Bioprecipitation of Heavy Metals and Radionuclides with Calcium Carbonate in Aqueous Solutions and Particulate Media

Ahmed Jadaan Mugwar

Geoenvironmental Research Centre
Cardiff School of Engineering
Cardiff University

Thesis submitted in candidature for the degree of
Doctor of Philosophy at Cardiff University
December 2015

DECLARATION

This work has not been submitted in substance for any other degree or award at this or any other university or place of learning, nor is being submitted concurrently in candidature for any degree or other award.

Signed ..... **(Ahmed Mugwar)**

Date 22/12/2015

STATEMENT 1

This thesis is being submitted in partial fulfilment of the requirements for the degree of doctor of educational psychology.

Signed ..... **(Ahmed Mugwar)**

Date 22/12/2015

STATEMENT 2

This thesis is the result of my own independent work/investigation, except where otherwise stated. Other sources are acknowledged by explicit references. The views expressed are my own.

Signed ..... **(Ahmed Mugwar)**

Date 22/12/2015

STATEMENT 3

I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loan, and for the title and summary to be made available to outside organisations.

Signed ..... **(Ahmed Mugwar)**

Date 22/12/2015

ACKNOWLEDGMENTS

In the Name of **Allah**, the All- Merciful, the All-Compassionate.

The praise is due to **Allah** for giving me the strength, patience and perseverance to complete this work in its best though my chronic illness.

I am extremely grateful to my supervisor **Dr Michael Harbottle** for providing me this opportunity and introducing me to this novel idea. Thanks for his patience, guidance and invaluable advice throughout this work. I am forever thankful for his professional and personal mentorship. Also I would thank my second supervisor **Dr Devin Sapsford**.

I am really grateful to all staff in haematology clinic/ Heath Hospital/ Cardiff, in particular **Dr Steven Knapper** for helping me to overcome my severe illnesses.

Also I am grateful to Ministry of Higher Education and Scientific Research in my great country **IRAQ** for financial support during my study.

I would like to express my gratitude also to all admin staff in the research office and I am grateful to the technical staff of the School of Engineering, in particular Jeff, Len, Harry and Ravi. I am thankful for their assistance.

I am thankful for all my friends in Iraq and UK who have prayed, helped for me throughout this work.

Finally, and most of all, I would like to express my deepest gratitude and appreciation to my mother, my brothers, and all my relative. Special thanks for my wife **Eman** for her tears and support in my times of pain and to my children **Abdullah** and **Fatima** for their praying, help, sacrifices, and patience during my study.

I dedicate this thesis to **My Lord on the Earth** for permanent inspiration and supporting, also this work is dedicated to the memory of my father, **Jadaan,** I miss him every day, if he alive would be proud of me.

ABSTRACT

The possibility of utilisation of microbially induced calcium carbonate precipitation as a potential remedial technology for sequestering divalent metallic contaminants in subsurface environment was explored. Contamination by heavy metals and radionuclides is a significant environmental problem. Incorporating metals in calcium carbonate minerals is a mechanism that may offer long-term removal of metallic cations. Stimulating native ureolytic bacteria by supplying required nutrient and chemicals to create alkaline conditions suitable for calcium carbonate precipitation may accelerate biomineralisation processes in polluted soil. This study reports the ability of a bacterium in soils, *Sporosarcina pasteurii*, to remediate range of heavy metal and radionuclide (by using non-radioactive proxies) concentrations in aqueous solutions and particulate media by inducing urea hydrolysis and calcium carbonate precipitation. However, bacterial activity is limited by heavy metals toxicity and availability, hence, initially the minimal inhibitory concentration (MIC) of metals to bacterial activity was estimated, without the presence of a metal precipitation mechanism (only metals ions and nutrient broth). Metals inhibited bacteria in the following order Cd >Zn >Cu >Pb >Sr. The bacterial cells showed an improved growth and metal toxicity resistance in presence of urea containing medium over their presence in urea-free medium. Cd, Pb and Sr were fully removed, whilst Zn and Cu were partially removed by this technique. Removal of heavy metals was strongly correlated with full removal of calcium and high pH increase then white precipitate was produced, suggesting that urea hydrolysis by bacterial cells did play a role. Finally, it was explored the effects of ground conditions (transport and availability of heavy metals, preferential flow and heterogeneous hydraulic conductivity) on biomineralisation process in porous media in different sand fractions (fine, medium and coarse). High removal rates were achieved in porous media especially for cadmium and strontium. It is resulted that the heterogeneity of hydraulic conductivity plays a main role in distributing bacterial activity and subsequent precipitation processes in porous media. XRF, XRD, SEM and EDX spectrum tests for sand samples showed the presence of calcite and aragonite in precipitation crystals. The gained results demonstrate that microbially induce calcium carbonate technique is a means of sequestration of soluble heavy metals via co-precipitation with calcium carbonate precipitation that can be useful for divalent heavy metal and radionuclides bioremediation.

Table of Contents

List of Tables

List of Figures

Chapter 1: Introduction

1.1 Heavy Metals and Radionuclides Bioprecipitation

Contamination by heavy metals and radionuclides is a significant environmental problem. Presence of these toxicants in concentrations higher than their natural limits may pose threat for human health. Due to the fact that subsurface environment is dynamic system metals can spread out horizontally and vertically in subsurface depend on nature of contaminated zone. Heavy metals can cause long-term effects as they are non-biodegradable and persistent environmental pollutants. The behaviour of heavy metals and radionuclides in the subsurface environment is controlled by soil and groundwater chemistry, mainly redox potential and pH, which strongly affect their solubility, precipitation and sorption to the solid phase of soil (Wernick and Themelis 1998; National Research Council 2003; Dragović *et al.* 2008).

Heavy metals and radionuclides are naturally occurring but anthropogenic sources (industrial, agricultural, domestic and accidents) have altered their geochemical cycles (Dixit *et al.* 2015). Contaminants resulting from anthropogenic sources are found to be more mobile and available than those from natural sources in environment. Natural attenuation may not be enough to remediate excessive amounts of heavy metals or radionuclides (Wernick and Themelis 1998; Ruggiero *et al.* 2005; Megharaj *et al.* 2011).

Biological remediation is being investigated as a more active method (economically and practically) than traditional means to remediate this type of contamination. Bioremediation or bioprecipitation of heavy metals and radionuclides represents all the processes in which microorganisms are exploited to transfer the

contaminants present in soils, sediments and water to less or nontoxic products. Bioremediation can be more efficient than traditional treatment methods in contaminated soils treatment (e.g. soil excavation and disposal to landfill and pump and treat) (Wernick and Themelis 1998; Ruggiero *et al.* 2005; Megharaj *et al.* 2011).

Microbially induced calcium carbonate precipitation is a bio-geochemical process involving precipitation of calcium carbonate and possibility of co-precipitation of heavy metals and radionuclides within soil matrices (Fujita *et al.* 2000). This technology has been studied extensively in different fields. The applications in biocementation of porous media, bioclogging of soil voids and crack healing have received a great deal of attention (DeJong *et al.* 2006; Whiffin *et al.* 2007; DeJong *et al.* 2010; Harkes *et al.* 2010; Dejong *et al.* 2013). However, apart from strontium mineralisation, bioprecipitation of heavy metals and radionuclides with carbonate minerals has received relatively little attention though such minerals have the potential to sorb and sequester a range of metallic cations. Generally, generation of conditions favouring precipitation of calcium carbonate is through urea hydrolysis by a specific microorganism, which produces carbonate and ammonium ions and an elevated pH (Fujita *et al.* 2000). A wide range of microorganisms is capable of producing urease enzymes that cause urea hydrolysis (Ehrlich 1996; Warren *et al.* 2001). Microbial cells may offer their cell wall as nucleation site for the calcium carbonate precipitation, as well as their ability in creating conditions favouring calcium carbonate precipitation (Stocks-Fischer *et al.* 1999). Hence, bioprecipitation efficiency in contaminated sites depend on the capability of these microbes to grow and operate in presence of toxic contaminants. The process of calcium carbonate biomineralisation has been reported to be faster (Al-Thawadi 2011) and more extensive (Ferris *et al.* 1995) than that achievable by chemical means alone.

Nevertheless, limited studies were reported on heavy metal and radionuclides toxicity to other organisms.

Microbially-induced calcite precipitation has been reported in only a very limited fashion for removal of heavy metal ions from aqueous solution. Applicability of heavy metals removal by this technology that based on understanding metals availability and toxicity to bacteria and ground conditions effects on the bioprocesses is lacking. This study investigates the possibility of using this technique to remove a range of heavy metals and radionuclides by considering many factors that may affect the metal removal mechanism (metal toxicity to the bacteria that induce calcium carbonate precipitation, bioavailability of heavy metals in subsurface environment, metal-calcium carbonate interaction and ground conditions influence). Improving information and understanding of the parameters that relate to this technology can lead to increase its efficiency and to enable upscaling to suit in-situ applications. Parameters that were considered in this study are metal toxicity to the bacteria (*Sporosarcina pasteurii*) and effects of metal cations availability, sand particle sizes and the influence of the homogeneity and heterogeneity in hydraulic conductivity on bioprecipitation processes.

1.2 Statement of Objectives

This study aims to explore the ability of a specific microorganism to induce removal of divalent contaminants from polluted water and soils by exploiting the potential of calcium carbonate precipitation of sequestering a range of heavy metals and radionuclides. Batch and column experiments were carried out in triplicates to satisfy the objectives of this study. The overall objectives of this research were:

1- Investigate the extent that a urease-positive bacterium, *Sporosarcina pasteurii* can tolerate a range of heavy metals (zinc, cadmium, lead copper and strontium). This is considered an indicator for the ability of the bacteria to grow and operate in presence of metallic toxicants. In this part bacteria were allowed to grow in a medium that contained only nutrient broth and the target metal. Also, to estimate the minimum inhibitory concentration (MIC) for each tested metal to the bacteria growth in this part of study.

2- Explore the potential of bioprecipitation with calcium carbonate to remove these metals from aqueous solutions and the variations in performance with different metals. This study hypothesises that metal precipitation technique provides a protective mechanism for the bacteria by reducing the overall metal concentrations to tolerable levels, tested concentrations of above heavy metals were increased in calcium carbonate bioprecipitation experiments. This is important to investigate the influence of the metal removal mechanism on the organism's inhibition by high concentrations of the different metals.

3- Monitor how the heterogeneity of subsurface can affect the bioprecipitation processes. Different values of hydraulic conductivity may lead to non-homogenous distribution of calcium carbonate precipitation in porous media. In addition, how precipitated mineral can control solute transfer in porous media, such effect occurs when calcium carbonate precipitation clog pore throats.

1.3 Organization of the Thesis

The thesis has been divided into seven chapters including chapter 1 (Introduction):

Chapter 2: Literature review, is a summary of the literature that dealt with microbially induced calcium carbonate precipitation and co-precipitation of heavy metals and radionuclides. It also includes a discussion of the factors that may affect the behaviour of these bioprocesses.

Chapter 3: Methodology, includes a listing of all methods and materials that were used in this study to perform all experiment. It also includes describing the characteristics of used microorganism and sand fractions.

Chapter 4: Bacteria inhibition by metals toxicity, investigates how toxicity of a range of heavy metals (zinc, cadmium, copper, lead and strontium) affects growth of urease-positive bacterium, *Sporosarcina pasteurii*. It also determines the minimum inhibitory concentration (MIC) for each tested metal to the bacteria growth. According to bacteria tolerability, metals concentrations were specified in next experiments (bioprecipitation in aqueous solutions).

Chapter 5: Heavy metals bioprecipitation in aqueous solutions, explores the potential of calcium carbonate minerals to co-precipitate the tested heavy metals in aqueous solutions. It also, examines the ability of *S. pasteurii* to grow and operate in presence of high metals concentrations. Moreover, how the bioprecipitation mechanism can provide a protection to ureolytic microorganisms by decreasing the concentration of toxic metals. This was done through a serious of batch experiments.

Chapter 6: Bioprecipitation of heavy metals in particulate media, to discover the effects of heavy metals availability in soil, sand particle sizes, and heterogeneity of subsurface on bioprecipitation processes. This was done by a series of batch and column experiments. Finally, compares the performances of calcium carbonate bioprecipitation in aqueous solutions and particulate media.

Chapter 7: Conclusion and recommended future work, to present conclusions from this research study and recommendations for future work.

Chapter 2: Literature Review

2.1 Introduction

Contamination of subsurface with heavy metals and radionuclides is a significant environmental problem. Presence of these toxicants in concentrations more than their natural values may pose a real risk to biosphere after exposure (Duruibe *et al.* 2007; Singh *et al.* 2011). Environmental contamination with heavy metals and radionuclides has been treating by different physical, chemical and biological methods. Choosing any technique depends on the nature of metallic pollutants, contaminated environment and prearranged budget. However, most of physical and chemical handling processes are not cost effective, removing of pollutants in some methods is not completely, can damage soil structure and generating of toxic wastes need extra treatments and taking long time. Biological methods have may replace physical and chemical processes in this field especially when the biological treatments rely on the native organisms (Dixit *et al.* 2015). Many studies have referred to the potential of biological processes in remediating contaminated sites and their rules in hastening the sequestering of heavy metals and radionuclides for long term. The outputs of these studies imply promise for the stabilisation of polluted sites, which include soils and groundwater (Gadd 2000; Barkay and Schaefer 2001; Martinez *et al.* 2007; Prakash *et al.* 2013).

Recently extensive attentions have been paid on using biological treatments of pollutant rather than physical and chemical solutions. Many studies devoted their research to find organism less affected by the toxicity of heavy metals and radionuclides, where these processes have to be mediated by a live organism. Organisms can meditate different actions toward heavy metals and radionuclides, like

biosorption, bioaccumulation and biomineralisation (Dhami *et al.* 2013; Lauchnor *et al.* 2013; Phillips *et al.* 2013; Prakash *et al.* 2013; Reddy 2013; Kang *et al.* 2014). As heavy metals are non-degradable elements bioremediation processes work on either chemically transfer the formulas in which metals are toxic to non or less toxic, for example, reducing of toxic Cr(VI) to less toxicity Cr(III), or by decrease their bioavailability when sequester them for long term inside mineral structures, e.g. co-precipitating with carbonate minerals (Gadd 2000; Lloyd and Lovley 2001; Gadd 2002; Gadd 2010).

Apart from strontium, heavy metals and radionuclides precipitation with microbially induced calcium carbonate has received less attention. Strontium biomineralisation has studied extensively in lab and field scales (Ferris *et al.* 1987; Ferris *et al.* 1995; Warren *et al.* 2001; Fujita *et al.* 2004; Fujita *et al.* 2008; Fujita *et al.* 2010; Lauchnor *et al.* 2013). This metal shows no resistance to be precipitated or co- precipitated with calcium carbonates minerals, strontium is thought to behave in a comparable way as calcium in term of precipitating with carbonate (Warren *et al.* 2001). Its neither toxic to bacteria or inhibitor to calcium carbonate precipitation. Strontium biomineralisation features may not found in other heavy metals or radionuclides, where many metallic toxicants either toxic to microorganisms and/or inhibitor for calcium carbonate recrystallisation (Ferris *et al.* 1987; Ferris *et al.* 1995; Warren *et al.* 2001; Fujita *et al.* 2004; Fujita *et al.* 2008; Fujita *et al.* 2010; Lauchnor *et al.* 2013). The physical properties of strontium isotopes (radioactive and non-radioactive) are similar, as well as their identical chemical properties. Bunzl *et al.* (1999) and Tsukada *et al*. (1998) referred to the possibility of predicting the behaviour of a radioactive isotope by monitoring the stable one in the environment. Hence the study of nonradioactive strontium ions behaviour can mimic the behaviour of

radioactive isotope in the environment, however, metal radioactivity has to be considered (Mitchell and Ferris, 2005).

This chapter reports studies found in literature that dealt with microbially induced calcium carbonate precipitation and co-precipitation of heavy metals and radionuclides with these minerals in aqueous solutions and soil. Nevertheless, microbially-induced calcium carbonate precipitation technology has previously been considered in only a very limited fashion for removal of heavy metal pollution from aqueous solution. Hence, improve understanding of the parameters that relate to this technology can lead to increase its efficiency and to enable upscaling to suit in-situ applications.

2.2 Heavy metals and Radionuclides Contaminations

Heavy metals are elements with a specific gravity of or more than 5 g/cm^3. Metals are naturally occurring and non-degradable materials in the environment. Many metals within trace concentration are essential for microorganisms. The concentration and availability of heavy metals in subsurface environment depend on soil formation and local geology of parent materials. Pollution of soil and groundwater with heavy metals and radionuclides is of concern; especially when their concentrations become above natural levels. High concentrations of metals and radionuclide in environment may relate to the natural sources or manmade releasing of wastes from industries and mines. (McNeal and Balistrieri 1989; Duruibe *et al.* 2007; Young Jang *et al.* 2008; Viggi *et al.* 2009; Singh *et al.* 2011; Tchounwou *et al.* 2012; Prakash *et al.* 2013). Anthropogenic activities (industrial, agricultural, domestic and accidents) may consider the main source of subsurface and groundwater pollution with heavy metals and radionuclides. Metallic contaminants resulting from anthropogenic sources are

found to be more mobile and available than that resulting from natural sources in environment. Existence of heavy metals and radionuclides in food chain is of concern issues, which in turn caused many types of health problems, e.g. kidney cancer. Due to their persistence (non-degradable) and toxicity metals are posing real risk to environment (Marsily 1986; Wernick and Themelis 1998; Madsen 2003; Hashim *et al.* 2011; Lema *et al.* 2014). Several technologies (chemical, biological and physic-chemical) are working on remediation of metals and radionuclides released to soils and groundwater, to mitigate their potential risk.

Chemical remediation techniques mainly aim to reduce mobility and toxicity of heavy metals by converting them to less effect materials. This can be done by reduction, oxidation or neutralisation reactions. Although chemical treatment methods of heavy metals can be used for large contaminated area, but the used materials can destroy soil matrix. If the treatment ex-situ the exposure to extracted, handled and transformed toxicants may cause many health problems. Add to this many added chelates are cost effective (Kurniawan *et al.* 2006; United States Environmental Protection Agency 2006). Permeable reactive barriers (PRB) are a physio-chemical heavy metals treatment. Its media are placed across contaminations plume in subsurface environment, with less permeability than surrounding soil to enforce groundwater directing and passing through them. PRB media work on removing contaminations from groundwater by adsorption, degrading, precipitation or transforming, hence, loss of permeability due to precipitation is possible. However, this method can apply for shallow plumes (Kurniawan *et al.* 2006; United States Environmental Protection Agency 2006; Wilkin *et al.* 2008).

Biological treatments of heavy metals and radionuclides in contaminated soils or groundwater are achieved by exploiting specific microorganisms or plants. The vital factor in this kind of remediation is the ability of mediating organisms or plant to tolerate the toxicity of heavy metals or radionuclides. Immobilisation of heavy metals and radionuclides by microorganisms involves long term sequestration of metallic ions in groundwater as sulphides, phosphates or carbonates precipitations (Prakash *et al.* 2013). These processes include injecting required reagents, such as, nutrients, necessary chemical reactants and carbon source like molasses, acetate, lactate, and urea (United States Environmental Protection Agency 2006). Many in-situ remediation techniques work on deliver fluids containing treatment agents then recover and treat fluids containing pollutants. Therefore, the crucial issue in these methods is the hydraulic conductivity of contaminated soil, which play main role in affecting the delivered, and collected fluids (United States Environmental Protection Agency 2006). Hence, low permeability or clogging of contaminated site consider an obstacle in maintain any treatment process. In addition, heavy metals bioremediation in contaminated soils is especially challenging because of many metal cations high affinity to adsorption sites on the surfaces of soil particles (Dhami *et al.* 2013).

2.3 Exploiting Bacteria to Precipitate Metals and Radionuclides

2.3.1 Sulphate-Reducing Bacteria (SRB)

Sulphate-Reducing Bacteria are heterotrophic, utilize organic carbon and sulphate as energy source, they are ubiquitous in environment and common in anaerobic conditions. SRB use sulphate as a terminal electron acceptor and organic matter as a terminal electron donor in bioprecipitation processes. This process defines as dissimilatory sulphate reduction. When sulphide produced in the presence of heavy

metals cation, the precipitation of metal-sulphide occurs due to the reaction between metal and sulphide ions, which in turn, reduce the concentration of metals and sulphide. Many field studies show that native SRB in polluted environments with metals and radionuclides capable to stimulate reduction of sulphate anaerobically, which in turn form insoluble sulphide solids of metals or radionuclides (Postgate 1979; Lee and Saunders 2003; Doshi 2006; Costa *et al.* 2008; Satyawali *et al.* 2010; Torres *et al.* 2012).

Choosing terminal electron donor or carbon source is the key in bioprecipitation processes. Many electron donors can be used to induce sulphate-reducing processes. Some of them lead to delay the induction period of processes. Furthermore, some of carbon sources are expensive (ethanol, lactate and hydrogen), whilst others are cheap (molasses and acetate). Therefore, using mixture for many carbon sources may be cost-effective and important in decreasing the induction period (Diels *et al.* 2010; Viggi *et al.* 2010). Szewczyk *et al.* (2007) found the most effective electron acceptor to induce SRB is ethanol, while molasses is good carbon source for SRB induction, but whey was not significant. Young Jang *et al.* (2008) showed in batch and column experiments that methanol mixed with glucose is an optimal carbon source to enhance the indigenous SRB in contaminated soils with arsenic and other heavy metals (Cd and Pb), also when applying extra carbon source and sulphate high rate of sulphate reduction occurred and in turn leads to remove high quantity of heavy metals. Using molasses, as carbon source supports growth of SRB, where pH increased and redox potential decreased, even though, it was not as efficient as lactate (Teclu *et al.* 2009). On the other hand, Szewczyk *et al.* (2007) consider ethanol the most effective carbon source to stimulate the bioprecipitation of metals with SRB. However, using high concentration of carbon source leads to produce methane in microenvironment in turn

acidity will increase. Acidity condition enhances releasing heavy metals ions from solid phase into aqueous phase. Conditions that support starting sulphate-reducing processes, are when SO_4^{2-} > 100 mg/L, pH between 4-8, redox potential <-200 mV and 25-35^{o}C is the best temperature range to incubate the bacteria (Postgate 1984; Diels *et al.* 2010).

2.3.2 Metals-Phosphate Bioprecipitation

Metal-phosphate bioprecipitation techniques include enzymatically mediate biomineralisation of heavy metals and radionuclides as metal phosphate. The role of bacteria (such as *Citrobacter species*) and other microorganisms to enhance metal precipitation arises when ionic product of metals and phosphate concentrations in bulk solution does not exceed the solubility product. Therefore, liberation of additional concentrations of phosphate leads to exceed the solubility products even in presence of low metal or radionuclide concentrations, which in turn promote the mineralisation (Macaskie *et al.* 1987; Macaskie 1990; Montgomery *et al.* 1995; Lloyd 2002; Valsami-Jones 2004; Das *et al.* 2008).

Unanimous agreement in the literature with respect to the ability of many microbes to precipitate heavy metals and radionuclides, as crystalline minerals, through uptake of these toxic materials onto their cell surfaces. When these microorganisms liberate high concentrations of HPO_4^{2-} by phosphatase activity, a class of enzymes that catalyse the hydrolysis of phosphate monoesters to give inorganic phosphate and an alcohol, result in exceeding in solubility products would be mineralise metals or radionuclides. Organic phosphate substances, like glycerol 2-phosphate, used as phosphate donor for precipitation such as $PbHPO_4$, $CdHPO_4$ or UO_2HPO_4 (Macaskie *et al.* 1987; Montgomery *et al.* 1995; Barkay and Schaefer 2001;

Boswell *et al.* 2001; Valsami-Jones 2004; Martinez *et al.* 2007; Dunham-Cheatham *et al.* 2011).

2.3.3 Metals-calcium carbonate Bioprecipitation

Calcium carbonate bioprecipitation is a bio-geochemical process involves precipitation of calcium carbonate and possibility of co-precipitation of heavy metals and radionuclides within soil matrices. Bioprecipitation of carbonate minerals has received relatively little attention although the potential of such minerals to sorb and sequester a range of metals has previously been considered. This method will be discussed in more details in the next sections.

2.4 Biomineralisation of Calcium Carbonate

Mineralisation is a widespread phenomenon in nature where minerals are formed by abiotic or biotic processes. When specific living organisms meditate this process precipitation formation can be much faster than that abiotic precipitation, this due to the effect of produced enzymes by the living organisms. Calcium carbonate precipitation is one of biomineralisation phenomenon where calcium and carbonate ions react to produce low soluble crystal in alkaline condition environments. These minerals are found in oversaturated environments with calcium and carbonate ions in waters and soils (Silva-Castro *et al.* 2015; Tobler *et al.* 2015). Precipitation of calcium carbonate is often stimulated by microorganisms. The process depends on many factors such as the concentration of calcium and carbonate ions (or their chemical sources), their concentration should be saturated. pH value of the microenvironment, where the mineralization processes take a place, needs to be in alkaline range. The activity of microorganisms plays a crucial role in speeding the recrystallisation

process. Nucleation sites is thought to be vital in initiating mineralisation processes, bacterial cells are expected to play the role of nucleation sites (Gadd 2000; Fujita *et al.* 2010; Dhami *et al.* 2013)

Ureolytic bacteria are able to produce enzymes (urease, urea amidohydrolase) that hydrolyses urea in water into carbonate and ammonia ions in short time. The hydrolysis process is 10^{14} times faster than chemical hydrolysis (Lloyd and Sheaffe 1973; Benini *et al.* 1999; Hausinger 2004). Urease is secreted by many organisms and plants, amounts produced are different from organism to another. Urease enzyme can be existed in two states intracellular and extracellular. Intracellular state when exists inside ureolytic microorganism cells, whilst extracellular state when releases from dead plants and microorganism cells, which is then adsorbed to soil colloids (Lloyd and Sheaffe 1973; Todar 2005). Urea hydrolyses intracellularly when urea molecules are inside bacteria or fungi cells. The enzyme is widespread in soil and groundwater, in the same context, ureolytic bacteria are around 50% of total soil bacteria (Lloyd and Sheaffe 1973; Todar 2005).

Equations below from (2.1) to (2.5) describe calcium carbonate precipitation after catalysing by a microorganism. Process starts when urea hydrolyses by urease enzyme secreted by specific organisms, then forming of carbonate and ammonia ions, in turn pH increases to reach a value more than 9.0. When supersaturating state occurred for calcium and carbonate ions at high pH environment, recrystallisation of calcium carbonate happens. The efficiency of process is considered 100% when all dissolved calcium and carbonate ions transfer into solid state calcium carbonate precipitate (Stocks-Fischer *et al.* 1999; DeJong *et al.* 2006; DeJong *et al.* 2010; Dhami *et al.* 2013). Also, Figure 2.1 is an overview shows how bacteria that mediate calcium

carbonate mineralisation hydrolysis urea and offer their cells as nucleation sites for recrystallisation for precipitation processes.

$$H_2N(CO)NH_2 + H_2 \xrightarrow{urease} NH_2COOH + NH_2 \quad \text{.....2.1}$$

$$NH_2COOH + H_2O \longrightarrow NH_3 + H_2CO_3 \quad \text{.....2.2}$$

$$H_2CO_3 \longleftrightarrow 2H^2 + 2CO_3^{2-} \quad (carbonate\ ions\ producing) \text{ .....2.3}$$

$$NH_3 + H_2O \longleftrightarrow NH_4^+ + OH^- \quad (pH\ increase) \text{ .....2.4}$$

$$Ca^{2+} + CO_3^{2-} \longleftrightarrow CaCO_3 \quad (precipitation) \text{ .....2.5}$$

Where K_{SP} (solubility product) in Equation (2.5) is 3.8×10^{-9}.

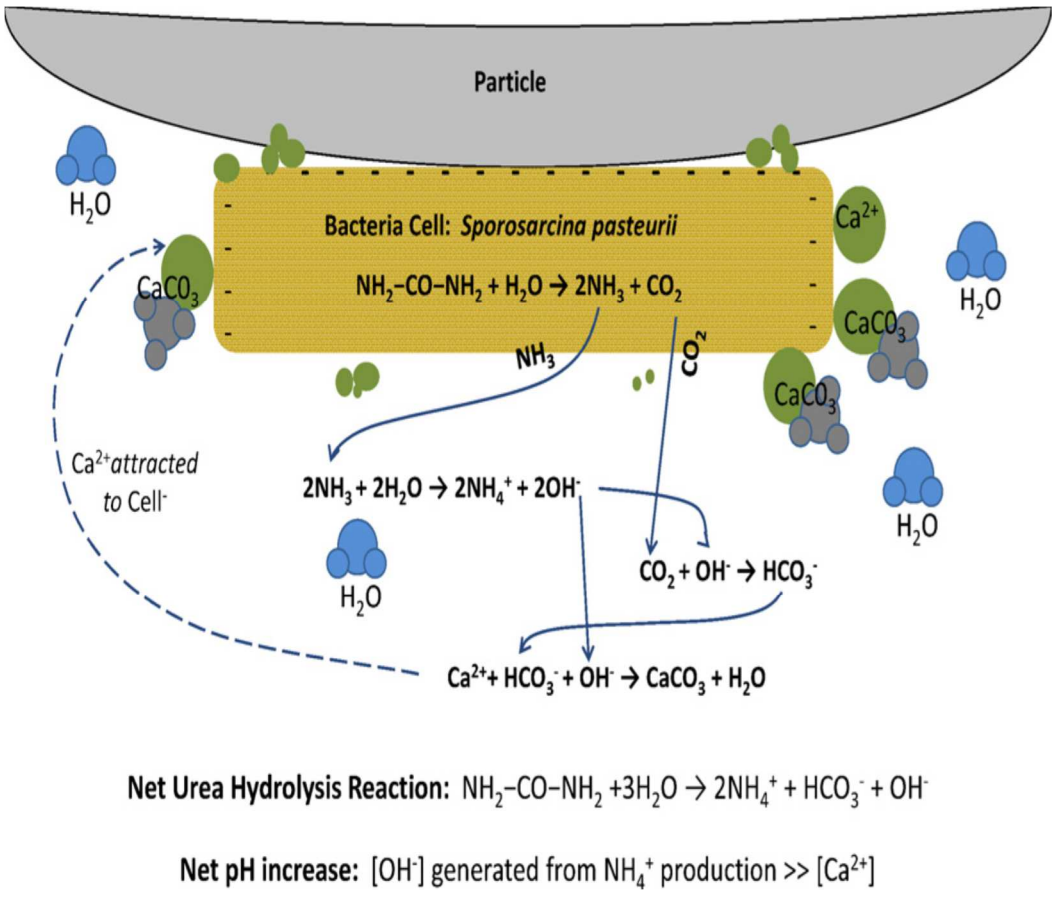


Figure 2.1. Overview shows how bacteria that mediate calcium carbonate mineralisation hydrolysis urea and offer their cells as nucleation sites for recrystallisation for precipitation processes (DeJong et al. 2010).

Bacterial cells themselves often act as nucleation points for the formation of minerals due to their typically overall negative charge attracting metal ions and therefore creating a zone of high metal ion concentration (e.g. calcium) (Tobler *et al.* 2011). The continuous precipitation of calcium carbonate around bacteria can inhibit or reduce their ureolytic ability due to encapsulation of the cells, hence, any decreasing in cell numbers may limited mineralisation processes. In addition, release of ammonia and ammonium ions is of potential environmental concern and subsequent biological action may cause acidification and potential dissolution of the precipitate (Fujita *et al.* 2010).

Calcium carbonate can precipitate in one of three anhydrous polymorphs calcite, aragonite and vaterite, where calcite is the most thermodynamically stable polymorph of calcium carbonates precipitation then aragonite. In addition, calcium carbonate can be produced in hydrate crystalline phases monohydrocalcite ($CaCO_3 \cdot H_2O$) and ikaite ($CaCO_3 \cdot 6H_2O$). However, hydrous phases are rare in nature, but they may play an important role during microbially calcium carbonate formation. Calcite is a widespread form of calcium carbonate because all other unstable forms transform to this polymorph with time. The possibility of producing a specific polymorph of calcium carbonate is controlled by recrystallisation conditions such as the degree of initial supersaturation, pH, temperature, adsorption of organic or inorganic impurities while crystallisation and solubility of that possible crystal (Brecevic and Kralj 2007; Ni and Ratner 2008). The size and morphology of precipitated calcium carbonate influence the mineral solubility and hence long-term immobilisation of co-precipitated contaminants. For example, strontium incorporation into a calcite lattice may result in a reduction in the durability of metal immobilisation by generating small and more soluble crystals (Mitchell and Ferris 2006; Tobler *et al.* 2011; Cuthbert *et*

al. 2012; Tobler *et al.* 2014). Calcite can take a shape of a hexagonal-rhombohedral structure, whilst aragonite may take orthorhombic or needles shapes. Vaterite which is the least thermodynamically stable polymorph of calcium carbonate has been reported to be spherical shape after formation. Kang *et al.* (2014) reported that mineral crystals produced by ureolytic bacteria can take various shapes, such as, roughly rhombohedral, spherical, or needle-shaped, size of the produced solids is generally from 10-50 μm. Figure 2.2 shows polymorphs of calcium carbonate precipitation.

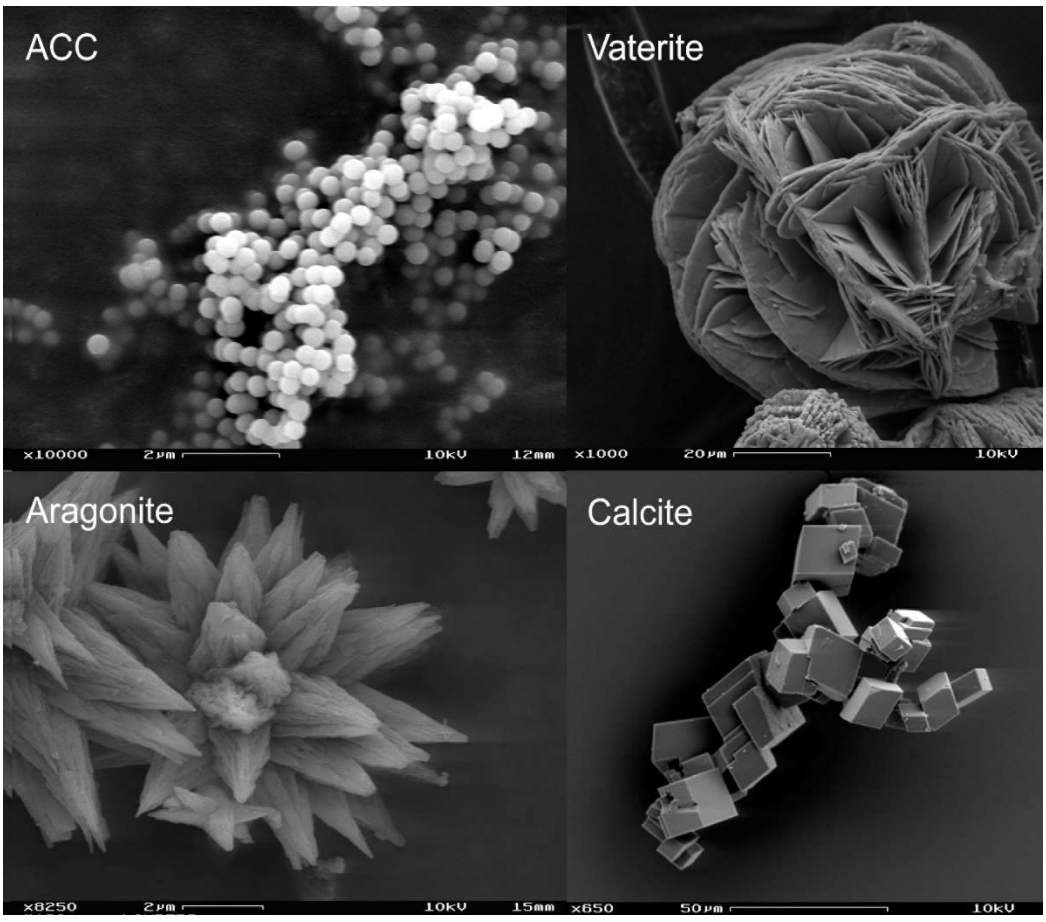


Figure 2.2. Three polymorphs of calcium carbonate precipitation, calcite, aragonite, vaterite and hydrous phase ACC (amorphous calcium carbonate). Adapted from (http://www.ruhr-uni-bochum.de/sediment/forschung.html).

Many studies reported the mineralogy of calcium carbonate precipitation induced by ureolytic bacteria as aragonite, calcite and vaterite (Rivadeneyra *et al.* 1996;

Stocks-Fischer *et al.* 1999; Fujita *et al.* 2000; Fujita *et al.* 2004; Mitchell and Ferris 2005; Fujita *et al.* 2008; Chahal *et al.* 2011; Abo-El-Enein *et al.* 2012; Achal *et al.* 2012a; Achal *et al.* 2012b; Vahabi *et al.* 2015; Wei *et al.* 2015). Most of these studies reported that the produced crystals were predominantly calcite. However, Rivadeneyra *et al.* (1996) reported that calcium carbonate crystals produced in their experiments were polymorphous aragonite. Also, Achal *et al.* (2012a) and Achal *et al.* (2012b) in their studies of heavy metals biomineralisation with calcium carbonate precipitation found that XRD spectra showed presence of aragonite as well as calcite and vaterite precipitated in soil samples. Hausinger (2004) noticed that zinc ions influenced calcium carbonate bioprecipitation where the polymorphs produced were monohydrocalcite and vaterite, whilst calcite was mineralised in a medium free of metal ions. Most of research that studied microbially induced calcium carbonate precipitation technique to be used for purposes other than heavy metals remediation, such as crack healing, soil improvement and groundwater control, they described precipitation solids of calcium carbonate as calcite (DeJong *et al.* 2006; Whiffin *et al.* 2007; DeJong *et al.* 2010; Harkes *et al.* 2010; Dejong *et al.* 2013). Fujita *et al.* (2008) stated that XRD analysis indicated that the precipitated crystals were predominantly calcite, during strontium co-precipitation process with calcium carbonate precipitation. Suggesting that co-precipitation of heavy metal divalent by microbially induced calcium carbonate precipitation technique can influence the shape of resulting crystalline polymorphs of carbonate.

Furthermore, there are temporal changes that occur due to the continual formation of calcium carbonate over time including chemical changes such as removal of mineral components (e.g. calcium) from solution and production of new materials such as ammonium. These conditions may also vary in the longer term, e.g.

ammonium produced via urea hydrolysis may become oxidised leading to acidic conditions (Reed *et al.* 2010). Physically the resulting precipitation in porous media can block pore throats, which leads to obstruction of the transport and mixing of delivered reactants (Gebrehiwet *et al.* 2012). Fujita *et al.* (2010) found that urea hydrolysis processes in situ are not homogeneous, mainly due to the heterogeneous distribution of microorganisms. The heterogeneous distribution of bacterial activity in the subsurface environment may therefore lead to non-uniform mineralisation of target contamination, and so pose problems for design of biomineralisation treatment systems in particulate media (Mockaitis *et al.* 2012).

2.5 Applications of Calcium Carbonate Mineralisation

Microbially induced calcium carbonate techniques have been studied extensively in different fields. Applications in biocementation of porous media, bioclogging of soil voids and crack healing have received high attention (DeJong *et al.* 2006; Whiffin *et al.* 2007; DeJong *et al.* 2010; Harkes *et al.* 2010; Dejong *et al.* 2013). However, apart from strontium mineralisation, bioprecipitation of divalent metals with carbonate minerals has received relatively little attention though such minerals have the potential to sorb and sequester a range of heavy metals and radionuclides. Understanding and developing this technique may lead to upscale lab finding to in situ applications for long-term sequestration of heavy metals and radionuclide in contaminated subsurface environment (Fujita *et al.* 2004; Fujita *et al.* 2008; Fujita *et al.* 2010).

In term of ground improvement DeJong *et al.* (2006) stated that microbially induce calcium carbonate can be applied in many applications, such as, improve subsurface prior to tunnelling, construction settlement decreasing, slope stabilisation and dams.

Whiffin *et al.* (2007) reported that precipitation of calcium carbonate can improve soil strength without making it impermeable, by balancing urea hydrolysis and reagents delivery via the flow rate in soil to create calcium carbonate precipitation where it is required. In addition, many mechanical properties of soil (cohesion, friction, rigidity and permeability) can be improved by this technique (Harkes *et al.* 2010).

Microbially induce calcium carbonate precipitation offers a potential for in-situ sequestration and subsequent reduction in the bioavailability of heavy metals and radionuclides in the subsurface environment. Calcium carbonate minerals are known to sorb and form solid solutions with a range of target elements, and are readily produced by the actions of common microorganisms on simple chemical precursors (Ehrlich 1996; Buekers *et al.* 2007; Achal *et al.* 2009; Khosravi and Alamdari 2009; Fujita *et al.* 2010; Achal *et al.* 2012a; Achal *et al.* 2012b; Kang *et al.* 2014). In addition, the widespread of urease bacteria in subsurface environment, and the formation of calcium carbonate is a common phenomenon in nature, makes heavy metal sequestering by using the precipitation of this mineral an attractive technique for many researchers (Fujita *et al.* 2000). Traditional methods of using pump and treat are found to be less effective in remediating contaminated groundwater with heavy metals (Fujita *et al.* 2008; Dhami *et al.* 2013). Also, using other techniques other than bioprecipitation may be not preferable, for example, it was found that the treatment of strontium contaminated sites in USA by using pump and treat scheme lead to enhance the sorption of contaminants on solid phase, while they should be dissolved in extracted solutions. Hence, the treatment takes long time may reach up to decay time in case of radioactive strontium (half-life =29 years) (Fujita *et al.* 2010).

Co-precipitation of a number of heavy metals of interest with calcium carbonate has been reported, both in natural and engineered situations and through biotic and abiotic processes, including arsenic (Achal *et al.* 2012a), cadmium (Kang *et al.* 2014), zinc and nickel (Buekers *et al.* 2007), copper (Khosravi and Alamdari 2009a), lead and a range of others contaminants, including radionuclides, such as strontium (Fujita *et al.* 2000; Fujita *et al.* 2004; Fujita *et al.* 2008; Fujita *et al.* 2010), cobalt and uranium (Ehrlich 1996; Fujita *et al.* 2010). Buekers *et al.* (2007) also reported that cadmium and zinc may adsorb to, and diffuse into, calcium carbonate minerals.

Strontium has previously been a target for remediation via calcite biomineralisation, this due to its presence on certain sites as a radioactive heavy metal contaminant. It is not redox sensitive, and its availability in the subsurface environment is primarily affected by sorption and abiotic precipitation in high pH environment with some minerals.

Many research achieved by Fujita *et al.* (2000), Fujita *et al.* (2004), Fujita *et al.* (2008) and Fujita *et al.* (2010) aimed to improve remediation technique of metallic contaminations by microbially induced calcium carbonate in subsurface environment. Fujita *et al.* (2000) investigated the availability of microorganisms, in Eastern Snake River Plain (ESRP) aquifer in Idaho, that enable to hydrolysis urea for exploiting them in heavy metals sequestration by microbially induced calcium carbonate. Fujita *et al.* (2004) considered their study important contribution to what had been achieved in this field (Ferris *et al.* 1995; Warren *et al.* 2001). This study concluded that strontium incorporation into calcium carbonate can be increased at high rate of calcite precipitation, which is resulted from the high rate of urea hydrolysis that catalysed by bacteria. In addition, the research reported that the incorporation of strontium ions

into calcium carbonate occurs in the form of solid solution, which means high stability of this metal inside calcite structure.

2.6 Precipitation and Co-Precipitation of Heavy Metals and Radionuclides with Calcium Carbonate

Bioremediation of heavy metals and radionuclides in subsurface environment has recently received wide attention and many studies on several bacteria types have been conducted. Metallic contaminants bioprecipitation with calcium carbonate is considered a potential in which suitable metallic cations are incorporated into the mineral lattice (Warren *et al.* 2001; Dhami *et al.* 2013). Urea hydrolysis leads to elevated pH and an increase in carbonate ions, both of which increase the likelihood of calcium carbonate precipitation in presence of calcium ions (Gebrehiwet *et al.* 2012). The ability of calcium carbonate precipitation to co-precipitate a number of heavy metal species, including cadmium, zinc, copper, lead and strontium, makes sequestration of such contamination by co-precipitation with this mineral an attractive technique (Fujita *et al.* 2000). Ammonium ions can exchange heavy metals ions and other ions like calcium on grain surfaces in subsurface environment, and this in turn increases the bioavailability of these heavy metals (Wu *et al.* 2011). On other hand, the carbonates ions promote the precipitation of calcium carbonate and co-precipitation of heavy metals in high pH environments (Fujita *et al.* 2004; Fujita *et al.* 2010; Wu *et al.* 2011). Figure 2.3 shows a conceptual diagram explains different stages of the process, urea hydrolysis by urease enzyme that secreted by *S. pasteurii* strains, cations exchange, calcium carbonate precipitation and heavy metal co-precipitation with crystals precipitation.

The ability of ureolytic bacteria (*S. pasteurii*) to induce the precipitation of calcium carbonate minerals offers a bioremediation means by which divalent heavy metals and radionuclides can be sequestered for long-term in subsurface environment. The remediation mechanism is based on capturing possibility of metallic cations during calcium carbonate recrystallization processes. Providing that solid solution can be created from calcium carbonate and heavy metals cations, when these cations replace calcium ions in the lattice structure, see equation 2.6 (Wu *et al.* 2011).

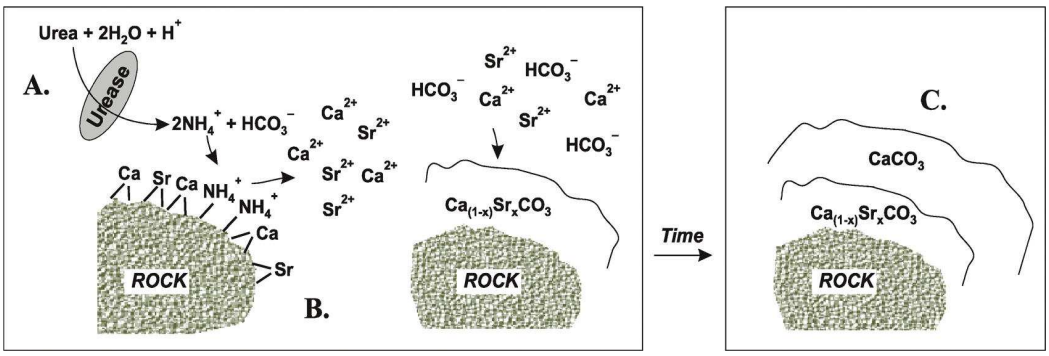


Figure 2.3. Conceptual diagram explains A- urea hydrolysis by urease enzyme that secreted by S. pasteurii strains. B- Cations exchange, Calcium carbonate precipitation and heavy metal co-precipitation with crystals. C- Precipitation and co-precipitation processes (Fujita et al. 2010).

$$xMe^{2+} + (1-x)Ca^{2+} + 2HCO_3^- \longleftrightarrow Ca_{(1-x)}Me_xCO_3 + H_2O + CO_2 \quad \ldots\ldots 2.6$$

Where*:* Me^{2+} is the metallic cations.

2.6.1 Abiotic Co-Precipitation

Limited studies have referred to the role of divalent metals and calcite interaction in sequestering these toxic material for long term in subsurface. The interaction includes uptake metal cation from solution which probably leads to make solid-solution in calcium carbonate minerals. Solid solutions of metals and carbonate minerals are formed when divalent metals substitute calcium ions in the lattice

structure of calcium carbonate (Stipp *et al.* 1992; Fujita *et al.* 2000). Some cations are found relatively readily substitute calcium ions in carbonate minerals, such as, strontium and cadmium, whilst some of them may not like zinc (Zachara *et al.* 1991; Fujita *et al.* 2000; García-Sánchez and Álvarez-Ayuso 2002). Tesoriero and Pankow (1996) found in their investigation a great affinity of cadmium ions to be co-precipitated by calcite precipitation. According to the calculations in this study the distribution coefficients (Equation 2.7) for divalent metal ions for partition from solution into calcium carbonate of strontium, barium and cadmium were 0.021±0.003, 0.012±0.005 and 1240±300, respectively. The high value of cadmium distribution coefficient reflects the strong retarding of its migration with groundwater in subsurface environment saturated with calcium carbonate.

$$D_{Me} = (X_{MeCO_{3(S)}}/[Me^{2+}])/(X_{CaCO_{3(S)}}/[Ca^{2+}])\ldots\ldots\ldots 2.7$$

where: D_{Me} is the distribution coefficients, X values are solid-phase mole fractions and $[Ca^{2+}]$ and $[Me^{2+}]$ are aqueous molal concentrations for calcium and heavy metal, respectively (Tesoriero and Pankow 1996).

In addition, other studies reported the importance of carbonate minerals as sorbents for divelant heavy metals in nature. Davis *et al.* (1987) found that cadmium adsorption on calcium carbonate is irrevesible unless large change in pH occurs. In soil and groundwater calcite has significant role in sequstering many divelant metals (Zachara *et al.* 1991). It was reported that calcite can decrease metal ions in aqueous solutions (Stipp *et al.* 1992; Sturchio *et al.* 1997; Parkman *et al.* 1998; Shahwan *et al.* 2005; Adekola *et al.* 2012). Calcite minerals was suggested to be used for a cost-effective treatment to purify industrial effluents from metallic cations (García-Sánchez and Álvarez-Ayuso 2002). Khosravi and Alamdari (2009b) achieved 78%

removal efficiency of copper from field brine by co-precipitation process with calcium carbonate, when copper concentration decreased from 0.27 ppm to 0.06 ppm. However, in Khosravi and Alamdari (2009b)'s study only 5% of copper total concentration was incorporated with crystal lattice during the precipitation process, whilst 95% of that concentration was removed through the adsorption on calcium carbonate surfaces. However, Buekers *et al.* (2007) thought that copper removal in soil was poorly related to the carbonate presence, however, the decreasing of cadmium, zinc and nickel concentrations in soil may attribute to presence of calcium carbonate minerals, also, removal of these metal ions in soil increase with pH and carbonate concentration.

2.6.2 Biotic Co-Precipitation

Apart from strontium, no field studies have done *in situ* to remediate heavy metals or radionuclides by using *S. pasteurii* bacterium induced calcium carbonate (according to available literature). Warren *et al.* (2001) reported the possibility of precipitate strontium, Uranium dioxide and copper ions by *S. pasteurii* induced calcium carbonate mineralisation. The removal of strontium by this technique was very effective (95%), whilst only 30% of uranium dioxide were sequestered. In contrast, copper removal was very poor, authors attributed that to toxicity of the heavy metal to the bacteria which in turn inhibited urease production and associated urea hydrolysis and then calcium carbonate formation. Achal *et al.* (2012a) investigated the role of microbially induced calcium carbonate by arsenic tolerant bacterium *Sporosarcina ginsengisoli* to sequester this highly toxic metalloid from contaminated soil. Achal *et al.* (2012a) considered the key in this remediation processes is the ability of this bacteria to produce significant amount of urease enzyme, that is important to

initiate calcium carbonate precipitation, though high concentration of arsenic was used, up to 50 mM.

In a study by Li *et al.* (2013a) high removal proportions for heavy metals (zinc, cadmium, copper and lead) were conducted by *S. pasteurii* induced carbonate mineralisation. However, no calcium was supplied in precipitation medium where produced minerals were pure metal carbonates. Also, no control samples were considered in this experiment though abiotic precipitation could not be ruled out in kind of experiments. Kang *et al.* (2014) examined the removal of cadmium ions in aqueous solution by using microbially induced calcium carbonate precipitation technique, they achieved high rate removal (99.95%) of the metal after 48 hours.

Strontium has previously been a target for remediation via calcite biomineralisation (Ferris *et al.* 1987; Fujita *et al.* 2000; Fujita *et al.* 2004; Fairbrother *et al.* 2007; Fujita *et al.* 2008; Fujita *et al.* 2010; Gebrehiwet *et al.* 2012; Lauchnor *et al.* 2013; Tobler *et al.* 2014), this due to its presence on certain sites as a radioactive heavy metal contaminant. It's not redox sensitive, and its availability in the subsurface environment is primarily affected by sorption and abiotic precipitation in high pH environment with some minerals (Fujita *et al.* 2000). In the case of radioactive strontium, the sequestering of its ions inside the calcite structure would reduce almost all its radioactivity, because metal cations substitute relatively readily for calcium ions in a calcium carbonate structure. Mitchell and Ferris (2005) recommended using microbially induce calcite precipitation and strontium precipitation in groundwater remediation.

Mitchell and Ferris (2005) achieved a set of experiments on non-radioactive isotope of strontium to mimic the radioactive isotope, to study the partitioning of this

metal with artificial groundwater. They determined the calcium carbonate mineralisation increases with calcite saturation state regardless of test temperature (10, 15 or 20 ºC). These outcomes are consistent with earlier studies by Lorens (1981) and Mucci and Morse (1983). Mitchell and Ferris (2006) proved the effect of strontium ions when incorporated with calcium carbonate structure that catalysed by *S. pasteurii*. The crystals produced by biomineralisation processes without strontium were up to 1000 nm in diameter, whilst crystals produced by mineralisation of calcium carbonate in a medium containing strontium were smaller up to 850 nm. However, this may result in a reduction in the durability of metal immobilisation by generating small and more soluble crystals.

Fujita *et al.* (2004) concluded that strontium incorporation into calcium carbonate can be increased at high rate of calcite precipitation, which is resulted from the high rate of urea hydrolysis that catalysed by bacteria. Furthermore, the research reported that the incorporation of strontium ions into calcium carbonate occurs in the form of solid solution, which means high stability of this metal inside calcite structure.

A field study was done by Fujita *et al.* (2008) in Eastern Snake River Plain (ESRP) aquifer in Idaho, started with injecting dilute molasses three times over two weeks to stimulate overall bacterial cells growth in groundwater through a single well experiment. This led to increase the cells up to 2 orders of magnitude. Then to initiate calcium carbonate precipitate in high rates, and promote immobilising of metallic contaminates such as strontium, urea was injected for only one time. Fujita *et al.* (2008) proved that introducing molasses and urea consecutively can stimulate the hydrolysis of urea and promote calcium carbonate precipitation. However, the authors reported the limited information that were gained regarding the distribution of

precipitation and variations in permeability in treated field. Fujita *et al.* (2000) studied the capability of indigenous microorganisms at the Hanford 100-N Area in Washington, to catalyse urea hydrolysis and promote calcite precipitation and then immobilise strontium cations that contaminated the site. However, they found that the effects of heterogeneity in subsurface environment can pose a great challenge when design any treatment system based on microbially induced calcium carbonate. The spatial differentiation in subsurface environment controls the distribution of bacterial activity, and in turn the bioprecipitation processes that associated with bacteria.

Lauchnor *et al.* (2013) did an important study where the differences between pulsed and continuous flow systems of biomineralisation medium into porous media reactors were stated. This study aimed to achieve *S. pasteurii* induced calcium carbonate precipitation and strontium co-precipitation by using two injection strategies (pulsed and continuous). As pulsed scenario involved high velocity fluid injection and then no-flow over sequential periods for 60 days, it led to increase the mass and efficiency of calcium carbonate precipitation compared with continuous flow. Pulsed flow system can prevent extensive plugging over long period and control the distribution of bacterial activity. However, the efficiency of strontium co-precipitation from both flow systems was almost similar. Fujita *et al.* (2004) studied the distribution coefficient of strontium in calcium carbonate enhanced by ureolytic bacteria. They found this factor is higher than natural calcite which is due to precipitation of calcite induced by the ureolytic bacteria is more rapid. Fujita *et al.* (2004) concluded that the high rate precipitation of calcium carbonate can motivate the co-precipitation of strontium with calcite lattice, which has been proven in many earlier studies (Ferris *et al.* 1995; Warren *et al.* 2001; Gebrehiwet *et al.* 2012).

2.7 Factors Affecting Co-Precipitation of Heavy Metals and Radionuclides with Calcium Carbonate

The co-precipitation of heavy metals with calcium carbonate is controlled by a number of factors. Among these factors are bacteria type and ability to hydrolyse urea, bacterial cells, nutrient and reagents concentrations, pH and temperature, injection of nutrients and chemicals into contaminated soils, distribution of bacterial cells in soil, precipitation rate, bioavailability and toxicity of heavy metals and the effect of subsurface heterogeneity on bacterial cells and solute transport (Lorens 1981; Pingitore Jr and Eastman 1986; Dixit *et al.* 2015). Toxicity of heavy metals may hinder *Sporosarcina pasteurii* to play its role in hydrolysing urea to derive calcite precipitation in presence of calcium ions (Soejima *et al.* 2010). The high affinity of cations to be absorbed on granular surfaces of soil makes them unavailable for precipitation processes. Moreover, the inherent heterogeneity of subsurface environment leads to non-homogenous distribution of the bioprecipitation activities.

Contaminants usually are moving in subsurface environment taking plume shape over long distances (vertically and horizontally). Potential hazards (toxic) of metals and radionuclides, in subsurface environment, arises when their presence in solution as ions, such as Zn^{2+}, Pb^{2+}, Cr^{2+}, As^{2+}, Cd^{2+}, UO_2^{2+}. Soluble metal ions travel with groundwater flow through soil matrix. The mobility of metals and radionuclides depend on many factors, redox reaction, pH, precipitation, dissolution, sorption to solid phase in soil, ion exchange and others. Redox potential and pH consider the most effective factors, because they are strongly affect the species formation, precipitation and sorption to solid phase (Marsily 1986; Faure 1991; Wernick and Themelis 1998;

Maier *et al.* 2000; Lee and Saunders 2003; Tsezos 2007; Viggi *et al.* 2009; Hashim *et al.* 2011).

2.7.1 Type of Bacteria

Choosing suitable type of bacteria is crucial matter in achieving efficient biomineralisation processes. This choosing based on the amount of urease enzyme (in case of using urea as a nitrogen source in carbonate bioprecipitation), which is very important in catalysing urea hydrolysis, can be produced by that urease positive bacteria, such as genera *Bacillus, Sporosarcina, Sporolactobacillus, Clostridium* and *Desulfotomaculum*. The tolerability to heavy metals and radionuclides toxicity should be considered when choosing specific bacteria to bioremediation processes. Urease positive strains *Sporosarcina pasteurii* formerly *Bacillus pasteurii* is a widespread, gram-positive, endospore-forming soil bacterium and non-pathogenic and produce high amount of urease enzyme which is up to 1% of cell dry weight (Hausinger 1993). For this reason and for high resistance to chemical and physical agents, the strain is widely used in research. Many studies stated that the rate and amount of calcium carbonate mineralisation are associated with urease concentration (Fujita *et al.* 2000; Fujita *et al.* 2004; DeJong *et al.* 2006; Whiffin *et al.* 2007; Harkes *et al.* 2010; Dejong *et al.* 2013). Stocks-Fischer *et al.* (1999) concluded that the *Bacillus pasteurii* can raise the pH solution to alkaline range (8-9) favouring the mineralisation of calcium carbonate, as well as the bacterial cells can act as a nucleation sites for calcium carbonate precipitation (Ercole *et al.* 2007, Mitchell and Ferris 2006).

2.7.2 Concentrations and Distribution of Chemicals and Biomass

The concentrations of required reagents (calcium and carbonate or their sources) and nutrients are vital parameters to be considered. Without supplying sufficient chemicals or nutrients can lead to terminate or deactivate the bioprocesses. The initial concentration of mediated microorganisms should be considered as well to maintain the mineralisation processes. Efficient supplying of energy sources and nutrients are important to sustain active number of bacterial cells (Ferris *et al.* 1987; DeJong *et al.* 2010; Al Qabany *et al.* 2012; Phillips *et al.* 2013). Fujita *et al.* (2008) and Tobler *et al.* (2011) reported that the urea hydrolysis and calcium carbonate precipitation are increased with increasing the number of microorganisms and the initial urea concentration. Whiffin (2004) investigated the effect of urea, calcium and ammonium concentrations on *S. pasteurii* urease activity. The author noticed that high concentrations of urea (>1.5 M) and calcium (2 M) can inhibit urease activity, whilst the presence of high concentration of ammonium (3 M) may not affect the enzyme. Okwadha and Li (2010) stated that calcium carbonate deposition is proportional with the concentrations of urea, calcium and bacterial cells. They considered 666 mM urea, 250 mM calcium and 2.3×10^8 cells/ ml bacterial cell concentrations (at 25ºC) are the optimum conditions to produce calcium carbonate.

The introducing methods of these materials into the contaminated site or simulation system should ensure, to high extent, effective and efficient distribution in treated medium. Injection of reagents, catalysis and bacteria all one time favour rapid crystal growing near injection points, which in turn clog the locations around these injection points. Whiffin *et al.* (2007) and Harkes *et al.* (2010) recommend that injection of bacteria solution to be before chemical and nutrient solutions, may lead

to homogeneous distribution of $CaCO_3$ precipitation. Al Qabany *et al.* (2012) mentioned that one of the factors that stimulate bacterial cells adsorption in soil is the salinity of bacteria solution. Torkzaban *et al.* (2008) showed that the more salinity bacteria solution, the more adsorption of bacteria cell on soils particles. The author reported that injection bacteria suspension into soil then washing out these soils with low salinity solution resulting in mobilise the bacteria from solid phase to liquid phase, which lead to heterogeneous distribution of biological activity.

2.7.3 Temperature and pH

Temperature and pH are parameters influence bacteria function and urease activity in calcium carbonate precipitation processes. The optimum growth temperature for *Bacillus Megaterium* in soil that makes it suitable for calcium carbonate precipitation was found to be 30°C (Benini *et al.* 1999). Baskar *et al.* (2006) found optimum temperature for calcite precipitation by *Bacillus thuringiensis* and *Bacillus pumilis* is 25°C. Optimum growing pH for *S. pasteurii* is 9.0 (Vig *et al.* 2003; Whiffin 2004).

Urease enzyme works actively on hydrolysing urea at a certain range of pH values. Stocks-Fischer *et al.* (1999) and Evans *et al.* (1991) stated that, based on the results of their study, urease activity increased with pH, where highest activity was noticed at pH of 8.0. More precisely, urease activity is significantly higher at the pH values where calcium carbonate mineralisation is favourable. Urease enzyme may also influence by temperature of the medium. Mitchell and Ferris (2005) reported the positive relationship between the rate of urea hydrolysis and temperature, where optimum temperature range at which urease enzyme be active is from 20-37ºC. However, urease enzyme can be inhibited at high temperature degrees. The rate of calcium carbonate precipitation increases 5 times when increase temperature from 15

to 20ºC, due to increase urease activity. Moreover, urease enzyme activity of *S. pasteurii* increases with temperature range from 20-50ºC, whilst no change occurs between 50-70ºC, then activity decreases after increasing temperature above 70ºC (Okwadha and Li 2010; Li *et al.* 2013b).

2.7.4 Type of Soil

Soil engineering properties (cohesion, friction, stiffness and hydraulic conductivity) are very important to be considered when studying microbial induced carbonate precipitation and co-precipitation of heavy metals and radionuclides. Each site has its specific properties, which depends on historical sedimentary conditions (Harkes *et al.* 2010). Differentiation in soil fractions size means different value for hydraulic conductivity which in turn leads to heterogeneous distribution of chemicals and nutrient. Hence, biomineralisation process may not take place in fine fractions of soil due to very low hydraulic conductivity or it take long time in coarse sand fractions where hydraulic conductivity very high which leads to flush out the reagents or bacterial cells in these kind of soils. In addition, incidence of preferential flow can lead to transfer these material away from they were needed (Harkes *et al.* 2010; Al Qabany *et al.* 2011; Dixit *et al.* 2015). Also, soil adsorption capacity can limit heavy metals availability in porous media, this in turn leads to limit metals biomineralisation.

2.7.5 Bioavailability of Heavy Metals and Radionuclides

Bioprecipitation processes may be affected by the available amount of heavy metals. Metals in subsurface environment are subjected to physical, chemical and biological processes lead to affect their concentrations and change speciation. Among these processes are complexation, precipitation, adsorption on solid phase, and to be

taken up by microorganisms. Behaviour of metallic ions is a function of conditions in their new environment (Scow and Johnson. 1997; Alexander 1999; National Research Council 2003a). Nonetheless, the available quantity of any metal ions will subjected to increase or decrease in concentration at any time due to soil type, cation exchange capacity, pH change, metal speciation, ageing, absorption or desorption, competition with other sorbed ions, nature of contaminants have been released and the nature of the microorganisms in the polluted site (Vig *et al.* 2003). National Research Council (2003b) reported that the total concentration of contaminants may be not reflect the actual risk behind these contaminants as their bioavailability are low. Bioavailability of specific contaminant, in liquid or solid phase of soil, is that fraction which is available to be taken by microorganisms.

Adsorption is considered the main process that leads to loss cations from soil aqueous solutions, especially at low concentrations of heavy metals. Fe, Mn and Al oxides and organic matter are the main adsorbents for many metallic cations in soil. Metals adsorption to Fe, Mn and Al oxides would be occurred by chemisorption. Many research correlated the adsorption of heavy metal ions to the presence of organic matter in soil (Bradl 2004; Choi 2006; Lafuente *et al.* 2008; Usman 2008; Gunawardana *et al.* 2015). Fe_2O_3, Al_2O_3, $CaCO_3$ and organic matter have high adsorption capacity to adsorb cadmium (Stietiya and Wang 2014). Fe_2O_3 has a great influence on cadmium geochemical behaviour. $CaCO_3$ is able to adsorb heavy metals actively. Organic matter occurs in soil as colloids or coating soil grains. The mechanisms by which the adsorb heavy metals are physical adsorption, electrostatic attraction, hydrogen bonding and co-ordination complexes. Their presence affects heavy metals distribution in soil (Bradl 2004; Lafuente *et al.* 2008; Stietiya and Wang 2014; Zhao *et al.* 2014). Cadmium ions in soil can make surface complexes on silicate,

iron and manganese oxides and organic matter layers. Removing metal oxides and organic matter makes soils with less affinity for retaining of cadmium ions. Calcium carbonate minerals and organic matter in soil with high pH (>6) are able to adsorb cadmium strongly. Cadmium chemisorption at low concentration by $CaCO_3$ is possible and very rapid, while at high concentration of this heavy metal the possibility of $CdCO_3$ is more possible (Bradl 2004; Choi 2006; Stietiya and Wang 2014; Zhao *et al.* 2014). Park *et al.* (2011) estimated the high affinity of inorganic compounds to retain cadmium ions. Zinc is adsorbed preferentially to Fe, Mn and Al oxides in soil. Many research proved the high affinity of zinc ions to inorganic compounds and organic matter (McLean *et al.* 1992; Gunawardana *et al.* 2015).

2.7.6 Toxicity of Heavy Metals to Microorganisms

Bioprecipitation processes depend mainly on microorganisms activity in presence of the target heavy metals inhibition of this activity leads to terminate the action of bacteria-based bioremediation. Microorganisms show variability in their response to heavy metals toxicity. Minimal inhibitory concentration (MIC) of a metal is the concentration that inhibits the growth of a microorganism by >70% compared to its optimum growth (Ruggiero *et al.* 2005). One of the important factors that affects bacterial tolerance to heavy metal, is the bioavailability or solubility of that metal. This availability may increase with decreasing solution pH. The possibility of passing the outer membrane of any microorganism by metal ions increases with increasing the solubility (Nies and Silver 1995; Nies 1999).

Bacteria have evolved many mechanisms to detoxify heavy metals when they entering the cell, through efflux, complexation, or reduction of metal ions or to use them as terminal electron acceptors in anaerobic respiration. Boularbah *et al.* (1992)

concluded that gram-positive bacteria have a developed resistance to cadmium accumulation in cells, metal toxicity can be reduced in presence of manganese ions. The outer membrane plays an important role as a block toward heavy metals cations. Many studies estimated MIC of many metals to *Bacillus sp.*, but not *S. pasteurii* (Ruggiero *et al.* 2005; Murthy *et al.* 2011; Karakagh *et al.* 2012; Samanta *et al.* 2012; Ankita *et al.* 2013; Farshid *et al.* 2013; Rathnayake *et al.* 2013).

Among many heavy metals cadmium was found the most toxic to bacteria. Cadmium is one of the deleterious heavy metals to bacteria; this natural metal contaminates soils after releasing from zinc ores or other industries, such as, electroplating, electronic parts and mining. Cd has no known biological function to bacteria, toxic to microorganisms even with very low concentrations.

2.7.7 Subsurface Heterogeneity and Preferential Flow

Spatial heterogeneity is an inherent character of the soil, it plays a main role in creating great differences in distribution the physical, chemical and biological activities in soil. Many factors are involved in shaping this diversity, such as, geological and biological processes and soil using (Kamnev *et al.* 2000; Franklin and Mills 2009; Vos *et al.* 2013). Soil in microscale is a large number of microenvironments that vary in their physical, chemical and biological conditions. This variation profoundly depends on spatial heterogeneity of underground environment, also may be affected by temporal heterogeneity (Ranjard *et al.* 2000; Sullivan *et al.* 2013). The diversity in soil biotic and abiotic conditions leads to unevenly distribution of microorganisms within soil texture. As these microenvironments are habitats for the soil biota, the ability of the microorganisms to resist heavy metals will be determined by the conditions of those habitats. Some

microenvironments maintain the activity of the bacteria due to availability of necessary nutrients, which is in turn increase the chances of bacteria resistance to heavy metals.

Organic matters in soil are the carbon and energy source for the endogenous microorganisms. The biggest part of organic matters in soil is available within very fine fractions (2-20μm) and clay fraction (<2μm), which makes these microenvironments good habitats for the bacteria (Ranjard and Richaume 2001). Soil water circulation within soil microenvironments transfers nutrients and other substances to the microorganisms that live in soil pores, therefore, soil desiccation may bring about decreasing in bacteria numbers (Treves *et al.* 2003; Holden and Fierer 2005; Or *et al.* 2007). Holden and Fierer (2005) found that it is more important to consider spatial rather than temporal heterogeneity in estimation the concentration and mobility of heavy metals in contaminated soils.

The infiltration of water and dissolved solutes takes different pattern through the soil. When the flow wets most of the soil matrix parts, this type is defined as "matrix flow", which is slow, even and takes an average flow path through the soil. In contrast, there is another type of flow faster than the matrix flow and uneven denoted bypass, macropore or "preferential flow". This type of flow occurs because of the presence of macropores, fractures, fissures, wormhole, which represent the heterogeneity of the natural soil texture. The formation of the macropores and subsurface channels is due to root channels, worm holes, and the erosion factors within the soil matrix (Beven and Germann 1982; Beven 1991; Rosenbom and Undersogelse 2005; Wang 2008). The preferential flow arises in all types of flow, and may occur in homogeneous soil structures of sandy and other types of soils. In addition, the velocity of preferential

flow reduces the opportunity for sorption by soil solid phase (Skopp 1981; Beven and Germann 1982; Brusseau and Rao 1990; Wang *et al.* 1998; Al Hagrey *et al.* 1999).

Many experiments have demonstrated the presence of preferential flow phenomena when they found different concentrations of pollutants in different depths of contaminated soil, this may have attributed to the variety of the paths taken by water, which holds the solutes, and then may reach a small part of soil matrix in the unsaturated zone (Kung 1990; Al Hagrey *et al.* 1999). The concern arises from preferential flow is the potential of transporting nutrients and trace metals directly to the groundwater aquifer (Wang *et al.* 2000; Morales *et al.* 2010). However, the preferential flow transports a great amount of oxygen, moisture and nutrients, which are important to soil microorganisms to undergo the degradation of pollutants, and this transported amount is greater than that available in the rest of soil matrix (Pivetz and Steenhuis 1995; Morales *et al.* 2010). Bundt *et al.* (2001) estimated that the carbon concentration in the preferential paths was up to 70% greater than in matrix flow.

Due to heterogeneous structure of the subsurface environment, high and low permeable regions can be found. Macropore network (root, fractures, fissures and wormhole) represent the regions with high permeability and the pathways of preferential flow. The regions with low permeability can be affected by diffusion exchange. Therefore, the solute movement with preferential flow is faster (Zhang and Xu 2003). Zhang and Xu (2003) found that the difference in heavy metals concentrations between exterior and interior of clayey soil higher than that in loam soil. This attributed to that the preferential flow is dominant in transferring the solutes in clayey soils, hence, the preferential flow contact exterior surface only in these soils. In contrast, in loam soils even flow is dominant which can distribute heavy metals and

radionuclides, to some extent, uniformly. The ability of heavy metals and radionuclides diffusion in soil structure affects their bioavailability. Zhang and Xu (2003) showed, as well, that the preferential flow is responsible for the differences in heavy metals concentrations between ped interior and exterior in contaminated soils, where some heavy metal has low mobility by diffusion.

2.7.8 Calcium Carbonate and Cations Interaction

Long–term sequestering of heavy metals with calcium carbonate precipitation is controlled by many parameters. Radius similarity of metallic cations with calcium, the geometry of adsorption complexes is among the parameters that found to control interaction mechanism (Sturchio *et al.* 1997; Elzinga *et al.* 2006). However, cadmium and calcium have almost identical atomic radius and metal desorption from hydrate layer is slow, whilst zinc remains hydrate until the incorporation occurs into the structure by calcium carbonate recrystallization. In term of structure compatibility between calcium carbonate and metal carbonate, cadmium forms a similar octahedral carbonate structure, whilst lead thought to make distorted octahedral surface complexes and zinc adsorption complexes have a tetrahedral geometry. Structural incompatibility may reduce the long term stability of divalent metals with calcium carbonate precipitation (Hausinger 2004; Teng and Zhao 2012). The co-precipitation of strontium with calcite controls by a number of factors, the concentration of the metal ions, the barium ions will act as competitive cation, and the precipitation rate, where the co-precipitation increase with increasing this rate (Lorens 1981; Pingitore Jr and Eastman 1986). The strontium incorporation into calcite lattice may result non-long term immobilisation of this metal, by generating small and more soluble crystals of minerals (Mitchell and Ferris 2006; Tobler *et al.* 2011; Cuthbert *et al.* 2012).

Chapter 3: Methodology

3.1 Introduction

Batch and column experiments were conducted in this study to investigate the applicability of microbially-induced calcium carbonate precipitation to co-precipitate heavy metals and radionuclides (by using non-radioactive proxies) in aqueous solutions and particulate media. This chapter defines the chemicals, sand, apparatus and procedures (for preparing and accomplishing experiments) that were used in this study. The microorganisms that were used here and their growth medium were described in this chapter as well. Table 3.1 presents the main experiments in this research. All experimental works carried out in triplicates (test and control samples).

Table 3.1. Main experiments conducted in this study.

Experiment	Purpose	Medium
Cell Number-Optical Density Relationship	Estimate the number of bacterial cells (cell/ml) from the optical density value of bacterial suspension to know the viable cell number that may able to grow and operate in an experiment.	*S. pasteurii* optimal medium.
Heavy Metals Adsorption Isotherms Experiment.	Explore heavy metals affinity to be adsorbed on sand grain.	Heavy metals + calcium + sand.
Bacteria Inhibition by Metals Toxicity.	Estimate minimal inhibitory concentration (MIC) of tested heavy metals that cause growth inhibition for the bacteria.	Heavy metal + nutrient broth + bacterial cells
Precipitation in aqueous solutions.	Investigate co-precipitation of heavy metals with calcium carbonate precipitation in through series of batch experiments.	Heavy metal + Precipitation medium + bacteria.
Precipitation in particulate medium.	Investigate co-precipitation of heavy metals in porous media through a series of batch and column experiments.	Heavy metal + Precipitation medium + sand + bacteria.

3.2 Strains and Culture Media

Organisms that were used in all experiments of this study was *Sporosarcina pasteurii*, a gram-positive, no capsule, facultative anaerobe and endospore-forming, formerly known as *Bacillus pasteurii* (Warren *et al.* 2001). This strain obtained from the National Collection of Industrial & Marine Bacteria (Aberdeen, UK; NCIMB8221/ATCC6453), was used due to its ability to hydrolysis urea in aqueous solutions (Fujita *et al.* 2000). *Sporosarcina pasteurii* strain was grown at 30°C for 24 hours in autoclaved solution containing Oxoid CM001 nutrient broth (13 g/L) and deionized (DI) water amended with 0.2μm filter-sterilised urea (final concentration 20 g/L) (Fujita *et al.* 2000). Stocks were kept at 5°C to minimise the growth of the bacteria and to be used for preparing a fresh culture of the strain. Stocks of this bacteria were prepared weekly to keep the strain active over the study period.

Preparation of bacteria inoculum was conducted by aseptically extracting 1mL from *S. pasteurii* stock described above and transferring it into an autoclaved 50mL of Oxoid CM001 nutrient broth (13 g/L) amended with 20 g/L filter-sterilised urea medium and incubated overnight at 30°C. Bacterial pellets were harvested by centrifuging the grown cultures at 1450 RCF for 20 minutes, then, to remove any used medium, the pellets were washed with phosphate-buffered saline (PBS: 8.3 mM Na_2HPO_4, 1.6 mM NaH_2PO_4, 145 mM NaCl, pH 7.2). Cells were again pelletised by centrifugation prior to resuspension in the working media defined later.

3.3 Heavy Metals Stock Solution Preparation

Appropriate amount of metal salts was dissolved in deionized (DI) water were added to give the required range of contaminant final concentrations. Then pH of all

solution, was corrected to 6.5 with hydrochloric acid in order to ensure solubility of all contaminants. Zinc chloride ($ZnCl_2$), cadmium sulphate ($3CdSO_4.8H_2O$), hydrate copper chloride ($CuCl_2.2H_2O$), lead chloride ($PbCl_2$) and strontium chloride ($SrCl_2.6H_2O$), were used to prepare dissolved free ions of zinc, cadmium, copper, lead and strontium, respectively. Metals solutions were prepared concurrently with other requirements (bacteria and chemicals) of each experiment. Metal solutions were autoclaved at 126°C for 25 minutes before introducing aseptically in relevant experiment.

3.4 Sand Preparation

Leighton Buzzard sand (Hepworth Minerals and Chemicals Ltd) was used in this study and was sieved according to British Standard, Figure 3.1 shows the particle size distribution for the used sand. It was washed by soaking in 1M hydrochloric acid, then in 1M sodium hydroxide for 24 hours each with periodic stirring, to remove inorganic carbonate. Finally, the sand was rinsed with deionised water until reaching pH 7.0 (sand pH was determined using soil-water ratio of 1:1, see section 3.5 for more details). Sand was then separated into three fractions by dry sieving [fine (63-300 μm), medium (300-425 μm) and coarse (600-1180 μm)]. It was autoclaved for sterilisation before using in any experiment.

Table 3.2 lists characteristics and the most important parameters of used sand in this study, as can be seen from the table the amount of organic matter and inorganic compounds that attach with medium sand fraction much lower than that with fine and coarse fractions.

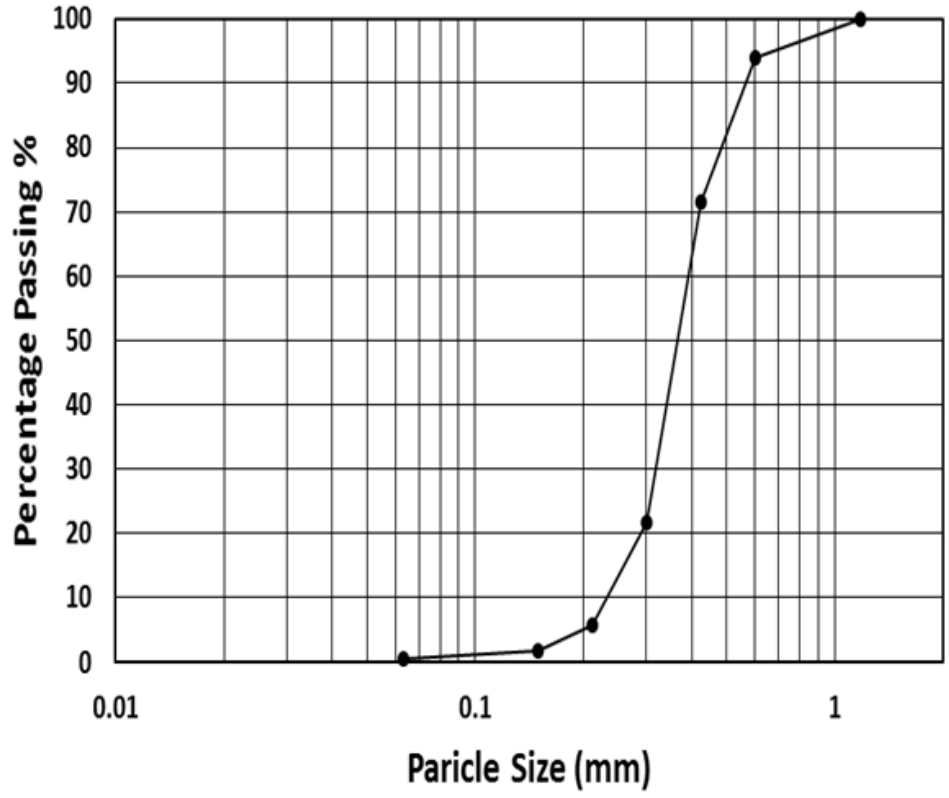


Figure 3.1. Particle size distribution for sand used (subsequently divided into fine, medium and coarse.

3.5 Analytical Methods

3.5.1 pH

Measuring pH values for liquid samples were done by using SevenMulti™ pH meter, for best reading this device was calibrated weekly and the probe was rinsed with deionised water. Sand samples pH were determined by using sand-water ratio 1:1, which was done by adding 5 grams of sand and 5 mL of deionised water to a 50ml beaker. The mixture was stirred vigorously for 10 minutes before allowing to stand without agitation for 30 minutes, pH value then was taken immediately after well stirring (Kalra 1995).

Table 3.2. Characteristics and parameters of sand that used in this study

characteristics	Fine Sand	Medium Sand	Coarse Sand	Analysis Method
Particle density (g/cm^3)	2.55	2.58	2.55	Particle density determination
Porosity (%)	35.31	36.27	35.42	mass-volume relationship
Organic Matter (%) ±SD	0.748 0.045	0.185 0.021	0.793 0.113	Lost on Ignition
SiO_2 (%) ±SD	95.517 0.171	98.761 1.105	96.977 1.052	XRD
$CaCO_3$ (%) ±SD	0.270 0.005	0.031 0.004	0.295 0.20	XRD
Fe_2O_3 (%) ±SD	3.630 0.071	0.982 0.057	2.628 0.101	XRD
K_2O (%) ±SD	0.062 0.006	0.029 0.002	0.027 0.005	XRD
MgO (%) ±SD	0.057 0.003	0.012 0.001	0.023 0.002	XRD
MnO_2 (%) ±SD	0.007 0.001	0.001 0.000	0.007 0.000	XRD
Al_2O_3 (%) ±SD	0.384 0.016	0.153 0.009	0.259 0.021	XRD
P_2O_5 (%) ±SD	0.037 0.001	0.010 0.002	0.047 0.011	XRD
Na_2O (%) ±SD	0.016 0.006	0.012 0.003	0.021 0.003	XRD
TiO_2 (%) ±SD	0.019 0.003	0.008 0.000	0.014 0.003	XRD
Solubility in water	Insoluble			Sand Data Sheet
Persistence	Resistance to biodegradation			Sand Data Sheet

3.5.2 Calcium and heavy metals concentrations

Collected liquids samples from relevant experiment were 0.2 μm filtered and diluted as required, then place in 35 ml vessels designed to test ion concentrations by Induction Coupled Plasma Spectroscopy (ICP, Optical Emission Spectrometer, Optima 2100 DV, PerkinElmer). This machine was used for measuring the heavy metals and calcium concentrations, sample in this analytical tool is aerosolised into the inductively coupled plasma, where the sample is excited and ionised at high

temperature plasma (up to 10000K). Due to this excitation a specific emission spectrum is created which belongs to a tested metal (Trevizan and Nóbrega 2007).

3.5.3 Optical density

Concentration of suspended *S. pasteurii* cells in working solutions was measured by taking optical density value at 600nm, samples were not filtered. Samples were collected (2 ml) aseptically in a disposable plastic UV cuvette (Fisherbrand). HITACHI U 1900 UV VIS Spectrophotometer was used to measure samples optical density or turbidity. Spectrophotometer was zeroed by using the same solution but free of the bacterial cells.

3.5.4 XRF Analysis

An Olympus–Innov-X, X-5000 X-ray fluorescence (XRF) was used to investigate the concentrations of calcium and heavy metal ions that were retained in sand fractions due to the bioprecipitation processes. Samples were first washed thoroughly with deionised water, then dried at 105ºC. Samples were then powdered and placed in XRF sample holder. XRF was calibrated before using, results were given in ppm units.

3.5.5 XRD Analysis

X-ray diffraction (XRD) analysis was performed on a Philips PW3830 X-ray diffractometer to determine the crystal structure of calcium carbonate (calcite, aragonite or vaterite) obtained in bioprecipitation processes in sand fractions. Samples were grounded and mounted on glass slides and placed in a sample holder and analysis

was conducted at angles from 20-80° 2θ with 2 seconds dwell time. The diffractometer was operated at 40 keV and 30 mA, at a step size of 0.02° 2θ.

3.5.6 SEM Analysis

Philips XL-30 ESEM (FEI K LTD., Cambridge UK) scanning electron microscope (SEM), fitted with secondary, gaseous secondary and backscattered electron detectors and a fully standardised EDX, X-ray analysis system (Oxford Instruments, High Wycombe, UK) were used to analyse a selection of sand test samples. Samples were mounted on aluminium stubs by using adhesive graphite tape and sputter-coated with carbon using K450 sputter-coater (Emitech, Kent, UK) prior to analysis to determine the most suitable imaging method for detection of precipitation pattern. Quantitative analysis of the chemical composition of samples was also conducted on the samples using an energy dispersive microanalysis of X-ray spectrometer (EDS) at an accelerating voltage of 20 keV. This was done to confirm that the crystals observed in the images were actually calcium carbonate crystals precipitated on the sand grains and to detect whether there was any precipitation of heavy metals in the samples.

3.5.7 Speciation Analysis

Visual MINTEQ is a Windows version of the US EPA code MINTEQA2, was used to simulate metal speciation in solutions in study experiments. Visual MINTEQ model outcomes depend on inputted data of metal and calcium concentrations and final pH, as well as on chemicals substances and their concentrations that were used in bioprecipitation experiments in this study. This model is useful in calculating the percentages of species that generated from the reactions between a specific metal and

the chemicals that represent calcium carbonate medium ingredients (but not the nutrient broth). This to compare the results that were gained from the experiments and the expected results from this model. Though Visual MINTEQ model cannot handle the biological processing due to the lack of equilibrium in such processes, but its data base can give a proper prediction about the resulted species from the chemical reactions (Gustafsson, 2013).

Though Visual MINTEQ cannot reproduce biological phenomena due to a lack of equilibrium in such processes, it has been used to predict final metal speciation using knowledge of major ion concentrations present, ambient temperature, ionic strength plus the final pH.

3.6 Cells Number-Optical Density Relationship

This section was implemented to estimate the number of alive *S. pasteurii* cells in a bacterial suspension that may able to induce precipitation processes. Although, Ramachandran *et al.* (2001) found an equation to determine *S. pasteurii* cells number in solutions, but it thought that concentration of cells suspension in a solution is controlled by experiment conditions. Hence, this section aims to elicit a relationship by which can estimate number of bacterial cells suspending in each working solution before using these solutions in the experiments of this study.

CTC (5-cyano-2, 3-ditolyl tetrazolium chloride) was used in this study to determine the number of suspended *S. pasteurii* cells in a solution. CTC staining is reliable, simple and quick method for bacteria counting, for this reasons CTC was the main method that used in this part (Bartosch *et al.* 2003; Créach *et al.* 2003). *S. pasteurii* cells were cultured in their normal growth medium and conditions, and

allowed to grow over 24 hours in two periods of time. Values of OD_{600} were determined for the culture every hour by using the Hitachi U1900 UV VIS spectrophotometer. Optical density values determined by spectrophotometer reflect the turbidity that resulted from both live and dead cells. At stationary phase cell division matches cell death, though high value of optical density can be recorded at this phase but the number of live cells may be lower. Therefore, discovering live cells number can be by other methods rather than spectrophotometer, such as, CTC. Samples were taken at different periods after measuring their optical density values to determine the number of live cells by CTC method.

CTC is a redox dye able to penetrate bacteria membrane (this leads to kill the bacteria. The dye will precipitate in live cells after reduction by electron transfer system of bacteria cell to give an insoluble red high fluorescent precipitation under microscope (Yu *et al.* 1995; Sieracki *et al.* 1999; Yamaguchi *et al.* 2003). Based on many tests were implemented to find best dilution that gives countable number of cells (30-300) under the microscope, bacteria samples (that were selected to be stained with CTC-DAPI) were diluted to 10^{-2} and 10^{-3}. 10 mg CTC (5-Cyano-2, 3-di-(p-tolyl) tetrazolium chloride, Sigma Aldrich, UK) was dissolved in 1.6mL deionised water to prepare 20mM working solution, stored in 4°C and used within two weeks. To prepare a DAPI stock solution, 5mg DAPI (4′, 6-Diamidino-2-phenylindole dihydrochloride, Sigma, Aldrich, UK) was dissolved in 5mL 0.2µm filtered deionised water. This stock solution stored in a temperature less than 0°C to be used within several weeks. 1 mL of this stock solution was added to 10 mL 0.2µm filtered deionised water to make DAPI working solution and stored in 4°C, which used within few weeks.

Number of total cells (live and dead) was determined by using CTC-DAPI double staining technique. Where 1mL of diluted *S. pasteurii* was placed in 2ml micro-centrifuge tube and amended with 100µL of CTC solution, for 2mM CTC final concentration, where is recommended CTC concentrations is 2-5mM. Then micro centrifuge tubes were mixed gently by vortex mixer and incubated at 30°C for four hours. After the incubation period, the CTC added bacterial suspension was amended with 100µm DAPI for 10µm/mL final concentration. The micro centrifuge tubes then incubated at room temperature for 10 minutes (Yu *et al.* 1995; Boulos *et al.* 1999; Sieracki *et al.* 1999; Créach *et al.* 2003; Yamaguchi *et al.* 2003; Tammert *et al.* 2008) 2008). Stained bacterial suspension samples then immediately filtered through black 0.2 µm pore-size polycarbonate filters (Nucleopore) at a vacuum pressure by using vacuum filter assembly.

The filter then was air-dried and placed on a glass slide to be analysed under the microscope. A Nikon ECLIPSE LV100 microscope equipped with Nikon DS-Fi1digital camera, was used for epifluorescence counts. Actively respiring cells that stained with CTC were determined and enumerated by red fluorescence under a blue 450-490nm excitation filter and 520nm barrier filter. Whilst CTC-DAPI cells (total number) were viewed with 330-380nm excitation filter and 420nm barrier filter.

The number of cells (*N*, cell/ml) in a suspension was found from the following equation (Boulos *et al.* 1999):

$$N = \frac{R \times A}{F \times D \times V} \quad \text{..... 3.1}$$

Where:

R: is the average number of bacterial cells per field.

A: is the surface of filtration (mm^2).

F: is the area of microscopic field (mm^2) (depends on the used objective).

D: is the dilution factor.

V: is the volume of the sample (ml).

3.7 Heavy Metals Adsorption Isotherms Experiment

This part of study discovered the capacity of sand fractions to adsorb heavy metal cations through determining their equilibrium concentrations in the sand. This done according to the Environmental Protection Agency batch-type test procedure for estimating soil adsorption of chemicals (Roy *et al.* 1992). Three sand fractions fine, medium and coarse (as adsorbents) and eight concentrations of tested heavy metals (as solutes) were used in this experiment. Metal solutions and sand fractions were prepared according to sections 3.3 and 3.4, respectively. However, 50 mM calcium was added to heavy metal solutions, to find if it competes metal cations adsorption on sand grains (same amount of calcium that was used with precipitation experiments).

Soil: solution ratio used in adsorption isotherms experiment was 1:10 (Roy *et al.* 1992), hence, 4 g from each sand fraction was equilibrated with 40 mL of heavy metal solutions containing different concentrations of heavy metals. Sand samples either fine, medium or coarse were aseptically weighted (4 g) in 50 mL sterilised polypropylene centrifuge tubes. The medium in this experiment were prepared by dissolving calcium chloride in required amount of deionised water for final concentration of 50 mM. After autoclaving calcium chloride solution 30 ml were

poured in the tubes that contain 4g sand. Heavy metals salt solutions were then added aseptically to give a final total volume of 40 ml. Metal salts added to calcium chloride solutions to give a range of contaminant concentrations as shown in Table 3.3.

Table 3.3. Zinc and cadmium concentrations that were used to study the adsorption capacity of used sand fractions in this study

Metal	Concentration (mM)							
Zinc	0.1	0.5	1	1.5	2	3	5	10
Cadmium	0.01	0.05	0.1	0.3	0.5	1	3	5

Samples were placed on an orbital shaker (120 RPM) for 1 hour before incubating at 30°C for 72 hours that was to be consistent with conditions of the bioprecipitation experiments. Samples prepared in triplicate. After equilibrating time, sand solution samples were centrifuged at 1450 RCF for 20 minute. Then 10 ml of each solution was poured in ICP bottles for measuring the concentrations of heavy metals and calcium. Zn, Cd and Ca concentrations were measured in aqueous solutions by using Induction Coupled Plasma Spectroscopy (ICP, Optical Emission Spectrometer, Optima 2100DV, PerkinElmer). pH values for the solutions were measured as well.

3.8 Bacteria Inhibition by Metals Toxicity Experiment

The aim of this experiment was to estimate the capability of *S. pasteurii* to survive in presence of a range of metallic concentrations for zinc, cadmium, copper, lead and strontium. Also to estimate minimum inhibitory concentrations (MICs) that inhibits *S. pasteurii* growth. Concentrations were chosen to demonstrate the limiting concentrations where growth was inhibited for each metal. In this part of study, the

bacterial cells allowed to grow in a medium containing only nutrient broth and heavy metals, no urea was used in this experiment to avoid any precipitation of metal ions which may lead to decrease the total concentrations of contaminants. Microcosms were prepared by suspending fresh bacteria pellets in 600 mL of autoclaved Oxoid CM0001 nutrient broth (13 g/L), then, aseptically, aliquots of 6 ml were dispensed into 10 ml acid washed and sterilised screw cap glass tubes. All solutions were then amended with sterilised metals salts solutions to give final volume of 8 ml. however, metal salts solutions were added to give a range of contaminant concentrations as shown in Table 3.4. The pH was then corrected to 6.5 with hydrochloric acid. The final bacterial optical density at 600 nm wavelength incident light (OD_{600}) was 0.064 (equivalent to approximately 1×10^7 cells / ml) in each tube. The medium used in this experiment denoted as *M1*.

Table 3.4. Initial concentration of heavy metals that were tested in toxicity experiment.

Metal	Concentration (mM)						
Zinc	0	0.01	0.02	0.05	0.1	0.2	0.5
Cadmium	0	0.005	0.01	0.02	0.05	0.1	0.15
Copper	0	0.01	0.02	0.05	0.1	0.2	0.5
Lead	0	0.01	0.05	0.1	0.2	0.5	1
Strontium	0	0.1	0.5	1	2	5	10

In this experiment the total turbidity in solutions results from bacterial cells and heavy metals presence in solutions. So to eliminate the turbidity that may result from metal presence, samples containing only nutrient broth and the heavy metal were prepared in same fashion to measure their optical density. Bacteria growing in a

medium containing 13 g/l nutriment broth and free of heavy metals or at 0 metal concentration was called full growth of bacteria.

All microcosms were incubated at 30ºC for 72 hours, after which 2 ml from each sample was pipetted aseptically and placed in a cuvette to measure the optical density by the Hitachi U1900 UV VIS spectrophotometer. The spectrophotometer was zeroed with autoclave 13 g/L nutrient broth before taking any reading. Measuring optical density (OD_{600}) values was to check bacterial growth under the stress of different concentrations of heavy metals. Each metal concentration was tested in triplicate.

This experiment only explored the absolute toxicity of various metal concentrations (similar to (Ruggiero *et al.* 2005)), and did not explore temporal issues such as increased lag. Typically, the majority of growth of *S. pasteurii* in these conditions occurs within 24 hours, the experimental duration was increased to account for growth hindrance due to the presence of metals.

To find the response of *S. pasteurii* cells to heavy metal concentrations a value denoted *R* represents the fraction of full growth was calculate as shown below:

$$R = \frac{ODB - ODM}{ODF} \quad \text{.....3.2}$$

Where: -

R (normalised): fraction of full growth for any metal at any concentration

ODB: optical density (OD_{600}) for samples containing bacteria, nutrient and heavy metals (medium *M1*), the value represents turbidity of solution due to bacteria growing under the effect of heavy metals.

ODM: optical density for samples containing only nutrient and heavy metals, the value represents turbidity of solution due to heavy metal presence.

ODF: optical density at zero metal concentration for samples containing only bacteria and nutrient without heavy metals, the value represents the turbidity due to bacteria growing in a medium free of heavy metals, this considered full growth of bacteria.

3.9 Calcium Carbonate Precipitation Experiment

This part of the study is to define nutrients, chemicals and preparing procedures that were used in microbially induced process of calcium carbonate precipitation experiment. Nutrient broth and chemical concentrations that were used to prepare calcium carbonate precipitation medium (CCPM) are presented in Table 3.5. There is almost unanimity in literature regard using 3 g/l Oxoid CM001 nutrient broth when using *S. pasteurii* strains to mediate inducing calcium carbonate precipitation. Different initial concentrations of urea and calcium were used in such studies. In CCPM urea ($CO(NH_2)_2$) is nitrogen and energy source for the bacteria, and its hydrolysis produces ammonium and bicarbonate ions. The calcium chloride is the calcium ions source for the calcium carbonate formation.

Table 3.5. Nutrient broth and chemical concentrations that were used to prepare CCPM in this study.

Ingredient	Manufacturer	Concentration	Sterilising
Oxoid CM0001 nutrient broth	Oxoid tLd, Basingstoke, UK	3 g/L	Autoclaving
Calcium Chloride Dehydrate	Fisherbrand	See Table 3.6	Autoclaving
Urea	Fisherbrand	See Table 3.6	0.2µm filter
Ammonium Chloride	Fisherbrand	10 g/L or 187mM	0.2µm filter
Sodium Bicarbonate	Fisherbrand	2.12 g/L or 25mM	0.2µm filter
Concentrated HCl	Fisherbrand	Adjusted to pH 6.5	Aseptically

Equations from 3.3 to 3.7 below describe calcium carbonate precipitation after catalysing by a microorganism. The process starts with hydrolysing urea by urease enzyme then producing of carbonate and ammonia ions, in turn pH increases. When supersaturating state occurred calcium and carbonate ions at high pH environment, recrystallisation of calcium carbonate happens. The efficiency of process is considered 100% when all dissolved calcium and carbonate ions transfer into solid state calcium carbonate precipitate (Stocks-Fischer *et al.* 1999; DeJong *et al.* 2006; DeJong *et al.* 2010; Al Qabany *et al.* 2012; Dhami *et al.* 2013).

$$H_2N(CO)NH_2 + H_2 \xrightarrow{urease} NH_2COOH + NH_2 \quad \text{.....3.3}$$

$$NH_2COOH + H_2O \longrightarrow NH_3 + H_2CO_3 \text{.....3.4}$$

$$H_2CO_3 \longleftrightarrow 2H^2 + 2CO_3^{2-} \quad (carbonate\ ions\ producing) \text{.....3.5}$$

$$NH_3 + H_2O \longleftrightarrow NH_4^+ + OH^- \quad (pH\ increase) \text{ ... .3.6}$$

$$Ca^{2+} + CO_3^{2-} \longleftrightarrow CaCO_3 \quad (K_{SP} = 3.8 \times 10^{-9})\ (precipitation) \text{ ... .3.7}$$

Where K_{SP}: is the solubility product in Equation (2.5).

To optimise calcium and urea concentrations in this study current experiment included investigating 12 tests of initial calcium and urea concentrations, as shown in Table 3.6. Nutrient broth, ammonium chloride and sodium bicarbonate concentrations in all tests were 3 g/l, 10 g/l and 2.12 g/l, respectively. For each test 25 ml of nutrient broth and calcium chloride were prepared in deionised water then autoclaved at 126°C for 25 minutes under 1.4 bar pressure. After that washed bacterial pellets re-suspended in autoclaved broth-calcium solution and placed aseptically in sterile 50 ml polypropylene centrifuge tubes (Fisherbrand). Urea, ammonium chloride and sodium bicarbonate were sterilised by 0.2µm syringe filter (Fisherbrand) and added aseptically to the 50 ml tubes to give a total final volume of 30 ml. The final OD_{600} was 0.082 (equivalent to approximately 1.3×10^7 cells / mL) and final calcium and urea concentrations as shown in Table 3.6. All tubes were incubated for 24 hours at 30ºC and all samples prepared in triplicates. Medium containing nutrient broth, calcium and urea is denoted as *M2.*

Table 3.6. Initial concentrations of calcium and urea that were tested to find optimum concentrations to prepare CCPM in this study.

		Calcium concentration (mM)					
		10	20	25	30	40	50
Urea concentration (g/L)	2	Test-1	Test-2	Test-3	Test-4	Test-5	Test-6
	20	Test-7	Test-8	Test-9	Test-10	Test-11	Test-12

3.10 Heavy Metals Bioprecipitation in Aqueous Solutions Experiments

After examining MICs for zinc, cadmium, copper, lead and strontium, in section 3.8, this experiment was designed to discover the ability of *S. pasteurii* in sequestering metallic contaminants with calcium carbonate precipitation. This study hypothesises that metal precipitation technique provides a protective mechanism for the bacteria by

reducing the overall metal concentrations to tolerable levels, hence, tested concentrations in this experiment were increased to be more than those in toxicity experiment.

Calcium carbonate precipitation medium (*M2*) was prepared as described in previous section (by using optimum concentrations of urea and calcium found in section 3.9). Microcosms were prepared by re-suspension the pelletised and washed cells in the urea-amended medium (*M2*) before 25 ml was placed aseptically in sterile 50 ml polypropylene centrifuge tubes and amended with heavy metals to give a total final volume of 30 ml. The final OD_{600} was 0.111 (equivalent to approximately 1.7×10^7 cells / mL) and final metal concentrations are shown in Table 3.7.

Table 3.7. Heavy metal initial concentrations that were applied in heavy metals precipitation in aqueous solution experiment

Metal	Concentration (mM)						
Zinc	0	0.1	0.2	0.5	2	5	10
Cadmium	0	0.015	0.03	0.1	0.5	1	3
Copper	0	0.05	0.1	0.5	1	3	5
Lead	0	0.05	0.1	0.1	0.5	1	3
Strontium	0	1	3	5	10	20	30

However, beside test samples same number of controls were, synchronously, prepared in an identical fashion, but *S. pasteurii* cells were killed by autoclaving the initial bacterial suspensions prior to incorporation *M2* medium. Test and control samples had the same nutrient broth, calcium, urea and heavy metal concentrations but the test samples included live cells of *S. pasteurii* whilst controls involved killed-cells of the bacteria. All samples then incubated at 30ºC for 7 days.

Additionally, to check if the abiotic precipitation can lead to remove heavy metal ions in period up to 14 days, samples were prepared in the same fashion but no bacterial cells were added to the media. Chemical or abiotic precipitation can occur if metal ions react with carbonate ions (carbonate ions can be produced from abiotic urea hydrolysis in water). Furthermore, presence of calcium carbonate minerals can lead to remove heavy metal ions from contaminated solutions.

Samples (5 ml) were obtained aseptically from each microcosm after 0, 1, 3 and 7 days, filtered past a 0.2μm membrane filter and sub-divided for analysis. Calcium and heavy metal ion concentrations were determined by ICP-OES (Optima 2100 DV, PerkinElmer) for the drawn samples after measuring their pH. The weight change of microcosm tubes over the experiment was recorded in order to determine the mass of precipitation seen adhering to the tube. Following completion of the experiment, tubes were emptied, carefully rinsed with deionised water and dried in an oven at 30ºC (to prevent heat damage to tubes) to constant weight before final weighing. Because of the small masses involved, loss of precipitate upon rinsing and drying may contribute to errors. The tubes were weighted before starting the experiment, the net precipitation weight was found from the difference in two weights (after and before).

3.11 Heavy Metals Bioprecipitation in Particulate Media Experiments

This section was designed to investigate the ability of *Sporosarcina pasteurii* bacteria to induce the precipitation of calcium carbonate and co-precipitation of heavy metals in porous media. The biomineralisation of heavy metals in soil may be influenced by a number of physical, chemical and biological factors. Presence of inorganic compounds and organic matters in soil may lessen metals availability through chemical and physical adsorption processes, where metal ions have to be

dissolved in solutions for bioprecipitation processes. Bacteria ability to initiate calcium carbonate precipitation may be affected by many factors such as metallic toxicity and nutrients delivering. Add to this, the heterogeneity of subsurface environment can play a main role in controlling biomineralisation processes. These effects were studied here through batch and column experiments.

3.11.1 Bioprecipitation in Porous Media by No-Flow System (Static) Experiments

The precipitation of heavy metals in porous media by using system without fluid flow was in investigated in this part of the study. This section includes precipitation with and without heavy metals. This experiments were conducted to examine the bioprecipitation process in a static system (no fluid flow was used in this experiment) and then compare with bioprecipitation in sand by dynamic system (fluid flow) and in aqueous solutions. Microcosms were prepared by re-suspension the pelletised and washed cells in the urea-amended medium (*M2*) then were placed aseptically in sterile 50 ml polypropylene centrifuge tubes and amended with heavy metals to give a total final volume of 17 ml (equivalent to the pore volume, see Table 3.2 for more details). The final metal concentrations and final OD_{600} are shown in Table 3.8. Then aseptically adding of 80g dry, autoclaved sand from individual fractions (fine, medium and coarse), which was wet-pluviated into the CCPM to minimise air voids and encourage uniform bacterial distribution. However, further homogenising of CCPM in sand was done by using vortex mixer. Metals concentrations were chosen base on previous and preliminary experiments. Multiple samples of each sand fraction were prepared such that specimens could be destructively sampled at 3 and 7 days. Control samples were prepared in an identical

fashion, medium used in these samples was free of urea (containing similar concentrations of nutrient broth, calcium and heavy metal but no urea added, denoted as *M3*). All samples then incubated at 30ºC.

The response of heavy metal ions to bioprecipitation process in sand by *S. pasteurii* was discovered by measuring the free ions of zinc, cadmium, strontium and calcium in soil solution, pH was measured as well. Tubes were pierced at the base by hot needle, solutions were extracted from the bottom of tubes under vacuum pressure. Figure 3.2 shows the extraction system that were used to extract solutions from sand tubes in this section. Solutions were then filtered past a 0.2µm membrane filter and sub-divided for analysis. Calcium and heavy metal ion concentrations were determined by ICP-OES (Optima 2100 DV, PerkinElmer).

Table 3.8. Final metal concentrations and final optical density and equivalent cell number in bioprecipitation in porous media by no-flow system (Static) experiments.

Tested element	Final concentration	Final OD_{600} of the medium before adding sand (equivalent to approximately)
Calcium	50 mM	0.106 (1.6×10^7 cells/ ml)
Strontium	30 mM	0.094 (1.4×10^7 cells/ ml)
Zinc	2 mM	0.094 (1.4×10^7 cells/ ml)
	5 mM	0.101 (1.5×10^7 cells/ ml)
	10 mM	
Cadmium	10 mM	0.101 (1.5×10^7 cells/ ml)

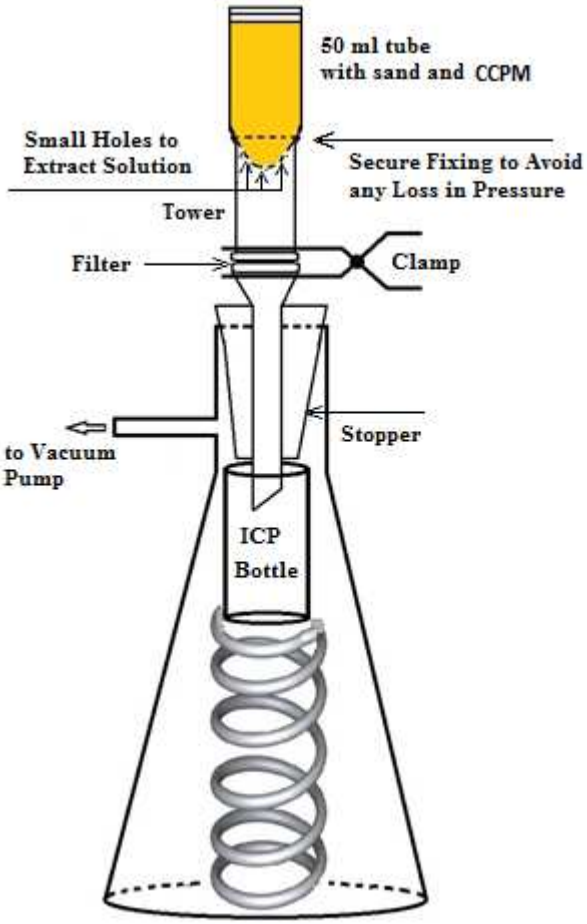


Figure 3.2. Schematic diagram shows the system by which solutions were extracted from sand tubes to measure ion concentrations and pH

3.11.2 Bioprecipitation in porous media by flow system (Dynamic)

The experiments in this part were designed to investigate the bioprecipitation processes in porous media under the effects of ground conditions. This done through dynamic system (with fluid flow) comprised of sand columns and peristaltic pumps to inject solutions into these columns by using pulsed injection strategy. Sand columns in this part were made of clear acrylic tubes (inner diameter 26mm and length 200mm). A stand was designed to this experiment with dimensions can fit into the incubator contained clamps to hold up to 18 sand columns. After positioning sand columns vertically on the stand, 4 mm clear tubes were used to connect inlets and

outlets of columns with peristaltic pumps and collection flasks, respectively, pumps also were connected to medium flasks by similar tubes. Dynamic system includes columns, tubes, connectors, pumps and flasks was sterilised by pumping Virkon solution (Rely^{+}On^{TM}, multi-purpose disinfectant) into the system then was washed by large quantities of autoclaved deionised water. The system was sterilised before starting any experiment. Dynamic systems used in this experiments are shown in Figure 3.3 and Figure 3.4.

Washed pellets of *S. pasteurii* were suspended in PBS, optical density was recorded for the bacterial suspension after zeroing the spectrophotometer with PBS. A volume of 35 ml (equivalent to pore volume of sand column) of the bacterial suspension were poured in each column before aseptically adding 150g dry, autoclaved sand from individual fractions (fine, medium and coarse), which was wet-pluviated into the bacterial suspension to minimise air voids and encourage uniform bacterial distribution. Thin layers of glass wool were placed at the top and bottom of the sand to prevent soil grains from traveling with injected fluids flow and to avoid any block may be occurred in the tubes or connectors. Then, couple of hollow rubber stoppers contain connectors, to be connected to the system tubes, were fitted into the ends of each column. All columns were prepared in triplicate in an identical fashion but were contained either fine, medium or coarse sand fraction. Control samples were prepared in an identical fashion. The system was then incubated at 30ºC for different periods of time depends on experiment purpose.

Liquids including precipitation medium (or urea-treated samples which denoted *M2*) and control samples medium (or urea-free samples and heavy metals which denoted *M3*) were placed aseptically in medium flasks and injected into sand columns

by the peristaltic pumps by using pulsed injection strategy. Lauchnor *et al.* (2013) showed that pulsed injection strategy (followed by stagnation periods, no-flow) can be more efficient than continuous injection in precipitating calcium and heavy metals via microbial-induced calcium carbonate precipitation. In addition, pulsed injection can lead to promote homogeneous calcium carbonate precipitation along sand column. The strategy involved injection 1.5 pore volume (55 ml) at pumping rate of the fluids into the columns was 5 ml/min. Collected samples, after each running, were then filtered past a 0.2µm membrane filter and sub-divided for analysis. Calcium and heavy metal ion concentrations were determined by ICP-OES (Optima 2100 DV, PerkinElmer), pH was measured as well. Medium and collection flasks were acidic washed sterilised (by autoclave) after each run of the system.

3.11.2.1 Homogenous Fluid Flow Experiment

In this part number of sand columns equals to the number of pumps, fine, medium and coarse sand fractions in columns receive same amount of solution (homogenous injection of CCPM into sand columns), as shown in Figure 3.3. This section included two tests first one was investigating precipitation of cadmium without aging *S. pasteurii* cells with cadmium ions. In this test CCPM was amended with cadmium ions and injected into sand columns. Followed by no fluid flow period (24 hours) then only CCPM was injected for 6 days. The final bacterial optical density at 600 nm wavelength incident light (OD_{600}) was 0.121 (equivalent to approximately 1.8×10^7 cells / ml) in each column and final cadmium concentration in each column was 20 mM.

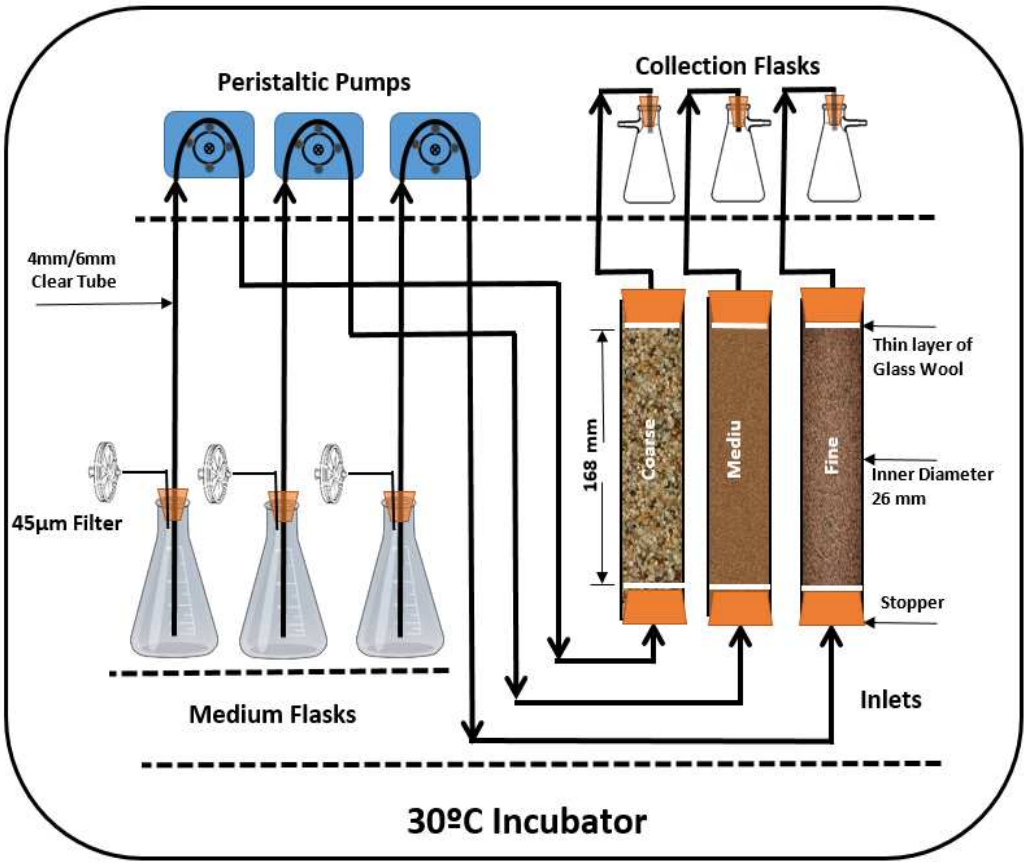


Figure 3.3. Schematic diagram illustrates the installation of homogenous fluid flow system in dynamic experiment.

Additional test included aging bacterial cells with cadmium ion for 24 hours in porous media before injecting CCPM. In this experiment 1.5 pore volume of known concentration of cadmium solution was injected into sand columns. CCPM was then injected into sand columns after 24 hours. Followed by no fluid flow period, then only CCPM was injected for the rest of experiment time. The final bacterial optical density at 600 nm wavelength incident light (OD_{600}) was 0.099 (equivalent to approximately 1.5×10^7 cells / ml) in each column and final cadmium concentration in each column was 20 mM.

3.11.2.2 Heterogeneous Fluid Flow Experiment

In this part of study, the effect of heterogeneous hydraulic conductivity on bioprecipitation processes was investigated. Soil physically, chemically and biologically is heterogeneous. Spatial heterogeneity is an intrinsic property of subsurface environment; liquids flow is affected by spatial changes in porous media. The variation in hydraulic conductivity in soil may lead to find different concentrations of biomass, substrates and pollutants within soil matrices. Porous media are rarely homogeneous, liquids transport in porous media varies spatially and temporally. This experiment was designed to mimic, to some extent, a heterogeneous permeability section of soil. Coarse fraction sand allows solutions to flow faster than other fractions due to its relative high hydraulic conductivity.

The system of this experiment involved 27 sand columns divided in 3 groups (*G1*, *G2* and *G3*), each group included 3 sets, each set contained 3 sand columns (fine, medium and coarse sand fractions), as shown in Figure 3.4. All groups prepared in the same fashion. Group 1 (*G1*) contained 9 sand columns (triplicates of fine, medium and coarse sand fractions) represented control (no cadmium ions used) to investigate the behaviour of precipitation processes in heterogeneous porous media without metal toxicity. Group 2 (*G2*) (test samples) was prepared in the same fashion of *G1* but 0.03 mM cadmium concentration was injected 24 hours before pumping calcium carbonate medium. Group 3 (*G3*) represent another control to investigate the flow of liquids into sand columns without calcium carbonate precipitation influence (no urea was added), this group involved injecting 0.03 mM cadmium concentration at the same time with *G2*. All three columns in one set received liquids through a single inlet, represent layered heterogeneity. For each set 1.5 pore volume (165 ml) was injected into a single

inlet of the three columns. Hydraulic conductivity values for sand fraction before treatment were 7.3×10^{-5}, 3.0×10^{-4} and 4.8×10^{-4} m/s for fine, medium and coarse sand fractions, respectively.

This experiment included also aging bacterial cells with cadmium ions in porous media where 1.5 pore volume of known concentration of cadmium solution was injected into each sand column of groups *G2* and *G3* (each column received same amount of cadmium solution). Cadmium ions left with bacterial cells for 24 hours before injecting *M2* or *M3* media. The final bacterial optical density at 600 nm wavelength incident light (OD_{600}) was 0.089 (equivalent to approximately 1.4×10^{7} cells / ml) in each column and final cadmium concentration in each column was 20 mM. Groups were prepared and ran as following: -

- Group-1 or *G1* (control-1): sand columns containing bacterial cells were injected with CCPM or *M2* medium for 32 days, no cadmium ions were injected. This to monitor the behaviour of calcium carbonate precipitation without metal effects.
- Group-2 or *G2* (test samples): sand columns containing bacterial cells were injected with 0.03 mM of cadmium ions and aged for 24 hours before injecting CCPM or *M2* medium for 32 days. This group contains the main samples to investigate the ability of this technique to co-precipitate metals under the heterogeneity in ground conditions.
- Group-3 or *G3* (control-2): sand columns containing bacterial cells were injected 0.03 mM of cadmium ions and aged for 24 hours before injecting *M3* medium (urea-free medium) for 32 days. This group without urea effect to monitor behaviour of calcium and cadmium ions under these conditions.

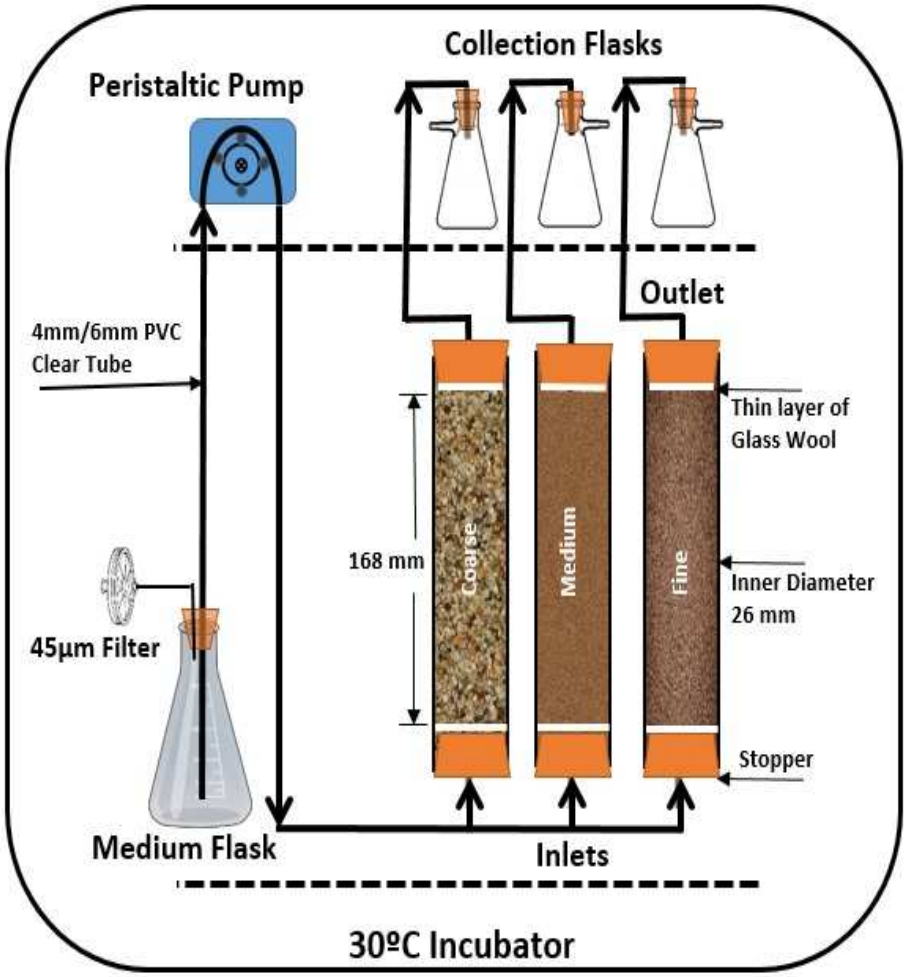


Figure 3.4. Schematic diagram illustrates the installation of Heterogeneous fluid flow system in dynamic experiment.

Chapter 4: Bacteria Inhibition by Metals Toxicity

4.1 Introduction

This chapter discusses toxicity of a range of heavy metals concentrations (zinc, cadmium, lead, copper and strontium), in aqueous solution, to the growth of a urease-positive bacterium, *Sporosarcina pasteurii*. This study hypothesises that the ability of this organism to breakdown urea and thus alter the chemistry of its environment provides it with a form of 'defence mechanism', as if sufficient numbers initially survive and hydrolyse urea, contamination in the vicinity will be reduced allowing enhanced microbial activity and further metal removal. The chapter includes two sections first one discusses *S. pasteurii* cells growing with time where optical density changes in bacteria suspension due to cells propagation. By counting bacterial cells (cells/ ml) at specific time and at known optical density value, then a relationship of [cells number-optical density] can be built. In second section the metals inhibition effect to *S. pasteurii* cells growing is explored.

4.2 Viable Bacterial Cell Counts Experiment

This section was implemented to estimate the initial number of *S. pasteurii* cells before suspending with working media in the experiments of this study. The increase in growing rate of the bacterial cells with time was investigated by measuring the turbidity in the bacterial suspension by spectrophotometer at 600nm (Chapter 3, section 3.6). Increased turbidity in the bacterial suspension is an indicator of cell number increase. However, at stationary phase cell division matches cell death, though high value of optical density can be recorded at this phase but the number of live cells may be lower. This study is mainly interested in live cells only.

4.2.1 Optical Density vs time

Spectrophotometer measures optical density for a bacterial suspension, the reading reflects absorb or scatter light by live and dead cells in that solution (presuming only bacterial cells exist). As bacteria continue propagating, optical density increase with time. The relationship between optical density and *S. pasteurii* cells number were found over 24 hours in two periods of time. Figure 4.1 shows increase in absorbance with increase cell number in bacterial suspension. As it clear from the figure the initial cultured number in bacteria medium showed very little increase with time up to 4 hours, but after that started to show a significant increasing. Probably the first 4 hours were the lag phase of the bacteria growth, and the exponential phase started after that and displayed sharp increase especially after 8 hours. Errors bars (standard deviation values) demonstrate that the dispersion of optical density values from their average at each hour increase with time or with cells number increasing, samples were tested in triplicate.

4.2.2 Cell Number-Optical Density Relationship

Estimating initial *S. pasteurii* cells number in their suspension was done by an expression (equation 4.1) was elicited from comparing optical density values and cell numbers that were found by CTC staining method at the corresponding interval during *S. pasteurii* cells growth. Equation 4.2 represent Ramachandran *et al.* (2001) equation. Figure 4.2 presents the relationship between number of *S. pasteurii* cells and optical density values in a bacterial suspension.

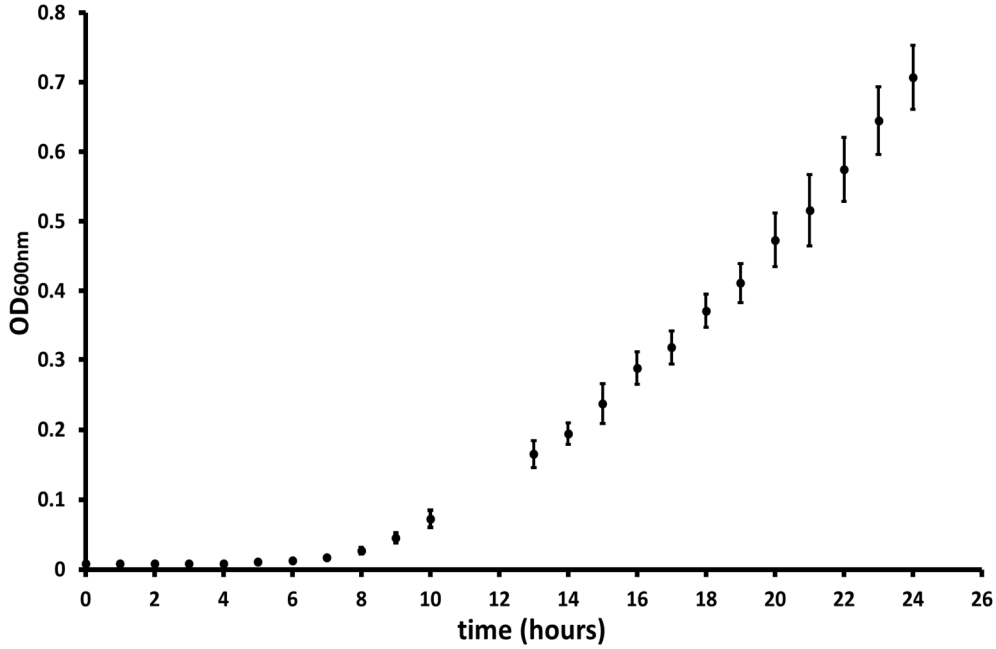


Figure 4.1. S. pasteurii cells growth over time in their normal medium shows increasing in bacterial cells with time and as a result optical density values were increased. The gab between hours 10 and 13 is due to cells OD values were measured over 24 hours in two periods of time. [Error bars: ± 1 SD, n=3].

$$N = 1.0x10^{8}x(OD_{600})^{0.8201} \quad \text{.....4.1}$$

$$N = 8.59x10^{7}x(OD_{600})^{1.3627} \quad \text{(Ramachandran } et\ al. \text{ 2001) .....4.2}$$

By comparing the formula created in this study with Ramachandran *et al.* (2001) equation it found that the former gives higher number of cells. However, it noticed that the difference in cell numbers from both formulas decrease with increase optical density values. At optical density 0.01 cell number that can be gained from this study formula is 14 times more than Ramachandran *et al.* (2001) equation, whilst at OD of 0.36 cell number is only 2 times more. At OD of 1.32 both formulas give almost similar number of cells/ml. Hence, the difference can be significant at low optical density values. The variance in estimation initial bacterial cells number and corresponding optical density may be related to the used bacteria strain activity, counting methods and experiment conditions, such as, incubation period and

temperature. In addition, the counting methods may affect the final counted number of cells. Ramachandran *et al.* (2001) used plate cell-counting method whilst in this study CTC staining method was used for counting.

Data were fitted by *curved power trendline*, that is best used with such data sets (where cells number >0) and to compare measurements increase with time. Ramachandran *et al.* (2001) used the same *trendline* to create his expression (equation 4.2) that was used to compare the equation found in this study. However, it noticed that both formulas may not suitable to calculate viable cells number in stationary phase of bacteria growth curve, reason behind this is both give increasing cell number with increase optical density. At stationary phase cell division matches cell death, though high value of optical density can be recorded at this phase but the number of live cells may be lower.

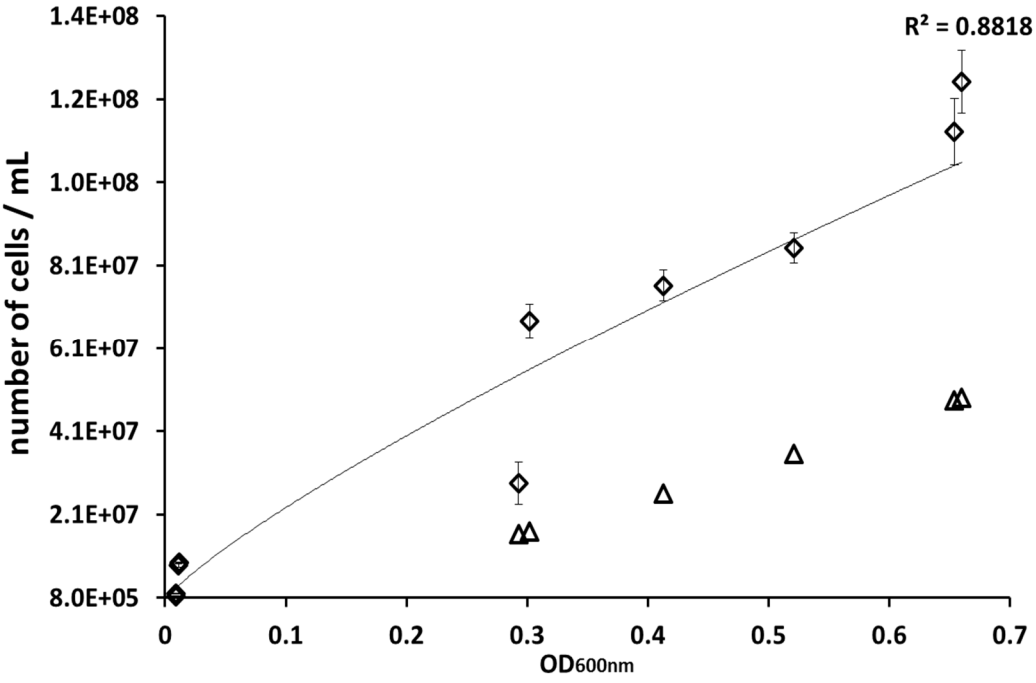


*Figure 4.2.Relationship between number of S. pasteurii cells and optical density values in a bacterial suspension, also data were compared with Ramachandran et al. 2001 (**Δ**). [Error bars: ± 1 SD].*

4.3 *S. pasteurii* Response to Metallic Toxicity

This section discusses the experimental results of the toxicity of a range of heavy metals (zinc, cadmium, lead, copper and strontium), in aqueous solution, on the growth of a urease-positive bacterium, *Sporosarcina pasteurii*. Generally metallic contaminants may prolong bacterial lag phase, bring about growth rate reduction or cause complete inhibition for the bacteria (Ruggiero *et al.* 2005). Such effects will be problematic when using bacteria in heavy metals precipitation processes. Heavy metal effects on the *S. pasteurii* cells growth in *M1* medium (contained only nutrient broth and a range of contaminant concentrations) were monitoring by measuring the optical density (OD_{600}) for samples after incubation period. Bacteria growth in their optimal medium (nutrient broth and urea) reaches more than 10^8 cells/mL after 24 hours of growing. However, in case of metallic toxicity, preliminary tests demonstrated that more time is required to discover the maximum tolerated concentration by *S. pasteurii* cells in *M1* medium. This may because the presence of heavy metals lead to prolong the lag phase for bacteria growing and lowering normal growing rates (Ruggiero *et al.* 2005). Hence, toxicity of metal cations was estimated the after 72 hours to allow for some growth delays.

The possible bioavailable portion of total heavy metal concentration in diluted solutions was predicted by using the geochemical equilibrium speciation model, MINTEQ. Table 4.1 presents the equilibrium concentration of heavy metals in dilute solutions. The MINTEQ calculations confirmed the presence of free heavy metal cations that dissolved in aqueous solutions. However, free metal ions were predominant over other species that resulted from dissolving heavy metal salts in deionised water. However, metal salts that were used here zinc, copper, lead and

strontium chlorides and cadmium sulphate. Cadmium sulphate can act as a weak acid leads to decrease the solution pH value to lower than 6, which may contribute to increase metal solubility.

The response of *S. pasteurii* cells to heavy metals toxicity is shown Figure 4.3. Ruggiero *et al.* (2005) defined the toxic concentration or MIC of a metal in solution as that concentration inhibits the growth of a microorganism by >70% compared to normal growing (organism growth measured by optical density). Accordingly, cadmium exhibited the greatest toxicity with MIC 0.03-0.06 mM, whilst the bacteria resisted strontium with concentrations up to 10 mM. From measured optical density values and after comparing with optical density value at full growth (bacteria that were grown in nutrient broth but without heavy metal) it be able to specify heavy metal minimum inhibitory concentrations (MIC; defined as a 70% reduction in growth as measured by optical density).

Table 4.2 present MICs estimated in this study. The bacteria responses to metallic toxicity, in term of propagation, were showed inhibition in the following order Cd >Zn >Cu >Pb >Sr. Lower values of optical density reflect that the bacteria could not able to keep growing in presence of increased concentrations range of heavy metals.

Table 4.1. Equilibrium concentration of heavy metals in dilute solutions that were predicted by using the geochemical equilibrium speciation model, MINTEQ.

Zinc concentration Final pH Species (%)	Cadmium concentration Final pH Species (%)	Copper concentration Final pH Species (%)	Lead concentration Final pH Species (%)	Strontium concentration Final pH Species (%)
0.01mM 6.773 Zn^{2+} (99.19) $ZnOH^{+}$(0.77) $Zn(OH)_2$(0.04)	0.015mM 6.897 Cd^{2+}(99.64) $CdSO_4$(0.28) $CdOH^{+}$(0.08)	0.01mM 6.068 Cu^{2+}(93.75) $CuOH^{+}$(5.97) $Cu(OH)^2$(0.03) $Cu_2(OH)_2^{2+}$(0.25)	0.01mM 6.348 Pb^{2+} (95.35) $PbOH^{+}$(4.6) $PbCl^{+}$(0.05)	0.1mM 6.918 Sr^{2+}(99.99) $SrCl^{+}$(0.01)
0.02mM 6.686 Zn^{2+}(99.33) $ZnOH^{+}$(0.63) $ZnCl^{+}$(0.01) $Zn(OH)_2$(0.03)	0.03mM 6.878 Cd^{2+}(99.37) $CdSO_4$(0.55) $CdOH^{+}$(0.08)	0.02mM 6.245 Cu^{2+}(95.69) $CuOH^{+}$(4.06) $Cu(OH)^2$(0.01) $Cu_2(OH)_2^{2+}$ (0.23)	0.05mM 6.019 Pb^{2+}(97.55) $PbOH^{+}$(2.2) $PbCl^{+}$(0.25)	0.5mM 6.917 Sr^{2+} (99.89) $SrCl^{+}$(0.11)
0.05mM 6.555 Zn^{2+}(99.49) $ZnOH^{+}$(0.47) $ZnCl^{+}$(0.03) $Zn(OH)_2$(0.01)	0.06mM 6.846 Cd^{2+}(98.84) $CdSO_4$(1.09) $CdOH^{+}$(0.07)	0.05mM 5.864 Cu^{2+}(97.17) $CuOH^{+}$(2.58) $Cu(OH)_2^{2+}$(0.23) $CuCl^{+}$(0.02)	0.1mM 5.99 Pb^{2+}(98.37) $PbOH^{+}$(1.35) $PbCl^{+}$(0.28)	1mM 6.916 Sr^{2+}(99.78) $SrCl^{+}$(0.22)
0.1mM 6.429 Zn^{2+}(99.60) $ZnOH^{+}$(0.35) $ZnCl^{+}$(0.05)	0.15mM 6.769 Cd^{2+}(97.29) $CdSO_4$(2.65) $CdOH^{+}$(0.06)	0.1mM 5.667 Cu^{2+}(98.12) $CuOH^{+}$(1.65) $Cu(OH)_2^{2+}$(0.19) $CuCl^{+}$(0.04)	0.2mM 5.836 Pb^{2+}(98.48) $PbOH^{+}$(0.95) $PbCl^{+}$(0.56) Pb_2OH^{3+}(0.01)	2mM 6.915 Sr^{2+}(99.57) $SrCl^{+}$(0.43)
0.2mM 6.302 Zn^{2+}(99.63) $ZnOH^{+}$(0.26) $ZnCl^{+}$(0.11)	0.3mM 6.746 Cd^{2+}(94.89) $CdSO_4$(5.03) $Cd(SO_4)_2^{2-}$(0.02) $CdOH^{+}$(0.06)	0.2mM 5.545 Cu^{2+}(98.45) $CuOH^{+}$(1.25) $Cu(OH)_2^{2+}$(0.23) $CuCl^{+}$(0.07)	0.5mM 5.676 Pb^{2+}(98.25) $PbOH^{+}$(0.57) $PbCl^{+}$(1.15) Pb_2OH^{3+}(0.03)	5mM 6.911 Sr^{2+}(98.94) $SrCl^{+}$(1.06)
0.5mM 6.12 Zn^{2+}(99.57) $ZnOH^{+}$(0.17) $ZnCl^{+}$(0.26)	0.45mM 6.721 Cd^{2+}(92.70) $CdSO_4$(7.20) $Cd(SO_4)_2^{2-}$(0.04) $CdOH^{+}$(0.06)	0.5mM 5.324 Cu^{2+}(98.86) $CuOH^{+}$(0.76) $Cu(OH)_2^{2+}$(0.20) $CuCl^{+}$(0.18)	1mM 5.491 Pb^{2+}(97.32) $PbOH^{+}$(0.37) $PbCl^{+}$(2.27) Pb_2OH^{3+}(0.04)	10mM 6.900 Sr^{2+}(97.91) $SrCl^{+}$(2.09)

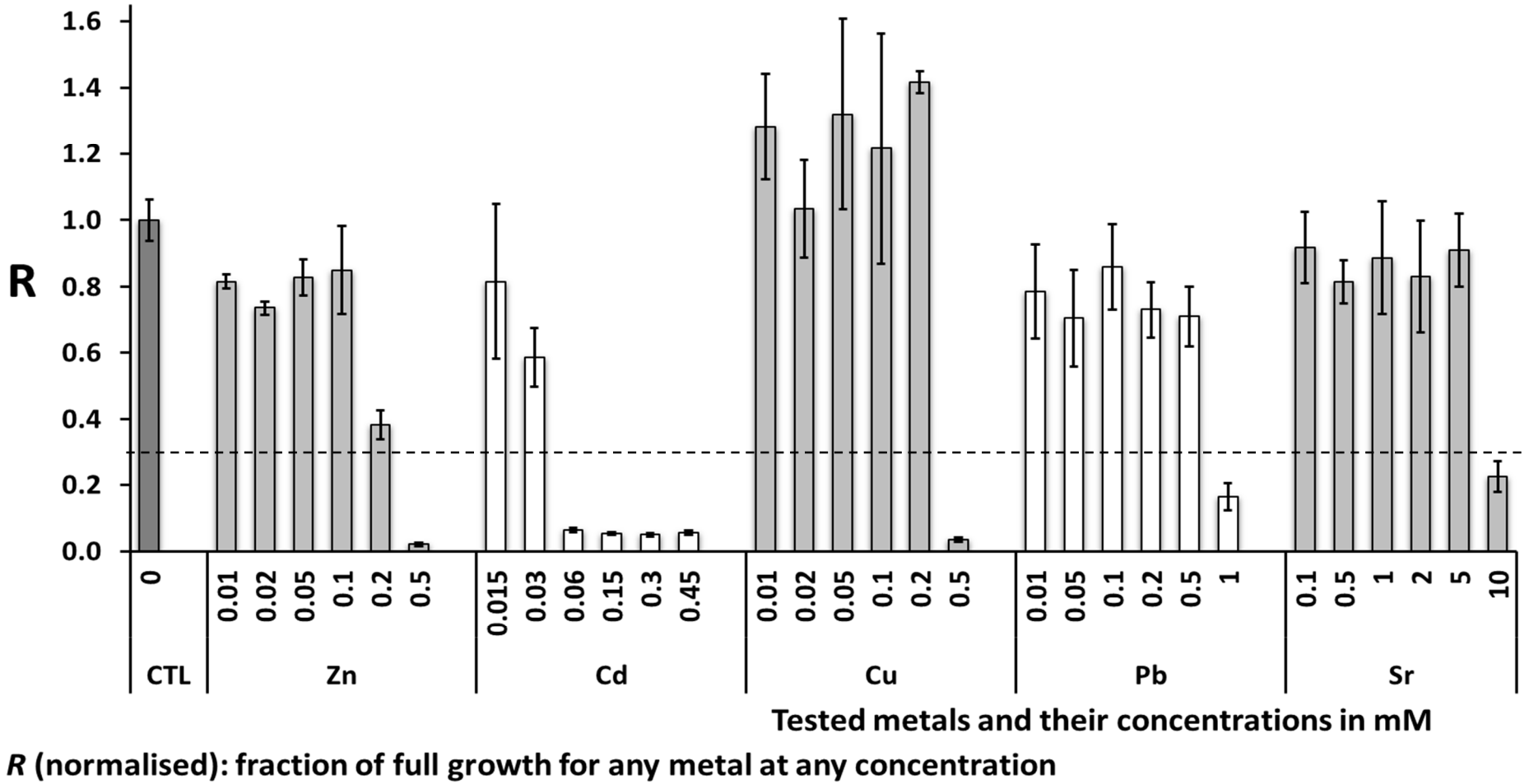


Figure 4.3. Response of S. pasteurii cells that were cultured at various metal concentrations (mM) after 3 days incubation at 30ºC. Dashed line represents MIC value (70% reduction in growth). [Error bars ± 1 SD, n=3].

Table 4.2. Minimum inhibitory concentrations (MIC) of heavy metals to S. pasteurii growth in solutions.

Metal	Zinc	Cadmium	Copper	Lead	Strontium
MIC (mM)	0.2-0.5	0.03-0.06	0.2-0.5	0.5-1	5-10

It is likely that zinc, cadmium, copper, lead or strontium ions entered *S. pasteurii* cells by chemiosmosis gradient mechanism. Metallic cations movement results from the concentration difference across the membrane without any energy consuming from ATP (Adenosine triphophate), ions movement by this mechanism can be quick and unselected for specific ion. Accordingly, many heavy metal cations with high concentrations can be inside the cells cause toxicity problems (Bauda and Block 1990; Nies 1999; Jaysankar *et al.* 2008; Rathnayake *et al.* 2013). Nevertheless, *S. pasteurii* cells may tolerate metal ions at concentrations equal or less than MICs by many mechanisms include removing heavy metals from the cell by efflux system, reduce heavy metal ions valence to less toxic states and accumulation and complication of heavy metals inside the cell (Roane 1999; Murthy *et al.* 2011).

No studies were found in the literature that were specified for the tolerance of *S. pasteurii* strains to metallic toxicant to compare with. The inhibitory concentrations values were found here are specified for this study and should not generalise for the bacteria strain. But it can consider basic or reference values of inhibitory concentration for *Sporosarcina pasteurii* with considering the experiment conditions.

4.3.1 Zinc Toxicity

Zinc is one of the essential elements for microbial growth, at trace concentrations zinc stimulates bacteria growth. In spite of that zinc does not involve in biological redox reaction but it is important in forming complexes and is a component of different enzymes (Nies 1999). In this experiment *S. pasteurii* cells tolerated zinc concentrations <0.5mM by using their efflux mechanisms. Possibly zinc ions can affect bacteria by disrupting cell membrane or inhibiting their enzyme activity (Rajendran *et al.* 2003). As it clear from Figure 4.3 bacterial growth is fairly consistent up to 0.1 mM before the growth affected by higher concentrations of the metal.

The inhibitory concentration of zinc for *S. Pasteurii* strains found in this study (0.2-0.5 mM) is consistent with other studies found MICs of zinc to other *Bacillus* species (Mgbemena *et al.* 2012; Nweke *et al.* 2007). However, it is lower than that estimated by Freitas *et al.* (2008) (2 mM) for *Bacillus* sp. that isolated from a steel plant wastes, this may be attributed to aging with contaminants for long time may improve microorganisms tolerance to heavy metals.

4.3.2 Cadmium Toxicity

Cadmium concentration that inhibited *S. pasteurii* growth is in the range from 0.03-0.06 mM (Figure 4.3), which is low concentration compare to other heavy metals. Defence mechanisms toward heavy metals seem did not help the bacteria to detoxify even low cadmium concentrations. The detoxifying mechanism in gram-positive bacteria is based on efflux the cadmium ions outside the cell. Cadmium ions may toxify bacteria by inhibiting cell division or enzyme activity, cell membrane

disruption and protein denaturation (Rajendran *et al.* 2003). This reflects the high sensitivity of this strain to this metal. The effect of cadmium is similar to that described by Ruggiero *et al*. (2005) for *D. radiodurans*, where it is seen to impact growth at far lower concentrations than other metals tested.

By reviewing inhibitory concentrations of cadmium to *Bacillus species* or gram-positive bacteria that found by many studies, it noticed that cadmium MIC estimated in this study is close to that found by Rathnayake *et al.* (2009). However, 0.4-16 mM was the estimated range of MICs of cadmium to *Bacillus species* or gram-positive bacteria that found by many other (Farshid *et al.* 2013) studies (Roane and Kellogg 1996; Hu *et al.* 2007; Murthy *et al.* 2011; Samanta *et al.* 2012; Ankita *et al.* 2013). This high variance may relate to bacteria type resistance.

4.3.3 Copper Toxicity

As it clear from Figure 4.3, *S. pasteurii* cells exhibited growth rates (0.01-0.2 mM) higher than that in metal-free medium, before inhibition at copper concentration of 0.5 mM. The metal showed different trend (the response was different at each concentration) in affecting *S. pasteurii* cells at concentrations range 0.01-0.2mM. MIC (0.2-0.5 mM) of copper encountered to be similar to that for zinc in this study. Again no studies in literature to compare with but MICs that estimated for other *Bacillus sp.* showed how these values can be different from study to other (Tamil Selvi *et al.* 2012; Oladipo and Adeoye 2013; Chatterjee *et al.* 2014; Khusro *et al.* 2014).

4.3.4 Lead Toxicity

The response of *S. pasteurii* cells to a range of lead concentrations (0.01-1 mM) is shown in Figure 4.3. Bacteria tolerates lead toxicity may be attributed to physical, chemical and biological factors. Lead chloride (the salt used here as lead ions source) is less in soluble than other metal salts, it is noticed that lead chloride is readily to re-precipitate in the bottom of tubes when is left without shaking. However, MINTEQ data Table 4.1 predicts high dissolution of lead chloride salt. This imply that bacterial cells may not subject to total lead concentration because the bioavailability of lead ions in solutions can decrease with time due to precipitation. One of the important factors that affects bacteria tolerance to heavy metal, is the bioavailability or solubility of that metal. This availability increased with decreasing solution pH. The possibility of passing the outer membrane of any microorganism by metal ions increases with increasing the solubility. Therefore, the minimum inhibitory concentration of lead found in this study (1mM) may not resulted from total added lead concentration. The relative high MIC of lead may attribute to its low solubility, so the bacteria did not subject to total lead concentration but only soluble or available portion. This conclusion is consistent with Nies (1999) outcomes.

The inhibitory lead concentration estimated in this study is compatible with some previous studies but for different bacteria (Roane and Kellogg 1996; Nath *et al.* 2012; Tamil Selvi *et al.* 2012; Ankita *et al.* 2013).

4.3.5 Strontium Toxicity

The physical properties of strontium isotopes (radioactive and non-radioactive) are almost similar. Bunzl *et al.* (1999), Tsukada *et al.* (1998) and Tsukada

et al. (2005) referred to the possibility of predicting the behaviour of a radioactive isotope by monitoring the stable one in the environment. Hence, the study of non-radioactive Sr^{2+} ions behaviour will mimic the behaviour of radioactive isotope in the environment (Mitchell and Ferris 2005). As can be seen from Figure 4.3 *S. pasteurii* cells evidently uninhibited by high concentrations of strontium less than 10 mM (0.1-5 mM). The high tolerance of strontium by the strain may attributed to many reasons. In spite of it has not any known benefit for organisms life, some findings suggest that strontium can suffice enzymes systems requirements for calcium, which makes strontium non-toxic (Nielsen 1986).

Owing to their lower sensitivity to strontium, research has not paid significant attention for *S. pasteurii* toxicity by this metal. However, tremendous amount of attention has been given to use ureolytic bacteria for precipitating this heavy metals. Therefore, it is not surprise that the bacteria tolerated Sr concentrations up to 10 mM, because other bacteria tolerate more than this. Many studies showed high resistance of many organisms to strontium toxicity (Ghazvini *et al.* 2007; Ben Salem *et al.* 2012; Ozer *et al.* 2012)

Chapter 5: Heavy Metals Bioprecipitation in Aqueous Solutions

5.1 Introduction

This chapter describes the ability of *S. pasteurii* to cause precipitation or co-precipitation of a number of metallic contaminants (zinc, cadmium, lead, copper and strontium) in aqueous solutions through generation of carbonate ions and elevated pH. Batch experiments were carried out to find whether bioprecipitation technique is applicable for long-term removal of above heavy metal cations with calcium carbonate precipitation. The metals were tested separately with calcium carbonate precipitation medium. Concentrations of heavy metal and calcium ions and solutions pH values were measured at periods of 0, 1, 3, and 7 days. The chosen concentrations of heavy metals were based on toxicity experiments and preliminary tests.

In each case, live-cell and killed-cell (autoclave sterilisation) experimental data are presented side by side. Normalised metal and calcium concentrations, relative to initial values, are presented, indicating the extent of removal over time at the concentrations tested. In addition, pH data are presented. The conjunction of calcium removal and pH data provides an indication of whether calcium carbonate precipitation was occurring.

Visual MINTEQ, a Windows version of the US EPA code MINTEQA2, was used to compare the results that were gained from the experiments and the expected results from this model. Though Visual MINTEQ model cannot handle the biological processing due to the lack of equilibrium in such processes, but its data base can give a proper prediction about the resulted species from the chemical reactions (Gustafsson, 2013).

5.2 Calcium and Urea Concentrations

This experiment was done to optimise calcium and urea concentrations to be used in precipitation medium. The initial concentrations of calcium and urea in precipitation medium were chosen from a range of concentrations (2 and 20 g/L for urea and 10, 20, 25, 30, 40 and 50 mM for calcium). Figure 5.1 shows the reaction between different concentrations of urea and calcium in presence of urease enzyme which produced by *S. pasteurii* in the solutions. Urease enzyme leads to hydrolyse urea in water to produce ammonium and carbonate ions. Carbonate and calcium ions react later at high pH to produce calcium carbonate precipitation (white precipitate was seen at the end of the experiment in all samples). This process can remove or decrease calcium and carbonate ions from the solutions. Simply, to precipitate all the dissolve Ca ions, it needs enough number of CO_3 ions in the same solution.

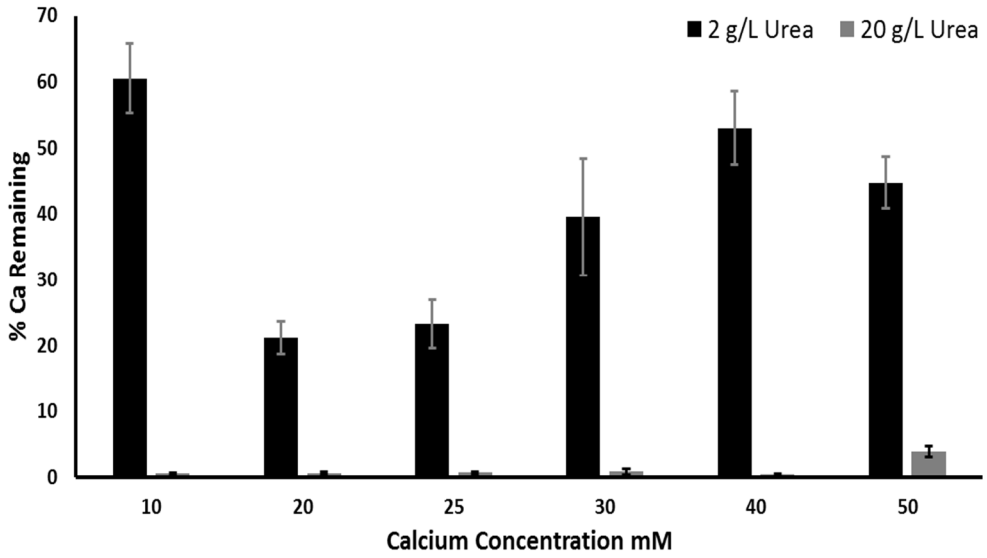


Figure 5.1. The effect of urea concentration on calcium precipitation with S. pasteurii. [Error bars: ±1 SD, n=3]

As it clear from the figure, urea concentration of 2 g/l was not enough to precipitate all calcium ions dissolved in solution. This may mean carbonate ions ran

out before removing calcium ions. The amount of calcium removed with 2 g/l urea was not similar to each calcium concentration. Compared to other calcium concentrations significant proportion was recovered at 10 mM calcium concentration, which is unexpected, this may relate to bacterial activity (bacteria was less activity in these samples). With 20 g/l calcium ions removal was up to 99.5% at all concentrations (apart from highest calcium concentration) of dissolved calcium ions in solutions probably by precipitation reactions. At highest concentration 4% calcium ions were left, hence, mathematically optimal calcium concentration with 20 g/l urea is 48 mM (96%×50mM=48mM). Accordingly, 20 g/l (333 mM) urea and 50 mM calcium were chosen for all precipitation media in this study. pH in 20 g/l urea samples reached up to 9, whilst in 2 g/l samples was less.

The selected urea initial concentration (20 g/l) was used in other microbially calcite precipitation studies (Fujita *et al.* 2000; DeJong *et al.* 2006; Achal *et al.* 2009; Lauchnor *et al.* 2013), but with different initial calcium concentration. Stocks-Fischer *et al.* (1999) proved that the precipitation rate and ammonia production rate are virtually the same for initial calcium concentrations of 12.6, 25.2 and 50.4 mM.

5.3 Zinc

In this experiment the ability of *S. pasteurii* cells to induce calcium carbonate precipitation and co-precipitation of zinc ions was explored. Figure 5.2 shows the response of *S. pasteurii* to different concentrations of zinc. Complete removal of zinc from solution in the 7 days period occurs up to a concentration of 0.5 mM in the presence of live cells, which indicates an improved resistance to metal toxicity over that seen in earlier experiments (Chapter 4, Figure 4.3: toxicity response seen at concentrations greater than 0.2 mM). This improvement is probably due to role of

urea hydrolysis by the bacterial cells that induce calcium carbonate precipitation and metal removal.

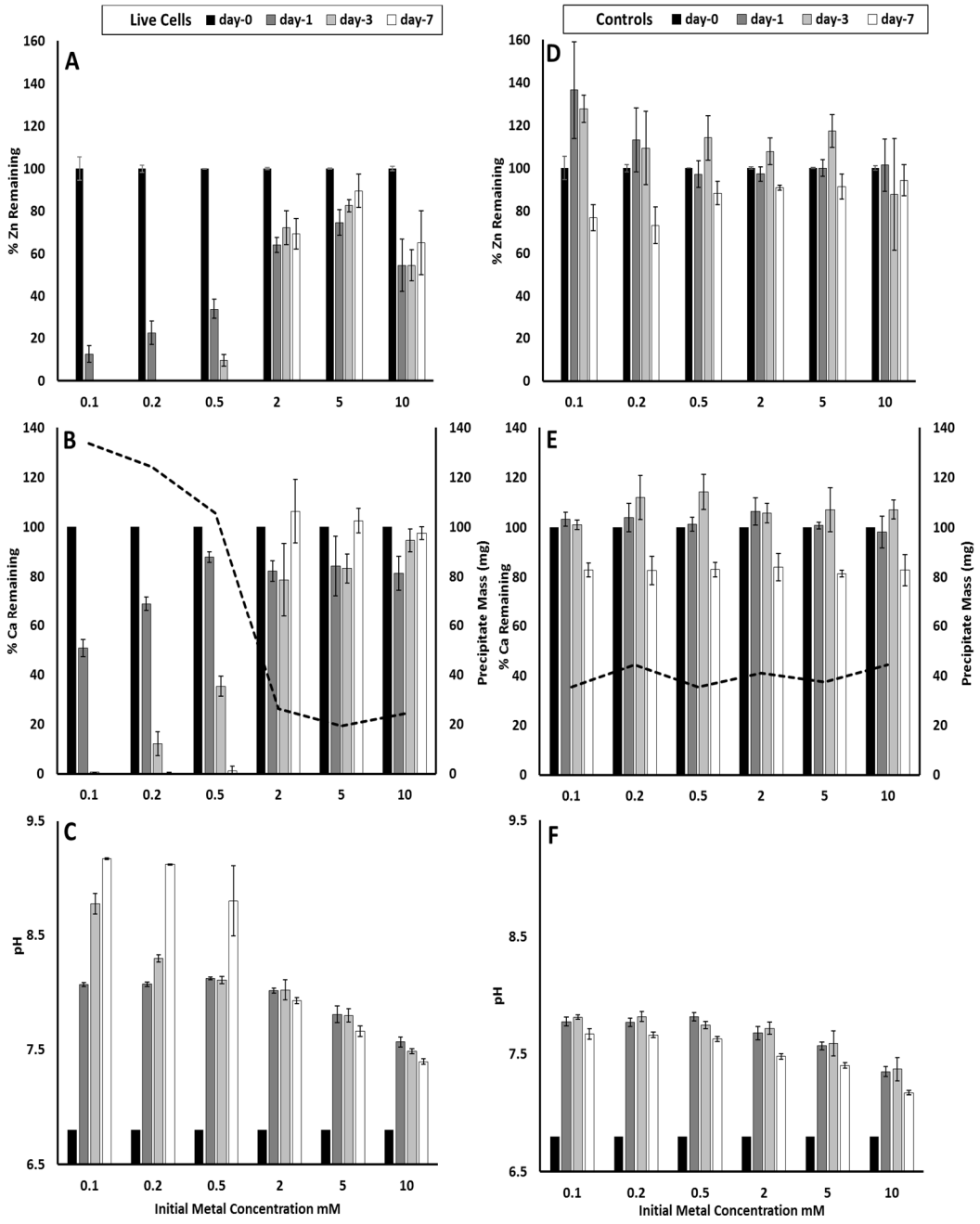


Figure 5.2. Impact of S. pasteurii activity on zinc removal (A, D), calcium removal (B, E) and pH (C, F) at a range of zinc concentrations, with live (A-C) and killed (D-F) cells at 30ºC with urea growth medium. Dashed lines on (B) and (E) represent precipitate mass after day-7. [Error bars: ±1 SD, n=3]

Increase in pH and removal of calcium and zinc from solution are associated; the pH change is expected to be driven by urea hydrolysis by urease enzyme. These changes are evident at a zinc concentration of 0.5 mM or below, whilst at 2 mM or above the pH in live cell experiments is consistent with killed cell controls and shows a slight decrease with increasing zinc concentration. The rate of removal of both zinc and calcium also decreases with increasing zinc concentration. Calcium removal at higher zinc concentrations (10 mM) is generally low, albeit with some variability at different days. This implies that *S. pasteurii* cells were inhibited by metal ions or could not survive in presence of zinc concentration above 0.5 mM. The increasing in pH values in these samples and control samples may relate to abiotic reactions (chemical reactions may have led to change the pH value). Zinc recovery in controls has some variability at lower concentrations, possibly due to abiotic precipitation or inherent difficulties in preparing and analysing low-concentration experiments. It's not clear in this experiment if any biosorption was occurred by zinc ions sorption on bacterial biomass (live or dead), if so would be responsible of partial metal decreasing in solutions.

At zinc concentration equal or greater than 2 mM, metal toxicity to bacterial activity may affected urea hydrolysis process, also zinc ions can inhibit crystal growth of calcium carbonate (McBride 1980; Comans and Middelburg 1987; US EPA 1992). Such effects seem to prevent occurrence of any calcium and zinc precipitation at those concentrations, though the toxicity may have the more effect. However, as it clear from Figure 5.2 few amount of calcium has precipitated with zinc concentrations more than 2 mM.

Dashed lines on (B) and (E) in Figure 5.2 shows the amount of precipitate per each sample from the test and control samples (mg/L). The amount of precipitates that generated in live cells experiments at 0.1, 0.2 and 0.5 mM zinc concentrations much higher than those produced at zinc concentrations more than 0.5 mM and control samples. It's clear that the precipitate amounts decrease with increasing zinc concentration up to 0.5 mM, which reflects the toxicity effect of zinc ions on the bacteria. Increased precipitate mass correlates well with elevated removal of calcium in solution, although there is a consistent minimum mass of precipitate even when there was no calcium removal. Loss of calcium ions from solutions associated with pH increasing. The highest precipitation amounts in live-cells samples occurred at zinc concentrations up to 0.5 mM, which were accompanied with almost full removal of calcium ions. Whilst the amount of precipitates and calcium removal in control samples were consistent.

Analysis of expected zinc and calcium speciation at chemical equilibrium in live and killed cells experiments was performed using Visual MINTEQ. Table 5.1 and Table 5.2 illustrate the main species expected to be present under the prevailing conditions in live and killed cells samples. Decreasing amounts of calcium carbonate and increasing amounts of ionic calcium species are expected as zinc concentration increases and pH decreases. This correlates broadly with the increasing calcium remaining in solution with zinc concentration (Figure 5.2).

However, in live cells samples, the prediction for zinc species results in entirely ionic/dissolved species apart from at the two highest zinc concentrations where small but increasing amounts of zinc carbonate are expected. This does not agree with Figure 5.2-A, where at lower zinc concentrations no dissolved zinc was found at the end of the experiment. This suggests that a mechanism not included in the Visual MINTEQ model had a significant role, likely to be sorption or co-precipitation of metals with increasing amounts of calcium carbonate. At higher concentrations (2 and 5 mM), increasing amounts of dissolved zinc were found but at 10 mM there was a further decrease, which may indicate a decreasing effect of this mechanism partly counteracted by the increasing precipitation of zinc carbonates. Precipitation of relatively small amounts of zinc and calcium carbonates may therefore explain the small decreases in dissolved zinc and calcium seen in control specimens at pH similar to that observed at 10 mM with live cells.

Visual MINTEQ model calculations for control experiment data according to zinc concentration and final pH after 7 days (Table 5.2) illustrate the possibility of zinc carbonate formation, though in small amounts. This amount increased with zinc concentrations although pH was decreasing. Contrary, the predicted amount of calcium carbonate decreased with zinc concentration increase and pH decrease. Based on Visual MINTEQ model results and providing that the precipitate amounts in Figure 5.2 were accumulated from zinc and calcium carbonates, it can be assumed some consistency between the experiment outcomes and Visual MINTEQ model calculations.

Table 5.1. Zinc and calcium predominant species in calcium carbonate medium calculated by visual MINTEQ according to zinc concentration and final pH, for the test samples (live-cells experiments).

Zinc (mM) (Final pH)	Zn species name	% of total concentration	Ca species name	% of total concentration
0.1 (9.17)	$Zn(CO_3)_2^{2-}$	0.037	Ca^{2+}	2.644
	$Zn(NH_3)_4^{2+}$	97.59	$CaCl^+$	1.39
	$Zn(NH_3)_3^{2+}$	2.344	$CaNH_3^{2+}$	1.364
	$Zn(NH_3)_2^{2+}$	0.025	$Ca(NH_3)_2^{2+}$	0.217
			$CaHCO_3^+$	8.632
			$CaCO_3$	85.752
0.2 (9.12)	$Zn(CO_3)_2^{2-}$	0.037	Ca^{2+}	2.893
	$Zn(NH_3)_4^{2+}$	97.455	$CaCl^+$	1.52
	$Zn(NH_3)_3^{2+}$	2.475	$CaNH_3^{2+}$	1.411
	$Zn(NH_3)_2^{2+}$	0.028	$Ca(NH_3)_2^{2+}$	0.213
			$CaHCO_3^+$	9.536
			$CaCO_3$	84.426
0.5 (8.80)	$Zn(CO_3)_2^{2-}$	0.051	Ca^{2+}	5.03
	$Zn(NH_3)_4^{2+}$	96.11	$CaCl^+$	2.642
	$Zn(NH_3)_3^{2+}$	3.758	$CaNH_3^{2+}$	1.594
	$Zn(NH_3)_2^{2+}$	0.065	$Ca(NH_3)_2^{2+}$	0.156
	$ZnCO_3$	0.014	$CaHCO_3^+$	17.293
			$CaCO_3$	73.284
2 (7.93)	Zn^{2+}	0.02	Ca^{2+}	14.194
	$Zn(NH_3)_4^{2+}$	79.756	$CaCl^+$	7.448
	$Zn(NH_3)_3^{2+}$	16.498	$CaNH_3^{2+}$	0.85
	$ZnCO_3$	1.239	$Ca(NH_3)_2^{2+}$	0.016
	$ZnHCO_3^+$	0.177	$CaHCO_3^+$	49.307
			$CaCO_3$	28.186
5 (7.66)	Zn^{2+}	0.169	Ca^{2+}	16.731
	$Zn(CO_3)_2^{2-}$	1.428	$CaCl^+$	8.778
	$Zn(NH_3)_4^{2+}$	63.071	$CaNH_3^{2+}$	0.554
	$Zn(NH_3)_3^{2+}$	23.591	$CaHCO_3^+$	56.565
	$ZnCO_3$	5.474	$CaCO_3$	17.365
	$ZnHCO_3^+$	1.456		
10 (7.40)	Zn^{2+}	1.003	Ca^{2+}	18.856
	$Zn(CO_3)_2^{2-}$	2.323	$CaCl^+$	9.892
	$Zn(NH_3)_4^{2+}$	36.412	$CaNH_3^{2+}$	0.349
	$Zn(NH_3)_3^{2+}$	24.399	$CaHCO_3^+$	60.666
	$ZnCO_3$	17.024	$CaCO_3$	10.235
	$ZnHCO_3^+$	8.238		

Table 5.2. Zinc and calcium predominant species in calcium carbonate medium calculated by visual MINTEQ according to zinc concentration and final pH, for the control samples (killed-cells experiments).

Zinc (mM) (Final pH)	Zn species name	% of total concentration	Ca species name	% of total concentration
0.1 (7.67)	Zn^{2+}	0.156	Ca^{2+}	16.442
	$Zn(CO_3)_2^{2-}$	1.432	$CaCl^{+}$	8.699
	$Zn(NH_3)_4^{2+}$	63.797	$CaNH_3^{2+}$	0.557
	$Zn(NH_3)_3^{2+}$	23.338	$CaHCO_3^{+}$	56.536
	$ZnCO_3$	5.273	$CaCO_3$	17.76
	$ZnHCO_3^{+}$	1.37		
0.2 (7.66)	Zn^{2+}	0.168	Ca^{2+}	16.525
	$Zn(CO_3)_2^{2-}$	1.471	$CaCl^{+}$	8.744
	$Zn(NH_3)_4^{2+}$	62.972	$CaNH_3^{2+}$	0.547
	$Zn(NH_3)_3^{2+}$	23.554	$CaHCO_3^{+}$	56.755
	$ZnCO_3$	5.553	$CaCO_3$	17.423
	$ZnHCO_3^{+}$	1.476		
0.5 (7.63)	Zn^{2+}	0.211	Ca^{2+}	16.767
	$Zn(CO_3)_2^{2-}$	1.593	$CaCl^{+}$	8.908
	$Zn(NH_3)_4^{2+}$	60.391	$CaNH_3^{2+}$	0.519
	$Zn(NH_3)_3^{2+}$	24.148	$CaHCO_3^{+}$	57.365
	$ZnCO_3$	6.466	$CaCO_3$	16.435
	$ZnHCO_3^{+}$	1.842		
2 (7.48)	Zn^{2+}	0.606	Ca^{2+}	17.91
	$Zn(CO_3)_2^{2-}$	2.192	$CaCl^{+}$	9.635
	$Zn(NH_3)_4^{2+}$	45.242	$CaNH_3^{2+}$	0.397
	$Zn(NH_3)_3^{2+}$	25.313	$CaHCO_3^{+}$	59.905
	$ZnCO_3$	12.852	$CaCO_3$	12.15
	$ZnHCO_3^{+}$	5.173		
5 (7.40)	Zn^{2+}	0.998	Ca^{2+}	18.542
	$Zn(CO_3)_2^{2-}$	2.388	$CaCl^{+}$	10.221
	$Zn(NH_3)_4^{2+}$	36.21	$CaNH_3^{2+}$	0.343
	$Zn(NH_3)_3^{2+}$	24.263	$CaHCO_3^{+}$	60.659
	$ZnCO_3$	17.215	$CaCO_3$	10.233
	$ZnHCO_3^{+}$	8.33		
10 (7.17)	Zn^{2+}	2.978	Ca^{2+}	20.211
	$Zn(CO_3)_2^{2-}$	2.161	$CaCl^{+}$	11.587
	$Zn(NH_3)_4^{2+}$	13.408	$CaNH_3^{2+}$	0.222
	$Zn(NH_3)_3^{2+}$	15.138	$CaHCO_3^{+}$	61.837
	$ZnCO_3$	28.289	$CaCO_3$	6.143
	$ZnHCO_3^{+}$	23.247		

Zinc sorbs strongly to calcium carbonate minerals (Zachara *et al.* 1991; Garcia-Sanchez and Alvarez-Ayuso 2002; Elzinga *et al.* 2006) though is readily desorbable due to its high hydration energy and because sorbed zinc is only slowly absorbed into a solid solution unless recrystallization occurs. Garcia-Sanchez and Alvarez-Ayuso (2002) report sufficient sorption capacity on calcite to full sorb zinc at all but the

largest concentration tested in this study. Here, however, calcium carbonate crystals are being formed concurrently and so co-precipitation as a Ca-Zn-CO_3 solid solution (as noted by Buekers *et al.* (2007)) is likely, possibly alongside sorption and subsequent encapsulation. Zinc forms a tetrahedral carbonate structure (Teng and Zhao 2012) which may reduce long term stability of any co-precipitate due to a mismatch with the microstructure of calcium carbonate (the calcium carbonate matrix in calcite is octahedral). Zinc has been reported to accelerate heterogeneous nucleation (Zeppenfeld 2010) so may lead to a larger number of smaller crystals forming, which may reduce long term sequestration of encapsulated zinc, although this was not directly observed.

The experimental data do not indicate whether sorption, co-precipitation or a combination of the two are responsible for removal of zinc, as there is good correlation between calcium removal (and therefore presumably formation of carbonate) and loss of zinc.

5.4 **Cadmium**

Removal of cadmium in live cell experiments again correlates well with pH change and calcium removal (Figure 5.3). Almost all tested cadmium concentrations were removed by day 3 at all concentrations tested, with a decrease in removal rate evident at higher concentrations (1.5 mM). Considering use different media, this is a substantial improvement on resistance of *S. pasteurii* to cadmium toxicity (Chapter 4, Figure 4.3), with the organism able to remove the metal from solution at concentrations at least 25 times that at which growth was impaired when urea was not present (MIC).

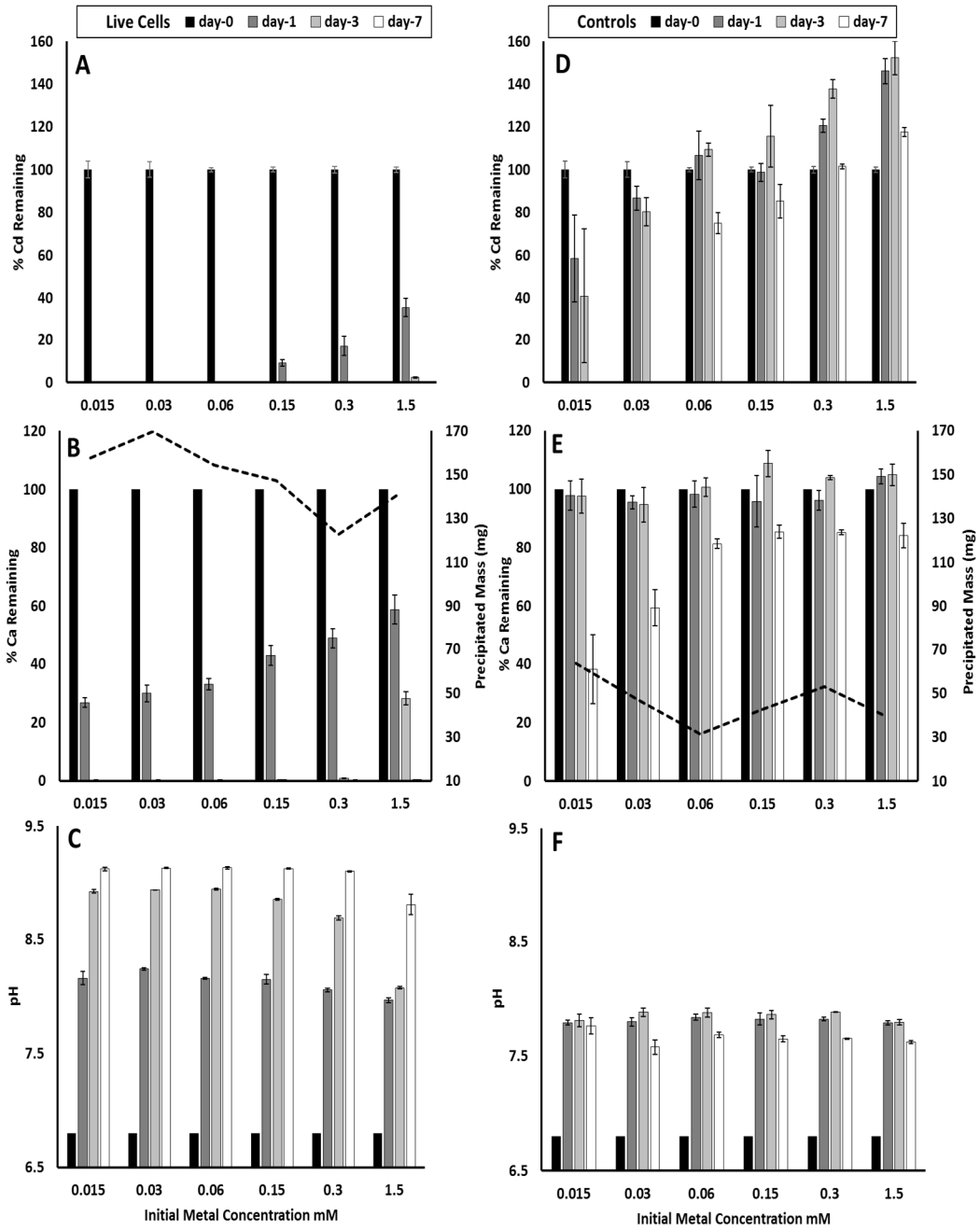


Figure 5.3. Impact of S. pasteurii activity on cadmium removal (A, D), calcium removal (B, E) and pH (C, F) at a range of cadmium concentrations, with live (A-C) and killed (D-F) cells at 30ºC with urea growth medium. Dashed lines on (B) and (E) represent precipitate mass after day-7. [Error bars ±1 SD, n=3].

In live cells experiment pH increased gradually over seven days to reach up to 9.14. In previous experiment where no cadmium added (section 5.2), only calcium carbonate medium (bacteria, urea and calcium), *S. pasteurii* needed less than 24 hours

to induce pH increasing up to 9, due to urea hydrolysis in water. This reflects the effect of cadmium ions in weakening bacterial activity to produce urease enzyme in solution.

In control experiments, there were no observed changes in pH as expected, although there was some reduction in calcium concentration over time particularly at low cadmium concentrations, correlated with loss of cadmium. At low cadmium concentrations (0.015 and 0.03 mM) full removal of metal ions was noticed. This correlation may indicate occurrence of similar mechanism of calcium precipitation to that with live cells samples driving completely and partly cadmium removal in control samples. *S. pasteurii* is nominally a spore-forming organism and so spores may have survived the autoclaving process (Stolp 1988; Todar 2005), with subsequent regeneration leading to microbial activity in controls. No significant pH increase was seen, although it may be that a small change in pH was sufficient to cause a degree of precipitation but that this was then buffered by the mineralisation process. The slow change in calcium and cadmium concentrations could be attributed to delays in spore regeneration and subsequent growth. There was excess recovery of cadmium at times, which was unexpected.

Removal of cadmium ions in this study may due to adsorption on precipitated calcium carbonate crystals or precipitated as metals carbonate. At low metal concentrations adsorption on calcium carbonate precipitation surface is more potential, while at higher concentration metal carbonate precipitation is dominant (Papadopoulos and Rowell 1989). Davis *et al.* (1987) reported that the adsorption of cadmium ions on calcite is a pH dependent process, also cadmium ions can exchange with calcium ions in hydrate layers after 24 hours of reaction. Also, according to Papadopoulos and Rowell (1989) and García-Sánchez and Álvarez-Ayuso (2002)

cadmium sorption mechanism on calcium carbonate is almost similar to zinc, but cadmium has affinity to be adsorbed on calcite higher than zinc, also cadmium desorption is slow (Zachara *et al.* 1991). At 1.5 mM cadmium concentration *S. pasteurii* response was different from other concentrations, more time was needed to precipitate cadmium and calcium ions and minimum pH value recorded when compares with other live cells samples.

The precipitation amounts of calcium carbonate in cadmium experiment are shown in Figure 5.3 (B and E). In live cells samples calcium carbonate precipitation occurred in conjunction with calcium removal from solutions. At the first three cadmium concentrations where cadmium ions were removed after 24 hours, the amount of precipitates were higher than that at the other three concentrations. The precipitates produced in control samples (killed cells) were much less than live cells samples. Precipitated amounts in both experiments (live and killed cells) probably were responsible for cadmium removal from solutions. Many studies referred to that cadmium chemisorption at low concentration by $CaCO_3$ is possible and very rapid, while at higher concentration of this heavy metal the possibility of $CdCO_3$ is more probable (Bradl 2004; Choi 2006; Stietiya and Wang 2014; Zhao *et al.* 2014).

As with zinc, there is a degree of commonality between Visual MINTEQ and experimental data for removal of calcium from solution, with the former indicating a decreasing proportion of precipitated calcium with increasing cadmium (Table 5.3). In experiments, although the final results indicated no calcium in solution, the rate at which it was removed does decrease as cadmium concentration increases (Figure 5.3, B). In addition, although Visual MINTEQ predicts that all cadmium should remain in

solution, no cadmium was actually found (Figure 5.3, A). Again, it is thought that sorption and co-precipitation with calcium carbonate are the main reasons for this.

Analysis of expected cadmium and calcium speciation at chemical equilibrium for cadmium control experiment was performed using Visual MINTEQ (Table 5.4) as well. The small amount of precipitates that were expected by the model against each concentration are consentient with the control experiment results (Figure 5.3, E).

Cadmium is similar in atomic radius to calcium and forms a similar octahedral structure with carbonate (Teng and Zhao 2012). It therefore can substitute for calcium relatively easily in carbonate minerals, forming solid solutions and co-precipitates readily. On existing surfaces of calcite, rapid sorption as a stable complex can form an epitaxial layer, preventing further sorption of the metal and limiting dissolution of the mineral (Chada *et al.* 2005; Du *et al.* 2011) although Stipp *et al.* (1992) and Buekers *et al.* (2007) found that movement from surface layers into the mineral was possible in a relatively short period. Sorption to other, more openly structured forms of calcium carbonate such as aragonite is not affected as an epitaxial layer does not form (Prieto *et al.* 2013) although these minerals are relatively soluble and over time may recrystallize as calcite, incorporating the metal as a solid solution.

As with zinc, however, the non-equilibrium status of the carbonate minerals (with growth over the experiment) may prevent formation of a coherent epitaxial layer due to continuous deposition of new material. In such cases, cadmium carbonate may precipitate initially as otavite, even at low, non-saturated concentrations (Tesoriero and Pankow 1996), in preference to calcium carbonate due to the lower solubility of the former; this may then be encapsulated by more calcium-rich material (Prieto *et al.* 2013).

Table 5.3. Cadmium and calcium predominant species in calcium carbonate medium calculated by visual MINTEQ according to cadmium concentration and final pH, for the test samples (live-cells experiments).

Cadmium (mM) (Final pH)	Cd species name	% of total concentration	Ca species name	% of total concentration
0.015 (9.12)	$CdCl^+$	0.01	Ca^{2+}	2.893
	$Cd(NH_3)_4^{2+}$	67.858	$CaCl^+$	1.52
	$Cd(NH_3)_3^{2+}$	25.949	$CaNH_3^{2+}$	1.413
	$Cd(NH_3)_2^{2+}$	2.93	$Ca(NH_3)_2^{2+}$	0.213
	$CdCO_3$	0.194	$CaHCO_3^+$	9.535
	$Cd(CO_3)_2^{2-}$	2.969	$CaCO_3$	84.424
0.03 (9.13)	$Cd(NH_3)_4^{2+}$	68.113	Ca^{2+}	2.841
	$Cd(NH_3)_3^{2+}$	25.754	$CaCl^+$	1.493
	$Cd(NH_3)_2^{2+}$	2.876	$CaNH_3^{2+}$	1.403
	$CdNH_3^{2+}$	0.072	$Ca(NH_3)_2^{2+}$	0.214
	$CdCO_3$	0.19	$CaHCO_3^+$	9.348
	$Cd(CO_3)_2^{2-}$	2.972	$CaCO_3$	84.696
0.06 (9.14)	$Cd(NH_3)_4^{2+}$	68.361	Ca^{2+}	2.791
	$Cd(NH_3)_3^{2+}$	25.562	$CaCl^+$	1.467
	$Cd(NH_3)_2^{2+}$	2.823	$CaNH_3^{2+}$	1.394
	$CdNH_3^{2+}$	0.07	$Ca(NH_3)_2^{2+}$	0.215
	$CdCO_3$	0.186	$CaHCO_3^+$	9.164
	$Cd(CO_3)_2^{2-}$	2.975	$CaCO_3$	84.962
0.15 (9.13)	$Cd(NH_3)_4^{2+}$	68.111	Ca^{2+}	2.842
	$Cd(NH_3)_3^{2+}$	25.756	$CaCl^+$	1.494
	$Cd(NH_3)_2^{2+}$	2.876	$CaNH_3^{2+}$	1.403
	$CdNH_3^{2+}$	0.072	$Ca(NH_3)_2^{2+}$	0.214
	$CdCO_3$	0.19	$CaHCO_3^+$	9.347
	$Cd(CO_3)_2^{2-}$	2.97	$CaCO_3$	84.683
0.3 (9.10)	$CdCl^+$	0.011	Ca^{2+}	3
	$CdCl_2$	0.01	$CaCl^+$	1.576
	$Cd(NH_3)_4^{2+}$	67.329	$CaSO_4$	0.032
	$Cd(NH_3)_3^{2+}$	26.353	$CaNH_3^{2+}$	1.431
	$Cd(NH_3)_2^{2+}$	3.046	$Ca(NH_3)_2^{2+}$	0.211
	$CdCO_3$	0.202	$CaHCO_3^+$	9.915
	$Cd(CO_3)_2^{2-}$	2.964	$CaCO_3$	83.834
1.5 (8.81)	$CdCl^+$	0.045	Ca^{2+}	4.949
	$CdCl_2$	0.042	$CaCl^+$	2.599
	$Cd(NH_3)_4^{2+}$	57.096	$CaSO_4$	0.021
	$Cd(NH_3)_3^{2+}$	33.066	$CaNH_3^{2+}$	1.596
	$Cd(NH_3)_2^{2+}$	5.655	$Ca(NH_3)_2^{2+}$	0.159
	$CdCO_3$	0.437	$CaHCO_3^+$	16.992
	$Cd(CO_3)_2^{2-}$	3.419	$CaCO_3$	73.683

Table 5.4. Cadmium and calcium predominant species in calcium carbonate medium calculated by visual MINTEQ according to cadmium concentration and final pH, for the control samples (killed-cells experiments).

Cadmium (mM) (Final pH)	Cd species name	% of total concentration	Ca species name	% of total concentration
0.015 (7.76)	Cd^{2+}	0.602	Ca^{2+}	15.7
	$CdCl^{+}$	11.855	$CaHCO_3^{+}$	54.382
	$Cd(NH_3)_3^{2+}$	18.18	$CaCO_3$	21.018
	$Cd(NH_3)_2^{2+}$	24.257		
	$CdCl_2$	10.988		
	$CdCO_3$	10.267		
	$Cd(CO_3)_2^{2-}$	7.218		
0.03 (7.58)	Cd^{2+}	0.996	Ca^{2+}	17.146
	$CdCl^{+}$	19.596	$CaHCO_3^{+}$	58.445
	$Cd(NH_3)_3^{2+}$	9.056	$CaCO_3$	17.923
	$Cd(NH_3)_2^{2+}$	18.024		
	$CdCl_2$	18.163		
	$CdCO_3$	11.035		
0.06 (7.69)	Cd^{2+}	0.773	Ca^{2+}	16.369
	$CdCl^{+}$	15.207	$CaHCO_3^{+}$	56.339
	$Cd(NH_3)_3^{2+}$	13.712	$CaCO_3$	18.111
	$Cd(NH_3)_2^{2+}$	21.839		
	$CdCl_2$	14.095		
	$CdCO_3$	10.885		
0.15 (7.65)	Cd^{2+}	0.839	Ca^{2+}	16.607
	$CdCl^{+}$	16.52	$CaHCO_3^{+}$	56.989
	$Cd(NH_3)_3^{2+}$	12.192	$CaCO_3$	17.097
	$Cd(NH_3)_2^{2+}$	20.759		
	$CdCl_2$	15.312		
	$CdCO_3$	11.003		
0.3 (7.65)	Cd^{2+}	0839	Ca^{2+}	16.604
	$CdCl^{+}$	16.524	$CaHCO_3^{+}$	56.952
	$Cd(NH_3)_3^{2+}$	12.19	$CaCO_3$	17.086
	$Cd(NH_3)_2^{2+}$	20.758		
	$CdCl_2$	15.316		
	$CdCO_3$	11.00		
1.5 (7.62)	Cd^{2+}	0.908	Ca^{2+}	16.816
	$CdCl^{+}$	17.881	$CaHCO_3^{+}$	57.289
	$Cd(NH_3)_3^{2+}$	10.754	$CaCO_3$	16.04
	$Cd(NH_3)_2^{2+}$	19.604		
	$CdCl_2$	16.573		
	$CdCO_3$	11.034		

Again, it is unclear to what extent sorption or co-precipitation are prevalent here, as both are linked to increasing amounts of calcium carbonate. However, Garcia-Sanchez and Alvarez-Ayuso (2002) quote a sorption capacity for cadmium on calcite of 10 mg/g, which is more than enough to sorb all the cadmium present in these experiments given the amounts of precipitate produced (assuming the precipitate is

calcite). Li *et al.* (2013) again found tolerance to and rapid removal (> 90% over 48 hours, mostly within 2 hours) of cadmium at much higher concentrations than tested here with *S. pasteurii* (10.9 mM compared to 1.5 mM) although complete removal was observed here at the highest concentration tested and so no toxicity limit was observed. High cadmium removal rates (99.95%) in aqueous solutions were observed by Kang *et al.* (2014) by using calcite-formation bacteria, metal concentration was 7.3 mM.

5.5 Copper

Experiments with copper contamination again indicate that microbial pH amendment leads to commensurate calcium removal (Figure 5.4). In this case, activity was seen up to a concentration of 1 mM copper. Despite the formation of calcium carbonate, and the elevated pH, a significant proportion (>50%) of copper ions remain in solution and so the toxicity of the system is not significantly reduced.

Full removal of calcium ions (up to a concentration of 1 mM copper) and recovering more than 50% of copper ions after 7 day may indicate that copper ions were not incorporated with calcium carbonate precipitate. Also, it is less likely to copper to precipitate as $CuCO_3$ due to instability of this carbonate (Franklin and Morse 1981). It seems that *S. pasteurii* survived under toxicant copper concentrations up to 1mM, where pH increased due to urea hydrolysis and almost full removal of calcium ions had occurred. However, bacteria showed less tolerance in toxicity experiment (Chapter 4, Figure 4.3) inhibited at copper concentration more than 0.2 mM, indicating that bacterial cells have more protection when present in precipitation medium (urea, calcium and other chemicals) though copper ions were not fully removed.

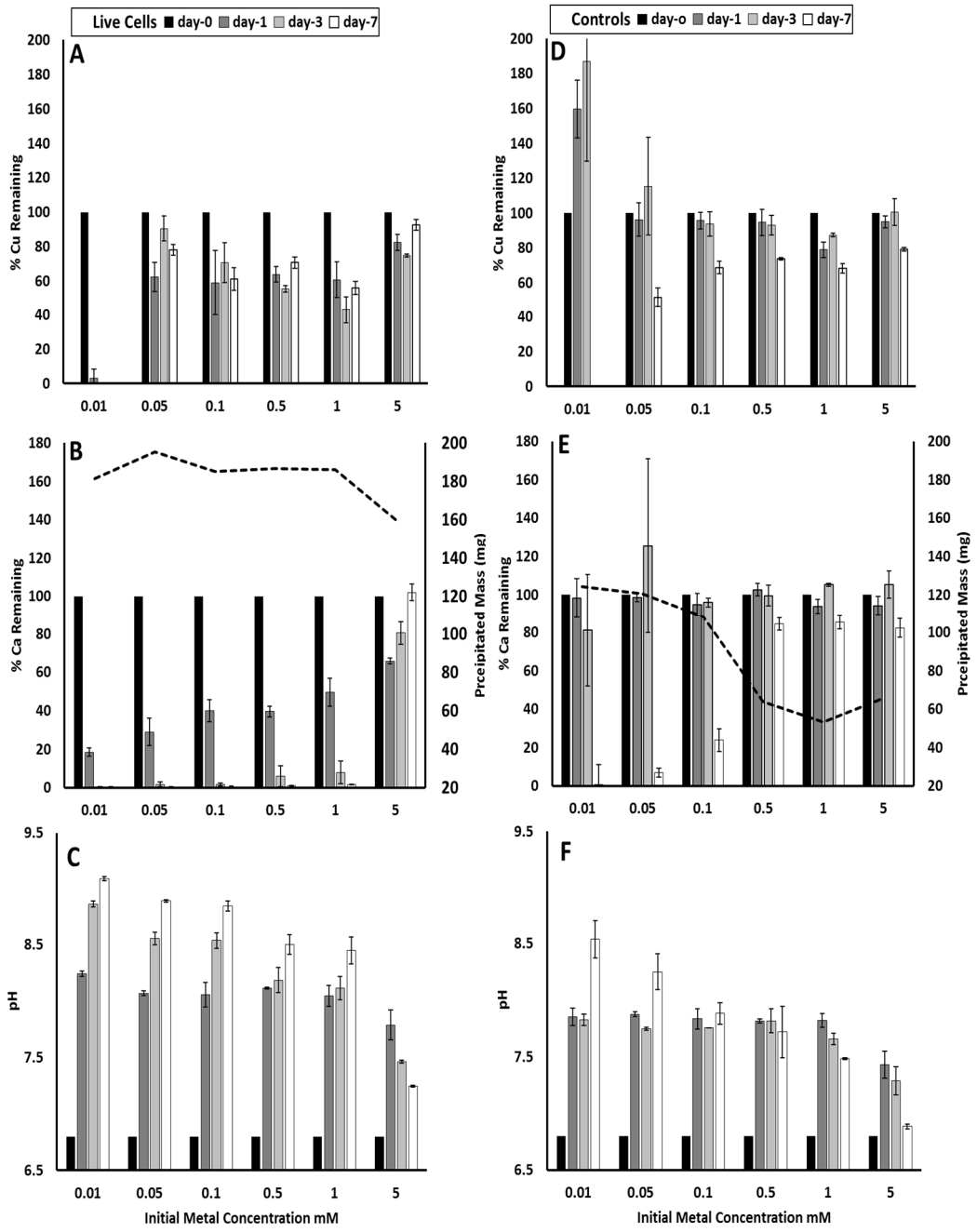


Figure 5.4. Impact of S. pasteurii activity on copper removal (A, D), calcium removal (B, E) and pH (C, F) at a range of copper concentrations, with live (A-C) and killed (D-F) cells at 30ºC with urea growth medium. [Error bars ± 1 SD, n=3].

High affinity of copper ions for $CaCO_3$ surfaces were reported, but presence of these ions into the lattice of calcium carbonate leads to distort its structure (Franklin and Morse 1981; Compton and Pritchard 1999; Schosseler *et al.* 1999). The partial loss of copper ions from solutions may also attributed to reaction with other species,

or copper ions precipitated as clusters in the structure of produced calcium carbonate (Nassrallah-Aboukais *et al.* 1996). However, this is not predicted by MINTEQ model.

Nonetheless, the copper ions affected the biomineralisation processes. Calcium removal from solution with increasing copper concentration is shown to gradually become slower (Figure 5.4, B) though is complete apart from at the highest concentration (5 mM) where almost all of the copper remained in solution after 7 days. Also the remaining amount of calcium ions in solutions after 7 days increased with increase the metal concentration. Add to this, final pH value for each copper samples decreased with increasing its concentration. This may have attributed to decreasing happened in *S. pasteurii* activity to produce urease enzyme with increasing copper concentration.

Copper is reported to impact upon both microbial activity in mineralising carbonates as well as the formation of carbonate crystals themselves. Warren *etal.* (2001) found that a copper concentration of 0.75 mM hindered microbially mediated removal (by *S. pasteurii* strain) of the copper through the metal's toxicity. This compares well to the upper limit of observed microbial effects here (in the form of increased pH), at concentrations of 0.5-1 mM.

Calcium removal in the presence of copper was not notably hindered apart from at the highest concentration of 5 mM, but the amount of copper concurrently removed was less than observed with other metals. Schosseler *et al.* (1999) found that the form of copper carbonate complexes on the surfaces of calcite and vaterite can hinder further sorption as well as growth or dissolution of the mineral, and at high concentrations an epitaxial layer may form. This may account for the relatively limited

removal of copper in the experiments presented here even when carbonate minerals are expected to be present.

Low levels of copper ions may form a solid solution upon recrystallization, with up to 0.2 ppm copper reported by Khosravi *et al.* (2009), although the tetrahedral structure that arises around copper ions (Teng and Zhao 2012) can destabilise the mineral (Schosseler *et al.* 1999). Zeppenfeld (2010) found that at very low concentrations copper hindered abiotic calcite formation, but at the range of concentrations of the present study, $Cu(OH)_2$ formed, which catalysed nucleation of calcite crystals. Growth was still inhibited, at an increasing level as copper concentration increased, suggesting the creation of larger numbers of smaller crystals. Li *et al*. (2013) achieved good removal of copper ions up to a concentration of 15.4 mM (compared to 0.01 mM here) with *S. pasteurii*.

As happened in other experiments in this study, spores of *S. pasteurii* may have survived the autoclaving process (Stolp 1988; Todar 2005), with subsequent regeneration leading to microbial activity in controls. This could led to limited pH increase and corresponding calcium concentration decrease was seen in killed cell controls. In copper control experiment this was seen at 0.01 and 0.05 mM concentrations. At these concentrations copper removal was 100% and 50%, respectively. Whilst almost all no calcium ions were recovered and pH increased up to 8.5.

The amount of precipitates produced in copper experiment are presented in Figure 5.4 (B and E). The precipitates in live samples exceeded their corresponding ones in killed samples. This may be attributed to bacterial activity that led to hydrolysis urea

and increasing pH which in turn leads to calcium removal and calcium carbonate precipitation.

Modelling in Visual MINTEQ predicted a gradual reduction of the amount of calcium carbonate with increasing copper concentration, with a significant drop (and corresponding increase in ionic species) at 5 mM (Table 5.5), mimicking the experimental data. Copper is expected to remain in solution in all cases, apart from a small amount of copper carbonate in the 5 mM experiment. This is very different from the experimental data (Figure 5.4, A) which has total removal at the lowest concentration, substantial removal (~30-60%) at intermediate concentrations and almost no removal at 5 mM.

Visual MINTEQ model for control samples (Table 5.6) predicted copper species in entirely ionic/dissolved species apart from at the highest copper concentrations where small of copper carbonate was produced. The removal of calcium ions from the solutions was inversely proportional to calcium carbonate precipitate amounts. Also the precipitation amounts of calcium carbonate decreased with copper concentration increase. The experiment data for controls showed decreasing in calcium carbonate precipitation with copper concentration increase up to 1 mM. At 5 mM copper concentration the precipitate amount increased which may due to copper carbonate precipitation according to Visual MINTEQ model.

Table 5.5. Copper and calcium predominant species in calcium carbonate medium calculated by visual MINTEQ according to copper concentration and final pH, for the test samples (live-cells experiments).

Copper (mM) (Final pH)	Cu species name	% of total concentration	Ca species name	% of total concentration
0.01 (9.09)	$Cu(NH_3)_4^{2+}$	97.756	Ca^{2+}	3.052
	$Cu(NH_3)_3^{2+}$	2.22	$CaCl^{+}$	1.604
	$Cu(CO_3)_2^{2-}$	0.014	$CaNH_3^{2+}$	1.44
			$Ca(NH_3)_2^{2+}$	0.21
			$CaHCO_3^{+}$	10.115
			$CaCO_3$	83.578
0.05 (8.89)	$Cu(NH_3)_4^{2+}$	97.102	Ca^{2+}	4.329
	$Cu(NH_3)_3^{2+}$	2.865	$CaCl^{+}$	2.274
	$Cu(NH_3)_2^{2+}$	0.016	$CaNH_3^{2+}$	1.572
	$Cu(CO_3)_2^{2-}$	0.016	$Ca(NH_3)_2^{2+}$	0.176
			$CaHCO_3^{+}$	14.75
			$CaCO_3$	76.898
0.1 (8.85)	$Cu(NH_3)_4^{2+}$	96.931	Ca^{2+}	4.631
	$Cu(NH_3)_3^{2+}$	3.033	$CaCl^{+}$	2.432
	$Cu(NH_3)_2^{2+}$	0.018	$CaNH_3^{2+}$	1.586
	$Cu(CO_3)_2^{2-}$	0.017	$Ca(NH_3)_2^{2+}$	0.168
			$CaHCO_3^{+}$	15.845
			$CaCO_3$	75.338
0.5 (8.50)	$Cu(NH_3)_4^{2+}$	94.571	Ca^{2+}	7.899
	$Cu(NH_3)_3^{2+}$	5.333	$CaCl^{+}$	4.146
	$Cu(NH_3)_2^{2+}$	0.057	$CaNH_3^{2+}$	1.501
	$Cu(CO_3)_2^{2-}$	0.037	$Ca(NH_3)_2^{2+}$	0.088
			$CaHCO_3^{+}$	27.647
			$CaCO_3$	58.718
1.0 (8.45)	$Cu(NH_3)_4^{2+}$	94.049	Ca^{2+}	8.44
	$Cu(NH_3)_3^{2+}$	5.836	$CaCl^{+}$	4.43
	$Cu(NH_3)_2^{2+}$	0.069	$CaNH_3^{2+}$	1.457
	$Cu(CO_3)_2^{2-}$	0.043	Ca(NH3)22+	0.078
			$CaHCO_3^{+}$	29.588
			$CaCO_3$	56.006
5.0 (7.25)	$Cu(NH_3)_4^{2+}$	48.732	Ca^{2+}	19.557
	$Cu(NH_3)_3^{2+}$	39.708	$CaCl^{+}$	10.26
	$Cu(NH_3)_2^{2+}$	6.135	$Ca(NH_3)_2^{2+}$	0.257
	$CuNH_3^{2+}$	0.259	$CaHCO_3^{+}$	62.464
	$CuCO_3$	2.937	$CaCO_3$	7.46
	$CuHCO_3^{+}$	0.039		
	$Cu(CO_3)_2^{2-}$	2.186		

Table 5.6. Copper and calcium predominant species in calcium carbonate medium calculated by visual MINTEQ according to copper concentration and final pH, for the control samples (killed-cells experiments).

Copper (mM) (Final pH)	Cu species name	% of total concentration	Ca species name	% of total concentration
0.01 (8.54)	$Cu(NH_3)_4^{2+}$	94.951	Ca^{2+}	7.476
	$Cu(NH_3)_3^{2+}$	4.965	$CaCl^+$	3.925
			$CaHCO_3^+$	26.127
			$CaCO_3$	60.843
0.05 (8.25)	$Cu(NH_3)_4^{2+}$	91.41	Ca^{2+}	10.682
	$Cu(NH_3)_3^{2+}$	8.356	$CaCl^+$	5.606
			$CaHCO_3^+$	37.558
			$CaCO_3$	44.857
0.1 (7.88)	$Cu(NH_3)_4^{2+}$	82.54	Ca^{2+}	14.609
	$Cu(NH_3)_3^{2+}$	16.459	$CaCl^+$	7.666
			$CaHCO_3^+$	50.962
			$CaCO_3$	25.964
0.5 (7.72)	$Cu(NH_3)_4^{2+}$	76.404	Ca^{2+}	16.041
	$Cu(NH_3)_3^{2+}$	21.682	$CaCl^+$	8.416
			$CaHCO_3^+$	55.402
			$CaCO_3$	19.528
1.0 (7.49)	$Cu(NH_3)_4^{2+}$	64.572	Ca^{2+}	17.804
	$Cu(NH_3)_3^{2+}$	30.651	$CaCl^+$	9.341
			$CaHCO_3^+$	59.997
			$CaCO_3$	12.452
5.0 (6.88)	Cu^{2+}	0.032	Ca^{2+}	22.319
	$Cu(NH_3)_4^{2+}$	22.192	$CaCl^+$	11.707
	$Cu(NH_3)_3^{2+}$	42.022	$Ca(NH_3)_2^{2+}$	0.257
	$Cu(NH_3)_2^{2+}$	15.088	$CaHCO_3^+$	62.656
	$CuCO_3$	14.625	$CaCO_3$	3.192
	$CuHCO_3^+$	0.039		
	$Cu(CO_3)_2^{2-}$	4.081		

5.1 Lead

In the presence of lead up to 5 mM, live cell experiments exhibited large pH increases, with corresponding removal of calcium ions from solution and almost complete removal of lead ions (Figure 5.5). It is likely that the loss of lead ions allowed bacteria to operate in higher concentration scenarios than in metal toxicity experiments (Chapter 4, Figure 4.3), suggesting that urea hydrolysis did play a role.

Although there is at least a small role expected due to microbial carbonate precipitation (there is more lead removal with live cells) it may not be the most affected mechanism. This may also explain the ability of *S. pasteurii* to apparently resist higher concentrations of lead than other metals in the toxicity experiment. Complete removal of lead from solution with *S. pasteurii* and several other organisms, at similar concentrations (7.2 mM compared to 5 mM here), was reported by Li *et al.* (2013), although no controls were performed to confirm whether another mechanism was responsible. It is suggested that this 'perfect' removal may also include a contribution from mechanisms not related to carbonate precipitation.

However, with killed cell controls similar, albeit slightly slower, lead removal was observed, despite only limited pH change and corresponding calcium concentration decrease being observed (possibly due to regeneration of *S. pasteurii* spores at low concentrations of lead). Once more, precipitate mass broadly agrees with the degree of calcium removal, although the mass produced in live cell specimens was greater than that in controls even when calcium removal was comparable as shown in Figure 5.5 (B and E).

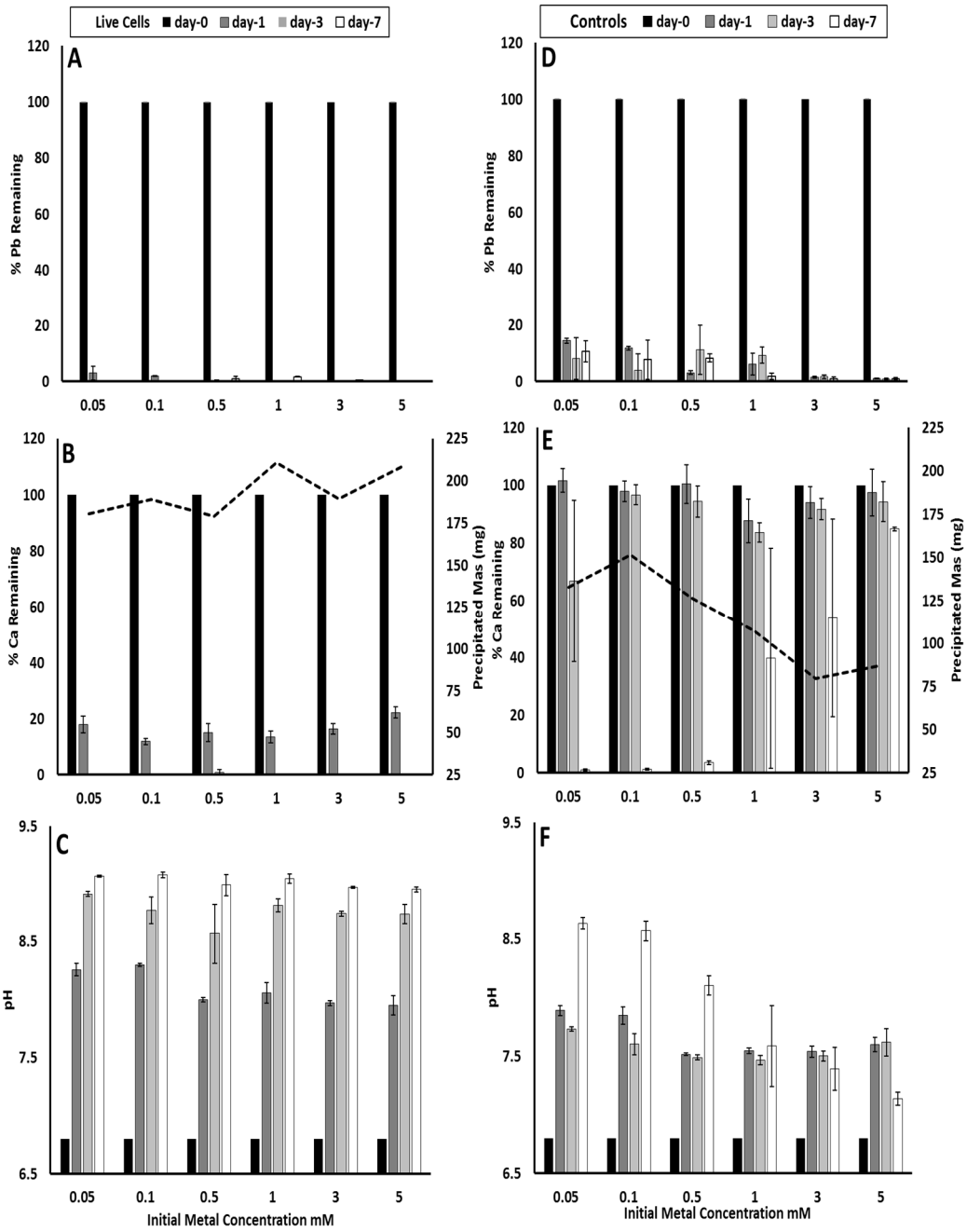


*Figure 5.5. Impact of S. pasteurii activity on lead removal (*A, D*), calcium removal (*B, E*) and pH (*C, F*) at a range of lead concentrations, with live (*A-C*) and killed (*d-f*) cells at 30ºC with urea growth medium. [Error bars ± 1 SD, n=3]*

As with other metals, lead ions can sorb strongly to calcium carbonate surfaces, particularly calcite, and can become incorporated into a solid solution in this mineral, albeit forming a distorted lattice (Chada *et al.* 2005; Rouff *et al.* 2006; Teng and Zhao 2012). However, here lead removal occurred prior to calcium carbonate formation, in

control specimens at least, and so this cannot be a full explanation. Although lead removal correlates well with calcium removal and pH change in live cell experiments, it does not in killed cell controls, indicating that some other mechanism is at work in the latter, and which is likely in the former too.

Lead ions precipitate in killed cell samples almost looks like an inverse correlation to calcium removal or pH increase, where almost full removal of lead ions occurred in 1, 3 and 5 mM of control samples, more than 40% of calcium ions remained in the solutions and no significant increase occurred in pH values. pH increase in control samples with lead concentrations of 0.05, 0.1 and 0.5 mM and the consequence decrease in calcium concentration, may be due that spores of *S. pasteurii* may have survived the autoclaving process (Stolp 1988; Todar 2005), with subsequent regeneration leading to microbial activity in controls.

Visual MINTEQ models based on final live-cell experiment conditions predict similar outcomes to experimental data (Figure 5.5, B) in that calcium carbonate concentrations are relatively constant, and that the majority of calcium is removed from solution (Table 5.7), primarily due to the lack of variability in pH. However, only a small amount of insoluble lead carbonate was predicted, whereas complete removal of lead ions was observed experimentally (Figure 5.5, A).

However, the prediction of lead species by visual MINTEQ revealed that lead ions do not react with available chemicals, at specific concentration and pH, to produce many complexes like zinc, cadmium and copper. Table 5.7 presents the species that can produced from reacting lead ions with precipitation medium chemicals. Where almost all lead ions were not precipitated but reacted only with carbonate or bicarbonate ions. Therefore, the loss in lead ions may down to be adsorbed on calcium

carbonate surfaces, especially this affinity has been proved (Comans and Middelburg 1987; Sturchio *et al.* 1997; Shahwan *et al.* 2005; Elzinga *et al.* 2006; Adekola *et al.* 2012; Callagon *et al.* 2014). Shahwan *et al.* (2005) concluded that lead ions retention on calcite is pH-dependent and the mechanism included co-precipitation and precipitation overgrowth (cerussite, hydrocerussite). However, Adekola *et al.* (2012) pointed out that lead ions uptake on calcite depends on precipitate particle size and there was less dependent on pH.

Visual MINTEQ model prediction for control samples (Table 5.8), predicts that the amount of calcium carbonate precipitates decreasing with lead concentration increase, whilst lead carbonate precipitates increase. The optimal pH for $PbCO_3$ precipitation was found to be 8.0–9.0 (Wang *et al.* 2006). Hence, it is uncertain why the model predicts increasing amount of lead carbonate with pH decrease. This suggests that a mechanism not included in the Visual MINTEQ model had a significant role, likely to be sorption or co-precipitation of metals with increasing amounts of calcium carbonate. According to MINTEQ calculations $CaCO_3$ and $PbCO_3$ were calcite and cerussite, respectively. Apart from calcite and cerussite, all other species are unsaturated.

Table 5.7. Lead and calcium predominant species in calcium carbonate medium calculated by visual MINTEQ according to lead concentration and final pH, for the test samples (live-cells experiments).

Lead (mM) (Final pH)	Pb species name	% of total concentration	Ca species name	% of total concentration
0.05 (9.07)	$Pb(CO3)_2^{2-}$	97.984	Ca^{2+}	3.164
	$PbCO_3$	2.005	$CaCl^+$	1.662
			$CaNH_3^{2+}$	1.457
			$Ca(NH_3)_2^{2+}$	0.207
			$CaHCO_3^+$	10.517
			$CaCO_3$	82.991
0.1 (9.08)	$Pb(CO3)_2^{2-}$	98.026	Ca^{2+}	3.108
	$PbCO_3$	1.963	$CaCl^+$	1.633
			$CaNH_3^{2+}$	1.449
			$Ca(NH_3)_2^{2+}$	0.209
			$CaHCO_3^+$	10.314
			$CaCO_3$	83.285
0.5 (8.99)	$Pb(CO3)_2^{2-}$	97.609	Ca^{2+}	3.649
	$PbCO_3$	2.375	$CaCl^+$	1.917
	$PbHCO_3^+$	0.013	$CaNH_3^{2+}$	1.52
			$Ca(NH_3)_2^{2+}$	0.196
			$CaHCO_3^+$	12.259
			CaCO3	80.459
1.0 (9.05)	$Pb(CO3)_2^{2-}$	97.891	Ca^{2+}	3.288
	$PbCO_3$	2.097	$CaCl^+$	1.727
			$CaNH_3^{2+}$	1.478
			$Ca(NH_3)_2^{2+}$	0.205
			$CaHCO_3^+$	10.931
			$CaCO_3$	82.37
3.0 (8.97)	$Pb(CO3)_2^{2-}$	97.485	Ca^{2+}	3.807
	$PbCO_3$	2.498	$CaCl^+$	2
	$PbHCO_3^+$	0.014	$CaNH_3^{2+}$	1.545
			$Ca(NH_3)_2^{2+}$	0.194
			$CaHCO_3^+$	12.721
			$CaCO_3$	79.733
5.0 (8.95)	$Pb(CO3)_2^{2-}$	97.359	Ca^{2+}	3.965
	$PbCO_3$	2.623	$CaCl^+$	2.083
	$PbHCO_3^+$	0.015	$CaNH_3^{2+}$	1.566
			$Ca(NH_3)_2^{2+}$	0.191
			$CaHCO_3^+$	13.197
			$CaCO_3$	78.996

Table 5.8. Lead and calcium predominant species in calcium carbonate medium calculated by visual MINTEQ according to lead concentration and final pH, for the control samples (killed-cells experiments).

Lead (mM) (Final pH)	Pb species name	% of total concentration	Ca species name	% of total concentration
0.05 (8.64)	$Pb(CO3)_2^{2-}$	94.935	Ca^{2+}	6.469
	$PbCO_3$	4.998	$CaCl^+$	3.396
			$CaHCO_3^+$	22.492
			$CaCO_3$	65.941
0.1 (8.57)	$Pb(CO3)_2^{2-}$	94.109	Ca^{2+}	7.168
	$PbCO_3$	5.801	$CaCl^+$	3.763
			$CaHCO_3^+$	25.009
			$CaCO_3$	62.105
0.5 (8.10)	$Pb(CO3)_2^{2-}$	84.102	Ca^{2+}	12.369
	$PbCO_3$	15.223	$CaCl^+$	6.491
	$PbCl_2$	0.011	$CaHCO_3^+$	43.374
			CaCO3	36.674
1.0 (7.59)	$Pb(CO3)_2^{2-}$	59.178	Ca^{2+}	17.113
	$PbCO_3$	35.733	$CaCl^+$	8.978
	$PbCl_2$	0.084	$CaHCO_3^+$	58.21
			$CaCO_3$	15.21
3.0 (7.39)	$Pb(CO3)_2^{2-}$	45.169	Ca^{2+}	18.615
	$PbCO_3$	44.731	$CaCl^+$	9.766
	$PbCl_2$	0.171	$CaHCO_3^+$	61.192
			$CaCO_3$	10.088
5.0 (7.14)	$Pb(CO3)_2^{2-}$	27.528	Ca^{2+}	20.387
	$PbCO_3$	51.667	$CaCl^+$	10.694
	$PbCl_2$	0.375	$CaHCO_3^+$	62.879
			$CaCO_3$	5.83

5.2 Strontium

In this section the ability of live *S. pasteurii* to induce calcium carbonate precipitation in the presence of a range of strontium concentrations (0-30 mM), and therefore co-precipitate strontium, was investigated. The changes in strontium and calcium concentrations, and pH, are shown in Figure 5.6. In live cell experiment pH increased over the testing period, reaching around 9.2 in all specimens by 7 days, thought to be due to the action of the *S. pasteurii* urease enzyme on urea. Calcium was

rapidly removed in all samples, with approximately 50% remaining in solution after one day and being undetectable after 3 days at all tested concentrations; this was concurrent with generation of white crystalline precipitates in each case. The decrease in concentration of calcium ions in solution was faster than that of strontium ions, with the amount lost in 24 hours greater than that of strontium by a factor of 1.45 (±0.1). Strontium was almost fully removed after 3 days, and not detectable after 7 days. In previous experiments, without strontium but otherwise identical, the majority of calcium ions were removed from solution in the first 24 hours with a pH value of up to 9.0. The delay of calcium removal in this experiment is thought to be related to the presence of strontium ions in solution. *S. pasteurii* cells were found to tolerate far higher strontium concentrations in this experiment with urea than in aqueous solution without it. It is therefore suggesting that the co-precipitation of strontium with calcium carbonate offered a mechanism of protection against metal toxicity.

In killed-cell controls, pH had only slightly increased after three days and there was little loss of Ca or Sr from solution. The slight pH increase may be partially due to abiotic hydrolysis of urea in solution, which is slower than the biotic hydrolysis (United States Environmental Protection Agency, 2002), but by day 7 the pH had increased substantially. It is thought that the autoclave procedure used to sterilise samples in this part of the study was insufficient to deactivate bacterial spores, which then regenerated and had similar but delayed effects to live cells by day 7 (Stolp 1988; Todar 2005). The minimum inhibitory concentration in the presence of the urea medium has not been established, but appears to be greater than 30 mM for this system, more than three times the previous value in toxicity experiment.

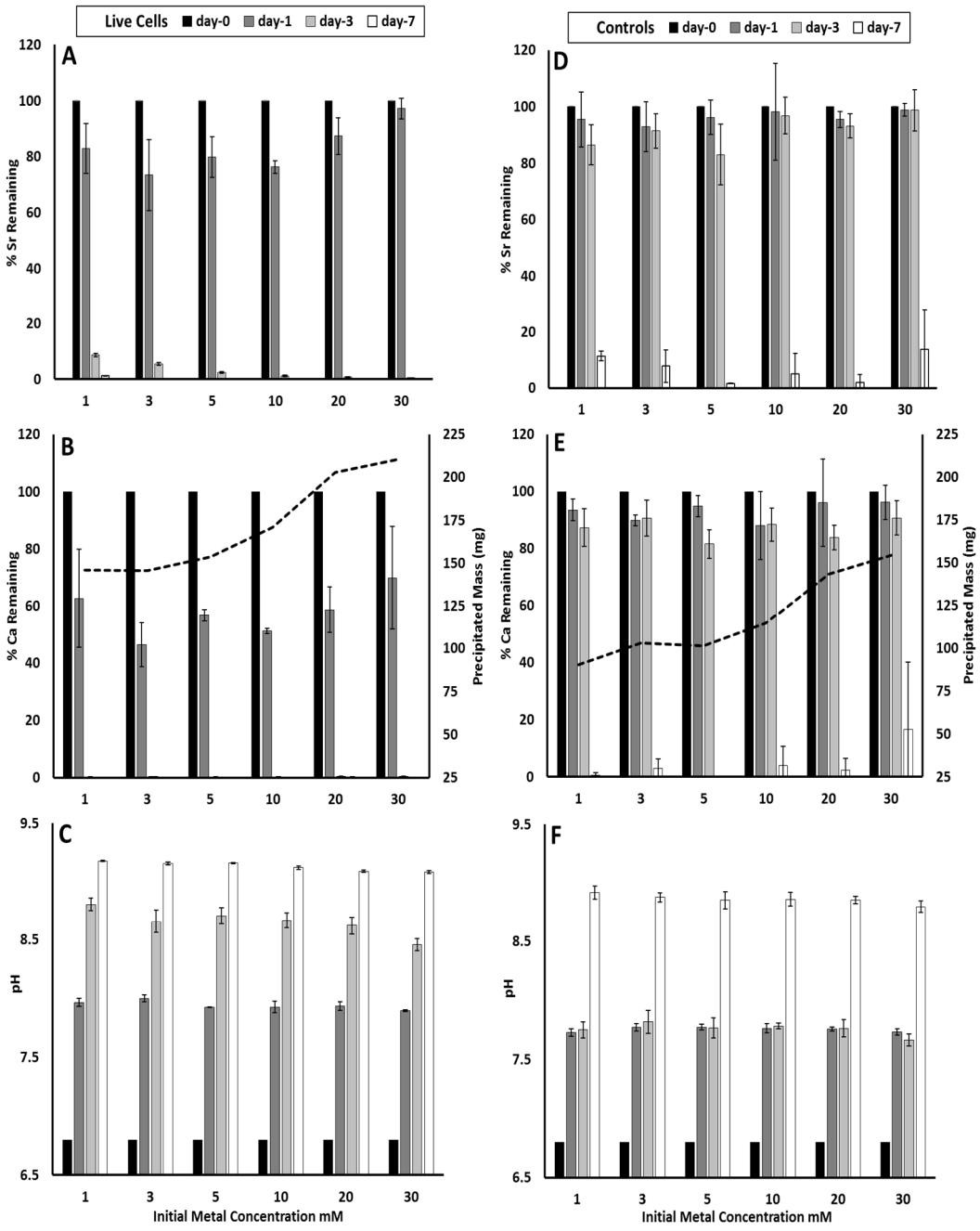


Figure 5.6. Impact of S. pasteurii activity on strontium removal (A, D), calcium removal (B, E) and pH (C, F) at a range of strontium concentrations, with live (A-C) and killed (D-F) cells at 30ºC with urea growth medium.[Error bars ±1 SD, n=3]

Figure 5.6 (B, E) shows the amounts of produced precipitate in live and control samples for strontium experiment. As it clears the amount of precipitate in live cell and kill-cell experiments increased with strontium concentration. There were no signs that strontium inhibited calcium carbonate mineralisation at all tested concentrations.

Visual MINTEQ model for test (Table 5.9) and control (Table 5.10) samples predicted strontium carbonate with calcium carbonate precipitation (strontianite and calcite, respectively, according to the model). The amounts of $SrCO_3$ and $CaCO_3$ decreased with increase strontium concentration. For the metal carbonate decreasing with increase its concentration, is a behaviour had not occurred with other tested metals. Experiment results indicated opposite behaviour, where precipitate amounts were increasing with strontium concentration. This suggests that a mechanism not included in the Visual MINTEQ model had a significant role, likely to be sorption or co-precipitation of metals with increasing amounts of calcium carbonate.

Table 5.9. Strontium and calcium predominant species in calcium carbonate medium calculated by visual MINTEQ according to strontium concentration and final pH, for the test samples (live-cells experiments).

Strontium (mM) (Final pH)	Sr species name	% of total concentration	Ca species name	% of total concentration
1 (9.18)	Sr^{2+}	5.12	Ca^{2+}	2.605
	$SrCl^{+}$	1.704	$CaCl^{+}$	1.369
	$SrNH_3^{2+}$	1.685	$CaNH_3^{2+}$	1.359
	$SrCO_3$	68.171	$CaHCO_3^{+}$	8.459
	$SrHCO_3^{+}$	23.319	$CaCO_3$	85.989
3 (9.16)	Sr^{2+}	5.296	Ca^{2+}	2.717
	$SrCl^{+}$	1.763	$CaCl^{+}$	1.428
	$SrNH_3^{2+}$	1.706	$CaNH_3^{2+}$	1.387
	$SrCO_3$	67.174	$CaHCO_3^{+}$	8.802
	$SrHCO_3^{+}$	24.061	$CaCO_3$	85.446
5 (9.16)	Sr^{2+}	5.328	Ca^{2+}	2.734
	$SrCl^{+}$	1.773	$CaCl^{+}$	1.437
	$SrNH_3^{2+}$	1.716	$CaNH_3^{2+}$	1.396
	$SrCO_3$	67.135	$CaHCO_3^{+}$	8.798
	$SrHCO_3^{+}$	24.047	$CaCO_3$	85.413
10 (9.12)	Sr^{2+}	5.709	Ca^{2+}	2.982
	$SrCl^{+}$	1.98	$CaCl^{+}$	1.633
	$SrNH_3^{2+}$	1.759	$CaNH_3^{2+}$	1.456
	$SrCO_3$	65.016	$CaHCO_3^{+}$	9.51
	$SrHCO_3^{+}$	25.535	$CaCO_3$	84.198
20 (9.09)	Sr^{2+}	6.124	Ca^{2+}	3.246
	$SrCl^{+}$	2.21	$CaCl^{+}$	1.85
	$SrNH_3^{2+}$	1.823	$CaNH_3^{2+}$	1.531
	$SrCO_3$	63.232	$CaHCO_3^{+}$	10.056
	$SrHCO_3^{+}$	26.61	$CaCO_3$	83.093
30 (9.08)	Sr^{2+}	6.4	Ca^{2+}	3.412
	$SrCl^{+}$	2.399	$CaCl^{+}$	2.02
	$SrNH_3^{2+}$	1.883	$CaNH_3^{2+}$	1.591
	$SrCO_3$	62.432	$CaHCO_3^{+}$	10.22
	$SrHCO_3^{+}$	26.885	$CaCO_3$	82.527

Table 5.10. Strontium and calcium predominant species in calcium carbonate medium calculated by visual MINTEQ according to strontium concentration and final pH, for the control samples (killed-cells experiments).

Strontium (mM) (Final pH)	Sr species name	% of total concentration	Ca species name	% of total concentration
1 (8.92)	Sr^{2+}	7.171	Ca^{2+}	4.125
	$SrCl^{+}$	2.385	$CaCl^{+}$	2.167
	$SrCO_3$	54.688	$CaHCO_3^{+}$	13.962
	$SrHCO_3^{+}$	34.041	$CaCO_3$	77.998
3 (8.88)	Sr^{2+}	7.555	Ca^{2+}	4.443
	$SrCl^{+}$	2.513	$CaCl^{+}$	2.333
	$SrCO_3$	52.437	$CaHCO_3^{+}$	15.006
	$SrHCO_3^{+}$	35.789	$CaCO_3$	76.451
5 (8.86)	Sr^{2+}	7.772	Ca^{2+}	4.622
	$SrCl^{+}$	2.585	$CaCl^{+}$	2.428
	$SrCO_3$	51.285	$CaHCO_3^{+}$	15.543
	$SrHCO_3^{+}$	36.652	$CaCO_3$	76.626
10 (8.86)	Sr^{2+}	7.887	Ca^{2+}	4.693
	$SrCl^{+}$	2.623	$CaCl^{+}$	2.465
	$SrCO_3$	51.181	$CaHCO_3^{+}$	15.520
	$SrHCO_3^{+}$	36.578	$CaCO_3$	75.513
20 (8.85)	Sr^{2+}	8.218	Ca^{2+}	4.923
	$SrCl^{+}$	2.733	$CaCl^{+}$	2.585
	$SrCO_3$	50.408	$CaHCO_3^{+}$	15.748
	$SrHCO_3^{+}$	36.865	$CaCO_3$	74.877
30 (8.80)	Sr^{2+}	8.941	Ca^{2+}	5.518
	$SrCl^{+}$	2.973	$CaCl^{+}$	2.898
	$SrCO_3$	47.400	$CaHCO_3^{+}$	17.118
	$SrHCO_3^{+}$	38.895	$CaCO_3$	72.540

5.3 Overall Discussion

Microbial activity in the presence of urea-amended medium has been demonstrated to occur at metal concentrations higher than the minimum inhibitory concentrations (MIC) determined for each of the metals considered here (Chapter 4). In the cases of zinc, cadmium and strontium, the amount and rate of pH increase and Ca removal have been demonstrated to correlate strongly with significant removal of these metals from solution. However, a mechanism other than carbonate precipitation

appears to have contributed to lead removal, whilst only limited removal of copper was noted *via* this method, leading to microbial inhibition at similar concentrations to those observed in toxicity experiments without urea.

It is suggested that at metal concentrations above the MIC there may be a low level of microbial activity that allows a diminished rate of urea hydrolysis, leading to a small amount of carbonate, and metal, precipitation. This in turn reduces the overall toxicity, allowing enhanced microbial activity and therefore further biomineralisation; once this process commences, provided sufficient nutrients and feed are provided, similar remedial outcomes to those seen at lower concentrations might be expected. Therefore, as long as the conditions permit the initial survival of a viable population of cells, remediation may continue even at relatively high metal concentrations.

In the presence of urea, calcium and suitable nutrients, *S. pasteurii* can grow rapidly, altering the pH and causing calcium carbonate precipitation. Their cell walls act as nucleation sites for calcium carbonate formation, and this in turn can lead to the bacteria becoming embedded within the solid matrix which is likely to kill cells by prevent access to nutrients and energy sources (Tobler *et al.* 2011). Due to the presence of a large number of cells, providing a small population remain following the precipitation of the mineral, the removal of metals from solution will enhance the environment for the remaining live cells. The technique provides a protective mechanism for the bacteria by reducing the overall metal concentrations to tolerable levels.

However, when bacterial cells were not added to check the abiotic or chemical precipitation of heavy metals, only very few amounts of metal ions were removed from solutions. Chemical or abiotic precipitation of heavy metals can occur from a

reaction of metal and carbonate ions or due to co-precipitate with abiotic precipitation of calcium carbonate minerals in presence of calcium ions. Carbonate ions can be produced from abiotic hydrolysis of urea in water which is much slower than biotic hydrolysis. This is clear from the abiotic experiments that were done in this study. The results are presented in Appendix A. pH in abiotic experiments did not increase much (8.1 after 14 days), which seems not enough to precipitate metal ions in solutions. As it clear from the figures in Appendix A pH decreased with metal concentration increase (which was more clear in zinc, copper and lead samples). This may be due to acidity effects of higher concentrations of metal salts (metal chloride).

The mechanism of removal here is probable to be a combination of a form of carbonate precipitation or co-precipitation of the metal ions in conjunction with calcium alongside or following sorption to calcium carbonate crystals. It is possible that the form of calcium carbonate produced by this organism in this manner are aragonite and calcite, based on crystal structures observed in other experiments in this study (Chapter 6) and previous studies (Fujita *et al.* 2000; Achal *et al.* 2012; Kang *et al.* 2014). As noted by Tesoriero and Pankow (1996), divalent metal ions can be removed from solution as a solid solution with calcium carbonate. Results from basic Visual MINTEQ models of the experiments suggest that the heavy metals would be expected to be largely in soluble form at the end of each experiment. The removal process is therefore thought to be microbially driven through biomineralisation of calcium carbonate, leading either to co-precipitation as heavy metal ions associate with a growing calcium carbonate lattice or through sorption to calcium carbonate surfaces, possibly followed by encapsulation through continued mineralisation. In the latter case, the continued generation of calcium carbonate precipitate would continue to provide new surface area for sorption, preventing formation of a coherent epitaxial

layer and increasing the potential for removal from solution. Unlike many previous studies (e.g. Elzinga *et al.* (2006)) where interaction of heavy metals and calcium carbonate surfaces were explored under equilibrium conditions, the dynamic conditions experienced here with prolonged removal of metals alongside precipitate generation are more similar to conditions found by Schosseler *et al.* (1999), where dissolution and recrystallization allowed sequestration of metals in solid solution within calcium carbonate.

The form of calcium carbonate crystals does not appear to be strongly dependent on whether they are formed through abiotic or biotic means (Mitchell and Ferris 2006), although there is evidence to suggest that microbial action may lead to larger crystals, albeit with greater variation in size, and an increased rate of formation. In addition, Lea *et al.* (2001) report inhibition of crystal dissolution in the presence of heavy metals due to the greater insolubility of their carbonates relative to calcium. Larger crystals containing heavy metals in solid solution may therefore be more recalcitrant and act as a potentially durable method of sequestering heavy metal contamination.

Elevation of pH and removal of calcium from solution are shown to be strongly linked to remove zinc (0.1-0.5 mM), cadmium (0.015-1.5 mM), copper (0.01 mM) and strontium (1-30 mM). This reflect the ability of *S. pasteurii* cells to keep their activity in producing urease enzyme and urea hydrolysis over those metallic concentrations. Whilst in lead experiment (0.05-5 mM) the loss of lead ions allowed bacteria to operate in higher concentration scenarios than in metal toxicity experiments (Chapter 4), suggesting that urea hydrolysis did play a role. Contrast of this at zinc concentrations (2-10 mM) the bacteria could not show the same activity

in hydrolysing urea and in result increasing solutions pH. While at copper concentrations (0.05-1 mM) the bacteria was able to keep the required activity for urea hydrolysis, and precipitate calcium ions but more than 50% of copper ions have been recovered after 7 days.

pH of all heavy metal samples (apart from zinc concentrations 5 and 10 mM and copper concentration 5mM) increased only to around 8.0 after day-1 and not full removal of calcium ions was observed. However, in previous experiments (section 5.2) without heavy metals but otherwise identical, the majority of calcium ions were removed from solution in the first 24 hours with a pH value of up to 9.0, suggesting that presence of heavy metal cations caused delay in bacterial activity. Noticeably most of heavy metal concentrations had similar effect on bacterial activity, regarding pH change which relate to urea hydrolysis. However, after day-3 there were obvious clear variety in pH values.

Metal response to be removed from solution by *S. pasteurii* activity and carbonate precipitation mechanism was not similar for all heavy metals. Calcium and metal ions were removed after day-1, 3 or 7 depend on metal concentration, however, some concentrations of zinc and copper were not removed. The removal of metal ions mainly depends on bacterial activity to enhance urea hydrolysis and precipitate calcium carbonate precipitation. It also depends on the metallic cation affinity to be adsorbed on formed calcium carbonate surfaces. In case of copper experiments (0.05-5 mM) in spite of significant amount of calcium ions were removed from solutions but more than 50% of copper ions remained in solution. Mitchell and Ferris (2005) reported the presence of strontium ions in precipitation medium can slightly reduce calcite precipitation rates. Once more, calcium removal rates depend on bacterial

activity, which means if cells less affected by metallic toxicity can hydrolyse urea in high rates.

Results revealed that every metal has its own trend in responding to be bioprecipitated in calcium carbonate medium, and this behaviour depends on the concentration and physical and chemical properties of that metal. This also may depend on ionic radii of metals compared to calcium [atomic radii in angstrom are: Cu (0.73), Zn (0.74), Cd (0.97), Sr (1.12), Pb (1.19) and Ca (0.99)] (Barbalace 1995). According to Schosseler *et al.* (1999), Sturchio *et al.* (1997), Shahwan *et al.* (2005), Elzinga *et al.* (2006) and de Nooijer *et al.* (2007) cations that have an identical ionic radius to that of calcium are most likely to adsorb on calcite surface during recrystallisation. Whilst metals have atomic radii greater than calcium are more likely to precipitate as metals carbonate, this can be seen in visual MINTEQ outcomes regarding lead and strontium.

S. pasteurii is nominally a spore-forming organism and so spores may have survived the autoclaving process, with subsequent regeneration leading to microbial activity in controls (Stolp 1988; Todar 2005). This microbial activity may have led to increase pH values of low concentrations of copper (0.01-0.05 mM) and lead (0.05-0.5 mM) up to 8.6 and strontium (1-30 mM) up to 9 after day-7. This change in pH led to precipitate metal and calcium ions at these concentrations. This implies that if the bacteria spores resisted increasingly heat when sterilised by autoclave, but they could not tolerate the toxicity of any zinc and cadmium tested concentrations or high copper and lead concentrations.

In spite of no significant pH increase in cadmium control samples, full removal of cadmium ions and more than 40% of calcium ions decreasing were seen at

concentrations of 0.015 and 0.03 mM at day-7. This thought to be due to an abiotic precipitation. The MINTEQ model (Table 5.4) predicted precipitation amounts (up to 30%) may result from the precipitation of calcium carbonate, cadmium carbonate and cadmium chloride (white crystalline compound, hygroscopic solid that is highly soluble in water).

It's not clear in this study if any biosorption was occurred by metal ions sorption on bacterial biomass (live or dead), if so would be responsible of partial metal decreasing in solutions.

Figure 5.7 to Figure 5.11 present comparison between the results that were gained from the bioprecipitation experiments after 7 days and the expected results from Visual MINTEQ model, for both live and killed cell experiments. The experiments and model shared the same pH value and chemical concentrations, this to discover whether experimental results are predictable by this model. The bacterial activity makes the main differences in resulted species when enhance urea hydrolysis and pH increase in solutions. Therefore, in the absence of this activity, due to bacterial cells inhibition by toxic metals, the gained results may match expected results to high extent.

In case of zinc (Figure 5.7), generally, MINTEQ outcomes and experimental data agree reasonably well in terms of calcium removal from solution, resulted calcium carbonate precipitation and also the behaviour of zinc in control samples. In addition, at zinc concentrations more than 2 mM in live cell experiment, microbial urea hydrolysis was limited due to metal toxicity the expected and actual data were agreed to some extent as well at those concentrations.

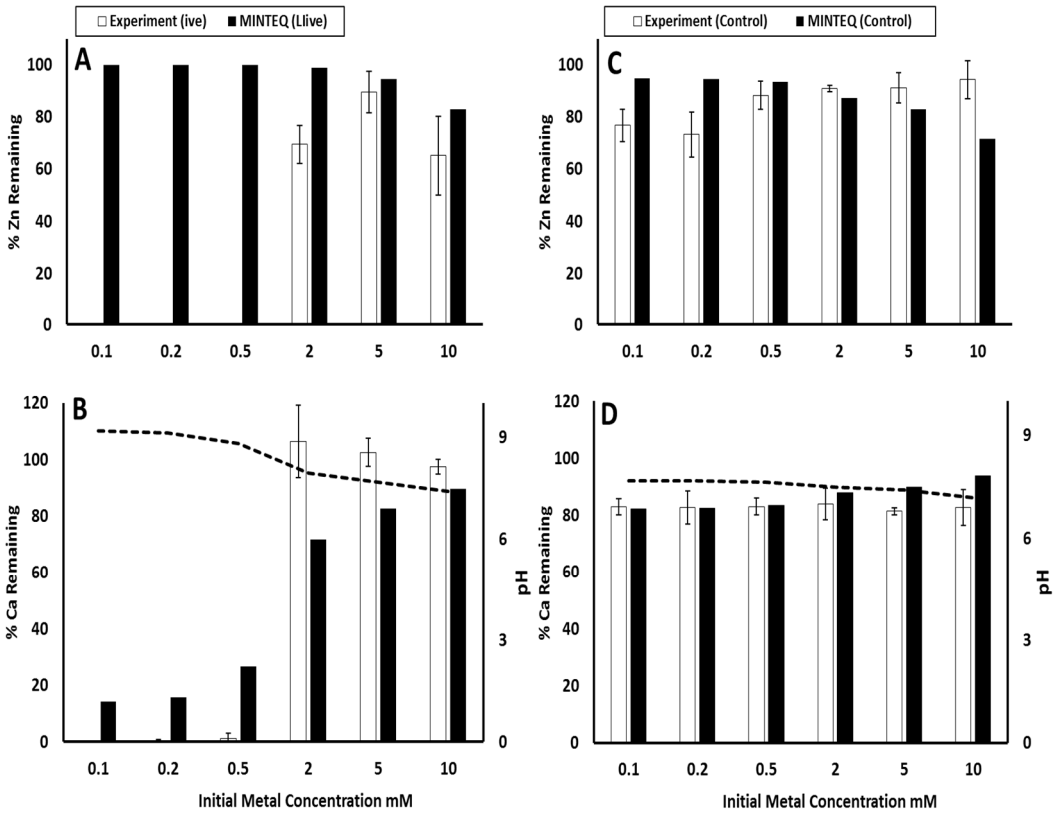


Figure 5.7. A comparison between the results that were gained from the zinc experiments after 7 days and the expected results from Visual MINTEQ model. A&B for live-cells and C&D for killed-cells experiments. Dashed lines on (B) and (D) represent pH values. [Error bars ±1 SD].

In cadmium experiment predicted final concentrations by MINTEQ model calculations (Figure 5.8), were unexpected in terms of cadmium removal from solutions. The model expected full recover of cadmium ions from live cell samples whilst more than 20% of metal ions precipitated in killed cell specimens. Calcium removal in cadmium precipitation experiment was probably predicted by MINTEQ according to final pH value of the sample. Generally, MINTEQ outcomes and experimental data agree reasonably well in terms of calcium removal from solutions in cadmium experiment.

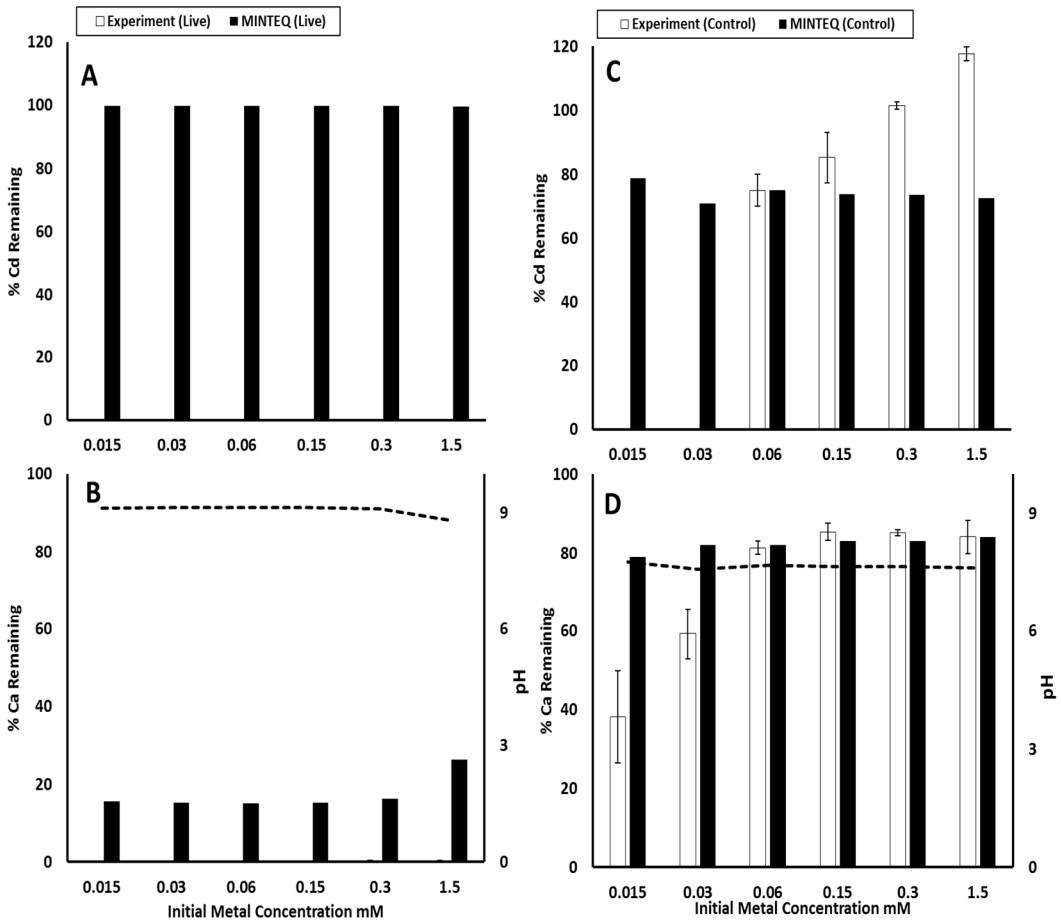


Figure 5.8. A comparison between the results that were gained from the cadmium experiments after 7 days and the expected results from Visual MINTEQ model. A&B for live-cells and C&D for killed-cells experiments. Dashed lines on (B) and (D) represent pH values. [Error bars ±1 SD].

As it clear from Figure 5.9 both experimental and predicted data show not full removal of copper ions from live and killed samples, this apart from lowest copper concentration. Again because calcium removal is correlated with solution pH MINTEQ predicted calcium removal from high pH samples. However, experimental data display full calcium removal whilst it is partially in MINTEQ results.

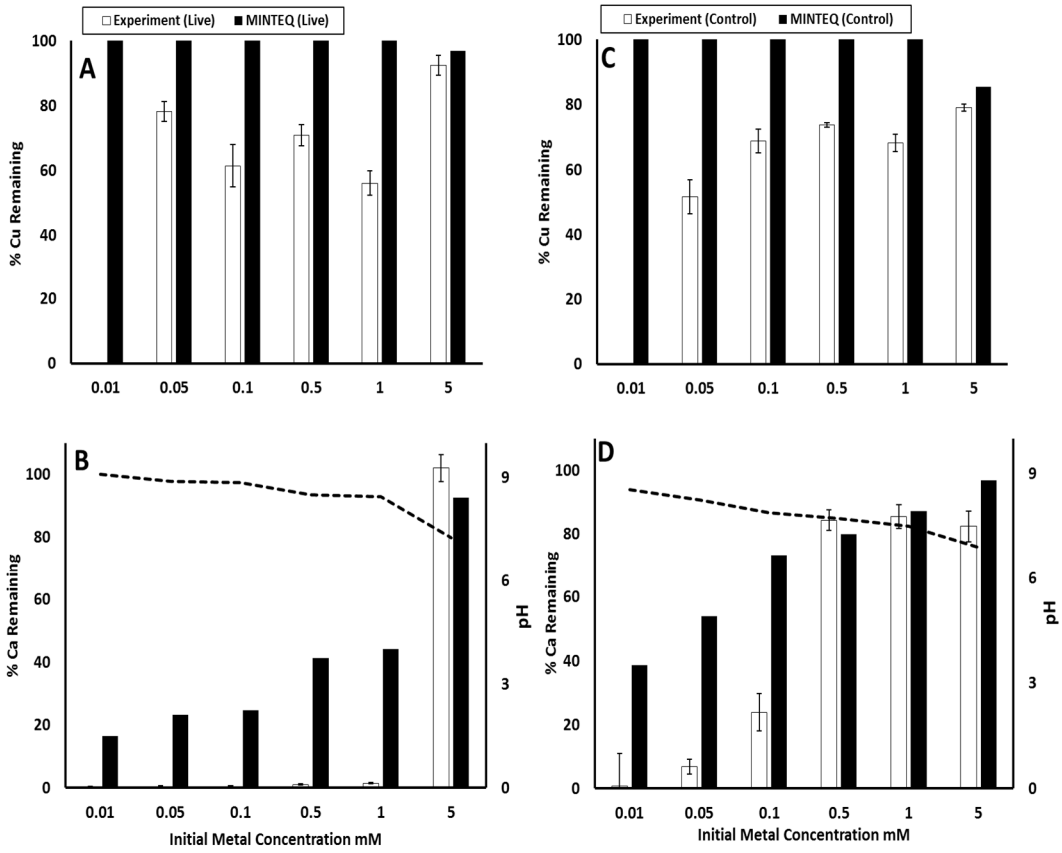


Figure 5.9. A comparison between the results that were gained from the copper experiments after 7 days and the expected results from Visual MINTEQ model. A&B for live-cells and C&D for killed-cells experiments. Dashed lines on (B) and (D) represent pH values. [Error bars ±1 SD].

In case of lead experiment (Figure 5.10) MINTEQ expected no removal of lead ions from live cell samples, whilst metal removal increased with its concentration in control samples. The model predicted $PbCO_3$ precipitation increase with lead concentration in killed cell samples, which is responsible for metal removal according to the model. High lead removal rates were noticed in experimental data in live and killed cell experiments, but it is not clear if the behaviour of lead removal was as described by MINTEQ in control samples at high metal concentrations, or other mechanism. Visual MINTEQ models of final live-cell experiment conditions predict similar outcomes to experimental calcium data in that the majority of calcium is removed from solution with live cells whilst increasing amounts remain in controls.

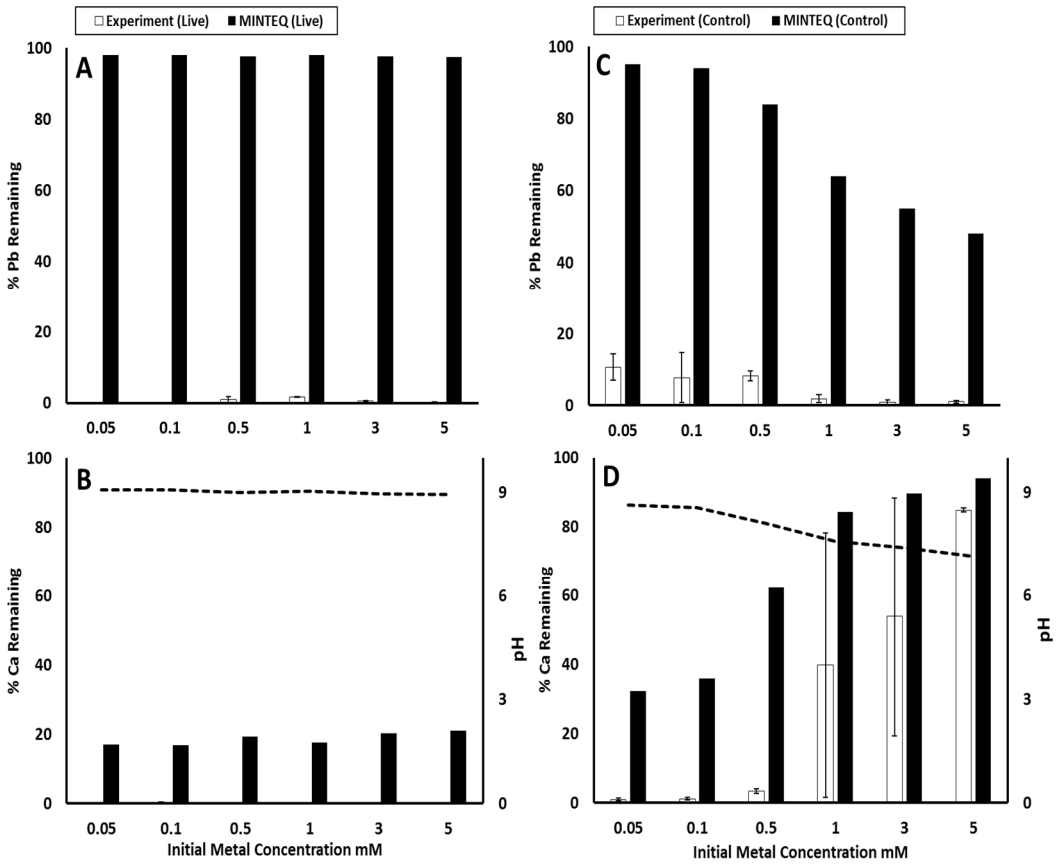


Figure 5.10. A comparison between the results that were gained from the lead experiments after 7 days and the expected results from Visual MINTEQ model. A&B for live-cells and C&D for killed-cells experiments. Dashed lines on (B) and (D) represent pH values. [Error bars ±1 SD].

Experimental and expected results in case of strontium (Figure 5.11) experiments demonstrated high removal of metal and calcium ions from solutions, but in actual results the removal was higher.

According to the results gained in this study, not all tested heavy metals are good candidates to be remediated with *S. pasteurii*. Zinc and copper did not full precipitate with calcium carbonate. This may be attributed to many reasons, such as the differentiation in ionic radii and the inhibition of calcium carbonate crystal growth by some heavy metals (McBride 1980; Comans and Middelburg 1987; US EPA 1992).

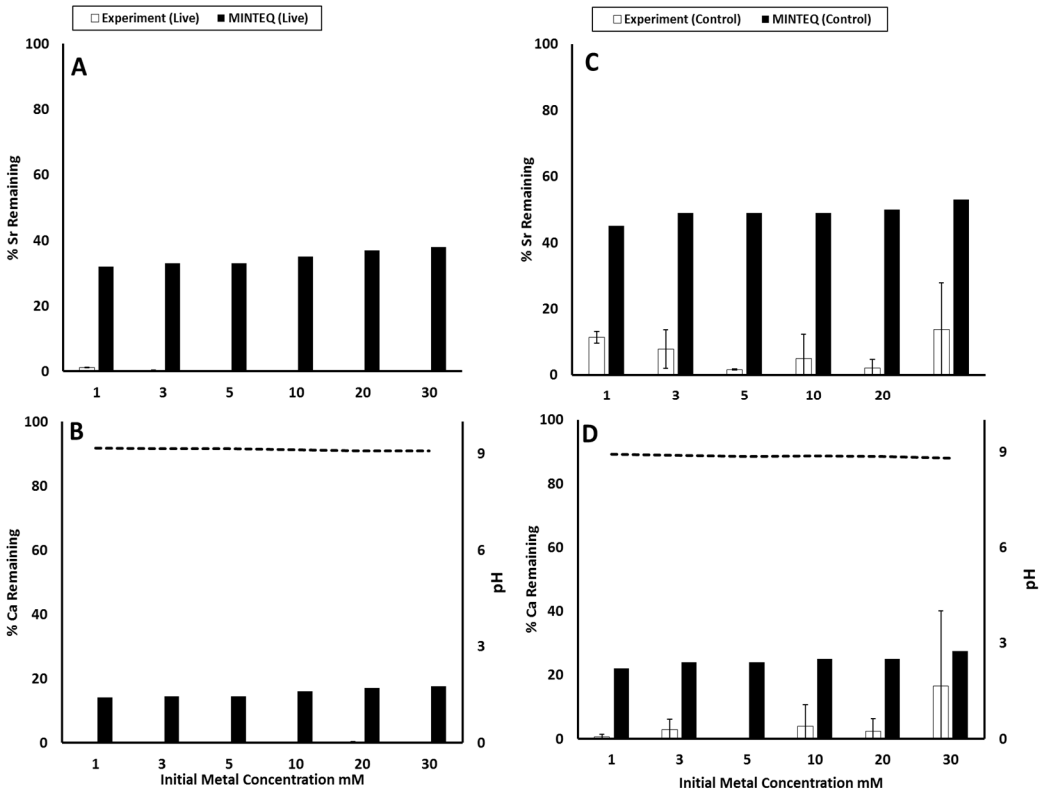


Figure 5.11. A comparison between the results that were gained from the strontium experiments after 7 days and the expected results from Visual MINTEQ model. A&B for live-cells and C&D for killed-cells experiments. Dashed lines on (B) and (D) represent pH values. [Error bars ±1 SD].

Chapter 6: Bioprecipitation of Heavy Metals in Particulate Media

6.1 Introduction

The ability of *Sporosarcina pasteurii* bacteria to induce precipitation of calcium carbonate and co-precipitation of strontium, zinc and cadmium ions in porous media has been explored in this chapter. The biomineralisation of heavy metals in soil may be influenced by a number of physical, chemical and biological factors. Presence of inorganic compounds and organic matters in soil may lessen metals availability through chemical and physical adsorption processes, where metal ions have to be dissolved in solutions for bioprecipitation processes. Heavy metals affinity to be adsorbed on sand grains was investigated in this chapter. Bacteria ability to initiate calcium carbonate precipitation may be affected by many factors such as metallic toxicity and nutrients delivering. In addition, the heterogeneity of subsurface environment can play a main role in controlling biomineralisation processes. This may be due to possible uneven distribution of injected nutrients and chemicals into contaminated sites.

The effects of sand particle size, metal ions adsorption capacity of sand fractions and heterogeneity in hydraulic conductivity were studied through static (no fluid flow) and dynamic (with fluid flow) experiments. The results from these two systems were compared with each other and to bioprecipitation in aqueous solution experiment alone. All bioprecipitation systems were working under the same experimental conditions in this study. In static system the microcosms were 50 mL sterilised polypropylene tubes filled with sand, working solutions (*M2* or *M3*, that were defined in Chapter 3) amended with heavy metals and bacterial cells. In dynamic system (fluid flow) working solutions (*M2* or *M3*) and heavy metal solutions were injected into sand

columns by using peristaltic pump. Bacterial cells were mixed with sand fractions by using PBS before packing sand in columns. All experiments included test the bioprecipitation in three sand fractions (fine, medium and coarse) in triplicate. Control samples prepared in the same fashion as main samples but different working solutions were used. Table 6.1 presents the experiments that were conducted to investigate the bioprecipitation processes in particulate media.

Table 6.1. Presents name and system of experiments that were done in this chapter and tested heavy metals with samples number.

<table>
<tr><th colspan="3">Experiment Name</th><th>System Used</th><th>Heavy Metal Tested</th></tr>
<tr><td colspan="3">Adsorption Isotherms</td><td>50 mL sterilised polypropylene tubes</td><td>Zinc, cadmium</td></tr>
<tr><td colspan="3">Bioprecipitation in porous media by no-flow system (Static)</td><td>50 mL sterilised polypropylene tubes</td><td>Strontium , Zinc, cadmium</td></tr>
<tr><td rowspan="3">Bioprecipitation in porous media by flow system (Dynamic)</td><td rowspan="2">Homogenous porous media</td><td>Without aging bacterial cells with cadmium ions</td><td rowspan="3">200 mm Height, 26 mm diameter acrylic columns</td><td rowspan="3">cadmium</td></tr>
<tr><td>With aging bacterial cells with cadmium ions for 24 hours in sand porous media</td></tr>
<tr><td>Heterogeneous porous media</td><td>With aging bacterial cells with cadmium ions for 24 hours in sand porous media</td></tr>
</table>

The choice of strontium, zinc and cadmium for this part of study, was based on the results that gained from bioprecipitation in aqueous solutions experiments (Chapter 5). Results indicated that copper removal is not as significant with this

mechanism as it is for other metals, though bacterial cells operation was efficient in presence of metal ions (up to 1 mM). In spite of that lead removal in solution was noticed at concentrations up to 5 mM, but still uncertain if the metal ions were removed by biotic or abiotic mechanisms. The possibility of that strontium, zinc and cadmium were co-precipitated with calcium carbonate precipitation is higher.

6.2 Adsorption Isotherms

Adsorption affinities of cadmium and zinc on adsorption sites of fine, medium and coarse sand fractions were estimated in this part of study. In preliminary experiment of metals precipitation in particulate media, sand particles showed high affinity to adsorb zinc and cadmium ions at normal pH, whilst the affinity to strontium and calcium ions was almost non-existent. This may due to ability of zinc and cadmium to make stronger complexes with inorganic compounds (e.g. ferrous and manganese oxides) and organic matter than strontium and calcium (McLean *et al.* 1992; Gunawardana *et al.* 2015).

The obtained data from these experiments was fitted with Langmuir and Freundlich adsorption isothermal models to check which model that is more applicable with obtained results. The assessment was based on the regression coefficient (R^2) values obtained after plotting the models according to obtained data. The conceptual basis of semi empirical Langmuir model is the formation of a monolayer adsorbate molecules on the external surface of the adsorbent, after that no additional uptake of molecules may occur. Freundlich model is an empirical adsorption model can apply for multilayer adsorption, this model considers the heterogeneity of adsorption sites and the interaction between the adsorbate (Bradl 2004; Coles and Yong 2006; Chen *et al.* 2010; Foo and Hameed 2010; Dada *et al.*

2012; Dawodu *et al.* 2012; Pan *et al.* 2012; Armagan and Toprak 2013; Desta 2013; Sun *et al.* 2014).

Based upon these assumptions, Langmuir represented the following equation:

$$q_e = \frac{q_m b C_e}{1 + b C_e} \quad \text{.....6.1}$$

Linear form of Langmuir equation is:

$$\frac{C_e}{q_e} = \frac{C_e}{q_m} + \frac{1}{q_m b} \quad \text{.....6.2}$$

Freundlich model is represented by the following equation:

$$q_e = K_f C_e^{\frac{1}{n}} \text{.....6.3}$$

Linear form of Freundlich equation is:

$$lnq_e = lnK_f + \frac{lnC_e}{n} \text{.....6.4}$$

Where:

C_e: (mg/l) is the equilibrium concentration of adsorbate (heavy metals).

q_e: (mg/g) the metal amount adsorbed per unit mass of the adsorbent at equilibrium.

q_m: (mg/g) is the maximum adsorption capacity.

b: (L/mg) is the Langmuir isotherm constant.

n: is a dimensionless constant describing the adsorption intensity, which indicates favourable sorption. The slope *1/n* indicates the effect of concentration on the adsorption capacity and represents adsorption intensity

K_f: (L/g) is the Freundlich isotherm constant describing the adsorption capacity of the adsorbent.

6.2.1 Cadmium Adsorption Experiment

Figure 6.1(A) presents the adsorption isotherms of cadmium ions on the particles of fine, medium and coarse sand fractions. The three sand fractions (fine, medium and coarse) displayed high affinity to adsorb cadmium ions from solution. Apart from highest cadmium equilibrium concentration, the uptake of the metal increased with equilibrium concentration. The adsorption capacity of fine sand fraction is higher than capacity for the other fractions. This may due to high adsorption surface area and the inorganic compounds and organic matter contents are higher than the other fractions (see Chapter 3, Table 3.2 for more details). Coarse sand fraction has lower specific surface area than medium fraction sand but the former included higher amounts of inorganic compounds and organic matter (inorganic compounds and organic matter quantities are present in Chapter 3, Table 3.2). This may justify the high adsorption capacity of coarse sand when compare with medium sand fractions. The proportion of adsorption generally decreased with cadmium equilibrium concentrations (Figure 6.1, B). This may relate to limited adsorption sites on sand grains.

Cadmium equilibrium sorption data were fitted into Langmuir and Freundlich isotherms models. Modelling of experimental adsorption isotherm data is an essential means for predicting the mechanisms of adsorption. Linear plots are shown in Figure

6.2. For Langmuir model the values of b and q_m were calculated from the slope and intercept respectively. Value of n for Freundlich model was obtained from the slope and intercept respectively. All parameters are presented in Table 6.2. Based on R^2 values Langmuir isotherm model fits more the results obtained from fine and medium sand fractions experiment, but Freundlich isotherm model fits more results gained from coarse sand fraction. Calculated low values of b (Table 6.2) indicate favourable adsorption processes of cadmium ions on sand grains for all fractions (fine, medium and coarse). n value (calculated from Freundlich model) for all sand fractions was more than 1, indicates that cadmium ions were adsorbed physically and adsorption was favourable on fine, medium and coarse sand fractions. Physical adsorption includes intermolecular forces (Van der Waals forces), which do not contain an important change in the electronic orbital patterns of the species (Alagumuthu *et al.* 2010; Al-Anber 2011).

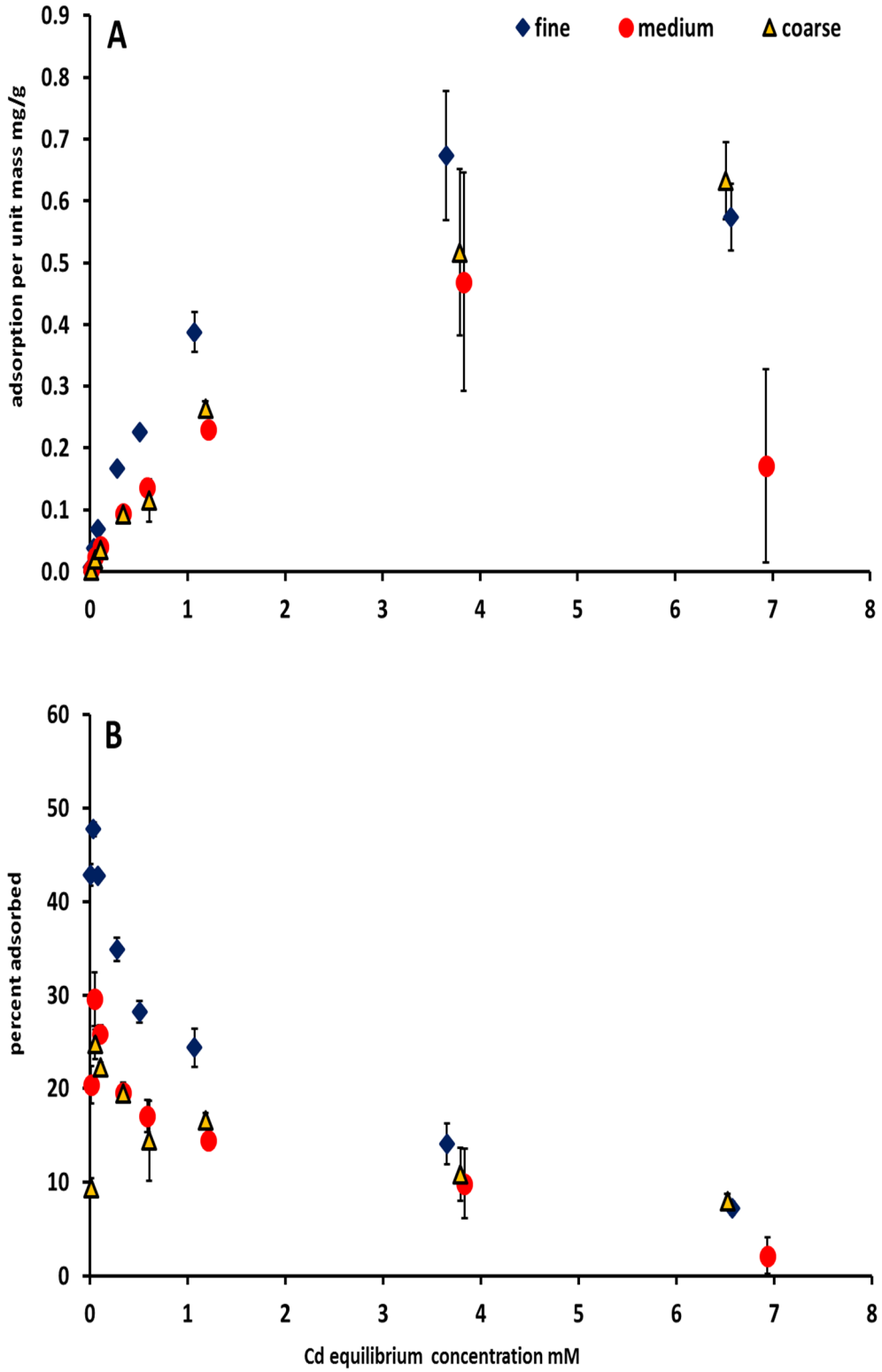


Figure 6.1. Adsorption isotherms of cadmium ions on three sand fractions samples (pH adjusted to 6.5), equilibrium adsorption capacity (A) and the percentage of adsorption (B). [Error bars represent ±1SD, n=3].

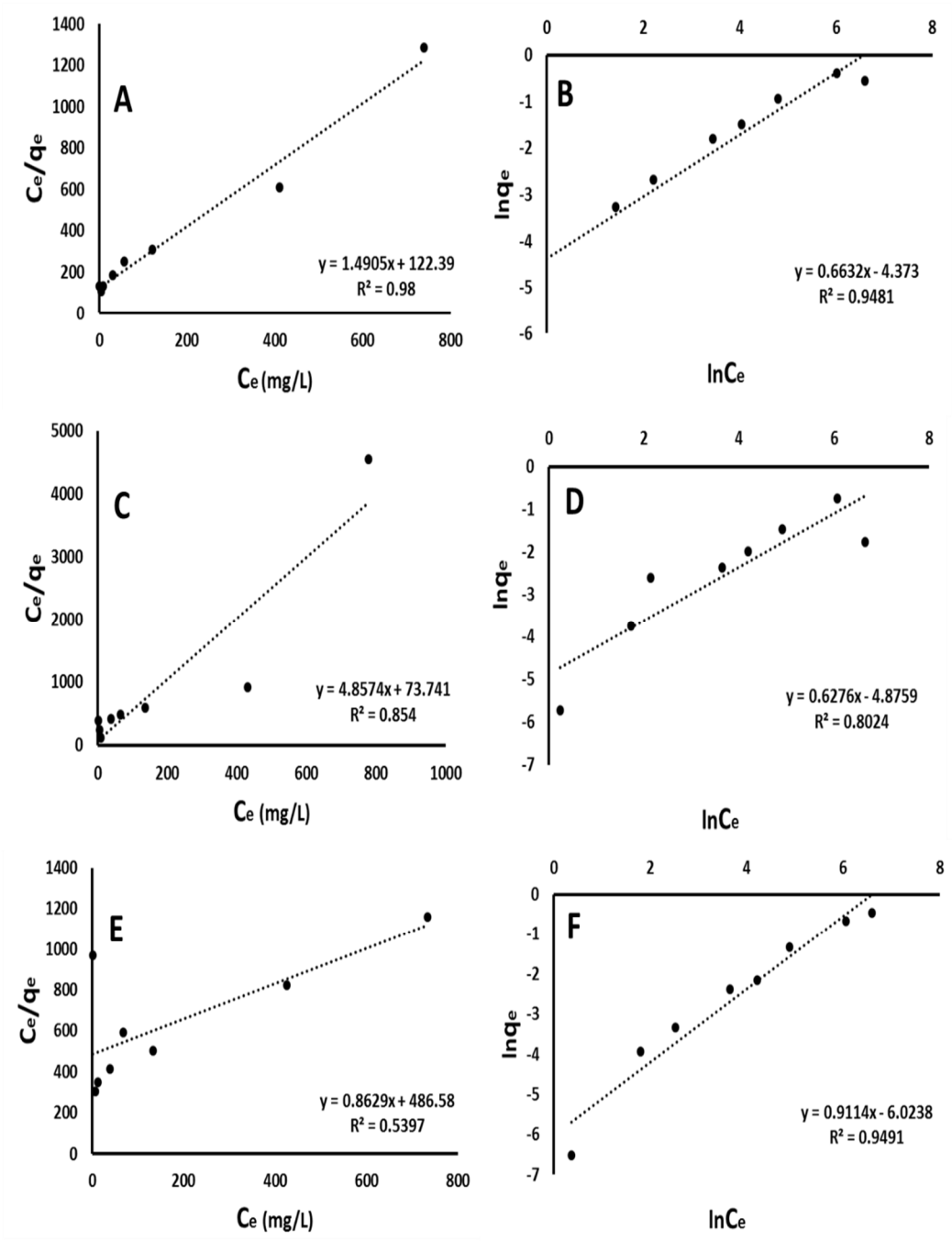


Figure 6.2. Langmuir and Freundlich isotherms of adsorption of cadmium on fine sand (A&B), medium sand (C&D) and coarse sand (E&F), respectively.

Table 6.2. Equilibrium parameters of cadmium adsorption on sand fractions, obtained from Langmuir and Freundlich isotherms Models.

Sand	Langmuir model		
	q_m (mg/g)	b (L/mg)	R^2
Fine sand	0.671	0.0122	0.980
Medium sand	0.206	0.0659	0.854
Coarse sand	1.159	0.0018	0.539
Sand	Freundlich model		
	q_m (mg/g)	n	R^2
Fine sand	0.671	1.508	0.948
Medium sand	0.206	1.594	0.802
Coarse sand	1.159	1.097	0.949

6.2.2 Zinc Adsorption Experiment

Figure 6.3 shows the relationship between the zinc equilibrium concentrations and the equilibrium adsorption capacity (A) and the percentage of adsorption (B). Apart from highest zinc concentration, metal adsorption on sand fractions increased with concentration. The capacity of fine sand fraction to adsorb zinc ions was higher than capacity for the other fractions. This may due to fine fraction offers relatively larger surface area than other fractions, hence higher adsorption occurred at equilibrium. Also, the amount of adsorption sites may affect the adsorption capacity of sand fraction. Many research proved the high affinity of zinc ions to inorganic compounds and organic matter Zinc is adsorbed preferentially to Fe, Mn and Al oxides in soil (McLean *et al.* 1992; Gunawardana *et al.* 2015). At zinc concentration of 10 mM the adsorption per unit mass was less than that at 5 mM, for fine and medium sand fraction, whilst for coarse sand fraction the adsorption at 5 and 10 mM was almost the same. This may be due to adsorption sites are different in each sand fraction.

Generally, the affinity of sand fractions to adsorb zinc ions is higher than that for cadmium. This may be due to the fact that zinc makes complexes with inorganic

compounds and organic matter more strongly than those made by cadmium. Also, above pH 5.5 value zinc ions are subjected to nonexchangeable retention on Fe and Mn oxides (Stahl and James 1991; McLean *et al.* 1992; Gunawardana *et al.* 2015).

To predict the mechanism of zinc ions adsorption on sand fractions metal equilibrium sorption data were fitted into Langmuir and Freundlich isotherms models. Figure 6.4 presents the linear plots of the models. Also Table 6.3 lists the calculated parameter of Langmuir and Freundlich isotherms models. Based on R^2 values Langmuir isotherm model fits more the results obtained from all sand fractions. Calculated low values of *b* (Table 6.3) indicate favourable adsorption processes of cadmium ions on sand grains for all fractions (fine, medium and coarse). In addition, *n* value (calculated from Freundlich model) for all sand fractions was more than 1, indicates that zinc ions were adsorbed physically and adsorption was favourable on fine, medium and coarse sand fractions.

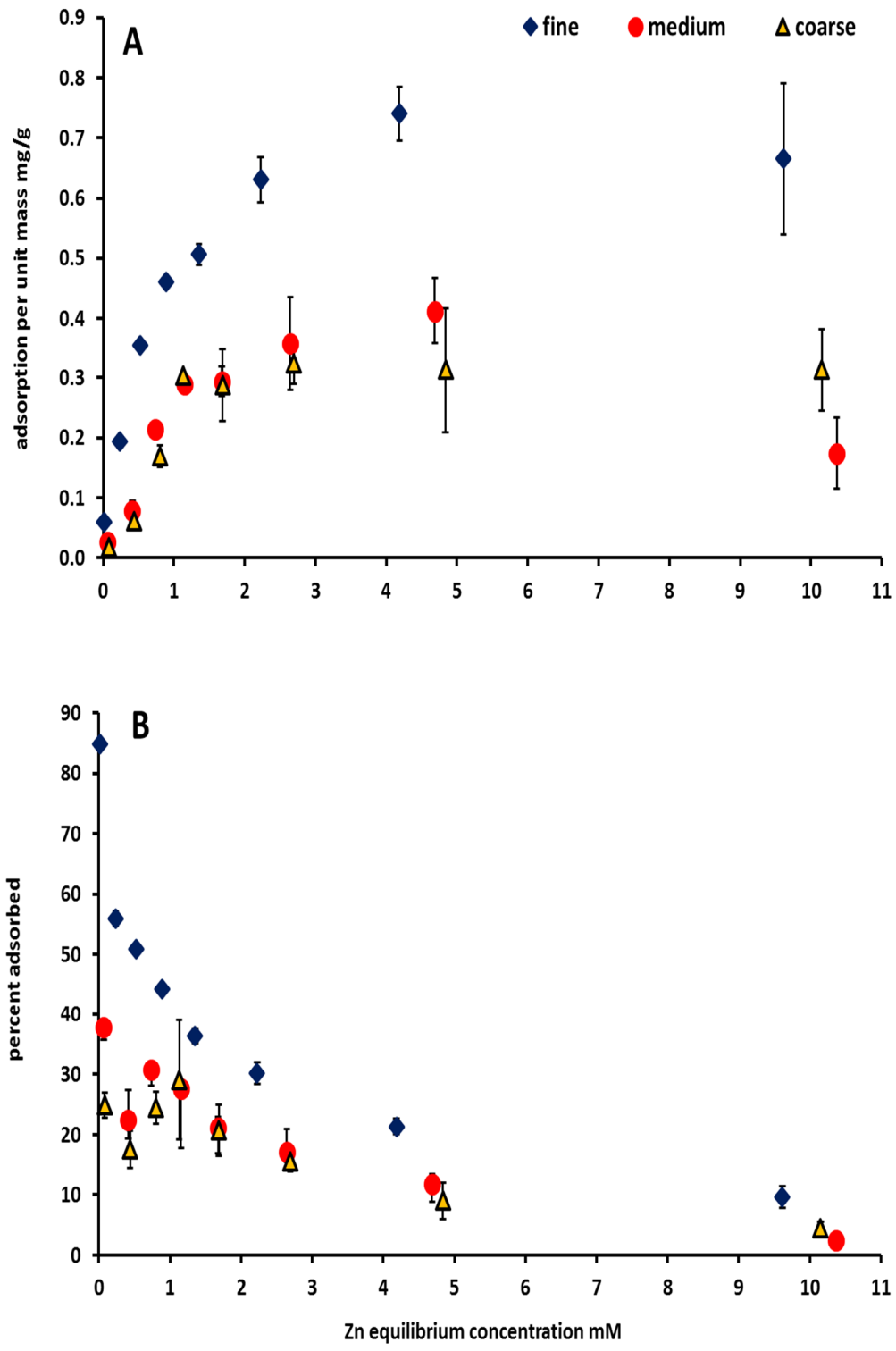


Figure 6.3. Adsorption isotherms of zinc ions on three sand fractions samples (pH adjusted to 6.5), equilibrium adsorption capacity (A) and the percentage of adsorption (B). [Error bars represent ±1SD, n=3].

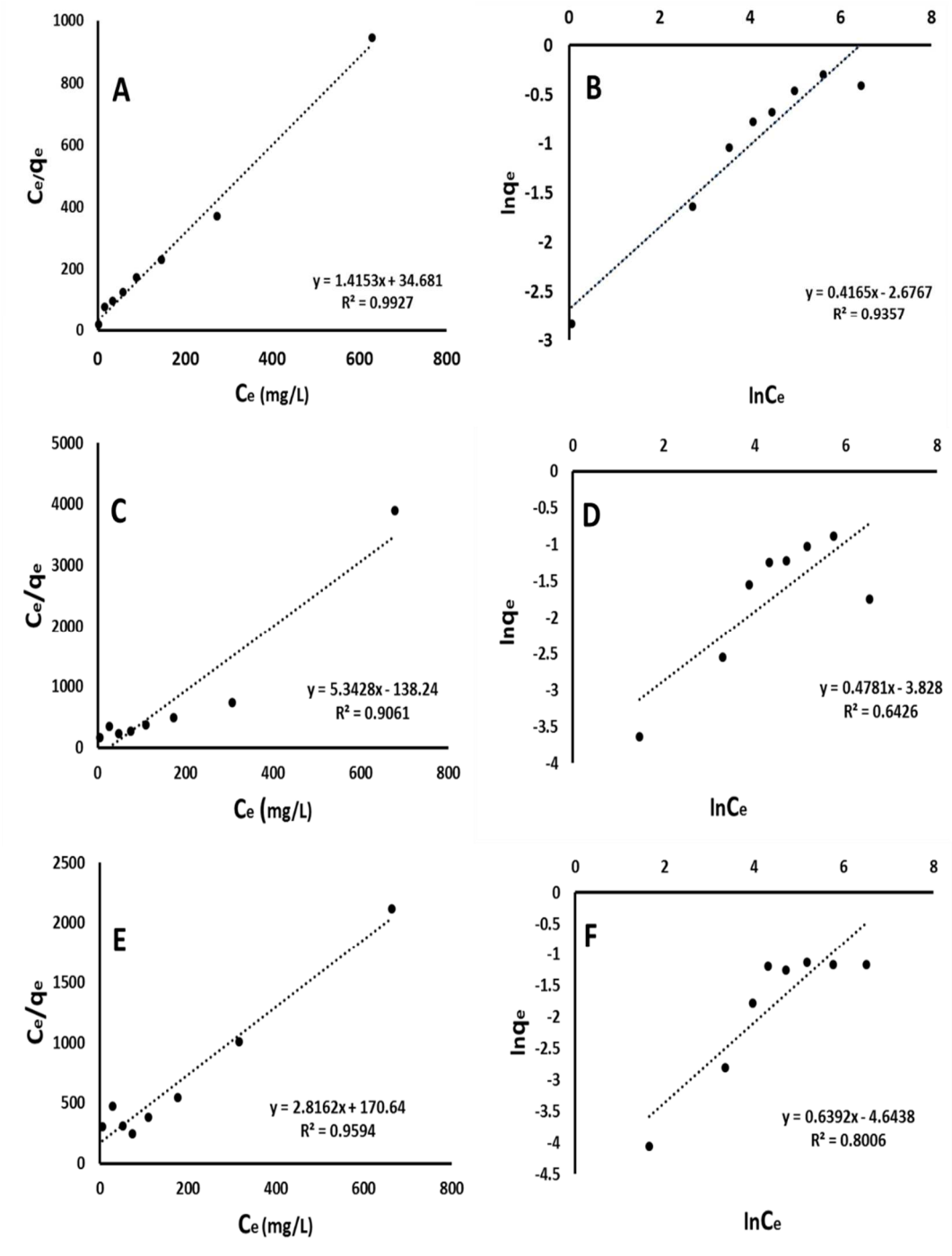


Figure 6.4. Langmuir and Freundlich isotherms of adsorption of zinc on fine sand (A&B), medium sand (C&D) and coarse sand (E&F), respectively.

Table 6.3. Equilibrium parameters of zinc adsorption on sand fractions, obtained from Langmuir and Freundlich isotherms Models.

sand	Langmuir model		
	q_m (mg/g)	b (L/mg)	R^2
Fine sand	0.707	0.041	0.993
Medium sand	0.187	0.039	0.906
Coarse sand	0.355	0.017	0.959
sand	Freundlich model		
	q_m (mg/g)	n	R^2
Fine sand	0.707	2.4	0.936
Medium sand	0.187	2.09	0.643
Coarse sand	0.355	1.565	0.801

6.3 Bioprecipitation in Porous Media by No-Flow System (Static)

The precipitation of heavy metals in porous media by using system without fluid flow was investigated in this section. This section includes precipitation with and without heavy metals. When bioprecipitation processes take place in porous media can be affected by sand grains size and availability of heavy metals. Such effects did not influence the bioprecipitation of heavy metals in aqueous solutions. The outcomes of the static system experiments were considered to design bioprecipitation experiments by using fluid flow or dynamic systems.

6.3.1 Calcium Carbonate Precipitation in Porous Media without Heavy Metals (precipitation in static experiment)

The first experiment in this section was to examine the ability of *S. Pasteurii* strain to induce calcium carbonate precipitation in particulate media without presence of heavy metals. Figure 6.5 shows calcium precipitation response to bacterial activity in fine, medium and coarse sand fractions. As it clear more than 99% of calcium ions (50 mM) were removed from soil solution after day-3. Calcium removal occurred in conjunction with pH increase, up to 9 in fine sand and more than 9 in other fractions. Gradually increase in pH over 14 days reflects continuing bacterial activity, in spite

of their cell walls may act as nucleation sites for calcium carbonate formation, and this in turn can lead to the bacteria becoming embedded within the solid matrix which is likely to kill cells by prevent access to nutrients and energy sources (Tobler *et al.* 2011). A similar effect is seen in fine, medium and coarse sand fractions regarding the ability of *S. pasteurii* to enhance calcium carbonate precipitation. pH increased with coarseness of sand grains under these static conditions, although over a very small range. This may be attributed to buffering capacity of fine sand (Liu *et al.* 2008).

In control samples no significant calcium removal after 7 days, but after 14 days calcium removal was only 20, 10, and 8 % in fine, medium and coarse sand fractions, respectively. This decreasing may occurred due to pH increase in control samples, which in turn increase sand capacity to adsorb cations or possible abiotic precipitation. Small pH increase in controls samples of fine, medium and coarse sand fraction was almost identical after 14 days. This may belong to the buffering capacity of used sand fractions in recovering their original pH after acidic washing (which was noticed by a side experiment). However, the presence of nutrient broth may affect medium pH value where its original pH after sterilisation was 7.4.

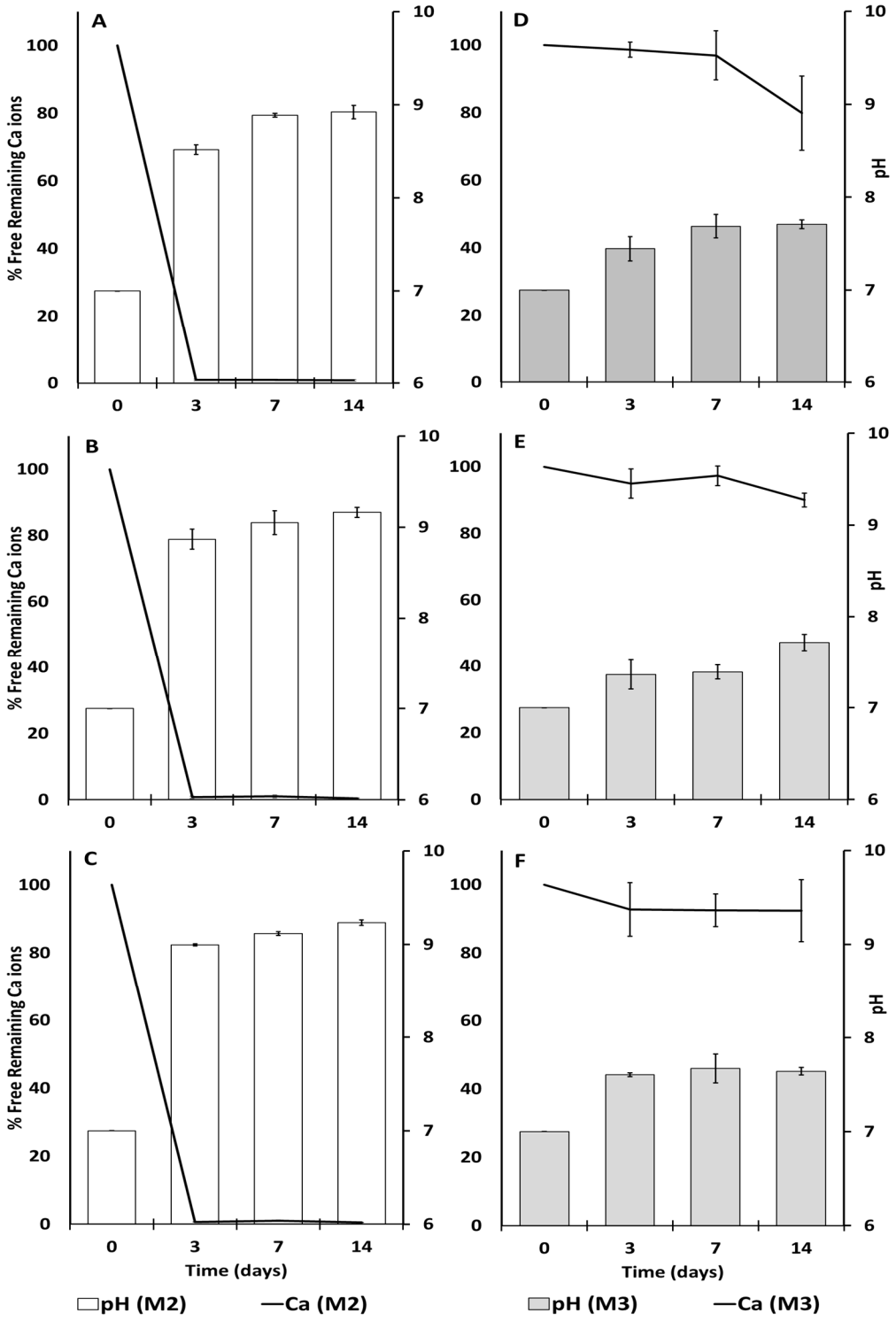


Figure 6.5. Response of calcium (50 mM) precipitation and pH to activity of S. pasteurii in soil pore fluid of the fine sand (A and D), medium sand (B and E) and coarse sand (C and F), with test samples (with urea growth medium, M2) (A-C) and controls (without urea growth medium, M3) (D-F), at 30ºC. [Error bars represent ±1SD, n=3].

6.3.2 Strontium Precipitation in Porous Media (in static experiment)

The ability of *S. pasteurii* cells to precipitate calcium carbonate seems not affected by presence of strontium ions (30 mM) or by porous media conditions. This comparing to precipitation without heavy metals (Figure 6.5) and precipitation of strontium (30 mM) in aqueous solutions (Chapter 5). Figure 6.6 presents experimental data obtained from precipitation of strontium in the three sand fractions (static system). The bacterial activity and the subsequent calcium carbonate mineralisation and strontium sequestration from soil solution were not influenced by the variety of sand fraction. This probably due to the immobility of fluid inside sand fractions for 7 days, and, to some extent, the homogeneity in distributing the bacterial cells, precipitation medium and strontium ions within sand pores.

With urea present, pH increased to around 9. There was little discernible difference in pH change, or recovery of strontium or calcium ions, between sand grain sizes, with perhaps a small increase in Sr removal with increasingly coarse grain size. Without urea in control samples, there was little loss of strontium (around 20% after 3 and 7 days) whilst when urea was present removal was almost complete. Calcium ions removal in controls was very similar in all sand fractions after 3 days, then reduced further to around 50% after 7 days, perhaps due to abiotic precipitation. A slight increase in pH in no-urea controls may be due to buffering capacity of sand recovered its original pH

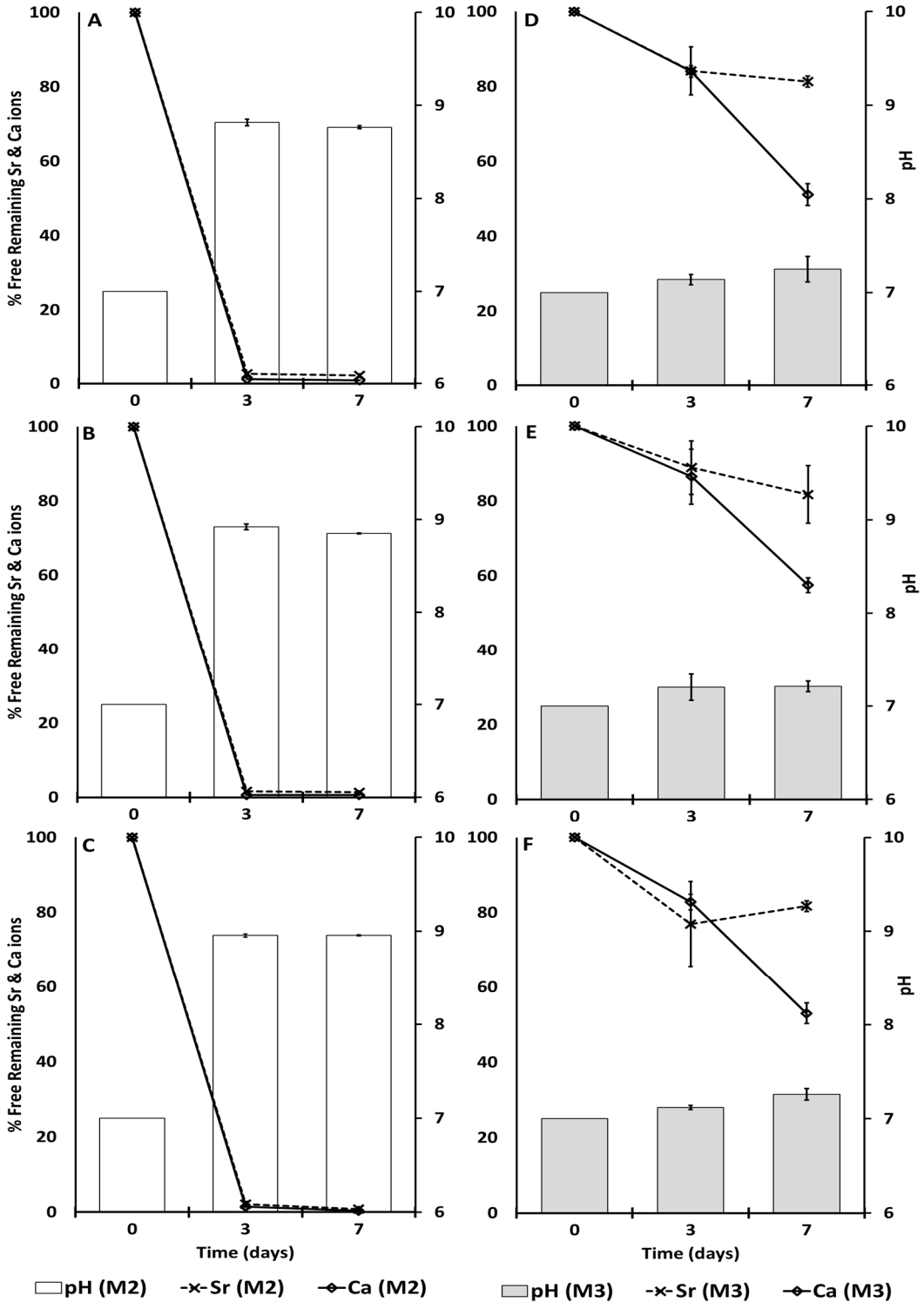


Figure 6.6. Response of strontium (30 mM) and calcium precipitation and pH to activity of S. pasteurii in soil pore fluid of the fine sand (A and D), medium sand (B and E) and coarse sand (C and F), with test samples (with urea growth medium, M2) (A-C) and controls (without urea growth medium, M3) (D-F), at 30ºC. [Error bars represent ±1SD, n=3].

Strontium ions recovery after 7 days in controls indicates the low affinity of these ions to be adsorbed on the three sand fractions. The lack of sorption in controls indicates that sorption is not a major mechanism here, suggesting that the removal of strontium when urea is present arises due to the presence of microorganisms, increase of pH, and subsequent precipitation. The loss of strontium ions in sand fractions (control samples) may due to presence different amounts of inorganic compounds and organic matter in sand fractions. It is likely that strontium ions in main samples were either precipitated or co-precipitated with calcium carbonate (Achal *et al.* 2012b; Gebrehiwet *et al.* 2012). There is a possibility of proportion of strontium ions was adsorbed on bacterial biomass, but this kind of removal was not covered by this study.

However, the results of this assay are consistent with many research studied regarding the ability of *S. pasteurii* to induce strontium remediation in soil (Fujita *et al.* 2000; Fujita *et al.* 2004; Mitchell and Ferris 2005; Mitchell and Ferris 2006; Fujita *et al.* 2008; Fujita *et al.* 2010; Brinza *et al.* 2013; Lauchnor *et al.* 2013). All these studies mentioned no obvious toxicity effects for the bacteria or inhibition influence to calcium carbonate recrystallisation of this metal. Fujita *et al.* (2004), based on X-ray diffraction results, referred to that strontium ions not only adsorbed on calcite surface but present deeply within the particles. In addition, strontium cations are present as a solid solution in calcium carbonate precipitations samples.

6.3.3 Zinc Precipitation (in static experiment)

In this section zinc co-precipitation with calcium carbonate induced by *S. pasteurii* strain was investigated. Three zinc concentrations were tested (2, 5, 10 mM) to test metal bioavailability on bacterial activity to hydrolyse urea. In preliminary experiments zinc concentrations less than 2 mM exhibited very low availability in the

sand fractions (fine, medium and coarse). Adsorption of heavy metals on sand grains allow bacteria to operate as no presence of toxic cations. Hence, zinc concentrations were increased to increase their availability in porous media.

Figure 6.7, Figure 6.8 and Figure 6.9 present the results of bioprecipitation experiments of 2, 5 and 10 mM zinc concentrations, respectively, in porous media of fine, medium and coarse sand fractions. As it clear bacterial activity affected by increasing zinc concentration, compared to bacterial activity when no heavy metals present. Almost all zinc ions were removed from urea-treated samples (*M2* medium) at all metal concentrations after day-3, this apart from medium sand samples at zinc concentration of 5 mM, 11% was recovered. Calcium removal or precipitation was correlated with pH values. Full calcium removal occurred at zinc concentrations of 2 mM (after day-7) and 5 mM (after day-3) in urea-treated samples. At 10 mM concentration high proportions of calcium ions were recovered from fine and coarse sand fractions. In urea-treated samples pH decreased with zinc concentrations in fine and medium sand fractions after day-3. Change in pH is an indicator of urea hydrolysing in solutions by bacterial activity or urease enzyme.

In control samples (no urea was added, medium *M3*) zinc recovery increased with metal concentration in fine sand fractions after day-3. Whilst almost same amounts of zinc ions were recovered from medium and coarse sand factions at all metal concentrations after day-3. All calcium ions were recovered from all control samples at all concentrations, also, no significant change in pH was seen, apart from fine and medium sand fractions at zinc concentration 2 mM. Fine sand fractions showed high adsorption capacity than other fractions.

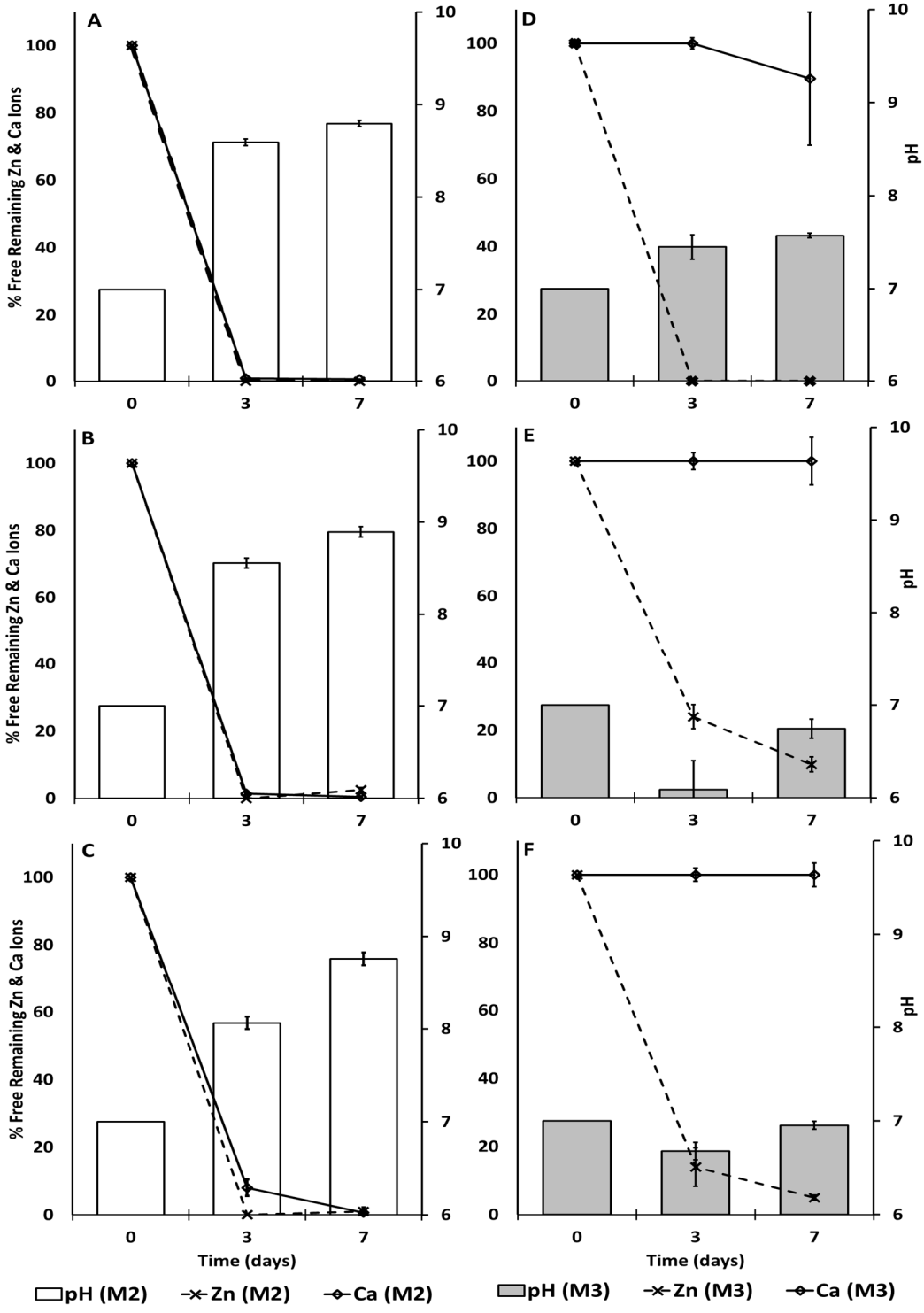


Figure 6.7. Response of zinc (2 mM) and calcium precipitation and pH to activity of S. pasteurii in soil pore fluid of the fine sand (A and D), medium sand (B and E) and coarse sand (C and F), with test samples (with urea growth medium, M2) (A-C) and controls (without urea growth medium, M3) (D-F), at 30ºC. [Error bars represent ±1SD, n=3].

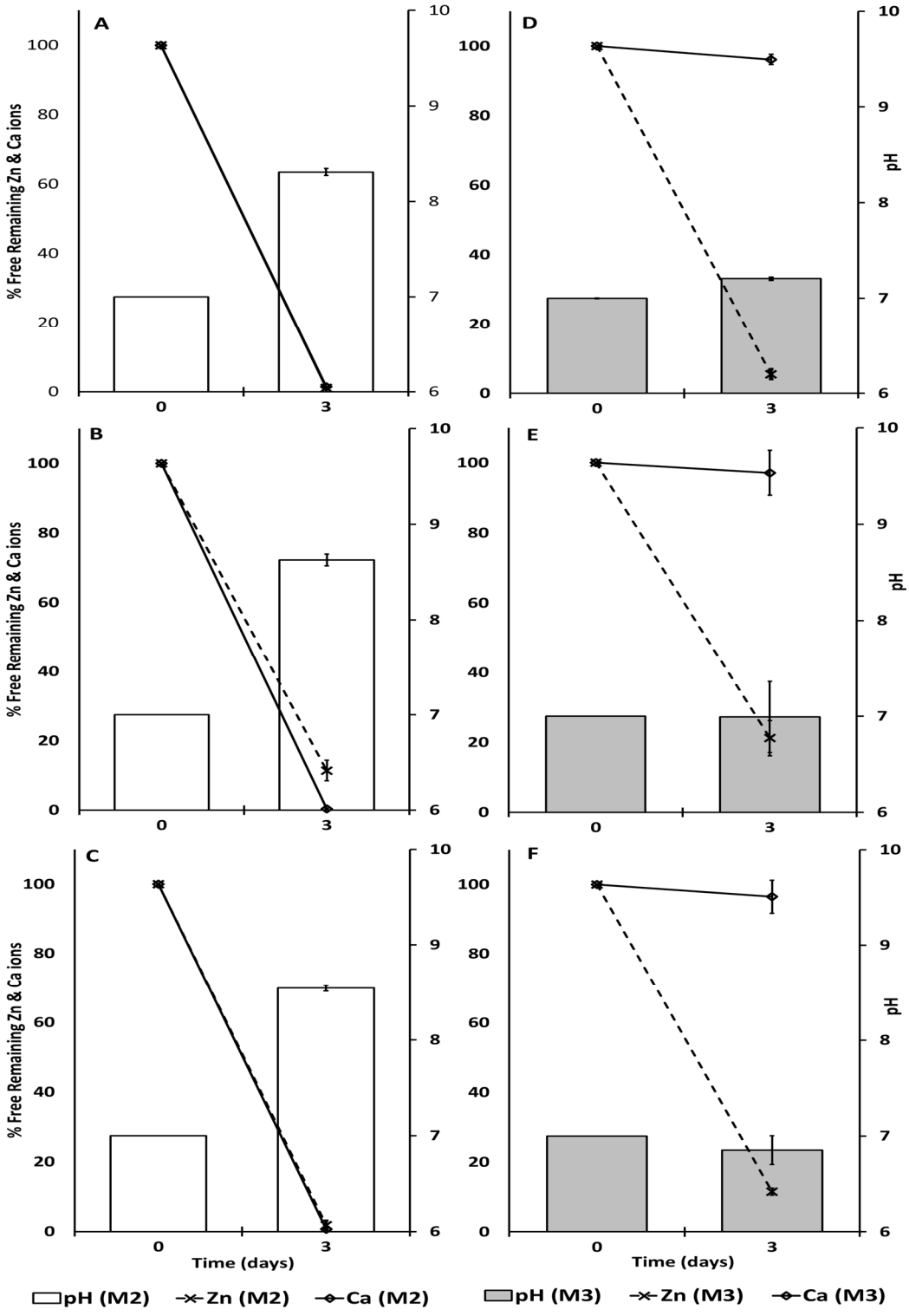


Figure 6.8. Response of zinc (5 mM) and calcium precipitation and pH to activity of S. pasteurii in soil pore fluid of the fine sand (A and D), medium sand (B and E) and coarse sand (C and F), with test samples (with urea growth medium, M2) (A-C) and controls (without urea growth medium, M3) (D-F), at 30ºC. [Error bars represent ±1SD, n=3].

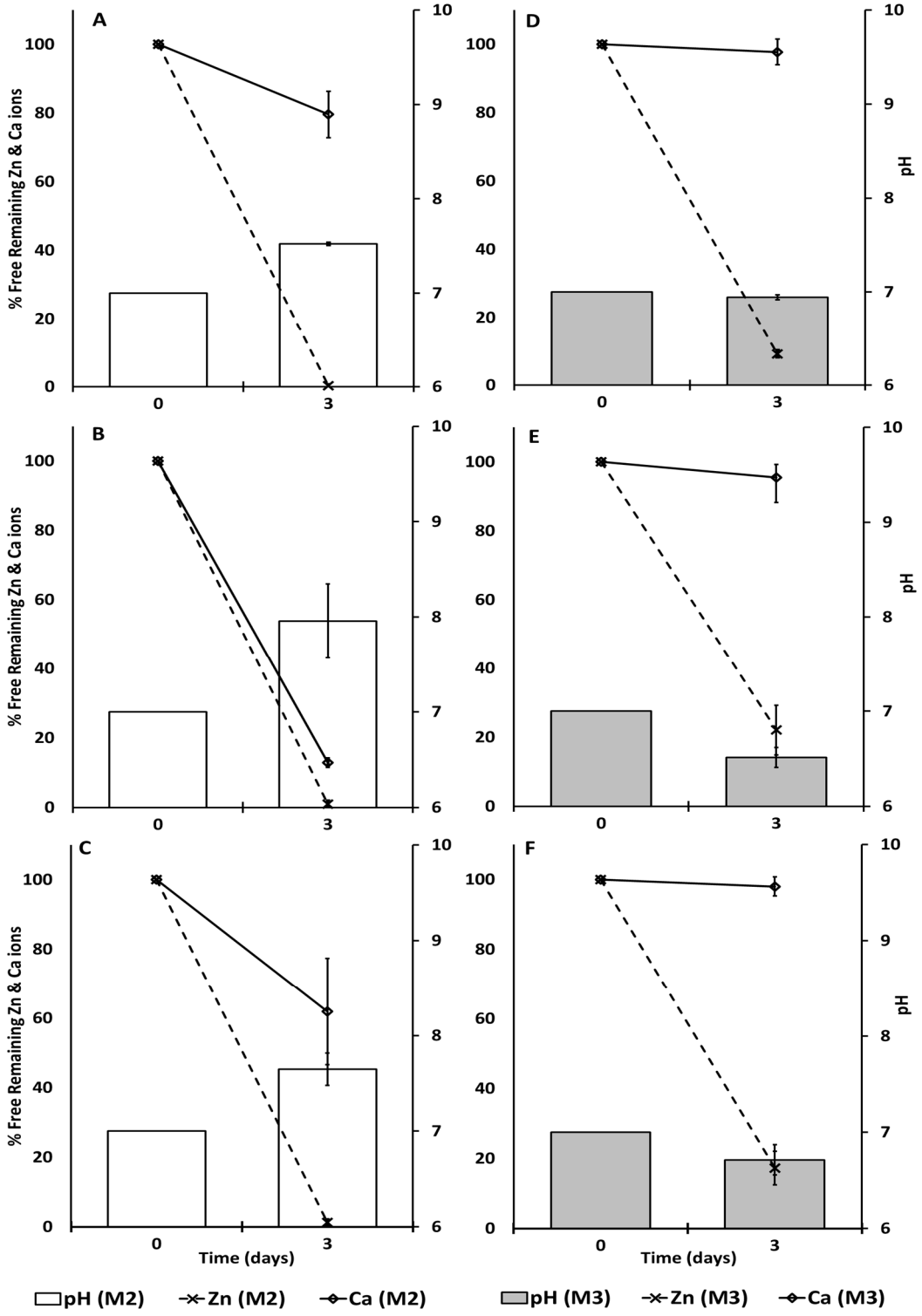


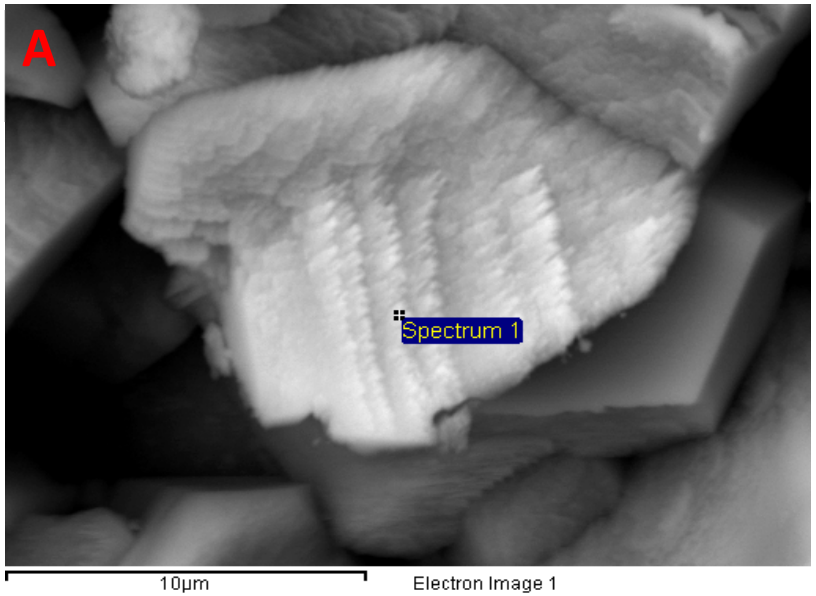
Figure 6.9. Response of zinc (10 mM) and calcium precipitation and pH to activity of S. pasteurii in soil pore fluid of the fine sand (A and D), medium sand (B and E) and coarse sand (C and F), with test samples (with urea growth medium, M2) (A-C) and controls (without urea growth medium, M3) (D-F), at 30ºC. [Error bars represent ±1SD, n=3].

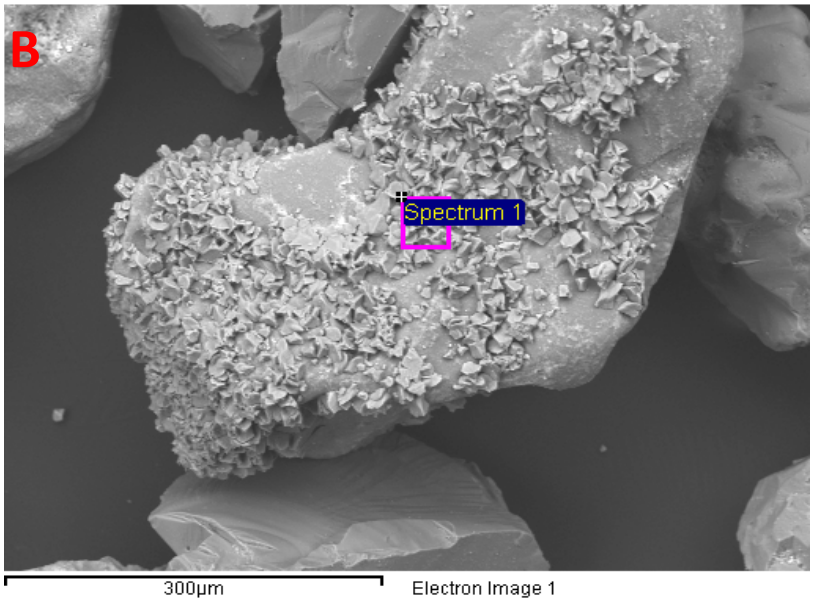
In 2 and 5 mM zinc concentration experiments pH increase may reflect significant bacterial activity that caused urea hydrolysis. Zinc ions removal from urea-treated samples may attribute to precipitation with calcium carbonate and adsorption on sand grains mechanisms. Hence, it may not possible here to identify the amount of zinc that adsorbed on sand grains or precipitated with calcium carbonate. In zinc adsorption isotherm experiment (section 6.2.2) metal ions showed less affinity to be adsorbed on sand fractions than their adsorption affinity in precipitation experiments (high adsorption zinc affinity in control samples is more clearly). This may due to pH change and the effects of presence of bacterial cells, nutrient broth and other chemicals, in addition, adsorption affinity may increase with pH increase (Abdus-Salam and M'civer 2012).

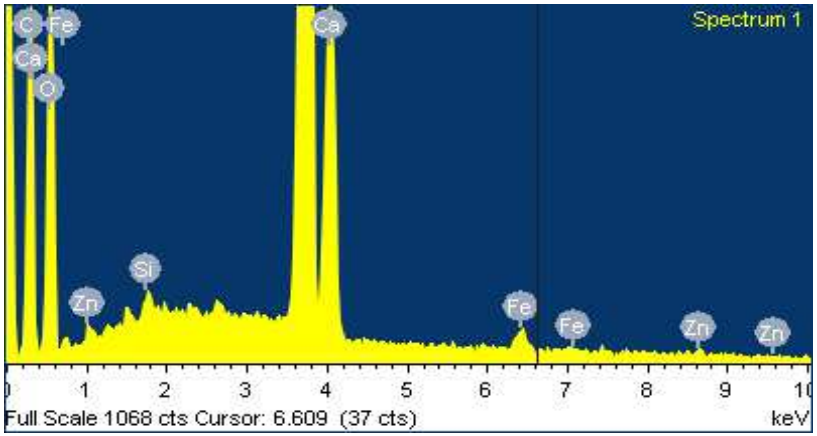
High removal proportion of zinc ions in control samples from 2 and 5 mM experiments after day-3 may mean the metal ions adsorption to sand grains is the main mechanism that responsible for metal removal. The available quantity of zinc and calcium ions in control samples seemingly depended on sand pH, sand grain sizes, inorganic compounds and organic matter. In control samples of the three experiments sand fractions were not similar in retaining zinc ions, which may reflect different sand fraction capacities to adsorb heavy metals. In fine sand specimens pH values were more than other sand fractions, albeit in small change. This again may due to acidity effect of zinc chloride salt, such effect was proven by visual MINTEQ model (pH of a solution containing zinc chloride and calcium chloride decrease with increase zinc chloride concentration). In 10 mM zinc experiment (Figure 6.9), bacterial activity in urea-treated samples seems to be affected by high concentration of zinc, pH values were less than that in 2 and 5 mM experiments after 3 days. Zinc ions were almost completely removed from urea-treated samples. Calcium was recovered in fine (60%)

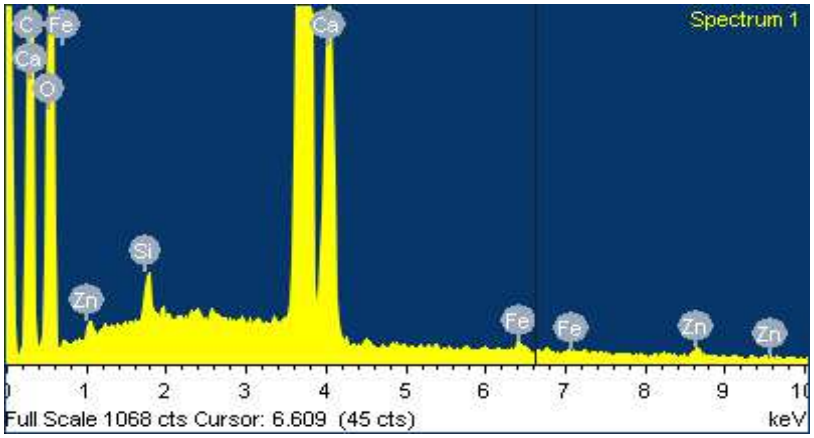
and coarse (80%) sand fractions, whilst only 13% recovered in medium sand samples. In 2 and 5 mM experiment calcium was removed after day-3, hence, in this experiment bacterial cells probably were inhibited by zinc ions.

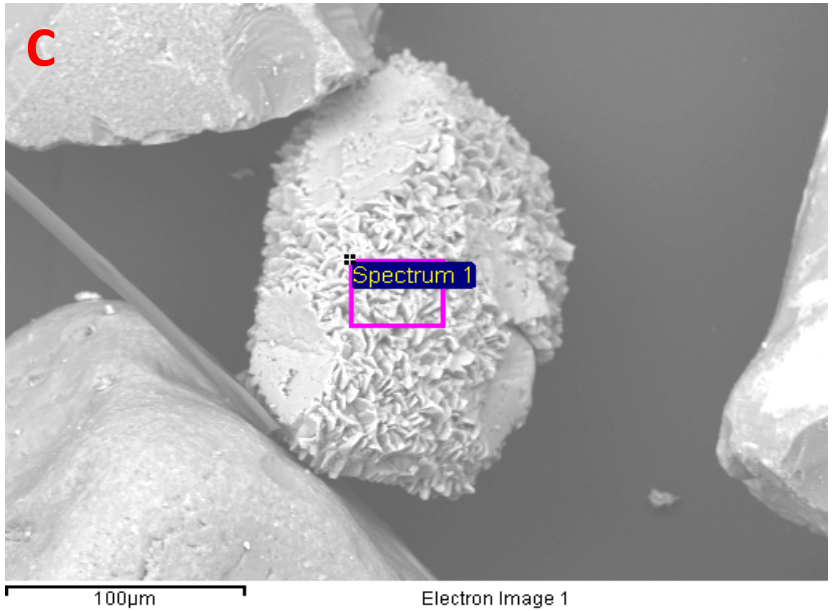
Figure 6.10 (A, B, C, D, E and F) illustrates the SEM and EDX spectrums of calcium carbonate precipitation that resulted from bioprecipitation processes of 2 mM zinc concentrations. A, B, C, D and E show clearly the presence of zinc and calcium in precipitated calcium carbonate on sand particles, albeit calcium is much higher than zinc. Whilst F spectrum was taken outside precipitate zone shows clearly no presence of zinc or calcium. Accordingly, suggesting that zinc co-precipitated with calcium carbonate precipitates on sand particles. Kang *et al.* (2014) reported that mineral crystals were produced by ureolytic bacteria that induced calcium carbonate precipitation, as roughly rhombohedral, spherical, or needle-shaped, size of the produced solids is generally from 10-50 μm. The resulted precipitations are surface layers may not follow specific shape. Bacteria mechanism that leads to select a polymorph during calcium carbonate biomineralisation processes is not completely understood. Hammes *et al.* (2003) suggested that the crystal phase formation and morphology of precipitated calcium carbonate are strain specific. However, Sondi and Salopek-Sondi (2005) reported that variances in the amino acid sequences of urease enzymes (secreted by different type of strains) have influences on polymorphs precipitation during calcium carbonate bioprecipitation processes. In addition, vaterite or calcite polymorph formation can be affected by specific proteins in extracellular polymeric secretions (EPS) that produced by different types of bacteria (Kawaguchi and Decho 2002). Reasons mentioned above may responsible for different shapes of precipitation produced in this experiment.

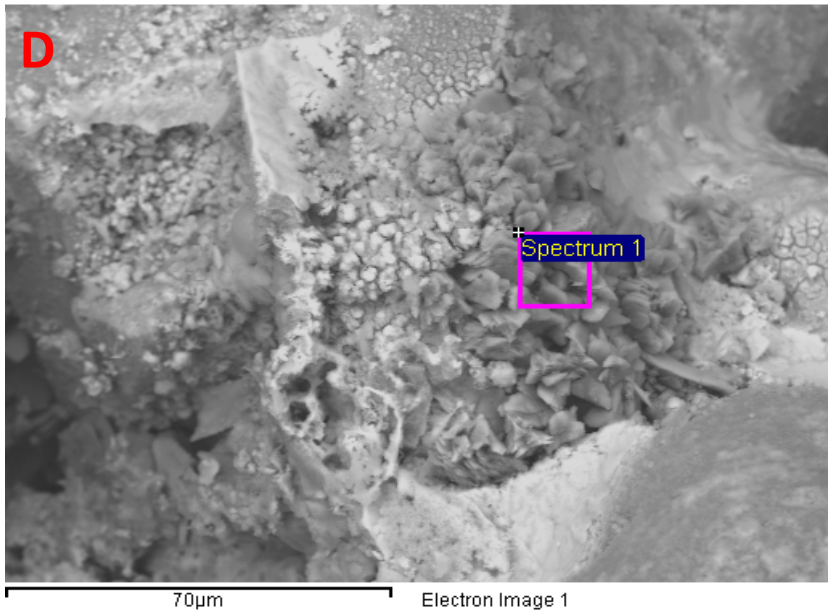

A
Spectrum 1
10µm
Electron Image 1

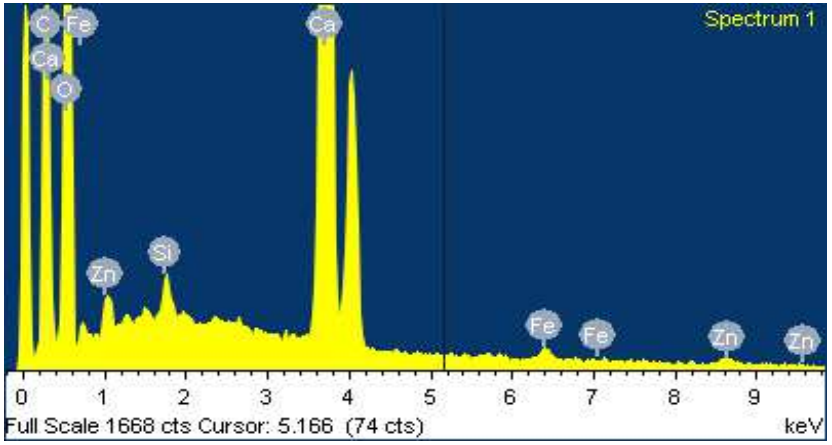

B
Spectrum 1
300µm
Electron Image 1

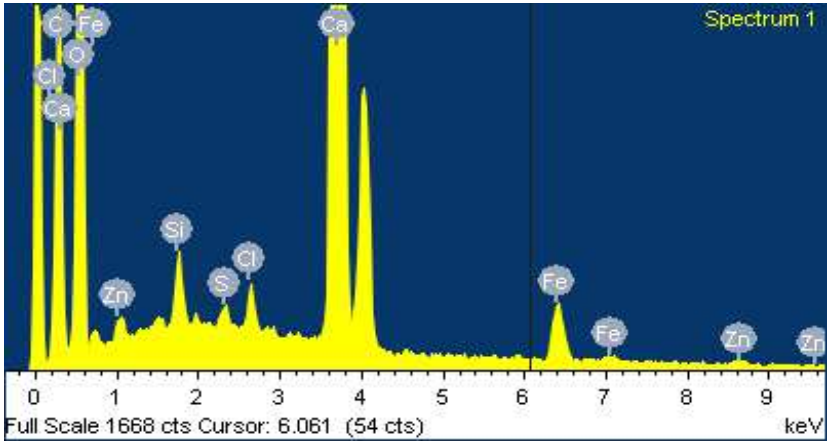

Spectrum 1
C
Fe
Ca
Ca
O
Si
Zn
Fe
Fe
Zn
Zn
0 1 2 3 4 5 6 7 8 9 10
Full Scale 1068 cts Cursor: 6.609 (37 cts)
keV


Spectrum 1
C
Fe
Ca
Ca
O
Si
Zn
Fe
Fe
Zn
Zn
0 1 2 3 4 5 6 7 8 9 10
Full Scale 1068 cts Cursor: 6.609 (45 cts)
keV


C
Spectrum 1
100µm
Electron Image 1


D
Spectrum 1
70µm
Electron Image 1


Spectrum 1
C
Fe
Ca
O
Zn
Si
Ca
Fe
Fe
Zn
Zn
0 1 2 3 4 5 6 7 8 9
Full Scale 1668 cts Cursor: 5.166 (74 cts)
keV


Spectrum 1
C
Fe
O
Cl
Ca
Ca
Si
Cl
S
Zn
Fe
Fe
Zn
Zn
0 1 2 3 4 5 6 7 8 9
Full Scale 1668 cts Cursor: 6.061 (54 cts)
keV

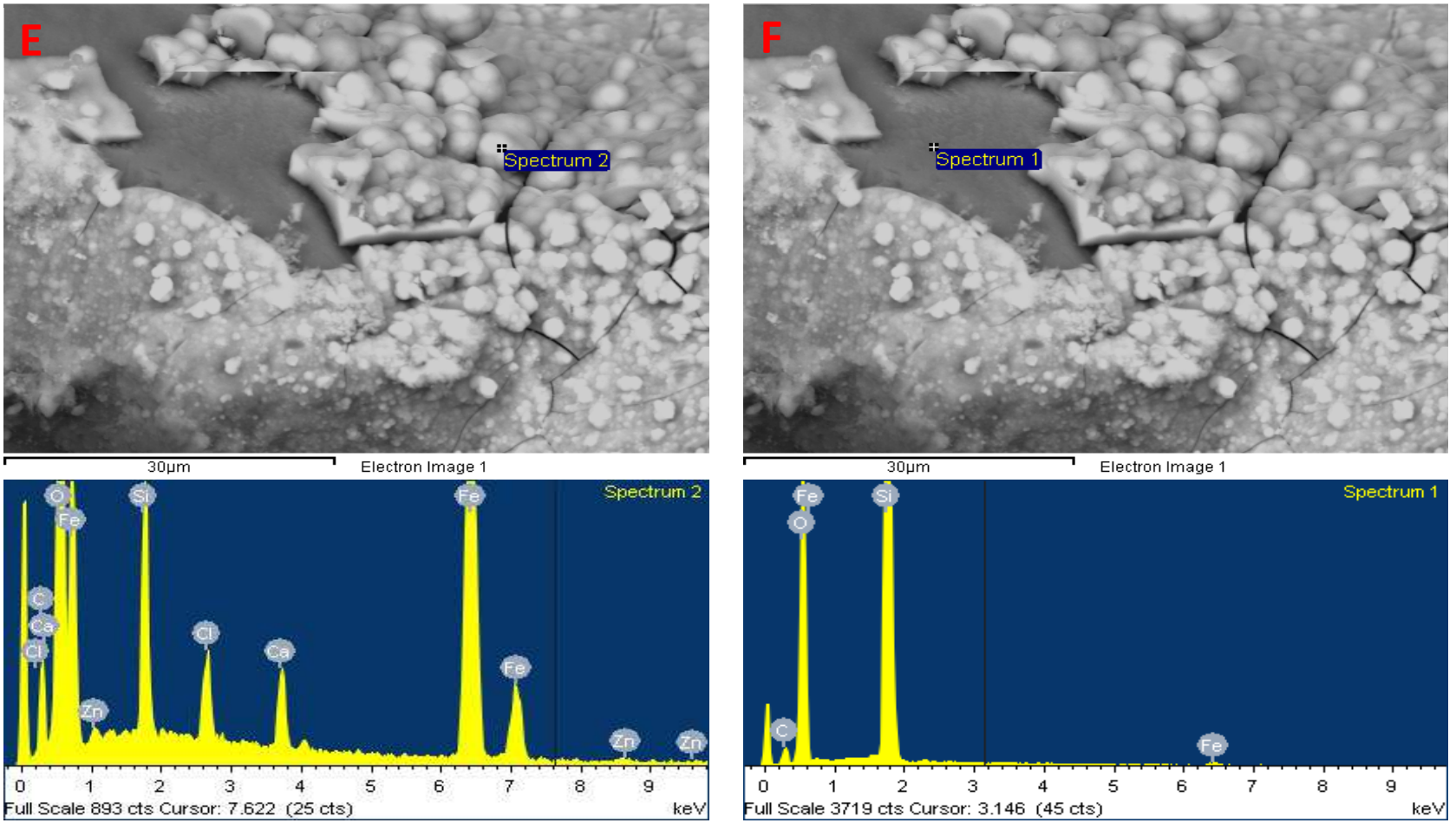


Figure 6.10. SEM and EDX spectrum of calcium carbonate precipitation on sand particles induced by S. pasteurii in presence of zinc ions (2 mM) by using urea-containing medium (static system experiment). A, B, C, D and E represent the spectrums that taken inside the precipitate, whilst F is inside.

6.3.4 Cadmium Precipitation (in static experiment)

Cadmium precipitation in sand depends on the available portion of total metal concentration. Figure 6.11 shows the response cadmium, calcium and pH to *S. pasteurii* activity in fine, medium and coarse sand fractions pores. High removal rates (99.8%) of cadmium ions from solution were observed in urea-treated samples following incubation for 3 days. Sequestering of the soluble 10 mM of this heavy metals was probably via adsorption on sand grains and precipitation with calcium carbonate. Regarding calcium ions more than 99% were removed from contaminated soil water in urea-treated samples. Calcium removal probably due to precipitation as calcium carbonate, especially this removal was conjunction with pH increase from its initial value (7) to up to 8.8. However, there were no obvious trends depend on sand fraction size.

Fluids extracted from control samples had lower pH values, 7.5 for fine and coarse sand fractions and 6.5 for medium size, at these value of pH almost all calcium recovered. Cadmium ions adsorbed on fine sand grains were higher than other fractions. However, cadmium ions adsorbed on coarse sand fraction were higher than medium sand fraction. Metal ions were added as cadmium sulphate ($3CdSO_4.8H_2O$), this formula can give weak acid effect on containing solution. Visual MINTEQ model predicts pH value of 6.5 for a solution containing 20 mM cadmium concentration resulted from dissolving cadmium sulphate in water (same as pH value in medium sand samples). Hence, suggesting that fine and coarse sand fractions containing materials able to recover sand original pH value may not available in medium sand fraction.

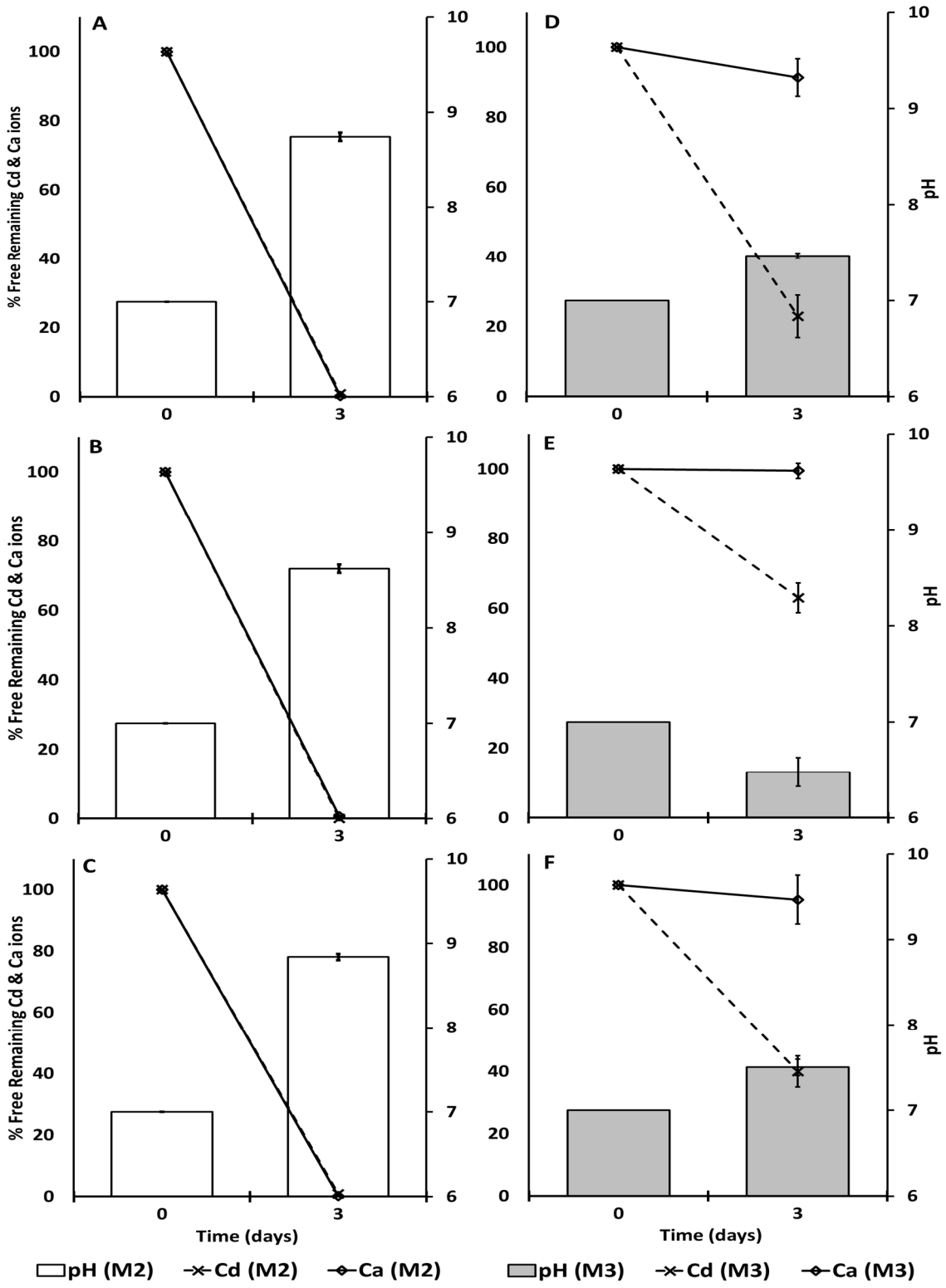


Figure 6.11. Response of cadmium (10 mM) and calcium precipitation and pH to activity of S. pasteurii in soil pore fluid of the fine sand (A and D), medium sand (B and E) and coarse sand (C and F), with test samples (with urea growth medium, M2) (A-C) and controls (without urea growth medium, M3) (D-F), at 30ºC. [Error bars represent ±1SD, n=3].

Cadmium ions showed less affinity to be adsorbed on sand grains than zinc (section 6.3.3). Cadmium ions have great affinity to be adsorbed on this poorly crystalline Fe oxides (like ferrihydrite), this adsorption increases with increase pH value (Jae-Young *et al*. 2002; Mustafa *et al*. 2004; Fortin and Langley 2005; Mustafa *et al*. 2006; Abdus-Salam and M'civer 2012). This affinity may be responsible for cadmium decreasing in extracted liquids from control sand samples. Metal adsorption depends on Fe oxides amount and pH value for the sand fractions. Figure 6.11 shows that more of cadmium ions in control samples were adsorbed on fine and coarse then medium sand fractions.

In terms of cadmium toxicity to it seems that *S. pasteurii* cells survive and operate at high metal concentration in sand fractions. In control samples 23% (2.3 mM), 63% (6.3 mM) and 40% (4.0 mM) were recovered from fine, medium and coarse sand fractions, respectively. Providing that these cadmium amounts (that recovered from control samples) were available in porous media of urea-treated sand samples at pH values similar to that in controls, hence, *S. pasteurii* cells induced calcium carbonate precipitation and probably cadmium co-precipitation, in presence of high amounts of metal ions.

6.4 Bioprecipitation in porous media by flow system (Dynamic)

Based on static condition tests results (section 6.3.2) strontium ions were readily precipitated or co-precipitated with calcium carbonate induced by *S. pasteurii* strain. This due to strontium ions pose less toxicity to the bacteria even at high concentrations and the metal cations easily incorporate into calcium carbonate lattice by replacing calcium ions (Fujita *et al.* 2004; Fujita *et al.* 2010; Achal *et al.* 2012b; Lauchnor *et al.* 2013; Newsome *et al.* 2014). Contrary zinc ions (section 6.3.3) have two effects on

heavy metals biomineralisation with calcium carbonate precipitation processes, very toxic to *S. pasteurii* strain even at low concentration. Cadmium ions showed good response to be precipitated or co-precipitated with calcium carbonate induced by *S. pasteurii* strain. The rest of this chapter presents and discusses the bioprecipitation experiments of calcium and cadmium only. These experiments included monitoring bioprecipitation processes in dynamic system (with fluid flow) included sand columns and peristaltic pumps to inject solutions into these columns by using pulsed injection strategy. This is important to investigate the bacteria ability to maintain urea hydrolysis when the cells are decreased in their numbers due to act as nucleation sites for calcium carbonate formation, and this in turn can lead to the bacteria becoming embedded within the solid matrix which is likely to kill cells by prevent access to nutrients and energy sources (Tobler *et al.* 2011).

The rate, magnitude and direction of seepage of fluid flow in porous media is important in maintaining bioprecipitation processes in subsurface environment. Transportation of nutrients and chemicals that are essential for biomineralisation processes in porous media is controlled by physical and chemical properties of soil. The permeability is the most important physical property of soil which governs the materials transmit with fluid flow. A section of soil can consider homogeneous when the permeability between two points is constant, whilst that section is considered heterogeneous when the permeability is different between that two points. Generally, flow in homogenous porous media is faster than that in heterogeneous porous media (Vadasz 1993; Alabi 2011).

The effect of homogeneity and heterogeneity of subsurface media on bioprecipitation processes was investigated in this part of study. Spatial distribution

of hydraulic conductivity may control groundwater flow and direction which in turn affects solute transport. In homogenous experiment same amounts of solutions containing calcium carbonate precipitation medium were injected into fine, medium and coarse sand columns (see Figure 3.3, Chapter: 3 for more details). In heterogeneous experiment fine, medium and coarse sand columns were connected to one inlet supplies all columns (see Figure 3.4, Chapter 3 for more details). Permeability of sand fractions controls solutions amount that received by each sand column.

6.4.1 Homogeneous Fluid Flow Experiments

The ability of *Sporosarcina pasteurii* bacteria to induce the precipitation of calcium carbonate and co-precipitation of cadmium ions in porous media has been explored in a dynamic system involved homogenous fluid flow of precipitation media into sand columns already containing bacterial cells. To try to mimic the nature, bacterial cells suspension (bacteria and PBS) was mixed with sand fractions in columns before pumping any solutions. When cells adsorbed to sand grains this help in minimising cells flushing out of columns with liquids flow, cells were left in sand for 2 hours before injection of any precipitation medium. In addition, the ability of *S. pasteurii* to tolerate cadmium ions toxicity during their presence in porous media was investigated by aging bacterial cells with cadmium ions in sand columns for 24 hours before injecting any growth or precipitation media.

First experiment of this section examined the precipitation processes of calcium carbonate without using cadmium, which is important to study bacteria behaviour in inducing urea hydrolysis in absence of cadmium toxicity effect to be compared then with bacteria response in presence of toxic ions. Then the response of *S. pasteurii* cells

to precipitate cadmium ions without aging with metal ions (inject precipitation medium amended with cadmium ions into sand columns) was studied before testing the effect of aging with metal ions on bacterial activity. Experiment system is shown in Figure 3.3, Chapter 3 for more details.

6.4.1.1 Calcium bioprecipitation without Heavy Metal (Homogenous Dynamic System)

In this experiment, laboratory-scale porous media flow cells we used to examine the potential *S. pasteurii* initiate urea hydrolysis and then calcium carbonate precipitation in sand columns. Calcium carbonate medium was injected for 10 days, whilst in static experiment this medium was mixed with sand fractions. This is to investigate the continuous formation of calcium carbonate precipitates in porous media effects on reducing hydraulic conductivity and then delivering nutrients, calcium and urea to bacterial cells in sand porous media.

Figure 6.12 shows activity of *S. pasteurii* in soil porous media to precipitate calcium carbonate in fine, medium and coarse sand fractions, calcium carbonate medium (*M2*) was injected every 2 days for 10 days. The indications of bacterial activity were pH and calcium ion concentration in extracted solutions after each injection. Calcium ion concentration was zero in all extracted solutions which means full precipitate of calcium in sand porous media. In fine and medium sand specimens values of pH were almost identical over test period while coarse sand pH increased with time. After 48 hours pH values reached up to 9.3 in fine and medium sand fractions, whilst it did not increase more than 8.4 in coarse sand fraction. Coarse sand samples needed 10 days to reach approximately same pH values as other sand fractions. Variation in bacterial activity occurred in sand samples, though same

amount of bacterial suspension was added to each sand fraction. It is possibly that bacterial cells were reduced after first injection of calcium carbonate medium in coarse sand columns, which is may relate to different hydraulic conductivity for each sand fraction (see Table 3.5, Chapter 3 for more details). It is possible that coarse sand fraction has less ability to retain bacterial cells than other sand fractions (Fontes *et al.* 1991), which led to flush out significant amounts of bacterial cells and hence, bacterial activity was reduced.

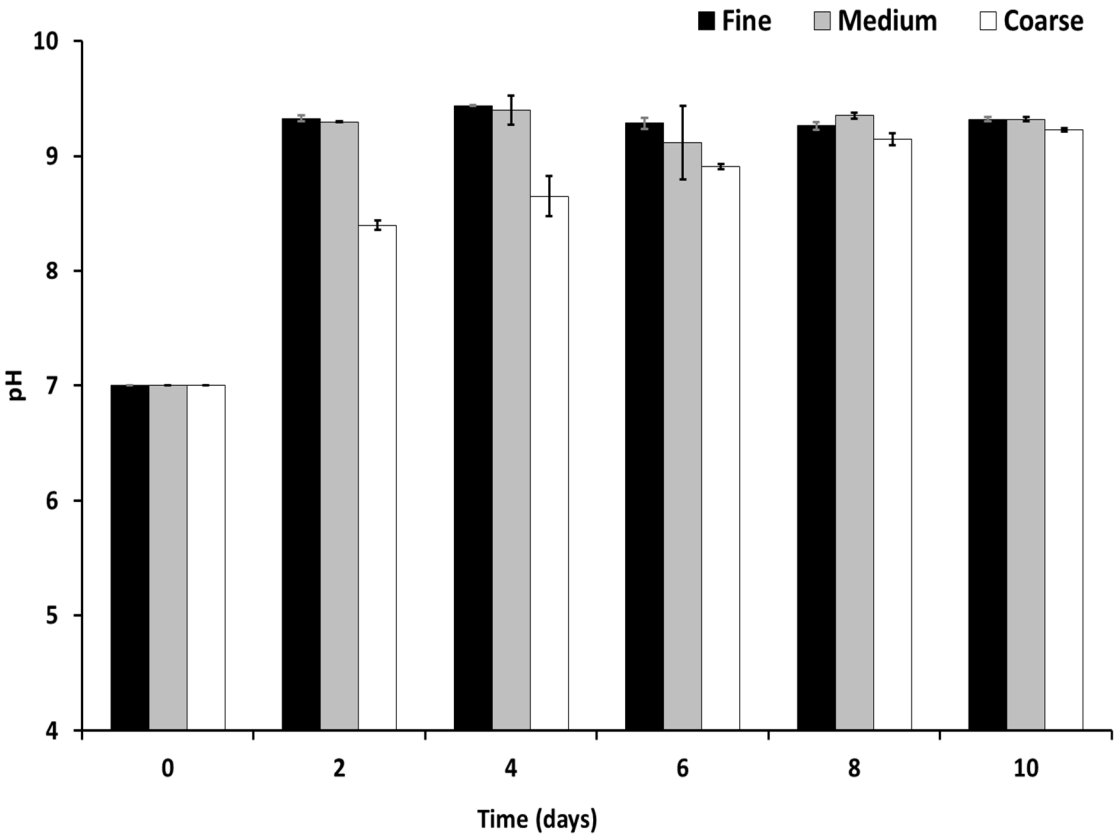


Figure 6.12. Response of pH to activity of S. pasteurii in soil porous media fluid of calcium carbonate precipitation in fine, medium and coarse sand fractions, calcium carbonate medium (M2) was injected every two days for 10 days, at 30ºC. [Error bars represent: ±1SD, n=3].

6.4.1.2 Cadmium Bioprecipitation (Homogenous Dynamic System)

Two experiments were conducted in this section to demonstrate how cadmium toxicity can affect *S. pasteurii* growth and operation to precipitate metal in porous media, and whether ground conditions can affect bacteria tolerability to metal toxicity. In first experiment precipitation medium was amended with cadmium ions before injecting through sand columns, whilst, in second one cadmium ions were injected into sand columns to explore bacteria ability to tolerate metal toxicity during their presence in porous media. In second experiment precipitation medium was pumped through sand columns after 24 hours.

6.4.1.2.1 Without Aging Bacterial cells with Cadmium Ions

In this experiment precipitation medium (*M2*) was amended with 20 mM cadmium concentration (final concentration) before injecting into sand columns of test samples. Whilst medium *M3* (urea-free medium) was also amended with 20 mM cadmium concentration and injected into control samples. Cadmium injection was only at day-0, in the following days (1-6), only *M2* (for test sampled) and *M3* (for control samples) were injected into sand columns without any cadmium ions. Using high concentration of the metal is based on bacteria tolerability to 10 mM concentration in static experiment (section 6.3.4). The results of this experiment are shown in Figure 6.13.

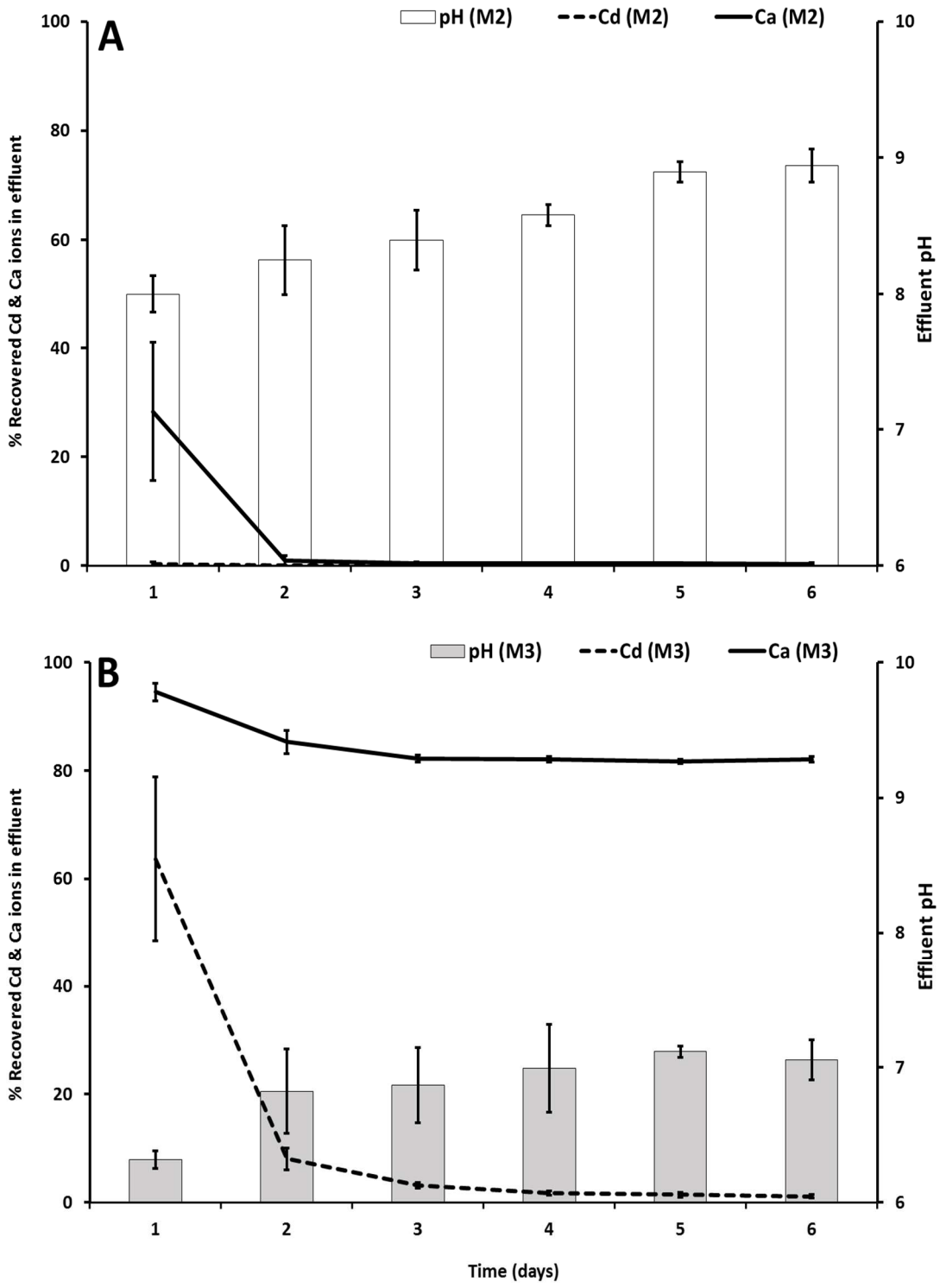


Figure 6.13. Response of cadmium, calcium and pH to activity of S. pasteurii in soil porous media in medium sand fractions, with urea growth medium, M2(A) and controls M3(B) (without urea growth medium), at 30ºC. M2 and M3 media were injected every two days for 10 days, at 30ºC. [Error bars represent: ±1SD, n=3].

In urea-treated samples pH increased gradually up to 9 after day-6, which is probably due to *S. pasteurii* cells activity in hydrolysing urea. Removing of cadmium ions from solutions at day-1 may relate to precipitation with calcium carbonate, though the role of adsorption mechanism that may responsible for metal ions loss as well. Comparing to precipitation without heavy metal experiment (Figure 6.12) where pH in medium sand fractions increased up to 9.2 after first injection of precipitation media, whilst in this test pH was 8.3 in day-2. Such variation in response may reflect the role of cadmium ions presence in delaying bacterial activity. Suggesting that adsorption of cadmium ions on sand grains from day-1 may allowed bacterial cells to operate in less-toxic environment. Daily pH increase over test period is an indicator of bacterial cells were utilising daily injected growth medium to increases their number and consequent activity. This activity led to full removal of calcium ions from solution probably by precipitation as calcium carbonate.

In control samples pH decreased from 7 to 6.3 at day-1, this may attribute to acidity effect of Cadmium Sulphate ($3CdSO_4.8H_2O$), MINTEQ model predicted pH value of 6.3 when dissolving 20 mM of cadmium and 50 mM calcium chloride in water. Increase pH for the next days may due to daily injection of *M3* medium (included nutrient broth with pH of 7.4) or sand ability of buffering pH. Recovering of 64 % of cadmium ions at day-1 in urea-free samples may down to pH decrease, which may have led to increase metal availability. In cadmium bioprecipitation experiment by static system (Figure 6.11) cadmium amount that recovered from control samples (urea-free) of medium sand fraction was 63 %. Hence, cadmium ions showed almost similar affinity to adsorb on medium sand fraction under static and dynamic conditions. After day-6 total recovered amount of cadmium ions was 79 % of that injected at day-0. This suggests that flushing sand columns with working

solutions may help cadmium ions desorption from sand grains especially if pH value is less than 7. High recovery of calcium ions from control samples was noticed daily over test period.

XRF (x-ray fluorescence) test showed cadmium (345.3 ± 3.3 ppm) and calcium (5223 ± 69 ppm) were retained in sand samples that treated with *M2* (urea-treated medium), whilst no presence of cadmium and few amount of calcium were discovered in control samples. Adsorption and precipitations mechanisms can lead to retain metal and calcium cations in sand porous media, but probably precipitation with calcium carbonate is the mechanism is more responsible for cadmium and calcium ions retaining in sand porous media.

Same amounts of cadmium and calcium were injected into control samples but because no precipitation occurred only small amount cadmium and calcium ions were held in sand samples. In addition, adsorption mechanism did not lead to retain metal and calcium ions on sand grains, though Mustafa *et al.* (2004) refer to that cadmium ions can be taken up on adsorption sites from pH value of 5 (control samples pH was around 7). This may be due to daily liquids injection into sand columns led to flush out the cadmium and calcium ions, however, calcium ions showed poor affinity to be adsorbed on sand grains in previous experiments.

X-ray diffraction (XRD) patterns were used to illuminate the phase of the synthesised calcium carbonate (ran for 45 minutes for each sample). XRD analysis showed that all calcium carbonate crystals, that induced by *S. pasteurii*, in tested samples are calcite and aragonite (Figure 6.14). XRD for untreated sand samples showed no presence of these crystals. Such finding leads to suggest that crystalline polymorphs of calcium carbonate were induced by the activity of *S. pasteurii* strain.

Polymorphs of calcium carbonate produced in this study are consistent with many studies (Rivadeneyra *et al.* 1996; Stocks-Fischer *et al.* 1999; Fujita *et al.* 2000; Fujita *et al.* 2004; Mitchell and Ferris 2005; Fujita *et al.* 2008; Chahal *et al.* 2011; Abo-El-Enein *et al.* 2012; Achal *et al.* 2012a; Achal *et al.* 2012b; Vahabi *et al.* 2015; Wei *et al.* 2015).

Figure 6.15 illustrates the SEM and EDX spectrums of calcium carbonate precipitation that resulted from bioprecipitation processes of 20 mM cadmium concentrations. Spectrum 2 (taken inside the precipitate) shows clearly the presence of calcium, whilst a spectrum 1 that was taken outside the precipitates shows no presence of calcium only the sand elements. However, no cadmium peaks were emerged in EDX spectrum, probably because beam energy that used to focus X-ray on samples, was up to 20 KeV, whiles cadmium needs more than 23 KeV.

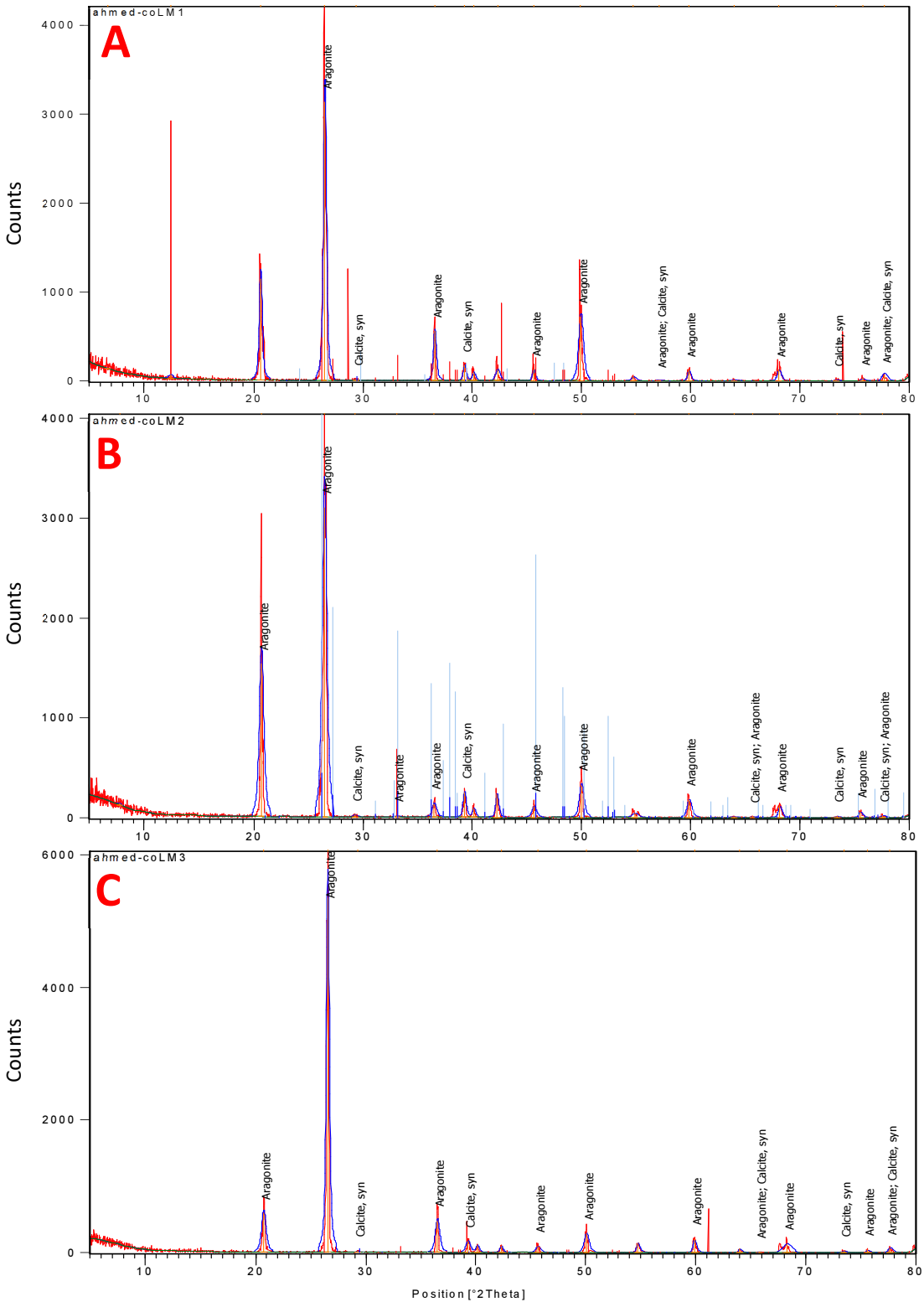


Figure 6.14. X-ray diffraction patterns of medium sand samples after cadmium bioprecipitation (20 mM) processes in homogeneous dynamic experiment without aging cadmium ions with bacterial cells. Samples treated with M2 (urea-containing medium) A, B and C represent samples from different columns. Test shows presence of Aragonite and calcite precipitations.

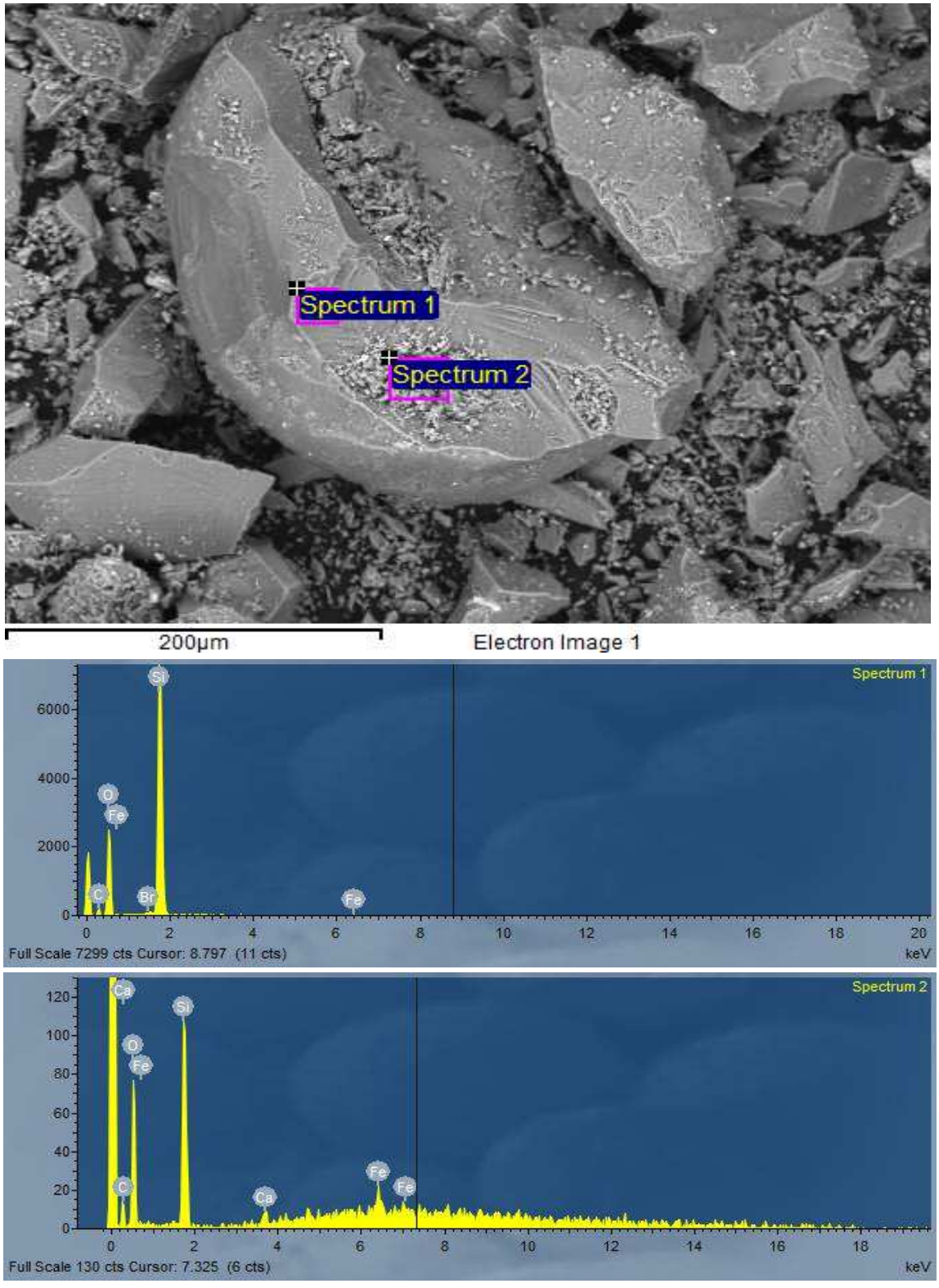


Figure 6.15. SEM and EDX spectrum of calcium carbonate precipitation on sand particles induced by S. pasteurii in presence of cadmium ions (20 mM) by using urea-containing medium (homogenous dynamic system experiment). Spectrum 1 and 2 represent the spectrum that taken outside and inside the aggregates, respectively.

6.4.1.2.2 Ageing Bacterial cells with Cadmium Ions

The ability of *S. pasteurii* cells to tolerate cadmium ions during their presence in porous media was explored in this part of the study. This is to investigate if ground condition can affect bacteria ability to resist heavy metals. Microorganisms and heavy metals transport and distribution in porous media may be affected by physical properties of soil. Bacterial cells in this experiment were left with cadmium ions for 24 hours in sand columns without supplying any nutrient or energy sources. Bacterial cells mixed with sand fractions (fine, medium and coarse) by using only PBS, then a solution (55 ml) containing 0.03 mM final concentration of cadmium was injected into each sand column. This concentration is MIC that found in cadmium toxicity experiment (Chapter 3, Figure 4.3), also, choosing this concentration is based on preliminary experiments, where bacteria could not tolerate higher concentration under similar conditions. Aging *S. pasteurii* cells with toxic metal can give an indication of the extent that bacterial activity may affected by toxic ions. However, calcium carbonate precipitation medium was injected into sand columns after 24 hours to investigate bacterial cells ability to hydrolyse urea and induce calcium carbonate precipitation in porous media.

Figure 6.16 presents the results of this experiment. Generally different response can be seen of bacterial activity in the three sand fractions, although medium and coarse sand fraction almost have same response. Very small proportion (1 %) of cadmium ions were recovered in the effluent, metal ions either adsorbed on sand grains or precipitated calcium minerals. Mustafa *et al.* (2004) reported that at low cadmium concentration its affinity increase to adsorption sites on sand grains with pH increase and overcome the competition from calcium ions. The small added amount of cations may readily have absorbed on sand grains.

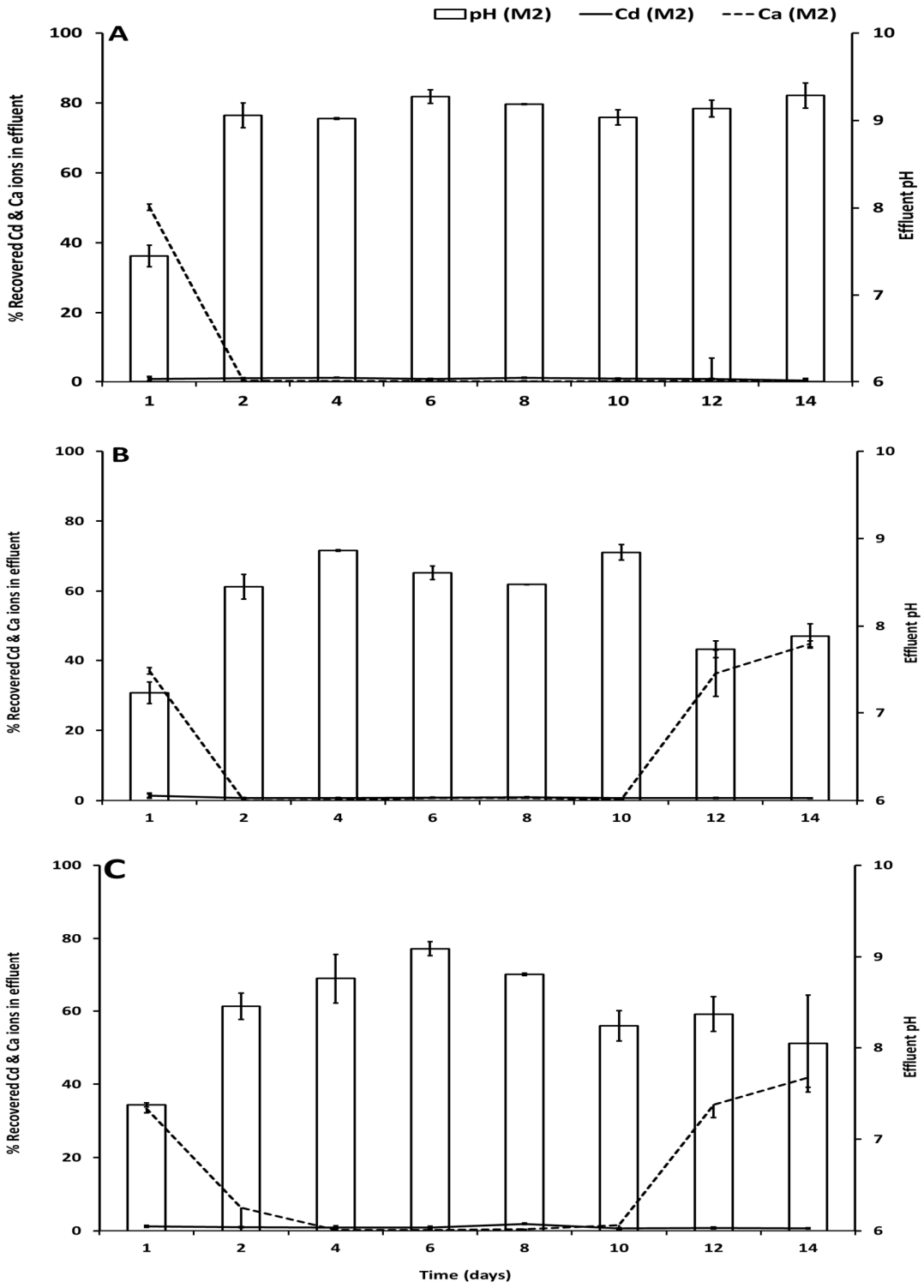


Figure 6.16. Response of cadmium (0.03 mM), calcium and pH to activity of S. pasteurii in porous media in fine (A), medium (B) and coarse (C) sand fractions, after aging bacterial cells with cadmium ions for 24 hours. Urea growth medium (M2) was injected from day-1 to day-14, at 30ºC. [Error bars represent: ±1SD].

The amount of recovered calcium ions was well correlated with pH of solutions, where the latter depends on bacterial activity in hydrolysing available urea. Calcium ions removal occurs rapidly after day-1 in conjunction with pH increase indicating precipitation of calcium carbonate. Calcium removal and pH increase are indicators for bacterial cells activity, whilst calcium ions show low affinity to adsorption sites on sand grains. Bacterial cells effectiveness by presence of cadmium ions in porous media seems not to the same degree in fine, medium or coarse sand fractions. *S. pasteurii* activity depends on survival cells number, survival cells number in fine sand fraction seems higher than that in other fractions, where pH value in fine sand samples reached 9 at day-2 and maintained high pH value (up to 9.3) over experiment time. This may attribute to high adsorption capacity of fine sand particles allowed bacterial cells to hydrolyse urea without affecting by metal toxicity. In medium and coarse sand specimens pH values after injection of *M2* medium were unstable. Suggesting that bacterial activity was affected by aging with cadmium ions in porous media, or presence of bacterial cells in sand fractions coarser than fine sand facilitate their flushing out with injected liquids which in turn led to reduce their number and activity. In the previous experiment (Figure 6.13) cadmium ions were injected with precipitation medium bacteria showed high activity in removing metal ions. Hence, the presence of precipitation medium allowed bacterial cells to operate at higher performance. This reflects the role of urea hydrolysis mechanism in protecting bacterial cells from metal toxicity.

Pore size and grain size may also have affected bacterial cells attach to sand particles and their transport in porous media. Bacterial cells transport in porous media may increase in large particles medium due to lower specific surface area and higher hydraulic conductivity of the coarse grained porous medium. Also occurrence of

preferential flow in soils may enhance bacterial cells transport in porous media, sand with greater permeability causes a preferential flow path for solute transport in soils (Gerlach 2001; Al-Abed and Chen 2002; Kapetas *et al.* 2012). Accordingly, *S. pasteurii* cells in fine sand may subjected to less influence regarding flushing out with injected liquids which may led to keep their original number or abled to increase it. Whilst in medium and coarse sand samples the bacterial activity was affected by either cadmium toxicity or flushing out with extracted solutions or both.

6.4.2 Flow in Heterogeneous Porous Media Experiments

In this part of study, the effect of heterogeneous hydraulic conductivity on bioprecipitation processes was investigated. Soil physically, chemically and biologically is heterogeneous. Spatial heterogeneity is an intrinsic property of subsurface environment; liquids flow is affected by spatial changes in porous media. Ground water is the carrier of bacteria, chemicals, nutrients and contaminations in soil. The differentiation in hydraulic conductivity in soil may lead to find different concentrations of biomass, substrates and pollutants within soil matrices. Porous media are rarely homogeneous, liquids transport in porous media varies spatially and temporally. Heterogeneity variation in porous media can have significant influence on liquids transport processes within soil. In case of biomineralisation processes such effect leads to uneven distribution of calcium carbonate precipitation. Information of solute transport in heterogeneous porous media is vital to monitor pollutant fate and transport in subsurface environment. Such influence can be estimated from hydraulic conductivity changes over time in soil sections (Zhao and Toksoz 1994; Leij and Van Genuchten 1995; Simmons *et al.* 2001; Wu *et al.* 2014; Majdalani *et al.* 2015). Using microbial induced calcium carbonate technique to remediate a contaminated subsurface environment, may change the original degree of heterogeneity of that

environment. Resulted minerals may segregate many regions by clogging porous throats which in turn leads to change the direction of liquids flow and hydraulic conductivity values for treated soil.

This experiment was designed to mimic, to some extent, a heterogeneous permeability section of soil. Coarse fraction sand allows solutions to flow faster than other fractions due to its relative high hydraulic conductivity. Hydraulic conductivity may decrease due to precipitation of calcium carbonate minerals in porous media. This decreasing enforces solutions to divert their paths to less resistance regions. Arising preferential flow paths (due to calcium carbonate precipitation in porous media over time) may result to uneven distribution of injected liquids along the sand column.

The system of this experiment involved 27 sand columns divided in 3 groups (*G1*, *G2* and *G3*), each group included 3 sets, each set contained 3 sand columns (fine, medium and coarse sand fractions). All groups prepared in the same fashion. Group 1 (*G1*) contained 9 sand columns (triplicates of fine, medium and coarse sand fractions) represented control (no cadmium ions used) to investigate the behaviour of precipitation processes in Heterogeneous porous media without metal toxicity. Group 2 (*G2*) (test samples) prepared in the same fashion of *G1* but 0.03 mM cadmium concentration was injected 24 hours before pumping calcium carbonate medium. Group 3 (*G3*) represent another control to investigate the flow of liquids into sand columns without calcium carbonate precipitation influence (no urea was injected into this group columns), this group involved injecting 0.03 mM cadmium concentration at the same time with *G2*. All three columns in one set received liquids through a single inlet, represent layered heterogeneity (see Figure 3.4, Chapter: 3 for more

details). For each set 1½ pore volume (165 mL) was injected into a single inlet of the three columns.

6.4.2.1 Hydraulic Conductivity Effect

Figure 6.17 presents the average of outflow volumes from each test group, outflow volumes controlled by hydraulic conductivity of sand fractions. Initial calculated hydraulic conductivity values for sand fraction before treatment were 7.3×10^{-5}, 3.0×10^{-4} and 4.8×10^{-4} m/s for fine, medium and coarse sand fractions, respectively. It seems that injecting working solutions into sand columns led to change initial hydraulic conductivity values. Liquids started to flow in coarse sand columns (higher hydraulic conductivity) then with time flow headed to medium and fine sand columns. Such effects happened in urea-treated samples (groups *G1* and *G2*), which is possible due to decreasing occurred in coarse sand permeability because the bacterial activity in inducing calcium carbonate precipitation.

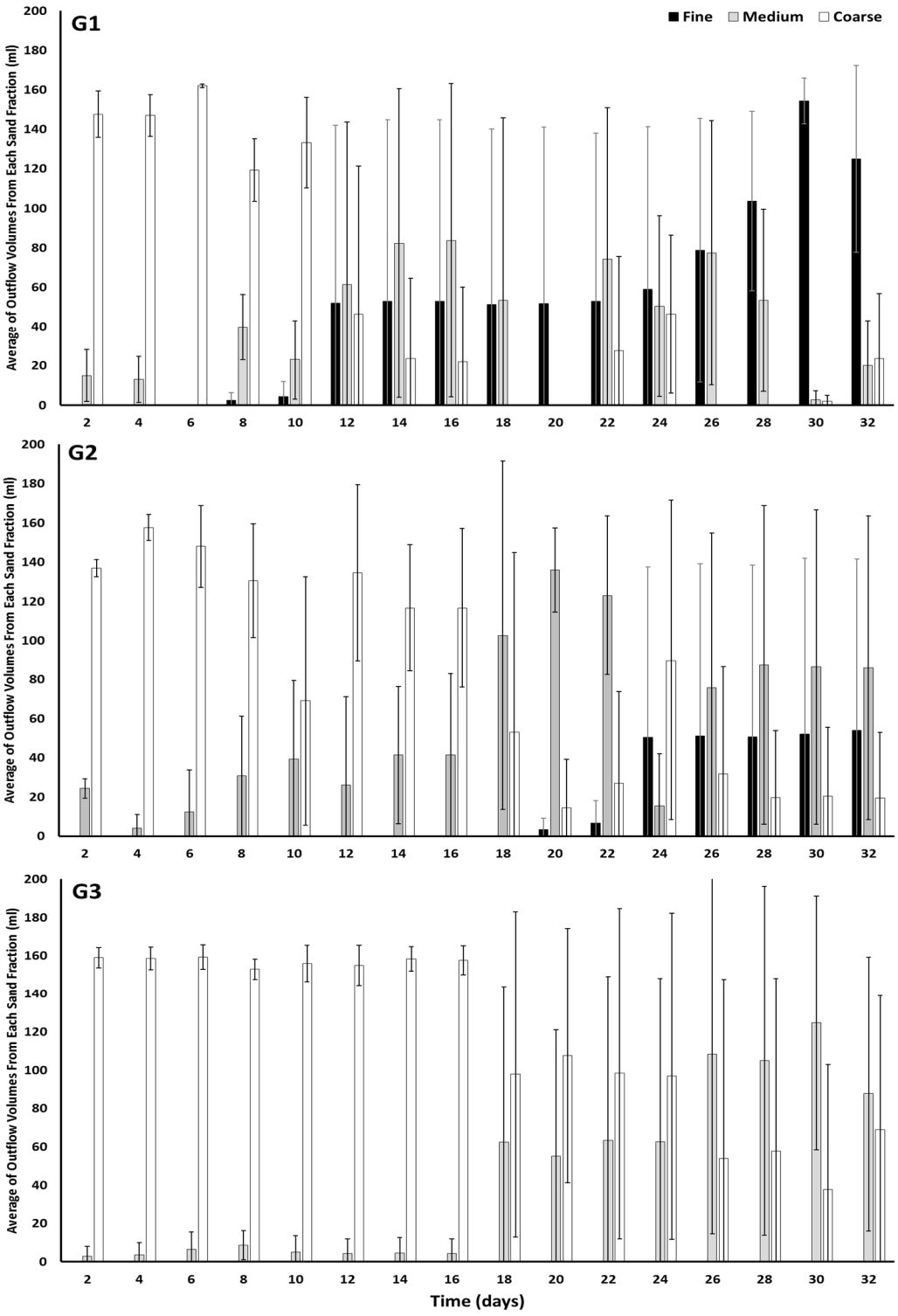


Figure 6.17. Average of outflow volumes (in milliliters) from each sand fraction (fine, medium and coarse) in each group (triplicates). G1 (urea-containing and free of cadmium medium), G2 (urea-containing with cadmium medium) and G3 (urea-free medium with cadmium medium). At 30ºC. [Error bars represent: ±1SD, n=3].

In group *G1*, where no cadmium ions used only calcium carbonate precipitation medium was used, liquids headed to coarse sand samples in quantities much higher than these flowed in medium sand fractions. Probably due to decreasing occurred in permeability of coarse sand from day-8 solutions diverted their direction to medium and fine sand fractions, though in small quantities at days 8 and 10. Which implies hydraulic conductivity in coarse sand had reached a value less than medium sand fraction. This decreasing in hydraulic conductivity probably because occurrence of calcium carbonate precipitation in porous media led to clog flow paths. At days-12, 14 and 16 (Figure 6.17, *G1*) injected liquids flowed in fine, medium and coarse sand fractions at the same time, this may due to the variation in hydraulic conductivity values was small. It is possibly that calcium carbonate precipitation increased with time in porous media and injected liquids diverted their flow wherever permeability allowed them to flow in sand columns. At days 30 and 32 the majority of solutions were running in fine sand specimens. However, from day-18 to day-28 coarse sand samples gave no outflow solutions, small quantities observed in days 30 and 32.

In group *G2* same effect of hydraulic conductivity happened but the diversion of flow from coarse sand to other fractions took longer time than group *G1*. This implies the decreasing in permeability of coarse sand was slow, where required 20 days before flow diverted to fine sand fraction. Suggesting that cadmium ions may cause delayed in bacterial activity, however, bacterial cells finally seem resisted cadmium toxicity and induced calcium carbonate precipitation in porous media. As can be noticed from Figure 6.17, *G2* flow diverted to medium and fine sand specimens after decreasing occurred in coarse sand hydraulic conductivity.

The injected media into sand columns in group *G3* contained only broth, cadmium (0.03 mM) and calcium (50 mM). The majority of liquids were running in coarse sand columns up to day-18. In spite of no calcium carbonate precipitation expected in this group (no urea injected) but the permeability of coarse sand decreased to allow solution diverting to medium sand columns. Suggesting that continuous injection and accumulation of broth and calcium ions into coarse sand fraction may led to decrease coarse sand hydraulic conductivity. Also bacterial cells may utilised injected broth to form biofilm in porous media which led to decrease permeability.

6.4.2.2 *pH* and Concentrations of Calcium and Cadmium

Bacterial activity in porous media after 24 hours aging with cadmium ions in porous media was investigated by measuring pH and cadmium and calcium concentrations for outflow solutions. Figure 6.18 shows pH values for outflow solution in each test group. High pH values in groups *G1* and *G2* reflect bacterial activity in hydrolysing urea in porous media, whilst pH values in group *G3* are expected as no urea was added in this group. Generally, according to pH values bacterial activity in *G1* (without metal toxicity effect) was higher than *G2* (with metal toxicity effect). Such preference may because the presence of cadmium ions with bacterial cells in porous media.

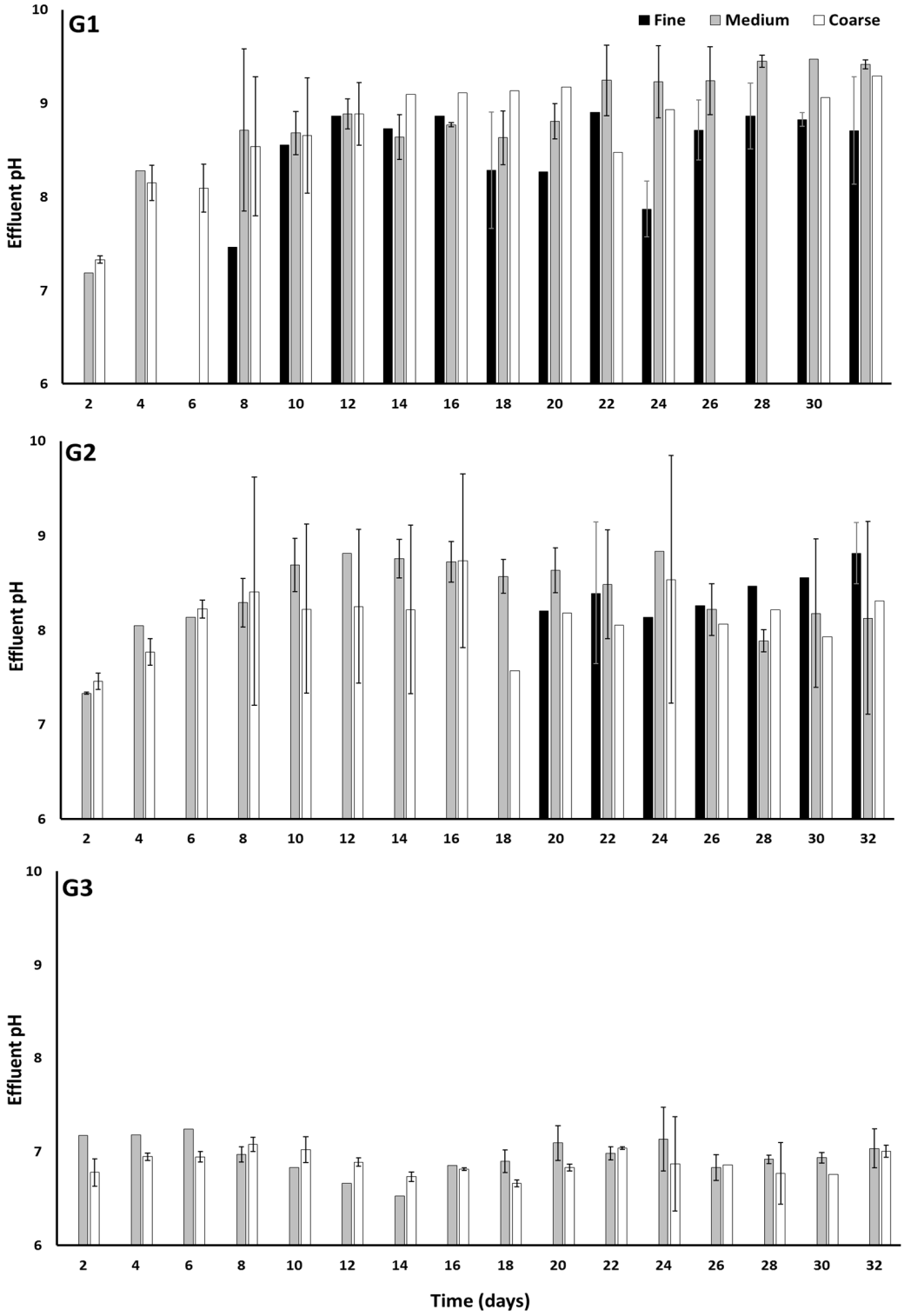


Figure 6.18. Values of pH for outflow solutions from each sand fraction (fine, medium and coarse) in each group (triplicates). G1 (urea-containing and free of cadmium medium), G2 (urea-containing with cadmium medium) and G3 (urea-free medium with cadmium medium). At 30ºC. [Error bars represent: ±1SD, n=3].

In group *G1*, where no heavy metals added, it seems that bacterial cells were active in all sand columns and as soon as received calcium carbonate precipitation medium (*M2*) started to hydrolyse urea and increase pH (Figure 6.18, *G1*). *M2* medium diverted to fine sand columns from day-8 (Figure 6.17), at day-10 pH increased from 7.5 to 8.5. In spite of flow (carries precipitation medium) diverted to fine sand columns in group *G2* from day-20 to the end of the test, bacterial cells seem were not totally inhibited by aging with cadmium ions and managed to hydrolyse urea and increase pH in porous media. Such response continued to the end of experiment. Bacterial activity in other sand fractions also showed same response in hydrolysing urea and increase pH. However, pH values decreased at the last week of experiment time, especially in medium and coarse sand samples. This decreasing may attribute to bacterial cells number had decreased due to aging with cadmium ions and may the survived cells then embedded by expecting calcium carbonate precipitation in porous media, which may have led to further reducing. Calcium ions were removed in high proportion from solutions (Figure 6.19, *G2*), this removal probably due to precipitation with calcium carbonate precipitation in porous media.

In group *G3* (heavy metals with urea-free medium) pH of medium and coarse sand samples were around 7. Such response was expected as no urea was injected. Little decreasing and increasing in pH values over experiment period may down to inject calcium chloride solution that can cause acidic effect can lead to decrease pH, this decreasing may then counteracted by sand buffering to increase pH. Medium and coarse sand fractions showed low affinity for calcium ions sorption especially pH did not increase above normal value, 90 % of calcium ions were recovered by outflow solutions (Figure 6.19, *G3*).

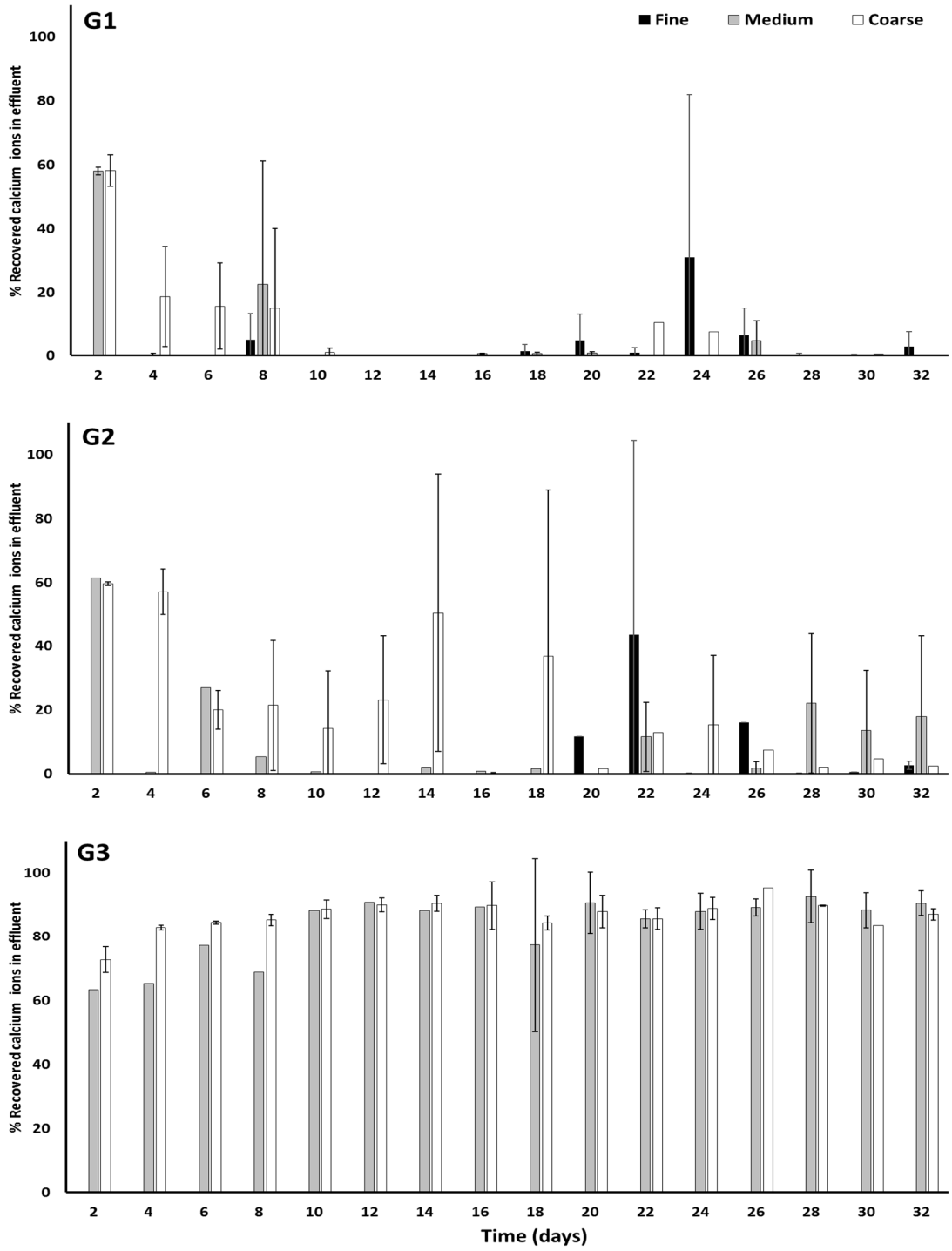


Figure 6.19. Percentages of calcium ions that recovered by outflow solutions from each sand fraction (fine, medium and coarse) in each group (triplicates). G1 (urea-containing and free of cadmium medium), G2 (urea-containing with cadmium medium) and G3 (urea-free medium with cadmium medium). At 30ºC. [Error bars represent: ±1SD, n=3].

In all test groups calcium removal was conjunction with pH increase in sand samples. Again because pH value of sand samples in group *G3* around 7 most of calcium ions were recovered. Contrary removal of calcium ions in groups *G1* and *G2* was a consequence of pH increase, this removal probably due to precipitate with calcium carbonate. However, cadmium recovery from groups *G2* and *G3* was not more than 1 %. Losing of cadmium ions may be due to the adsorption on sand grains, which can be happened at pH value from 5 (Mustafa *et al.* 2004).

6.4.2.3 XRF, XRD, SEM and EDX Analyses

Figure 6.20 presents the results obtained from XRF (x-ray fluorescence) test for fine, medium and coarse sand samples after treating with *M2* and *M3* media in heterogeneous dynamic experiment of cadmium bioprecipitation with calcium carbonate minerals. As it clear samples treated with urea-containing media (*G1* and *G2*) held significant amount of calcium than samples treated with urea-free medium (*G3*). Also, *G1* samples retained calcium ions more than *G2*, which may attribute to influence of cadmium ions in *G2*. Adsorption and precipitations mechanisms can lead to retain metal and calcium cations in sand porous media. In all the previous experiments calcium showed low affinity to be adsorbed on sand grains, suggesting that precipitation with calcium carbonate was the main mechanism that responsible for calcium ions retaining in sand porous media. XRF test did not show presence of cadmium ions in *G2* and *G3*, this may because small amount of the metal was used (0.03 mM or 3.4 mg/l). Same amounts of cadmium and calcium were injected into group *G3* samples but because no precipitation occurred only small amount calcium ions were held in sand samples. In addition, adsorption mechanism seems did not lead to retain calcium ions on sand grains, however, calcium ions showed poor affinity to be adsorbed on sand grains in previous experiments.

The amount of calcium in groups *G1* and *G2* (treated with urea-containing media) may give good conception of the calcium carbonate precipitations amount that occurred in each part (top, middle and bottom) of sand columns. Due to calcium carbonate precipitation in porous media changes occurred in permeability of sand fractions with time, played significant role in diverting flow from sand fraction to other. Generally, non-uniform distribution of calcium carbonate precipitations was found in each part (top, middle and bottom) of sand columns in groups *G1* and *G2*. Comparing Figures 6.19 and 6.20 can reveal that the amounts of calcium are decreased in extracted solutions due to retaining or precipitating in sand fractions (*G1* and *G2*), whilst high amount of calcium was recovered in *G3* samples because no calcium carbonate was enhanced to precipitate in this group.

Figure 6.21 illuminates the phase of the synthesised calcium carbonate in treated sand samples. XRD analysis showed that all calcium carbonate crystals, that induced by *S. pasteurii*, in tested samples are calcite and aragonite. However, XRD for untreated sand samples showed no presence of these crystals. Hence suggesting that crystalline polymorphs of calcium carbonate were induced by the activity of *S. pasteurii* strain. Li *et al.* (2013) reported that XRD results showed the presence of cadmium carbonate after bioprecipitation processes in aqueous solutions. The author did not use calcium in biomineralisation experiment (only urea and metal chloride).

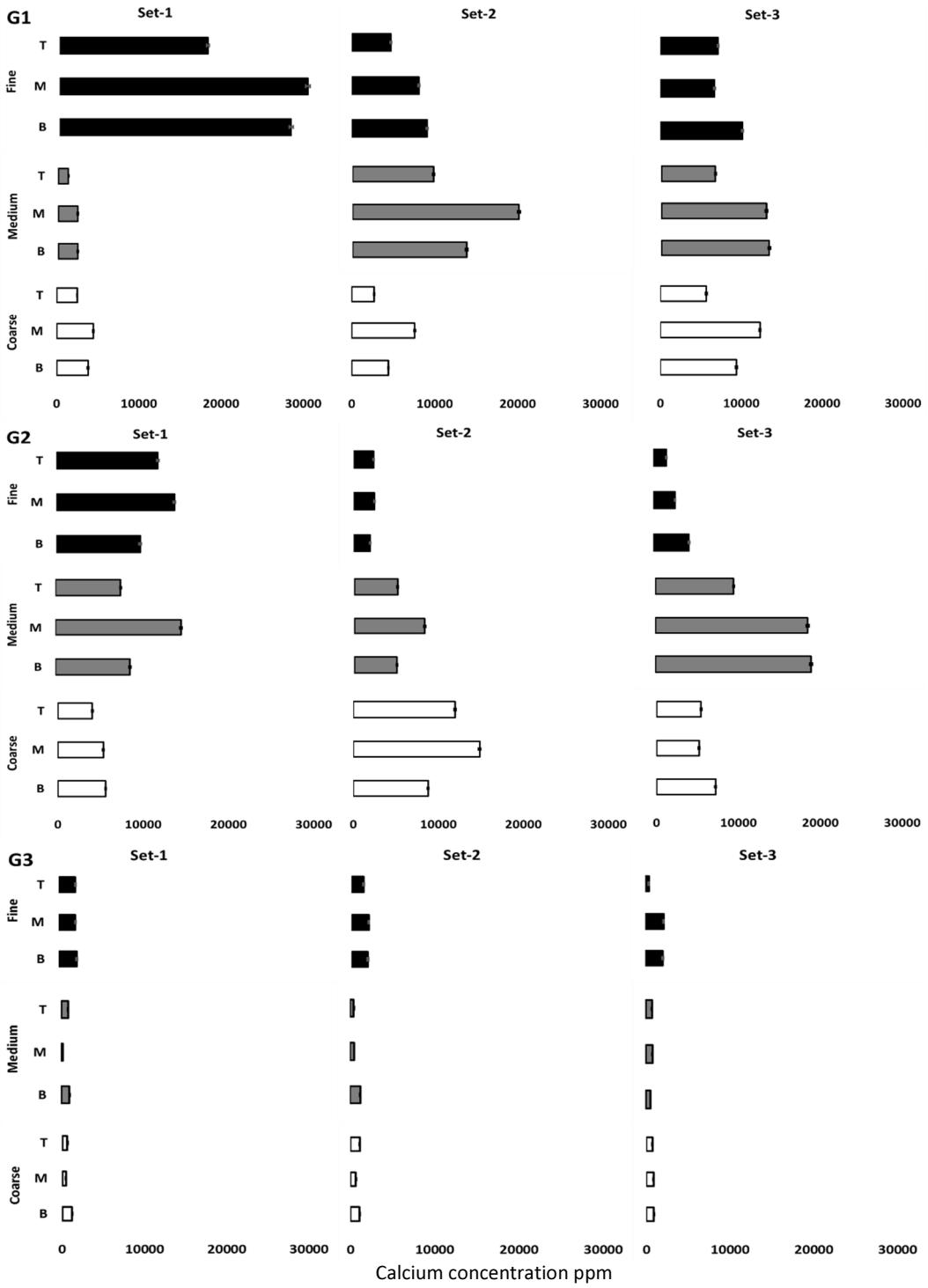


Figure 6.20. XRF test results of sand samples (fine, medium and coarse) represent the amount of calcium ions (ppm) that were retained in sand porous media of all fractions (where **T**, **M** *and* **B** *represent top, middle and bottom parts of sand columns, respectively). G1 (urea-containing medium and free of cadmium ions), G2 (urea-containing medium with cadmium ions) and G3 (urea-free medium with cadmium ions). At 30ºC. [Error bars represent: ±1SD].*

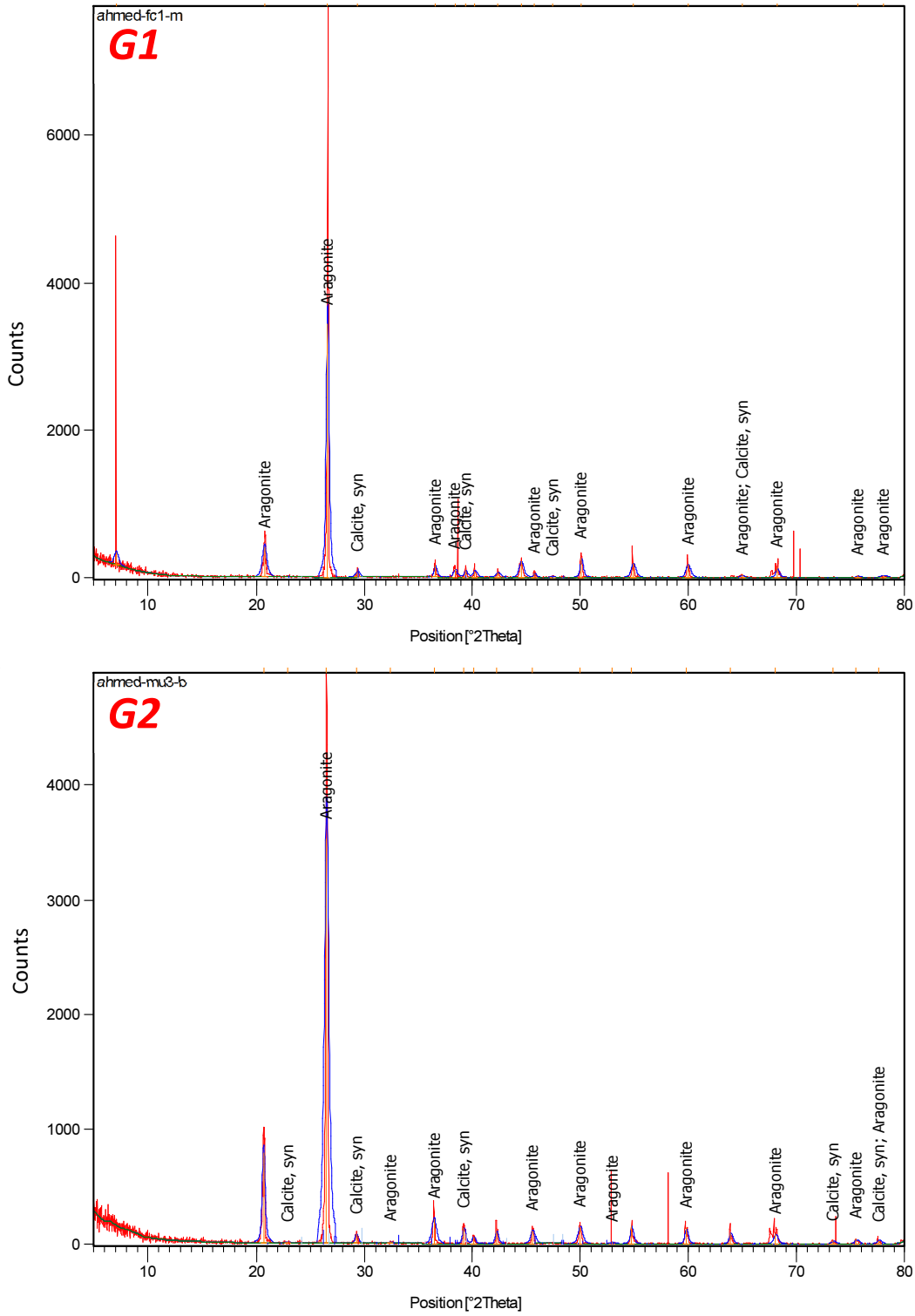


Figure 6.21. X-ray diffraction patterns of selected sand samples from G1 and G2 after bioprecipitation processes in heterogeneous dynamic experiment. Bacterial cells in G2 were aged with cadmium ions (0.03 mM) for 24 hours. Samples treated with M2 (urea-containing medium). Test shows presence of Aragonite and calcite precipitations.

Figure 6.22 and Figure 6.23 present images for selected sand samples from group *G1* and *G2*, respectively. Generally, can notice that crystals precipitated in *G1* (no cadmium ions were present) are almost similar in their shape (rhombohedral) and size (5-20 μm). Whilst the solids in *G2* (0.03 mM cadmium ions were present) were accompanied by smaller particles of precipitates.

Figure 6.24 shows the EDX spectrum of calcium carbonate precipitation on sand particles induced by *S. pasteurii*. It can be noticed that calcium peaks in spectrum taken in sand samples of groups *G1* and *G2*. However, no cadmium peaks were emerged in EDX spectrum, probably because beam energy that used to focus X-ray on samples, was up to 20 Kev, whiles cadmium needs more than 23 Kev.

Apart from cadmium, experiments in groups *G1* and *G2* were done in similar conditions, hence, producing crystals with different shape and size in *G1* and *G2* may lead to suggest that presence of cadmium ions may influenced the polymorph, shape and size of resulted crystals. Such effect does not appear to have previously been noticed.

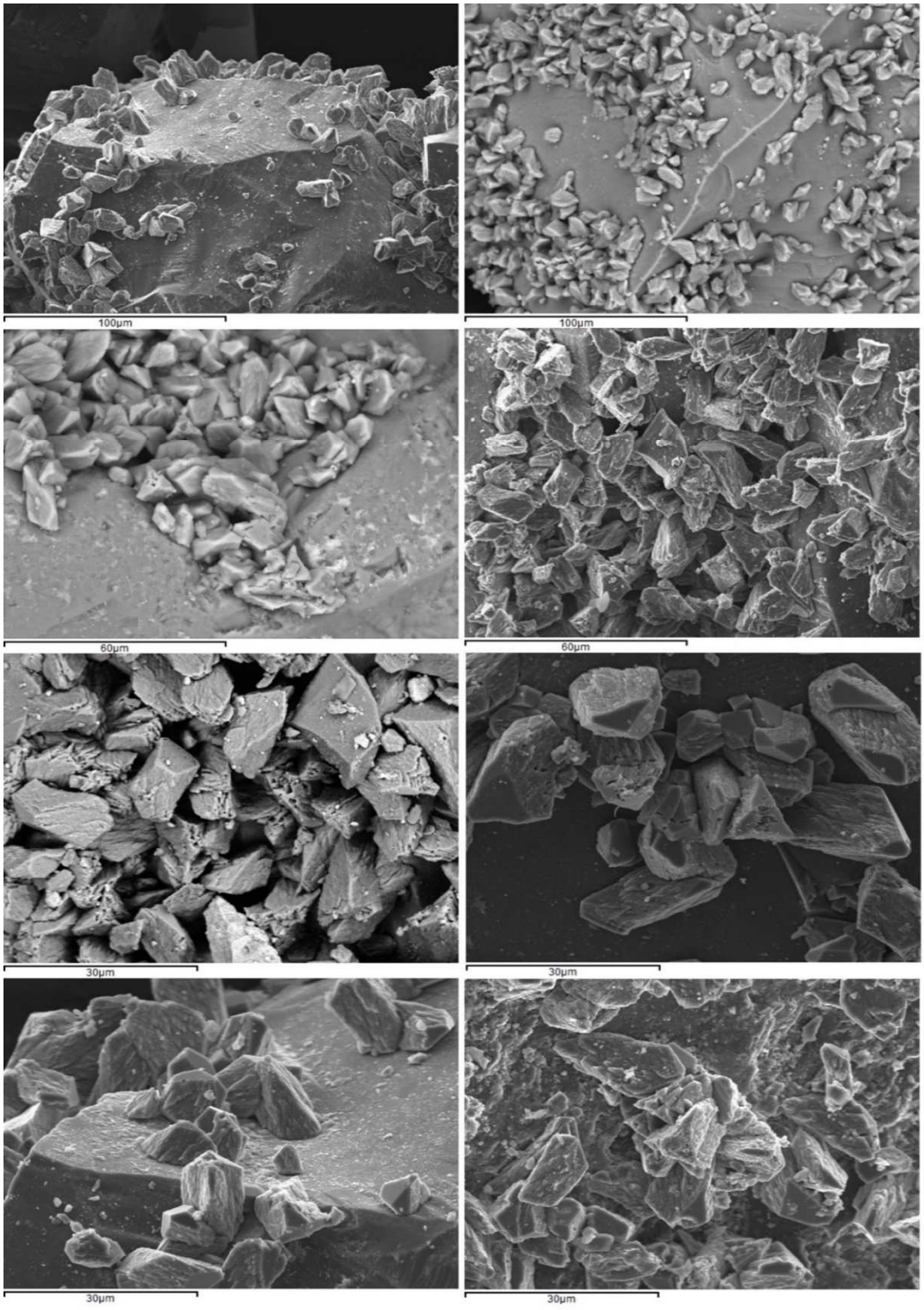


Figure 6.22. SEM test of calcium carbonate precipitation on sand particles induced by S. pasteurii by using urea-containing medium, where no cadmium ions were used in this test (heterogeneous dynamic system experiment). The images are for selected sand samples from group G1.

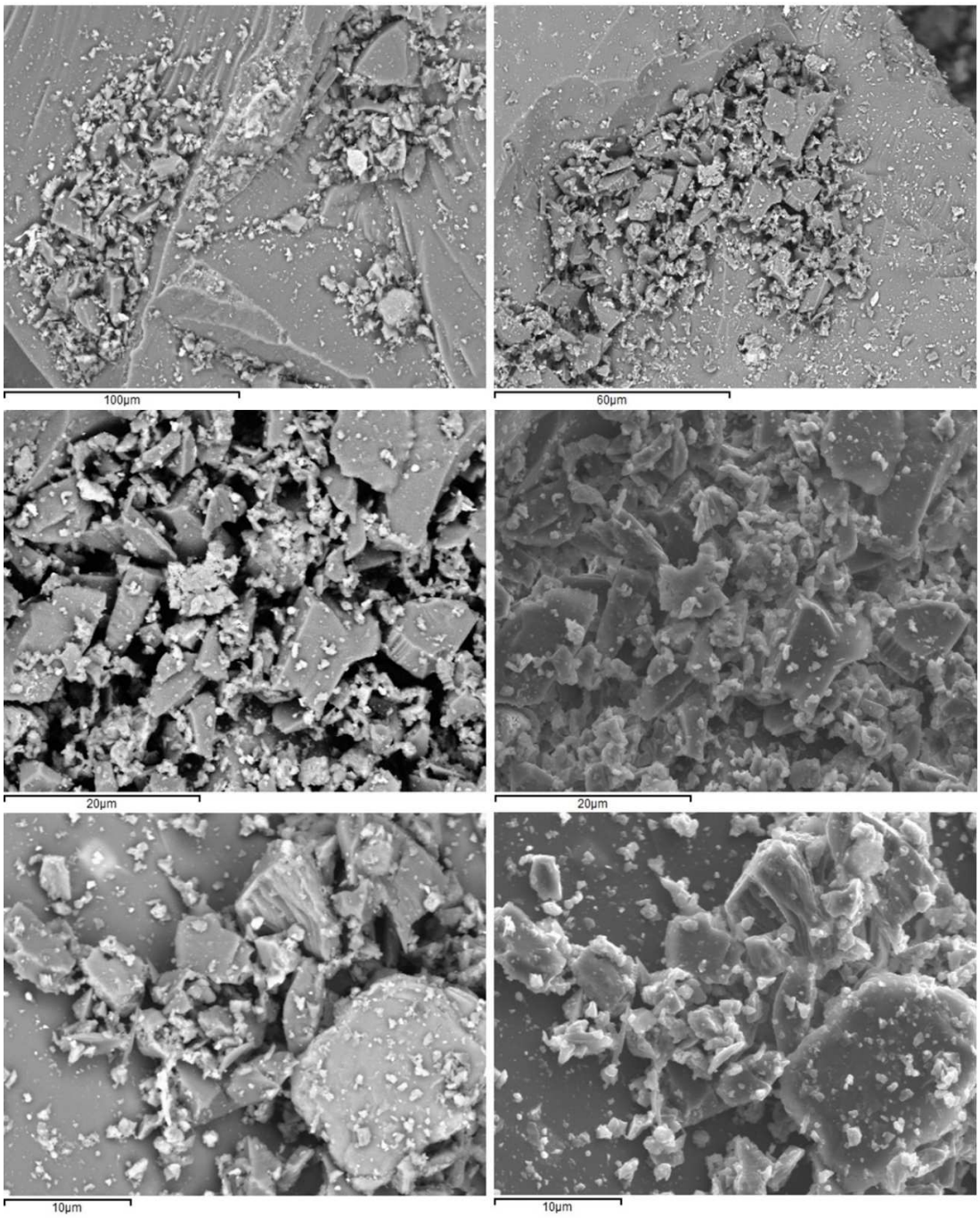


Figure 6.23. SEM of calcium carbonate precipitation on sand particles induced by S. pasteurii in presence of cadmium ions (0.03 mM) by using urea-containing medium (heterogeneous dynamic system experiment). The images are for selected sand samples from group G2.

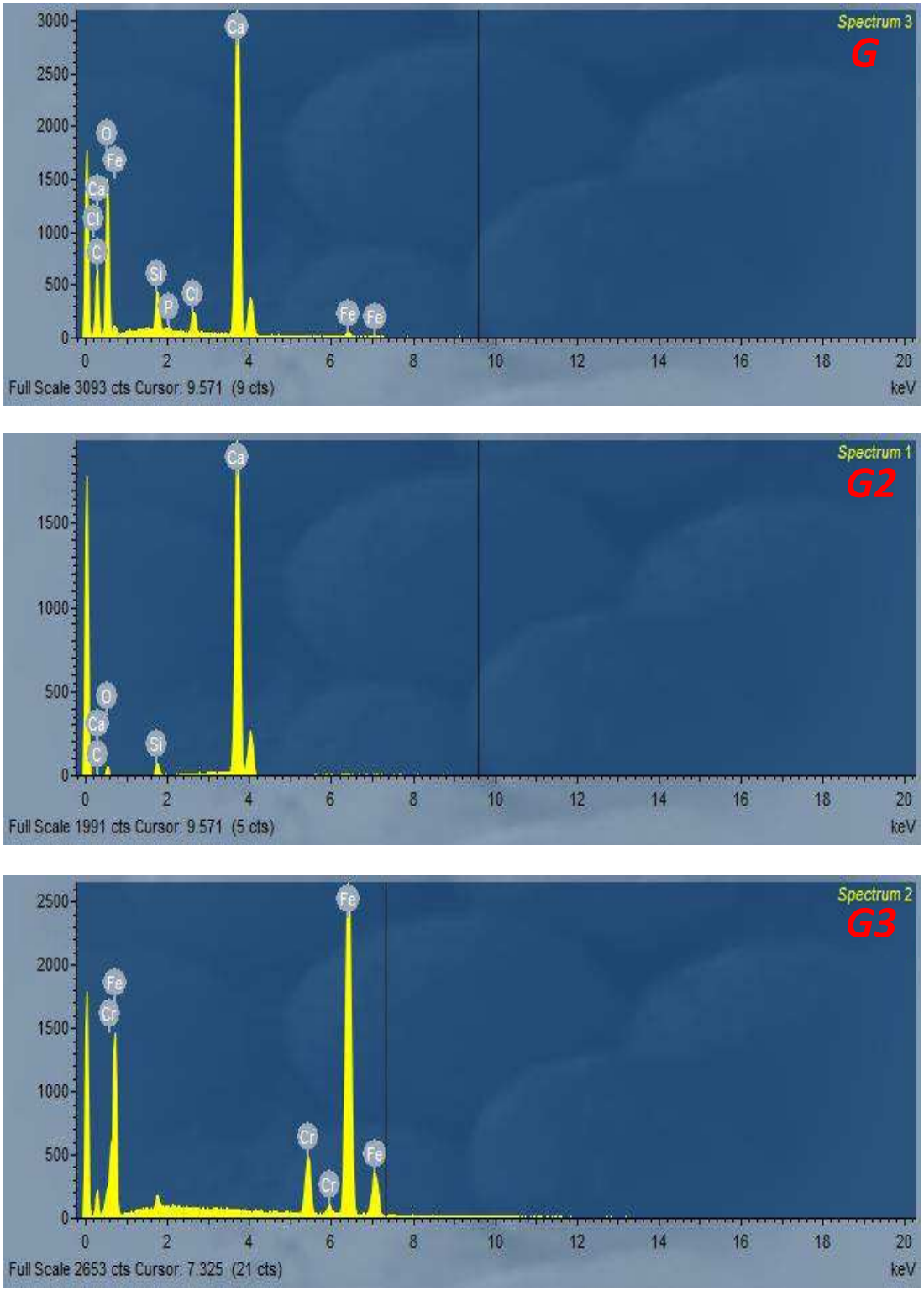


Figure 6.24. Selected EDX spectrum of calcium carbonate precipitation on sand particles induced by S. pasteurii (heterogeneous dynamic system experiment), by using urea-containing medium in groups G1 and G2. Whilst urea-free medium was used in group G3. Also cadmium ions (0.03 mM) were used in groups G2 and G3.

6.5 General discussion

In this chapter the ability of ureolytic bacterium *Sporosarcina pasteurii* to produce alkaline conditions encouraging calcium carbonate precipitation and metals divalent co-precipitation in porous media of fine, medium and coarse sand fractions was investigated. Also, the factors that may affect the bioprocesses, such as metal bioavailability in sand, the effect of metal toxicity on bacterial activity and the influence of different hydraulic conductivity of sand fractions were studied. Bioprecipitation processes were explored in static (without fluid flow) and dynamic (with fluid flow) system experiments. Dynamic system involved homogenous and heterogeneous liquids flow.

Calcium and strontium showed low affinity to be adsorbed on sand fractions (fine, medium and coarse). High proportion of injected calcium and strontium ions in control samples (urea-free samples) were recovered in extracted and outflow solutions in static and dynamic experiments, respectively. Results from adsorption isotherms experiments indicated that zinc and cadmium have high affinity to be taken up on adsorption sites in sand fractions. Hence, biomineralisation of these heavy metals depends on their availability in pore water, though their high availability may cause toxicity to the bacteria. Generally, adsorption is the main process lead to loss cations from soil aqueous solutions, especially at low concentrations of heavy metals. Fe, Mn and Al oxides, clay minerals and organic matter are the main adsorbents for cadmium ions in soil. Metal oxides contain surface functional groups rather than permanent charge sites (Bradl 2004; Choi 2006; Lafuente *et al.* 2008; Usman 2008; Gunawardana *et al.* 2015). As can be seen from Table 3.1 (Chapter 3) the amount of Fe oxides in medium sand fraction is less than that in other fractions, this may the reason behind

that medium sand showed less affinity to adsorb zinc and cadmium than other fractions.

S. pasteurii showed ability to precipitate strontium, zinc and cadmium in porous media of different sand fractions through calcium carbonate mineralisation processes. This ability was investigated through static (without fluid flow) and dynamic (with fluid flow) system experiments. Adsorption of metal ions on sand particles reduces cations availability which in turn lead to moderate metal toxicity effects on the bacterial activity to hydrolyse urea. Increasing pH of sand samples due to urea hydrolysis can also lead to increase the sand capacity of metals adsorption.

Firstly, the co-precipitation of strontium, zinc and cadmium was investigated in static system experiments. The microcosms in this part of study were 50 mL sterilised polypropylene tubes. Strontium bioprecipitation with calcium carbonate processes were running readily when compared to other divalent metals were tested in this study, in terms of metal availability, toxicity to the bacteria and can co-precipitates readily with calcium carbonate lattice during recrystallisation (Achal *et al.* 2012b; Lauchnor *et al.* 2013). Strontium displayed no resistance to precipitate by microbially induce calcium carbonate procedure at concentration up to 30 mM. The same response of the bacteria was noticed in strontium precipitation experiment in aqueous solutions (Chapter 5). Zinc precipitation was investigated in 3 concentrations (2, 5 and 10 mM) in static system experiments to test metal availability on bacterial activity. Bacterial activity to hydrolyse urea was decreased with zinc concentration, though most of zinc ions thought to be adsorbed on sand grains. Bacterial cells showed activity in presence of 2, 5 and 10 mM zinc higher than their activity in aqueous solutions for similar concentrations.

Full removal of cadmium ions (10 mM) was noticed in sand porous media in static system experiment compared to 1.5 mM metal removal in aqueous solutions. Although zinc and cadmium showed high toxicity to bacteria (Chapters 4 and 5) but adsorption of metal cations on sand particles seems allowed bacteria to operate in concentrations higher than those in aqueous solutions experiments. Cadmium showed response to precipitate with calcium carbonate precipitation better than zinc in aqueous solutions and particulate media experiments, though cadmium toxicity to *S. pasteurii* cells was higher than zinc (MIC is 0.03-0.06 mM for cadmium, whilst it is 0.2-0.5 mM for zinc).

Recovered zinc and cadmium concentrations in control samples (injected with urea-free media, *M3*) of static experiments indicated that the availability of cadmium ions was much higher than zinc especially in medium sand fractions. This may also mean the availability of cadmium was higher than zinc in urea-treated samples before pH increase due to urea hydrolysis. Hence, bacterial activity in presence precipitation medium (urea and calcium) to precipitate cadmium ions was higher than that for zinc precipitation. As this study hypothesises that metal precipitation technique provides a protective mechanism for the bacteria by reducing the overall metal concentrations to tolerable levels, it seems the removal of cadmium ions by carbonate precipitation is more efficient than zinc. Although zinc ions were reported to sorb strongly to calcite carbonate minerals (Zachara *et al.* 1991; Garcia-Sanchez and Alvarez-Ayuso 2002; Elzinga *et al.* 2006) but they may readily desorb due to zinc high hydration energy and it being only slowly absorbed into solid solution unless recrystallization occurs. In addition, zinc forms a tetrahedral carbonate structure (Teng and Zhao 2012) which may reduce long term stability of any co-precipitate due to a mismatch with the microstructure of calcium carbonate (in calcite this is octahedral). Zinc has been

reported to accelerate heterogeneous nucleation (Zeppenfeld 2010) so may lead to a larger number of smaller crystals forming, which may reduce long term sequestration of encapsulated zinc, although this was not directly observed. Hence, zinc ions may not fully have removed or removed slowly by calcium carbonate mineral (contrary to cadmium ions), which leads to prolong bacterial cells subjecting time to toxic cations then may reduce their activity. However, cadmium is similar in atomic radius to calcium and forms a similar octahedral carbonate structure (Teng and Zhao 2012). It therefore can substitute for calcium relatively easily in carbonate minerals, forming solid solutions and co-precipitates readily. On existing surfaces of calcite, rapid sorption as a stable complex can form an epitaxial layer, preventing further sorption of the metal and limiting dissolution of the mineral (Chada *et al.* 2005; Du *et al.* 2011) although Stipp *et al.* (1992) and Buekers *et al.* (2007) found that movement from surface layers into the mineral was possible in a relatively short period.

In other experiments *S. pasteurii* strain grew and operated in presence of high cadmium concentrations (20 mM) in porous media but in presence of precipitation medium (urea and calcium). Bacterial cells seemed affected by cadmium toxicity when aging (for 24 hours) with 0.03 mM metal concentration in porous media before injecting precipitation medium. Suggesting that presence of bacterial cells and metal ions in porous media without any growing medium may inhibit bacterial activity. Such effect may occur in nature when cadmium contaminants in subsurface environment can kill many kind of bacteria if they could not show any defence mechanism. In presence of precipitation medium bacteria can tolerate high concentration of heavy metals especially when carbonate precipitation mechanisms can decrease toxic ions rapidly. Such effect was notice with cadmium precipitation more efficient than for zinc.

Sand particle sizes had affected bacterial activity when aging bacterial cells with cadmium ions in sand fractions. Bacterial cells showed higher activity in fine sand fraction than other fractions. It is possible that medium and coarse sand fractions have less ability to retain bacterial cells than fine sand (Fontes *et al.* 1991), which led to flush out significant amounts of bacterial cells and hence, bacterial activity was reduced. Also, may due to that metal ions availability in medium and coarse sand fractions is higher than that in fine sand, the more available toxic cations, the more inhibition may occur to bacterial cells.

Layered heterogeneous system was created by injecting working solutions into sand columns (fine, medium and coarse) from one inlet. This was to consider the effect of different hydraulic conductivity of sand fractions in distributing bacterial activity and precipitation process, and how the precipitation of calcium carbonate in porous media can change sand initial permeability. Results showed that precipitation processes were affected by heterogeneity of solution flow. Flow headed to a specific sand fraction depending on its hydraulic conductivity. Firstly, flow ran in coarse sand columns then after the permeability reduced due to precipitation processes in porous media flow diverted to other sand fractions. Bacteria showed high activity after aging with cadmium ions (0.03 mM) for 24 hours before injecting precipitation medium in fine sand fraction. Such activity from *S. pasteurii* reflects their ability to survive in presence of small amount of cadmium (0.03 mM) especially in fine sand fractions.

Working solutions were injected into sand columns every 48 hours (48 hours stagnation period). It is possible that every set of sand columns (contained fine, medium and coarse sand columns connected to one inlet) functions as communicating vessels at stagnation period. Hence, when the liquid settle, it balances out to the same

level in all of the columns regardless of hydraulic conductivity in each column. Such effect can lead to move precipitation medium from column to another and then to maintain bacterial activity in each column.

The effect of heterogeneity of subsurface environment can affect the solute transport in porous media, the fate of pollutants can be predicted when understanding the parameters that may influence contaminants distribution in soil. Microbially induce calcium carbonate precipitation means that depend on native ureolytic bacteria may affected by heterogeneous distribution of bacteria and contaminants (Chrysikopoulos *et al.* 1992; Zhao and Toksoz 1994; Simmons *et al.* 2001; Majdalani *et al.* 2015). Cherblanc *et al.* (2007) and Zhao and Toksoz (1994) reported that heterogeneity of hydraulic conductivity plays a main role in solute transport with groundwater. Also, this spatial variation can lead to inconsistent dispersion over wide range at field scale. Wu *et al.* (2014) indicated that under saturated flow conditions, advection controls solute transport in both high and low hydraulic conductivity domains. Sand with greater permeability causes a preferential flow path for solute transport in the heterogeneous soils. Preferential flow may occur in different types of soils. Many reasons can lead to arise preferential flow such as root channelling and earthworm digging as biological activities or due to presence of cracks and fractures as hydrological processes, also tillage, boring and digging soil can cause this phenomena (Kung 1990; Ritsema *et al.* 1993; Bundt *et al.* 2001; Rosenbom and Undersøgelse 2005; Merdun 2012).

It was noticed that hydraulic conductivity of coarse sand was decreased by inducing calcium carbonate precipitation in porous media. After that flow diverted to more permeability media which are fine and medium sand fractions. Nevertheless,

this does not mean that coarse sand was totally clogged but its permeability reduced to a value less than other fractions. It noticed that after days of carbonate precipitation in fine and medium sand fractions the flow ran again in the coarse sand column after permeability reduced in these sand fractions. Continuous outflow of solutions from sand column without clogging may occurred under the effect of preferential flow. Such flow can occur in high permeability sand (Wu *et al.* 2014).

The effect of sand particles size on heavy metals bioprecipitation processes was not obvious in static system, this apart from the capacity of different sand fractions (fine, medium and coarse) to adsorb metallic cations. This may due to no fluid flow may lead to desorb metal ions and bacterial cells from adsorption sites on sand grains depending on sand adsorption capacity. Retaining of bacterial cells, metal ions and nutrients on sand grains decreases with sand coarseness (Fontes *et al.* 1991; Sessitsch *et al.* 2001), which could lead to flush out significant amounts of bacterial cells and then bacterial activity may be reduced. Generally, a decrease in particle size causes an increase in the solid's total surface area.

Precipitation in sand samples were examined by using XRD, SEM and EDX spectrum. All these tests confirmed that aragonite and calcite are the predominant calcium carbonate polymorphs produced. However, different morphologies were resulted from co-precipitation zinc and cadmium with calcium carbonate. Rhombohedral and sphere shapes were produced in zinc experiments, whilst only rhombohedral shapes were produced in cadmium and calcium carbonate precipitation without heavy metals experiments. Sizes of produced shapes were ranged from 5-35 μm. Generally, this finding consistent with many studies in this field (Fujita *et al.* 2000; Achal *et al.* 2012a; Achal *et al.* 2012b; Li *et al.* 2013; Kang *et al.* 2014).

Chapter 7: Conclusion and Suggestions for Further Research

7.1 Conclusion

current study investigated the ability of a bacterium, *Sporosarcina pasteurii,* to remove a range of heavy metals and radionuclides (by using non-radioactive proxies) in an aerobic system through calcium carbonate precipitation in aqueous solutions and particulate media. Microbially-induced calcite precipitation has been reported in only a very limited fashion for metallic cations removal from aqueous solution. Applicability of heavy metals removal by this technology that based on understanding metals availability and toxicity to bacteria and ground conditions effects on the bioprocesses is lacking. the study examined the possibility of a range contaminants removal by this technique and considering many parameters that may effects the metal removal mechanism. Parameters that were considered in this study are metal toxicity to the bacteria (*Sporosarcina pasteurii*) and effects of metal cations availability, sand particle sizes and the influence of the homogeneity and heterogeneity in hydraulic conductivity on bioprecipitation processes. An improve understanding of the parameters that relate to this technology can lead to increase its efficiency and to enable upscaling to suit in-situ applications.

Toxicity experiments were designed to evaluate the ability of *S. pasteurii* to operate and grow in the presence of the target heavy metals (zinc, cadmium, copper, lead and strontium). These heavy metals are causing inhibition to bacteria growth, such as, an increased lag phase or reduced growth rate, however, bacteria may tolerate metals toxicity. Such effects can be problematic for heavy metals and radionuclides removal when using microbially induced calcium carbonate precipitation mechanism. In these experiments bacteria allowed to resist heavy metals toxicity in a medium

contained only nutrient broth (13 g/l) and the target metal for 72 hours at 30ºC (urea and calcium were not present). Soluble forms of all metals were used (metal solubility was examined by Visual MINTEQ model), hence metal ions were almost completely bioavailable to bacterial cells.

Results gained from toxicity experiments showed that heavy metals (zinc, cadmium, copper and lead) are toxic to the bacteria (*Sporosarcina pasteurii*) at concentrations known as minimal inhibitory concentrations (MIC). MIC was defined as a 70% reduction in bacteria growth as measured by optical density. Cadmium exhibited the greatest toxicity (MIC = 0.03-0.06 mM), whilst zinc and copper were similar in their toxicity effect (MIC = 0.2-0.5 mM, for zinc and copper). However, bacteria showed growth rate at 0.2 mM copper concentration much higher than at similar concentration of zinc, but *S. pasteurii* cells were almost completely inhibited at zinc and copper concentration of 0.5 mM. Bacteria were able to grow at higher initial lead concentrations (MIC = 0.5-1 mM). Strontium is found only toxic at very high concentrations (MIC = 5-10 mM). Bacteria showed variety in response to each heavy metals.

Bacteria showed variety in their response to metals toxicity, this may depend on bacteria mechanism to detoxify metals toxicity. Cadmium toxicity effect was expected as it has no essential biological function to bacteria, also it was found extremely toxic to microorganisms even at low concentration. Zinc and copper, though essentials metal at trace concentrations, but they appear toxic at higher concentrations. Interestingly, there was significant growth of *S. pasteurii* cells at lead concentrations up to 0.5 mM, though the metal is non-bioessential for bacteria and was reported as very toxic metal. Strontium showed low toxicity effect to bacterial cells, this may due

to the metal is not redox sensitive. No studies were found in literature that dealt with metals toxicity effects on *S. pasteurii* strains. Also, it thought such effects are species-specific response, in addition, growth media and abiotic metal response may also have an impact on bacteria response. Hence, comparing the results of toxicity experiments in this study with other studies for different microorganisms but same heavy metals may be impractical.

The ability of *S. pasteurii* to cause precipitation of the metallic contaminants *via* urease hydrolysis was then demonstrated through batch experiments. Precipitation medium in these experiments contained bacterial cells, nutrient broths, urea and calcium. Bacteria live-cells were used in main samples, whilst killed-cells (cells were killed by autoclaving) were used in control samples, both live and killed-cells samples were prepared in the same fashion. Results from metals bioprecipitation in aqueous solutions experiments showed an improvement to *S. pasteurii* resistance to heavy metals toxicity. The presence of urea and calcium ions in precipitation medium offers bacteria that possess the urease enzyme a protective mechanism against metals toxicity. Urea hydrolysis leads to an elevated pH and enhanced carbonate ion concentration in solution, making metal carbonate precipitation more likely. Initial precipitation reduces metal bioavailability, leading to enhanced bacterial growth and activity, which in turn causes further precipitation. A significantly increased minimum inhibitory concentration results, at least three times greater than the original value that was found in toxicity experiments.

Heavy metals showed different responses to be precipitated with calcium carbonate precipitation. Final metal concentrations were as follows: zinc (0-10 mM), cadmium (0-1.5 mM), copper (0-5 mM), lead (0-5 mM) and strontium (0-30 mM).

Metal concentrations were increased (compared to toxicity experiments) because this study hypothesises that metal precipitation technique provides a protective mechanism for the bacteria by reducing the overall metal concentrations to tolerable levels. Heavy metal ions were almost completely bioavailable to bacterial cells (metals availability were checked by Visual MINTEQ model). Cadmium, lead and strontium were completely removed from solutions at all tested concentrations after 3 days. Whilst zinc removal was limited, full metal removal was up to 0.5 mM. Copper removal was only at lowest concentration (0.01 mM). Metals removal occurred in conjunction with pH increase, calcium removal and white precipitate indicating co-precipitation with calcium carbonate.

S. pasteurii is nominally a spore-forming organism and so spores may survive autoclaving and subsequently regenerate. This may indicate that a similar mechanism of calcium precipitation driving pH change, calcium and metal removal also occurred in control samples (killed-cells samples) but only very slowly. Such effects happened at low concentrations of cadmium, copper and lead, however, it occurred at all strontium concentrations. Suggesting that bacterial spores were able to survive autoclaving but not high metal concentrations.

By comparing the results from live and killed-cells experiments can notice the role of the bacteria live cells in removing metallic ions of zinc (up to 0.5 mM), cadmium and strontium ions from solutions, whilst, bacteria were able to remove only the lowest concentration of copper. In lead experiment high proportion of metal ions were removed in control samples, albeit slower, despite limited pH change and calcium concentration decrease. This again may be due to spore regeneration at low lead concentrations, the removal of lead is not correlated with this and increases at higher

lead concentrations; an abiotic mechanism is therefore suspected. Such a mechanism will allow greater microbial activity at higher lead concentrations. It is unclear, therefore, whether pH increase and calcium removal were the cause of lead removal, or facilitated by it. The loss of lead allowed bacteria to operate at higher concentrations than in earlier experiments, and live cell experiments did have an improved response compared to killed cell controls, suggesting that urea hydrolysis did play a role.

It noticed that two main factors control metal removal with microbially induce calcium carbonate precipitation mechanism. First one is bacteria ability to tolerate metal toxicity in presence of urea and calcium, this is important to initiate calcium carbonate precipitation. Second factor is the possibility of metal-$CaCO_3$ interaction that can lead to permanent sequester metal ions from solution and reduce metal availability. Metal-$CaCO_3$ interaction when divalent contaminants substitute calcium ions in the crystal lattice of calcium carbonate. In the same context, the structure compatibility between metal carbonate and calcium carbonate is important for permanent metal adsorption on calcium carbonate precipitate. Calcium ions present in an octahedral coordination in the structure of calcium carbonate. Hence metals that can form octahedral surface complexes will be ideally for calcium substitution in calcium carbonate structure and to be adsorbed permanently. However, other coordination (tetrahedral) may lead to reduce long term stability of any co-precipitate due to a mismatch with the microstructure of calcium carbonate.

It was found in this study that elevation of pH and removal of calcium from solution are shown to be strongly linked to removal of cadmium and strontium and zinc (not at higher zinc concentrations), but only partially linked to removal of lead

and copper. Strontium was found readily substitute calcium ions in calcium carbonate lattice structure. Cadmium is similar in atomic radius to calcium and forms a similar octahedral carbonate structure. The case of zinc this may be due to the metal adsorption complexes have a tetrahedral geometry, which is not matching the octahedral coordination of cations in calcium carbonate. Also it is found that copper carbonate complexes on the surfaces of calcite and vaterite can hinder further sorption as well as growth or dissolution of the mineral, and at high concentrations an epitaxial layer may form. This may have led to limited removal of copper in the experiments presented here even when carbonate minerals are expected to be precipitated. Lead ions were reported to sorb strongly to calcium carbonate and can form a solid solution in this mineral, albeit with a distorted lattice. However, the experimental and visual MINTEQ model data suggest an abiotic mechanism is at least partially responsible for the observed results although a proportion of metal removal is thought to be due to microbial carbonate precipitation. This may also explain the ability of *S. pasteurii* to apparently resist higher concentrations of lead than other metals in the toxicity experiment.

According to the results that obtained from bioprecipitation in aqueous solutions experiments, strontium, zinc and cadmium were chosen to be investigated in bioprecipitation in particulate media experiments. In batch and column tests the ability of *S. pasteurii* to induce metals removal in porous media of three sand fractions (fine, medium and coarse) was explored. Firstly, strontium (30 mM), zinc (2, 5 and 10 mM) and cadmium (10 mM) were tested in static system (no fluid flow). Microcosms in this system were sterilised 50 mL polypropylene tubes were filled with precipitation medium and sand fractions before incubating at 30ºC. Main samples (urea-treated samples) were treated with similar precipitation medium in previous experiments,

whilst, control samples were treated with urea-free medium. Sand fractions of urea-treated and control samples contained live bacterial cells. Working solutions were amended with heavy metals before mixing with sand.

Strontium removal (urea-treated samples) in porous media of fine, medium and coarse sand fractions was as efficient as that in aqueous solutions. Recovering high proportion of strontium and calcium ions from control or urea-free samples, indicated the role of urea hydrolysis in metals removal. Strontium and calcium showed low affinity to adsorb on sand grains, which means they were available to bacteria. However, zinc and cadmium displayed high affinity to take up on sand particles. Decreasing the availability of zinc and cadmium due to adsorption on sand grains allowed bacteria to grow and operate in metal concentrations much higher those in aqueous solution experiments. At 2 and 5 mM zinc concentrations bacteria were able to operate and remove available zinc ions, whilst at 10 mM bacteria appeared to be inhibited by this amount of metal. Although, the bioavailability of cadmium ions (at 10 mM) seemed higher than zinc, bacteria were able to cope with high concentration of cadmium and induce precipitation of metal with calcium carbonate precipitation. This may due to cadmium ions are adsorbed on calcium carbonate surfaces more rapid than zinc. Hence, their availability decreased quicker than zinc in presence of calcium carbonate precipitation. According to EDX spectrum precipitation of calcium carbonate appears led to co-precipitate zinc ions, though the adsorption of zinc to sand particles seems dominant removal mechanism especially at high pH. It is still uncertain which mechanism (adsorption on sand particles or co-precipitation with calcium carbonate) is more responsible for metals removal. However, in case of strontium co-precipitation with calcium carbonate is probably the mechanism that

responsible for metal removal, because it was noticed that strontium has low affinity to be adsorb on sand grains.

In dynamic system (fluid flow) bacterial cells were mixed with sand fine, medium and coarse sand fractions before packing in columns. Columns were connected to peristaltic pumps to inject metal solution and precipitation medium into sand columns by using pulsed injection strategy. The first experiment involved injecting precipitation medium amended with high cadmium concentration (20 mM). This was to examine bacteria ability to precipitate metal ions in presence of precipitation medium. First dynamic experiment only considered the medium sand fraction, but cells were able to tolerate and full removal of all cadmium ions from liquid phase. Bacterial survival in particulate media was found to be greater than in aqueous solution alone, indicating a protection mechanism due the presence of solid particles such as reduced contaminant bioavailability through physical structure, preferential flow paths or cell adhesion to surfaces. The induced precipitation of cadmium also allowed survival at much higher total concentrations than when a bioprecipitation mechanism was not present.

In other experiment were all sand sizes considered, bacteria mixed with sand before packing in the columns. Cadmium solution (0.03 mM, final concentration) was pumped through columns to contaminate sand fractions and test bacteria ability to tolerate cadmium ions in porous media when bioprecipitation mechanism is not present. Precipitation medium was then injected into urea-treated samples after 24 hours for 14 days, whilst, urea-free medium was pumped into control samples that were prepared in the same fashion of main samples. Bacteria showed higher resistance in fine sand fraction, this may attribute to high adsorption capacity of fine sand

particles allowed bacterial cells to hydrolyse urea without affecting by metal toxicity. It also may be the presence of bacterial cells in sand fractions coarser than fine sand facilitate their flushing out with injected liquids which in turn led to reduce their number and activity.

In previous dynamic experiments the injection of working solutions was homogenous (similar amounts of fluid run in fine, medium and coarse sand fraction). The effect of heterogeneous hydraulic conductivity and preferential flow on bioprecipitation processes in different grain sizes was studied in other experiments. It is resulted that the heterogeneity of hydraulic conductivity plays a main role in distributing bacterial activity and subsequence precipitation processes in porous media. Heterogeneous hydraulic conductivity was considered by injecting solutions into fine, medium and coarse sand fractions from one inlet. Also, it is observed that precipitation of calcium carbonate in porous media affected solute transport, when it leads to decrease the permeability. It is thought that precipitating of calcium carbonate in sporadic parts of sand columns due to heterogeneity in bioprocesses activity may lead to arise preferential flow that can responsible for solute transport across sand sections. Clogging preferential flow paths by calcium carbonate precipitation can be slower than pore throat in soil, due to preferential flow allows solute transport much faster than matrix flow.

The effect of sand particles size on heavy metals bioprecipitation processes was not obvious in static system, apart from the capacity of different sand fractions (fine, medium and coarse) to adsorb metallic cations. This may due to no fluid flow may lead to desorb metal ions and bacterial cells from adsorption sites on sand grains depending on sand adsorption capacity. Retaining of bacterial cells, metal ions and

nutrients on sand grains decrease with sand coarseness which led to flush out significant amounts of bacterial cells and then bacterial activity may be reduced. Generally, a decrease in particle size causes an increase in the solid's total surface area.

Precipitation in sand samples were examined by using XRD, SEM and EDX spectrum. All these tests confirmed that aragonite and calcite are the predominant calcium carbonate polymorphs produced. However, different morphologies resulted from co-precipitation of zinc and cadmium with calcium carbonate. Rhombohedral and sphere shapes were produced in zinc experiments, whilst only rhombohedral shapes were resulted in cadmium experiments. Sizes of produced shapes were ranged from 5-35 μm. The gained results from this study demonstrate that microbially induce calcium carbonate technique is a means of sequestration of soluble heavy metals via co-precipitation with calcium carbonate precipitation that can be useful for divalent heavy metal and radionuclides bioremediation. Though, zinc and copper are partially removed by this remedial technology (only at low concentrations), but some low metals concentrations may not be removable by traditional methods.

However, the most obvious finding to emerge from this study is that ureolytic bacteria (*S. pasteurii* in this study) able to permanent sequester heavy metal cations *via* co-precipitation with calcium carbonate minerals in contaminated soil. This suggestion is based on that resulted minerals from bioprecipitation processes (discovered by SEM, XRD and EDX) are aragonite and calcite, which are the most stable polymorphs of calcium carbonate and offer long-term removal of heavy metals. Results gained from dynamic system experiment, where calcium carbonate precipitation medium injected into sand columns by using peristaltic pumps, may

more mimicking the subsurface environment, when consider hydraulic conductivity and solute transport in porous media effects.

The ability of ureolytic bacteria to induce the precipitation of calcium carbonate minerals offers an opportunity to develop an in-situ bioprecipitation technique for soils and groundwater contaminated with divalent metals or radionuclides. The experiments in this study by using ureolytic bacteria (*S. pasteurii*) have demonstrated that microbially mediated calcium carbonate precipitation can enhance the capture of toxic heavy metals. Urease enzyme is widespread in subsurface, so it may possible to rely on native microorganisms in contaminated sites rather than on introduction of any non-native species.

Conditions in nature are different and uncontrolled compared to laboratory conditions. In this study the response of metals to be precipitated with calcium carbonate precipitation was studied separately for each metal. Whilst, in nature many metals can act at the same time and place. Also, their bioavailability can be varied from environment to other. In addition, ground conditions can be changed spatially and temporally in subsurface. Although, this study did not include these kind of environmental conditions all together, but studying each influence separately is required. This study covered many effects that are considered vital for bioprecipitation processes.

7.2 Recommendation for Future work

It is recommended that further research to be undertaken to improve understanding the influences that may limit applying bioprecipitation of heavy metals in contaminated sites. Although, the results of this study indicate that calcium carbonate precipitation induced by ureolytic organism is effective and may provide a useful

remediation strategy for contaminated sites, but additional estimation of this approach under conditions more closely mimicking environmental conditions is required. In addition, more examining works at large scale should be carried out to determine the efficiency of in-situ bioprecipitation for sequestration of divalent contaminants. This study has covered important factors that can affect heavy metals bioremediation, such as, metals toxicity and availability, metal-mineral interaction and the effects of ground conditions, but still more research is required to determine the efficacy of this technology with other divalent metals and radionuclides (e.g., $^{90}Sr^{2+}$, UO_2^{+2}, Co^{2+}) that may contaminate subsurface. In addition, introducing the influence of radioactivity on ureolytic organisms can give impression to what extent divalent radionuclides can be co-precipitated with carbonate minerals. Usually the contamination of environments occurs with more than one metal with different availability and effects. Hence, studying the influence of multi metals on ureolytic organisms is required.

It is not completely clear in this study to what extent that co-precipitation with calcium carbonate minerals mechanism contributed in removing heavy metal cations in presence of adsorption on sand grains mechanism. Hence, it recommended conducted the bioprecipitation of heavy metals experiments in a medium has low adsorption capacity to adsorb metal ions from solutions. In addition, investigating the effects of ground conditions on bioprecipitation processes was in one dimensional system, if such effects can be explored in two or three dimensional systems would be more mimicking to nature. Especially contaminants usually are moving in subsurface environment taking plume shape over long distances (vertically and horizontally). Monitoring, predicting and controlling calcium carbonate bioprecipitation distribution in porous media are essential to increase heavy metals bioremediation in contaminated

sites. Heterogeneous distribution of calcium carbonate can lead to exclude some sections in soil without complete treatment due to decreasing in permeability. Also, studying preferential flow phenomenon in more details (e.g. with tracking) would be more beneficial in investigating how can affect heavy metals biomineralisation in subsurface.

References

Abdus-Salam, N. and M'civer, F. A. 2012. Synthesis, Characterization and Application of 2-Line and 6-Line Ferrihydrite to Pb(II) Removal from Aqueous Solution. *J. Appl. Sci. Environ. Manage* 16(4), pp. 327-336.

Abo-El-Enein, S. A., Ali, A. H., Talkhan, F. N. and Abdel-Gawwad, H. A. 2012. Utilization of microbial induced calcite precipitation for sand consolidation and mortar crack remediation. *HBRC Journal* 8(3), pp. 185-192.

Achal, V., Mukherjee, A., Basu, P. C. and Reddy, M. S. 2009. Strain improvement of Sporosarcina pasteurii for enhanced urease and calcite production. *Journal of industrial microbiology & biotechnology* 36(7), pp. 981-988.

Achal, V., Pan, X., Fu, Q. and Zhang, D. 2012a. Biomineralization based remediation of As(III) contaminated soil by Sporosarcina ginsengisoli. *Journal of Hazardous Materials* 201–202(0), pp. 178-184.

Achal, V., Pan, X. and Zhang, D. 2012b. Bioremediation of strontium (Sr) contaminated aquifer quartz sand based on carbonate precipitation induced by Sr resistant Halomonas sp. *Chemosphere* 89(6), pp. 764-768.

Adekola, F. A., Salam, N. A., Adegoke, H., yabode , A., Adesola, M. and Adekeye, J. I. D. 2012. REMOVAL OF Pb(II) FROM AQUEOUS SOLUTION BY NATURAL AND SYNTHETIC CALCITES. *Bull. Chem. Soc. Ethiop* 26(2), pp. 195-210.

Al-Abed, S. and Chen, J.-L. 2002. Transport of Trichloroethylene (TCE) in Natural Soil by Electroosmosis. In: Smith, J. and Burns, S. eds. *Physicochemical Groundwater Remediation*. Springer US, pp. 91-114.

Al-Anber, M. A. 2011. Thermodynamics Approach in the Adsorption of Heavy Metals, Thermodynamics - Interaction Studies - Solids, Liquids and Gases, Dr. Juan Carlos Moreno PirajÃ¡n (Ed.), ISBN: 978-953-307-563-1, InTech, DOI: 10.5772/21326. Available from: http://www.intechopen.com/books/thermodynamics-interaction-studies-solids-liquids-and-gases/thermodynamics-approach-in-the-adsorption-of-heavy-metals.

Al-Thawadi, S. M. 2011. Ureolytic Bacteria and Calcium Carbonate Formation as a Mechanism of Strength Enhancement of Sand. *Journal of Advanced Science and Engineering Research* 1, pp. 98-114.

Al Hagrey, S. A., T.Schubert-Klempnauer, D.Wachsmuth, J.Michaelsen and R.Meissner. 1999. Preferential flow: first results of a full-scale flow model. *Geophys. J. Int.* 138, pp. 643-654.

Al Qabany, A., Mortensen, B., Martinez, B., Soga, K. and DeJong2, J. 2011. Microbial Carbonate Precipitation: Correlation of S-Wave Velocity with Calcite Precipitation. In: *Geo.Frontiers 2011: Advances in Geotechnical Engineering Proceedings of the Geo.Frontiers 2011 Conference.* Engineering Department, University of Cambridge,Department of Civil and Environmental Engineering, University of California. ASCE, pp. 3993-4001.

Al Qabany, A. A. A., Soga, K. and Santamarina, J. C. 2012. Factors Affecting Efficiency of Microbially Induced Calcite Precipitation. *Journal of Geotechnical and Geoenvironmental Engineering*.

Alabi, O. O. 2011. Fluid Flow in Homogeneous and Heterogeneous Porous Media. *EJGE* 16, pp. 61-70.

Alagumuthu, G., Veeraputhiran, V. and Venkataraman, R. 2010. Adsorption Isotherms on Fluoride Removal: Batch Techniques. *Archives of Applied Science Research* 2(4), pp. 170-185.

Alexander, M. 1999. *Biodegradation and Bioremediation*. 2nd ed ed. San Diego: Academic Press, p. 453 p.

Ankita, S., Singh, V., Jadon, S. and Bhadauria, S. 2013. Heavy Metal Tolerance of Three Different Bacteria Isolated From Industrial Effluent. *ijprbs logoINTERNATIONAL JOURNAL OF PHARMACEUTICAL RESEARCH AND BIO-SCIENCE* 2(2), pp. 137-147.

Armagan, B. and Toprak, F. 2013. Optimum Isotherm Parameters for Reactive Azo Dye onto Pistachio Nut Shells: Comparison of Linear and Non-Linear Methods. *Pol. J. Environ. Stud.* 22(4), pp. 1007-1011.

Barbalace, K. 1995. *Periodic Table of Elements - Sorted by Ionic Radius. EnvironmentalChemistry.com* [Online]. Available at: http://EnvironmentalChemistry.com/yogi/periodic/ionicradius.html.[Accessed: 8/3/2015].

Barkay, T. and Schaefer, J. 2001. Metal and radionuclide bioremediation: issues, considerations and potentials. *Current Opinion in Microbiology* 4(3), pp. 318-323.

Baskar, S., Baskar, R., Mauclaire, L. and McKenzie2, J. A. 2006. Microbially induced calcite precipitation in culture experiments: Possible origin for stalactites in Sahastradhara caves, Dehradun, India. *CURRENT SCIENCE* 90(1), pp. 58-64.

Bauda, P. and Block, J. C. 1990. Role of envelopes of gram-negative bacteria in cadmium binding and toxicity. *Toxicity Assessment* 5(1), pp. 47-60.

Ben Salem, I., Sghaier, H., Trifi, H., Héni1, S., Khwaldia, K., Saidi, M. and Landoulsi2, A. 2012. Isolation and characterization of a novel Micrococcus strain for bioremediation of strontium in radioactive residues. *African Journal of Microbiology Research* 6(4), pp. 851-858.

Benini, S., Rypniewski, W. R., Wilson, K. S., Miletti, S., Ciurli, S. and Mangani, S. 1999. A new proposal for urease mechanism based on the crystal structures of the native and inhibited enzyme from Bacillus pasteurii: why urea hydrolysis costs two nickels. *Structure* 7(2), pp. 205-216.

Beven, K. and Germann, P. 1982. MACROPORES AND WATER-FLOW IN SOILS. *Water Resources Research.* 18(5), pp. 1311-1325.

Beven, K. J. 1991. Modeling preferential flow: An uncertain future?P.1-11. In T. J. Gish and A. Shirmahammadi (ed.). Preferential flow. Proceedings Natl. Symp. ASAE, Chicago, IL. .16-17 Dec. 1991. ASAE, St. Joespeph, MI.

Boswell, C. D., Dick, R. E., Eccles, H. and Macaskie, L. E. 2001. Phosphate uptake and release by <i>Acinetobacter johnsonii</i> in continuous culture and coupling of phosphate release to heavy metal accumulation. *Journal of Industrial Microbiology & Biotechnology* 26(6), pp. 333-340.

Boularbah, A., Morel, J. L., Bitton, G. and Guckert, A. 1992. Cadmium biosorption and toxicity to six cadmium-resistant gram-positive bacteria isolated from contaminated soil. *Environmental Toxicology and Water Quality* 7(3), pp. 237-246.

Boulos, L., Prevost, M., Barbeau, B., Coallier, J. and Desjardins, R. 1999. LIVE/DEAD BacLight : application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. *J Microbiol Methods* 37(1), pp. 77-86.

Bradl, H. B. 2004. Adsorption of heavy metal ions on soils and soils constituents. *Journal of Colloid and Interface Science* 277(1), pp. 1-18.

Brecevic, L. and Kralj, D. 2007. On Calcium Carbonates: from Fundamental Research to. *Application. Croatia Chem. Acta CCACAA* 80 ((3-4)), pp. 467-484.

Brinza, L., Quinn, P. D., Schofield, P. F., Mosselmans, J. F. W. and Hodson, M. E. 2013. Incorporation of strontium in earthworm-secreted calcium carbonate granules produced in strontium-amended and strontium-bearing soil. *Geochimica et Cosmochimica Acta* 113(0), pp. 21-37.

Brusseau, M. L. and Rao, P. S. C. 1990. Modeling solute transport in saturated solid: A review. *Geoderma* 46, pp. 169-192.

Buekers, J., Van Laer, L., Amery, F., Van Buggenhout, S., Maes, A. and Smolders, E. 2007. Role of soil constituents in fixation of soluble Zn, Cu, Ni and Cd added to soils. *European Journal of Soil Science* 58(6), pp. 1514-1524.

Bundt, M., Widmer, F., Pesaro, M., Zeyer, J. and Blaser, P. 2001. Preferential flow paths: biological 'hot spots' in soils. Soil Biol. *Biochem* 33, pp. 729-738.

Bunzl, K., Schimmack, W., Schramel, P. and Suomela, M. 1999. Effect of sample drying and storage time on the extraction of fallout 239+240Pu, 137Cs and natural 210Pb as well as of stable Cs, Pb and Mn from soils. *Analyst* 124(9), pp. 1383-1387.

Callagon, E., Fenter, P., Nagy, K. L. and Sturchio, N. C. 2014. Incorporation of Pb at the calcite (104)-water interface. *Environ Sci Technol* 48(16), pp. 9263-9269.

Chada, V. G. R., Hausner, D. B., Strongin, D. R., Rouff, A. A. and Reeder, R. J. 2005. Divalent Cd and Pb uptake on calcite {1014} cleavage faces: an XPS and AFM study. *Journal of Colloid and Interface Science* 288(2), pp. 350-360.

Chahal, N., Rajor, A. and Siddique, R. 2011. Calcium carbonate precipitation by different bacterial strains. *African Journal of Biotechnology* 10(42), pp. 8359-8372.

Chatterjee, A. K., Chakraborty, R. and Basu, T. 2014. Synthesis of Cu2O micro/nanocrystals with tunable morphologies using coordinating ligands as structure controlling agents and antimicrobial studies. *CrystEngComm* 16(42), p. 9866.

Chen, S. B., Ma, Y. B., Chen, L. and Xian, K. 2010. Adsorption of aqueous Cd2+, Pb^{2+}, Cu^{2+} ions by nano-hydroxyapatite: Single- and multi-metal competitive adsorption study. *GEOCHEMICAL JOURNAL* 44(3), pp. 233-239.

Cherblanc, F., Ahmadi, A. and Quintard, M. 2007. Two-domain description of solute transport in heterogeneous porous media: Comparison between theoretical predictions and numerical experiments. *Advances in Water Resources* 30(5), pp. 1127-1143.

Choi, J. 2006. Geochemical modeling of cadmium sorption to soil as a function of soil properties. *Chemosphere* 63(11), pp. 1824-1834.

Chrysikopoulos, C. V., Kitanidis, P. K. and Roberts, P. V. 1992. Macrodispersion of sorbing solutes in heterogeneous porous formations with spatially periodic retardation factor and velocity field. *Water Resources Research* 28(6), pp. 1517-1529.

Coles, C. A. and Yong, R. N. 2006. Use of equilibrium and initial metal concentrations in determining Freundlich isotherms for soils and sediments. *Engineering Geology* 85(1–2), pp. 19-25.

Comans, R. N. J. and Middelburg, J. J. 1987. Sorption of trace metals on calcite: Applicability of the surface precipitation model. *Geochimica et Cosmochimica Acta* 51(9), pp. 2587-2591.

Compton, R. G. and Pritchard, K. L. 1999. Kinetics of the Langmuirian Adsorption of Cull Ions at the CaIcite/Water Interface. *J. CHEM. SOC. FARADAY TRANS.* 86(1), pp. 129-136.

Costa, M., Martins, M., Jesus, C. and Duarte, J. 2008. Treatment of Acid Mine Drainage by Sulphate-reducing Bacteria Using Low Cost Matrices. *Water, Air, & Soil Pollution* 189(1), pp. 149-162.

Créach, V., Baudoux, A.-C., Bertru, G. and Rouzic, B. L. 2003. Direct estimate of active bacteria: CTC use and limitations. *Journal of Microbiological Methods* 52(1), pp. 19-28.

Cuthbert, M. O., Riley, M. S., Handley-Sidhu, S., Renshaw, J. C., Tobler, D. J., Phoenix, V. R. and Mackay, R. 2012. Controls on the rate of ureolysis and the morphology of carbonate precipitated by S. Pasteurii biofilms and limits due to bacterial encapsulation. *Ecological Engineering* 41(0), pp. 32-40.

Dada, A. O., Olalekan, A. P., Olatunya, A. M. and DADA, O. 2012. Langmuir, Freundlich, Temkin and Dubinin–Radushkevich Isotherms Studies of Equilibrium Sorption of Zn^{2+} Unto Phosphoric Acid Modified Rice Husk. *IOSR Journal of Applied Chemistry (IOSR-JAC)* 3(1), pp. 38-45.

Das, N., Vimala, R. and Karthika, P. 2008. Biosorption of heavy metals-An overview. *Indian Journal of Biotechnology (IJBT)* 7, pp. 159-169.

Davis, J. A., Fuller, C. C. and Cook, A. D. 1987. A model for trace metal sorption processes at the calcite surface: Adsorption of Cd2+ and subsequent solid solution formation. *Geochimica et Cosmochimica Acta* 51(6), pp. 1477-1490.

Dawodu, F. A., Akpomie, G. K. and Abuh, M. A. 2012. Batch Sorption of Lead(II) From Aqueous Stream by "Ekulu" Clay-Equilibrium, Kinetic and Thermodynamic Studies. *INTERNATIONAL JOURNAL OF MULTIDISCIPLINARY SCIENCES AND ENGINEERING, VOL. 3, NO. 10, OCTOBER 2012* 3(10), pp. 32-37.

de Nooijer, L. J., Reichart, G. J., DueÃ±as-BohÃ³rquez, A., Wolthers, M., Ernst, S. R., Mason, P. R. D. and van der Zwaan, G. J. T. 2007. Copper incorporation in foraminiferal calcite: results from culturing experiments. *Biogeosciences* 4, pp. 493-504.

Dejong, J. T., Fauriel, S. and Laloui, L. 2013. Biogeochemical processes and geotechnical applications: progress, opportunities and challenges. *Geotechnique - London-* 63(4), pp. 287–301.

DeJong, J. T., Fritzges, M. B. and Nüsslein, K. 2006. Microbially Induced Cementation to Control Sand Response to Undrained Shear. *Journal of Geotechnical and Geoenvironmental Engineering* 132(11), pp. 1381-1392.

DeJong, J. T., Mortensen, B. M., Martinez, B. C. and Nelson, D. C. 2010. Bio-mediated soil improvement. *Ecological Engineering* 36(2), pp. 197-210.

Desta, M. B. 2013. Batch Sorption Experiments: Langmuir and Freundlich Isotherm Studies for the Adsorption of Textile Metal Ions onto Teff Straw (Eragrostis tef) Agricultural Waste. *Journal of Thermodynamics* 2013, pp. 1-6.

Dhami, N. K., Reddy, M. S. and Mukherjee, A. 2013. Biomineralization of calcium carbonate polymorphs by the bacterial strains isolated from calcareous sites. *J Microbiol Biotechnol* 23(5), pp. 707-714.

Diels, L., Geets, J., Dejonghe, W., Roy, S. V. and Vanbroekhoven, K. eds. 2010. *Heavy Metal Immobilization In Groundwater By In Situ Bioprecipitation: Comments And Questions About Efficiency And Sustainability Of The Process*. Proceedings of the Annual International Conference on Soils, Sediments, Water and Energy.

Dixit, R., Wasiullah, Malaviya, D., Pandiyan, K., Singh, U., Sahu, A., Shukla, R., Singh, B., Rai, J., Sharma, P., Lade, H. and Paul, D. 2015. Bioremediation of Heavy Metals from Soil and Aquatic Environment: An Overview of Principles and Criteria of Fundamental Processes. *Sustainability* 7(2), p. 2189.

Doshi, S. M. 2006. *Bioremediation of Acid Mine Drainage Using Sulfate-Reducing Bacteria.* Washington, D.C.: U.S. Environmental Protection Agency.

Dragović, S., Mihailović, N. and Gajić, B. 2008. Heavy metals in soils: Distribution, relationship with soil characteristics and radionuclides and multivariate assessment of contamination sources. *Chemosphere* 72(3), pp. 491-495.

Du, Y., Lian, F. and Zhu, L. 2011. Biosorption of divalent Pb, Cd and Zn on aragonite and calcite mollusk shells. *Environmental Pollution* 159(7), pp. 1763-1768.

Dunham-Cheatham, S., Rui, X., Bunker, B., Menguy, N., Hellmann, R. and Fein, J. 2011. The effects of non-metabolizing bacterial cells on the precipitation of U, Pb and Ca phosphates. *Geochimica et Cosmochimica Acta* 75(10), pp. 2828-2847.

Duruibe, J. O., Ogwuegbu, M. O. C. and Egwurugwu, J. N. 2007. Heavy metal pollution and human biotoxic effects. *International Journal of Physical Sciences* 2(5), pp. 112-118.

Ehrlich, H. L. 1996a. How microbes influence mineral growth and dissolution. *Chemical Geology* 132(1-4), pp. 5-9.

Ehrlich, H. L. 1996b. How microbes influence mineral growth and dissolution. *Chemical Geology* 132(1–4), pp. 5-9.

Elzinga, E. J., Rouff, A. A. and Reeder, R. J. 2006. The long-term fate of Cu2+, Zn2+, and Pb2+ adsorption complexes at the calcite surface: An X-ray absorption spectroscopy study. *Geochimica et Cosmochimica Acta* 70(11), pp. 2715-2725.

Evans, D. J., Evans, D. G., Kirkpatrick, S. S. and Graham, D. Y. 1991. Characterization of the Helicobacter pylori urease and purification of its subunits. *Microbial Pathogenesis* 10(1), pp. 15-26.

Fairbrother, A., Wenstel, R., Sappington, K. and Wood, W. 2007. Framework for Metals Risk Assessment. *Ecotoxicology and Environmental Safety* 68(2), pp. 145-227.

Farshid, K., Moghtaderi, Y. and Jahromi, A. R. 2013. Isolation and identification of cadmium-resistant bacteria in Soltan Abad river sediments and determination of tolerance of bacteria through MIC and MBC. *European Journal of Experimental Biology* 3(5), pp. 268-273.

Faure, G. 1991. *Principles and applications of inorganic geochemistry : a comprehensive textbook for geology students*. New York; Toronto; New York: Macmillan Pub. Co. ; Collier Macmillan ; Maxwell Macmillan International.

Ferris, F. G., Fratton, C. M., Gerits, J. P., Schultze-Lam, S. and Lollar, B. S. 1995. Microbial precipitation of a strontium calcite phase at a groundwater discharge zone near rock Creek, British Columbia, Canada. *Geomicrobiology Journal* 13(1), pp. 57-67.

Ferris, F. G., Fyfe, W. S. and Beveridge, T. J. 1987. Bacteria as nucleation sites for authigenic minerals in a metal-contaminated lake sediment. *Chemical Geology* 63(3–4), pp. 225-232.

Fontes, D. E., Mills, A. L., Hornberger, G. M. and Herman, J. S. 1991. Physical and chemical factors influencing transport of microorganisms through porous media. *Applied and Environmental Microbiology* 57(9), pp. 2473-2481.

Foo, K. Y. and Hameed, B. H. 2010. Insights into the modeling of adsorption isotherm systems. *Chemical Engineering Journal* 156(1), pp. 2-10.

Franklin, M. L. and Morse, J. W. 1981. *Interaction of copper with the surface of calcite*.

Franklin, R. B. and Mills, A. L. 2009. Importance of spatially structured environmental heterogeneity in controlling microbial community composition at small spatial scales in an agricultural field. *Soil Biology and Biochemistry* 41(9), pp. 1833-1840.

Freitas, D. B., Reis, M. P., Lima-Bittencourt, C. I., Costa, P. S. and Assis, P. S. 2008. Chartone-Souza, E. and Nascimento, A. M. 2008. Genotypic and phenotypic diversity of Bacillus spp. isolated from steel plant waste. *BMC Res Notes* 1, p. 92.

Fujita, Y., Ferris, F. G., Lawson, R. D., Colwell, F. S. and Smith, R. W. 2000. Calcium Carbonate Precipitation by Ureolytic Subsurface Bacteria. *Geomicrobiology Journal* 17(4), pp. 305-318.

Fujita, Y., Redden, G. D., Ingram, J. C., Cortez, M. M., Ferris, F. G. and Smith, R. W. 2004. Strontium incorporation into calcite generated by bacterial ureolysis. *Geochimica et Cosmochimica Acta* 68(15), pp. 3261-3270.

Fujita, Y., Taylor, J. L., Gresham, T. L. T., Delwiche, M. E., Colwell, F. S., McLing, T. L., Petzke, L. M. and Smith, R. W. 2008. Stimulation Of Microbial Urea Hydrolysis In Groundwater To Enhance Calcite Precipitation. *Environmental Science & Technology* 42(8), pp. 3025-3032.

Fujita, Y., Taylor, J. L., Wendt, L. M., Reed, D. W. and Smith, R. W. 2010. Evaluating the Potential of Native Ureolytic Microbes To Remediate a 90Sr Contaminated Environment. *Environmental Science & Technology* 44(19), pp. 7652-7658.

Gadd, G. M. 2000. Bioremedial potential of microbial mechanisms of metal mobilization and immobilization. *Current Opinion in Biotechnology* 11(3), pp. 271-279.

Gadd, G. M. 2002. Chapter 6 Microbial interactions with metals/radionuclides: The basis of bioremediation. In: Keith-Roach, M.J. and Livens, F.R. eds. *Radioactivity in the Environment*. Vol. Volume 2. Elsevier, pp. 179-203.

Gadd, G. M. 2010. Metals, minerals and microbes: geomicrobiology and bioremediation. *Microbiology* 156(3), pp. 609-643.

García-Sánchez, A. and Álvarez-Ayuso, E. 2002. Sorption of Zn, Cd and Cr on calcite. Application to purification of industrial wastewaters. *Minerals Engineering* 15(7), pp. 539-547.

Gebrehiwet, T., Redden, G., Fujita, Y., Beig, M. and Smith, R. 2012. The Effect of the CO3 2- to Ca2+ Ion activity ratio on calcite precipitation kinetics and Sr2+ partitioning. *Geochemical Transactions* 13(1), pp. 1-11.

Gerlach, R. 2001. *TRANSPORT AND ACTIVITY OF DISSIMILATORY METAL-REDUCING BACTERIA IN POROUS MEDIA FOR THE REMEDIATION OF HEAVY METALS AND CHLORINATED HYDROCARBONS.* Dissertation, Montana State University.

Ghazvini, T. M., P., Ghorbanzadeh Mashkani, S. and Ghafourian, H. 2007. Bio sorption of strontium from aqueous solution by New Strain *Bacillus sp.* GTG-83. In: *WM'07: Waste Management Symposium 2007 - Global Accomplishments in Environmental and Radioactive Waste Management: Education and Opportunity for the Next Generation of Waste Management Professionals.* united state.

Gunawardana, C., Egodawatta, P. and Goonetilleke, A. 2015. Adsorption and mobility of metals in build-up on road surfaces. *Chemosphere* 119, pp. 1391-1398.

Gustafsson, J. P., 2013. Visual MINTEQ 3.0/3.1 ed.: KTH, Sweden, since 2000.

Hammes, F., Boon, N., de Villiers, J., Verstraete, W. and Siciliano, S. D. 2003. Strain-Specific Ureolytic Microbial Calcium Carbonate Precipitation. *Applied and Environmental Microbiology* 69(8), pp. 4901-4909.

Harkes, M. P., van Paassen, L. A., Booster, J. L., Whiffin, V. S. and van Loosdrecht, M. C. M. 2010. Fixation and distribution of bacterial activity in sand to induce carbonate precipitation for ground reinforcement. *Ecological Engineering* 36(2), pp. 112-117.

Hashim, M. A., Mukhopadhyay, S., Sahu, J. N. and Sengupta, B. 2011. Remediation technologies for heavy metal contaminated groundwater. *Journal of Environmental Management* 92(10), pp. 2355-2388.

Hausinger, R. P. 1993. *Biochemistry of nickel* [Online]. New York: Plenum Press. Available at: http://catalog.hathitrust.org/api/volumes/oclc/28889256.html.

Hausinger, R. P. 2004. Metabolic Versatility of Prokaryotes for Urea Decomposition. *Journal of Bacteriology* 186(9), pp. 2520-2522.

Holden, P. A. and Fierer, N. 2005. Microbial Processes in the Vadose Zone. *Vadose Zone Journal* 4(1), pp. 1-21.

Hu, Q., Dou, M.-n., Qi, H.-y., Xie, X.-m., Zhuang, G.-q. and Yang, M. 2007. Detection, isolation, and identification of cadmium-resistant bacteria based on PCR-DGGE. *Journal of Environmental Sciences* 19(9), pp. 1114-1119.

Jaysankar, D., Ramaiah, N. and Vardanyan, L. 2008. Detoxification of toxic heavy metals by marine bacteria highly resistant to mercury. *Marine Biotechnology* 10(4), pp. 471-477.

Kalra, Y. P. 1995. Determination of pH of soils by different methods: collaborative study. *JOURNAL OF AOAC INTERNATIONAL* 78(2), pp. 310-324.

Kamnev, A. A., Kuzmann, E., Perfiliev, Y. D., Vértes, A., Ristić, M., Popović, S. and Musić, S. 2000. Composite Ferric Oxyhydroxide-Containing Phases Formed in Neutral Aqueous Solutions of Tryptophan and Indole-3-Acetic Acid. *Journal of Radioanalytical and Nuclear Chemistry* 246(1), pp. 123-129.

Kang, C. H., Han, S. H., Shin, Y., Oh, S. J. and So, J. S. 2014. Bioremediation of Cd by microbially induced calcite precipitation. *Appl Biochem Biotechnol* 172(4), pp. 1929-1937.

Kapetas, L., Ngwenya, B. T., Macdonald, A. M. and Elphick, S. C. 2012. Thermodynamic and kinetic controls on cotransport of Pantoea agglomerans cells and Zn through clean and iron oxide coated sand columns. *Environ Sci Technol* 46(24), pp. 13193-13201.

Karakagh, R. M., Chorom, M., Motamedi, H., Kalkhajeh, Y. K. and Oustan, S. 2012. Biosorption of Cd and Ni by inactivated bacteria isolated from agricultural soil treated with sewage sludge. *Ecohydrology & Hydrobiology* 12(3), pp. 191-198.

Kawaguchi, T. and Decho, A. W. 2002. A laboratory investigation of cyanobacterial extracellular polymeric secretions (EPS) in influencing CaCO3 polymorphism. *Journal of Crystal Growth* 240(1–2), pp. 230-235.

Khosravi, J. and Alamdari, A. 2009a. Copper removal from oil-field brine by coprecipitation. *Journal of Hazardous Materials* 166(2-3), pp. 695-700.

Khosravi, J. and Alamdari, A. 2009b. Copper removal from oil-field brine by coprecipitation. *J Hazard Mater* 166(2-3), pp. 695-700.

Khusro, A., Preetam, R. J. P. and Panicker, S. G. 2014. Multiple heavy metals response and antibiotic sensitivity pattern of Bacillus subtilis strain KPA. *Journal of Chemical and Pharmaceutical Research* 6(8), pp. 532-538.

Kung, K. J. S. 1990. PREFERENTIAL FLOW IN A SANDY VADOSE ZONE .2. MECHANISM AND IMPLICATIONS. *Geoderma* 46(1-3), pp. 59-71.

Kurniawan, T. A., Chan, G. Y. S., Lo, W.-H. and Babel, S. 2006. Physico–chemical treatment techniques for wastewater laden with heavy metals. *Chemical Engineering Journal* 118(1–2), pp. 83-98.

Lafuente, A. L., González, C., Quintana, J. R., Vázquez, A. and Romero, A. 2008. Mobility of heavy metals in poorly developed carbonate soils in the Mediterranean region. *Geoderma* 145(3–4), pp. 238-244.

Lauchnor, E. G., Schultz, L. N., Bugni, S., Mitchell, A. C., Cunningham, A. B. and Gerlach, R. 2013. Bacterially induced calcium carbonate precipitation and strontium coprecipitation in a porous media flow system. *Environ Sci Technol* 47(3), pp. 1557-1564.

Lea, A. S., Amonette, J. E., Baer, D. R., Liang, Y. and Colton, N. G. 2001. Microscopic effects of carbonate, manganese, and strontium ions on calcite dissolution. *Geochimica et Cosmochimica Acta* 65(3), pp. 369-379.

Lee, M.-K. and Saunders, J. A. 2003. Effects of pH on Metals Precipitation and Sorption. *Vadose Zone Journal* 2(2), pp. 177-185.

Leij, F. J. and Van Genuchten, M. T. 1995. Approximate analytical solutions for solute transport in two-layer porous media. *Transport in Porous Media* 18(1), pp. 65-85.

Lema, M. W., Ijumba, J. N., Njau, K. N. and Ndakidemi, P. A. 2014. Environmental Contamination By Radionuclides And Heavy Metals Through The Application Of Phosphate RocksDuring Farming And Mathematical Modeling Of Their Impacts To The Ecosystem. *International Journal of Engineering Research and General Science* 2(4), pp. 852-863.

Li, M., Cheng, X. and Guo, H. 2013. Heavy metal removal by biomineralization of urease producing bacteria isolated from soil. *International Biodeterioration & Biodegradation* 76(0), pp. 81-85.

Li, W., Chen, W.-S., Zhou, P.-P., Cao, L. and Yu, L.-J. 2013c. Influence of initial pH on the precipitation and crystal morphology of calcium carbonate induced by microbial carbonic anhydrase. *Colloids and Surfaces B: Biointerfaces* 102(0), pp. 281-287.

Liu, Y. Y., Zhao, Y. S., Dong, J., Liu, P., Zhu, Z. G. and Sun, Y. 2008. [pH buffering capacity of geological media on landfill leachate]. *Huan Jing Ke Xue* 29(7), pp. 1948-1954.

Lloyd, A. B. and Sheaffe, M. J. 1973. Urease activity in soils. *Plant and Soil* 39(1), pp. 71-80.

Lloyd, J. R. 2002. Bioremediation of metals; the application of micro-organisms that make and break minerals. *MICROBIOLOGYTODAY* 29, pp. 67-69.

Lloyd, J. R. and Lovley, D. R. 2001. Microbial detoxification of metals and radionuclides. *Current Opinion in Biotechnology* 12(3), pp. 248-253.

Lorens, R. B. 1981. Sr, Cd, Mn and Co distribution coefficients in calcite as a function of calcite precipitation rate. *Geochimica et Cosmochimica Acta* 45(4), pp. 553-561.

Macaskie, L. E. 1990. An immobilized cell bioprocess for the removal of heavy metals from aqueous flows. *Journal of Chemical Technology & Biotechnology* 49(4), pp. 357-379.

Macaskie, L. E., DEAN, A. C. R., CHEETHAM, A. K., JAKEMAN, R. J. B. and SKARNULIS, A. J. 1987. Cadmium Accumulation by a Citrobacter sp.: the Chemical Nature of the Accumulated Metal Precipitate and its Location on the Bacterial Cells. *Journal of General Microbiology* 133(3), pp. 539-544.

Madsen, E. L. 2003. *Report on bioavailability of chemical wastes with respect to the potential for soil bioremediation*. National government publication : English ed. Washington: National Center for Environmental Research. United States. Environmental Protection Agency. Office of Research and Development; Cornell University. Dept. of Microbiology.

Maier, R. M., Ian L. Pepper and Gerba, C. P. 2000. *Environmental Microbiology*. illustrated edition ed. Elsevier Science & Technology Academic Press Inc.

Majdalani, S., Chazarin, J. P., Delenne, C. and Guinot, V. 2015. Solute transport in periodical heterogeneous porous media: Importance of observation scale and experimental sampling. *Journal of Hydrology* 520, pp. 52-60.

Marsily, G. D. 1986. *Quantitative Hydrogeology.Groundwater Hydrology for Engineers*. Academic Press INC(LONDON).

Martinez, R. J., Beazley, M. J., Taillefert, M., Arakaki, A. K., Skolnick, J. and Sobecky, P. A. 2007. Aerobic uranium (VI) bioprecipitation by metal-resistant bacteria isolated from radionuclide- and metal-contaminated subsurface soils. *Environmental Microbiology* 9(12), pp. 3122-3133.

McBride, M. B. 1980. Chemisorption of Cd2+ on Calcite Surfaces1. *Soil Sci. Soc. Am. J.* 44(1), pp. 26-28.

McLean, J. E., Bledsoe, B. E. and United States Environmental Protection Agency. 1992. *Behavior of metals in soils: Ground Water Issue*. Washington, DC; [Ada, Okla.]: Technology Innovation Office, Office of Solid Waste and Emergency Response] ; [Superfund Technology Support Center for Ground Water, Robert S. Kerr Environmental Research Laboratory].

McNeal, J. M. and Balistrieri, L. S. 1989. *Geochemistry and occurrence of selenium: an overview*. American Society of Agronomy: Madison, WI.

Megharaj, M., Ramakrishnan, B., Venkateswarlu, K., Sethunathan, N. and Naidu, R. 2011. Bioremediation approaches for organic pollutants: A critical perspective. *Environment International* 37(8), pp. 1362-1375.

Merdun, H. 2012. Investigation of Interactive Effects on Water Flow and Solute Transport in Sandy Loam Soil Using Time Domain Reflectometry. *Sensors (Basel, Switzerland)* 12(7), pp. 9749-9772.

Mgbemena, I. C., Nnokwe, J. C., Adjeroh, L. A. and Onyemekara, N. N. 2012. Resistance of Bacteria Isolated from Otamiri River to Heavy Metals and Some Selected Antibiotics. *Current Research Journal of Biological Sciences* 4(5), pp. 551-556.

Mitchell, A. C. and Ferris, F. G. 2005. The coprecipitation of Sr into calcite precipitates induced by bacterial ureolysis in artificial groundwater: Temperature and kinetic dependence. *Geochimica et Cosmochimica Acta* 69(17), pp. 4199-4210.

Mitchell, A. C. and Ferris, F. G. 2006a. Effect of strontium contaminants upon the size and solubility of calcite crystals precipitated by the bacterial hydrolysis of urea. *Environ Sci Technol* 40(3), pp. 1008-1014.

Mitchell, A. C. and Ferris, F. G. 2006b. The Influence of Bacillus pasteurii on the Nucleation and Growth of Calcium Carbonate. *Geomicrobiology Journal* 23(3-4), pp. 213-226.

Mockaitis, G., Rodrigues, J. A., Foresti, E. and Zaiat, M. 2012. Toxic effects of cadmium (Cd(2)(+)) on anaerobic biomass: kinetic and metabolic implications. *J Environ Manage* 106, pp. 75-84.

Montgomery, D. M., Dean, A. C. R., Wiffen, P. and Macaskie, L. E. 1995. Phosphatase production and activity in Citrobacter freundii and a naturally occurring, heavy-metal-accumulating Citrobacter sp. *Microbiology* 141(10), pp. 2433-2441.

Morales, V. L., Parlange, J. Y. and Steenhuis, T. S. 2010. Are preferential flow paths perpetuated by microbial activity in the soil matrix? A review. *Journal of Hydrology* 393(1–2), pp. 29-36.

Mucci, A. and Morse, J. W. 1983. The incorporation of Mg2+ and Sr2+ into calcite overgrowths: influences of growth rate and solution composition. *Geochimica et Cosmochimica Acta* 47(2), pp. 217-233.

Murthy, S., Bali, G. and Sarangi, S. K. 2011. Effect of lead on metallothionein concentration in leadresistant bacteria Bacillus cereus isolated from industrial effluent. *African Journal of Biotechnology* 10(71), pp. 15966-15972.

Mustafa, G., Singh, B. and Kookana, R. S. 2004. Cadmium adsorption and desorption behaviour on goethite at low equilibrium concentrations: effects of pH and index cations. *Chemosphere* 57(10), pp. 1325-1333.

Nath, S., Bibhas Deb and Indu Sharma. 2012. Isolation and Characterization of Cadmium and Lead Resistant Bacteria. *Global Advanced Research Journal of Microbiology* 1(11), pp. 194-198.

National Research Council. 2003. *Bioavailability of Contaminants in Soils and Sediments: Processes, Tools, and Applications*. Washington, DC: The National Academies Press, p. 432.

Newsome, L., Morris, K. and Lloyd, J. R. 2014. The biogeochemistry and bioremediation of uranium and other priority radionuclides. *Chemical Geology* 363(0), pp. 164-184.

Ni, M. and Ratner, B. D. 2008. Differentiation of Calcium Carbonate Polymorphs by Surface Analysis Techniques – An XPS and TOF-SIMS study. *Surface and interface analysis : SIA* 40(10), pp. 1356-1361.

Nies, D. H. 1999. Microbial heavy-metal resistance. *Appl Microbiol Biotechnol* 51(6), pp. 730-750.

Nies, D. H. and Silver, S. 1995. Ion efflux systems involved in bacterial metal resistances. *J Ind Microbiol* 14(2), pp. 186-199.

Nweke, C. O., Alisi, C. S., Okolo, J. C. and Nwanyanwu, C. E. 2007. Toxicity of Zinc To Heterotrophic Bacteria From A Tropical River Sediment. *Applied Ecology and Environmental Research* 5(1), pp. 123-132.

Okwadha, G. D. O. and Li, J. 2010. Optimum conditions for microbial carbonate precipitation. *Chemosphere* 81(9), pp. 1143-1148.

Oladipo, I. C. and Adeoye, I. O. 2013. Synthesis and Antimicrobial Study of Some Transition Metal Complexes of Picolinic Acid Hydrazide. *Journal of Pharmaceutical and Biological Sciences* 1(4), pp. 34-39.

Or, D., Smets, B. F., Wraith, J. M., Dechesne, A. and Friedman, S. P. 2007. Physical constraints affecting bacterial habitats and activity in unsaturated porous media – a review. *Advances in Water Resources* 30(6–7), pp. 1505-1527.

Ozer, G., Ergene, A. and Icgen, B. 2012. Biochemical and Molecular Characterization of Strontium-resistant Environmental Isolates of Pseudomonas fluorescens and Sphingomonas paucimobilis. *Geomicrobiology Journal* 30(5), pp. 381-390.

Pan, S., Shen, H., Xu, Q., Luo, J. and Hu, M. 2012. Surface mercapto engineered magnetic Fe3O4 nanoadsorbent for the removal of mercury from aqueous solutions. *J Colloid Interface Sci* 365(1), pp. 204-212.

Papadopoulos, P. and Rowell, D. L. 1989. The reactions of copper and zinc with calcium carbonate surfaces. *Journal of Soil Science* 40(1), pp. 39-48.

Park, J. H., Lamb, D., Paneerselvam, P., Choppala, G., Bolan, N. and Chung, J. W. 2011. Role of organic amendments on enhanced bioremediation of heavy metal(loid) contaminated soils. *J Hazard Mater* 185(2-3), pp. 549-574.

Parkman, R. H., Charnock, J. M., Livens, F. R. and Vaughan, D. J. 1998. A study of the interaction of strontium ions in aqueous solution with the surfaces of calcite and kaolinite. *Geochimica et Cosmochimica Acta* 62(9), pp. 1481-1492.

Phillips, A. J., Gerlach, R., Lauchnor, E., Mitchell, A. C., Cunningham, A. B. and Spangler, L. 2013. Engineered applications of ureolytic biomineralization: a review. *Biofouling* 29(6), pp. 715-733.

Pingitore Jr, N. E. and Eastman, M. P. 1986. The coprecipitation of Sr2+ with calcite at 25°C and 1 atm. *Geochimica et Cosmochimica Acta* 50(10), pp. 2195-2203.

Pivetz, B. E. and Steenhuis, T. S. 1995. Biodegradation and bioremediation: soil matrix and macropore biodegradation of 2, 4-D. *J. environ. Quality* 24, pp. 564-570.

Postgate, J. R. 1979. *THE SULFATE REDUCING BACTERIA*.

Postgate, J. R. 1984. *The Sulphate-Reducing Bacteria*. Cambridge University Press, p. 224.

Prakash, D., Gabani, P., Chandel, A. K., Ronen, Z. and Singh, O. V. 2013. Bioremediation: a genuine technology to remediate radionuclides from the environment. *Microbial Biotechnology* 6(4), pp. 349-360.

Prieto, M., Astilleros, J. M. and Fernandez-Diaz, L. 2013. Environmental Remediation by Crystallization of Solid Solutions. *Elements* 9(3), pp. 195-201.

Rajendran, P., Muthukrishnan, J. and Gunasekaran, P. 2003. Microbes in heavy metal remediation. *Indian J Exp Biol* 41(9), pp. 935-944.

Ranjard, L., Nazaret, S., Gourbière, F., Thioulouse, J., Linet, P. and Richaume, A. 2000. A soil microscale study to reveal the heterogeneity of Hg(II) impact on indigenous bacteria by quantification of adapted phenotypes and analysis of community DNA fingerprints. *FEMS Microbiology Ecology* 31(2), pp. 107-115.

Rathnayake, I., Mallavarapu, M., Bolan, N. S., Krishnamurti, G. S. R. and Naidu, R. 2013. Heavy metal toxicity to bacteria – Are the existing growth media accurate enough to determine heavy metal toxicity? *Chemosphere* 90, pp. 1195-1200.

Rathnayake, I., Mallavarapu, M., Bolan, N. S. and Naidu, R. 2009. Tolerance of heavy metals by Gram positive soil bacteria. *International journal of environmental science and engineering* 2(4), pp. 191-195

Reddy, M. S. 2013. Biomineralization of calcium carbonates and their engineered applications: a review. *Frontiers in Microbiology* 4.

Reed, D. W., Smith, J. M., Francis, C. A. and Fujita, Y. 2010. Responses of Ammonia-Oxidizing Bacterial and Archaeal Populations to Organic Nitrogen Amendments in Low-Nutrient Groundwater. *Applied and Environmental Microbiology* 76(8), pp. 2517-2523.

Ritsema, C. J., Dekker, L. W., Hendrickx, J. M. H. and Hamminga, W. 1993. PREFERENTIAL FLOW MECHANISM IN A WATER REPELLENT SANDY SOIL. *Water Resources Research* 29(7), pp. 2183-2193.

Rivadeneyra, M. A., Ramos-Cormenzana, A., Delgado, G. and Delgado, R. 1996. Process of Carbonate Precipitation by Deleya halophila. *Curr Microbiol* 32(6), pp. 308-313.

Roane, T. M. 1999. Lead Resistance in Two Bacterial Isolates from Heavy Metal-Contaminated Soils. *Microb Ecol* 37(3), pp. 218-224.

Roane, T. M. and Kellogg, S. T. 1996. Characterization of bacterial communities in heavy metal contaminated soils. *Can J Microbiol* 42(6), pp. 593-603.

Rosenbom, A. E. and Undersøgelse, D. D. o. G. G. 2005. *Preferential Flow and Transport in Variably Saturated Fractured Media: Ph.d.-thesis*. GEUS, Geological Survey of Denmark and Greenland.

Rouff, A. A., Elzinga, E. J. and Reeder, R. J. 2006. The Effect of Aging and pH on Pb(II) Sorption Processes at the Calcite-Water Interface. *Environmental Science and Technology* 40, pp. 1792-1798.

Roy, W. R., Illinois State Geological, S., Risk Reduction Engineering, L. and United, S. 1992. *Technical resource document: batch-type procedures for estimating soil adsorption of chemicals*. Washington, D.C. : Cincinnati, Ohio: Office of Solid Waste and Emergency Response, U.S. Environmental Protection Agency ; Risk Reduction Engineering Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, pp. xi, 100 p.

Ruggiero, C. E., Boukhalfa, H., Forsythe, J. H., Lack, J. G., Hersman, L. E. and Neu, M. P. 2005. Actinide and metal toxicity to prospective bioremediation bacteria. *Environmental Microbiology* 7(1), pp. 88-97.

Samanta, A., Bera, P., Khatun, M., Sinha, C., Pal, P., Lalee, A. and Mandal., A. 2012. An investigation on heavy metal tolerance and antibiotic resistance properties of bacterial strain Bacillus sp. isolated from municipal waste. *Journal of Microbiology and Biotechnology Research* 1(2), pp. 178-189.

Satyawali, Y., Schols, E., Van Roy, S., Dejonghe, W., Diels, L. and Vanbroekhoven, K. 2010. Stability investigations of zinc and cobalt precipitates immobilized by in situ bioprecipitation (ISBP) process. *Journal of Hazardous Materials* 181(1–3), pp. 217-225.

Schosseler, P. M., Schweiger, A. and Wehrli, B. 1999. Uptake of Cu{sup 2+} by the calcium carbonates vaterite and calcite as studied by continuous wave (CW) and pulse electron paramagnetic resonance. *Journal Name: Geochimica et Cosmochimica Acta; Journal Volume: 63; Journal Issue: 13-14; Other Information: PBD: Jul 1999*, pp. Medium: X; Size: pp. 1955-1967.

Scow, K. M. and Johnson., C. R. 1997. Effect of sorption on biodegradation of soil pollutants,. *Advances in Agronomy,* 58, pp. 1-58.

Sessitsch, A., Weilharter, A., Gerzabek, M. H., Kirchmann, H. and Kandeler, E. 2001. Microbial Population Structures in Soil Particle Size Fractions of a Long-Term Fertilizer Field Experiment. *Applied and Environmental Microbiology* 67(9), pp. 4215-4224.

Shahwan, T., Zunbul, B. and Akar, D. 2005. Study of the scavenging behavior and structural changes accompanying the interaction of aqueous Pb2+ and Sr2+ ions with calcite. *GEOCHEMICAL JOURNAL* 39(4), pp. 317-326.

Sieracki, M. E., Cucci, T. L. and Nicinski, J. 1999. Flow cytometric analysis of 5-cyano-2,3-ditolyl tetrazolium chloride activity of marine bacterioplankton in dilution cultures. *Appl Environ Microbiol* 65(6), pp. 2409-2417.

Silva-Castro, G. A., Uad, I., Gonzalez-Martinez, A., Rivadeneyra, A., Gonzalez-Lopez, J. and Rivadeneyra, M. A. 2015. Bioprecipitation of Calcium Carbonate Crystals by Bacteria Isolated from Saline Environments Grown in Culture Media Amended with Seawater and Real Brine. *BioMed Research International* 2015, p. 12.

Simmons, C. T., Fenstemaker, T. R. and Sharp Jr, J. M. 2001. Variable-density groundwater flow and solute transport in heterogeneous porous media: approaches, resolutions and future challenges. *Journal of Contaminant Hydrology* 52(1–4), pp. 245-275.

Singh, R., Gautam, N., Mishra, A. and Gupta, R. 2011. Heavy metals and living systems: An overview. *Indian Journal of Pharmacology* 43(3), pp. 246-253.

Skopp, J. 1981. Comment on 'micro-, meso- and macroporosity of soil. *Soil Science Society of America Journal* 45, p. 1246.

Soejima, T., Itoh, M. and Imamura, S. 2010. Column Experiment on Nitrate Purification with Polylactic Acid and Iron Powder. *Water Air and Soil Pollution* 213(1-4), pp. 485-492.

Sondi, I. and Salopek-Sondi, B. 2005. Influence of the primary structure of enzymes on the formation of CaCO2 polymorphs: a comparison of plant (Canavalia ensiformis) and bacterial (Bacillus pasteurii) ureases. *Langmuir* 21(19), pp. 8876-8882.

Stahl, R. S. and James, B. R. 1991. Zinc Sorption by B Horizon Soils as a Function of pH. *Soil Sci. Soc. Am. J.* 55(6), pp. 1592-1597.

Stietiya, M. H. and Wang, J. J. 2014. Zinc and cadmium adsorption to aluminum oxide nanoparticles affected by naturally occurring ligands. *J Environ Qual* 43(2), pp. 498-506.

Stipp, S. L., Hochella, M. F., Parks, G. A. and Leckie, J. O. 1992. Cd^{2+} uptake by calcite, solid-state diffusion, and the formation of solid-solution: Interface processes observed with near-surface sensitive techniques (XPS, LEED, and AES). *Geochimica et Cosmochimica Acta* 56, pp. 1941-1954.

Stocks-Fischer, S., Galinat, J. K. and Bang, S. S. 1999. Microbiological precipitation of CaCO3. *Soil Biology and Biochemistry* 31(11), pp. 1563-1571.

Stolp, H. 1988. *Microbial Ecology: Organisms, Habitats, Activities*. Cambridge University Press.

Sturchio, N. C., Chiarello, R. P., Cheng, L., Lyman, P. F., Bedzyk, M. J., Qian, Y., You, H., Yee, D., Geissbuhler, P., Sorensen, L. B., Liang, Y. and Baer, D. R. 1997. Lead adsorption at the calcite-water interface: Synchrotron X-ray standing wave and X-ray reflectivity studies. *Geochimica et Cosmochimica Acta* 61(2), pp. 251-263.

Sullivan, T. S., McBride, M. B. and Thies, J. E. 2013. Soil bacterial and archaeal community composition reflects high spatial heterogeneity of pH, bioavailable Zn, and Cu in a metalliferous peat soil. *Soil Biology and Biochemistry* 66, pp. 102-109.

Sun, Y., Wu, Q.-T., Lee, C. C. C., Li, B. and Long, X. 2014. Cadmium Sorption Characteristics of Soil Amendments and its Relationship with the Cadmium Uptake by Hyperaccumulator and Normal Plants in Amended Soils. *International Journal of Phytoremediation* 16(5), pp. 496-508.

Szewczyk, A., I., Z. and G., M. eds. 2007. *The effect of various carbon sources on stimulating bioprecipitation of heavy metals in the saturated zone*. Proceedings of the 10th International Conference on Environmental Science and Technology. Kos island, Greece, 05-07 sep. University of the Aegean.

Tamil Selvi, A., E.Anjugam, Devi, R. A., B.Madhan, S.Kannappan and B.Chandrasekaran. 2012. Isolation and Characterization of Bacteria from Tannery Effluent Treatment Plant and Their Tolerance to Heavy Metals and Antibiotics. *ASIAN J. EXP. BIOL. SCI.* 3(1), pp. 34-41.

Tammert, H., Olli, K., Sturluson, M. and Hodal, H. 2008. Bacterial biomass and activity in the marginal ice zone of the northern Barents Sea. *Deep Sea Research Part II: Topical Studies in Oceanography* 55(20–21), pp. 2199-2209.

Tchounwou, P. B., Yedjou, C. G., Patlolla, A. K. and Sutton, D. J. 2012. Heavy Metals Toxicity and the Environment. *EXS* 101, pp. 133-164.

Teclu, D. G., Laing, M. D. and Wallis, F. M. eds. 2009. *BIOREMEDIATION OF CONTAMINATED WATER SOURCES WITH SULPHATE-REDUCING BACTERIA*. the International Mine Water Conference. Pretoria, South Africa, 19th – 23rd October 2009.

Teng, H. H. and Zhao, L. 2012. Surface Behavior of Calcite upon Uptake of Cd2+ and Pb2+. *Geological Journal of China Universities* 18(2), pp. 193-202.

Tesoriero, A. J. and Pankow, J. F. 1996. Solid solution partitioning of Sr2+, Ba2+, and Cd2+ to calcite. *Geochimica et Cosmochimica Acta* 60(6), pp. 1053-1063.

Tobler, D. J., Cuthbert, M. O., Greswell, R. B., Riley, M. S., Renshaw, J. C., Handley-Sidhu, S. and Phoenix, V. R. 2011. Comparison of rates of ureolysis between Sporosarcina pasteurii and an indigenous groundwater community under conditions required to precipitate large volumes of calcite. *Geochimica et Cosmochimica Acta* 75(11), pp. 3290-3301.

Tobler, D. J., Cuthbert, M. O. and Phoenix, V. R. 2014. Transport of Sporosarcina pasteurii in sandstone and its significance for subsurface engineering technologies. *Applied Geochemistry* 42(0), pp. 38-44.

Tobler, D. J., Rodriguez-Blanco, J. D., Dideriksen, K., Bovet, N., Sand, K. K. and Stipp, S. L. S. 2015. Citrate Effects on Amorphous Calcium Carbonate (ACC) Structure, Stability, and Crystallization. *Advanced Functional Materials* 25(20), pp. 3081-3090.

Todar, K. 2005. Todar s online textbook of bacteriology: the genus Bacillus. *University of Wisconsin- Madison, Department of bacteriology*.

Torkzaban, S., Tazehkand, S. S., Walker, S. L. and Bradford, S. A. 2008. Transport and fate of bacteria in porous media: Coupled effects of chemical conditions and pore space geometry. *Water Resour. Res.* 44(4), p. W04403.

Torres, L. G., Lopez, R. B. and Beltran, M. 2012. Removal of As, Cd, Cu, Ni, Pb, and Zn from a highly contaminated industrial soil using surfactant enhanced soil washing. *Physics and Chemistry of the Earth, Parts A/B/C* 37–39, pp. 30-36.

Treves, D. S., Xia, B., Zhou, J. and Tiedje, J. M. 2003. A two-species test of the hypothesis that spatial isolation influences microbial diversity in soil. *Microb Ecol* 45(1), pp. 20-28.

Tsezos, M. 2007. Biological Removal of Ions: Principles and Applications. *Advanced Materials Research* 20 - 21, pp. 589-596.

Tsukada, H., Shibata, H. and Sugiyama, H. 1998. Transfer of radiocaesium and stable caesium from substrata to mushrooms in a pine forest in Rokkasho-mura, Aomori, Japan. *Journal of Environmental Radioactivity* 39(2), pp. 149-160.

United States Environmental Protection Agency. 2006. *In situ treatment technologies for contaminated soil [electronic resource]*. [Washington, DC]: U.S. Environmental Protection Agency, Office of Solid Waste and Emergency Response [and] Office of Research and Development.

US EPA. 1992. Groundwater Issue- Behavior of Metals in Soils, EPA/540/S-92/018, by Joan McLean and Bert E. Bledsoe.

Usman, A. R. A. 2008. The relative adsorption selectivities of Pb, Cu, Zn, Cd and Ni by soils developed on shale in New Valley, Egypt. *Geoderma* 144(1–2), pp. 334-343.

Vadasz, P. 1993. Fluid flow through heterogeneous porous media in a rotating square channel. *Transport in Porous Media* 12(1), pp. 43-54.

Vahabi, A., Ramezanianpour, A. A., Sharafi, H., Zahiri, H. S., Vali, H. and Noghabi, K. A. 2015. Calcium carbonate precipitation by strain Bacillus licheniformisAK01, newly isolated from loamy soil: a promising alternative for sealing cement-based materials. *Journal of Basic Microbiology* 55(1), pp. 105-111.

Valsami-Jones, E. 2004. *Phosphorus in environmental technologies*. London: IWA Publishing.

Vig, K., Megharaj, M., Sethunathan, N. and Naidu, R. 2003. Bioavailability and toxicity of cadmium to microorganisms and their activities in soil: a review. *Advances in Environmental Research* 8(1), pp. 121-135.

Viggi, C. C., Pagnanelli, F., Cibati, A., Uccelletti, D., Palleschi, C. and Toro, L. 2010. Biotreatment and bioassessment of heavy metal removal by sulphate reducing bacteria in fixed bed reactors. *Water Research* 44(1), pp. 151-158.

Viggi, C. C., Pagnanelli, F. and Toro, L. 2009. Sulphate bioreduction for the treatment of polluted waters: solid versus liquid organic substrates. *Journal of Chemical Technology & Biotechnology* 84(6), pp. 859-863.

Vos, M., Wolf, A. B., Jennings, S. J. and Kowalchuk, G. A. 2013. Micro-scale determinants of bacterial diversity in soil. *FEMS Microbiol Rev* 37(6), pp. 936-954.

Wang, L. K., Hung, Y. T., Lo, H. H. and Yapijakis, C. 2006. *Hazardous Industrial Waste Treatment*. CRC Press.

Wang, Z. 2008. Study on the Phenomenon of Preferential Flow in Seepage Process of Reservoir Porous Medium.

Wang, Z., Feyen, J. and Rietsma, C. J. 1998. Susceptibility and predsctibility of conditions for preferential flow. *Water Resour* 34(9), pp. 2169-2182.

Wang, Z., Wu, Q. J., Wu, L., R., C.J., D. and L.W., F., J. 2000. Effects of soil water repellency on infiltration rate and flow instability. *Journal of Hydrology* 231, pp. 265–276.

Warren, L. A., Maurice, P. A., Parmar, N. and Grant Ferris, F. 2001. Microbially Mediated Calcium Carbonate Precipitation: Implications for Interpreting Calcite Precipitation and for Solid-Phase Capture of Inorganic Contaminants. *Geomicrobiology Journal* 18(1), pp. 93-115.

Wei, S., Cui, H., Jiang, Z., Liu, H., He, H. and Fang, N. 2015. Biomineralization processes of calcite induced by bacteria isolated from marine sediments. *Brazilian Journal of Microbiology* 46(2), pp. 455-464.

Wernick, I. K. and Themelis, N. J. 1998. RECYCLING METALS FOR THE ENVIRONMENT. *Annual Review of Energy and the Environment* 23, pp. 465-497.

Whiffin, V. S. 2004. *Microbial CaCO3 precipitation for the production of biocement.* Murdoch university.

Whiffin, V. S., Paassen, v., Harkes, L. A. and P., M. 2007. Microbial Carbonate Precipitation as a Soil Improvement Technique. *GEOMICROBIOLOGY JOURNAL* 24(4), pp. 417-423.

Wilkin, R. T., Acree, S. D., Beak, D. G., Ross, R. R., Lee, T. R. and Paul, C. J. 2008. *Field application of a permeable reactive barrier for treatment of arsenic in ground water [electronic resource*. Ada, Okla: U.S. Environmental Protection Agency, Office of Research and Development, National Risk Management Research Laboratory.

Wu, L., Gao, B., Tian, Y. and Munoz-Carpena, R. 2014. Analytical and experimental analysis of solute transport in heterogeneous porous media. *J Environ Sci Health A Tox Hazard Subst Environ Eng* 49(3), pp. 338-343.

Wu, Y., Ajo-Franklin, J. B., Spycher, N., Hubbard, S. S., Zhang, G., Williams, K. H., Taylor, J., Fujita, Y. and Smith, R. 2011. Geophysical monitoring and reactive transport modeling of ureolytically-driven calcium carbonate precipitation. *Geochemical transactions* 12(1), p. 7.

Yamaguchi, N., Sasada, M., Yamanaka, M. and Nasu, M. 2003. Rapid detection of respiring Escherichia coli O157:H7 in apple juice, milk, and ground beef by flow cytometry. *Cytometry A* 54(1), pp. 27-35.

Young Jang, H., Chon, H.-T., Lee, J.-U. and Sager, M. eds. 2008. *In Situ Metal Precipitation Based on Biological Sulfate Reduction by Indigenous Bacteria from Soil Contaminated with Arsenic and Heavy Metals*. Geoscience Resources and Environments of Asian Terranes (GREAT 2008). Bangkok, Thailand, 24 - 26 November 2008.

Yu, W., Dodds, W. K., Banks, M. K., Skalsky, J. and Strauss, E. A. 1995. Optimal staining and sample storage time for direct microscopic enumeration of total and active bacteria in soil with two fluorescent dyes. *Appl Environ Microbiol* 61(9), pp. 3367-3372.

Zachara, J. M., Cowan, C. E. and Resch, C. T. 1991. Sorption of divalent metals on calcite. *Geochimica et Cosmochimica Acta* 55(6), pp. 1549-1562.

Zeppenfeld, K. 2010. Prevention of CaCO3 scale formation by trace amounts of copper (II) in comparison to zinc (II). *Desalination* 252(1–3), pp. 60-65.

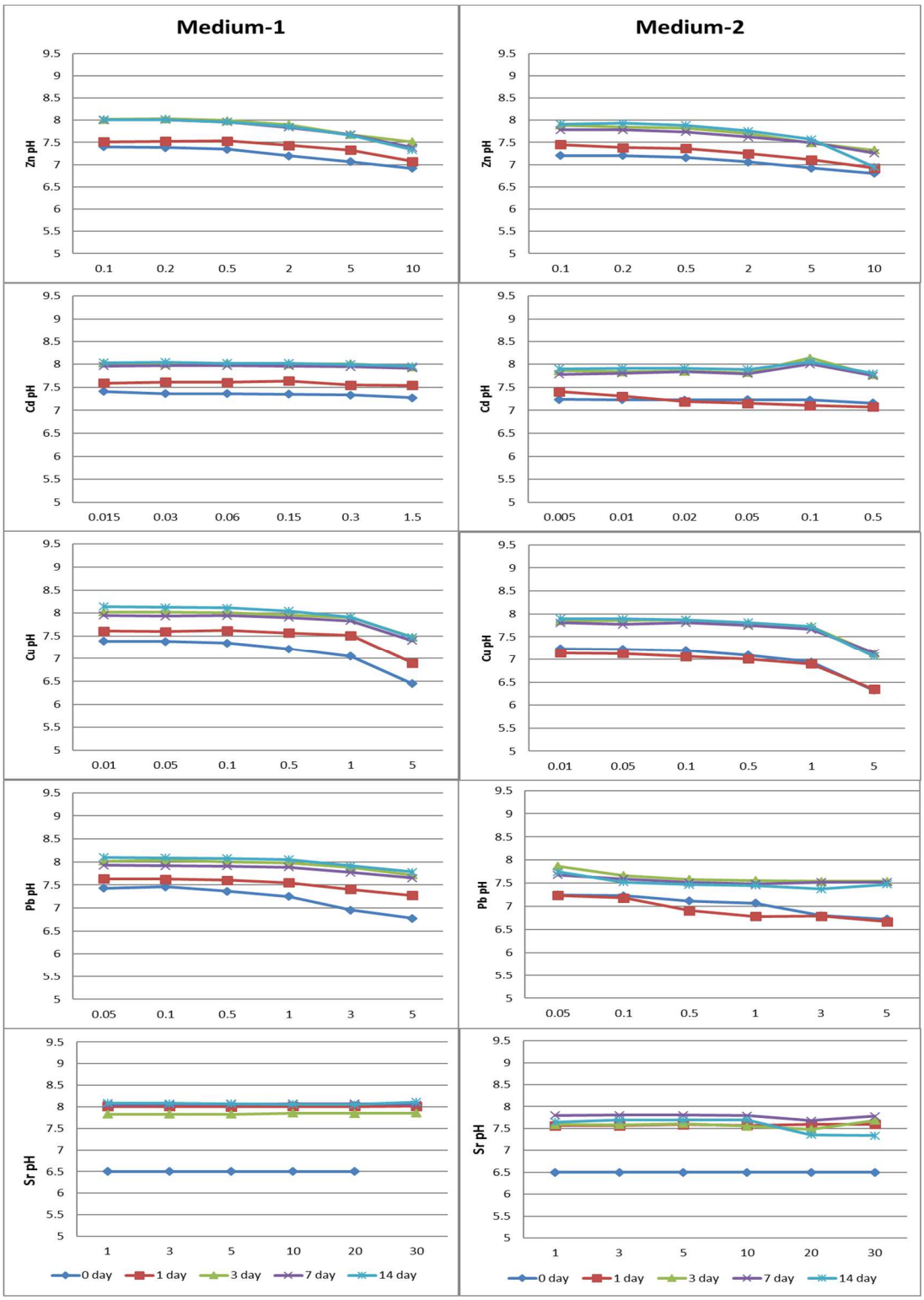
Zhang, M. K. and Xu, J. M. 2003. Difference of lead, copper and zinc concentrations between interiors and exteriors of peds in some contaminated soils. *Chemosphere* 50(6), pp. 733-738.

Zhao, X., Jiang, T. and Du, B. 2014. Effect of organic matter and calcium carbonate on behaviors of cadmium adsorption-desorption on/from purple paddy soils. *Chemosphere* 99, pp. 41-48.

Zhao, X. and Toksoz, M. N. 1994. *Solute Transport In Heterogeneous Porous Media*. Cambridge, MA 02139: Massachusetts Institute of Technology. Earth Resources Laboratory.

Appendix: A

Heavy metals abiotic precipitation experiments:



A1-1. Results of abiotic precipitation of heavy metals in solution (no bacterial cells were added), medium-1 consisted of 3g/l nutrient broth, 20 g/l urea, 2.12 g/l sodium bicarbonate and 10g/l ammonium chloride, medium-2 consisted of medium-1 and 50mM calcium.

Appendix: B

Publications:

Conferences

MUGWAR, A. J. & HARBOTTLE, M. J. 2014. Biomineralisation of metals in soil – effect of metal toxicity and precipitation as a protective mechanism. *In:* BOUAZZA, A., YUEN, S. & BROWN, B. (eds.) *7th International Congress on Environmental Geotechnics.* Melbourne, Australia.

MUGWAR, A. J. & HARBOTTLE, M. J. 2014. Metal Bioprecipitation in Particulate Media–Effect of Particle and Flow Heterogeneity. In Situ Remediation '14 Conference, London 2nd–4th September 2014 (Abstract ID: 17).

Journal

Mugwar, A. J. and Harbottle, M. J. 2016. Toxicity effects on metal sequestration by microbially-induced carbonate precipitation. *Journal of Hazardous Materials* 314, pp. 237-248.

LESSONS, LEARNINGS & CHALLENGES

PROCEEDINGS OF THE

7th INTERNATIONAL CONGRESS ON ENVIRONMENTAL GEOTECHNICS

10-14 NOVEMBER 2014 | MELBOURNE, AUSTRALIA

Edited by

Abdelmalek Bouazza
Monash University, Victoria, Australia

Samuel T S Yuen
University of Melbourne, Victoria, Australia

Bruce Brown
Bruce Brown Consulting Pty Ltd, Australia

Organised by

International Society of Soil Mechanics and Geotechnical Engineering (ISSMGE)
TC 215 Enviromental Geotechnics
Engineers Australia (EA)

Supported by

Australian Geomechanics Society (AGS)
International Geosynthetics Society (IGS)
Melbourne Convention Bureau (MCB)
City of Melbourne

Published by

Engineers Australia (EA)

Created by

Causal Productions

ISBN 978-1-922107-23-7

Biomineralisation of metals in soil – effect of metal toxicity and precipitation as a protective mechanism

A. J. Mugwar[1] and M. J. Harbottle[2]

[1]*PhD student, School of Engineering, Cardiff University, Cardiff, CF24 3AA, United Kingdom, and Lecturer, College of Engineering, Al-Muthanna University, Samawah-Iraq; mugwaraj@cardiff.ac.uk; ahm19712000@yahoo.com*

[2]*Lecturer, School of Engineering, Cardiff University, Cardiff, CF24 3AA, United Kingdom; Tel. +44 2920 875759; harbottlem@cardiff.ac.uk*

ABSTRACT

Biomineralisation offers the potential for in-situ sequestration and subsequent reduction in the bioavailability of heavy metals and radionuclides in the subsurface environment. Calcium carbonate minerals are known to sorb and form solid solutions with a range of target elements, and are readily produced by the actions of common microorganisms on simple chemical precursors. The ability of a commonly used urea-degrading, calcium carbonate-precipitating bacterium, *Sporosarcina pasteurii*, to tolerate the presence of a model contaminant, strontium, was determined in aqueous solution, with reduction in growth only seen at concentrations of 10 mM. Its ability to remove strontium from solution via calcium carbonate precipitation was then determined, and here *S. pasteurii* was shown to be able to remove 99% (+/- 1%) strontium from solution at concentrations up to 30 mM. This suggests that biomineralisation of metallic elements may afford a protective mechanism for the bacteria through providing a means to reduce the overall concentrations to tolerable levels. Finally, we explored the effects of ground conditions on mineralisation and strontium sequestration in different sand fractions (fine, medium and coarse), in a series of batch experiments. Almost all (97-99%) strontium present was removed from aqueous solution after three days, whereas no precipitation was observed in control samples over the same period. The amount of strontium removed increased with coarseness of sand grains under these static conditions, although over a very small range.

Keywords: Biomineralisation, *Sporosarcina pasteurii*, Strontium, Urease, calcium carbonate

1. INTRODUCTION

The natural formation of calcium carbonate occurs both through abiotic and biotic processes. The process can be harnessed for engineering processes (again, biologically or otherwise) for various purposes, including soil cementation and precipitation and removal of a range of toxic or otherwise problematic chemical species. A common mechanism for engineering calcium carbonate precipitation is through microbial urea hydrolysis, ordinarily brought about by *urease* enzymes; bacteria with the ability to break down urea *via* this mechanism are widespread in the subsurface environment (Lloyd & Sheaffe, 1973). This, and the ability of calcium carbonate precipitation to co-precipitate a number of heavy metal species, including cadmium, lead and strontium, makes sequestration of such contamination by co-precipitation with this mineral an attractive technique (Fujita *et al.*, 2000).

Urea hydrolysis leads to elevated pH and an increase in carbonate ions, both of which increase the likelihood of metal carbonate precipitation (Gebrehiwet *et al.*, 2012). Forms of calcium carbonate, including calcite, may offer a stable environment over long periods depending on the local environment. The co-precipitation of heavy metals with calcium carbonate is controlled by a number of factors, including bacterial urease activity, metal ion concentration, and the precipitation rate (Lorens, 1981; Pingitore Jr and Eastman, 1986). Cells themselves often act as nucleation points for the formation of minerals due to their typically overall negative charge attracting metal ions and therefore creating a zone of high metal ion concentration (e.g. calcium). The continuous precipitation of calcium carbonate around bacteria can inhibit or reduce their ureolytic ability due to encapsulation of the cells, and so growth in cell numbers is necessary to maintain mineral production. In addition, release of ammonia and ammonium ions is of potential environmental concern and subsequent biological action may cause acidification and potential dissolution of the precipitate.

The size and morphology of precipitated calcium carbonate influences the mineral solubility and long-term immobilisation of co-precipitated contaminants. For example, strontium incorporation into a calcite lattice may result in a reduction in the durability of metal immobilisation by generating small and more soluble crystals (Mitchell and Ferris, 2006; Tobler *et al.*, 2011; Cuthbert *et al.*, 2012). Due to the inherent heterogeneity of subsurface environment, the spatial distribution of calcium carbonate precipitation within porous media remains highly variable, particularly at the microscopic scale. In

ISBN 978-1-922107-23-7

addition, there are temporal changes that occur due to the continual formation of calcium carbonate over time including chemical changes such as removal of mineral components (e.g. calcium) from solution and production of new materials such as ammonium. These conditions may also vary in the longer term, e.g. ammonium produced *via* urea hydrolysis may become oxidised leading to acidic conditions (Reed *et al.*, 2010). Physically the resulting precipitation can block pore throats, which leads to obstruction of the transport and mixing of delivered reactants (Gebrehiwet *et al.*, 2012). Fujita *et al.* (2010) found that urea hydrolysis processes in situ are not homogeneous, mainly due to the heterogeneous distribution of microorganisms. The heterogeneous distribution of activity in the subsurface environment may therefore lead to non-uniform mineralisation of target contamination, and so pose problems for design of biomineralisation treatment systems in particulate media.

Strontium has previously been a target for remediation *via* calcite biomineralisation (Fujita *et al.*, 2000; Fujita *et al.*, 2008; Fujita *et al.*, 2010; Achal *et al.*, 2012; Lauchnor *et al.*, 2013), due to its presence on certain sites as a radioactive heavy metal contaminant. It is not redox sensitive, and its availability in the subsurface environment is primarily affected by sorption and abiotic precipitation in high pH environment with some minerals. Studies have shown the ability of microorganisms to precipitate this metal as strontium carbonate (Newsome *et al.*, 2014). Precipitation of elevated levels of strontium in the subsurface environment as $SrCO_3$ can occur after an increase in groundwater pH due to oversaturation with carbonate. With low Sr concentration, the metal ions can be incorporated within the structure of calcium carbonate as a solid solution (Fujita *et al.*, 2004).

In this study the tolerance of *S. pasteurii* to strontium (Sr) as a representative heavy metal, and its ability to subsequently remediate this metal, is investigated. Firstly, the ability of *S. pasteurii* to tolerate Sr in aqueous growth medium was examined. Next, this is compared to the tolerance of the organism to strontium in aqueous media containing the necessary minerals for calcium carbonate precipitation and Sr co-precipitation *via* urea hydrolysis. Finally, the ability of this bacterium to induce calcium carbonate precipitation and co-precipitation of strontium in different sand fractions (fine, medium and coarse) was explored, as an initial study into the effects of ground heterogeneity on heavy metal biomineralisation.

2. METHODS

2.1 Culture Media and Growth

Due to its high urease activity in aqueous solutions *S. pasteurii* (National Collection of Industrial & Marine Bacteria, Aberdeen, UK; NCIMB8221 / ATCC6453), was used in this study. *S. pasteurii* is a widespread, gram-positive, endospore-forming soil bacterium. This strain was grown at 30°C for 24 hours in autoclaved Oxoid CM001 nutrient broth (13 g/L) amended with 20 g/L filter-sterilised urea (Fujita *et al.*, 2000). Bacterial pellets were harvested by centrifuging the grown cultures at 1450 RCF for 20 minutes, then the pellets were washed with phosphate-buffered saline (PBS: 8.3 mM Na_2HPO_4, 1.6 mM NaH_2PO_4, 145 mM NaCl, pH 7.2). Cells were again pelletised by centrifugation prior to re-suspension in the working media defined below.

2.2 Tolerance of *S. pasteurii* to strontium in aqueous solution

The minimum inhibitory concentration of strontium for *S. pasteurii* was determined. Bacteria were re-suspended in autoclaved nutrient broth (denoted medium M1, containing Oxoid CM001, 13 g/L). Six millilitres of this suspension was placed in sterile 10mL screw cap glass centrifuge tubes, and 2 mL of a sterile strontium chloride hexahydrate ($SrCl_2.6H_2O$) solution added aseptically to give a range of final metal concentrations (0, 0.1, 0.5, 1, 2, 5, 10 mM). The final bacterial optical density at 600 nm wavelength incident light (OD_{600}) was 0.064 (equivalent to approximately $2x10^6$ cells / mL). The pH was corrected to 6.5 in order to ensure solubility of the metal salt and each concentration was tested in triplicate. The range of strontium concentrations was specified according to preliminary tests to determine to what extent the bacteria can resist strontium. The tubes were incubated at 30ºC for 72 hours, after which the OD_{600} of the solution was measured by UV/visible spectrometry (Hitachi U1900).

2.3 Strontium Bioprecipitation in Aqueous Solution

A urea-containing medium (denoted medium M2: Oxoid CM001 nutrient broth - 3 g/L; $CaCl_2$ - 50 mM; autoclaved prior to addition of 0.2µm filter-sterilised urea [to give 20 g/L], NH_4Cl [to give 10 g/L] and

Na_2CO_3 [to give 2.12 g/L] – Fujita *et al.*, 2000) was amended with strontium chloride as above to give a range of strontium concentrations (1, 3, 5, 10, 20, 30 mM). *S. pasteurii* was prepared as described above – killed-cell controls were prepared by autoclaving bacterial suspensions. Following pelletisation and washing, both live and killed cells were re-suspended in the urea/strontium media to give a final bacterial OD_{600} of 0.111 (equivalent to approximately $5x10^6$ cells / mL) and then 30 mL placed aseptically into 50 mL sterilised polypropylene tubes. The microcosms were incubated at 30ºC for 7 days. Each concentration was again tested in triplicate. Strontium and calcium concentrations, and pH, were determined after 0, 1, 3 and 7 days. At each time a 5 mL aliquot was removed from each sample aseptically, then filtered (0.2 µm) to remove cells and any suspended precipitates. Half of the filtered sample was used to measure the pH. The rest was diluted as necessary using deionised water and analysed for strontium and calcium concentrations by ICP-OES (Optical Emission Spectrometer, Optima 2100 DV, Perkin Elmer).

2.4 Strontium Bioprecipitation in Soil

Leighton Buzzard sand (Hepworth Minerals and Chemicals Ltd - see Figure 1 for grain size distribution) was washed by soaking in 1M hydrochloric acid, then in 1M sodium hydroxide for 24 hours each with periodic stirring, to remove inorganic carbonate. Finally, the sand was rinsed with deionised water until it reached pH 7.0. It was then separated into three fractions by dry sieving (fine (63-300 µm), medium (300-425 µm) or coarse (600-1180 µm)). Sand fractions were autoclaved and dried at 30°C before use. Cultures of *S. pasteurii* prepared as above were suspended in medium M2 amended with 10 mM strontium. In all cases, 17 ml of the requisite medium containing suspended cells (equivalent to the pore volume) was added to sterile 50 ml polypropylene tubes prior to addition of 80g dry, autoclaved sand from individual fractions, which was wet-pluviated into the bacterial suspension to minimise air voids and encourage uniform bacterial distribution. This resulted in a final dry density of 1.65 g/cm^3 with final bacterial densities were calculated to be $2.55x10^6$ cells per gram of sand. Multiple samples for each of the three sand fractions were prepared such that triplicate specimens could be destructively sampled at 3 and 7 days. Control samples were prepared in an identical fashion, but a urea-free medium (denoted M3), otherwise identical to M2, was used instead. The microcosms were then incubated at 30ºC. At each sampling time, specimens were removed from the incubator and the solutions extracted under vacuum from the soil then filtered through a 0.2 µm membrane filter prior to testing of pH, strontium and calcium concentration as described above.

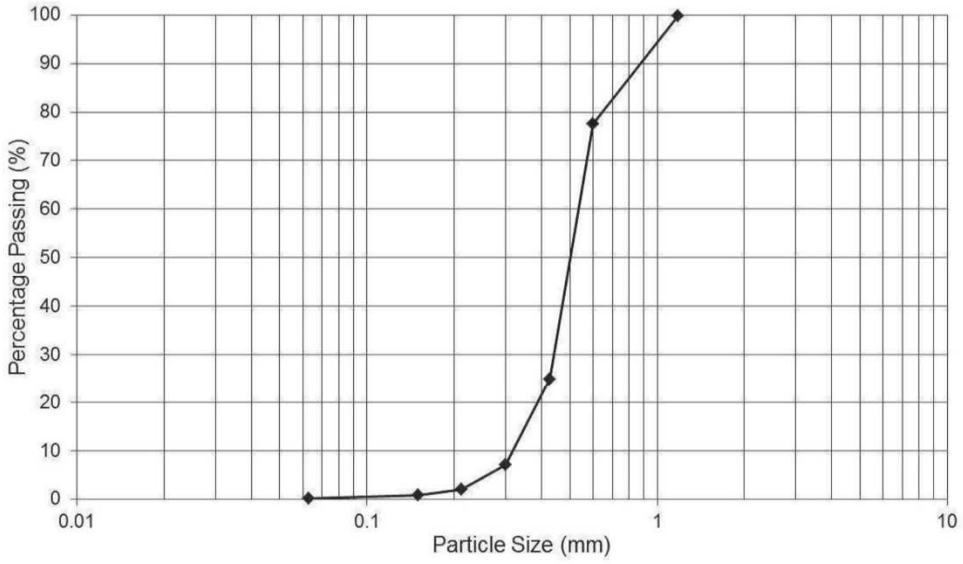


Figure 1. Particle size distribution for sand used (subsequently divided into fine, medium and coarse fractions as described).

3. RESULTS AND DISCUSSION

Figure 2 shows the tolerance of *S. pasteurii* to strontium in aqueous solution, presenting the OD_{600} absorbance divided by the absorbance without strontium. Ruggiero *et al.* (2005) defined the toxic concentration of metals in solution as the concentration that inhibits the growth of a microorganism by >70% compared to controls. A concentration of 10 mM hindered growth of the bacteria (Figure 2); it is apparent that the minimum inhibitory concentration is between 5 and 10 mM.

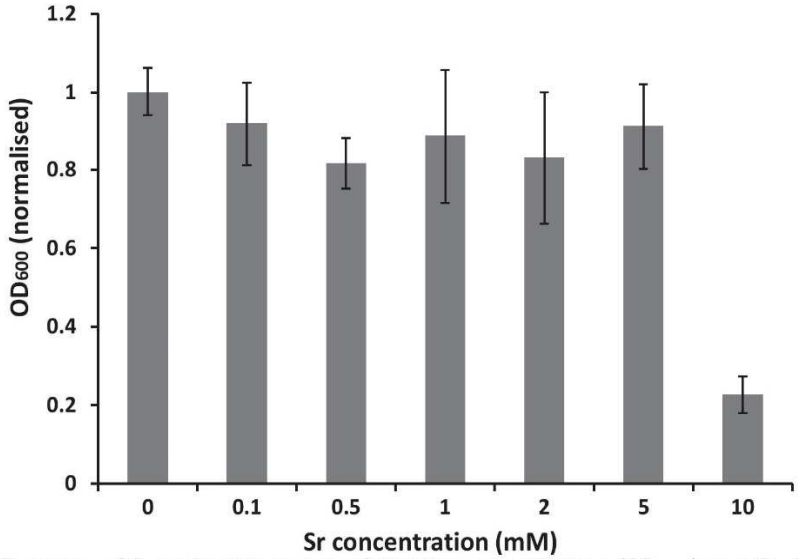


Figure 2. Response of S. pasteurii to a range of strontium concentrations (OD_{600} (normalised) = OD_{600} at any Sr concentration / average OD_{600} at zero Sr concentration. Error bars represent ±1SD)).

In the second part of this study, the ability of live *S. pasteurii* to induce calcium carbonate precipitation in the presence of a range of strontium concentrations (0-30 mM), and therefore co-precipitate strontium, was investigated. The changes in strontium and calcium concentrations, and pH, are shown in Figure 3. The pH increased over the testing period, reaching around 9.2 in all specimens by 7 days, thought to be due to the action of the *S. pasteurii* urease enzyme on urea. Calcium was rapidly removed in all specimens, with approximately 50% remaining in solution after one day and being undetectable after 3 days; this was concurrent with generation of white crystalline precipitates in each case. The decrease in concentration of calcium ions in solution was faster than that of strontium ions, with the amount lost in 24 hours greater than that of strontium by a factor of 1.45 (±0.1). Strontium was almost fully removed after 3 days, and not detectable after 7 days. In previous experiments, without strontium but otherwise identical, the majority of calcium ions were removed from solution in the first 24 hours with a pH value of up to 9.0. The delay of calcium removal in this experiment is thought to be related to the presence of strontium ions in solution. However, *S. pasteurii* cells were found to tolerate far higher Sr concentrations in this experiment with urea than in aqueous solution without it. It is therefore thought that the co-precipitation of Sr with calcium carbonate offered a mechanism of protection against metal toxicity.

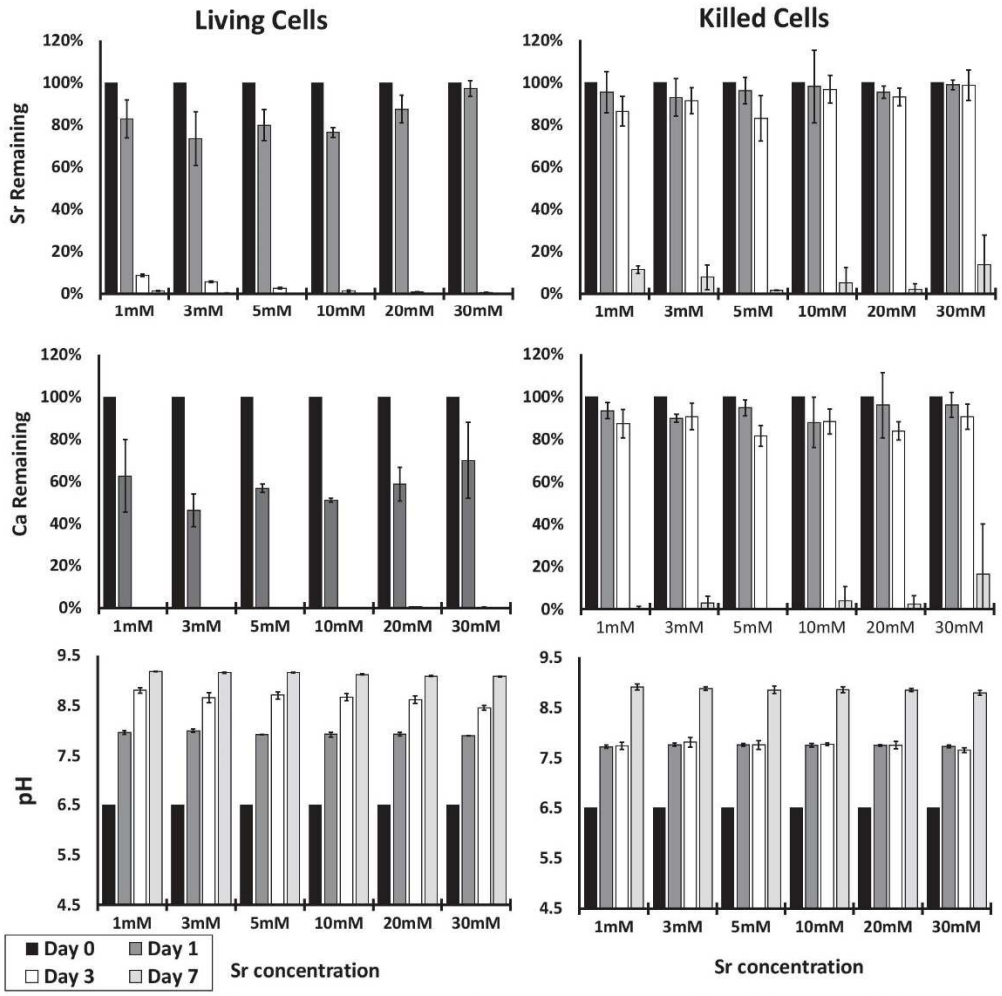


Figure 3. Response of Sr and Ca concentrations, and pH, to activity of S. pasteurii (error bars represent ±1SD)).

In killed-cell controls, pH had only slightly increased after three days and there was little loss of Ca or Sr from solution. The slight pH increase may be partially due to abiotic hydrolysis of urea in solution, which is slower than the biotic hydrolysis (United States Environmental Protection Agency, 2002), but by day 7 the pH had increased substantially – it is thought that the autoclave procedure used to sterilise samples in this part of the study was insufficient to deactivate bacterial spores, which then regenerated and had similar but delayed effects to live cells by day 7 (Stolp, 1988; Todar, 2008-12). The minimum inhibitory concentration in the presence of the urea medium has not been established, but appears to be greater than 30 mM for this system, more than three times the previous value.

Similar experimental data have been obtained from precipitation in sands (Figure 4). Without urea, there was little loss of Sr (around 20% after 3 and 7 days) whilst when urea was present removal was almost complete. Ca removal was very similar after 3 days, but reduced further to around 50% after 7 days, perhaps due to abiotic interactions with sand grains or cell surfaces. A slight increase in pH in no-urea controls may be due to chemical interaction with the sand grains. With urea present, pH increased to around 9. There was little discernible difference in pH change, or recovery of Sr or Ca, between sand grain sizes, with perhaps a small increase in Sr removal with increasingly coarse grain size. It is also shown that in particulate media, the presence of urea degradation allows survival at concentrations greater than the minimum inhibitory concentration seen in aqueous solution alone.

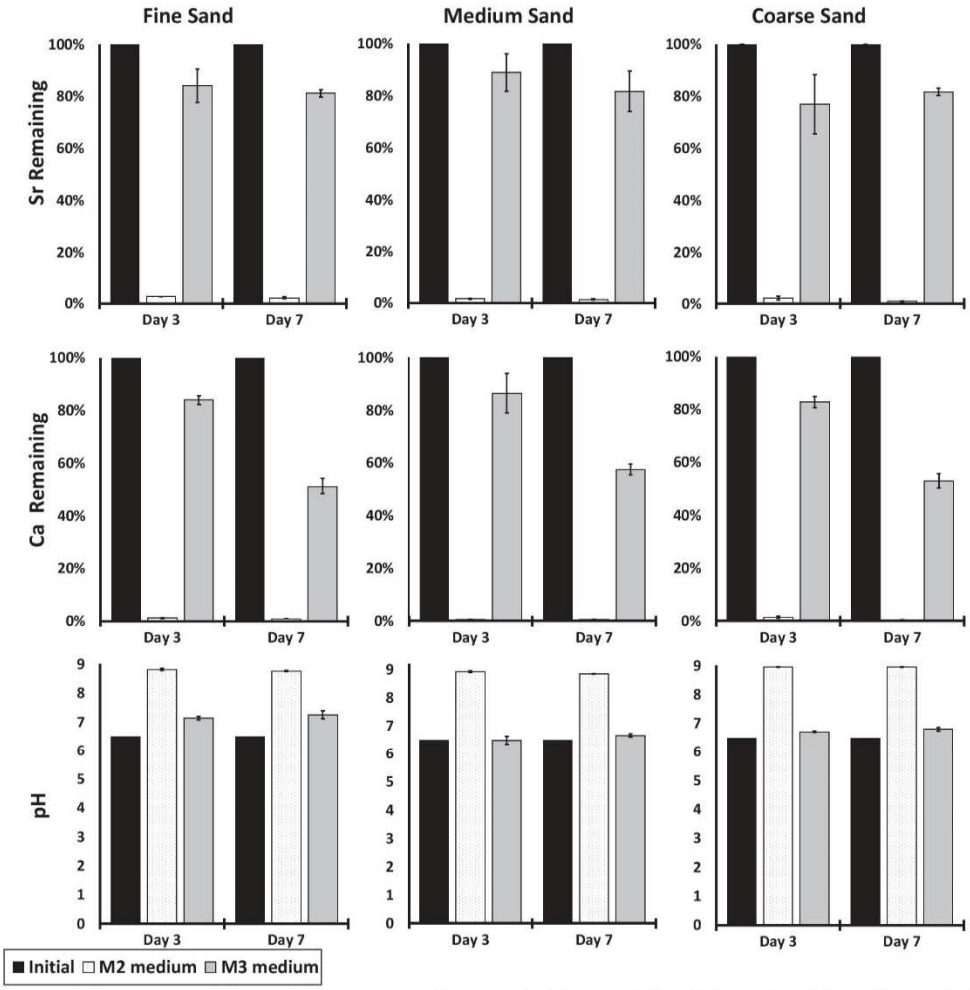


Figure 4. Response of Sr and Ca concentrations, and pH, to activity of S. pasteurii in soil pore fluid (error bars represent ±1SD))

It should be noted that these static experiments would not have been subject to the effects of heterogeneity through preferential flow or other dynamic effects, which will play a significant role in the heterogeneity of the delivery of subsurface remediation.

In the presence of urea, calcium and suitable nutrients, *S. pasteurii* will grow rapidly, altering the pH and causing calcium carbonate precipitation. However, their cell walls act as nucleation sites for calcium carbonate formation, and this in turn can lead to the bacteria becoming embedded within the solid matrix which is likely to kill cells by prevent access to nutrients and energy sources (Tobler *et al.*, 2011). However, due to the presence of a large number of cells, providing a small population remain following the precipitation of the mineral, the removal of metals from solution will enhance the environment for the remaining live cells. The technique provides a protective mechanism for the bacteria by reducing the overall metal concentrations to tolerable levels.

4. CONCLUSION

The sequestering of heavy metals in the subsurface environment by bacterially induced calcium carbonate precipitation provides potential long-term removal of heavy metals in contaminated subsurface environments with simple requirements and potential for use in a wide range of

environments. It has been shown that provision of urea and other simple chemical species also offers bacteria that possess the urease enzyme a protective mechanism against metal toxicity. Urea degradation leads to an elevated pH and enhanced carbonate ion concentration in solution, making metal carbonate precipitation more likely. Initial precipitation reduces metal bioavailability, leading to enhanced bacterial growth and activity, which in turn causes further precipitation. A significantly increased minimum inhibitory concentration results, at least three times greater than the original value. A similar effect is seen in particulate media. However, no significant effect of particle size was seen, under the static flow conditions tested.

5. ACKNOWLEDGEMENTS

The authors thank the Iraqi Ministry of Higher Education and Scientific Research for funding the studentship of the first author.

REFERENCES

Achal, V., Pan, X., Zhang, D. 2012. Bioremediation of strontium (Sr) contaminated aquifer quartz sand based on carbonate precipitation induced by Sr resistant *Halomonas* sp.. Chemosphere, **89** (6): 764-768.

Cuthbert, M. O., Riley, M. S., Handley-Sidhu, S., Renshaw, J. C., Tobler, D. J., Phoenix, V. R., Mackay, R. 2012. Controls on the rate of ureolysis and the morphology of carbonate precipitated by *S. Pasteurii* biofilms and limits due to bacterial encapsulation. Ecological Engineering, **41**: 32-40.

Fujita, Y., Ferris, F. G., Lawson, R. D., Colwell, F. S., Smith, R. W. 2000. Calcium carbonate precipitation by ureolytic subsurface bacteria. Geomicrobiology Journal, **17** (4): 305-318.

Fujita, Y., Redden, G. D., Ingram, J. C., Cortez, M. M., Ferris, F. G., Smith, R. W. 2004. Strontium incorporation into calcite generated by bacterial ureolysis. Geochimica et Cosmochimica Acta, **68** (15): 3261-3270.

Fujita, Y., Taylor, J. L., Gresham, T. L. T., Delwiche, M. E., Colwell, F. S., McLing, T. L., Petzke, L. M., Smith, R. W. 2008. Stimulation of microbial urea hydrolysis in groundwater to enhance calcite precipitation. Environmental Science & Technology, **42** (8): 3025-3032.

Fujita, Y., Taylor, J. L., Wendt, L. M., Reed, D. W., Smith, R. W. 2010. Evaluating the potential of native ureolytic microbes to remediate a ^{90}Sr contaminated environment. Environmental Science & Technology, **44** (19): 7652-7658.

Gebrehiwet, T., Redden, G., Fujita, Y., Beig, M., Smith, R. 2012. The effect of the CO_3^{2-} to Ca^{2+} ion activity ratio on calcite precipitation kinetics and Sr^{2+} partitioning. Geochemical Transactions, **13** (1): 1-11.

Lauchnor, E. G., Schultz, L. N., Bugni, S., Mitchell, A. C., Cunningham, A. B., Gerlach, R. 2013. Bacterially induced calcium carbonate precipitation and strontium coprecipitation in a porous media flow system. Environmental Science & Technology, **47** (3): 1557-1564.

Lloyd, A. B., Sheaffe, M.J. 1973. Urease activity in soils. Plant and Soil, **39**: 71-80.

Lorens, R. B. 1981. Sr, Cd, Mn and Co distribution coefficients in calcite as a function of calcite precipitation rate. Geochimica et Cosmochimica Acta, **45** (4): 553-561.

Mitchell, A. C., Ferris, F. G. 2006. Effect of strontium contaminants upon the size and solubility of calcite crystals precipitated by the bacterial hydrolysis of urea. Environmental Science & Technology, **40** (3): 1008-1014.

Newsome, L., Morris, K., Lloyd, J. R. 2014. The biogeochemistry and bioremediation of uranium and other priority radionuclides. Chemical Geology, **363**: 164-184.

Pingitore Jr, N. E., Eastman, M. P. 1986. The coprecipitation of Sr^{2+} with calcite at 25°C and 1 atm. Geochimica et Cosmochimica Acta, **50** (10): 2195-2203.

Reed, D. W., Smith, J. M., Francis, C. A., Fujita, Y. 2010. Responses of ammonia-oxidizing bacterial and archaeal populations to organic nitrogen amendments in low-nutrient groundwater. Applied and Environmental Microbiology, **76** (8): 2517-2523.

Ruggiero, C. E., Boukhalfa, H., Forsythe, J. H., Lack, J. G., Hersman, L. E., Neu, M. P. 2005. Actinide and metal toxicity to prospective bioremediation bacteria. Environmental Microbiology, **7** (1): 88-97.

Stolp, H. 1988. Microbial Ecology: Organisms, Habitats, Activities. Cambridge University Press, Cambridge.

Tobler, D. J., Cuthbert, M. O., Greswell, R. B., Riley, M. S., Renshaw, J. C., Handley-Sidhu, S., Phoenix, V. R. 2011. Comparison of rates of ureolysis between *Sporosarcina pasteurii* and an indigenous groundwater community under conditions required to precipitate large volumes of calcite. Geochimica et Cosmochimica Acta, **75** (11): 3290-3301.

Todar, K. 2008-2012 The Genus *Bacillus* [Online]. Available at: http://textbookofbacteriology.net/Bacillus_3.html [Accessed: 15 April].

United States Environmental Protection Agency 2002. Urea Tolerance Reassessment Eligibility Decision (TRED). Federal Register: April 15, 2002. Volume 67, Number 72.

METAL BIOPRECIPITATION IN PARTICULATE MEDIA – EFFECT OF PARTICLE AND FLOW HETEROGENEITY

A.J. Mugwar[1] and M. J. Harbottle[2]
[1]PhD student, School of Engineering, Cardiff University, Cardiff, CF24 3AA, United Kingdom, and Lecturer, College of Engineering, Al-Muthanna Un., Iraq; mugwaraj@cardiff.ac.uk; ahmedmugwar@gmail.com
[2]Lecturer, School of Engineering, Cardiff University, Cardiff, CF24 3AA, United Kingdom; Tel. +44 2920 875759; harbottlem@cardiff.ac.uk

INTRODUCTION

- Bioprecipitation of carbonate minerals has received relatively little attention although the potential of such minerals to sorb and sequester a range of metals has previously been considered.
- The bacterial hydrolysis of urea locally increases the pH and promotes the deposition of calcium carbonate and sequestration of cadmium ions from soil solution.
- The ability of *Sporosarcina pasteurii* bacteria to induce the precipitation of calcium carbonate and co-precipitation of cadmium ions in porous media has been explored.
- Exp. No.1 : The bacteria cells were aged (3 days) with Cd ions in three sizes of sand in presence of $CaCO_3$ medium to examine their resistance and the effects of sand sizes on $CaCO_3$ precipitation and Cd ions removal.
- Exp. No.2 : the bacteria cells were suspended with only medium sand in columns (Fig.1), then Cd ions and $CaCO_3$ media were injected into the columns, to inspect how the flow can change over time and if this will affect the precipitation of heavy metals

BASIC INFORMATION

- Sand
  - Fine : 63-212 µm
  - Medium: 300-425 µm
  - Coarse : 600-1180µm
- Cd Concentration:
  - Exp. No.1 (static system) : 10 mM (1124 mg/L)
  - Exp. No.2 (dynamic system) : 21 mM (2350.5 mg/L)
- Injected Bacteria Concentration:
  - Exp. No.1 : 7.6 x 10^6 cells/mL
  - Exp. No.2 : 5.8 x 10^6 cells/mL

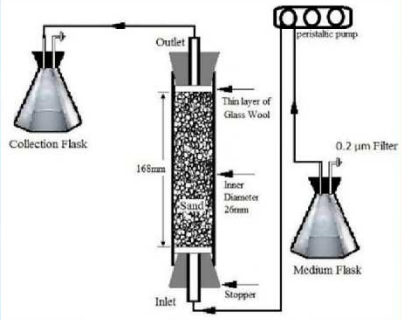


Figure 1. Schematic of sand column setup

MECHANISM

The reactions involved in this process include:

- The bacteria enhance urea hydrolysis in water which causes an increase in pH :

$$H_2N(CO)NH_2 + H^+ + 2H_2O \rightarrow 2NH_4^+ + HCO_3^-$$

- NH_4^+ promotes pH increase and desorption of cations from grain surfaces:

$$Solid - (Me^{2+}) + 2NH_4^+ \Leftrightarrow Solid - (NH_4^+)2 + Me^{2+}$$

- HCO_3^- promotes precipitation of calcium carbonate and co-precipitation of heavy metals.

$$xMe^{2+} + (1-x)Ca^{2+} + 2HCO_3^- \Leftrightarrow Ca_{(1-x)}\,Me_xCO_3 + H_2O + CO_2$$

RESULTS

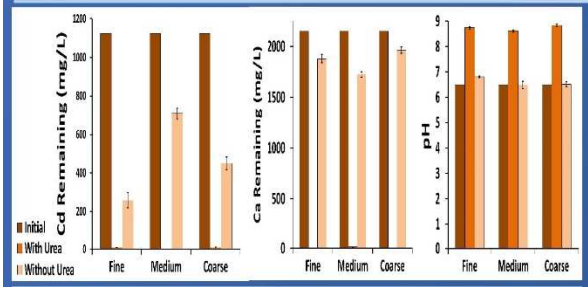


Figure 2. Changes in Cd and Ca concentrations in soil pore solution and pH change in static experiment (Exp. No.1), error bars represent ±1SD (n=3)

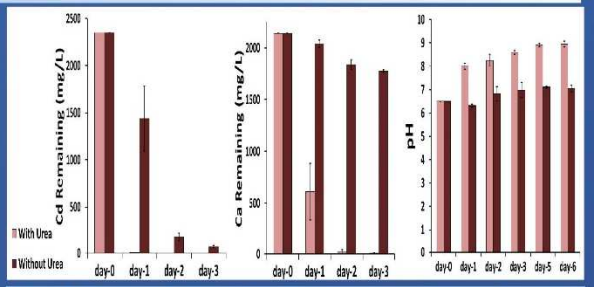


Figure 3. Changes in Cd and Ca concentrations in soil pore solution and pH change over time-dynamic experiment (Exp. No.2) error bars represent ±1SD (n=3)

CONCLUSIONS

- Complete Cd removal occurs rapidly in conjunction with pH increase and Ca removal indicating co-precipitation with $CaCO_3$ (Fig 2 and 3).
- Cd removal is correlated with the extent of Ca removal (Fig 2 and 3), that there is a slower, incomplete removal mechanism without microbial activity.
- There are no obvious trends dependent on sand fraction size in Exp. No.1 (static system), Fig 2.
- The process occurs over time when the growth medium is replenished regularly but Cd removal is rapid (though pH takes longer to increase, perhaps due to dilution with incoming medium), Fig 3.
- The increasing in pH in the Exp. No.2 (dynamic system) of the experiments was continuous over 6 days, this reflect the activity of the bacteria cells after complete removal of Cd ions.
- The reduction in hydraulic conductivity is might be due to heterogeneous $CaCO_3$ precipitated in sporadic parts of column, it is thought that nutrient distribution was by preferential flow.

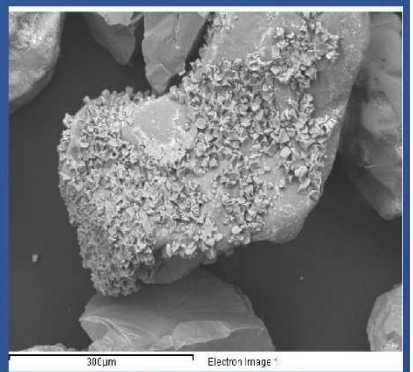
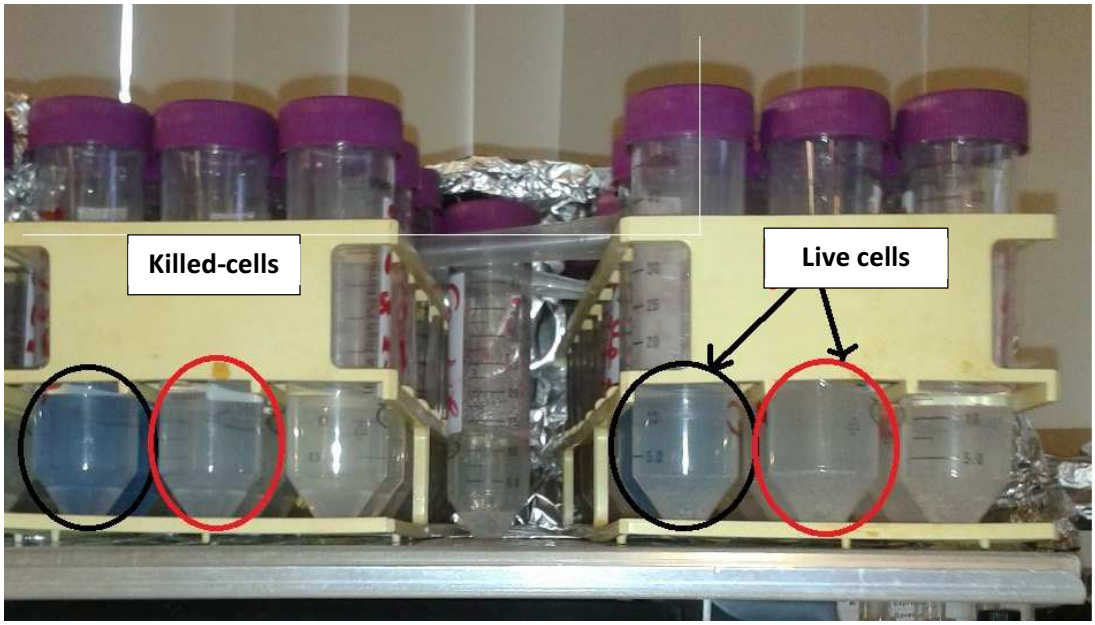


Figure 4. Scanning electron microscopy images of bioprecipitation in sand

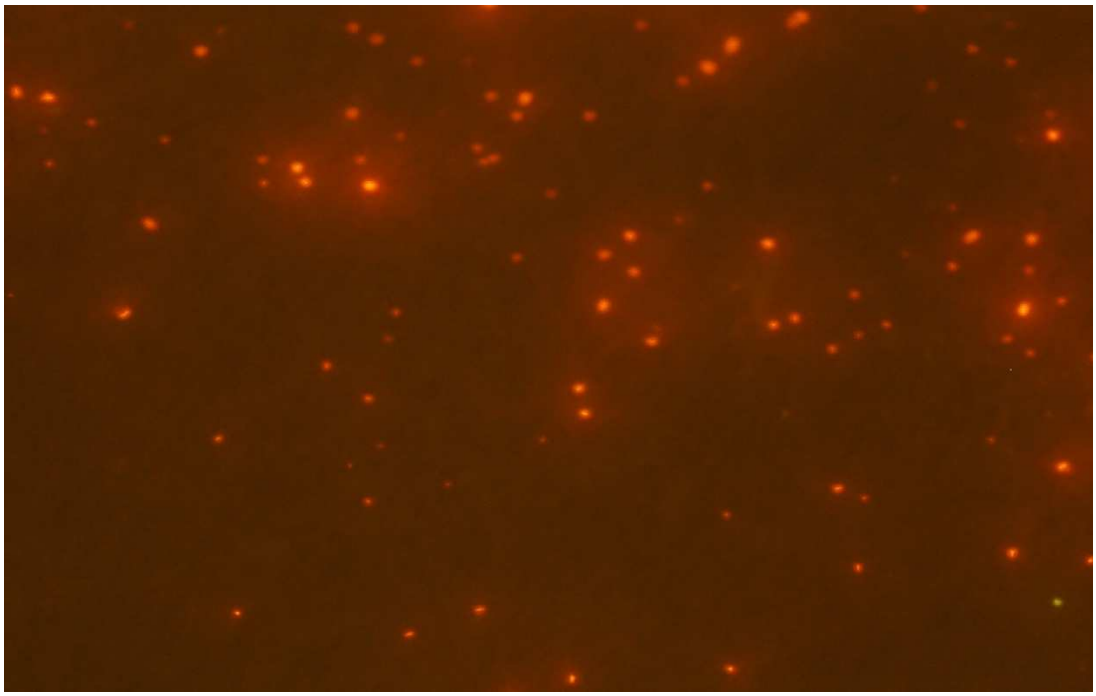
In Situ Remediation '14 Conference, London
2nd – 4th September 2014 (Abstract ID:17)

Appendix: C

Selected Photos from the Work

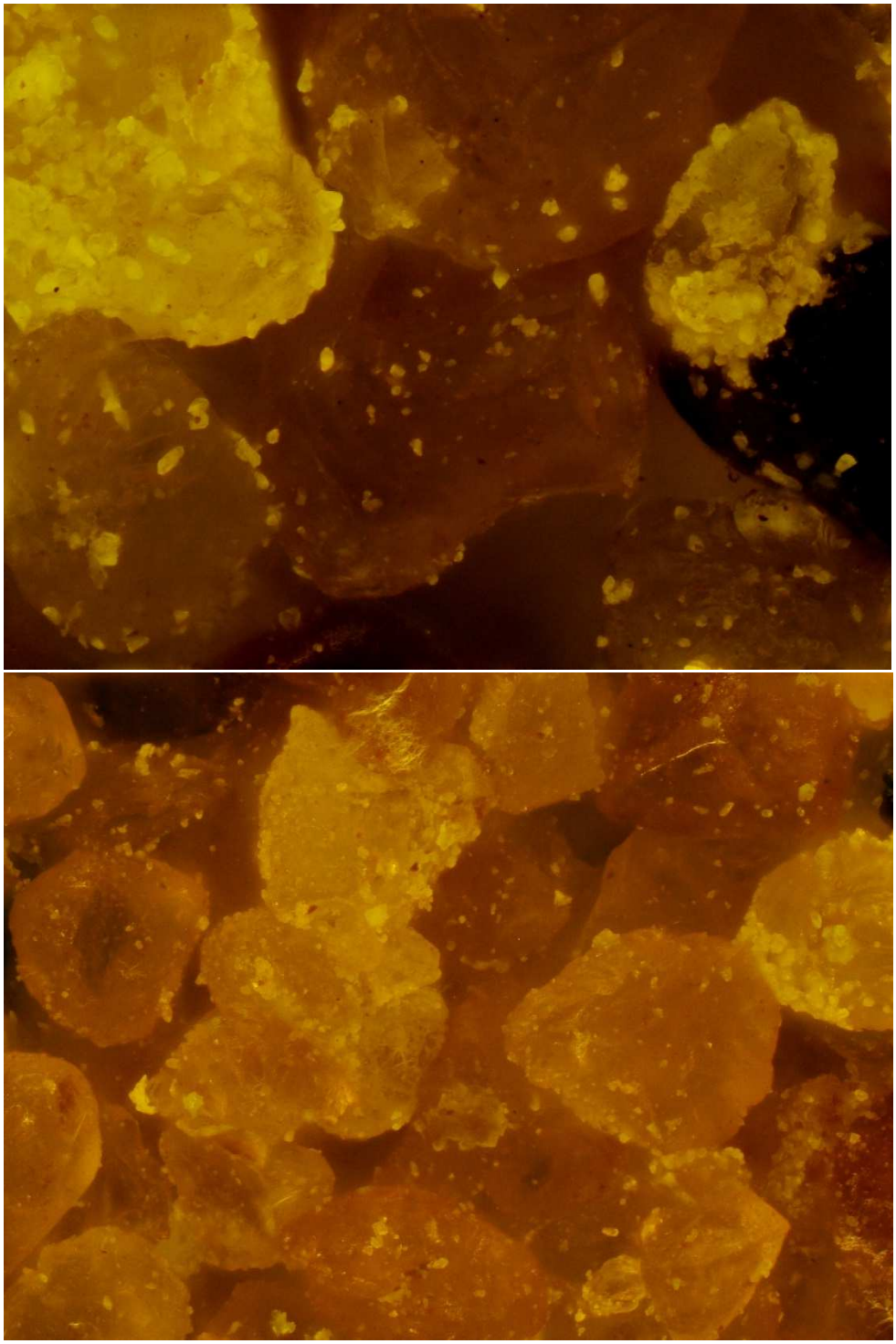


A2.1. Precipitation in aqueous solutions (copper experiment). Red and black circles represent similar concentrations of copper in live and killed-cells experiments. Different in colours between live and killed cells samples may due to decreasing in dissolved copper ions in solutions. Photo was taken after day-1.



A2.2. Counting bacteria by CTC method under Nikon ECLIPSE LV100 microscope

A2.3. Dynamic system in heterogeneous fluid flow inside the incubator experiment.



A2.4. Precipitation of calcium carbonate in sand display by Nikon ECLIPSE LV100 microscope equipped with Nikon DS-Fi1digital camera.