



SELF-HEALING GEOTECHNICAL STRUCTURES VIA
MICROBIAL ACTION

A thesis submitted for the degree of Doctor of Philosophy

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September 2017

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Acknowledgments

I would like to give many thanks to my supervisors Dr Michael Harbottle and Dr Diane Gardner for giving me the opportunity to work on such an exciting project and for providing me with assistance throughout the experimental work and writing of the thesis.

I would like to thank all the technical staff from the laboratories of the Cardiff School of Engineering for being always so helpful and patient with me. Many thanks to Steffan Jones and Marco Santonastaso for providing general assistance with machines, and for constructing experimental apparatus.

I would also like to thank my colleagues – Rob, Martins, and Ollie for our discussions during lunchtime that helped me greatly during the hard times in the lab.

And finally, I would like to express my deepest gratitude and appreciation to Neli, Peshoti, Savina, Ben, Maria, and Sava and thank them for their support. I would not have made it without you.

Abstract

Recent years have seen the growing interest in the novel field of biogeotechnologies. Of particular interest to researchers has been the microbially induced calcium carbonate precipitation (MICP) for geotechnical (stabilisation of soils) and geoenvironmental (immobilisation of contaminants) applications. The MICP process results in the formation of a brittle monolith from sand cemented by the microbial calcium carbonate which will, however, be subjected to chemical and physical deterioration over time. This will bring the need for repair of the earth structure which can be highly difficult and costly. This study investigates the possibility of incorporating a self-healing mechanism in the MICP that will allow the initially formed monolith to be healed after damage. MICP via a spore-forming, ureolytic organism, *Sporosarcina ureae*, was evaluated for its self-healing potential. The most commonly used organism in biocementation, namely *Sporosarcina pasteurii*, was found to not form spores. Experiments in aqueous solution showed that encapsulated (in calcium carbonate) sporulated bacteria can survive harsh conditions, starvation for periods of 6 months (while encapsulated within the mineral) and be able to germinate once exposed from the mineral matrix with availability of nutrients. The revived bacteria were then able to form the precipitation of further calcium carbonate. Additionally, the bacterium was able to go through cycles of self-healing multiple times without its activity being significantly affected. Also presented are experiments in particulate media which showed the ability of the organism to cause the cementation of sand columns. *S. ureae* was able to respond when a physical or chemical damage occurred to the monolith by germinating and producing again calcium carbonate which restored the functionality of the bio-cemented sand columns.

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1 Introduction

Growing population and urbanisation together with technological advances in civil engineering in recent years have driven the building of ever more complex and large infrastructure projects. Geological materials and soils in particular form a significant portion of construction materials and are also very often a substantial component of civil engineering infrastructure. As part of this infrastructure, geotechnical structures, though not visible, are usually far more extensive than buildings. Geotechnical structures are part of the critical infrastructure, (transportation networks, power and water supply) and examples of such include motorway cuttings and embankments, tunnelling, dams, levees, and waste repositories. In order to meet design requirements, today's structures are designed according to the "damage prevention" paradigm, as outlined by Van der Zwaag (2007). This type of approach requires initial overdesign and the execution of regular maintenance. To enhance their performance, geotechnical structures are subjected to ground improvement techniques. As an example, improvement in terms of hydraulic conductivity reduction, stabilisation, or strengthening can be achieved through a variety of artificial cementation methods available in the current geotechnical practice, also known as chemical grouts (Karol 2003). Chemical grouting is widely used both as a preventative and remedial technique in ground improvement. Such grouts involve the injection of chemicals, such as silicates which harden and form a cementing/bonding or a plugging agent in the soil. The resulting stabilised material however is prone to deformation under load or erosion processes in the subsurface (e.g. cracking, piping, formation of slip surfaces) which can lead to the overall structures` performance being impaired.

Problems with the poor state of infrastructure are faced worldwide - it has been estimated that to bring US strategic infrastructure back to an adequate standard, an

investment of \$3.9 trillion will be needed (American Society of Civil Engineers 2017). Similarly in the UK, the cost of maintaining, renovating, and growing transport infrastructure alone has been estimated to be £17.5 billion per year for the next two decades (Mills et al. 2011). In the transport sector alone, repairing slope instabilities in geotechnical structures (e.g. embankments and cuttings) costs the UK economy a significant amount - £20 million per year- according to a report by ARUP (2010). In addition to the high costs associated with the maintenance of earth structures, repair works can sometimes be highly difficult to perform, they can also cause disruption to users and the local communities. The repair of a slope of a road embankment or a cutting, for example, may include excavation (if failure is deeper than just erosion on the surface), removal, transportation to a landfill and replacement of the failed fill material. The repair works would also require lane closure and would incur traffic disruption. Therefore, new ways to prolong the service life of geotechnical structures and reduce the need for maintenance works are desirable.

In recent years, a new field of biogeotechnologies has emerged which has shown great potential for geoenvironmental and geotechnical applications. In particular, a process called microbially induced calcium carbonate precipitation (MICP) which harnesses microorganisms (native to soils) to produce, through their metabolism, a mineral phase (calcium carbonate) within the soil which creates a monolithic structure. The microbial bio-cementation process has been adopted in research for remediating contaminated land and groundwater through the capture of contaminants (heavy metals and radionuclides) (Fujita et al. 2010; Mugwar and Harbottle 2016) in the mineral, for reduction of permeability in rock and soil for selective extraction of petroleum products from the subsurface (Ferris and Stehmeier 1992; Gollapudi et al. 1995) or for deep underground storage of carbon dioxide (Cunningham et al. 2009; Mitchell et al. 2010),

for prevention of soil erosion for re-vegetation of arid land (Stabnikov et al. 2011a; Gomez et al. 2015), and for improvement of ground properties (soil stabilisation) for construction (DeJong et al. 2006; Whiffin et al. 2007; van Paassen et al. 2010). The microbial bio-cementation process is considered a more environmentally friendly alternative to grouting as it does not involve the use of synthetic materials pumped into the subsurface, rather it can harness bacteria present in the ground by stimulating it with harmless chemical precursors (a source of carbon and calcium).

One unexpected use of bio-mineralising bacteria has been for closure of structural cracks in cementitious materials and concrete (Jonkers 2007; Wiktor and Jonkers 2011; Wang et al. 2014; Sharma et al. 2017). Such bacteria has either been sprayed along with nutrients on the surface of concrete or incorporated as a part of the concrete mix in attempts to make the material self-healing by blocking cracks and preventing the ingress of corrosive substances to the reinforcement. Hardened concrete, however, is a highly aggressive environment for living organisms due to its high pH (12-13) and restricted pore space. In order for the bacteria to survive the mixing process, as well as the challenging conditions in the hardened concrete, researchers have selected bio-mineralising bacteria which can form spores. The remarkable abilities of spores to survive various extreme environments and prolonged starvation periods meant that the sporulated bacteria could endure the harsh concrete surroundings and grow back into a vegetative bacterium once a crack exposed it to more favourable conditions. The vegetative bacteria could then fill the cracks with the precipitation of calcium carbonate. The microbial self-healing technique in concrete is still to be perfected, however. Despite the efforts of many in the field, the extent to which spores survive in the concrete is limited especially in the long run, the presence of various healing agents within the concrete matrix renders a decrease in the strength properties of the hardened

concrete, and self-sealing (blocking of cracks), as opposed to self-healing (strength regain), is observed in most cases.

Soils, as opposed to concrete, are natural hosts of a significant number of bacteria and are a much “friendlier” environment. Drawing inspiration from self-healing concrete research, this project will explore the potential of microbially induced calcium carbonate precipitation to be augmented as a self-healing mechanism in soils for geoenvironmental and geotechnical applications. Self-healing abilities in earth structures would allow them to respond to damage and deterioration by initiating a healing process which repairs the damage and can restore the properties of the structure to the pre-damage condition. The microbial bio-cementation process can be altered in order to provide multiple cycles of healing and do so in the long-term through the use of spore-forming organisms. An initially created monolithic structure by the bacteria in the soil would encompass a number of encapsulated (in their own mineral tomb) sporulated organisms. The spores of the bacteria would stay dormant in the mineral until damage in the form of deterioration of the calcium carbonate (for example, a physical crack or acid dissolution) would expose them to the environment and to nutrients after which they will germinate back to vegetative life. The vegetative bacteria would then be able to “heal” the damage through the production of further calcium carbonate. The process of encapsulation, dormancy and subsequent damage scenarios could in theory be repeated indefinitely. This would allow initially bio-cemented structures to continually repair themselves and therefore prevent the escape of bio-cemented soil and groundwater contaminants, stored CO₂, or alleviate cracks in earth structures. Such biological processes would therefore reduce the need for maintenance and repair and subsequent disruption, they would also improve safety and prolong the service life of structures.

In the UK, a research project, called Materials for Life (M4L), funded by EPSRC (grant number EP/K026631/1), was initiated and it focused on the development of self-healing systems in construction materials. The work presented here is part of the M4L project and focuses on augmenting MICP for the development of self-healing mechanisms in soils.

1.1 Main aim and objectives of the research

The main aim of the work described here was to demonstrate the potential of self-healing MICP using a spore-forming, calcium carbonate precipitating organism. The following objectives were established to achieve this aim:

- i. to select an appropriate spore-forming, mineral-precipitating organism; to explore the self-healing process in idealised conditions in aqueous solution, through cycles of sporulation, carbonate precipitation, damage to the precipitate, and regeneration; to demonstrate long-term survivability of the sporulating organism in crystallised form;
- ii. to explore the ability of the organism to bring about MICP and monolith formation and investigate optimum conditions for doing so in the shortest timescale;
- iii. to demonstrate the ability of sporulated organisms to survive the initial soil bio-cementation process and subsequent damage (either physical or chemical), germinate upon exposure to favourable conditions and cause healing of the damaged mineral monolith.

1.2 Outline of the thesis

This thesis reports the work undertaken to investigate the potential of microbially induced calcium carbonate precipitation (MICP) as a self-healing mechanism in soils.

A review of the state of the art of bio-cementation and microbial self-healing is presented in Chapter 2. The literature review focuses on bio-mineralisation applications in cementitious materials, geoenvironmental and geotechnical engineering. Self-healing cementitious materials and self-healing soils, both autogenous and autonomous, have been discussed. Focus has been given to the microbial self-healing process with prerequisites for its realisation and applications in the areas of cementitious materials and geotechnical engineering.

Chapter 3 outlines the analytical methods used both in experiments in aqueous solution and particulate media. The developed apparatus and methods used for the bacterial treatment of soil are also presented.

Results from the selection of appropriate bacteria, as well as an investigation of the self-healing principles and repetition of the process in idealised conditions in aqueous solution are presented in Chapter 4.

Batch experiments (complemented with a geochemical model), as well as static and dynamic column experiments to optimise the conditions for MICP using *S. ureae* and reduce the bacterial treatment time to achieve adequate levels of bio-cementation are presented in Chapter 5.

Chapter 6 presents two experiments which explored the potential of initially bio-cemented sand columns to heal different forms of damage. The first experiment determined the healing response of the columns to chemical deterioration (acidic flow) and the second explored the response to mechanical damage.

Finally, conclusions of the work and recommendations for future work are presented in Chapter 7.

2 Literature Review

2.1 Introduction

This chapter reviews studies concerned with the action of bacteria as a geological agent in the subsurface and its geotechnical implications, and a particular process, called microbially induced calcium carbonate precipitation (MICP), with its different applications. Also reviewed here are publications demonstrating self-healing of concrete and of soils and earth structures, both autogenic (natural) and autonomous (engineered).

2.2 Bio-mineralisation

Microbes have been associated with the formation of a variety of biogenic minerals in both subsurface and marine environments (Lowenstam 1981; Ehrlich 1996; Schultze-Lam et al. 1996) and it has been postulated that they have impacted Earth's lithosphere since the Precambrian era (Ehrlich 1998; Castanier et al. 1999; DeJong et al. 2013). In their research, Boquet et al. (1973) isolated 210 organisms which formed calcium carbonate crystals and argued that under suitable conditions, most bacteria can in fact form mineral precipitation. In addition, Sarikaya (1999) and Lowenstam and Weiner (1983) (cited in Rebata-Landa (2007)) reported more than 60 biogenic minerals produced by bacteria. They include hydroxides, oxides, carbonates, sulphates, sulphides, phosphates and clays, and those aggregate into amorphous, pre-crystalline or crystalline structures - the degree of crystallinity usually depending on the stage of crystal growth (Schultze-Lam et al. 1996; Castanier et al. 1999), as well as on the environmental conditions and the particular species present (Cacchio et al. 2003; Mitchell and Santamarina 2005; Konhauser 2006). Metal cations most commonly found associated with biogenic minerals are calcium (e.g. formation of calcium carbonate), iron (e.g. formation of ferric hydroxide) and manganese (e.g. formation of manganese hydroxide) (Konhauser 2006).

Different studies have reported the extensive scale to which microbes can alter the biogeochemical processes and lead to extensive precipitations of minerals - one of the earliest studies, presented by Black (1918), concluded that the presence of vast calcium carbonate formation in the Great Bahama Bank was brought about by mangrove swamp anaerobic bacteria. Furthermore, Konhauser et al. (2002) suggests that the most plausible mechanism to explain the Precambrian banded iron formations is their precipitation by iron-oxidising bacteria. In fact, Castanier et al. (1999) specified that most limestones are considered to be formed as a result of the activity of heterotrophic bacteria. It is considered that chemical reactions, leading to formation of minerals, can be extremely slow (or even impossible) to occur in an abiotic fashion, but when catalysed by bacteria they can occur momentarily (Konhauser 2006). As an example, the process of urea hydrolysis (described in detail later in this section) is 10^{14} times faster when catalysed by the enzymatic activity of bacteria as compared to the uncatalysed reaction (Jabri et al. 1995). The factor which governs the catalysis of chemical reactions and mineral precipitation on very large scales is that bacteria are ubiquitous in the subsurface. There are 10^9 to 10^{12} organisms per kilogram of soil near the surface, i.e. in the first 1 meter (Mitchell and Santamarina 2005), and 10^6 to 10^{11} organisms per kilogram at greater depths of 1 to 8 meters (Whitman et al. 1998). For these reasons Ehrlich (1998) used the term important “geologic agents” and DeJong et al. (2013) - “active geotechnical engineers” to describe bacteria and emphasise the significance of their activity in the subsurface.

Microbial mineral precipitation can occur via different mechanisms - “biologically controlled” (active), “biologically induced” or influenced (passive) (terms defined by Lowenstam (1981)) bio-mineralisation (Phillips et al. 2013). Bio-mineralisation processes are usually used by microbes to form protection from predation, physical

damage, or toxic substances in the surrounding environment and, consequently, increase their competitiveness within microbial communities (Ehrlich 1996). In biologically controlled mineralisation the organism controls mineralisation through the development of an organic cast (either extra- or intra-cellularly) onto which chosen ions of appropriate concentrations are introduced and minerals start to form. In passive bio-mineralisation minerals form as a result of the metabolic activity of the microbes and its by-products (which alter the chemistry of the system), as well as the presence of the microbes themselves acting as reactive surfaces for crystal nucleation (Ehrlich 1996; Konhauser 2006; Phillips et al. 2013). According to Konhauser (2006), the majority of biogenic minerals are formed passively, whereas Phillips et al. (2013) make the point that a combination of the two mechanisms can often be present in a system.

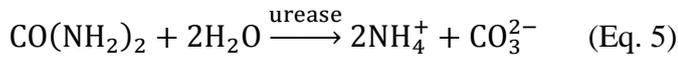
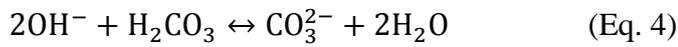
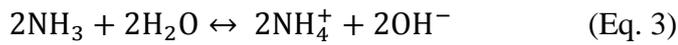
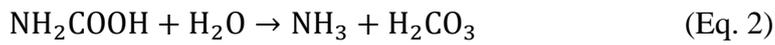
MICP through ureolysis (degradation of urea or uric acid via a urease enzyme) is one heterotrophic passive process which results in calcium carbonate formation in terrestrial environments. There are several other pathways which, in the presence of calcium and organic matter, can cause calcium carbonate precipitation: (i) ammonification of amino acid, (ii) dissimilatory reduction of nitrate, and (iii) dissimilatory reduction of sulphate (Ehrlich 1998; Castanier et al. 1999; Mitchell and Santamarina 2005; Madigan et al. 2009). The ease of control and high amounts of carbonate produced in a relatively short period of time has made ureolysis the preferred pathway for use by many researchers (Stocks-Fischer et al. 1999; DeJong et al. 2006; De Muynck et al. 2010). It has to be noted, however, that ureolysis has certain disadvantages - production of ammonia in the subsurface which can be potentially harmful (Bremner and Krogmeier 1989; Nolan et al. 1998), or acidification of the subsurface due to ammonia oxidation by microbial communities (Reed et al. 2010). Therefore, if bio-cementation is to be applied in the field there should be careful consideration for and the appropriate design of the

metabolic process employed and the subsequent removal of potentially harmful by-products.

2.2.1 MICP via urea hydrolysis

The presence of urea in soils has been attributed to the degradation of purines, pyrimidines, and arginine by microorganisms (Vogels and Van der Drift 1976; Cunin et al. 1986; Witte 2011). Urea is also produced synthetically and is one of the most commonly used soil fertilisers (McKenzie 1998). In addition to certain higher plants (Witte 2011), actinomycetes, fungi, and moulds (Seneca et al. 1962), between 17 and 30 % of bacterial populations in soil (including aerobic, micro-aerophilic and anaerobic) contain a urease enzyme (Lloyd and Sheaffe 1973). The hydrolysis of urea, catalysed by urease, is the end stage of the mineralisation of organic nitrogen in the subsurface (Benini et al. 1999). In the presence of organic matter, calcium, and urea in the pore fluid of soils bacterial urease triggers a chain of chemical reactions (Stocks-Fischer et al. 1999; De Muynck et al. 2010). Urea is first degraded to ammonia and carbamate (Eq. (1)) and subsequently further hydrolysed to ammonia and carbonic acid (Eq. (2)). After equilibrating in water these products form bicarbonate, ammonium, and hydroxide ions (Eqs. (3) and (4)). The reaction in Eq. (5) summarises the process - urea degradation results in the release of ammonia and carbonate which is accompanied by an increase in pH (to ~ 9) and carbonate concentration in the locality of the bacterial cell. This alkaline microenvironment together with calcium present in solution creates conditions oversaturated with respect to CaCO_3 which favours the precipitation of calcium carbonate around or on the cell wall (Eq. (6)) (Schultze-Lam et al. 1996; Stocks-Fischer et al. 1999).





It is important to note that secondary polymers attached to the peptidoglycan macromolecules in the cell wall of Gram-positive bacteria and the lipopolysaccharide (LPS) in the outer membrane of Gram-negative bacteria are highly anionic. They impart a net electronegative charge and make bacterial cell walls very interactive with metal ions which consequently make them ideal nucleation sites for heterogeneous crystal formation. Bacteria attract metal cations (Ca^{2+}) and subsequently counter ions (CO_3^{2-}) to precipitate as calcium carbonate on their surface (Schultze-Lam et al. 1996; Stocks-Fischer et al. 1999; Phillips et al. 2013). The process leads to a significant crystal growth around the cell wall effectively entombing the bacteria (DeJong et al. 2006; Rodriguez-Navarro et al. 2007). A diagram depicting the chemical conversions catalysed by bacteria and the associated precipitation is presented in Figure 2-1.

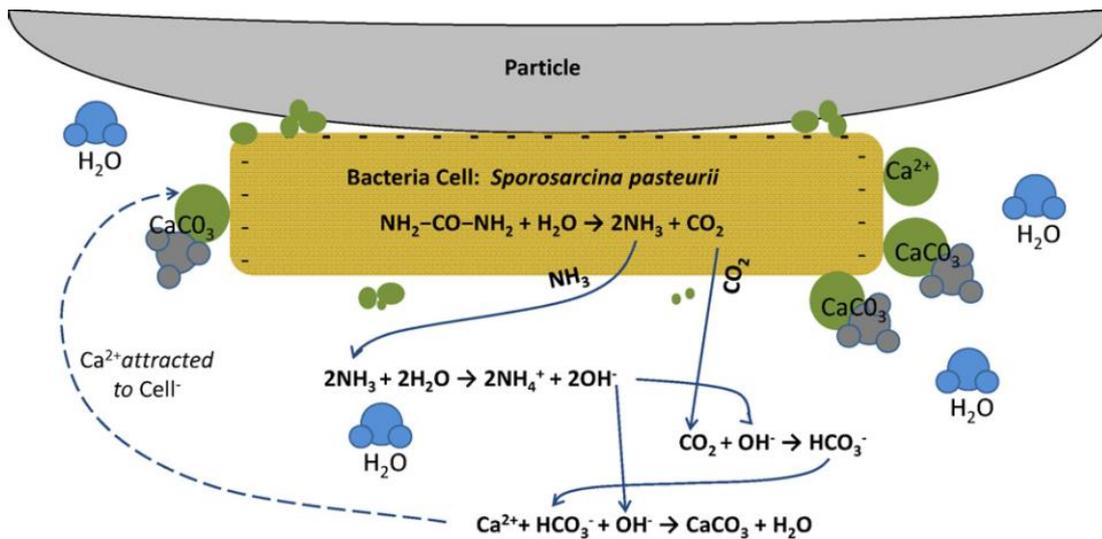


Figure 2-1 Microbially induced calcium carbonate precipitation overview (figure reproduced from DeJong et al. 2010)

The three most commonly found anhydrous polymorphs of calcium carbonate in nature are: (i) calcite (most thermodynamically stable); (ii) aragonite (metastable); and (iii) vaterite (least stable of the polymorphs) (Brecevic and Kralj 2007). Crystallographically calcite belongs to the hexagonal-rhombohedral system (though more than 300 calcite variations have been reported), aragonite to the orthorhombic system (often displays needle like structure), and vaterite belongs to the hexagonal system and is spherulitic or disc-like (Chakraborty et al. 1994). The hydrated phases of calcium carbonate are very often amorphous or poorly crystalline.

Although in biologically controlled processes by higher organisms there is a remarkable control on the polymorph type, crystal orientation, and mineral composition (e.g. molluscs forming their shells; otolith formation in the ear bone of teleost fish), it is somewhat dubious whether such control exists in biologically induced processes (Falini et al. 1996; Falini et al. 2005; Rodriguez-Navarro et al. 2007).

The morphology and physico-chemical properties of the precipitate formed during induced bio-mineralisation depend upon many factors, such as the initial supersaturation

of ions in solution, type of organic matrix present, pH, and temperature (Chakraborty et al. 1994; Konhauser 2006; Brecevic and Kralj 2007). It has been reported that amorphous calcium carbonate can be seen in the early stages of precipitation by microorganisms. As growth and agglomeration of bacterial precipitates occurs they transform to more crystalline structures and stable forms, such as calcite (Castanier et al. 1999).

In publications where induced bio-mineralisation was used some report the formation of aragonite (Rivadeneira et al. 1996), vaterite (Rodriguez-Navarro et al. 2007), or a combination of the three polymorphs (Achal et al. 2012). Polymorphism of the calcium carbonate formed was attributed to the specific strain of bacteria, as well as the presence of other metal ions in solution. The majority of publications, however, report predominant precipitation of calcite (DeJong et al. 2006; Whiffin et al. 2007; Tobler et al. 2011; Cuthbert et al. 2012; Al Qabany and Soga 2013; Chu et al. 2013). Although the bio-precipitation of stable forms of calcium carbonate is preferable when MICP is applied for the geotechnical improvement of soil, the effect of polymorphism on the durability and resistance of the precipitated mass to erosion by groundwater flow, physical stresses, and acidic conditions, is an area of research yet to be explored.

2.3 Applications of MICP

A wide range of applications for the microbial cementation process have been envisioned by researchers. For example, as a protective treatment for ornamental stone to self-healing cementitious materials, geoenvironmental remediation mechanisms for contaminated soil and groundwater, optimised oil recovery, as a stabilisation method for liquefiable soils, through to prevention of desertification by surface stabilisation of erodible soils. A diagrammatical representation of some of these applications is shown in Figure 2-2. In the following sections, the applications of MICP have been divided

into such related to cementitious materials and concrete, geoenvironmental, and geotechnical engineering and each has been discussed in more detail below.

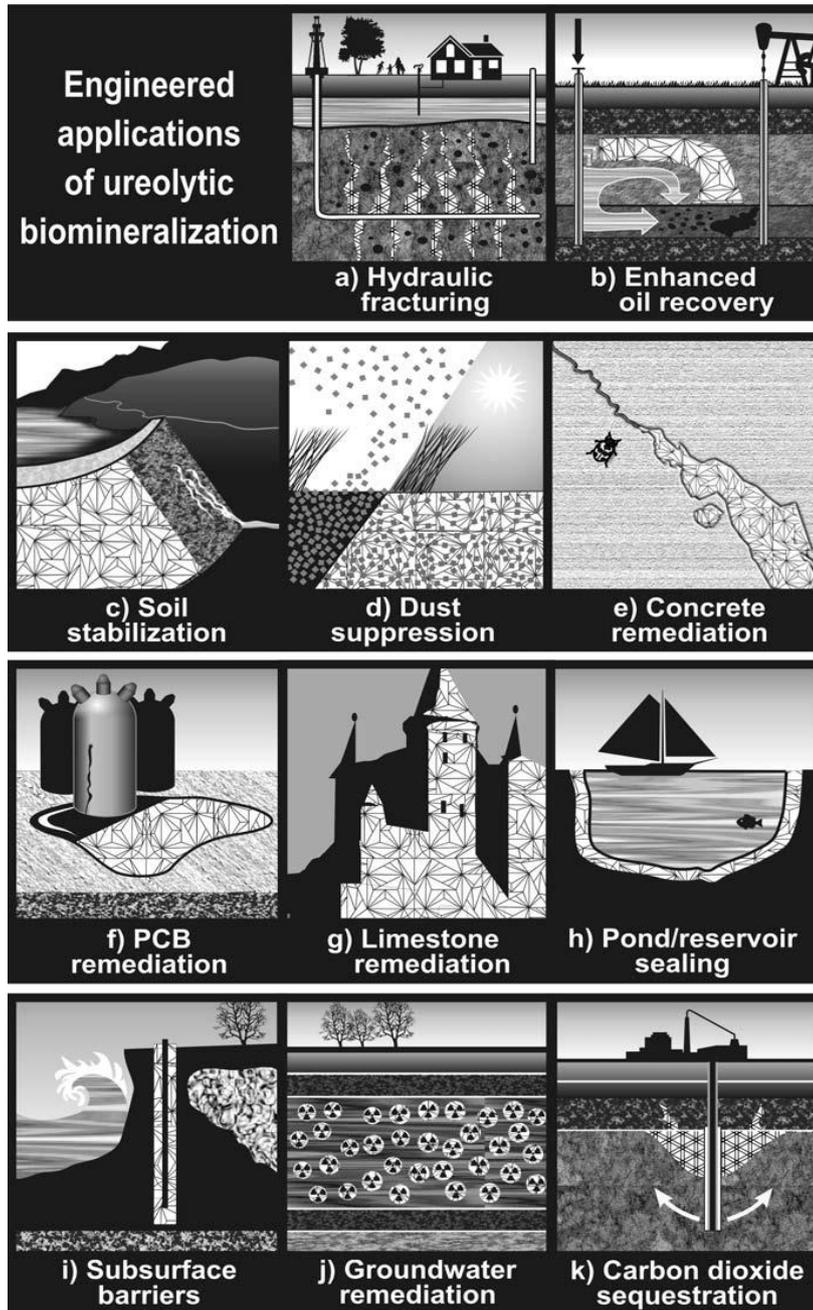


Figure 2-2 MICP engineering applications (figure reproduced from Phillips et al. (2013, p.716))

2.3.1 Cementitious materials applications of MICP

The first applications of MICP in the field of civil engineering were used to protect limestone monuments (Adolphe et al. 1990). A comprehensive review of the different approaches adopted by various research groups was published by De Muynck et al. (2010). They categorised the research into bio-deposition, bio-cementation, and self-healing cementitious materials. Bio-deposition is concerned with the deposition of a protective carbonate layer by calcinogenic bacteria on the surface of ornamental stone for its protection from weathering and erosion processes. The bio-deposition method was termed Calcite Bioconcept and was patented by Adolphe et al. (1990), cited in Le Metayer-Levrel et al. (1999). The promise of the technique led to its further exploration, as well as evaluation of different approaches by many others (Tiano 1995; Le Metayer-Levrel et al. 1999; Tiano et al. 1999; Rodriguez-Navarro et al. 2003; Dick et al. 2006; Tiano et al. 2006; De Muynck et al. 2008a; Chunxiang et al. 2009; Rodriguez-Navarro et al. 2012). The microbial bio-deposition technique was evaluated by Okwadha and Li (2011) for the surface remediation of polychlorinated biphenyls-contaminated (PCB) concrete surfaces as an alternative to epoxy-coating systems. Concrete elements near machinery and motors, which run on PCB-based oils, may become contaminated as a result of leaks and spills. The microbial carbonate precipitation technique showed significant promise for the containment of the contamination - the “biosealed” concrete surfaces underwent a reduction in permeability of between 1 to 5 orders of magnitude resulting in an almost complete reduction of leaching of PCB-contaminated water. The MICP “biosealant” was also shown to be thermally stable at temperatures of up to 840° C, typical of temperatures measured in the surroundings of machines containing PCB-based oils.

Microbial carbonate production has also shown significant promise for crack closure (self-sealing), improvement of strength and even self-healing of concrete and mortars (Bang et al. 2001; Ramachandran et al. 2001; Bachmeier et al. 2002; Day et al. 2003; Ghosh et al. 2005; Ramakrishnan et al. 2005; Jonkers 2007; Jonkers and Schlangen 2007; De Belie and De Muynck 2009; Jonkers et al. 2010). Microbial self-healing in cementitious materials will be discussed in more detail in section 2.4.2.

2.3.2 Geoenvironmental applications of MICP

Amongst the first applications of MICP in the field of geo-environmental engineering was the plugging of water-rich porous media to ease the extraction of oil from oil reservoirs as proposed by Ferris and Stehmeier (1992) and Kantzas et al. (1992). The microbial plugging process yielded significant success in laboratory conditions resulting in the complete plugging (no flow) through sand-packed columns within days of treatment. Gollapudi et al. (1995), Stocks-Fischer et al. (1999), and Fujita et al. (2000) were among the first to envision the implementation of microbial carbonate precipitation for the containment of groundwater contaminants, leaching prevention, and consolidation of rock. For example the co-precipitation of calcite with radionuclide groundwater contaminants, such as strontium (Sr-90), has been studied in natural systems (Ferris et al. 1995), in artificial microbiological media (Warren et al. 2001) and in media resembling natural groundwater (Fujita et al. 2004). Warren et al. (2001) presented promising results of 95 % capture of strontium (at a concentration of 1 mM) into calcite in artificial media, whilst Fujita et al. (2004) showed a 70 % drop in strontium within an hour of start of experiment (at lower concentrations of 0.1 mM) in media resembling natural groundwater. The findings indicated strong evidence that this emerging technology could provide a rapid and effective immobilisation mechanism for radionuclides in contaminated groundwater. Several researchers have also noted that

there is a ubiquitous amount of ureolytic organisms, as well as supersaturated conditions with respect to calcium carbonate, present in natural systems. Therefore stimulation of indigenous communities (as opposed to introduction of foreign microorganisms) can generate favourable conditions for the co-precipitation of calcium carbonate and radionuclides and provide an effective long-term remediation solution (Ferris et al. 2003; Mitchell and Ferris 2006a; Fujita et al. 2008, 2010). A significant body of research can also be found related to the co-precipitation of other heavy metals with calcium carbonate (e.g. zinc, cadmium, lead, copper, arsenic and chromium - another group of common contaminants) in groundwater and soil. Bioremediation technologies, such as the co-precipitation method, have shown promise as a more reliable, cost-effective, and efficient alternative to current treatment methods for contaminated groundwater and soil (e.g. pumping of the polluted water to be treated ex-situ or soil excavation and disposal to a landfill) (Megharaj et al. 2011).

The parameters which influence MICP when used for metal remediation include: the tolerability of the bacterial species to the concentrations of different metals; the heterogeneity of the soil system and consequent availability of nutrients to the bacteria; CaCO_3 precursors (Kurmaç 2009; Achal et al. 2012; Mugwar 2015; Mugwar and Harbottle 2016). It is interesting to note that, as outlined by Mugwar and Harbottle (2016), bacterial cells are able to function at higher concentrations of metals when ureolytic mineral precipitation is occurring. Bio-mineralisation aids in removing metals from solution thereby reducing the concentration of bioavailable metals. This mechanism may be advantageous in situations where the concentration of metals is hindering the bacteria present by reducing the toxicity and allowing further bacterial activity. This, in a sense, complements the findings of Lowenstam (1981) and

Konhauser (2006) according to which mineral precipitation may serve many biological functions including a protective mechanism to the cell.

Another promising application for MICP in the field of geoenvironmental engineering has been developed in a series of publications by Mitchell et al. (2008), Cunningham et al. (2009), Mitchell et al. (2009), Mitchell et al. (2010), Cunningham et al. (2011), Mitchell et al. (2013), Phillips et al. (2013), and Phillips et al. (2016). The potential for microbial biofilms and mineralisation processes to be used for the containment of carbon dioxide in deep geological aquifers was investigated. The bio-mineralisation process and biofilm formation would ensure effective plugging of potential leakage zones for the stored carbon dioxide. However, the elevated temperature and pressure conditions in such aquifers mean that carbon dioxide will be present in its supercritical form (SC-CO₂) which may adversely affect or suppress the effectiveness of the bacterial cells. In a preliminary investigation by Mitchell et al. (2008) they showed that planktonic cultures (single-cells, not associated with other cells in a biofilm) exhibited 3 times higher death rates when subjected to high-pressure, high-temperature CO₂ (or SC-CO₂) compared to biofilm cultures. These findings were developed further and in their publications Cunningham et al. (2009), Mitchell et al. (2009), Mitchell et al. (2010), Cunningham et al. (2011), and Phillips et al. (2013) examined the ability of engineered biofilms to form crystalline structures through ureolysis. Bio-mineralising biofilms would have a higher resilience to SC-CO₂ as well as potentially provide a more effective plugging of the zones of preferential flow overlaying the storage aquifer. It has to be added that as well as biofilms extracellular polymeric substances (EPS), which are commonly associated with biofilms, may play an important role in carbonatogenesis by either limiting or promoting the precipitation process (Dupraz et al. 2009; Zhu and Dittrich 2016). The EPS matrix contains acidic functional groups which are negatively

charged and attach strongly with mono- and divalent ions such as calcium and magnesium. Calcium binding also plays a role in maintaining the gel structure of the EPS. Both these factors limit the availability of calcium ions and inhibit the precipitation of calcium carbonate in the proximate environment around the EPS. However, EPS degrades easily while releasing the previously bound calcium which in turn may create supersaturated conditions with respect to CaCO_3 . The degradation of EPS would also release organic carbon which can be rapidly metabolised by bacteria resulting in CaCO_3 formation (Dupraz et al. 2009).

2.3.3 Geotechnical applications of MICP

Microbial processes and MICP in particular have recently gained a significant popularity among geotechnical engineers also. Research using the bio-mineralisation process has focused on a range of different applications such as improving bearing capacity, liquefaction resistance, stability of slopes, soil erosion control, dam and levee safety, stabilisation prior to tunnelling, and development of alternative geotechnical engineering solutions in general. Mitchell and Santamarina (2005) were among the first to draw attention to the emerging field of bio-geotechnical engineering. They reviewed the many different biogeochemical processes that bacteria facilitate in the subsurface and noted how they can considerably alter the geotechnical properties of soil highlighting the possible implications for new ground modification methodologies. A series of comprehensive investigations on MICP as a soil improvement method have been undertaken by various research groups from around the world (DeJong et al. 2006; Rebata-Landa and Santamarina 2006; Whiffin et al. 2007; van Paassen et al. 2010; Al Qabany and Soga 2013; Burbank et al. 2013; Chu et al. 2013). Following is a discussion of the publications related to the two main geotechnical improvements when MICP is applied to soils which are: (i) stabilisation and liquefaction prevention and (ii) reduction

in permeability. Additionally, publications of first attempts and challenges of up-scaling the MICP process from the laboratory to on-site applications are also discussed.

2.3.3.1 Soil stabilisation and liquefaction prevention

DeJong et al. (2006) were among the first researchers to comprehensively review and describe the microbially induced cementation process. They also demonstrated experimentally the effect of the microbial treatment on the geotechnical properties of soil which was modified from its loose and collapsible state to cemented behaviour. The behaviour of bacterially treated sand was significantly modified from its non-cemented state and was comparable to gypsum-treated sand in their response to compressive triaxial testing. DeJong et al. (2006) also discussed potential monitoring techniques such as measurement of shear/compression wave velocities and resistivity using bender element devices. Such geophysical methods are non-destructive and can accurately predict the evolution of the microbial bio-cement in a body of soil, its quantity, and effect on geotechnical properties. These or similar techniques (e.g. seismic wave monitoring) have proven highly useful and have been utilised in research for MICP of soils by many researchers (van Paassen et al. 2010; Wu et al. 2011; Bernardi et al. 2014).

Whiffin et al. (2007) published another significant work on MICP for sand stabilisation called BioGrout (following from a patent by Kucharski et al. (2008)). The bio-cementation of a 5 m-long column of sand was tested which resulted in precipitation forming along the entire column length; however they noted the presence of a significant precipitation gradient. In attempts to overcome this precipitation gradient Whiffin et al. (2007) developed a protocol for the retention of bacterial cells within the sand using a two phase injection of bacteria in a low-strength ionic solution followed by the injection of a fixation fluid (50 mM calcium chloride solution) with a high ionic

strength. A similar study with a different bacterium (*Escherichia coli*) was performed by Torkzaban et al. (2008) in which it was concluded that the retention or release of soil bacteria is strongly dependent on the ionic strength of the injected solutions. The aim of the two-phase injection developed by Whiffin et al. (2007) was to retain as much as possible from the injected bacteria within the soil and therefore achieve uniform precipitation of calcium carbonate. In a following publication Harkes et al. (2010) investigated the bacterial adhesion to soil further and showed that differing injection strategies (flow rates and batch or continuous flow) can be used along with weak or strong ionic solutions to manipulate the distribution of bacteria. It has been shown that solutions of ionic strength of 1 mM salt concentration promote the detachment and solutions of higher than 50 mM ionic strength promote the retention of bacterial cells to the grain surfaces of soil (Torkzaban et al. 2008; Harkes et al. 2010). Although the two-phase injection technique has proven effective for MICP in small-scale sand columns a more comprehensive study is needed in large-scale or field conditions with additional parameters examined as well such as precipitated content and evolution of strength. An important finding by Cheng et al. (2016) revealed that factors such as rainfall during or immediately after the MICP treatment of soil or oil contamination in the soil may render the two phase injection and fixation method unsuccessful.

In support of the work by Whiffin et al. (2007) and Harkes et al. (2010), although showing the opposite principle, are the publications of Cunningham et al. (2007), Al Qabany et al. (2012), and Al Qabany and Soga (2013). They showed that the use of lower ionic strength solutions (Al Qabany et al. 2012; Al Qabany and Soga 2013) or even bacterial starvation (Cunningham et al. 2007) can lead to the decreased attachment of bacteria near injection and leading to a more homogeneous distribution of calcite in

the treated soil. A further discussion on methods to influence bacterial distribution for the optimisation of MICP is presented in the section 2.3.4.

Other publications which followed from and build upon the work of DeJong et al. (2006), Whiffin et al. (2007), and Harkes et al. (2010), and are concerned with the geotechnical improvement of particulate media (mainly sandy soils) include: reinforcement of liquefiable sands and slope stabilisation (Van der Ruyt and Van Der Zon 2009; DeJong et al. 2010), bore hole stabilisation (van der Star et al. 2011), and stabilisation of railroad tracks and reinforcement of calcarenite room and pillar mines (van Paassen 2009). Although these publications are not directly relevant to the literature presented here and will not be discussed further they are important demonstrations of the workings of the MICP for various geotechnical problems.

Although the works on MICP in geotechnics, which have been presented so far, have shown significant promise and success most of them faced a similar issue with the blocking of soil pore space at injection points due to the accumulation of calcium carbonate in the soil and consequent non-homogeneous distribution of the biogenic precipitation. For this reason much of the research performed on MICP for soil stabilisation to date has concentrated on controlling the factors influencing the bacterial healing and its homogeneous distribution. Important developments to overcome heterogeneity when MICP is applied either in the laboratory or *in situ* were made by Burbank et al. (2011, 2012, 2013). By collecting samples of different soils and treating them in columns (in their study they used syringes as columns) with nutrients to stimulate indigenous bacteria they demonstrated that a variety of soils (fine, poorly graded tidal sand; organic soil; mined alluvial, poorly graded sand; and quartz poorly graded silica sand) contain urease-active bacteria which are able to tolerate high concentrations of ammonia (Burbank et al. 2012). Also, when stimulated with

enrichment solutions containing sources of carbon (e.g. molasses) and calcium the indigenous communities were able to precipitate significant amounts of biogenic calcite *in-situ*. Using vertical infiltration in an area of about 1 m² the underlying soil was treated with enrichment and bio-mineralisation solutions for a period of about 30 days. At the end of the treatment cone penetrometer tests as well as amount of calcium carbonate analysis were performed. Accumulation of precipitation in the pore space close to injection was not observed and, in fact, there was a higher calcite content at depth away from the injection point (~1 % by weight near surface and 1.8-2.4 % below 90 cm) and the corresponding strength, measured by a cone penetrometer varied from 1 to 6 MPa (Burbank et al. 2011). The researchers note that the higher amount of precipitation at a distance from injection may be attributed to an increased ureolytic activity at depth as well as the downward transportation of fine CaCO₃ particles with the infiltrating solution (i.e. eluviation). A very similar approach was used by Cheng and Cord-Ruwisch (2014) for the biogenic treatment of fine silica sand by surface percolation. However, they introduced laboratory cultured ureolytic bacteria followed by solutions to induce cementation. They reported a relatively homogeneous distribution of the biogenic calcite but only in depths up to 20 cm. They also noted a dramatic decrease in ureolytic activity and subsequently much lower precipitation values and strengths at greater depths. Although in their study Burbank et al. (2011) did not achieve homogeneous distribution of the precipitation they overcame the persistent problem of CaCO₃ accumulation and clogging near the point of injection when bio-augmentation is used. Therefore their findings suggest that stimulating the natural ureolytic communities may lead to better overall treatment penetration especially when applied on the large-scale.

A significant work presenting up-scaling of MICP for the purposes of preventing liquefaction in a sandy soil was undertaken by van Paassen et al. (2010). A large body of sand ($\sim 100 \text{ m}^3$) was biologically treated with the use of an injection-extraction system of 6 wells which resembled systems used with chemical grouting. The treatment involved pumping of bacterial solution followed by a medium promoting attachment of cells to sand particle surfaces (as described in Harkes et al. (2010)) and a series of 10 urea and calcium solutions (cementation solutions) over a period of approximately 2 weeks. Geophysical (seismic) monitoring during the treatment as well as geotechnical tests at the end of the treatment helped to evaluate the effectiveness of the MICP process on the change in sand properties. The result of the treatment was as much as 40 m^3 of the sand cemented together with a shape closely following the flow pattern from the injection to the extraction wells (Figure 2-3), as much as 110 kg of calcium carbonate precipitated per m^3 of soil (or 6.8 % by weight) on average in the cemented area, and an increase in the unconfined compressive strength (UCS) of 0.7 to 12.4 MPa (for a reference, UCS of uncemented sand can be taken as zero). However, the cementation was achieved through the injection of a substantial amount of reagents ($\sim 96 \text{ m}^3$) and high concentrations of 1 molar calcium and urea in the medium (equal to approximately 110 and 60 kg/m^3 of each, respectively), less than half of which were utilised.

A significant outcome from their study was the remarkably close correlation between the measured stiffness and the calculated one (from the seismic wave measurements). This means that in field applications geophysical methods can be used both as means of monitoring the development of the bio-cementation (not possible with conventional geotechnical tests) as well as estimating the final geotechnical parameters with a reliable accuracy. The work presented by van Paassen et al. (2010) is also a significant evidence

that MICP is possible on a larger scale, can be achieved through injection-extraction systems used in conventional chemical grouting, and can be monitored through seismic methods. However, challenges with the excessive amount of reagents lost in the process are still to be resolved.



Figure 2-3 MICP treatment on a 100m³ of sand (figure reproduced from van Paassen et al. (2010))

2.3.3.2 Formation of a bio-cemented surface layer in soil

In certain scenarios, as outlined by Stabnikov et al. (2011) and Chu et al. (2013), the heterogeneous distribution and a greater accumulation of calcite on the surface of the treated soil is desirable if the purpose of the bacterial treatment is to create a stiff layer or an impermeable barrier. Ureolysis driven calcite precipitation has been adopted for the formation of a stiff crust layer on the surface of soil (Stabnikov et al. 2011b; Chu et al. 2013) which can be used for the construction of aquaculture ponds in sand, dust control, re-vegetation in arid land, and reservoir sealing. The microbial surface treatment was applied to a model sand pond of approximately 0.2 m² area and it resulted in the formation of a low permeability layer (~ 2 cm thickness) of consolidated sand. Chu et al. (2013) make important observations on the water conductivity, compressive

and tensile strengths of the calcified layer of sand. They noted significant reductions in permeability (by 1000-fold), increases in compressive (by 50-fold), and tensile (by 250-fold) strengths.

Gomez et al. (2015) attempted to up-scale the microbial surface treatment (for creating a stiff surface layer of soil) for the prevention of erosion in loose sand deposits. There were 4 test areas of soil (each 12 m²) which were treated with bacteria and varying concentrations of substrates by surface watering with a hose. Similarly to Chu et al. (2013) they report the formation of a 0.64 to 2.5 cm thick layer of calcified sand following the bacterial treatment and also a penetration of the treatment up to depths of about 30 cm when lower concentrations of substrates (urea and calcium) were used which is in agreement with Harkes et al. (2010) and Al Qabany and Soga (2013). Unlike the high-concentration bio-treated test plots, the plot treated with low-level concentrations exhibited little deterioration after a very harsh winter with temperatures of – 41° C. The study by Gomez et al. (2015) is an important evidence that MICP can also be applied in large scale for the surface consolidation of soils and that the treatment does not deteriorate even after a prolonged period under extreme environmental conditions.

2.3.4 Factors affecting spatial homogeneity of MICP

Regardless of the application for which MICP would be used, up-scaling and field applications would require a proper control over the result of the biological treatment. The effort of many has been focused on the optimisation of ureolytic MICP and characterisation of its control parameters at small- and large-scale laboratory and field investigation. A review on important MICP control parameters examined by researchers was provided by Phillips et al. (2013) and was used as a basis for the development of this section of the literature review. The factors which govern the bio-mineralisation

process are many and can be broadly categorised into the activity of the ureolytic process which is dependent on the source of enzymatic activity (type of bacteria), the reaction kinetics and availability of nutrients and chemical precursors to the microorganisms or free enzymes, and type of soil and subsurface characteristics. These factors will be discussed in more detail in the following sections.

2.3.4.1 Biomass at onset of MICP

Various concentrations of bacteria (biomass) have been used by different researchers in laboratory conditions. Most of them vary from 10^5 to 10^9 cfu (colony forming units) per ml of solution. These numbers reflect the populations of microorganism existing in natural soils. In microcosm experiments with *S. pasteurii* cells Mitchell and Ferris (2006b) found that the greater bacterial concentrations lead to the formation of larger and more insoluble crystals. Harkes et al. (2010) report an increase in urease activity and hydrolysis rate with increasing bacterial concentrations which was confirmed in an investigation by Okwadha and Li (2010). The latter report a 30 % increase in precipitated calcium carbonate when the cell concentration was increased from 10^6 to 10^8 cells/ ml.

2.3.4.2 Source of enzymatic activity

Due to its highly active urease enzyme, *S. pasteurii* has been the chosen bacterium for bio-cementation by many researchers. As outlined previously, a control over the strain and biomass is relatively easy in laboratory conditions however bacteria introduced to natural soils may face predation and competition from other organisms. This led several researchers to examine the possibility of using free urease enzymes as opposed to viable cells. A study by Bachmeier et al. (2002) found that enzyme induced precipitation is possible as the enzyme remains stable and active at a range of temperatures (4 – 60 °C), however a protective matrix, such as polyurethane (PU), is essential. In contrast,

Mortensen et al. (2011) reported no change in overall ureolytic activity upon cell lysis (i.e. release of free enzymes) in aqueous solution batch tests. However, the test was performed only for a period of several hours and the activity of the free enzymes was not examined for longer time periods at which their activity may reduce or cease. When applied to soil Zhao et al. (2014) conclude that bacterially-treated samples result in a much greater compressive strength (5 times more) than enzyme-treated ones.

Elucidation as to why this happened is lacking detail and is somewhat inconclusive, however.

An alternative source of enzymatic activity may also be natural microbial communities which can be stimulated to induce precipitation in field applications. As previously discussed, in publications by Fujita et al. (2008, 2010), Burbank et al. (2011), and Tobler et al. (2011) it was concluded that natural ureolytic communities can perform similarly to laboratory strains and the bio-stimulation technique (as compared to bio-augmentation) results in a greater depth at which the mineral can be formed.

2.3.4.3 Concentration of reactant chemicals

Among the most influential factors which can be easily controlled in laboratory and on-site applications of MICP are the concentrations of reactants. Application of concentrations of 6 mM to 1.5 M of urea, and 25 μ M to 1.5 M of calcium have been used (Warren et al. 2001; Ferris et al. 2003; DeJong et al. 2006; De Muyne et al. 2008b; Chunxiang et al. 2009; Okwadha and Li 2010; van Paassen et al. 2010; Burbank et al. 2011; Mortensen et al. 2011; Stabnikov et al. 2011; Cheng et al. 2016). In two studies by Al Qabany et al. (2012) and Al Qabany and Soga (2013) they found that the use of lower chemical concentrations and input rates (0.042 mol/l/h) results in an increased efficiency, more homogeneous distribution of CaCO_3 , and greater compressive strength of the treated soil. Although using lower concentrations in a large-

scale MICP treatment may require the injection of larger volumes of liquids and therefore make the process slower and more expensive it would prevent other problems connected to the clogging of pore space in the injection zones and inhomogeneous distribution of CaCO_3 when high substrate concentrations are used.

2.3.4.4 Transport of bacteria/ substrates

A very common method to study MICP treatment of soil in the laboratory is injection of fluids in one-dimensional sand packed columns. The flow rates at which bacteria, as well as the subsequent nutrients and chemicals, are supplied to the soil to induce precipitation have a profound effect on the homogeneity, attachment, and bridging of the biogenic crystallisation between soil particles.

As noted by Whiffin et al. (2007), on-field applications of MICP would require the use of low injection speeds for the fluids to avoid inducing physical stresses in the soil, resulting in possible fracturing. In their 5-meter-long column experiment they used a constant low flow rate of 0.35 l/h and, as previously outlined, observed a significant calcite precipitation gradient - high near the injection point and low further away from injection. This was attributed to a high chemical conversion rate in the vicinity of the injection point which meant most of the substrates were depleted at the injection area of the column limiting the availability of nutrients further away from the injection point. Harkes et al. (2010), Cunningham et al. (2011), and Mortensen et al. (2011) increased the flow rate to approximately 0.6 l/h and observed a better distribution of CaCO_3 which was due to the alteration in the balance of supply and conversion (i.e. reaction rates) of substrates (calcium and urea) (Phillips et al. 2013). It has to be noted that the reaction rates, or the speed at which biomass metabolises substrates and converts them to CaCO_3 , are also influenced by the substrates' concentrations. These were previously discussed in more detail in section 2.3.4.3.

2.3.4.5 Soil characteristics

The conditions in a specific soil and its engineering properties are often affected by historical sedimentary conditions (Harkes et al. 2010). Soil characteristics such as moisture content and saturation, particle size distribution, porosity, hydraulic conductivity may play a crucial role in the design, implementation, and success of a bio-engineered soil treatment (DeJong et al. 2013).

It has been shown that an MICP treatment performed to a soil under a low degree of saturation (20 %) results in stronger samples despite the lower CaCO_3 contents as compared to fully saturated samples (100 %) (Cheng et al. 2013). For a partially saturated soil the capillary and adsorptive forces result in the formation of a layer of moisture covering the individual soil particles while forming a menisci shape at the contact points. The rest of the pore space is taken up by air. For a fully saturated condition all of the pore space is filled with liquid. Therefore in the partially saturated condition the precipitation of CaCO_3 is expected to occur where the moisture is, i.e. at the contact points, thereby resulting in "cementing" of particles and increased strength (Tuller et al. 1999; Cheng et al. 2013).

Also important for the bio-treatment of soil are the mechanical interactions between bacterial cells and soil with varying particle sizes and at various burial depths. The survivability of bacteria has been shown to be greatly affected by the type of soil (fine to coarse grained sediments) and the overburden pressure (i.e. depth of soil). Rebata-Landa and Santamarina (2006) and Rebata-Landa (2007) found that there are three possible states for bacteria to be in. They can be "active and mobile", "trapped inside pores", and "dead" when confinement results in puncturing or squeezing. However, spore-forming species of bacteria may remain dormant even at great burial depths and significant confinement (Rebata-Landa and Santamarina 2006).

When assessing the applicability of a bio-engineered treatment based on the gradation of soil it has been shown that bio-treatments are appropriate for soils ranging from coarser clay soils, silt, through to sandy soils, i.e. the lower limit to bacterial cells transport is dependent on the pore and pore throat sizes which should be not less than 0.4 μm (Mitchell and Santamarina 2005; DeJong et al. 2006). In summary, bio-engineering solutions would be applicable to soils that have a sufficient pore space and pore throats to maintain microbial activity and substrate (nutrient and cementation solutions) mobility.

2.3.4.6 Soil heterogeneity and preferential flow

One of the most significant challenges for up-scaling any biogeochemical process has been outlined to be the subsurface heterogeneity and its major effect on uniform distribution of a bio-treatment material (DeJong et al. 2013). Heterogeneity (spatial, as well as temporal) is an inherent characteristic of natural soils and occurs, both in a large- and small-scale, as a result of a number of factors including physical, chemical, geological, and biological (Franklin and Mills 2009). The spatial heterogeneity of a soil has an effect on the flow of water and dissolved solutes. Subsurface flow can be described as “matrix flow” which is evenly distributed and occurs slowly through an average flow path, and “preferential flow” which occurs unevenly and fast through regions of enhanced flux. Such zones of higher permeability are due to stratification and presence of different soil fractions, erosion processes creating cracks and fissures, as well as wormholes or root channels (Abu-Ashour et al. 1994; Rosenbom et al. 2005). In relatively uniform man-made structures, such as embankments and earth dams, preferential flow may arise as a consequence of a physical damage, i.e. seepage, erosion, piping, or tension cracks (Knappett and Craig 2012). The size of the bacterial cells (usually around 0.5 to 2 μm) means they can be viewed as colloids (i.e. less than

10 μm) and are therefore affected by the same physical and chemical processes for solute transport through porous media, i.e. advection, dispersion, diffusion and sorption (Bradford et al. 2002; DeJong et al. 2006). For a bio-engineered treatment the transport of bacteria (if it is bio-augmented), nutrients, other substrates, and dissolved oxygen required for bio-precipitation of minerals would therefore be largely governed by preferential flow in the subsurface. As with other chemical grouting methods bio-grouting would firstly occur favourably within zones of higher permeability (DeJong et al. 2013). Although subsurface heterogeneity has been viewed mainly as a negative factor to a successful, homogeneously distributed MICP, studies by Martinez (2012) (cited in DeJong et al. (2013)) and Mugwar (2015) suggest that the MICP process may be “self-equilibrating”. That is CaCO_3 precipitation in heterogeneous systems occurs initially preferentially in zones of higher permeability (i.e. coarser soils) thereby reducing their hydraulic conductivity and sequentially re-directing to zones of lower permeability (i.e. medium and fine fractions). This effect of self-equilibration may mean that an MICP treatment would transform an initially heterogeneous soil to a more homogeneous one (in terms of geotechnical properties such as hydraulic conductivity) as coarser zones of higher conductivity would become less permeable, then the treatment would convert to the medium fractions, and finally into the fine fractions until a steady state is eventually reached.

2.3.4.7 Temperature and salinity

A positive relationship between ureolysis rates and temperature has been derived, with onset of calcite precipitation lagging at lower temperatures of 10 to 15 °C when compared to 20 to 30 °C (Ferris et al. 2003; Mitchell and Ferris 2005; Dupraz et al. 2009a). Furthermore free urease was shown to retain its ureolytic activity at significant ranges of temperatures, varying from 4 to 60 °C, especially when immobilised in a

protective carrier such as polyurethane (Bachmeier et al. 2002). Whiffin (2004) and van Paassen (2009) report an observed increase in urease activity up to a temperature of 60 °C. Even though temperature can play an important role on the ureolysis rates and time for precipitation onset it does not affect the pH increase and calcite precipitation rates (Ferris et al. 2003; Dupraz et al. 2009a). The kinetics of CaCO₃ precipitations are mostly affected by the salinity of the medium used to induce precipitation. It has been shown that increased salinities can lead to an increased alkalinity and calcite precipitation rates (Ferris et al. 2003; Dupraz et al. 2009a) as well as an increased adsorption of bacterial cells to the soil grains (Harkes et al. 2010). The salinity of the cementation media used in MICP applications can be easily controlled by adding or reducing the concentrations of substrates (e.g. calcium chloride). However, in field applications this should be done in an efficient and cost-effective manner ensuring minimum loss of substrates and optimised yield of bio-precipitation for the input concentrations.

2.3.4.8 Chemical saturation state and pH

In biologically influenced precipitation, as well as in abiotic conditions, the most important factors governing the onset of the crystallisation process are the saturation state of calcium carbonate and the activities (i.e. concentrations) of calcium and carbonate ions in solution (Ferris et al. 2003; Mitchell and Ferris 2005; Dupraz et al. 2009b; Tobler et al. 2011). Precipitation of a mineral begins once the critical saturation state is reached. The critical saturation state is an empirical value which determines how highly supersaturated the solution has to be (e.g. with respect to CaCO₃) before precipitation begins. In biological mineralisation the critical saturation state is greatly affected by pH changes which are governed by ureolysis rates, biomass, and presence of nucleation points, for example in the form of bacterial cells (Phillips et al. 2013).

Gollapudi et al. (1995) experimentally determined the relationship between rising pH during MICP with calcium carbonate precipitation and numbers of viable bacteria. They found that even though calcite is progressively being precipitated at rising pH (from 7 to approximately 9.8 in experiments with *S. pasteurii*) the number of bacteria increases up to a maximum at pH 8.6 and then gradually decreases. As reported by Stocks-Fischer et al. (1999) the bacterial urease enzyme has a low affinity to urea degradation at pH 7 and a high affinity to urea degradation at pH above 7.7. Peak hydrolysis activity was found to occur at around 8.5 where calcium carbonate precipitation is thermodynamically favourable. It should be noted that extreme pH (highly acidic or alkaline) can be harmful to bacterial cells and hinder their enzymatic and metabolic activity. Hence, encapsulation in a carrier, protective material is sometimes necessary as seen in applications of bacteria in concrete and cementitious materials where conditions are highly alkaline (pH 12.5 – 13.5) (Bang et al. 2001; Jonkers et al. 2010; Wang et al. 2012).

2.4 Microbial self-healing

2.4.1 Definitions of and prerequisites for self-healing

Historically, building materials have been designed to meet a certain predefined criteria and to last a definite amount of time and their design is based on the theory of “damage prevention”. To achieve longevity and durability their design and production involves careful control over the extent to which they can deform under loading or in time (Van der Zwaag 2007). Additionally, to achieve such durability in building materials (a typical design life of a structure is 50 years) usually expensive maintenance regimes have to be accounted for (Joseph et al. 2011).

In recent years, materials scientists have realised that a new philosophy in the design of materials is needed - one for “damage management”. Materials would undergo micro-

cracking but be able to repair themselves and restore their initial performance. Such materials would have a self-healing capacity through the inclusion of different healing components within them that activate when damage occurs. In the field of self-healing materials two different mechanism for self-repair have been defined, namely autogenous (natural) and autonomous (engineered) (Van der Zwaag 2007). These functions of man-made materials have mostly been investigated by materials scientists and have been attributed to polymers, ceramics, and metals used in metallurgy, construction, and the automobile and aerospace industries (Van der Zwaag 2007). Additionally, depending on whether or not a human intervention is needed for the self-healing mechanism to get activated self-healing can be either active or passive, respectively.

2.4.1.1 Autogenous and autonomous self-healing

The ability of natural materials to self-heal is called autogenous self-healing. Such materials can respond to environmental factors, sense damage, and replace and repair their structure repeatedly. Man-made materials, in contrast, often have a limited ability to change under environmental circumstances. Autogenous self-healing in anthropogenic materials has been observed to occur simply by coincidence without it being intentionally developed at the production stage. Examples of autogenous self-healing in some man-made materials are: in chromate coatings used for corrosion prevention where a damage and exposure of the ferrous substrate leads to a chemical reaction in which the chromate layer self-repairs and continues to protect the metal; and Roman mortar's ability to close its cracks. The closing of cracks phenomena occurs due to a chemical reaction and re-precipitation, crystallization, and growth of minerals, such as calcium carbonate and calcium silicate products because of the moisture ingress in those cracks (Van der Zwaag 2007).

Neville (2002) described the autogenous self-healing in concrete as the phenomenon of crack closure and restoration due to the presence of chemical compounds on the faces of the crack. The contact of such compounds with moisture triggers chemical reactions which result in the formation of filling materials closing the cracks (e.g. calcium hydroxide, calcium carbonate, and calcium silicate hydrates (CSH)). Studies which investigated autogenous self-healing in cementitious materials (mortar and concrete) include: Yang et al. (2011), Van Tittelboom et al. (2012), and Palin et al. (2015).

Autonomous self-healing, in contrast, involves the intentional manufacturing of components into a material to bring about mechanisms for healing of damage. For example engineering of such mechanisms in cementitious materials is often inspired by nature and the self-healing processes of the human body and include: shape-memory polymers, micro- and macro-capsules filled with a healing agent or vascular networks within concrete for the delivery of crack healing agents (e.g. methyl methacrylate, polyurethane, superglues, epoxy resins, sodium silicate, bacteria, and nutrients) (Van Tittelboom et al. 2011; Litina and Kanellopoulos 2014; Davies et al. 2015; Sharma et al. 2017; Kanellopoulos et al. 2017; Teall et al. 2017).

A differentiation should be made between self-sealing and self-healing processes. While the former is a reduction in permeability (in the cracks) after the damage, the latter implies recovery of mechanical properties (i.e. strength regain). Very often, especially in research publications for self-healing of concrete, a process which leads to blocking of cracks is incorrectly described as a self-healing process even though assessment on the mechanical properties is vague or undetermined.

More detail to the microbial self-healing in concrete is given in Section 2.4.2.

2.4.1.2 Prerequisites for microbial self-healing

For the microbial bio-cementation mechanism, MICP, to be modified into a self-healing one, the microorganism must be able to:

- i. bring about MICP and monolith formation due to its metabolic activity
- ii. survive prolonged periods under potentially adverse or hostile conditions due to its spore forming abilities
- iii. monitor continually its surrounding environment for occurrence of damage to the previously formed monolith and “heal” such damage
- iv. perform cycles of i, ii, and iii multiple times

Section 2.2 discussed the various metabolic pathways that lead to bio-mineralisation. In summary, as outlined by Boquet et al. (1973), under suitable conditions most bacteria can in fact form mineral precipitation. Therefore this section of the literature review will focus on the ability of microorganisms to form spores, survive prolonged periods in a senescent form under limiting conditions, and germinate back to vegetative life once favourable conditions occur. The implications of spore formation for microbial self-healing are also discussed.

Since the discoveries of Cohn (1876), Koch (1876), and Tyndall (1877), cited in Nicholson et al. (2000), it is now known that bacteria have developed a remarkable mechanism to endure unfavourable (for vegetative life) conditions through the formation of endospores. Spore formation is triggered in vegetatively growing bacteria when availability of nutrients (various forms of carbon, nitrogen, or phosphate) in their surrounding environment is exhausted. A spore is present in a dormant state in which metabolic processes are almost fully inactive (in order to preserve energy) until more favourable conditions occur in the environment (Nicholson et al. 2000; Setlow 2006). In

the dormant state bacterial spores are incredibly resistant to various physical extremes (even for prolonged periods) such as radiation, wet and dry heat, oxidising agents, desiccation, and vacuum. They are, in fact, considered to be the most resilient form of life found on Earth and this is attributed to a number of factors such as the genetic make-up of the species, the spore coat and spore core, water and mineral content in the core, and the ability of the spore to repair damage during germination (Nicholson et al. 2000). There are claims that *Bacillus* spores have been resuscitated after conservation in amber for 25-40 million years (Cano and Borucki 1995) and also in brine inclusion within a salt crystal aged 250 million years (Vreeland et al. 2000). Among the bacteria living in soils spore-forming bacteria are widespread - species include anaerobic heterotrophs, halophiles, microaerophilic lactate fermenters, anaerobes, sulphate reducers and phototrophs. Moreover, some bacteria are believed to enter spore form and spend a major part of their life in a dormant state before forming a vegetative organism again. This is particularly true for subsurface bacteria as soils pose significant challenges to its inhabiting micro-life such as heat/cold cycles (including freezing and thawing), physical stresses and contact with harsh chemicals, drought and desiccation, predation by other organisms, space confinement, and limited availability of electron acceptors and nutrients. Endospores are metabolically inactive; however they continually monitor their surroundings for available nutrients. Once nutrients are present the spores rapidly germinate into metabolically active vegetative cells once again.

2.4.2 Self-healing cementitious materials

In attempts to develop self-healing concrete bacterial cells were applied as a constituent of the concrete mix or as surface treatments in which they were applied along with nutrients on the exterior of concrete or mortar specimens (De Muynck et al. 2008a;

Chunxiang et al. 2009). Serious challenges arose from applying vegetatively growing bacteria within the concrete matrix such as physical strains during concrete mixing, the highly alkaline environment ($\text{pH} > 12$), a limited supply of oxygen and water, as well as a space confinement in the hardened concrete. In attempts to overcome those challenges some significant advances were made by self-healing materials research groups at Delft and Ghent universities. Preliminary studies conducted by Jonkers (2007), Jonkers and Schlangen (2007), and Jonkers et al. (2010) used spore-forming, extremophile (alkali-resistant, $8 < \text{pH} < 11$) bacteria embedded in mortar specimens along with an organic mineral precursor (e.g. calcium lactate). The sporulated bacterium was used in order to resist the physical and chemical stresses associated with mixing, space confinement, and high pH. Once a crack occurred in the concrete it would allow ingress of oxygen and nutrients to the spores and cause their germination and consequent precipitation of minerals in the crack. Although a significant amount of biogenic crystallisation could be observed in the cracks of young specimens (7 days) the direct contact of the spores to the “hostile” concrete environment meant they were only viable for a short term of 7 to 10 days. There was formation of crystallisation in the cracks of older specimens (28 days) however the majority of this crystallisation was produced abiotically, i.e. as a result of autogenous healing. In subsequent studies performed by Jonkers (2011) and Wiktor and Jonkers (2011) bacterial spores and nutrients were embedded into porous expanded clay particles and mixed in mortar-cement specimens. The presence of the clay matrix provided a protection to the spores so their viability increased from days to months. Additionally, a significant crack closure was yielded even of 100 days-aged specimens with bacteria-based mortar specimens closing cracks which were almost three times as wide (0.46 mm) as the ones (0.18 mm) closed by control samples. It should be noted that, as previously discussed, the closure of cracks should be referred to

as self-sealing rather than self-healing as no strength testing was again performed to assess mechanical recovery. The disadvantage of the method, presented by Wiktor and Jonkers (2011), was however that the expanded clay particles replaced half of the volume of the aggregates (sand and gravel) in the concrete mix which resulted in 50 % reduction in compressive strength of these samples compared to control samples without clay particles.

In a series of publications by Wang et al. (2012a; 2012b; 2013; 2014) they placed the focus on comparison of various cargo materials for the encapsulation of *Bacillus sphaericus* (a sporulating species of ureolytic bacteria) for self-healing concrete. The materials used for the encapsulation of spores included diatomaceous earth (DE), silica gel, polyurethane foam, hydrophilic gel (hydrogel), and microcapsules. Though certain cargo materials, such as the silica gel, were found to be more compatible with bacteria and maintain their viability for longer and better, the use of polyurethane foam was found to yield a better healing effect in terms of strength regain and permeability of mortar specimens (Wang et al. 2012b). The use of diatomaceous earth (DE), hydrogel, and microcapsules as carriers healed cracks with widths of 0.17 mm, 0.5 mm, and 0.97 mm, respectively. The higher healing potential of the microcapsules contributed to the robustness of the material in terms of flexibility at high humidity and brittleness at lower humidity. Such properties allow the microcapsules to survive the mixing process of concrete (while wet) but break easily (and release their contents thereafter) when cracking in the hardened concrete occurs. In terms of crack healing potential the addition of spore-containing microcapsules and nutrients in the mortar specimens yielded 48 to 80 % closure of cracks sized up to 970 μm , while control specimens (ordinary mortar with no additions; mortar with microcapsules; or mortar with microcapsules and nutrients) yielded 18 to 50 % closure of cracks widths up to 250 μm .

In summary, there is a significant effect of autogenous healing on the crack closure potential in mortar specimens, as seen in the studies by both Wiktor and Jonkers (2011) and Wang et al. (2014), and even though the addition of encapsulated bacteria and nutrients improved the healing efficiency of cracks this was paralleled by a significant negative impact of these additions on the mechanical properties of the mortar.

Microbial-based self-healing of concrete has shown much potential to reduce cracks in mortar and concrete however there are still aspects of these healing systems to be investigated. For example the addition of microcapsules or other carrier materials (expanded clay particles) have shown to impact the strength of mortar negatively in some cases to up to 50 % reduction in compressive strength. The extent to which concrete performance is recovered not only in terms of permeability reduction in the cracks (self-sealing) but also mechanical properties (self-healing) is an important aspect to be investigated. The majority of studies so far have demonstrated the encapsulation of the spores, their survival of the mixing and hardening of concrete, germination upon cracking, and deposition of minerals in the crack. However further investigations are needed to assess the viability of the bacteria in the longer term especially after repeated cycles of damage and healing. As outlined previously in 2.4.1.2 the ability of the bacterial cells to go through multiple damage and healing scenarios is of considerable importance in ensuring the long-term performance of a self-healing structure.

Knowledge on this ability of sporulating, calcite precipitating microorganisms would also largely govern the extent to which a self-healing mechanism requires external intervention and maintenance, for example whether addition of new bacteria will be needed after a specific number of healing cycles is reached.

2.4.3 Self-healing in soils and geotechnical structures

2.4.3.1 Autogenous self-healing in earth structures

Similarly to cement-based materials, autogenous self-sealing can also be observed in natural soils and geo-materials. Soils' self-sealing potential can be realised in terms of hydraulic conductivity reduction due to closure of macro-cracks. Certain fine-grained, very low plasticity soils or highly-swelling soils (bentonite) can self-seal with regard to hydraulic conductivity under freeze-thaw conditions. Freeze and thaw cycles can cause the formation of interconnected macropores which channel moisture flow and can increase significantly the hydraulic conductivity of the soil. For low-plastic soils fissures and fractures can be closed due to moisture flow which causes particle erosion and subsequent blocking of the void. In swelling soils the availability of moisture in the crack causes the particles to swell and therefore clog the cracks. These distinctive processes were investigated and described in detail by Eigenbrod (2003) and were explored for several different geotechnical and geoenvironmental applications such as self-sealing/-healing of landfill liners, earth dam or embankment clay-seepage liners (Shi and Booth 2005; Kakuturu and Reddi 2006; Sari and Chai 2013; Wang et al. 2013). Furthermore, several studies have commented on the possible action of penetrating plant or tree roots acting as reinforcement to potential landslide sites and chemical changes leading to the accumulation and precipitation of mineral phases which can block fissures and cracks in soil and prevent moisture ingress and further opening such cracks (Chandler 1969; Hutchinson 1969; Fleming and Johnson 1994; Anson and Hawkins 1998; Schmidt et al. 2001; Bromhead and Clarke 2003; Cornforth 2005; Tiwari et al. 2005, cited in Mesri and Huvaj-Sarihan 2012). Although such factors may lead to an increase in the factor of safety for historical landslides Mesri and Huvaj-

Sarihan (2012) note that their effect is uncertain in the long-term and is not documented well enough by field evidence to be considered a self-healing mechanism.

Also, as outlined by Li and Yang (2007) and Al-Tabbaa and Harbottle (2015), the downside of autogenous self-healing is that it is often sporadic, unreliable, with an unknown repeatability, and limited to small-scale damage situations. Therefore, engineered, autonomous self-healing systems are needed in the reliable prevention and repair of damage in geotechnical structures.

2.4.3.2 Engineered self-healing in earth structures

Though soils and geological materials are ubiquitous in construction, especially in infrastructure, and damage in such structures can be enormously difficult and costly to repair, research and development of autonomous self-healing mechanisms in particulate materials has been very limited thus far.

A few publications and patents have touched upon the self-healing abilities of “flexible” chemical grouts which can re-shape and repair micro- and macro-cracks in the subsurface after a damage (Harriet 1987; Ahrens 1999; Beihoffer et al. 2009). The patent by Beihoffer et al. (2009) describes the manufacture of a layered geomembrane which can provide an effective impermeable barrier for high-conductivity salt water (to be used in waterproofing structures and foundation surfaces near such high-conductivity water) and can self-repair in the case of a rupture of the outer layer. The principle of work of the self-repairing geo-membrane is based on an outer layer of highly-impermeable cover sheet and an inner highly-swellable mat of cross-linked polymers. In the case of a rupture of the cover sheet the water entering the second layer of the geo-membrane reacts with the swellable mat and seals the damaged area.

The emergence of the field of bio-geotechnics for soil stabilisation, however, has the potential to reform entirely the way infrastructure is built and operates toward more sustainable and economic ways (DeJong et al. 2013). Biological methods for soil improvement and stabilisation can also provide a built-in healing mechanism. First attempts at demonstrating the damage healing capacity of bio-cementing bacteria in soil were performed by Montoya and DeJong (2013) and Harbottle et al. (2014). The two studies examined the response of MICP-treated sand (using *S. pasteurii* bacterium) to damage when nutrients were further supplied. Both studies adopted similar approaches of performing a bio-treatment of sand after which the shear strength characteristics of the soil improved. Following a damage event (triaxial compression or shear vane) which lead to bio-cement degradation, the soil was further supplied with nutrients to aid the recovery of metabolic activity of the bacteria already present in the samples. The healing treatment resulted in the soil recovering or exceeding its initial shear strength (achieved after the first bio-treatment). In both studies, however, the second treatment with nutrients was performed directly after the first treatment and the shearing, rather than after a prolonged period which suggests that the bacteria remaining in the samples from the initial treatment was still viable and produced more calcite to heal degraded bonds and so recovered the pre-shearing condition.

Preliminary work has also been carried out (Duraisamy 2016) which demonstrated the potential of another ureolytic spore-former, *Bacillus megaterium*, to bring about damage recovery to pile foundations. Spore formation was however not specifically studied, for example with microscopic evidence and quantification of sporulation kinetics in *B. megaterium*. It is also unclear whether the bacterium sporulated readily or required sporulation media and special conditions. Therefore, it is unknown whether the bacterium had formed spores during the process of bio-cementation. Additionally, as

Duraisamy (2016) supplied the bacterium with nutrients immediately after the damage was initiated (at the end of the bio-treatment) in the bio-cemented soil specimens the “healing” effect may not necessarily, similarly to above, be attributed to the spores as the bacteria were likely to have been still active.

Though these publications did not investigate spore presence and possibility for reactivation of the microbial treatment in the long-term they demonstrated that significant mechanical damage recovery and healing in the short term is possible via MICP processes.

2.5 Summary

Bio-mineralisation processes have been widely used in research for soil stabilisation and immobilisation of contaminants in soil and groundwater as well as for alternative solutions for carbon dioxide storage. Such processes have been shown to work in small-scale laboratory conditions using laboratory-grown strains of bacteria but also at larger scale field conditions using indigenous organisms as well as under various extreme environmental conditions. A limited number of studies showed that recovery of mechanical properties due to healing is possible after a physical damage has been introduced in an initially bio-cemented body of soil; however the effect was only studied in the short-term and could be ascribed to active vegetative bacteria introduced to the samples originally.

The concept of biological self-healing has been studied much more widely in a different class of construction materials - mortars and concrete - which do not naturally host microbes and are, in fact, much more hostile and challenging for microbial vegetative life. Nevertheless, calcium carbonate precipitating bacteria have been applied to concrete in attempts to develop self-healing mechanism through sealing of microcracks

and thereby preventing the ingress of corrosive substances to the reinforcement. For applications in cementitious materials bacterial strains have been specifically isolated from extreme environments to endure alkaline, also load, and dehydration extremities through the formation of spores. In such applications the placement of the bacterial spores in a protective carrier material (e.g. microcapsules) is essential for their survival in the concrete matrix.

MICP processes in soils have been successfully applied to create a brittle monolithic material consisting of soil particles, bonded by calcium carbonate, which will however undergo deformation in time under physical or chemical stresses in the subsurface. Such damages can include cracks and shear surfaces in the bio-stabilised soil, thereby resulting in the formation of fluid passages and loss of mechanical performance. MICP processes can be altered to contain a self-healing mechanism similarly to the previously described self-healing of concrete. Soil, as opposed to concrete, is a natural habitat for a ubiquitous number of bacteria, including those that can precipitate minerals, as well as exist in a senescent state in a spore form. Therefore a spore-forming bacteria in the soil can produce a bio-mineral in the process of which it will become entrapped in its own crystal and will turn into a dormant state of life (i.e. form a spore). Such mineral-encapsulated spores in the soil will be able to react to a damage (e.g. cracking) in their immediate environment which will release them from the capsule and allow them to turn to vegetative life once again (if there are suitable conditions available) and heal the damage. This mechanism has the potential for providing multiple cycles of healing in the long-term as bacterial spores have been shown to survive extended periods of time (hundreds of years) without losing their abilities to germinate and form a vegetative bacteria. Research into the possibility for MICP processes to be applied as a self-healing

instrument for geotechnical and geoenvironmental applications is therefore relevant and novel.

3 Experimental Apparatus, Materials and Methods

3.1 Bacterial strains and materials

This section describes the strains of bacteria used in this study as well as the methods and media used for sporulation of the different strains.

The type and origins of the soil used in the particulate media experiments, the testing methods for its classification, and preparation prior to use in experiments, are also presented.

3.1.1 Bacterial strains

The bacterial strains used in this study were selected based on the “prerequisites” for self-healing which were previously outlined in section 2.4.1.2. These prerequisites served as the criteria for the laboratory bacterial strains. They can be briefly summarised in the following: the bacteria must be able to cause mineral precipitation; must be able to survive prolonged periods of encapsulation (in the mineral) and starvation due to its spore forming abilities; must be able to survive damage to the mineral and consequently heal the damage; finally the bacterial strain must be able to undergo cycles of the previous four in both aqueous and particulate media.

3.1.1.1 *Sporosarcina pasteurii*

S. pasteurii is an aerobic bacteria isolated from soil which possesses a highly active urease enzyme. The organism was formerly known as *Bacillus pasteurii* (Yoon et al. 2001). A freeze-dried culture was obtained from the National Collection of Industrial and Marine Bacteria, UK (NCIMB 8221, ACDP Group 1). The bacteria was maintained either in a glycerol stock at -20° C or a liquid culture stored at 4° C (for up to two weeks) and were used for inoculation of liquid media. Glycerol stock was prepared

occasionally (every 3-6 months) from exponentially growing bacteria (1:1 ratio bacteria and glycerol) and was frozen at -20° C for long-term storage. The medium used for culturing contained per litre of deionised water: 13 g Oxoid CM0001 nutrient broth and 20 g urea (filter sterilised). Flasks containing bacterial solution were placed in a Stuart SI600 orbital shaking incubator at 150 rpm for 24 hours. When cultures were used experimentally they were grown until they reached late exponential phase determined by measurement of optical density (section 3.2.3). For the induction of calcium carbonate precipitation the bacteria were re-suspended in a medium (referred to as “cementation medium” in this work) which contained per litre of deionised or tap water (depending on experiment): 3 g Oxoid CM0001 nutrient broth, 10 g NH₄Cl, 2.12 g NaHCO₃, 7.35 to 147 g CaCl₂.2H₂O (depending on experiment) and 20 g urea (filter sterilised); pH was adjusted to 6.5. This medium was adopted from DeJong et al. (2006), and originally presented by Stocks-Fischer et al. (1999)). Prior to utilising the strain in experiments the fully grown cultures were pelleted by centrifuging at 3200 RCF for 20 min in a Heraeus Varifuge 3.0 Centrifuge (Heraeus Medical GmbH; Wehrheim, Germany). The supernatant was discarded and exchanged with the same volume of phosphate buffered saline (PBS) which contained per litre of deionised water: 8 g NaCl, 1.42 g Na₂HPO₄, 0.24 g KH₂PO₄, pH 7.2. Prior to further use cultures were centrifuged twice in fresh PBS in order to remove metabolic wastes from the bacterial suspension.

Due to limited information on the exact conditions in which *S. pasteurii* forms spores various methods and media were examined for use in this study. In summary, *S. pasteurii* was subjected to various media which do not advocate growth and the bacterium was expected to therefore sporulate. A table summarising the different methods is presented herein (Table 3-1).

Table 3-1 Table summary of sporulating media and methods used with *S. pasteurii*

Media	Details of procedure	Components of medium	Reference
Growth medium with prolonged incubation	Bacterium was inoculated in medium and incubated at 30° C or at room temperature for up to 2 weeks	13 g Oxoid CM0001 nutrient broth, 20 g urea (filter sterilised)	Schaeffer et al. (1965)
Growth medium amended with manganese (with prolonged incubation)	Bacterium was inoculated in medium and incubated at 30° C or at room temperature for up to 2 weeks	13 g Oxoid CM0001 nutrient broth, 20 g urea (filter sterilised)	Atlas (1995) DSMZ (2017)
Highly alkaline sporulation medium used for various strains	Bacterium initially cultivated in growth medium for 4 to 5 hours and transferred to highly alkaline medium; incubated for up to a week	0.1 g yeast extract, 0.2 g NH ₄ Cl, 0.02 g KH ₂ PO ₄ , 0.225 g CaCl ₂ , 0.2 g KCl, 0.2 g MgCl ₂ .6H ₂ O, 0.01 g MnSO ₄ .2H ₂ O, 1 ml Trace Element Solution SL12B, 5.16 g Citric Acid Trisodium Salt, 4.2 g NaHCO ₃ , 5.3 g Na ₂ CO ₃ , pH was close to 10	De Belie and De Muynck (2009) Jonkers et al. (2010)
Complex medium used with <i>B. subtilis</i>	Bacterium initially cultivated in growth medium for 4-5 hours and transferred to the complex medium; OR bacterium directly inoculated in complex medium and incubated for up to a week	8 g Oxoid CM0001 nutrient broth, 1 g KCl, 0.25 g MgSO ₄ .7H ₂ O, 0.002 g MnCl ₂ .4H ₂ O. After adjusting the pH to 7.0 and autoclaving, sterile CaCl ₂ and FeSO ₄ were added to 5x10 ⁻⁴ M and 1x10 ⁻⁶ M, respectively; MnCl ₂ .4H ₂ O, 0.002. After adjusting the pH to 7.0 and autoclaving, sterile CaCl ₂ and FeSO ₄ were added to 5x 10 ⁻⁴ M and 1x10 ⁻⁶ M, respectively	Schaeffer et al. (1965)
MacDonald sporulation medium used with <i>S. ureae</i>	Bacterium initially cultivated in growth medium for 4-5 hours and transferred to the complex medium; OR bacterium directly inoculated in complex medium and incubated for up to a week	2 g yeast extract, 3 g peptone, 4 g glucose, 1 g K ₂ HPO ₄ , 3.238 g NH ₄ Cl, 0.13 g CaCl ₂ .2H ₂ O, 0.92 g MgSO ₄ .7H ₂ O, 0.112 g MnSO ₄ .7H ₂ O, 0.001 g FeSO ₄ .7H ₂ O, 0.018 g ZnSO ₄ .7H ₂ O, CuSO ₄ .5H ₂ O, pH adjusted to 9	Zhang et al. (1997), originally from MacDonald and MacDonald (1962)

3.1.1.2 *Bacillus cohnii*

B. cohnii is an alkaliphilic spore-forming organism which has been used in research for self-healing concrete due to its high pH tolerability (Wiktor and Jonkers 2011; Sharma et al. 2015). The bacterial species were provided by Dr Trupti Sharma from Bath University; purchased from DSMZ culture collection Germany (DSM 6307). The bacteria was maintained in a glycerol-stock at -20° C (for long-term storage) or a liquid culture stored at 4° C (for up to two weeks) and were used for inoculation of liquid media. *B. cohnii* was grown in Luria-Bertani medium which contained per litre of deionised water: 10 g tryptone, 5 g yeast extract, 5 g NaCl, 4.2 g NaHCO₃, 5.3 g Na₂CO₃ (Atlas 1995). The last two components were added by filter sterilisation after autoclaving; pH of the medium was 9.7.

Modified B4 medium was used for calcium carbonate precipitation which contained per litre of deionised water: 4 g yeast extract, 10 g dextrose, 2.5 g calcium acetate, with pH adjusted to close to 9 (Boquet et al. 1973).

Induction of sporulation in *B. cohnii* was achieved through a highly alkaline mineral medium which contained per litre of deionised water: 0.1g yeast extract, 0.2 g NH₄Cl, 0.02 g KH₂PO₄, 0.225 g CaCl₂, 0.2 g KCl, 0.2 g MgCl₂.6H₂O, 0.01 g MnSO₄.2H₂O, 1 ml Trace Element Solution SL12B, 5.16 g Citric Acid Trisodium Salt, 4.2 g NaHCO₃, 5.3 g Na₂CO₃, pH was close to 10 (Jonkers et al. 2010).

Prior to utilising in experiments the bacterial suspension of *B. cohnii* was pelletised and washed twice in a PBS solution for removal of metabolic wastes.

3.1.1.3 *Sporosarcina ureae*

Another urease-positive, spore forming, aerobic organism used was *S. ureae*. The bacterium was obtained from the National Collection of Industrial and Marine Bacteria, UK (NCIMB 9251, ACDP Group 1) as a freeze-dried culture. The bacterium was maintained in a glycerol-stock at -20° C (for long-term storage) or a liquid culture stored at 4° C (for up to two weeks) and was used for inoculation of agar plates or liquid cultures. The medium used for culturing (DSMZ 2017) contained per litre of deionised water: 5 g peptone, 3 g meat extract and 20 g urea (filter sterilised).

For the induction of calcium carbonate precipitation the bacteria were re-suspended in a “cementation medium” (as previously defined in 3.1.1.1). When induction of sporulation was required the procedure specified in Zhang et al. (1997) was followed, using a McDonald medium (Atlas 1995, originally by MacDonald and MacDonald 1962). *S. ureae* inoculum was initially introduced to its growth medium and was incubated for 4 to 5 hours until exponential phase began. Then the bacterium was pelletised, washed in PBS, and transferred to McDonald medium and incubated for another 15 to 20 hours. MacDonald medium contained per litre of deionised water: 2 g yeast extract, 3 g peptone, 4 g glucose, 1 g K₂HPO₄, 3.238 g NH₄Cl, 0.13 g CaCl₂·2H₂O, 0.92 g MgSO₄·7H₂O, 0.112 g MnSO₄·7H₂O, 0.001 g FeSO₄·7H₂O, 0.018 g ZnSO₄·7H₂O, CuSO₄·5H₂O, pH adjusted to 9.

Prior to utilising in experiments, the bacterial suspension of *S. ureae* was pelletised and washed twice in a fresh PBS solution for removal of metabolic wastes.

3.1.2 Soil Classification and sand column preparation

The soil used for all experiments in this research was a silica sand from Leighton Buzzard (Hepworth Minerals and Chemicals Ltd). A range of classification tests were

performed on the sand prior to utilising it in experimental work. The methods for soil classification were conducted according to the British Standards Institution, BS 1377: 1990 - Parts 1, 2 and 4 (British Standards Institution (BSI) 1990a; 1990b; 1990c). Results from the classification tests are presented in section 5.2.

3.1.2.1 Particle Size Distribution

The particle size distribution curve was determined by the Dry Sieving Method described in BS 1377 - Part 2:1990 using sieves of sizes: 1.18, 1.00, 600, 425, 300, 212, 150 and 0.063mm.

3.1.2.2 Specific Gravity of Soil Particles

The Specific Gravity of the silica sand was determined by the Small Pyknometer Method described in BS 1377 - 2:1990. The specific gravity of the soil particles was taken as the average from three measurements.

3.1.2.3 Maximum/Minimum Dry Density and Optimum Moisture Content

The relationship between maximum dry density and optimum moisture content for the silica sand was determined using the 2.5 kg Rammer Method according to BS 1377 - 4: 1990. The method included the compaction of the soil into a mould with a known volume at a range of different moisture contents (from 2 to 20 % approximately). The point at which the curve peaked on the graph of dry density to moisture content was taken as the maximum dry density and the corresponding moisture content was taken as the optimum moisture content.

Additionally, minimum dry density of the soil was determined according to BS 1377 - 4: 1990 using a 1 l glass measuring cylinder. The values for maximum and minimum

dry density of the sand were used to calculate the theoretical maximum and minimum void ratios and the mass of soil for a given target relative density in the sand column experiments.

3.1.2.4 Soil preparation

The sand was washed with 3 % of HCl in deionised water overnight to remove carbonates then thoroughly washed with deionised water through a 0.063 mm sieve, its pH adjusted to neutral with NaOH, followed again by thorough washing with deionised water. Prior to utilising in experiments the sand was dried at 105° C and sterilised by autoclaving (121° C, 1.3 bar for 15 min).

3.2 Analytical techniques

A range of analytical methods were used to evaluate microbiological activity and its effect on the surrounding environment in both aqueous solution and soil pore water. Sections 3.2.1 to 3.2.11 describe the microbiological techniques and microscopy methods, as well as chemical analyses and geotechnical tests of soils, used throughout this work.

3.2.1 pH monitoring

pH measurements of freshly grown bacterial cultures and of discharged liquid from sand samples were routinely performed as an indication of bacterial activity. Aqueous samples were syringe filtered (0.2µm filter pore size) to remove bacterial cells and precipitates present. pH was measured to 3 decimal places using a Mettler Toledo Seven Excellence pH meter (Switzerland).

3.2.2 Calcium ion concentration in aqueous samples

Aqueous samples were syringe filtered (0.2µm filter pore size) to remove bacterial cells and precipitates present. Calcium ions concentration was then determined using Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) Optima 2100 DV ICP-OES Instrument (Perkin Elmer Inc., Waltham, US). Samples with an unknown concentration were measured against calcium calibration solutions in the range of 1 to 100 mg/l at a wavelength of 317.933 nm. All samples were diluted prior to ICP-OES analysis so they would fall in the specified range.

3.2.3 Optical Density

A spectrophotometric method for determination of cell concentrations in bacterial cultures was used. Optical density was determined by placing 1 ml of the solution under test in an optical polystyrene (PS), 1.6 ml cuvette before recording the optical density (light absorbance) of the specimen at a wavelength of 600 nm in a Hitachi UV-1900 UV-visible wavelength spectrophotometer (Tokyo, Japan). The absorbance measured at a 600 nm wavelength (OD600) is a logarithmic ratio of the transmitted light and the absorbed or scattered light by the specimen. Optical density (or turbidity) of bacterial suspensions is a reflection of number of bacterial cells present in the solution. As bacterial cell numbers increase so does the optical density of the suspension. OD600 measurements were used to assess bacterial growth, to determine cell numbers at onset of experiments, and to construct growth curves of bacteria.

3.2.4 Optical density and cell counts correlation

The reading for optical density (which includes both dead and live cells) could be correlated to number of live cells in suspension determined by cell counts. Direct visualisation under an epi-fluorescent microscope of actively respiring bacteria was enabled by the use of a redox dye, called CTC (5-Cyano-2, 3-di-(p-tolyl) tetrazolium

chloride). CTC was obtained from Sigma-Aldrich, Dorset, UK. The bacterial staining procedure was taken from Rodriguez et al. (1992). A batch solution of the CTC dye was first prepared in deionised water to usually 10-fold the required concentration and was then mixed with diluted in PBS bacterial solution. The required final concentration of CTC was 2-5 mM. The samples were incubated at 30° C for 3-4 hours which allowed the live cells to reduce the dye to fluorescent CTC-formazan. An aliquot of 10 µl was placed on a glass microscopic slide and was covered with an 18 x 18 mm glass cover slip. The slides were observed under a Nikon Eclipse LV100 fluorescent microscope (Tokyo, Japan) with a 20x lens. The CTC-formazan present in the living cells was excited at 365 nm and emitted red/orange light at 650nm wavelength. The number of live cells in the original solution was back-calculated using the following equation (modified from Boulos et al. (1999)):

$$N = \frac{n \times A \times D \times 100}{a} \quad (\text{Eq. 7})$$

Where:

N - Number of cells per ml of bacterial suspension

n - Average of number of cells counted from 10 random locations on the glass slide

A - Area of glass cover slip, mm²

D - Dilution factor

a - area of field under the microscope (depends on the objective used), mm²

It should be noted that a countable number of cells (either by flow cytometry or under the microscope) is considered to be between 30 and 300 (Humphries 1974). Therefore, appropriate dilutions of the bacterial suspensions were prepared in order to fall in the

specified range when the stained smears were examined under the microscope.

Correlation between optical density and viable number of cells for *S. ureae* is presented in section 4.3.1.

3.2.5 Schaeffer-Fulton spore staining

Spore staining of bacterial cultures was performed according the method proposed by Schaeffer and Fulton (1990) with the use of a Schaeffer and Fulton Spore Stain Kit (purchased from Sigma Aldrich, Dorset, UK). The staining procedure involved preparing diluted bacterial suspensions and air-drying smears of those onto glass slides. The smears were heat-fixed with a gas burner. The slides were covered with blotting paper (cut in size just to cover the smear) and a malachite green solution was poured on top. The slides were steamed for 3-5 minutes (time required for the dye to penetrate through the spore wall) followed by thorough washing off the dye with tap water. A second staining solution – safranin - was then poured and left to stay for 30 sec before washing. The slides were dried with tissue paper and observed under a 100x Nikon oil immersion lens with transmitted illumination. Bacterial cells appeared bright red and spores appeared green under the microscope.

3.2.6 Phase-contrast microscopy

Phase contrast microscopy was used to show sporulation in bacterial cultures. It was undertaken in the Bioimaging Unit, Cardiff School of Biosciences. Unstained microbiological samples were examined under phase contrast optics with a 40x oil immersion lens using an Olympus BX41 phase contrast microscope (Olympus, Tokyo, Japan). Due to the differences in density of the cells and spores, the phase contrast method allows observable differentiation between sporulated and non-sporulated cells.

3.2.7 Scanning Electron Microscopy

Dried and powdered calcium carbonate, produced by bacteria, was visualised by a dual beam Scanning Electron Microscope (SEM) model XB1540 (Carl Zeiss, Germany) located at the Cardiff School of Engineering. Samples were coated with Au/Pd (80/20) using a sputter coater (Agar Scientific, Stansted, UK).

3.2.8 Powder X-Ray Diffraction

The mineralogy of the newly formed bacterial material was determined by an X-Ray Diffraction (XRD) Instrument PW1710 Phillips (Amsterdam, Netherlands). Prior to running the XRD analysis samples were oven-dried at 105 ° C and powdered with a mortar and pestle, they were then placed in a zero-background silicon sample holders. The goniometer was calibrated using a silicon standard. Samples were analysed using PANalytical software (Almelo, Netherlands), with goniometer start and end angles at 5.00 and 80.00 2 ° Theta, step size of 0.020 2 ° Theta and scan step time of 0.5 s. The generator was adjusted at 40 mA and 35 kV.

3.2.9 Determination of precipitated mass

In aqueous solution

For the determination of amount of CaCO₃ precipitated by bacteria, grown cultures were suspended in cementation medium and incubated in a shaking incubator for up to 7 days. The precipitate which formed was pelletised using a centrifuge (20 min at 3200 RCF) its supernatant was then exchanged with deionised water to wash out metabolic wastes and nutrients. The centrifuging and deionised water washing was repeated up to 4 times. The contents at the bottom of the tubes were oven-dried at 105 ° C overnight and dry mass recorded as amount of CaCO₃ precipitate. It has to be noted that the described method assumes the mass measured after oven drying to be only due to mass

of precipitate. In reality biomass would also be present. However, it was considered negligible in quantity and was thereafter disregarded.

In sand columns

Following bacterial healing and strength testing of the sand columns, distribution of precipitate within the specimens was determined according to a modification of the method outlined in BS 1377 - 3: 1990 (British Standards Institution 1990c), with an ignition temperature of 900° C (Heiri et al. 2001). Samples of approximately 10 g of sand were taken from several locations along the length of the column (inlet, middle, and outlet) and dried at 105° C overnight (for removal of moisture). The mass of the container (m1) and container with the sample before ignition (m2) were recorded. The samples were put in a furnace at 900° C for 24 hours to allow for all calcium carbonate, biomass, and volatile substances in the sand, to decompose. The furnace was turned off and the samples were allowed to cool down before the mass was again measured (m3, mass of container and sample after ignition). The amount of precipitated CaCO₃ (m4 in percent by mass) was then determined according to the following:

$$m4 = \frac{m2-m3}{m2-m1} \times 100 \quad (\text{Eq. 8})$$

The mass lost after ignition at 900 ° C was used as a measure of the amount of calcium carbonate precipitated due to the bacterial treatment.

3.2.10 Hydraulic conductivity of sand columns

The permeability of sand columns was measured using a modified Falling Head Permeability Test (Head, 1994). A falling head test, as opposed to a constant head test, was chosen as an appropriate method for hydraulic conductivity measurement of the sand samples. Pure sand samples (before the bio-treatment was initiated) were suitable

for a constant head test as their hydraulic conductivity was between 4 and 6×10^{-5} m/s. However their conductivity dramatically decreased to between 0 and 2×10^{-5} m/s when significant bio-cementation was achieved. Therefore, a falling head test was more applicable and was performed for all samples when bio-treatment was taking place.

A 6mm diameter, 60 cm-long standpipe tube was connected to the sand column samples via silicone tubing which allowed for in-situ testing of the samples in the incubator.

Hydraulic conductivity was derived from the drop in the level of water per unit time in the standpipe tube. The falling head standpipe tube is opened to atmospheric pressure at the top, hence - the falling of the head of water in the duration of the test. The test was performed bottom up in the columns as this was the direction of flow used with the peristaltic pump. A schematic of the test set up is shown in Figure 3-1.

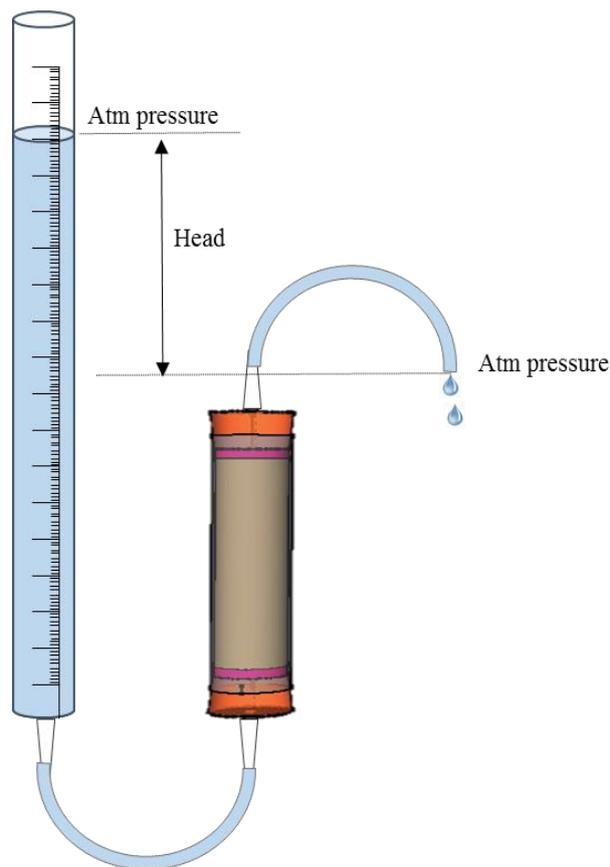


Figure 3-1 Falling head test of sand columns

Flow rate (Q) was measured through the drop in water in the standpipe cylinder which allows for hydraulic permeability (k) calculation using Darcy`s law. Hydraulic conductivity was calculated using the following equation:

$$k = 3.84 \times 10^{-5} \frac{a \times L}{A} \log_{10} \frac{h_1}{h_2} \times \frac{1}{t} \quad (\text{Eq. 9})$$

Where:

k – Hydraulic conductivity of sand in column, m/s

a – Cross-sectional area of standpipe, mm^2

A – Cross-sectional area of sand column, mm^2

L – Length of sand column, mm

h_1 – Point on standpipe at start of test, mm

h_2 – Point on standpipe at end of test, mm

t – Time for fluid to drop from point h_1 to h_2 , min

It has to be noted that this method for measurement and calculation of hydraulic permeability of sand samples disregards head losses due to friction of the whole system which contains flexible rubber tubes (tubing length was minimised (<0.5 m)) and connections and assumes the head losses are only due to the resistance of the soil mass.

In order to estimate head losses in the flexible rubber tubes the Darcy-Weisbach relationship for clean new pipes was used (Chadwick and Morfett 1986):

$$h_L = \frac{\lambda L}{D} \times \frac{V^2}{2g} \quad (\text{Eq. 10})$$

Where:

h_L – Head loss, m

λ - Frictional factor related to the roughness inside the pipe (taken as 0.006 for flexible rubber tubing (Chadwick and Morfett 1986))

L – Pipe length, m

D – Pipe diameter, m

V – Average velocity of fluid in the pipe, m/s

g – Gravitational acceleration, m/s^2

For a flexible rubber tube of 0.5 m length and 3 mm diameter, carrying water, the head loss, calculated by the Darcy-Weisbach equation, was found to be 1.12×10^{-6} m, or 1.12 μm . The head losses in the tubes were therefore considered negligible and were not considered in the hydraulic conductivity calculations.

The fluid used for permeability testing was sterile PBS, in order to avoid inducing of osmotic stresses to the cells caused by deionised water.

3.2.11 Unconfined compression of sand columns

Unconfined compressive strength testing was performed on the bacterially treated sand samples ($d=64\pm 0.5$ mm, $h=37.5\pm 0.5$ mm). A Stepless Compression Test Machine Wykeham Farrance (England) was used, along with a 500 N capacity load cell, and a spring LVDT (linear variable displacement transformer) to measure vertical strains.

Loading was performed at a rate of 1.3 mm/min (in accordance with 7.2 of BS 1377 - 7: 1990; (British Standards Institution 1990e)) until 20 % vertical strain. The confining effect from the membrane was found to be a maximum of 2 kPa at 20 % strain

according to Fig. 11 from BS 1377 - 7: 1990. Measurements were taken every second using a data logger (each test was between 13 and 15 minutes in length).

3.3 Experimental structure

3.3.1 Introduction

The experimental structure described in the subsequent sections followed the aims and objectives of the project outlined in chapter 1 - the overall aim was to develop self-healing capabilities in soils using microorganisms. The experiments were constructed in such a way as to understand the basic science behind self-healing using experiments in idealised conditions (aqueous solution) and then to attempt at transferring the self-healing mechanism to a more complicated environment such is sand.

Initially, a selection was performed for a suitable organism which can cause the precipitation of CaCO_3 due to its metabolic activity. Precipitation abilities were studied in suitable “bio-cementation” induction media based on each organism`s metabolic and nutritional requirements. Parameters such as pH, available calcium ion in solution, visual observation of precipitation, microscopic observation, as well as crystallographic properties of the minerals served to analyse and verify the activity of the bacteria and ability to cause crystallisation. The organism had to be able to survive prolonged periods of starvation and harsh conditions due to its spore-forming abilities. Similarly to bio-precipitation, spore formation was studied in media which support sporulation and is specific for each strain. Spore-forming abilities were studied through the use of spore-staining and various microscopic methods. Once a spore- and CaCO_3 -forming organism was selected, the self-healing process was studied in idealised conditions in aqueous solutions. Self-healing involved the cycling of sporulation, carbonate precipitation, damage to precipitate to expose the spores, germination and regeneration of metabolic

activity. Additionally, long-term survivability of the crystallised spores was also examined. Similarly to above, parameters such as pH and calcium ion, and optical density served to analyse and verify the workings of the process.

Secondly, the ability of the chosen organism to cause monolith formation in sand (i.e. sand and bio-precipitation forming a solid body) within an acceptable time period for a laboratory study (1 to 2 months) was examined. To accelerate the bio-consolidation process, a range of substrate concentrations (calcium and urea) in the cementation media were studied. Batch experiment, complemented by a geochemical model, as well as dynamic flow column experiments were used to determine the most suitable concentrations for optimised sand treatment periods. Available calcium ion in solution, pH, mass loss on ignition, and geotechnical parameters (hydraulic conductivity and unconfined compression testing) were used for analysis.

Finally, the self-healing process was examined in an initially bio-cemented sand in sand columns. The ability of the sporulating bacteria to survive the bio-cementation process and in response to a damage to germinate, regenerate into vegetative bacteria, and cause healing of the damaged monolith was examined. Two types of damage were studied – chemical (acidic flow) and physical (breaking due to compression loading). Damage healing was investigated through pH and calcium changes in the sand pore fluid along with geotechnical changes such as hydraulic conductivity, mass loss on ignition (measurement of precipitated carbonate), and unconfined compression testing.

3.3.2 Self-healing in aqueous solution

3.3.2.1 Organism selection

This section describes the selection of suitable bacteria to satisfy the objectives of the project (chapter 1). The three strains, described previously in section 3.1, were subjected

to sporulation media and their spore-forming abilities were assessed using the Schaeffer-Fulton method for spore staining (section 3.2.5) and phase-contrast microscopy (section 3.2.6). The results from the organism selection experiments are presented in section 4.2.

3.3.2.2 Growth kinetics of *S. ureae*

The growth kinetics of *S. ureae*, when inoculated in growth and cementation media, were investigated. Growth curves were constructed using optical density measurements (OD₆₀₀, section 3.2.3) and these were correlated to viable cells in solution (section 3.2.4). The results from the growth curve experiment with *S. ureae* are presented in section 4.3.

3.3.2.3 CaCO₃ formation with *S. ureae*

X-Ray Diffraction (XRD, section 3.2.8) and Scanning Electron Microscopy (SEM, section 3.2.7) were the analytical methods used to analyse and categorise the crystalline material formed by *S. ureae* in cementation medium in aqueous solution. The results of the XRD and SEM analysis are presented in section 4.4.

3.3.2.4 Regeneration of bio-cement entombed spores

The aim of this experiment was to demonstrate that a bacterial spore, encapsulated in carbonate precipitate in the process of bio-cementation, is able to respond to damage of the precipitate, germinate into a vegetative bacterium and regenerate its ureolytic activity to precipitate further calcium carbonate. To demonstrate that only spores were able to go through the process of bio-cementation and extreme conditions (autoclaving was used to simulate extreme conditions in laboratory conditions) and germinate once they were exposed from the crystal, a range of different control conditions were

included. Additionally, testing of both a sporulating and non-sporulating organisms was carried out. The three conditions were as follows:

a) Testing the response of pure cultures (i.e. without precipitation) of both the sporulating and non-sporulating organisms to sterilisation (autoclaving)

Cells regeneration (growth) was studied after sterilisation (autoclaving) without carbonate precipitation (control). Sterilised culture (autoclaved at 121°C and 1.3 bar for 15 minutes) was re-suspended in 10 ml fresh growth medium and incubated at 30°C / 150 rpm for up to a week.

b) Testing the response of bio-cemented cultures (sporulating and non-sporulating) to damage (dissolving in acid) without autoclaving

Cells were grown from unsterilised microbially induced calcium carbonate crystals. Freshly grown, sporulated cells were suspended in 10 ml cementation medium, and incubated at 30°C / 150 rpm for 7 days. Samples of the resulting crystals were transferred to microcentrifuge tubes, repeatedly centrifuged, washed in PBS and immersed in an ultrasonic water bath for 10-15 seconds to remove crystal surface-associated cells/spores. To determine effects of encapsulated cells/spores, half of the crystal samples were dissolved in 0.1 M hydrochloric acid for 1-2 hours whereas the remainder were untreated. Following this, samples were re-suspended in growth medium.

c) Testing the response of bio-cemented cultures (sporulating and non-sporulating) to damage (dissolving in acid) with autoclaving

Cells were grown from sterilized microbially induced calcium carbonate crystals. The method was as for b), although after crystals were washed and sonicated they were autoclave sterilised.

Samples were then incubated at 30°C in the respective growth medium and regularly monitored for microbial growth using optical density measurements. The results of the spore regeneration experiment are presented in section 4.5.

3.3.2.5 Secondary calcite precipitation

This experiment investigated the ability of the regenerated spores from the experiment outlined in sections 3.3.2.4, to form calcium carbonate. The regenerated culture was pelleted by centrifuging, washed twice in PBS and re-suspended in cementation media. The pH of the regenerated culture and of non-inoculated controls was monitored daily for five days and it served as an indication of revived ureolytic activity (which would result in the precipitation of CaCO₃). Samples of 1 ml were taken with a sterile pipette and filter-sterilised before pH measurement. The results from the experiment are presented in section 4.6.

3.3.2.6 Long-term survivability of entombed spores

Freshly grown, sporulated cells of *S. ureae* were suspended in 10 ml cementation medium and incubated at 30°C / 150 rpm for 7 days. The samples were centrifuged, washed in PBS and maintained in PBS at room temperature for 3 and 6 months. Samples of the resulting crystals were transferred to microcentrifuge tubes, repeatedly centrifuged, washed in PBS, and immersed in an ultrasonic water bath for 10-15 seconds to remove crystal surface-associated cells/spores. All samples were then autoclaved. Half of the samples were dissolved in 0.1 M HCl, while the rest remained untreated. Following this, samples were re-suspended in growth medium and bacterial growth monitored via optical density measurements. Results from the long-term survivability of entombed spores experiment are presented in section 4.7.

3.3.3 Optimisation of MICP with *S. ureae*

3.3.3.1 Metabolism of *S. ureae* and *S. pasteurii* - a comparison

In this experiment, the ureolytic potential of *S. ureae* was compared against a well-documented and commonly used, highly ureolytic strain - *S. pasteurii*. The ureolytic potential of both bacteria was measured by the degree of pH increase and aqueous calcium conversion to calcium carbonate (the analytical techniques were described in sections 3.2.1 and 3.2.2). The metabolism of the two strains was examined in four different conditions. Firstly, idealised conditions with the use of a shaking incubation (which allows for oxygen availability) for bacterial growth and metabolism were examined. Then, idealised conditions with the presence of a potentially hindering factor (sand) in a small quantity, and static conditions (less oxygen available) with the presence of sand. And finally, the metabolism of the bacteria was studied in fully-saturated sand in static conditions (least oxygen available).

- i. Aqueous solution with shaking incubation (optimised condition)
- ii. Aqueous solution with a presence of sand (in 2:1 weight ratio) with shaking incubation
- iii. Aqueous solution with a presence of sand (in 2:1 weight ratio) with static incubation
- iv. In fully saturated (loose) sand; static incubation

The aim of this experiment was to determine how well the selected organism performed as compared to a model highly ureolytic strain. It helped to establish important information on the time the organism needed for substrate conversion to calcium carbonate and extend to which bio-cementation was hindered when less than ideal conditions were present. The results of the experiment are presented in section 5.3.

3.3.3.2 Effect of substrate concentrations and solvents

In this experiment a range of calcium and urea concentrations in the cementation medium were tested, as well as the type of solvent used - tap or deionised water, to determine the response of the bacterium and its ability to precipitate CaCO_3 in various concentrations of substrates. As outlined previously in chapter 1, the concentrations of calcium and urea play a significant role in the amount of precipitation that can be formed and its effect on the geotechnical properties of the treated soil. Additionally, higher concentrations have an effect on the amounts of CaCO_3 per unit time and therefore on the period needed for the bio-treatment to take place. This study examined the ability of the bacterium to function and precipitate CaCO_3 in an increasingly challenging environment (in terms of concentrations of calcium and urea) with the aim of achieving greatest amounts of precipitation. The concentrations of calcium and urea were based on the range of concentrations in previous published research of MICP in soils – 0.06 to 1.5 M of urea, and 25 μM to 1.5 M of calcium have been used (Warren et al. 2001; Ferris et al. 2003; DeJong et al. 2006; De Muynck et al. 2008b; Chunxiang et al. 2009; Okwadha and Li 2010; van Paassen et al. 2010; Burbank et al. 2011; Mortensen et al. 2011; Stabnikov et al. 2011; Cheng et al. 2016). In the first part of the experiment a batch experiment was performed in static conditions in both aqueous solution and in the presence of sand. Chemical indications (pH and aqueous calcium), as well as mass loss on ignition (as a measurement of amount of precipitate, section 3.2.9), were used to assess the effect of the different concentrations of substrates in the cementation medium on the activity of *S. ureae*. The results on the pH increase due to the bacterial metabolism from the batch experiment were compared against a geochemical simulation made using Visual MINTEQ 3.1 (Gustafsson 2016).

3.3.3.2.1 Batch experiment

Freshly grown cells of *S. ureae* were suspended in 10 ml cementation medium in triplicates (with varying concentrations of calcium and urea shown in Table 3-2 and either tap or de-ionised water as a solvent) in 50 ml tubes. To test conversion of substrates to CaCO₃ the tubes were incubated in a shaking incubator at 30°C / 150 rpm for 7 days. Similarly, freshly grown cells were suspended in 10 ml cementation medium (varying concentrations and solvents) and sterile sand (see 3.1.2.4) to the top, and then incubated at 30°C / 150 rpm for 7 days. Triplicates of control samples contained identical media with a lack of urea.

Table 3-2 Concentrations of calcium and urea in batch experiment and MINTEQ model

Substrates	M0	M50	M150	M300	M1000
Calcium mM	0	50	150	300	1000
Urea mM	333	333	333	333	1000

After the incubation period of 7 days all aqueous samples and discharge liquids from the sand samples were tested for pH and aqueous calcium. A system, developed by Mugwar (2015), for the effective extraction of solutions from the sand samples, was used (Figure 3-2). A drilled 1 mm hole (covered with glass wool) on the bottom of the sand-filled tubes allowed for the extraction of solutions through a vacuum extraction system. The solutions were vacuumed through a 0.2 µm pore-size membrane which retained bacterial cells and precipitates. A metal spring, inserted into the conical vacuum flask, allowed the positioning of the discharge bottle under the flow of liquids. The discharged liquids were then analysed for pH and calcium. Amount of calcium carbonate precipitated in the aqueous samples was measured after drying at 105 °C; sand samples were tested for mass loss on ignition, as a measure of the amount of calcium carbonate present (section 3.2.9). Results from the batch experiment are presented in section 5.4.1.

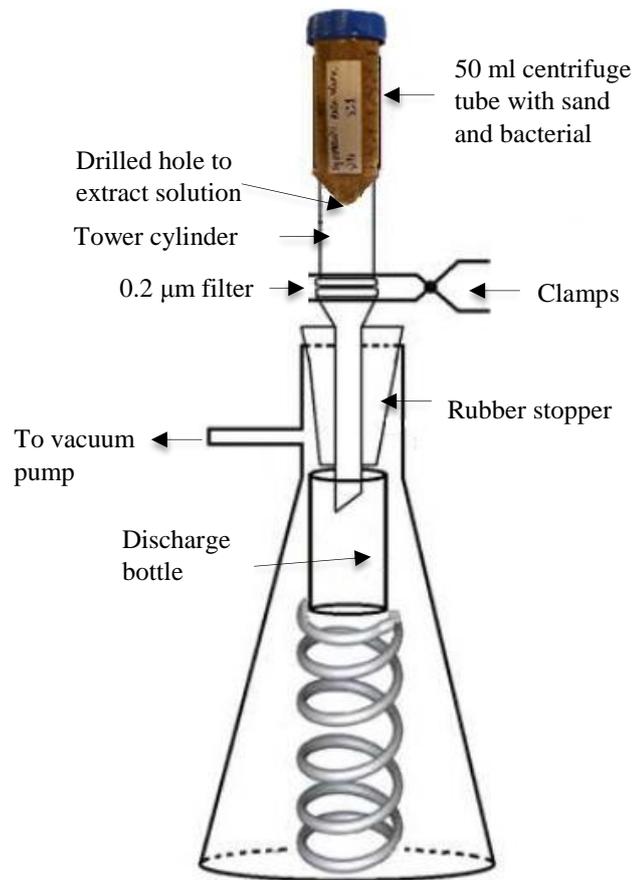


Figure 3-2 Schematic of system to extract solutions from sand-filled centrifuge tubes (taken from Mugwar (2015))

3.3.3.2.2 Geochemical model

Simulation of the pH of solutions with various composition and concentrations of calcium and urea in the cementation media was achieved with the Windows version of the Visual MINTEQ software, Version 3.1 (Gustafsson 2016). The model allowed for chemical equilibrium for the calculation of metal speciation and solubility equilibria. It was used to compare the predicted pH of cementation media with different concentrations of calcium and urea and compare that pH to the results from the batch experiment. As pH is one of the governing parameters of the MICP process, the development of a model to predict alkalinity (as a function of calcium and urea concentrations in the substrates and urea degradation) may be used in the future for the

design and implementation of MICP treatments and eliminate the need for tedious and time-consuming laboratory tests. The use of a fairly simple geochemical model would save time and has the potential to predict with accuracy the outcome of a bio-cementation treatment.

It should be noted that Visual MINTEQ is not able to handle non-equilibrium reactions such as biologically catalysed reactions. Therefore to simulate the degradation of urea (urea hydrolysis), the compound was presented as ammonium and hydroxide ions assuming complete degradation (Equations 1 – 3, p.10-11). The concentrations of substrates (in the cementation medium) modelled are shown in Table 3-2. The remaining components of the cementation medium - sodium bicarbonate and ammonium chloride were kept constant in concentration (same as in cementation medium, section 3.1.1.1); effect of nutrient broth was not included. Modelled results are shown in section 5.4.2.

3.3.3.3 Effect of substrate concentrations - sand column experiment

The activity of the bacterium (in terms of pH, calcium conversion and amount of precipitation) was determined under the various concentrations and type of solvents in the static batch experiment in the previous section. To examine the effect of the substrate concentrations on the geotechnical properties of bio-treated sand, a sand column experiment was performed. It allowed investigating both ureolytic activity with pH, calcium conversion and amount of precipitation, as well as hydraulic conductivity and unconfined compression strength.

3.3.3.3.1 Sand column preparation and bacterial treatment

Sand (prepared according to 3.1.2.4) was wet pluviated (sand was poured in the liquid to prevent entrapment of air bubbles within the soil and achieve full saturation) from a

height of 5 cm into one and a half pore volumes of a suspension of *S. ureae* in PBS and vibrated until a target of 95 % relative density was reached. Split-mould sand columns (diameter 38 mm; length 66 mm) were prepared in 0.2 mm latex rubber membranes with 3D-printed plastic discs top and bottom (diameter 38 mm; thickness 6 mm; 57 holes of 2 mm diameter) (Figure 3-3). These discs ensured a more uniform flow distribution across the sand specimen and minimised clogging of the inlet or outlet. A layer of glass wool around the discs prevented sand from escaping through the holes of the discs. Rubber stoppers with ports at the inlet and outlet provided connection to the peristaltic pump (Watson Marlow, Type 205U, Cornwall, UK). The pump allowed the aseptic injection of sterile solutions at a rate of 2 ml/min. The columns were supported with the acrylic split-mould held together with zip ties. A layer of tracing paper between the latex membrane and the acrylic tubes prevented adhesion between the two. The use of an acrylic split mould which comprised of two halves of a cylinder, maintained by zip ties, allowed the containment of sand and solutions within a membrane during the bacterial treatment. Once the bacterial treatment was terminated, the acrylic split-mould could be opened without compromising the integrity of the sand samples which were subsequently placed under compression testing machine.

All components of the experimental apparatus (including latex membrane and flexible rubber tubes) were sterilised using 1 % Virkon solution (Rely⁺OnTM, multi-purpose disinfectant) and thoroughly washed with sterile de-ionised water prior to start of the experiment.

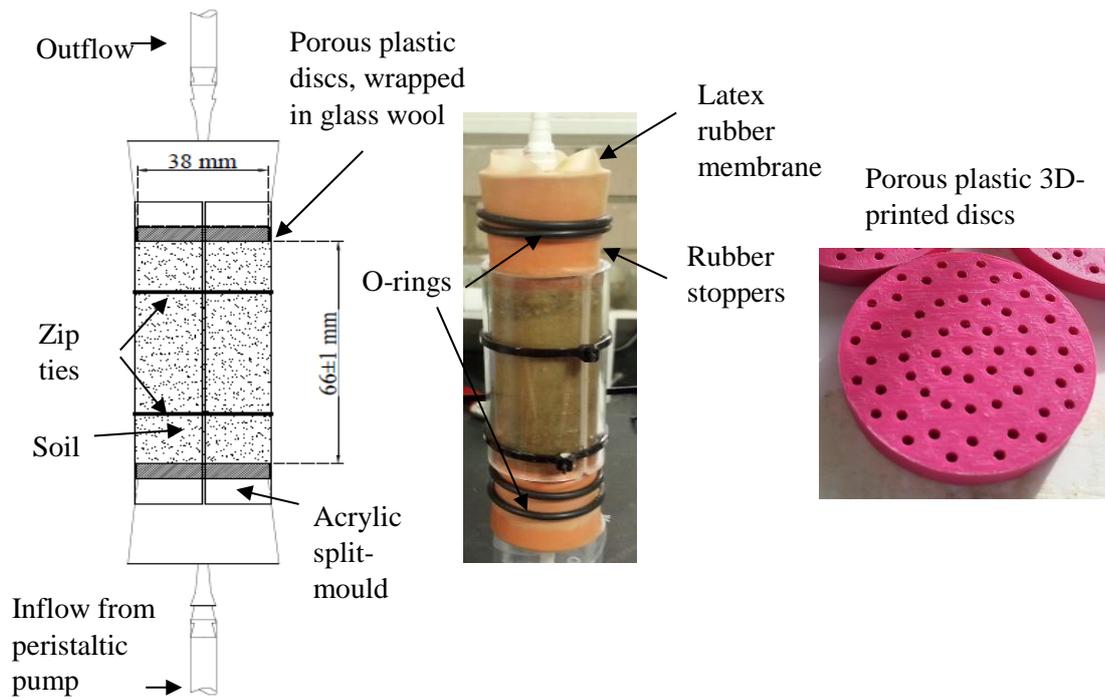


Figure 3-3 Split-mould sand column apparatus

3.3.3.3.2 Substrate concentrations, chemical and geotechnical tests

Cementation medium with varying concentrations of calcium – 50, 150, and 300 mM (urea was kept at 333 mM) was supplied to the sand columns (in triplicates), and it was compared against triplicates of control sand columns, treated with cementation medium lacking urea. The lack of urea in the medium did not allow growth of *S. ureae* cells and metabolic conversion of substrates into calcium carbonate. The bacterial treatment was performed in 39 days (6 injections). After each injection of cementation medium there was a static incubation period of 6 to 7 days. At the end of the incubation period the old liquids were replaced with PBS and the discharge liquid from each column was tested for pH and calcium (sections 3.2.1 and 3.2.2). Subsequently, each column was connected to the falling head apparatus (section 3.2.10) for hydraulic conductivity testing. The columns were then connected to the peristaltic pump once again for injection of fresh cementation medium followed again by a static incubation period and

chemical and hydraulic conductivity tests. At the end of the bacterial treatment all columns were washed with deionized water, drained, and air-dried to a constant mass at 30°C (for approximately 3 weeks) to minimise effects of moisture on compressive behaviour whilst maintaining membrane integrity. Subsequently, the split moulds were removed and each specimen physically characterised by unconfined compression testing (section 3.2.11). Results from the experiment are presented in section 5.5.

3.3.4 Self-healing in particulate media

The two experiments presented in chapter 6 were aimed at investigating whether initially bio-cemented soil in sand columns, which contained sporulated bacteria encapsulated in the monolith, could respond to various forms of damage via the action of spores. The first experiment examined the effect of a chemical deterioration to the monolith, which exposed the spores, followed by a supply of nutrients, which allowed the spores to germinate and heal the damage. The second experiment examined the effect of a physical damage for spore exposure and subsequent damage healing. Damage healing was analysed by recovered metabolic activity (pH and calcium), amount of precipitate reproduced, and recovery of mechanical strength (unconfined compression), as well as hydraulic conductivity measurements.

3.3.4.1 Healing after chemical deterioration

These experiments comprised nine acrylic columns (length 68 mm; inner diameter 26 mm), sealed with rubber stoppers and with glass wool at either end to prevent escape of sand and to minimise clogging at the inlet or outlet. Ports in the stoppers were connected to the pump. A diagram of the experimental apparatus can be seen in Figure 3-4. All components of the experimental apparatus (including flexible rubber tubes which transferred media) were sterilised using 1 % Virkon solution (Rely⁺OnTM, multi-

purpose disinfectant) and thoroughly washed with sterile de-ionised water prior to start of the experiment.

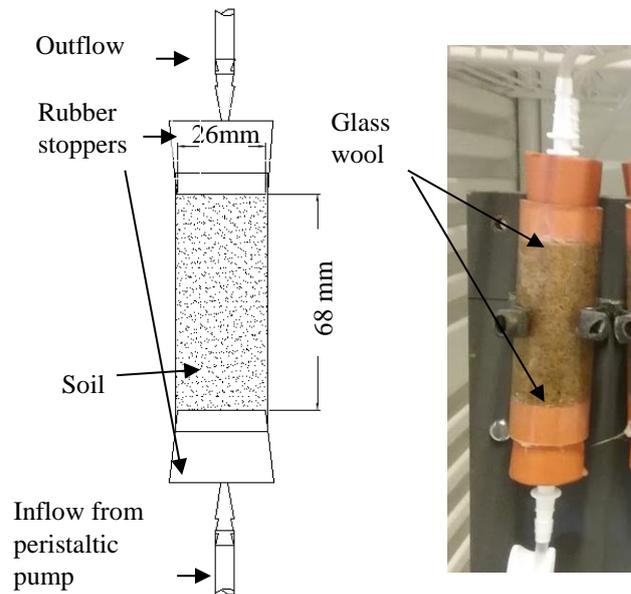


Figure 3-4 Sand column apparatus for self-healing after chemical deterioration experiment

Sand (prepared as described in 3.1.2.4) was wet pluviated from a distance of 5 cm (to achieve full saturation) into one and a half pore volumes of a suspension of *S. ureae* in PBS and vibrated until a target of 95 % relative density was reached. Cells had been previously exposed to the sporulation medium to ensure the presence of spores.

Cementation medium was supplied over a period of 29 days (5 injections) to allow the initial cementation of the sand to take place. Three columns were dismantled for measurement of loss on ignition (a measure of carbonate precipitation). Chemical deterioration of healed sand in the remaining columns was carried out by injecting one pore volume of 0.1 M hydrochloric acid, leaving for 2 hours and then repeating 3 times. A further three columns were then dismantled to determine carbonate precipitate content. The remaining columns were then sterilised to kill off viable cells by injecting two pore volumes of 0.5% hydrogen peroxide solution and leaving for 1-2 hours. Spore

viability was found to be unaffected by this process. The contents of the remaining three columns were transferred aseptically to sterile flasks and incubated at 30°C in a shaking incubator due to difficulties in stimulating growth *via* flow in the columns. These were supplied with two pore volumes of cementation medium (replaced 6 times over 26 days). After this period, the extent of carbonate precipitation was determined by loss on ignition. A timeline of the experiment is shown in the table below (Table 3-3). Results from the experiment are presented in section 6.2.

Table 3-3 Timeline of the healing after chemical deterioration experiment

Day of experiment	Procedure
Initial bacterial treatment stage	
0	Experimental apparatus set up; injection of nutrients in columns;
0-7	Static incubation
7	pH and calcium of columns discharge liquids; injection of nutrients
7-13	Static incubation
13	pH and calcium of discharge liquids; injection of nutrients
13-19	Static incubation
19	pH and calcium of discharge liquids; injection of nutrients
19-24	Static incubation
24	pH and calcium of discharge liquids; injection of nutrients
24-29	Static incubation
29	pH and calcium of discharge liquids; DI-water injection for washing off residual nutrients and debris
	Columns 1-3 disassembled for testing of mass loss on ignition
	Columns 4-6 injected with HCl and then dismantled for mass loss on ignition testing
	Columns 7-9 sequentially injected with HCl and then with H ₂ O ₂ for dissolving of calcium carbonate and sterilisation; columns transferred aseptically to flasks
Healing stage	
29	Nutrient supply to flasks
29-34	Shaking incubation (for better aeration)
34	pH and calcium of discharge liquids; injection of nutrients
34-39	Shaking incubation
39	pH and calcium of discharge liquids; injection of nutrients
39-44	Shaking incubation
44	pH and calcium of discharge liquids; injection of nutrients
44-48	Shaking incubation
48	pH and calcium of discharge liquids; injection of nutrients
48-50	Shaking incubation
50	pH and calcium of discharge liquids; injection of nutrients
50-57	Shaking incubation
57	pH and calcium of discharge liquids; DI-water injection for washing off debris and residual nutrients; mass loss on ignition of contents of columns 7-9

3.3.4.2 Healing after physical deterioration

The experiment comprised ten split-mould sand columns (shown in Figure 3-3), prepared as described in section 3.3.3.3.1. Sand was wet pluviated into one and a half pore volumes of a suspension of *S. ureae* in PBS and vibrated until the targeted relative density was reached. Cells had been previously exposed to the sporulation medium to ensure the presence of spores. After an initial cementation period of 38 days (7 injections), all columns were washed with deionized water, drained and air-dried to constant mass at 30°C (approximately 3 weeks) to minimise effects of moisture on compressive behaviour whilst maintaining membrane integrity. Subsequently, the split moulds were removed and each specimen physically deteriorated by unconfined compression testing (according to 3.2.11), followed by removal of viable cells with hydrogen peroxide (the columns were sterilised to kill off viable cells by injecting two pore volumes of 0.5 % hydrogen peroxide solution and leaving for 1-2 hours). As before, spore viability was found to be unaffected by the process. The sand specimens were replaced in the split moulds for support and stored at 30°C for the healing stage of 22 days (5 injections). Odd-numbered specimens were supplied with cementation medium whilst even numbered specimens were supplied with deionised water as controls. Prior to each injection in both stages, and at the end of each stage, hydraulic conductivity was determined for each sand column according to the procedure, specified in 3.2.10. A timeline of the experiment is shown in the table below (). Results from the experiment are presented in section 6.3.

Table 3-4 Timeline of the healing after physical deterioration experiment

Day of experiment	Procedure
Initial bacterial treatment stage	
0	Experimental apparatus set up; testing initial hydraulic conductivity injection of nutrients in columns;
0-7	Static incubation
7	Hydraulic conductivity testing; pH and calcium of discharge liquids; injection of nutrients
7-13	Static incubation
13	Hydraulic conductivity testing; pH and calcium of discharge liquids; injection of nutrients
13-19	Static incubation
19	Hydraulic conductivity testing; pH and calcium of discharge liquids; injection of nutrients
19-24	Static incubation
24	Hydraulic conductivity testing; pH and calcium of discharge liquids; injection of nutrients
24-29	Static incubation
29	Hydraulic conductivity testing; pH and calcium of discharge liquids; injection of nutrients
29-33	Static incubation
33	Hydraulic conductivity testing; pH and calcium of discharge liquids; injection of nutrients
33-37	Static incubation
37	Hydraulic conductivity testing; pH and calcium of discharge liquids; DI-water injection for washing off residual nutrients and debris
37-63	26 days of drying of sand columns in incubator until constant mass
63	Unconfined compression testing
Healing stage	
63-64	Assemble of sand specimens in split moulds; gradual re-saturation with DI-water
64	Injection and retention of sterilisation solution (H ₂ O ₂) in columns; testing of initial hydraulic conductivity; injection of nutrients
64-68	Static incubation
68	Hydraulic conductivity testing; pH and calcium of discharge liquids; injection of nutrients
68-73	Static incubation
73	Hydraulic conductivity testing; pH and calcium of discharge liquids; injection of nutrients
73-78	Static incubation
78	Hydraulic conductivity testing; pH and calcium of discharge liquids; injection of nutrients
78-82	Static incubation
82	Hydraulic conductivity testing; pH and calcium of discharge liquids; injection of nutrients
82-86	Static incubation
86	Hydraulic conductivity testing; pH and calcium of discharge liquids; DI-water injection for washing off residual nutrients and debris
86-112	26 days of drying of sand columns in incubator until constant mass
112	Unconfined compression testing

4 Microbial self-healing in idealised conditions

4.1 Introduction

The prerequisites for an ideal microbial self-healing mechanism (when applied to soil) have been identified in section 2.4.1.2. This chapter, however, discusses the basic principle of the self-healing process explored through experiments in idealised conditions. Therefore, the requirements from 2.4.1.2 can be amended to serve as the objectives for the current chapter. As such it can be stated that for self-healing, a microorganism must be able to:

- i. bring about MICP formation due to its metabolic activity
- ii. survive prolonged periods of starvation and under adverse or hostile conditions in a spore form within the crystal of its own making
- iii. monitor continually its surrounding environment for occurrence of deterioration (loss mass of crystal) to the previously formed crystal; germinate upon its exposure from the crystal and cause the formation of further crystallisation
- iv. perform cycles of i, ii, and iii multiple times.

This chapter will present the selection of a suitable organism based on these objectives. Firstly, spore formation was studied in three candidate bacteria through microscopic analysis. The growth kinetics of the chosen bacterium is discussed, along with crystallographic and microscopical analysis of the mineral precipitate produced by the same bacterium. The response of the bacterial spores, encapsulated in their own mineral matrix, to deterioration of the mineral and subsequent exposure to nutrients, is also presented. Additionally, in order to assess the ability of the bacterium to repeat the

process of bio-precipitation and deterioration of mineral, further bio-precipitation cycles were explored alongside the long-term survivability of the mineral-encapsulated spores.

4.2 Organism selection

Spore-forming alkaliphiles have been previously used in concrete applications. Some of these include *Bacillus pseudofirmus*, *Bacillus halodurans*, *Bacillus cohnii*, *Bacillus alkalinitrilicus*, and *Bacillus sphaericus* (Jonkers and Schlangen 2007; De Muynck et al. 2008b; Wiktor and Jonkers 2011; Ducasse-Lapeyresse et al. 2013; Sharma et al. 2017).

In geotechnical research applications, the requirement for bacteria to form spores was not needed as the soil environment is generally “friendlier” and allows bacterial growth and metabolism. Furthermore, the focus of most publications on bio-cementation of soil has been the “one-off” improvement of soil’s properties due to the bacterial treatment, and as such sporulation for bacterial survival in the long-term has not been investigated.

Strains for MICP in geotechnical applications include *Sporosarcina pasteurii*, *Bacillus megaterium*, *Bacillus lentus*, *Brevibacterium ammoniagenes*, *Sporosarcina ureae*, *Sporosarcina ginsengisoli*, *Idiomarina isuliasalsae* (DeJong et al. 2006; Achal et al. 2012; Soon et al. 2013; Oliveira et al. 2014; Sarmast et al. 2014; Canakci et al. 2015).

Three candidate bacteria were investigated in this study - *Sporosarcina pasteurii*, *Sporosarcina ureae* and *Bacillus cohnii*. All three have been described as having mineral precipitating abilities, have been used in MICP-related research (DeJong et al. 2006; Jonkers and Schlangen 2007; Sarmast et al. 2014), and have also been described as spore-formers (Spanka and Fritze 1993; Zhang et al. 1997; Yoon et al. 2001).

4.2.1 *Sporosarcina pasteurii*

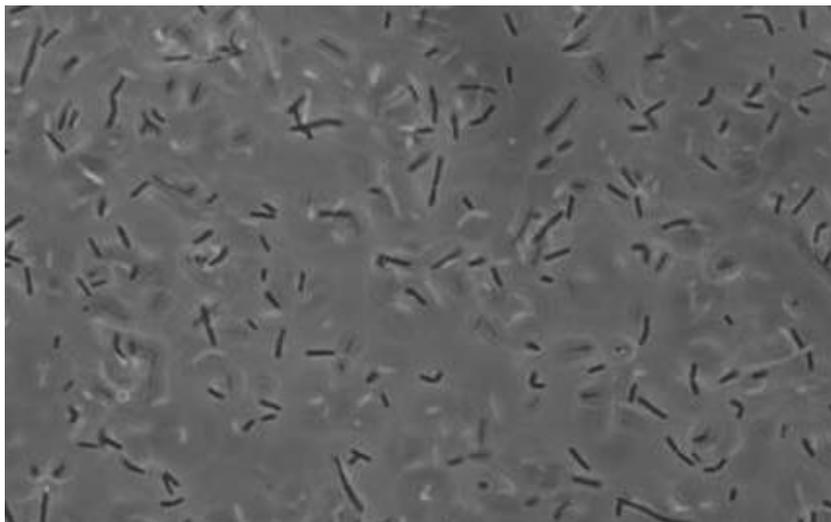
S. pasteurii (section 3.1.1.1) was the primary choice of bacterium for this study as it has been widely used in research for soil stabilisation due to its highly active urease enzyme and effectiveness in bio-cementing soil (DeJong et al. 2006; van Paassen et al. 2010; Tobler et al. 2011; Al Qabany and Soga 2013; Cheng et al. 2013). Experiments showed that the strain required less than 24 hours for the conversion of calcium and urea substrates into calcium carbonate when a starting concentration of 50 mM calcium was used (presented in chapter 5). *S. pasteurii* is commonly described as a spore-former in books of microbiology (Madigan et al. 2009) and was re-classified from the *Bacillus* to the *Sporosarcina* genus (i.e. forms spores) by Yoon et al. (2001). However, comprehensive studies on the sporulation of *S. pasteurii* are extremely limited, and lack details regarding the conditions in which it sporulates and microscopical evidence of this sporulation. As outlined by Setlow (2006) the study of sporulation in the laboratory is undertaken using media which does not readily support growth, i.e. in conditions which are limiting to the bacteria, therefore causing it to turn into survival mode and form spores. All media and methods which were attempted for *S. pasteurii* sporulation were described in Table 3-1 and include prolonged incubation in the growth and manganese amended medium in optimum and room temperature (i.e. lower than optimum temperature for this strain), as well as the medium used for inducing sporulation in *B. cohnii* (Jonkers et al. 2010) and other *Bacillus* strains (Nicholson and Setlow 1990). Schaeffer-Fulton spore staining and phase contrast microscopy were used to monitor if spores formed (described in sections 3.2.5 and 3.2.6).

Although most media resulted in growth reduction due to their decreased concentrations of nutrients, compared to growth in *S. pasteurii* growth medium, sporulation could not

be detected with any of them. Micrographs of what appear to be spore-free smears of bacteria are shown in Figure 4-1.



5 μm (a)



5 μm (b)

Figure 4-1 Monitoring cultures of *S. pasteurii* for sporulation; (a) Schaeffer-Fulton spore stain, (b) Phase contrast microscopy

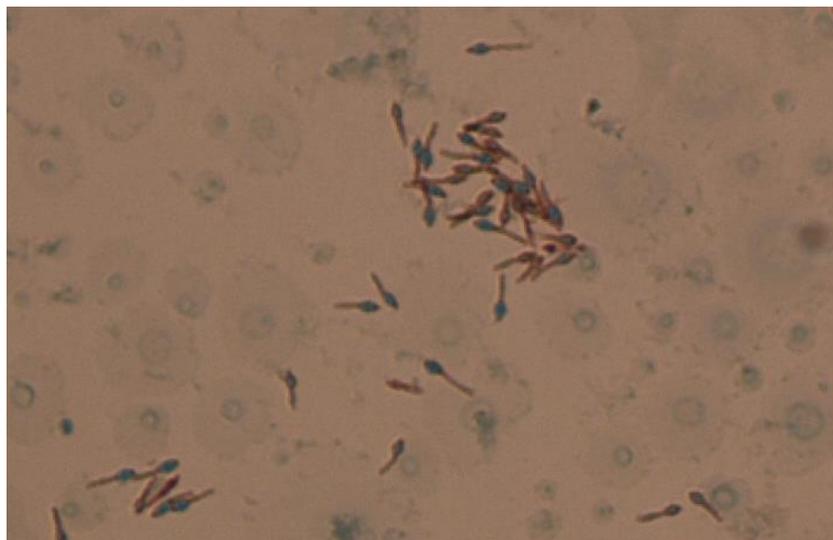
The images show smears of *S. pasteurii* grown in manganese-amended growth medium, however all other sporulation methods produced a similar result. Figure 4-1a shows an optical microscope image of a smear of bacteria, treated sequentially with safranin and

malachite green which stain viable cells in red and spores in green, respectively. The micrograph clearly shows the presence of red-stained rod-shaped cells; however no green spores can be seen. Additionally, a phase contrast micrograph, depicted in Figure 4-1b confirms the lack of spores. Under phase contrast microscopy spores appear as bright objects compared to darker grey cells. Again, only cells could be seen in the *S. pasteurii* cultures, irrespective of the medium and conditions in which they were cultivated.

4.2.2 *Bacillus cohnii*

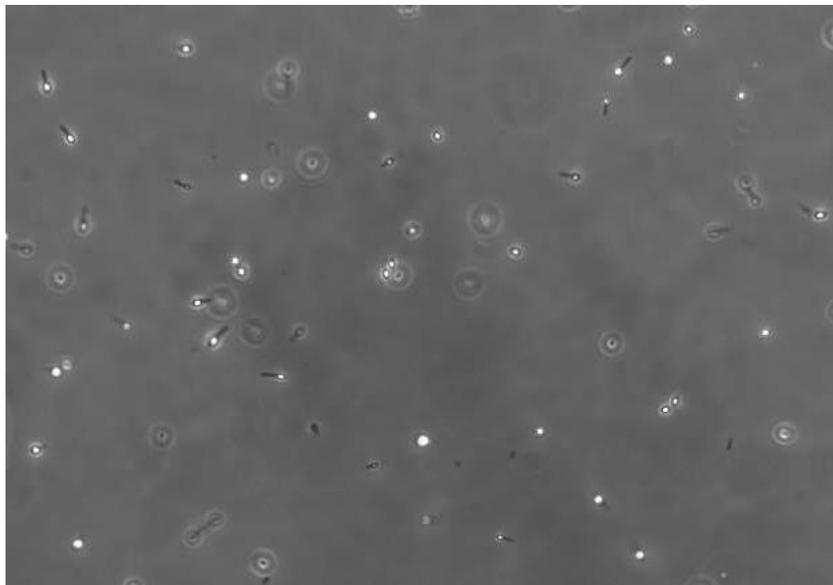
Another strain used in this study was *B. cohnii* (section 3.1.1.2) - an alkaliphilic organism, found naturally in alkaline soil which is a spore former and under suitable conditions precipitates calcium carbonate. It has been widely used in research for self-healing concrete (Jonkers et al. 2010; Sharma et al. 2015) due to its high tolerance to alkaline conditions, copious spore formation and rapid germination abilities.

The bacterium was grown in a highly alkaline medium (section 3.1.1.2) which resulted in the sporulation of up to 86 % of the cells in the *B. cohnii* culture. An optical microscope and phase contrast micrographs of sporulated cells can be seen in Figure 4-2. Well defined green coloured spores which appear swollen and terminally located in the cell, as well as free spores, can be observed in Figure 4-2a. Also, the phase contrast micrographs clearly show the bright spores either free or associated with cells (Figure 4-2b). However, it was found that this strain required a prolonged period of 16 to 18 days to form calcium carbonate in solution (results not presented) which would make it highly impractical for the experiments in particulate media in this project.



5 μm

(a)



5 μm

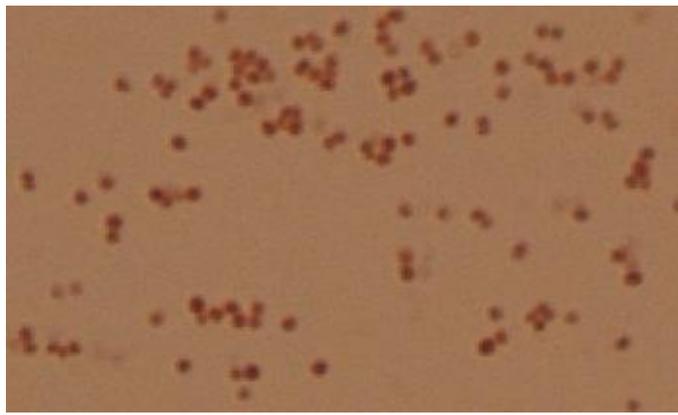
(b)

Figure 4-2 Monitoring cultures of *B. cohnii* for sporulation; (a) Schaeffer-Fulton spore stain, (b) Phase contrast microscopy

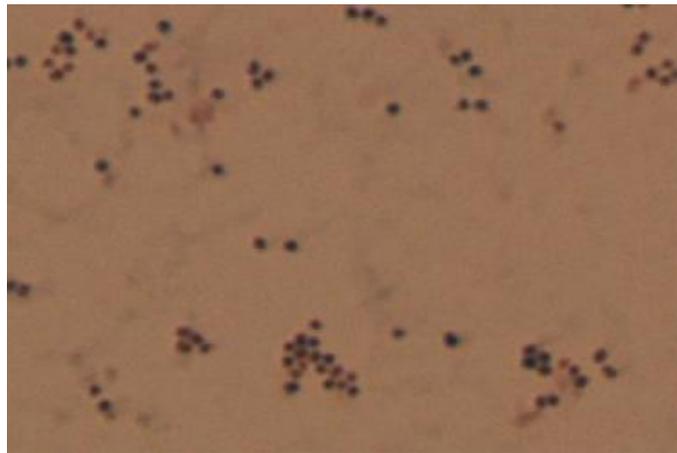
4.2.3 *Sporosarcina ureae*

S. ureae (section 3.1.1.3) is another ureolytic strain which is very similar to *S. pasteurii* but with a less active urease enzyme (Morsdorf and Kaltwasser 1989; McCoy et al. 1992). Experiments in the current study showed that the strain required between 5 and 7 days for the conversion of calcium and urea substrates to calcium carbonate when 50

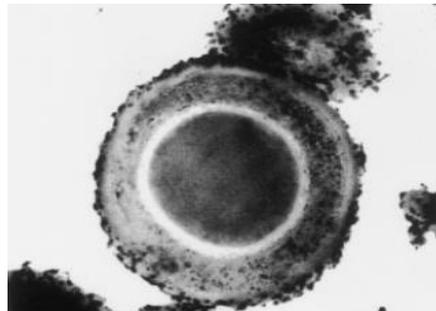
and 150 mM of calcium were used (presented in chapter 5). A body of information on the sporulation conditions and characteristics of this strain exists (Mazanec et al. 1966; Zhang et al. 1997; Chary et al. 2000). To induce sporulation the bacterium was initially inoculated in its growth medium and at the onset of exponential growth was transferred to the medium originally defined by MacDonald and MacDonald (1962). *S. ureae* is a 0.5 to 1 µm coccus which made microscopic observation of spores challenging - differentiation between the viable cell and spore could not be easily achieved the way it is done in a rod-shaped cell with a spore, located terminally. Thereafter, spore staining was performed up to 5 hours from onset of culturing *S. ureae* in its growth medium, i.e. before spore formation had initiated which can be seen in Figure 4-3a - red coccoid cells can be observed. In contrast, (Figure 4-3b) shows a stained culture which is approximately 20-24 hours old and had been grown in sporulation medium - most cocci cells appear green-blue in colour which is as a result of the presence of spores stained by the malachite green dye. An SEM micrograph, reproduced from Chary et al. (2000) (Figure 4-3c) shows an ultrathin section of an early stage of a sporulating cell of *S. ureae* in detail - a spore enveloped by a thick cortex (white material) within a mother cell.



5 μm (a)



5 μm (b)



0.5 μm

Figure 4-3 Monitoring cultures of *S. ureae* for sporulation; (a) Schaeffer-Fulton spore stain of a fresh, non-sporulated culture, (b) Schaeffer-Fulton spore stain of a sporulated culture, (c) SEM image of an ultrathin section of a sporulating *S. ureae* cell (reproduced from Chary et al. (2000))

Table 4-1 summarises the findings from the selection process in terms of bio-cementation characteristics and the presence of sporulation. Due to the inability of *S. pasteurii* to form spores and the prolonged incubation period for *B. cohnii* to form bio-cement *S. ureae* was considered the most appropriate organism for implementing self-healing MICP and was the model organism used in all subsequent experiments in this study.

Table 4-1 Bio-cementation characteristics and sporulation in *S. pasteurii*, *B. cohnii* and *S. ureae*. Summary of findings.

Strain	Spore formation	Metabolic activity	Further comments
<i>Sporosarcina pasteurii</i>	Does not form spores	Ureolysis	Very strong urease enzyme; rapid assimilation of substrates and CaCO ₃ precipitation - less than 24 hours
<i>Bacillus cohnii</i>	Forms spores	Calcium lactate to calcium carbonate metabolic conversion	Prolonged period of incubation required for precipitation (16-18 days);
<i>Sporosarcina ureae</i>	Forms spores	Ureolysis	Weaker urease enzyme; period of incubation required for substrate assimilation 5 to 7 days

4.3 Growth kinetics of *S. ureae*

The aim of this experiment was to determine the growth kinetics of the strain of *S. ureae* which would give vital information, such as the time of exponential growth start, as well as number of cells at late exponential phase. Control over these factors would govern sporulation induction, as well as ureolytic activity in particulate media experiments. The experiment was performed according to the procedure presented in section 3.2.4. Bacterial growth in a given volume of liquid medium is characterised by several distinctive stages, according to Monod (1949): lag phase (maturation of the cell in preparation for reproduction), acceleration phase (increase of growth rate), exponential (or log) phase (growth rate is constant), retardation phase (growth rate

decrease), stationary phase (no growth), phase of decline (cells start to die off). As the doubling time of *S. ureae* is approximately 20 minutes and the initial volume of inoculum was 2 % of the total volume of medium the time it required for the culture to reach the stationary phase and phase of decline took approximately 24-30 hours. In order to plot the entire period of 24 hours two cultures in triplicates were started from the same source of bacteria. The first one was started in the morning and was monitored every 1 to 2 hours for 10 hours (0 to 10-hour time points on the curve) and the second one was started in the evening and monitored on the next day for the 13- to 24-hour time points. In this manner the full growth curve was plotted. This experiment is based on the assumption that both cultures (morning and evening ones) were nominally the same as they were started from the same source and had equal starting number of cells, all cultures were also maintained under identical culture conditions. In reality, as this is a living organism all cultures from the morning and evening ones and their replicates may be expected to vary to a certain extent.

Optical density measurements of the bacterial cultures were taken every 1 to 2 hours in the 24-hour period from the onset of inoculation. Figure 4-4a shows the increase in turbidity with time which was due to the increase of number of cells (assuming only bacteria were present in the suspensions) in growth and cementation medium.

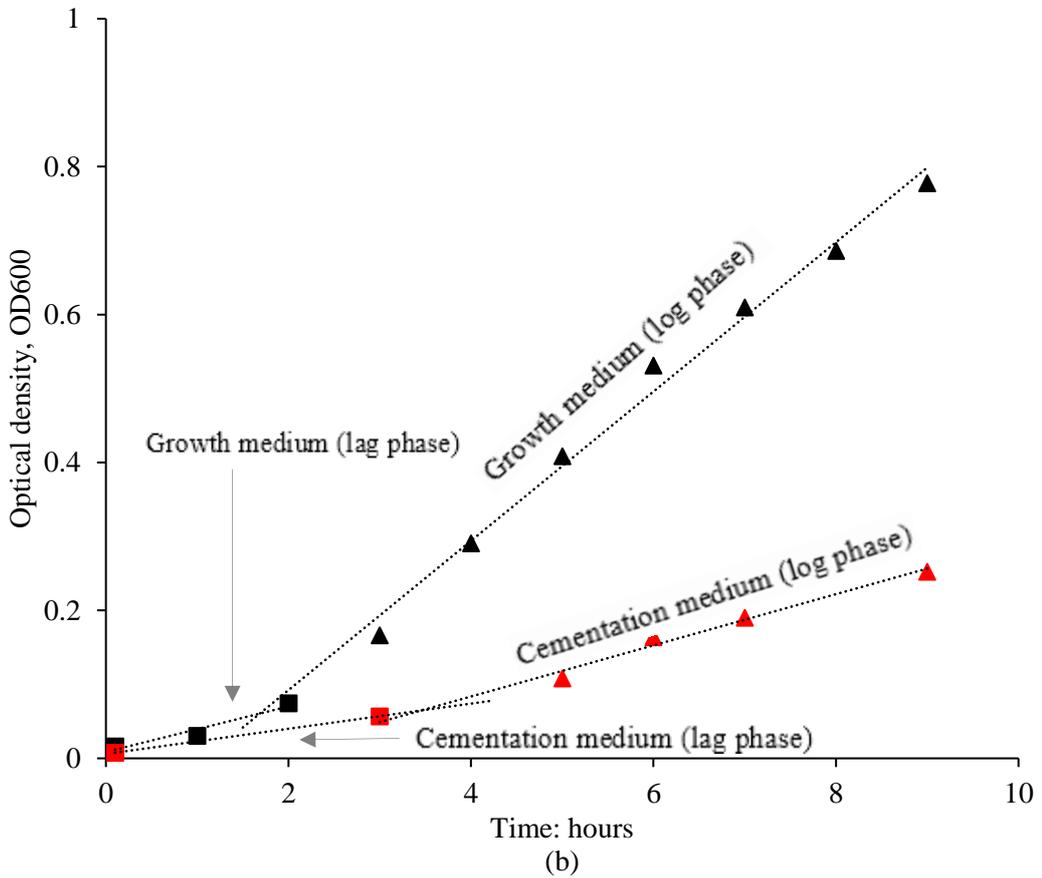
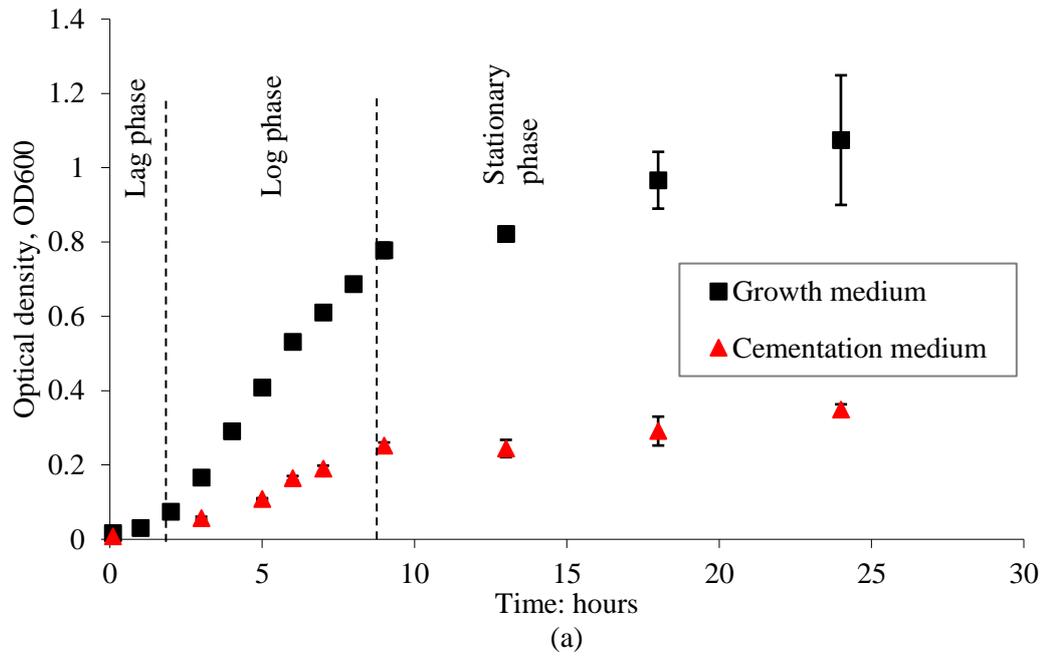


Figure 4-4 *S. ureae* cells growth (measured by optical density increase) over time in their normal growth medium and in the cementation medium (a). Estimation of lag and log phases and specific growth rate from the first 10 hours (b). (Error bars: ± 1 SD, $n=3$)

Growth of *S. ureae* was monitored in two different types of media - growth and cementation medium (components of media can be found in 3.1.1.3). This was done in order to determine whether any growth occurred when *S. ureae* was subjected to cementation medium and if so, the extent to which the growth of the bacterium was hindered in such a medium relative to the optimum conditions in the growth medium.

Figure 4-4a shows that initially, up to around 2-4 hours, there was very little growth, probably as the newly introduced cells were adjusting to the new environment and starting to mature (lag phase). Exponential growth in both media started between 2 and 4 hours and ended at approximately 9 hours (log phase). Stationary phase can be observed from 10 hours onwards. Theoretically, the stationary phase of growth would be approximately parallel to the x-axis because cell doubling would be matched by cell death. However, the measurement of turbidity would continue to increase (albeit at a slower rate) as it is related to the biomass in suspension (both dead and live cells).

Increasing error bars (standard deviation values) can be observed with increasing time and increasing biomass, especially in the growth medium curve. This may be attributed to the natural differences in the replicates of bacterial cultures which at the beginning of their growth were low in numbers but potentially had a different number of cells with a doubling potential. As doubling (i.e. growing) of the cultures continued and cell numbers increased the variation between the replicates would have also increased.

Figure 4-4b shows the first ten hours of the growth curve with straight lines fitted using linear trendline on Excel for the lag and log phases. The figure shows that when the bacterium was exposed to the cementation medium, which contained a high concentration of calcium (150 mM) and a reduced amount of the nutrients, the lag time was increased from 2 to 3.5 hours (an approximate value given by the intercept of the straight lines through the lag phase and log phase data points). Also, the specific growth

rate (calculated by the slope of the log phase) and the final optical density (at stationary phase) were reduced by 25 and 65 %, respectively.

4.3.1 Optical density - viable cell counts relationship for *S.ureae*

The growth curve of *S. ureae* using optical density measurements and cell counts of viable bacteria was constructed for the bacteria grown in growth medium. The relationship of the two parameters in the bacterial suspension was studied up until late exponential stage (Figure 4-5). This was determined by the OD600 vs. time curve which flattened after approximately 8 to 13 hours. Beyond this point cell division is matched by cell death and while optical density may be increasing some of the cells would be dying off so the discrepancy between optical density and viable cell counts increases. It should also be noted that although here it is referred to cells dying off this may mean a cease (or reduction) in metabolic activity of the vegetative cells which are turning into spores. Due to the reduced metabolic activity of the spores (including respiration) they would appear to be dead when stained with CTC however they would still be capable of returning back to a vegetative cell if nutrients become available.

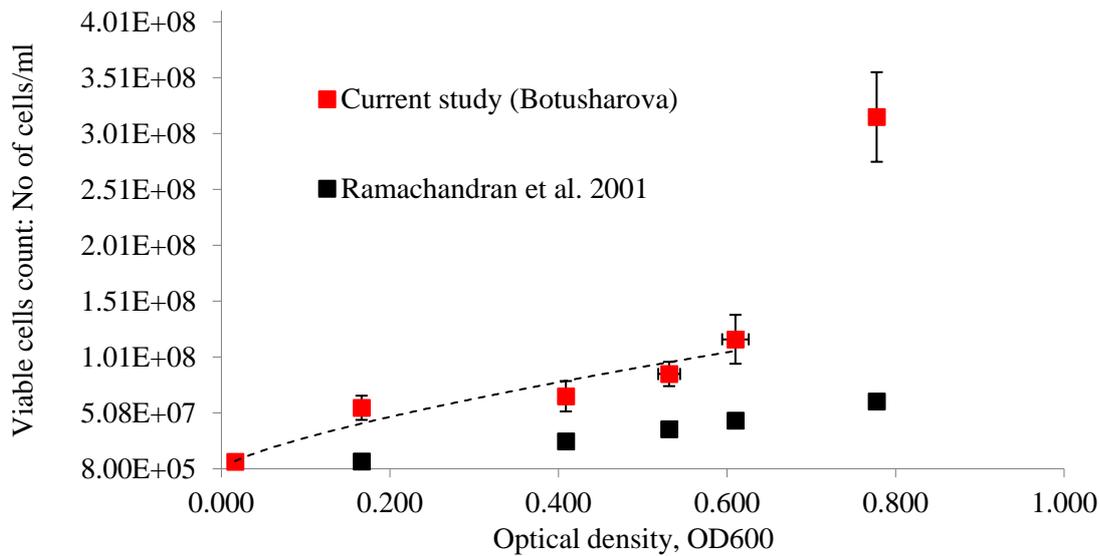


Figure 4-5 Relationship between number of viable *S. ureae* cells and the optical density in a suspension. (Error bars: ± 1 SD, $n=3$)

The two sets of data were correlated by a *curved power trendline*, to accommodate for the different stages of growth on the graph, which was an approach also used by Ramachandran et al. (2001) and Mugwar (2015). Although these two studies utilised a different strain of bacterium (*S. pasteurii*), the above mentioned approach proved effective for the correlation of the two sets of data in this study also. The curved power trendline also gave the highest coefficient of determination, R^2 , of 0.905. A relationship was established between optical density and viable cell counts such as that shown in the equation:

$$N = 2 \times 10^8 (OD600)^{0.7273} \quad (\text{Eq. 11})$$

where N is the number of viable cells in a suspension. It should be noted that the relationship, which was derived in the above, would only be usable for cultures when they are in the region from inoculation up to late exponential phase. Additionally, while a largely linear trend can be observed in the initial stage, in the last point on the graph a rapid increase in number of viable cells can be seen, however this is not matched by a

corresponding increase in the optical density. Such a phenomenon would not be possible and arose probably due to an error in performing the experimental protocol. The last point on the graph was therefore eliminated from the correlation. Possible explanations for the unexpected rise in viable bacteria include an incorrect dilution of the samples in the last measurement point, or improper mixing of the settled bacteria in the CTC suspension which could have led to the pipetting of an aliquot of a higher concentration of bacteria from the bottom of the vial. The possible error may also explain the increased variability (as represented by error bars) in the last point on the graph.

The equation, derived here, was used in all consequent experiments to estimate the number of viable bacteria from the measurement of optical density of a suspension.

4.4 Calcium carbonate precipitation with *S. ureae*

When *S. ureae* was grown and re-suspended in cementation medium, a crystalline material formed in the solution after approximately a week. Samples of this material were characterised by X-Ray Diffraction for phase identification and were also observed under SEM.

4.4.1 X-Ray Diffraction

To determine the type of calcium carbonate polymorph produced by *S. ureae*, crystals formed from a bacterial suspension were collected, dried, powdered, and analysed by x-ray diffraction (XRD) (as described in section 3.2.8). The scatter produced by the samples in this study were analysed and the results indicated that the samples contained pure calcite (Figure 4-6).

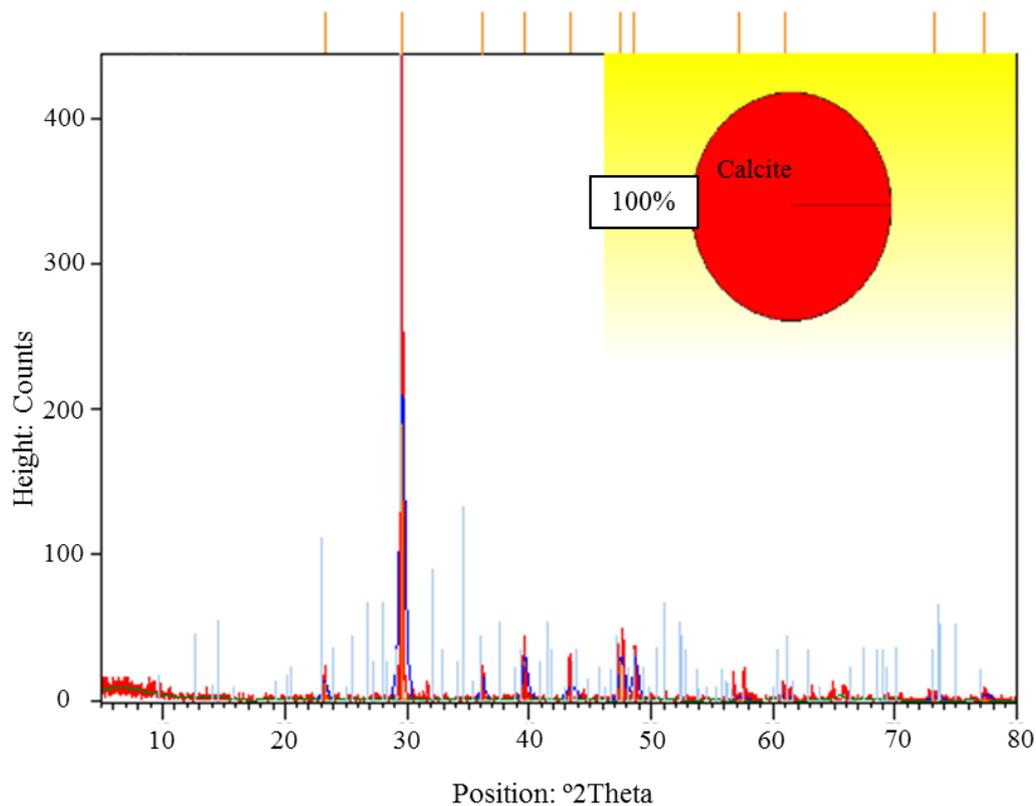


Figure 4-6 X-Ray Diffraction analysis of calcium carbonate produced by *S. ureae*

4.4.2 SEM analysis of CaCO₃

Calcium carbonate crystals produced by *S. ureae* in solution were visually examined under a Scanning Electron Microscope (SEM) (as described in section 3.2.7). The resultant micrographs are shown in Figure 4-7. Large crystal conglomerations were observed which ranged between 200 and 400 μm in size (Figure 4-7a, b). Upon examination of the close-ups of the two conglomerates (magnified area indicated by red arrows), one shows clusters of relatively small crystals (Figure 4-7a, c, e, g) ($\sim 10 \mu\text{m}$), and one shows a large angular massif (Figure 4-7b, d, f, h), both closely associated with bacterial cells. Also, cells become apparently cemented in a “cocoon” of calcium carbonate in the process of precipitation (term used by Castanier et al. (1999)). Figure 4-7h depicts a crack in the crystal which is an evidence of the rigid matrix formed around the cells.

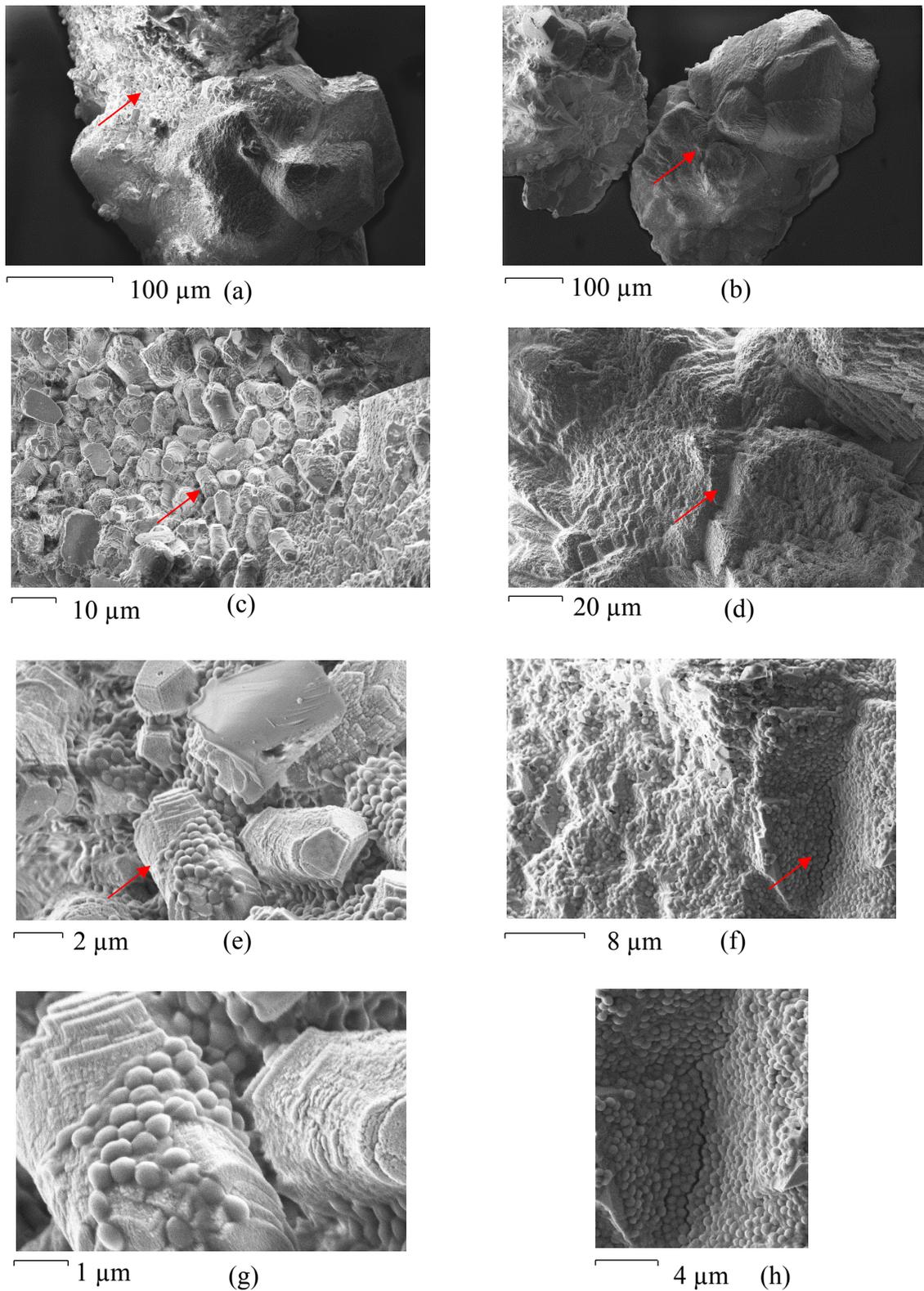


Figure 4-7 SEM images of calcite crystals, formed in solution by *S. ureae*. Small regular crystals clustered to form a big block (a), with close-up in (c), (e) and (g). A large angular massif (b), covered in cocci cells and a crack, with close-up in (d), (f), and (h).

4.5 Regeneration of bio-cement entombed spores

This section discusses the results from an experiment in aqueous solution which investigated the ability of a sporulated organism to firstly produce bio-cementation in the course of which it becomes entrapped within the bio-cement. Regeneration of the spores was examined after the bio-cement enveloping them was broken down (dissolved in weak acid) and nutrients were supplied to the spores. The protocol outlined in section 3.3.2.4 was followed. Autoclaving was the method used to kill viable cells from the surface of the bio-cement in laboratory conditions. In real-life situations, e.g. in the subsurface, prolonged periods of starvation, space confinement, and physical/ chemical stresses may lead to a dramatic decrease in viable cell numbers and cause the formation of spores. In essence, autoclaving was used in the laboratory to simulate the worst case scenario whereby all vegetative cells are lost over time.

When pure cultures (without bio-cementation) of both organisms were autoclaved (Figure 4-8a and b) neither of them survived the process confirmed by the subsequent lack of growth when placed in the growth medium. This indicates that both cells (*S. pasteurii*) and spores (*S. ureae*) are killed by autoclaving. Cultures of both sporulating and non-sporulating organisms, precipitated calcium carbonate (which was not sterilised) and demonstrated growth (regardless of whether the CaCO₃ matrix was broken down or not) when they were presented to a nutrient-rich medium (Figure 4-8c, d). This indicates that the crystal washing process did not affect some cells as undissolved and dissolved crystals led to similar growth rates. When *S. ureae* crystals were dissolved and re-suspended in growth medium, the final optical density was greater than for dissolved *S. pasteurii* crystals. This may have been due to differences in the growth kinetics of the two strains, or a higher degree of survival of the sporulating species (during the bio-cementation and subsequent dissolution processes) and a higher

culturability of the spores, as suggested by O'Sullivan et al. (2015). Although the non-sporulating species (*S. pasteurii*) also survived the bio-cementation and dissolution process such a phenomenon is not expected to be long-lasting. This short-term survival could explain the healing effect of *S. pasteurii* on previously cemented sands described by Montoya and DeJong (2013) and Harbottle et al. (2014), however the presence of bacterial cells alone cannot guarantee the same effect in the longer-term. Finally, when the carbonate crystals were autoclaved (Figure 4-8e, f) the non-sporulating *S. pasteurii* did not regenerate and grow upon re-suspension in growth medium. This was the case for both the dissolved and undissolved crystals which indicates that the vegetative cells did not survive the sterilisation process regardless of whether they were on the surface of the crystal or encased within it. The sporulated *S. ureae*, in contrast, exhibited growth only when the crystal was broken down. This suggests that only the encapsulated spores survived calcification and autoclaving (i.e. adverse conditions) and germinated, but only when they were exposed from the calcium carbonate matrix due to the inflicted damage (acid dissolution) and were supplied with nutrients.

It has previously been shown by O'Sullivan et al. (2015) that certain spore-forming species of bacteria are able to survive multiple autoclaving cycles. Furthermore, mineral precipitation has been described primarily as a defence mechanism used by bacteria to enhance their competitiveness and aid their survival in hostile conditions (Ehrlich 1996). It should be noted, however, that while encapsulated spores survived autoclaving, this was not the case for non-sporulating cells encapsulated in calcite. Therefore, the combined effect of the cells sporulating and the protective properties of the mineral surrounding them played a role in surviving autoclaving.

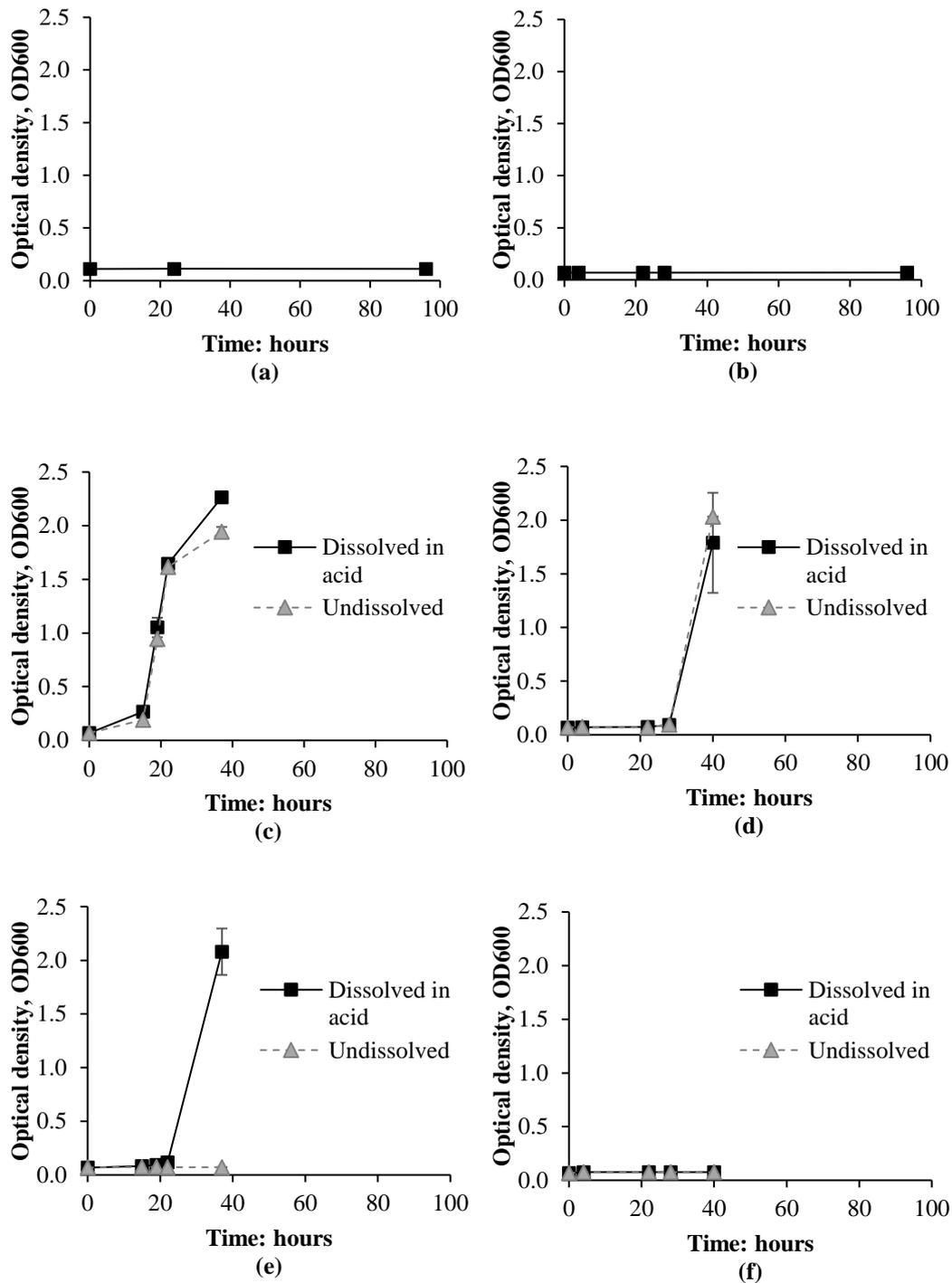


Figure 4-8 Growth of *S. ureae* (sporulating organism, Graphs a, c, and e) and *S. pasteurii* (non-sporulating organism, Graphs b, d, and f) following sterilisation (a and b); carbonate crystal formation (c and d) or carbonate crystal formation and sterilisation (e and f). (Error bars: ± 1 standard deviation (SD), n=3)

4.6 Secondary calcite precipitation

Upon regeneration of the spores from the experiment in the previous section (set (e)), 5 separate samples were harvested and introduced to cementation medium, to examine their precipitation abilities (Figure 4-9), according to the procedure outlined in 3.3.2.5. In the first cycle pH increase (indication of urea hydrolysis) was observed up to approximately 8 on average from 5 samples. In 2 out of the 5 samples, the pH increased more rapidly up to between 8.5 and 9, whereas in the other 3 samples, the pH developed slower and at 120 hours pH values in the range of 7.5 to 8 were recorded. This led to a significant variation in the results which is represented by the error bars on the graph. This could have also led to some of the samples' decreased metabolic activity and resulting weaker pH response. However, in all 5 samples, regardless of the degree to which their pH changed, calcite crystal formation could be observed.

In addition, the ability of this system to respond to multiple cycles of damage has been explored. The cycle was repeated using secondary calcium carbonate precipitates containing spores and regeneration and calcite precipitation was again observed (in 4 out of 5 samples). However, a smaller increase was observed in the pH which could be due to a fatigue of the bacterium after undergoing autoclaving multiple times, as well as possible enzyme damage (heat denaturation) inflicted to the spores again as a result of the autoclaving, as previously suggested by Setlow and Setlow (1998).

In contrast to the bacterial suspensions in the first and second cycle, abiotic controls (i.e. non-inoculated cementation medium) showed no change in pH over 120 hours.

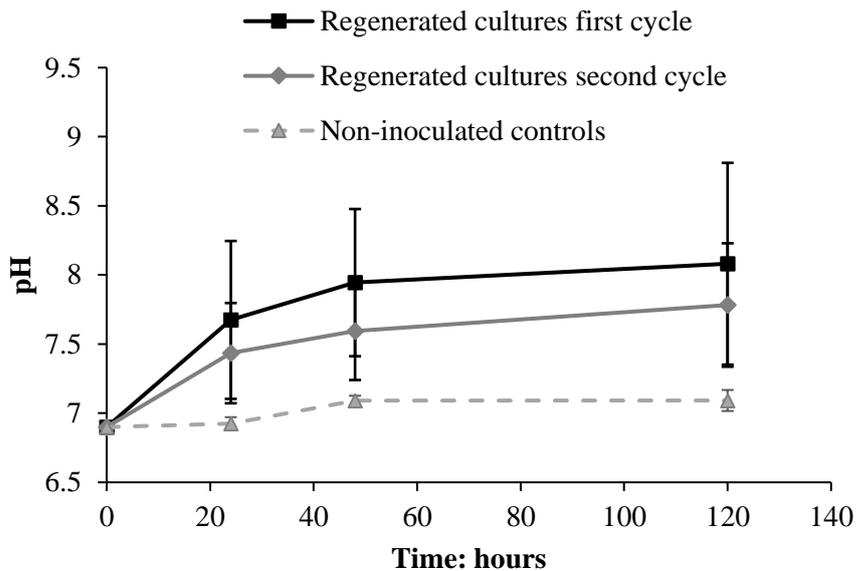


Figure 4-9 pH increase (as an indication of ureolysis) induced by regenerated spores of *S. ureae* in cementation medium. (Error bars: ± 1 SD, $n=5$).

The results from the experiments, shown in Figure 4-8e and Figure 4-9, indicate that the spores are able to return to their vegetative form after the extreme process of autoclaving and damage to the mineral and that they also retain their calcite precipitating abilities. Also, they are able to repeat the formation of calcium carbonate after undergoing 2 cycles of encapsulation, adverse conditions and break-down. This demonstrates the fundamental concepts behind self-healing process, that the bacteria are not only able to perform damage healing once, but even after going through various extremes multiple times, they survive and retained their metabolic activity which allowed them to form precipitation and heal damage repeatedly.

4.7 Long-term survivability of the entombed spores

The aim of this experiment was to examine the ability of calcite-entombed spores of *S. ureae* to survive prolonged periods. The bacterial calcite, produced in solution was stored in sterile containers at room temperature for 3 and 6 months. The crystals were

cleaned, sterilised, dissolved and suspended in a nutrient solution (procedure described in 3.3.2.6) after 3 and 6 months of storage and regeneration was monitored via optical density. As can be seen from Figure 4-10, spores of *S. ureae* germinated and regenerated which lead to an increase in the optical density of the bacterial suspension to above 1.5 after 40 hours of incubation.

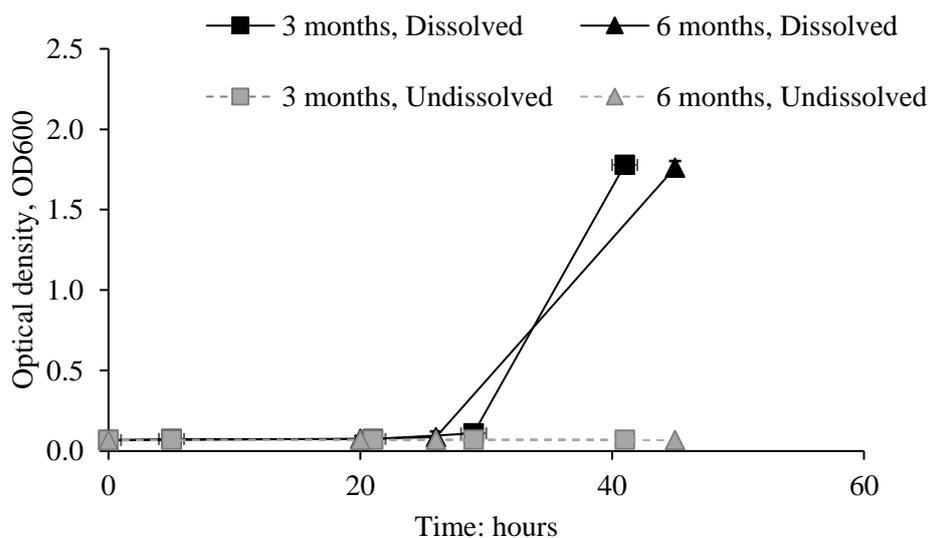


Figure 4-10 Long-term survivability of *S. ureae* spores encapsulated in CaCO_3 . (Error bars: ± 1 SD, n=5).

There was an increased lag time of approximately 20 to 30 hours before regeneration began, as compared to fresh cultures which normally start exponential growth between 2 and 5 hours. The prolonged lag could have been as a result of the time it took the spores to detect a change in the environment, i.e. availability of nutrients, and return to their vegetative state. Also, spores stored for 3 months exhibited a higher specific growth rate compared to the spores after 6 months. It is important to notice that regeneration was only observed when the spores were released from their calcite cocoon - undissolved calcite crystals suspended in nutrients did not show regeneration (as

represented by grey dashed lines, Figure 4-10). This is in agreement with the experimental results from section 4.5.

4.8 Discussion of results

The apparent inability of *S. pasteurii* to form spores (as seen by microscopic evidence) has not been documented previously. Sporulation in bacteria is a complex process involving a chain of reactions which only occur under specific conditions (Schaeffer et al. 1965; Nicholson et al. 2000; Driks 2002; Setlow 2006). Therefore the reasons for *S. pasteurii* not sporulating may lie in the specific physiological properties of this particular strain and the culture conditions under which it sporulates. It is also possible that the strain has been incorrectly assigned to sporulating genera (*Sporosarcina*). Nevertheless, more research is needed by specialists in the field in order to determine the reasons behind the apparent inability of *S. pasteurii* to sporulate.

Due its inability to sporulate *S. pasteurii* was not used further in self-healing experiments. It proved useful, however, in serving as a negative non-sporulating control in the regeneration experiments in section 4.5. It was also used as a baseline very strong ureolytic organism in the optimisation study of MICP with *S. ureae* (presented in chapter 5).

Several reasons led to the elimination of *B. cohnii* as a suitable candidate for further use also. As it was found from bio-cementation experiments, cultures of this bacterium which were inoculated in B4 medium (section 3.1.1.2) were only observed to start the formation of a crystalline material in solution after approximately 2 weeks of incubation. Similar observations have been previously made in studies by Boquet et al. (1973) and Sharma et al. (2017) (personal communication with the latter). Additionally, *B. cohnii* is a non-ureolytic organism (Spanka and Fritze 1993) and therefore it is unable

to participate in the hydrolysis of urea. It is also an obligate alkaliphile with an optimum pH for growth of 9.7. In the study by Spanka and Fritze (1993) they demonstrated that *B. cohnii* does not grow in a medium with a pH of 7. Therefore, in order for this organism to precipitate calcium carbonate, it had to be incubated in a medium with an artificially adjusted pH and maintained in an incubator for about 2 weeks. In contrast, a ureolysis driven calcium carbonate precipitation (previously described in chapter 2) causes the rise in pH as a result of the bacterial metabolism, which in turn favours precipitation, and has been shown to be a much more rapid process - for example assimilation of nutrients by *S. pasteurii* takes 1-2 days (Whiffin et al. 2007; van Paassen et al. 2010). Also, as seen by previous researches, the number of nutrient injections in a soil may vary significantly depending on the desired level of cementation produced by the MICP process - from 10-15 (van Paassen et al. 2010; Gomez et al. 2015), 36 (Cunningham et al. 2011), or even 190 (Ferris and Stehmeier 1992). Hence, a fast metabolic assimilation of nutrients by the cells is of a significant importance, specifically when the MICP is applied to soil. While urea hydrolysis is a process which can be easily controlled via alteration of biomass and substrate concentrations (calcium and urea, as discussed previously in chapter 2), the alteration in the concentrations of the components (particularly calcium and the carbon source) in the B4 medium did not significantly change the time for precipitation with *B. cohnii* (work not presented). Therefore, the use of *B. cohnii* was not deemed viable in this study.

S. ureae showed strong sporulation abilities and calcium carbonate precipitation (albeit with a longer period for incubation compared to *S. pasteurii*) and was therefore chosen for further work. A very typical growth curve was constructed for the two growth conditions in which *S. ureae* was tested (normal growth medium and cementation medium) with fairly well-defined lag, log and stationary phases. The reduction in

growth rate and final optical density (a proxy of number of bacteria in suspension) when the bacterium was subjected to cementation medium was expected and was most probably brought about by the crystallisation occurring around the bacterial cell walls which leads to the partial or complete enclosure of the bacteria and limits nutrients needed for cell multiplying. Therefore, although the bacteria may have still been able to multiply and increase in numbers, its inclusion within the CaCO₃ would have limited this ability. This is in agreement with Cuthbert et al. (2012) who described the MICP process as “self-limiting” because of the encapsulation of the microbial communities by precipitation.

Calcium carbonate was precipitated in the form of calcite, as was found by XRD analysis. The presence of this polymorph of CaCO₃ was consistent with many other studies of MICP (DeJong et al. 2006; Whiffin et al. 2007; Tobler et al. 2011; Cuthbert et al. 2012; Al Qabany and Soga 2013; Chu et al. 2013). It should be noted that in a publication by De Muynck et al (2008a) it was found that the type of polymorph depends on the nutrients supplied to the bacteria. Examining effects of the type of nutrients on the polymorphism of biogenic CaCO₃ was outside the scope of this study, however it would be an important factor in taking MICP to larger-scale applications. The predominant precipitation of calcite by *S. ureae* is desirable, in that this polymorph is the most stable and consequently would be more resilient to deterioration processes.

SEM microscopic observation of the microbially produced crystals revealed that bio-cementation exhibited different shapes and forms. The images show what appear to be early biomineral aggregates in clusters which were formed by “cocooned” cells and exhibited angular shapes. Such clusters appear to have further grown with more precipitation deposited on the surface, thereby forming progressively more crystalline structures. A possible explanation to the presence of different shapes of calcium

carbonate formed by bacteria has been given by Castanier et al. (1999). In bacterial mineralisation, the predominant force behind crystallisation at the initial phases is governed mainly by the strong activity of bacteria (in a nutrient-rich environment). Although early crystals formed by bacteria show some evidence of angularity, they rarely exhibit an exact crystallographic structure. As carbonate formation progresses and the bacterial activity starts to weaken (due to nutrient exhaustion or crystal encapsulation), the primary aggregates start to form secondary accumulations of mineral which show increasingly more crystalline structures. This second phase of carbonatogenesis occurs in a partially abiotic manner (due to the weak nutrient environment) and therefore crystallographic physical rules overtake, thereby producing more regular crystalline structures.

To demonstrate without ambiguity that spores can survive the processes of bio-cementation and break down of the mineral phase, a range of different controls had to be accommodated in the experiment. The response of the bacteria (both sporulating and non-sporulating) had to be examined at various extremes, i.e. pure cultures (without bio-cementation) exposed to sterilisation, bio-cemented cultures tested without sterilisation, and a combination of the two (sterilised bio-cemented cultures). In agreement with previous studies, vegetative cells and spores did not survive autoclaving (Perkins et al. 2004) when bio-mineralisation did not take place in the cultures. However, it should be noted that certain extremophile sporulating bacteria has been shown to survive autoclaving (O'Sullivan et al. 2015). The results from the non-sterilised bio-cemented cultures (specifically when performed with *S. pasteurii*) may be correlated to the regeneration and healing seen in the studies by Montoya and DeJong (2013) and Harbottle et al. (2014). Although this organism was shown not to be spore-forming, it was nevertheless able to survive the bio-cementation and break-down process and

recover the mass of calcium carbonate which can be regarded as healing in the aqueous solution experiments. However, regeneration and further precipitation after sterilisation can only be seen in *S. ureae* cultures, and not with *S. pasteurii*. This is a clear indication that the sporulating organism is more durable and resistant to various environmental extremes following which it can bring about further precipitation.

Though previous research on microbially enhanced concrete differs significantly from the experiments in this study in terms of testing conditions and strains of bacteria used, a correlation between the results from this project and self-healing concrete publications can be drawn. It could be argued that, in essence, experiments performed by Jonkers and Schlangen (2007) and Sharma et al. (2017) for testing the survivability of spores in cement stone are very similar to the experiments, undertaken in this work. In their investigations, spore suspensions of *B. pseudofirmus* were immobilised in cement stone samples (as opposed to the naturally produced mineral in the experiments from this project), viability was assessed after rupturing the samples and: i) inoculating them in nutrients to induce bio-cementation (Jonkers and Schlangen 2007), or ii) plating out on agar plates for viable bacterial counts (Sharma et al. 2017). Similarly to the results presented in this chapter, they found spores regenerated and produced biominerals after being exposed from the cement stone matrix and also had the ability to survive for 3 months (which was the duration of the test performed by Sharma et al. (2017)). The longer survival period (6 months), which the spores of *S. ureae* endured in this project, could have been due to the “friendlier” environment in which they were stored and differences in spore properties of *S. ureae* compared to *B. pseudofirmus*. In this study, a culture of *S. ureae* was suspended in a cementation medium to induce precipitation until exhaustion of the medium, the bacterium was then stored in a calcified form at room temperature for 6 months. Additionally, while the spores of *B. pseudofirmus* were

subjected to a pH of about 12-13 in the cement stone samples, the calcium carbonate mineral enveloping the spores of *S. ureae* buffered the pH to around 7-8 (results not presented) which is a less challenging environment. As discussed in chapter 2, in publications on self-healing mortar performed by Wang et al. (2012a; 2012b; 2013; 2014) they encapsulated spores of *B. sphaericus* in various cargo materials (diatomaceous earth, silica gel, hydrogel or microcapsules) in order to enhance their survivability in the highly alkaline pH in the mortar. The outcomes of this study suggest, however, that such a protective carrier can in fact be the biomineral produced by the cells themselves. Such naturally produced capsules would probably be more compatible with the bacteria and may prove to provide a more effective guard than those created artificially. However, more research is needed in order to assess and compare the functions of a naturally formed bio-carrier with other materials.

Although repeated healing of damage is of paramount importance in achieving improved durability due to self-healing, such experimental work in microbial self-healing has not yet been undertaken. Spores, extracted from salt and amber (Cano and Borucki 1995; Vreeland et al. 2000), as well as mortar and concrete (Jonkers and Schlangen 2007; Sharma et al. 2017), can be rejuvenated even after a significant period of time and have been shown to preserve their enzymatic activities. However, it has been somewhat unclear as to how many times a bacteria can go through adverse conditions and sporulation, followed by germination in favourable conditions. Here the principle of multiple self-healing cycles was demonstrated three times from a single starting culture in aqueous solution experiments. Although referred to as “self-healing”, the process was not strictly autonomic as sporulation of the bacterium, as well as the bio-cementation was achieved in an artificial manner through the provision of specific media. In natural conditions (e.g. subsurface) bacteria would sporulate when exposed to

environmental stress and such artificial media may not be required since natural groundwater may provide a sufficient supply of nutrients and required substrates (a source of carbon and calcium) for the bacteria to produce bio-cementation.

Alternatively, encapsulation of the cementation medium may be adopted, similarly to the nutrient supply achieved in concrete applications (Wang et al. 2014; Kanellopoulos et al. 2017) to allow for enough amount of nutrients needed to cause healing of a damage. Depending on what material the capsules are made of, as well as their size (micro- or microcapsules), they may be injected using conventional grouting systems with pumps and wells in the subsurface, or they may be pre-mixed with the soil in man-made earth structures.

Nevertheless, evidence of the ability of the microorganisms to perform repeated healing cycles, albeit with the external intervention provided in this study, has been provided.

This evidence has formed the baseline for progression of this research project into applying microbial self-healing in a more challenging environment in particulate media which is discussed in chapter 6.

4.9 Summary

One of the objectives of this thesis was to investigate self-healing in optimum conditions through a bacterium which forms precipitation metabolically and is able to form spores. In this chapter, a suitable bacterium was selected among three potential candidate strains. Sequentially eliminating *S. pasteurii*, because it did not form apparent spores under the range of different conditions it was tested, as well as *B. cohnii*, because of the extended time it needed for precipitation. The most appropriate organism was shown to be *S. ureae*, as it was successfully able to form calcite (albeit with a prolonged incubation period) due to hydrolysis of urea by its enzymatic activity and when

subjected to a sporulation medium it also formed copious amount of spores in a short period of time.

The requirement for a microbial self-healing agent to heal damage was investigated through a regeneration experiment in this study. Initially sporulated cultures of *S. ureae* were shown to be able to produce bio-cement, in the process of which the bacteria became enclosed within the precipitate. An important aspect of this study was the use of a rigorous sterilisation procedure for removal of viable cells, as well as the use of various controls, which ensured that only the effect of the spores - embedded in calcite - was studied. The results from the regeneration experiment revealed that the bio-cemented *S. ureae* spores were resistant to the bio-mineralisation and subsequent sterilisation processes and maintained their viability during both stages. The protection, provided by the bio-cement played a role in their survival. Previous studies on MICP in cementitious materials (outlined previously in chapter 3) have used a range of artificial carrier materials for the protection of bacterial spores, whereas this study suggests that a naturally produced bio-mineral can perform a similar function.

Also discussed was the ability of the crystal-embedded bacteria to survive prolonged periods and form further precipitation when released from the crystal and supplied with nutrients. This ability of the bacterium would allow healing of damage in a bio-cemented body of sand which is discussed later in chapter 6. Also, the encapsulated bacteria were shown to still be viable when released from the crystal after 6 months of encapsulation. Other literature reports that spores of bacteria were rejuvenated after much longer periods (years) which suggests that *S. ureae* may also be capable of this given the right conditions. Long-term survival is also important in facilitating the durability of the self-healing process and it will ensure the activation of the mechanism even after a prolonged “passive” period of no damage.

Most importantly, this chapter presented cycles of bio-cementation by the sporulated bacterium followed by breakdown of the mineral, rejuvenation of spores, and further formation of bio-cement. The repetition of the above-mentioned sequence using *S. ureae* was an aspect of this work which has not been demonstrated before. Although the process was demonstrated with one strain of bacterium it can be speculated that a similar performance could be expected from other bio-mineralising (regardless of the metabolic process that they facilitate) spore-forming bacteria. Also, the concept could be transferred to materials to which MICP can be applied (i.e. cementitious and earth materials).

Even though performing cycles of healing in this study required human intervention throughout and was demonstrated at optimum conditions for the bacterium, the outcomes of the experiments provided vital information for the governing processes of self-healing at small-scale. Future studies could potentially explore the microbial self-healing process in a less “ideal” environment, when subjected to natural subsurface conditions. That is, the growth, sporulation, and bio-cementation kinetics of a bacterium in media which resembles natural groundwater, for example. Such a study could determine the minimum requirements for maintenance and would investigate the possibility of a true, autonomous and active self-healing process. This was, however, outside the scope of this thesis and despite this holding great interest for the author, was not investigated further.

5 Optimisation of MICP with *S. ureae*

5.1 Introduction

As outlined earlier in chapter 2, substrate concentrations are some of the factors which greatly affects MICP and is easily controlled in laboratory conditions. This chapter presents a comparison between the ureolytic activities of the most commonly used microorganism in MICP so far – *S. pasteurii* – and the bacterium (*S. ureae*) selected earlier in this study (chapter 4). A batch experiment coupled with a theoretical model is presented and the metabolism and calcium carbonate precipitating ability of *S. ureae* under a range of concentrations of calcium in both tap and deionised water in aqueous solution or sand is studied. A geochemical model, MINTEQ, was used to compare predicted bacterial influence on the pH with the test values from the batch experiment. Additionally, the various concentrations in the cementation media were examined in a dynamic flow system of soil columns and their effect was discussed in terms of the geotechnical improvement of the soil.

5.2 Soil properties

All experiments, which involved the use of particulate media, employed a silica sand obtained from Hepworth Minerals and Chemicals Ltd. Prior to utilisation in bacterial experiments a range of soil classification tests were performed. The sand was acid washed and sterilised as described in section 3.1.2.4.

5.2.1 Particle size distribution

Details of the techniques used to obtain the particle size distribution curve can be found in section 3.1.2.1. The particle size distribution of the silica sand is shown in Figure 5-1. According to BS the soil was classified as a well-graded sand ($d_{10}= 100 \mu\text{m}$, $d_{90}= 820 \mu\text{m}$, $C_U= 4.90$, $C_C= 1.43$).

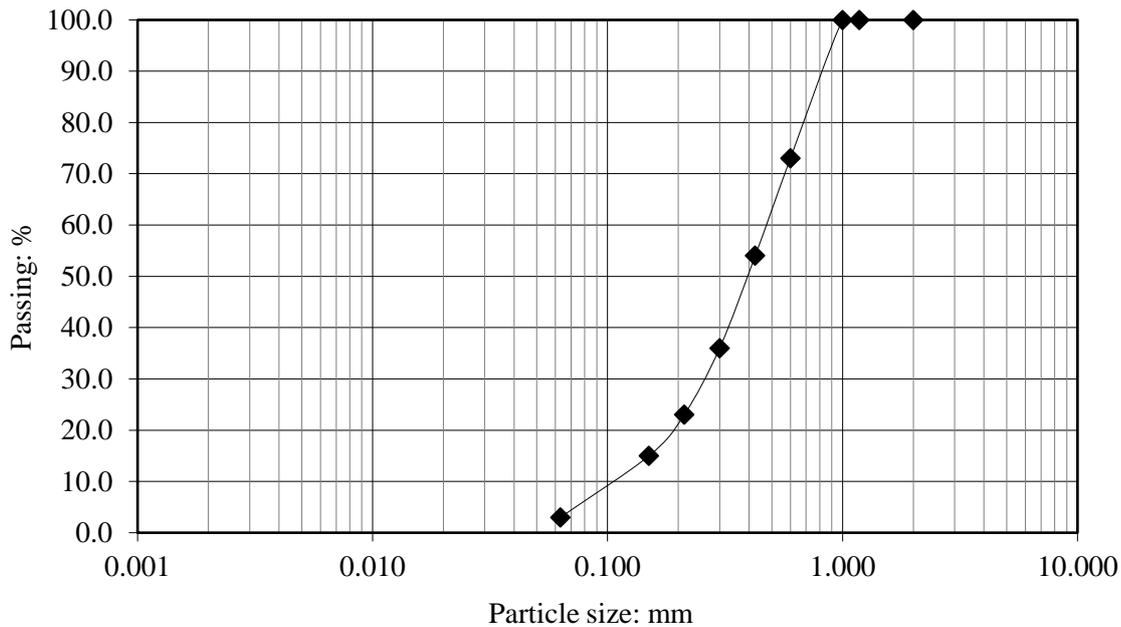


Figure 5-1 Particle size distribution of silica sand

5.2.2 Specific gravity of soil particles

The specific gravity (Gs) of the soil particles was determined to be 2.73 (using the method in 3.1.2.2).

5.2.3 Maximum/Minimum Dry Density and Optimum Moisture Content

The maximum dry density was found to be 1.742 g/cm^3 which corresponded to an optimum moisture content of 10 %. The minimum dry density was found to be 1.493 g/cm^3 . The values for maximum dry density and optimum moisture content were obtained from Figure 5-2. The data was fitted using a polynomial curve of second order ($R^2=0.9828$). The details of the methods used are given in 3.1.2.3.

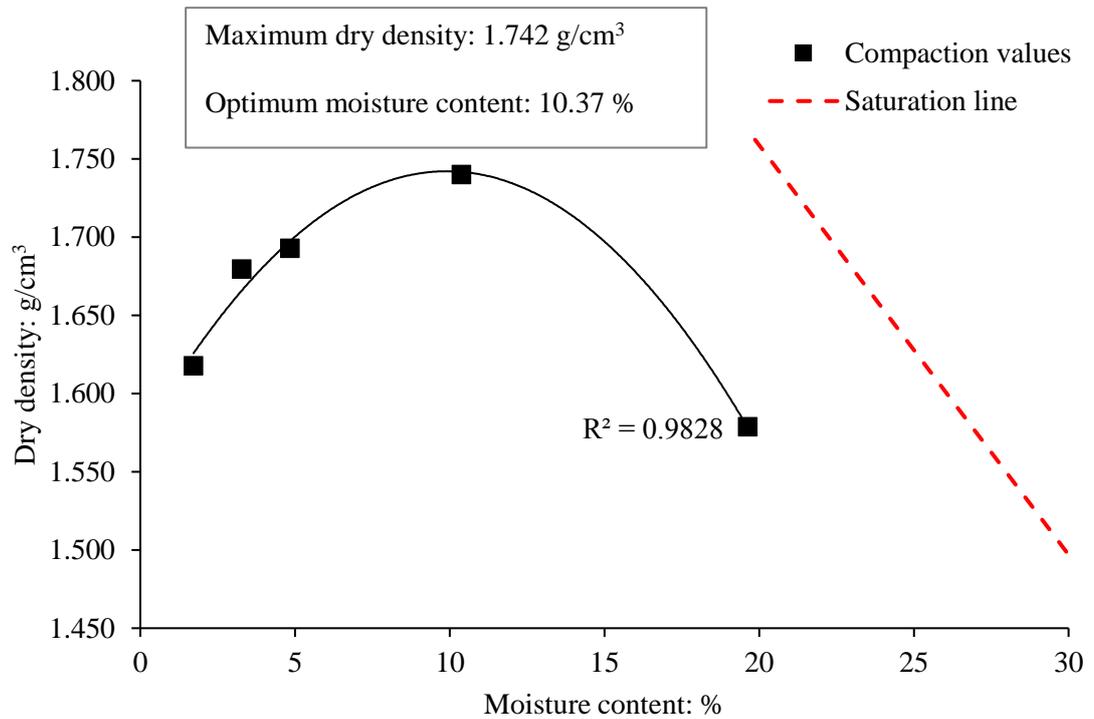


Figure 5-2 Moisture content/ dry density curve

5.3 Metabolism of *S. ureae* and *S. pasteurii* - a comparison

In order to evaluate the ureolytic activity of *S. ureae*, a batch test was performed according to the method specified in 3.3.3.1. *S. ureae* was compared against a highly ureolytic organism *S. pasteurii*. Ureolytic activity was evaluated through the organism's ability to raise the pH of the cementation medium while simultaneously utilising the free calcium in solution (converted into CaCO₃). Measurements of pH and calcium were performed according to the methods specified in 3.2.1 and 3.2.2. The experiment was performed with the two strains of bacteria under four situations in an increasingly challenging environment: i) aqueous solution with shaking incubation (optimised condition); ii) aqueous solution with a presence of sand (in 2:1 mass ratio) with shaking incubation; iii) aqueous solution with a presence of sand (in 2:1 mass ratio) with static incubation; iv) in fully saturated (loose) sand with static incubation.

The results from the experiment are presented in Figure 5-3a (*S. ureae*) and Figure 5-3b (*S. pasteurii*). An initial relatively rapid increase in pH to about 8 was observed in the *S. ureae* samples together with a very slow, gradual decrease in calcium (of 10 to 40 %) over the 5 to 7 days in which *S. ureae* was subjected to the cementation medium. Static or shaking incubation made no significant improvement to the behaviour of *S. ureae*. The lowest pH increase (to about 8 after 7 days) was observed in condition “iv” in the fully saturated sand. However, the highest calcium conversion also occurred in “iv”. This suggests that the presence of the sand may have either limited the bacterial activity or it may have buffered the pH, while contributing to the adsorption of calcium ions to the sand particle surfaces. Vapours, gases, cations and anions in solution get adsorbed to solid matter when they come in contact (Stowell 1927). Another research study, performed with the same sand under very similar conditions, reports similar observations for calcium adsorption to the soil (Mugwar 2015).

In contrast to *S. ureae*, *S. pasteurii* rapidly increased the pH to 9-9.5 in all conditions (i-iv) and depleted all available calcium in solution within 24 hours of the start of the test. Similarly to *S. ureae*, the lowest increase in pH was found in the fully saturated sand (pH 9.2, iv), although in contrast to *S. ureae*, the calcium was reduced to zero after 24 hours of testing.

For the conditions in which the two strains were tested in this study the different responses of both in terms of pH and aqueous calcium (which are indicators of bacterial ureolytic activity) suggest that the two have different urease enzymatic activities.

Although *S. pasteurii* and *S. ureae* are closely related (MacDonald and MacDonald 1962; Pechman et al. 1976), their urease enzymes have been shown to be chemically and structurally different at the molecular level, thereby the two strains have different urease activities (McCoy et al. 1992). In a study by Hammes et al. (2003), in which 12

different ureolytic isolates of bacteria were tested for their CaCO₃-precipitating abilities, they found that considerable differences occur in the morphology and size of crystals, as well as rates of ureolysis among the different strains. Two of the isolates which were determined to be closest relatives to *Bacillus pasteurii* (currently known as *S. pasteurii*, section 3.1.1.1) displayed a 10-fold increase in ureolytic activity when they were in the presence of 30 mM of calcium. Hammes et al. (2003) attributed this effect to the role of calcium as a facilitator of a better “trans-membrane transport” and improved “intracellular signalling process”. Additionally, Tobler et al. (2011) used media ranging from 50 to 500 mM calcium and found that the activity of *S. pasteurii* was not affected by the increasing concentration. Others (Whiffin 2004; van Paassen et al. 2010; Sarmast et al. 2014) have adopted concentrations of up to 1.5 M of calcium and have observed the precipitation of copious amounts of CaCO₃ by *S. pasteurii*. However, it is unknown whether calcium plays a similar role in “trans-membrane transport” and cell signalling in *S. ureae* ureolysis. It is also unclear what concentration of calcium this bacterium can tolerate. The results from the experiment, presented here, indicate that *S. ureae* has a weaker urease when compared to *S. pasteurii* and it requires a much longer incubation period for substrate (calcium and urea) metabolism.

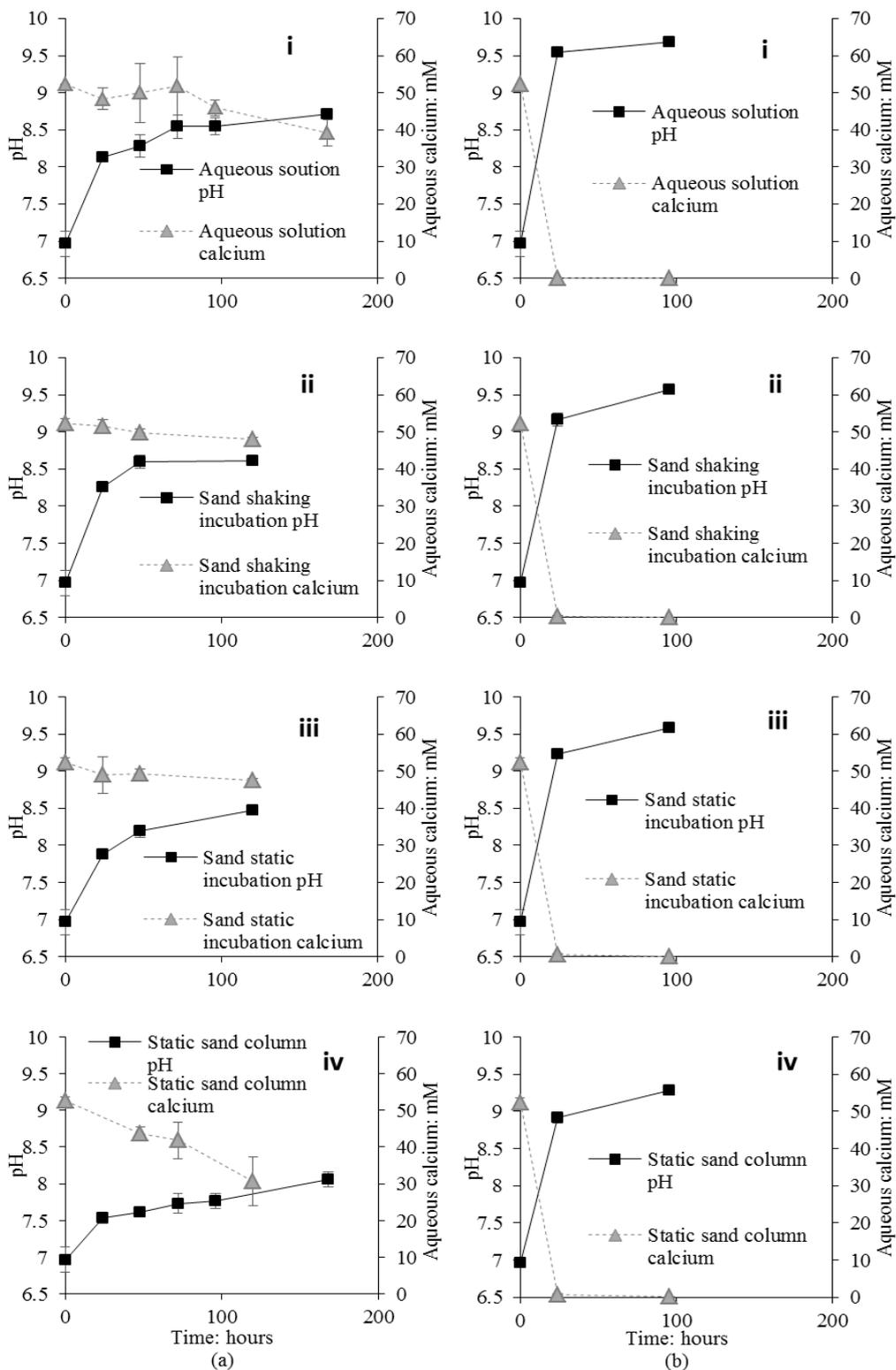


Figure 5-3 pH and calcium changes in (a) *S. ureae* and (b) *S. pasteurii*; (Error bars: ± 1 SD, n=3). Roman numeral indicate conditions of incubation: i. aqueous solution with shaking incubation; ii. aqueous solution with added sand under shaking incubation; iii. aqueous solution with added sand under static incubation; iv. saturated sand under static incubation

5.4 Effect of substrate concentrations and solvents

The following sections present the results of an investigation into the influence of calcium and urea concentrations and types of solvent (deionised or tap water) in the cementation medium on the precipitation of calcium carbonate induced by *S. ureae* in both aqueous solution and in sand. With regard to the slower metabolism of *S. ureae*, the issue with using this bacterium for the MICP treatment of soils is mainly related to the time needed for CaCO₃ precipitation to take place. According to Chu et al. (2014), a minimum of 1.3 % calcium carbonate precipitation is needed for a measurable change in geotechnical properties of the soil. A stoichiometric calculation was performed based on 1 mole of calcium ions forming 1 mole of calcium carbonate (Eq. 6, p.11). According to the calculation, a biological treatment using *S. ureae* and a cementation medium with 50 mM calcium (Stocks-Fischer et al. 1999; DeJong et al. 2006) of 1 kg of soil (density of 1.73 g/cm³), to reach a target of 3 % precipitation would involve a minimum of 19 injections of at least 1 pore volume of liquid (assuming all calcium ions in solution are metabolised to CaCO₃ after every injection). Each injection would be followed by 5-7 days of static period which would render the treatment 115 days long, or 16 weeks. Such a period of time is impracticable for investigations of limited duration and therefore ways of shortening the treatment period were investigated. As indicated previously in chapter 2, some of the easiest parameters to control are the concentrations of substrates in the cementation medium (calcium in particular). To illustrate, if in the example presented above the concentration of calcium was increased 6 times (to 300 mM) the treatment times would reduce correspondingly (again assuming substrates are fully converted to CaCO₃ at each injection). Various researchers have adopted a range of calcium concentrations in their media – from 25 µM to 1.5 M, therefore the concentrations which were tested in the current research were in the same range.

Additionally, geochemical software (MINTEQ v 3.1) was used to study the effect of the concentrations of calcium and urea in the medium on the theoretical pH increase in solution. The details on performing the batch experiment and the geochemical theoretical study are outlined in section 3.3.3.2.1.

5.4.1 Batch experiment

A batch experiment is presented in which exponentially growing *S. ureae* was suspended in a cementation medium with varying concentrations of substrates (Table 3-2) and incubated for 7 days. Control samples had no urea added to them. At the end of the incubation period measurements of pH (Figure 5-4) aqueous calcium ion (Figure 5-5), and mass of precipitate (Figure 5-6) served as indicators of bacterial activity in the various media tested.

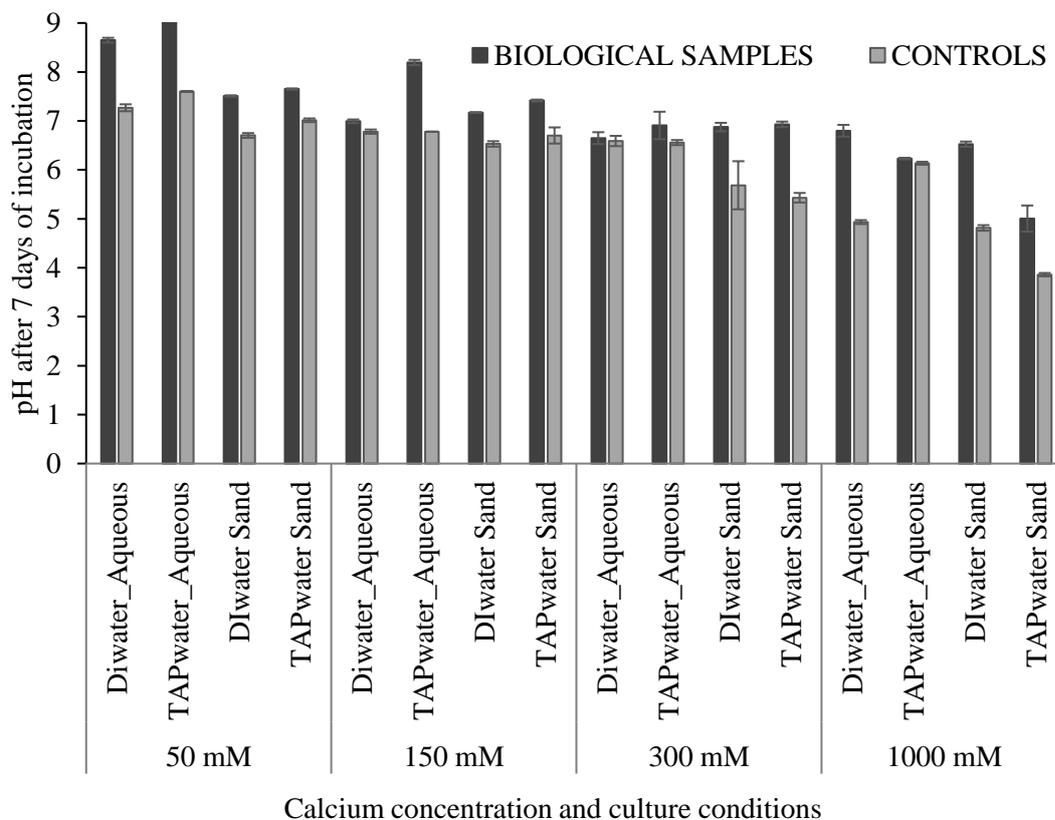


Figure 5-4 pH after 7 days of incubation; (Error bars: ± 1 SD, n=3)

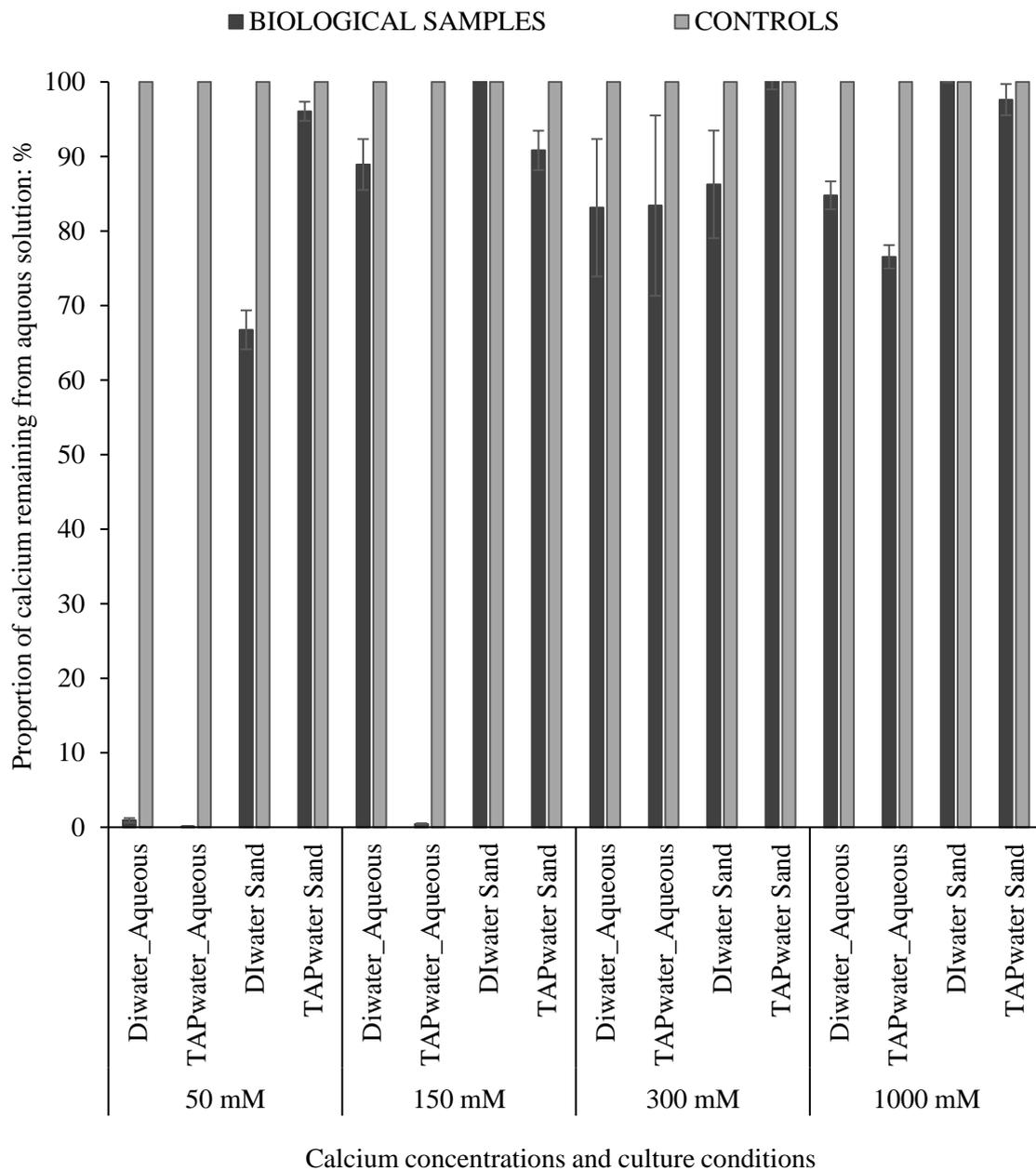


Figure 5-5 Aqueous calcium after 7 days of incubation; (Error bars: ± 1 SD, n=3)

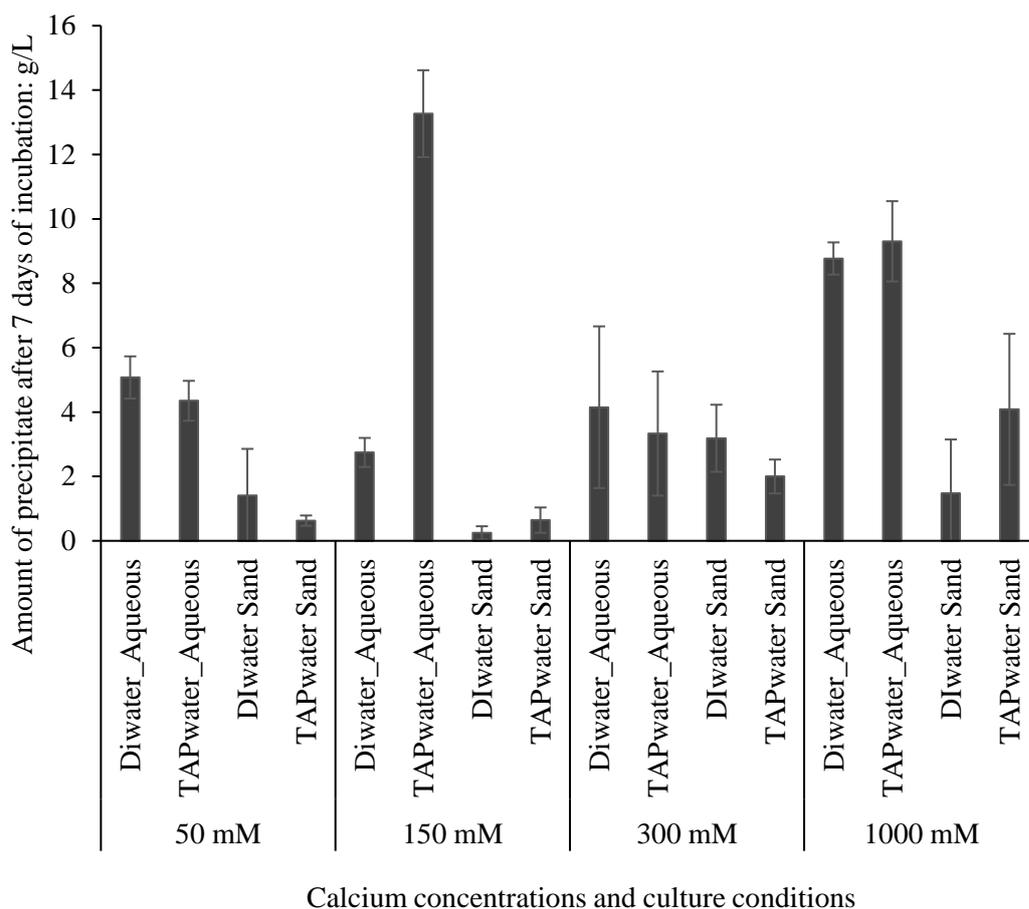


Figure 5-6 Mass of precipitate after 7 days of incubation; (Error bars: ± 1 SD, n=3)

Highest pH of 8.6-9.1 can be observed in the 50 mM aqueous biological samples with their sand counterparts showing significantly lower pH of around 7.5. Similarly, in all other instances (150, 300, and 1000 mM) the aqueous samples exhibited a higher pH than their sand counterparts. However, the 7-day pH in all samples was buffered by the increasing concentration of calcium, particularly in the 300 and 1000 mM solution. The pH in the biological samples of 150, 300 and 1000 mM concentrations were between pH6.9-8.1, pH6.6-6.9, and pH5-6.7, respectively.

It should be noted that in terms of reduction of pH and aqueous calcium the 50 mM calcium medium in both tap and deionised water yielded much better results in this experiment (as compared to the previous experiment in section 5.3 (Figure 5-3a, i-iv).

This could have come as a result of a more active starting culture (starting cultures were regularly replenished from the frozen stock and were observed to occasionally show stronger or weaker activities).

In addition to the buffering effect of the increasing calcium concentration on pH, it was observed that control samples of sand with cementation medium showed a decreased “natural” pH as compared to the same controls without sand. This effect was also imposed with the increasing concentration. The phenomenon can be explained with a chemical interaction and exchange of ions with the solute and the silica sand acting as an adsorbing agent. Calcium cations have a tendency to adsorb on the surface of the sand particles (Stowell 1927) creating a possibility of an imbalance of chlorine anions in the solution and resulting in the lowering of the pH. This mechanism would also explain the stronger effect seen in samples of 300 and 1000 mM concentration which would bring about more adsorption of calcium ions to the sand and a greater imbalance.

The sand samples were saturated by the bacterial suspension in cementation medium therefore the cells were occupying the volume of the pores in the soil and their metabolism and growth may have been limited due to a reduced supply of oxygen. Such factors have been shown to adversely affect bacterial populations in finer soil environments (Rebata-Landa and Santamarina 2006; Whiffin et al. 2007; Ivanov and Chu 2008) and may to an extent explain the reduced activity of *S. ureae* when in sand in this experiment. Aqueous calcium in the samples after 7 days also indicates a somewhat negative influence of the presence of sand on the calcium conversion to CaCO₃. While calcium was fully depleted in the biological aqueous samples of 50 mM this was not the case in their sand counterparts. The same effect, albeit not as strong, is observed in 150 and 300 mM biological samples, and to an extent, in the 1000 mM samples. Quantity of converted calcium reached almost 100 % in the majority of the

aqueous biological samples for 50 and 150 mM (apart from 150 mM in deionised water), and between 15 and 25 % for the aqueous samples for 300 and 1000 mM. Calcium conversion in the biological sand samples was significantly reduced, however, and was between 0 and 35 % for the former two concentrations and between 0 and 15 % for the latter two.

The effect of using deionised or tap water as a solvent for the cementation medium was also examined. The results indicate a positive effect of the tap water on the pH and the aqueous calcium depletion of the biological samples in both aqueous solution and sand, particularly for 150 and 1000 mM. The influence is especially strong in the 150 mM calcium medium in which there is a significant difference in the final pH and calcium decrease compared to samples of the same concentration in deionised water. The 150 mM concentration in aqueous solution in tap water resulted in full depletion of aqueous calcium and yielded the highest amount of calcium carbonate of 13 g/L as compared to all other testing conditions (Figure 5-6). The influence of tap water as a solvent may be explained with the presence of trace metals and minerals (Dinelli et al. 2012) which can contribute to the better overall bacterial activity (Pregerson 1973). It is, however, difficult to explain why the same positive effect in terms of calcium carbonate precipitation was not observed in the 150mM samples in sand with tap water - slightly higher amounts of precipitated calcium carbonate were observed in both the 50mM tap and deionised water samples in sand, despite the lower concentration. The 300 mM samples precipitated similar amounts of calcium carbonate as the 50 mM ones (ranging from less than 1 to approximately 5 g/L) despite the former having a 6 times larger concentration of calcium in solution.

Although the 1000 mM medium had a significant buffering effect on the pH which was between 4 and 6 in control samples and rose to a maximum of 7 in the biological

samples (Figure 5-4), there was an observed precipitation ranging from 2 to 10 g/L. Similarly to the observations made by van Paassen (2009) (who however used *S. pasteurii* as a catalyst for the MICP) the pH was buffered by high concentrations of calcium in the medium, however precipitation still occurred even at neutral pH.

The results from pH and calcium so far give indication that concentrations of 300 mM and above may be either too high for the bacterium to utilise for precipitation or may be hindering its activity. This is quite an important observation as there is very limited information on MICP with *S. ureae* and especially on the exact conditions under which *S. ureae* can function and precipitate large amounts of CaCO₃. Therefore, the results from the experiments in this chapter will help to elucidate under what range of calcium and urea concentrations this bacterium is active and can perform ureolysis.

All control samples remained clear of precipitation and therefore the mass of precipitate was only determined for the biological samples. There was, however, an observed white flocculated amorphous material formed as a suspension in the control samples, specifically in the 300 mM and 1000 mM aqueous samples which was considered to be formed abiotically due to the high concentration of calcium in solution. Despite this apparent abiotic precipitation it did not noticeably affect the calcium ion concentration in solution, however.

Additionally, calcium conversion efficiency (Figure 5-7) was calculated for each medium concentration as the ratio of actual calcium carbonate precipitated (taken from Figure 5-6) to the maximum theoretical yield. For example, the maximum yield for 150 mM calcium concentration is approximately 15 g/L calculated based on 1 mole of calcium ion (input) reacting to form 1 mole of CaCO₃ (according to Eq. 6, p.11).

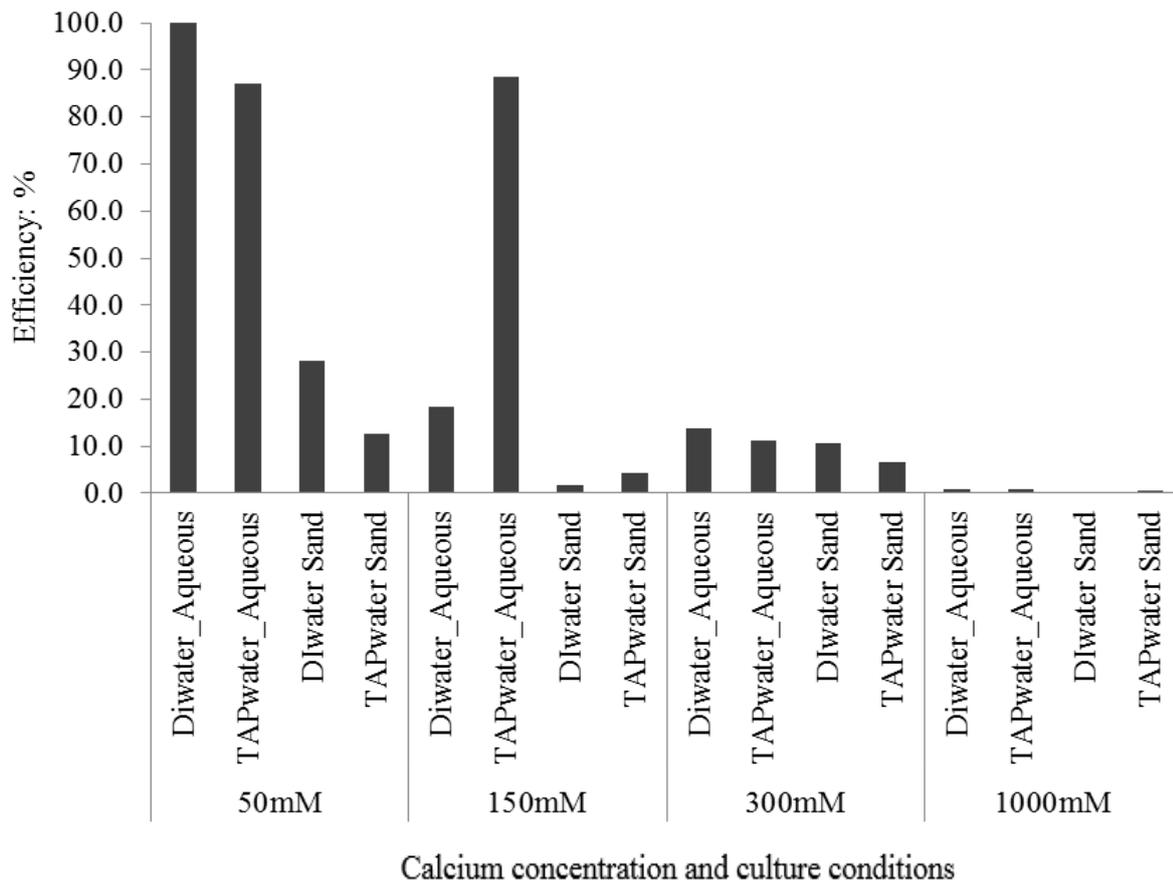


Figure 5-7 Calcium conversion efficiency in the batch experiment

It can be seen that the 50 mM aqueous solution with both tap and deionised water, as well as 150 mM aqueous solution with tap water approach the maximum calcium conversion efficiency. The presence of sand in both these concentrations resulted in a dramatic reduction in efficiency. Very low efficiencies were achieved when the bacteria were subjected to 300 mM calcium media (6 to 13 %) and 1000 mM (0.1 to 0.9 %) which suggests that these concentrations are possibly too high for *S. ureae*. As a result of the weakened ureolytic activity a significant portion of the medium would be left unused by the bacterium which would render it highly impractical for laboratory and especially for in-situ applications. Optimisation studies concerned with tolerable concentrations of calcium in the cementation medium are abundant with regard to MICP with *S. pasteurii*. This bacterium has been shown to be very adept and to be

hydrolysing urea and precipitating calcium carbonate at a remarkable range of concentrations of calcium in the cementation medium – from as low as 25 μM to as high as 1.5 M (Warren et al. 2001; Ferris et al. 2003; DeJong et al. 2006; De Muynck et al. 2008b; Chunxiang et al. 2009; Okwadha and Li 2010; van Paassen et al. 2010; Burbank et al. 2011; Mortensen et al. 2011; Stabnikov et al. 2011; Cheng et al. 2016). Literature concerned with using *S. ureae* for MICP is very limited, however. A comparison study for the bio-cementation in sand columns with *S. pasteurii* and *S. ureae* was carried out by Sarmast et al. (2014). In their study the two bacteria were tested under high concentrations of 0.5, 1, and 1.5 M of Ca^{2+} and urea media and an incubation period of up to 12 days, however in contrast to *S. pasteurii*, no visibly detectable calcium carbonate could be observed in the *S. ureae*-treated sand columns. The study by Sarmast et al. (2014) is in agreement with the results from the batch experiment, presented here, and confirms that *S. ureae* has a less robust enzymatic activity than *S. pasteurii* and is not able to function properly at calcium substrate concentrations of 0.3-0.5 M and above.

Although a large number of parameters were examined and analysed in order to assess the effect of the various concentrations of calcium on the MICP with *S. ureae*, the results are still somewhat inconclusive. For example, while the 50 mM concentration performed well in aqueous solution (with deionised and tap water alike) both in terms of calcium conversion and precipitation efficiency, the results from the 150 mM concentration are not as clear (calcium conversion in aqueous solution with deionised and tap water were significantly different). Also, in all instances MICP in sand, as compared to aqueous solution, was much less effective and resulted in low precipitation levels. Although the batch experiment gave some indication about the range of Ca^{2+} concentrations at which the bacterium can operate a further study was needed to show

the activity of the bacterium under dynamic flow conditions in packed soil columns.

Such a study is presented in section 5.5.

5.4.2 Geochemical model

The use of a geochemical software (MINTEQ V3.1; (Gustafsson 2016)) was used to predict the evolution of pH in the media of various concentrations of calcium (0, 50, 150, 300 and 1000 mM) and urea (333 mM for the former four and 1000 mM for the latter) which mimics the experimental concentrations. It should be noted that the 0 mM calcium concentration in the model mimics the control samples in the experiment which had no urea added to them. The program calculated pH as a function of the mass and charge balance. Since the model can only deal with equilibrium reactions (biologically catalysed reactions are non-equilibrium ones), the calculated pH was presented as a function of the extent of the hydrolysed urea. Ureolysis was simulated by presenting urea in its degraded form, i.e. as ammonium and hydroxide ions. The following reaction was used as an example of full degradation of urea (assuming 1 mole of urea reacts to form 2 moles of ammonium, 2 moles of hydroxide, and 1 mole of carbon dioxide):

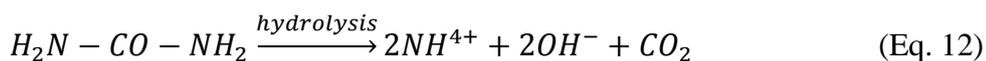


Figure 5-8a shows the buffering effect of the increasing concentration of calcium in the solution, regardless of the amount of urea degraded which is similar to the results from the batch experiment in section 5.4.1 (Figure 5-4). An increase in the concentration of calcium from 0 to 300 mM results in the calculated pH decrease of 0.5 at all levels of hydrolysed urea.

Similarly to the experiment in 5.3 (Figure 5-3), a rapid increase in pH is observed initially (from 7 to above 8) followed by a slower gradual increase and a plateau. This effect was as a result of the logarithmic nature of the pH scale.

It should be noted that the 1000 mM calcium-urea cementation medium had a very significant effect on the calculated pH (pH ~1) without urea degraded which is not in agreement with the observed experimental results presented in Figure 5-4 (note control samples of 1000 mM) where a pH of between 4 and 6 was measured. This disparity between the experimental and calculated values for pH in the 1000 mM medium was a result of the MINTEQ program not being able to operate with solutions of large ionic strength (i.e. higher than 1 M) (Gustafsson 2016).

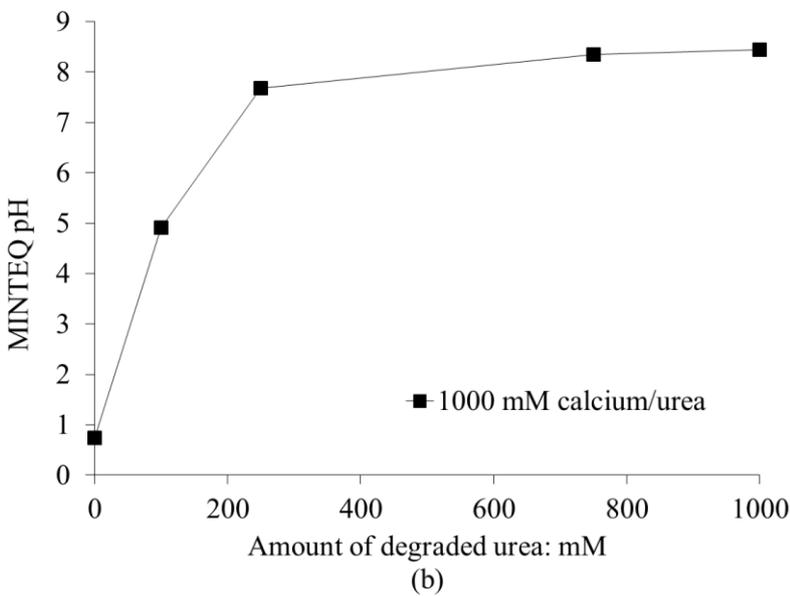
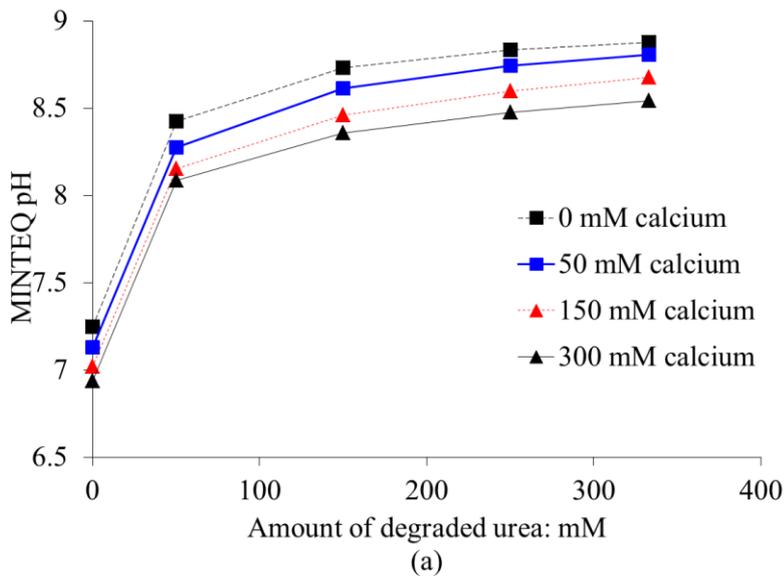


Figure 5-8 Calculated pH as a function of hydrolysed urea and calcium concentration in the cementation medium (based on MINTEQ software)

The experimental results from Figure 5-4 and the calculated pH from Figure 5-8 are compared graphically in Figure 5-9. The graphs of the experimental pH were plotted by taking the values from the control and the corresponding biological samples in tap water (in both aqueous solution and sand) from Figure 5-4. This type of solvent appeared to result in a better bacterial activity suggesting higher ureolysis rates as seen by pH (Figure 5-4) and would therefore be more suitable (than the deionised water samples)

for comparison with the model which assumes full hydrolysis of urea. The control samples represented the pH of the medium before any hydrolysis had taken place, and similarly, the biological samples represented the state of the solution after urea has been hydrolysed. In a similar fashion the values from the initial (at 0 mM hydrolysed urea) and final (at 333 or 1000 mM hydrolysed urea) theoretical pH were plotted. It should be noted that the experimental pH from the biological samples (specifically for 300 and 1000 mM calcium and in sand samples) does not necessarily present the state at full hydrolysis (amount of hydrolysed urea was not measured in the batch experiment). This is because, as was seen from the experimental results in 5.4.1, the presence of sand and the higher calcium concentrations hindered the activity of *S. ureae*, thereby not allowing the bacterium to metabolise the substrates completely. Nevertheless, this approach proved useful for the validation of the theoretical model with the experimental results.

An almost identical correlation can be observed between the rates of pH increase for the 50 and 150 mM calcium concentrations for the calculated and experimental lines (aqueous solution; Figure 5-9a). When in the presence of sand (Figure 5-9b) these two concentrations exhibit a 2 times slower rate of pH increase which, as previously outlined, may have been due to the buffering of the pH because of the presence of sand, thereby resulting in a disparity between experimental and theoretical lines. A significant discrepancy can be seen for the 300 and 1000 mM theoretical and experimental curves for both the aqueous solution and sand (a and b).

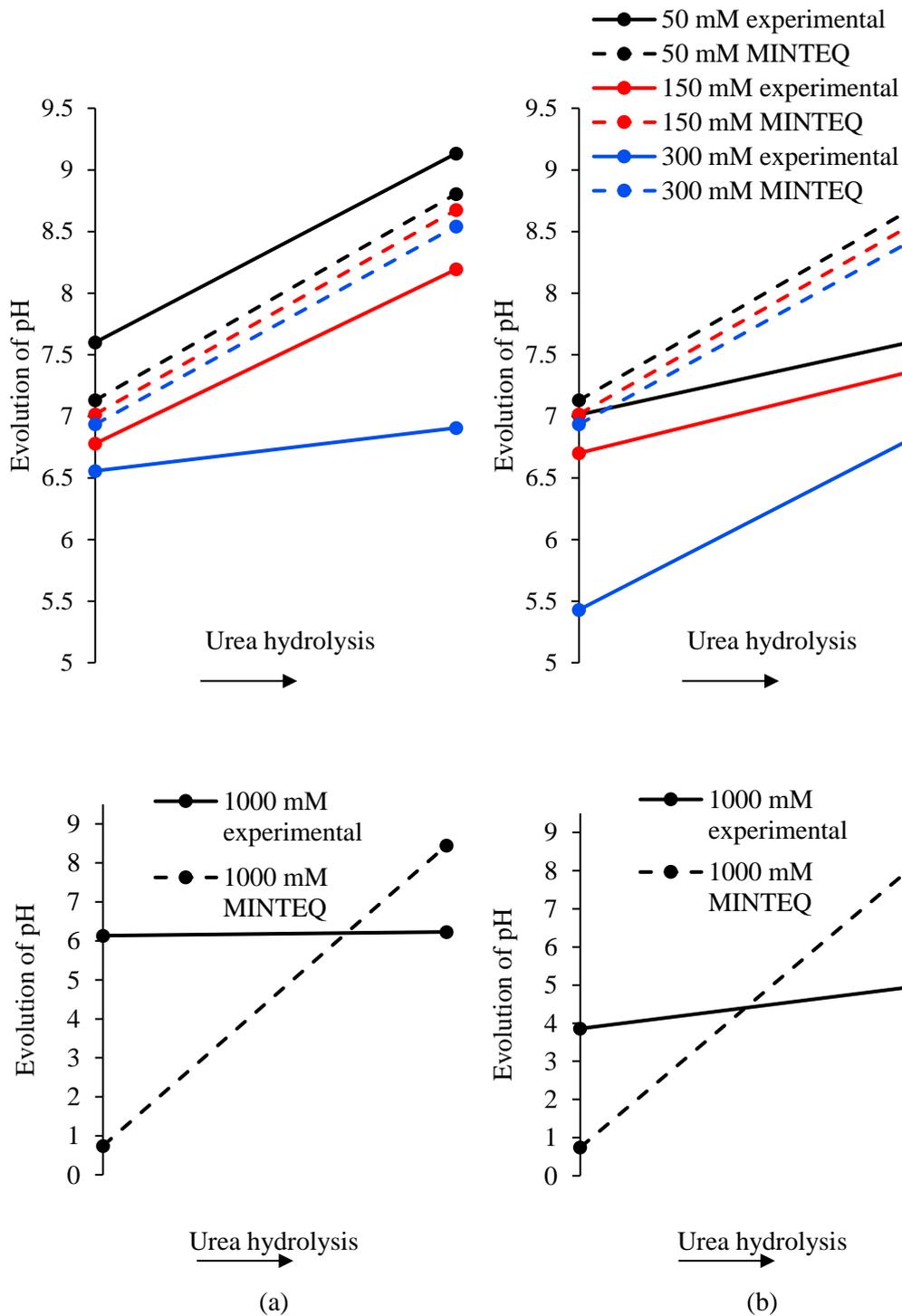


Figure 5-9 Comparison between calculated pH (MINTEQ) and experimental pH from (a) aqueous samples with tap water and (b) sand samples with tap water

The difference arose because of the much weaker activity of the bacteria in the higher concentrations which suggests that urea hydrolysis was weakened and therefore the lines of the experimental results for 300 and 1000 mM in aqueous solution and sand are

almost parallel to the x-axis. This was not true for the 300 and 1000 mM calcium samples in sand which have a starting pH of 5.5 and 4 (Figure 5-9b) compared to the aqueous solution samples which have starting pH of 6.5 and 6, respectively. In fact, control samples for all concentrations in sand exhibited a lower pH than the corresponding control samples in aqueous solution. This effect was previously discussed in 5.4.1 and was ascribed to a chemical and ion exchange interaction between the solvent (containing calcium chloride) and the sand as an adsorbent which resulted in lowering of the pH of the cementation medium in the sand.

The results from the comparison between theoretical and experimental results (Figure 5-9) demonstrate a close correlation between the two when smaller concentrations of calcium were used and the bacterium was in idealised conditions (aqueous solution). This is because, on one hand, *S. ureae* appears to be able to actively carry out hydrolysis with solutions of 50 and 150 mM in aqueous solution, and on the other, the MINTEQ software could accurately predict the pH during ureolysis in solutions of ionic strength of less than 1 M (i.e. for the 50, 150, and 300 mM). However, when the reaction was occurring in the presence of sand, the rate of pH increase was about twice as slow, thereby rendering the predicted pH inaccurate. A significant disparity arose when comparing 300 and 1000 mM calcium in cementation media with the theoretical model because of the apparent inability of *S. ureae* to metabolise substrates at such high concentrations, and because of the high ionic strength of the 1000 mM calcium solution at which the program does not operate correctly.

The model served to demonstrate that certain MICP parameters (such as pH) can be modelled for idealised conditions and this can be done relatively easy with the use of geochemical equilibrium software. However, further studies are required to determine the extent to which bacterial activity is reduced in particulate media conditions in order

to predict the evolution of pH in this environment more accurately. Additionally, further research is needed to develop a more sophisticated theoretical model which would predict other important MICP parameters, such as calcium carbonate content, based on type of bacterial strain, injected biomass, type and gradation of soil, and subsurface conditions. Such a model could eventually aid in designing an on-site MICP treatment based on the specific properties and design requirements for the site.

5.5 Effect of substrate concentrations – column experiment

To further supplement the results presented in section 5.4.1, concerned with identifying the optimum conditions in terms of substrate concentrations (Ca^{2+} and urea) for MICP using *S. ureae*, a dynamic column experiment was also performed. Concentrations of 50, 150 and 300 mM calcium in the concentration medium, as well the use of tap water as a solvent was employed in this experiment. The details of the sand column preparation are described in 3.3.3.3 and the experiment outline can be found in 3.3.3.3.2. Each concentration was tested with replicates of biological samples and control samples (in which urea was omitted from the cementation medium) – 50 and 150 mM concentrations were tested in duplicates, and 300 mM concentration was tested in triplicates. The cementation medium was supplied over a period of 35 to 38 days (6 injections) to allow bio-cementation of the sand to take place. The evolution of pH, aqueous calcium, and hydraulic conductivity measurements were conducted before each new injection of nutrients and the results from those are presented in Figure 5-10 to 5-12. Additionally, measurements of mass loss on ignition (indication of calcium carbonate content) as well as compression testing were also conducted on the sand columns and the results of these are presented in Figure 5-14 to 5-16.

5.5.1 pH and aqueous calcium results

Columns, treated with 50 mM solution (Figure 5-10a), showed the highest increase in pH after each injection (from 8 in the initial to close to 9.5 in the final injection), whereas 150 and 300 mM columns (Figure 5-10b and c) showed a lower pH of 7.5 to 8.5 for the former and below 7.5 for the latter. The low pH in the control samples (between 5 and 6) for the 300 mM concentration indicates that the bacteria has to compensate for the buffering effect of the high concentration of calcium, and therefore the end pH in the biological samples is of the order of 7.5.

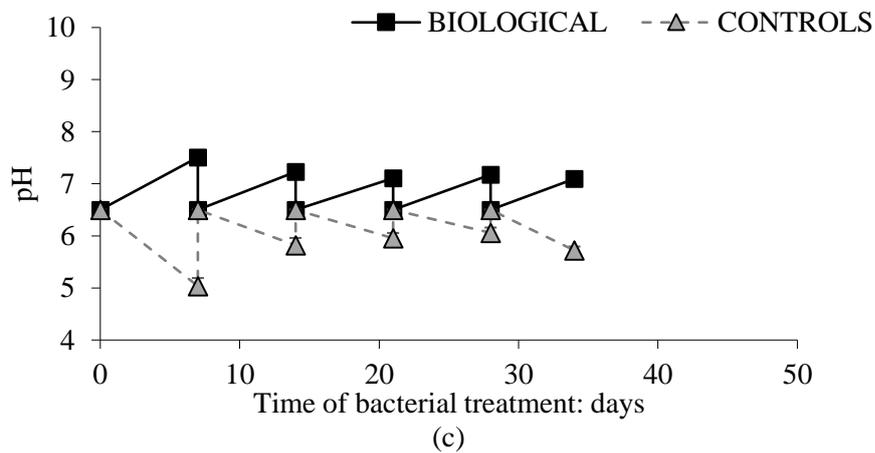
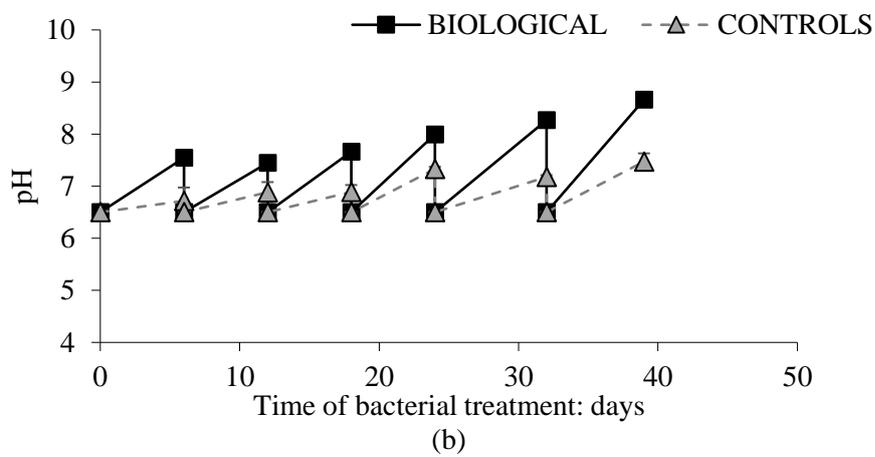
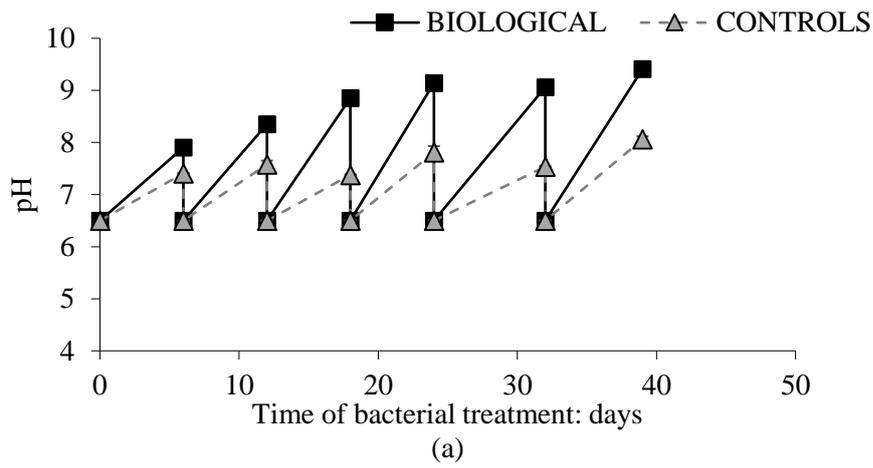


Figure 5-10 pH evolution during bacterial treatment of sand with various concentrations of substrates; (a) 50 mM calcium; (b) 150 mM calcium; (c) 300 mM calcium; (Error bars: ± 1 SD, n=2 or 3)

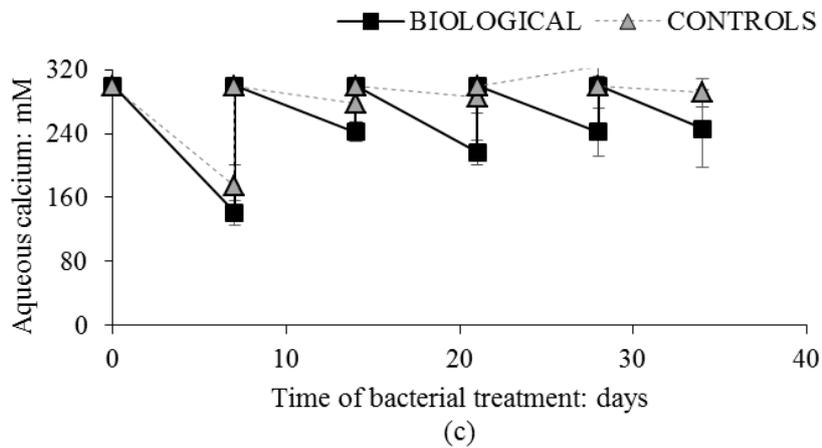
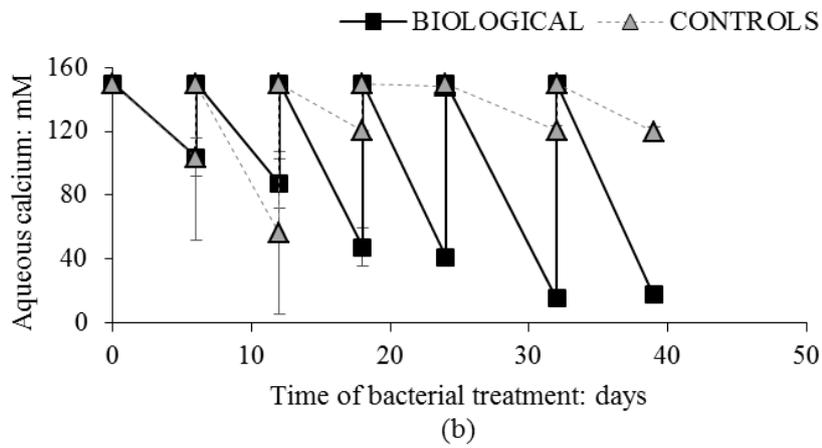
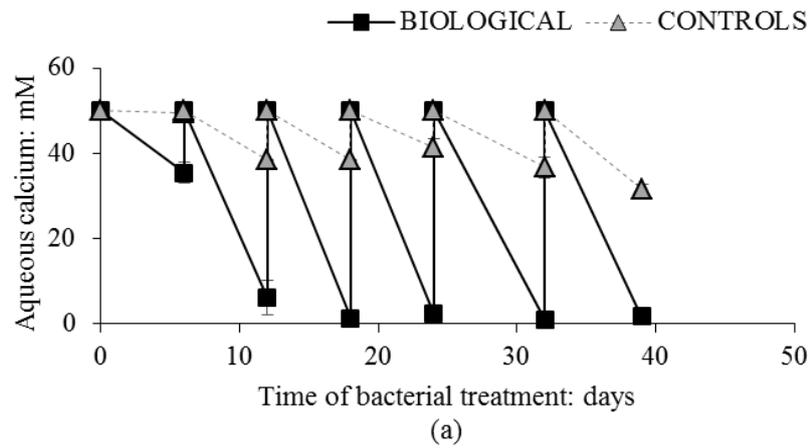


Figure 5-11 Aqueous calcium during bacterial treatment with various concentrations of substrates; (a) 50 mM calcium; (b) 150 mM calcium; (c) 300 mM calcium; (Error bars: ± 1 SD, n=2 or 3)

Similarly to the batch experiment in 5.4.1, the observed drop in pH in the control samples of 300 mM can be attributed to be as a result of the increased concentration of calcium and chloride and the interactions of these ions in the cementation solution with the silica sand (discussed previously in 5.4.1). In general, control samples in all cases differed significantly from the biological samples, indicating biological activity, albeit to different degrees, was present in all concentration treatments.

A common trend was observed in both 50 and 150 mM samples with pH gradually increasing after each subsequent injection which suggests that the bacteria in the columns adjusted to the conditions and progressively hydrolysed more urea. This is confirmed by the aqueous calcium results (Figure 5-11a, b) for 50 and 150 mM, where a similar trend of initially small amount of calcium reduction (~30 %) is observed followed by almost full reduction at the final injections. Such behaviour of the bacteria has been previously documented and is ascribed to the demand on bacterial populations to yield maximal growth of the fittest cells (Darwinian concept) by adapting to changing environments in terms of pH, temperature, and salinity in the growth medium (Ehrenberg and Kurland 1984; Pavlov and Ehrenberg 2013). In this study, the adaptation effect was observed for the two lower concentrations of calcium in the medium (50 and 150 mM); however this was not the case with 300 mM which showed a stable low conversion of calcium of between 20 and 30 % throughout the experiment (Figure 5-11c). This is in agreement with the results from 5.4.1 and indicates that such a concentration of calcium is hindering the activity and metabolism of *S. ureae* and results in low levels of ureolysis and subsequent low conversion of calcium.

A small drop in calcium at each injection was also observed for the control samples in all concentrations. This was considered to be due to the calcium adsorption to the sand grains, as discussed previously. A significant reduction in calcium (comparable to the

one in the biological samples) after the first injection can be seen in the 150 and 300 mM treatments (Figure 5-11a and c). This reduction could be a result from both adsorption of the calcium to the sand grain surfaces and the potential for the bacteria in the control columns (initially grown in urea containing medium prior to placement in the columns) to still have intracellular urea present which is subsequently hydrolysed resulting in the conversion of calcium. This effect, however, is not apparent in later injections because all of the available intracellular urea was probably exhausted in the first cycle.

5.5.2 Hydraulic conductivity results

Hydraulic conductivity results are shown in Figure 5-12. A decrease in conductivity of 20, 16, and 17 % can be seen for the 50, 150, and 300 mM biological samples, respectively, while control samples remain relatively unchanged. However, control samples for 150 mM concentration exhibited a significant variability in the replicates, as represented by the error bars. Upon dismantling the sand columns at the end of the experiment it was observed that one of the 150 mM control sand columns had slightly blocked at the inlet due to the cylinder end sealing method failing and allowing sand grains to enter the porous disc. This could explain the drop in hydraulic conductivity of this particular column sample and therefore affecting the results of the control samples.

It should also be noted that blocking of the holes of the bottom (at inlet) plastic porous discs was observed in all biological samples (Figure 5-13a) which may have contributed to the drop in hydraulic conductivity. It is possible that due to gravity flow more cells were present at the bottom of the columns (near the flow inlet) which would have also contributed to a larger accumulation of calcium carbonate in the pore space at the bottom than near the top of the columns. Upon dismantling of the sand columns at the

end of the experiment some of the holes of the plastic porous discs were filled with a white powder material (Figure 5-13a) which was analysed by X-Ray Diffraction (section 3.2.8) and found to be calcite. Additionally, at the end of the bacterial treatment, white powder material can be seen covering the bottom rubber stopper but not the top one (Figure 5-13b).

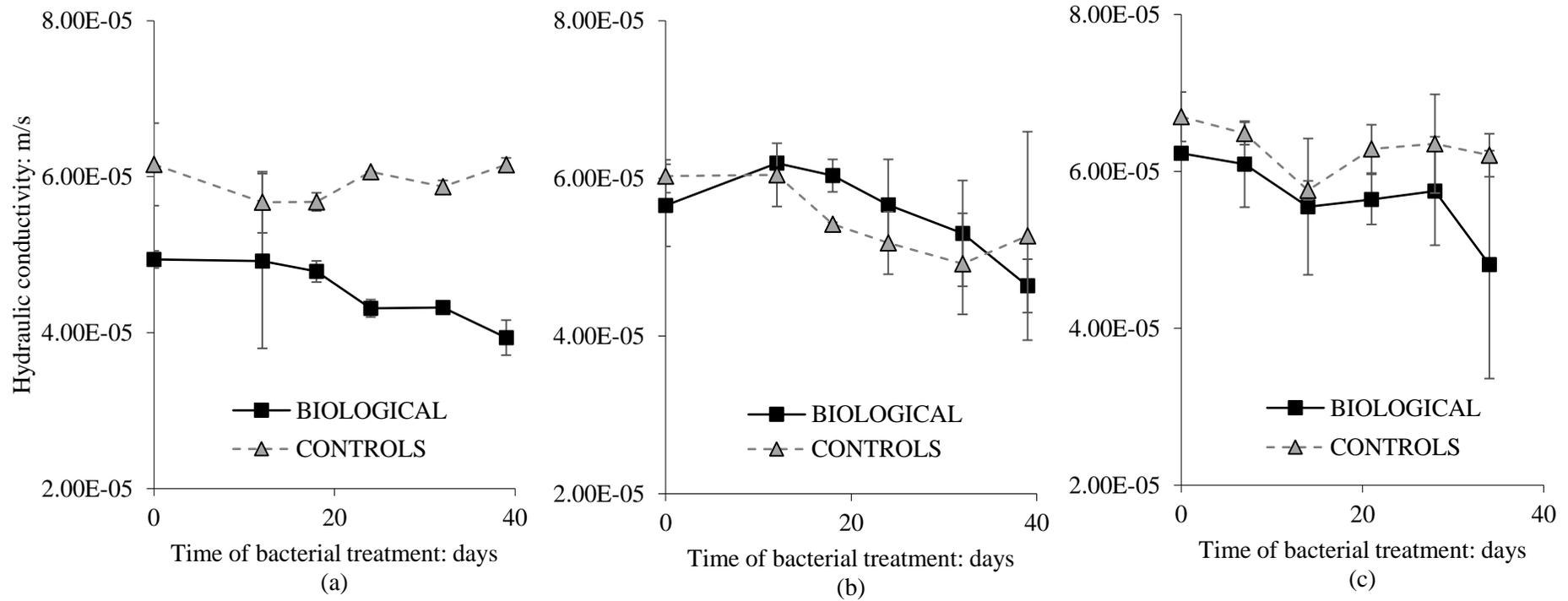


Figure 5-12 Hydraulic conductivity of columns during bacterial treatment with: (a) 50 mM calcium; (b) 150 mM; (c) 300 mM; (Err. bars: ± 1 SD, n=3)

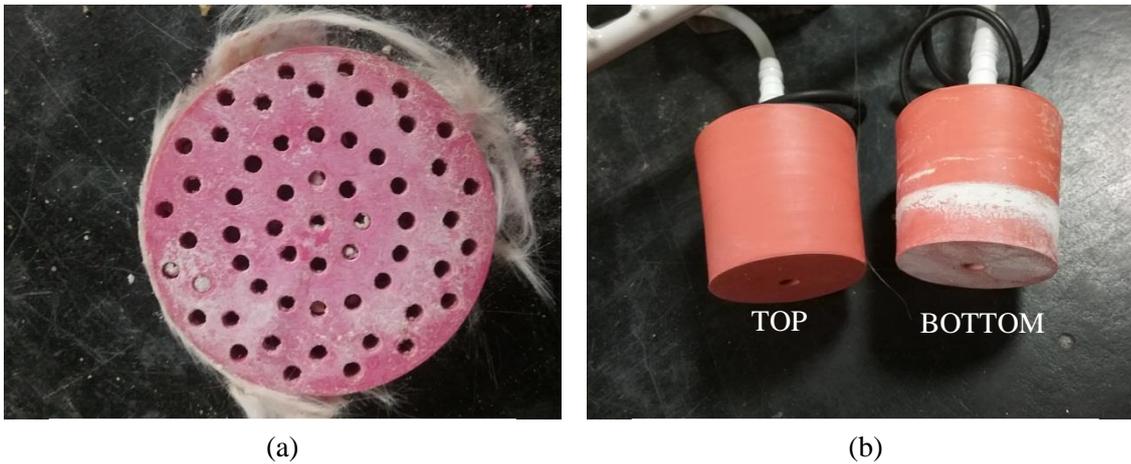


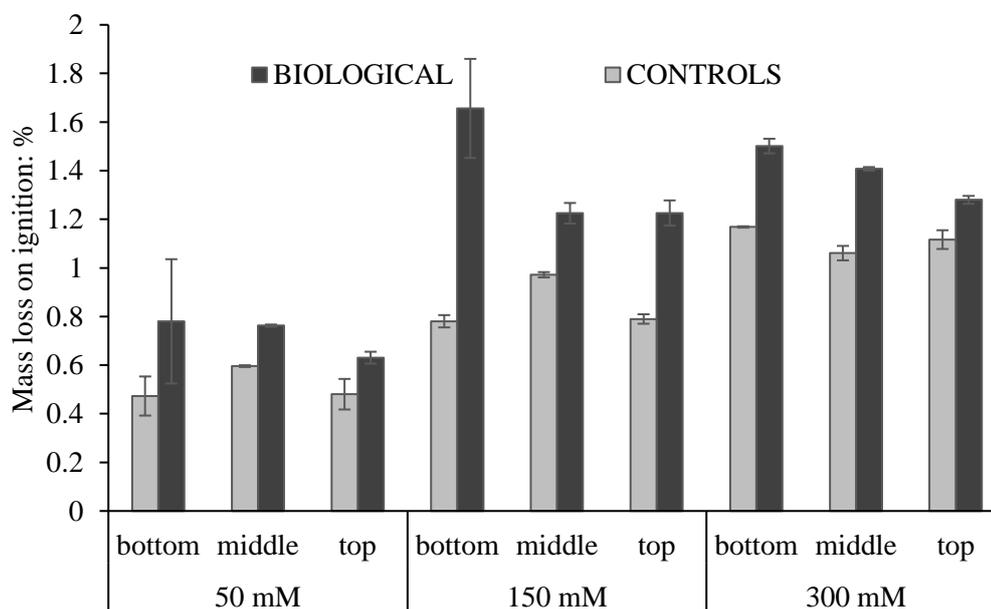
Figure 5-13 Bottom (inlet) porous plastic 3-D printed disc (a), and rubber stoppers with holes (b) from a bacterially treated sand column

In summary, a certain degree of hydraulic conductivity reduction (~17 to 20 %) was observed in all samples in this experiment and these results agree with observations from previous studies in which MICP was applied for soil stabilisation (van Paassen 2009; Al Qabany et al. 2012). In their publication, Al Qabany et al. (2012) determined that among the most influential factors for the reduction of the hydraulic conductivity of a bio-treated soil is the amount of calcium carbonate precipitated.

5.5.3 Quantity of precipitate

The results from the percent mass loss on ignition (indicative of mass of calcium carbonate precipitated as a portion of the total mass of sand in the columns) are shown in Figure 5-14 for both biological and control samples. As can be seen, there was a mass lost in the control samples which ranged from 0.42 to 0.6, 0.78 to 0.97, and 1.06 to 1.16 %, respectively for 50, 150, and 300 mM. The reduction in mass after ignition in the control samples is assumed to be due to the biomass in the columns, volatiles present, as well as a certain degree of abiotic precipitation. The latter could also explain the increase in mass lost with the increasing calcium concentration in the medium which

would lead to a higher level of abiotic precipitation due to supersaturation with respect to calcium carbonate (Rodriguez-Navarro et al. 2007; Phillips et al. 2013).



Calcium concentration and position of measurement in sand column

Figure 5-14 Mass loss on ignition (as a measure of amount of calcite precipitated) in sand columns treated with various concentrations of substrates in the cementation medium; (Error bars: ± 1 SD, n=2 or 3).

In order to determine the effect of the bacterium on the precipitation the average difference between biological and control samples was calculated (Figure 5-15) and it gives an indication of the mass of calcium carbonate which was precipitated in the biological samples. This approach disregards any difference in biomass between the biological and control samples that arose during the experiment as it was assumed to be insignificant. Percent masses of between 0.15 to 0.3, 0.26 to 0.88, and 0.16 to 0.32 % can be observed for the three concentrations respectively. It is interesting to note that the 50 and 300 mM treatments yielded similar results despite the significant difference in concentrations between the two (Figure 5-15). This is in agreement with the results

from the batch experiment from section 5.4.1 and could be explained on one hand with the low concentration of 50 mM which leads to precipitation but at low levels due to the insufficient amount of substrates, and on the other, with the hindering effect of the 300 mM on the bacterial activity which leads to only a partial utilisation of the substrates in the medium. Therefore, given the raised amounts of CaCO_3 in the 150 mM, which reached up to 0.88 %, it can be concluded that this concentration provides sufficient amount of substrates to precipitate more than double the mass as compared to the other two concentrations while not hindering the activity of the bacterium. It should be noted that concentrations of calcium carbonate in soil of up to 1.3 % are not expected to bring about a significant alteration in the geotechnical properties of the bio-treated soil, as outlined by Chu et al. (2014) and as seen from experimental results in other studies (Al Qabany et al. 2012; Al Qabany and Soga 2013; Cheng et al. 2013). The relatively low precipitated amounts of calcium carbonate (less than 1.3 %) would also explain the minor reduction in hydraulic conductivity seen from Figure 5-12.

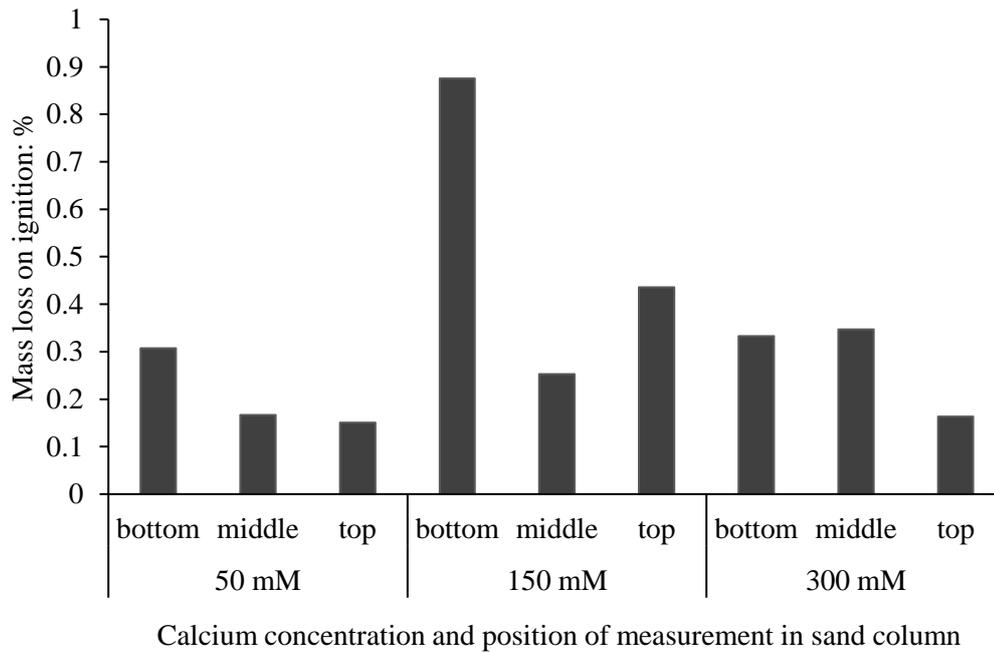


Figure 5-15 Average difference in mass loss on ignition between biological and control sand columns

Higher amounts of CaCO_3 were precipitated at the bottom of the biological columns as compared with samples taken from the top of the columns (Figure 5-15). In the 50 and 300 mM concentrations there are observed differences between the mass lost at bottom and top of the columns of approximately 0.17 % for both. The difference between the precipitated amount from the bottom and top of the 150 mM columns is 0.43 % suggesting a calcification gradient. A calcification gradient is a very common occurrence in MICP treatment of soil and has been reported by many (Whiffin et al. 2007; Harkes et al. 2010; Al Qabany et al. 2012; Cheng et al. 2013; Gomez et al. 2015). Non-uniform spatial distribution of CaCO_3 has been attributed to a number of factors including the type and heterogeneity of the soil and preferential pathways for nutrients, flow rate for nutrient injections, and concentrations of substrates in the cementation medium.

5.5.4 Unconfined compression of sand columns

After the end of the bacterial treatment the sand columns were repeatedly injected with deionised water to flush out any remaining bacteria and media. The columns were then air-dried to a constant mass at 30° C (approximately 3 weeks) before strength testing was carried out. This was done in order to preserve rubber membrane integrity and to minimise effects of moisture on compressive behaviour of the sand samples. The columns were then strength tested with an unconfined compression testing apparatus (section 3.2.11). Stress-strain curves for all biological and control samples were recorded and plotted in Figure 5-16. Both 50 mM and 300 mM biological and control samples exhibited relatively similar peak stresses of 7 to 15 kPa and stress-strain curves typical of un-cemented sand. This indicates that the bacterial treatment in these columns did not result in enough formation of CaCO₃ between sand particles to act as a bond between them, rather than just filler in the void space. The formed precipitation in the 50 and 300 mM treatments (between 0.15 to 0.3 % by mass of sand, Figure 5-15) was too insignificant to provide an improvement in the stress-strain behaviour of the sand.

In contrast, a 70 % increase in the average peak strength of the biological samples, relative to the controls, can be observed in the 150 mM bio-treatment. The 150 mM sand columns also exhibited a brittle type of response with an initially steep increase in stress up until peak stress was reached and a mechanical break occurred. Such a brittle type of response is brought about by the formation of inter-particle calcification which bonded the sand grains, and once peak strength was reached the bio-cementation deteriorated (DeJong et al. 2006). The improvement in the mechanical behaviour of the 150 mM samples is assumed to be related to the higher amount of precipitation in those columns.

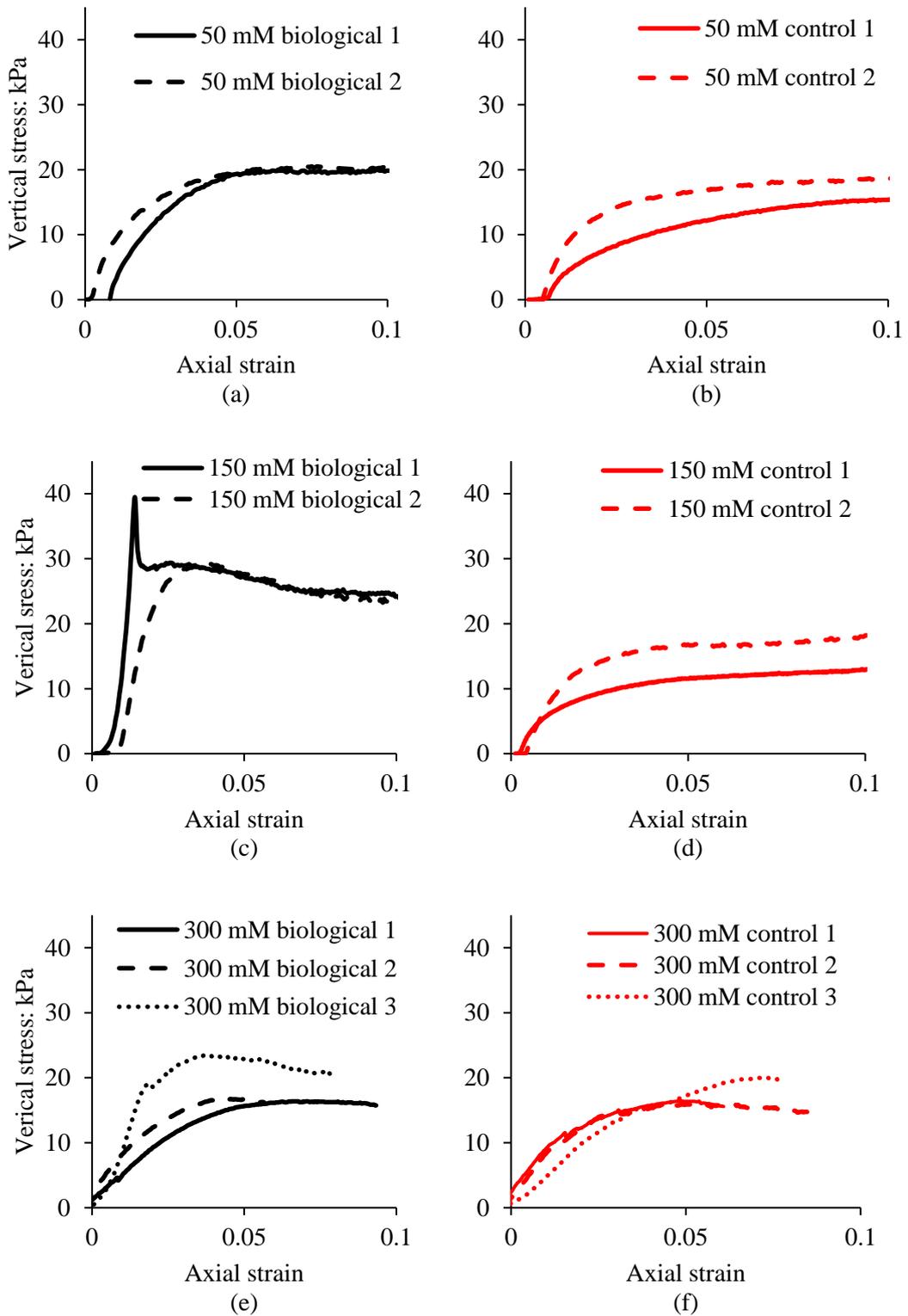


Figure 5-16 Stress-strain curves of sand columns treated with various concentrations of substrates in the cementation medium. Results from unconfined compression testing; (50 and 150 mM were tested in duplicates, and 300 mM was tested in triplicate)

5.6 Summary

In the comparison of MICP with *S. ureae* and *S. pasteurii* it became evident that the former bacterium exhibits substantially weaker metabolic potential. The increase in pH and the assimilation of calcium from solution occurred after a prolonged incubation period. This would bring about issues with the time-scale for the bio-treatment of soil when *S. ureae* was used as the catalysis for MICP. The use of a batch experiment coupled with a geochemical model helped in elucidating the parameters which could be easily altered (i.e. increasing the substrate concentrations in the cementation media) in order to accelerate the bio-treatment of sand. *S. ureae* functioned well for concentrations of up to 300 mM and the calculated pH (as a vital parameter of MICP) exhibited very similar trends to those from the tests data. The pH changes with the same concentrations but in the presence of particulate media were less well modelled mainly due to the hindering effect of the sand on the bacteria. An important finding was that tap water had a positive effect on the metabolism of the bacterium for most concentrations of calcium in which *S. ureae* was tested. Dynamic flow sand column tests showed that for a period of 38 days and 5 to 6 injections of cementation media, the bacteria stayed active and even improved its MICP performance progressively by adjusting to the conditions in the columns (especially in concentrations of up to 150 mM). The 50 mM and 300 mM treatments did not improve the strength of the columns due to the small quantities of calcium carbonate precipitated. The 150 mM calcium in the medium resulted in improved mechanical behaviour with 60 % increase in the peak stress of the columns and a change in the behaviour of the sand from un-cemented to brittle-type. The results from the dynamic column experiments demonstrated that a measureable strength improvement was possible with *S. ureae* MICP and it was achieved within an acceptable time-scale.

6 Self-healing in particulate media

6.1 Introduction

The following chapter presents the results from two experiments which investigated the response of spore-forming bacteria, *S. ureae*, in initially bio-cemented sand to chemical and physical damage and its ability to heal such damages. The experiments here follow from and build upon the experiments presented in chapter 4 which demonstrated the cycles of sporulation, mineral formation, break-down and regeneration of bio-mineral in aqueous solution.

6.2 The potential for self-healing after a chemical deterioration

This experiment comprised 9 acrylic sand columns, prepared as described in section 3.3.4.1 with a summary of the experimental procedure described in Table 3-3. The aim of this experiment was to demonstrate the potential for calcium carbonate precipitation following chemical damage to an existing carbonate mineral phase within soil, achieved through spores of *S. ureae*. Aqueous calcium, pH, and mass loss on ignition (as a measurement of mass of calcium carbonate) served as indicators of damage healing by the regenerated spores.

6.2.1 pH and aqueous calcium in initial treatment and healing stages

Figure 6-1a and c illustrate cycles of pH increase (to above 8) and calcium concentration decrease in the initial bacterial treatment (by 90-95% apart from the last injection [average 73%]), respectively, between each injection, suggesting ureolysis and calcium carbonate precipitation. Urea-free controls were not included in this experiment. Such abiotic controls were used in previous experiments (section 5.5) and showed that without urea, no effect on the pH and calcium was observed (see control columns in Figure 5-9 and Figure 5-10). The bacterial activity was observed to slightly

decrease in the final two injections, possibly due to encapsulation and limitation of nutrients to the microorganisms in the columns and partial clogging at the injection points. Once the initial bacterial treatment was stopped, 3 (columns 1-3) out of the 9 columns were dismantled and tested for mass loss on ignition (i.e. amount of CaCO_3), another 3 columns (columns 4-6) were injected with 0.1 M hydrochloric acid to dissolve the calcite formed during the initial treatment stage, then dismantled for mass loss on ignition. The final 3 columns (columns 7-9) were also acid-treated and subsequently injected with hydrogen peroxide for removal of vegetative bacteria. Samples from the liquid in columns 7-9 were stained with CTC and examined under a fluorescent microscope (section 3.2.4) which showed no apparent presence of viable (or actively respiring cells). Columns 7-9 were then further treated with growth media. However, as the pH response was very weak, it was assumed that due to the effect of acidic flow and hydrogen peroxide treatments the number of spores may have been low. Additionally, due to non-uniform flow there may have been little availability of nutrients to the spores. These effects may have contributed to the spores not regenerating as expected. Additionally the number of spores available was potentially reduced due to the washing effect of the acidic and hydrogen peroxide treatments. In order to stimulate spore regeneration and growth by improving the availability of nutrients to the organism, the contents of columns 7-9 were aseptically transferred to flasks with media added in a 2:1 ratio relative to the sand mass. The regeneration of spores from the material in columns 7-9 is presented in Figure 6-1b and d in terms of pH increase and aqueous calcium decrease. Both parameters show a weaker metabolic response when compared to the initial cementation but still indicate that microbial ureolysis was taking place, and therefore that encapsulated organisms had survived the initial precipitation and its

subsequent deterioration. On average, the pH rose above 7.7, and 9 to 42 % reduction in aqueous calcium was observed.

There is an observed irregularity in the graphs of the healing stage (Figure 6-1b and d) between day 15 and 21 where two injections were made in the period of 6 days thereby not allowing enough incubation time for nutrient metabolism. The insufficient period can particularly be seen affecting the aqueous calcium results with 8 and 12 % reduction compared to 31% in the last injection with a 5-day incubation time.

The overall weakened pH and calcium conversion response at the healing stage may have been due to larger fluid volumes in the samples in the flasks diluting the observed response, as well as the eroding effect of the acid and hydrogen peroxide treatments, and as no new bacteria were supplied, it is possible that the metabolic potential (i.e. number of active bacteria) in the sand was reduced. Even though some growth may have occurred in the healing stage, the overall reduced numbers of bacteria along with the encapsulating and limiting effect of bio-cementation may have resulted in the generally weaker metabolic activity.

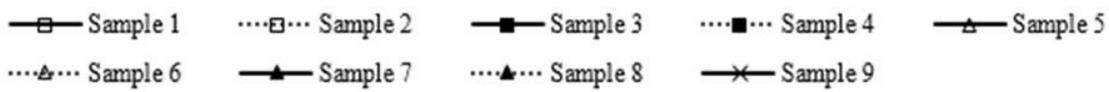
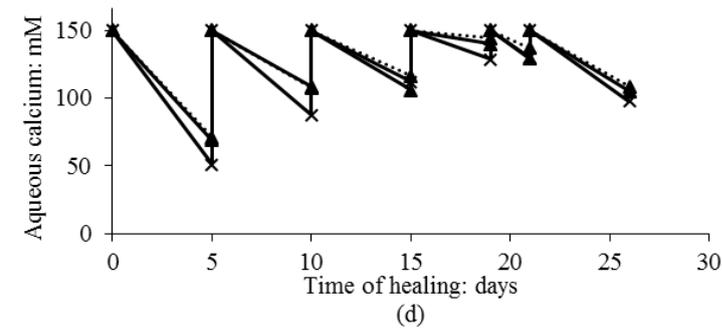
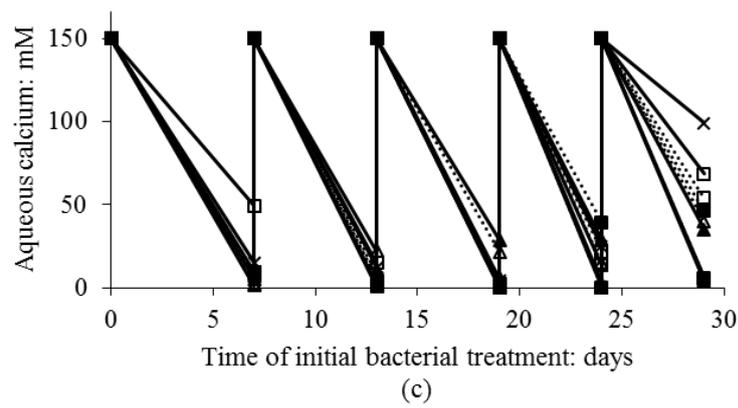
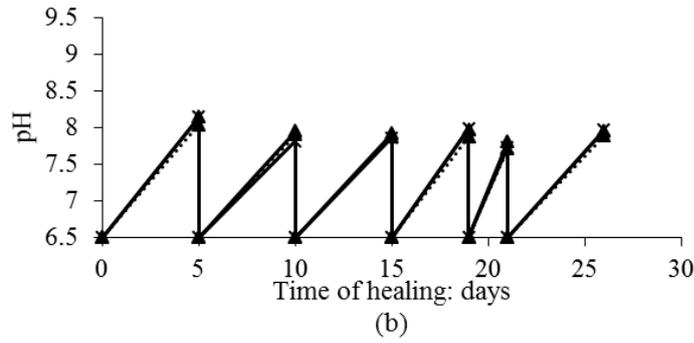
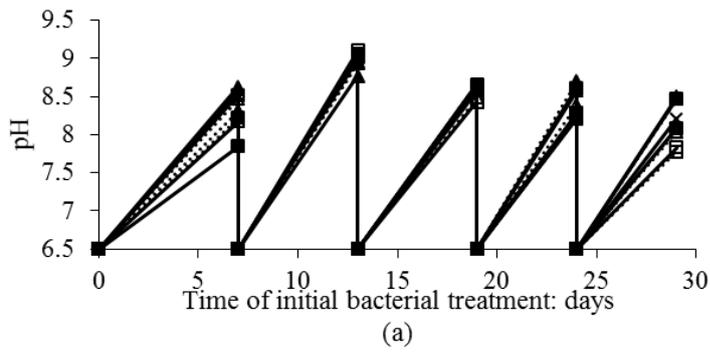


Figure 6-1 pH (a, b) and aqueous calcium concentration (c, d) in initial bacterial treatment (a, c) and healing (b, d) stages in the healing after a chemical deterioration experiment

6.2.2 Mass loss on ignition

The initial bacterial treatment of the columns resulted in the precipitation of 1.6 to 1.9 % calcium carbonate by mass of sand (columns 1-3, Figure 6-2). According to stoichiometry calculations, the total amount of calcium injected in the columns in the initial treatment was 750 mM which (if all converted to form calcium carbonate) would have resulted in per cent mass of precipitate in the sand of ~2.4 %. Therefore, the initial treatment resulted in efficiency (in terms of calcium to calcium carbonate conversion) of 66 to 79 %. Treatment with acid resulted in approximately 22% lost mass of carbonate precipitate (columns 4-6, Figure 6-2). Despite the weaker response in terms of pH and aqueous calcium conversion at the healing stage (Figure 6-1), a significant amount of approximately 1.4 % additional precipitate (on average of the three samples) was recovered and reached values of 2.3 to 4 % (columns 7-9, Figure 6-2). Similarly to before, stoichiometric calculations show that the efficiency of calcium to calcium carbonate conversion in the healing stage was reduced to around 28 %, possibly due to the larger volume of liquids used in the healing stage (about 5 times more than in the initial bacterial treatment) and the reduced metabolic potential in the sand.

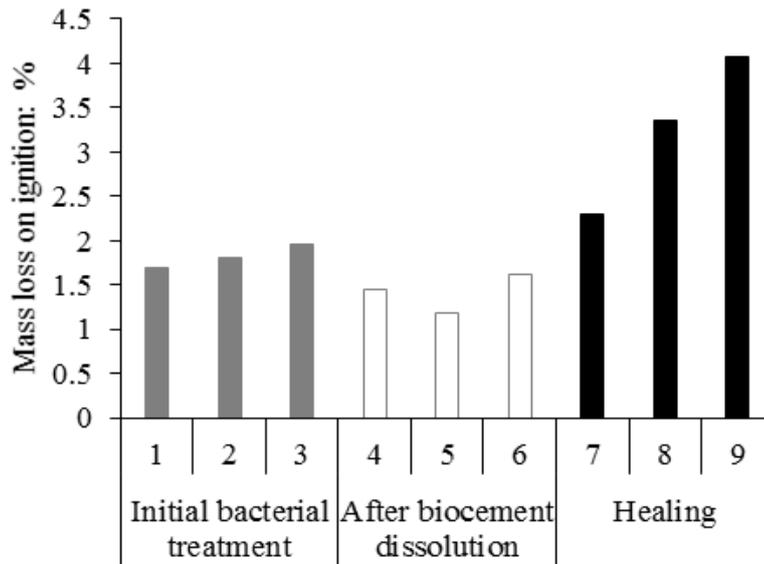


Figure 6-2 Mass of CaCO_3 initially formed (columns 1-3); mass of CaCO_3 present after acid dissolution (columns 4-6); mass of CaCO_3 present after CaCO_3 formation in healing stage (columns 7-9).

6.3 The potential for self-healing after a physical deterioration

This experiment comprised 10 split mould sand columns, prepared as described in 3.3.4.2 with a summary of the experimental procedure and testing regimes shown in Table 3-4. Similarly to the experiment presented in 6.2, the aim of this experiment was to explore the potential for damage healing in+ initially bio-cemented soil columns, however in terms of mechanical performance recovery after physical damage. The 10 split-mould sand columns (as shown in Figure 3-3) were all bacterially treated for 38 days. They were then physically “damaged” *via* unconfined compression tests and subsequently returned to split-mould apparatus in the incubator for further treatment. Five of the broken columns (odd-numbered) were sterilised with hydrogen peroxide for removal of vegetative cells and were then treated with cementation medium for another 22 days. The rest of the columns (even-numbered) were also H_2O_2 -sterilised and treated with deionised water to serve as controls. Aqueous calcium, pH, hydraulic conductivity,

unconfined compression and mass loss on ignition served as indicators of the success of the damage-healing process.

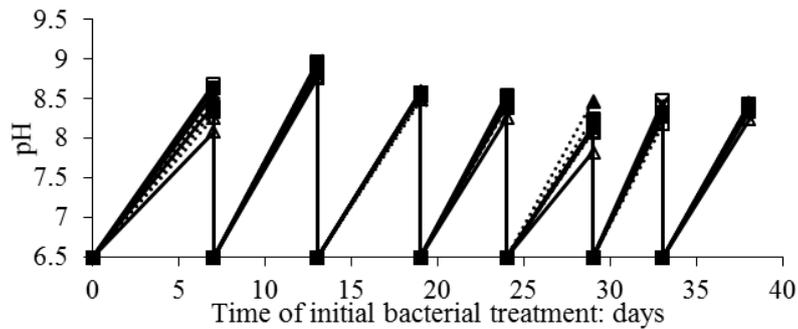
6.3.1 pH and aqueous calcium in initial treatment and healing stages

In the initial bacterial treatment stage, all specimens demonstrated a rise in alkalinity (to above pH8.1) and removal of aqueous calcium (average 91 to 98 %) following each injection (Figure 6-3a and c), slightly higher than that observed in the previous experiment. The improved response of the columns in this experiment can be attributed to a modification made to their design. The accommodation of porous plastic discs in the split mould sand columns was observed to prevent blocking at inlet and improve homogeneity of the flow of liquids in the column.

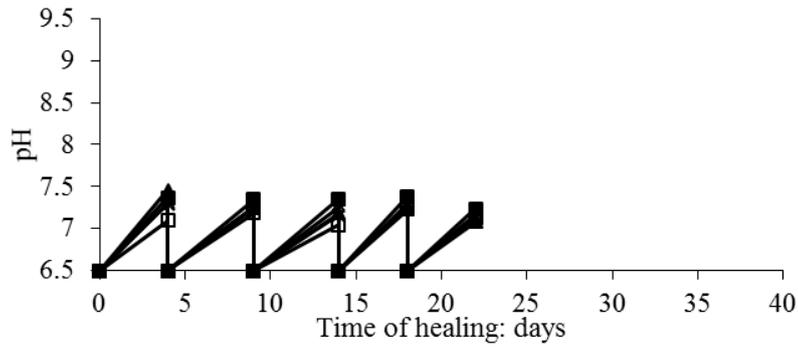
It should be noted, that in the experiment in this section, the metabolic response in the bacterial treatment in terms calcium conversion was seen to occur very strongly (almost full depletion of calcium) from the first injections and throughout the initial bacterial treatment. In contrast, calcium conversion in the column experiment in the previous chapter (section 5.5) was seen to occur in a somewhat weak manner in the initial injections of the treatment and to progressively strengthen due to bacterial adjustment to the conditions in the columns. As the two experiments from sections 5.5 and 6.3 were conducted in identical ways, a possible explanation for the difference in the bacterial activities may be due to a more active starting inoculum culture in the latter which responded better to the conditions in the columns and therefore actively converted calcium to CaCO_3 from the start of the test.

The metabolic response with respect to pH in the healing stage (Figure 6-3b) was weaker than the initial bacterial treatment, and also when compared to the healing stage from the previous experiment in section 6.2. An average pH increase to above 7 can be

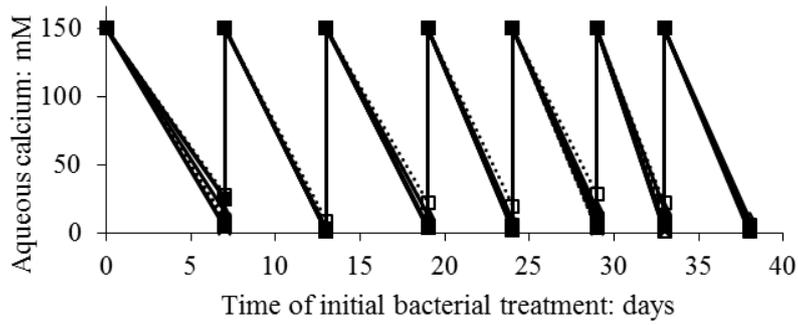
observed, as compared to average of 8-8.5 from the previous experiment. The weaker healing response in this test may be explained with the nature of the initiated physical damage in this experiment which may have led to a smaller proportion of the available spores in the columns to get exposed (e.g. only those on the face of a shear plane or a crack). In contrast, the acid dissolution in the previous experiment (6.2) was assumed to have broken up a larger surface area of calcium carbonate and therefore exposed more spores. Calcium conversion was not affected by the weaker pH, however, with 30 to 58% observed in the healing stage (Figure 6-3d).



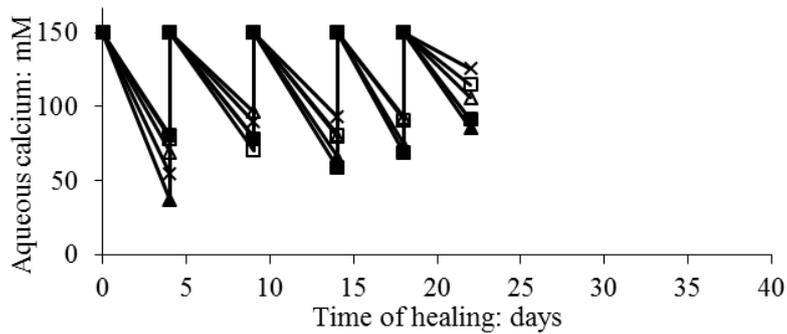
(a)



(b)



(c)



(d)

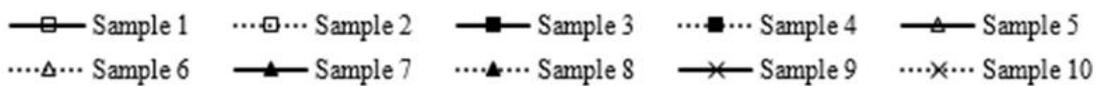
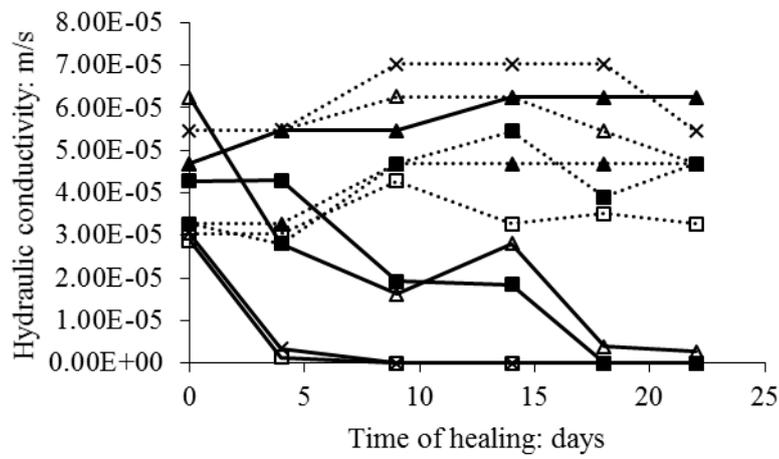
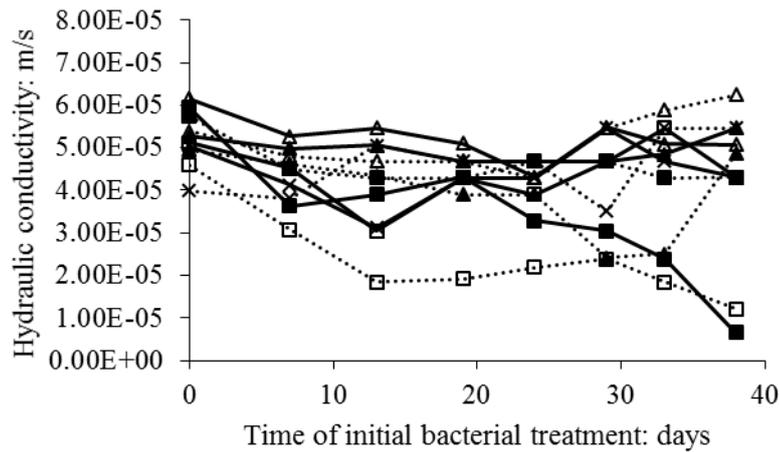


Figure 6-3 pH (a, b) and aqueous calcium concentration (c, d) in initial cementation (a, c) and healing (b, d) stages in the healing after physical deterioration experiment.

6.3.2 Hydraulic conductivity in initial and healing stages

The hydraulic conductivity of the columns remained largely unchanged over the initial bacterial treatment stage, apart from columns 2 and 3 in which there was a significant drop observed (Figure 6-4a). It is assumed that if the treatment continued then all columns would eventually become blocked due to the formation of bio-cement in the pore spaces of the sand. However, natural heterogeneity and preferential flow would have resulted in the different temporal precipitation patterns and hence the variance in the hydraulic conductivities.



—□— Sample 1 ...□... Sample 2 —■— Sample 3 ...■... Sample 4 —▲— Sample 5
 ...△... Sample 6 —▲— Sample 7 ...▲... Sample 8 —×— Sample 9 ...×... Sample 10

Figure 6-4 Hydraulic conductivity during the initial cementation stage (a), and during the healing stage (b). In the latter, odd-numbered columns were injected with cementation medium whilst even-numbered columns were injected with deionised water.

Following unconfined compression testing, the majority of columns exhibited either a similar or an increased value in hydraulic conductivity which was indicative of the damage caused. The significant drop after the initial treatment in columns 2 and 3 in particular was reversed. This suggests that the bio-cementation degraded during the unconfined compression test and cracks/fissures were created in the columns which served as preferential channels for water flow, thereby increasing the hydraulic

conductivity. Over the course of the healing stage (Figure 6-4, b), even-numbered columns treated with deionised water exhibited broadly constant hydraulic conductivity. Hydraulic conductivity was substantially reduced in all odd-numbered columns (treated with cementation medium), apart from column 7, within 22 days of healing (Figure 6-4, b).

6.3.3 Unconfined compression after initial and healing stages

The stress-strain results from the unconfined compression testing of the 10 columns after the initial cementation and healing stages are shown in Figure 6-5 and Figure 6-6. Images of the specimens after unconfined compression testing (at both stages of the experiment) can be seen in Figure 6-7.

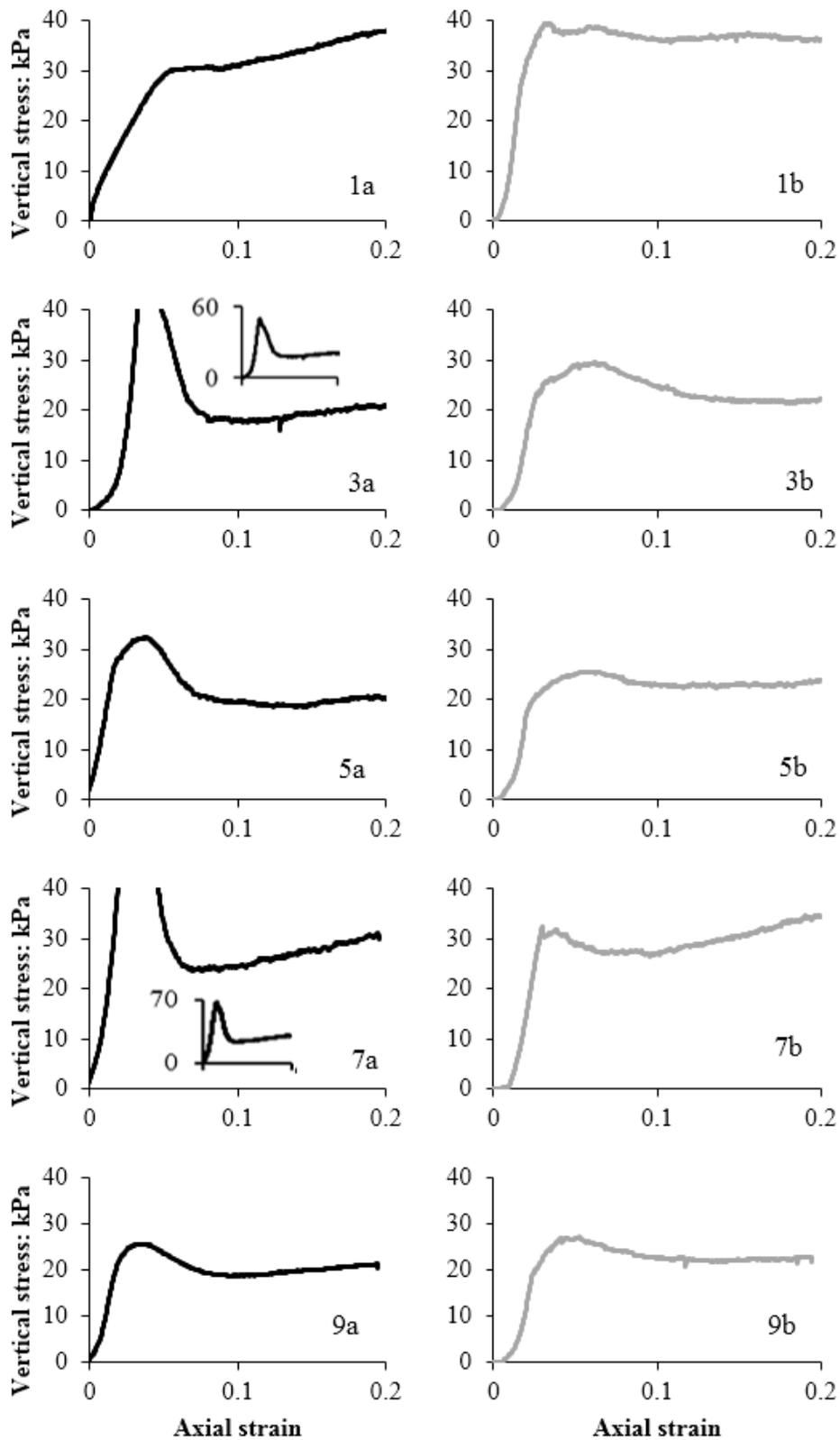


Figure 6-5 Unconfined compression of odd-numbered specimens after the initial bacterial treatment ('a' plots) and healing stages ('b' plots). Odd-numbered specimens were injected with cementation medium at the healing stage. For consistency of scale, the peaks of columns 3 and 7 are displayed on inset figures.

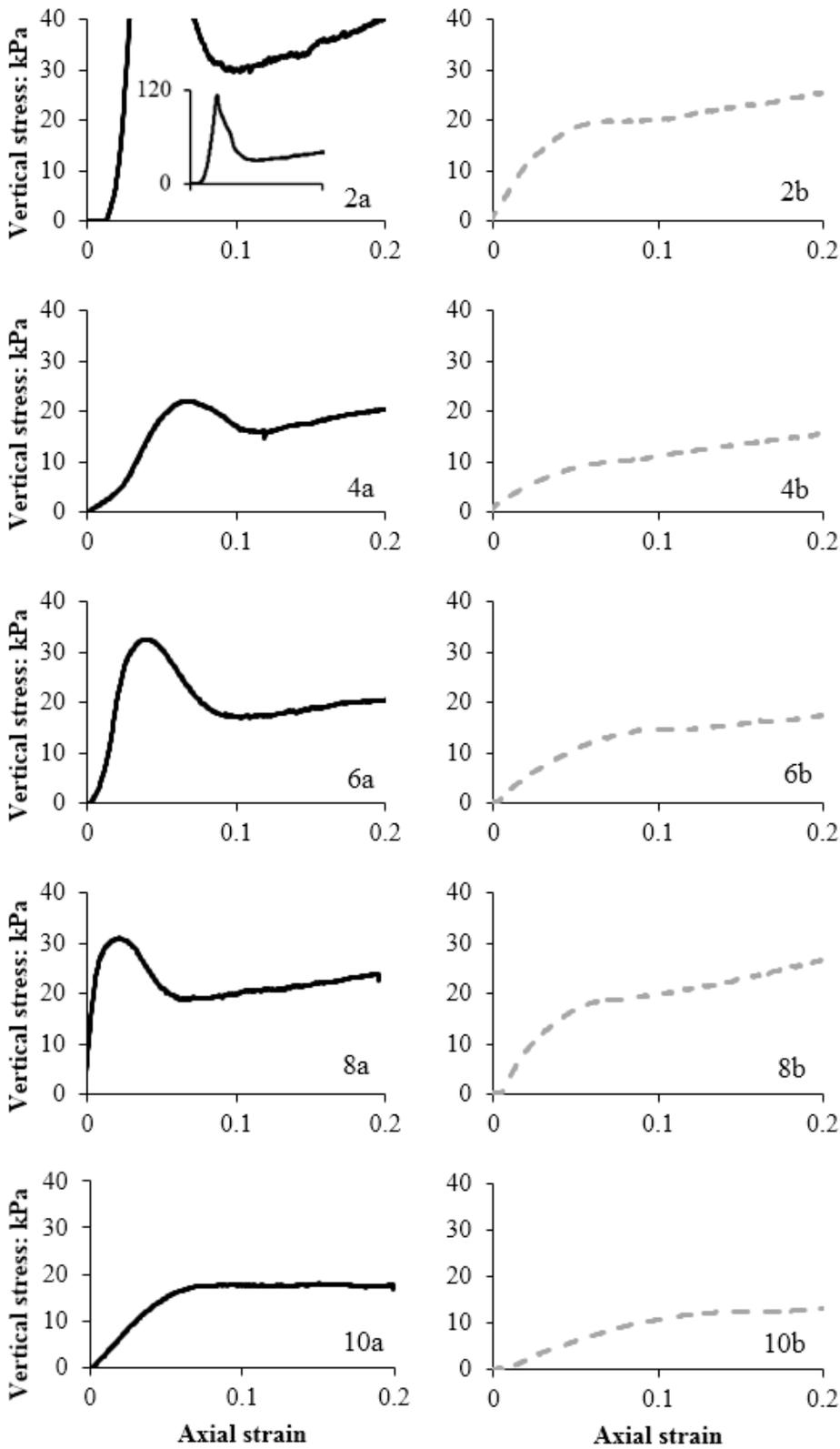


Figure 6-6 Unconfined compression of even-numbered specimens after the initial bacterial treatment ('a' plots) and healing stages ('b' plots). Even-numbered specimens were injected with water at the healing stage. For consistency of scale, the peak of specimen 2 is displayed on inset figure.

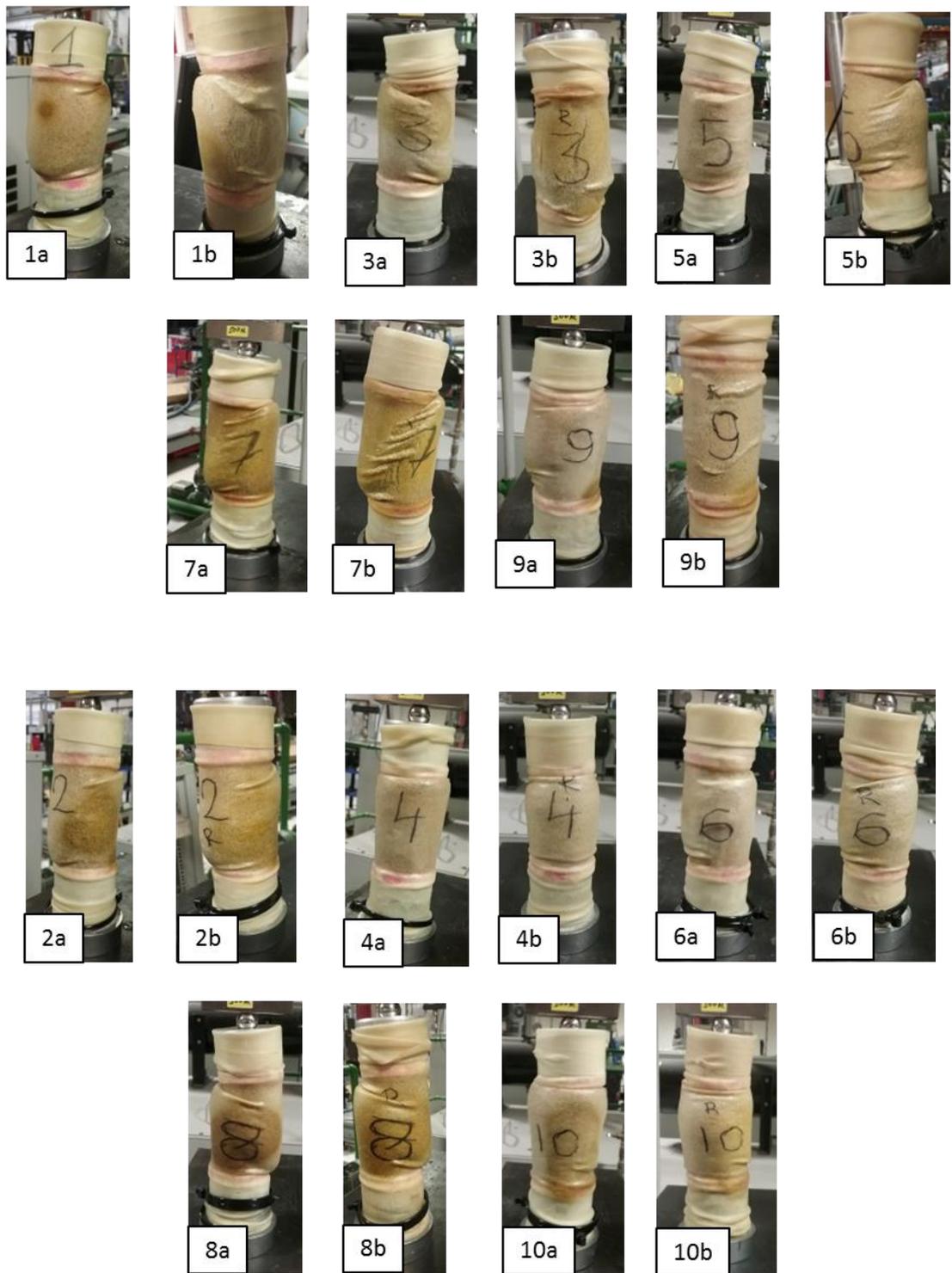


Figure 6-7 Images of the specimens after unconfined compression testing - after the initial bacterial treatment ('a' plots) and healing stages ('b' plots).

Although a large variation in the peak strength of the 10 columns was measured (from 18 to 120 kPa), a brittle type of response with a defined peak was observed for all of the columns after the initial cementation stage (Figure 6-5 and Figure 6-6, 'a' plots), indicative of cementation in the sand. Large peaks were observed in columns 2 and 3, reflecting the large decrease in hydraulic conductivity. Column 7 exhibited one of the largest peak strengths and most brittle behaviours of all 10 columns (Figure 6-5, 7a) and it is possible that during the compression testing of this column a large shear plane was created which could not be filled by bio-cement in the healing stage and therefore it continued to channel water flow. This could explain the different behaviour of this particular column in terms of hydraulic conductivity.

The response to the unconfined compressive strength can be further characterised by the form of failure of the specimen after testing. In the specimens which exhibited high peak strengths (particularly columns 2, 3, and 7 after the initial bacterial treatment), the failure mode tended to be via the formation of a diagonal shear plane through the specimen. In contrast, columns 1, 4, 9, and 10 which failed at low peak stresses tended to exhibit a barrel shape at failure suggesting less cementation. For example, under triaxial testing a sand cemented with Portland cement would develop cracks near the surface which would propagate toward the middle and eventually lead to the formation of a shear band diagonally (Abdulla and Kioussis 1997). It has previously been shown that similar brittle behaviour is observed in gypsum- and calcite-cemented sands (Ismail et al. 2002; Lee et al. 2009) and in microbially cemented sands (DeJong et al. 2006). Additionally, it is common for all types of artificial cementation in sand to rapidly degrade at peak stress under compressive or tensile loading after which the sand returns to its un-cemented behaviour.

When unconfined compression testing was again performed after the healing stage, all columns injected with cementation medium responded in a similar brittle fashion (Figure 6-5, 'b' plots), with peak strengths over and above the residual strength from the initial stress-strain curves. In contrast, all columns injected with deionised water returned to a typical un-cemented sand behaviour without a pronounced peak and less rapid stress build-up (Figure 6-6 'b' plots).

6.3.4 Mass loss on ignition after initial bacterial treatment and healing stages

Figure 6-8 shows the loss on ignition from samples across each column at the end of testing, as a measure of calcium carbonate precipitation. Although a portion may be due to biomass, this would be relatively small (approximately 0.5 %, see abiotic samples in Figure 5-13). Typically, the mass loss from odd-numbered, treated columns with healing (1.7-2.75%) was greater than from untreated columns without healing (1.49-1.84% on average).

As outlined previously in section 6.3.1, there was an observed difference in the bacterial activity in this experiment and the experiment from section 5.5. The greater calcium conversion activity from the onset of the initial bacterial treatment seen in this experiment may have also contributed to the greater mass of precipitate forming (1.7-2.75 % as compared to 0.6 to 1.8 % in the experiment from chapter 5, data from mass loss on ignition). Additionally, the initial bacterial treatment here comprised of 7 injections, whereas in the previous experiment it was 6 injections.

Amount of precipitated mass from Figure 6-8 may explain the data on hydraulic conductivity and shear strength from before. As previously outlined, in order for the bio-treatment to result in a significant hydraulic conductivity reduction, concentrations of CaCO_3 in the soil need to exceed 1.3 % (Chu et al. 2014). Figure 6-8 (even-numbered

columns) shows that the percent mass loss on ignition results (indicative of mass of calcium carbonate) varied from 1.6 to 1.8 % on average. Therefore, although masses of calcium carbonate exceeded this threshold value of 1.3 %, it was only by 0.3 to 0.5 % and the effect on hydraulic conductivity reduction was only apparent in 2 columns. Consequently, the significant reduction in conductivity of the columns at the healing stage can be explained with the mass of calcium carbonate accumulated (Figure 6-8, odd-numbered columns) which exceeded 2 % at the bottom of all columns.

Stoichiometric calculations show that calcium to calcium carbonate conversion efficiencies (calculated as the actual to theoretical yield from the mass of input calcium) in the initial bacterial treatment and in the healing stages were between 74 - 84 % and 52 - 64 %, respectively. In both stages, this experiment showed better calcium conversion efficiency than the chemical deterioration experiment in 6.2 which may be due to the improved column design and specifically the use of the 3D-printed porous discs which aided in a more homogeneous flow of liquids.

Similarly to the observations from previous dynamic column experiments (chapter 5) the precipitation produced in the healing process appeared to be more prevalent at the base of the columns, nearer the inlet. In contrast, the even-numbered columns did not exhibit a consistent behaviour with a precipitation gradient which was most likely due to the washing effect of the injected water at the healing stage.

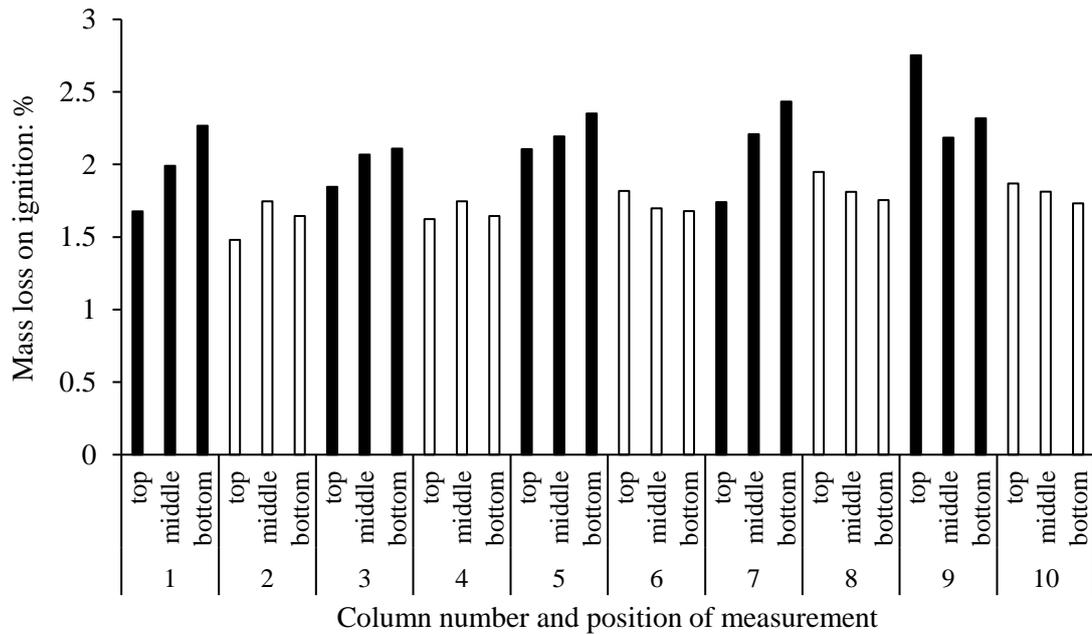


Figure 6-8 Loss on ignition as an indication for amount of calcium carbonate precipitated in sand columns at end of experiment.

There is a reasonable consistency between the mass loss on ignition and the results from the hydraulic conductivity and unconfined compression testing data after the healing stage (Figure 6-9). The graphs were plotted using the data from the mass loss after ignition (amount of CaCO_3) from the bottom of the 10 columns, the final data from hydraulic conductivity, and the peak strengths taken from the stress-strain graphs after the healing stage. The increasing amount of precipitation has a positive effect on shear strength of samples, and a negative effect on hydraulic conductivity. As can be seen, an increase in CaCO_3 content from approximately 1.5 % to 2.4 % results in more than a 130 % increase in peak strength, and almost 80 % reduction in hydraulic conductivity. The irregularity in the second plot is from column 7 which exhibited no significant change in hydraulic conductivity at the healing stage despite of the increased amount of precipitation. The possible explanation for this was previously outlined in sections 6.3.2 and 6.3.3.

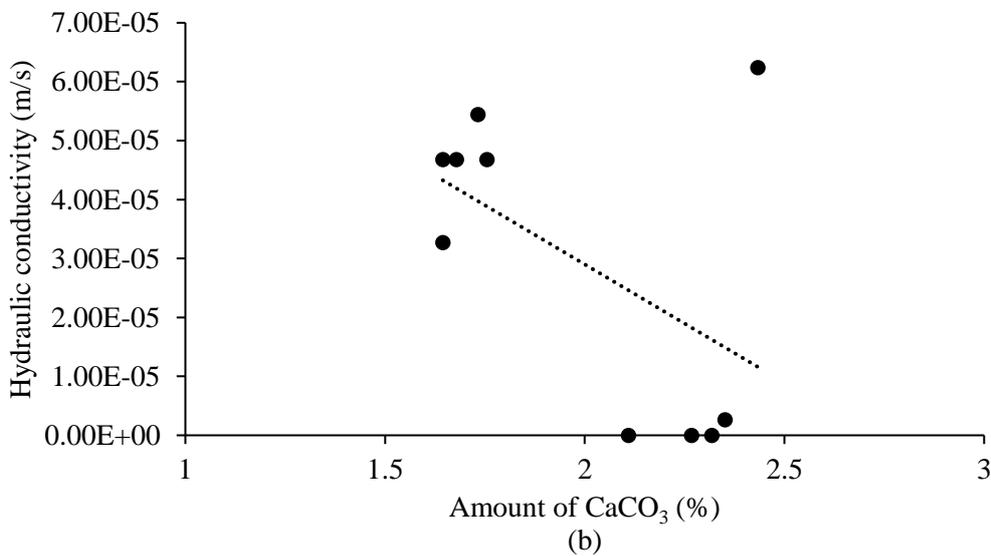
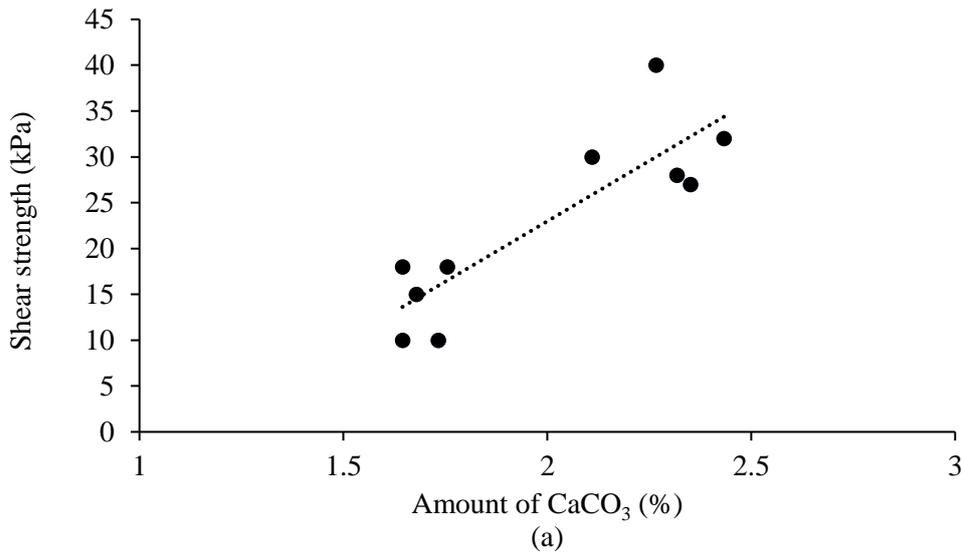


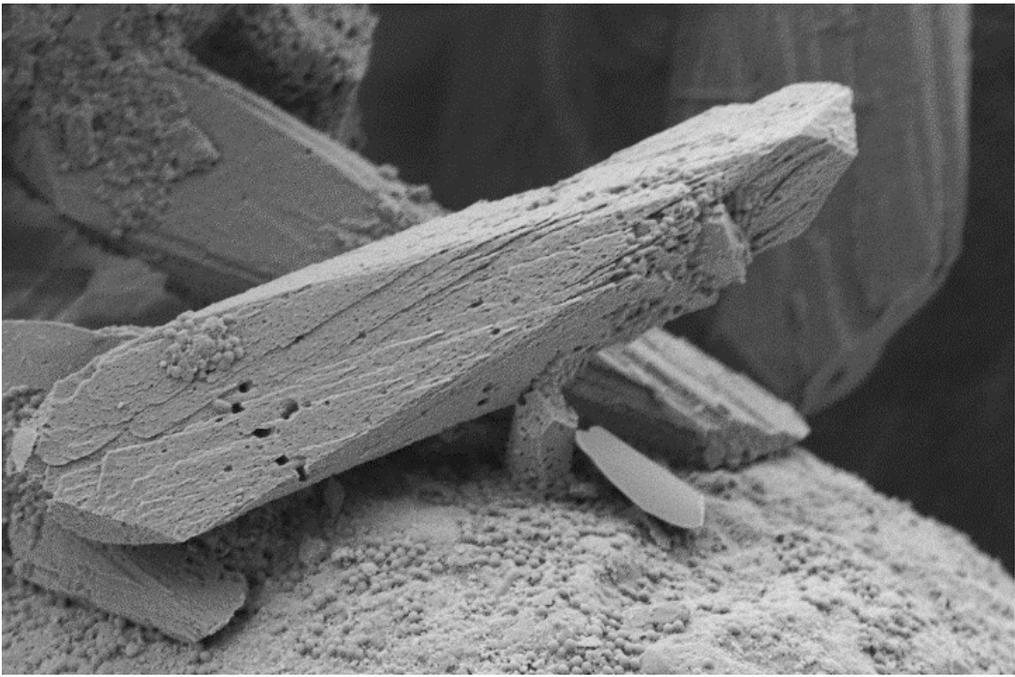
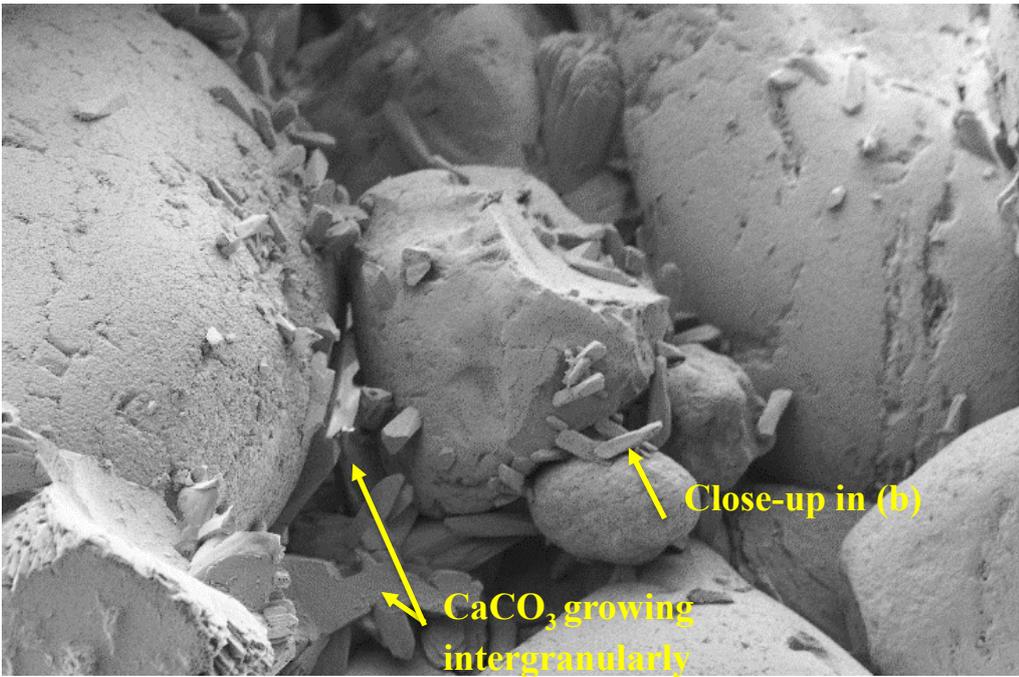
Figure 6-9 Correlation between amount of CaCO₃ precipitated in the sand columns and peak strength as measured by unconfined compression (a), and hydraulic conductivity (b)

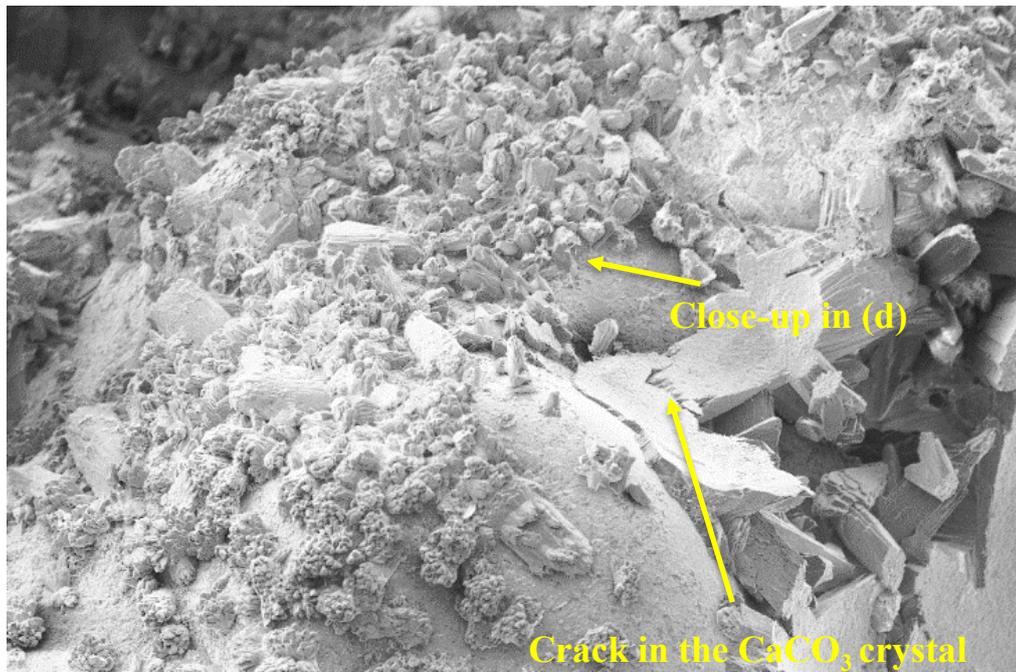
6.3.5 Scanning Electron Microscopy

SEM imaging of samples taken after the initial bacterial treatment of the sand columns show that calcium carbonate crystals exhibited different shapes and forms and occurred in a number of ways within the sand. Some crystals appeared to only seemingly be resting on sand grains (central sand grain in Figure 6-10a, with a close-up in b). Such crystals may have formed initially associated with the sand grains but due to physical

strains under the water flow to have separated and flowed in the pore space.

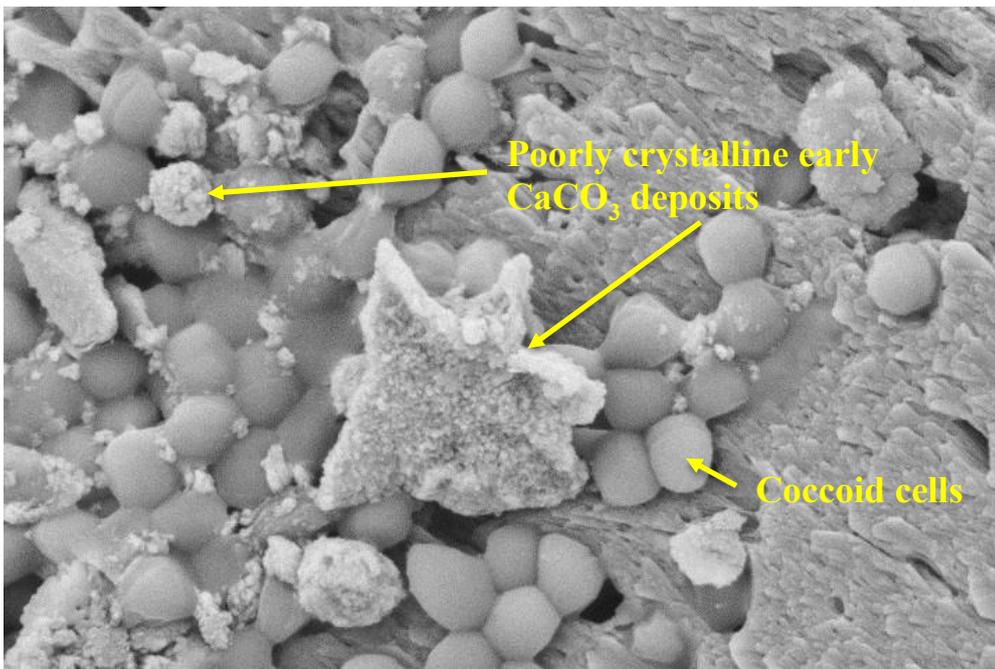
Alternatively, they may have crystallised abiotically in solution. Such type of crystallisation may be present in the pore space and act as filler thereby reducing the hydraulic conductivity of the sand. It may also increase strength due to the increased surface area and therefore friction, however they will not be as effective as the ones bonding grains. In contrast, Figure 6-10a also depicts the formation of a CaCO_3 conglomerate growing in-between sand grains (bottom left corner indicated by arrows on the image). Figure 6-10c shows sand grains almost fully covered by precipitation as well as crystallisation which appeared to have grown as a solid mass between the grains. Such mineralisation is associated with the strength improvement in sands due to the formation of hinges at the contact points of particles (Cheng et al. 2013). The presence of various kinds of cementation occurring at different growth locations may have also contributed to the large variation observed in the hydraulic conductivity (Figure 6-4) and compressive strength results (Figure 6-5 and Figure 6-6) of the columns, especially after the initial bacterial treatment stage.





100 μm

(c)



2 μm

(d)

Figure 6-10 SEM images of CaCO₃ formed by *S. ureae* within sand. Crystals “resting” on sand grains (a) with a close-up in (b), and solid mass of calcium carbonate growing on the surface and in-between sand grains, almost fully covering and bonding them together (c) and close-up in (d)

Close-up of the surface of the sand grain (Figure 6-10d) revealed the presence of coccoïd cells, associated with an angular massif underneath them, and what appear to be amorphous calcium carbonate deposits. The presence of this type of poorly crystalline material was previously discussed in chapter 4 and was attributed to the role of the bacteria in forming the primary calcium carbonate mould (with a weak crystalline structure) onto which the deposits grow and become increasingly more crystalline (Castanier et al. 1999).

Figure 6-10c shows what appears to be a crack in the deposit (indicated by a yellow arrow) which may form under various forms of stress. Such type of physical deterioration may arise as soon after peak strength is reached under compression.

DeJong et al. (2010) described diagrammatically the possible failure mechanisms that may occur in bio-cemented sand and these are shown in Figure 6-11. Failure may occur within the bacterial deposit as a de-bonding of the deposit from the silica grain, or a combination of the two (which is the most probable scenario in reality).

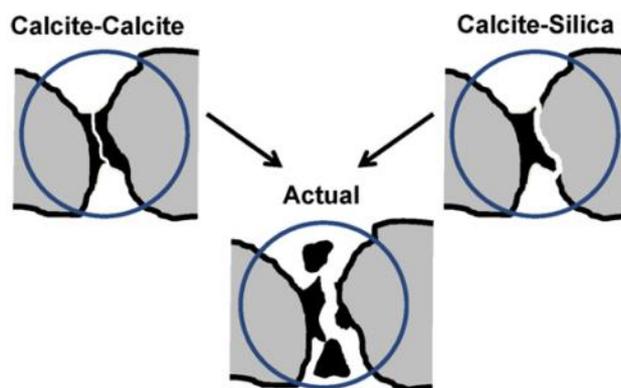


Figure 6-11 Mechanisms of deterioration within a bacterially mediated sand under shear or compression loading (reproduced from DeJong et al. (2010))

The compression testing of the columns from this experiment would have led to the formation of any of the above-described failure mechanisms. The opening of cracks

within the bacterial deposits (Figure 6-10c) suggests that the cells, which were involved in its formation and were encapsulated in the process, would have become exposed. As is seen from the SEM images, especially Figure 6-10d, there is an abundant presence of bacterial cells associated with calcium carbonate. Therefore, provided there is a nutrient-rich environment and the sporulated cells were able to survive the encapsulation and deterioration processes, a healing of the damaged area due to the regenerated bacterial activity is a valid assumption.

The type of crystallisation that would occur in the regeneration process may have an effect on the extent to which healing is achieved. That is, if the predominant crystallisation is occurring in solution and is simply acting as a filler material, then self-sealing will be occurring primarily. However, if the newly formed crystallisation occurs as a bonding agent between the de-bonded faces then a mechanical recovery would be expected. The hydraulic permeability and strength results from the experiment indicate that crystallisation, specifically in the healing stage, was occurring as a sealer of pore voids but also as a bonding agent providing mechanical recovery.

6.4 Overall discussion

The experiment in section 6.2 was helpful in demonstrating the regeneration abilities of bio-cemented spores within sand with the use of simple metabolic activity markers - calcium consumption and pH. This experiment was essentially analogous of the experiment in chapter 4 (section 4.5) in that sporulated *S. ureae* was present in its bio-cemented form but in a monolithic structure with sand. In summary, although the response of the regenerated spores was considerably weaker when compared to freshly introduced cultures in sand, there was strong evidence that the spores of this bacterium can survive a series of potentially damaging events (bio-cementation, acid dissolution,

hydrogen peroxide treatment) but retain their ability to germinate, reproduce and continue hydrolysing urea. Regained urea hydrolysis in the regenerated cultures was indicated by the increased calcium carbonate content relative to the amount after acid dissolution. Studies have previously discussed the various forms of damage that extreme conditions can cause to pores, and even though they can survive extreme events and be able to reproduce, damages can be imposed on protein and enzymatic activity of the germinated cells (Nicholson et al. 2000). Recovery of enzymatic activity in the survived spores was a vital aspect for demonstrating that calcium carbonate can be precipitated again in the damaged area and the process can therefore be repeated. The results from the experiment in section 6.2 were also important in demonstrating that a bio-cemented soil can respond positively to the deteriorative processes of acidic flow. A table summary of the most important parameters and main findings of the experiment in Section 6.3 is shown below (Table 6-1).

Table 6-1 Summary of major findings from experiments in Section 6.2 (Healing after chemical deterioration)

Stage of experiment	Source of ureolytic activity	Measured chemical and geotechnical parameters		
		<i>pH</i>	<i>Aq. calcium</i>	<i>Mass loss on ign.</i>
Initial bacterial treatment stage	Artificially sporulated fresh culture of <i>S. ureae</i> which was introduced in the columns at beginning of experiment	Strong pH response after each nutrient injection to ~8.5-9	Almost full consumption of calcium after each injection	Mass loss on ignition measurements indicated the formation of ~1.5 % CaCO ₃ in the soil
Healing stage	Mineral-encapsulated spores released during treatment with the acidic solution	Weaker pH increase to ~8; still indicating regenerated bacterial activity	Weaker calcium consumption after each injection	CaCO ₃ precipitation reached up to 4 % calcification with a significant recovery of the lost mass of calcite at the deterioration phase

The improved design of the sand column apparatus in the second experiment (section 6.3) allowed for the monitoring of additional geotechnical parameters to assess recovery of mechanical properties due to regeneration of the spores. A table summary of the most important parameters and main findings of the experiment in Section 6.3 is shown below (Table 6-2).

The nature of the initially formed bio-cementation within the sand meant that compression testing resulted in the physical breaking of the interparticle precipitation, thereby exposing the previously entombed spores/cells of *S. ureae*. After killing of the

vegetative cells and supply of fresh cementation medium, the spores regenerated and produced further cementation which provided both a sealing and a healing effect, as measured by hydraulic conductivity and compressive strength. In this study, much effort was put into ensuring that the healing effect was only due to the spores entombed within the calcium carbonate and this was done through a selective sterilisation of vegetative cells only with hydrogen peroxide. In a complex, multi-component environment, such as the bio-cemented sand, the heterogeneity of the system posed a significant difficulty in ensuring the contact of the sterilising agent with all vegetative cells, which were potentially present. However, the use of prolonged reaction times for the chemical resulted in the substantial effectiveness in killing respiring (vegetative) cells. It is also expected that the 2- to 3-week period in which the sand columns were air-dried would have additionally greatly contributed to the killing of such cells. It is therefore assumed with a high degree of confidence that spores were the predominant trigger behind the regeneration and healing effect seen in the experiments from this chapter.

Table 6-2 Summary of major findings from experiments in Section 6.3 (Healing after physical deterioration)

Stage of experiment	Source of ureolytic activity	Measured chemical and geotechnical parameters				
		<i>pH</i>	<i>Aq. calcium</i>	<i>Hydraulic conductivity</i>	<i>Mass loss on ign.</i>	<i>Unconfined compression</i>
Initial bacterial treatment stage	Artificially sporulated fresh culture of <i>S. ureae</i> which was introduced in the columns at beginning of experiment	Strong pH response after each nutrient injection to ~8.5-9	Almost full consumption of calcium after each injection	Majority of samples remained unchanged	No measurements of mass loss on ignition taken after first stage	Large variation of peak strengths of 10 samples, however all exhibiting brittle behaviour
Healing stage	Mineral-encapsulated spores released during first UC testing	Weaker pH increase to ~7.5	Approx. half of total calcium consumed after each injection	Strong contrast between healed and control samples with former ones reducing to up to 100 % their initial hydraulic conductivity	Majority of sand columns accumulated over 2 % calcification at their bases	Strong contrast between healed and control specimens with former ones exhibiting recovered brittle behaviour while the latter returning to un-cemented behaviour

It was also interesting to see that a similar healing effect can be achieved in MICP-cemented sands via the spores of the bacteria, as was seen with active bacteria (Montoya and DeJong 2013; Harbottle et al. 2014; Duraisamy 2016). As discussed previously, however, Montoya and DeJong (2013) and Harbottle et al. (2014) used a non-sporulating organism which healed a physical damage in the soil but the effect would probably last only so long as the longevity of the cells. The spores and their remarkable longevity and resistance to various physical and chemical stresses can provide a much more reliable and potentially durable self-healing mechanism.

In research for microbial self-healing so far, especially in concrete applications, self-sealing, as opposed to self-healing, has been the predominant means for reducing damage due to cracking (Jonkers and Schlangen 2007; Ducasse-Lapeyresse et al. 2013; Sharma et al. 2017). The test data in this chapter indicates that as well as the crack and pore filling effect, which leads to hydraulic conductivity reduction, i.e. sealing, the regenerated bacteria were able to produce precipitation that “welded” and provided an effective bonding in the shear zones. The former was indicated by the dramatic decrease of hydraulic conductivity (to almost zero in all columns) at the healing stage, while the latter was represented by the recovery of brittle behaviour and elevated peak strengths to above the residual conditions (after the first compression testing). The combination of these two mechanisms – sealing and healing – may prove advantageous in certain situations. For example, earth structures in which water flow and seepage creates fissures and cracks that may lead to slip failures (e.g. seepage in tension cracks leading to landslides; piping in earth dams) can benefit from a bacterial treatment that can provide both a blocking/sealing effect of a crack, as well as a `welding` effect to prevent it from opening (or slipping) further.

The results from both experiments in this chapter suggest with some certainty that under laboratory conditions MICP-cemented sands through the use of sporulating bacteria can be healed from both chemical and physical damage and their properties can be restored fully from the original level of treatment. However, further research is needed to examine the response of the encapsulated organisms to multiple damage scenarios. As was seen, bacterial activity was significantly weaker at the healing stage and it is possible that a repetition of the self-healing cycle may further reduce activity. Therefore, if this issue can be overcome, a significantly better healing could be expected. Also, issues with the homogeneous distribution of flow of nutrients may pose a significant challenge especially in large-scale or site projects. Further study is also needed to elucidate how MICP can be advanced into a truly self-healing process with a minimal need for intervention. Although, as previously discussed, groundwater flow may provide chemicals and nutrients needed for bacterial growth and possibly precipitation of minerals, the exact extent to which this may be occurring is unknown. It is also possible to provide nutrients in an encapsulated form, similarly to the approach taken by many researchers developing self-healing concrete (Wang et al. 2012; Wang et al. 2013). Alternatively, chemical grouting technology with pumps and extraction wells (Karol 2003; van Paassen et al. 2010), paired with geophysical sensing methods (van Paassen et al. 2010; Wu et al. 2011) may be customised to detect damage in MICP-modified soil structures and to automatically activate a system for the delivery of healing agents (nutrients, as well as microorganisms) to regions that require repair when needed.

7 Conclusions and recommendations for future work

The completed work addresses the fundamental basis for self-healing geotechnical structures using bacteria. The ability of microbes to go through cycles of bio-cementation, damage and healing of damage in both idealised conditions and in particulate media was demonstrated. Additional work was also undertaken for the optimisation of the MICP process, achieved through *S. ureae*. The main conclusions from this work, based on the overall aims and objectives set out in the first chapter are presented herein.

7.1 Conclusions

- The first objective of selecting a suitable microorganism was achieved. *S. ureae* was chosen among three candidate strains. The microorganism contains a urease enzyme, can bring about microbially induced calcium carbonate precipitation (MICP) and was shown to form copious spores.
- The self-healing cycle was demonstrated in idealised conditions with the ability of *S. ureae* to go through cycles of sporulation, mineral precipitation, mineral break down, regeneration of bacterial activity, and production of further calcium carbonate.
- The initially entombed (in calcium carbonate) bacteria in sporulated form were shown to survive a period of up to 6 months and retain its ability to regenerate when exposed from the crystal.
- *S. ureae*-induced MICP in sand columns was shown to lead to most satisfactory results in terms of geotechnical improvement of soil for an acceptable time period for healing under moderate concentrations of 150 mM calcium in the cementation medium. This concentration lead to the highest increase in the

amount of calcium carbonate precipitated and unconfined compressive strength for a treatment period of 5 weeks as compared to 50 and 300 mM calcium in the cementation medium.

- The primary objective of augmenting MICP in soil with the capability to self-heal has been achieved. An initial bio-treatment of the soil resulted in the formation of a soil-calcium carbonate monolith which was either chemically or physically deteriorated. The damage exposed the previously encapsulated sporulated bacteria which healed the damage through the formation of further calcium carbonate in the impaired area thereby returning the soil to its pre-damage condition.

The primary choice of organism for this study was the commonly used (in MICP research) *S. pasteurii*. Despite the various sporulation media (see section ...) in which this organism was tested, it did not apparently (as visualised by a microscope) sporulate. Due to its inability to sporulate *S. pasteurii* could not be used for self-healing MICP, it was very useful, however, in functioning as a non-sporulating control in the self-healing experiments, as well as a highly-ureolytic model organism in the optimisation study for MICP with *S. ureae*.

Sporulated cells of *S. ureae* were shown to form precipitation in aqueous solution in the form of calcite (most stable polymorph of calcium carbonate). In the process of precipitation, the microorganisms became entombed within the crystal. The encapsulated spores survived sterilisation by autoclaving and subsequent damage to the mineral, upon which they regenerated to vegetatively growing bacteria. In effect, the encapsulation of the spores within their own crystals acted as a protective mechanism against the extreme temperature in the autoclave. To demonstrate unambiguously that

only bio-cement encapsulated spores can survive the autoclaving process, a range of distinctive controls, as well as rigorous sterilisation protocols, were accommodated. Controls in the form of encapsulated non-sporulating cells (*S. pasteurii*) and non-encapsulated spores did not survive autoclaving and did not regenerate upon nutrient exposure thereafter. It is important to note that despite the inability of *S. pasteurii* to sporulate, this organism was able to survive the bio-cementation process and breakdown of the precipitation and reproduce calcium carbonate when nutrients were available. This effect has previously been shown in other studies (Montoya and DeJong 2013; Harbottle et al. 2014) and is likely to occur in natural conditions, however, as previously outlined, the activity of vegetative bacteria cannot be relied upon as the effect may not be enduring. Sporulating organisms, such as *S. ureae*, in contrast are much more durable and resistant to extreme conditions and can be more reliable for long-term use.

Regenerated spores from the primary precipitates were able to reproduce precipitation when nutrients were supplied, effectively recovering mass of precipitate lost and “healing” the damage. The self-healing cycle was repeated up to 3 times, albeit with some reduction in the metabolic activity due to the multiple regenerations. The repetition of the self-healing cycle is a novel concept which has not been demonstrated previously. Although here the process was demonstrated with one particular strain of bacteria it suggests that microbial self-healing for application in both soils and cementitious materials can be achieved with other organisms and metabolic pathways they facilitate, too. Crystal encapsulated spores were also shown to survive prolonged periods of up to 6 months, and once the calcium carbonate crystals were broken down (in acidic conditions) the spores regenerated, albeit with a prolonged lag time as compared to inoculum from fresh cultures.

A comparison between the metabolic activities of *S. ureae* and *S. pasteurii* revealed a significantly weaker metabolic potential of the former strain. This was the reason behind the significant period needed for obtaining measurable improvement in geotechnical properties in MICP-treated sand columns using *S. ureae*. In order to reduce the treatment time an optimisation study of MICP with *S. ureae* was performed in which a range of calcium concentrations in the cementation medium were tested. A concentration of 150 mM calcium in the cementation medium, along with the use of tap water as the solvent was shown to improve effectiveness in terms of calcium ion to calcium carbonate conversion. This concentration was sufficiently high to produce a significant amount of precipitation for alteration of soil properties (non-cemented to cemented brittle behaviour), without it hindering the bacterial metabolic activity. In contrast, the lower concentration (50 mM) was not sufficient for an adequate amount of calcium carbonate precipitation, and the 300 and 1000 mM concentrations were too high for precipitation with *S. ureae*.

Additionally, a geochemical model was used to predict the evolution of pH as function of urea hydrolysis and increasing calcium concentrations (a parameter of significant importance to the effectiveness of MICP) and was compared against batch test data for the range of calcium concentrations (50, 150, 300, and 1000 mM) which were tested. There was a considerable correlation between the model and experimental results for concentrations of up to 300 mM which demonstrates that relatively easy to use geochemical software can predict (though with not a very high accuracy in this study) the pH and potentially aid in designing on-site MICP treatments in the future.

The sporulated bacterial cells of *S. ureae* were shown to be able to produce a substantial amount of bio-cementation in a body of sand within 5 weeks thereby creating a monolithic structure. The bio-cemented columns were exposed to a chemical (acid

deterioration) or a physical damage (axial compression loading), both of which may be expected under typical conditions in the subsurface (erosion, low pH groundwater and stresses due to applied loads). The spores, exposed from the damaged monolith, were able to regenerate, however with an apparent slower metabolic response as compared to the initial treatment. Despite the slower response at the healing stage, the bacteria produced substantial extra bio-cementation, sufficient to effectively heal the damage. Damage recovery was recognised both as a “sealing” (i.e. hydraulic conductivity reduction), as well as a “healing” phenomena (mechanical strength recovery). Mechanical recovery was observed after healing took place in the physical deterioration experiments. Most healed sand columns exhibited an improved mechanical performance with an increase in the strength relative to the residual strength after the initial compression testing. In some specimens the healed strength reached the undamaged peak strength. Permeability measurements and mass of precipitate also indicated damage recovery, whereby healed sand columns showed an increase in mass of precipitate and a significant reduction in hydraulic conductivity compared to the post-damage condition.

Microbial self-healing in this study was demonstrated in small-scale sand column experiments and it required human intervention throughout with the supply of artificial media and at optimum conditions for the bacterium. Parameters, such as the heterogeneity of the soil, distribution of bacteria, fluid flow and delivery of nutrients were controlled in the laboratory; however they may play a crucial role when self-healing is applied to a larger scale. The principles behind microbial self-healing will still hold at field scale provided proper control is executed on the above mentioned parameters. Existing grouting technology may perhaps prove useful in the delivery of fluids and bacteria to the site, paired with geophysical methods to monitor bio-

cementation development, detect damages to the monolith and initiate healing processes.

7.1 Recommendations for future work

The work presented in this thesis demonstrated the ability of sporulating, calcite precipitating microbes to alter geotechnical properties of soil and to respond to and heal damage. However, a substantial body of work is still required for a microbial self-healing in soils to be fully understood and implemented on field applications.

In the final experiments of this work in which self-healing was explored in particulate media the healing phenomena was mainly attributed to the action of spores. Despite the rigorous sterilisation procedure which was adopted in the experiments, there was still a possibility that the action of vegetative bacteria (surviving from the initial treatment) was not fully eliminated at the healing stage. This was due to difficulty in ensuring full contact with the sterilisation solutions with the sand in the columns as a result of natural heterogeneity of the soil. Further work could elucidate this issue through the use of a full range of controls similar to those adopted in the aqueous solution experiments- for example, use of non-sporulating bacteria with a similar ureolytic potential to *S. ureae*. The adoption of such controls was not possible in the current study due to limitation with the experimental facilities and time restrictions.

In the majority of the experiments the use of artificial media aided in all aspects of the microbial self-healing process, thereby rendering it only semi-autonomous. For example, sporulation was enhanced with sporulation media, calcium carbonate precipitation and germination of the spores was achieved through nutrient and cementation media. In a natural setting the key issues arise with the occurring of sporulation in sufficient quantities and in the right time, with the germination of spores once they are exposed

from the monolith, and with the presence of sufficient chemical precursors in the surrounding environment for the germinated cells to produce further precipitation for damage recovery. A study is needed to establish whether sporulation, precipitation, and germination occur inherently in a natural subsurface media (typical groundwater), to what extent, and what are the minimum requirements and additives needed for these processes to take place. If any of these processes do not occur to a sufficient level naturally, then they may be engineered. For example, the use of microcapsules for the delivery of chemical precursors as well as bacteria (Wang et al. 2014; Kanellopoulos et al. 2017) has proved useful in self-healing concrete applications and may be used in soils, too. Alternatively, conventional grouting technology with injection-extraction systems (Karol 2003; van Paassen 2009) may be used for the transport of liquids and bacteria where and when needed to heal damage in a bio-treated soil.

Repeatability of the self-healing cycle, as well as the long-term survivability of mineral-encapsulated bacteria was only demonstrated in aqueous solution experiments in this project. Further work is needed to establish repeated self-healing cycles and survival of monolith (bio-cemented soils) encapsulated spores which will ensure the long-term durability of geotechnical structures.

It is worth investigating the possibility of bio-stimulation, as opposed to the bio-augmentation mechanism used in this project. Issues faced with the heterogeneous distribution of the bio-mineral in soil may be overcome by stimulating organisms naturally present in large numbers and in depth in the subsurface. Homogeneously distributed bio-cementation, as well as the presence of natural bacteria in a soil will in turn improve the possibility of self-healing in case of monolith deterioration. The principles of microbial self-healing were investigated in idealised conditions and soil columns experiments in this study. In the future, the mechanisms established in the

small-scale study here can be scaled-up to demonstrate the workings on a larger scale. Perhaps, small-scale geotechnical centrifuge testing with the use of natural heterogeneous soils may be required to overcome issues with non-uniform bio-cementation, repeatability of self-healing and minimum requirements for maintenance of a geotechnical structure.

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