UNIVERSITY OF LIVERPOOL

Calcite-precipitating indigenous bacteria in landfills and their application towards ground improvement

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

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ABSTRACT

This project offers a new outlook on landfills as potential locations from which calcite precipitating bacteria could be isolated and/or stimulated for ground improvement purposes. The research presented in this thesis confirms the presence of indigenous calcite-precipitating bacteria in landfills and investigates their potential application for ground improvement. For instance, this technique can be employed to reduce the permeability of the soil at landfill sites to prevent or minimise possible contamination of ground water from leachate. The novel research presented here, describes the isolation and identification of seven unique indigenous calcite-precipitating bacteria from landfill leachate and groundwater. In addition, their proof-of-principle application for ground improvement within geomaterials has been successfully demonstrated.

A review of existing literature has highlighted the need for identifying indigenous calcite precipitating bacteria in harsh environments such as landfills. Most studies focussed on a number of known calcite precipitating bacteria that are readily available from cell culture collection laboratories and only a few researchers have tried to investigate the potential calcite precipitation of indigenous bacteria. A culture independent technique was implemented to investigate a selected landfill environment in detail, sampling its leachate, soil and groundwater. An urban sampling site located at a significant distance away from the landfill was selected for contrasting purposes. Using NGS, it was found that the bacterial consortia consisted of up to 16 phyla in which Proteobacteria, Actinobacteria and Bacteroidetes were found to be dominant in both sampling sites. Only *Chloroflexi* was detected at urban site and *Pseudomonas* was the dominant (67-93%) genus in landfill leachate. Arsenic concentrations were $1.11x10^3$ µg/L and $1.78x10^3$ µg/L for the landfill raw leachate (RL) and fresh leachate (FL2), respectively. Similarly, the mercury concentrations measured were 10.9 µg/L and 7.37 µg/L, for RL and FL2, respectively. These values were

higher than the recommended values of those heavy metals in the Chinese State Environmental Protection Administration (SEPA) standards for leachate in landfills. Shannon diversity index and Chao 1 richness estimate showed RL and FL2 lacked bacterial richness and diversity when compared with other samples. A total of two indigenous bacteria from the landfill groundwater and five from the landfill leachate were selected and isolated using a nutrient broth media. The isolated bacteria were studied for their ability to mediate calcite precipitation. The media consisted of calcium chloride, urea and nutrient broth. The biomineralisation experiment was conducted at a starting pH of 7.5 at 30°C for 168 hours. The results from the isolates proved that indigenous calcite precipitating bacteria can be found in environments ranging from landfill leachate to groundwater. As a qualitative assessment, SEM images showed the difference in crystal morphology between the bacterial and abiotic solutions having spherical shaped crystals and trigonal shaped crystal formations, respectively. For quantitative analysis, carbonate titration experiments were performed following the biomineralisation experiment to determine the amount of carbonate precipitated by each bacterial strain during the biomineralisation process. The most prolific bacteria (bacteria that precipitated the most calcium carbonate) from landfill leachate and landfill groundwater were determined based on the amount of carbonate precipitated by the bacteria. The carbonate titration experiment revealed that the seven selected bacteria precipitated between 4.66 to 6.1 g/L of carbonate which was three times more than that observed for the abiotic solution. One prolific bacterium isolated from landfill groundwater and two prolific bacteria from landfill leachate were further investigated in column-based porous media studies. Porous media studies were conducted using specially designed polyvinyl chloride columns and sand was used as the porous media to determine the effects of biocementation exerted by the bacteria through biomineralisation. The experimentally generated compressive strength of the bacterial columns ranged from 150-260 kPa. Improved permeability ranging

from 10^{-6} to 10^{-7} m/s was observed in the bacterial columns. Superior cementation between sand particles was observed under SEM in the columns where the bacteria were added.

In summary, calcite-precipitating bacteria have been shown to survive even in contaminated leachate conditions. The extent of their calcite-precipitating abilities is shown through laboratory and porous media experiments. This means that the biohazard from the landfill is not necessarily a barrier towards remediation and applying microbially induced calcite precipitation (MICP) methodology along with monitored natural attenuation would prove to be beneficial in treating the leachate along with prevention of its interaction with groundwater.

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CHAPTER 1 Thesis Overview

The main chapters of this thesis are extracts of manuscripts that have been submitted to journals for publication and are currently under review. This introductory chapter provides an outline of this thesis along with the background literature which underpins the argument for the importance of this research project. Synthesis of the literature leads to the presentation of the aims and objectives and an overview of the structure of the work completed over the course of this research project.

1.1 Introduction

This thesis forms a major part of a research project entitled "Stimulated microbial activity for sustainable geotechnical remediation of unlined/uncontrolled municipal solid waste disposal sites" funded by Xi'an-Jiaotong Liverpool University. The objective of the above mentioned research project is to investigate the potential beneficial use of *in situ* bacteria for their ability to precipitate minerals within inter-particle spaces of soils and thus reduce the soil permeability while increasing soil strength in environments such as landfills. The results presented within this thesis indicate that bacteria which are indigenous to the soils and groundwater in and around a landfill site are able to perform this function. In hindsight, the work presented here demonstrates the potential of mineral precipitation by indigenous bacteria isolated from environments such as landfills for potential geotechnical engineering applications such as soil strengthening and improved permeability.

1.1.1 Scope

The focus of this thesis is to analyse and assess biomineralisation as a potential environmental friendly solution for ground improvement in high permeability geomaterials. The interaction of wastewater/leachate discharge from industries and landfill leachates with groundwater results in a reduction in the availability of potable groundwater resources around the world (Mor et al., 2006). Leachates in particular, pose a serious threat to water quality due

to their complexity (polyaromatic and polycyclic hydrocarbons) and the diverse composition of such pollutants. Depending on the location, history and operational context of a landfill, its leachate may consists of discharges from industrial processes, household wastes, landfill leachates (organic and inorganic compounds including polyaromatic hydrocarbons (PAHs), non-aqueous phase liquids (NAPLs), heavy metals); effluent from septic tanks, sewers and sewage treatment (organic matter, pathogenic micro-organisms), and agricultural sources (pesticide, herbicide and chemical fertiliser derivatives, livestock wastes and slurry/sludge-based fertilisers which may contain pathogenic micro-organisms) (Zhang et al., 2010, Zhang et al., 2013b). Although the concentration of the pollutants stated above may vary based on geographical location, the potential exists for the interaction of leachate with groundwater resources, limiting the amount of these resources that are safe for future use (Zhang et al., 2010, Zhang et al., 2013a, Zhang et al., 2013b). Landfills are usually designed with thick and often costly liners to contain leachate and prevent contamination of groundwater (Rowe and Rimal, 2008). Currently, landfill designers have moved towards implementing three-layer liners to prevent the interaction of leachate/industrial waste with groundwater. The landfill system shown in Figure 1-1 details the installations that are made to track the interaction of leachate with groundwater through gas testing and monitoring wells.

Even after the liner implementation, surface run off is a possibility which cannot be ignored as shown in the schematic diagram Figure 1-1. But in some cases, these solutions could cause more damage than prevention. For example, increasing storm activity might result in an increase in rainwater run off to the nearby sewers leading to an overflow of the sewer system (Longe and Enekwechi, 2007) . Potential breaches in the capture system in the form of holes or cracks during the liner implementation may also be problematic. Finally, there can be contamination from illegal activity; even though legal landfills are increasing, waste dumping in illegal landfills needs to be considered.

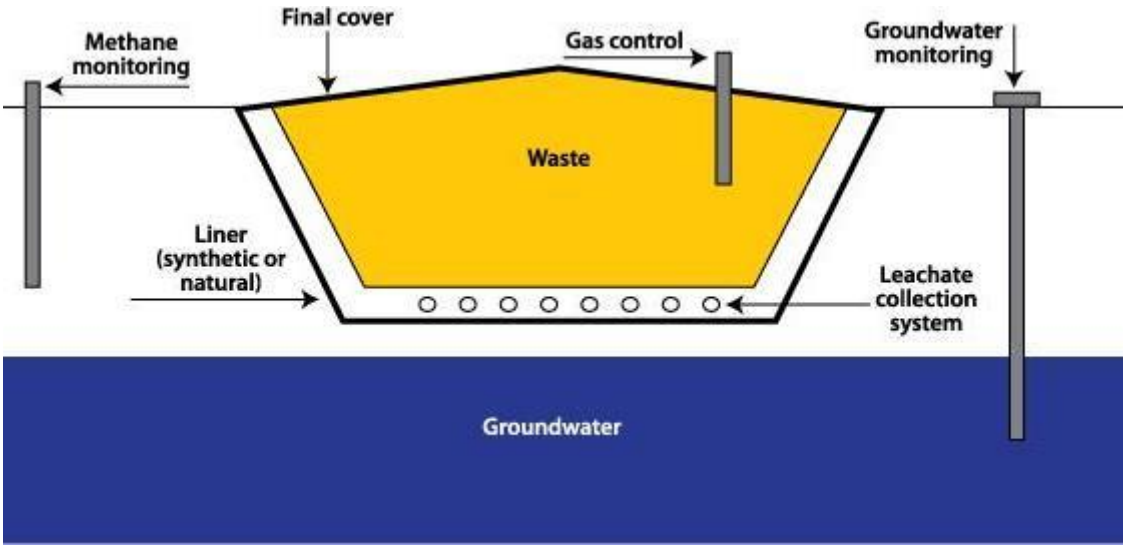

Figure 1-1 Leachate interacting with groundwater due to poor soil permeability which causes groundwater contamination.

1.1.2 Current techniques implemented to prevent contamination

Where such groundwater contamination exists, there are a range of methods that are commonly used for remediation (Table 1-1). The approach that is used depends on the type of contamination, the scale of the contaminated area and the concentration of the contaminant within that area. Sometimes current technology does not allow for the appropriate treatment of the contaminant due to cost ineffectiveness (Ivanov and Chu, 2008). The conventional approach would be to use some form of containment (normally a cut-off wall) to ensure that the contamination does not spread to other areas. Where the contaminant is treatable, but is spread over a large area, cut-off walls could be used in order to control the direction of fluid flow to ensure that the contaminants in the groundwater are directed to a treatment location.

Appropriate containment is a common approach used for the remediation of landfill leachates for the following reasons:

- Leachates from landfills tend to be very complex in comparison to other wastewater systems due to the wide diversity of sources which generate the leachate.

- The source cannot normally be remediated, i.e. once a landfill is in place, it tends to stay in place.
- The pH, organic matter and biological activity of the leachate dictate the biological methods that can be implemented for remediation and biomineralisation.
- The extent of the contaminated region tends to be inaccessible, i.e. it is difficult to remediate below a landfill.

The installation of cut-off walls however, has a disadvantage in that it normally requires the use of large quantities of cement products. The production process for cement manufacture produces 829 million metric tons of CO_2 which makes this an environmentally unfriendly and ultimately unsustainable solution to contamination containment (Anbu et al., 2016).

Table 1-1 List of different types of remediation methods identifying the potential type of application of biomineralisation (Ivanov and Chu, 2008, Azubuike et al., 2016).

Process	***In situ* (No Excavation)**	***Ex situ* (Excavated, but may be treated on- or off-site)**
Destruction/ Transformation	• Bioremediation	• Thermal incineration • Composting / biopile
Separation	• Soil vapour extraction (may include air sparging, bioventing, bioaugmentation, biostimulation) • Pump and treat • Injection and recovery • Phytoremediation	• Physical separation • Soil washing • Steam stripping
Containment	• Cut-offs / Diaphragm wall • Permeable Reactive Barrier • Stabilisation • Biomineralisation	• Landfill • Solidification

1.1.3 Biomineralisation-as a potential method for containing leachate

Biomineralisation is an emerging process in the area of ground improvement that presents promising potential characteristics for ground improvement to mitigate contamination. In this

case, ground improvement refers to soil strengthening and reduced permeability. The process involves the interaction of CO_2 released by the bacteria with water resulting in the formation of carbonic acid (H_2CO_3). Carbonic acid may lose protons to form bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) aids in the production of calcium carbonate (Dhami et al., 2013).

$$CO_2\,(g) \longleftrightarrow CO_2\,(aq.)$$

$$CO_2\,(aq.) + H_2O \longleftrightarrow H_2CO_3$$

$$H_2CO_3 \longleftrightarrow H^+ + HCO_3^-$$

$$HCO_3^- \longleftrightarrow CO_3^{2-} + H^+$$

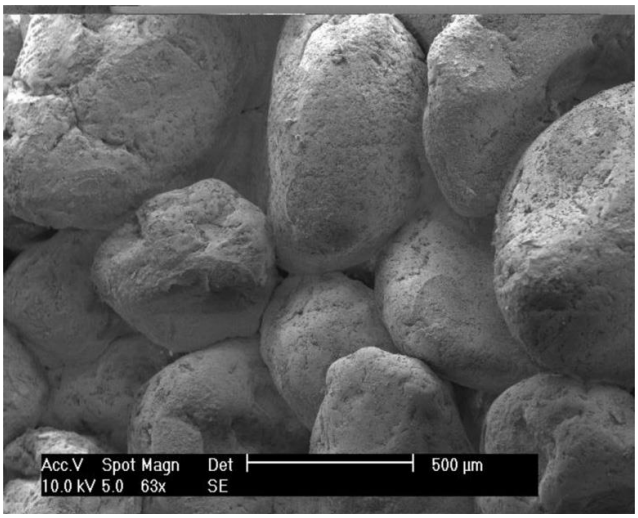

Figure 1-2 Cementation of calcite observed between sand particles. Adapted from (Cheng and Cord-Ruwisch, 2012).

The precipitation of calcium carbonate forms a cementation bond by combining and holding the sand particles together. This cementation increases the strength and lowers the permeability of sand.

Biomineralisation is environmental friendly when compared with “containment” methods presented in Table 1-1. The crystals formed due to precipitation will lead to cementation

bonds between soil particles which will then trap the leachate within a low permeability zone (Figure 1-2) (Cheng and Cord-Ruwisch, 2012, Whiffin et al., 2007).

At present, most extensively studied biomineralising bacteria have been purchased from culture collection laboratories (Benzerara et al., 2004, Rivadeneyra et al., 2004, Lian et al., 2006, Sanchez-Roman et al., 2007, Sánchez-Román et al., 2011). This would mean that external injection of bacteria into the ground would be required for biomineralisation to take place. This approach to ground improvement has a specific risk in that the bacteria may not survive well in the natural soil environment. This is especially true for environments that contain biotoxic contaminants such as landfill leachates. One of the key areas of investigation of this thesis is identifying if indigenous bacteria inhabiting the source or sites of contamination have the potential for carbonate precipitation. More information on the investigation of indigenous bacteria and their biomineralising ability is discussed in detail in Chapters 3 and 4. It is considered that the use of indigenous bacteria is a better approach for containment and remediation of toxic sites as it implies minimal disturbance to the existing microbial consortia and limited competition between this consortia and the injected species. With the use of indigenous bacteria, the risks in biomineralisation can be mitigated as the bacteria's survivability within the contaminated environment is already known. In addition, should such bacteria exist, there is a potential for stimulating indigenous bacteria *in situ* by adding the necessary nutrients to help in calcite precipitation directly to the soil while requiring minimal microbiological work (no requirement for isolation, identification and sub culturing of such bacteria) thus substantially minimising the cost of the biomineralisation process (Ivanov and Chu, 2008). The identification of, and exploration of the potential for such indigenous bacteria forms the majority of this thesis.

Previous studies of bacteria isolated from mine tailing soil (Achal and Pan, 2014), karst soil (Li et al., 2011), caves (Rusznyak et al., 2012), an abandoned expressway (Kang et al., 2014b)

and fresh water lakes (Zamarreňo et al., 2009b) have shown that biomineralising bacteria do exist in a wide range of environments, which are not restricted to any specific geographical terrain. This range of biomineralising bacteria has been shown to be efficient in calcite precipitation under laboratory conditions (within what is later termed as flask experiments). From the literature review (Chapter 2) it is evident that landfills have not been explored for the existence of biomineralising bacteria. Biomineralisation could be a promising method to prevent leachate from contaminating groundwater. Thus, landfills present an opportunity for investigating the existence of indigenous biomineralising bacteria where there also exists a potential engineering application. Hence, this study focuses on identifying if indigenous bacteria in regions like landfills are capable of calcite precipitation. To achieve this, seven indigenous bacteria were isolated from landfill leachate and groundwater samples from the landfill. Two bacteria were isolated from landfill groundwater and five bacteria were isolated from landfill leachate. Fresh leachate samples were collected from a pipe that drains the body of the landfill. Raw leachate is the name used to describe fresh leachate which was stored in a tank located in a separate location of the landfill that is used for leachate processing. Initially, the bacteria were studied under laboratory conditions in comparison to an abiotic solution (solution with no bacteria) and later quantitative (carbonate titration) and qualitative analyses (SEM and XRD) were performed to confirm the relative extent to which the indigenous bacteria can precipitate calcite. The solution containing the bacteria was 2.91 to 3.24 times superior in calcite precipitation in comparison to an abiotic solution. Subsequently, the three most efficient bacteria were chosen for engineering studies using sand as the medium and a purpose-made PVC pipe setup. The three most efficient bacteria were obtained from landfill leachate and groundwater samples. Two bacteria were obtained from landfill fresh leachate and one bacterium was obtained from landfill groundwater. Reduced permeability, carbonate precipitation and strength were observed in the bacterial system in comparison to an abiotic

system. The character of the intergranular improvement was assessed using Scanning Electron Microscopy (SEM) which was used to identify the improved cementation bonds between sand particles in the bacterial system.

Improvements in strength of the biomineralised system have been previously identified (Chu et al., 2012). The improvements in ground strength imply that biomineralisation can also have an additional range of potential applications where it is used for the modification and improvement of the mechanical properties of the soil. Currently, existing methodologies such as soil mixing, grouting and lime treatment are used for both lowering permeability and increasing the strength of soil. Each methodology presents a positive scope in improving the ground, but all such techniques have the potential to cause harm towards the microbial consortia which makes them non-environmental friendly and also, they are not time and cost effective. Soil mixing in particular has the potential to change the chemical environment of the soil so much that the bacteria which were once responsible for the immobilization of heavy metals or nitrogen fixation might not exist anymore. Soil mixing, grouting and lime treatment also require the manufacturing of cement products which are responsible for the generation of large quantities of CO_2 which has the previously discussed negative environmental consequences. For the isolated bacteria applied in the sand columns, strength index tests were made using a pocket penetrometer to confirm biocementation bonds between sand particles. An increase in strength was observed for effective biomineralisation samples.

Finally, as a “proof of concept” a highly permeable soil was collected from the field and the indigenous bacteria present in the soil were stimulated for carbonate precipitation at 15°C. The experiment was carried out in the UK by collecting soil samples from Formby national park. This approach was used to show that indigenous carbonate precipitating bacteria do exists in geographical conditions that are very different to those present in China.

1.2 Aims and Objectives

The investigation presented here has been conducted with the purpose of isolating, identifying and applying indigenous carbonate precipitating bacteria from a landfill environment for engineering purposes. Thus, this work investigates a less hazardous and more environmental friendly approach to ground improvement and permeability reduction in comparison to either the external injection of bacteria (also biomineralisation) or more traditional ground improvement methods such as grout injection into soil. In particular, this research is focused on contamination sourced from landfill leachate as this presents a particularly complex problem due to the mixture of contaminants that leachates commonly contain. The points shown below are detailed step-by-step approaches undertaken to fulfil the aims and objectives that are shown below,

1) **Investigate the microbial communities and heavy metal concentrations in a landfill and urban site (fulfilled in Chapter 3).**

To study the effect of heavy metal concentrations, present in an environment on the microbial community. The heavy metal concentrations of two sites were measured using inductively coupled plasma-mass spectrometry (ICP-MS) and next generation sequencing (NGS) was used to assess the microbial diversity for both sites. The potential influence of heavy metals towards microbial community was analysed and contrasted for samples from a landfill and an urban site. The sites investigated were 27 kilometres apart from each other (Figure 1-3). The urban site was used for agricultural purposes before urbanization. Geographical co-ordinates of the landfill cannot be disclosed due to privacy and ethical reasons. Both sites are located in Suzhou, Jiangsu, China and more details about the site specifications are provided in Chapter 3. Statistical techniques including cluster analysis and assessment of richness indices were used to compare both sites to identify whether the heavy metals have an effect on the sites microbial community diversity and composition.

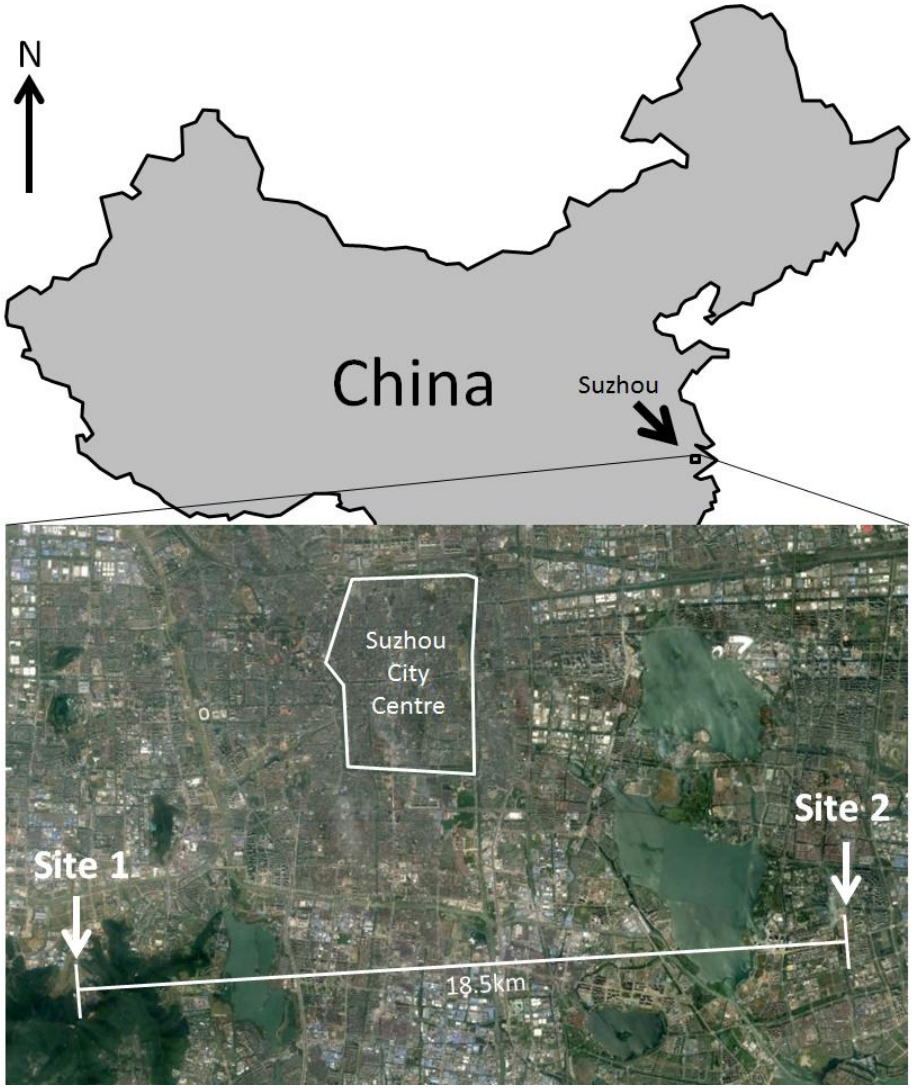

Figure 1-3 Distance between the landfill (Site 1) and urban site (site 2).

2) **To identify if indigenous carbonate precipitating bacteria that inhabit environments such as landfills could potentially be used for engineering applications. (fulfilled in Chapters 4)**

To demonstrate this, individual bacteria were initially isolated using the streak plate method and sequencing was carried out to identify the bacterial species. Laboratory biomineralisation experiments were conducted in conical flasks containing calcium chloride, urea and nutrient broth (along with bacteria) to study whether the bacteria are capable of carbonate

precipitation. Comparisons with an abiotic solution including both qualitative analysis via electron microscope imaging, X-ray diffraction and quantitative analysis via carbonate titration were used to assess the extent of the bacteria's ability to precipitate carbonate minerals.

3) **To apply the identified indigenous carbonate precipitating bacteria in treated sand columns to assess their effectiveness. (fulfilled in Chapter 5)**

To demonstrate that indigenous bacteria can precipitate calcite within a soil (standard granular) environment, multiple pilot scale sand column precipitation experiments were carried out. The experiments were performed using the three most efficient carbonate precipitating bacteria.

4) **To test the potency of the identified indigenous carbonate precipitating bacteria in treated soil columns experiments (Shown in Appendix)**

Soil columns were constructed using a unique design containing a 50 ml syringe. The soil utilised were high permeability silty sand soils and the indigenous bacteria were stimulated using the same chemicals that were used during the sand column experiments. No external bacteria were added to these soil columns. Thus, any biomineralisation observed were the result of existing indigenous soil bacteria.

To achieve the Aims and objectives shown in Section 1.2, a flow chart was designed to follow a step by step methodical approach to attain the results. Figure 1-4 shows the approach that was taken for achieving the desired result for Chapter 4. Figure 1-5 shows the approach that was taken for achieving the desired results for Chapter 5.

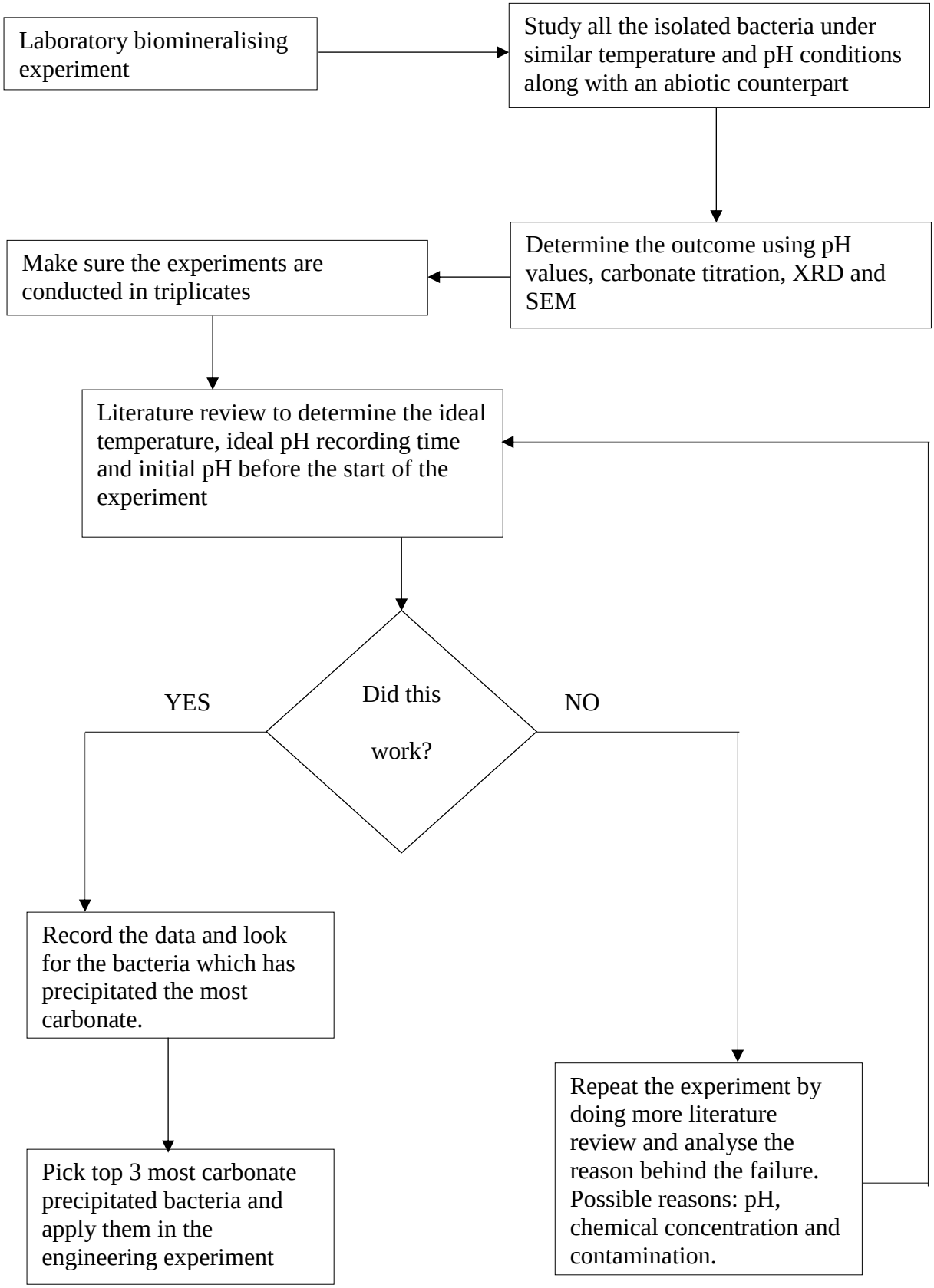

Figure 1-4 Flow chart displaying the thought process behind the laboratory experiments for the biomineralising bacteria.

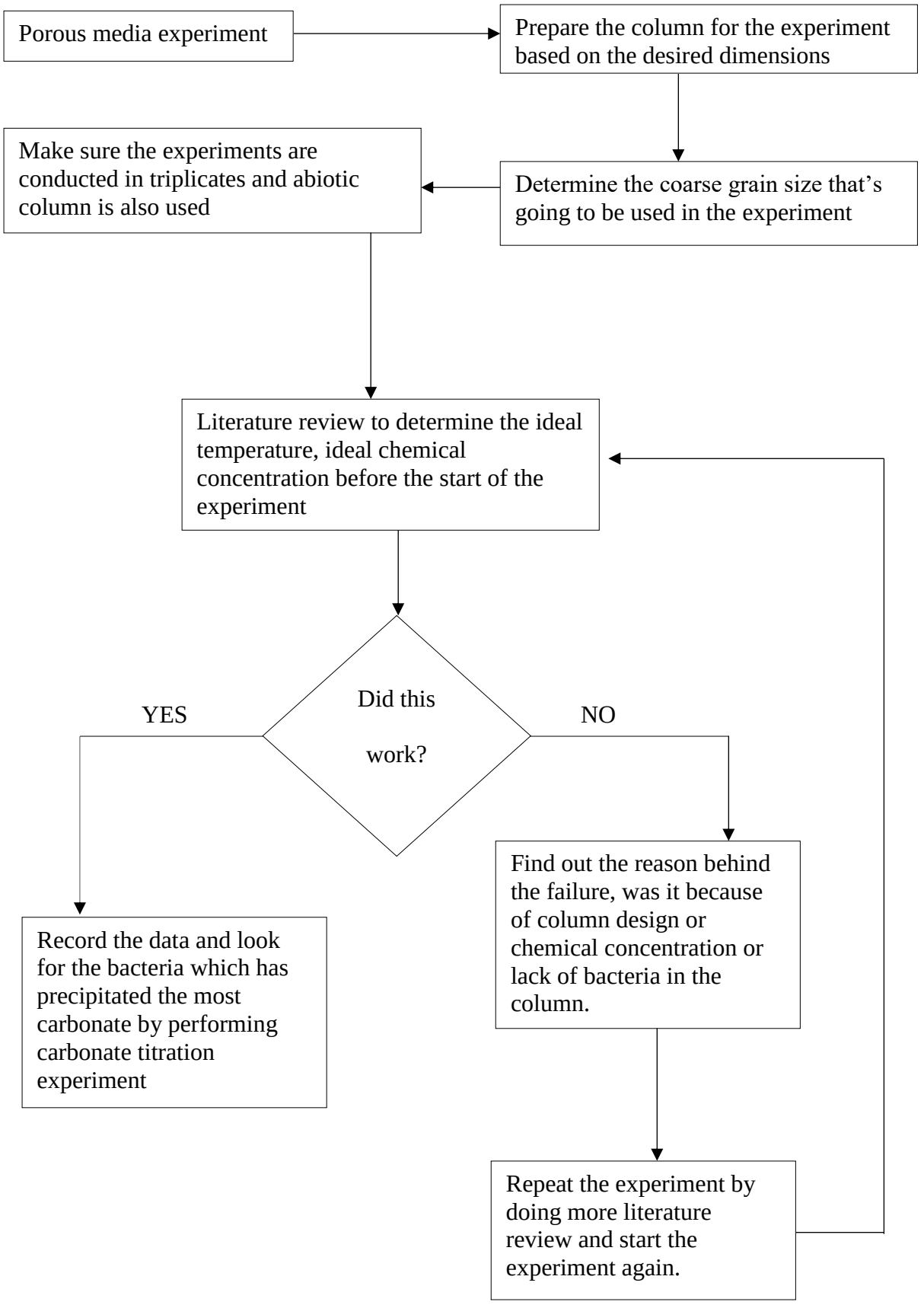

Figure 1-5 Flow chart displaying the thought process behind the porous media experiments for the biomineralising bacteria.

1.3 Structure of the Thesis

This thesis consists of seven chapters, including three distinct pieces of research work and additional appendices which provide the findings when addressing the objectives listed above. Chapters 1 and 2 provide the introduction to the thesis; this includes a brief review of the relevant literature, the aims and objectives and structure of the work that has been undertaken in order to meet the research objectives of this thesis. Chapters 3-5 describe in detail key elements of the research which have been carried out, with the results and interpretation. Each of Chapter 3-5 is a self-contained, complete piece of research forming part of the wider project. Each chapter is written as a manuscript which has either been published at or submitted to a peer-reviewed journal for publication. Chapter 6 includes a discussion and synthesis of Chapters 3-5 and a review of work that has been carried out as background work in the preparation of Chapters 3-5. A discussion is presented as a part of each of the three pieces of research work presented in Chapters 3-5. The discussion in Chapter 6 is written to show how the work presented in Chapters 3-5 connects together in order to meet the overall aims of the project. Chapter 7 concludes the thesis with a summary of the main outcomes, a summary of the results achieved and the key conclusions which can be drawn from those results. This section also includes recommendations for how this research work could be expanded by future researchers. Details of the submitted papers associated with each chapter of this thesis are presented here:

Chapter 3

Published with Canadian Journal of Microbiology: Adharsh Rajasekar, Raju Sekar, Eduardo-Medina Roldan, Jonathan Bridge, Charles K.S. Moy and Stephen Wilkinson. 2018. Next-generation sequencing showing potential leachate influence on bacterial communities around a landfill in China.

Chapter 4

Accepted by Canadian Journal of Microbiology: Adharsh Rajasekar, Stephen Wilkinson, Raju Sekar, Jonathan Bridge, Eduardo-Medina Roldan, Charles K.S. Moy. 2018. Biomineralisation performance of bacteria isolated from a landfill in China**.**

Chapter 5

Submitted to Engineering Geology: Adharsh Rajasekar, Stephen Wilkinson, Jonathan Bridge, Charles K.S.Moy, Raju Sekar and Eduardo Medina-Roldan. 2018. The geotechnical application of MICP using locally extracted Bacterial Strains.

In all cases, the author of this thesis conducted the research work and prepared the manuscript along with all figures and tables. Co-authors named in the manuscripts provided supervisory roles in manuscript structure and content during preparation for submission. Given the structure of this thesis, the review of the relevant literature surrounding this research work is divided between Chapters 2-5 such that the most relevant elements of the literature are presented independently for each piece of this research project. The literature review presented in Chapter 2 is a brief overview of the subject area as a whole, which forms a background context to the project. The details of this are enhanced within each of the other chapters. This approach allows each of the chapters to be read independently, but it does result in some elements of the literature being repeated in later chapters.

CHAPTER 2 LITERATURE REVIEW

The review of literature presented in this section includes the processes causing biomineralisation, how these processes may be applied for engineering purposes and the need for the use of such environmental friendly methodologies in civil and environmental engineering. The ground improvement methodology presented in this thesis along with the "proof of concept" identifies and suggests that indigenous bacteria capable of biomineralisation can be found in landfill environments. In a broader sense, this thesis suggests that such bacteria are not restricted to any particular geographical terrain. This thesis also forms the basis for stating that utilizing the indigenous bacteria for biomineralisation as a means for ground improvement should provide a more environmental friendly solution than equivalent existing technologies.

2.1 Introduction

The process of biomineralisation involves precipitation of minerals by bacteria via the alteration of the chemical environment. This occurs by a sequence of chemical reactions and physiological pathways which results in the precipitation of a range of different forms of solid mineral structure (Rajasekar et al., 2017).

The key areas of interest for the study of biomineralisation are: 1) the role of bacteria, 2) the influence of chemical environment 3) the types of calcium sources 4) the application of biomineralisation in engineering 5) the current ground improvement technologies their advantages and limitations 6) the advantages and disadvantages of biomineralisation 7) the justification for the application of biomineralisation in engineering. All of these are discussed in detail below.

2.2 Role of bacteria in biomineralisation

Bacteria have the potential to induce carbonate (particularly $CaCO_3$) precipitation through processes such as photosynthesis (Bundeleva et al., 2012), ammonification (Okwadha and Li, 2010, Lian et al., 2006), ureolysis (Okwadha and Li, 2010), sulfate reduction (Deng et al., 2010), methane oxidation (Reeburgh, 2007) and denitrification (Martin et al., 2013) as shown in Figure 2-1. Calcite, aragonite and vaterite are all crystal precipitates that have been observed as a result of these processes (Sanchez-Roman et al., 2007). Other minerals that are precipitated via biomineralisation through passive surface-mediated mineralisation include Fe, Mn and other metal oxides, e.g., ferrihydrite ($5Fe_2O_3 \cdot 9H_2O$), hematite (α-Fe_2O_3) and goethite(α-FeOOH); metal sulfates, phosphates, and carbonates; phosphorite; Fe and Fe-Aluminosilicates and metal sulfides (Dhami et al., 2013).

There are two defined modes of biomineralisation: biologically induced and biologically controlled biomineralisation. In the first case (biologically induced biomineralisation), chemical variations, a result of the metabolic activity of microorganisms lead to precipitation in the external environment surrounding the bacteria (Barat et al., 2008). In this case, the bacteria induces the precipitation by altering the chemistry of the environment as a result of its metabolic activity, making it an active not a passive process (Bazylinski et al., 1995).

During biologically controlled biomineralisation, bio-minerals not directly associated with cellular structures or a specific nucleation site intracellularly or on the cell wall of bacteria can be observed (Benzerara et al., 2004). Investigations of the morphology of recent and ancient natural phosphate deposits have indicated that precipitation occurs on bacterial cell surfaces, which is a good example of naturally occurring biologically controlled biomineralisation (Barat et al., 2011).

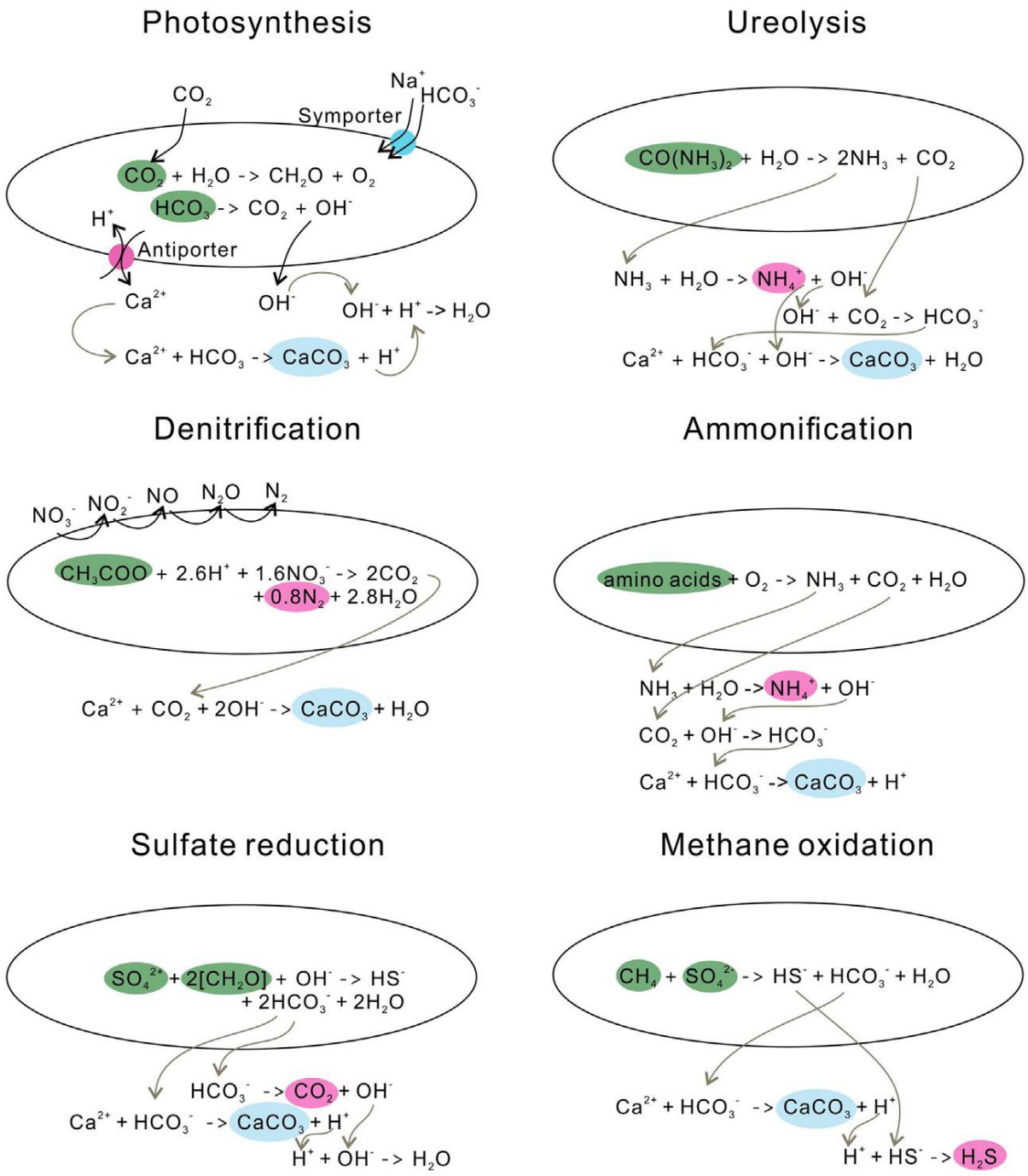

Figure 2-1 Various chemical processes that have shown the ability to generate supersaturated environments essential for calcite precipitation. Adapted from (Zhu and Dittrich, 2016).

2.2.1 Photosynthesis

Photosynthesis lead to the precipitation of stromatolites through calcification of cyanobacterial mats during most of the Precambrian times (Aloisi, 2008). Photosynthetic microbes, particularly cyanobacteria, are found to the main reason behind the formation of carbonate rocks on earth (Altermann et al., 2006). Cyanobacterial stromatolite, a laminated

calcareous fossil, was found in various environments (Goh et al., 2010). The process essentially leads to calcite precipitation by conducting an HCO_3^- /OH^- exchange process across the cell membrane, resulting in an increase of pH in the microenvironment around cells (Figure 2-1). The calcification process begins when the symporter transports Na^+ and HCO_3^- into the cells along with CO_2. Later CO_2 is synthesized through HCO_3^- break down forming OH^-, resulting in an alkaline environment. A higher HCO_3^- concentration leads to higher formation of CO_2 and also $CaCO_3$ (Figure 2-1). Shiraishi (2012) found that higher $CaCO_3$ was attained with a high Ca^{2+} concentration and low ionic strength with optimum pH and dissolved inorganic carbon.

2.2.2 Ureolysis

Similar to the photosynthetic microorganisms, ureolytic bacteria impact the concentration of the DIC and the pH of an environment, yet through urea hydrolysis. For example, *Sporosarcina pasteurii* (Wei et al., 2015) hydrolyze urea to produce ammonia and carbonic acid (De Muynck et al., 2013) (Figure 2-1). The subsequent hydrolysis of ammonia increases the pH by producing OH−, and the dissociation of carbonic acid generates bicarbonates (Knoll, 2003). In this process, alkalinity is increased through urea hydrolysis at a cost of generating the unfavorable by-product ammonia. Therefore, the reaction favors the precipitation of calcium carbonate in the presence of calcium in solution. Urea is a nitrogen source for a variety of microorganisms belonging to different genus or species, including fungi (*Aspergillus* sp., *Coprinus* sp., *Neurospora* sp., *Penicillium* sp., and *Ustilago* sp.), *Bacillus* (*Bacillus lentus*, *B. pasteurii*, *B. sphaericus*, and *B. subtilis*), *Lactobacillus* (*Lactobacillus reuteri, L. animalsi, L. fermentum, Streptococcus salivarius, S. mitior, S. thermophilus*, and *Staphylococcus epidermis*), *Nitrosomonas* and *Nitrosospira* species, purple sulfur and non-sulfur bacteria (*Rhodobacter cap-sulatus*), cyanobacteria (*Anabaena cycadae, A. cylindrica, A. doli-olum, A. variabilis, Anacystis nidulans, Spirulina maxima, Nostoc*

calcicola, *N. muscorum*, and *Brevibacterium ammoniagenes*), and Actinomycetes (*Streptomyces aureofaciens*) (Hasan, 2000). Microbial urease activity is greatly influenced by temperature, pH (with an optimal of 7–8.7 except for acid-urease activity), concentration of urea and the end product ammonia, carbon source, and incubation period (Hasan, 2000). The genus *Bacillus* is the most common ureolytic bacteria used for biotechnology. They typically are Gram-positive, aerobic, and rod-shaped prokaryotic cells with a size of 1–10 μm (Martin et al., 2012; Wong, 2015). They are particularly interesting for MCP technology due to their capability of producing CO_2 paralleled by increasing pH in the surrounding environment (Sel et al., 2015; Wei et al., 2015). Both respirations by cell and the decomposition of urea provide a source of CO_2. For example, *Bacillus diminuta*, one of the most effective carbonatogenic bacterium, was isolated from decayed building stones (Jroundi et al., 2010). Other *Bacillus* species, such as *B. lentus*, found in soil, marine, and sediments (Proom and Knight, 1955; Belliveau et al., 1991; Siefert et al., 2000), are widely used in industry to produce alkaline protease (Jørgensen et al., 2000).

2.2.3 Methane Oxidation

Anaerobic oxidation of methane favors the precipitation of calcium carbonate, whereas aerobic oxidation of methane leads to the dissolution of carbonates by increasing acidity (Reeburgh, 2007). In the anaerobic oxidation, methane is oxidized to bicarbonate, and sulfate is reduced to HS^-. In the presence of Ca^{2+}, calcium carbonate is precipitated, and hydrogen sulfide is generated (Figure 2-1). As indicated by methane profiles, radiotracers, and a stable carbon isotope, a large portion of the methane is converted to CO_2 through anaerobic oxidation in marine sediments (Stadnitskaia et al., 2008).

2.2.4 Ammonification of Amino Acids

Microbial metabolisms through ammonification of amino acids produces CO_3^{2-} and NH_3 (Figure 2-1). Release of NH_3 generates OH^- around cells and leads to a high local

supersaturation which facilitates the precipitation of calcium carbonate, and consequently, precipitates calcite or vaterite (Gonzalez-Munoz et al., 2010). Some species, which are representative of Myxobacteria, e.g., *Myxococcus xanthus* serve as a nucleation template for carbonate precipitation (Jimenez-Lopez et al., 2007). They are Gram-negative, aerobic, non-pathogenic, heterotrophic, and rod-shaped that have the ability to utilise/consume amino acids as their sole energy source (Gonzalez-Munoz et al., 2003).

2.2.5 Denitrification

Denitrifiers are typically facultative anaerobes, which provides more options to use them for application of carbonate precipitation during field experiments. Under anaerobic conditions, nitrate is consumed by microorganisms to oxidize organic compounds for energy and cell growth (Martin et al., 2013). Due to its highly negative standard Gibbs free energy ($\Delta G°$), denitrification can be expected to dominate where nitrate and organic carbon is present and O_2 is limited (DeJong et al., 2010). The denitrification process increases the pH in the surrounding medium by consuming H^+, and produces CO_2, which favors carbonate precipitation (Figure 2-1). Till now, only a few studies have investigated the direct link between denitrification and calcium carbonate formation and observed calcium carbonate crystals precipitating around the cells during denitrification. The by-product generated during this process is N_2, which is harmless (Van Paassen et al., 2010). However, toxic nitrite and nitrous oxide might accumulate if any of four different enzymes (nitrate reductase, nitrite reductases, nitric oxide reductase and nitrous oxide reductases) involved in the denitrification process are inhibited (Van Paassen et al., 2010).

2.2.6 Sulfate Reduction

Sulfate-reducing bacteria (SRB) reduce sulfate to sulfide while oxidizing organic carbon to bicarbonate which leads to an environment with high pH and saturation state (Baumgartner et al., 2006). SRB utilize a variety of electron donors, including hydrogen, formate, acetate,

propionate, butyrate, ethanol, methanol, and lactate. With an increase in HCO_3^- concentration and the release of Ca^{2+}, it favors $CaCO_3$ precipitation (Figure 2-1). SRB are an important component of mineral precipitation because SR causes an increase in alkalinity relative to aerobic respiration. In addition, even the metabolically inactive SRB cells, e.g., *D. desulfuricans* strain G20, may lead to calcium carbonate precipitation by providing heterogeneous nucleation sites (Bosak and Newman, 2005). Baumgartner et al. (2006) successfully observed carbonate precipitation by SRB in microbial mats underneath the surface layers where cyanobacteria are active.

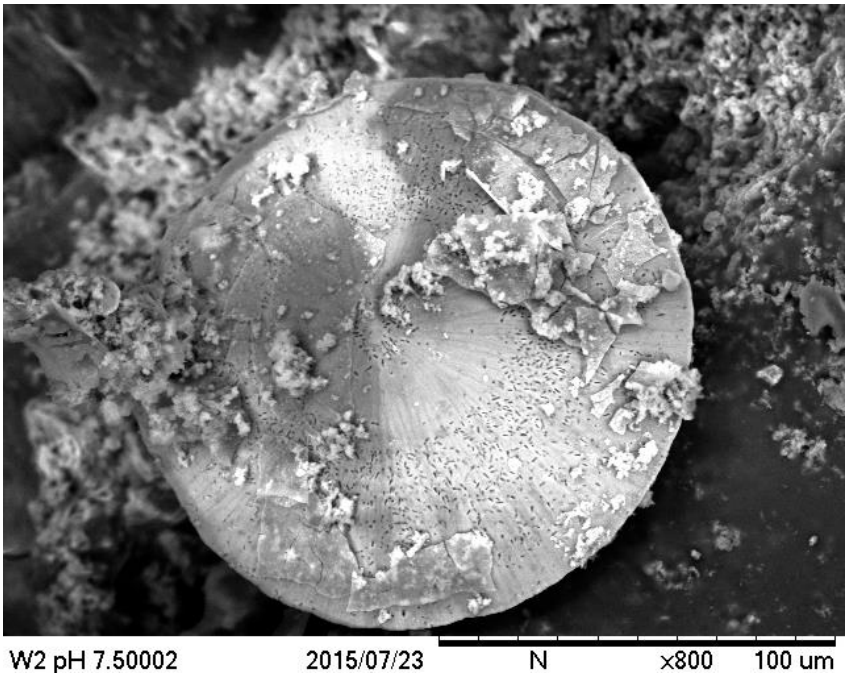

Figure 2-2 Bacterial imprints in a calcite crystal (discussed further in Chapter 3).

As part of this process, bacterial imprints are commonly found on the surface of calcite crystals (Figure 2-2).

Observations from Chapter 4 indicate that the crystal structures of the abiotic and bacterial solutions are different. Bacterial solutions have a spherical shaped crystal structure compared to the trigonal structure formed in the abiotic solution. Literature sources also identify that bacteria influence the shape of the precipitated crystal as for example, spherical crystals

(Rivadeneyra et al., 2006, Sanchez-Roman et al., 2007) and polyhedral crystal formation (Lian et al., 2006). But the process behind how the bacteria influence the shape of the crystal formation is not currently explored.

2.3 Influence of environmental factors

Calcium carbonate precipitation is a rather straightforward chemical process governed mainly by three key factors: (1) pH, (2) temperature, (3) a catalyst (e.g. urease or carbonic anhydrase) and (4) the calcium concentration/sources.

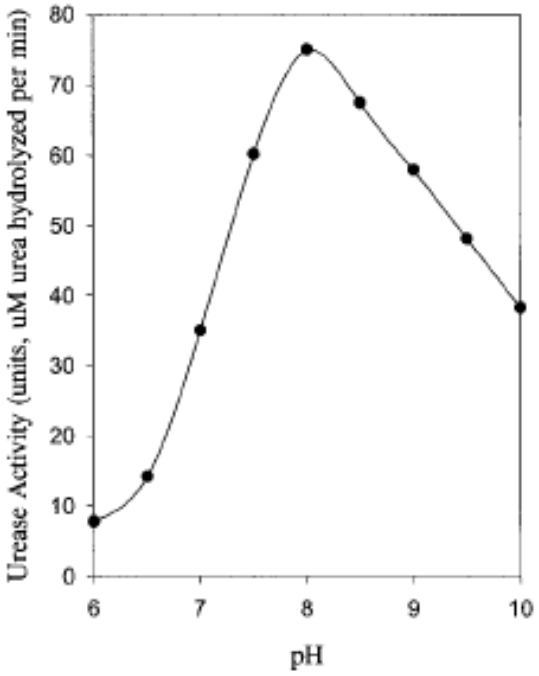

Figure 2-3 Effect of pH on urease activity. Adapted from (Stocks-Fischer et al., 1999)

2.3.1 pH

Several studies have been carried out to assess the relative impact of different environmental conditions on the biomineralisation process. Investigations of the precipitation of minerals by different groups of bacteria have identified that pH influence carbonate precipitation (Helmi et al., 2016, Ferris et al., 2003). The precipitation of calcite in a solution can be tracked by measuring the pH as the urease enzyme will only be active at pH values which support urea hydrolysis. Many researchers have reported that the optimum pH for urease is 8.0 and above

8.0, the enzyme activity decreases by half (Stocks-Fischer et al., 1999, Gorospe et al., 2013) (Figure 2-3).

Aerobic bacteria release CO_2 via cell respiration, which is paralleled by an increase in pH due to ammonia production (Ivanov and Chu, 2008). Upon the decrease of pH, the carbonate starts to dissolve (Hammes and Verstraete, 2002).

Most calcite precipitation occurs under alkaline conditions from pH 8.7 to 9.5 (Ferris et al., 2003, Stocks-Fischer et al., 1999, Sanchez-Roman et al., 2007). Stabnikov et al. (2013) investigated the activity of halophilic and alkaliphilic urease producing bacteria at high concentrations of inorganic salt and pH above 8.5. The results show that halophilic and alkaliphilic bacteria are more tolerant to conditions in which other urease producing bacteria cannot survive. This shows that certain groups of bacteria capable of surviving and precipitating calcium carbonate do exist and can survive extreme conditions such as high concentrations of inorganic salt concentration and high alkaline pH.

2.3.2 Temperature

Temperature dictates the growth of bacteria and the ability of urease enzyme to facilitate the precipitation of calcium carbonate. Experimental studies tested on carbonate precipitating isolates were able to grow and precipitate carbonate crystals at temperatures between 10 and 40°C (Zamarreňo et al., 2009b). However, at 40°C, only certain bacteria were able to grow and precipitate crystals. Recent findings indicate that significant calcite precipitation only occurs between 20-37°C (Helmi et al., 2016). The optimum temperature for efficient carbonate precipitation is 35°C (Zamarreňo et al., 2009b, Helmi et al., 2016). The optimum temperature was proved by the fact that the bacteria were able to stimulate optimum urea hydrolysis and superior calcium carbonate precipitation.

2.3.3 Effect of urease towards calcite precipitation

Urease producing bacteria (UPB) are the most commonly used bacteria for precipitation studies and the conversion of urea to ammonia is the proposed mechanism for carbonate precipitation (Table 2-1). Upon addition of urea to the bacteria, dissolved inorganic carbon (DIC) and ammonium (AMM) are released in the microenvironment (Figure 2-4A). Calcium ions in the solution are attracted to the bacterial cell wall due to the negative charge of the latter creating calcium supersaturation locations (Figure 2-4B). This causes bacterial encapsulation due to a limited amount of nutrient transfer, which results in cell death (Figure 2-4C). The imprints of bacterial cells involved in carbonate precipitation remain in the carbonate crystals (Figure 2-4D). The word "commonly used" is substantiated by the table shown below.

Table 2-1 List of urease producing bacteria that are capable of calcite precipitation.

Number	Name of the bacteria	Authors
1	*Bacillus megaterium*	(Stocks-Fischer et al., 1999, Lian et al., 2006)
2	*Sporoscarcina pasteurii*	(Okwadha and Li, 2010, Tobler et al., 2011)
3	*Bacillus* sp. VS1	(Chu et al., 2012, Chu et al., 2013, Chu et al., 2014)
4	MCP-11 (*Bacillus sphaericus*)	(Cheng and Cord-Ruwisch, 2012, Cheng and Cord-Ruwisch, 2013)
5	*Bacillus sp.* CR2	(Achal and Pan, 2014)
6	*Sporosarcina pasteurii* WJ-2	(Kang et al., 2014b)
7	*Methylocystis parvum* OBBP	(Ganendra et al., 2014)
8	*Lysinibacillus sphaericus* CH5	(Kang et al., 2014a)
9	*Bacillus licheniformis*	(Helmi et al., 2016)

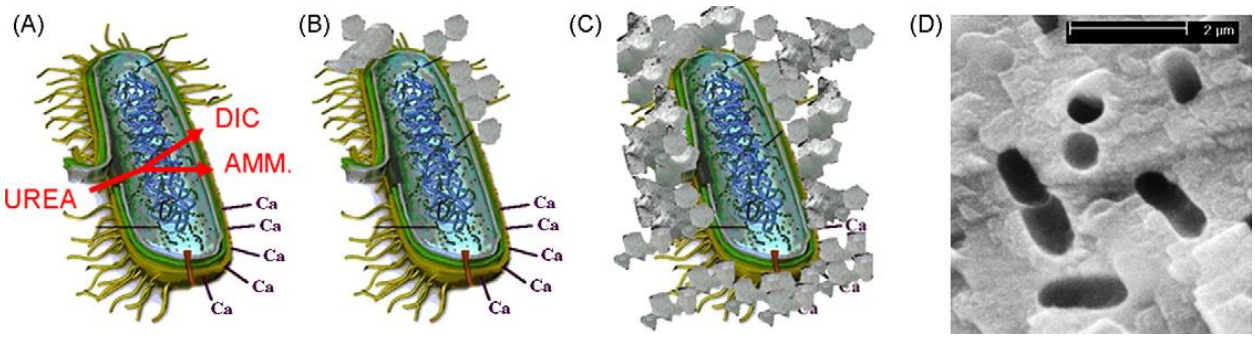

Figure 2-4 Simplified representation of the process that happens during ureolytic induced carbonate precipitation. Adapted from (Muynck et al., 2010a).

The conversion from urea to NH_4^+ results in a pH increase which favours the precipitation of calcium carbonate. The chemical reactions and the pathways of urease producing bacteria are discussed in more detail in Chapters 4.

An alternative precipitation pathway via the enzyme carbonic anhydrase (CA) (EC4.2.1.1) has been suggested and is an area of ongoing research for calcite precipitation (Li et al., 2013b). CA is a zinc-containing metalloenzyme that is ubiquitously distributed in organisms which are responsible for eukaryotic biological processes such as photosynthesis, respiration, transport of CO_2 and ion, calcification and acid–base balance (Li et al., 2007). The mechanism by which CA contributes to the formation of calcium carbonate was put forward by Li et al. (2010) as shown below,

$$HCO_3^- + H^+ \xrightarrow{CA} H_2O + CO_2$$

$$Ca^{2+} + 2HCO_3^- \longrightarrow CaCO_3 + H^+ + HCO_3^- \longrightarrow CaCO_3\downarrow + H_2O + CO_2$$

If CO_2 is the source of dissolved inorganic carbon (DIC), CA may catalyze its conversion into HCO_3^-, and the overall reactive equations are as follows:

$$CO_2 + H_2O \xrightarrow{CA} HCO_3^- + H+$$

$$Ca^{2+} + HCO_3^- \longrightarrow CaCO_3\downarrow + H^+$$

In previous studies, it was hypothesized that CA from bacteria acts as an activator to convert CO_2 to bicarbonate which helps in calcite precipitation (Li et al., 2007). Li et al. (2007) stated that not only urease but CA also plays an important role in bacterially induced $CaCO_3$ precipitation. Carbonic anhydrase produced by bacteria may also promote $CaCO_3$ precipitation by inhibiting calcium deposition in the presence of CA inhibitors in the experimental systems with or without the bacteria (Li et al., 2013a). It should be noted that the application of CA for biocementation and biogrouting has not yet been assessed.

2.3.4 Types of calcium sources investigated

One of the most basic inputs required for the precipitation of a calcium carbonate via biomineralisation is a calcium source. Calcium chloride is the most dominantly used calcium source for calcite precipitation. However, there have been studies which have attempted to identify the difference in precipitation caused by multiple (more than 1 in the same study) calcium sources (Achal and Pan, 2014, Gorospe et al., 2013, Xu et al., 2015). Achal and Pan (2014) showed that the pH of the media when calcium chloride is added is far more important than other calcium sources for calcite precipitation. Xu et al. (2015) performed kinetic studies and found that calcium nitrate is the least effective calcium source for calcite precipitation. SEM images from these studies have shown calcium salts along with the bacteria also play a role in determining the crystal structure for each individual calcium precipitate (Figure 2-5).

These studies have also shown that the type of calcium source determines the amount of urease activity that takes place. For example, calcium lactate promotes superior urease activity compared to calcium nitrate (Xu et al., 2015). An earlier study by Achal and Pan (2014) investigated the efficiency of five different calcium sources; calcium chloride, nitrate, oxide, acetate and reported that calcium oxide was the most underachieving calcium source when it comes to calcite precipitation, as it lacked in promoting urease activity when compared to calcium nitrate (Figure 2-6).

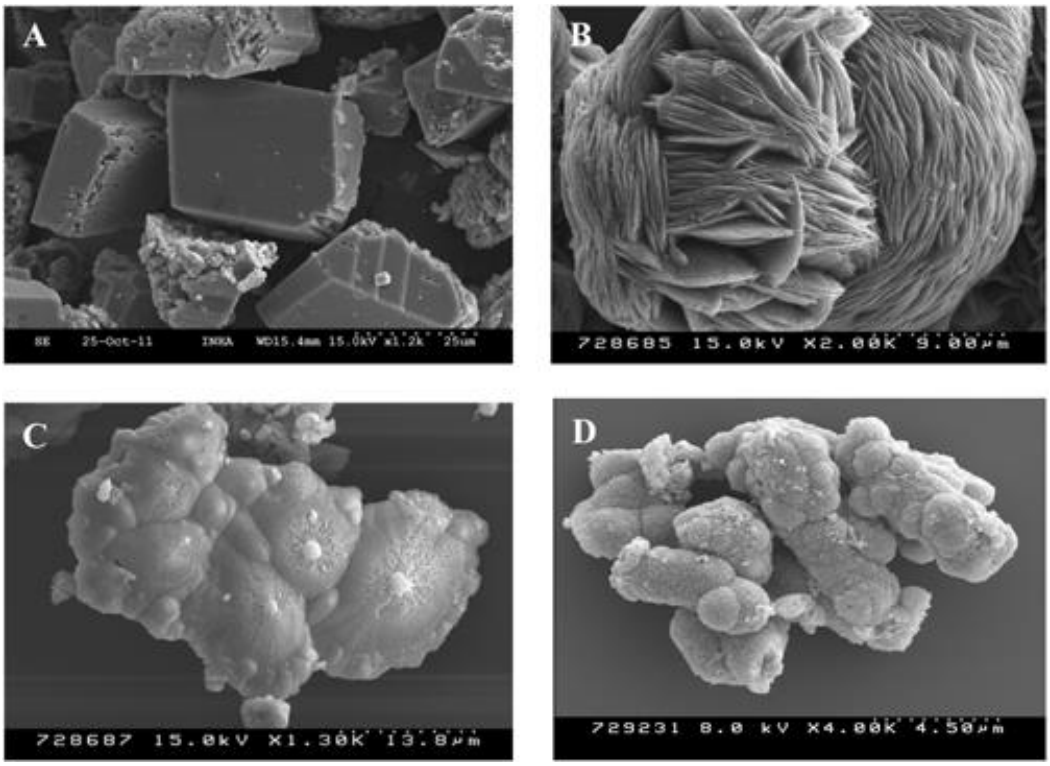

Figure 2-5 SEM images of showing the difference in shape of the calcium carbonate precipitated when calcium chloride (A), calcium acetate (B), calcium lactate (C), and calcium gluconate (D) were used. Adapted from (Gorospe et al., 2013).

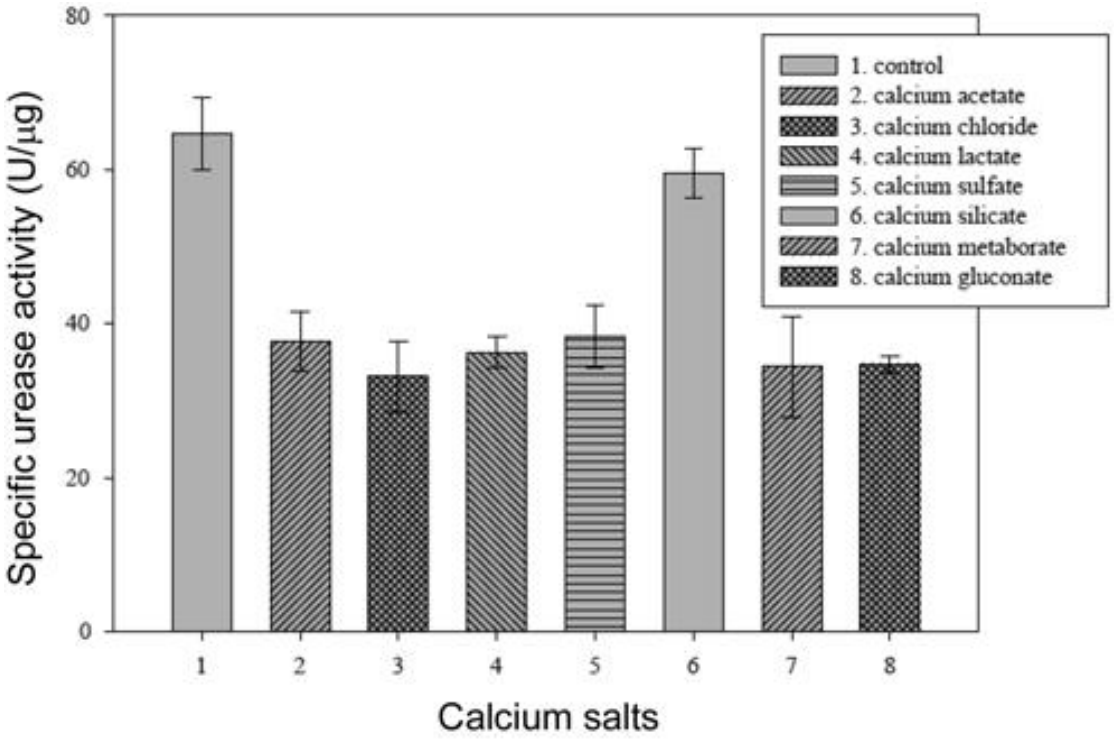

Figure 2-6 Effects of different calcium salts on the urease activity of *S. pasteurii*. Adapted from (Gorospe et al., 2013).

The use of calcium chloride promoted rhombohedral shape, this is a characteristic of the most stable form of calcium carbonate. Conversely, using calcium acetate induced a different shape, a lettuce like or lamellar shape, which is a type of vaterite, a metastable form of calcium carbonate (Fig. 2B) [16]. The crystal shape while using calcium lactate and calcium gluconate (Figs. 2C and 2D, respectively), as a source of calcium ions, gave a spherical shape, which is another form of vaterite. The morphological differences of the crystals formed may be strain-specific, having different levels of urease activity (Hammes et al., 2003). Also, the composition of the bacterial media or culture may affect the crystal morphology. Moreover, crystal growth can be inhibited or altered by the adsorption of proteins and organic and inorganic components to the specific crystallographic planes of the growing crystal (Muynck et al., 2008).

Calcium nitrate has additional issues which would prevent it from being used as a calcium source for engineering purposes. Calcium nitrate is highly soluble and it contributes to the bio-deterioration (conversion of nitrate to ammonia or nitrite) of building materials such as concrete (Anbu et al., 2016).

2.4 Application of biomineralisation in engineering

It has been recognised that the ability to control the precipitation of a solid material within a granular soil environment (via biomineralisation) could be a useful tool for engineers (Ivanov and Chu, 2008). This and other similar approaches to engineering have been collected under the name microbial geotechnology. This is a novel branch of geotechnical engineering which harnesses two key processes widely observed in biologically active porous media (wastewater filters; soils; lake sediments) – the filling of pores by cells, cell exudates, and cell-mineral aggregates which reduces permeability (bio clogging), and the microbial-induced

or microbial-controlled precipitation of minerals (biomineralisation) which yields considerable increases in strength and lowering permeability.

Table 2-2 Example engineering experiments conducted using bacteria capable of biomineralisation in sand columns.

Size of sand grain (*μ*m)	**Concentration of calcium chloride/urea**	**Strength achieved**	**Permeability achieved**	**Bacteria used**	**References**
125–250	1.1/1.1 M	200-570 Kpa	$1x10^{-5}$ m/s	*Sporoscarcina pasteurii*	(Whiffin et al., 2007)
125 to 175	100/333 mM	Not measured	$1x10^{-6}$ m/s	*Sporoscarcina pasteurii*	(Martinez et al., 2013)
420	0.75/1.5 M	215-932 Kpa	$1x10^{-7}$ m/s	*Bacillus* sp. VS1	(Chu et al., 2013)
110 to 175	1/1 M	Not measured	Not measured	*Sporoscarcina pasteurii*	(Harkes et al., 2010)

Many experiments have been conducted to study the potential application of biomineralisation in engineering (Table 2-2).

It should be noted that the bacteria used in most cases is *Sporoscarcina pasteurii (*previously known as *Bacillus pasteurii)* and it is one of the most studied biomineralising bacteria to date. The concentration of calcium chloride and urea ranged from 100 mM to 1.5 M for all these experiments (Table 2-2). Permeability is the only major improvement which is consistently substantiated from all experiments, even given that a few focused on measuring ammonium concentration as an indicator of biomineralisation instead of permeability. Strength varies a lot in each experiment and this inconsistency could be due to the heterogeneous distribution of the cementation solution throughout the produced columns.

Thus, the main envisaged engineering applications for biomineralisation is permeability reduction to reduce flow or control the direction of flow, and modification of the strength and stiffness of soils for ground improvement applications (Ivanov and Chu, 2008). In terms of the reduction in permeability, this could be applied in the remediation of contaminated land either for containment of contaminants or to control the movement of contaminants towards an area for treatment (Brad et al., 2013). This could potentially be applied in the near surface environment to reduce infiltration which could be used for example for slope stabilization. In addition, in terms of ground improvement this could be used for the strengthening of fill materials to reduce consolidation settlements, or for soil enhancement for structural applications. They could be also applied for the immobilisation of heavy metals, treatment of solid-phase capture of inorganic contaminants and atmospheric CO_2 capture (Mahanty et al., 2013). Results from the experiments outlined earlier have provided sufficient evidence that biomineralisation could be used for such engineering applications (Table 2-2). Biomineralisation has the potential to become a part of standard civil engineering practice in addition to its potential use in environmental and ecological engineering works.

2.5 Current alternatives to biomineralisation

The methodologies mentioned below are currently applied for ground improvement across the world. These techniques are conventional and have been in practice for a long time. Most of these techniques can be applied to soil which is either sandy or silty. It has to be noted that biomineralisation cannot be applied in soil which are rich in clay or has a low permeability. The tables below (Table 2-3;Table 2-4;Table 2-5) compare the current conventional ground improvement methodologies against biomineralisation. The main intention is to support the hypothesis that biomineralisation is a cost effective and eco-friendly technique for ground improvement along while preserving the microbial consortia of a given environment.

Table 2-3 Alternatives that are currently used in the field for ground improvement (Part 1) (Nicholson, 2014)

Name	**Process**	**Advantages when compared with biomineralisation**	**Disadvantages when compared with biomineralisation**
Surcharging	Addition of an excess load of material to existing ground to improve strength and reduce permeability.	If the transportation cost is low, this is a cheap option. Highly dependent on the geographical location. Surcharging can be applied to soil with low permeability.	Transportation costs makes it highly expensive. Time consuming and causes damage to the microbial consortia. May transfer heavy metals from one region to another, leading to indirect increase of heavy metal concentration.
Accelerated consolidation-sand drains	Installing high permeability sand drains in soil to reduce water movement in ground.	Helps in mitigating the transport of wastewater to nearby groundwater zones. It can be applied in soft and silt clay.	Installation of sand drains might lead to new cracks in the soil which might counteract the initial cause. It is not time and cost effective. The reinforcement effect of sand drains may reduce the effectiveness of preloading the sub soil

Table 2-4 Alternatives that are currently applied in the field for ground improvement (Part 2)(Nicholson, 2014)

Name	**Process**	**Advantages when compared with biomineralisation**	**Disadvantages when compared with biomineralisation**
Accelerated consolidation – electro-osmosis	Using electro osmosis to create new drains and improve soil compaction.	Application of a electric field to move the soil and water separately and create a new water drain to empty any potential contaminants.	This process can be applied only to places which have uncontrolled water movement. This does not prevent any contamination of groundwater, as soil compaction doesn't necessarily confirm mitigation in this technique.
Vibro-compaction/vibro-replacement	Levelling grounds using compaction where the soil is thick.	This technique is effective towards ground levelling for construction purposes.	The vibration that occurs during the levelling can create cracks in non-target zones. Levelling of soil could affect the microbial consortia that inhabit the soil.
Soil Mixing	Creating soil pile columns by mixing cement with soil.	This process can be applied in target zones where the permeability and strength can be improved.	Time consuming process and very expensive. Disturbing the microbial consortia by mixing with cement is not eco-friendly.

Table 2-5 Alternatives that are currently applied in the field for ground improvement (Part 3) (Nicholson, 2014)

Name	**Process**	**Advantages when compared with biomineralisation**	**Disadvantages when compared with biomineralisation**
Lime treatment	Spraying or spreading of lime on the top of a wet soil.	Helps increase the strength of the soil as the hydration process of lime converts wet soil to dry soil. It increases the soil strength early within days of adding lime and continuously improves. It can be applied in sandy and clay soils.	Cannot be applied to dry soil which makes it highly inefficient in terms of applicability in dry zones. Soil with high humic acid content can cause potential damage to the lime treatment process.
Grouting (Chemical, cement, slurry, jet, compensation)	Injection of slurry or a liquid solution into a soil or rock formation is termed as grouting. The injected material is referred to as the grout. The process of grouting was developed primarily as a technique for making vertical seepage barriers beneath dams and hydraulic structures by injecting cement slurry into the void space of river bed material.	It can be used to improve the permeability and strength of any high permeability zone. Can be applied to sandy and clay soils.	The pressure applied in the ground to inject the grout can create new fractures that can lead to the potential of increased high permeability in non-target zones.
Freezing	A typical ground freezing system for a shaft or tunnel consists of a series of freeze	It's applied in tunnels to prevent coarse material run off.	Changing the temperature of the soil could inhibit the growth of the microbial consortia and also its not cost effective

	pipes installed along the perimeter of the proposed excavation, extending into the subsurface strata. To freeze an area, freeze pipes are installed in a grid pattern and extend into the subsurface strata.		

2.6 Limitations/Disadvantages of biomineralisation

It has been suggested that biomineralisation may not be completely environmental friendly. The term environmental friendly is based on the use of *in situ* bacteria and chemical sources such as calcium and urea in the ground for biomineralisation. The ammonium and nitrate that are generated during the ureolysis-driven process could be toxic to human health and soil microbial consortia at high concentrations also poses a risk to health (Van Paassen et al., 2010). Additionally, if ammonium enters building materials such as concrete it can be converted into nitric acid by bacteria. The chemical reaction between calcium and nitric acid can lead to the formation of calcium nitrate, which is reported as a highly soluble component that aides with the bio-deterioration of building materials (Ganendra et al., 2014). Ganendra et al. (2014) recently investigated the MICP process using calcium formate produced by *Methylocystis parvus* OBBP and found it to be advantageous over ureolysis-driven processes because the calcium formate did not release the ammonia to the air or produce nitric acid when applied to building materials such as concrete, resulting in decreased risk of air pollution and bio-deterioration of the materials. Calcium formate hasn't necessarily been studied in porous media as this is less of a concern for geotechnical applications.

Another disadvantage of MICP is that microbial processes are usually slower and more complex than chemical process. Lian et al. (2006) reported the precipitation of calcite in the abiotic solution to be faster than in a bacterial solution. Although the rate of precipitation won't be higher than the bacterial solution due to lack of CO_2, the abiotic solution is a little faster during the initial rate of precipitation. The slow rate of precipitation in the bacterial solution is due to the microbial activity as it is completely dependent on environmental factors such as temperature, pH, concentration of electron donors and acceptors, and concentration and diffusion rates of nutrients and metabolites (Ivanov and Chu, 2008). In addition, the process is also dependant on the bacteria that's involved in the precipitation

process, as shown in Chapter 3 and 4, since certain bacteria can initiate precipitation due to their quick adaptation to the chemical environment. Therefore, a lot of optimization and analysis of MICP have been performed in anticipation of this approach being implemented on a commercial scale (Helmi et al., 2016, Li et al., 2013a, Li et al., 2013b, Okwadha and Li, 2010).

The application of biomineralisation in the field has not been investigated without the usage of laboratory grade nutrient sources. Since most (not all) engineering experiments published so far have been conducted with laboratory gradient sources such as a calcium source, urea and nutrient broth (Chu et al., 2012, Chu et al., 2013, Chu et al., 2014), this makes it challenging as the cost of these chemicals are expensive when applied in the field (Ivanov and Chu, 2008). The efficiency of the process might be affected if lower grade materials are used. In addition to this limitation, Muynck et al. (2010a) showed that application of MICP treatment depends heavily on the price of the microorganisms and the nutrients with an averaging cost ranging between \$11–19/m^2. This makes MICP economically challenging when compared to traditional methods (Ivanov and Chu, 2008). Finally, additional investigations to improve the technology and reduce unwanted by-products, such as nitrate and ammonia are needed to enable use of MICP on a commercial scale.

2.7 Potential benefits of biomineralisation

Given the major economic cost of biomineralisation is in the cultivation of the microorganisms, there is a possible cost saving to be made if existing onsite bacteria can be utilised for the biomineralisation process. Utilization of such bacteria could reduce the cost requirement for biomineralisation. In this project there has been a specific focus on the potential for biomineralisation to be applied in the remediation of contaminated land, especially for the containment of contaminant plumes of landfill leachates. This overall focus has shaped the direction of the investigations which formed the emphasis in this research

project. This is especially true concerning the types of microbes that have been investigated for this research. This is discussed in detail in Chapter 3. A brief overview of this subject is presented here.

There are several possible mechanisms that can result in leachates leaving the landfill environment and entering the surrounding groundwater, for example, breach of the landfill liner which sometimes happens due to exposure of the landfill liner to excessive rainfall/flooding; in older landfill where the landfill liner was not installed and; unlicensed or illegal waste dumping where liners are rarely in place. In addition, soil of higher permeability is known to favour the percolation of leachate to the groundwater. Once leachate enters the groundwater the direction of groundwater flow is often controlled by pumping for water abstraction; hence the leachate plume travels towards the water abstraction resulting in contamination of groundwater with leachate (Figure 2-7).

Many treatments are being experimentally assessed for removing pollutants present in the leachate; however, the degree of treatment required is dependent upon the nature and concentration of the contaminants in the leachate (Xie et al., 2012, Kalcikova et al., 2014, Fernandes et al., 2013). Many such treatments involve the utilisation of bacteria present within the leachate to breakdown the waste and resulting leachates.

Due to the rapid growth of industrialization, the heavy metals that accumulate in environments like landfills generate health problems for living organisms including microorganisms, animals and humans. Some heavy metals are crucial to human health in small concentrations, but toxic in the greater concentrations released by industry (Fu and Wang, 2011).

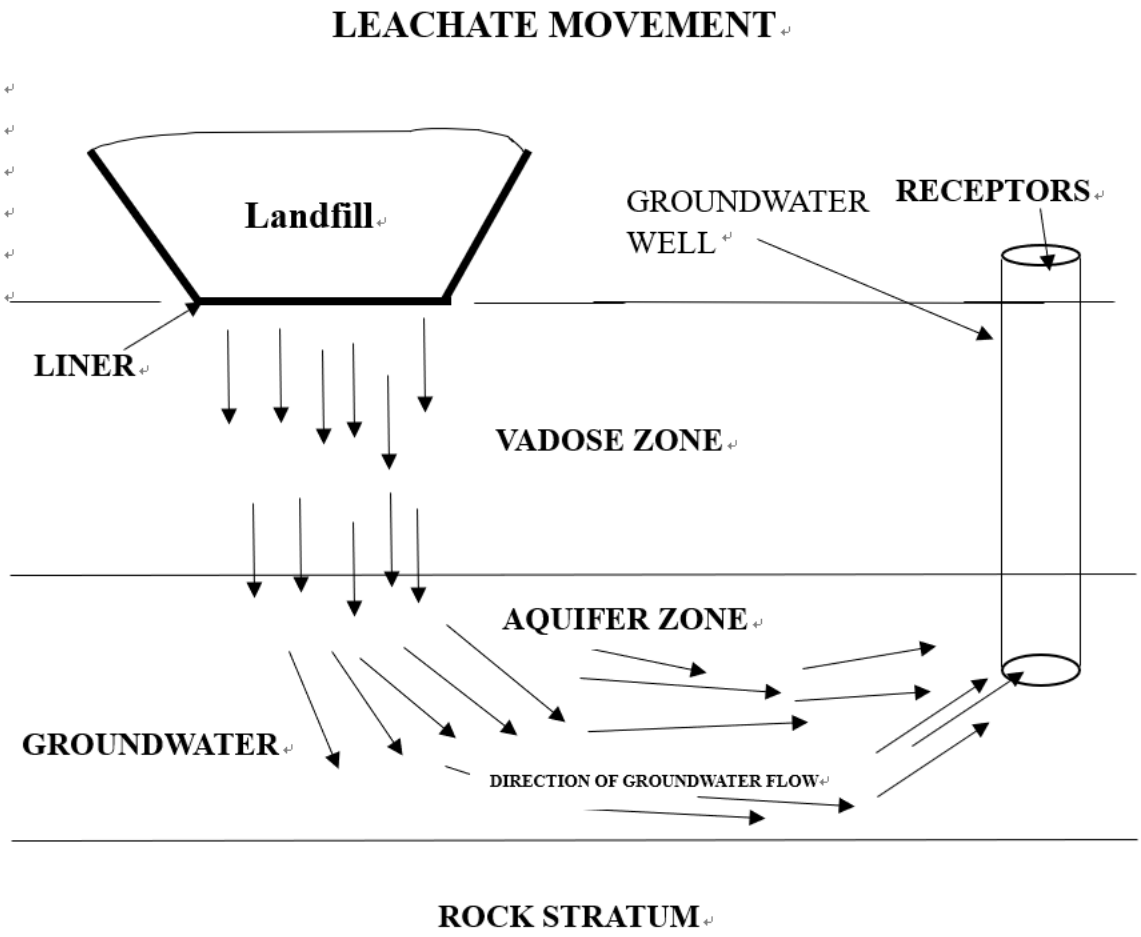

Figure 2-7 Leachate plume movement governed by groundwater flow which widens the contamination.

The level of toxicity is based on the concentrations of the particular heavy metals that are released; therefore, heavy metal ion contaminants are a serious problem in the environment and their removal from contaminated soil and wastewater requires attention.

Common heavy metals such as cadmium, chromium, cobalt, copper, arsenic, lead, nickel, selenium, silver, zinc, mercury, antimony and thallium are naturally occurring, but become concentrated as a result of anthropogenic activities (Perez-Leblic et al., 2012). Conventional treatments such as adsorption, chemical precipitation, electrochemical treatment, evaporation, filtration, ion exchange, membrane technology, oxidation/reduction and reverse osmosis have been used to remove heavy metals from contaminated environments (Gunatilake S.K., 2015) . Unfortunately, these traditional methods often do not remove the metals successfully because

they are ineffective, costly, and consume high amounts of chemicals and energy (Fu and Wang, 2011). However, these methods are also not effective because they result in the release of immobilized or adsorbed heavy metals back to the environment (Achal et al., 2012). Therefore, alternative methods such as MICP are now applied to remove the heavy metals effectively, economically and in an eco-friendly manner (Kang et al., 2014b, Kang et al., 2014a, Kang et al., 2015, Anbu et al., 2016, Achal et al., 2012).

In the MICP process, calcites can incorporate heavy metals (e.g., Cd^{2+}) onto their surfaces via substitution of suitable divalent cations (Ca^{2+}) in the calcite lattice, after which these compounds are changed from soluble heavy metals to insoluble forms i.e., detoxifying the heavy metals (Figure 2-8).

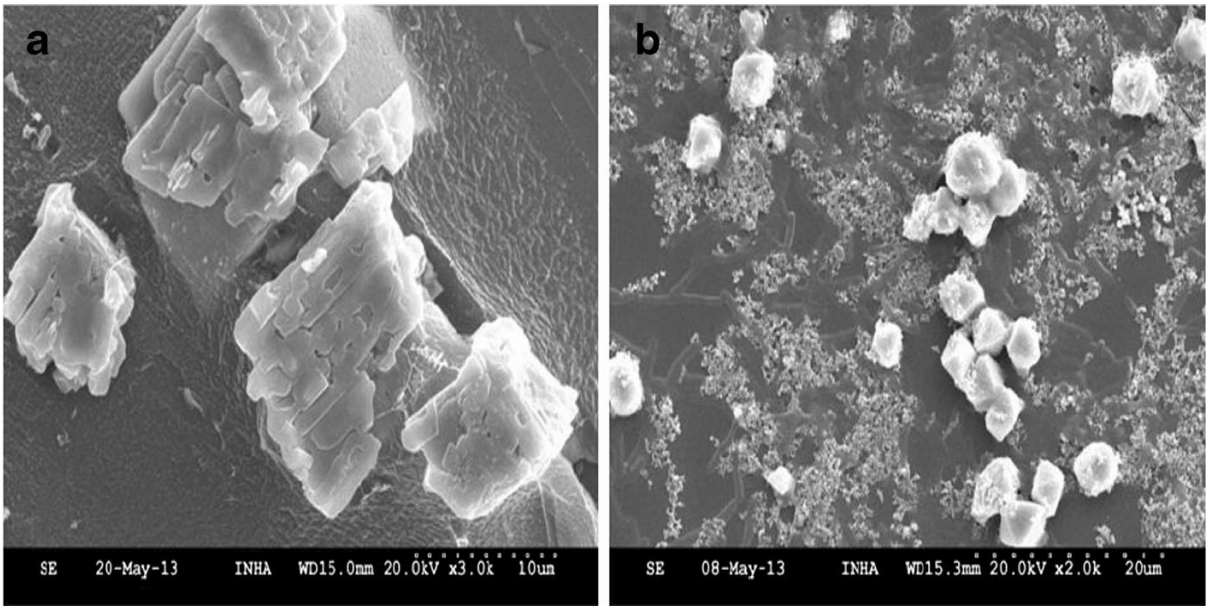

Figure 2-8 SEM images heavy metal cadmium trapped in crystal form $CdCl_2$ (a) $CdCO_3$ (b) by *Lysinibacillus sphaericus* CH-5. Adapted from (Kang et al., 2014a).

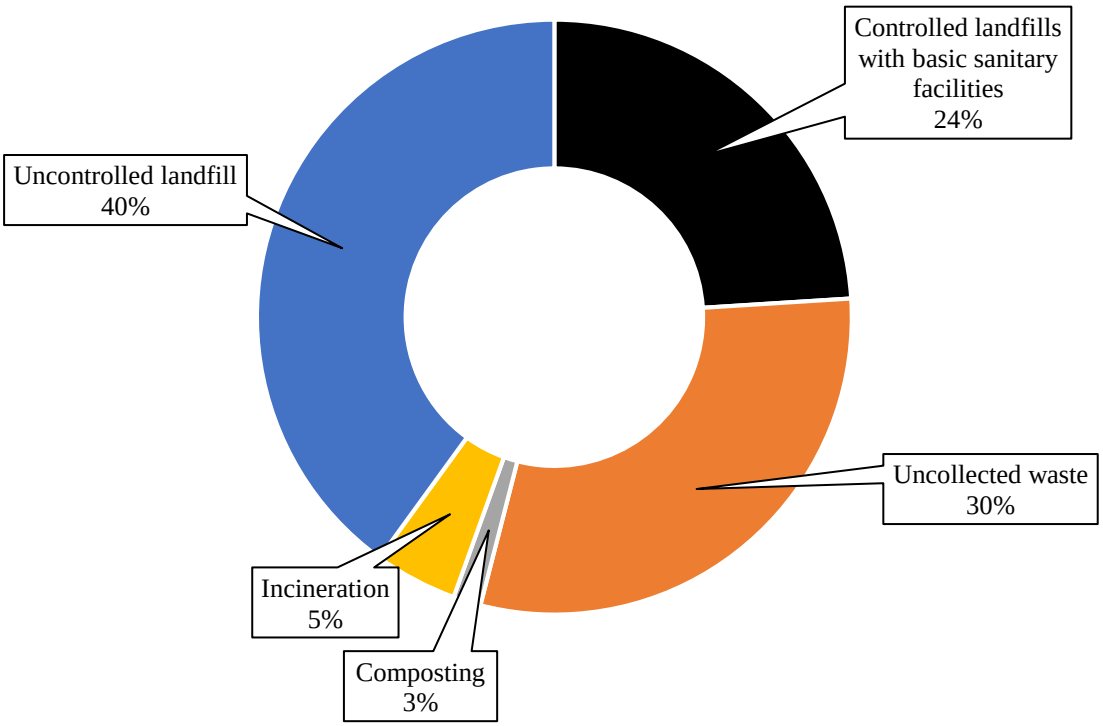

Figure 2-9 Waste distribution in China, modified from Zhang et al. (2010)

Given the current waste distribution (Figure 2-9), landfills pose a threat to water quality; this problem is exacerbated with diminishing water reserves, in addition to concerns that contaminated groundwater may lead to a range of waterborne diseases such as cholera, typhoid fever and amoebeasis. Therefore, techniques such biomineralisation may provide a cost-effective, environmental friendly and efficient way to contain leachate from landfills or remediate the surrounding contaminated land.

In order to utilize the bacteria that are present in environments like leachate, soil and groundwater the environment has to be studied. Culture dependent and culture independent techniques are common methodologies that are applied to analyse and isolate target bacteria. The particularities are described below:

2.8 Culture dependent/independent microbiology

Bacteria are considered important bioindicators because they interact with plants during their entire life cycles and can act as precise and fast indicators of environmental changes. Bacteria account for approximately half of the total carbon of the global biomass and play fundamental roles in biogeochemical cycles (e.g. C and N). Without human intervention/interference bacteria have been maintaining, rehabilitating and restoring ecosystems - cleaning water, soil and maintaining soil fertility for several decades (Andreote et al., 2009). However, the vast majority of bacteria (99%) present in natural environments have not yet been cultured. These uncultured bacteria are often referred to as a 'black box' containing an undiscovered consortium that represents an unexploited potential that could provide to be novel and valuable catalysts, enzymes and building blocks for ground improvement, remediation and engineering (Wang et al., 2012).

Traditionally, microorganisms are characterized by their physiological and biochemical properties. These methods have disadvantages because of the difficulty of isolating many microorganisms, since less than 1% of natural environmental bacteria are capable of being cultured. Traditional microbiological methods such as enumeration using different general and selective media can provide (i) quantitative data on the occurrence of different groups of microorganisms and (ii) isolated pure cultures for experimental fermentations. However, this classical culture-dependent approach only reveals the culturable microbes. Culture-dependent methods present several disadvantages, above all for determining bacterial numbers; they are known to be biased because bacteria can only be cultivated if their metabolic and physiological requirements can be reproduced in vitro (Zheng et al., 2012). Problems with using culturing for consortium analysis arise from the fact that an artificial homogenous medium typically allows growth of only a small fraction of the organisms. In addition, when

complex microbial communities are under investigation, enumerating bacteria by traditional microbial culturing techniques may produce erroneous results. Culturing fails to reproduce the ecological niches and symbiotic relationships encountered in complex natural environments required to support the full spectrum of microbial diversity. Apart from selectively allowing growth of some species and suppressing growth of others, the consortium composition of the culturable fraction is distorted during culturing because replication times vary, with fast-growing species efficiently out-competing others (Carraro et al., 2011).

The identification of microorganisms using physiological and biochemical methods is blatantly difficult, time-consuming and the results are often ambiguous. However, microbial consortium analysis in most previous studies has been performed using a culture-dependent method, and there are many micro-organisms that have not been detected due to a detection bias of the medium and/or the physiological status of the micro-organism (such as the viable but non-culturable state, VBNC state)(Li et al., 2017). It is well established that only approximately 1% of bacteria on Earth can be readily cultivated *in vitro* – the so called 'great plate count anomaly', based on the observation that microscopic counts are considerably larger than the equivalent total viable counts. There are currently estimated to be 61 distinct bacterial phyla, of which 31 have no cultivable representatives. Because the majority of bacteria and archaea remain unculturable, the diversity of complex bacterial communities is inevitably underestimated using standard cultivation methods. Furthermore, microorganisms that are highly valuable and beneficial to the entire ecosystem could go unexplored if they are unculturable (Wang et al., 2012).

Traditional culture-based approaches were applied for characterisation and enumeration of endophytic and phyllosphere bacteria, though these the culture-based approach relies highly on the medium used for isolation and also the incubation temperature. Traditional cultivated separation methods are universally used in microorganism isolation and identification, but it becomes useless when it comes to culture independent microorganisms (Jackson et al., 2013). In most reports, the isolation of bacterial cultures from various sources was limited to a part of the fermentation process; hence, a systematic analysis on bacterial counts and diversity has not been conducted over the consecutive stages of the fermentation process (Ramos et al., 2010). Additionally, the identification of isolates in many studies has been based on phenotypic characterizations that have led to potential misidentification due to the ambiguity of variable characteristics. Although the analysis of the composition of natural microbial populations has a long tradition in microbial ecology, microbiologists have been constrained by the use of traditional methods, since the component organisms have had to be grown in the laboratory. Consequently, a collection of bacterial isolates of a habitat is unlikely to represent the *in-situ* diversity (Jackson et al., 2013).

However, it has been hypothesized that 99% of microorganism's recognizable in nature usually are not cultivated using standard techniques. Out of over 40 known prokaryotic phyla, only half have cultured representatives (Wang et al., 2012). For a large number of habitats in which most of the microorganisms are unknown and unculturable, a culture-dependent method biases our view on microbial diversity. It is well documented for example, that stressed or injured cells are not recovered in selective media and that cells present in low numbers are very often inhibited by microbial populations numerically more abundantly. For these reasons, it is crucial to have tools that allow monitoring of the microbial populations without cultivation (Cocolin et al., 2007). Several newly emerging methods for monitoring

specific microbial genotypes in environmental samples and for analysing microbial consortium structure at the genetic level, which do not require the culturing of the microorganisms from the samples, depend upon the efficient recovery of DNA as an essential part of the procedures. These methods are advantageous because they avoid problems associated with enumeration procedures that depend upon culturing of organisms from environmental samples (Steffan et al., 1988). Since the 1980s, the application of molecular ecological methods, especially those based on surveys of genes after PCR amplification, has promoted culture-independent investigations of the microbial communities in diverse environments (Feng et al., 2016).

Most of the molecular studies have relied on sequences of the small subunit ribosomal RNA (SSU rRNA/16S rRNA) gene. PCR based molecular techniques such as denaturing/temperature gradient gel electrophoresis (DGGE/TGGE), restriction fragment length polymorphisms (RFLP), terminal restriction fragment length polymorphisms (T-RFLP), and quantitative PCR (qPCR) have been used widely for studying microbial communities. Meanwhile, non-PCR-based molecular techniques such as fluorescence *in situ* hybridization (FISH) and microarray were also developed and applied to this field of research (Zheng et al., 2012). Researchers have propelled the emergence of many exciting fields such as metagenomics, metatranscriptomics, metaproteomics, and single-cell genomics. Some of these traditional methods (e.g., DGGE, TRFLP, clone libraries) that analyze the 16S rRNA genes have been widely applied over the past 20 years and have already been reviewed extensively (Fukui et al., 2012). Along with the previously mentioned methodologies next generation sequencing (NGS) methods such as pyrosequencing and Illumina are also currently applied to study the microbial consortium of the environment.

The steps involved for studying microbial consortium using CIMs include sampling, DNA extraction, amplification of gene fragment(s) from environmental samples, distinguishing different fragments, analysis of experimental results, and summary of microbial consortium (Figure 2-10). The major difference among the genomic fingerprinting techniques is the isolation of the different fragments. The separation of heterogeneous products is carried out by various electrophoretic separation techniques (D/TGGE), with or without restriction enzyme digestion (RFLP, T-RFLP). For the genomic fingerprinting techniques, it is impossible to avoid the PCR biases completely. However, DNA microarray, FISH, and qPCR are based on oligonucleotide probes and primers that target the ribosomal RNA sequences or other genes in different hybridization procedures; thus, they avoid the PCR step and its associated inherent bias (Weidner et al., 1996). However, FISH, qPCR, and microarray technologies are based on only known samples and will not detect those that do not have corresponding probes on the array. The strategy of PCR-mediated amplification of targeted genes, using either rRNA or rDNA isolated from an environmental habitat, followed by gene cloning, sequencing, and comparative data analysis has been used successfully on samples from landfills. Although different CIMs have their own limitations, people can reduce this bias by taking some necessary measures (Kesmen and Kacmaz, 2011).

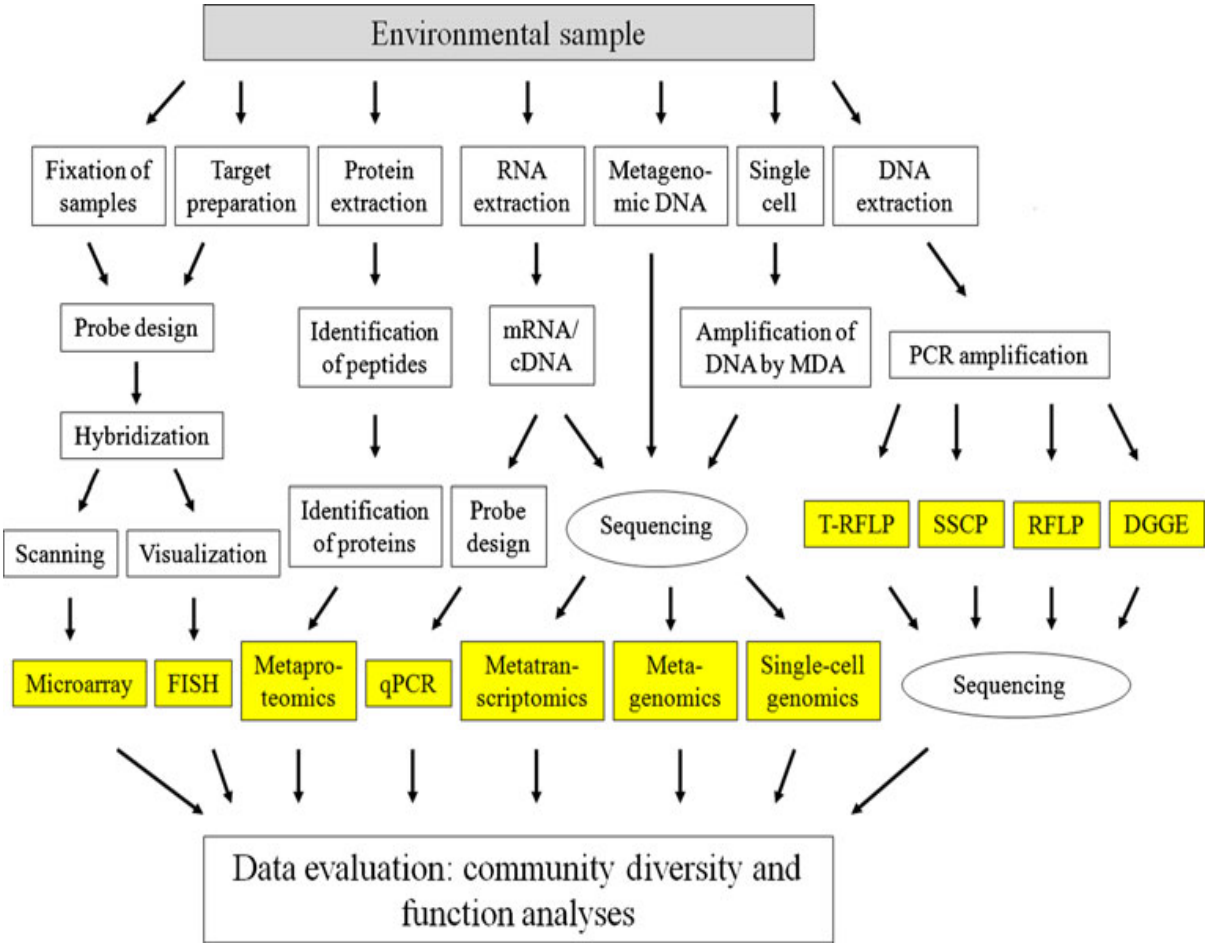

Figure 2-10 Experimental procedures of different CIMs. The arrows indicate different procedures applied in most studies of microbial consortium diversity using CIMs. The yellow boxes indicate different CIMs. Adapted from (Su et al., 2012),

In recent years, increasing studies used two or more CIMs and/or complemented with culture-dependent method to reduce biases caused by only one method. Also, the application of two CIMs could be beneficial in providing in depth information on certain samples. In addition, more than one set of primers or genes can be used in the experiment procedures to avoid a certain bias resulting from primer mismatch or biased representation by the specific gene(s) in the sample. For example, microbial diversity in the Movile Cave (Romania) was studied using bacterial and archaeal 16S rRNA gene sequence and functional gene analyses, including RuBisCO, soxB, and amoA (Chen et al., 2009). Moreover, optimization of amplification program also can reduce bias. This simple modification to the protocol may ensure that sequence richness encountered in clone libraries more closely reflects genetic

diversity in the original sample. This method has been used to study the microbes in the human gut and soil. Other techniques such as nested PCR and touch-down PCR also can minimize the potential PCR amplification bias.

CHAPTER 3 Next-generation sequencing showing potential leachate influence on bacterial communities around a landfill in China

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3.1 ABSTRACT

The impact of contaminated leachate on groundwater from landfills is well known but specific effects on bacterial consortia are less well-studied. Bacterial communities in landfill and an urban site located in Suzhou, China were studied using Illumina high-throughput sequencing. A total number of 153944 good quality reads were produced and sequences assigned to 6388 operational taxonomic units (OTUs). Bacterial consortia consisted of up to 16 phyla including *Proteobacteria* (31.9 to 94.9% at landfill, 25.1 to 43.3% at urban sites), *Actinobacteria* (0 to 28.7% at landfill, 9.9 to 34.3% at urban sites), *Bacteroidetes* (1.4 to 25.6% at landfill, 5.6 to 7.8% at urban sites), *Chloroflexi* (0.4 to 26.5% at urban sites only) and unclassified bacteria. *Pseudomonas* was the dominant (67-93%) genus in landfill leachate. Arsenic concentrations in landfill raw leachate (RL) ($1.11x10^3$ µg/L) and fresh leachate (FL2) ($1.78x10^3$ µg/L), and mercury concentrations in RL (10.9 µg/L) and FL2 (7.37 µg/L) were higher than Chinese State Environmental Protection Administration (SEPA) standards for leachate in landfills. Shannon diversity index and Chao 1 richness estimate showed RL and FL2 lacked richness and diversity when compared with other samples. This is consistent with stresses imposed by elevated arsenic and mercury and has implications for ecological site remediation by bioremediation or natural attenuation.

3.2 INTRODUCTION

Municipal landfill waste compositions can range from food wastes to high-strength detergents, solvents and pharmacological products comprising a broad spectrum of xenobiotic and recalcitrant toxic compounds with potential harmful ecological impacts (Köchling et al., 2015, Song et al., 2015a). Although modern landfills in well-regulated economies are highly engineered and monitored, older or informal (unplanned, uncontrolled) landfills worldwide are sources of leachate which, unless correctly collected and treated, can cause serious reductions in the quality of water bodies and groundwater sources (Li et al., 2014, Zhang et al., 2013a). Previous studies have indicated a diverse range of heavy metal concentrations in leachates (Song et al., 2015b, Zhang et al., 2013a). Heavy metals have been previously shown to directly influence the bacterial community composition of various environments (Muller et al., 2001, Vishnivetskaya et al., 2011, Sandaa et al., 1999, Mor et al., 2006, Yao et al., 2017). Long term studies have shown a strong influence of mercury towards the bacterial community of a river basin and soil (Muller et al., 2001).

To study complex microbial ecosystems such as leachate, molecular techniques have several advantages over culture-based techniques as they allow the analysis of uncultured organisms and provide higher resolution measurements closer to the complete microbial profile (Staley et al., 2011). Analysing the microbial community around a landfill can potentially determine whether the leachate is being transported through the landfill liner into the natural soil and groundwater, via changes in the diversity and composition of bacterial consortia as different species are more or less tolerant of elevated pollutant concentrations (Wang et al., 2017, El-Salam and Abu-Zuid, 2015, Vukanti et al., 2009).

Previous studies on heavy metal influence towards microbial communities were performed using PCR-DGGE and GS 454 FLX pyrosequencing (Muller et al., 2001, Yao et al., 2017, Vishnivetskaya et al., 2011). Next generation sequencing (NGS) methods can assist in the identification of very rare taxa in the landfill samples (Köchling et al., 2015, Song et al., 2015a). NGS provides efficient, multiple level details of the operational taxonomical units (OTUs), richness and diversity, so it can be used to identify both similarities and differences between sites. Furthermore, the rapidity and portability of NGS methods and apparatus, for example, Nanopore (Oxford Nanopore Technologies, Oxford, UK) mean that sequencing of microbial consortia now presents a potentially rapid, low-cost option for the detection of leachate impacts on natural groundwater consortia and hence mapping of contaminant plumes based on ecological, rather than chemical, indicators (Brown et al., 2017).

Understanding the environmental conditions and bacterial community is of upmost importance when it comes to cleaning up the contaminants by employing techniques such as biodegradation. It is a microbial process that degrade contaminants found in the environment. Over the past 20 years, in-situ biodegradation has successfully been applied to various environments with different level of degrading abilities depending on the bacteria (Meckenstock et al., 2015). The process requires careful identification of the degrading bacteria prior to implementation. Generally, constant monitoring of the microbial activity is also required to ensure constant and consistent microbial activity over time. For example, Adetutu et al. (2015) utilised biostimulation (BS), biostimulation-bioaugmentation (BS-BA) and monitored natural attenuation (MNA) approaches to bioremediate groundwater polluted with trichloroethene (TCE). Next-generation sequencing was an effective technique to study the microbial community dynamics throughout while performing the dechlorination process.

In the present work, we investigated the potential for NGS to identify potential impacts on soil and groundwater bacterial communities due to heavy metal-rich landfill leachate in a conurbation in Suzhou, Jiangsu province, China. The objectives of this study were i) to characterize the composition of the bacterial communities of a selected landfill (leachate, soil and groundwater) and a non-landfill site in same conurbation, hereby referred to as "urban" (soil and groundwater); ii) to compare the unique and dominant bacterial taxa among the landfill and urban samples; and iii) to investigate and compare the bacterial diversity and heavy metal concentration of the soil and groundwater samples from a landfill and urban site. The study not only adds to the knowledge in respect of leachate impacts on subsurface consortia under urban areas, but assesses the potential of NGS for rapid monitoring of environmental impacts from landfills, and has implications for the design and implementation of biological remediation options such as natural attenuation or *in situ* microbially-induced carbonate precipitation.

3.3 MATERIALS AND METHODS

3.3.1 Sample locations

The selected landfill (located at 31°14'18.31" N 120°33'3.09" E) began operation in 1993 and receives about 1,500 tons/day of household wastes and industrial wastes from the Suzhou conurbation. A new landfill was constructed in 2006 on the surface of the older landfill (Rong et al., 2011). The urban site samples were collected from an area that was previously used for agriculture prior to reclamation for industrial development. The two sites are approximately 27 km from each other. The two sites are approximately 27 km from each other. Suzhou is situated on top of a 200 m deep sequence of Quaternary sediments. The depth of drift reduces to 0m directly to the West and South West of the City. At depth the bedrock is composed of Devonian quartzite and shales of the Wutong Formation, the sandstones shales and quartzites of the Maoshan Group and zones of Carboniferous limestone (the karstic features of which

are known commercially as Taihu Stone, exposed at Dongting Mountain and in Linwu Cave) which forms the hills to the south and west of the city. This sequence is intruded by the Suzhou Granite which is exposed to the West of the city centre. The variable erosive bedrock surface, has been infilled by alluvial and lacustrine sediments of the lower flood plains of the Yangtze River. The subsurface materials vary from clays to silty sands (Shi et al., 2012). The structure of the quaternary strata below ground varies at the very large scale, due to the movement of the rivers and changes in the extent and location of the lakes with time. However, the extent of variation has been limited by the volume of materials being deposited within a geologically short period of time. Some of the silty/sandy subsurface zones are a result of reworking of loess by the Yangtze River. The silty sands have sufficient porosity to act as aquifer materials (Ma et al., 2011). Pumping works from these aquifers have caused the collapse of their porous structure resulting in approximately 1 m of settlement across the region increasing to 1.4 m towards city centres, and reducing to 0m towards the locations of large permanent lakes (Shi et al., 2012). Details regarding Suzhou landfill construction and waste were briefly discussed by Rong et al. (2011).

The landfill sampling comprised of two leachates, soil from three different locations around the landfill (samples LS1, LS2 and LS3) and one groundwater from the landfill monitoring well (samples BHGW) (Table 3-1). Leachate samples were either fresh (FL2, collected from an outlet pipe that runs beneath the landfill) or raw leachate (RL, sampled from a leachate pond). Soil samples were collected using a Spiral auger at 30cm depth. The first soil location was near the leachate pond; the second was close to agricultural land on the boundary of the site; and the third soil location was close to the groundwater monitoring borehole. The groundwater was collected at an approximate depth of 4 meters using a hand-held slow flow peristaltic pump. The samples were collected from well below the groundwater surface such

that any residual floating matter would not be collected. Groundwater and leachate were collected in sterile high-density polyethylene plastic bottles and soil samples were collected in a sterile plastic zip lock bags and transported to the laboratory under ambient temperature conditions, then stored in a cold room (4°C) prior to analysis.

To contrast the bacterial community from the landfill, soil (samples USS1 and USSur1) and groundwater (samples USGW) samples were collected from the urban site. Two samples from the two different locations in an urban area were selected for the soil sampling which were 200 meters apart. The groundwater borehole was chosen for the groundwater sampling. Ground water was collected at a depth of 4 meters. The first location of the soil sampling was located closer to the urban site groundwater and the second location of the soil sample was an isolated location.

Table 3-1 **Collection and description for landfill samples.**

Samples acronyms	Sample name	Reason for collection
RL	Raw Leachate	Due to its long-term storage in the landfill that might influence variation in the microbial diversity.
FL2	Fresh Leachate	Provides an in depth understanding on the microbial diversity when compared with raw leachate
LS1	Landfill soil location 1	Closer to the landfill which might provide data on any leakage from leachate.
LS2	Landfill soil location 2	Closer to the agricultural land; data can be used to compare with landfill soil location 1.
LS3	Landfill soil location 3	Closer to the groundwater monitoring borehole; data can be used to compare the permeability of the landfill.
BHGW	Landfill groundwater monitoring borehole	Only functioning borehole used to check the contamination levels of the groundwater.
USGW	Urban site groundwater	Accessible borehole close to the soil locations.
USS1	Urban site soil sample 1	Location of the soil sampling was located closer to the urban site groundwater. It was collected from the surface.
USSur1	Urban site soil sample 2	Isolated soil location 500 m away from USS1 and USGW. It was collected 30 cm depth.

3.3.2 Physicochemical analysis of soil and water samples

The following heavy metals were analysed for all samples: mercury (Hg), arsenic (As), cadmium (Cd), copper (Cu), lead (Pb), zinc (Zn) and chromium (Cr). The heavy metals were analysed at Tsingcheng Environment Company in Suzhou, China. Mercury and arsenic were analysed using Atomic Fluorescence Spectroscopy (AFS 2100, Haiguang Instruments Co. Ltd); zinc, lead and copper were analysed using Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP 710, Agilent Technologies); cadmium was analysed using

graphite furnace-Atomic Absorption Spectroscopy (240Z, Agilent technologies) and chromium was analysed using Flame-Atomic Absorption Spectroscopy (ICP 710, Agilent technologies). The pH of soil, groundwater and leachate samples was measured using a Suntex® TS 3000 pH/Temp portable probe in the Department of Environmental Science at XJTLU. The samples were stored at +4°C prior to analysis.

3.3.3 Preparation and extraction of DNA from soil, leachate and groundwater samples

3.3.3.1 Preparation of samples for DNA extraction

One liter of groundwater was filtered on a 0.22 µm pore size polycarbonate membrane filter (Millipore, USA) using a vacuum pump. Samples were filtered and the filters were placed in sterile Petri dishes and stored at -20°C until they were used for DNA extraction. Due to the nature of the sample (high turbidity), 50 ml of leachate was centrifuged at 5000 rpm for 5 minutes and both the pellet and the supernatant were collected. The supernatant was filtered in a 0.22 µm membrane filter (Millipore, USA) and both pellet and membrane filter were used for DNA extraction. Soil samples were weighed (0.25 g) and used for DNA extraction.

3.3.3.2 DNA extraction

The genomic DNA from all the samples was extracted using a commercial DNA extraction Kit (MO BIO Power soil® DNA kit, USA) according to the manufacturer protocol. 50 µl of elution buffer was used to elute the DNA samples and these were frozen at −20 °C until further processing for bacterial community analysis. The DNA was quantified using Nanodrop (Thermo Scientific, Waltham, MA, USA) and examined by agarose gel electrophoresis (1% w/v).

3.3.4 Bacterial community analysis by next-generation sequencing

The bacterial diversity and community composition of soil, leachate and groundwater samples were studied by NGS using the Illumina MiseqPE250 platform. NGS was carried out

at Shanghai Majorbio Pharmaceutical Technology Limited, China. 16S rRNA genes (V4 region) were amplified by PCR using 515F (5'barcoded GTGCCAGCMGCCGCGG3') and 806R (5'GGACTACHVGGGTWTCTAAT3') primer sets. PCR reactions contained in 20 µl: 4 µl of 5× FastPfu Buffer, 2 µl of 2.5 mM dNTPs, 0.8µl of forward and revers primers (5 µM), 0.4 µl of FastPfu polymerase, 10 ng of template DNA and DD water up to 20 µl. PCR conditions: a ABI GenAmp 9700 thermocycler was used. Initial denaturation 3 minutes at 95°C was followed by 28 cycles of 30 s at 95°C, 30 s at 55°C and 30 s at 72°C; final extension was carried out at 72°C for 10 min. The purified amplicons were pooled and sequenced on an Illumina MiSeq platform. Chimeric sequences were removed and the operational taxonomic units (OTUs) were clustered with 97% similarity cutoff using UPARSE (Edgar, 2013). The phylogenetic affiliation of each 16S rRNA sequence was analysed by RDP classifier against the SILVA data base (Pruesse et al., 2007). The sequences were submitted to National Centre for Biotechnological Information (NCBI) Short Read Archive (SRA) database under the accession numbers SAMN06339740 to SAMN06339748.

3.3.5 Data analyses

The diversity within each sample (alpha diversity) was calculated by Shannon (H') and Simpson (D) diversity indices, abundance-based coverage estimator (ACE) and Chao 1 richness estimator using MOTHUR (http://www.mothur.org). The diversity between samples were compared (beta diversity) by non-metric multidimensional scaling (NMDS) and cluster analysis by using QIIME. The relationship between the environmental parameters (pH and heavy metals) and bacterial community was assessed by redundancy analysis (RDA) or canonical correspondence analysis (CCA) by using R language vegan package.

3.4 RESULTS

3.4.1 pH and heavy metals

Table 3-2 and Table 3-3 shows that the soil samples from the landfill and urban site were slightly acidic while landfill groundwater (BHGW), raw leachate (RL) and fresh leachate (FL2) sample were alkaline. To ensure accuracy in the results, two samples were collected for the landfill sites. The two readings labelled as $^{(1)}$ and $^{(2)}$ were taken from the same pool at slightly different location and interval. The Arsenic concentrations in RL and FL2 were 11.1-12.3 to 17.8-18.4 times higher than the Chinese SEPA guideline concentration value for landfill of 100 µg/L – Class V (Yang et al., 2008). Mercury concentrations were an order of magnitude higher in RL and FL2 samples and in the BHGW (landfill groundwater) than the Chinese SEPA guideline values (Yang et al., 2008). Heavy metal concentrations of the soil samples from the landfill were within the guideline range (Table 3-3). The As concentration of urban site soil 1 and 2 (USS1 and USSUR1) was at the threshold tolerance value of the guideline range. The heavy metal concentration of Hg in BHGW was found to be 340 times higher than USGW.

Table 3-2 pH and heavy metal composition in landfill leachate (RL & FL2) and ground water samples (BHGW) and urban site groundwater sample (USGW) respectively; (1) represents the first reading and (2) represents the second reading. ND = Not detected

	pH	Mercury (µg/L)	Arsenic (µg/L)	Cadmium (µg/L)	Copper (µg/L)	Lead (µg/L)	Zinc (µg/L)	Chromium (µg/L)
RL[1]	7.78	11.42	$1.11x10^3$	ND	ND	ND	ND	0.508
RL[2]	7.9	11.42	$1.23x10^3$	ND	ND	ND	ND	0.581
FL2[1]	8.12	7.37	$1.78x10^3$	ND	0.107	0.027	ND	0.586
FL2[2]	8.3	8.20	$1.84x10^3$	ND	ND	ND	ND	0.541
BHGW[1]	8.2	12.7	ND	ND	0.048	ND	0.186	0.015
BHGW[2]	8.25	5.59	ND	ND	ND	ND	0.062	0.011
USGW	7.75	0.037	ND	ND	ND	0.078	0.030	ND

Table 3-3 pH and heavy metal composition of samples obtained from landfill (LS1, LS2 & LS3) and urban site (USS1 & USSUR1) soil respectively; (1) represents the first reading and (2) represents the second reading. ND = Not detected

	pH	Mercury (mg/kg)	Arsenic (mg/kg)	Cadmium (mg/kg)	Copper (mg/kg)	Lead (mg/kg)	Zinc (mg/kg)	Chromium (mg/kg)
LS1[1]	6.71	0.175	0.766	ND	69.5	10.3	49.1	62.3
LS1[2]	6.87	0.152	0.854	ND	75.3	10.1	81.4	67.3
LS2[1]	6.63	0.150	0.937	ND	79.3	5.72	55.7	70.4
LS2[2]	6.42	0.184	0.726	ND	77.2	7.90	71.2	71.5
LS3[1]	7.1	0.146	0.998	ND	79.5	6.91	76.9	73.8
LS3[2]	6.95	0.143	0.907	ND	71.8	8.73	64.8	68.2
USS1	6.82	0.075	11.3	0.169	5.8	27.6	64.9	43.45
USSUR1	6.74	0.058	9.28	0.137	7.57	26.3	63.6	50.2

3.4.2 Bacterial diversity

Table 3-4 shows the number of reads obtained from the landfill samples varied from 13611 to 20464 and in urban site, it ranged from 14015 to 22643. The maximum reads obtained from LS3 and lowest from LS2 in the landfill environment. In urban site, USSUR1 had the lowest reads compared to other urban samples. OTU values ranged from 139 to 1018 for the landfill samples compared to 168 to 1167 in the urban site samples. FL2 had the lowest number and BHGW had the highest number of OTUs. In the urban site, USGW had the lowest OTU read compared to USS1 which had the highest OTU read of 1224. The bacterial richness and diversity (Shannon *H'* index) of the urban soil samples (USS1 and USSUR1) were the highest of all the samples. Species diversity estimates obtained for the abundance-based coverage estimators (ACE) and the Chao1 index was higher in the urban site soil samples when compared to the landfill soil samples, despite As concentrations an order of magnitude higher in the urban site soil samples than in the landfill soil samples. Furthermore, the landfill groundwater (BHGW) had more bacterial diversity than the urban groundwater (USGW) by every metric despite the Hg concentration in BHGW being more than 340 times higher than USGW (Table 3-2).

Table 3-4 Bacterial diversity based on 16S rRNA gene retrieved by NGS from a landfill and an urban site. ACE = Abundance based coverage estimators

Sample ID	Number of Reads	Number of OTUs	ACE index	Chao 1 richness estimate	Shannon diversity index (H')	Simpson diversity index (D)	Coverage
RL	15386	154	159	164	2.06	0.3716	0.999
FL2	15746	139	174	163	0.98	0.6584	0.997
LS1	15313	996	1109	1103	5.77	0.0089	0.989
LS2	13611	647	892	862	3.43	0.125	0.983
LS3	20464	875	1080	1112	4.49	0.0516	0.989
BHGW	20141	1018	1201	1259	5.6	0.0093	0.988
USGW	22643	168	189	190	2.65	0.177	0.999
USS1	16625	1224	1331	1332	6.1	0.0056	0.989
USSUR1	14015	1167	1322	1328	5.94	0.0079	0.983

3.4.3 Bacterial community structure

Figure 3-1 shows the bacterial community composition at phylum level in both landfill and urban site samples. Among all the phyla, only *Proteobacteria* and *Bacteroidetes* were found to be present in all the samples. The phylum *Proteobacteria* was dominant in all the samples from landfill site with their abundance ranging from 31.4% to 94.9% in the landfill samples. Across the urban site, their abundance ranged from 25.1% to 43.3% with USGW possessing a lower abundance compared to the USS1 and USSUR1. *Bacteroidetes* abundance ranged from 1.42% to 25.64% among the landfill samples with FL2 having the lowest abundance and LS2 the highest. In the urban site, samples they ranged from 5.69% to 7.86% in abundance with USGW having the higher presence of *Bacteroidetes*. Members of phylum *Actinobacteria*

were found in all the samples except the leachate samples. The relative abundance of *Actinobacteria* ranged from 12.6 % to 28.6% and from 9.9% to 34.3% for the landfill site and urban site, respectively. USGW was again found to be higher for *Actinobacteria*. *Chlamydiae* was only found in USGW at 24.1%. *Firmicutes* and *Thermotogae* were only found in the RL sample with 6.4% and 8.2% abundance, respectively.

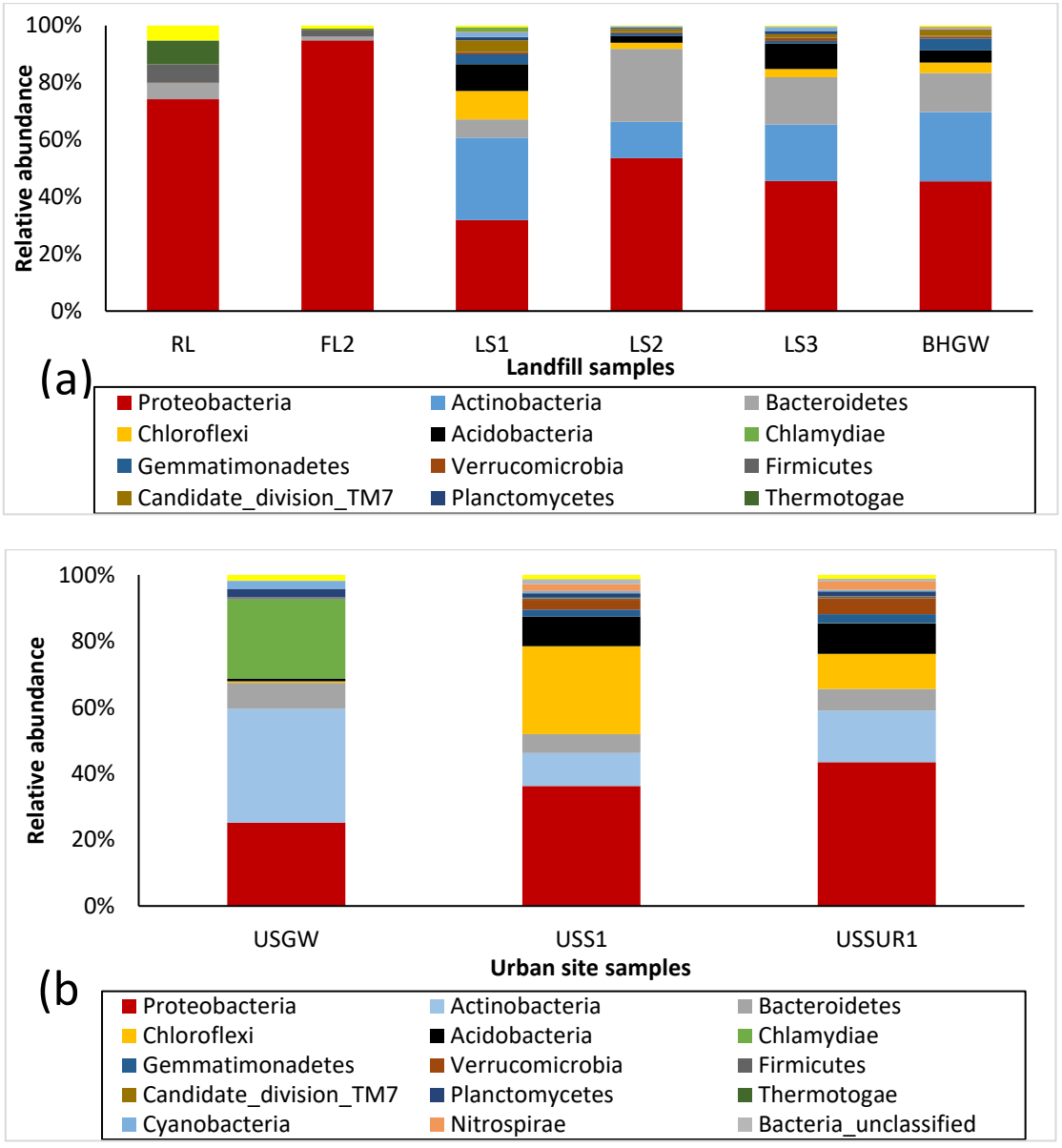


Figure 3-1 Phylum level bacterial community composition observed in the samples collected from a landfill site (*a*) and an urban site (*b*). FL2, fresh leachate; RL, raw leachate; LS1, LS2, and LS3, landfill soil; BHGW, landfill groundwater; USGW, urban site groundwater; USS1 and USSUR1, urban site soil samples.

Figure 3-2 shows that at the order level, *Pseudomonadales* and *Sphingobacteriales* were present in all samples. *Pseudomonadales* were dominant in the landfill samples at RL (69.96 %), FL2 (92.97 %), LS2 (25.29 %) and LS3 (16.11 %). In LS2 and LS3, either *Xanthomonadales* (11.04% and 14.09%) or *Flavobacteriales* (20.88% and 10.55%) were the second or third dominant orders observed. However, in USGW samples, *Frankiales* (34.06%) and *Chlamydiales* (24.09%) were dominant and their abundance was either <1% or absent in other samples from both sites. *Sphingobacteriales* were found to be the second dominant order at 8.5% for BHGW and 7.81% for USGW. *Flavobacteriales* were present in higher percentages in LS2 (20.88%) and LS3 (10.55%) but their abundance were found to be less than <2% in other samples.

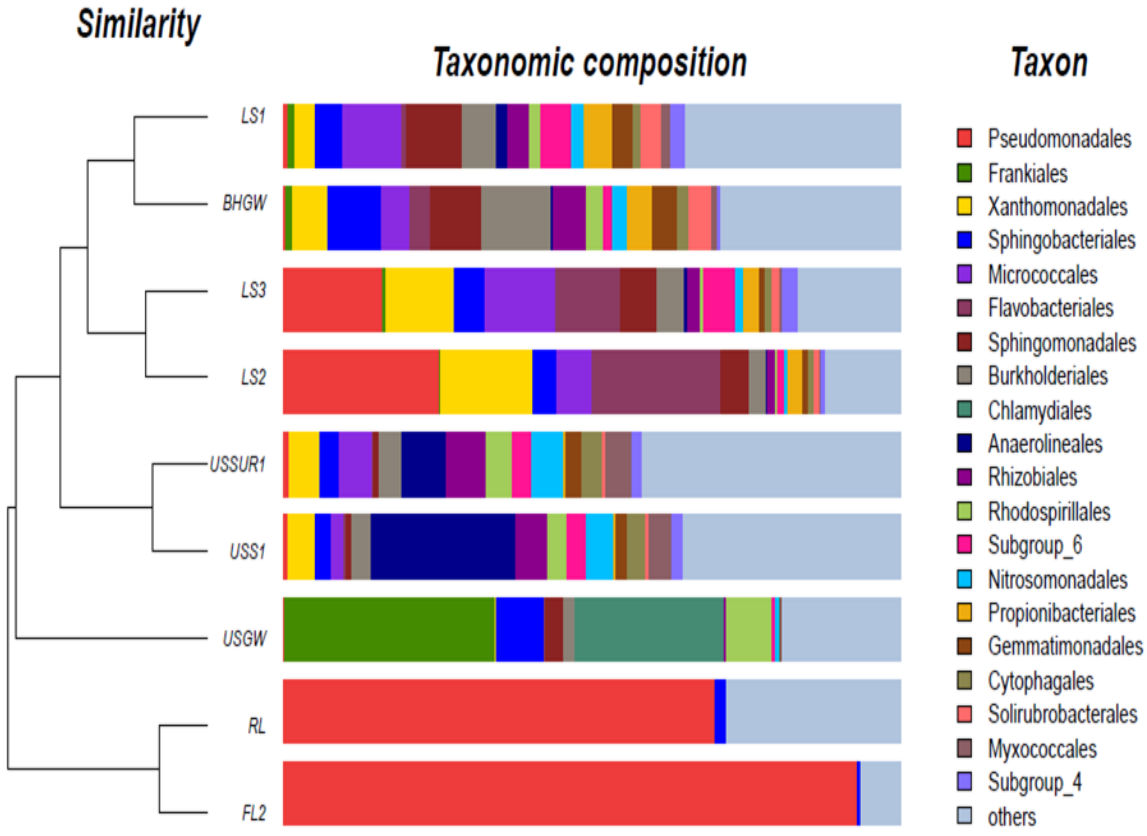
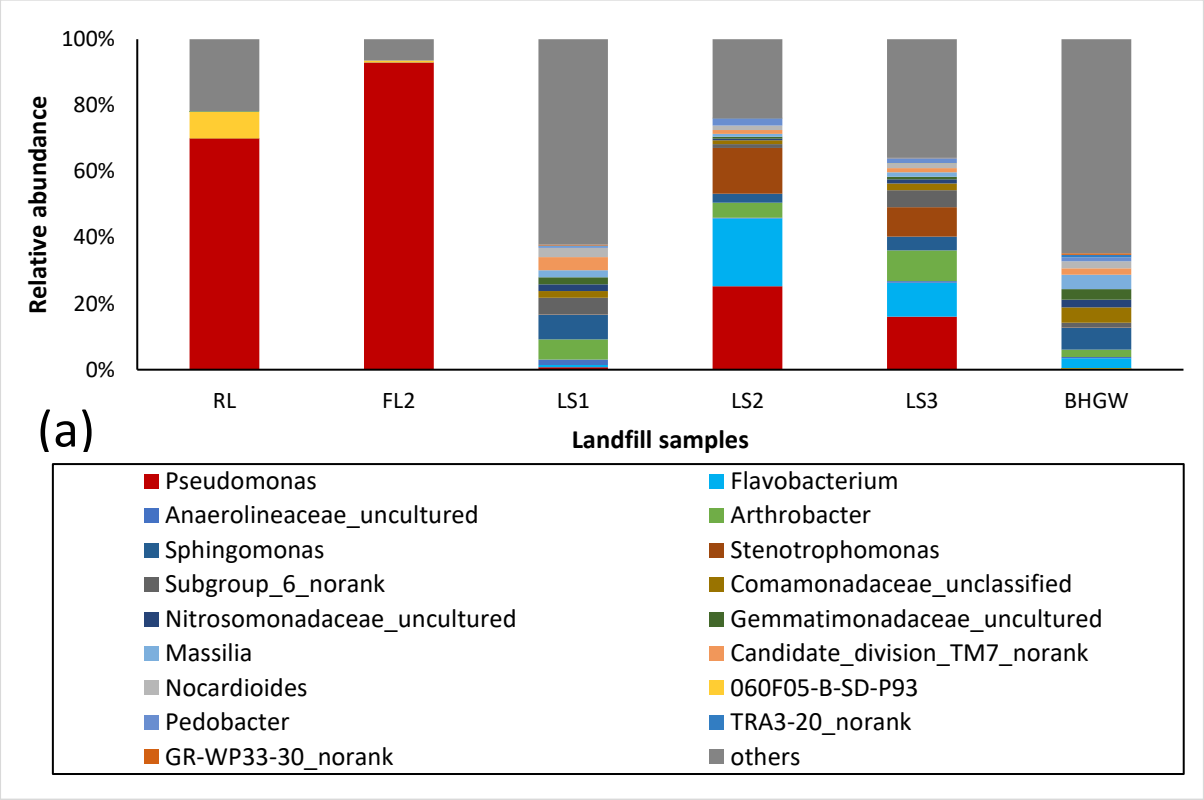


Figure 3-2 Bacterial community composition and cluster analysis at the order level in samples collected from a landfill site and an urban site. FL2, fresh leachate; RL, raw leachate; LS1, LS2, and LS3, landfill soil locations; BHGW, landfill groundwater; USGW, urban site groundwater; USS1 and USSUR1, urban site soil samples.

At genus level, the bacterial communities from the two sites were more diverse and unique. Figure 3-3a shows that *Pseudomonas* was the most dominant genus observed in FL2 and RL with a relative abundance of 92.9 and 69.9%, respectively. This genus was also dominant in LS2 and LS3 but their relative abundance was less (16-25%) as compared to leachate samples. *Sphingomonas* (6.5%) was found to be dominant in BHGW. In contrast the urban site samples (Figure 3-3b) show *Sporichthyaceae_unclassified* (34%) to be dominant followed by *Candidatus_Rhabdochlamydia* (24%) and *Sediminibacterium* (5.83%) in USGW sample. *Thiobacillus*, Anaerolineaceae_uncultured and Nitrosomonadaceae_uncultured were dominant in USS1 and USSUR1 samples.



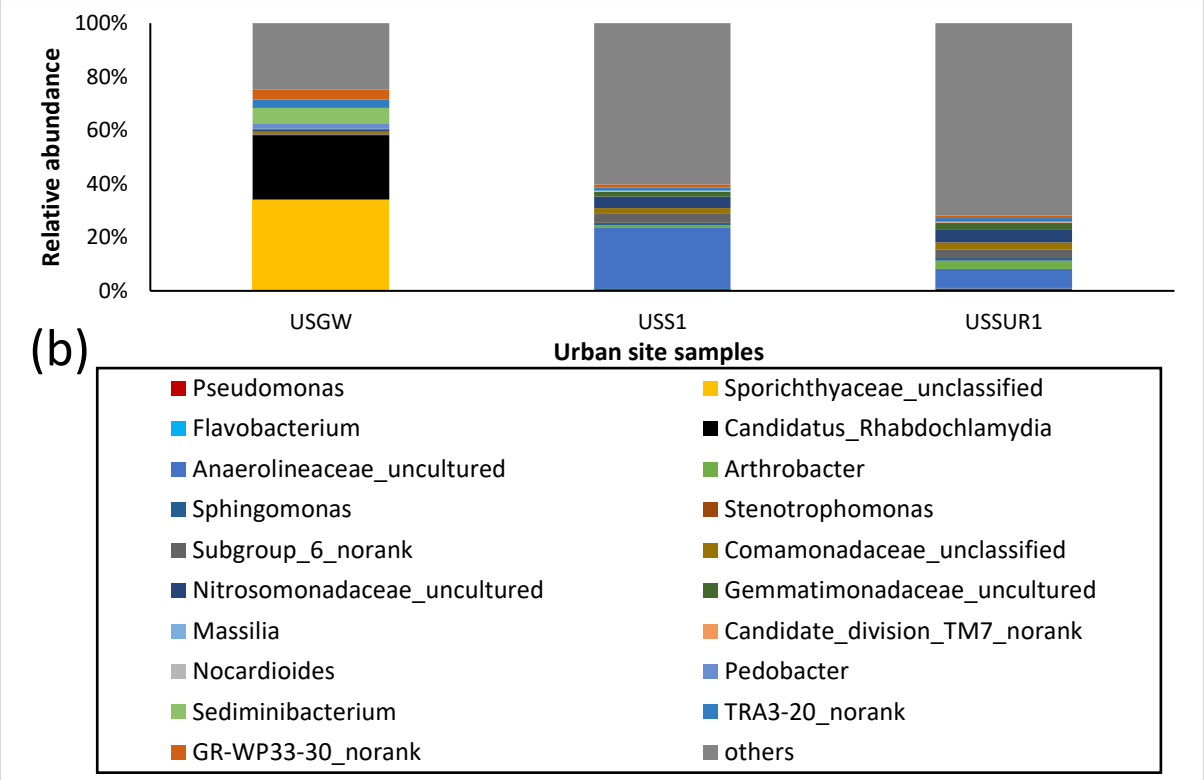


Figure 3-3 Genus level bacterial community composition observed in the samples collected from landfill site (*a*) and an urban site (*b*). FL2, fresh leachate; RL, raw leachate; LS1, LS2, and LS3, landfill soil; BHGW, landfill groundwater; USGW, urban site groundwater; USS1 and USSUR1, urban site soil samples.

Cluster analysis and NMDS was performed on the landfill and urban site samples (Figure 3-4 and Figure 3-5). Figure 3-4a indicates a high level of similarity among the LS1, LS2 and LS3, BHGW, USS1 and USSUR1 samples. RL, FL2 and USGW are shown to be unique compared to the rest of the samples. Cluster analysis shown in Figure 3-5 support the results observed for RL, FL2 and USGW in Figure 3-4a. Figure 3-4b shows the least level of similarity observed among RL, FL2, LS1, LS2, LS3, USS1 and USSUR1 samples.

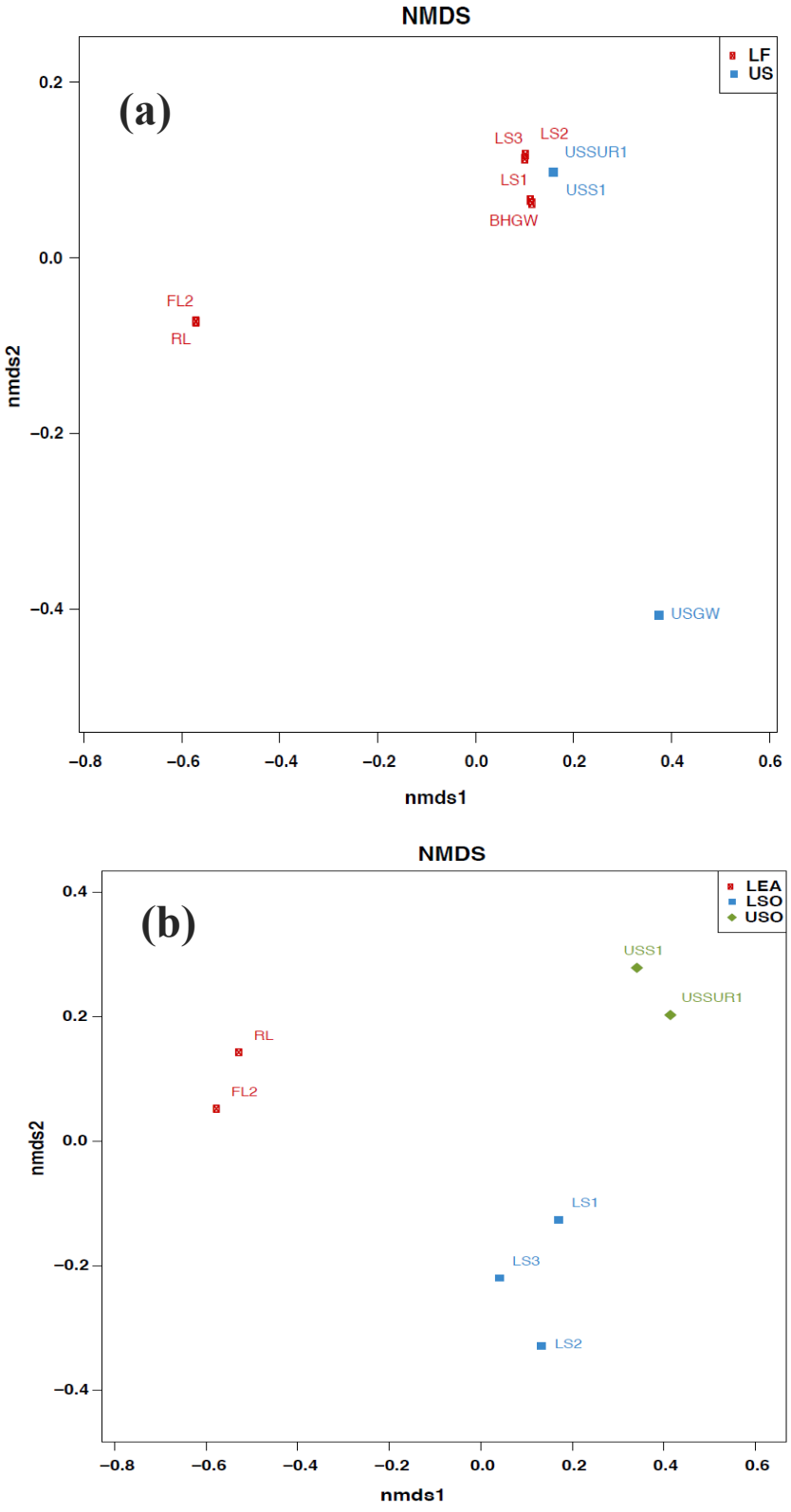
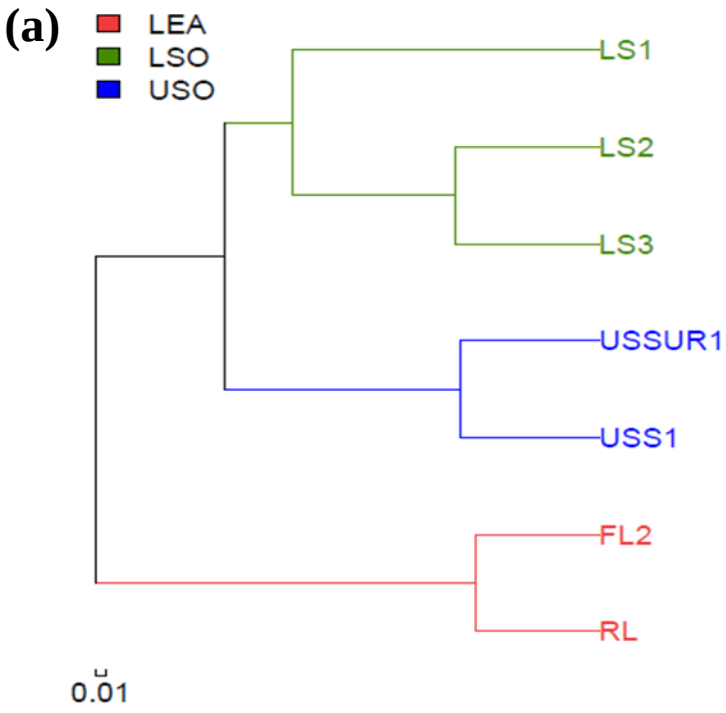


Figure 3-4 Nonmetric multidimensional scaling (NMDS) analysis of sequences. (*a*) LF and US; (*b*) LEA, LSO, USO. FL2, fresh leachate; RL, raw leachate; LS1, LS2, and LS3, landfill soil locations; BHGW, landfill groundwater; LF, combination of all landfill samples; USGW, urban site groundwater; USS1 and USSUR1, urban site soil samples; US, combination of all urban sites.



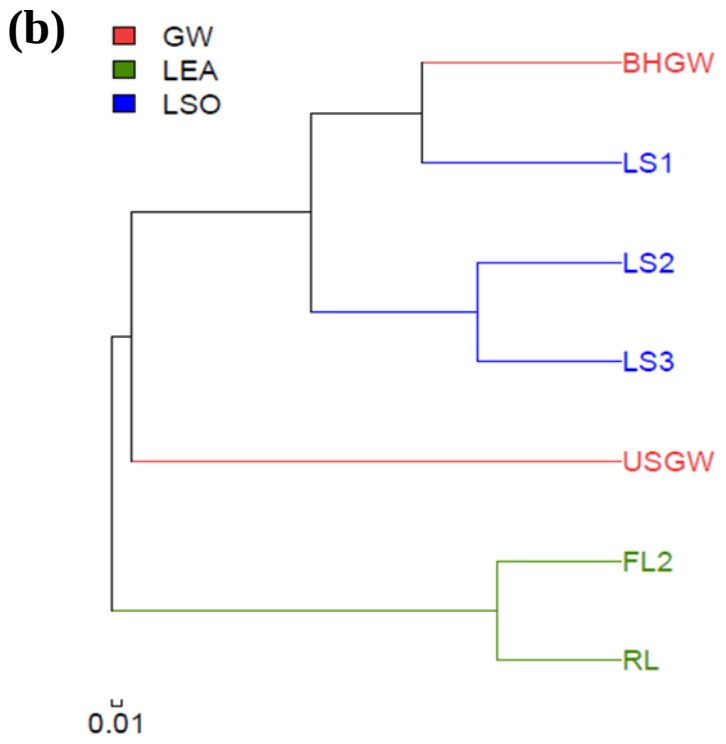


Figure 3-5 Cluster analysis based on order level bacterial abundance. (*a*) LEA, USO, LSO; (*b*) GW, LEA, LSO. FL2, fresh leachate; RL, raw leachate; LS1, LS2, and LS3, landfill soil; BHGW, landfill groundwater; USGW, urban site groundwater; USS1 and USSUR1, urban site soil samples; GW, combination of groundwater from both sites.

To study the relationship between environmental parameters and bacterial community composition, both multivariate redundancy analysis (RDA) and canonical correspondence analysis (CCA) were performed and compared since the length of the first axis gradient were between 3.0 and 4.0. Figure 3-6 shows the RDA plot of the influence of As, Pb, Hg and pH on the soil samples from the different locations. The USS1 and USSUR1 samples were mainly correlated with the As and Pb content in the soil. The LS3 samples exhibited the reverse pattern and were correlated with the pH and Hg concentration in the soil. Canonical correspondence analysis (CCA) was performed to determine the possible linkages between the bacterial communities and environmental parameters by examining the leachate and groundwater samples. Canonical correspondence analysis (CCA) showed a negative correlation between As, pH, Hg and the bacterial community of the samples, indicating that they had the biggest impacts on the bacterial community of these samples (Figure 3-7). Arsenic was the major factor that negatively correlated with bacterial communities from FL2 and RL samples. CCA identified both pH and heavy metals in the samples as a major environmental factor in affecting bacterial communities.

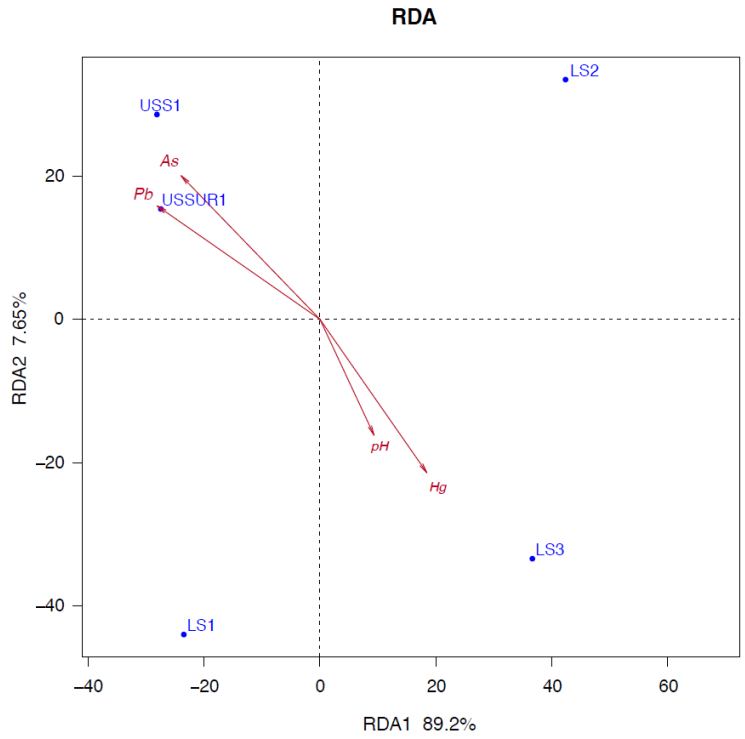


Figure 3-6 Redundancy analysis (RDA) of soil bacterial communities in landfill and urban site soil samples. RDA1 explained 89.2% and RDA2 explained 7.65% of the total variance. LS1, LS2, and LS3, landfill soil locations; USS1 and USSUR1, urban site soil samples.

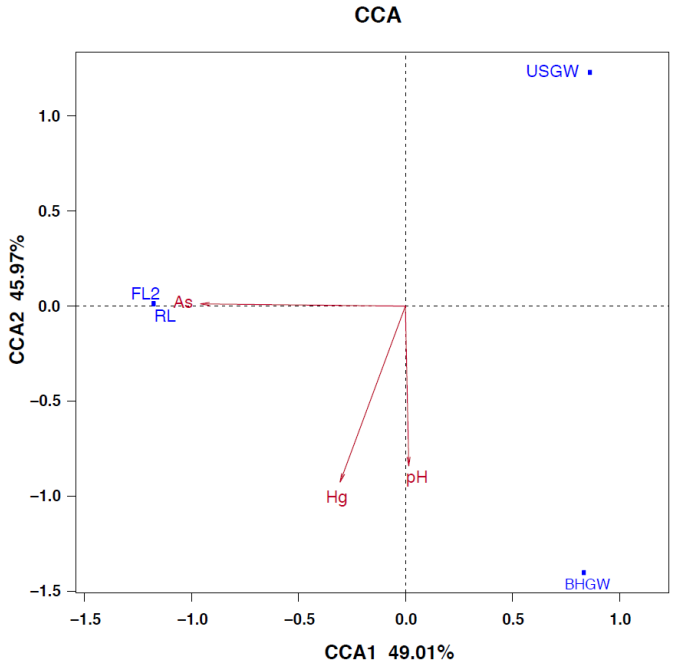


Figure 3-7 Canonical correspondence analysis (CCA) of bacterial communities in RL, FL2, BHGW, and USGW. CCA1 explained 49.01% and CCA2 explained 45.97% of the total variance. FL2, fresh leachate; RL, raw leachate; BHGW, landfill groundwater; USGW, urban site groundwater.

3.4.4 DISCUSSION

3.4.4.1 Comparison of pH and heavy metals between sites

The pH of leachate samples RL and FL2 were 7.78 and 8.12, respectively (Table 3-2). This range of pH has been reported in other landfill leachate studies conducted in China (Song et al., 2015a, Song et al., 2015b, Li et al., 2014). Since this landfill has an onsite incinerator, the alkaline pH could be attributed to the disposal of ash in the landfill. The pH of BHGW and urban site groundwater (USGW) was also alkaline at 8.2 and 7.75, respectively (Table 3-2). The pH values of landfill and urban site soil were between 6.6 and 7.1 which indicate that the samples are slightly more acidic in nature than the natural groundwater (Table 3-3). The pH values of the soil are not surprising given the sites were previously used as agricultural lands (Zou et al., 2015) and the regional presence of limestone formations.

The heavy metal concentrations for As and Hg were above the guidelines range in both leachate samples (Table 3-2). These hazardous ranges of As and Hg could be due to the solid waste decomposition (mostly from waste water and MSW) and indicates the age of the landfill (more than 10 years old) (Zhang et al., 2013a, Huang et al., 2013, Huang et al., 2003). The Hg level in BHGW was 340 times higher when compared with USGW, indicating a possible percolation of mercury from the landfill leachate to landfill groundwater. Very low concentrations in LS1, LS2 & LS3 indicating Hg-bearing leachate and groundwater are not interacting with the soils. On this chemical evidence, it might be concluded that at this site, the near surface environment around the landfill remains relatively uncontaminated and leachate was not percolating directly to the groundwater below the water table (Roling et al., 2001) (Wang et al., 2011).

The concentration of As in RL & FL2 was very high in comparison to other landfills in Jiangsu province which was between 0.03 to 0.113 mg/L (Yang et al., 2008). Given that both sites were agricultural land prior to rapid urbanisation in the late 20th century, agri-chemical residues within the soil at USS1 & USSUR1 could explain the elevated arsenic levels (Zou et al., 2015). The remaining heavy metals were analyzed from both sites and are typical of soils in urban contexts subject to uncontrolled disposal of consumer and industrial chemicals, road runoff and deposition of airborne pollutants (Mor et al., 2006). (Wijesekara et al., 2014). This context of high background contamination presents the key challenge for both chemical and microbiological investigation of leachate impacts.

3.4.4.2 Analysis of bacterial community structure in landfill

3.4.4.2.1 Comparison OTU and community composition among samples

Figure 3-4 and Figure 3-5 shows OTU based NMDS and cluster analysis plots which demonstrate the level of similarity among the samples from both sites. When aggregated together, similarity between landfill soil samples (LSO) and urban site soil samples (USO) was high when compared against the similarity between groundwater samples from both sites (Figure 3-4a). Landfill groundwater (BHGW) consortia were also closely similar with the soil samples. The reason behind the low similarity between the groundwater samples could be due to the poor diversity and richness of the urban groundwater (USGW) (Table 3-3). It is also clear that the bacterial communities in the raw and fresh leachate were markedly distinct from any of the soil or groundwater communities; this is evident at both genus and order level (Figure 3-2 and Figure 3-3). On the basis of bacterial community analysis, the dramatic differences between leachate and environmental samples offer the potential for fingerprinting the presence of leachate contamination through identification of leachate-specific DNA in environmental samples. Although such detailed mapping was not possible in this study, we

note that all three landfill soil samples contained *Pseudomonas*, in common with the leachate samples, which was not present in soils or groundwater from non-landfill locations. This may indicate surface or in-soil transport of leachates not evident from the heavy metals analysis.

3.4.4.2.2 Dominant phyla and genera in both sites

Leachate samples RL and FL2 had the least diverse phyla detection, in contrast to other landfill leachate studies (Song et al., 2015a, Wang et al., 2017). The high concentration of As and Hg in RL and FL2 could have inhibited the growth of other phyla, whereas *Pseudomonas* spp. have recently been identified as key members of arsenotrophic consortia in contaminated groundwater environments in Bangladesh (Sultana et al., 2017). The low diversity in leachate samples, compared with samples taken from within the landfill (e.g.,(Wang et al., 2017) may also be due to the concentration of landfill microbiota within surface-attached biofilms rather than in mobile planktonic forms (Costerton and Wilson, 2004). Landfill and urban site soil and groundwater samples shared most of the phyla except for *Chlamydiae*; which was only found in USGW. As far as we are aware, this is the first study to observe significant presence of *Chlamydiae* in urban groundwater microbial consortia; interestingly, given the high levels of lead and zinc in the urban soils, the phyla has previously been isolated in groundwater samples affected by lead-mine tailings (Zhang et al., 2008) .

Proteobacteria were most dominantly found in leachate samples from landfills (Song et al., 2015a, Song et al., 2015b) and aquifer sediments (Wan et al., 2012). It has been reported that members of *Proteobacteria* involved in the degradation of aromatic oils such as polycyclic aromatic hydrocarbons (Vukanti et al., 2009). These bacteria have been found to lose dominance in older leachate samples (Köchling et al., 2015) and they were detected at highly abundant levels in aged refuse from Shanghai landfills (Xie et al., 2012). *Actinobacteria* was

found in the soil and groundwater samples from both sites but not in the leachate samples. This was not expected as *Actinobacteria* has previously been found in leachate samples (Vukanti et al., 2009). The high arsenic and mercury concentrations of leachate could perhaps have restricted their growth. *Actinobacteria* are responsible for organic matter degradation contributing to carbon turnover (Song et al., 2015b). Since landfills receive waste ranging from households to industries, the amount of organic matter present in the soil could be a reason behind their presence in landfill soil compared to urban site soil. *Bacteroidetes* was observed in abundance at BHGW being twice as much as USGW. While LS2 & LS3 had three times the dominance as USS1 & USSUR1 which could possibly indicate early stages of organic matter degradation within the landfill samples as they commonly contain more soluble and easily degradable material (Schmidtova and Baldwin, 2011). *Bacteroidetes* tend to become more dominant than Proteobacteria as the waste in the landfill ages (Köchling et al., 2015). *Firmicutes* was only found to be dominant in the leachate samples which suggest that they are able to withstand and survive the toxic heavy metal concentrations found in the leachate. They have also been found in other toxic chemical environments such as sewers and drainage (Rodrigues et al., 2014).

Environmental factors may have fundamental impacts on the structure and function diversity of bacterial communities in landfill. Analysis from RDA showed that LS1, LS2, LS3 and BHGW were not influenced by pH and heavy metals, where USS1 and USUR1 were shown to be lightly influenced by As and Pb (Figure 3-6). In this study, analysis from CCA has shown that higher concentrations of As and Hg influence the bacterial community of leachate. pH was also shown to significantly influence the bacterial community of leachate (Figure 3-7). The findings from this paper are consistent with previous results that show that heavy metals influence the bacterial community of landfill (Yao et al., 2017).

3.4.5 Potential of NGS for fingerprinting leachate interactions with soil and groundwater

In this study, Illumina MiSeq technique was used to investigate the bacterial community in samples collected from landfill and urban sites. Bacterial richness and abundance were found to vary significantly among the landfill and urban site samples. Further bacterial analysis revealed lack of diversity in leachate samples when compared with soil and groundwater samples. OTU data from NGS could be used in mapping the interactions between the samples at a site. In our study, OTU data helped in understanding the similarity among the samples from both sites. More studies are now being published using MiSeq methodology since it offers high-resolution microbial community data which helps us in understanding the influence of external factors such as heavy metals towards soil and groundwater microbial consortia. Further study needs to be conducted to understand the long-term effects of leachate interactions with soil and groundwater in a landfill to observe the changes in microbial community.

CHAPTER 4 **Biomineralisation performance of bacteria isolated from a landfill in China.**

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4.1 Abstract

This paper reports an investigation of carbonate precipitation by seven indigenous bacteria isolated from a landfill in China. To assess their biomineralisation potential, the bacteria were studied within a medium supplemented with 25 mM calcium chloride and 333 mM urea. The experiments were carried out at 30°C for 7 days at 130 rpm. Scanning Electron Microscopic (SEM) and X-ray diffraction (XRD) showed variations in calcium carbonate polymorphs and mineral composition induced by all bacterial strains. The amount of carbonate precipitation was quantified using titration and pH variation showed that each bacteria had an adaptation period within the environment to achieve carbonate precipitation through ureolysis. The amount of carbonate precipitation varied among isolates with the lowest being *Bacillus aerius rawirorabr15* (LC092833) precipitating 1.53 times more carbonate than the abiotic solution. *Pseudomonas nitroreducens* szh_asesj15 (LC090854) was found to be the most efficient, precipitating 3.2 times more carbonate than the abiotic solution. Our results indicate that bacterial carbonate precipitation occurred through ureolysis and variations in pH, carbonate polymorphs and precipitation was mainly due to the differences in urease expression and response to the alkaline environment. These results provide benchmark data for bioremediation potential of indigenous bacteria for containment of contaminants in landfills.

4.2 Introduction

Since the early 1970's, it has been known that diverse microbial species involve in the precipitation of carbonates in various natural environments, including soils, geological formations, oceans, and saline lakes (Boquet et al., 1973). This bio-mediated process is a type of carbonate precipitation known as microbially induced carbonate precipitation (MICP). The ability of these bacteria to precipitate carbonates, in particular from laboratory cultures, has been widely studied (Rivadeneyra et al., 2006, Sanchez-Roman et al., 2007, Rivadeneyra et al., 2000, Rivadeneyra et al., 2004, Han et al., 2013, Kang et al., 2014b). The precipitation process is complex and it is influenced by the types of bacteria, calcium source and urease activity along with a range of abiotic factors (Rivadeneyra et al., 2006, Gorospe et al., 2013, Achal and Pan, 2014, Dhami et al., 2014). Both active and passive mechanisms have been proposed to explain how bacterium mediate the precipitation process (Hammes and Verstraete, 2002, Silva-Castro et al., 2013). In many studies, the actual role of bacteria in the mechanism of carbonate formation is still not fully understood; although it is clear that each bacteria precipitate carbonate based on their adaptation to the environment and their urease activity within a given system (Zamarreňo et al., 2009a).

In order to show that indigenous carbonate precipitating bacteria inhabit landfills, bacteria need to be isolated from the environment and assessed for their potential for carbonate precipitation. Microorganisms identified and isolated from natural environments like soil and groundwater have been shown to precipitate polymorphs of calcium carbonates, principally Calcite and Vaterite (Lian et al., 2006, Achal and Pan, 2014, Zamarreňo et al., 2009a, Zamarreňo et al., 2009b). Previous studies have isolated carbonate precipitating bacteria from mine tailing soils (Achal and Pan, 2014), caves (Rusznyak et al., 2012), abandoned highways (Kang et al., 2014b) and freshwater (Zamarreňo et al., 2009b). These studies have implied that carbonate precipitating bacteria are not geographically restricted to any

particular terrain. While diverse point sources do not indicate that such bacteria are universal, it implies that they might be more widespread, and less restrictive than currently thought.

Given that landfills are complex microbial systems inhabited by bacteria that remediate or degrade toxic compounds (Staley et al., 2011) and given the wide range of environments from which carbonate precipitating bacteria have previously been isolated, it is possible that such bacteria would be present in a landfill. Stimulating carbonate precipitation in indigenous bacteria is cost effective and potentially more sustainable than using bacteria obtained from culture collections. Since indigenous microbes are already adapted to the environmental conditions of a landfill; they could be used for contaminant/heavy metal immobilization (Ivanov and Chu, 2008, Miot et al., 2009, Kang et al., 2014b, Amidi and Wang, 2015). For example, Kang et al. 2014a and Ma et al. 2009 have used biomineralisation to trap heavy metals such as cadmium. Achal et al. (2012) utilised this technique to immobilise arsenic and Kang et al. (2015) assessed the containment of lead. The main mechanism for trapping heavy metals is through a reduction in the permeability of the porous medium via the growth of minerals within the pore space. In addition, it has been shown that some heavy metals can precipitate to form carbonates. For example, calcium or Ca^{2+} can be replaced by Cd^{2+} or Pb^{2+} to form $CdCO_3$ or $PbCO_3$ instead of $CaCO_3$ entrapping the corresponding heavy metal. In addition there is some evidence for the formation of arsenic carbonate complexes (Kang et al., 2014a, Kang et al., 2015). Finally, the increase in the pH of the soil/groundwater reduces the mobility of metals via a buffering effect.

This study aims to establish (i) that carbonate precipitating bacteria inhabit landfills; (ii) the bio-mediated process to form carbonates is different among bacteria from the same environment; and (iii) the performance of the isolated from ground water and leachate towards biomineralisation. The results offer benchmarking data for further studies towards

techniques such as bioremediation or contaminants containment in such an aggressive environment.

4.3 Material and Methods

4.3.1 Sampling and Storage

The landfill (31°30.34′N 120°55.87′E) is located in Suzhou, Jiangsu, China; 27 km from an urban area. The landfill is located on the slope of a limestone hill which is an alkali natural environment. In addition, there is a waste incinerator onsite and therefore ash forms a high proportion of the waste disposed of within the landfill. Only a portion of the waste is incinerated as the volume of waste each day is greater than the capacity of the incinerator. The groundwater samples were collected at 4 m depth, approximately 1.9 m below the water table. Samples were collected using sterile high-density polyethylene (HDPE) sealable plastic bottles in triplicate and stored at 4°C prior to bacterial isolation. Fresh leachate was collected from a pipe that drains the body of the landfill. Raw leachate is the name used to describe leachate which was stored in a tank located in a separate location. Note that such tanks may develop leaks into the subsurface soil generating the potential for groundwater contamination. Groundwater and leachate was collected using a hand-held peristaltic pump with a 10 mm diameter tube. Individual sterilised tubes were used for groundwater and leachate collection.

4.3.2 Isolation and identification of bacterial isolates

Prior to this stage, a detailed investigation of the bacterial consortia was carried out using Next-generation sequencing (Rajasekar et al., 2018). This provided an indication of the bacterial strains present and their richness. Heavy metal toxicity from leachate and groundwater was shown to have a negative influence towards the bacterial communities.

Following this, the bacterial isolates were obtained using the following procedure: Raw and fresh leachate samples with serial dilutions were spread onto nutrient agar plates and

incubated at 30°C for 24 hours until visible colonies were obtained. The bacterial isolates were purified by repeated streaking and then transferred into nutrient broth. The spread plate method was also used for bacterial isolation from an undiluted 100 µl groundwater aliquot. Isolates were purified by repeated streaking. The bacterial isolates were grown in nutrient broth (which consists of beef extract and peptone), the cells were harvested and pellets were directly transferred to the bead columns for DNA extraction. The genomic DNA was extracted using PowerSoil® DNA isolation kit (MO BIO, USA) following the manufacturer's instructions. The 16S rRNA genes were amplified using PCR with 10 mM concentration of 27F and 1492R primers (Muyzer et al., 1993). A final volume of 50 µL was used in the PCR assay, which contains 10X PCR buffer (5 µL), 10 mmol/L dNTPs (1 µL), 25 mmol/L $MgCl_2$ (4 µL), forward and reverse primers 10 mM each (2µL), Taq polymerase (2 U), DNA template (1 µL), and 37 µL of double-distilled water. The PCR cycling conditions were as follows: initial denaturation at 94 °C for 4 minutes followed by 30 cycles of denaturation at 94 °C for 1 minute, annealing at 55 °C for 35 seconds, extension for 1 minute at 72 °C; after 30 cycles final extension at 72 °C for 10 minutes. The PCR products were verified by agarose gel (1.5% wt/v) electrophoresis and purified using a PCR purification kit (Axygen®, CA, USA). The purified PCR products were sequenced at a sequencing facility (Sangon Biotech Co Ltd) in Shanghai, China using the 27F primer. The partial sequences were compared using BLAST queuing system (Altschul et al., 1990) to identify their closest relatives and tentative phylogenetic positions. The sequences were later submitted to DNA Data Bank of Japan (DDBJ) for acquisition of unique accession numbers for the sequences (LC090023, LC092830, LC092831, LC092832, LC092833, LC090854 and LC090855).

4.3.3 Urease activity assay

The isolates were tested for their urease activity on urea agar media described previously (Hammes et al., 2003). The isolates tested positive for urease enzyme.

4.3.4 Biomineralisation assay

The composition of the biomineralisation media consisted of 25 mM calcium chloride solution (purity ≥98%), 333 mM of urea solution (purity ≥ 97 %) and 0.8 g of nutrient broth (BD, Difco™, USA) per 150 ml. Similar concentrations were also used in previous research to effectively identify carbonate precipitating bacteria (Kang et al., 2014b, Muynck et al., 2010a, Helmi et al., 2016, Muynck et al., 2010b, Achal and Pan, 2014). Achal and Pan (2014) observed high urea hydrolysis when 25 mM calcium chloride and 333 mM urea were used for studying calcium carbonate precipitation compared to other calcium sources. The initial pH was 9.1 and 1 M HCl was added to adjust the final pH to 7.5. Each chemical solution was individually autoclaved and filter-sterilized to avoid any contamination before mixing. Two mL of the bacterial culture (grown overnight in nutrient broth at 30 °C for 24 hours) were added to 150 mL of the biomineralisation media and incubated in a rotary shaker at 120 rpm for 7 days at 30 °C. The biomineralising media without bacterial isolates was used as a blank. The pH of the bacterial and abiotic solutions were recorded using Suntex® TS1 pH meter once every 24 hours. The pH was checked under a laminar hood to avoid any contamination. After 7 days of incubation, the solution was vacuum filtered through a 0.6 μm Whatman membrane filter paper. The filtrate was later placed in a Petri dish and air dried at 37°C for 24 hours and checked for calcium carbonate precipitation (see below). All experiments were carried out in triplicate.

4.3.5 Scanning Electron Microscopy

The filter papers with residues were placed in Petri dishes individually and transported to the imaging facility. Double sided carbon tape was applied to standard 5 mm electron microscope stubs and fragments of the filter paper residue were transferred onto the double-sided carbon tape for imaging within the Hitachi TM3000 (Japan) microscope. Five mm stubs were used to allow easy transportation and storage of samples for future observation; an adaptor was used

to allow the stubs to be inserted on top of the Hitachi TM3000 stage. The samples were imaged uncoated, under relatively low vacuum conditions. Due to the low magnification used, no charging errors were observed during imaging. Images were taken at magnifications between 400× and 1500× to allow the identification of crystals formed due to biomineralisation.

4.3.6 X-ray powder diffraction (XRD) analysis

Filter paper samples in Petri dishes were transported to the chemical analysis laboratory. Part of the residue on the filter paper was scraped using a razor blade. The scraping process produced a powder sample which was brought together and transferred onto the sample holder. The upper surface was then carefully flattened using a glass slide. The sample was then placed into the X-ray diffractometer (Advanced D8, Bruker, Germany) in order to identify the chemical composition of the precipitated crystals. The sample holder was rotated during measurement to ensure good sampling of the crystal lattices within the powder sample.

4.3.7 Carbonate titration analysis

The total carbonate recovered from media was quantified using the titration method (Maulood et al., 2012). The residue deposited on the filter paper after the filtration was used for carbonate analysis.

4.4 Results and Discussion

Two indigenous bacterial strains isolated from the landfill groundwater were identified as *Pseudomonas nitroreducens* szh_asesj15 (LC090854) and *Sphingopyxis* sp. szh_adharsh (LC090855) by 16S rRNA gene sequencing (Table 1). *Pseudomonas* belongs to γ-Proteobacteria and has commonly been found in landfills (Kalwasinska and Burkowska, 2013). *Sphingopyxis* belongs to α-Proteobacteria, and members of this genus are extremely resistant towards soil contamination such as that from high heavy metal concentrations (Choi

et al., 2010). *Sphingopyxis* sp., have previously been isolated from crude oils (Kim et al., 2014) and drinking water systems (Gomez-Alvarez et al., 2016). Subsequently, five indigenous *Bacillus* genus belonging to Firmicutes phylum were isolated from landfill leachate. Among the five bacterial strains, two were isolated from raw leachate and three from fresh leachate samples (Table 4-1). Bacteria belonging to Firmicutes phylum have been isolated from other toxic environments such as copper mines and sewers in Brazil (Rodrigues et al., 2014). Several isolates were not used for further experiments due to their hazardous nature, repetition or due to lack of urease enzyme.

Table 4-1 Accession numbers for bacteria isolated from Landfill raw, fresh leachate and groundwater respectively

Source	Accession number	Name of bacteria	Percentage identity	Closest relative in Genbank with accession number
Landfill leachate (raw)	LC090023	*Bacillus licheniformis* SZH2015_A	98%	*Bacillus licheniformis* LRF2X **(**KX364925)
Landfill leachate (raw)	LC092830	*Bacillus pumilus* szhxjlu2015	98%	*Bacillus pumilus* Bp02 (KJ438145)
Landfill leachate(fresh)	LC092831	*Bacillus* sp. xjlu_herc15	97%	*Uncultured Bacillus* sp. *clone* CBHOS-08 ***(***EU371582*)*
Landfill leachate (fresh)	LC092832	*Bacillus licheniformis* adseedstjo15	98%	*Bacillus licheniformis* LRF2X **(**KX364925)
Landfill leachate (fresh)	LC092833	*Bacillus aerius* rawirorabr15	99%	*Bacillus aerius* CCMMB945 (KF879282)
Landfill groundwater	LC090854	*Pseudomonas nitroreducens* szh_asesj15	98%	*Pseudomonas nitroreducens* TA-E11 **(**KX682023)
Landfill groundwater	LC090855	*Sphingopyxis* sp. szh_adharsh	99%	*Sphingopyxis* sp. AX-A (Jq418293)

4.4.1 Analysis of pH in bacterial and blank solutions

The maximum pH measurements for all of the bacterial isolates exceeded that of the blank (Figure 4-1& Figure 4-2). This was expected since the blank did not have the urease enzyme.

The pH surge within 24 hours of the experiment observed in the leachate isolates was quite different when compared with the groundwater isolates. Even among the leachate bacteria, pH variations could be observed. This indicated that each bacterium undergoes different rates of ureolysis for carbonate precipitation. During the first 24 hours of incubation, the pH of the *Bacillus pumilus szhxjlu2015* and *Bacillus aerius rawirorabr15* decreased from their initial pH values (Figure 4-1). This was probably due to the different adaption time by the bacteria to the environment for urea hydrolysis (Lian et al., 2006). Bacteria such as *Bacillus subtilis* have been shown to pump out protons through their cell walls during respiration (Mera et al., 1992). These protons will presumably occupy the negatively charged cell surface sites and lower the pH of the local environment. This early reduction in pH has also been observed previously (Rivadeneyra et al., 2006, Sanchez-Roman et al., 2007). In comparison to Figure 4-1, Figure 4-2 shows an increase in pH from 7.5 to ~8.4 for the groundwater bacteria during the first 24 hours following inoculation into biomineralisation medium. It has been reported that certain carbonate precipitating bacteria begin the process of urea hydrolysis within 24 hours for carbonate precipitation (Achal and Pan, 2014). For the groundwater bacteria, the pH increased almost linearly over 144 hours presumably because of the consistent enzymatic hydrolysis of urea and higher CO_3^{2-} precipitation and upon depletion of the dissolved urea results in a reduction in pH (Stocks-Fischer et al., 1999).

$$CO(NH_2)_2 + 3H_2O \rightarrow 2NH_4^+ + HCO_3^- + OH^-$$

A similar trend was also observed for leachate bacteria after 48 hours (Figure 4-1). All the leachate bacteria are in their linear progressive state (consistent increase in pH) indicated by the bacterial enzymatic hydrolysis of urea leading to an increased production in [OH^-] ions which contributes to the pH increase.

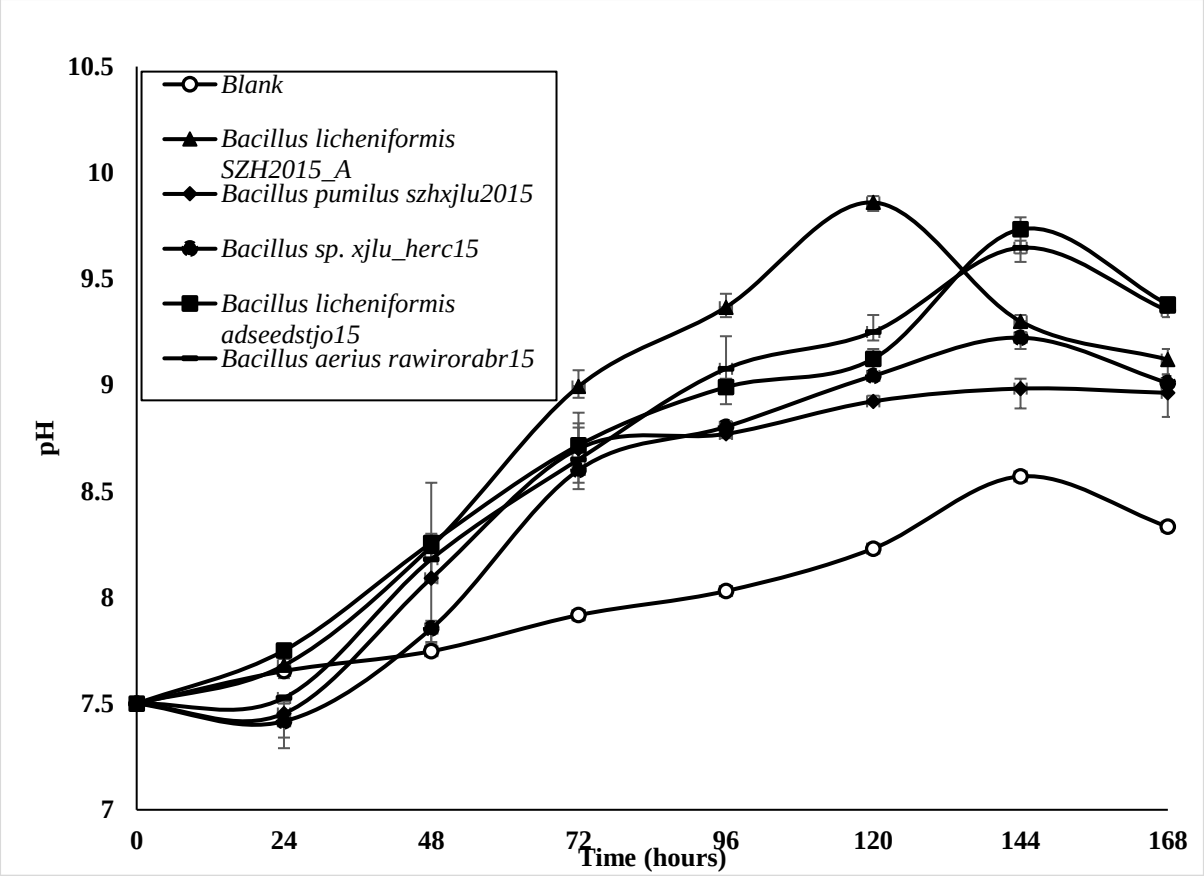

Figure 4-1 pH curves of isolated bacteria from leachate and blank (abiotic) solution performed in triplicate measured during the experiment. Data points are means of experiments performed in triplicate and error bars represent the variations obtained with triplicate pH readings.

Dupraz et al. (2009) have shown enhanced carbonate precipitation in saline aquifers within the bacterial solution in comparison to the abiotic solution. Hommel et al. (2015) re-modelled the whole MICP process and stated that whole-cell-catalysed ureolysis is the driving force of MICP, increasing pH to the pKa of NH_3-NH_4^+ by the production of NH_3. At this pH, a substantial amount of carbonate is present in the solution (the pKa of HCO_3^{2-} -CO_3^{2-} is approximately one order of magnitude higher), which in turn, in the presence of calcium ions, can lead to a supersaturation of carbonate in the solution, thereby promoting the precipitation of calcium carbonate. The forward reaction is catalysed by microbes, thus allowing the

generation of a higher peak pH in the bacterial solutions in comparison to the control (Fujita et al., 2008). The reduction in pH can be explained using two chemical reactions, the precipitation of calcium carbonate and the conversion of ammonium to ammonia:

$$Ca^{2+}+HCO_3^-+OH^- \rightarrow CaCO_3+H_2O$$

$$NH_4^+ + OH^- \rightarrow NH_{3(gas)} + H_2O$$

The pH values from this study can be explained using the theory proposed by Sanchez-Roman et al., 2007 for ureolysis. They reported that the activity of urease is optimum at a pH of 8.5, leading to superior carbonate precipitation (Gorospe et al., 2013, Stabnikov et al., 2013, Chu et al., 2014). They indicated that the metabolic activity of the bacteria is extremely important and it varies from one bacteria to another. Each bacteria supplies the ions necessary for the formation of the minerals, namely NH_4^+ and CO_3^{2-} for carbonates. Moreover, the appropriate microenvironment is created for precipitation, i.e. increased pH and/or ionic concentration. This increased pH environment was also observed in our study for all the bacteria. This demonstrates that bacteria are not simply heterogeneous nuclei for precipitation but are also active mediators in the process.

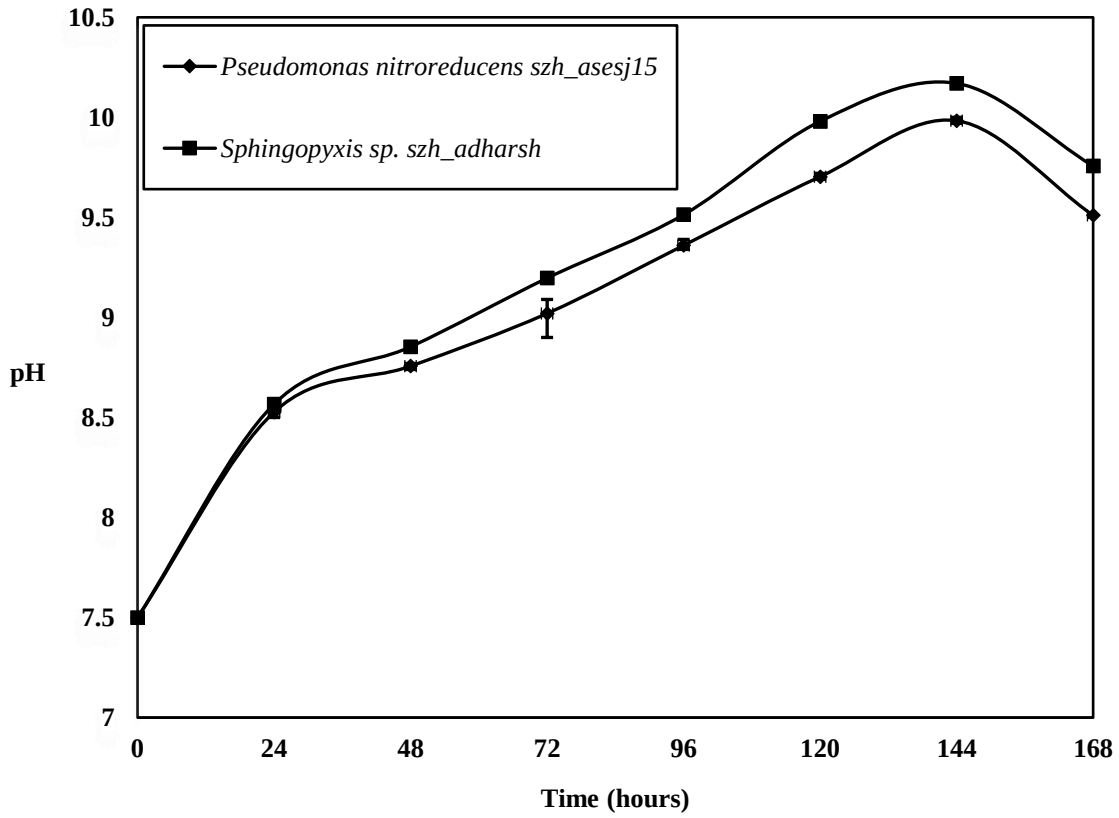

Figure 4-2 pH curves of the bacteria isolated from groundwater performed in triplicate measured within the biomineralisation media. Data points are means of experiments performed in triplicate and error bars represent the variations obtained in the triplicate pH readings.

Furthermore, the bacterial degradation of peptones and yeast extract takes place, supplying NH_4^+ leading to an increase of pH, as observed in our experiments. The metabolic activity occurring in the media, together with the concentration of ions in the cellular envelopes, will drive local oversaturation of such ions, leading to carbonate precipitation. The pH change in the abiotic solution was also reported previously (Ferris et al., 2003, Gorospe et al., 2013, Achal and Pan, 2014) and it is attributed to the very slow hydrolysis of urea which is speculated to be 10^{14} slower than a biotic hydrolysis of urea.

The presence of bacteria can induce the precipitation of minerals in microenvironments by the combination of two mechanisms (1) modifying the conditions of their surrounding environments through ureolysis and/or the concentration of ions in the bacterial cell envelope

(Li et al., 2013a); and (2) cell walls acting as nucleation sites for the growth of the carbonate crystals (Li et al., 2011).

4.4.2 Morphology of crystals in bacterial and control solutions

Previous SEM studies of carbonates formed due to MICP have identified that spherical crystal forms are commonly observed in samples containing bacteria in comparison to the normal rhombohedral crystal form (trigonal system) in non-bacterial samples (Sánchez-Román et al., 2011, Stocks-Fischer et al., 1999, Lian et al., 2006, Jimenez-Lopez et al., 2007, Rivadeneyra et al., 2004). It has been suggested that spherical crystals are a result of the higher rate of crystal formation which is occurring due to the action of the ureolytic bacteria (Stocks-Fischer et al., 1999). The SEM images obtained for the seven bacterial isolates also showed this spherical crystal morphology (Figure 4-3 A, B, C, D, E; Figure 4-4 A and B). Very similar observations have been made for the well-studied ureolytic bacteria, *Bacillus megaterium* (Lian et al., 2006). Further to this, the full range of observations displayed in Figure 4-3 and Figure 4-4 indicate that the bacterial strains influence both the crystal morphology and growth patterns. Similar observations have been individually reported across a range of studies for other biomineralising organisms (Rivadeneyra et al., 2000, Rivadeneyra et al., 2004, Lian et al., 2006, Jimenez-Lopez et al., 2007). The main reason for the changes in morphology is probably due to the differences in ureolysis rates influenced by the bacterial density (Rodriguez-Navarro et al., 2012) and the saturation index of the solution (Bosak and Newman, 2005, Sanchez-Roman et al., 2007, Mitchell and Ferris, 2006).

Fused spherical crystals were observed in *Bacillus licheniformis* SZH2015_A (Figure 4-3A) & *Bacillus aerius* rawirorabr15 (Figure 4-3E) samples, where the spherical crystals have grown together and become interlocked. (Xu et al., 2015) suggesting that calcium source are highly influential in the clumping or fusing of crystals. This type of crystal formation is highly desirable for soil applications, as it can generate very low permeability zones within a

soil allowing pore necks to become sealed. At a larger scale, clumping of large numbers of calcite crystals is produced by *Bacillus licheniformis* adseedstjo15 (Figure 4-3E). Clumping of crystals occurs when the expansion of crystals displaces and entrains smaller growing crystals. This leads to the formation of an interlocking framework that enables bacteria to slowly establish contact with nearby crystals surfaces and develop colonies on them (Wang et al., 2013). The structure which forms is not a completely fused crystal, although it is likely to contain fused crystals. Such structures will have the effect of reducing permeability, but not to the extent of a fully interlocking crystalline structure.

Bacterial imprints were also identified on the surface of calcite crystals for *Sphingopyxis* sp. szh_adharsh (Figure 4-4B). These results suggested that the bacteria might serve as nucleation sites for calcite precipitation, which is in agreement with observations with other carbonate precipitating bacteria (Lian et al., 2006, Li et al., 2011). The bacterial cell surface could induce mineral deposition by providing nucleation sites due to ion composition on its surface (Lian et al., 2006). Ion composition is referred to as the negatively charged functional groups that are present on the bacterial cell walls which attract Ca^{2+} to induce a local supersaturation so that calcite nucleation takes place on the cell surfaces. No spherical calcite forms were observed in the blank sample (Figure 4-4F).

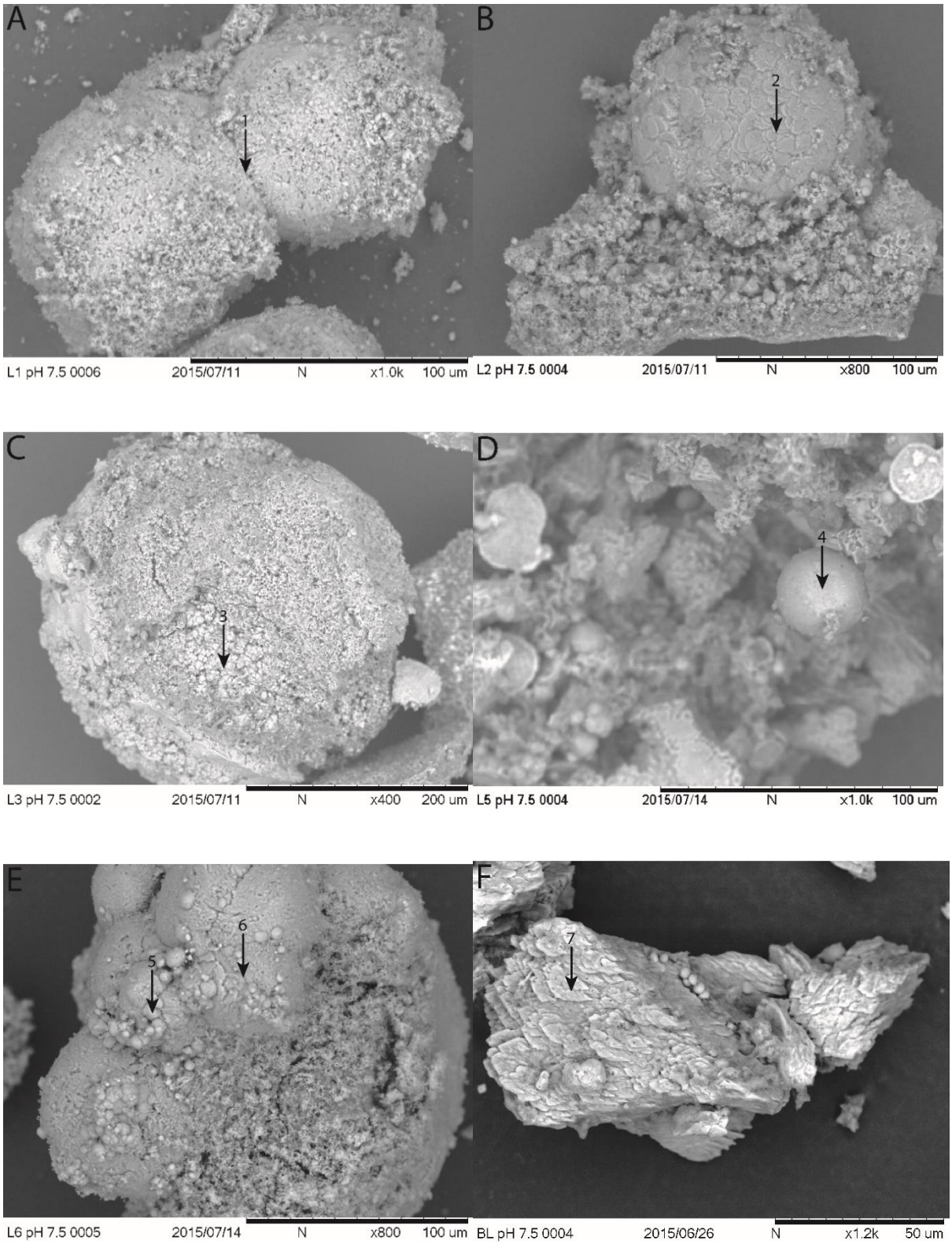

Figure 4-3 Spherical calcite crystals found in solutions containing **(A)** *Bacillus licheniformis* SZH2015_A, (1) fusing of two calcite crystals. **(B)** *Bacillus pumilus* szhxjlu2015, (2) fibrous patterns on the surface of a spherical calcite crystal. **(C)** *Bacillus* sp. xjlu_herc15, (3) very small calcite crystals(<30μm) on the surface of a single calcite crystal. **(D)** *Bacillus*

licheniformis adseedstjo15, (4) single spherical calcite crystal connected with non-spherical calcite crystals. **(E)** *Bacillus aerius* rawirorabr15, (5) small calcite crystals (50-75µm) fused together on the top of a calcite crystals, (6) minor cracks observed on the surface of a calcite crystals and non-spherical calcite crystal with platy overlapping layers on the surface of the calcite crystal observed in. **(F)** abiotic solution showing rhombohedral crystal forms.

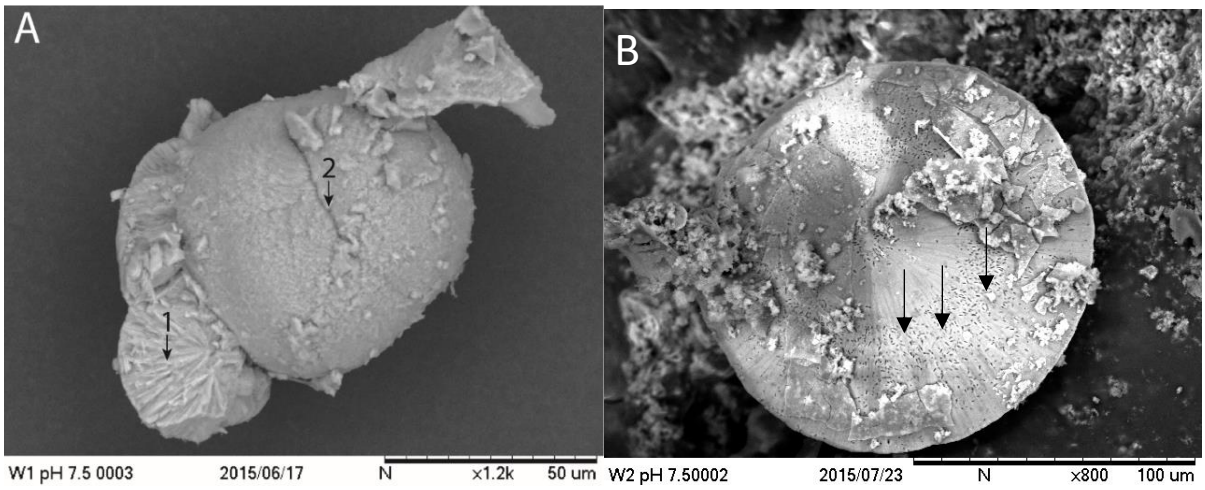

Figure 4-4 Scanning electron micrographs showing mineral precipitates formed in the presence of *Pseudomonas nitroreducens* szh_asesj15 (A) Radiating growth structures in the crystal (1) and internal fusing lines on a spherical calcite crystal (2). (B) Arrows indicate bacterial imprints on the surface of calcite crystals formed in the presence of *Sphingopyxis* sp. szh_adharsh.

4.4.3 X-Ray diffraction analysis (XRD)

XRD analysis was used to measure the composition, structure and microstructure of the crystal compounds. Calcium carbonate crystals were precipitated by all the bacterial isolates in this study (Figure 4-5 & Figure 4-6). Calcite and vaterite were produced in all samples. The results, especially from the use of calcium chloride, concur with the previous reports in which calcite and vaterite were produced (Gorospe et al., 2013). Zamarreňo et al. (2009a) reported that precipitation of calcite and vaterite were also influenced by the bacteria and the carbonate precipitation media. To our knowledge, our study indicates that bacteria rather than calcium chloride caused differences in the morphology of calcium carbonate polymorphs (Figure 4-5 & Figure 4-6). This is a very important finding because it suggests each bacteria precipitate calcium carbonate polymorphs in a slightly different way in the same media.

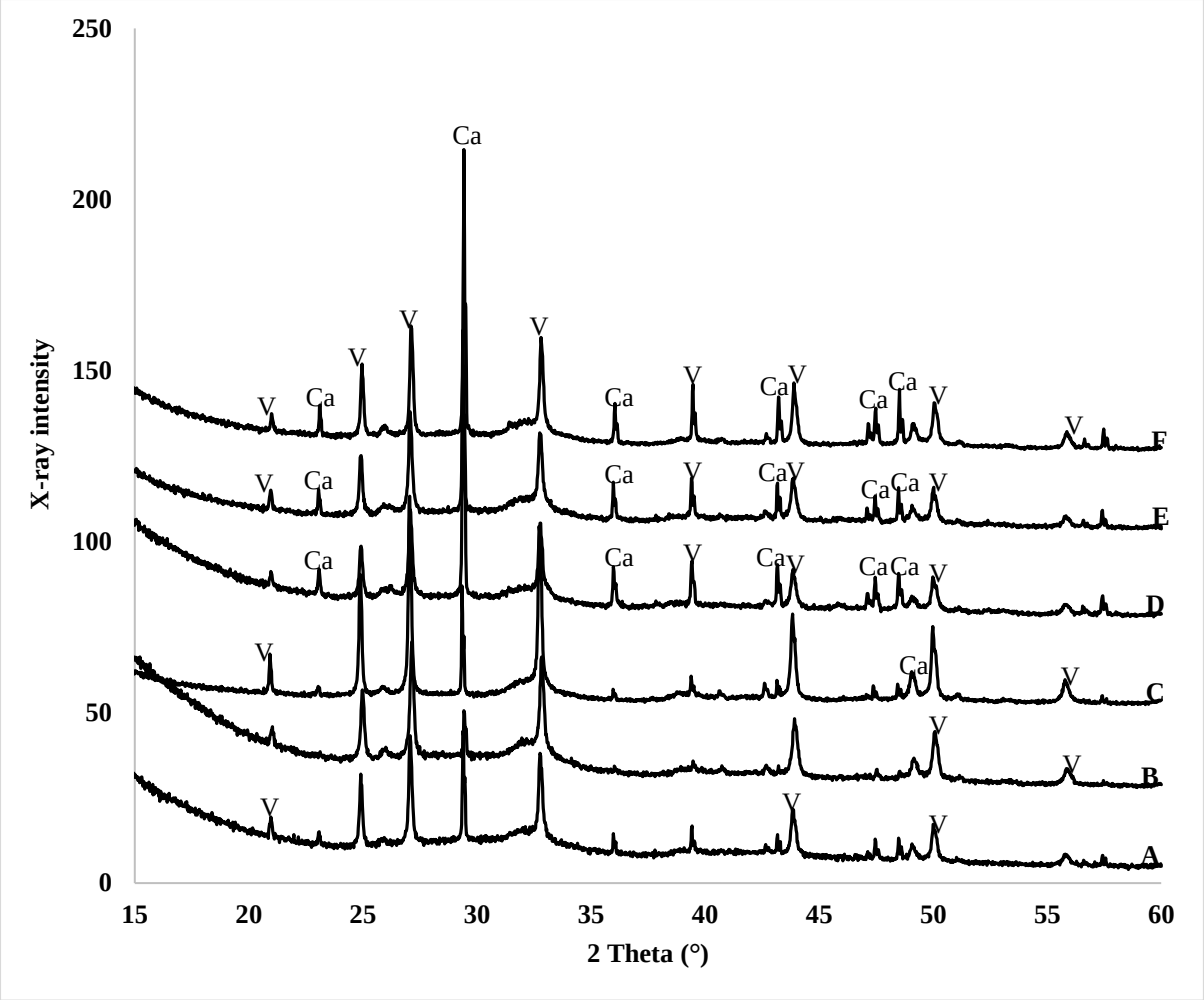

Figure 4-5 XRD spectra indicating multiple calcite and vaterite peaks in all five bacterial isolates and the blank. (A) *Bacillus licheniformis* SZH2015_A; (B) *Bacillus pumilus* szhxjlu2015; (C) *Bacillus sp.* xjlu_herc15; (D) *Bacillus licheniformis* adseedstjo15; (E) *Bacillus aerius* rawirorabr15 and (F) abiotic solution. (Ca= Calcite; V= Vaterite).

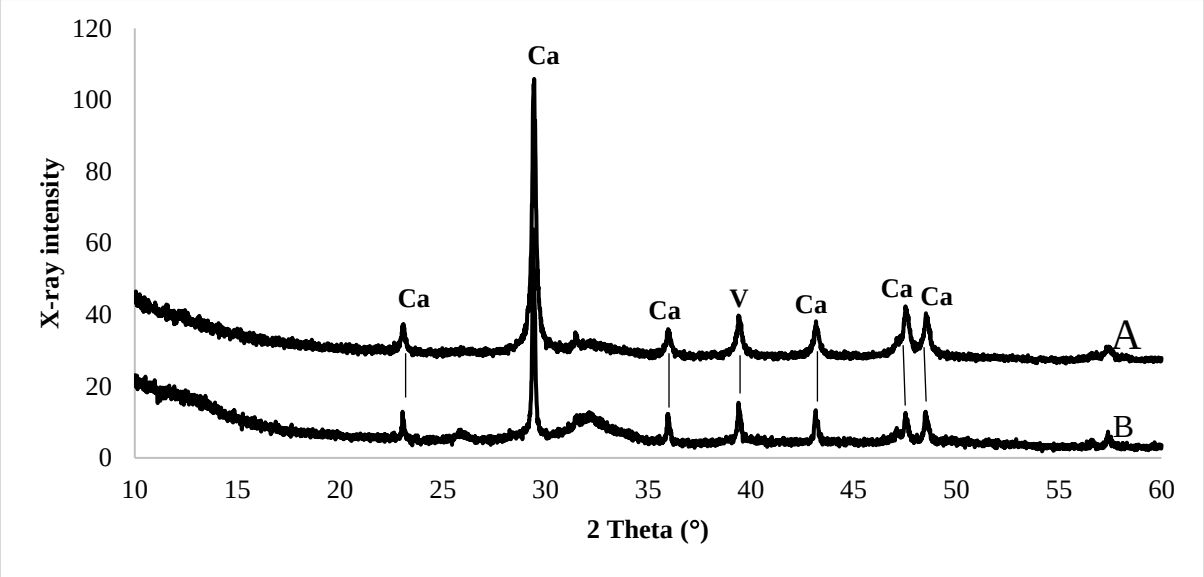

Figure 4-6 XRD spectra showing multiple calcites and a single vaterite peak for the bacterial samples. A = *Pseudomonas nitroreducens* szh_asesj15; B = *Sphingopyxis* sp. szh_adharsh. Ca=Calcite and V=Vaterite respectively.

4.4.4 Carbonate titration

Titration was performed to calculate and compare the efficiency of carbonate precipitation by each bacteria. The final quantities of precipitated calcium carbonate were confirmed through titration with 0.5 M HCl. Previous studies have shown that urease production increases the pH resulting in a superior carbonate precipitation (Achal and Pan, 2014). Observations in our study differ from this conclusion, as the pH of *Bacillus sp.* xjlu_herc15 reached a higher pH than *Pseudomonas nitroreducens* szh_asesj15. However, *Bacillus sp.* xjlu_herc15 precipitated 0.8 grams of carbonate compared to *Pseudomonas nitroreducens* szh_asesj15 which precipitated 0.9 grams (Figure 4-7). Although *Bacillus sp.* xjlu_herc15 took time to adapt to the environment in comparison to the other bacteria, it still managed to precipitate a superior quantity of carbonate compared to the other five bacteria. Given that pH rise is correlated with urease activity, *Bacillus sp.* xjlu_herc15 has shown to have superior enzyme activity compared to other bacteria from the landfill between 48 to 144 hours. For all of the bacterial samples, the amount of precipitation was higher than that of the abiotic (blank) solution. The variation in effectiveness ranged from 1.53 to 3.2 times more $CaCO_3$

precipitation per 150 ml retained on the filter paper compared to the abiotic (blank) sample (Figure 4-7).

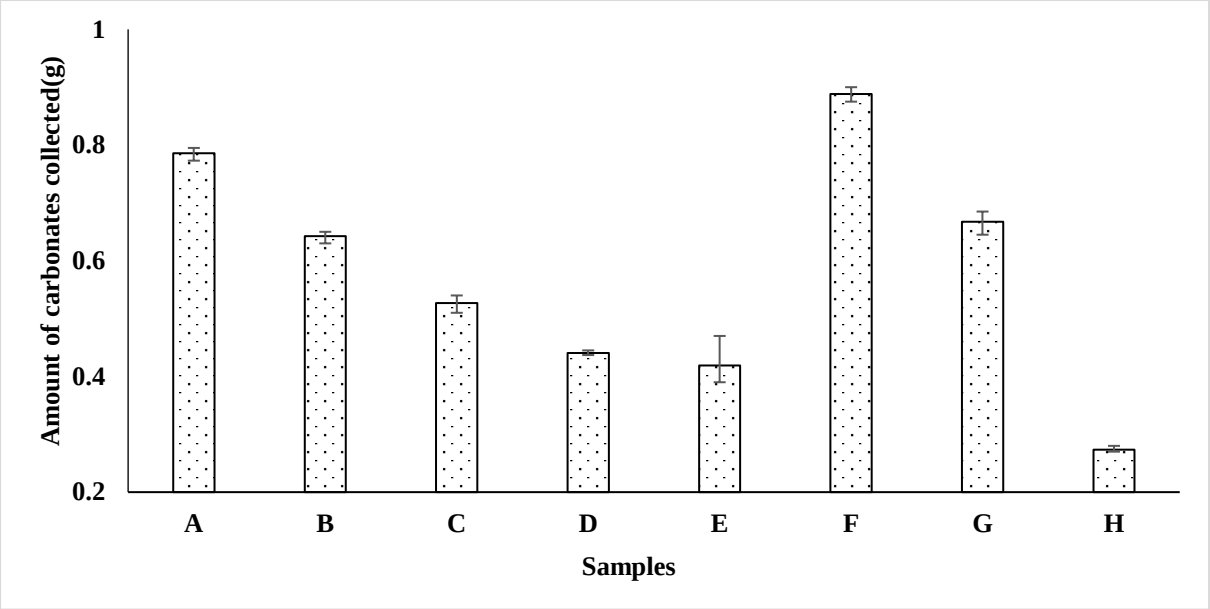

Figure 4-7 Calcium carbonate precipitation with error bars for individual bacterial solutions (A) *Bacillus sp.* xjlu_herc15 (B) *Bacillus licheniformis* adseedstjo15 (C) *Bacillus licheniformis* SZH2015_A (D) *Bacillus aerius* rawirorabr15 (E) *Bacillus pumilus* szhxjlu2015 (F) *Pseudomonas nitroreducens* szh_asesj15 (G) *Sphingopyxis* sp. szh_adharsh and (H) abiotic solution.

No carbonate precipitation was found in the abiotic samples reported by (Sanchez-Roman et al., 2007, Achal and Pan, 2014) but recent studies conducted by (Zamarreño et al., 2009a, Okyay and Rodrigues, 2015) reported carbonate precipitation under abiotic conditions. Okyay and Rodrigues (2015) suggested that the interaction of CO_2 with the abiotic media results in the precipitation of carbonate.

4.5 Conclusions

Studies based on MICP have shown that the composition of the culture medium and pH can change the type and amount of calcium carbonate precipitated. This study focuses mainly on the biomineralisation potential of indigenous bacteria from a landfill and its surroundings. Hence, we provide strong evidence of such possibility and present data showing the precipitation performance of a range of newly identified bacterial strains. Analysis of the

microbially induced calcium carbonate produced was achieved using a combination of carbonate titration, SEM and XRD methods. Each bacteria, irrelevant of their environment, influenced the morphology and amount of calcium carbonate precipitation. Bacterial strain was identified as more important than pH in terms of the amount of carbonate being precipitated by the bacteria. Even though, urease activity does promote carbonate precipitation, it doesn't appear to influence the amount of carbonate that will be precipitated by the bacteria. This approach makes it ideal for biostimulation of these bacteria in the landfill for environmental remediation purposes. Therefore, the authors hope that the findings from this study will potentially lead to an optimistic implication for the design of future engineering applications involving microbially induced calcite precipitation, such as sand consolidation, soil improvement, and bioremediation.

CHAPTER 5 The geotechnical application of MICP using locally extracted bacterial strains

Currently under review with Engineering Geology: Adharsh Rajasekar, Stephen Wilkinson, Jonathan Bridge, Raju Sekar, Eduardo-Medina Roldan, Charles K.S.Moy 2018.

5.1 Abstract

Microbially induced calcite precipitation (MICP) is a promising soil stabilizing technique that utilizes the metabolic pathways of bacteria to form calcite precipitation throughout the soil matrix, leading to an increase in soil strength and stiffness. The bacteria used creates an environment which leads to calcite precipitation through urea hydrolysis, these calcite crystals adsorb onto the surface of sand grains. In this paper, the effect of three indigenous bacteria isolated from a landfill (leachate and groundwater) for MICP in sand was studied. One bacteria from landfill groundwater *Pseudomonas nitroreducens* szh_asesj15 and two bacteria from landfill leachate *Bacillus sp.* xjlu_herc15 and *Bacillus licheniformis* adseedstjo15 were applied for the biocementation of soil samples. These bacteria were all capable of reducing permeability and increasing strength of sand columns through MICP. An abiotic sand column was also studied to allow the comparative assessment of bacterial performance. The unconfined compressive strength of sand treated with the bacteria was 4-5 times higher than the abiotic column. The permeability of sand varied with the content of precipitated calcium. The percentage of void space filled was ~7% higher in the bacterially treated soil in comparison to the abiotic column. The data presented in this study indicates the widespread nature of calcite precipitating bacteria across several sites within the city of Suzhou. This gives us an indication of their application they can be applied for geotechnical engineering purposes such as soil remediation or strengthening.

5.2 Introduction

Biocementation is a product generated through a combination of bacteria and a cementation solution (primarily consisting of a calcium source) within a porous medium. The use of microbially induced carbonate precipitation (MICP) for geotechnical and environmental engineering purposes such as reducing the permeability of soil or increase in shear strength of soil through biocementation has been proposed (Chu et al. 2012; Chu et al. 2013; DeJong et al. 2006; Stabnikov et al. 2013). Improvement of soil mechanical properties by MICP is currently of particular interest to engineers and microbiologists and has been demonstrated by several researchers at varying scales (DeJong et al. 2006; DeJong et al. 2010; Paassen et al. 2010; Van Paassen et al. 2010).

As discussed earlier, one specific biological process which has been identified as highly beneficial for engineering is MICP. It is a type of bio-mediated process induced by micro-organisms. At the scale of the micro-organism however, precipitation is commonly induced by a chemical change in the local micro-environment. For example, chemical reactions that result in a pH change can generate precipitation in the presence of the dissolved ions. The most commonly observed and reported reaction is urea hydrolysis, where urease enzyme catalyses the conversion of urea to ammonium which results in an increase in pH (Muynck et al. 2010). A study by Gorospe et al. (2013) found that calcium chloride is the ideal calcium source for superior calcite precipitation since it promotes superior urea hydrolysis when compared with calcium nitrate, silicate and oxide.

Where MICP occurs within the soil environment the precipitate forms in the voids between particles. If the resulting crystals are of sufficient size they can generate bridges between particles (biocementation). The growth of these crystal will reduce the void space and increase the density of the soil. As a result of this the permeability of the soil can be greatly

reduced. If the crystal bridges connect particles together then they can also act as cements which have the ability to transfer stresses, and also increase the contact area between particles. This effect increases both the strength and stiffness of the resulting soil (Cheng et al. 2013). This observed modification of the mechanical properties of soils generates the potential for biomineralisation to be applied for ground improvement purposes. In addition, the resultant reduction in permeability implies that biomineralisation could be used to generate artificial geological barriers to flow. Such barriers could be used to control flow and control hydraulic gradients, or alternatively could be used for subsurface containment of dissolved materials. Ivanov and Chu (2008) present a good summary of the possible geotechnical applications of biomineralisation techniques.

Existing published experiments assessing the capabilities of organisms for MICP are commonly conducted at room temperatures (22 – 25°C), also high pressures are used (Chu et al. 2014; Harkes et al. 2010; Martin et al. 2013; Whiffin et al. 2007).

The aim of this work is to assess the behaviour of the isolated bacteria and their carbonate precipitating ability under more realistic temperatures in order to provide a better assessment of field behaviour.

5.3 Materials and Methods

5.3.1 Site Identification and Sampling

Given the inference that MICP capable organisms are likely to be present in high pH environments, such environments near the urban centre were sought for sampling. Within Suzhou there is a large energy from waste incinerator (Wu et al., 2011), which produces a ash which is disposed of alongside excess waste within a local landfill. Given the normally broad microbial diversity of landfills, and the high pH generated by the high levels of ash, it was thought that this landfill would contain MICP capable organisms. Rajasekar et al. (2018)

studied the impact of heavy metal towards the microbial diversity of the landfill from which the biomineralising bacteria were isolated. The heavy metals were shown to have a potential impact towards microbial diversity and the isolation of these bacteria possibly show their resistance to grow in unfavourable conditions. In addition, given the issues of urease activity reported in agricultural soil (discussed earlier), groundwater samples were obtained from a groundwater monitoring well at Xi'an Jiaotong-Liverpool University (XJTLU).

The groundwater samples were collected at approximately 4 meters depth from the two sites using a hand-held slow flow peristaltic pump. The samples were collected from well below the groundwater surface such that any residual floating matter would not be collected. Soil samples were collected using a hand auger from three different locations in the landfill which were approximately 400 meters apart from each other. The first location was near the leachate pond, the second location was close to agricultural land on the boundary of the site and the third location was close to the groundwater monitoring borehole. In addition, soil samples were collected from two locations on an ex-agricultural urban site (XJTLU), which were approximately 500 meters apart. The samples of fresh leachate (which were collected from an outlet pipe that runs beneath the landfill) and raw leachate (which was stored in a leachate pond) were collected from the same landfill site.

The groundwater and leachate were collected in sterile high-density polyethylene plastic bottles and the soil samples were collected in a sterile plastic zip lock bags and transported to the laboratory under ambient temperature conditions, then stored in a cold room prior to analysis.

5.3.2 Culturing and identification of microbes

A culture dependant technique was used for the isolation of bacteria from the landfill samples then individual bacteria were isolated using the serial dilution method and the isolates were

grown in a nutrient broth containing beef extract and peptone. Seven different bacteria were isolated from the samples and later harvested in pellet form by centrifuging the growth media at 30°C for 48 hours. The pellets were transferred to bead columns for DNA extraction; the extraction was performed with a DNA isolation kit. The DNA was later amplified using PCR and sent for sequencing. The genome sequences obtained were submitted to the National Center for Biotechnology Information (LC092832, LC092831, LC090854). Only one hazardous bacteria was found after analysing the genome sequences and it was killed by autoclaving the bacterial sample.

5.3.3 Initial assessment of biomineralisation capability

The isolated bacteria were placed in conical flasks (standard flask experiments) with a media containing 25 mM calcium chloride, 333 mM urea and nutrient broth (Muynck et al., 2008, Muynck et al., 2010b, Helmi et al., 2016). The bacteria were studied for 7 days and following this the media was filtered through a 0.45 µm Whatman filter paper, using vacuum filtration. The amount of carbonate in the media was quantified through titration. The three bacteria with the highest carbonate precipitation were chosen for assessment in soil column experiments.

The bacteria extracted in the field were isolated from a biomineralisation promoting media (as described above). Thus, they all have an ability to promote biomineralisation. Most MICP experiments obtain organisms from a culture collection, such organisms are good at producing bio-cements. As outlined above, the isolated organisms are the best of the *in-situ* organisms, it is anticipated that while these will produce cements, these will not be as extensive as those produced by organisms from culture collections.

5.3.4 Simple bioreactor construction

The design of the soil column used to assess biocementation was modified from a previous study (Harkes et al., 2010). Pure silica sand (ISO 900:2001) of 0.425 mm was used for the experiments (Figure 5-1). All columns used in this study were made of Poly Vinyl Chloride (PVC) tubing with an internal diameter of 5.5 cm and length 20 cm. Sand columns were packed with dry silica sand and non-continuous vibration was applied at the start of the experiment. The bottom of the column was covered with a scouring pad layer, to act as a filter to avoid sand loss. A tube of 20 mm inner diameter was connected to the outlet of the soil column. A u-bend arrangement was used to maintain saturation in the sand column at all times (Cheng and Cord-Ruwisch, 2012).

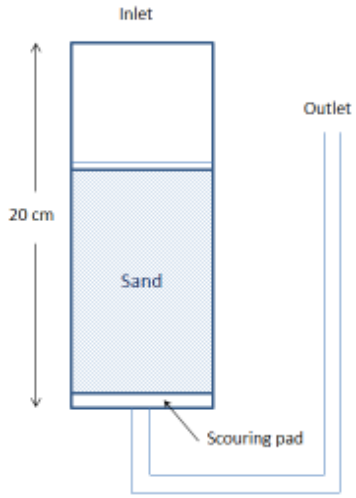

Figure 5-1 Column design for soil experiments modified from (Harkes et al., 2010).

5.3.5 Experimental process

The sand columns were positioned vertically with the top part of the column being open to the atmosphere and bottom part being connected to a 20 mm inner diameter tube. Reagents (bacterial suspension and cementation solution) were introduced from the top of the columns. The cementation solution consists of 500 mM calcium chloride and 500 mM urea (Soon et al., 2013). The transport of media through the column occurred due to gravity and diffusion. The

experiment was conducted at a temperature of 17°C, and at atmospheric pressure. The cementation solution was replaced every 2 days.

5.3.6 Measurement procedures

In previous experiments reported in the literature, difficulties have occurred with the extraction of samples from bioreactors due to the developed brittleness of the samples (Cheng and Cord-Ruwisch, 2012, Cheng et al., 2013, Chu et al., 2014). Given the conditions for the experiments reported in this paper and that the microbes used were not the optimum microbes, the relative brittleness and fragility of the samples was expected. The cements formed between the sand and the sides of the column were quite strong. Attempts were made to cut out the sample, and to extract the sample using gradually applied piston pressure, however all such attempts did not produce an unbroken cylindrical sample for uniaxial/triaxial testing. Such breakages were especially problematic for the non-microbial (blank) sample. After several attempts to extract whole samples, *in-situ* measurements were made inside the bio-reactor using a pocket penetrometer (presented as converted UCS values). While such measurements are inherently variable, they allow a comparison between the microbial and non-microbial samples to confirm the approximate extent of the increase in strength caused by MICP within the sand. In addition to this, *in-situ* estimates of permeability were also made using a falling head procedure between two marks on the inside of the column above the sand.

Following strength testing, small samples of sand agglomerations from all of the bioreactors were transferred to the Department of Civil Engineering's imaging laboratory. Electron microscope stubs were covered with sticky double-sided carbon tabs, and the sand samples were placed onto them. These stubs were placed uncoated into the Hitachi TM3000 scanning electron microscope to allow a qualitative assessment of the extent of mineral precipitation that had occurred.

Finally, the extent of precipitation of calcium carbonate was assessed via titration of HCl using approximately 10 grams of the cemented sand from the column.

5.4 Results and Discussion

The microbes used in the column experiments were identified and isolated using standard flask experiments (Stocks-Fischer et al., 1999). The three microbes which produced most carbonates during the flask experiments were used during the column experiments.

During the soil column experiments the liquid media was replaced regularly, so care must be taken to ensure that the microbes are not washed through the soil column. The experiments were carried out at room temperature and without the agitation which was applied to the conical flasks inside the incubator. However even under these non-ideal conditions an increase in soil strength was observed for all biotic samples in comparison to the abiotic blank (Figure 5-2). The increase in strength varied from 3 to 5 times that of the blank sample. Given the nature of the strength tests 5 measurements were made to generate an assessment of the variability. Error bars are provided indicating the maximum and minimum measurements.

In parallel to this strength change the permeability of the soil decreased by an order of magnitude in comparison to the blank. The blank samples had a highly variable flow rate, both due to measurements inaccuracies (high flow rate) and due to real variabilities in cementation.

The growth of crystals causes a reduction in the void ratio of the soils alongside the creation of agglomerated/cemented particles. This is the primary cause of both strength and permeability changes. Whilst the reduction in permeability of the biotic samples reached the same levels for all three organisms, the increase in strength was more variable (Figure 5-3).

This implies that while the volume of impeding materials is similar for all three organisms, the ability of the grown crystals to transfer stress is different. The observed morphology of biomineral crystals formed varies between organisms. This variation in morphology can be a result of the interaction of the organism with the crystals, or it can be a secondary effect caused by the extent of the chemical changes in the soil environment which has been generated by the organism (Zamarreňo et al., 2009b, Sanchez-Roman et al., 2007, Silva-Castro et al., 2013). This is an ongoing area of research. The structural capacity of biomineral masses formed by different organisms is worth broader consideration across a wider range of biomineralizing organisms. A partial assessment of this can be made by observing the microstructure of the resulting soil columns.

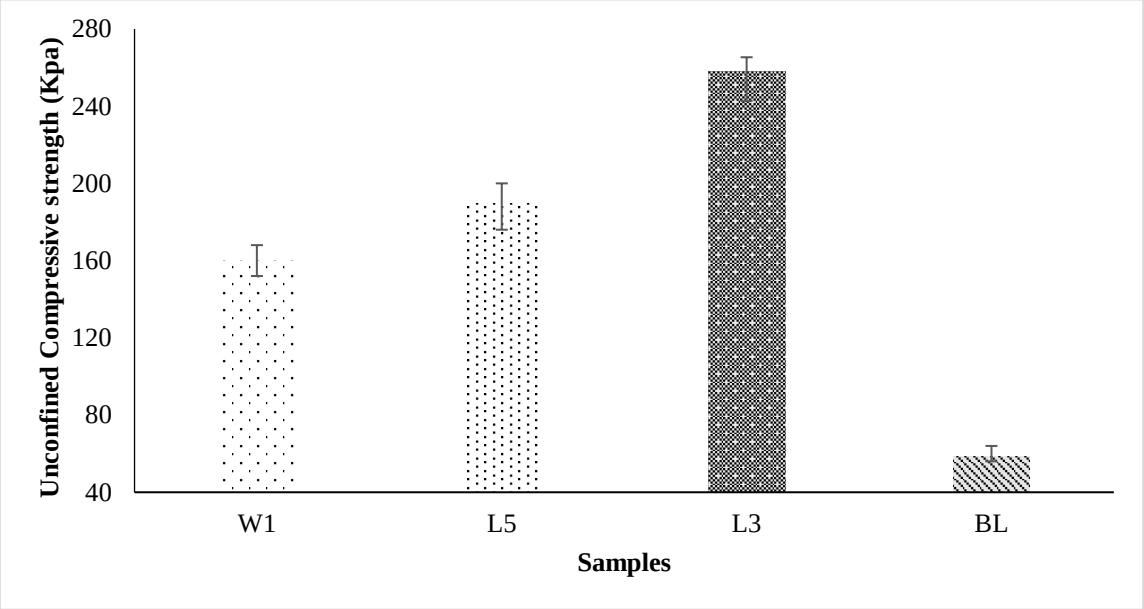

Figure 5-2 Measurments of soil strength W1= *Pseudomonas nitroreducens* szh_asesj15; L3= *Bacillus sp. xjlu_herc15*; L5= *Bacillus licheniformis adseedstjo15* and BL= blank (without bacteria) respectively.

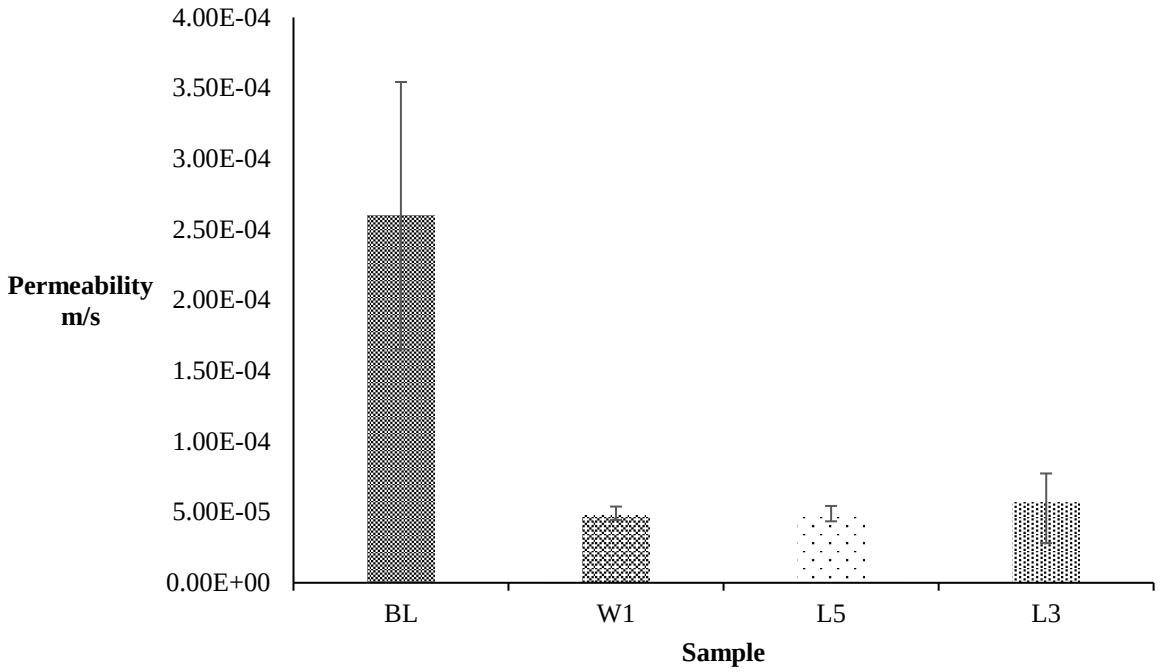

Figure 5-3 Measurements of soil permeability W1= *Pseudomonas nitroreducens* szh_asesj15; L3= *Bacillus sp. xjlu_herc15*; L5= *Bacillus licheniformis adseedstjo15* and BL= blank (without bacteria) respectively.

The precipitation of calcium carbonate occurs both in the biotic and abiotic sand columns. However clear differences in crystal extent are observed between the biotic and abiotic samples indicating that the rate of growth and extent of the crystals is enhanced by the presence of the micro-organisms (Figure 5-4 A, B and C). As the particles have been imaged after the sample has been broken using the pocket penetrometer it is likely that the observed cracks are formed during the mechanical tests. However as predicted by the permeability tests the observable pore crystal density is approximately equivalent for all of the microbe samples. For the abiotic sample (Figure 5-4 D) the crystals appear to be more widely spaced. Some grains appear well covered where as others display very limited crystal growth. The exact cause of enhanced nucleation on some grains compared to others is unclear.

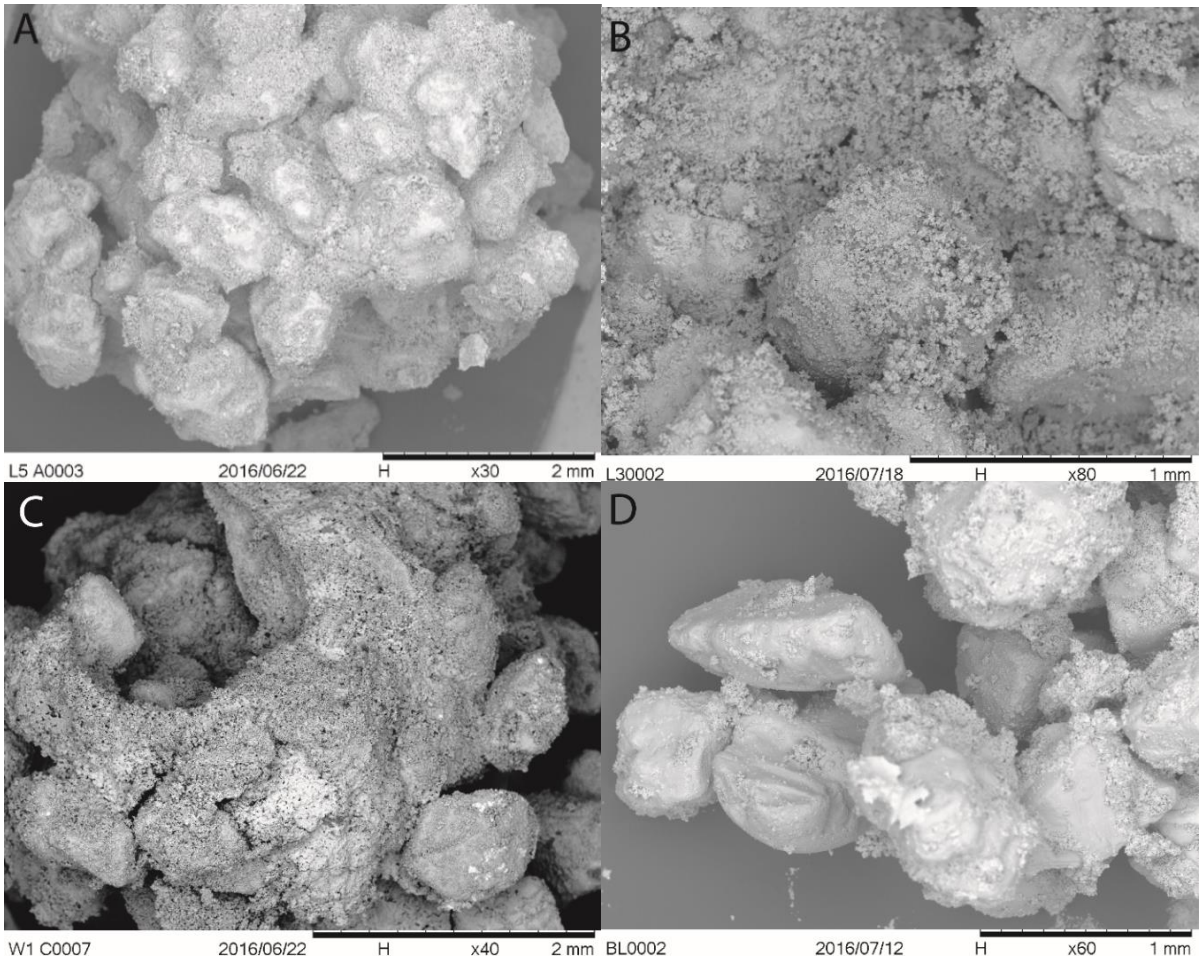

Figure 5-4 SEM images showing superior cementation between sand particles achieved by the application of MICP in the samples containing bacteria (A, B and C). Poor cementation observed in the abiotic sample (D).

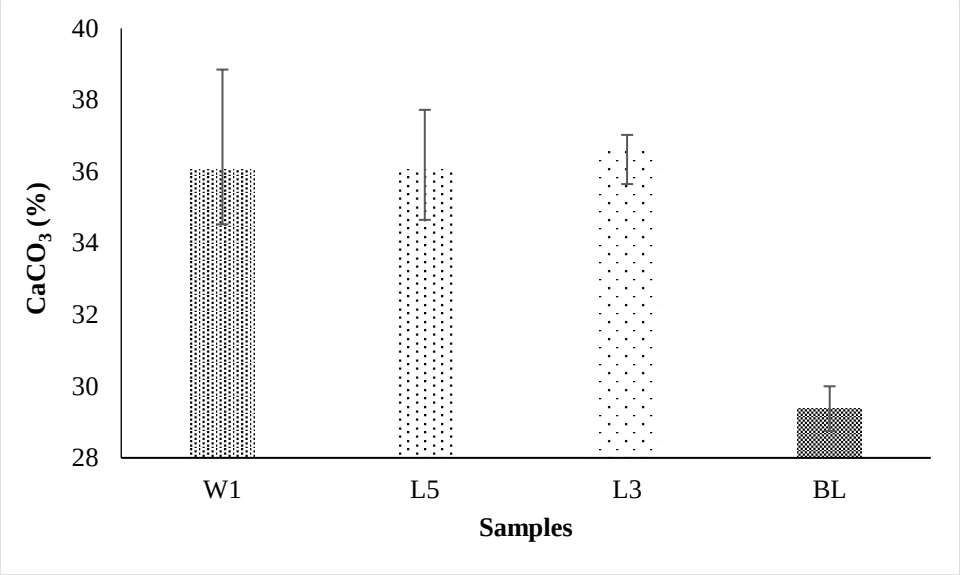

Figure 5-5 Percentage of void space filled with $CaCO_3$ W1= *Pseudomonas nitroreducens* szh_asesj15; L3= *Bacillus sp. xjlu_herc15*; L5= *Bacillus licheniformis adseedstjo15* and BL= blank (without bacteria) respectively.

It may be a function of surface roughness/mineralogy, as grains with limited coverage often display crystal growth within crevices in the grain surface. Given the differential growth of crystals observed in the electron microscope and the permeability data, the higher percentage of $CaCO_3$ in the biotic samples in comparison to the abiotic sample is as expected (Figure 5-5). It is however slightly surprising that the difference is only ~7% of the void space. This suggests that in terms of engineering the distribution produced by the organism is significant for the modification of permeability and strength.

Given that all of the organisms have produced an improvement in comparison to the blank sample, they all aid biomineralisation. However, the variations in the strength of the resultant soils suggests that not all bacteria act in the same way. This may be a function of the organism alone, it may also be a function of the interaction of the organism with the environment. It is likely that each organism would have different optimum conditions for precipitation. This suggests perhaps that different organisms may act in an optimum way in

different environments. An assessment of this would be of use for applied microbial geotechnology in different regions across the world.

5.5 Conclusions

Applied microbial geotechnology is an approach to engineering which has a strong potential to enhance the sustainability of geotechnical practice. The microbes used in this study were not the optimum microbes for biomineralisation; they were locally derived from soil and leachate samples. However even such microbes have been shown to be able to produce a beneficial effect on the engineering properties of soils. Up to a 500% increase in strength and an approximately 75% reduction in permeability relative to the abiotic samples were observed. The results of this study suggest that bacteria which might be useful for engineering purposes may be more commonly available within the ground than might currently be assumed. The variability in the resultant strength produced by the bacteria does however suggest that the studied microorganisms are not equal, and there will be an optimum organism for each environment.

The utilisation of *in situ* organisms within this work was an attempt to assess the existence of potentially beneficial microorganisms within the natural engineering environment and to alleviate some of the fears over their usage especially fear over use of exogenous organisms. Using organisms that are already on a site is of much less concern than importing new organisms. It is also possible that utilising *in situ* organisms can reduce the cost of application. However, the level of improvement will be highly dependent on the organisms on site and will not be as extensive as that produced using the optimum organisms purchased from a culture collection.

Finally, the results of this work indicate that the solution to an engineering problem has the potential to be already be present in the form of a micro-organism beneath the engineering

site. The authors hope that the assessment and incorporation of biogeotechnological techniques in engineering will continue to gain momentum.

CHAPTER 6 Discussion and Conclusion

6.1 Discussion

This Chapter discusses the findings from Chapters 3-5 and explains how they are complimentary in achieving the objectives set out in this thesis.

In order to determine if and how indigenous bacteria found in contaminated regions such as landfills can have biomineralisation potential, the following three steps were undertaken;

i. **Investigation of possible impacts of the landfill contamination towards the microbial consortia.**

   Leachate, soil and groundwater samples from landfill sites were collected and compared with a selected urban site to identify the diversity and composition of the microbial consortia. Heavy metal analyses provided an insight to understand whether their presence and concentration had any effect towards the microbial consortia. Comparison of the microbial communities between the two sites provided details of the bacteria that are capable of surviving under landfill conditions.

ii. **Identification of potential indigenous bacteria capable of carbonate precipitation.**

   A culture dependant technique was implemented to isolate the bacteria from the landfill samples. A media consisting of a calcium source, nitrogen source (possibly urea or ammonium chloride) and nutrient broth was formulated to study the ability of bacteria to precipitate carbonate. The experimental data was compared against an abiotic solution and carbonate titration was used to compare the carbonate precipitation between the bacteria and the abiotic solution.

iii. **Potential of selected bacteria for engineering applications.**

   The carbonate precipitating bacteria was further applied in a porous media (in this case, sand) at a temperature ranging between 15-25°C. Permeability was monitored

regularly to check the changes of the column in liquid permeation. Upon completion, scanning electron microscopy was used to investigate the nature of the cementation bonds between the sand particles. Penetrometer test was utilised to probe the uniaxial compressive strengths of the sand samples.

These approaches have been discussed in Chapter 3-5 and their contribution to the thesis is discussed below;

In Chapter 3, NGS was performed to analyse the bacterial communities between a landfill and a reference (less contaminated) site. The reference site was an urban area away from the landfill without the possibility of leachate contamination. The reason for this approach was to study whether heavy metals have an impact on the bacterial community and also to identify the heavy metal contamination levels in the landfill. In addition, landfills are unique environments with specific nutrient composition and microbial diversity, this makes comparability an important aspect for this research work. To analyse the impact of heavy metals, the bacterial richness and diversity was compared between samples from a landfill and neutral site. Statistical analysis was performed by the authors to compare bacterial diversity among multiple locations. The leachate lacked in terms of richness and diversity when compared to its counterparts. Richness and diversity was calculated based on OTUs. The lack of richness and diversity could be explained as the levels of arsenic and mercury were higher in the leachate in comparison to the other samples. The arsenic and mercury concentrations were also higher than the recommended limits provided in the European landfill standards as described in Chapter 2. Cluster analysis was performed to study the similarities of OTUs between both sites; leachate was found to have almost no similarities with the other samples. The landfill soil and groundwater samples shared similar OTU patterns as shown in Chapter 3. The results from this chapter identified that the diversity and microbial consortia in landfills varied highly in comparison to an urban site.

In order to show that indigenous carbonate precipitating bacteria inhabit environments similar to landfills, bacteria were isolated from them and studied for carbonate precipitation. Chapter 4 shows the investigation of the ability of carbonate precipitation of bacteria that were isolated from landfill leachate and groundwater. Given that the literature review identified that landfills are one of the most unexplored places for carbonate precipitating bacteria, this research shows the potential for identifying such bacteria in landfills. Previous studies were limited to isolated biomineralising bacteria from mine tailing soil (Achal and Pan, 2014), Aeolian sediments (Lian et al., 2006), caves (Rusznyak et al., 2012) and freshwater (Zamarreňo et al., 2009b). The isolation of bacteria from contaminated zones such as landfills showed that carbonate precipitating bacteria could be found in these environments and they could help in solving geotechnical engineering problems such as permeability reduction. The carbonate precipitating bacteria were shown to alter the chemical environment to favour the precipitation of calcium carbonate. One of the major indications was the sudden increase in pH after 24 hours in the bacterial system (Chapter 4). Similar chemical reagents studied in the literature were used in these experiments, since it would be easier to compare with other literature to identify the efficiency of carbonate precipitation by these bacteria. pH, SEM, XRD and carbonate titration experiments identified that the isolated bacteria from landfill leachate and groundwater had the ability for carbonate precipitation (Chapter 4).

In Chapter 5, two bacteria from landfill leachate and one from landfill groundwater (Chapter 4) were chosen for further studies (those identified to be most efficient carbonate precipitating bacteria). Porous media studies were performed using sand and PVC pipe columns to investigate their ability for permeability reduction and strength enhancement. SEM images were used to identify the cementation between sand particles which is the foundation for the reduction in permeability within a soil environment. The final permeability was in the range of 10^{-6} m/s, which is close to the permeability of silty clay. The results from

Chapter 5 in terms of permeability and strength were very encouraging as they proved that the carbonate precipitating bacteria could potentially be used for solving engineering problems.

6.1.1 Fulfilled objectives

The objectives mentioned in 1.2 were successfully achieved and the process of implementation of individual objectives and their overall contribution to the project is shown below;

Objective 1, the microbial diversity and heavy metal analyses of a landfill and an urban site were considered in Chapter 3. NGS was used to compare the microbial communities between the two sites and ICP-MS was used to compare the heavy metals between the two sites. These studies were mainly performed to collect preliminary data and characterise the landfill to gain an understanding regarding the composition of microbial communities and heavy metals.

Objectives 2 and 3 were addressed in Chapters 4 and 5, respectively. Bacteria from landfill groundwater and leachate were isolated and a culture dependent approach was employed to investigate their carbonate precipitation capabilities. SEM, XRD and carbonate titration techniques were utilised to study seven selected bacteria. SEM provided visual analysis of the crystals structural difference resulting from the bacteria and the abiotic solution. XRD provided the composition of the crystals that were precipitated in both solutions. Carbonate titrations helped prove that the bacteria are capable of precipitating calcite more than three times than the abiotic system.

Chapter 5 included studying of the prolific bacteria from Chapter 4 for their application in porous media for cementation between the sand particles. The objectives were achieved successfully with carbonate precipitating bacterial columns achieving permeability in the

range of 10^{-6} m/s and strength between 160-250kPa. SEM images showing superior cementation bonds between the sand particles

Chapters 3-5 provided evidence that the landfill site had more contaminants than the urban site. The isolated bacteria were also shown to be capable of carbonate precipitation which makes landfill a site to be explored for identifying carbonate precipitating bacteria. Application of these bacteria in the porous media columns have shown to decrease permeability.

6.2 Conclusion

The core hypothesis behind this work was that carbonate precipitating bacteria can be found in contaminated zones like landfills and they can potentially be stimulated for ground improvement purposes. Most of the research in the literature relies heavily on bacteria bought from cell culture laboratories. Although the bacteria bought from laboratories are efficient, they are not environmental friendly and cost effective. In addition, injection of external bacteria into a contaminated zone poses high survival risks to the bacteria. However, this situation needs to change and by studying the indigenous bacteria for calcite precipitation in the contaminated zones; microbial consortia will be preserved and unaltered. The work presented here shows that indigenous bacteria capable of carbonate precipitation can be found in toxic environments like landfill, which as cited in the literature consists of wide variety of pollutants whose sources cannot always be tracked.

The landfill that was studied in this project had an alkaline pH due to the limestone hill that was located near the landfill (Chapter 3). In addition, there was a waste incinerator onsite and ash from the incinerator therefore forms a high proportion of the waste disposed of within the landfill. However, this project does not propose that biomineralising bacteria are available only in landfills which have an alkaline pH. As indicated in Chapter 3, the hazardous arsenic

and mercury concentrations in the landfill could inhibit the growth of several bacteria in the landfill. Comparing the richness index revealed leachate to be the least diverse environment when compared to soil and groundwater. This work shows that even in zones as toxic as leachate, biomineralising bacteria do exist. Thus, landfills present an opportunity for the identification of potentially beneficial bacteria (especially for biomineralisation purposes).

Several techniques were used to confirm that the isolated bacteria are capable of calcite precipitation. Chapters 4 and 5 demonstrate the ability of these bacteria in laboratory and engineering experiments. Carbonate precipitation by abiotic solutions proved that the presence of CO_2 is vital to the precipitation of carbonate which may combine with calcium to form calcium carbonate. This idea was refuted by Sanchez-Roman et al., (2007), Achal and Pan, (2014); but previous studies on biomineralisation proved that abiotic solutions can precipitate carbonate but not as effectively as the bacterial solution (Stocks-Fischer et al., 1999, Lian et al., 2006). This study agrees with the latter, having shown with multiple observations that the abiotic solution precipitates calcite. Two bacteria were isolated from landfill groundwater, *Pseudomonas nitroreducens* szh_asesj and *Sphingopyxis sp.* szh_adharsh. Five bacteria were isolated from landfill leachate, *Bacillus licheniformis* SZH2015_A, *Bacillus pumilus* szhxjlu2015**,** *Bacillus sp.* xjlu_herc15, *Bacillus licheniformis* adseedstjo15 and *Bacillus aerius* rawirorabr15. In this work, carbonate titration studies compared the abiotic solution against the seven bacteria and it was found that bacteria precipitated 0.7 to 0.9 grams per 150 ml of carbonate which was three times more than abiotic solution (Chapters 4 and 5). The carbonate precipitating bacteria were shown to alter the chemical environment (pH change) to favour the precipitation of calcium carbonate (Chapters 4 and 5). *Pseudomonas nitroreducens* szh_asesj, *Bacillus sp. xjlu_herc15* and *Bacillus licheniformis adseedstjo15* were chosen for the porous media experiments due to their higher carbonate precipitation efficiency.

The carbonate precipitation data obtained from the seven isolated bacteria during the conical flask experiments was analysed to determine the superior and/or efficient bacteria. The three efficient bacteria from the seven bacteria were studied in engineering experiments where sand was used as a porous medium. The main aim of the experiment was to investigate the bacteria's ability to lower permeability and increasing soil strength (Chapter 5). The experiments provided evidence that the calcite precipitation was able to significantly improve the permeability and strength when compared to the abiotic system (Chapter 5). This work also shows that there is no universal concentration that could be used to stimulate carbonate precipitation as each bacterium is very unique on their carbonate precipitation (Chapter 5). The compressive strength of the bacterial columns ranged from 160-250 kPa. Improved permeability in the range of 10^{-6} m/s was observed in the bacterial columns. Superior cementation between sand particles was observed under SEM in the columns where the bacteria were added.

SEM images showed superior cementation between the sand particles compared to the abiotic solution, thus these bacteria are highly efficient in terms of lowering permeability (Chapter 5).

The most significant novelty was the isolation of these bacteria from environments that are toxic and their ability to precipitate calcite at a superior efficiency when compared with other bacteria in the literature. The potential of stimulating such bacteria in a contaminated zone could help in the mitigation of leachate interacting with the groundwater.

6.3 Applications

The MICP process is an effective and environmental friendly technology that can be applied to solve various environmental engineering problems, including remediation of heavy metals and radionuclides, bioconsolidation, biocement, CO_2 sequestration and other applications. In terms of environmental engineering, it could be used to reduce high permeability zones to prevent groundwater contamination. Bio-cementation is another field that has brought civil

engineers and microbiologist working together to create structures that have improved tensile and compressive strength. CO_2 sequestration is another potential applicability to slow down climate change; the stored CO_2 can be used to precipitate calcite in regions that require ground improvement.

6.4 Recommendations for future work

From the work performed during this study, several avenues of future research have been identified:

- **Time frame studies.** A broad experiment can be setup with multiple columns to study the step by step progression of calcite precipitation, which could provide a better idea of the mechanism that's followed by each bacterium. This study will help in identifying a time frame for media exchange for efficient carbonate precipitation.
- **Organic waste.** It would allow to minimise the cost of reagents that are being currently used for carbonate precipitation. Agricultural and food-processing wastes could be used to replace the laboratory grade reagents. Past studies including the one mentioned in this thesis have shown that replacing laboratory grade reagents by organic waste would be cost effective.
- **Carbon dioxide capture.** Global warming is a major environmental issue occurring primarily in response to increasing concentrations of CO_2 in the earth's atmosphere. Currently, the concentration of CO_2 in the earth's atmosphere is about 400 ppm and it seems to be increasing exponentially. MICP could be an effective method for the removal of CO_2 from the environment. In this method, CO_2 is converted into carbonate minerals that can form different crystals such as calcite, aragonite, dolomite and magnesite. This method is safer and more eco-friendly than conventional methods of sequestering CO_2 from the atmosphere.
- **Modelling calcite precipitation.** Simulations can be performed to analyse the rate of calcite formation in the ground using software which couples multiphysics and finite element software. By inserting the external CO_2 factor from the atmosphere in the simulation, the efficiency of biomineralisation used for ground improvement can be checked.

CHAPTER 7 **REFERENCES**

ACHAL, V. & PAN, X. 2014. Influence of calcium sources on microbially induced calcium carbonate precipitation by *Bacillus* sp. CR2. *Applied Biochemistry and Biotechnology,* 173**,** 307-317.

ACHAL, V., PAN, X., FU, Q. & ZHANG, D. 2012. Biomineralization based remediation of As(III) contaminated soil by *Sporosarcina ginsengisoli*. *Journal of Hazardous Materials,* 201-202**,** 178-184.

ADETUTU, E. M., GUNDRY, T. D., PATIL, S. S., GOLNESHIN, A., ADIGUN, J., BHASKARLA, V., ALEER, S., SHAHSAVARI, E., ROSS, E. & BALL, A. S. 2015. Exploiting the intrinsic microbial degradative potential for field-based in situ dechlorination of trichloroethene contaminated groundwater. *Journal of Hazardous Materials,* 300**,** 48-57.

ALOISI, G. 2008. The calcium carbonate saturation state in cyanobacterial mats throughout Earth's history. *Geochimica et Cosmochimica Acta,* 72**,** 6037-6060.

ALTERMANN, W., KAZMIERCZAK, J., OREN, A. & WRIGHT, D. T. 2006. Cyanobacterial calcification and its rock-building potential during 3.5 billion years of Earth history. *Geobiology,* 4**,** 147-166.

ALTSCHUL, S. F., GISH, W., MILLER, W., MYERS, E. W. & LIPMAN, D. J. 1990. Basic Local Alignment Search Tool. *Journal of Molecular Biology,* 215**,** 403-410.

AMIDI, S. & WANG, J. 2015. Surface treatment of concrete bricks using calcium carbonate precipitation. *Construction and Building Materials,* 80**,** 273-278.

ANBU, P., KANG, C.-H., SHIN, Y.-J. & SO, J.-S. 2016. Formations of calcium carbonate minerals by bacteria and its multiple applications. *Springer plus,* 5**,** 1-26.

ANDREOTE, F. D., CARNEIRO, R. T., SALLES, J. F., MARCON, J., LABATE, C. A., AZEVEDO, J. L. & ARAUJO, W. L. 2009. Culture-independent assessment of Rhizobiales-related alphaproteobacteria and the diversity of Methylobacterium in the rhizosphere and rhizoplane of transgenic eucalyptus. *Microb Ecol,* 57**,** 82-93.

AZUBUIKE, C. C., CHIKERE, C. B. & OKPOKWASILI, G. C. 2016. Bioremediation techniques–classification based on site of application: principles, advantages, limitations and prospects. *World Journal of Microbiology and Biotechnology,* 32**,** 180-198.

BARAT, R., MONTOYA, T., BORRAS, L., FERRER, J. & SECO, A. 2008. Interactions between calcium precipitation and the polyphosphate-accumulating bacteria metabolism. *Water Research,* 42**,** 3415-3424.

BARAT, R., MONTOYA, T., SECO, A. & FERRER, J. 2011. Modelling biological and chemically induced precipitation of calcium phosphate in enhanced biological phosphorus removal systems. *Water Research,* 45**,** 3744-3752.

BAUMGARTNER, L. K., REID, R. P., DUPRAZ, C., DECHO, A. W., BUCKLEY, D. H., SPEAR, J. R., PRZEKOP, K. M. & VISSCHER, P. T. 2006. Sulfate reducing bacteria in microbial mats: Changing paradigms, new discoveries. *Sedimentary geology,* 185**,** 131-145.

BAZYLINSKI, D. A., FRANKEL, R. B., HEYWOOD, B. R., MANN, S., KING, J. W., DONAGHAY, P. L. & HANSON, A. K. 1995. Controlled Biomineralization of Magnetite (Fe3O4) and Greigite (Fe3S4) in a Magnetotactic Bacterium. *Applied and Environmental Microbiology,* 61**,** 3232–3239.

BENZERARA, K., MENGUY, N., GUYOT, F., SKOURI, F., DE LUCA, G., BARAKAT, M. & HEULIN, T. 2004. Biologically controlled precipitation of calcium phosphate by Ramlibacter tataouinensis. *Earth and Planetary Science Letters,* 228**,** 439-449.

BOQUET.E, A.BORONAT & A.RAMOS-CORMENZANA 1973. Production of Calcite (Calcium Carbonate) crystals by soil bacteria is a general phenomenon. *Nature,* 246**,** 527-529.

BOSAK, T. & NEWMAN, D. K. 2005. Microbial kinetic controls on calcite morphology in supersaturated solutions. *Journal of Sedimentary research,* 75**,** 190-199.

BRAD, T., OBERGFELL, C., BREUKELEN, B. M. V., STRAALEN, N. M. V. & RÖLING, W. F. M. 2013. Spatiotemporal Variations in Microbial Communities in a landfill Leachate Plume. *Groundwater Monitoring & Remediation,* 33**,** 69-78.

BROWN, B. L., WATSON, M., MINOT, S. S., RIVERA, M. C. & FRANKLIN, R. B. 2017. MinION^{TM} nanopore sequencing of environmental metagenomes: a synthetic approach. *GigaScience,* 6**,** 1-10.

BUNDELEVA, I. A., SHIROKOVA, L. S., BÉNÉZETH, P., POKROVSKY, O. S., KOMPANTSEVA, E. I. & BALOR, S. 2012. Calcium carbonate precipitation by anoxygenic phototrophic bacteria. *Chemical Geology,* 291**,** 116-131.

CARRARO, L., MAIFRENI, M., BARTOLOMEOLI, I., MARTINO, M. E., NOVELLI, E., FRIGO, F., MARINO, M. & CARDAZZO, B. 2011. Comparison of culture-

dependent and -independent methods for bacterial community monitoring during Montasio cheese manufacturing. *Res Microbiol,* 162**,** 231-9.

CHEN, Y., WU, L., BODEN, R., HILLEBRAND, A., KUMARESAN, D., MOUSSARD, H., BACIU, M., LU, Y. & MURRELL, J. C. 2009. Life without light: microbial diversity and evidence of sulfur- and ammonium-based chemolithotrophy in Movile Cave. *ISME Journal,* 3**,** 1093-1104.

CHENG, L. & CORD-RUWISCH, R. 2012. In situ soil cementation with ureolytic bacteria by surface percolation. *Ecological Engineering,* 42**,** 64-72.

CHENG, L. & CORD-RUWISCH, R. 2013. Selective enrichment and production of highly urease active bacteria by non-sterile (open) chemostat culture. *Journal of Industrial Microbiology and Biotechnology,* 40**,** 1095-1104.

CHENG, L., CORD-RUWISCH, R. & SHAHIN, M. A. 2013. Cementation of sand soil by microbially induced calcite precipitation at various degrees of saturation. *Canadian Geotechnical Journal,* 50**,** 81-90.

CHOI, J. H., KIM, M. S., JUNG, M. J., ROH, S. W., SHIN, K. S. & BAE, J. W. 2010. Sphingopyxis soli sp. nov., isolated from landfill soil. *International Journal of Systematic and Evolutionary Microbiology,* 60**,** 1682-1686.

CHU, J., IVANOV, V., NAEIMI, M., STABNIKOV, V. & LIU, H.-L. 2014. Optimization of calcium-based bioclogging and biocementation of sand. *Acta Geotechnica,* 9**,** 277-285.

CHU, J., STABNIKOV, V. & IVANOV, V. 2012. Microbially Induced Calcium Carbonate Precipitation on Surface or in the Bulk of Soil. *Geomicrobiology Journal,* 29**,** 544-549.

CHU, J., STABNIKOV, V., IVANOV, V. & LI, B. 2013. Microbial method for construction of an aquaculture pond in sand. *Géotechnique,* 63**,** 871-875.

COCOLIN, L., DIEZ, A., URSO, R., RANTSIOU, K., COMI, G., BERGMAIER, I. & BEIMFOHR, C. 2007. Optimization of conditions for profiling bacterial populations in food by culture-independent methods. *Int J Food Microbiol,* 120**,** 100-9.

COSTERTON, W. J. & WILSON, M. 2004. Introducing Biofilm. *Biofilms,* 1**,** 1-4.

DEJONG, J. T., MORTENSEN, B. M., MARTINEZ, B. C. & NELSON, D. C. 2010. Bio-mediated soil improvement. *Ecological Engineering,* 36**,** 197-210.

DENG, S., DONG, H., LV, G., JIANG, H., YU, B. & BISHOP, M. E. 2010. Microbial dolomite precipitation using sulfate reducing and halophilic bacteria: Results from Qinghai Lake, Tibetan Plateau, NW China. *Chemical Geology,* 278**,** 151-159.

DHAMI, N. K., REDDY, M. S. & MUKHERJEE, A. 2013. Biomineralization of calcium carbonates and their engineered applications: a review. *Frontiers in Microbiology,* 4**,** 1-13.

DHAMI, N. K., REDDY, M. S. & MUKHERJEE, A. 2014. Synergistic Role of Bacterial Urease and Carbonic Anhydrase in Carbonate Mineralization. *Applied Biochemistry and Biotechnology,* 172**,** 2552-2561.

DUPRAZ, S., PARMENTIER, M., MÉNEZ, B. & GUYOT, F. 2009. Experimental and numerical modeling of bacterially induced pH increase and calcite precipitation in saline aquifers. *Chemical Geology,* 265**,** 44-53.

EDGAR, R. C. 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nature Methods,* 10**,** 996-1001.

EL-SALAM, M. M. A. & ABU-ZUID, G. I. 2015. Impact of landfill leachate on the groundwater quality: A case study in Egypt. *Journal of Advanced research,* 6**,** 579-586.

FENG, X., DONG, H., YANG, P., YANG, R., LU, J., LV, J. & SHENG, J. 2016. Culture-Dependent and -Independent Methods to Investigate the Predominant Microorganisms Associated with Wet Processed Coffee. *Curr Microbiol,* 73**,** 190-5.

FERNANDES, H., VIANCELLI, A., MARTINS, C. L., ANTONIO, R. V. & COSTA, R. H. 2013. Microbial and chemical profile of a ponds system for the treatment of landfill leachate. *Waste Management,* 33**,** 2123-2128.

FERRIS, F. G., V.PHOENIX, FUJITA, Y. & SMITH, R. W. 2003. Kinetics of calcite precipitation induced by ureolytic bacteria at 10 to 20°C in artificial groundwater. *Geochimica et Cosmochimica Acta,* 67**,** 1701-1722.

FU, F. & WANG, Q. 2011. Removal of heavy metal ions from wastewaters: A review. *Journal of Environmental Management,* 92**,** 407-418.

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 and technology,* 42**,** 3025-3032.

FUKUI, Y., YOSHIDA, M., SHOZEN, K.-I., FUNATSU, Y., TAKANO, T., OIKAWA, H., YANO, Y. & SATOMI, M. 2012. Bacterial communities in fish sauce mash using culture-dependent and -independent methods. *The Journal of General and Applied microbiology,* 58**,** 273-281.

GANENDRA, G., MUYNCK, W. D., HO, A., ARVANITI, E. C., HOSSEINKHANI, B., RAMOS, J. A., RAHIER, H. & BOON, N. 2014. Formate Oxidation-Driven Calcium Carbonate Precipitation by *Methylocystis parvus* OBBP. *Applied and Environmental Microbiology,* 80**,** 4659–4667.

GOH, F., BARROW, K. D., BURNS, B. P. & NEILAN, B. A. 2010. IdentiWcation and regulation of novel compatible solutes from hypersaline stromatolite-associated cyanobacteria. *Archives of Microbiology,* 192**,** 1031-1038.

GOMEZ-ALVAREZ, V., PFALLER, S. & REVETTA, R. P. 2016. Draft Genome Sequence of Two Sphingopyxis sp. Strains, Dominant Members of the Bacterial Community Associated with a Drinking Water Distribution System Simulator. *Genome Annoucements,* 4**,** 2.

GONZALEZ-MUNOZ, M. T., FERNANDEZ-LUQUE, B., MARTINEZ-RUIZ, F., BEN CHEKROUN, K., ARIAS, J. M., RODRIGUEZ-GALLEGO, M., MARTINEZ-CANAMERO, M., DE LINARES, C. & PAYTAN, A. 2003. Precipitation of Barite by Myxococcus xanthus: Possible Implications for the Biogeochemical Cycle of Barium. *Applied and Environmental Microbiology,* 69**,** 5722-5725.

GONZALEZ-MUNOZ, M. T., RODRIGUEZ-NAVARRO, C., MARTINEZ-RUIZ, F., ARIAS, J. M., MERROUN, M. L. & RODRIGUEZ-GALLEGO, M. 2010. Bacterial biomineralization: New insights from Myxococcus-induced mineral precipitation. *Geological Society London Special Publications,* 336**,** 31-50.

GOROSPE, C. M., HAN, S.-H., KIM, S.-G., PARK, J.-Y., KANG, C.-H., JEONG, J.-H. & SO, J.-S. 2013. Effects of Different Calcium Salts on Calcium Carbonate Crystal Formation by *Sporosarcina pasteurii* KCTC 3558. *Biotechnology and Bioprocess Engineering,* 18**,** 903-908.

HAMMES, F., BOON, N., VILLIERS, J. D., VERSTRAETE, W. & SICILIANO, S. D. 2003. Strain-Specific Ureolytic Microbial Calcium Carbonate Precipitation. *Applied and Environmental Microbiology,* 69**,** 4901-4909.

HAMMES, F. & VERSTRAETE, W. 2002. Key roles of pH and calcium metabolism in microbial carbonate precipitation. *Re/Views in Environmental Science & BioTechnology,* 1**,** 3-7.

HAN, Z., YAN, H., ZHOU, S., ZHAO, H., ZHANG, Y., ZHANG, N., YAO, C., ZHAO, L. & HAN, C. 2013. Precipitation of calcite induced by Synechocystis sp. PCC6803. *World Journal of Microbiology and Biotechnology,* 29**,** 1801-1811.

HARKES, M. P., VAN PAASSEN, L. A., BOOSTER, J. L., WHIFFIN, V. S. & VAN LOOSDRECHT, M. C. M. 2010. Fixation and distribution of bacterial activity in sand to induce carbonate precipitation for ground reinforcement. *Ecological Engineering,* 36**,** 112-117.

HELMI, F. M., ELMITWALLI, H. R., ELNAGDY, S. M. & EL-HAGRASSY, A. F. 2016. Calcium carbonate precipitation induced by ureolytic bacteria *Bacillus licheniformis*. *Ecological Engineering,* 90**,** 367-371.

HOMMEL, J., LAUCHNOR, E., PHILLIPS, A., GERLACH, R., CUNNINGHAM, A. B., HELMIG, R., EBIGBO, A. & CLASS, H. 2015. A revised model formicrobially induced calcite precipitation: Improvements and new insights based on recent experiments. *Water Resources Research,* 51**,** 1-21.

HUANG, K., GUO, J., LIN, K. F., ZHOU, X. Y., WANG, J. X., ZHOU, P., XU, F. & ZHANG, M. L. 2013. Distribution and temporal trend of polybrominated diphenyl ethers in one Shanghai municipal landfill, China. *Environmental Science Pollution Research International,* 20**,** 5299-5308.

HUANG, L.-N., CHEN, Y.-Q., ZHOU, H., LUO, S., LAN, C.-Y. & QU, L.-H. 2003. Characterization of methanogenic Archaea in the leachate of a closed municipal solid waste landfill. *FEMS Microbiology Ecology,* 46**,** 171-177.

IVANOV, V. & CHU, J. 2008. Applications of microorganisms to geotechnical engineering for bioclogging and biocementation of soil in situ. *Reviews in Environmental Science and Bio/Technology,* 7**,** 139-153.

JACKSON, C. R., RANDOLPH, K. C., OSBORN, S. L. & TYLER, H. L. 2013. Culture dependent and independent analysis of bacterial communities associated with commercial salad leaf vegetables. *BMC Microbiology,* 13**,** 274-300.

JIMENEZ-LOPEZ, C., JROUNDI, F., RODRÍGUEZ-GALLEGO, M., ARIAS, J. M. & GONZALEZ-MUÑOZ, M. T. 2007. Biomineralization induced by Myxobacteria. *Communicating Current Research and Educational Topics and Trends in Applied Microbiology***,** 143-154.

KALCIKOVA, G., BABIC, J., PAVKO, A. & GOTVAJN, A. Z. 2014. Fungal and enzymatic treatment of mature municipal landfill leachate. *Waste Management,* 34**,** 798-803.

KALWASINSKA, A. & BURKOWSKA, A. 2013. Municipal landfill sites as sources of microorganisms potentially pathogenic to humans. *Environmental Science Processes & Impacts,* 15**,** 1078-1086.

KANG, C.-H., HAN, S.-H., SHIN, Y., OH, S. J. & SO, J.-S. 2014a. Bioremediation of Cd by Microbially Induced Calcite Precipitation. *Applied Biochemistry and Biotechnology,* 172**,** 2907-2915.

KANG, C. H., CHOI, J. H., NOH, J., KWAK, D. Y., HAN, S. H. & SO, J. S. 2014b. Microbially induced calcite precipitation-based sequestration of strontium by *Sporosarcina pasteurii* WJ-2. *Applied Biochemistry and Biotechnology,* 174**,** 2482-2491.

KANG, C.-H., OH, S. J., SHIN, Y., HAN, S.-H., NAM, I.-H. & SO, J.-S. 2015. Bioremediation of lead by ureolytic bacteria isolated from soil at abandoned metal mines in South Korea. *Ecological Engineering,* 74**,** 402-407.

KESMEN, Z. & KACMAZ, N. 2011. Determination of lactic microflora of kefir grains and kefir beverage by using culture-dependent and culture-independent methods. *J Food Sci,* 76**,** M276-83.

KIM, J., KIM, S. J., KIM, S. H., KIM, S. I., MOON, Y.-J., PARK, S.-J., KAHNG, H.-Y. & CHUNG, Y.-H. 2014. Draft Genome Sequence of Sphingopyxis sp. Strain MWB1, a Crude-Oil-Degrading Marine Bacterium. *Genome Annoucements,* 2**,** 2.

KÖCHLING, T., SANZ, J. L., GAVAZZA, S. & FLORENCIO, L. 2015. Analysis of microbial community structure and composition in leachates from a young landfill by 454 pyrosequencing. *Applied Microbiology and Biotechnology,* 99**,** 5657-5668.

LI, J., LIU, L., YANG, D., LIU, W. L., SHEN, Z. Q., QU, H. M., QIU, Z. G., HOU, A. M., WANG, D. N., DING, C. S., LI, J. W., GUO, J. H. & JIN, M. 2017. Culture-dependent enumeration methods failed to simultaneously detect disinfectant-injured and genetically modified Escherichia coli in drinking water. *Environ Sci Process Impacts,* 19**,** 720-726.

LI, W., CHEN, W.-S., ZHOU, P.-P., ZHU, S.-L. & YU, L.-J. 2013a. Influence of initial calcium ion concentration on the precipitation and crystal morphology of calcium carbonate induced by bacterial carbonic anhydrase. *Chemical Engineering Journal,* 218**,** 65-72.

LI, W., CHEN, W. S., ZHOU, P. P., CAO, L. & YU, L. J. 2013b. Influence of initial pH on the precipitation and crystal morphology of calcium carbonate induced by microbial carbonic anhydrase. *Colloids and Surfaces B: Biointerfaces,* 102**,** 281-287.

LI, W., LIU, L.-P., ZHOU, P.-P., CAO, L., YU, L.-J. & JIANG, S.-Y. 2011. Calcite precipitation induced by bacteria and bacterially produced carbonic anhydrase. *Current science,* 100**,** 502-508.

LI, W., LIU, L., CHEN, W., YU, L., LI, W. & YU, H. 2010. Calcium carbonate precipitation and crystal morphology induced by microbial carbonic anhydrase and other biological factors. *Process Biochemistry,* 45**,** 1017-1021.

LI, W., YU, L. J., WU, Y., JIA, L. P. & YUAN, D. X. 2007. Enhancement of Ca2+ release from limestone by microbial extracellular carbonic anhydrase. *Bioresource Technology,* 98**,** 950-953.

LI, Y., LI, J. & DENG, C. 2014. Occurrence, characteristics and leakage of polybrominated diphenyl ethers in leachate from municipal solid waste landfills in China. *Environmental Pollution,* 184**,** 94-100.

LIAN, B., HU, Q., CHEN, J., JI, J. & TENG, H. H. 2006. Carbonate biomineralization induced by soil bacterium *Bacillus megaterium*. *Geochimica et Cosmochimica Acta,* 70**,** 5522-5535.

LONGE, E. O. & ENEKWECHI, L. O. 2007. Investigation on potential groundwater impacts and influence of local hydrogeology on natural attenuation of leachate at a municipal landfill. *International Journal of Environmental Science and Technology,* 4**,** 133-140.

MA, F. S., YANG, Y. S., YUAN, R. M., YAO, B. K. & GUO, J. 2011. Effect of regional land subsidence on engineering structures: a case study of the 6 km long Su-tong Yantze River Bridge. *Bulletin of Engineering Geology and the Environment,* 70**,** 449-459.

MAHANTY, B., KIM, S. & KIM, C. G. 2013. Assessment of a biostimulated or bioaugmented calcification system with Bacillus pasteurii in a simulated soil environment. *Microbial Ecology,* 65**,** 679-688.

MARTIN, D., DODDS, K., BUTLER, I. B. & NGWENYA, B. T. 2013. Carbonate precipitation under pressure for bioengineering in the anaerobic subsurface via denitrification. *Environmental Science and Technology,* 47**,** 8692-8699.

MARTINEZ, B. C., DEJONG, J. T., GINN, T. R., MONTOYA, B. M., BARKOUKI, T. H., HUNT, C., TANYU, B. & MAJOR, D. 2013. Experimental Optimization of Microbial-Induced Carbonate Precipitation for Soil Improvement. *Journal of Geotechnical and Geoenvironmental Engineering,* 139**,** 587-598.

MAULOOD, P. M., ESMAIL, A. O., DOHUKI, M. S. S. & DARWESH, D. A. 2012. Comparison between Calcimetric and Titrimetric Methods for Calcium Carbonate Determination. *Open Journal of Soil Science,* 2**,** 263-268.

MECKENSTOCK, R. U., ELSNER, M., GRIEBLER, C., LUEDERS, T., STUMPP, C., AAMAND, J., AGATHOS, S. N., ALBRECHTSEN, H.-J., BASTIAENS, L., BJERG,

P. L., BOON, N., DEJONGHE, W., HUANG, W. E., SCHMIDT, S. I., SMOLDERS, E., SØRENSEN, S. R., SPRINGAEL, D. & BREUKELEN, B. M. V. 2015. Biodegradation: Updating the Concepts of Control for Microbial Cleanup in Contaminated Aquifers. *Environmental science and technology,* 49**,** 7073-7081.

MERA, M. U., KEMPER, M., DOYLE, R. & BEVERIDGE, T. J. 1992. The membrane-induced proton motive force influences the metal-binding ability of Bacillus subtilis cell walls. *Applied and Environmental Microbiology,* 58**,** 3837-3844.

MIOT, J., BENZERARA, K., OBST, M., KAPPLER, A., HEGLER, F., SCHADLER, S., BOUCHEZ, C., GUYOT, F. & MORIN, G. 2009. Extracellular iron biomineralization by photoautotrophic iron-oxidizing bacteria. *Applied and Environmental Microbiology,* 75**,** 5586-5591.

MITCHELL, A. C. & FERRIS, F. G. 2006. The Influence of *Bacillus pasteurii* on the Nucleation and Growth of Calcium Carbonate. *Geomicrobiology Journal,* 23**,** 213-226.

MOR, S., RAVINDRA, K., DAHIYA, R. P. & CHANDRA, A. 2006. Leachate characterization and assessment of groundwater pollution near municipal solid waste landfill site. *Environmental Monitoring and Assessment,* 118**,** 435-456.

MULLER, A. K., WESTERGAARD, K., CHRISTENSEN, S. & SORENSON, S. J. 2001. The effect of long-term mercury pollution on the soil microbial community. *FEMS Microbiology Ecology,* 36**,** 11-19.

MUYNCK, W. D., COX, K., BELIE, N. D. & VERSTRAETE, W. 2008. Bacterial carbonate precipitation as an alternative surface treatment for concrete. *Construction and Building Materials,* 22**,** 875-885.

MUYNCK, W. D., BELIE, N. D. & VERSTRAETE, W. 2010a. Microbial carbonate precipitation in construction materials: A review. *Ecological Engineering,* 36**,** 118-136.

MUYNCK, W. D., VERBEKEN, K., BELIE, N. D. & VERSTRAETE, W. 2010b. Influence of urea and calcium dosage on the effectiveness of bacterially induced carbonate precipitation on limestone. *Ecological Engineering,* 36**,** 99-111.

MUYZER, G., WAAL, E. C. D. & UITIERLINDEN, A. G. 1993. Profiling of Complex Microbial Populations by Denaturing Gradient Gel Electrophoresis Analysis of Polymerase Chain Reaction-Amplified Genes Coding for 16S rRNA. *Applied and Environmental Microbiology,* 59**,** 695-700.

NICHOLSON, P. 2014. *Soil Improvement and Ground Modification Method*, United States of America, Butterworth-Heinemann

OKWADHA, G. D. & LI, J. 2010. Optimum conditions for microbial carbonate precipitation. *Chemosphere,* 81**,** 1143-1148.

OKYAY, T. O. & RODRIGUES, D. F. 2015. Biotic and abiotic effects on CO2 sequestration during microbially-induced calcium carbonate precipitation. *FEMS Microbiology Ecology,* 91**,** 1-13.

PEREZ-LEBLIC, M. I., TURMERO, A., HERNANDEZ, M., HERNANDEZ, A. J., PASTOR, J., BALL, A. S., RODRIGUEZ, J. & ARIAS, M. E. 2012. Influence of xenobiotic contaminants on landfill soil microbial activity and diversity. *Journal of Environmental Management,* 95**,** 285-290.

PRUESSE, E., QUAST, C., KNITTEL, K., FUCHS, B. M., LUDWIG, W., PEPLIES, J. & GLOCKNER, F. O. 2007. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Research,* 35**,** 7188-7196.

RAJASEKAR, A., MOY, C. K. S. & WILKINSON, S. 2017. MICP and Advances towards Eco-Friendly and Economical Applications. *8th International Conference on Environmental Science and Technology (ICEST 2017).* Spain: IOP Conference Series: Earth and Environmental Science.

RAJASEKAR, A., SEKAR, R., MEDINA-ROLDAN, E., BRIDGE, J., K.S.MOY, C. & WILKINSON, S. 2018. Next-generation sequencing showing potential leachate influence on bacterial communities around a landfill in China. *Canadian Journal of Microbiology,* 64**,** 537-549.

RAMOS, C. L., DE ALMEIDA, E. G., PEREIRA, G. V., CARDOSO, P. G., DIAS, E. S. & SCHWAN, R. F. 2010. Determination of dynamic characteristics of microbiota in a fermented beverage produced by Brazilian Amerindians using culture-dependent and culture-independent methods. *Int J Food Microbiol,* 140**,** 225-31.

REEBURGH, W. S. 2007. Oceanic Methane Biogeochemistry. *Chemical reviews,* 107**,** 486-513.

RIVADENEYRA, M. A., DELGADO, G., SORIANO, M., RAMOS-CORMENZANA, A. & DELGADO, R. 2000. Precipitation of carbonates by Nesterenkonia halobia in liquid media. *Chemosphere,* 41**,** 617-624.

RIVADENEYRA, M. A., MARTÍN-ALGARRA, A., SÁNCHEZ-NAVAS, A. & MARTÍN-RAMOS, D. 2006. Carbonate and Phosphate Precipitation by Chromohalobacter marismortui. *Geomicrobiology Journal,* 23**,** 1-13.

RIVADENEYRA, M. A., PARRAGA, J., DELGADO, R., RAMOS-CORMENZANA, A. & DELGADO, G. 2004. Biomineralization of carbonates by Halobacillus trueperi in solid and liquid media with different salinities. *FEMS Microbiology Ecology,* 48**,** 39-46.

RODRIGUES, V. D., TORRES, T. T. & OTTOBONI, L. M. 2014. Bacterial diversity assessment in soil of an active Brazilian copper mine using high-throughput sequencing of 16S rDNA amplicons. *Antonie Van Leeuwenhoek,* 106**,** 879-90.

RODRIGUEZ-NAVARRO, C., JROUNDI, F., SCHIRO, M., RUIZ-AGUDO, E. & GONZÁLEZ-MUÑOZ, M. T. 2012. Influence of Substrate Mineralogy on Bacterial Mineralization of Calcium Carbonate: Implications for Stone Conservation. *Applied and Environmental Microbiology,* 78**,** 4017-4029.

ROLING, W. F. M., VAN BREUKELEN, B. M., BRASTER, M., LIN, B. & VAN VERSEVELD, H. W. 2001. Relationships between Microbial Community Structure and Hydrochemistry in a Landfill Leachate-Polluted Aquifer. *Applied and Environmental Microbiology,* 67**,** 4619-4629.

RONG, F., ZHAOGUI, G. & TUGEN, F. 2011. Analysis of Stability and Control in Landfill Sites Expansion. *Procedia Engineering,* 24**,** 667-671.

ROWE, R. K. & RIMAL, S. 2008. Aging of HDPE Geomembrane in Three Composite Landfill Liner Configurations. *Journal of Geotechnical and Geoenvironmental Engineering,* 134**,** 906-916.

RUSZNYAK, A., AKOB, D. M., NIETZSCHE, S., EUSTERHUES, K., TOTSCHE, K. U., NEU, T. R., FROSCH, T., POPP, J., KEINER, R., GELETNEKY, J., KATZSCHMANN, L., SCHULZE, E. D. & KUSEL, K. 2012. Calcite biomineralization by bacterial isolates from the recently discovered pristine karstic herrenberg cave. *Applied and Environmental Microbiology,* 78**,** 1157-1167.

S.K., GUNATILAKE. 2015. Methods of Removing Heavy Metals from Industrial Wastewater. *Journal of Multidisciplinary Engineering Science Studies,* 1**,** 12-18.

SANCHEZ-ROMAN, M., RIVADENEYRA, M. A., VASCONCELOS, C. & MCKENZIE, J. A. 2007. Biomineralization of carbonate and phosphate by moderately halophilic bacteria. *FEMS Microbiology Ecology,* 61**,** 273-284.

SÁNCHEZ-ROMÁN, M., ROMANEK, C. S., FERNÁNDEZ-REMOLAR, D. C., SÁNCHEZ-NAVAS, A., MCKENZIE, J. A., PIBERNAT, R. A. & VASCONCELOS, C. 2011. Aerobic biomineralization of Mg-rich carbonates: Implications for natural environments. *Chemical Geology,* 281**,** 143-150.

SANDAA, R.-A., TORSVIK, V., ENGER, O., DAAE, F. L., CASTBERG, T. & HAHN, D. 1999. Analysis of bacterial communities in heavy metal contaminated soils at diferrent levels of resolution. *FEMS Microbiology Ecology,* 30**,** 237-251.

SCHMIDTOVA, J. & BALDWIN, S. A. 2011. Correlation of bacterial communities supported by different organic materials with sulfate reduction in metal-rich landfill leachate. *Water Research,* 45**,** 1115-1128.

SHI, X., FANG, R., WU, J., XU, H., SUN, Y. & YU, J. 2012. Sustainable development and utilization of groundwater resources considering land subsidence in Suzhou, China. *Engineering Geology,* 124**,** 77-89.

SHIRAISHI, F. 2012. Chemical conditions favoring photosynthesis-induced CaCO3 precipitation and implications for microbial carbonate formation in the ancient ocean. *Geochimica et Cosmochimica Acta,* 77**,** 157-174.

SILVA-CASTRO, G. A., UAD, I., RIVADENEYRA, A., VILCHEZ, J. I., MARTIN-RAMOS, D., GONZÁLEZ-LÓPEZ, J. & RIVADENEYRA, M. A. 2013. Carbonate Precipitation of Bacterial Strains Isolated from Sediments and Seawater: Formation Mechanisms. *Geomicrobiology Journal,* 30**,** 840-850.

SONG, L., WANG, Y., TANG, W. & LE, Y. 2015a. Bacterial community diversity in municipal waste landfill sites. *Applied Microbiology and Biotechnology,* 99**,** 7745-7756.

SONG, L., WANG, Y., ZHAO, H. & LONG, D. T. 2015b. Composition of bacterial and archaeal communities during landfill refuse decomposition processes. *Microbiological research,* 181**,** 105-111.

SOON, N. W., LEE, L. M., KHUN, T. C. & LING, H. S. 2013. Improvements in engineering properties of soils through microbial-induced calcite precipitation. *KSCE Journal of Civil Engineering,* 17**,** 718-728.

STABNIKOV, V., JIAN, C., IVANOV, V. & LI, Y. 2013. Halotolerant, alkaliphilic urease-producing bacteria from different climate zones and their application for biocementation of sand. *World Journal of Microbiology and Biotechnology,* 29**,** 1453-1460.

STADNITSKAIA, A., NADEZHKIN, D., ABBAS, B., BLINOVA, V., IVANOV, M. K. & DAMSTE, J. S. S. 2008. Carbonate formation by anaerobic oxidation of methane: Evidence from lipid biomarker and fossil 16S rDNA. *Geochimica et Cosmochimica Acta,* 72**,** 1824-1836.

STALEY, B. F., SAIKALY, P. E., DE LOS REYES, F. L., 3RD & BARLAZ, M. A. 2011. Critical evaluation of solid waste sample processing for DNA-based microbial community analysis. *Biodegradation,* 22**,** 189-204.

STEFFAN, R. J., GOKSOYR, J., BEJ, A. K. & ATLAS, R. M. 1988. Recovery of DNA from Soils and Sediments. *Applied and Environmental Microbiology,* 54**,** 2908-2915.

STOCKS-FISCHER, S., GALINAT, J. K. & BANG, S. S. 1999. Microbiological precipitation of CaCO3. *Soil Biology and Biochemistry,* 31**,** 1563-1571.

SU, C., LEI, L., DUAN, Y., ZHANG, K.-Q. & YANG, J. 2012. Culture-independent methods for studying environmental microorganisms: methods, application, and perspective. *Applied Microbiology and Biotechnology,* 93**,** 993-1003.

SULTANA, M., MOU, T. J., SANYAL, S. K., DIBA, F., MAHMUD, Z. H., PARVEZ, A. K. & HOSSAIN, M. A. 2017. Investigation of Arsenotrophic Microbiome in Arsenic-Affected Bangladesh Groundwater. *Ground Water,* 55**,** 736-746.

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**,** 3290-3301.

VAN PAASSEN, L. A., DAZA, C. M., STAAL, M., SOROKIN, D. Y., VAN DER ZON, W. & VAN LOOSDRECHT, M. C. M. 2010. Potential soil reinforcement by biological denitrification. *Ecological Engineering,* 36**,** 168-175.

VISHNIVETSKAYA, T. A., MOSHER, J. J., PALUMBO, A. V., YANG, Z. K., PODAR, M., BROWN, S. D., BROOKS, S. C., GU, B., SOUTHWORTH, G. R., DRAKE, M. M., BRANDT, C. C. & ELIAS, D. A. 2011. Mercury and Other Heavy Metals Influence Bacterial Community Structure in Contaminated Tennessee Streams. *Applied and Environmental Microbiology,* 77**,** 302-311.

VUKANTI, R., CRISSMAN, M., LEFF, L. G. & LEFF, A. A. 2009. Bacterial communities of tyre monofill sites: growth on tyre shreds and leachate. *Journal of Applied Microbiology,* 106**,** 1957-1966.

WAN, R., ZHANG, S. & XIE, S. 2012. Microbial community changes in aquifer sediment microcosm for anaerobic anthracene biodegradation under methanogenic condition. *Journal of Environmental sciences,* 24**,** 1498-1503.

WANG, J., LIN, Z., LIN, K., WANG, C., ZHANG, W., CUI, C., LIN, J., DONG, Q. & HUANG, C. 2011. Polybrominated diphenyl ethers in water, sediment, soil, and biological samples from different industrial areas in Zhejiang, China. *Journal of Hazardous Materials,* 197**,** 211-219.

WANG, X., CAO, A., ZHAO, G., ZHOU, C. & XU, R. 2017. Microbial community structure and diversity in a municipal solid waste landfil. *Waste Management,* 66**,** 79-87.

WANG, Y.-Y., YAO, Q.-Z., ZHOU, G.-T. & FU, S.-Q. 2013. Formation of elongated calcite mesocrystals and implication for biomineralization. *Chemical Geology,* 360-361**,** 126-133.

WANG, Y., CHEN, Y., ZHOU, Q., HUANG, S., NING, K., XU, J., KALIN, R. M., ROLFE, S. & HUANG, W. E. 2012. A culture-independent approach to unravel uncultured bacteria and functional genes in a complex microbial community. *PLoS One,* 7**,** e47530.

WEIDNER, S., ARNOLD, W. & PUHLER, A. 1996. Diversity of Uncultured Microorganisms Associated with the Seagrass Halophila stipulacea Estimated by Restriction Fragment Length Polymorphism Analysis of PCR-Amplified 16S rRNA Genes. *Applied and Environmental Microbiology,* 62**,** 776-771.

WHIFFIN, V. S., VAN PAASSEN, L. A. & HARKES, M. P. 2007. Microbial Carbonate Precipitation as a Soil Improvement Technique. *Geomicrobiology Journal,* 24**,** 417-423.

WIJESEKARA, S. S. R. M. D. H. R., MAYAKADUWA, S. S., SIRIWARDANA, A. R., SILVA, N. D., BASNAYAKE, B. F. A., KAWAMOTO, K. & VITHANAGE, M. 2014. Fate and transport of pollutants through a municipal solid waste landfill leachate in Sri Lanka. *Environmental Earth Sciences,* 72**,** 1707-1719.

WU, K., SHI, H. & GUO, X. 2011. Utilization of municipal solid waste incineration fly ash for sulfoaluminate cement clinker production. *Waste Management,* 31**,** 2001-2008.

XIE, B., XIONG, S., LIANG, S., HU, C., ZHANG, X. & LU, J. 2012. Performance and bacterial compositions of aged refuse reactors treating mature landfill leachate. *Bioresource Technology,* 103**,** 71-77.

XU, J., DU, Y., JIANG, Z. & SHE, A. 2015. Effects of Calcium Source on Biochemical Properties of Microbial $CaCO_3$ Precipitation. *Frontiers in Microbiology,* 6**,** 1-7.

YANG, K., ZHOU, X.-N., YAN, W.-A., HANG, D.-R. & STEINMANN, P. 2008. Landfills in Jiangsu province, China, and potential threats for public health: Leachate appraisal and spatial analysis using geographic information system and remote sensing. *Waste Management,* 28**,** 2750-2757.

YAO, X.-F., ZHANG, J.-M., TIAN, L. & GUO, J.-H. 2017. The effect of heavy metal contamination on the bacterial community structure at Jiaozhou Bay, China. *Brazilian Journal of Microbiology,* 48**,** 71-78.

ZAMARREŇO, D. V., INKPEN, R. & MAY, E. 2009a. Carbonate crystals precipitated by freshwater bacteria and their use as a limestone consolidant. *Applied and Environmental Microbiology,* 75**,** 5981-5990.

ZAMARREŇO, D. V., MAY, E. & INKPEN, R. 2009b. Influence of Environmental Temperature on Biocalcification by Non-sporing Freshwater Bacteria. *Geomicrobiology Journal,* 26**,** 298-309.

ZHANG, D. Q., TAN, S. K. & GERSBERG, R. M. 2010. Municipal solid waste management in China: status, problems and challenges. *Journal of Environmental Management,* 91**,** 1623-1633.

ZHANG, H.-B., SHI, W., YANG, M.-X., SHA, T. & ZHAO, Z.-W. 2008. Bacterial Diversity at Different Depths in Lead-Zinc Mine Tailings as Revealed by 16S rRNA Gene Libraries. *Journal of Microbiology,* 45**,** 479-484.

ZHANG, Q. Q., TIAN, B. H., ZHANG, X., GHULAM, A., FANG, C. R. & HE, R. 2013a. Investigation on characteristics of leachate and concentrated leachate in three landfill leachate treatment plants. *Waste Management,* 33**,** 2277-2286.

ZHANG, W.-J., ZHANG, G.-G. & CHEN, Y.-M. 2013b. Analyses on a high leachate mound in a landfill of municipal solid waste in China. *Environmental Earth Sciences,* 70**,** 1747-1752.

ZHENG, X. W., YAN, Z., HAN, B. Z., ZWIETERING, M. H., SAMSON, R. A., BOEKHOUT, T. & ROBERT NOUT, M. J. 2012. Complex microbiota of a Chinese "Fen" liquor fermentation starter (Fen-Daqu), revealed by culture-dependent and culture-independent methods. *Food Microbiol,* 31**,** 293-300.

ZHU, T. & DITTRICH, M. 2016. Carbonate Precipitation through Microbial Activities in Natural Environment, and Their Potential in Biotechnology: A Review. *Frontiers in Microbiology,* 4**,** 1-21.

ZOU, J., DAI, W., GONG, S. & MA, Z. 2015. Analysis of spatial variations and sources of heavy metals in farmland soils of Beijing suburbs. *PLoS One,* 10**,** 1-13.

Appendix A: Optimisation of parameters for conical flask and engineering experiments.

The aim of this appendix is to show the trial experiment that was conducted to successfully optimise the experimental parameters for conducting the conical flask and engineering experiment shown in Chapter 4 and 5.

Optimisation of parameters for conical flask experiment

The experiment presented below was performed for the optimizing of calcite precipitation for Chapter 4. The experiments were performed under sterile conditions. The calcium chloride and urea solution were filter sterilized before the start of the experiment to avoid any contamination. The experiments were conducted in a Grant-bio environmental shaker incubator.

Figure A-1 Filter paper containing the filtrate stored inside a petri dish.

The pH was measured in a laminar hood and the probe was cleaned with 70% ethanol and Nanopure water before measuring the pH. The abiotic solution was studied separately to avoid any possible interaction with the bacterial solutions. Upon the completion of experiment, the abiotic and individual bacterial solutions were vacuum filtered using 0.6µm Whatman® filter paper. Later, the filter paper which contains the filtrate was stored in a petri dish for SEM and XRD experiments (Figure A-1). All of the experiments were done in triplicate. The experimentation technique followed for SEM and XRD are discussed in Chapter 2 and 3. The bacterium in Table 6-1 were isolated from a landfill environment.

Experiment 1

Table A-1 shows the optimisation of chemical concentration, initial pH and pH measurement that led to the data found in Chapter 4.

Table A-1 Successful biomineralisation experiment conducted using bacteria isolated from landfill leachate.

$CaCl_2$ / Urea	**Starting pH**	**Type of bacteria studied**	**Duration**	**pH measurement**
25 mM/333 mM	7.5	Abiotic *Bacillus sp.* xjlu_herc15	1 week	Every 24 hours

This experiment was conducted to investigate the precipitation ability of the bacteria isolated from landfill groundwater and leachate. In this experiment the pH was measured every 24 hours. To ensure that the bacteria do precipitate calcite, an abiotic solution was studied in parallel to identify any subtle changes in precipitation.

The calcium chloride and urea solutions were autoclaved separately to avoid premature precipitation. The pH was recorded every 24 hours to track any changes that happen due to precipitation. All the experiments were performed in triplicate. The experiment was conducted with the environmental shaker incubator set at 30°C and 130 rpm.

Outcome:

This experimental outcome was successful as the ability of the bacterium to precipitate calcite was obtained in a convincing manner. Major changes in pH were observed during this study for the bacterial solutions. The changes include variations in pH from 0-48 hours; with pH increasing from 7.5 to 8.7. Differences in crystal morphology were observed between the bacterial and abiotic solutions. With bacterial solutions precipitating spherical calcite and abiotic solutions precipitating trigonal shaped crystals.

Summary:

An experiment dedicated only to abiotic solution (not shown here) was performed after those for Chapter 4 to verify whether the abiotic solution does precipitate calcite to avoid any contamination/ experimental errors. The abiotic solution was still observed to precipitated calcite. The experiments parameters from this experiment were applied for the remaining bacteria isolated from landfill leachate and groundwater to test for calcite precipitation.

Optimisation of parameters for engineering experiment

Experiment 1

The experiment presented below was performed for optimizing the parameters for calcite precipitation shown in Chapter 5.

Table A-2 shows the successful experiment that led to the optimisation of chemical concentration, media replacement and column design for Chapter 4.

$CaCl_2$/Urea	**Type of bacteria studied**	**Media replacement**	**Duration**
50 mM/ 300 mM	*Bacillus licheniformis* SZH2015_A *Bacillus pumilus* szhxjlu2015 *Bacillus sp.* xjlu_herc15 Blank	Every 24 hours	2 weeks

Experimental setup:

Cross sectional diagram of the experimental set up is shown below (Figure A-2). PVC column with a tube attached to the end of the column which goes parallel to the sand column. The tube is placed in such a way that the liquid level is constantly maintained in the column. The tube is supposed to serve as a reservoir for calcium, urea and broth. The bacteria and broth were added every time during the liquid exchange.

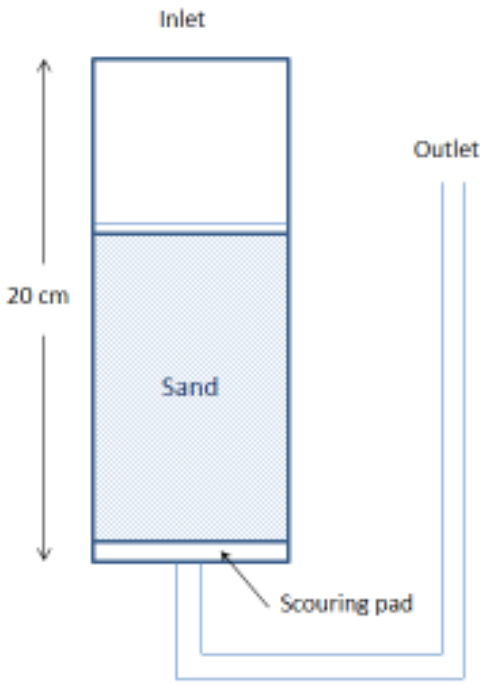

Figure A-2 Cross section of the experimental set up.

Outcome:

This experiment turned out to be a success with improvement in permeability for the bacterial samples. The blank didn't show any major changes in permeability. The scanning electron microscope images showed cementation between sand particles (Figure A-3). Sand particles held together when the base of the column was cut off (Figure A-4).

Conclusion:

Since this experiment turned out to be positive one for permeability and strength, the experimental setup was used for the work shown in Chapter 5 with changes to concentration for calcium chloride and urea.

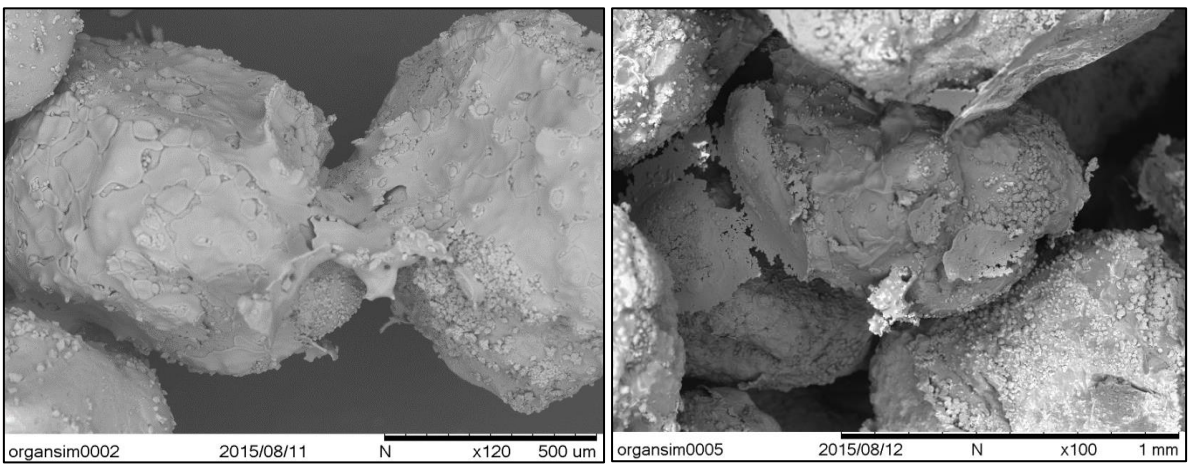

Figure A-3 Cementation bond formed between sand particles in the column that was treated with bacteria.

Figure A-4 Saturated cementation bonds that cover the entire sand particles in the column.

Appendix B: Stimulation of *in situ* MICP in soil through indigenous bacteria.

The aim of this Appendix is to show that indigenous carbonate precipitating bacteria found in soil can be simulated to precipitate calcite for permeability improvement.

Title: Stimulation of indigenous carbonate precipitating bacteria for ground improvement

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Abstract

Calcite minerals are precipitated in soil through biomineralisation which can be either organic or inorganic in nature. Biomineralisation can be employed to improve ground conditions in its natural state. Usually, studies of applied biomineralisation are highly interdisciplinary involving expertise from engineers, chemists and microbiologists. In this paper, we study the potential of biomineralisation from indigenous bacteria present in soil. The soil samples were collected from a high permeable zone and the bacteria that inhabit the soil were stimulated at a temperature of 15°C. A cementation solution consisting of 500mM calcium chloride, urea and nutrient broth at a pH of 7.5 was added to the soil samples. Inorganic precipitation was found to be dominant and was more efficient when compared to organic precipitation. Carbonate precipitation data indicated that inorganic precipitation were 1.37 times better at carbonate formation in comparison to organic precipitation. Scanning Electron Microscopy analysis identified cementation bonds formed between soil particles. It was deducted that organic precipitation is dependent on temperature and may take an extended time at such low temperature. The preliminary data presented in this paper suggests that the implementation of biomineralisation with in-situ microbes is promising but requires further laboratory and field investigation before being considered for engineering application.

Introduction

Bacterial application towards engineering purposes is attracting the attention of microbiologist and geotechnical engineers worldwide. Biomineralisation has recently gained popularity among researchers for its applicability towards ground improvement applications. Microbial activity that alters the chemical environment favoring mineral formation is known as Biomineralisation (Stocks-Fischer et al., 1999, Bazylinski et al., 1995). Microbially induced calcite precipitation (MICP) is a biochemical mechanism which is driven by the microorganism upon interacting with a chemical solution rich in calcium. Research in MICP has shown that microbially released CO_2 interacts with the biomineralisation solution favoring carbonate formation. The carbonate combines with the calcium ion (Ca^{2+}) leading to the precipitation of calcium carbonate.

In the biomineralisation process, bacteria commonly serve as nucleation sites for the precipitation of calcium carbonate. One of the important enzymes that's been shown to mediate high percentage of carbonates is urease. Urease (UA; EC 3.5.1.5) is a nickel-containing metalloenzyme found in a wide range of microorganisms (Dhami et al., 2014). Presence of urease initiates a process commonly known as ureolytic activity which aides in the conversion of urea to ammonia and CO_2 (Equation 1). Ureolytic activity promotes superior precipitation of carbonate (Equation 2 and 3). Ca^{2+} ions bind to the negatively charged bacteria surfaces, thus creating a neutral environment for Ca^{2+} adsorption (Equation 4). Bacterial cells are very important for the precipitation of CaCO3, because the bacteria provide nucleation sites (heterogeneous nucleation) and affect the specific types of minerals formed (Figure B-2). Okwadha and Li (2010) found that high concentration of bacterial cells increases the amount of calcite precipitation by MICP, which happens because of the increase in the urease concentration for urea hydrolysis.

$$CO(NH_2)_2 + 3H_2O \rightarrow 2NH_4^+ + 2OH^- + CO_2 \uparrow \quad (1)$$

$$CO_2 + H_2O \leftrightarrow HCO_3^- + H^+ \quad (2)$$

$$HCO_3^- + H_2O \leftrightarrow H^+ + CO_3^{2-} \quad (3)$$

$$Cell - Ca^{2+} + CO_3^{2-} \rightarrow Cell - CaCO_3 \downarrow \quad (4)$$

Inorganic precipitation involves the precipitation of minerals in the absence of bacteria and relies heavily on the available atmospheric CO_2 (Equation 2). CO_2 helps in helps in the formation of bicarbonate, which later becomes carbonate (Equation 3), which later forms calcium carbonate.

Studies on abiotic systems identified calcite precipitation to be less effective in comparison to bacterial system (Stocks-Fischer et al., 1999). The precipitation rate is especially high during the early stages of the precipitation process. Since bacteria take time to adjust to the environmental conditions, an abiotic approach could possibly be superior where pre-treatment has not taken place.

This paper presents a study that investigates the carbonate precipitating bacterial stimulation from a soil sampled from Formby, UK and the main objective is to provide a preliminary analysis on the possibility of *insitu* application of biomineralisation towards ground improvement.

Materials and Methods

Sample collection

The soil sample was collected from Formby national park, United Kingdom. The soil was collected at 30cm depth using an auger and transported to the lab inside zip lock bags. The soil was stored at 4°C prior to analysis.

Experimental system

Soil was added to a 50ml syringe column and liquid exchange was performed using a peristaltic pump with a 10 mm ID tube at 5 rpm (Figure B-1). Cementation liquid consists of 500mM of Calcium chloride and Urea. Nutrient broth was added along with the calcium chloride and urea. The liquid exchange was performed every 2 days. The experiment was conducted at 15°C. The experiment was terminated when no liquid was collected during the liquid exchange. The experiments were performed in triplicates to study organic and inorganic precipitation. For inorganic precipitation, the soil was autoclaved to remove biological matter.

Carbonate titration and SEM imaging

Carbonate titration was performed using the protocol followed by (Maulood et al., 2012) and SEM imaging was performed using the Zeiss EVO50 scanning electron microscope at the University of Wolverhampton.

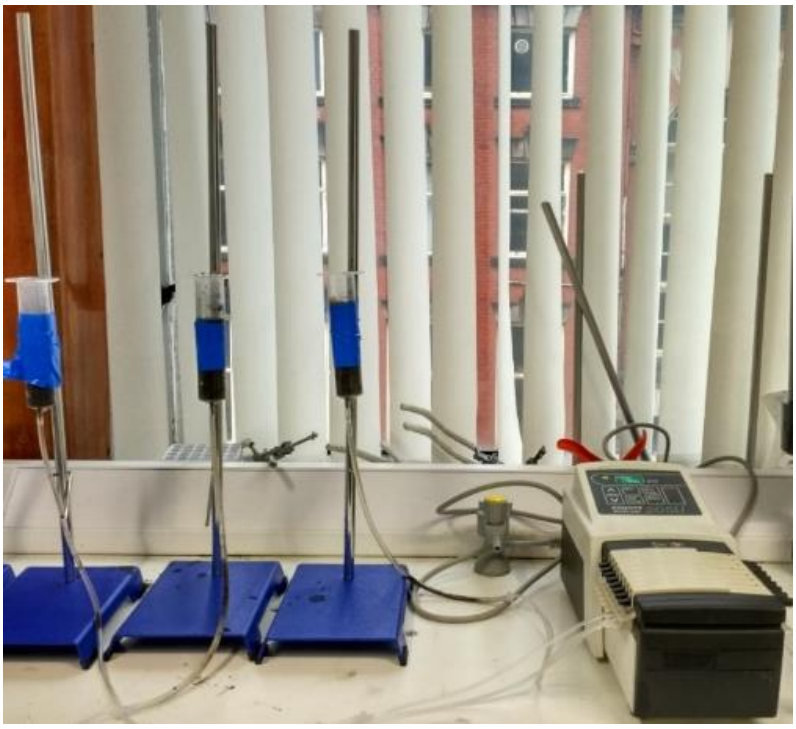

Figure B-1. Experimental setup for the precipitation process.

Results and Discussion

The experiments for organic and inorganic precipitation was conducted at 15°C. A temperature of 20°C is normally required to obtain an efficient calcite precipitation (Zamarreňo et al., 2009b, Helmi et al., 2016). However, the experiments conducted for this study were designed to mimic real *in situ* conditions, thus the lower temperature of 15°C was maintained.

SEM images of particles after mineralization were used to identify the differing precipitation patters between the organic and inorganic samples (Figure B-2 and B-3). The organic precipitation produced snowflake like crystal formations on the top of the soil particles, such patterns being absent in the inorganic samples. The cementation bonds observed between soil particles due to organic precipitation are denser in comparison to the inorganic precipitation.

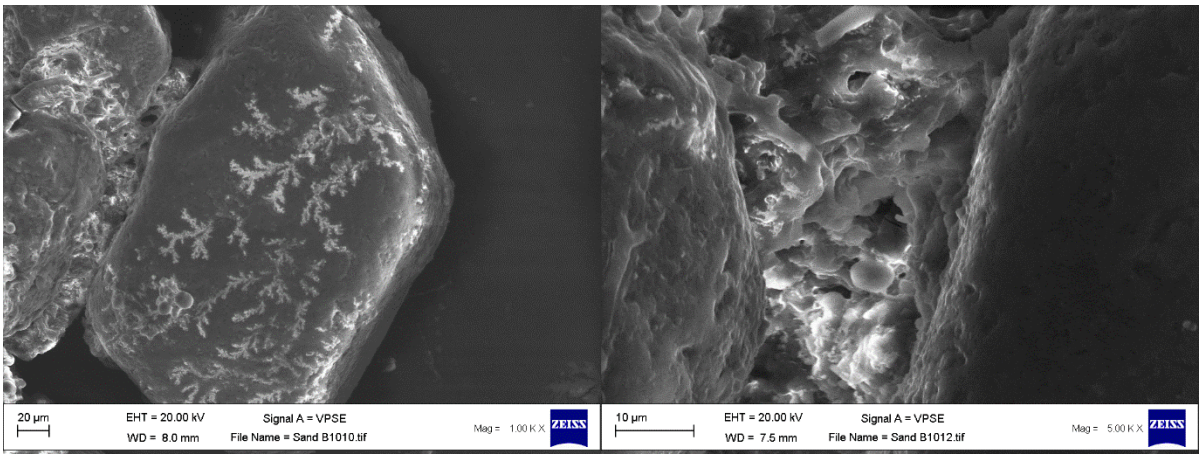

Figure B-2. SEM images showing the cementation bonds through organic precipitation process.

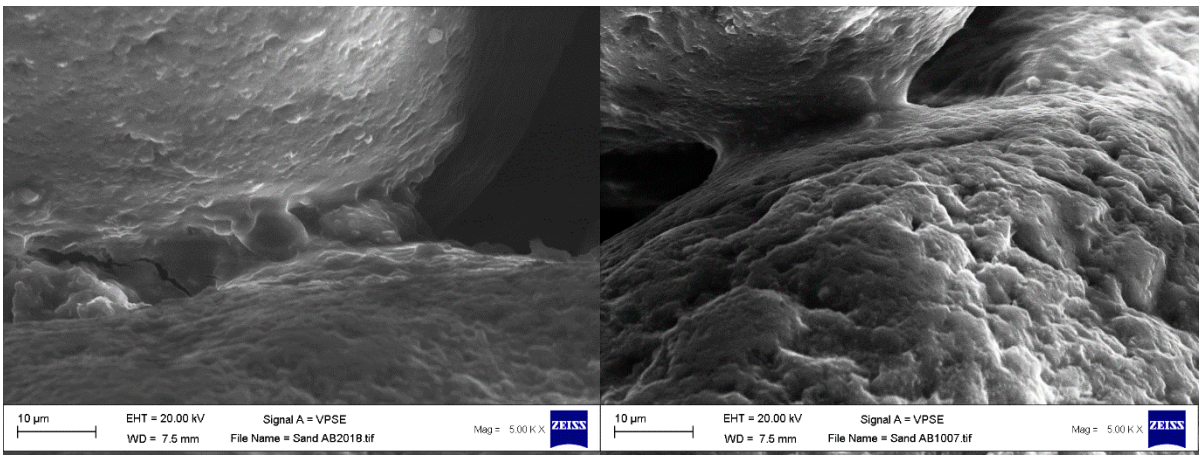

Figure B-3. SEM images showing the cementation bonds through inorganic precipitation process.

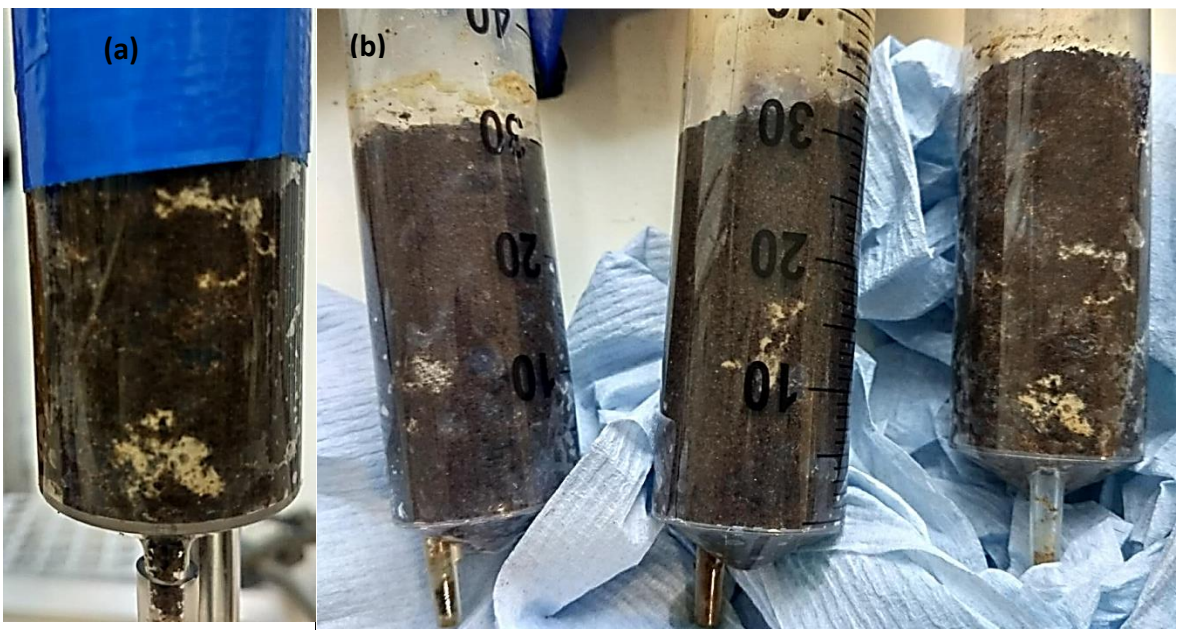

Figure B-4. Calcium carbonate formations observed between soil particles during organic precipitation experiment (a) and observed in all three columns after the termination of experiments (b).

Before the drying of soil samples for carbonate titration, visible calcium carbonate formations were visible in the organic precipitation experiments. This was not observed for the inorganic precipitation experiments.

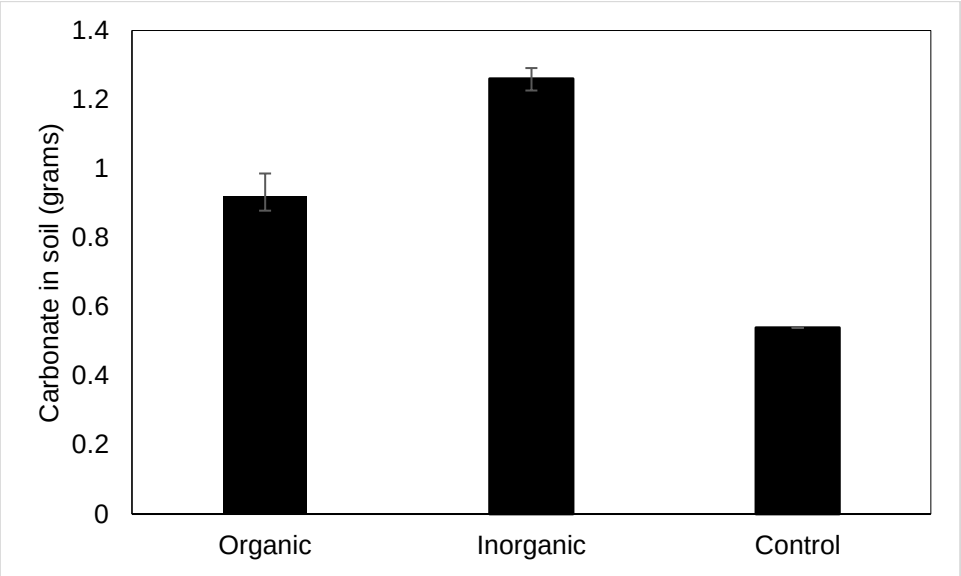

Figure B-5. Carbonate precipitation obtained from organic, inorganic and control soil experiments. All experiments were performed in triplicates.

Carbonate titration identified that inorganic precipitation process precipitated 1.37 times more carbonate than the organic precipitation (Figure B-5). The data comparison between the organic precipitation and control sample reveals that carbonate precipitating bacteria exist in the soil. The superior precipitation of carbonate through inorganic process is probably due to the additional time required for the organic process, since the bacteria take time to adjust to the environment.

Improvements that need to be made for future soil experiments

The preliminary data presented in this paper suggests that carbonate precipitating bacteria exists in soils and they could be stimulated for calcite precipitation. However, in this case, the organic precipitation was inferior in comparison to the inorganic precipitation, although both were superior to the control. Applied indigenous biomineralisation is still a new technique, a few limitations need to be addressed prior to implementation in the field;

MICP is a microbial processes which highly depends on temperature, pH, calcium concentration, DIC and the presence of nucleation sites (Ivanov and Chu, 2008). This makes it a complex and time-consuming process compared to the chemical process. Bacteria that's present in the soil can produce organic acids to counterbalance the pH increase associated with ammonia diffusion which results in the delay of crystallization. MICP has to be optimized for time effectiveness before it's used for *insitu* applications. Since the data shown in this study indicates that temperatures as low as 15°C could make organic precipitation less efficient in comparison to organic precipitation. Multiple parameter studies need to be conducted to optimize the efficiency and crystal productivity for organic precipitation.