the bioreactors except BSTc-5 which recorded the highest weekly removal efficiency in week 3 (48.21%) with a 56.62% overall removal efficiency which represent TPH reduction by 28,310mg/kg from 50,000mg/kg soil initial concentration in week 4 at 1011,07mg/day average removal rate. BSTc-1 showed TPH removal in week 1 with 19.82% weekly removal efficiency, which increased progressively by 7.11% and 10.17% in week 2 and week 3 to attain the removal efficiency of 58.39% in week 4 at 1042mg/day average removal rate.

However, BSTc-2, 3, and 4 showed a similar trend in relation to other treatments but recorded removal efficiency of less than 50% after week 4 (Fig. 4-2). The average removal efficiency of >15% was observed for these treatments (BSTc-2, 3, and 4) in week 1, but as BSTc-2 and BSTc-3 increased by >8% (9.58% and 10.16% respectively), BSTc-4 only recorded a 2.03% increase in removal efficiency in week 2. Also, the TPH removal efficiencies of treatment BSTc-2, 3 and 4 plateaued at 48.67%, 49.67%, and 48.81% with an average removal rate of 869.11, 886.96 and 871.61mg/day respectively attributed to >5% increase in removal efficiencies of treatments BSTc-2 and BSTc-4 in week 4, while BSTc-3 recorded 2.87% which is a decline from 10.16% increased efficiency in week 3 (Fig. 4-2). Fig. 2 showed that the control treatment, BATc recorded the least TPH removal efficiency from week 1 to week 4 (8.15 to 34.50%) which represents TPH removal of 17250mg/kg at 616.07mg/day average TPH removal rate *(See Appendix 1)*. This validates the assertion that TPH reduction from treatments supplemented

organic waste is due to the activities induced by these biostimulants. The treatments amended with nutrients showed similar TPH removal efficiencies from week 1 to week 4 in both single and mixed substrates.

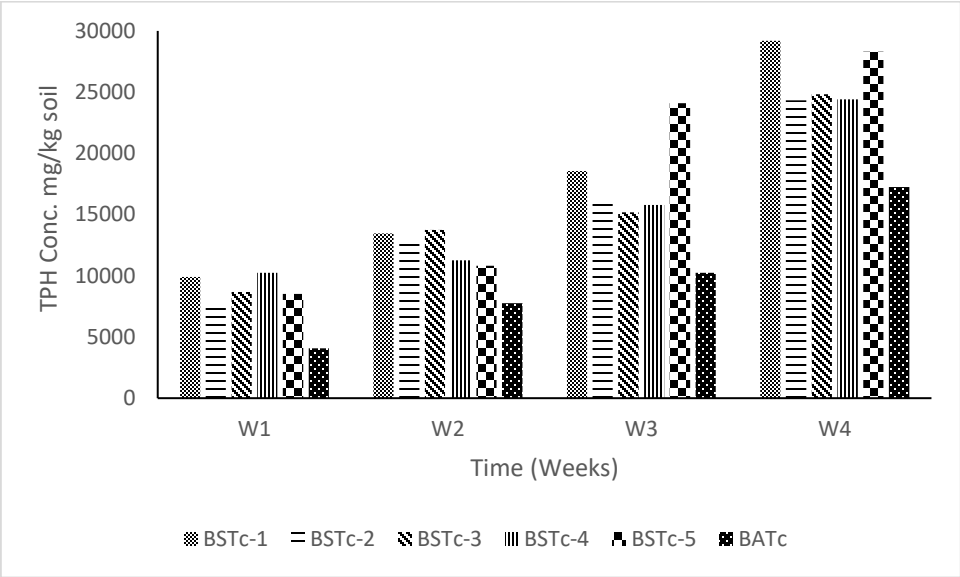


Figure 4-1. TPH Cumulative removal (mg/kg soil) from BST treatment

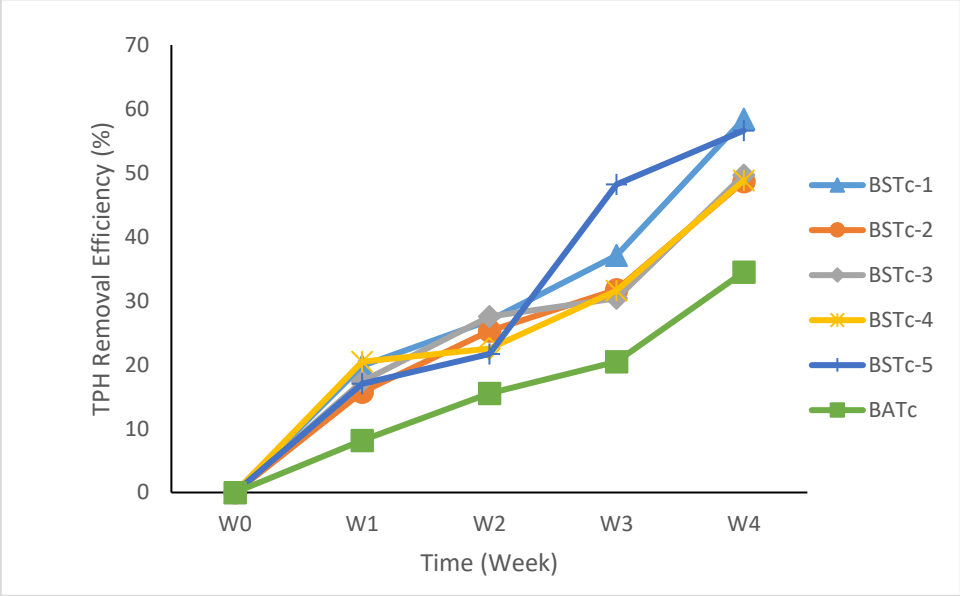


Figure 4-2. TPH Cumulative Removal Efficiency (%) from BST treatment

4.1.1.2 BSTc Discussion

The first week of BST treatment observed the modification of environmental condition for microbial activities by the organic substrate (BWW and MWW), accompanied by the acclimatization of oil-degrading microbes in the treatment system similar to the findings of (Mbah and Obahiagbon 2017), which resulted in appreciable TPH weekly removal efficiency according to time, within the first 14 days and beyond (Mbah and Obahiagbon 2017). The increase in the TPH removal efficiency recorded from week 1 to week 4 with the BSTc treatments suggests a possible correlation with an increase in microbial load as the introduction of nutrients provides a favourable condition that invigorates the native microorganisms in the soil for effective degradation which has been validated in different studies (Mao and Yue 2010; Chikere *et al.* 2012; Chijioke-Osuji, Ibegbulam-Njoku and Belford 2014b; Ofoegbu, Momoh and Nwaogazie 2014; Agarry and Latinwo 2015) as the microbial growth and metabolism is a factor of hydrocarbon reduction (Abioye 2011; Varjani and Upasani 2017; Liu *et al.* 2018; Chen *et al.* 2019), hence, microbial population growth is proportional to the rate of TPH removal (Agarry and Latinwo 2015; Mbah and Obahiagbon 2017).

Organic substrates like MWW and BWW simultaneously served as a bulking agent, pH buffering agent, organic substitute, fertilizer, and soil conditioner during the treatment of TPH contaminated soil to enhance biomass growth and metabolism which was also found to be a proactive natural surfactant that improves the solubilisation of petroleum pollutants (Liu *et al.* 2018) to increase the bioavailability of PHC to degrading microbes and degradation of hydrocarbon fractions (Adams, Niyomugabo and Sylvester 2017). Amenorfenyo *et al.* (2019) reported that wastewater contains organic nutrients (N and P) which also act as a carrier for immobilizing oil-degrading strains (Adekunle 2011) as microbes grow well in nutrient-wastewater by consuming organic nutrients and turning them into usable biomass for hydrogenase (Adekunle 2011). The treatments amended with BWW only (BSTc-1) showed slightly appreciable removal efficiency (58.39%) than MWW (BSTc-5) which recorded 56.62% while the use of mixed substrates (BWW+MWW) at varying ratios showed possible synergy between the wastewaters with an average removal efficiency of 49.05%, which is slightly below that of single substrate (57.50%). The significant removal efficiency recorded with BWW only

amended treatment can be attributed to the high nutrient content (N and P) (Jaiyeola and Bwapwa 2016; Bwapwa, Jaiyeola and Chetty 2017a; Gebeyehu *et al.* 2018; Kebede 2018; Amenorfenyo *et al.* 2019) responsible for appreciable microbial loads present in BWW, which is essential for increased biodegradation efficiency for optimum bioremediation (Amenorfenyo *et al.* 2019).

Also, in addition to these attributes, wastewater was reported to contain a range of microbes that were highly resistant to toxic contaminants and had exceptional organic contaminant degradation capability (Agarry and Latinwo 2015). The efficiency of bioremediation depends on microbial viability in the environmental natural system and the viability of these microbes is a limiting factor in bioremediation (Joo *et al.* 2008).

In agreement with the present BSTc study which recorded TPH average removal efficiency of 52.43% with 50,000mg/kg TPH initial concentration of contaminated soil, Mohajeri *et al.* (2017) recorded 43% TPH average removal efficiency using an organic substrate with 60,000mg/kg TPH contaminated soil while 3000 and 30,000mg/kg contaminated soil recorded 53.22% and 58.36% average removal efficiencies respectively after 90days study period. The result indicated that a high concentration of crude oil affects the bioremediation efficiency presumably due to the toxicity of excess crude oil (>30,000mg/kg) to the microbial community which inhibits or lowers metabolism as reported by Mohajeri *et al.* (2017). To buttress the above stated, Ofoegbu, Momoh and Nwaogazie (2014) reported that that rate of biodegradation of crude oil contaminated soil is dependent on the volume of contaminants or the degree of contamination, thus, the higher the contaminant the slower the rate of biodegradation and vice versa, hence, the significant variation in removal efficiencies of organic amendments.

Also, the dependence of biodegradation rate on the degree of contamination was reaffirmed by Naowasarn and Leungprasert (2016a) in the bioremediation of oil-contaminated soil using chicken manure (organic supplement) as the result of 5% (w/w) contaminated soil treatment gave the highest TPH reduction efficiency of >60% more than the 10% (w/w) and 20% (w/w) treatment at equal addition of organic supplement after 42days of the study period. It showed that a high concentration of contaminants plays an inhibitory role in the biodegradation of hydrocarbons (Kuyukina, Krivoruchko and Ivshina 2018). In contrast, the study by Ani *et al.*

(2018) and Obiakalaije, Makinde and Amakoromo (2015) reported that low concentration of contaminants showed a significant decline and low rate of biodegradation due to the limitation of the bioavailable target contaminant for the microbial intake which indicated that the available contaminant is practically insufficient for the microbial biodegradation activities. Invariably, it can be noted that the optimum concentration of contaminants and nutrients are required to enhance bioavailability to degrading microbes for effective remediation (Obiakalaije, Makinde and Amakoromo 2015).

Similarly, Ling and Isa (2006) and Chorom, Hosseini and Motamedi (2010) recorded 45-65.6%, and 45-60% (average of 55.3%, and 52.5%) using sewage sludge after 9 and 10 weeks study period respectively which corresponds to the results of the present study (48.67-58.43%, with 52.43% average removal efficiency). Also, Agarry (2018) recorded 65.5% TPH removal efficiency using organic and inorganic fertilizers while biodegradation efficiency >40% was recorded by Al-Kindi and Abed (2016) and Adekunle (2011) with organic waste after 96 days and 15 days of treatment accordingly and >60% was reported using aged refuse by Liu *et al.* (2018) and Chen *et al.* (2019) after 98 days and 30 weeks respectively. However, the high removal efficiency (96.58%) recorded by Mbah and Obahiagbon (2017) using after 8 weeks of treatment using plant waste can be attributed to the high microbial load in plant particulates associated with appreciable cellulose content which is high in C:N (Carbon and Nitrogen ratio). Also, Isitekhale *et al.* (2013) and Margesin and Schinner (1997b) reported TPH removal efficiency of >75% using the organic substrate for the biostimulation treatment. The high removal efficiency may be attributed to the low initial concentration of crude oil 300mg/kg and 4000mg/kg (when compared to the present study) respectively which results in less toxicity of contaminants to degrading microbes and an increase in the rate of biodegradation (Mohajeri *et al.* 2017). This demonstrates that the use of organic nutrients can stimulate the microbial environment, increase the mineralization rate and promote the activities of microorganisms for effective biodegradation as reported by Jia *et al.* (2016).

4.1.2 Bioventing of crude oil contaminated soils (BVTc)

4.1.2.1 BVTc Results

Crude oil removal from the contaminated soil was evident in all the bioreactors, both the vented and vented + nutrient amendment treatments with a decrease in TPH concentrations as shown in Fig. 4-3. Fig. 4-4 shows that the introduction of atmospheric air into the treatment reactors facilitated the bioremediation process in the first week of treatment which recorded (in week 1) >15% removal efficiencies in all treatments that received nutrient amendment + air (BSTc-2, BVTc-3, and BVTc-4), 11.25% for treatment that was only ventilated (BVTc-1) and <10% for the control treatment (that was neither amended with nutrients nor ventilated (BATc) in the first week of treatment. BVTc-1 (air) and BVTc-4 (wastewaters + air) maintained >9% average increase in TPH weekly removal efficiency from week 1 till the end of the treatment in week 4 with 54.93% and 61.47% which represents 27465mg/kg and 30735mg/kg TPH removal at 980.89 and 1097.68mg/day average TPH removal rate, respectively. However, BVTc-3 (MWW+air) TPH removal efficiency increased by 3.44% in week 2 with an average increase of >19% from week 3 to week 4 and was able to reduce TPH concentration to 19990mg/kg from the treatment at 1071.79mg/day average removal rate. BSTc-2 which was amended with BWW and ventilated recorded the highest weekly removal rate after week 1 (47.52%, 59.88%, 74.75% for week 2, 3, and 4 respectively) at an average TPH removal rate of 1334.82mg/day. In comparison to other treatments, the control treatment BATc recorded the least removal efficiency (34.5%) representing only 17,250mg/kg TPH removal from the treatment. The removal efficiencies of the BVTc treatment follows the trend BVTc-2> BVTc-4> BVTc-3> BVTc-1> BATc. The weekly TPH reduction efficiencies in all treatments amended with nutrients and/or ventilated was observed to show an appreciable progressive increase from week 1 to week 4 which is linked to the amendment *(See Appendix 2).*

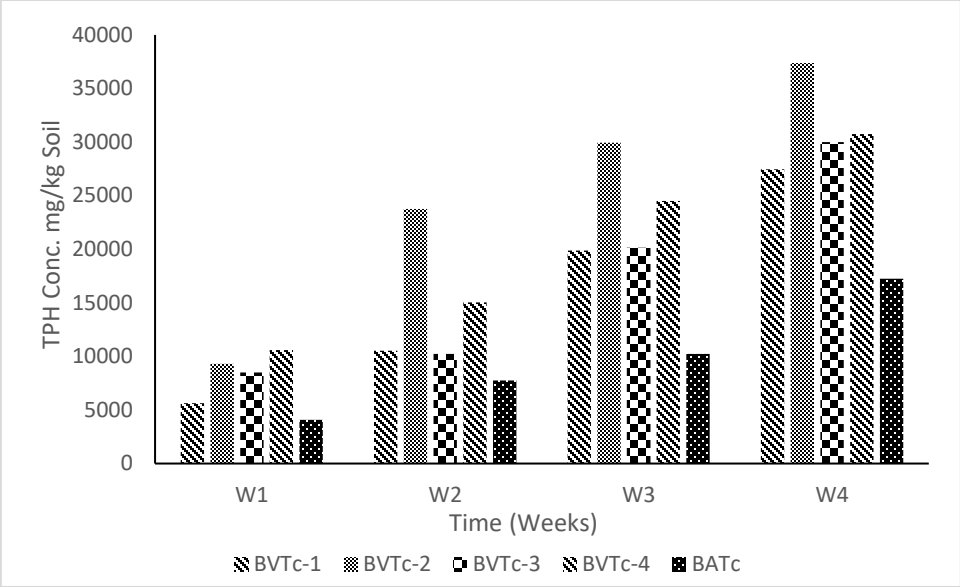


Figure 4-3. TPH Cumulative Removal (mg/kg soil) from BVTc treatment

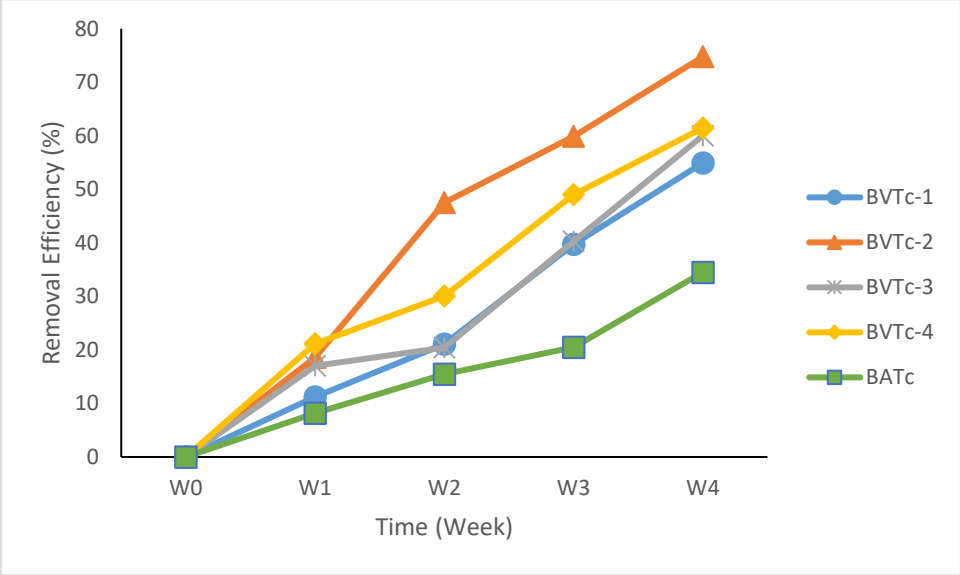


Figure 4-4. TPH Cumulative Removal Efficiency (%) BVTc treatment

4.1.2.2 BVTc Discussion

In BVTc treatments, the introduction of air into the system improved toxic conditions that increased biodegradation by providing an aerobic environment sufficient to stimulate and regenerate the autochthonous microorganism's activities (Couto and García-Frutos 2016) while

the presence of wastewaters in the bioventing system acts as biostimulants, and thus provides enough nutrient levels to boost the growth of the microbial community (Agarry and Latinwo 2015), leading to high energy demand by oil-degrading microbes, which enhanced hydrogenase and increased TPH biodegradation (Agarry and Latinwo 2015). Bioventing stimulates the indigenous microbial community by adequate air (oxygen) supply to enhance aerobic degradation of biodegradable contaminants (Leeson and Hinchee 1997; Chapelle 1999; Byun *et al.* 2005) through the oxidation process (Pardieck, Bouwer and Stone 1992; Troquet, Larroche and Dussap 2003). Muskus Morales, Santoyo Muñoz and Plata Quintero (2013) reported a higher biodegradation efficiency with bioventing more than bioaugmentation in a crude oil treatment while Agarry and Latinwo (2015) reported greater %TPH removal with combined bioventing and organic nutrient than bioventing used alone during the treatment period. The study by Thomé *et al.* (2014), buttresses the assertion that nutrient supplementation to a bioventing system renders greater TPH removal than a single utilization of each approach. This is evident in the present study, where air-injection + wastewater was able to increase the TPH removal efficiencies by 16.36%, 3.4% and 12.42% which represents 74.75%, 60.02% and 61.47% removal efficiencies (for BVTc-2, BVTc-3, BVTc-4 respectively) when compared to BSTc treatments (BSTc-1, BSTc-5 and avg. mixed substrates – treatments without air-injection) while bioventing without nutrient recorded 54.93% removal efficiency. Also, the overall average removal efficiency recorded for BVTc treatment was 10.36% greater than BSTc treatment (52.43%) which correlates with the study by Lee and Swindoll (1993), Møller *et al.* (1996) and Bulman, Newland and Wester (1993) which demonstrated that the addition of nutrients to bioventing rendered an appreciable increase in the rate of degradation of TPC (Lee and Swindoll 1993; Møller *et al.* 1996; Frutos *et al.* 2010).

The findings of this study and the ability to achieve an enhanced bioventing biodegradation with nutrient addition were in contrast with the investigation by Dupont, Doucette and Hinchee (1991) which reported that the addition of nutrient to BVT system was insignificant for the increase rate of biodegradation of TPH contaminated soil. However, a further study by Bulman, Newland and Wester (1993) demonstrated that the addition of nutrients to bioventing rendered an appreciable increase in the rate of degradation which showed that the ability of nutrient amendment to boost TPH removal efficiency is dependent on soil type and nature of nutrient

required for a successful bioventing process since some additives (nutrients) may trigger an increase in toxicity or hinder bioremediation process (Frutos *et al.* 2010).

However, soil type was reported as a crucial factor that influences the rate of degradation as Haghollahi, Fazaelipoor and Schaffie (2016) recorded the highest TPH reduction rate with sandy soil (70%) and a very low rate with clay soil (23.5%) during the study period. The degradation efficiency was increased to 57% by mixing clay with sandy soil. This low removal rate observed with clay soil is attributed to the low availability of oxygen in the soil environment since the increase in the volume of available oxygen increases the rate of degradation. Sandy soil has low total porosity with large individual pores which facilitates faster oxygen absorption into and through the soil, and the transport of carbon dioxide out of the soil which is attributed to the appreciable biodegradation recorded in the present study. Soils like clay with small pores have slower absorption of oxygen into the soil, and diffusion of carbon dioxide from the soil (Lin *et al.* 1999; Dorn and Salanitro 2000; Kwok and Loh 2003; Haghollahi, Fazaelipoor and Schaffie 2016). However, soil modification with organic wastes like BWW and MWW has shown an improvement in soil porosity and water holding capacity (Liu *et al.* 2018) required for effective bioremediation. This facilitates microbial growth, reduction in soil eco-toxicity, increase in soil pH and nutrients (N&P) which are indispensable for an improved rate of biodegradation (Abbasian *et al.* 2016; Al-Kindi and Abed 2016; Liu *et al.* 2018).

ln this study, TPH loss due to abiotic factors (sunlight, temperature, light, wind and water) were not taken into cognizance because they often played a marginal role in extracting petroleum hydrocarbons (Wild and Jones 1993). Similarly, Agnello *et al.* (2016) reported that abiotic factor facilitated desorption of pollutants to enhance the bioavailability of petroleum and hence, minimally contributes to direct TPH removal (Sun *et al.* 2013). Also, volatilization was not considered in the process because the contaminant has less volatile components and considering low operating temperature, and low flow intensities, these components have a negligible effect according to the findings of Fingas (2004) and Ma *et al.* (2014). To further reaffirm the above stated, Jia *et al.* (2016) noted that the removal of crude oil in soils in the sets of treatments was caused by volatilization and biodegradation, but biodegradation was the most effective removal process, accounting for more than 58% of the total removal and reached the highest

removal efficiency with nutrient adjustment as reported by (Agarry and Latinwo 2015; Jia *et al.* 2016).

The non-responsive nature of some hydrocarbons to biological degradation accounts for the low % removal of this recalcitrant group of hydrocarbons which occurs at difficult intervals in the same treatment environment, hence, reducing the TPH removal efficiency. According to Adams, Niyomugabo and Sylvester (2017), the biological method mechanism is targeted on degrading mainly aliphatic compounds, cyclic hydrocarbons, aromatics, and other heavy hydrocarbons which are resistant to bioattenuation. The difference in initial TPH concentration with bioattenuation indicates the microbial population degradation of the hydrocarbons (Agarry and Latinwo 2015; Adams, Niyomugabo and Sylvester 2017) which was supported by the GCMS study, which showed a substantial reduction in TPH concentration due to microbial degradation and the formation of oxidized hydrocarbons, such as alcohols, acids, carboxylic acids and esters, and the formation of low concentration amine compounds (*See Appendix 23-26*) due to the presence of organic nutrients in the soil (Adams, Niyomugabo and Sylvester 2017). However, the decrease in removal efficiencies suggests depletion of nutrients needed by microbes (Boopathy 2000).

The present bioventing study which recorded 54.93% (BVT) and 65.41% (BVT and wastewater amendment) average TPH removal efficiency after 28 days correlated with the study by Lee and Swindoll (1993), where bioventing with organic matter amendment boosted >60% removal efficiency after 70days study period. Also, the study by Balba *et al.* (1998) and Agarry and Latinwo (2015) reported 64.2% and 61.7% biodegradation efficiencies with bioventing after 12 months and 28 days treatment period respectively while appreciable removal efficiency of >75% was recorded with bioventing and organic amendment as reported by (Agarry and Latinwo 2015). Similarly, Thomé *et al.* (2014), Frutos *et al.* (2010) and Møller *et al.* (1996) recorded >70% TPH removal with bioventing and nutrient amendment technique after 112, 210 and 120 days treatment period accordingly while Jia *et al.* (2016) and Eslami and Joodat (2018) reported an average of 60% and 65% TPH removal efficiencies with bioventing and nutrient supplement after 91 and 40-50 days study period respectively. However, the low TPH removal efficiency of 45% recorded by Mao *et al.* (2009) with bioventing and nutrient amendment after

40 days treatment period can be attributed to the high concentration of crude oil (70,000mg/kg) which is toxic to the microbial community and decreased the rate of biodegradation (Mohajeri *et al.* 2017). Lee and Swindoll (1993) reported that bioventing with the nutrient amendment was effective in the removal of BTEX and recalcitrant PAHs, heavy hydrocarbon which validates bioventing suitability for the treatment of hydrocarbon ranging from light to medium (gasoline and diesel) to heavy hydrocarbons (such as fuel oils and other volatile, non-volatile HC and PAHs). It can be inferred that oil biodegradation improved significantly by providing the contaminated soil with nutrients, and oxygen (Lee and Swindoll 1993; Frutos *et al.* 2010).

The result of the bioattenuation (control treatment) from this study (34.5%) is also in agreement with the studies by Mohajeri *et al.* (2010), Agarry and Ogunleye (2012) and Liu *et al.* (2018) which recorded the lowest TPH removal efficiency from natural attenuation treatment with 9-12.6, 44.78% and 22-32% TPH removal efficiencies after 60, 98 and 42 days of treatment respectively which can be due to lack of nutrient required by indigenous microbes for metabolism. Meanwhile, the bioremediation techniques applied in this study, except the control (BATc) were able to reduce the initial concentration of crude oil below the extreme toxic level in the soil, as Baker (1976) reported that concentration of crude oil above 3%(w/w) is detrimental to soil biota, plant growth and development (Osuji, Egbuson and Ojinnaka 2005) and reduced agricultural productivity (Osuji and Nwoye 2007) as the control treatment was unable to appreciably reduce the concentration below toxic level due to non-application of lack of nutrients, which buttresses the positive effect of wastewater and air-injection as potential bioremediation strategy for the treatment of crude oil contaminated soils.

4.1.3 Bioaugmentation of Crude oil-contaminated soils (BAUc)

4.1.3.1 BAUc Results

The bioaugmentation treatment of crude oil contaminated soil using *P. aeruginosa* ATCC 15442 showed the accumulation of crude oil evident in all treatments inoculated with *P. aeruginosa* ATCC 15442 as shown in Fig. 4-6 within the first 2 weeks of the treatment and seems to have reduced in week 4 (Fig. 4-7) which is contrary to the control treatment with no visible changes in treatment (Fig. 4-5). Fig 4-8 showed that the reduction in TPH was visible

from week 1 with BAUc-2 recording the highest weekly removal efficiency throughout the treatment except in week 2, which observed a decline when compared with other treatments and reduced the initial concentration of 50,000mg/kg to 15260mg/kg (Fig. 4-8) which represents 69.48% TPH removal efficiency at 1318.75mg/day average removal rate in week 4. BAUc-3 showed the highest removal efficiency in week 2 by 20.14% increase in removal efficiency as against 10.88% and 9.43% for BAUc-1 and BAUc-2 respectively (Fig. 4-9). However, BAUc-3 was able to remove 32315mg/kg of hydrocarbon from the treatment corresponding to 65.03% TPH removal efficiency (which is 4.45% less than BAUc-2) in week 4 at 1161.25mg/day average removal rate. BAUc-1 observed a rapid removal from week 1 to week 3 with a slow process in week 4 which represents 10.81, 21.69, 39.55 and 42.02% weekly removal efficiencies respectively at an average removal rate of 750mg/day. The control treatment (BATc) recorded the lowest and slowest TPH reduction from week 1 till the end of the treatment removing 17250mg/kg at 616mg/day (*See Appendix 7-9*). It is evident from the BAUc treatment that *P. aeruginosa* ATCC 15442 was able to degrade hydrocarbons which facilitate the reduction in TPH as shown in treatment inoculated with *P. aeruginosa* ATCC 15442 only (BAUc-1) when compared to the control treatment which was neither inoculated nor amended with bacteria strain nor wastewater respectively. Also, the introduction of wastewaters increased the TPH removal efficiency in all treatments supplemented with BWW or MWW.

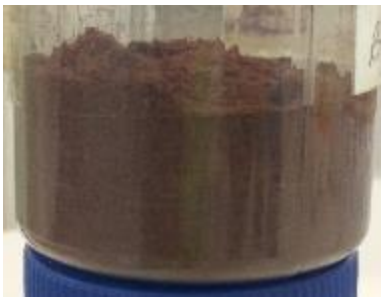


Fig. 4-5

Fig. 4-6

Fig. 4-7

Figure 4-5. Control Treatment (BATc) – No amendment. wk1

Figure 4-6. BAUc treatment amended with *P. aeruginosa* ATCC 15442 and BWW (BAUc - 2) wk1

Figure 4-7. BAUc treatment amended with *P. aeruginosa* ATCC 15442 and BWW (BAUc - 2) wk4

Fig. 4-6 showed the accumulation and immobilization of crude oil due to effect of biosurfactant which reduced in wk4 (Fig. 4-7) because of biodegradation while no visible changes or effect was observed in Fig. 4-5, the control treatment.

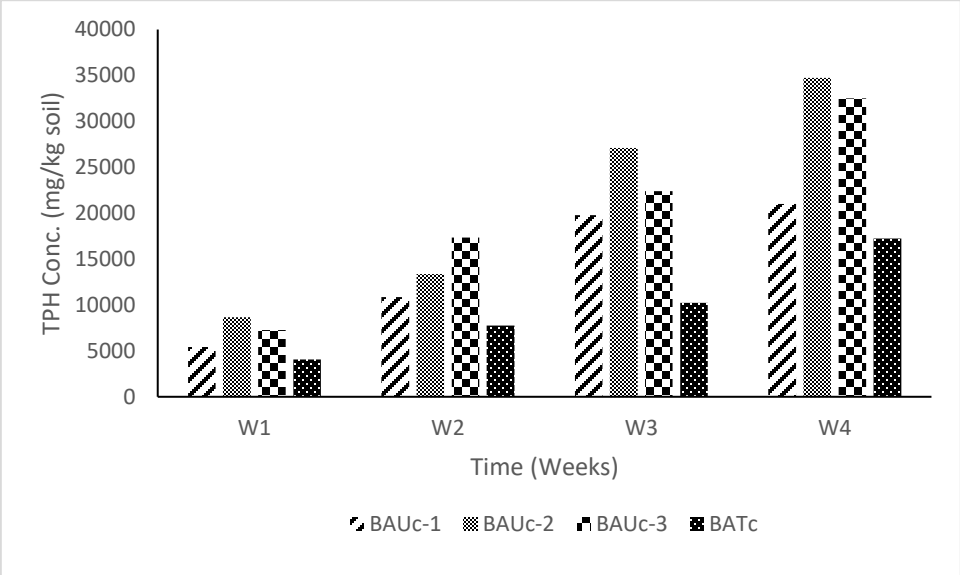


Figure 4-8. TPH Cumulative Removal (mg/kg) from BAUa treatment

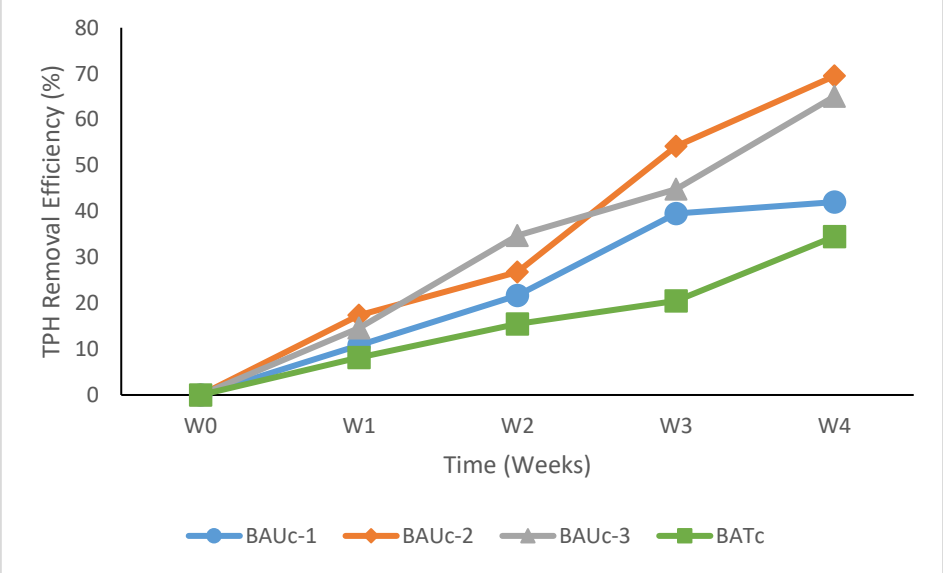


Figure 4-9. TPH Cumulative Removal Efficiency (%) from BAUa treatment

4.1.3.2 BAUc Discussion - Crude oil biodegradation Mechanism

The accumulation of crude oil as shown in Fig. 4-6 evident in treatments amended with *P. aeruginosa* ATCC 15442 after the first week of treatment can be attributed to the result of solubilisation and immobilization of crude oil to the degrading bacteria by the produced rhamnolipids (Ron and Rosenberg 2001) and the subsequent degradation of the accumulated crude oil (Guo-liang *et al.* 2005) results to the decrease in the concentration of the accumulated crude oil (Fig. 4-7) in week 4. Biodegradation of crude oil by *P. aeruginosa* ATCC 15442 was feasible through the production of rhamnolipids biosurfactant which is a low molecular weight glycolipid (Uzoigwe *et al.* 2015) which explains the reason for no visible changes in the control treatment (Fig. 4-5). Rhamnolipids by *Pseudomonas aeruginosa* is a well-known biosurfactant, which allows the biological absorption of crude oil by accumulated biomass (Beal and Betts 2000) which can have an impact on the actual removal of crude oil as evident in treatments inoculated with *P. aeruginosa* ATCC 15442. The rhamnolipid biosurfactants possess unique characteristics such as non-toxicity, biodegradability, biocompatibility, low concentration efficiency, and their production of natural substrates under mild environmental conditions attribute to their suitability for bioremediation purposes.

Guo-liang *et al.* (2005) and Uzoigwe *et al.* (2015) reported that crude oil adsorption to dead *P. aeruginosa* may be negligible in hydrocarbon degradation since *P. aeruginosa* utilizes crude oil as the sole energy source for biological activities through the synthesis of rhamnolipids. The amphiphilic nature of the rhamnolipids enables them to dissolve and immobilize hydrophobic solvents like crude oil to enhance bioavailability and degradation (Perfumo *et al.* 2006; Satpute *et al.* 2010; Smyth *et al.* 2010). This process is accomplished by reducing the surface and interface stress between the relatively high liquid-liquid phase and the formation of stable emulsion to enhance degradation. Reduction of surface and interface tensions is promoted by biosurfactant adsorption into various stages, leading to greater contact and mixing of non-similar phases (hydrocarbon-H_2O) (Uzoigwe *et al.* 2015). The more surface and interfacial pressures are lower, the higher the distribution of oil and the more bioavailable the hydrophobic substrates to degrading microbes (Uzoigwe *et al.* 2015). Since only approximately 0.02% of

crude oil is water-soluble, the emulsification of crude oil is essential to facilitate the treatment of polluted soil (Guo-liang *et al.* 2005).

The dependence of hydrocarbon degradation on solubility attributes to the need for emulsification (Ron and Rosenberg 2001). High molecular hydrocarbons and PAH have a low solubility (which increases the sorption to the surface) as a result of their recalcitrant existence – restricting their bioavailability to these bacteria that degrade them and inhibit biodegradation due to the strong relation or bonding of organic molecules to surfaces or soil (Ron and Rosenberg 2001). By desorbing organic substrate from soil or surface using biosurfactant, the apparent solubility is increased which it enhances growth rate to improve biodegradation. However, factors such as energy source, temperature, pH, availability of oxygen, agitation, the concentration of culture medium and condition of bacteria cultivation may affect the development of the biosurfactant as the desorption of hydrocarbons in polluted soil increases as rhamnolipids production increases which makes more insoluble substrates available for degradation (de Jesus Cortes-Sanchez, Hernandez-Sanchez and Jaramillo-Flores 2013).

It was demonstrated in the study by Rosenberg, Gottlieb and Rosenberg (1983) that oil-degrading bacteria experience starvation once the choice of their energy source (specific hydrocarbon fractions) is degraded. The degraded oil droplets develop a hydrophilic surface which makes binding or connecting the bacteria to depleted oil-droplet difficult to enhance the emancipation of cells from depleted oil droplets motivates them to search for new oil droplets or nutrients. A separation between bacteria and the depleted oil droplet increases dehydrogenase and enhance bacterial free movement with the view to mobilizing the necessary hydrocarbon fraction for energy which increases biodegradation efficiency and facilitates the bioremediation of crude oil-polluted areas. The bacteria strain, *P. aeruginosa* can degrade different hydrocarbon fractions effectively which attributes to its potential application in the treatment of varieties of petroleum hydrocarbons (Rahman and Gakpe 2008). The stability, eco-friendly and selective existence of biosurfactants (rhamnolipids) is responsible for its efficiency when compared to chemical and synthetic surfactants, which enhances mobilization of hydrophobic compounds for hydrocarbon bioremediation processes (Rahman and Gakpe 2008). Also, Zhang *et al.* (2011) noted that *P. Aeruginosa* can use diesel oil and crude oil as a substantive energy source for

growth while degrading effectively the major fractions of crude oil (n-alkanes and PAHs) (Rahman and Gakpe 2008).

4.1.3.3 Effect of organic amendment as an extra energy source for *P. aeruginosa* biodegradation

The amendment of organic substrates like BWW and MWW as additional carbon source enhanced the dehydrogenase which increases the pH of the treatment system to promote biodegradation of hydrocarbons (Amhakhian and Faleke 2014; Adekunle *et al.* 2017a) as shown in treatment BAUc-2 and BAUc-3, amended with wastewaters which recorded TPH removal efficiency (average of 25%) more than the treatment without wastewater amendment (BAUc-1) as shown in Fig. 4-9. The increase in pH provides conducive remediation environment for the microbial community to thrive as the optimal pH of 7 provides a favourable condition for the growth of microorganisms which facilitates the remediation process (Ani *et al.* 2018). Extreme pH values have been reported to negatively influence microbial growth and reduce their capacity to degrade target pollutants comfortably (Leahy and Colwell 1990). Imafidon and Ogirigbo (2018b); Amhakhian and Faleke (2014); Omokaro (2006) reported that organic nutrients can stimulate soil microbial activities which increased the removal efficiency of organic pollutant. This can be attributed to the ability of the organic substrate to establish a favourable environment for microorganisms to thrive (Amhakhian and Faleke 2014; Kuyukina, Krivoruchko and Ivshina 2018) as evident in this study where BWW and MWW served as an extra direct energy source for *P. aeruginosa* ATCC 15442 readily available for microbial utilization (Tsukamoto, Killion and Miller 2004).

In agreement with the present BAUc study which recorded 42-69% with an average of 58.84% TPH removal efficiency after 28 days, the study by Kumari, Regar and Manickam (2018), reported with *P. aeruginosa*, 67.1% hydrocarbon removal efficiency from contaminated soil after 45 days of treatment and Bezza and Chirwa (2015), using biosurfactant produced by *P. aeruginosa* for the treatment of PAH polluted soil recorded 62% after 8 days of treatment. Besides, microbial biodegradation of resins fractionated from Arabian light crude using *P. aeruginosa* isolated from emulsified mixed population recorded 50% removal efficiency from 5000ppm TPH concentration of crude oil after 7 days of treatment as reported by

Venkateswaran *et al.* (1995) while Tavassoli *et al.* (2012) reported 46% removal efficiency with *Pseudomonas spp.* According to the findings by Tavassoli *et al.* (2012), the increase in growth *P. aeruginosa* was evident while degrading fractions of hydrocarbons. Similarly, the study by Mukherjee *et al.* (2010b) further supports *P. aeruginosa* tolerance in oil-polluted soils as earlier reported by Venkateswaran *et al.* (1995) while using selected fractions of hydrocarbons as an energy source. Das and Mukherjee (2007) recorded 75% and 46.4% TPH removal efficiency for *P. aeruginosa* M and NM and B. *subtilis* respectively after 120 days. The study showed that *P. aeruginosa* strains were more effective than *B. subtilis* strain in TPH degradation process (Das and Mukherjee 2007; Karamalidis *et al.* 2010). Noordman and Janssen (2002) noted that the *P. aeruginosa* strain possesses an energy-dependent system which mediates the rapid absorption (in the presence of rhamnolipid) of hydrophobic components. Shin, Kim and Ahn (2006) reported that the application of *P. aeruginosa* biosurfactant (rhamnolipid) in the remediation of phenanthrene polluted soils through a combined solubilisation and biodegradation process showed substantial reduction in phenanthrene concentration. However, study by Conte *et al.* (2005) validated the effectiveness of organic surfactant (biosurfactant) in the removal of pollutants from polluted soils and reported that water could not extract pollutants entirely from the soil, whereas all organic surfactants had a 90% hydrocarbon removal efficiency from the soil.

The result of the present investigation recorded 42.02% removal efficiency with *P. aeruginosa* ATCC 15442 amendment only, while Song *et al.* (2006) reported 69% and 52% TPH removal efficiencies for *P. aeruginosa* S and *P. aeruginosa* Y strains respectively. The study showed that *aeruginosa S* strains degraded faster than *P. aeruginosa* Y. The variation in degradation efficiency of different *P. aeruginosa* strains can be attributed to cell surface hydrophobicity (CSH) as reported by Bouchez Naïtali *et al.* (1999) where *P.aeruginosa S* showed low cell hydrophobicity and decrease in broth's surface tension as the strain was able to grow on alkanes. On the contrary, *P. aeruginosa Y* observed significant cell surface hydrophobicity with no substantial variation visible in the surface tension. This showed that *P. aeruginosa* Y relates directly to oil droplets, while *P. aeruginosa* S engaged hydrocarbon droplets through biosurfactant-medium mode which correlate to a higher removal efficiency. However, the results noted that the biosurfactant-producing mode is more effective than the direct mode

degradation which suggest that biosurfactant could achieve an optimal reduction of hydrocarbon concentration (Gautam and Tyagi 2006). This may explain and contributes to the variation in biodegradation efficiencies with *P.aeruginosa* strains since the synthesized biosurfactant plays a vital role in petroleum biodegradation, and its deployment in the remediation of crude oil contaminated soil has great potential (Song *et al.* 2006).

Similar to the current BAUc study which recorded 65–69% removal efficiencies after 28 days using wastewater as an extra energy source for *P. aeruginosa*, the findings by Al-Hadhrami, Lappin-Scott and Fisher (1997) showed an increase in TPH removal efficiency using the organic substrate as an energy source for *P. aeruginosa* where the supplementation of organic nutrients to the treatment system recorded appreciable alkane biodegradation efficiency of 20-50% than mineral fertilizer which recorded 14-22% after 24 hours treatment period. The addition of a mineral fertilizer was ineffective in increasing respiration or biodegradation of alkane, but cane sugar molasses was able to effectively increase respiration and was associated with significant *n*-alkane breakdown. The use of molasses enhanced the oxidation rates two-fold over a carbon-free mineral fertilizer. This showed the effect and selectivity of energy source by bacteria (*P. aeruginosa*) for the promotion of the biodegradation rate of crude oil (Al-Hadhrami, Lappin-Scott and Fisher 1997).

Similarly to the result of this investigation which recorded 67.47% average TPH removal efficiency with *P. aeruginosa* ATCC 15442 and wastewater as organic nutrient after 28days, Abdulsalam *et al.* (2011), Qiao *et al.* (2013), and Benyahia and Embaby (2016) reported removal efficiencies of 66%, 46-64%, and 56-77% after 10weeks, 90 and 156 days treatment period respectively while Mohajeri *et al.* (2017) observed that amendment of organic nutrient to the microbial population increases the biodegradation efficiency with removal efficiencies of 73.89, 73.76 and 58.31% reported for initial oil concentrations of 3, 30 and 60g/kg soil respectively after 90 days study period which is correspond to the present study with the present study which recorded average removal efficiency of 67.47% for 50g/kg soil with *P. aeruginosa* and wastewater amendment after 28 days study period. The study reported that a high concentration of crude oil affects the rate of biodegradation. Ghaly, Yusran and Dave (2013) reported pyrene reduction with mycobacteria and organic nutrient supplement of 57.86%, and

84.29% for bioaugmentation (only bacteria) and bioaugmentation + biostimulation (bacteria + organic supplement) respectively after 10weeks study period. The high degradation efficiency with combined BAU and BST can be attributed to a low concentration of initial TPH (3g/kg or 700 mg/kg soil) when compared to the present study as reported by Mohajeri *et al.* (2017). However, many studies have shown a significant increase in removal efficiency using combined bioremediation technique of micro-organisms and nutrients (Mancera-López *et al.* 2008; Mohajeri *et al.* 2017), but the TPH removal efficiency comparisons of different petroleum-hydrocarbon-treatment techniques are still vague (Mohajeri *et al.* 2017). This validates the efficacy of bioaugmentation and biostimulation (*P. aeruginosa* inoculation and wastewater amendment) in the treatment of crude oil-contaminated site which recorded appreciable removal efficiency when compared with control treatment which received neither inoculation nor wastewater amendment.

4.2 Bioremediation Study of AMD Contaminated Soils

4.2.1 Metal Removal (XRF Results and Discussion)

4.2.1.1 Biostimulation of AMD Contaminated Soils (BSTa) – Results and Discussion

The biostimulation treatment started in the first week with the presence of black precipitate evident on the surface of the samples (Fig. 4-10) for all the BSTa treatments amended with wastewaters (except the control treatment which received no amendment as shown in Fig. 4-11). BSTa-1 treatments recorded >40% average removal efficiencies for all heavy metals (Fe, Al, Cu and Zn) in week 1 except for Mn which recorded <40% (Fig. 4-12). The treatment recorded an average metal removal efficiency of 51.12% with a significant reduction of Cu at 65.69%. Also, in treatment BSTa-2, Cu was mostly removed while Mn was least removed with 71.26 and 25.36% (Fig. 4-13) average removal efficiencies respectively and 48-60% removal efficiencies for other metals after the treatment with 52.75% average removal efficiency. BSTa-3 recorded the highest average removal efficiency of 58.45% with average individual metal removal efficiencies of 66.17, 59.38, 69.77, 60.53 and 36.41% for Fe, Al, Cu, Zn and Mn respectively (Fig. 4-14) in week 4.

BSTa-4 and BSTa-5 showed a slightly similar trend with >30% (Fig. 4-15) and >40% (Fig. 4-16) metal weekly removal efficiencies in week 1 and for the rest of the treatment period respectively except for Mn as shown in Fig 4-15 & Fig. 4-16. These treatments showed an appreciable reduction of Fe and Cu with 59.33% and 64.57% average removal efficiencies for BSTa-4 and BSTa-5 respectively. These treatments (BSTa-4 and BSTa-5) recorded an average of 49.38 and 49.82% metal removal efficiencies respectively at the end of the remediation period (week 4). The control treatment (BATa) showed the lowest metal removal efficiencies of 12 -33% (Fig. 4-17) with 22.70% average metal removal efficiency (*See Appendix 5-10*). The BSTa treatment recorded metal removal of 47-73%, 34-74%, 47-87, 29-70% and <40% for Fe, Al, Cu, Zn and Mn (*See Appendices 11-15*) with an increase in pH from 6.9 to 7.3 – 7.4. It can be deduced that the amendment of wastewater for the BSTa treatment of AMD contaminated soil showed an effective metal reduction in all the treatments (when compared to the control treatment) with average removal efficiencies of >45% except for Mn which showed a very slow removal process with <37% average removal efficiencies from the BSTa treatments.

Fig. 4-10. AMD soil + Biostimulant (wastewater)`

Fig. 4-11. Control Treatment (BAT)

Figure 4-10. Formation of Black ppt. as a result of *Biosulfidogenic process* (Martins *et al.* 2011b)

Figure 4-11. No Black ppt formed due to lack of organic amendment (Bai *et al.* 2013)

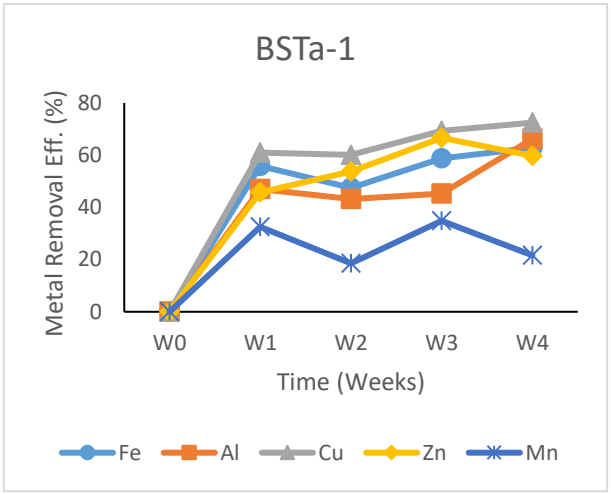


Fig. 4-12

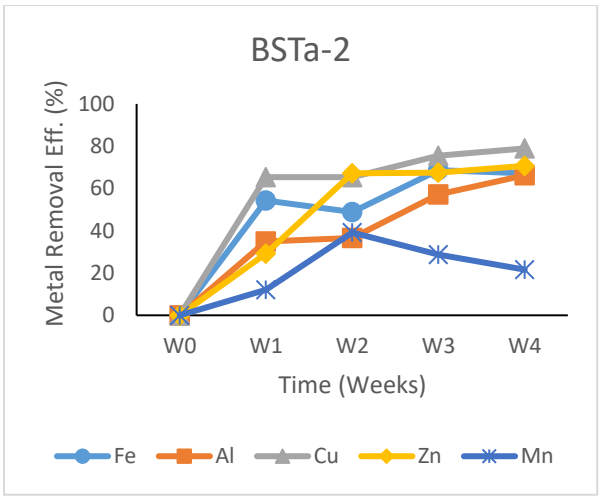


Fig. 4-13

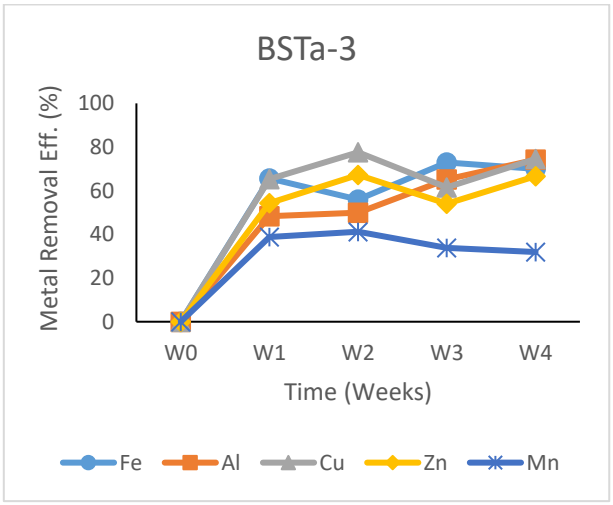


Fig. 4-14

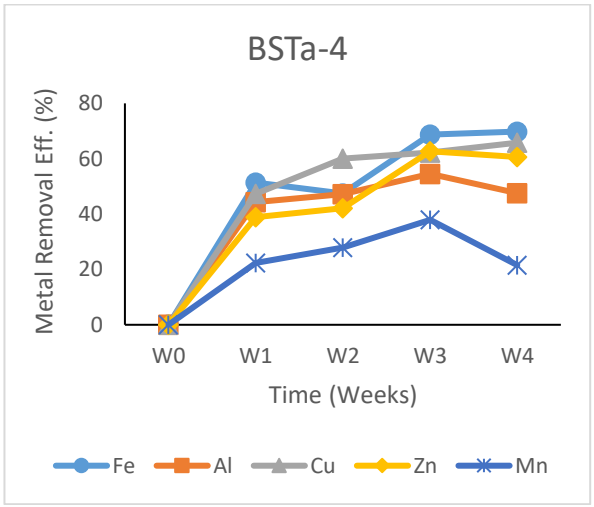


Fig. 4-15

Figure 4-12. BSTa-1 (BWW:MWW – 4:0) Metal Removal Efficiencies

Figure 4-13. BSTa-2 (BWW:MWW – 3:1) Metal Removal Efficiencies

Figure 4-14. BSTa-3 (BWW:MWW – 2:2) Metal Removal Efficiencies

Figure 4-15. BSTa-4 (BWW:MWW – 1:3) Metal Removal Efficiencies

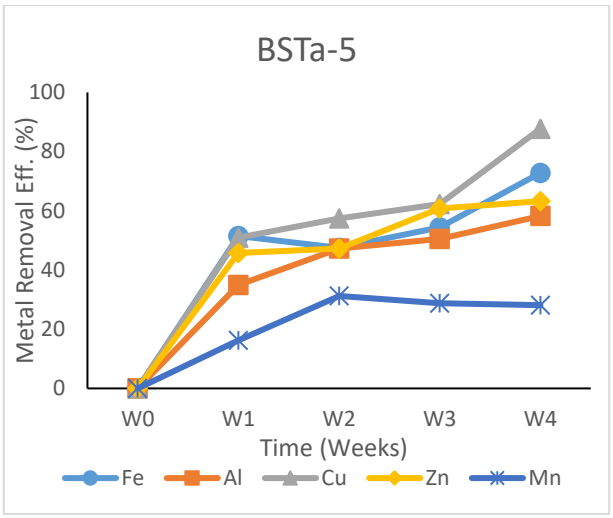


Fig. 4-16

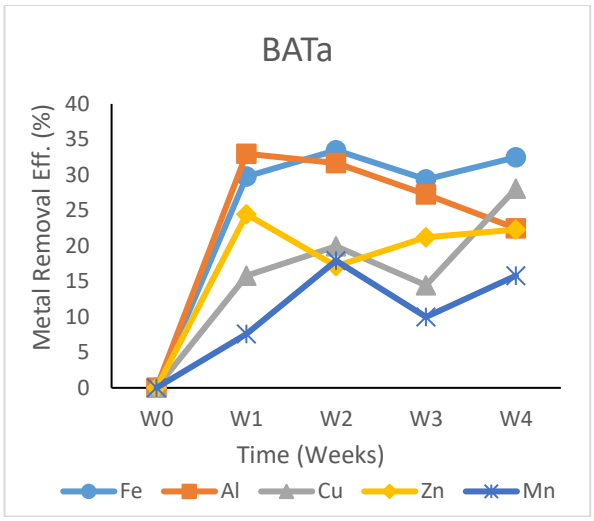


Fig. 4-17

Figure 4-16. BSTa-5 (BWW:MWW – 0:4) Metal Removal Efficiencies

Figure 4-17. BATa (BWW:MWW – 0:0) Metal Removal Efficiencies

The heavy metals removal was triggered by the sulfidogenic process facilitated by sulfate-reducing bacteria (SRB) which reduces sulfate to biogenic hydrogen sulfide (Chang, Shin and Kim 2000) while utilizing organic substrate (BWW and MWW) as an energy source as represented in Eqn. 4-1. The biogenic H_2S subsequently reacts with heavy metals in the treatment system to form metal sulfides (Eqn. 4-2) (Marchioretto, Bruning and Rulkens 2005; Adams, Lawrence and Bratty 2008; Muyzer and Stams 2008; Lu *et al.* 2011; Ňancucheo and Johnson 2012), while lowering dissolved SO_4^{2-} concentration and increasing the pH of the system to enhance remediation of AMD (Gibert *et al.* 2003; Kaksonen, Riekkola-Vanhanen and Puhakka 2003; Bhagat *et al.* 2004). This is only feasible if the environment is stimulated with organic nutrients to foster hydrogenases of reducing bacteria (Hammack, Dvorak and Edenborn 1993) which results to the occurrence of black precipitate as visible on the top layer of the samples amended with wastewaters (Fig. 4-10) after week 1 except for the control treatment (BAT) without black precipitate on the top layer (Fig. 4-11) which suggests the absence of organic amendment (Bai *et al.* 2013). The formation of black ppt. within the first week of treatment could be due to the increase in metabolic activities of reducing bacteria present in the wastewater which enhanced sulfate reduction and concomitant removal of heavy metals. In the

sulfidogenic process, certain bacteria are capable of reducing SO_4^{2-} and other sulfur oxyanions to H_2S when supplied with a suitable carbon source (Postgate 1984)

$$2CH_2O + SO_4^{2-} \rightarrow H_2S + 2HCO_3^- \quad \text{Equation 4-1}$$

$$H_2S + Me^{2+} + \rightarrow MeS(s) + 2H^+ \quad \text{Equation 4-2}$$

The precipitation of metals as sulfide is a recurring process that reverses the oxidation process that initially forms the AMD with metals of varying concentrations (Skousen *et al.* 2017). Al precipitates at pH >5 while Mn removal is a complex process because of the variable oxidation state but generally dependent on oxidation number of cations at pH of >7. Sometimes, a pH of 10.5 is required for complete removal of Mn as reported by Balintova and Petrilakova (2011) which accounts to the low removal of Mn in the current study with an average removal efficiency of 28.79% from BSTa treatment. Also, the uncatalyzed oxidation of Mn contributes to its low removal efficiency as Mn required to be oxidized from soluble Mn(II) to insoluble Mn(IV) to facilitate removal as MnO_2 precipitate (Johnson and Hallberg 2005; Sanchez-Andrea, Triana and Sanz 2012).

During the sulfidogenic process, heavy metals are reduced through precipitation as sulfides, hydroxides or carbonates, (Dvorak *et al.* 1992; Lyew and Sheppard 1997; Martins *et al.* 2011a; Bai *et al.* 2013; Deng and Lin 2013; van den Berg *et al.* 2016). Additional processes for metal removal include co-precipitation with these precipitates (Cravotta III and Trahan 1999; Song *et al.* 2001; Cravotta 2007), biosorption into the organic substrates used for the treatment purpose (Machemer and Wildeman 1992; Song *et al.* 2001; Gibert *et al.* 2003; Willow and Cohen 2003), exchange to precipitated Fe, organic materials, and soil-like materials (Skousen *et al.* 2017), while Fe and Al precipitate as hydroxides (Gilbert et al 2003). Alternatively, for Al and divalent transition metals, sorption to organic materials is important, whereas sorption to precipitated Fe and Mn contribute to the removal of trace metals (Zachara, Cowan and Resch 1991). However, divalent metals (Cu, Fe, Zn) can also be extracted by precipitation as sulfide minerals after microbial sulfide reduction (Stumm and Morgan 1996) as shown in Eqn. 4-3 for Fe below,

$$Fe^{2+} + H_2S + 2HCO_3^- \rightarrow FeS + 2H_2O + 2CO_2 \quad \text{Equation 4-3}$$

The effects on solution-phase acidity are simple and comprehensive for heavy metal removal during the treatment period by precipitation as metal hydroxides (Fe and Al) when compared to sulfide precipitation mechanism of metal removal which is much more complex than represented in the equation.4-3 and accounts to fluctuation in the removal efficiencies as shown in Fig. 4-12 to 4-16 (Stumm and Morgan 1996). However, removal of metals depends on the concentration of AMD, rate of biogenic H_2S production and the rate at which metal precipitate is formed but the reactivity caused by sulfate reduction contributes to various products of sulfide (since AMD comprises of varieties of heavy metals of different concentration) where the alkalinity of these reduced reactions, therefore, depends on the fate and nature of the synthesized biogenic H_2S or metal sulfides. The role of degrading bacteria in sulfate reduction and alkalinity generation has been criticized for the inability to explain the complicated nature of easily broken or displaced energy source (Skousen *et al.* 2017), the probable alkalinity contribution to Fe reduction and partial consideration of the fate of H_2S produced during the sulfate reduction process (Vile and Wieder 1993). The cycle is affected by seasonal variations where cold weather decreases alkalinity production levels according to Kuyucak, Chabot and Martschuk (2006), which (decreased in alkalinity levels) can be mitigated by choosing cold-hardy SRB varieties to enhance the sulfate reduction process (Nordwick *et al.* 2006; Janin and Harrington 2015).

The BSTa-1 (BWW only) treatment recorded slightly higher removal efficiency than BSTa-5 (MWW only) with an average metal removal efficiency of 51.13 and 49.82% respectively but BSTa-3 (BWW + MWW) treatment recorded the highest average metal removal efficiency which can be due to the possible synergy between the two wastewater effluents at the ratio 2:2. This suggests that an appropriate combination of organic substrates is effective in AMD bioremediation than the application of single substrate (Neculita, Zagury and Bussière 2007b; McCullough and Lund 2011). However, brewery wastewater contains an appreciable amount of chemical oxygen demand (COD) and biological oxygen demand (BOD) than the municipal wastewater, due to the presence of organic components in high quantity which attributes to its appreciable removal efficiencies recorded with this amendment (Jaiyeola and Bwapwa 2016; Amenorfenyo *et al.* 2019).

In agreement to the current BSTa study with the appreciable metal removal efficiencies of 47-73%, 34-74%, 47-87%, 29-70% for Fe, Al, Cu, and Zn except for Mn which recorded <40% after 28 days with pH increase from 6.9 to 7.3 – 7.4, Strosnider *et al.* (2013) recorded removal efficiencies of 99.8%, 99.8% 73.4% and 13% for Al, Fe, Zn and Mn respectively with pH increase from 2.6 to 6.79 after the 135 days treatment period using an organic substrate. Similarly, Hughes and Gray (2013) reported removal efficiencies of 52-84%, 47-61%, 74-86% and 58-90% for Al, Cu, Fe & Zn respectively in the co-treatment of AMD with MWW. Sanchez-Andrea, Triana and Sanz (2012) recorded a heavy metal reduction in AMD treatment using domestic wastewater as carbon source with 85%, 99%, 99.5%, and 99.5% for Fe, Co, Cu, Zn respectively (except for Mn – 13%) through co-precipitation of metals from AMD after 183 days treatment period. According to Strosnider *et al.* (2013), the removal of heavy metals in the process was linked to precipitation and co-precipitation as metal sulfides where Cd, Cu, Fe, Pb, Hg, Ni, and Zn are among the metals that precipitated as metal sulfides and Manganese and some of these metals may also be removed by co-precipitation with metal sulfide as reported by Logan *et al.* (2005).

Strosnider *et al.* (2013) observed the slow removal of Mn throughout the system till the pH increased (>6) which resulted in oxidation and hydrolysis with Mn reduction of 13.6%. Also, low reduction efficiency of the Mn which is sensitive to high toxicity was reported by Hughes and Gray (2013) that a high concentration of multi-metals AMD limits the removal efficiency of Mn and other metals. Similarly, Mn concentration remains unchanged in the treatment in the kaldnes stage but 20.6% of reduction of dissolved Mn was observed with treatment using limestone. Also, the removal of Mn was poor with activating sludge (Oliver and Cosgrove 1974; Chang *et al.* 2007) which can be attributed to the uncatalyzed Mn oxidation which is sensitive to high acidic concentration and tend to occur at pH > 9, since Mn tend to precipitate at high pH with complete removal at pH 10.5, giving Mn a solubility over a wide range of pH (Stumm and Morgan 1996; Hughes and Gray 2013). This explains Mn low removal from the present BSTa study with 28.74% average removal efficiency. However, the formation of Manganese carbonate ($MnCO_3$) was suggested as the removal mechanism of Mn in AMD treatment (Waybrant, Blowes and Ptacek 1998; Bamforth *et al.* 2006), also, Mn can be efficiently removed were the acidity is not limited to a particular pH range value; as tertiary treatment with alkali

and carbonate will be apt to achieve required Mn removal efficiency (Edenborn and Brickett 2002).

Also**,** in contrast to low Mn removal recorded in this investigation, the study by Hughes and Gray (2013) recorded high removal efficiency in the co-treatment of AMD with MWW as Mn and Zn removal were favourable at pH < 10 which recorded 93-95% for Mn and 58-90% for Zn. Similarly, the study by Deng and Lin (2013) reported high Mn removal efficiency among other metals using two-stage treatment of AMD and Municipal wastewater (MWW) which involved the batch mixing of the two wastes in pH of 6.2 – 7.9. The result reported adsorption and co-precipitation of heavy metals (Costa and Duarte 2005) as the metal removal approach with varying metal reduction efficiencies; Fe > 99%, Al ~ 100%, Mn 75 – 100%, Ca 52 – 81%, Mg 13 – 76% and Na 56 – 76%. Silva *et al.* (2012) reported that Mn removal efficiency of 99% was recorded after 2 weeks study period achievable with carbonate ions (introduced by treating AMD with limestone) which provided the pH within the alkaline range to facilitate Mn removal.

The rate of metal removal depends on many factors such as bioavailability of contaminants, temperature, and toxicity of heavy metals and the nature of the organic substrate (Boopathy 2000). The operating condition for the present study was adjusted to ambient condition (22 ± 3^{o}C) to suit microbial activities which account for significant metal removal efficiencies since high temperatures (50 – 80^{o}C) decreases cell viability in *P. aeruginosa* which hinder microbial growth (O'Toole, Ricker and Nuxoll 2015). The appreciable metal removal efficiency can also be attributed to the increase in dehydrogenase is due to the nature of carbon source (wastewater) used for the study, which is readily available and contains appreciable microbial population. (Tsukamoto, Killion and Miller 2004). Sanchez-Andrea, Triana and Sanz (2012) reported that the microbial community in the SO_4^{2-} and heavy metal removal includes, the fermentative bacteria (*Clostridium spp. Delftia spp., Paludibacter spp. (syntrophic bacterium)* which metabolizes complex substrate and sulfate-reducing bacteria (*Desulfovibrio spp, Desulfosporosinus spp.(Clostridia), Desulfomonile spp., Desulfotomaculum spp.*), citing *these bacteria.* as the most abundant species present in wastewater during the AMD treatment (Martins *et al.* 2011b; van den Berg *et al.* 2016). It can be that the use of wastewater for AMD

treatment was effective in heavy metals and sulfate reduction (Hughes and Gray 2013; Strosnider *et al.* 2013).

4.2.1.2 Bioventing of AMD contaminated soil (BVTa) – Results and Discussion

The bioventing (BVTa) treatment of AMD contaminated soils observed metal reduction with the introduction of atmospheric air into the bioreactor, which triggered the presence of black precipitate on the surface of the samples. BVTa-1 which was supplied with atmospheric air only recorded >30% removal efficiency for all metals in week 1 (Fig. 4-18) with 49.16% average removal efficiency after the treatment period (28 days). BVTa-2 and BVTa-3 amended with wastewaters (BWW and MWW respectively) and vented showed a general progressive trend in weekly removal efficiencies for all metals from week 1 with 59 – 72% and 60 – 67% removal efficiencies respectively (Fig. 4-19 & Fig. 4-20) and 65.25% and 63.56% average removal efficiencies after the week 4 respectively. The BVTa-4 treatment amended with wastewaters (BWW and MWW) and vented also recorded an increased metal weekly removal of >42% in week 1 with significant metal removal efficiencies of 75.78, 86.17, 93.42, 88.23, 80.85% recorded in week 4 for Fe, Al, Cu, Zn and Mn respectively (Fig. 4-21). The treatment (BVTa-4) showed an average metal removal efficiency of 70.20%. The control treatment (BATa) was unable to enhance metal reduction as the lowest removal efficiencies of 12 -33% with 22.70% average metal removal efficiency was recorded. The BVTa treatment increased the pH of the contaminated sample from 6.9 to 7.4 – 7.5 which accounts to metal removal efficiencies of 47-75%, 33-86%, 43-93%, 41-88%, and 30-81% for Fe, Al, Cu, Zn and Mn respectively (*See Appendix 11-14*)**.** The BVTa treatment showed that combined amendment of wastewater and air-injection facilitated the reduction of metal concentration (including Mn) in all treatments except the control treatment (Fig. 4-17).

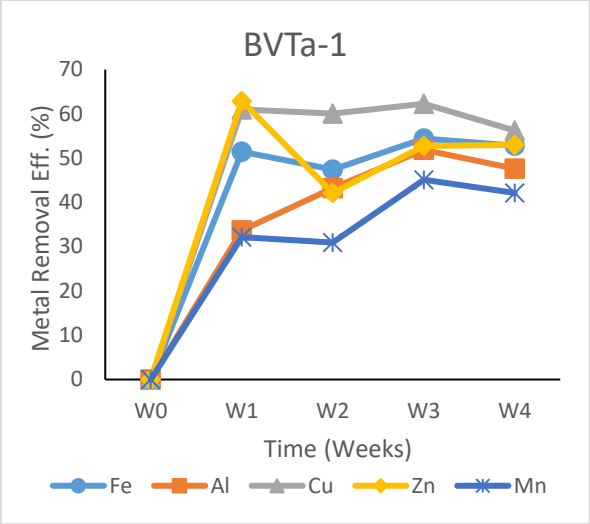


Fig. 4-18

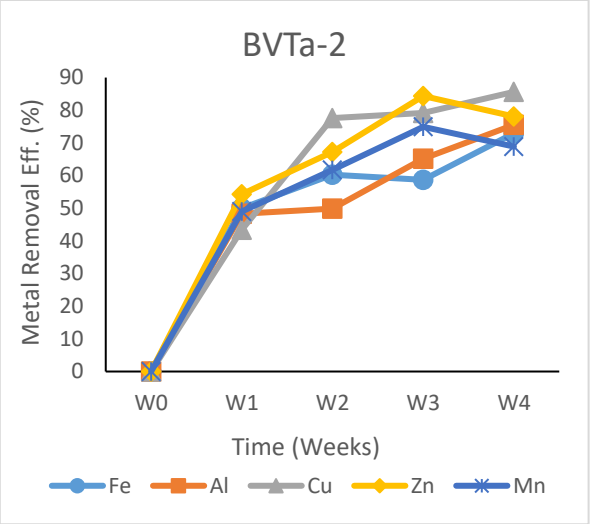


Fig. 4-19

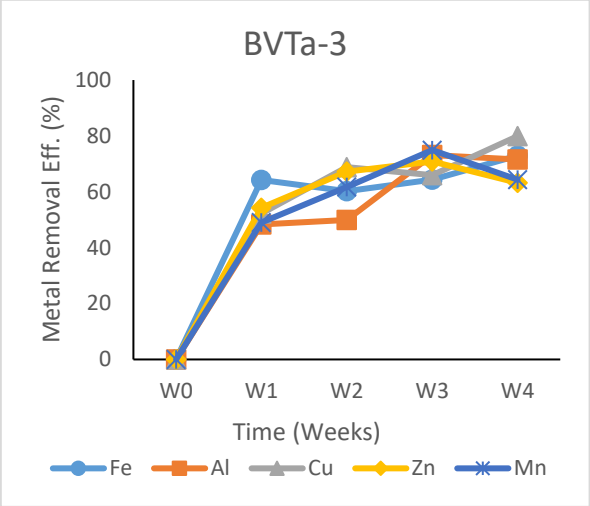


Fig. 4-20

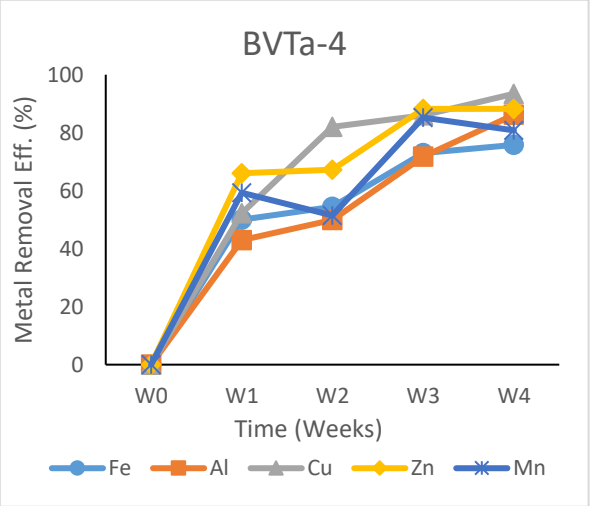


Fig. 4-21

Figure 4-18. BVTa-1 (air-injection only) - Metal Removal Efficiencies

Figure 4-19. BVTa-2 (air-injection + BWW) - Metal Removal Efficiencies

Figure 4-20. BVTa-3 (air-injection + MWW) - Metal Removal Efficiencies

Figure 4-21. BVTa-4 (air-injection + BWW + MWW) - Metal Removal Efficiencies

Metal Removal Mechanism

Metal removal occurred through the oxidation process for the BVTa study, as the treatment system was aerated, the oxidation reaction is triggered (catalysed by indigenous bacteria present

in BWW and MWW) where Fe^{2+}, main constituent of the AMD is oxidized to Fe^{3+}. Interaction between bacteria, O_2 and AMD (Berghorn and Hunzeker 2001), the presence of alkalinity (in form of wastewater) facilitated the oxidation process in the treatment system as Ackman and Kleinmann (1984) noted that the introduction of alkalinity into the aeration system invariably increases microbial activities and the rate of oxidation through neutralization of the AMD (Vadapalli *et al.* 2015) which is evident in the current investigation where treatment with wastewater + air-injection (BSTa-2, 3, 4) recorded >15% increase in average removal efficiency when compared to treatment with air-injection only (BSTa-1).

Precipitation occurs over the range of pH for various metals due to gradual oxidation of Fe^{2+} to Fe^{3+} by O_2 injected into the system and its precipitation in the form of $Fe(OH)_3$ as shown in Eqn. 4-4 (Jones *et al.* 2014) which enables Fe to be removed alongside other heavy metals (Cu, Zn, Al, & Mn) through co-precipitation with Fe (Ackman and Kleinmann 1984). Unlike the BSTa treatment, Mn oxidation was feasible (with BVTa) where soluble Mn^{2+} was oxidized to insoluble Mn^{4+} to facilitate Mn removal as MnO_2, (Eqn. 4-5). This process may be sensitive and sluggish at low pH (acidic condition) as reported by Nijjer, Thonstad and Haarberg (2000) or the presence of Fe^{2+} which either inhibit the process or triggered reverse oxidation (Skousen *et al.* 2017). However, the process can be accelerated by bacteria and surface catalysis (Stumm and Morgan 1996). Hence, the combination of oxidation and precipitation process is responsible for the significant Mn removal efficiency recorded with treatments amended with wastewaters (BWW and MWW) and vented (BVTa-2, BVTa-3 and BVTa-4) with >60% while BVTa-1 (only vented) recorded <40% average removal efficiency. The BVTa treatment showed effective Mn removal when compared to BSTa treatment.

$$Fe^{2+} + 1/4O_2 + 5/2H_2O \rightarrow Fe(OH)_3 + 2H^+ \qquad \text{Equation 4-4}$$

$$Mn^{2+} + 1/2O_2 + H_2O \rightarrow MnO_2 + 2H^+ \qquad \text{Equation 4-5}$$

Since the bioreactor was not subjected to continuous ventilation, another process that enhances the removal of heavy metals is the sulfidogenic reaction (Eqn. 4-1 & 4-2) feasible in the presence of organic substrates in the form of BWW and MWW, facilitated by bacteria. This process reduces sulfate to biogenic H_2S releasing elemental sulfur which precipitate metals as sulfides (Al, Zn, Cu, Co, Fe and Mn) (Marchioretto, Bruning and Rulkens 2005; Adams,

Lawrence and Bratty 2008; Muyzer and Stams 2008; Lu *et al.* 2011; Ňancucheo and Johnson 2012). However, alkalinity generated might not be proportional to the amount of ppt. formed since alkalinity depends on the rate of sulfate reduction and the extent of metal sulfide precipitation (Skousen *et al.* 2017). This attribute to the complexity of the sulfidogenic process, as pH increase is largely dependent on the production of alkalinity (Skousen *et al.* 2017). However, sorption to organic materials also contributes to the removal of heavy metals (Zachara, Cowan and Resch 1991).

With the metal removal efficiencies of 47-75%, 33-86%, 43-93%, 41-88% and 30-81% for Fe, Al, Cu, Zn and Mn respectively after 28 days with an increase in pH from 6.9 to 7.4, the current BVTa investigation agrees with the study by Balintova and Petrilakova (2011) which reported removal efficiencies of 97.16%, 95.23%, 92.9%, 89.49% and 88.72% for Fe, Cu, Al, Mn and Zn respectively after 72 hours treatment period where the bioremediation system was aerated by the introduction of H_2O_2 for the treatment of AMD to induced oxidation of Fe^{2+} to Fe^{3+} followed by precipitation of metal hydroxides from the AMD using NaOH (which introduces alkalinity into the system) as metal precipitation was evident. Similarly, Cravotta (2007) recorded rapid oxidation (of Fe^{2+}) after the aeration of the treatment system, as the metal reduction was observed from 96.9% for Fe and other metals (Al and Mn) removal rate of >80% within 24hours of treatment. Cravotta (2007) reported that continuous aeration will be suitable to decrease CO_2 and increase the pH value to accelerate Fe^{2+} oxidation for effective remediation.

Similarly, Peters and Bennett (1989) recorded >80% removal efficiencies for Cu, Pb and Zn using NaOH or Sulfide precipitation and air injection techniques. Zvimba *et al.* (2013) noted that the remediation of AMD using sludge and air injection with the introduction of alkalinity ($CaCO_3$) to neutralize the AMD promoted the oxidation of Fe^{2+} to Fe^{3+}. The oxidation of ferrous iron triggers the removal of other metals at >80% efficiency. In aerobically modified system, oxidation of Fe^{2+} is key to AMD treatment (Zvimba *et al.* 2013). Similarly, Ching (1986) investigation reported heavy metal removal efficiency of 76%, 98%, and 50% for Pb, Cu and Zn respectively with the venting system. The result by Ching (1986) represents a significant reduction in the initial concentration of heavy metals which confirmed that removal of metals was indeed possible by air flotation with heavy metals removals efficiency of more than 70%.

This BVTa study showed that combined air-injection and wastewater amendment enhanced the removal of all metals present with appreciably increase in pH.

4.2.1.3 Bioaugmentation of AMD contaminated soil (BAUa) – Results and Discussion

The treatment started on the first week with appreciable metal reduction observed in different bioreactors especially treatments inoculated and amended with *P. aeruginosa* ATCC 15442 and wastewaters respectively with black precipitate observed on the surface of treatments amended with P.A + wastewater only. BAUa-1 treatment inoculated with *P. aeruginosa* ATCC 15442 recorded average metal removal efficiency of >50% for Fe, Cu, Zn, and Mn, except for Al with <40% in week 4 (Fig. 4-22). BAUa-2 (*P. aeruginosa* ATCC 15442 and BWW) recorded the highest weekly removal efficiency in week 1 (>80% for Fe, Al, Cu, Zn except for Mn) and a decline from week 2 to week 4 with an average metal removal efficiency of 62.40% after the treatment (Fig. 4-23). However, the BAUa-3 inoculated and amended with *P. aeruginosa* ATCC 15442 and MWW respectively showed >40% removal for all metals except for Al (<40%) in week 1 and the removal efficiencies for the treatment appreciated in week 2 with a decline of >15% in the remaining 14 days (Fig. 4-24). The treatment reduced all metals (Fe, Al, Cu, Zn, and Mn) by >50% (average) to attain an average removal efficiency of 54.04%. The control treatment (BATa) observed a slow metal removal efficiency when compared to other treatments with an average metal reduction below 30% efficiency (Fig. 4-17). An increase in pH was observed in all BAUa treatment to 7.3 from 6.9**.** Metal removal efficiencies of 24-84%, 21-81%, 43-88%, 36-89%, and 31-64% for Fe, Al, Cu, Zn, and Mn respectively (*See Appendix 15-17*). Hence, it can be deduced that the bacteria strain, *P. aeruginosa* ATCC 15442 was effective in metal reduction as evident in BAUa-1 treatment while the introduction of wastewater increased the metal reduction efficiencies as shown in BAUa-2 and BAUa-3 treatments.

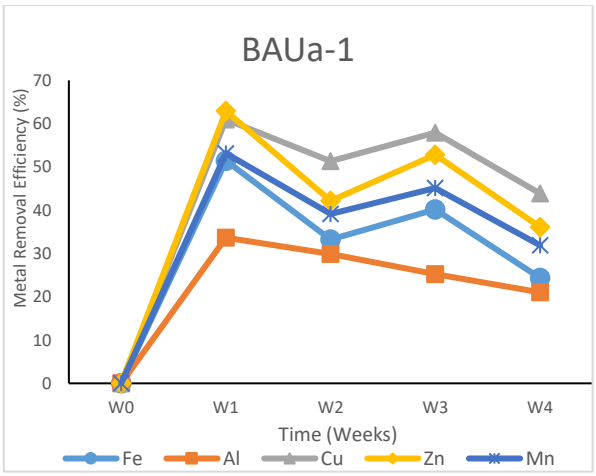


Fig. 4-22

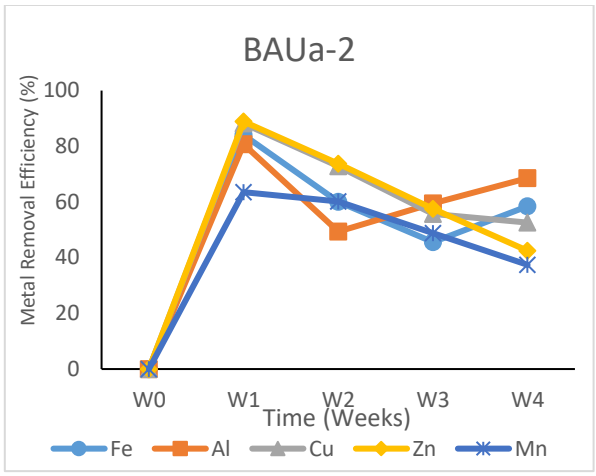


Fig. 4-23

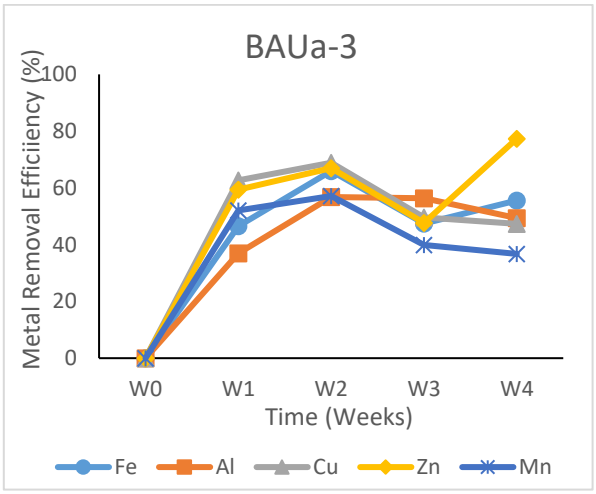


Fig. 4-24

Figure 4-22. Metal Removal Efficiencies *(P. A. inoculation only)*

Figure 4-23. Metal Removal Efficiencies *(P. A + BWW)*

Figure 4-24. Metal Removal Efficiencies *(P. A. + MWW)*

Mechanism of Metal sorption by P. aeruginosa

The reduction of metal concentration in treatment amended with *P. aeruginosa* ATCC 15442 showed the bacteria potential to remove varying metal contaminants. The ability of *P. aeruginosa* to sorb metals depends on the cell wall which consists of polysaccharides, proteins, lipids, functional groups such as carboxylates, hydroxyls, phosphates, and amino groups which

can bind metals effectively (Vijayaraghavan and Yun 2008; Abdi and Kazemi 2015). The adsorption of heavy metals on the microorganisms' outer cell surface is a defence approach adopted by microorganisms to prevent the toxicity by creating a polymeric coating which restricts metal from penetrating through the cell wall (Scott and Karanjkar 1992). According to Fomina and Gadd (2014), through surface complexation on cells and other external layers, biosorption can be performed as a passive absorption by cell and tissue fragments or through dead biomass or live cells (Gavrilescu 2004). *P. Aeruginosa* tolerance to heavy metals like Cu, Cd, Cr, Ni, Zn, Co, Fe, Mn, Mo makes it viable for the treatment of heavy metals in bioremediation process (Awasthi *et al.* 2015; Maitra 2016) as they can grow in the presence of heavy metals by using functional groups and metal chelating agents present on the cell wall to make metal binding (Srivastava, Agrawal and Mondal 2015).

In a heavy metal polluted environment, bacteria follow many growth mechanisms, including the accumulation and uptake, metal sorption, enzyme or oxidation-reduction, and extracellular precipitation. However, Abdelbary, Elgamal and Farrag (2019) reported that biosorption (passive process) and bioaccumulation (active process) (Chojnacka 2010) are the two prominent methods used by bacteria to adapt in the metal-rich environment and remove these heavy metals (Fourest and Roux 1992; Hussein *et al.* 2004; Kumar *et al.* 2014). It has been shown that *P. aeruginosa* uses the biosorption method for detoxing and reducing metal concentration in polluted sites using live or dead cells (Chang, Law and Chang 1997; Das, Vimala and Karthika 2008; Gabr, Hassan and Shoreit 2008; Timková, Sedláková-Kaduková and Pristaš 2018) by attracting heavy metals and then sorbing them gradually into the binding sites of their cell wall (Ayangbenro and Babalola 2017) and the amount of passive metal uptake is a factor of kinetic equilibrium and metal constituents regardless of the time taken for the metabolism in the system which involved different processes such as electrostatic interaction, ion exchange, precipitation, redox phase, and surface complexation. This explains the reason for an effective reduction in heavy metal concentration in treatment inoculated with *P. aeruginosa* (BAUa-1) when compared with the control treatment (BATa).

As metal removal mechanism, *P. aeruginosa* synthesis siderophore (pyoverdine (*iron carriers)* – a high-affinity iron-chelating compounds) as reported by Peek *et al.* (2012) which can bind

metal during bioremediation treatment. Peek *et al.* (2012) noted that siderophores are essential for the acquisition of iron by certain pathogenic bacteria like *P. aeruginosa* sp. The unavailability of Fe which is a significant nutrient for the *P. aeruginosa* bacteria prompted the secretion of siderophore which mobilize and transport Fe across cell membranes. These siderophores are released by *P. aeruginosa* to scavenge available Fe from the mineral phase in the treatment system by the gradual formation of soluble Fe^{3+} complexes (under acidic condition) for active transport and uptake mechanism (Miethke and Marahiel 2007; Hider and Kong 2010) which contributes to the effective reduction of Fe and other metals. Siderophore can chelate and detoxify other metal ions (aside Fe), such as Cu, Al, Pb, Mn, Zn, Cd, Cr, and Ur among others (Carrillo-Castañeda *et al.* 2002; Del Olmo, Caramelo and SanJose 2003) to enhance metal reduction as evident in the current study.

However, the black precipitate visible on the surface of the treatment suggests sulfidogenic process feasible due to the introduction of wastewater into the system which also contributes in metal reduction through precipitation as sulfides, hydroxides or carbonates, and sorption into organic materials (Dvorak *et al.* 1992; Lyew and Sheppard 1997; Martins *et al.* 2011a; Bai *et al.* 2013; Deng and Lin 2013; van den Berg *et al.* 2016). Silva *et al.* (2009) reported that metal removal mechanism using *P. aeruginosa* can be facilitated by the introduction of organic energy source for the bacteria for the maintenance of active, stable and sensitive microbial community needed for effective bioaccumulation or biosorption process for enhanced metal removal (Brown and Lester 1982; Silva *et al.* 2009). This vividly elucidates the increase in average metal removal efficiencies in treatments amended with *P. aeruginosa* and wastewater (BAUa-3 and BAUa-4) where the presence of an organic substrate in form of wastewater (BWW and MWW) increased the average metal removal efficiency in treatment BAUa-2 and BAUa-3 by 20.59% and 12.23% respectively when compared to the treatment inoculated with *P. aeruginosa* ATCC 15442 without nutrient amendment (BAUa-1) which recorded 41.81% average metal removal efficiency.

According to Sinha and Mukherjee (2009), the increase in *P. aeruginosa* KUCd1 survival rate can be linked to the amendment and availability of extra energy source during the treatment for rapid metabolism which facilitated metal removal rate to 89% while media treatment recorded

75% and nutrient-deficient media removes less than 20% Cd from the wastewater after 96 hours treatment period. These correspond to 41.81% (without extra nutrient) as against 58.22% (avg. removal with wastewater nutrient amendment) in the present study which represents 16.41% increase in removal efficiency after 28 days treatment period. The discrepancy and fluctuations in metal removal efficiencies observed during the treatment may be due to the complexation of metal in the multi-metal treatment systems which lowered the bioavailability of metals and hence increases toxicity (Stumm and Morgan 1996; Sinha and Mukherjee 2009)**,** depletion of organic substrates and/or lack of bioavailability of metals in the soil to degrading microbes. Also, the low metal removal recorded in the control treatment (BATa) in the present study is due to lack of inoculum or organic substrate (Boopathy 2000) and in some cases, long ageing presence of metals triggers strong bound of chemicals in the soil which affects the rate of removal.

The metal removal efficiencies of 24-84%, 21-81%, 43-88%, 36-89%, and 31-64% were recorded for Fe, Al, Cu, Zn, and Mn respectively after 28 days corresponds to the study by Awasthi *et al.* (2015) which reported removal efficiencies of 79.5, 52.4% and 61% for Cu, Zn and Fe after 48, 72 and 24hours treatment period respectively. The study showed significant removal of Cu from all treatments which signifies that the effect of Cu concentration on *P. aeruginosa* was negligible. The appreciable reduction efficiency of Cu and Zn as evident in this study can be due to the indispensable nature of Cu and Zn as essential nutrients required for bacterial growth and cell survival and excess may be detrimental to microbial growth (Teitzel *et al.* 2006; O'Brien, Hodgson and Buckling 2014), hence, appreciable adsorption is of these metals was evident at >48% average efficiency in BAUa-1 treatment inoculated with *P. aeruginosa* only after 28 days treatment period. Similarly, Juwarkar *et al.* (2008) reported heavy metal removal efficiencies of 86 – 96% with *P. aeruginosa* BS2 isolated from oil sludge after 96 hours while Chen *et al.* (2006), reported *Pseudomonas putida* (CZ1) strain's ability to remove about 87.2% of Cu and 99.8% of Zn during the active growth cycle. *P. putida* demonstrated high biosorption potential of Cu and Zn and ability to tolerate and grow in a significant concentration of metal because of high metal uptake in aerobic condition. Also, the high removal efficiency of Cu and Zn recorded in these treatments buttresses the positive effect of optimum concentration of Cu and Zn required for cell survival (Awasthi *et al.* 2015) as *P.*

aeruginosa react differently to different concentration of heavy metal with varying intake levels with respect to time and change in biomass (Awasthi *et al.* 2015).

However, according to Silva *et al.* (2009), metal removal using *P. aeruginosa* was dependent on the pH of the treatment system as, increase in sorption of Cu (95.0%) occurred at lower pH 6.25 which is the optimum adsorption pH for Cu and Zn removal was effective at pH 7.0 with an 87.7% removal efficiency after 72 hours of treatment while the low Mn removal (21.69%) can be due to high toxicity from the high Mn concentration (49mg/L) when compared to the present study with low Mn concentration which recorded 42.31% average removal efficiency (BAUa-1) amended with bacteria strain only. Mn possession of high atomic mass also affects the adsorption process (Chen *et al.* 2000) as evident in the present study. The findings from Silva *et al.* (2009) showed that *P. aeruginosa* adsorption from metals from individual solution is higher than multi-metal mixture and growth of *P. aeruginosa* was more visible in Zn followed by Cu, and Mn, which suggested *P. aeruginosa* resistance to high metal concentration, suitability for metal removal and the design of a treatment system using *P. aeruginosa* as a viable alternative approach for remediation purpose. The study showed that the application of *P. aeruginosa* ATCC 15442 with wastewater as an extra carbon source was effective for the reduction of multi-metals concentration in the soil and can serve as a potential remediation alternative.

4.2.2 Sulfate Removal

4.2.2.1 Sulfate Removal (BSTa) - Results

Results of the treatment carried out in airtight bioreactors (very low oxic condition) showed that the introduction of BWW and/or DWW triggered the bioremediation process with a reduction in sulfate level observed in bioreactors with 50.9% maximum removal efficiency recorded for BST-2 after the first week of treatment. (Fig. 2). This introduction of the organic substrate was preceded by the formation of black precipitates on the surface of the samples. BST-4 recorded the highest sulfate removal efficiency of 53.4% (421mg/kg removed) in week 4 followed by BST-3, BST-2, BST-1, BST-5 with 50.8%, 47.9%, 47.1% and 33.5% removal efficiencies representing 401, 378, 372, 265mg/kg (Fig. 4) sulfate removal from 789mg/kg initial

concentration respectively after wk 4. BST-1 and BST-4 showed a similar trend as both treatments progressively recorded an increase in sulfate weekly removal efficiency from wk.1 to wk.4 with 13.2% to 47.1% and 37.0% to 53.4% at 13.3mg/day and 16.1mg/day average removal rate respectively while BST-2 and BST-3 observed a significant decline in the sulfate weekly removal efficiency from wk1 to wk2 (50.9 to 30.2% and 47.4 to 32.1% respectively) but subsequently increased appreciably to 47.9%.and 50.8% for BST-2 and BST-3 after wk 4 at 14.4mg/day and 14.5mg/day average removal rate accordingly.

The removal efficiency of BST-5 treatment increased gradually from wk1 at 29.5% and climaxed in wk3 at 52.1% before observing a decline to 33.6% in wk 4 with an average removal rate of 14.7mg/day. All treatments with BWW alone (BST-1) and in combination with the DWW amendment (BST-2, 3, and 4) showed a significant reduction in sulfate when compared to DWW alone (BST-5). The control treatment, BAT recorded the least sulfate removal efficiency throughout the treatment with 24.8% in wk4 at 8.9mg/day average sulfate removal rate (*See Appendix 19*). This goes to confirm that the amended soils have activities that resulted from the introduced biostimulants. The treatments showed variations in sulfate reduction rates from wk1 one to wk4 due to variation in the composition of the stimulants used hence indicating that BWW is more effective than DWW as evident in higher removal efficiencies with BWW amended bioreactors after 28 days treatment period.

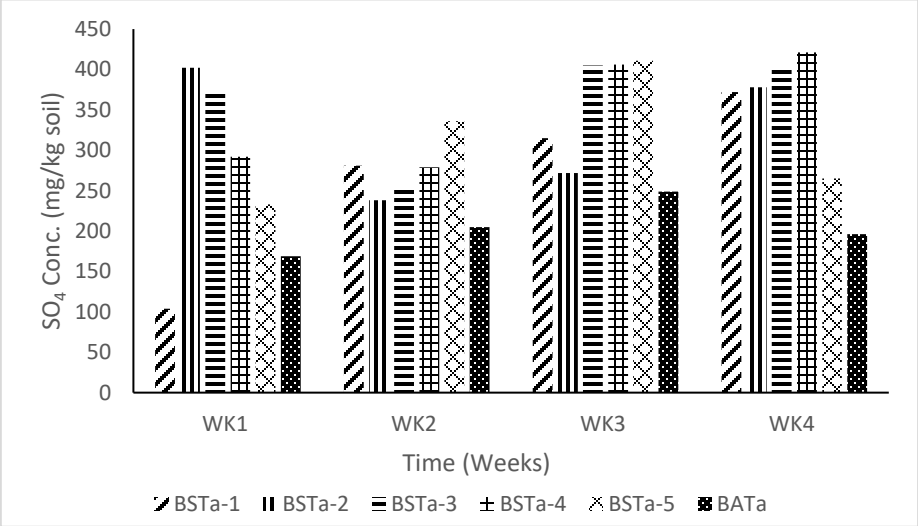


Figure 4-25. Sulfate Removal (mg/kg) from BSTa treatment

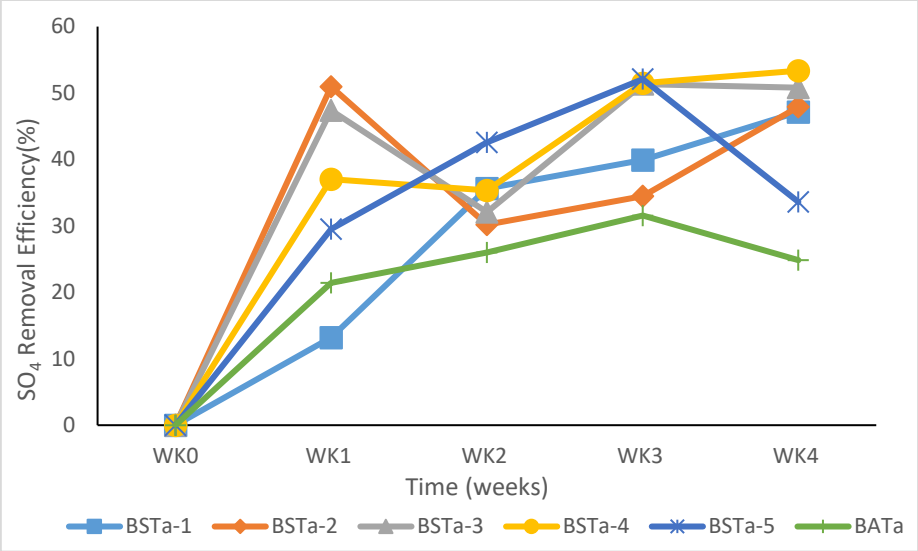


Figure 4-26. Sulfate Removal Efficiency (%) from BSTa treatment

4.2.2.2 Sulfate Removal (BVTa) – Results

Bioremediation treatment was evident in all treatments with air injection and wastewater amendment which triggered an increase in sulfate removal in all treatments from wk1 to wk2 with a decline in wk3 for treatments BVT-1, BVT-3 and BVT-4 except for BST-2 (Fig. 3) which recorded a progressive increase, and also showed the highest weekly removal efficiency from wk1 to wk4 with 4.4, 16.6, 31.7 and 41.9% weekly removal efficiencies at 11.8/day average removal rate representing 35, 131, 250 and 331mg/kg weekly SO_4 removal respectively from 789mg/kg initial concentration. Meanwhile, there was a decline in weekly SO_4 removal efficiencies in treatments BST-3 (from 39.3 to 31.6%) and BST-4 (from 34.9 to 26.5%) from wk2 to wk3 but as BVT-4 appreciated by 5% in wk4, BST-3 slightly decreased further by 3% at 9.9mg/day and 8.7mg/day average removal rate (Fig. 5) which resulted to 249mg/kg and 226mg/kg SO_4 removal respectively from the treatments. BVT-1 observed an increased weekly removal efficiency from wk1 and plateaued in wk3 with an average removal rate of 8.5mg/day, but subsequently experienced a decline in removal efficiency by 3.4% in wk4 which resulted to 26.86% removal efficiency. Treatments with BWW amendment only (BVT-2) and in combination with DWW (BVT-4) showed greater removal than DWW amended treatment (BVT-3) as removal efficiency after wk 4 follows the pattern BVT-2 >BVT-4 > BVT-3 > BVT-1. When compared to treatments that received nutrients amendment, the control treatment, BAT

recorded the least sulfate removal efficiency, with an increased efficiency from wk 1 to wk3 and a decline in wk 4 recording 24.8% removal efficiency representing 196mg/kg sulfate removal from the treatment. The average weekly sulfate reduction efficiency in all treatments was observed to show a progressive increase from wk1 to wk4 (*See Appendix 20*).

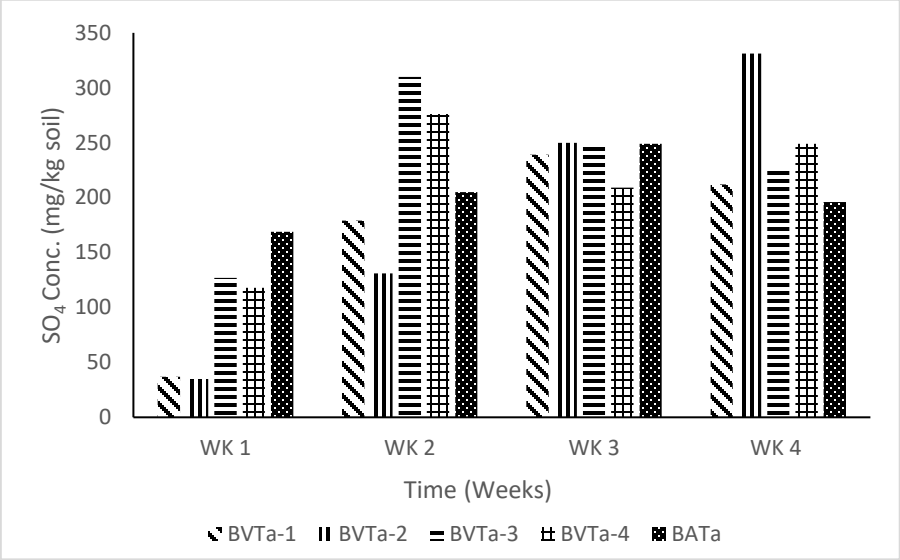


Figure 4-27. Sulfate Removal (mg/kg) from BVT treatment

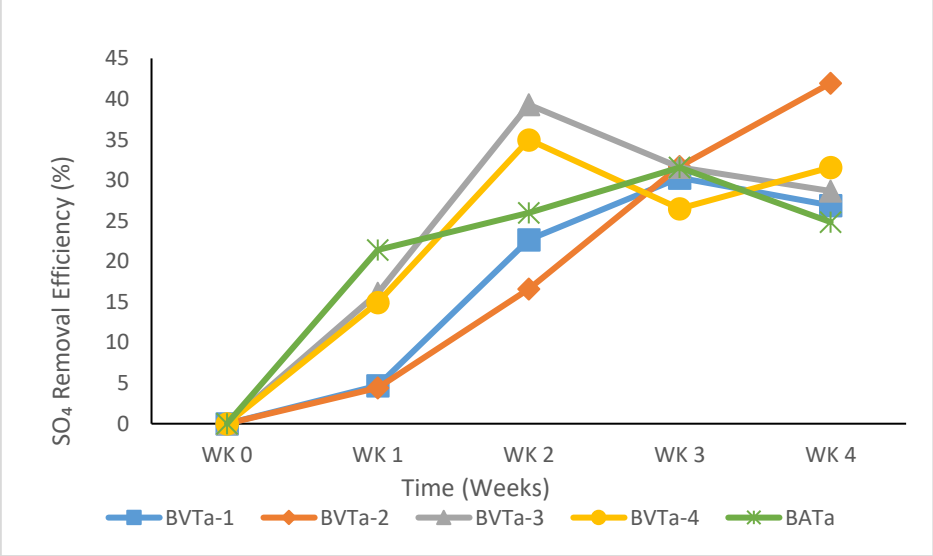


Figure 4-28. Sulfate Removal Efficiency (%) from BVT treatment

4.2.2.3 Sulfate Removal (BAUa) – Results

The sulfate removal in BAUa treatment started in the first week with >23% removal efficiency recorded in the first week of treatment with the formation of black precipitate on the surface of treatments amended with wastewater except for the control treatment (BATa) (Fig. 4-30). The treatment inoculated with bacteria strain, *P. aeruginosa* ATCC 15442 only (BAUa-1) observed a 15.54% increase in week 2 with a 7.52% declined in removal efficiency in week 3 which appreciated to attain 38.14% average removal efficiency in week 4 (Fig. 4-30). The treatment (BAUa-1) was able to cumulatively reduce sulfate concentration by 321mg/kg at 11.46mg/day average removal rate. However, BAUa-2 and BAUa-3 treatment where *P. aeruginosa* ATCC 15442 was amended with BWW and MWW respectively observed a 4.52% and 2.13% decline in week 3 and week 2 which attribute to a cumulative sulfate removal of 447mg/kg and 410mg/kg to represent 56.01% and 51.37% average removal efficiency at 15.96mg/day and 14.64mg/day average removal rate respectively. The control treatment (BATa) recorded the lowest cumulative sulfate removal of 249mg/kg at 8.89mg/day average removal rate (*See Appendix 21*). The bioaugmentation (BAUa) treatment showed the reduction of sulfate from AMD contaminated soils with *P. aeruginosa* ATCC 15442 inoculation and the ability of wastewater amendment to enhance sulfate removal.

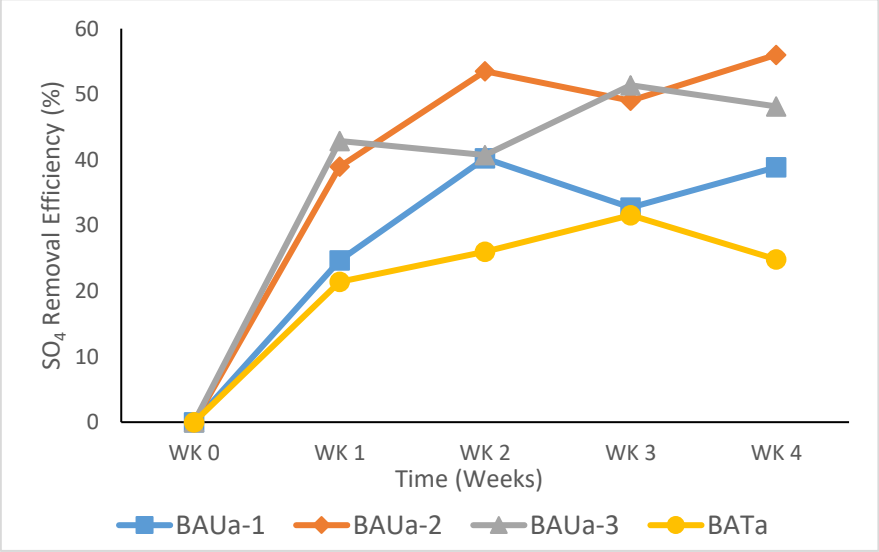


Figure 4-29. Sulfate Removal (mg/kg) from BAUa Treatment

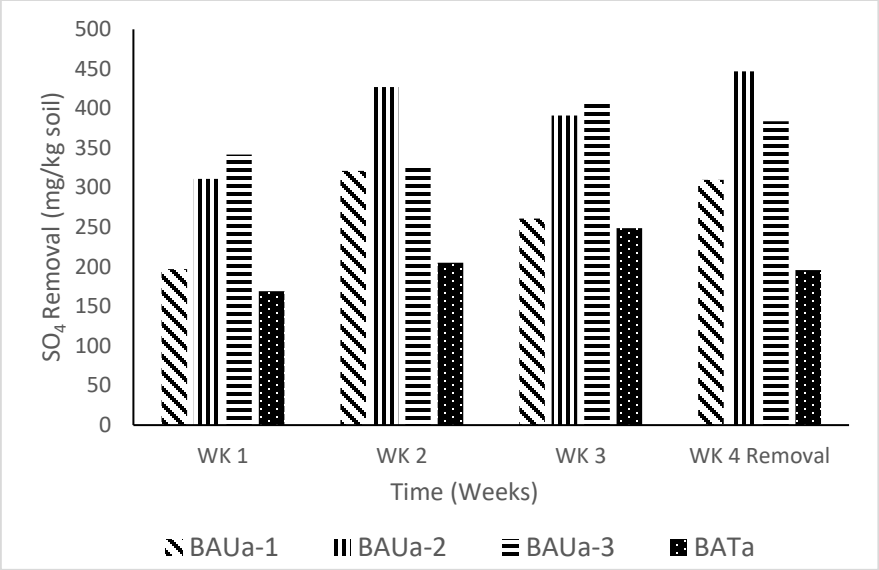


Figure 4-30. Sulfate Removal Efficiency (%) from BAUa Treatment

4.2.2.4 Sulfate Removal – *BSTa, BVTa and BAUa* Discussion

Sulfate removal in a low oxygen-induced environment promotes the sulfidogenic process which reduces sulfate to sulfide to enhance the formation of metal sulfide (black) ppt. (Chang, Shin and Kim 2000; Bhagat *et al.* 2004). This process was facilitated by organic nutrients (BWW and MWW) amended in the treatment which provided an energy source required by the sulfate-reducing bacteria to hasten the sulfate removal process (Gibert *et al.* 2003; Kaksonen, Riekkola-Vanhanen and Puhakka 2003; Bhagat *et al.* 2004).

The three treatment methods for sulfate removal (BSTa, BVTa and BAUa) recorded a reduction in sulfate concentration. However, removal efficiencies in BSTa and BAUa (especially bioreactors amended with wastewater) treatments were higher than BVTa due to the anaerobic environment provided by BSTa and BAUa treatment reactors for the sulfidogenic process, which reaffirms that sulfate reduction requires anoxic environment, for improved metabolism and tends to decreases with the injection of oxygen into the reactor (Fründ and Cohen 1992). Hence, the oxic condition activated in the BVTa treatment systems triggered by air injection inhibits the sulfidogenic process, reducing the activities of sulfate-reducing microbes which results in low sulfate removal in all air injected (BVTa) treatments (Jørgensen 1994; Liu *et al.* 2015) when compared to BSTa treatments. Meanwhile, the sulfate reduction in the treatments

can also be linked to the ability of wastewater amendment to provide enough nutrients for reducing microbes in anoxic and amid oxic conditions (Neculita, Zagury and Bussière 2007a; McCullough and Lund 2011). This is evident in the result recorded in all treatments with nutrients, and nutrients + air injection. It is observed that BWW showed slightly appreciable removal efficiency than MWW amendment in some BSTa, BVTa and BAUa treatments (using BWW only or BWW + MWW), which can be attributed to an increase in metabolic activities since BWW contains a significant amount of COD and BOD than MWW, due to the presence of organic components in sufficient quantity required for reducing bacteria (Bwapwa, Jaiyeola and Chetty 2017b; Amenorfenyo *et al.* 2019) and also the possible synergy between the organic substrates in the appropriate combination accounts for effective remediation as reported by Neculita, Zagury and Bussière (2007a) and McCullough and Lund (2011). While control experiment (BATa) and BVTa-1 (which was only vented) recorded the least average removal efficiency of 31.56% and 30.50% respectively due to lack of organic nutrients (Bai *et al.* 2013).

The BAUa treatment with the bacteria strain, *Pseudomonas aeruginosa* ATCC 15442 showed sulfate reduction as evident in BAUa-1 because the bacteria specie has been found to grow in organic or inorganic sulfur sources (Ismail *et al.* 2014) while utilizing the same as the sole sulfur source in the presence of glucose as an energy source. However, *Pseudomonas sp.* produced varying amounts of biosurfactants depending on the available sulfur sources. It has been reported that under pilot-scale, *P. Aeruginosa*, among other bacteria like *P. Stutzeri, Desulfovibrio Vulgaris, Desulfovibrio Desulfurican* were able to remove a reasonable amount of undesirable salts such as sulfate and nitrate from contaminated sites (Chen *et al.* 2008; Ajao, Adebayo and Yakubu 2011). In agreement with the present BAUa study which recorded 40–56% average sulfate removal efficiency after 28 days, Ajao, Adebayo and Yakubu (2011) reported 62.8 % removal efficiency for sulfate with *P. aeruginosa* and *B. Subtilis* after 15 days treatment period.

The introduction of wastewater (BWW and MWW) into the system as an extra energy source for *P. aeruginosa* ATCC 15442 facilitated the sulfate removal efficiency as evident in BAUa-2 and BAUa-3 treatment which increased the average removal efficiencies by 15.80% and 11.16% respectively (when compared to BAUa-1 inoculated with the bacteria strain only with 40.22%)

which is attributed to the possible synergy between *P. aeruginosa* and other microbial community present in wastewater (Agarry and Latinwo 2015). In addition, Chen *et al.* (2008) reported that the ability of *P. aeruginosa*, *(*heterotrophic denitrifiers) to effectively reduce sulfate can be attributed to the positive interaction with SRB present in wastewater to attain >70% sulfate removal efficiency after 10 days of treatment. The study reported that microbial community present in the sludge bed reactor used for the treatment comprises of *P. aeruginosa* and *sulfurospirillium sp* as heterotrophic; SRB (*Desulfomicrobium sp.*) as autotrophic and *Sulfurovum sp.* and *Paracoccus denitrifican* as denitrifiers. Also, at high concentrations of sulfate and nitrate, heterotrophic denitrifiers (*P. aeruginosa)* have been reported to outcompete autotrophic denitrifiers to reduce SRB activities while utilizing sulfate as electron acceptors (Hubert and Voordouw 2007; Kodama and Watanabe 2007) to enhance sulfate reduction.

However, the presence of SRB in wastewater triggers a sulfidogenic process which contributes to the increase in sulfate reduction in the treatments and collaboration with *P. aeruginosa* ATCC 15442 (for BAUa treatment) effective remediation was achieved (Chen *et al.* 2008). Sulfate reduction, especially with the use of organic carbon source or reducing bacteria, flourish in anaerobic conditions as no pure culture of SRB is known to grow or reduce sulfate in the presence of high oxygen concentration (Jørgensen 1994; Liu *et al.* 2015). Sequel to the lack of dissolved oxygen in a contaminated environment, sulfate availability enables sulfate reducers to thrive by utilization of sulfate as an electron acceptor in the anoxic environment to degrade organic matter resulting in the production of HS^- (Cresswell 2013). However, the availability of sufficient oxygen in the treatment system interfered with microbial activities of reducing bacteria which accounts to the decrease in sulfate removal efficiency in BVTa where injection of air (oxygen) resulted in temporary inhibition of SRB metabolism but did not cause cell death (Kjeldsen, Joulian and Ingvorsen 2004).

The study by Liu *et al.* (2015) reported that the growth of SRB and the sulfidogenic process decreases with an increase in oxygen leading to decrease in sulfate removal efficiency which is evident in the present study as the same treatment BSTa-2 (amended with BWW) with 47.15% average removal efficiency reduced by 5.2% when air was supplied in BVTa-2 and also reduced by 13.56% with BAUa-2 (*P. aeruginosa* + BWW) treatment. Moreover, the overall average

sulfate removal efficiency of 51.61% (BSTa) and 48.61% (BAUa) decreased to 34.53% (BVTa) which represent 17.02%, and 14.08% decline to cumulatively remove 408.20mg/kg, 383.45mg/kg and 272.5mg/kg sulfate from BSTa, BAUa and BVTa treatments, respectively. To further elucidate the effect of oxic condition in sulfate reduction, comparison of the removal efficiencies of treatments, BSTa with the organic nutrients; BWW (BSTa-1), MWW (BSTa-5) and combined BWW and MWW (BSTa-3) showed a decrease in sulfate weekly reduction efficiencies with corresponding BVT treatments (BVTa-2, BVTa-3 and BVTa-4) respectively (Fig. 4-26 & Fig. 4-28). This can be attributed to the increase in oxygen level in the BVTa treatment system since these bacteria that promote sulfate reduction are believed to operate optimally at a very low O_2 environment (Postgate 1984; Willow and Cohen 2003). The fluctuation and decrease in sulfate removal efficiencies observed during the treatment phases can be due to variations in wastewater effluents amendment (Sanchez-Andrea, Triana and Sanz 2012), the depletion of organic substrates or lack of bioavailability of contaminant to degrading microbes (Okabe, Nielsen and Characklis 1992; Boopathy 2000).

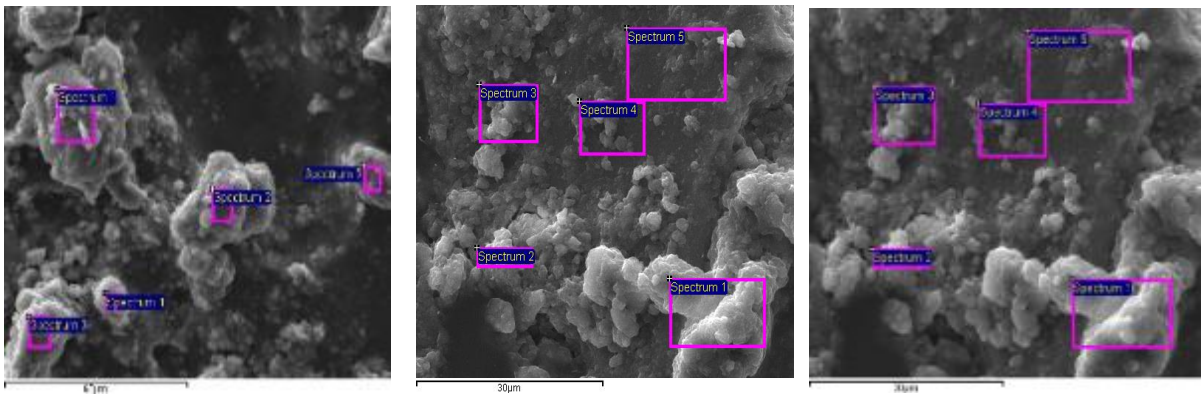
In agreement with the current study which recorded 47-58% and 30-42% average removal efficiencies for BSTa and BVTa respectively, studies by Luptáková *et al.* (2016), Muhammad *et al.* (2015), Gibert *et al.* (2003), Benner *et al.* (2002), Chang, Shin and Kim (2000) reported sulfate removal efficiency of 12 – 66% using the organic substrate as an energy source while Kijjanapanich *et al.* (2014) noted that the highest sulfate removal efficiency of 59% was recorded in the soil mixed with 40% organic mixture after the treatment period which correlates with the results of the current study. In accordance to the results of the present study, Fründ and Cohen (1992) recorded an increase in sulfate reduction with 68% efficiency in low O_2 concentrated bioreactor and a decrease to 45% in a high oxic environment. The study further reported that SRB activities were detected under oxic conditions during the day time and facilitated with the addition of carbon source but noted that sulfate reduction in dark zones is more favourable than in light sufficient zones (Fründ and Cohen 1992) and appreciable in reduced oxygen conditions (Postgate 1984; Willow and Cohen 2003), hence, air-injection treatments showed lower sulfate removal efficiencies than the organic waste amendment. Also, the study by Kjeldsen, Joulian and Ingvorsen (2004) reported a reduction in the growth of SRB by 20% in aerobic conditions but consequently, experienced quick proliferation potential of

reducing bacteria and microbial activities which were evident with the introduction of activated anaerobic sludge in the treatment system (Hastings and Emerson 1988). It can be inferred that the application of wastewater (brewery and municipal effluent) and *P. Aeruginosa* ATCC 15442 is effective for sulfate removal from AMD contaminated soil but air -injection tend to hinder the activities of the reducing bacteria which results to low efficiency.

4.2.3 Scanning Electron Microscopy (SEM) – Energy Dispersive X-Ray Spectroscopy (EDS) – Results and Discussion

SEM examination of samples from all treatments (BSTa, BVTa and BAUa) showed that the surface of samples was coated with pervasive layers (Fig. 4-31a, b & c respectively) which are made up of Fe, Al, S, and O with microelements and minor amounts of other metals as represented in the peaks of EDS spectrum (Fig. 4-33a, b & c respectively). These peaks were linked to the representative components of the AMD treatment system, which further revealed that Fe, Al, and other metals were precipitated in the form of metal sulfides. However, the EDS spectrum from the contaminated sample before the application of bioremediation methods showed the presences of Fe, Al, Zn, Mn, and other elements (Fig. 4-32) which (target metals) decreases or involved in the formation of complexes which results to non-detected nature during the treatment as evident in EDS spectrum from samples after treatments as shown in Fig. 4-33a, b & c for BSTa, BVTa and BAUa treatment respectively but the degree of metal reduction or removal efficiency was determined by XRF analysis (section 4.2.1) above.

It can be deduced (from the SEM result) that the black precipitate formed on the surface of the treatment is attributed to biosulfidogenic process (Pawlowska and Sadowski 2019) which reduces sulfate to elemental sulfur to facilitate the formation of metal sulfide ppt. (Dvorak et al. 1992; Lyew and Sheppard 1997; Gibert et al. 2003). However, metal complexation and precipitation were feasible during the treatment due to the formation of amorphous Fe-Al (oxy) hydroxide ppt. (Gibert *et al.* 2003; Gibert *et al.* 2005a, 2005b) since the presence of Fe, Al, S, O was evident as shown Fig. 4-33a, b & c which contributed to co-precipitation and removal of metals from the treatments (Song *et al.* 2001; Gibert *et al.* 2005b; Cravotta 2007).



(a) BSTc (b) BVTc (c) BAUc

Figure 4-31a,b & c. SEM result showed cryptocrystalline coating layer of spherulitic aggregates consists of Fe, Al, O, S, microelements, and minor amounts of other metals. (from BSTc, BVTc and BAUa treatment respectively)

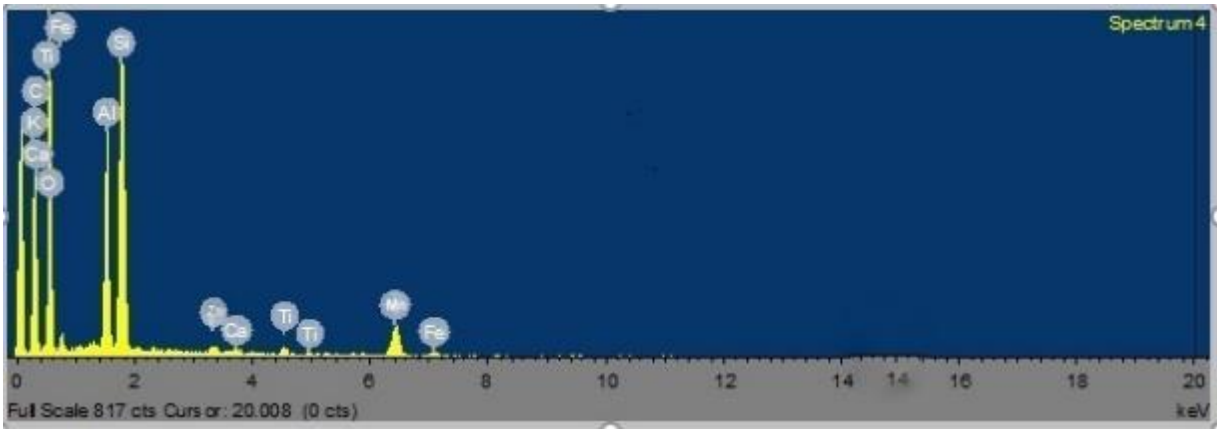


Figure 4-32. EDS spectrum of the contaminated sample before treatment which shows the presence of Fe, Al, Zn, Mn, microelements, and other elements *(before the application of bioremediation methods).*

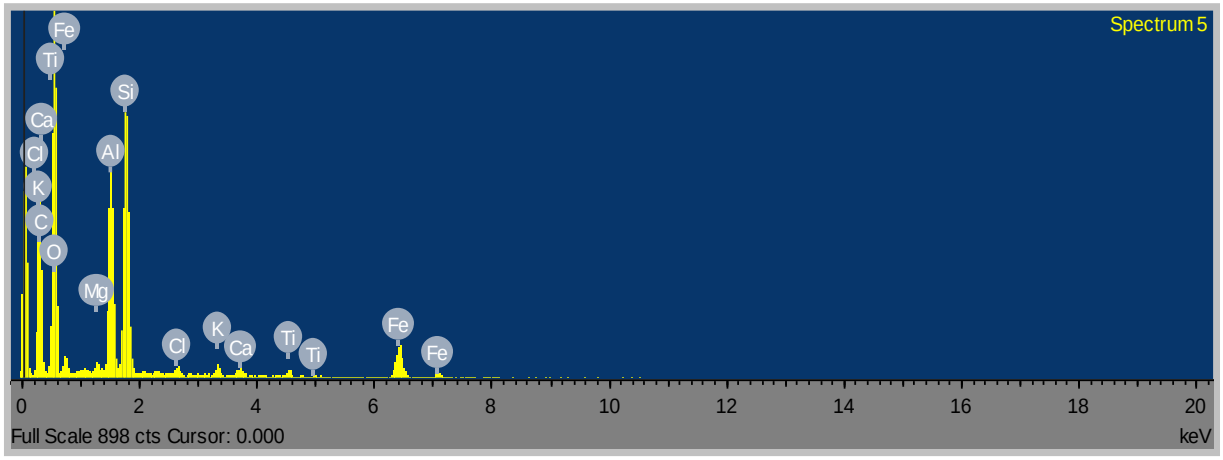


Figure 4-33a. BST treatment system

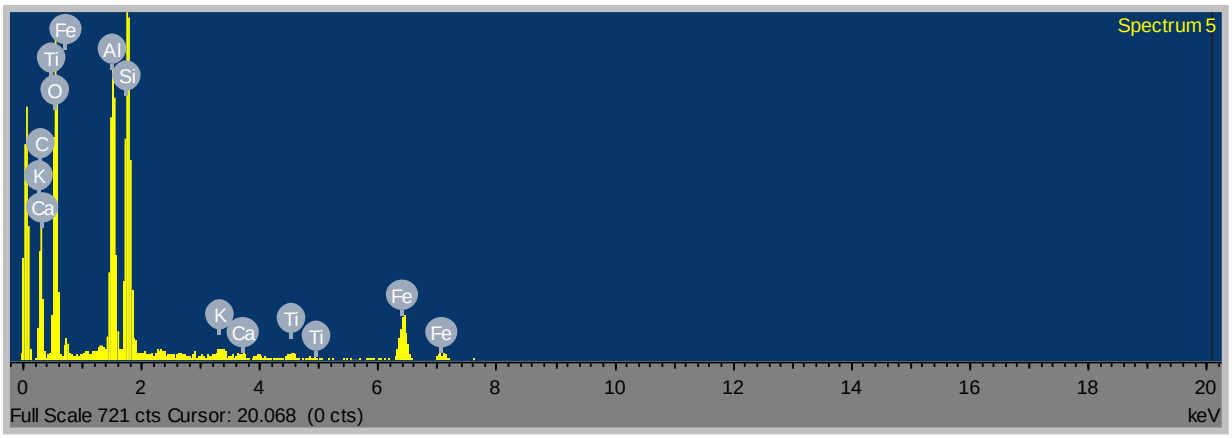


Figure 4-33b. BVTa treatment system

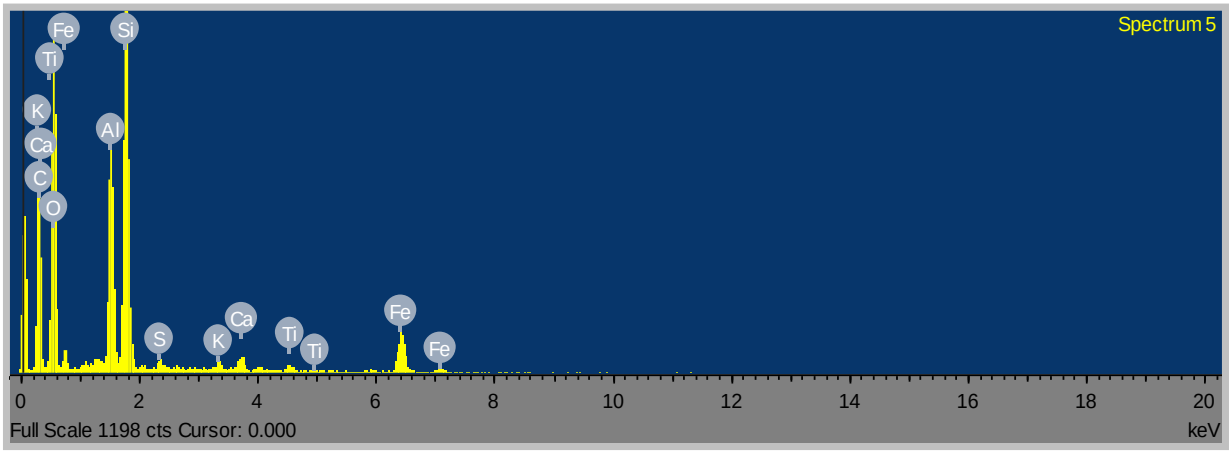


Figure 4-33c. BAUa treatment system

Figure 4-33a, b & c. EDS qualitative analysis of samples from the BSTa, BVTa and BAUa treatment respectively which showed the absence of some metals when compared to Fig. 24.

4.3 Comparative Analysis of Bioremediation techniques (BST, BVT, BAU & BAT) for the treatment of Crude oil and AMD Contaminated Soils

4.3.1 Evaluation of bioremediation of crude oil contaminated soils

The result of the treatments showed that bioventing treatment (BVTc) recorded the highest removal efficiency when compared to biostimulation (BSTc) and bioaugmentation (BAUc) treatments with 10.36% and 3.95% greater than average removal efficiencies of BSTc and BAUc respectively (Fig. 4-34). Also, BVTc treatment removed the highest TPH after week 4, reducing the initial TPH concentration from 50000mg/kg to 18603.75mg/kg (average residue)

at 1121.30mg/day average removal rate (Fig. 4-34). Meanwhile, BSTc and BAUc recorded average removal efficiencies of 938.29mg/day and 1050mg/day respectively. The removal efficiency of these treatments followed the trend BVTc > BAUc > BSTc. However, the control treatment recorded the least removal efficiency (34%) and was able to reduce the initial concentration to 32750mg/kg after 28 days treatment period (*see Appendix 4*).

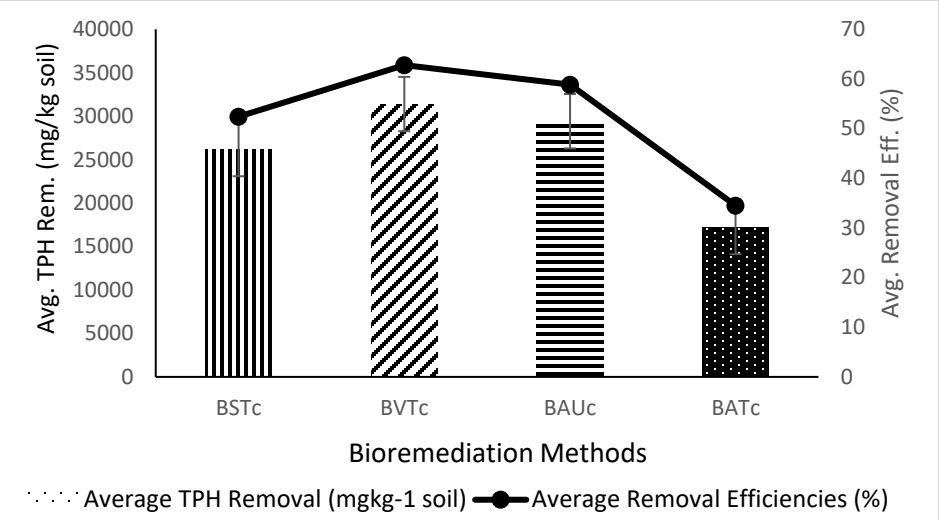


Figure 4-34. Evaluation of BSTc, BVTc, BAUc and BATc for the treatment of crude oil contaminated soils

The application of organic nutrients (wastewater) for the BSTc treatment ensured the availability of sufficient nutrients to the indigenous microbial population to facilitate biodegradation of hydrocarbons (Agarry and Latinwo 2015; Adekunle *et al.* 2017a; Mbah and Obahiagbon 2017; Liu *et al.* 2018; Amenorfenyo *et al.* 2019). However, since the concentration of hydrocarbon hindered soil ventilation which reduces oxygen level (Osuji and Adesiyan 2005), the injection of atmospheric air into the system provided the aerobic environment for the microbes to facilitate the oxidation process, and enhance hydrocarbon degradation (Couto and García-Frutos 2016) while the presence of wastewaters in the bioventing system acts as biostimulants, which boost nutrient levels (Lee and Swindoll 1993; Frutos *et al.* 2010; Thomé *et al.* 2014; Agarry and Latinwo 2015) for effective biodegradation of hydrocarbons as evident in the significant removal efficiency recorded with BVTc. Also, the application of combined *P. aeruginosa* ATCC 15442 and wastewater (BAUc) was more effective and recorded appreciable removal efficiencies than the use of *P. aeruginosa* ATCC 15442 inoculation only, since the

organic substrate provided an extra nutrient source for the microbes to enhance the metabolic process and degradation of hydrocarbon (Mancera-López *et al.* 2008; Ghaly, Yusran and Dave 2013; Mohajeri *et al.* 2017). Meanwhile, all these treatments except the BATc were able to reduce the initial concentration of crude oil below the extreme toxic level in the soil as Baker (1976) reported that concentration of crude oil above 3% is detrimental to soil biota, plant growth and development (Osuji, Egbuson and Ojinnaka 2005) and reduced agricultural productivity (Osuji and Nwoye 2007) while the control treatment was unable to reduce the concentration below toxic level (Fig. 4-34) due to non-application of bioremediation technique which buttresses the positive effect of wastewater, air-injection and *P. aeruginosa* ATCC 15442 as bioremediation strategy in the treatment of crude oil contaminated soils.

However, the present biostimulation *(BSTc) study* is in agreement with several studies (Adekunle 2011; Chijioke-Osuji, Ibegbulam-Njoku and Belford 2014b; Agarry and Latinwo 2015; Obiakalaije, Makinde and Amakoromo 2015; Adekunle *et al.* 2017c; Agarry 2018; Ani *et al.* 2018; Imafidon and Ogirigbo 2018b; Liu *et al.* 2018; Amenorfenyo *et al.* 2019; Chen *et al.* 2019) and the bioventing *(BVTc) study* is supported by Agarry and Latinwo (2015), Frutos *et al.* (2010), Muskus Morales, Santoyo Muñoz and Plata Quintero (2013), Jia *et al.* (2016), Lee and Swindoll (1993), Mao *et al.* (2009), Thomé *et al.* (2014), Møller *et al.* (1996) *while* Ghaly, Yusran and Dave (2013) Al-Hadhrami, Lappin-Scott and Fisher (1997), Mohajeri *et al.* (2017), Karamalidis *et al.* (2010), Venkateswaran *et al.* (1995), Guo-liang *et al.* (2005), Tavassoli *et al.* (2012) and Varjani and Upasani (2017) recorded similar results with the microbial inoculation *(BAUc study).*

4.3.2 Evaluation of bioremediation of AMD contaminated soils

4.3.2.1 Evaluation of bioremediation methods for metal removal

The biostimulation (BSTa) treatment recorded an appreciable metal removal through biosulfidogenic process which reduces sulfate to elemental sulfur in the presence of organic substrate (wastewater) to facilitate metal removal through precipitation (Muyzer and Stams 2008; Toogood 2012). BSTa treatment recorded an average of 51.23% (avg. range: 27-66%) metal removal efficiency as Fe, Al, Cu and Zn showed >50% (Fig. 4-35), Mn recorded the least

average removal efficiency of 27.12% due to the uncatalyzed Mn oxidation after the 28days treatment. The introduction of atmospheric air for the BVTa treatment ventilates the bioreactor which triggers oxidation process for metal removal, while the amendment of wastewater stimulates the microbial activities which increased the metal removal efficiency when compared to the treatment that was vented only. BVTa treatment showed average metal removal efficiency of 62.12% (avg. range: 56-69%) with >56% removal efficiency for all metals including Mn due to effective oxidation and precipitation process (Fig. 4-35). However, the bacteria-induced treatment (BAUa) showed that the bacteria strain, *Pseudomonas aeruginosa* ATCC 15442 was able to reduce metal concentration through biosorption process but the amendment of wastewater as an extra carbon source enhanced the removal of metals which recorded an average metal removal efficiency of 53.11% (avg. range: 47-60%). BAUa treatment recorded a slightly higher average metal removal efficiency than BSTa as Mn removal was also facilitated with avg. of 47.10% due to the synergy between *P. aeruginosa* and wastewater. The control treatment (BATa) showed the least average metal removal efficiency of 22.70% (Fig. 4-35) due to lack of biostimulants (*See Appendix 18*). Metal removal was most favourable with BVTa treatment which showed effective removal of all metals.

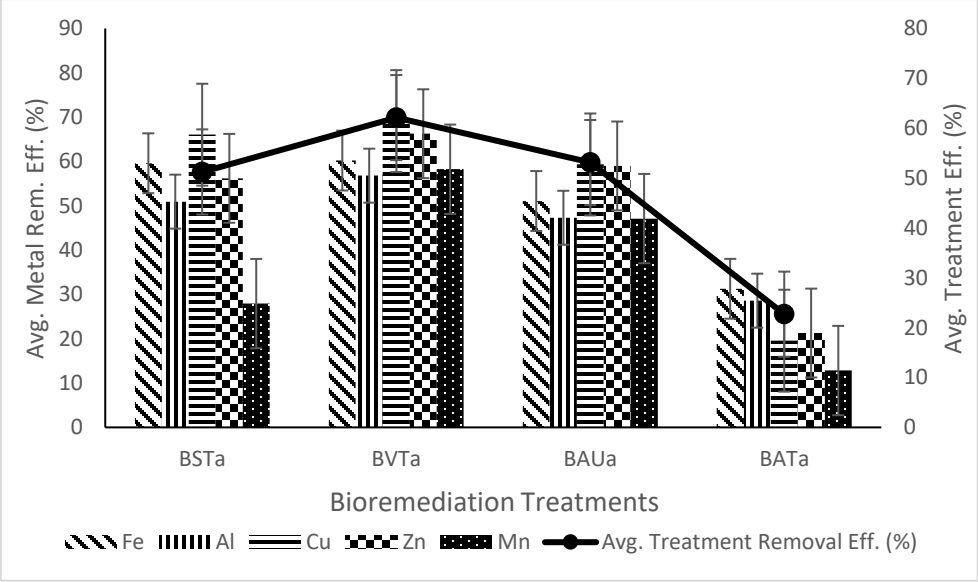


Figure 4-35. Evaluation of BSTc, BVTc, BAUc and BATc for metal removal from AMD Contaminated soils

The present *BSTa treatment* for AMD treatment was in agreement with several studies (Martins *et al.* 2008; Sanchez-Andrea, Triana and Sanz 2012; Hughes and Gray 2013; Strosnider *et al.* 2013) and *BVTa study* validates works by Balintova and Petrilakova (2011), Cravotta (2007), Peters and Bennett (1989) and Ching (1986) while the results of the current *BAUa investigation* is corresponds with studies by Sinha and Mukherjee (2009), Awasthi *et al.* (2015), Juwarkar *et al.* (2008), and Chen *et al.* (2006).

4.3.2.2 Evaluation of bioremediation methods for Sulfate removal

The use of organic substrate showed effective sulfate reduction through the provision of direct carbon source to the degrading bacteria which breathes sulfate in place of oxygen while utilizing organic nutrients as an energy source to reduce sulfate to biogenic hydrogen sulfide (Gibert *et al.* 2003; Kaksonen, Riekkola-Vanhanen and Puhakka 2003; Bhagat *et al.* 2004). The study showed that aerobic environment is not suitable for sulfate removal which is responsible for the decline in the removal efficiencies when atmospheric air was introduced into the system in BVTa treatment (Fig. 4-36) (Jørgensen 1994; Liu *et al.* 2015) which is 17.08% (135.52mg/kg) less than average removal efficiency for BSTa (Fig. 4-36). Hence, efforts to boost sulfate removal efficiency through air-injection proved abortive. However, the amendment of wastewater enhanced the efficiency of *P. aeruginosa* ATCC 15442 in BAUa treatment which recorded 49.21% (Fig. 4-36), (15.21% more than BVTa) and corresponds to the reduction of sulfate concentration to 405mg/kg (average residue) from 798mg/kg^{-1} initial concentration due to the ability of the bacteria to utilize wastewater as an extra carbon source for sulfate removal process (Brown and Lester 1982; Silva *et al.* 2009) (*See Appendix 22*).

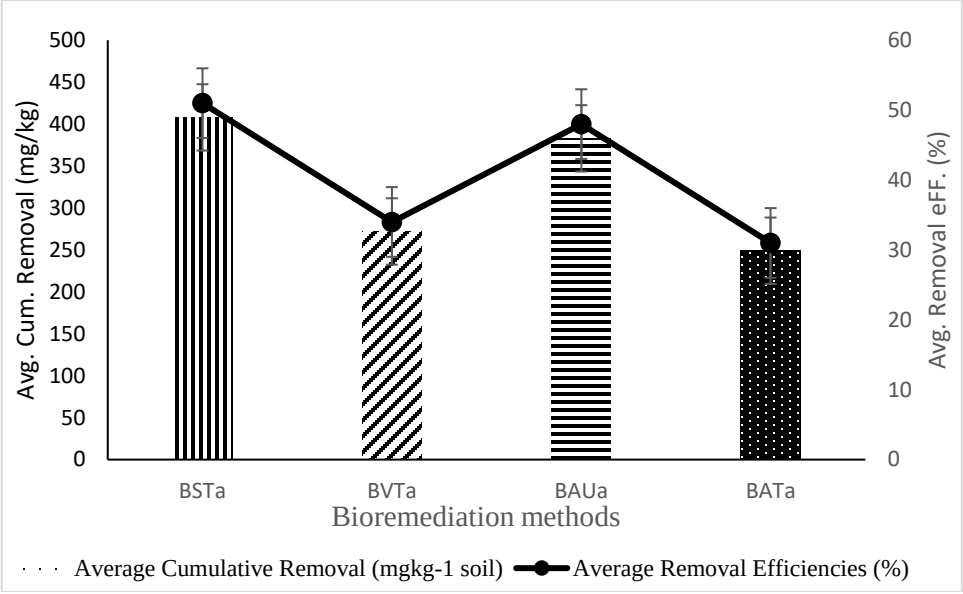


Figure 4-36. Evaluation of BSTc, BVTc, BAUc and BATc for sulfate removal from AMD Contaminated soils

These bioremediation treatments were able to appreciably reduce sulfate concentration in AMD contaminated soil except for BVTa and BATa due to high oxygen concentration which hinders SRB activities (Fründ and Cohen 1992) and BATa as a result of the lack of nutrient amendment (Bai *et al.* 2013) respectively. The present Biostimulation (BST) *study* is in agreement with studies by Luptáková *et al.* (2016), Muhammad *et al.* (2015), Gibert *et al.* (2003), Benner *et al.* (2002), Chang, Shin and Kim (2000), the result of Bioventing *(BVTa) treatment* correlated with studies by Fründ and Cohen (1992) and Kjeldsen, Joulian and Ingvorsen (2004) while present Bioaugmentation *(BAUa) study* for sulfate removal conforms with previous studies (Chen *et al.* 2008; Ajao, Adebayo and Yakubu 2011).

However, a real complex pyrite AMD soil with dynamic acid-generating capacity may affect bioremediation mechanism. Since AMD contains multiple combinations of acidity and metals, which attributes to the distinct feature of every AMD as no AMD is the same, the presence of heavy metals might be similar but with varying concentrations. The formation of complexes with varying pH by acid-generating capacity may pose a significant challenge to effective bioremediation on large scale. The rate of sulfate reduction can be affected by factors such as bioavailability of contaminants, complexity, high concentration, and toxicity of heavy metals. Moreover, temperature and seasonal variation which may result to extreme cold or hot weather

which affect microbial activities and hinder the application of bioremediation methods (Boopathy 2000). The adjustment of operating temperature the present study to the ambient condition was necessary to enhance microbial activities and performance since extreme temperatures hinder microbial growth while the moderate toxicity of AMD account to significant metal removal efficiency (Boopathy 2000).

CHAPTER FIVE

5.0 Conclusion and Recommendations

5.1 Conclusion

1. Biostimulation (BST) through wastewater amendment provided direct organic nutrients to the indigenous microbial population to facilitate TPH degradation and triggered the sulfidogenic process which reduced sulfate to biogenic hydrogen sulphide and subsequently removed metals through precipitation process which attributes to the black precipitate on the surface of the treatments.

2. Air-injection in bioventing (BVT) process, ventilated the bioreactor to induce aerobic process which resuscitated and revived the microbial activities and improved oxidation process for effective TPH and metal reduction.

3. The bacteria strain, *P. aeruginosa* ATCC 15442 used for this study showed great potential in utilization of crude oil as carbon source, adsorption of metals and sulfate but the introduction of wastewater as an extra energy source increased the removal process during the bioaugmentation (BAU) treatment of contaminated soils. The combined applications, BVT + wastewater and BAU + wastewater treatments were more effective than the single application of these methods for the reduction of TPH and metals concentrations than sulfate removal from the contaminated soil.

4. The reduction of TPH and metal concentration in the contaminated soils were most facilitated by bioventing process which recorded the highest average TPH and metal removal efficiencies of 62.79% and 62.12% respectively when compared with other treatments. BSTa and BAUa application favoured sulfate reduction because sulfidogenic process responsible for sulfate removal is more favourable in low oxic conditions and attempts to improve the removal rate through the injection of atmospheric air were not effective as bioventing treatment increases the oxygen concentration in the bioreactor which hinders the sulfidogenic process and reduces sulfate removal efficiency.

5. The BSTa and BAUa recorded 51.74% and 49.21% average sulfate removal efficiencies respectively as against 34.54% for BVTa. The high concentration of crude oil and varying

concentration of multi-metal composition of AMD contributes to toxicity and complexity of the metal reduction process respectively which inhibited *P. aeruginosa* and other microbial community activities and reduced the remediation efficiency.

6. Following the bioremediation techniques investigated for contaminant remediation, it can be deduced that TPH was facilitated by microbial degradation, biosorption and oxidation process while metal and sulfate were reduced through precipitation, co-precipitation, sorption into organic substrates, and biosorption by dead or live cells.

7. These bioremediation treatments having reduced these contaminants below the extreme toxic level depicts significant contaminants removal, except for the control treatment (BAT) which showed low removal efficiencies for all soil contaminants investigated. Hence, these bioremediation techniques can be used as a viable alternative for the restoration of polluted soil.

8. The study showed that wastewaters (brewery and municipal), air-injection and *P. aeruginosa* ATCC 15442 have the potentials for TPH, metals and sulfate removal from contaminated soils and could serve as a low-cost, and eco-friendly method for the treatment of polluted sites and alleviation of pollution aftermath in the affected areas. Hence, the application of BST, BVT and BAU to enhance the treatment of AMD and crude oil contaminated soil could be one of the severally sought bioremediation methods for the remediation of the environment contaminated with AMD or crude oil.

5.2 Recommendations

The application of wastewater should be considered for the remediation of crude oil and AMD polluted sites since it requires no pre-treatment before application for an optimum result which can also serve as an alternative measure for waste management. However, for the combined application of bioventing and biostimulation to be viable for sulfate reduction, the process should be controlled for effective reduction of metals and sulfate from the treatment since oxygen hinders sulfate reduction but accelerates heavy metal removal from AMD. Optimization of the process will be apt for effective remediation as further study is required to validate large scale application.

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APPENDICES

TPH Removal Results

Appendix 1. BSTc TPH Removal Efficiency (%)

Treatment	WK 0	WK 1	WK 2	WK 3	WK 4
BSTc-1	0	19.82	26.93	37.1	58.39
BSTc-2	0	15.69	25.27	31.73	48.67
BSTc-3	0	17.35	27.51	30.38	49.67
BSTc-4	0	20.48	22.51	31.59	48.81
BSTc-5	0	17.01	21.65	48.21	56.62
BATc	0	8.15	15.5	20.48	34.5

Appendix 2. BVTc TPH Removal Efficiency (%)

Treatment	WK 0	WK 1	1 2	WK 3	WK 4 l
BVTc-1	0	11.25	21.08	39.76	54.93
BVTc-2	0	18.61	47.52	59.88	74.75
BVTc-3	0	17.02	20.46	40.26	60.02
BVTc-4	0	21.16	30.07	48.98	61.47
BATc	0	8.15	15.5	20.48	34.5

Appendix 3. BAUc TPH Removal Efficiency (%)

Treatment	WK 0	WK 1	WK 2	WK 3	WK 4
BAUc-1	0	10.81	21.69	39.55	42.02
BAUc-2	0	17.34	26.77	54.18	69.48
BAUc-3	0	14.55	34.69	44.79	65.03
BATc	0	8.15	15.5	20.48	34.5

Appendix 4. Evaluation of the results of BSTc, BVTc, BAUc and BATc treatments

Treatments	Removal Efficiencies (%)	Average Removal Efficiencies (%)	Average TPH Removal ($mgkg^{-1}$ soil)	Average Removal Rate (mg/day)	Average Residue (mg/kg^{-1})
BSTc	48.67 – 58.39	52.43	26216.00	938.29	23784.00
BVTc	54.93 – 74.75	62.79	31396.25	1121.30	18603.75
BAUc	42.02 – 69.48	58.84	29421.67	1050.77	20578.33
BATc	34.50	34.50	17250.00	616.07	32750.00

Metal Removal (XRF Results)

Appendix 5. BSTa-1 Treatment (Metal Removal Efficiency, %)

Metals	W0	W1	W2	W3	W4
Fe	0	55,84046	47,4359	58,68946	62,82051
Al	0	46,94149	43,21809	45,21277	66,2234
Cu	0	60,96491	60,04386	69,29825	72,45614
Zn	0	45,69983	53,74368	66,61046	59,69646
Mn	0	32,57965	18,60226	34,8407	21,58273

Appendix 6. BSTa-2 Treatment (Metal Removal Efficiency, %)

Metals	W0	W1	W2	W3	W4
Fe	0	54,2735	48,8604	68,66097	67,09402
Al	0	34,9734	36,56915	57,18085	66,2234
Cu	0	65,35088	65,30702	75,4386	78,94737
Zn	0	29,17369	67,2344	67,48735	70,69309
Mn	0	12,02467	39,15725	28,6742	21,58273

Appendix 7. BSTa-3 Treatment (Metal Removal Efficiency, %)

Metals	W0	W1	W2	W3	W4
Fe	0	65,66952	55,98291	72,93447	70,08547
Al	0	48,27128	49,86702	65,15957	74,20213
Cu	0	65,35088	77,58772	61,57895	74,5614
Zn	0	54,26307	67,2344	53,99663	66,62732
Mn	0	38,74615	30,93525	33,81295	31,86023

Appendix 8. BSTa-4 Treatment (Metal Removal Efficiency, %)

Metals	W0	W1	W2	W3	W4
Fe	0	51,4245	47,4359	68,66097	69,80057
Al	0	44,28191	47,20745	54,52128	47,60638
Cu	0	47,36842	60,04386	62,2807	65,78947
Zn	0	38,92074	42,15852	62,68465	60,64435
Mn	0	22,30216	27,852	37,92395	21,58273

Appendix 9. BSTa-5 Treatment (Metal Removal Efficiency, %)

Metals	W0	W1	W2	W3	W4
Fe	0	51,4245	47,4359	54,41595	72,79202
Al	0	34,9734	47,20745	50,53191	58,24468
Cu	0	50,87719	57,40789	62,2807	87,7193
Zn	0	45,69983	47,21754	60,74199	63,25464
Mn	0	16,17677	31,22302	28,73587	28,1295

Appendix 10. BATa (Control) Treatment (Metal Removal Efficiency, %)

Metals	W0	W1	W2	W3	W4
Fe	0	29,77208	33,47578	29,34473	32,47863
Al	0	32,97872	31,64894	27,26064	22,4734
Cu	0	15,78947	19,95614	14,42982	28,07895
Zn	0	24,45194	17,16695	21,1973	22,29342
Mn	0	7,605344	17,91367	9,969168	15,79651

Appendix 11. BVTa-1 Treatment (Metal Removal Efficiency, %)

Metals	W0	W1	W2	W3	W4
Fe	0	51,4245	47,4359	54,41595	52,849
Al	0	33,64362	43,21809	51,8617	47,60638
Cu	0	60,96491	60,04386	62,2807	56,22807
Zn	0	62,90051	42,15852	52,78246	52,9511
Mn	0	32,16855	30,93525	45,11819	42,13772

Appendix 12. BVTa-2 Treatment (Metal Removal Efficiency, %)

Metals	W0	W1	W2	W3	W4
Fe	0	50	60,25641	58,68946	72,93447
Al	0	48,27128	49,86702	65,15957	75,53191
Cu	0	43,42105	77,58772	79,12281	85,61404
Zn	0	54,26307	67,2344	84,35076	78,09444
Mn	0	49,02364	61,76773	74,92292	68,8592

Appendix 13. BVTa-3 Treatment (Metal Removal Efficiency, %)

Metals	W0	W1	W2	W3	W4
Fe	0	64,24501	60,25641	64,38746	72,93447
Al	0	48,27128	49,86702	73,1383	71,54255
Cu	0	52,19298	68,81579	65,96491	79,91228
Zn	0	54,26307	67,2344	70,86003	63,25464
Mn	0	49,02364	61,76773	74,92292	64,40904

Appendix 14. BVTa-4 Treatment (Metal Removal Efficiency, %)

Metals	W0	W1	W2	W3	W4
Fe	0	50	54,41595	72,93447	75,78348
Al	0	42,95213	49,86702	71,80851	86,17021
Cu	0	52,19298	81,97368	85,96491	93,42105
Zn	0	66,06745	67,2344	88,1484	88,23103
Mn	0	59,30113	51,49024	85,20041	80,85303

Appendix 15. BAUa-1 Treatment (Metal Removal Efficiency, %)

Metals	W0	W1	W2	W3	W4
Fe	0	51,4245	33,19088	40,17094	24,35897
Al	0	33,64362	29,92021	25,26596	21,01064
Cu	0	60,96491	51,31579	57,89474	43,85965
Zn	0	62,90051	42,15852	52,78246	36,08769
Mn	0	53,13464	39,15725	45,11819	31,86023

Appendix 16. BAUa-2 Treatment (Metal Removal Efficiency, %)

Metals	W0	W1	W2	W3	W4
Fe	0	84,04558	60,11396	45,58405	58,40456
Al	0	80,71809	49,33511	59,44149	68,61702
Cu	0	88,15789	72,80702	55,70175	52,63158
Zn	0	88,87015	73,69309	57,50422	42,49578
Mn	0	63,5149	60,2261	48,81809	37,41007

Appendix 17. BAUa-3 Treatment (Metal Removal Efficiency, %)

Metals	W0	W1	W2	W3	W4
Fe	0	46,5812	65,95442	47,4359	55,55556
Al	0	36,96809	56,78191	56,38298	49,33511
Cu	0	62,7193	68,85965	49,5614	47,36842
Zn	0	59,35919	66,94772	47,72344	77,35245
Mn	0	52,10689	57,14286	39,87667	36,79342

Appendix 18. Evaluation BSTa, BVTa, BAUa and BATa for Average Metal Removal

Metals	BSTa	BVTa	BAUa	BATa
Fe	59,19	60,19	51,97	31,27
Al	50,03	56,79	47,29	28,59
Cu	66,03	69,1	59,72	19,56
Zn	56,19	66,25	58,98	21,28
Mn	27,12	58,24	47,1	12,82
Avg.Rem. from Treatments	51,71	62,12	53,01	22,7

Sulfate Analysis Results

Appendix 19. BSTa – Sulfate Removal Efficiency (%)

Treatment	WK 0	WK 1	WK 2	WK 3	WK 4
BSTa-1	0	13.18124	35.6147	39.92395	47.14829
BSTa-2	0	50.95057	30.16477	34.47402	47.90875
BSTa-3	0	47.40177	32.06591	51.3308	50.82383
BSTa-4	0	37.00887	35.36122	51.45754	53.35868
BSTa-5	0	29.53105	42.58555	52.09125	33.58682
BAT	0	21.41952	25.98226	31.55894	24.84157

Appendix 20. BVTa – Sulfate Removal Efficiency (%)

Treatment	WK 0	WK 1	WK 2	WK 3	WK 4
BVTa-1	0	4.68948	22.68695	30.29151	26.86946
BVTa-2	0	4.435995	16.6033	31.68568	41.95184
BVTa-3	0	16.09632	39.29024	31.55894	28.64385
BVTa-4	0	14.95564	34.98099	26.48923	31.55894
BATa	0	21.41952	25.98226	31.55894	24.84157

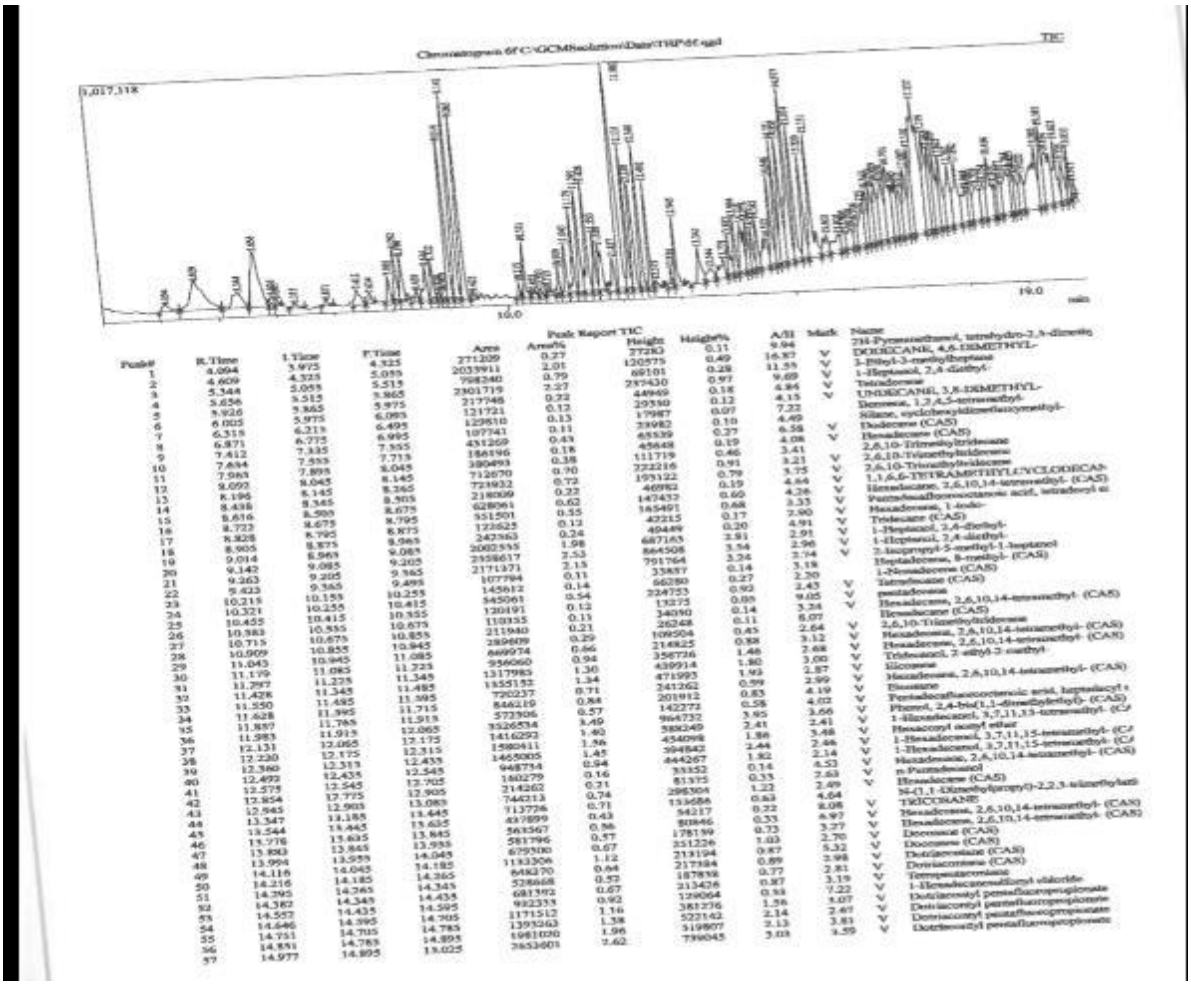
Appendix 21. BAUa – Sulfate Removal Efficiency (%)

Treatment	WK 0	WK 1	WK 2	WK 3	WK 4
BAUa-1	0	24,6867168	40,2255639	32,706767	38,847118
BAUa-2	0	38,9724311	53,5087719	48,997494	56,015038
BAUa-3	0	42,8571429	40,726817	51,378446	48,120301
BATa	0	21,4195184	25,982256	31,558935	24,841572

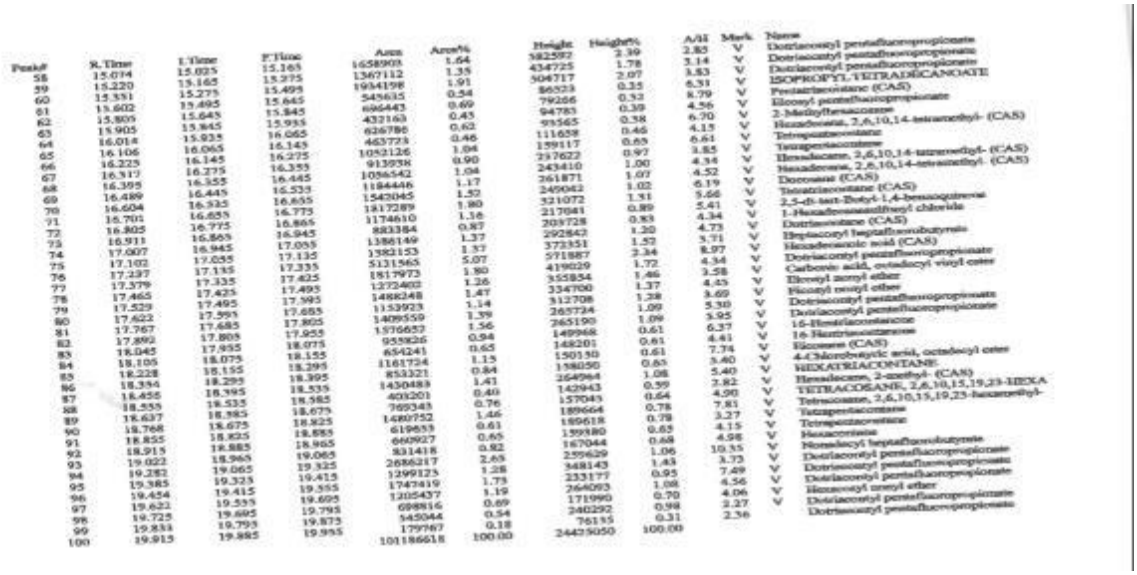
Appendix 22. Evaluation of BSTc, BVTc and BAUc and BATc for Sulfate removal

Treatments	Average Cumulative Removal ($mgkg^{-1}$ soil)	Average Removal Efficiencies (%)	Average Removal Rate (mg/day)	Average Residue (mg/kg^{-1})
BSTa	408	51	14	380.80
BVTa	272	34	9	516.50
BAUa	383	49	13	405.47
BATa	249	31	8	540.00

Appendix 23. GCMS Chromatogram before Treatment of contaminated soils

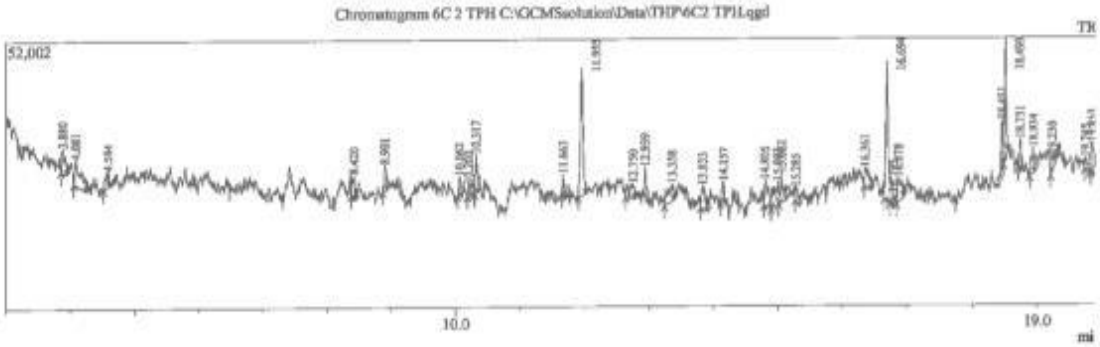


Peak#	R.Time	I.Time	F.Time	Area	Area%	Height	Height%	A/H	Mark	Name
1	4.094	3.975	4.325	271209	0.27	27230	0.11	9.94		2H-Pyran-2-one, tetrahydro-2,3-dimethyl-
2	4.609	4.325	5.095	2033911	2.01	120579	0.49	16.87	V	DODECANE, 4,6-DIMETHYL-
3	5.344	5.095	5.515	798240	0.79	68101	0.28	11.55	V	1-Ethyl-3-methylheptane
4	5.656	5.515	5.865	2301719	2.27	237430	0.97	9.69	V	1-Heptanol, 2,4-diethyl-
5	5.926	5.865	5.975	217948	0.22	44949	0.18	4.84	V	Tetradecene
6	6.005	5.975	6.085	121721	0.12	29350	0.12	4.15	V	UNDECANE, 3,8-DIMETHYL-
7	6.315	6.215	6.495	129810	0.13	17987	0.07	7.22		Benzene, 1,2,4,5-tetramethyl-
8	6.871	6.775	6.995	107743	0.11	33982	0.10	4.49		Silane, cyclohexyldimethoxymethyl-
9	7.412	7.335	7.715	431269	0.43	65539	0.27	6.58	V	Dodecene (CAS)
10	7.684	7.553	8.045	186196	0.18	45648	0.19	4.08	V	Hexadecene (CAS)
11	7.963	7.895	8.145	380493	0.38	111719	0.46	3.41		2,6,10-Trimethyltridecane
12	9.093	9.045	9.265	712670	0.72	222216	0.91	3.21	V	2,6,10-Trimethyltridecene
13	8.196	8.145	8.505	218009	0.22	193122	0.79	3.75	V	1,1,6,6-TETRAMETHYLCYCLODECA-
14	8.438	8.345	8.675	628061	0.62	46982	0.19	4.84	V	Hexadecane, 2,6,10,14-tetramethyl- (CAS)
15	8.616	8.505	8.795	551501	0.55	147452	0.60	4.26	V	Pentadecanoic acid, 14-methyl-, methyl es
16	8.722	8.675	8.875	123625	0.12	165491	0.68	3.33	V	Hexadecene, 1-iodo-
17	8.828	8.795	8.965	242363	0.24	42215	0.17	5.90	V	Tridecane (CAS)
18	8.905	8.875	9.085	2002555	1.98	49449	0.20	4.91	V	1-Heptanol, 2,4-diethyl-
19	9.014	8.965	9.205	2558617	2.53	687165	2.81	2.95	V	1-Heptanol, 2,4-diethyl-
20	9.140	9.085	9.365	2171371	2.15	864508	3.54	2.96	V	2-Isopropyl-5-methyl-1-heptanol
21	9.263	9.205	9.495	107794	0.11	791764	3.24	2.74		Heptadecane, 8-methyl- (CAS)
22	9.423	9.365	10.255	145612	0.14	33857	0.14	3.19		1-Nonadecene (CAS)
23	10.215	10.155	10.415	345061	0.34	66280	0.27	3.20		Tetratriacontane (CAS)
24	10.321	10.255	10.555	120191	0.12	224753	0.92	2.43	V	pentadecane
25	10.455	10.415	10.675	110335	0.11	13375	0.05	9.05	V	Hexadecane, 2,6,10,14-tetramethyl- (CAS)
26	10.583	10.555	10.855	211940	0.21	34090	0.14	3.24	V	Hexadecane (CAS)
27	10.715	10.675	10.945	289809	0.29	26248	0.11	6.07		2,6,10-Trimethyltridecane
28	10.909	10.855	11.085	669974	0.66	109504	0.45	2.64	V	Hexadecane, 2,6,10,14-tetramethyl- (CAS)
29	11.043	10.945	11.223	956060	0.94	214825	0.88	3.12	V	Hexadecane, 2,6,10,14-tetramethyl- (CAS)
30	11.179	11.085	11.345	1317983	1.30	356726	1.46	2.68	V	Tridecanol, 2-ethyl-3-methyl
31	11.297	11.223	11.485	1355153	1.34	439914	1.80	3.08	V	Eicosane
32	11.428	11.345	11.595	720237	0.71	471993	1.93	2.87	V	Hexadecane, 2,6,10,14-tetramethyl- (CAS)
33	11.550	11.485	11.715	846219	0.84	241262	0.99	2.99	V	Eicosane
34	11.628	11.595	11.915	572306	0.57	201912	0.83	4.19	V	Pentadecafluorooctanoic acid, heptadecyl e
35	11.857	11.715	12.065	3526534	3.49	142272	0.58	4.02	V	Phenol, 2,4-bis(1,1-dimethylethyl)- (CAS)
36	11.983	11.915	12.175	1416293	1.40	394732	3.95	3.66	V	1-Hexadecanol, 3,7,11,15-tetramethyl- (C/
37	12.131	12.065	12.315	1580411	1.56	380249	2.41	3.12	V	Hexacosyl acetyl ether
38	12.220	12.175	12.435	1465005	1.45	454098	1.86	3.48	V	1-Hexadecanol, 3,7,11,15-tetramethyl- (C/
39	12.380	12.315	12.545	948734	0.94	394542	2.44	2.46	V	1-Hexadecanol, 3,7,11,15-tetramethyl- (C/
40	12.493	12.435	12.705	160279	0.16	444267	1.82	2.14	V	Hexadecane, 2,6,10,14-tetramethyl- (CAS)
41	12.575	12.545	12.905	214262	0.21	33352	0.14	6.53	V	n-Pentadecanol
42	12.854	12.775	13.085	744213	0.74	81375	0.33	2.63	V	Hexadecane (CAS)
43	12.945	12.905	13.445	713726	0.71	298304	1.22	2.49	V	N-(3,3-Dimethylpropyl)-2,2,3-trimethylazi
44	13.347	13.185	13.635	437899	0.43	153686	0.63	4.64		TRICOSANE
45	13.544	13.445	13.845	563567	0.56	94217	0.22	8.08	V	Hexadecane, 2,6,10,14-tetramethyl- (CAS)
46	13.776	13.635	13.955	581796	0.57	80846	0.33	4.97	V	Hexadecane, 2,6,10,14-tetramethyl- (CAS)
47	13.893	13.845	14.045	679530	0.67	178139	0.73	3.27	V	Docosane (CAS)
48	13.994	13.955	14.185	1133506	1.12	251226	1.03	2.70	V	Docosane (CAS)
49	14.116	14.045	14.365	648270	0.64	213194	0.87	3.32	V	Dotriacontane (CAS)
50	14.216	14.185	14.545	528668	0.52	217584	0.89	2.98	V	Dotriacontane (CAS)
51	14.395	14.365	14.705	681592	0.67	187858	0.77	2.81	V	Tetratriacontane
52	14.557	14.545	14.905	932333	0.92	213426	0.87	3.19	V	1-Hexadecanesulfonyl chloride
53	14.646	14.705	15.025	1171512	1.16	129064	0.53	7.22	V	Dotriacontyl pentafluoropropionate
54	14.751	14.783	15.165	1393262	1.38	383276	1.56	3.07	V	Dotriacontyl pentafluoropropionate
55	14.851	14.895	15.275	1981020	1.96	522142	2.14	2.47	V	Dotriacontyl pentafluoropropionate
56	14.977	15.025	15.375	2653601	2.62	319807	2.13	3.81	V	Dotriacontyl pentafluoropropionate
57						739045	3.03	3.59	V	



Peak#	R.Time	I.Time	F.Time	Area	Area%	Height	Height%	A/H	Mark	Name
58	15.074	15.025	15.165	1658903	1.64	382592	2.39	2.85	V	Dotriacontyl pentafluoropropionate
59	15.220	15.165	15.375	1347112	1.33	434725	1.78	3.14	V	Dotriacontyl pentafluoropropionate
60	15.331	15.275	15.495	1934198	1.91	504717	2.07	3.83	V	Dotriacontyl pentafluoropropionate
61	15.602	15.495	15.645	545635	0.54	86523	0.35	6.31	V	ISOPROPYL TETRADECANOATE
62	15.805	15.645	15.845	696443	0.69	79266	0.32	8.79	V	Pentatriacontane (CAS)
63	15.905	15.845	15.935	432163	0.43	94783	0.39	4.56	V	Eicosyl pentafluoropropionate
64	16.014	15.935	16.065	626786	0.62	93565	0.38	6.70	V	2-Methylhexacosane
65	16.106	16.065	16.145	463773	0.46	111658	0.46	4.15	V	Hexadecane, 2,6,10,14-tetramethyl- (CAS)
66	16.223	16.145	16.275	1052126	1.04	159117	0.65	6.61	V	Tetratriacontane
67	16.317	16.275	16.355	913938	0.90	237622	0.97	3.85	V	Tetratriacontane (CAS)
68	16.395	16.355	16.445	1056542	1.04	243410	1.00	4.34	V	Hexadecane, 2,6,10,14-tetramethyl- (CAS)
69	16.489	16.445	16.533	1184446	1.17	261871	1.07	4.52	V	Hexadecane, 2,6,10,14-tetramethyl- (CAS)
70	16.604	16.533	16.655	1543045	1.52	249062	1.02	6.19	V	Docosane (CAS)
71	16.701	16.655	16.775	1817289	1.80	321072	1.31	5.66	V	Tetratriacontane (CAS)
72	16.805	16.775	16.865	1174610	1.16	217041	0.89	5.41	V	2,5-di-tert-Butyl-1,4-benzoquinone
73	16.911	16.865	16.945	883384	0.87	203728	0.83	4.34	V	1-Hexadecanesulfonyl chloride
74	17.007	16.945	17.055	1386149	1.37	292843	1.20	4.73	V	Dotriacontyl pentafluoropropionate
75	17.102	17.055	17.135	1382153	1.37	372351	1.52	3.71	V	Heptacosyl heptafluorobutyrate
76	17.237	17.135	17.335	5131565	5.07	571887	2.34	8.97	V	Hexadecanoic acid (CAS)
77	17.379	17.335	17.425	1817973	1.80	419029	1.72	4.34	V	Dotriacontyl pentafluoropropionate
78	17.465	17.425	17.495	1272402	1.26	355854	1.46	3.58	V	Carbonic acid, octadecyl vinyl ester
79	17.529	17.495	17.595	1488048	1.47	334700	1.37	4.45	V	Eicosyl acetyl ether
80	17.622	17.595	17.685	1153923	1.14	312708	1.28	3.69	V	Hexacosyl acetyl ether
81	17.767	17.685	17.805	1409559	1.39	265734	1.09	5.30	V	Dotriacontyl pentafluoropropionate
82	17.892	17.805	17.955	1576652	1.56	265190	1.09	5.95	V	Dotriacontyl pentafluoropropionate
83	18.045	17.955	18.075	959826	0.94	148968	0.61	6.37	V	16-Hentriacontanone
84	18.105	18.075	18.155	654241	0.65	148201	0.61	4.41	V	16-Hentriacontanone
85	18.228	18.155	18.295	1161724	1.15	150130	0.61	7.74	V	Eicosane (CAS)
86	18.394	18.295	18.395	853321	0.84	158650	0.65	5.40	V	4-Chlorobutyric acid, octadecyl ester
87	18.455	18.395	18.535	1430488	1.41	264964	1.08	5.40	V	HEXATRIACONTANE
88	18.555	18.535	18.585	403201	0.40	142943	0.59	2.82	V	Hexadecane, 2-methyl- (CAS)
89	18.637	18.585	18.675	769343	0.76	157043	0.64	4.90	V	TETRACOSANE, 2,6,10,15,19,23-HEXA
90	18.768	18.675	18.825	1480752	1.46	189664	0.78	7.81	V	Tetracosane, 2,6,10,15,19,23-hexamethyl-
91	18.855	18.825	18.885	619653	0.61	189618	0.78	3.27	V	Tetratetracontane
92	18.915	18.885	18.965	669927	0.66	159180	0.65	4.15	V	Tetratetracontane
93	19.022	18.965	19.065	831418	0.82	167044	0.68	4.98	V	Hexacosane
94	19.282	19.065	19.325	2686217	2.65	259629	1.06	10.35	V	Nonadecyl heptafluorobutyrate
95	19.385	19.325	19.415	1299125	1.28	348143	1.43	3.73	V	Dotriacontyl pentafluoropropionate
96	19.454	19.415	19.555	1743419	1.73	233177	0.95	7.49	V	Dotriacontyl pentafluoropropionate
97	19.622	19.555	19.695	1205437	1.19	264093	1.08	4.56	V	Dotriacontyl pentafluoropropionate
98	19.725	19.695	19.793	698916	0.69	171990	0.70	4.06	V	Hexacosyl acetyl ether
99	19.831	19.793	19.873	545044	0.54	240292	0.98	2.27	V	Dotriacontyl pentafluoropropionate
100	19.915	19.885	19.995	179767	0.18	76155	0.31	2.36	V	Dotriacontyl pentafluoropropionate
				101186618	100.00	24425050	100.00			

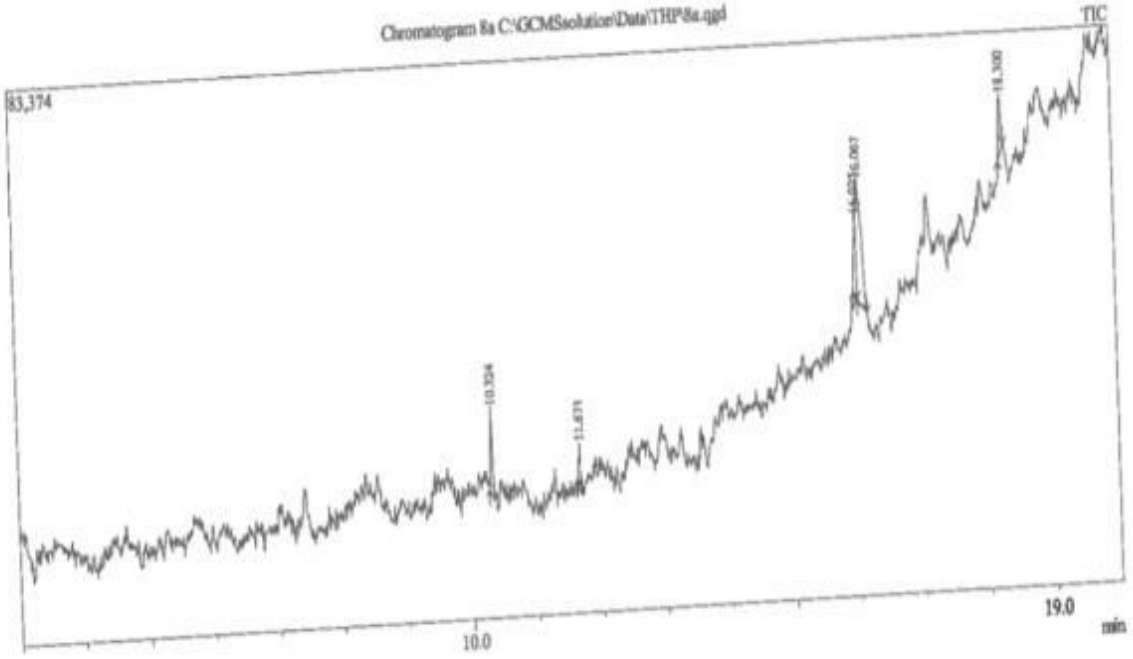
Appendix 24. Chromatogram after BSTc Treatment



Peak Report TIC

Peak#	R.Time	I.Time	F.Time	Area	Area%	Height	Height%	A/H	Mark	Name
1	3.880	3.850	3.955	16162	2.85	4681	2.37	3.45		1,3,5-Triazine-2,4-diamine, 6-hydroxy-N,
2	4.081	4.050	4.250	29563	5.22	4953	2.50	5.97		Silane, diphenyldi(hept-2-yloxy)-
3	4.584	4.510	4.600	10986	1.94	3049	1.54	3.60		Benzenamine, N-(6-bromo-n-hexanoyl)-4
4	8.420	8.375	8.450	10611	1.87	4634	2.34	2.29	V	1.beta.-iodo-2.alpha.-propyl-GA1 methyl
5	8.901	8.865	8.940	11072	1.95	5071	2.56	2.18		Tridecane (CAS)
6	10.062	10.030	10.085	7521	1.33	3860	1.95	1.95		N-OCTADECYL-B-4'-OH-PHENYL PR
7	10.200	10.185	10.245	6377	1.13	2350	1.19	2.71	V	(2S,4aR,7aS)-2-(t-butyl)-4a-(3'-chlorobut
8	10.317	10.295	10.345	15063	2.66	7660	3.87	1.97	V	Tetradecane (CAS)
9	11.663	11.640	11.700	5660	1.00	4298	2.17	1.32		Pentadecane (CAS)
10	11.955	11.895	12.025	73618	13.00	24789	12.53	2.97		PHENOL, 2,4-BIS(1,1-DIMETHYLETH
11	12.750	12.635	12.780	15539	2.74	3068	1.55	5.06		5-Chloro-9-oxo-9,10-dihydroacridine-4-c
12	12.939	12.915	12.990	11144	1.97	5523	2.79	2.02		Octadecane (CAS)
13	13.358	13.240	13.390	20007	3.53	3487	1.76	5.74		2-Hydroxy-dodecanoic acid, pyrrolidide
14	13.833	13.800	13.915	17881	3.16	4530	2.29	3.95		(S,S)-(-)-2-Methoxymethyl-1-(2-phenylth
15	14.157	14.115	14.180	9638	1.70	4417	2.23	2.18		Tricosane (CAS)
16	14.805	14.785	14.900	19682	3.47	5050	2.55	3.90		Leiocinol
17	15.003	14.900	15.020	23154	4.09	4638	2.34	4.99		Dimethylmalonic acid, monochloride, tet
18	15.062	15.020	15.140	17154	3.03	5751	2.91	2.98	V	1-Octanethiol (CAS)
19	15.285	15.275	15.360	6769	1.19	2743	1.39	2.47		Propionic acid, 3-iodo-, pentadecyl ester
20	16.361	16.345	16.460	16708	2.95	3617	1.83	4.62		DI-2-BENZOTHIAZOLE DISULFANE
21	16.694	16.655	16.745	65158	11.50	26890	13.59	2.42	V	Hexadecanoic acid, methyl ester (CAS)
22	16.795	16.745	16.810	12498	2.21	2518	1.27	4.96	V	trans-Manoconene-B
23	16.878	16.850	16.960	19577	3.46	4885	2.47	4.01		2-Propen-1-one, 3-(4-bromophenyl)-1-(2-
24	18.451	18.415	18.470	15998	2.82	9269	4.69	1.73		9,12-Octadecadienoic acid (Z,Z)-, methyl
25	18.499	18.470	18.560	43729	7.72	23544	11.90	1.86	V	9-Octadecenoic acid, methyl ester (CAS)
26	18.731	18.695	18.760	10906	1.93	5933	3.00	1.84		Methyl stearate
27	18.934	18.880	18.960	13625	2.41	5079	2.57	2.68		Cyclopropaneoctanoic acid, 2-[(2-pentyl
28	19.230	19.205	19.315	10564	1.86	3231	1.63	3.27		2-(4-METHOXY-3-METHYL-PHENYL
29	19.745	19.735	19.830	11757	2.08	2329	1.18	5.05		O,O'-Ethylenebis(p-chlorophenyldithioph
30	19.853	19.830	19.900	18326	3.24	5958	3.01	3.08	V	1-Isopropenyl-3-propenylcyclopentane

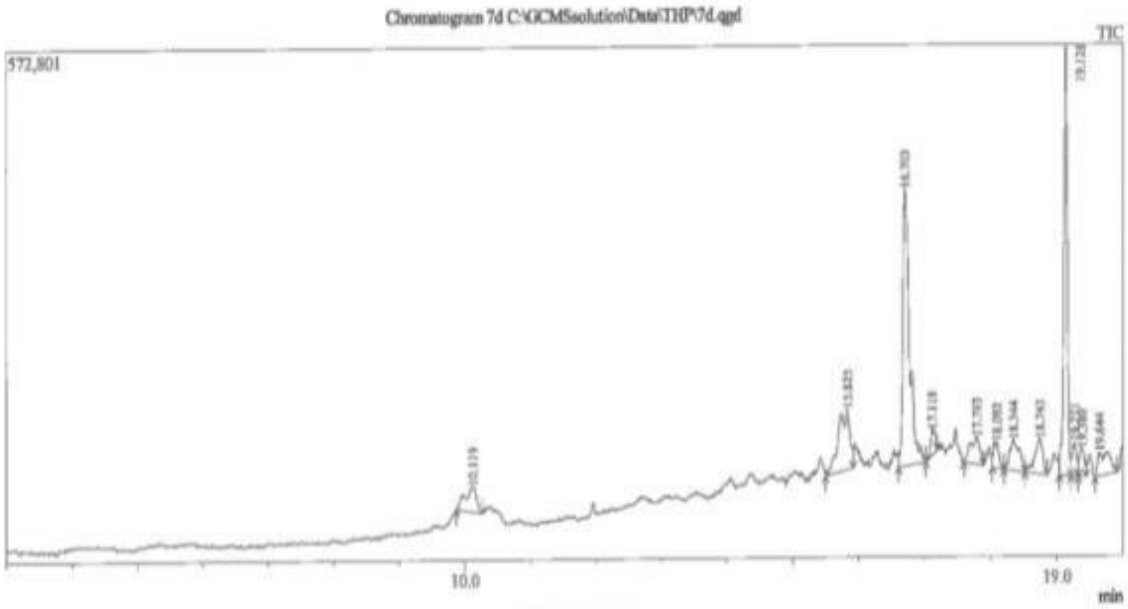
Appendix 25. Chromatogram after BVTc treatment



Peak Report TIC

Peak#	R.Time	L.Time	F.Time	Area	Area%	Height	Height%	A/H	Mark	Name
1	10.324	10.285	10.360	23266	12.53	13231	22.42	1.76		Tetradecane (CAS)
2	11.671	11.645	11.705	10325	5.56	6471	10.97	1.60		Hexadecane (CAS)
3	16.025	15.980	16.045	32555	17.53	12308	20.86	2.65		1,2-Benzenedicarboxylic acid, dioctyl est
4	16.067	16.045	16.190	97713	52.61	18261	30.95	5.35	V	1,2-Benzenedicarboxylic acid, bis(2-ethy
5	18.300	18.255	18.335	21862	11.77	8734	14.80	2.50		Dotriacontane (CAS)
				185721	100.00	59005	100.00			

Appendix 26. Chromatogram after BAUc treatment



Peak Report TIC

Peak#	R.Time	I.Time	F.Time	Area	Area%	Height	Height%	A/H	Mark	Name
1	10.119	9.865	10.265	345014	4.82	29035	2.53	11.88		PENTACOSANE
2	15.825	15.495	15.915	831139	11.62	67988	5.94	12.22	V	9-Octadecenamide, (Z)-
3	16.703	16.605	17.025	2177505	30.43	313377	27.36	6.95	SV	HEXATRIACONTANE
4	17.118	17.025	17.195	149899	2.09	28645	2.50	5.23		Heptacosyl heptafluorobutyrate
5	17.793	17.595	17.895	298366	4.17	31558	2.75	9.45		Eicosyl nonyl ether
6	18.092	18.025	18.195	158059	2.21	29584	2.58	5.34	V	Heptacosyl heptafluorobutyrate
7	18.344	18.195	18.515	349465	4.88	35233	3.08	9.92		2-Methylhexacosane
8	18.743	18.515	18.855	338009	4.72	39834	3.48	8.49	V	Nonadecyl heptafluorobutyrate
9	19.128	19.035	19.215	1836035	25.66	474487	41.42	3.87	V	HEXATRIACONTANE
10	19.271	19.215	19.325	175168	2.45	37017	3.23	4.73	V	Dotriacontyl pentafluoropropionate
11	19.380	19.325	19.445	169052	2.36	31834	2.78	5.31	V	14-.BETA.-H-PREGNA
12	19.644	19.575	19.915	327886	4.58	26937	2.35	12.17		14-.BETA.-H-PREGNA
				7155597	100.00	1145529	100.00			

Manuscripts Submitted

- Bioremediation of Acid Mine Drainage and Crude oil Contaminated Soils: *A Review*. Ifeanyi Anekwe and Yusuf Isa.
    - Submitted to Water, Air and Soil Pollution Journal. (under review)
- Evaluation of wastewater and bioventing system for the removal of sulfate from acid mine drainage contaminated soil. Ifeanyi Anekwe and Yusuf Isa
    - Submitted to Soil Sediment Contamination Journal. (under review)
- Application of Biostimulation and Bioventing System as Bioremediation Strategy for the treatment of Crude oil-contaminated soils. Ifeanyi Anekwe and Yusuf Isa
    - Submitted to Environmental Pollution Journal. (under review)
- Evaluation of the Application of Wastewater and Bioventing System for the Treatment of Crude oil Contaminated Soils. Ifeanyi Anekwe and Yusuf Isa
    - Submitted to the 18th International Conference on Science, Engineering, Technology and Waste Management (SETWM-20) (Conference paper)

NOTES