BIOREMEDIATION OF ARSENIC IN CONTAMINATED SOIL USING THE BACTERIUM, *ALCALIGENES FAECALIS*

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To God be the Glory, it is finished!

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Dear Ogunyemi ,

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Thank you for the above application which was presented to the Natural Sciences Ethics sub-Committee on 24th of May 2010. On behalf of the committee, I am pleased to give your project its final approval. Please note that the committee must be informed if any changes in the protocol need to be made at any stage.

I wish you all the very best with your project. The committee will be delighted to receive a copy of the final report.

Yours sincerely

Lucy Ghali

Dr Lucy Ghali
Chair of the Natural Sciences Ethics sub-Committee

DECLARATION

I **OGUNYEMI**, TOKUNBO confirm that this dissertation submitted for assessment is my own and is expressed in my own words. Any uses made within it of the works of other authors in any form (e.g. ideas, equations, figures, text, tables, programmes) are properly acknowledged at the point of their use. A full list of the references employed has been included.

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DR. Diane Purchase

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ABSTRACT

Arsenic contamination in soil is quite difficult in remediating due to the high financial implications and logistics involved. Attention therefore has now turned to the use of microbes and bioremediation as an efficient way of removing arsenic in the soil

This project was designed in order to determine the suitability and effectiveness of the bacterium, *Alcaligenes faecalis* in remediating arsenic from contaminated soil. 500 grams of soil were spiked with arsenic (III) solution to give a concentration of arsenic in soil of 1mg/g. Pure bacterial culture of *A.faecalis* was inoculated into the contaminated soil and arsenic concentrations were tested every week for four weeks.

Average arsenic concentrations in five grams of soil inoculated with the bacterium dropped from 3.404ppm to 0.3212ppm at the end of the four week period, which could also be expressed as a reduction from 680µg/g to 64µg/g in a gram of soil. This shows a 91% removal rate of arsenic (III) in the soil. Arsenic concentration in the control (containing only arsenic) however, maintained an average concentration of between 3.644ppm and 4.219ppm in the experiment.

Microbial counts in the soil containing arsenic did not show much difference in growth with a logged mean value of 8.183, while the microbial count in soil was 6.990, showing only a very slight change in growth pattern. The pH of the soil during their experiment ranged between 9.2 and 8.5 with the temperature constant at above 23^0 C

CHAPTER ONE: INTRODUCTION

1.1 Arsenic in the Environment

Remediation of contaminated land has long been a major problem for environmentalists the world over. Most often, the nature of the pollutant would most likely determine the method of remediation to be applied on the contaminated land. Pollutants may vary according by their physical and chemical composition as well as in specie and concentrations. Pollutants such as Arsenic, PAH and heavy metals to mention a few, are some of the most common types of pollutant in the environment today. Arsenic in particular has become one of the most problematic pollutants to deal with.

Arsenic occurs naturally in the environment in air, water and in soils. In the soils, it is a product of the weathering of the parent rock, especially sedimentary rock. It is mostly deposited in the environment through manmade activities (anthropogenic) such as through the mining and smelting of metals (Oehme, 1979). Other ways through which arsenic is deposited in the soil is through the introduction of arsenic into pesticides which are used in killing weevils on plants. Arsenic is also added to wood as a preservative and could enter the soil through this process. Gaseous emission fallout from the steel smelting industry is another immense contributor of arsenic in the soil.

Arsenic and its contamination in soil poses a lot of problems in the environment and to human's alike (Paul and Clark, 1996). This is because arsenic is unique in the case that it is not exactly a metal, being a group V element, it however mirrors characteristics of metals. Arsenic is infact a metalloid that shows the same properties as the element phosphorus. It has an atomic number of 33 and atomic mass of 74.92. It is notoriously poisonous, having been used as the poison of choice over the decades. It exhibits allotropy with its three most common forms being yellow, black and grey forms. The most common type however is the grey arsenic. The most common oxidation states of arsenic are the +3(arsenites), -3 (arsenides) and +5(arsenates). Like phosphorus, it also forms colourless, odourless crystalline oxides As_2O_3 and As_2O_5

Arsenic in soil possesses concentrations that vary widely, as they range from about 1 to 40 parts of arsenic to a million parts of soil (ppm) with an average level of 3–4 ppm. However, it has been discovered that soils within the areas of arsenic-rich geological deposits such as mining and smelting sites, or even agricultural areas which use or had used pesticides containing arsenic would contain much higher levels of arsenic (EPA, 2010).Concentrations of arsenic in

surface and groundwater range between 1 ppb to as high as 1,000 ppb in contaminated areas or where arsenic levels in soil are high (EPA, 2010).

Soil contaminated with arsenic is quite problematic to deal with owing to the elements ability to remain for long period of time in the soil. Soil naturally acts as a sort of sink for Arsenic compounds. This is further aggravated by their slow depletion through plant uptake as well as by leaching and soil erosion (Smith et al, 1998). Plants grown on arsenic contaminated land may also bio accumulate arsenic in their structure and if eaten by man could lead to a range of diseases as a result of arsenic poisoning. Animals also suffer the effects of arsenic poisoning as lives stock and cattle are most times poisoned by residual arsenic through dipping vats, filling places of spraying and dusting rigs as well as old orchards (Oehme, 1979.). Acute exposure to arsenic in most cases is as a result of arsenic containing pesticides, herbicides and dessicants (Oehme, 1979).

As mentioned earlier, probably the most difficult polluted land to remediate is land contaminated with heavy metals and metalloids such as arsenic. This is because heavy metals unlike some other form of pollutants cannot be destroyed. They can only be sequestered to a less toxic form. Remediating land contaminated with heavy metals has proved quite daunting owing to the reason stated above; however a particular method that has shown some level of success is through Bioremediation.

1.2 Bioremediation Process

“Bioremediation simply refers to the productive use of biodegradative processes to remove or detoxify pollutants that have found their way into the environment and threaten public health, usually as contaminants of soil, water or sediments” (Crawford and Crawford, 1996). The technique of bioremediation in relation to degradation of waste has been around for a long time, but only now has there been a deliberate attempt at harnessing its capabilities and applying them on a wider scale to solve more environmental pollution cases and effect restoration (Crawford and Crawford, 1996).
As earlier said, heavy metals are very much difficult to deal with in soil as they can only be detoxified and not totally destroyed as against the destruction of molecules obtained in the biodegradation of organic compounds. Evank and Dzombak (1997) put forward that the two most important approaches to bioremediation are immobilisation and mobilisation. The process of bioremediation however modifies, immobilizes or detoxifies the metal in order for remediation to take place (Alexander, 1999). It is important to note however that Bioremediation of most metals do not result in volatilisation (Alexander, 1999), but at times however would result in more or less complete remediation of the contaminated environment.

1.3 Bioremediation approach

Five general approaches to bioremediation were listed by Litchfield (1991) among which is the use of above ground bioreactors which are used to treat liquids, solids and soil either with suspended microorganisms, native microbial populations indigenous to the material being treated or problem specific designed genetically engineered microorganisms. Other bioremediation processes as described by Jerger and Exner (1994) include bioaugmentation, biofiltration, biostimulation, bioventing composting and land farming. The key process however, is the ultimate use of microorganisms in bioremediating contaminated land. An attempt shall be made to discuss these methods briefly before identifying the method to be used in the experiment.

Biosorption, which is one of the methods of bioremediation mentioned above simply refers to the passive uptake of metals by microbial cells. It is referred to as passive because no energy is used up while taking up the metals (Alexander, 1999). Biosorption makes use of a biomass which could range from bacteria, filamentous fungi, yeasts or algae.

Reductions of inorganic anions and cations have been well studied and documented over the years. Bautista and Alexander (1972) have also shown that soil bacteria and fungi are very much able to reduce ionic Arsenic, Vanadium, and Molybdenum. The principle behind the reduction of metals in soil is that reductions convert a higher oxidation state of the metal to a lower one, such as, Fe (III) to Fe (II), Mn (IV) to Mn (II) and As(V) to As (III) (Alexander, 1999). A key ingredient however for the reduction to occur is the availability of an energy source, either organic or inorganic in the absence of oxygen. The advantages of reduction of the metals are that in some metals it changes their water solubility and toxicity. An example is the case of arsenic which is reduced from As(V) to As(III) in which case the toxicity of arsenic rather increases.

Another method through which microorganisms transform metals is through the process of methylation. Microorganisms are able to methylate quite a number of elements forming their , mono-, di-, tri – and tetramethylated forms(Alexander, 1999). Some of the products however are highly toxic. In the case of arsenic, methylation forms methyl arsines which are a highly toxic form of the metal. Because of this, such a conversion is not an acceptable remediation strategy.

Studies by Osborne and Ehlrich (1976) have however shown that some bacterial genera are capable of oxidising Arsenic (III) to Arsenic (V). Williams and Silver (1984) went further to show that Arsenate is precipitated more readily out of the

water by using ferric ions. It is also important to note that the oxidation process could serve as an energy source for lithotrophic growth as against the metal acting as an electron acceptor in the case of reduction.

The presence of arsenic (III) and (V) and their distribution is a factor of their redox potential and pH conditions (Tallman and Shaikh, 1980). It has been discovered that under oxidising conditions Arsenic V is the specie most readily found to occur in surface waters. In contrast, under reducing conditions as is present in ground waters, Arsenic (III) is more thermodynamically stable and hence present as arsenious acid (Cullen and Raimer, 1989). Owing to the fact that Arsenic III may interact with other solid surfaces, it therefore becomes difficult to remove using the standard and conventional methods such as adsorption and precipitation. A lot of scientific works have been carried out on the best practicable method for the removal of arsenic in contaminated water and soil and they range from ion exchange, filtration, lime softening to reverse osmosis (Jekel, 1994). These unfortunately have yielded poor results as the methods out lined are more suitable for the removal of arsenic V. Quite a large number of bacteria have been used as agents of bioremediation of arsenic in soil and they cut across from aerobic to anaerobic and gram positive and negative bacteria. An example of such an organism is the bacterium, *Alcaligenes faecalis.*

1.4 *Alcaligenes Faecalis*

Alcaligenes faecalis is a gram negative, rod shaped motile obligate bacteria cell. It belongs to the kingdom bacteria and is sometimes found in the gut of patients suffering from cystic fibrosis. It derives its name *faecalis* due to the fact that it is normally found in the faeces. It was initially Petruschky (1896) that applied the name *Bacillus faecalis alcaligenes* to a peritrichous, non spore forming rod of intestinal origin, producing no acid in sugar broths and causing alkalinity in milk. It was however renamed *Alcaligenis faecalis* by Castellani and Chalmers (1919). *Alcaligenes faecalis* has long been known for its ability to oxidise As (III) to As (V).

This project shall examine the ability of *Alacaligenes faecalis* to bioremediate soil contaminated with Arsenic III

1.5 AIMS AND OBJECTIVES OF THE PROJECT

- To determine and assess the effectiveness of *Alcaligenes faecalis* in remediating arsenic from soil.
- To measure the removal of arsenic from soil by the bacterium *Alcaligenes faecalis*
- Investigate the impact of Arsenic on the growth pattern of the organism
- Evaluate the use of bioremediation in clean up of arsenic contaminated soil

Objectives

- Determine the concentration of Arsenic in soil before and after the experiment to estimate any possible changes in concentration
- Determine the number of organisms (Colony Forming Units) before and after exposure to Arsenic.
- Compare results from previous literature with results obtained in this project.

CHAPTER 2: LITERATURE REVIEW

A lot of research has been carried out on the bioremediation of heavy metals using soil microorganisms. This biological method of remediation is quite new and differs from the normal physical and chemical methods of remediation. Bioremediation could be used solely as a treatment method or with the inclusion of other methods such as filtration and sorption. However, it obeys the basic principle that results of bioremediation are achieved by either Oxidation or Reduction of the contaminant (Katsoyouannis and Zouboulis, 2002). Microorganisms are quite unique in the fact that they are able to accumulate heavy metals and radionuclide's in the environment in which they find themselves (Gadd and Griffiths, 1978). The level or amount of metals accumulated could either be large or moderate and would involve a range of mechanisms such as adsorption, precipitation as well as transport (Gadd and Griffiths, 1978).

Arsenic has direct impact on the microorganism in the soil and this is dependant on the amount or concentration of the metalloid. Its impact however varies from organism to organism. Most important to note is that the toxicity of arsenic is directly related to its valence state, with Arsenic (III) being at most a hundred times more toxic than arsenic (V)(Cervantes, 1994). The high toxicity of arsenic could be explained by its binding to protein sulfhydrl groups (Gebel, 2000). Because of this ability to bind to protein groups, Arsenic (III) inhibits enzyme reaction which may need free sulfhydryl groups and would lead to death and membrane

2.1 Microbial Metabolism of Arsenic

The role Arsenic plays at times is that of an electron acceptor or donor as well as being part of the transport chain in many forms of bacteria (Tsai et al., 2009). It is important to note however that because of the extreme toxicity of arsenic, specific uptake transporters have not evolved (Stolz et al. 2006). Adeniyi (2004) showed that microorganisms are able to transform contaminants using their metabolic systems. Investigations have revealed that both species of arsenic,(III) and (V) are taken up using the glycerol and phosphate transporter, this is attributed to their structure and chemical similarities. An example of such an uptake occurs in *E.coli* where two phosphate transporters are used for Arsenic (V) uptake with the dominant uptake pathway being Pst (Rosen and Lu, 2009). The glycerol transporter GIpf takes up the uncharged Arsenic III (Sander et al., 1997).

Most bacteria use a similar arsenic resistance mechanism which is based on the ars encoded on the chromosome or plasmid of the bacteria (Xu et al. 1998) wherever encoded, the fact remains the important component namely, a reductase enzyme (ArsC) is used in the reduction of arsenic (V) to arsenic (III). More interesting is the discovery of more ars genes which point to the suggestion of complex regulations and parallel evolution (Butcher et al. 2000). The basics or source of the inherent ability to reduce arsenic varies among many prokaryotes. *E. coli* uses GSH and Glutarexodin (Shi et al. 1999) while *Staphylococcus aerus* uses thiorexodin (Ji et al. 1992). The reduction step involves arsenic binding to a recognition domain comprising Arg residues, which eventually results in a disulphide bond between the cystein residues on ArsC and the reducing equivalents(Tsai et al. 2008). The eventual reduction of the disulphide bond results in the reduction of arsenic (V) to arsenic (III) (Silver and Phung, 2005). The components ArsR and ArsD act primarily as transcription repressor regulates the upper limit for operon activity (Rosen, 2002).

Both ArsR and ArsD have very high affinity for Arsenic III and as earlier described bind through their cysteine residues, an action that results in altered DNA binding for transcriptional activation (Rosen, 1999)

2.2 Metal Transformation

Metal transformation, a method by which microorganisms remediate heavy metal contaminated land can be divided into two major groups (Paul and Clark, 1996) namely reduction and oxidation. The oxidation of arsenic is very important in the process of arsenic removal as arsenic (V) has been found to be less soluble and can be recovered by various physical and chemical methods.(Liest et al. 2000). However oxidation of arsenic by microorganisms are carried out through the aid of the enzyme arsenic III oxidase. The enzyme arsenic III oxidase is a member of the DMSO reductase (Ellis et al. 2001). Apart from the intracellular reduction of arsenic V using the enzyme arsenate reductase, arsenate reduction could also be part of the anaerobic arsenate respiration in bacteria as seen in S*hewanella sp.* (Tsai et al., 2009). In this specie, the arsenate acts as the terminal electron acceptor.

2.3 Arsenic Methylation / Demethylation

Even though microorganisms can transform heavy metal and metalloid species by either reduction or oxidation, it is also possible for them to achieve this transformation by either methylation or demethylation. The microbial transformation of arsenic has been associated with reduced toxicity and is quite relevant to waste water treatment (Williams and Silver, 1984). The process of

methylation was originally observed as a detoxification process; however recent studies have since revealed that not all methylated arsenic products are less toxic (Bentley and Chasteen, 2002) The mode of arsine and methyl arsenicals generation is Arsenic V reduction and the addition of oxidative groups from different sources as methyl cobalamine in many bacteria forms (Dombrowski et al., 2005). Pongratz (1998) conducted studies on the speciation of arsenic in environmental samples of contaminated soil and discovered that arsenic (III) is a precursor to the formation of methylated arsenic species. Turpeinien et al.(1999) suggested that it is possible for arsenic from the soil to be biomethylated into monomethylarsenic acid and dimethylarsinic acid. It is important to note that methylated forms of arsenic are volatile(Rodriguez, 1999) and are easily released into the environment (Burau and Gao, 1997) where oxidation may convert them back to oxidised form of Arsenic V. Not enough literature is available on the pathways of the demethylation process however, although mono-methyl and di-methyl arsenic demethylation have been investigated as well as the use of methylated arsenicals as a carbon source is proven to be quite possible (Maki et al. 2004).

2.4 Arsenic Bioaccumulation

Eukaryotes such as fungi, yeast and algae are also active participants in the process of bioremediation of arsenic. The yeast, *Saccharomyces cerevisae* uptakes arsenic through three different pathways. Arsenic (V) because of the similarity to phosphate (Nidhubhghaill and Sadler, 1991) is taken up through a phosphate transporter Ph087p with two other transport systems for the uptake of Arsenic (III) also identified. Edvantoro (2004) studied the microbial formation of volatile arsenic in cattle dip soils contaminated with Arsenic. The study sought to test if the addition of nutrients in the form of manure and bioaugumentation from methylating fungi (*Penicillum sp* and *Ulocladium sp*) could increase the level of arsenic volatilisation in cattle dip soils that contained mixed arsenic (Edvantoro et al. 2004). Results indicated that an increase in the amount of manure introduced in the soil corresponded to a likewise decrease in arsenic in the contaminated land. The higher the manure levels the greater the loss of arsenic from the contaminated land. It is important to note that a manure supplement of 30% at 75% capacity showed the greatest reduction of arsenic in contaminated soil containing 1390mg/kg with the rate of arsenic loss and microbial respiration correlated with the levels of manure amendment. The experiment was hence able to prove the use of fungi in reducing the amount of arsenic in the soil.

Bioaccumulation of Arsenic by Fungi was investigated by Adeyemi (2009) in an attempt to investigate the possibility of bioaccumulation in fungi using three filamentous fungi such as *Serpulus humantioides, Aspergillus niger* and *Trametes versicolo.* The experiment was conducted to look into the organisms ability to accumulate and even probably solubilise arsenic from an agar

environment which contains non buffered mineral salts media spiked with 0.2, 0.4,0.6, 0.8 arsenopyrite. Factors recorded during the experiment included dry weights, growth rate, pH of media, arsenic accumulation and oxalate production by the fungi. Results from the experiment showed that there was no significant arsenopyrite underneath any of the growing fungal colonies or in their respective agar plates. The growth changes of the fungi on the agar plates spiked with different concentrations of arsenopyrite showed no specific pattern although growth was nevertheless stimulated in all fungi except *Aspergillus niger* having a concentration of 0.8 % (w/v). Also observed was the fact that the dry weight of all fungi did not show any particular pattern just as in the case of growth changes. However, the order of dry weights in each fungi were *A.niger>S. himantiodes>T. versicolo*. Overall the most effective fungi was the *T. versicolo* while the least effective was *Aspergillus niger.* Finally he concluded by suggesting that the amount of arsenic accumulated in the biomass of the fungi pointed towards a role for fungi in the bioremediation of arsenic contaminated soil.

The removal of Arsenic in soils has been studied extensively. Arsenic removal from contaminated soil through the process of anaerobic bioremediation was investigated by Chatain et al. (2005) and Ignatiadis and Battaglia-Brunet (2005), and they showed that mobilization of arsenic was obtainable through the stimulation of indigenous microorganism using a carbon and energy source under anaerobic conditions.
Katerina et al (2007), investigated the effectiveness of bioremediation over chemical treatment methods through the design of an experiment to measure the amount of arsenic removed in contaminated soil through chemical and biological methods using the microorganism *Desulfuromonas palmitatis.* The results indicated that the removal of arsenic from soil increased from 35% as recorded through chemical methods up to 90% when using the organism.

Santini et al (2000) discussed the growth of *Pseudomonas arsenitoxidans NT-26* chemoautotrophically using Arsenic III as the electron donor and Oxygen as the electron acceptor and carbon dioxide as the electron source. He discovered that the *pseudomonas* strain of bacteria gained energy necessary for growth from the arsenic (III). A similar result was also observed by Rhine et al. (2006) who observed anaerobic oxidation of arsenic III in contaminated soil using inorganic carbon as the carbon source and nitrate as the electron acceptor. Mophatra et al. (2008) discovered that it was possible for bacteria to volatilise arsenic while using cow dung as a carbon source. Results from both earlier experiments served to show that the heterotrophic oxidation of Arsenic (III) to the less toxic Arsenic (V) serves as detoxification reaction carried out by the bacterium cell (Wang and Zhao, 2009). Anderson et al. (1992) went further by stating from his experiments that bacteria overcame the toxicity of arsenic (III) through the reduction of arsenicals.

However, microbial growth isn't recorded all the time as during the oxidation of arsenic (III) to (V) by the organism as presented by Gihring et al. (2001), who discovered that even though the organisms *Thermus aquaticus* and *Thermus thermophilus* were indeed able to oxidise Arsenic (III) to Arsenic (V), they were however incapable of growing using Arsenic (III) as the sole energy source.

Various other forms of bacteria have also been used in the bioremediation of arsenic. Of particular interest is a study carried out by Takeuchi (2007) to investigate the effectiveness of marine and non marine bacteria on the removal of arsenic. The marine organisms *Vibro alginolyticus, Alteromonas macleodii and marinomonas communis* were grown in marine medium while the non marine bacteria, *Methytosinus trichosporium, eschericha coli, pseudomonas aeroginosa, bacillus subtilis and rhodcoccus* were grown in non marine medium at 30 °C. Arsenic stock solutions of 50,000 mg/l were made and diluted into concentrations of 0, 5, 50 and 250mg/l. *Marinomonas communis* showed the second highest arsenic resistance and was capable of removal of removing arsenic from the culture medium.

Yamamura et al. (2005) worked on isolating a novel dissimilatory arsenate reducing bacterium (DARB), *Bacillus sp.* SF-1 suspected to be much more capable of reducing arsenic in relation to other DARBS. The purpose for the experiment was to determine if the isolated *bacillus sp* SF-1 could efficiently extract Arsenic from Arsenic V containing soils. The set up for the experiment included spiking two different types of soil collected from forest and a paddy field with 1.5ml, 1Molar concentration of arsenic. The arsenic was added to 100g of soil in order to simulate a contaminated soil scenario. One gram of the soil sample was then transferred into 25ml serum bottles. The soils were dried in an autoclave and then 20ml of LM was added to the solution and stoppered. An aliquot of aerobically grown cell suspension was then inoculated into each bottle as arsenic reducing activity can be readily induced under anaerobic conditions. The total arsenic in the filtered samples was then measured with an atomic adsorption spectrophotometer. During extraction it was noticed that the bacterium *Bacillus* SF-1 had reduced arsenic (V) to the less adsorptive Arsenic III which was consequently extracted in the liquid phase.

Research on the removal of relatively high concentrations of arsenic has also been studied. In Japan, according to Soda et al. (2009), an experiment also using a dissmilatory arsente reducing bacterium, *Bacillus selenatarsenatis* was carried out via the use of a slurry bioreactor in treating high concentrations of arsenic contaminated soil. The extraction processes involved the use of synthetic arsenic contaminated soils with concentrations of 1100mg/kg from paddy fields and forests and actual contaminated soils with concentration levels of 2200mg /kg and 220mg/kg respectively. As earlier mentioned soil samples were

collected from paddy rice fields and forest and were dried at 60 °C, sieved through a 2mm sieve, then 1.5ml of 1M arsenic was added in to 100 grams of soil to give final arsenic in soil concentration of about 1100mg /kg. Actual contaminated soils were then obtained from different sites in Japan with concentrations of 2200mg/kg and 220mg/kg of arsenic. For the extraction experiment, a gram of the contaminated soil was then added into a 50ml bottle autoclaved for an hour and to it were added 20 ml of basal medium, soil were sterilised to exclude the activity of other microorganisms. Bacterial cells were then cultured in a basal salt medium containing 10 mg/l of glucose and left to stand for eight hours. A tier of the microbial culture was then inoculated into the bottles containing the contaminated soils. Upon analysis, there is seen to be a drop in arsenic concentrations form the actual contaminated soil from 2200 mg/kg to 1500mg/kg, also noticeable was a fall in the Colony forming units of *Bacillus selenatarsenatis* from an initial value of 10^7 CFU to 10^5 after 168hours.

Maheswari and Murugesan (2009), investigated the remediation of Arsenic in contaminated soil using the fungus *Aspergillus nidulans* that was isolated from an Arsenic contaminated site. Soil samples were collected and a prepared solution of arsenic was used in spiking the soil samples. Isolated fungus, *Aspergillus nidulans* was cultured and introduced into the soil and incubated at room temperature for five days, with dextrose being used as a carbon source. Arsenic tolerance was tested by growing *Aspegillus nidulans* SD broth with different concentrations of As(III)ion ranging from 20 – 500 mgL. *Aspergillus nidulans* was seen to show tolerance to the maximum concentration of 500mgL with the conclusion that *Aspergillus nidulans* is a suitable biosorbent for arsenic in soil.

2.5 Arsenic Oxidation

Studies have shown that the treatment of sewage containing high concentration of arsenic with oxidase producing bacteria, capable of oxidising As^{3+} to As^{5+} can improve arsenic removal methods owing to the fact that arsenate is more readily precipitated from waste water by Fe^{3+} than in arsenite . (Gadd and Griffiths, 1978). Santini et al.(2000) carried out further studies on the oxidation of arsenic in his scientific research of isolating a arsenite oxiding bacterium from Gold mine sites and discovered that as part of the organisms oxidation capability, a relationship existed between arsenic mobility and pH. Other organisms found to have the capability to oxidise Arsenic (III) to (V) includes *Psuedomonas* strain, which can derive metabolic energy from the oxidation of Arsenic (III)(Iilyatedinov and Abdrashitova,1981). Others include *Thermus aquaticus* and *Thermos thermophillus* were also found to oxidise Arsenic (III) to Arsenic (V), although they weren't able to synthesize growth with Arsenic (III) as the sole energy source thereby inferring that the ecological role of AS (III)

oxidation was for the detoxification of arsenic (Gihring et al. 2001). Masschelyn et al. (1991) conducted studies looking into the oxidation state of arsenic III as it affects solubility and bioavailability.

2.6 Arsenic Reduction

In the same vein, Arsenic (V) could also be reduced by dissimilatory reduction. Alexander (1977) and Woolson (1977) discussed that arsenic is lost in soil through the process of Arsenic (V) reduction. This occurred when microorganisms use Arsenic (V) as a terminal electron acceptor for anaerobic respiration. There are a number of bacterium which behave this way and they include *Sulfurospirillum barnesii*, *S. arsenophilum, Desulfotomaculum auripigmentum*, *Bacillus arsenicoselenatis*,*B. selenitireducens, Crysiogenes arsenatis, Sphingomonas* spp., *Pseudomonas* spp. and *Wolinella* spp. (Ahmann et al. 1994; Lovely and Coates, 1997; Newman et al. 1998; Stolz and Oreland, 1999; Oremland et al. 2000; Macur et al. 2001). Macur et al. (2001) conducted studies and reported that the microbial reduction of As (V) to As (III) enhanced from mine tailings. He discovered that *Sphingomonas, Caulobacter, Rhizobium* and *Pseudomonas* genera were able to rapidly reduce As(V) under anoxic conditions. The rate of reductions varied from 0.0004 to 0.401mM/d. upon the application of lime, the mean steady concentration of arsenic mobilised from non sterile treatment increased to 300mM after three days, while without any lime and sterilised was 20nM. Upon supply of organic carbon, the arsenic mobilised increased to 450nM. Drewniak et al. (2008) went further by isolating 22 arsenic hypotolerant cultivable bacterial strains from the walls of Gertruda Adit in the Zlotty Stok goldmine in southwest Poland containing arsenic biofilms. The range of tolerance for the bacteria isolated ranged from 5- 15mM for As (III) and 25 -500mM for As (V). His study further suggested that the detoxification process which is based on the reduction of arsenic V may be significant in arsenic mobilisation.

2.7 Arsenic Bioleaching

The recovery/remediation of arsenic from soil through the process of bioleaching while using microorganisms is currently an accepted biotechnology procedure (Wang and Zhao, 2009). The whole process relies on the capacity of micro organism to transform solid compounds into soluble and extractable elements which could then be recovered (Wang and Zhao, 2009). Deng and Liao (2002) studied the use of acidophilic iron and sulphur oxidising bacteria in leaching high concentrations of arsenic from the soil. The bioleaching of arsenic is most times as a result of reduction of the arsenic V to arsenic III which is much more mobile. The rate of arsenic removal from contaminated soils through bioleaching could increase through conditions that favour dissimilatory Iron III that result in dissolution of sorbing phases (Cummings et al. 1999).

Advancement on the above described process is biostimulation enhanced bioleaching. Seidal et al. (2002) postulated that elemental sulphur could be added as an energy substrate as well as an acid source in aerobic conditions to stimulate arsenic bioleaching from soils as well as from sediments. In particular Seidal et al. (2002) studied the *use of Thiobacillus sp* in the bioleaching of arsenic with concentrations of 753mg/kg from a highly polluted lake sediment under aerobic and anaerobic conditions. Without elemental sulphur and under aerobic conditions, arsenic solubility ranged between 0.6 and 3.5mg/kg. However, stimulating aerobic bioleaching using elemental sulphur there was an increase of soluble arsenic to 80 %(660mg/kg) in the speciation of As(III) and As(V). However, in anaerobic tests without the addition of sulphur, it was noticed that the arsenic solubility increased temporarily. Approximately about 9% of the total arsenic is soluble while arsenic III became the dominant specie (20mg/kg). However, in the late leaching state, total soluble arsenic decreased, with arsenic (V) becoming dominant with a concentration of 3.9mg/ kg. Arsenic leaching inhibition is therefore interpreted by fixation as insoluble sulfides, which proposes that immobilisation was propelled by disimillatory sulphur reduction.

Furthermore, carbon and nutrients sources addition are used to stimulate bacteria growth which eventually promotes arsenic bioleaching from soil. This notion was also supported by Mclean et al (2006), who showed that the addition of carbon and energy source could be used to increase arsenic leaching from solid phase. Kohler et al.(2001) discovered that arsenic ions and soluble organoarsenic compounds from

Contaminated increased upon the addition of nutrients and energy sources and were released by autochthonic bacteria as well as a mixture of pure cultures. Chatain et al.(2005) studied arsenic mobilisation under anaerobic conditions and discovered that anaerobic arsenic bioleaching from soils through indigenous bacteria was increased an amazing 28 fold through the addition of carbon sources as well as stating that the addition of mineral nutrients could accelerate respiratory reduction and decrease of redox potential.

2.8 Bioremediation Using *Alcaligenes faecalis*

The genus of bacteria, *Alcaligenes faecalis*, has been used in numerous studies on bioremediation. The bacterium is a gram positive rod shaped obligate aerobes, which are commonly found in soil. Originally named because it was most found in faeces, the bacterium is non –nitrate reducing oxidase positive organism with no pigmentation and grows optimally at a temperature of around 37 °C. The organism possesses a variety of distinct characteristics and for that purpose has been employed by scientists the world over in remediating various pollutants. Jiang et al. (2007) studied the biodegradation of phenol by *Alcaligenes faecalis* with interesting results. They exposed the organisms to a

wide range of phenol concentrations ranging from 0 to 1600 mg/l of phenol and observed its growth as well the concentration of phenol. They collected activated sludge from a municipal gas works in china and enriched for a ten week period with phenol as the only carbon source. The phenol concentration here ranged from 300 to 2000 mg/l. the samples of dilute activated sludge were then inoculated into shaking flasks with LB medium , with the late cultures plated into agar plates. A dominant culture type was then purified on completion of several transfers to the plate again. Upon activation of the strains, the cells were then harvested and used as innoculum. A volume of 2.5ml of the culture was then transferred into 50ml of the mineral medium which contained the various phenol concentrations from 0-200mg/l at intervals of 50mg/l as well as the other media containing concentrations of 200 -1800 mg/l, with intervals of 200mg/l. samples were then taken regularly for biomass and phenol concentration .

The results showed that the degradation of phenol was proportional to the innocula strength, with innocula containing large amounts of microorganism being able to degrade far greater quantities of phenol, up to 1600 mg/l in just 76hour.

Further researches have been carried out to investigate the suitability of the bacteria *Alcaligenes faecalis* to bioremediate arsenic contaminated soil. Phillips and Taylor (1976) tested the resistance of *Alacligenes faecalis* to arsenic solution in soil and discovered it was resistant to the concentration of 0.01 sodium arsenite. They discovered that the bacterium strain of *Alcaligenes faecalis* was capable of oxidising Arsenite to Arsenate. Results from their studies showed stochiometric conversion of arsenite to arsenate with evidence that the conversion was enzymatic with no stable arsylenated intermediate formed in the cell. No growth of *Alcaligenes* occurred during the experiment; however a constant rate 3.1 μ mol of arsenite oxidised/h per mg of protein was measured by an oxygen probe.

2.9 Bioremediation the future

The removal of Arsenic by microorganism has come a long way in the bioremediation process. Bioremediation has been proven to occur consistently either through the process of indigenous microorganisms in the contaminated media or through their direct and intentional introduction. Bioremediation of arsenic in the future lies in the development of engineered microbes. Engineered microbes are the next stage in the use of low cost green technology for removing arsenic (Singh et al. 2004).

Though a lot of research has gone into engineering microbes for the removal of metals such as cadmium and mercury through the expression of metal binding peptides like the Human MT (Pazirandeh,1995) and synthetic peptides, the

apparent low affinity of these peptides for arsenic make them ineffective for arsenic remediation. The development of a genetically modified microbe should be able to achieve two important things which are the ability to modify the naturally existing defence mechanisms and the development of hybrid pathways into one easily manipulated microorganism. An example of the initial attempts at engineering arsenic accumulation was displayed in plants. Arsc (arsenate reductase) a bacteria enzyme, and y-ECS(GSH Synthase) were expressed in *A. thaliana,* which later resulted in the accumulation of Arsenic V as GSH-Arsenic complexes(Singh et al. 2004). The results from that experiment has thrown open the possibility of engineering metabolism and eventual pathways for arsenic sequestration.

Other demonstrated cases include, the PC synthase of *A.haliana* expressed from E. coli (Pazirandeh,1995). It was seen that the engineered strain produced PC when exposed to different forms of arsenic, which then eventually led to the accumulation of arsenic (Tsai et al. 2008). Although it was noted that the level of GSH became limiting for the higher level of PC production and arsenic accumulation.

Also, the use of resting cells as an affinity biosorbent for arsenic removal has been investigated. The expression of AtPCS in *Saccharomyces cerevisae* which normally has a higher level of GSH, the engineered yeast strain accumulated high levels of arsenic and was efficient in arsenic removal in resting cell cultures (Singh et al. 2004).

3.0 METHODOLOGY

3.1 Soil Sample Collection and Preparation

Soil samples were collected from a Middlesex university garden. 10cm of Top Soil was collected and taken to the laboratory. The soil type was medium coarse sandy clay. Large particles of soil were removed to avoid clods and rocks which could be responsible for hot spots. The soil was passed through a 2mm pore size sieve and placed in a large clean container. 500 grams of the soil were then weighed using a weighing balance and kept in a large container before sterilizing.

3.2 Soil Sterilising and containers

Eight (8) plastic containers (Fischer pharmaceuticals) and their caps were sterilised in autoclave at 120^0C, 15 psi for an hour. The soil samples were then placed in the containers and sterilised in the autoclave at a temperature of 120^0 C in order to inactivate microbial action possibly in the soil.

3.3 Experiment Set Up

The set up for the experiment consisted of a total number of four pots with one set of replicates making a total number of eight pots. Pot A contained soil with water and was used as a blank, while Pot B contained Arsenic and the bacterium *A.faecalis,* Pot C contained only arsenic and soil while pot D contained only the bacterium *A.faecalis*. Each pot held 500 grams of soil.

3.4 Arsenic Stock Solutions

Arsenic stock solution was prepared by weighing out 1.320 gram of analytical grade of arsenic trioxide (Fischer scientific) in 50% Hydrochloric acid and then diluted to 1 litre. Arsenic working standard solutions were prepared from the stock when needed. Concentrations of arsenic III solution were prepared for the calibration of the ICP machine using the concentrations 0.5ppm, 1.0ppm, 2.0ppm, 5.0ppm, 10ppm for calibration. All reagents used were of analytical grade (AAS , Arsenic Solution)

3.5 Spiking of Samples

Three soil samples were spiked with the solution of arsenic III. The control, containing only arsenic and two soil samples with the organism *A. Feacalis*. The procedure for spiking the soil was a slight variation from method employed in Sill and Hindman (1974) as 200 gram of soil was carefully taken from each soil pot and poured into 500ml beakers which had

previously been acid cleaned. Arsenic (III) solution was then carefully poured into the soil and continuously mixed to form slurry. The slurry was then stirred for another five to ten minutes to properly evenly distribute the spiking solution. The soil was then returned back into the pots and the process repeated till all pots containing 500 grams of soil had been spiked resulting in the a final arsenic concentration in soil of 1 mg/g.

100ml of water was added to the control containing no arsenic and organism. The pots were then covered with sterile cotton plugs and left on a clean bench at a temperature of between 24 – 26^0C for four weeks.

3.6 Microorganism (*A.faecalis)* and Growth

The Microorganism *A.faecalis* was sourced from NCIMB limited and was cultured in 50 ml of Nutrient broth (peptone 5g/l, Meat extract 1g/l, yeast extract -2g/l, Sodium chloride -15g/l) for 48 hours at room temperature of 25^0 C

3.7 Serial Dilutions, Incubation and Innoculation.

The organism, *A.faecalis* was cultured for 48 hours in nutrient agar and then a 10^9 serial dilution was done. The spread plate plating method was used in transferring the organism to the solid agar (TSA) medium in sterilised Petri dishes. The Petri dishes were then placed in an incubator at 25^0C for two days. The samples were removed from the incubator and enumerated using a colony counter.

The inoculation of the bacterium into soil was done using 10^5 cfu/ml in an undefined nutrient broth containing 2% glucose. 5ml of the bacterial solution was inoculated into three soil pots and incubated for 48 hours at 25^oC. The soil pots were left to stand for two days and were then spiked with arsenic.

1 gram of soil sample was removed and serial dilution was done and plated out for the estimation of the weekly microbial count.

3.8 Arsenic Determination

Arsenic concentration was determined through the process of acid digestion. Yamamura et al. (2005). Five (5) grams of soil were placed in a filter paper and weighed on the weighing balance before being transferred into the oven digester corvettes. 10 ml of 70% analytical grade of Nitric acid (HNO_3) was added to the soil and was shaken for 2 minutes before being placed in the microwave digester (CEM-Microwave Digester) and the digestion method of EPA – 3051 -8 was selected. The soil containing Nitric acid was heated to a temperature of 175^0 C for five minutes with the process repeated again.

The soil was allowed to cool and filtered through GF filter paper. The solution was then made up with distilled water to the 100ml and passed into the Inductive Coupled Plasma Optical Emission Spectrometer(ICP-OES)for analysis.

3.9 Inductive Coupled Plasma

The flush pump rate was set to 100 (0-125rpm), Analysis pump rate 50(0-125 rpm), R.F power 1150 (750 -1350w), Nebuliser Gas flow 0.70 (0.0-1.5L/min), coolant gas flow 12(10-20L/min) and Auxillary gas flow 0.5 (0-2L/min)

4.0 RESULTS AND ANALYSIS

4.1 Arsenic Concentration

Concentration of Arsenic in the control (soil only) and soil containing *A.feacalis* was investigated using the ICP-OES. Concentration of Arsenic in five grams of soils was digested and run through the ICP-OES. Four samples of soil were taken from both the control and soil containing the organism *A.faecalis.* Analysis for Arsenic concentration in the soil samples was undertaken at 7-day intervals for four weeks. Concentration of Arsenic was measured in Parts Per Million (ppm) as expressed in the tables below.

Table 1: Showing Concentration of Arsenic in both Soil only and Soil containing *A.faecalis* from week 0-4 in Set up A.

SET UP A	WEEK 0 Concentration (ppm)	WEEK 1 Concentration (ppm)	WEEK 2 Concentration (ppm)	WEEK 3 Concentration (ppm)	WEEK 4 Concentration (ppm)
Arsenic in 5gms of Soil	3.875 3.668 3.402 4.544	4.945 3.492 3.506 4.933	3.395 3.381 4.896 3.389	3.368 3.704 3.703 4.278	3.374 4.292 3.306 3.603
Average concentration	**3.872**	**4.219**	**3.765**	**3.763**	**3.644**
Standard Dev	**0.488**	**0.831**	**0.754**	**0.378**	**0.450**
Arsenic and Alcaligenes in 5gms of soil	4.370 1.840 3.685 3.722	1.170 0.976 1.153 0.9902	0.9217 0.9365 1.039 0.9049	0.6212 0.6319 0.6134 0.6588	0.3473 0.3175 0.3111 0.3088
Average Concentration	**3.404**	**1.072**	**0.9505**	**0.6313**	**0.3212**
Standard Dev	**1.089**	**0.1034**	**0.060**	**0.040**	**0.008**

Soil and Water	Nil	Nil	Nil	Nil	Nil

From the table above, it can be seen that the average concentration of arsenic in the control for Set up A ranged from 3.644 ppm (week 3) to 4.219ppm (week 1) during the time under investigation. It is important to note that the concentration of Arsenic expressed in the table above were for the analysis of five grams of soil. In order to work back the concentration of arsenic in one gram of soil, the readings for the ICP-OES are divided by 5. This shows an average concentration of arsenic in 1 gram of soil of between 0.7288ppm and 0.8258ppm in the control. The average range for the concentration of Arsenic in soil containing the bacterium *A.faecalis* was between 0.3212ppm and 3.404ppm in five grams of soil respectively. As earlier mentioned, the average concentration of arsenic in one gram of soil ranged between 0.6808 and 0.064ppm, for the duration of the experiment.

Table 2: Showing Concentration of Arsenic in both Soil and Soil containing *A.faecalis* from week 0-4 in Set up B.

SET UP B (Replicate)	WEEK 0 Concentration (ppm)	WEEK 1 Concentration (ppm)	WEEK 2 Concentration (ppm)	WEEK 3 Concentration (ppm)	WEEK 4 Concentration (ppm)
Arsenic in Soil (2)	3.030 4.314 5.300 3.788	3.604 3.264 4.199 3.311	3.641 3.579 3.243 3.534	3.194 3.517 3.185 3.216	3.537 2.837 3.558 3.487
Average concentration	**4.108**	**3.5945**	**3.499**	**3.278**	**3.354**
Standard Deviaton	**0.954**	**0.430**	**0.1764**	**0.1599**	**0.346**
Arsenic in Alcaligenes (2)	3.642 3.710 4.314 4.111	1.015 0.9129 1.343 1.125	0.9134 0.9092 1.019 1.018	0.6385 0.6431 0.7258 0.4087	0.2570 0.2562 0.2467 0.3115
Average Concentration	**3.944**	**1.098**	**0.9649**	**0.6040**	**0.2678**
Standard	**0.322**	**0.1843**	**0.0619**	**0.1363**	**0.0295**

Deviatio					
Soil and Water	Nil	Nil	Nil	Nil	Nil

The output in Table 2 shows the concentration of Arsenic in soil in the replicate of the experiment, Set up B. Here, the concentration of Arsenic in soil only (Control) ranged from 3.278 to 4.108 ppm in five grams of soil, which could be recalculated in one gram of soil to be 0.6556 ppm and 0.8216 ppm respectively. The concentration of Arsenic in five grams of soil containing the *A.faecalis* ranged between 3.944 and 0.2678ppm. Again, in one gram of soil, this equates to between 0.7888 and 0.0536 ppm respectively.

In order to confirm that the concentrations of Arsenic recorded in both controls and the experiment in both set ups are significant, statistical tests are run on each data to find if there are genuine differences in the concentrations of arsenic recorded. To accomplish this, the statistical software, Minitab is used. The following tests below are designed to test the data and prove or disprove if there are genuine differences between them.

I. Descriptive statistics showing the mean, median, Standard deviation of the control and experiment

II. Test for Arsenic concentration in control and experiment at week 0

III. Test for the concentration of Arsenic in Control (set up A and B) at Wk 0 and wk 4 to ascertain if there is any significant difference in their concentration.

IV. Test for Arsenic in Alcaligenes and Soil in week 0 and week 4 to prove any change in concentration

V. Test for the Difference in Arsenic Concentration in Control (Arsenic in soil) and experiment (Arsenic in Soil with *A.faecalis*)

4.1.1 STATISTICAL ANALYSIS- TEST I

Table 3: Descriptive statistics of Control (Arsenic In Soil) and Experiment (Arsenic and *A.facelis)* in Set up A.

Variable	N	N*	Mean	SE Mean	StDev	Minimum	Q1	Median
Arsenic in Soil- A	20	0	3.853	0.129	0.576	3.306	3.396	3.636
Arsenic and *A.facelis-B*	20	0	1.274	0.269	1.203	0.309	0.615	0.929

Variable	Q3	Maximum
Arsenic in Soil- A	4.289	4.945

Arsenic and *A.facelis* 1.166 4.370

Table 4: Descriptive statistics of Control and Experiment in Set up B.

Variable	N	N*	Mean	SE Mean	StDev	Minimum	Q1	Median
Arsenic in Soil- B	20	0	3.567	0.121	0.539	2.837	3.223	3.526
Arsenic and *A.faecalis-B*	20	0	1.376	0.304	1.360	0.247	0.466	0.913

Variable	Q3	Maximum
Arsenic in Soil- A	3.632	5.300
Arsenic and *A.faecalis-B*	1.289	4.314

4.1.2 STATISTICAL ANALYSIS TEST II

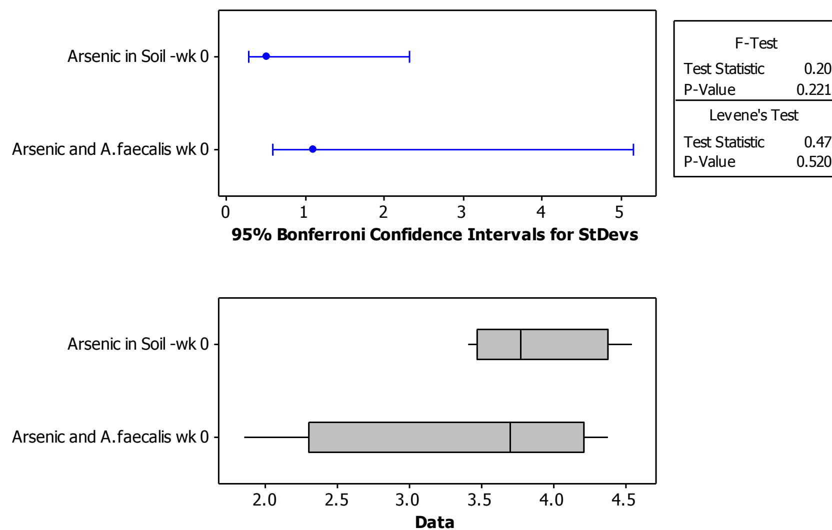

Figure 1: Test for equal variance between in Arsenic Soil and Arsenic and A.faecalis.

Table 5: Two sample T-test for Arsenic (week 0) in soil versus Arsenic and A.faecalis (week 0)

N Mean StDev SE Mean

Arsenic in Soil -wk 0 4 3.872 0.488 0.24
Arsenic and A.faecalis wk0 4 3.40 1.09 0.54

Difference = mu (Arsenic in Soil -wk 0) - mu (Arsenic and A.faecalis wk 0)
Estimate for difference: 0.476
95% CI for difference: (-1.178, 2.129)
T-Test of difference = 0 (vs not =): T-Value = 0.80 P-Value = 0.469 DF = 4

Table 5 shows the test for analysis of variance between Arsenic in soil and arsenic in Alcaligenes. The p-value result of 0.221 shows that the variances are equal. Further normality tests are done which shows that both sets of data are normal. The two sample T-test have a p-value of 0.469 showing that there is no difference between the arsenic concentration in soil and arsenic concentration in soil containing *A.faecalis* . This implies statistically with a 95% degree of confidence that the concentration of arsenic in soil and arsenic in *alcaligenes* are the same at week 0.

4.1.3 STATISTICAL ANALYSIS TEST III -

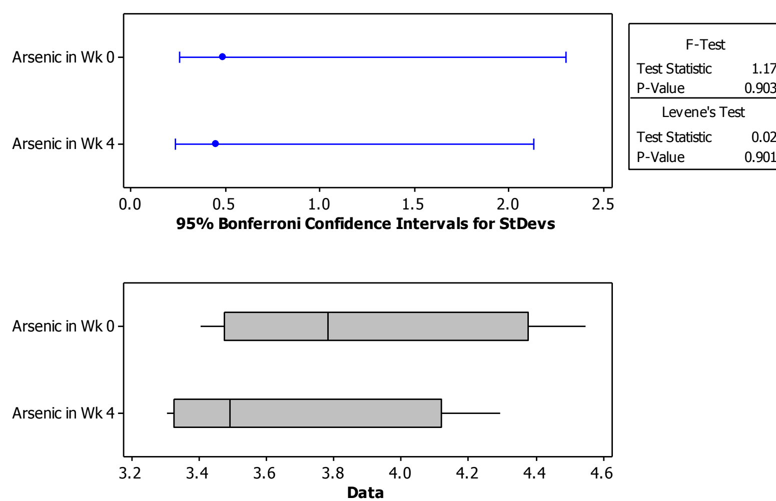

Figure 2: Test for Equal variance for Arsenic Concentration in control at Wk 0 and Wk 4(Set up A)

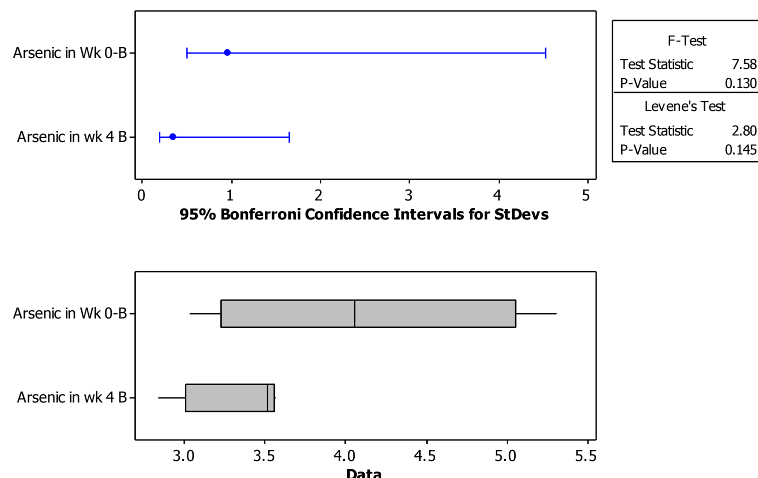

Figure 3: Test for Equal variance for Arsenic Concentration in Wk 0 and Arsenic in Wk 4(Set up b)

Table 6: Two sample T-test of Arsenic in soil at week 0 and week 4

	N	Mean	StDev	SE Mean
Arsenic in Wk 0	4	3.877	0.485	0.24
Arsenic in Wk 4	4	3.645	0.449	0.22

Difference = mu (Arsenic in Wk 0) - mu (Arsenic in Wk 4)

Estimate for difference: 0.232

95% CI for difference: (-0.618, 1.082)

T-Test of difference = 0 (vs not =): T-Value = 0.70 P-Value = 0.514 DF = 5

Table 7: Man- Whitney test of Arsenic in soil at week 0 and week 4

	N	Median
Arsenic in Wk 0-B	4	4.051
Arsenic in wk 4 B	4	3.512

Point estimate for ETA1-ETA2 is 0.767

97.0 Percent CI for ETA1-ETA2 is (-0.527,2.463)

W = 23.0

Test of ETA1 = ETA2 vs ETA1 not = ETA2 is significant at 0.1939

The tests for equal variances were carried out on the Arsenic concentrations in the control at week 0 and week 4 of both set up A and B. The variances in set up A were discovered to be almost equal with a p-value of 0.903. Since the p-value was higher than 0.05, the null hypothesis that they come from a sample with the same variance was accepted and a two sample t-test carried out. The two sample t-test had a significant p-value of 0.514, showing with a 95% degree of confidence, that the concentration of arsenic at week 0 and week 4 had no major difference. However, in set up B, a p-value of 0.145 was evidence to indicate that their variances were equal though the data were not normal. A Man-Whitney test (Table

7)was carried out and was significant at 0.1939, in which case it also shows that the there was no significant difference in the concentration of Arsenic in week 0 and week 4.

4.1.4 STATISTICAL ANALYSIS – TEST IV

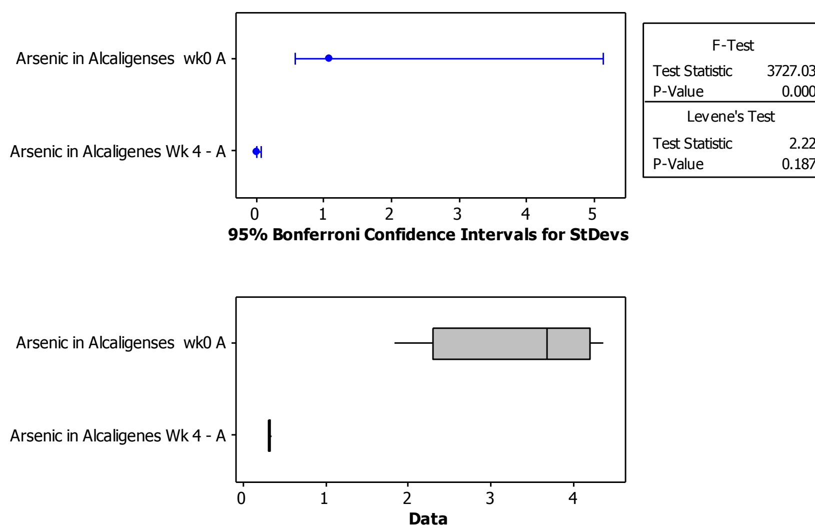

Fig 4: Test for equal variance for Arsenic and *alcaligenes* in soil. (Set up A)

Table 8: Man –Whitney Test of Arsenic and *Alcaligenes* at week 0 and week 4

	N	Median
Arsenic in Alcaligenes wk0 A	4	3.688
Arsenic in Alcaligenes Wk 4 - A	4	0.314

Point estimate for ETA1-ETA2 is 3.360

97.0 Percent CI for ETA1-ETA2 is (1.493,4.061)

W = 26.0

Test of ETA1 = ETA2 vs ETA1 not = ETA2 is significant at 0.0304

The test for equal variance between the concentration of Arsenic and Alcaligenes in Week 0 and week 4 presented interesting results. The P-value of 0.000 from the tests showed that the variances for the two data sets were not equal. A Mann-Whitney test was undertaken to further confirm this and it also showed that the concentration of Arsenic and Alcaligenes in Week 0 and week 4 were significant at 0.0304. In other words, there is a clear difference between the starting Arsenic concentration and the final concentration at the end of the experiment. The replicate, set up B , also confirmed the results with the same p-value of 0.0304.

4.1.5 STATISTICAL ANALYSIS TEST-V

In order to determine if there is any statistical evidence to show that the concentration of Arsenic in the control was different to the concentration of arsenic in the soil containing the bacterium *A.faecalis* . A thorough analysis of the data sets of arsenic in the control and arsenic in soil with the bacterium was undertaken. The sole aim for the analysis was to prove that there was significant difference in arsenic concentration in the control and experiment.

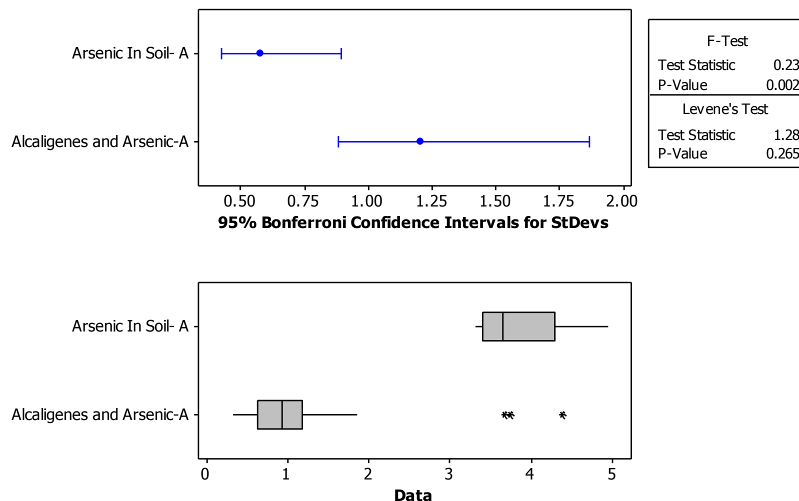

Figure 5: Test for Equal Variance of Arsenic in Soil and Arsenic and *A.faecalis* in soil

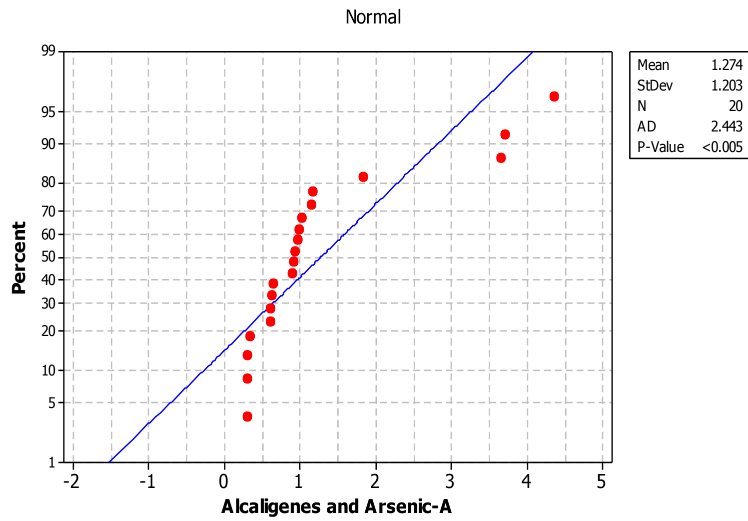

Figure 6: Normal probability plot of Arsenic and Alcaligenes in soil

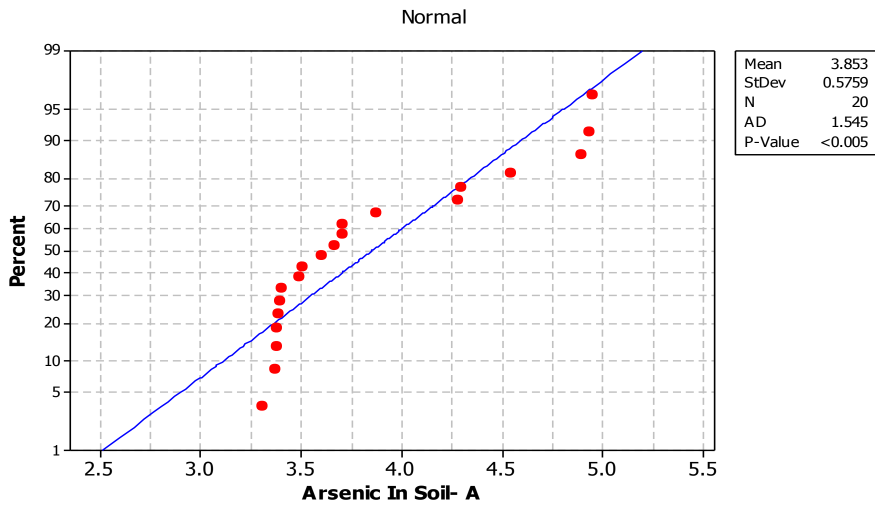

Figure 7: Normal probability plot of Arsenic in soil

Table 9: Mann-Whitney test for the concentration of arsenic in soil and soil containing *A.faecalis*

	N	Median
Arsenic in Soil- A	20	3.636
Alcaligenes and Arsenic-A	20	0.929

Point estimate for ETA1-ETA2 is 2.768
95.0 Percent CI for ETA1-ETA2 is (2.458, 3.108)

W = 571.0
Test of ETA1 = ETA2 vs. ETA1 not = ETA2 is significant at 0.0000

In figure 5, both variances of Arsenic concentration in the control and in soil containing *A.faecalis* were not equal. This is because, the P-value of 0.002 is less than the variance probability value of 0.05, in which case the alternate hypothesis which states that the variances are not equal is accepted. Having shown that the variances between the control and experiment are not equal, a normality test is carried out in order to check if the data is normally distributed. This is shown in figure 6 and 7. The irregular spreads of the outliers coupled with the very low p-values of 0.005 in both figures indicate that the data are not normal. Hence an analysis of difference using the Mann-Whitney test is carried out. The Mann- Whitney test for both samples was significant at 0.0000, which is less than 0.05; hence the alternate hypothesis that samples of arsenic concentration in soil (control) and arsenic concentration in soil containing *A.faecalis* are different is accepted. It can therefore be stated with a 95% degree of confidence that there is a statistically significant difference between the concentration of arsenic in soil and the concentration of arsenic in soil containing *A.faecalis* at the end of the experiment.
The same statistical tests were done on set up B, with the test being significant at 0.0004 further strengthening the earlier result.

4.2 MICROBIAL COUNT

Microbial count for the bacterium *A.faecalis* was done by serial dilution from 10^0 to 10^9 at seven day intervals throughout the duration of the experiment. Dilutions were then plated out on Trypticose Soy Agar (TSA) in replicates (Set up B) and incubated at 25^0 C for 48 hours in an incubator. Colonies were counted using the colony counter and enumerated and logged as shown below.

Table 10: Showing logged data of microbial counts in the control and experiment.

	SET UP A		SET UP B	
DURATION	ALCALIGENES + ARSENIC LOG (cfu/g)	ALCALIGENES +SOIL LOG (cfu/g)	ALCALIGENES + ARSENIC LOG (cfu/g)	ALCALIGENES +SOIL LOG (cfu/g)
WEEK 0	7.79239	7.84510	8.00860	7.92942
	8.07918	8.04139	8.32222	8.46240
WEEK 1	8.15229	8.08991	8.20683	7.46090
	8.54407	8.43136	8.56820	8.67555
WEEK 2	8.40140	7.98227	8.36361	7.94448

	8.78533	8.27875	8.762343	8.23045
WEEK 3	8.41330	7.8573	8.3962	7.8389
	8.86923	7.6989	8.8451	7.4771
WEEK 4	8.3562	7.5315	8.3636	6.4771
	8.7634	7.4771	8.7853	8.0000
Standard Deviation	**1.929**	**2.892**		

The microbial counts displayed in table 10 shows an interesting trend across the plated samples of soil containing arsenic and *A.faecalis* as well as soil containing only *A.faecalis* (control). From table 10, it can be seen that there was an initial increase in growth of the organism in soil containing arsenic, while there was a slight decrease in microbial count for the control containing no arsenic, over the four week period. However, there was no appreciable change in the microbial count in *A.faecalis* and arsenic.

In order to be able to properly analyse the data and compare if there were any statistical significant change in the microbial counts of organism in the control and the experiment, the total numbers are transformed by logging as shown in table 10 and statistical tests are carried out.

The tests are designed to show the following:

I. Descriptive statistics of the data showing the mean, median and standard deviation

II. Difference (if any)between the number of microbial count of arsenic and *A.faecalis* in soil and *A.faecalis* in soil at week 0

III. Difference between the microbial count of arsenic and *A.faecalis* in soil and *A.faecalis* in soil only.

IV. A bar Chart showing the standard Deviation of the microbial counts against Time.

Table 11: Descriptive statistics showing the mean, standard error, standard deviation of *A.faecalis* in soil and with arsenic.

4.2.1 STATISTICAL ANALYSIS TEST I-B

Variable	N	N*	Mean	SE Mean	StDev	Minimum	Q1	Median
Arsenic and Alcaligenes	22	0	8.183	0.411	1.929	0.000	8.008	8.479
Alcaligenes in Soil_	22	0	6.990	0.617	2.892	0.000	7.476	7.920

Variable	Q3	Maximum
Arsenic and Alcaligenes	8.966	9.954
Alcaligenes in Soil_	8.334	9.301

From table 11 above, the logged mean values of the microbial counts for the whole duration of the project are displayed with the average value for Arsenic and *Alcaligenes* in soil being 8.183 and *Alcaligenes* in soil only, 6.990.

4.2.2 STATISTICAL TEST –II-B

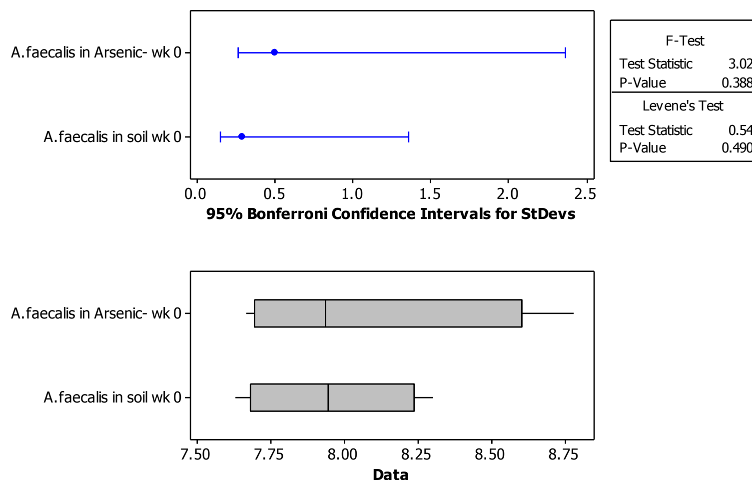

Figure 8: Showing the test for equal variance between A.faecalis in arsenic at week 0 and A.faecalis in soil at week 0

The equal variance test in figure 8 shows that both the control and the experiment had roughly the same initial microbial count at the beginning of the experiment. The P-value of 0.388 is much higher than the probability value for the null hypothesis of 0.05 in which case the null hypothesis that states they have the same variance is accepted.

Table 12: showing the two sample T-test for *A.faecalis* in arsenic vs. *A.faecalis* in soil

	N	Mean	StDev	SE Mean
A.faecalis in Arsenic- w	4	8.079	0.498	0.25
A.faecalis in soil wk 0	4	7.954	0.286	0.14

Difference = mu (A.faecalis in Arsenic- wk 0) - mu (A.faecalis in soil wk 0)
Estimate for difference: 0.125
95% CI for difference: (-0.672, 0.921)
T-Test of difference = 0 (vs not =): T-Value = 0.43 P-Value = 0.687 DF = 4

From the table above, a two sample test to show that there is no difference in between the organism in arsenic and the organism in soil was done at week 0 and the high p-value of 0.687 showed that there was indeed no difference.

4.2.3 STATISTICAL TEST- III-B

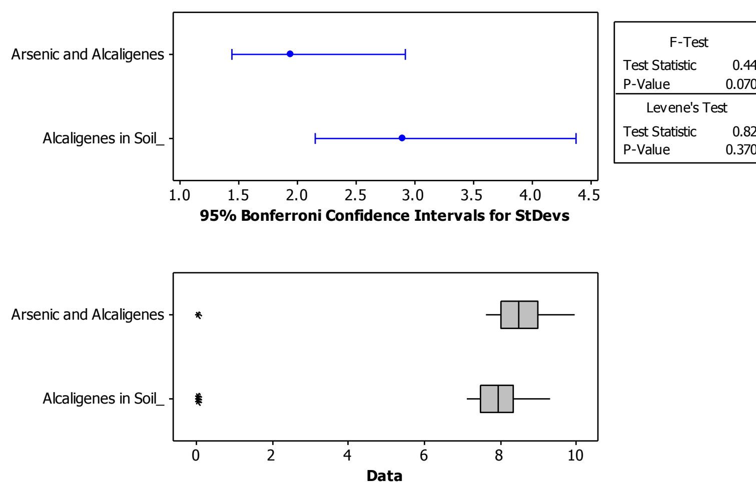

Figure 9: Showing the test for equal variance between *A.faecalis* in arsenic at week 0 and *A.faecalis* in soil at week 0

The major test to show if there was a difference between *A.faecalis* in arsenic and *A.faecalis* in soil was done. (Figure 9) The results of the variance test showed that the variances were only slightly the same with a p-value of 0.070.

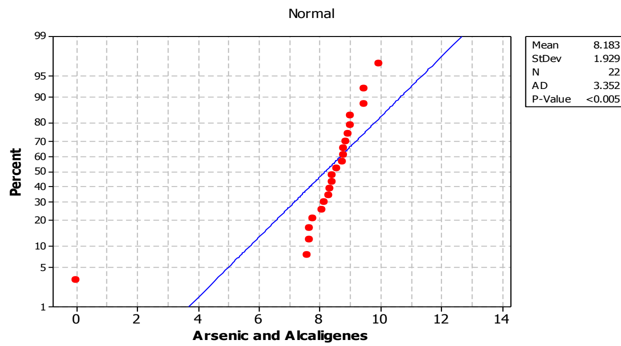

Figure 10: Normality plot of arsenic and *A. faecalis*

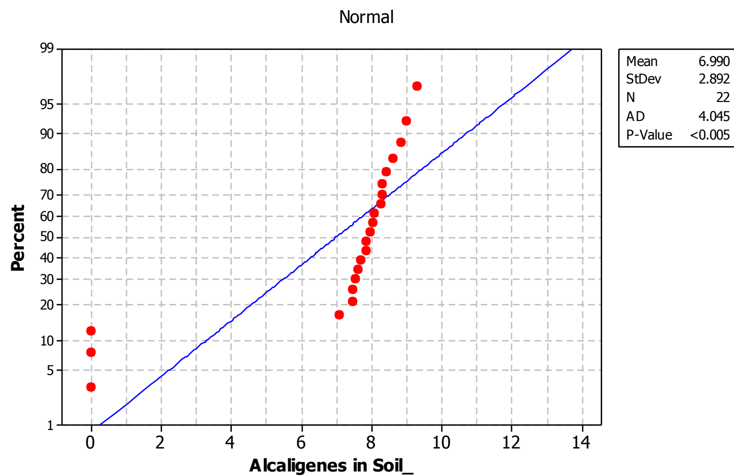

Figure 11: Normality plot of *A.faecalis* in soil

The normality test done on *A.faecalis* in soil and in arsenic showed that the data were not normal, hence the need to undertake a Mann-Whitney test on the data

Table 13: Showing the Mann-Whitnney test of Arsenic and *A. faecalis* and *A.faecalis* in soil.

	N	Median
Arsenic and Alcaligenes	22	8.479
Alcaligenes in Soil_	22	7.920

Point estimate for ETA1-ETA2 is 0.599
95.0 Percent CI for ETA1-ETA2 is (0.127, 1.087)
W = 602.5
Test of ETA1 = ETA2 vs ETA1 not = ETA2 is significant at 0.0120
The test is significant at 0.0120 (adjusted for ties)

The Mann-Whitney test showed that *A.faecalis* in arsenic and in soil were different but only just. The test was significant at 0.0120 which was lower than the p-value of 0.05 and so the alternate hypothesis that there is a difference in the microbial counts of both samples.

5.0 DISCUSSION

The results obtained at the conclusion of the experiment were quite interesting and were subjected to statistical analysis to further verify their authenticity. The average concentration of arsenic for the control of the experiment remained constant for the four week duration of the project and this was tested statistically using a two sample T-test with a p-value of 0.514, which indicated that concentrations were constant. A couple of factors could be responsible for this, ranging from Arsenic III mobility as well as its stability and other physical and chemical factors to be discussed in more detail later in this chapter. The values of arsenic concentration in soil inoculated with the bacterium *A.faecalis* at the start of the experiment and at conclusion showed a reduction from an average initial concentration of 3.404ppm to a final average concentration of 0.3212ppm (Table 1.) The difference in the concentration at the beginning and at the end of the experiment was proven statistically using the Mann-Whitney test which was significant at 0.0304(Table 8), showing that there was indeed a difference in between the concentration at the start and end of the experiment.

Further tests were then carried out to compare the values of concentration for the control and the value of concentration of arsenic in soil with *A.faecalis* . The reason for doing this was to verify if there was statistical evidence to show a difference in concentration between the concentrations of arsenic in the control and that of the experiment in order to further highlight evidence of sequestering. Having already established a significant difference in the initial and final concentration of arsenic in soil containing *A.faecalis* the test with the control shows that concentrations recorded were very different indeed.

The drop in concentration of arsenic in the soil containing *A.faecalis* throws up quite a number of questions, such as what happened to the arsenic and whether it was sequestered or some other chemical process occurred during the experiment that resulted in a drop in arsenic concentrations. The results from the set up A of the project were also similar to those produced in the replicate, set up B.

The microbial counts also showed some particular interesting sequence, reasons for which shall be discussed in detail later in this chapter. The total numbers of microbial counts were logged in order to be able to be analysed using statistical data. From the results, the control containing only *A.faecalis* had a mean log value of 6.990, while soil containing *A.faecalis*

and arsenic had a mean log value of about 8.183. Looking at the results of the total microbial count for the period of the project, it could be seen that microbial growth in the control decreased during the second and third week and was generally lower than microbial count which contained arsenic. Statistical tests were carried out to prove this case was true. The microbial count in the control sample at week 0 was tested with the count of the sample containing arsenic. A two sample t-test done returned a p-value of 0.687, which was much higher than probability of the null hypothesis of 0.05, which meant the counts of the control and the experiment were the same at the beginning of the experiment. This means both control and the experiment had the same amount of pure culture of *A.faecalis* at the start of the experiment which is crucial in determining if any change of the organism occurred by being able to compare any possible change in growth of the experiment with that of the control. Having ascertained that fact, another test designed to show if indeed there was any difference between the microbial count of A.faecalis in the control and that of the experiment.

A Mann-Whitney test was done on the microbial count between the control containing only the organism in soil and the experiment containing arsenic. The tests turned out to be significant at 0.01 (Table 13), which shows that there was a difference in the amount of microbial count between the two samples as earlier thought, however the amount of variation is quite small. Even though the Mann-Whitney test showed that there was a difference in the growth pattern of the microorganism in the control and experiment, it does not confirm if there was any really adverse change in the growth pattern of the microorganism in the soil contained in the arsenic as any likely change is bound to be very minimal. However, Table 10, clearly shows that the counts of the microorganism did not vary much during the experiment. This suggests that the microorganism coped well with level of arsenic in the soil, which was 1mg/g, possible reasons and factors for this are discussed later.

In order to be able to attempt a concise and detailed discussion of the possible reasons for the results obtained in this project, the discussion shall be divided into two parts. The first part shall attempt to look into the possible sequestering of arsenic as shown by the reduction in arsenic concentration, taking into account the physical and chemical mechanisms which operate in such an experimental study. Factors such as pH, oxidation state ,microbial metabolism, metal transformation and possible methylation. The second part of the discussion shall attempt to rationalise the results of the microbial counts presented in the results by looking into the effect of the toxicity of arsenic to the organism and the possibility of detoxification measures brought about by the organism.

5.1 Reduction in Arsenic Concentration

The final concentration of arsenic in soil on spiking 500 grams of soil with arsenic came to 1mg/g (1000mg/kg). This would appear quite high but in comparison to Soda et al. (2008) who experimented with an arsenic concentration of 1100mg/kg and 2200mg/kg with relatively successful results, it would appear the concentration used was adequate. The average initial concentration of arsenic in one gram of soil was 680µg/g at the start of the experiment and this dropped to 64µg/g indicating that a 91% drop in the concentration of arsenic in the soil. This suggests that about 617 µg/g of arsenic was acted upon by the organism, which is consistent with works done by Takeuchi et al. (2007) on the accumulation of 2290µg/g of arsenic by the bacterium *Marinomonas .communis* with an exposure time of just three days to the concentration of arsenic. The amount of arsenic (616 µg/g) acted upon by the organism is also within range of a 700mg/kg drop in arsenic recorded by Soda et al. (2009) while working on a dissimilate arsenate reducing bacterium. Even though metals or metalloids are not biodegradable, they can be transformed through methylation, sorption and complexation as well as changes in valence states.

5.2 Mobility and Oxidation State

It would appear that a lot of factors outside the action of the microbe, *A.faecalis* also contributed to the drop in concentration of the arsenic. It is common knowledge that the bioavailability, solubility and mobility of arsenic depends on its valence state Masschelyn et al. (1991) and that there is a relationship between arsenic mobility and the pH, Santini et al. (2000). The oxidation state of arsenic at the start of the experiment was $_{+}$ 3, while the pH of the soil at the start of the experiment was 9.2 and increased to 9.7 before gradually decreasing to 8.5. While it was not possible to determine the final oxidation state of the arsenic owing to it being beyond the scope of a three month study, it could be inferred that the mobility of arsenic III in the soil was increased by the initial increase in the pH of the soil which may invariably increase its solubility and bioavailability as arsenic sorption is strongly associated with pH range of 9-12, Masschelyn et al. (1991). This theory is also further supported by the work of Ignatiad and Battaghia-Brunet (2005), who studied the removal of arsenic from contaminated soils through the stimulation of indigenous microorganisms by the addition of carbon and energy sources with the discovery that arsenic mobilisation, was found to depend on the characteristics of soil samples such as the pH. In this work, the average pH was 8.96, indicating alkalinity, while the glucose contained in the nutrient broth and even the arsenic

could act as a carbon source. The oxidation state of arsenic also plays an important role. Arsenic III is quite much more mobile than arsenic V (Deng and Liao, 2002) and can be readily acted on by the microorganism much quicker than Arsenic V, sorption could occur by arsenic being bound to the cells of the organism, Chantain et al. (2005)

5.3 Methylation

Another quite possible scenario which may have occurred and resulted in the loss of concentration of arsenic in the soil sample is the process of methylation. This process is also linked to the mobility of arsenic in the soil as Arsenic III is a precursor to the formation of methylated arsenic species (Pongratz, 1998). Evank and Dzombak (1997), suggested that the two important approaches to bioremediation are immobilisation and mobilisation with the latter being more suited to microorganism. As earlier mentioned, the mobility of arsenic III could increase with the pH of the soil, hence microbial metal transformation processes such as methylation through the action of *A.faecalis* is possible. This thought is in line Cullen and Raimer (1989), that methylation is due exclusively to the presence of microorganism in the soil. The arsenic in the soil could be biomethylated to monometheylarsinic acid (MMAA), Dimethylarsinic acid (DMAA) and trimethyl arsine oxide (TMAO) as also confirmed by Turpeinien et al. (1999). Further evidence to support that methylation of arsenic could have occurred is presented by Gao and Burau(1997) who showed that water soluble arsenic species such as arsenic III could also be volatilised by microbes into gaseous arsines.

Possible pathways for the methylation of arsenic and eventual loss in concentration have been put forward by both Alexander (1977) and Woolson (1977). They postulated that arsenic is lost or sequestered in soil through the reduction of arsenic V to arsenic III, followed by a series of methylation reactions of arsenic III to eventually yield trimethyl arsine as a final product. Unfortunately, owing to the 3-month time constraint of the study, tests to confirm for the presence of the gases were not available. However, in this project, it is strongly inferred that methylation of arsenic and the production of arsines such as MMA, DMA and TMA are partly responsible for the drop in concentration of arsenic in the soil and may have occurred by evidence of the resultant drop in pH of the experiment . This statement is supported by Cullen and Raimer(1989) in stating that not only does methylation rates of arsines resulting in loss of arsenic vary greatly depending on the soil properties such as soil moisture, temperature and pH, but also on the abundance of microbial population in the soil.

5.4 Microbial metabolism

A unique avenue through which the drop in concentration of arsenic could be explained is by the process of microbial metabolism. Indeed, the very nature of bioremediation has its roots in the ability of microorganisms to detoxify the contaminant through the use of biochemical reactions or pathways that would result in the growth and reproduction of the organism (Adeniyi, 2004). In the case of this project, the organism is the *A.faecalis.* As earlier discussed in the literature, the organism *A.feacalis* has shown remarkable ability to oxidise arsenic as well as accumulate it and depending on the soil characteristics, even methylate it. According to Adeniyi (2004), the metabolic system in the microorganism enables it to use carbon electrons in the process of growth and cell multiplication while transforming the contaminant in the process. A unique angle is shown to the argument by Evanko and Dzombak(1997), that as part of the microorganisms interaction with contaminants, they could cause the metals or metalloid to be demobilised as the immobility of metals is caused by reactions that make the metals precipitate or be kept in a sold phase. This also brings us back to the argument of the oxidation state of arsenic as arsenic III is more mobile than Arsenic V, but A.faecalis could also readily oxidise arsenic III to arsenic V.

5.5 Volatilisation

This is yet another method by which arsenic concentration in the soil would have reduced and it involves the methylation of arsenic to form methyl arsenicals and then the release of these from the microbe as a gaseous product. (Rodriguez, 1999). Volatilisation however is based on the acting of the organism on a carbons substrate in the soil. The key factor here is the substrate that the microorganism acts upon. In this experiment, arsenic in the soil could be used as a carbon source as well as the glucose in the non defined media through which the microorganisms grow. This may account for the slight increase in microbial count for the organisms during the course of this experiment. This line of thought is also seen to be corroborated in the study of Mophatra et al. (2008) who discovered while working with cultures of methanogenic bacteria that just as the case with glucose in the soil, bacteria were able to volatilise 35% of arsenic at a substrate concentration of 25g/l, with the maximum uptake of arsenic at the end of the experiment found to be 1.08mg of arsenic /g of substrate.

5.6 Impact of Arsenic on *A.faecalis*

The impact of 1mg/g concentration of arsenic on the growth pattern of the organism is quite interesting. From the results and as earlier mentioned in the first part of this discussion, the growth rate of the organism does not change that much as the microbial counts in table 16 and bar chart in table 24 have clearly shown. The question therefore is why? There are a number of possible explanations for this and they are explained as below.

5.7 Metal transformation (Detoxification), pH and Temperature.

The most probable explanation for the growth of *A.faecalis* in the presence of such a high concentration of arsenic is the possible oxidation of Arsenic III to the less toxic arsenic V. This oxidation process is seen as a detoxification process as supported by Wang and Zhao (2009) when they discovered that oxidation of arsenic results in detoxification. However the growth pattern was not that significant as proved by the Mann-Whitnney Test (Table 23). A similar result was obtained from the study of Adeyemi (2009) who investigated the bioaccumulation of arsenic in fungi and discovered that no specific growth pattern was discerned in plates of the organisms spiked with arsenic. It is greatly inferred that the bacterium, *Alcaligenes faecalis* was able to detoxify the arsenic through a resistance mechanism. This thought is in line with the studies of Xu et al.(1998) who revealed that bacteria use a reductase enzyme (ArsC), which is encoded on the plasmid or chromosome of the bacteria. This enzyme serves as an arsenic resistance mechanism. However, as earlier mentioned the bacterium could have oxidised arsenic III into the more tolerable arsenic V through the enzyme arsenic III oxidase (Ellis et al. 2001).This statement appears more possible based on the research of Anderson et al.(1992) who also revealed that bacteria overcome the toxicity of arsenic III either by decreasing their intracellular concentrations through the reduction of intake or exporting the arsenicals.

The temperature of the set up was constant at between >25 ^{o}C , a temperature quite suitable for the bacterium (Phillips and Taylor, 1976) even though optimum growth is achieved at 37 ^{o}C. The pH of the soil though alkaline was suitable for the organism as it shows good tolerance for high alkalinity (Anderson et al, 1992). Overall, both temperature and pH, would be suitable for the process of detoxification of arsenic III to take place in the soil and help the organism tolerate the level of arsenic concentration.

5.8 Implications of Findings

The implications of the findings of this project are far reaching. The apparent ability of the organism, *A.faecalis* to reduce the arsenic concentration in the soil as shown in this project holds immense promise in the study of bioremediation and actual remediation of arsenic contaminated soils. Though the project was centred around soil, possible use of the organism, which has shown an enormous ability to tolerate high arsenic concentrations in other forms of

remediation studies with other heavy metal contaminants is highly possible. The role of microorganisms in bioremediation of arsenic is highly attractive

5.9 Limitations of the Project

There were some clear limitations in the course of the project. One of the major limitations was the inability to test for the speciation of arsenic left in the soil. This was due to the unavailability of an Inductive Coupled Plasma – Mass spectrophotometer in the laboratory. The absence of this immensely important analytical machine severely hampered further studies into arsenic speciation as well the analysis of possible arsine production in the soil.

Another limitation was the time frame for the period of the study. The three month period was too short for a thorough project to be executed. Other factors such as GSH and PC analysis could have been investigated but for the shortage of time.

6.0 **CONCLUSION**

In conclusion, this project was able to meet to an extent with the aims and objectives set out in the design of this work. From the results and discussion, there is enough statistical evidence to show that not only did the bacterium, *A. Faecalis,* show a high level of resistance to the 1mg/g concentration of arsenic in the soil, it was also able to transform and sequest a large amount of the metalloid, thereby indicating its suitability as an effective microbe in the remediation of arsenic from contaminated soil.

Apart from the organism's ability to transform and reduce the amount of arsenic in the soil, it also showed a remarkable ability to detoxify the metalloid as was evidence by its stable growth in soil even after the spiking of the soil with arsenic. This feature also showed that arsenic had very little effect on the organism as it was able to cope well with the concentration of arsenic. A big part of this detoxification ability can be attributed to the arsenic resistance mechanism and specific enzymes as confirmed by other literature and scientific studies.

The inherent ability of the organism to successfully bio remediate high concentrations of arsenic in a controlled laboratory experiment portends a great deal of potential in its use as a bioremediation agent, however there is still reason to be cautious as there is not sufficient literature and studies done to ascertain the microbes behaviour in a natural environment. A significant benefit of the bacterium however, is that it is found naturally in soil and water, however isolating strain may be a hindrance in simply bio stimulating it in the case of contaminated areas.

Finally, further studies and analysis still need to be undertaken to fully understand, comprehend and accurately predict the mechanisms of arsenic uptake and transformation of the organism, with the hope that a better scientific understanding will eventually see a large scale adaptation of the bacterium *Alcaligenes faecalis* in the bioremediation of Arsenic in Contaminated soils.

6.1 Recommendation and Suggestions

More studies using high concentrations of arsenic in bioremediation with microorganisms are encouraged. Compared with the number of studies using low concentrations of arsenic in soil, not enough work has been done using large arsenic concentration which is quite surprising

considering the fact that a lot of areas in the world have problems with high concentrations of arsenic and the seeming endless potential of microorganisms to remediate them.

The bacterium, *A.faecalis* is a very interesting and unique organism and although lots of studies have been carried out on the genus, a lot more could still be done in experimenting with arsenic and other metals. It is strongly believed that further studies should be done on the detoxification mechanisms of *A.faecalis* and bacteria in general. The process of microbial detoxification of arsenic is quite fascinating and should be further explored with particular insight into the arenic III oxidase enzyme, which particularly holds the key to future remediation studies

A lot more work should be carried out on the study of methylation of arsenic as this aspect of metalloid transformation is still relatively new. Detailed studies should be conducted.

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