Bioprecipitation of Calcite by *Sporosarcina pasteurii*: Developing Efficient Methodologies for Microbially Indurated Rammed Earth

By

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Abstract

Microbially-facilitated calcite precipitation has a high potential to reinforce unconsolidated soils, thus increasing their strength. Employing the use of microbially-facilitated precipitation of calcite will result in the creation of a new building material termed Microbially Indurated Rammed Earth (MIRE). This application of the bacterium *S. pasteurii* exploits the urease pathway to hydrolyze urea, resulting in the precipitation of calcite in Ca^{2+}-rich solutions. The resulting increase in strength derived from this process would meet standard building codes for residential structures in many states and could therefore supplement the building requirements of concrete. During the course of this study the maximum growth and optimal delivery method of a pure culture of the bacterium, *Sporosarcina pasteurii* was tested for its use as a natural binding agent. This study tested *S. pasteurii* for its cell density and growth rate using urea as a metabolite as studies have shown that bacterial cell concentration correlates to greater calcite precipitation. After ~70 hours, cell counts were taken to create growth curves for bacteria incubated at 35°C and 25°C. These data were applied to experiments on the bioprecipitation of calcite in a limestone soil as baseline parameters for the creation of MIRE. Over the course of this study it was determined that *S. pasteurii* grew most rapidly between 20-40 g L^{-1} urea at 35°C. Additionally, there was no significant difference in the spatial distribution of bacteria, critical for equal distribution of calcite cement, when the bacteria were delivered to MIRE soil as a freeze-dried pellet compared to freshly grown and harvested. Pilot tests of RE using *S. pasteurii* with urea and blood were performed. Compressive strength tests were done on standard engineering cylinders in order to test the efficacy of soil stabilized using MICP. These data provide the groundwork for meeting RE construction standards and building codes

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Chapter 1: Introduction

1.1 Modern Usage of Concrete

Concrete is an environmentally detrimental construction material. Processing the aggregate, and the use of Portland cement (the basic binding constituent in concrete), represents as much as 5% of all CO_2 emissions worldwide (Adam, 2012). Throughout this document Portland cement is the term that will be used to refer specifically to the binding agent within concrete. With environmental stewardship receiving increased emphasis as we move into a new millennium, it is desirable to reduce emissions wherever possible. Even small environmental improvements in construction material technology yielding a 1% reduction in global carbon dioxide emissions from the cement industry would equate to a reduction of approximately 20 million metric tons of CO_2 per annum (Alstom et al., 2013). Thus, decreasing the use of Portland cement-stabilized building materials, such as concrete, will aid in reducing our environmental footprint.

The relatively low cost of production (~4 dollars/square foot) coupled with concrete's strong physical properties have helped it become the most commonly used form of construction material worldwide (Crow, 2008). Concrete has been the dominant construction material for over 100 years because of its strong compressive strength and versatility. Concrete can be molded into many forms and used in a wide range of industrial and private applications including wellbore stability, bridge building, and the construction of high-rise structures. Concrete gains its versatility and strength from the cement that is used to bind the aggregate material together. Portland cement is the standard cement used in the concrete industry. Manufacturing of Portland cement

(excluding the mining and transportation) contributes 2.4% to greenhouse gases released into the atmosphere each year (EPA, 1998). Processing Portland cement involves burning fossil fuels to heat ground limestone to a temperature of 1400°C to produce "clinker". The clinker is ground to a fine powder to produce cement. The global demand for cement has increased 100% over the last decade and is projected to continue to increase in the future (International Cement Review, 2013). Therefore, finding an environmentally friendly way to bind the aggregate, and reach the desired strengths of concrete, could significantly decrease the need for Portland cement and increase the recyclability of construction materials (Kraus et al., 2013).

1.2 Rammed Earth

For the past several thousand years, rammed earth (RE), a construction material that applies a natural binding agent, such as blood, to a soil, has been used to produce residential structures in cities throughout the world (Jaquin, 2007). More recently there has been a resurgence of RE construction. Since the 1970's, and the heightened environmental awareness in subsequent decades, alternative, energy-efficient, forms of building have been becoming more common (Easton, 2007). Additionally, environmental subsidies for green building have been on the rise. These two factors combined are the underlying reason that RE construction has seen, and will see, a continued rise in usage.

Rammed earth has been used for thousands of years and many structures still remain standing today (Jaquin, 2007), some as old as 8000 years. This longevity is in stark contrast to standard concrete construction. As cracks develop in concrete structures, they lose their durability and must be repaired or replaced (Bažant and Xiang, 1997). An

additional benefit to RE construction is that the material can be created by using locally derived materials, or many materials that would otherwise be discarded, i.e., mine tailings (Reddy, 2004) and caliché soils (Sustainable Resources, 2014). In addition, RE can be recycled at any point during its lifetime adding to the overall sustainability of the material in construction (Kraus et al., 2013). The inherent sustainability and waste reducing capacity of RE make it an ideal candidate for the growing use of sustainable products in construction.

The single most important factor in building with RE is the selection of an appropriate medium (e.g., limestone soils, mine tailings supplemented with clay minerals, etc). While a variety of soils can be used to create RE, to achieve the maximum durability of the RE structure, the mix of sand- and clay-sized material must be near a ratio of 70:30 (Easton, 2007). This ratio can be artificially mixed (Hall and Djerbib, 2004) or found in subsurface, organic-free, soils and sediments (Easton, 2007). Once the ratio has been achieved and the soil/sediment is mixed and compacted, no other additive is necessary to achieve longevity (Jaquin et al., 2009). Additionally, the clay minerals must be composed of non-expanding clay minerals, such as illite, to avoid mechanical decay of the structure over time (Sustainable Resources, 2014). However, to increase the compressive strength to meet modern construction standards (i.e. NAREBA, 2009) and maintain the sustainability of RE, a structural stabilizer must be added. At present Portland cement is the most common stabilizer used in Stabilized Rammed Earth (SRE), which reduces the overall sustainability of the material. Alternatively, a natural binding agent, such as calcite, can be used to strengthen the material and achieve desired strengths in residential construction.

1.3 Microbially Induced Calcite Precipitation (MICP)

1.3.1 Background on MICP

Microorganisms occur naturally in soils and sediments and can facilitate the precipitation of minerals in two distinct manners: by acting as nucleation sites on their cell surfaces and through metabolic activity that perturbs aqueous geochemistry and shifts mineral equilibria. The isoelectric point is the point at which charged surfaces carry no net charge – above this point the surface-bound functional groups are deprotonated and negative, while below the point the groups are positive. Therefore, at higher solution pH the calcium ion (Ca^{2+}) tends to bind to cell surfaces more readily than at lower solution pH. The binding of cations to microbial cell surfaces acts as potential nucleation points for the nucleation and growth of minerals (Beveridge and Fyfe, 1985; Francios et al., 2011). Microorganisms are notably important in the microenvironment and because of their size and high surface area to volume ratio are adapted to interacting with metal ions in the environment. As a consequence of this interaction, several microbial metabolic processes result in the bioprecipitation of minerals and amorphous phases (Douglas and Beveridge, 1998). Formation of silica by cyanobacteria (Yee et al., 2003), iron oxides at acid mine drainage sites (Mann and Fyfe, 1989), and calcite precipitation in soils and groundwater throughout the world are commonly mediated by microbial processes (e.g., Barabesi et al., 2007).

Calcite precipitation is of particular importance and is a widespread phenomenon because of the large quantities of natural calcium in groundwater (Baskar et al. 2006). The geochemical processes driven by microbial metabolism and cell surface reactivity result in significant changes in the microenvironment surrounding the bacteria. These changes

reflect the specific microbiota present and this, in combination with solution chemistry, controls the composition and mineralogy of resulting bioprecipitates. Multiple metabolic processes result in the bioprecipitation of calcite (in the presence of dissolved Ca^{2+}), including sulfate reduction, denitrification, photosynthesis and ureolysis. Van Lith et al. (2003) conducted experiments showing that bacterial sulfate reduction in anoxic, low-temperature environments resulted in changes in the microenvironment surrounding the bacteria. These changes result in the bioprecipitation of high Mg-calcite. Paassen et al. (2010) conducted laboratory experiments in sand columns showing microbial precipitation of calcite resulting from denitrificaton. Metabolic processes may include increases in dissolved inorganic carbon (DIC) as organic carbon is utilized, and, an increased pH (Douglas and Beveridge, 1998). As pH increases the saturation state of calcite is reached and the kinetic rate of calcite precipitation increases rapidly. Microbially Induced Calcite Precipitation is a ubiquitous process in the environment, resulting in large-scale precipitation of biominerals (Boquet et al., 1973).

Although several metabolic pathways can lead to calcite precipitation, ureolysis, possesses exceptionally fast reaction kinetics and has been investigated due to its ability to precipitate large quantities of calcite in a relatively short period of time (Paassen et al., 2010; Tobler et al., 2011; Brookshaw et al., 2012). Ureolytic bacteria, such as *Sporosarcina pasteurii*, are common subsurface bacteria (DeJong et al., 2006) and readily utilize urea as an electron donor coupled to oxygen for energy. The bacteria hydrolyze urea and produce H_2CO_3, which subsequently equilibrates in water to CO_3^{2-}. As the reaction proceeds, OH^- is produced resulting in an increase in pH and concomitant increases in the activity of CO_3^{2-}. This process is a product of metabolism known as

ureolysis. Microbial ureolysis occurs in marine, soil, and karst environments (Boquet, 1973). The generalized geochemical pathway utilized by bacteria to hydrolyze urea is shown below (equations 1-4). Bacteria metabolize urea in water, which results in the production of ammonia and carbonic acid, and energy for the bacteria (1.1). The carbonic acid subsequently equilibrates in solution to produce carbonate (1.2). Supersaturation of carbonate minerals (in the presence of Ca^{2+} or other metals) occur because of the increased pH resulting from hydroxide ions being released during the production of ammonium from ammonia (1.3). The result of this metabolic reaction is the precipitation of calcite on the cell surface of bacteria (1.4).

Eq. 1.1: $CO(NH_2)_2 + 2H_2O \rightarrow 2NH_3 + H_2CO_3$

Eq. 1.2: $H_2CO_3 \rightarrow HCO_3^- + H^+ \rightarrow CO_3^{2-} + 2H^+$

Eq. 1.3: $NH_3 + H_2O \rightarrow NH_4^+ + OH^-$

Eq. 1.4: $Ca^{2+} + HCO_3^- \rightarrow CaCO_3 + H^+$

The reaction kinetics involved with the bioprecipitation of calcite via ureolysis are particularly fast. The reaction rate for Ureolytic Calcite Precipitation (UCP) is on the order of 0.8 mmol L^{-1} d^{-1} at the point of critical saturation (Ferris et al., 2004). Additionally, it has been shown that bioprecipitation of calcite is an effective means of stabilization in limestone soils (Ferris et al., 2004). Although there is potential for silica precipitation to stabilize unconsolidated clay-rich soils (Konhauser and Urrutia, 1999) the research thus far is not as robust as that of calcite precipitation (Fujita et al., 2000, Ferris et al., 2004, Tobler et al., 2011). Thus microbial precipitation of calcite was chosen for a binding agent in MIRE for this study, due to its durability and rapid formation.

1.3.2 MICP in *S. pasteurii*

Sporosarcina pasteurii inhabits a wide range of habitats, found in soils and aqueous environments throughout the world, and it is a common calcite precipitating bacteria; other examples include Proteus and Bacillus bacteria (ABIS Encyclopedia). Urease, the enzyme responsible for catalyzing the reaction that produces calcite, is ubiquitous in the environment (Mobley and Hausinger, 1989). *S. pasteurii* is an alkaliphilic, urease positive, facultative anaerobe. It precipitates large amounts of calcite in the presence of ammonia (from urea) and dissolved calcium, thus, making it a model for use in neutral to alkaline soils typically used in rammed earth. As *S. pasteurii* metabolized, the solution pH increases, increasing CO_3^{2-} activity, and created conditions that are supersaturated with respect to calcite when Ca^{2+} is present in solution. Bioprecipitation of calcite is rapid and eventually encapsulates the bacteria and ultimately results in the death of the bacteria (Ramachandran, 2001.) Additionally, recent studies suggest that precipitation of calcite increases with respect to higher bacterial cell counts (Tobler et al., 2011). Therefore, it is the goal of this study to optimize bacterial growth in order to precipitate large quantities of calcite.

S. pasteurii have been used in many modern applications to facilitate calcite bioprecipitation. These applications include solid-phase capture of heavy metals and radionuclides (Fujita et al., 2004), wastewater treatment (Hammes et al., 2003), carbon sequestration (Dupraz et al., 2009), concrete crack repair (De Muynck et al., 2010), and soil stabilization (Whiffin et al., 2007). In an effort to reduce shear during earthquakes and reduce the dangers of landslides and liquifacton, the process of urea hydrolysis and the associated calcite precipitation has also been examined for its ability to biostabilize

sandy soils (DeJong et al., 2010). Additionally, *S. pasteurii* has been investigated for its potential in stabilizing sand dunes and reducing the effective extent of desertification (Sarmast et al., 2014). These widespread uses of the bacterium *S. pasteurii* show the versatile applications of microbial calcite precipitation and the potential benefits of the high rates of urea hydrolysis (3.29 d^{-1}) producing calcite (Tobler et al., 2011).

The precipitation of calcite using *S. pasteurii* has been analyzed for its ability to sequester small amounts of CO_2. It has been shown that the hydrolysis of urea can trap atmospheric carbon dioxide in bioprecipitated calcite (Dupraz et al., 2009; Mitchell et al., 2010). Therefore, where cement stabilized Rammed Earth has a net increase in CO_2, Microbially Indurated Rammed Earth has the added benefit of sequestering existing CO_2 from the atmosphere in mineral form. Current calculations at a urea concentration of 20 g L^{-1} show that 37% of existing $CO_{2(aq)}$ in groundwater would be sequestered in calcite precipitated through urea hydrolysis (Mitchell et al., 2010.) Thus, further reducing atmospheric concentrations of carbon dioxide. For these reasons, it is not surprising that this bacterium has become the popular focus for microbial calcite precipitation in many modern day applications.

1.4 Microbially Indurated Rammed Earth

MIRE is a material that updates the ancient function of RE with the modern day application of microbial calcite precipitation. Kraus et al. (2013) states that MIRE provides the opportunity for Rammed Earth to develop into a natural replacement for Portland cement, which can meet modern building requirements. We hypothesize that bioprecipitation of calcite via ureolysis is a viable means of stabilizing RE. Creating this

updated material would improve the compressive strength of RE and allow broader applications, reducing the need for Portland cement. By increasing the compressive strengths of RE through the natural precipitation of calcite we can increase the usability of RE, while maintaining its inherent sustainability.

Our objective in this research is to determine the best delivery methods and growing conditions of *S. pasteurii* to maximize the distribution and amount of calcite precipitated in the pore spaces of an aggregate material. To be useful, these methods will require up-scaling and therefore our assessment of "best practices" takes into account both the practicality and quantification of these methods. A series of experiments were carried out in the laboratory to investigate mode of delivery and growth of *S. pasteurii*. Additionally, pilot tests of RE using *S. pasteurii* with urea and blood were performed. Compressive strength tests were done on standard engineering cylinders in order to test the efficacy of soil stabilized using MICP. These data provide the groundwork for meeting RE construction standards and building codes. Additionally, these data will help to reduce global carbon dioxide emissions through the development of an environmentally sustainable alternative to standard concrete construction in residential and private building.

Chapter 2: Methods

2.1 Experimental Setup

Experiments were performed in two phases; controlled laboratory experiments and MIRE experiments. First, laboratory experiments were conducted using a pure culture of *Sporosarcina pasteurii* to test the effects of urea concentration on the rate of growth and cell density of the bacteria. Additionally, experiments were conducted to determine the effects on growth and spatial distribution when the bacteria were delivered to a soil as a freeze-dried pellet instead of active, live cells. Prior to the MIRE experiments, maximum exponential growth was tested in relation to growth time, based on the assumption that the faster the bacteria reach their maximum growth potential the more calcite the bacteria will precipitate before the cylinders dry (Tobler et al., 2011).

Second, the above data provided baseline growth parameters for strength tests in MIRE. Growth parameters were developed in order to maximize the dispersion and efficiency of calcite precipitation throughout the MIRE cylinders. Other parameters included alkali soil medium, temperature of inoculation, growth time, initial Urea concentration, and initial soil water saturation. The cultured bacteria were introduced into soils to precipitate calcite into the pore spaces of an artificially compacted soil. An increase in compressive strength was expected as the bacteria metabolized a nitrogen source and precipitated calcite through the urease metabolic pathway.

The MIRE experiments were designed to test the compressive strength on standard engineering cylinders. The bacteria was introduced into the soil medium and allowed to grow and precipitate calcite. The calcite precipitate was used as stabilization in the MIRE

cylinders. The cylinders were then allowed to cure at room temperature for ASTM certified times of 7, 14, and 28 days. At which point, the cylinders were crushed to test the compressive strength gained.

2.2 Standard Microbial Growth and Preparation

All batch experiments were conducted on a stock culture of *Sporosarcina pasteurii* acquired from Robin Gerlach, Montana State University. The plate cultures were transferred to 1 L sterilized stock liquid growth solution containing 37 g brain heart infusion (BBL) and 20 g L^{-1} urea. A single culture was introduced into the growth solution. The bacteria were incubated at 35°C and mixed at 160 rpms until mid-exponential growth (approximately 96 hours). At this point 50mL of microbial solution was transferred to fresh media bottles containing growth solution to run Optical Density analysis at 600nm to produce growth curves of the bacteria (Sutton, 2006). Direct counts using DAPI were also performed (Yu et al 1995). Glycerol stocks were stored at -80 °C with a combination of 0.5 ml bacteria in solution and .5ml glycerin (Riesenberg et al., 1990). All laboratory experiments were completed using these methods.

Experiments were done in batches (31 in total), where one batch is defined as *S. pasteurii* grown in 500 ml stock solution to an optical density of approximately 0.5 absorbance units or ~$1.2x10^8$ cells. Two separate batches were grown to test the effects of differing amounts of urea on the growth of *S. pasteurii*. Two batches were used in the experimental setup to test the viability of the two delivery methods. The subsequent 25 batches were grown using the results from the preliminary four batches and used in the experimental design of the MIRE experiments.

2.2.1 Microbial enumeration

Each batch culture of bacteria was analyzed on a Genesys 20 Thermo Spectromic spectrometer to determine the growth of the bacteria in solution every hour. The Optical Density (OD) was tested at 600nm every hour until maximum growth was achieved. Optical Density is the percent absorbance of light through a medium, in this case measuring the microbial concentration. As the cell concentration increases the percent absorbance increases as well until the bacteria's stationary phase is reached (IUPAC, 1997). Stationary phase is defined as the growth phase where bacterial cell growth is equal to cell death. Growth curves were created for each batch of bacteria created for the MIRE experiments.

Direct microbial cell counts were also conducted using DAPI stains (e.g. Yu et al., 1995). DAPI (4',6-diamidino-2-phenylindole) stains the DNA of microbial cells making them visible for counting under UV light and 100X magnification. DAPI was carried out by diluting the microbial solution with DI water at dilutions of 1:1, 1:10, 1:100, and 1:1000. This was completed by sterile pipette and transferring an initial amount (1 ml) of microbial solution to a 10ml sterile falcon tube. DI water was then added to the tube and the solution mixed thoroughly. This process was completed three times in succession to a dilution of 1:1000. Upon completion of dilutions, 1ml of the 1:100 and 1:1000 dilutions were transferred, by sterile pipette, to a vacuum pump equipped with 25mm black, 0.2µm membrane filters. The vacuum pump was allowed to run for 15 minutes to ensure that all liquid was filtered from around the microbial cells. In a dark room, four drops of DAPI stain was added by syringe to each filter. The samples were then allowed to incubate for 15 minutes in the dark. After 15 minutes the vacuum pump was reconnected and the

excess DAPI stain was filtered off (~10 minutes). Each filter was then transferred, using flame-sterilized tweezers, to a microscope slide. One drop of mounting oil was added directly onto the filter and a cover slip emplaced, making sure no bubbles remain between the microscope slide and cover slip. To view DAPI one drop of immersion oil was added to the slides and, using a 100X objective on a Nikon E400 Epifluorescent microscope in a dark room, the microbial cells were counted at ten representative fields of view and averaged. To enumerate cells for the 1:1000 dilutions under 100X magnification, the following calculation was completed (Equation 2.1):

Eq. 2.1: B x 4676.2 x 1000

Where B is the average number of microbial cells per field of view when viewed under 100X magnification. 4676.2 is the actual number of fields of view on the area of the filter. Finally, the value is multiplied by 1000 because the initial 1ml sample was taken from a 1L batch and we are interested in the total cells L^{-1}. This procedure allows for an accurate measure of the number of cells that grow during the designated growth phase of the bacteria. To gather statistically significant values for the cell counts 10 fields of view were counted for each sample and averaged over the entire slide. This method gives an approximation of the absolute cell count in a sample (Yu et al., 1995).

2.2.2 Effects of Varying Urea Concentration on Microbial Growth

Bacteria were grown in urea concentrations of 20, 40, 60, and 80 g L^{-1} urea. Separate experimental runs were conducted at 25°C and 35°C. These experiments examined the growth potential and length of the lag phase of *Sporosarcina pasteurii*. Parameters for growth curve analysis were tracked throughout the growth phase (Appendix D). Growth

curves for each growth condition were created to examine the maximum growth potential of the bacterium that were freshly harvested using the equation:

Eq. 3.1 Optical Density = Asym-Drop*exp(-exp(lrc)*time^pwr)

Microbial cell counts were taken for each experiment on pure cultures of *S. pasteurii* grown in a solution composed of brain heart infusion and urea. Cells in mid-exponential phase were harvested and introduced to fresh growth solution and allowed to grow through exponential phase and reach their stationary phase.

2.2.3 Liquid vs. Freeze-dried Bacteria

To test the impact on ease of delivery to MIRE and growth, *S. pasteurii* was freeze-dried. To freeze-dry bacteria, the 1L media bottles containing *S. pasteurii* and growth solution were separated into 20 50ml samples and centrifuged. The supernatant was decanted leaving a microbial pellet with ~$1.4x10^4$ cells (or mid exponential growth where maximum growth, or stationary phase, is ~$1.2x10^8$ cells at 20g L^{-1}) and 5ml of growth solution was put into the 50ml falcon tube. The samples were then agitated to resuspend the microbial pellet and 1ml of skim milk was added to the microbial solution. The centrifuged bacteria were supplemented with 1 ml of sterile skim milk (Difco) to protect the viability of the microbial cells during the freeze-drying process (Zayed and Roos, 2004). The microbial solution plus 1ml of sterile skim milk was then flash frozen for 20 minutes at -80°C and then loaded onto a freeze drier (Labconco Freezone). The samples were left for 24 hours on the freeze drier. After 24 hours the samples were removed and stored, in sealed falcon tubes, at -80°C for later use in experiments.

To deduce the effect of the delivery method on a representative soil for MIRE experiments, 4 containers were set up (as shown in figure 1): 2 had bacteria delivered from freeze dried pellets and 2 had fresh bacteria added in growth solution. To test the microbial distribution in the soil an experimental design was created using four tubs with 4kg of Bonner Springs soil (description below) added to each tub. This experiment is important because success is determined by the bacterial distribution in the soil and the even precipitation of calcite throughout the MIRE cylinders. A freeze-dried microbial pellet containing ~$1.4x10^4$ cells was added to two of the tubs and ~$1.4x10^4$ cells of live bacteria in a liquid broth were added to the other two tubs. Additionally, 250 ml of Artificial Groundwater (AGW) (formulated using Ferris et al. (2004) and supplemented with 500 mM urea and 500 mM $CaCl_2$; Appendix B) was added to each tub. The soil and bacteria were mixed by hand over hand mixing until all of the soil was sufficiently wetted (~10 minutes) and was allowed to cure at room temperature in the sealed containers. The bacteria were allowed to grow for 2 days and 7 days. After 2 days, 5mg of soil was harvested every 2cm to a depth of 1cm for a total of 6 different points (Figure 1) along a transect in each of the 2-day containers and diluted to 20mL by adding 15ml of

Figure 1 Bonner Springs soil used in MIRE experiments. Image shows the experimental setup and sampling points used in all tubs during the comparative delivery experiment – Sample site 1 shown and red dots represent subsequent sampling points

DI water in 20ml falcon tubes. Further dilutions of 1:10, 1:100 and 1:1000 were done to prepare DAPI stains of the grown bacteria. After 7 days the process was repeated for the final 2 containers and DAPI stains were produced for both. DAPI stains were completed with the 1:1000 dilutions and cells counted for each of the six samples to determine the distribution of bacteria in the soil samples.

The variance in the samples was tested using a Bartlett's Test to determine the Homogeneity of Variance (P) in the Liquid versus Freeze-dried experiments. Homogeneity of Variance was based on Sokal and Rholf (1995). The cell counts were input into a Excel Spreadsheet that calculated the P-value, standard deviation, and means (McDonald, 2014). A P-value below 0.5 is considered a homogenous population.

2.3 MIRE Experiments

MIRE experiments were conducted using locally sourced soils from Bonner Springs soil in Lawrence, KS (Figure 1). Bonner Springs is a well-graded, sandy, coarse limestone soil. They were shipped and stored in a dry environment at 26°C in air-tight 55 gallon drums. Prior to use in MIRE experiments soils air dried for 24 hours and were autoclaved for 45 minutes at 141°C. Artificial groundwater was added to mimic the natural ionic strengths of typical, fresh groundwater. To accomplish this, artificial groundwater was created following after Ferris et al. (2004) and supplemented with 500 mM urea and 500 mM $CaCl_2$ (Appendix B). This formulation has been used in previous experiments using *S. pasteurii* (e.g. Ferris et al., 2004; Tobler et al., 2011). WebPhreeqC was used to calculate the Saturation Index for calcite in the artificial groundwater at various stages of the experiments.

2.3.1 MIRE

To test the compressive strength of standard engineering cylinders using MIRE, five experiments on the effects of biocalcification on the compressive strengths of soils were constructed (Table 1). Pure cultures of the bacterium *S. pasteurii* were grown to mid-exponential phase (based on the growth curves and cell counts established in 2.3). The bacteria were prepared in KUs Geomicrobiology lab into freeze-dried pellets and refrigerated until use in MIRE experiments. These bacteria were then hand mixed into the Bonner Springs soil with 250 mL of artificial ground water containing approximately 500 mM urea or 500 mM blood, and 500mM $CaCl_2$. Standard engineering cylinders (from ASTM International Standards) were then created using a pneumatic rammer.

Experiment	N-Source	Soil	Microbial Cells	Growth Solution	Goal
1	Urea	Ls	-	-	Experimental variability
2	Urea	Ls	Varied	250mL	Bacteria Concentration
3	Urea	Ls	1.46 cells*L^{-1}	250mL	Controlled MIRE Experiment
4	Urea	Ls	1.46 cells*L^{-1}	250mL	pH Testing
5	Blood	Ls	1.46 cells*L^{-1}	250mL	Alternate N-source

Table 1. MIRE experimental design. All experiments were done in triplicate. Growth Solution was a mixture of 500mM Ca^{2+} and 500 mM urea mixed into an artificial ground water (See 2.2.4). All bacterial samples were added as freeze-dried pellets.

To create the MIRE cylinders, the soil was spread out and allowed to dry for two days at room temperature before being autoclaved at 141°C. Following autoclaving, the soil was placed in an air tight 55-gallon drum for storage and use in MIRE experiments. In a sterile 3-gallon bucket, 8 kg of soil was added, along with one freeze-dried pellet of *S. pasteurii* (~$1.2x10^8$ cells). The dry contents were mixed thoroughly by hand. Upon completion of mixing the dry contents, 250mL of artificial groundwater containing 500mM urea and 500mM Ca^{2+} was added to the 3-gallon bucket. This was then mixed

thoroughly by placing the lid on the bucket and mixing in a rapid circular fashion until the dry soil was thoroughly saturated. Upon completion of mixing the dry and wet contents of MIRE together, ¼ of the saturated soil was placed in a 10x20 cm steel cylinder mold (ASTM approved) or PVC (Figure 2) and tamped down with a pneumatic rammer (set at 90 psi) by moving in a circular pattern around the perimeter of the mold 3 times. In order to ensure each lift forms into each other, the surface of each lift was scarified at right angles approximately at ~1/4 cm deep. This process was repeated 4 times to form 4 equal lifts. The cylinders were then removed from the molds and placed in a cylinder transport rack.

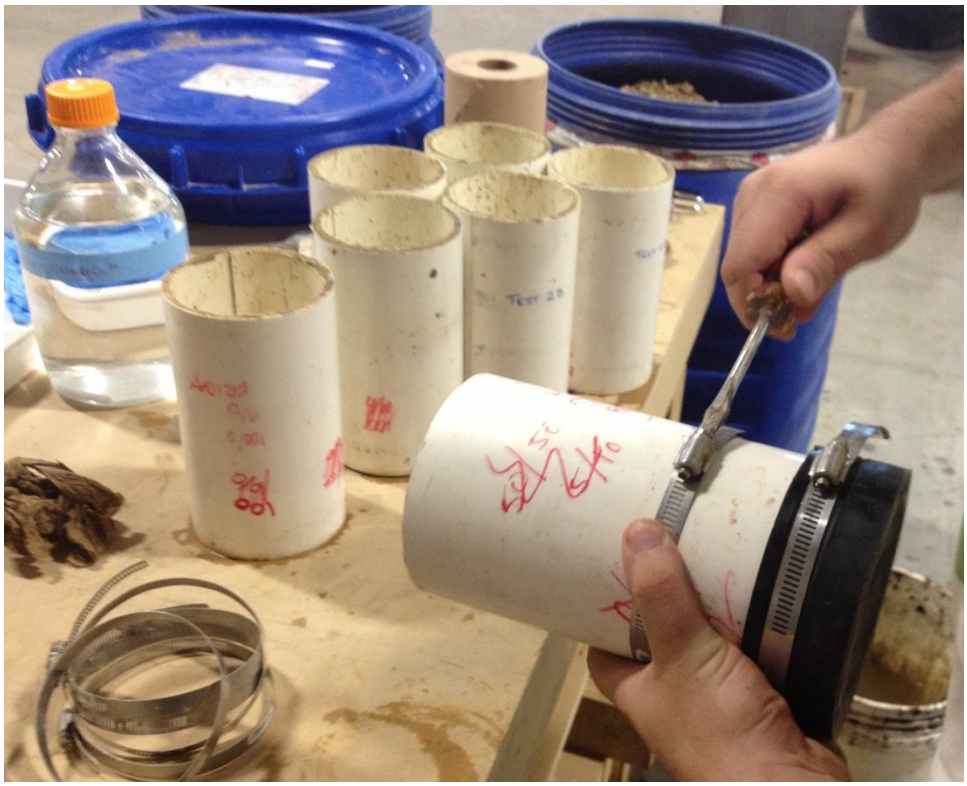

Figure 2 Preparation of MIRE cylinders.

The cylinders were set aside to cure, by storing them at room temperature and ambient humidity (~68% on average), allowing the bacteria to precipitate calcite into the pore spaces of the rammed earth at room temperature and ambient humidity. For each experiment 24 cylinders were created, 12 of which were control cylinders with no

bacteria and 12 experimental cylinders, which contained bacteria (Table 2). Cylinders were created for crush tests at 7, 14, 28, and 56 days. All experiments were done in triplicate and after 7 days the first set of test cylinders were placed in pneumatic crusher following ASTM standard protocols and the compressive strength recorded. The process of crushing the cylinders was repeated for 14 days, 28 days, and 56 days.

Control		**Experimental**	
Cylinders	Days	Cylinders	Days
3 cylinders	7	3 cylinders	7
3 cylinders	14	3 cylinders	14
3 cylinders	28	3 cylinders	28
3 cylinders	56	3 cylinders	56
3 cylinders	WRT	3 cylinders	WRT
1 cylinder	TS	1 cylinder	TS
3 cubes	WA	3 cubes	WA

Table 2 Test cylinders created for the use in MIRE experiments. Explanation of acronyms: WRT = Water Retention Curve, TS = Thin Sections, WA = Water Absorption.

2.3.2 Blood MIRE

Cylinders were prepared as above, however swine blood was used as a nitrogen source instead of urea in these experiments. Blood was acquired from Steve's Meat Market (32685 Lexington Ave, De Soto, KS 66018) and used within 4 hours of collection. The blood was diluted with 50ml of AGW to a ratio of 8:1 blood to AGW, and mixed thoroughly to represent a similar viscosity as urea + AGW. In this experiment, 500mM Ca^{2+} was added to the blood + AGW mixture and mixed into the soil as above. Two experiments were conducted. The first experiment was conducted with two groups, a blood MIRE and a control group. The blood MIRE group was conducted with $1.4x10^4$ microbial cells (the mid exponential growth point for the bacteria) and 4ml of blood. The second experiment was conducted with blood MIRE, control, and blood (with no

bacteria) groups. The duplicate experiment replicated from the previous experiment, however, an additional group was added with no bacteria added. This second experiment was conducted to test whether the bacteria played a crucial role in strengthening the cylinders, or, if any increase in strength was attributable to the effects of the blood drying in the cylinders. All experiments were performed in triplicate (Table 2).

2.3.3 MIRE Cylinder Dry Test

In order to determine if the bacteria had a sufficient amount of time to exit lag phase and begin exponential growth phase, it was necessary to determine the amount of time it took for cylinders to dry at room temperature and ambient humidity. A single test cylinder was created using the procedure above without bacteria or the addition of urea to the AGW. The cylinder was then allowed to cure at room temperature for one week. Typical lag phase for *S. pasteurii* is 24 hours and standard engineering compressive strength tests begin at 7 days. Therefore, a one-week dry test was conceived. Weight measurements were taken every 12 hours to determine the water loss over the course of the week.

2.4 Testing pH in MIRE cylinders

To test the soil pH in MIRE cylinders we took the pH of the initial soil mixture using a Mettler Toledo SevenMulti pH meter. Soil samples were prepared by adding DI water to dry soil and allowing time to fully saturate. Samples were then placed in paper towels and blotted dry so the samples were firm but saturated with water (USDA, 2014). Using a metal spatula, a half-syringe was packed to the 5 mL mark. 2.5mL of the prepared sample was put into a 30 mL falcon tube and 4 mL of 0.015 M $CaCl_2$ was added for a final concentration of $CaCl_2$ of 0.01 M. The mixture was then mixed, covered and allowed to

equilibrate for one hour. After one hour, the saturated soils were mixed again and pH was measured using a calibrated pH electrode and meter.

Chapter 3: Results

3.1 *S. pasteurii* Growth and Distribution

3.1.1 Effect of Urea Concentration on Cell Growth

Results of the of the urea experiments supplemented with 37 g L^{-1} brain heart infusion and urea concentrations at 20, 40, 60, and 80 g L^{-1} are shown in Figures 3 and 4. All experiments were allowed to grow through exponential phase to a stationary growth phase determined by the Optical Density slowly increasing and eventually stabilizing. The time it took for the bacteria to exit exponential phase was between 23 and 58 hours depending on urea concentration.

The urea experiment at 25°C (Figure 3) shows a lag time of approximately 24 hours. Upon exiting the lag phase, microbial concentrations in the 20 and 40 g L^{-1} runs increased to mid-exponential growth after 12 hours. Alternatively, microbial concentrations increased to mid-exponential growth in the 60 g L^{-1} runs after 1-2 hours. Results of the 25°C experiment show that, at the same starting microbial concentration, urea concentrations between 20 and 40 g L^{-1} entered exponential growth ~20 hours sooner than 60 g L^{-1}. The 60 g L^{-1}never reached the cell concentrations recorded in the 20 and 40 g L^{-1} (Figure 3). The bacteria reached stationary phase after 45 hours in the 20 and 40 g L^{-1} runs after a steady growth phase; however, the 60 g L^{-1} run abruptly exits exponential

phase into stationary phase after a short growth phase.

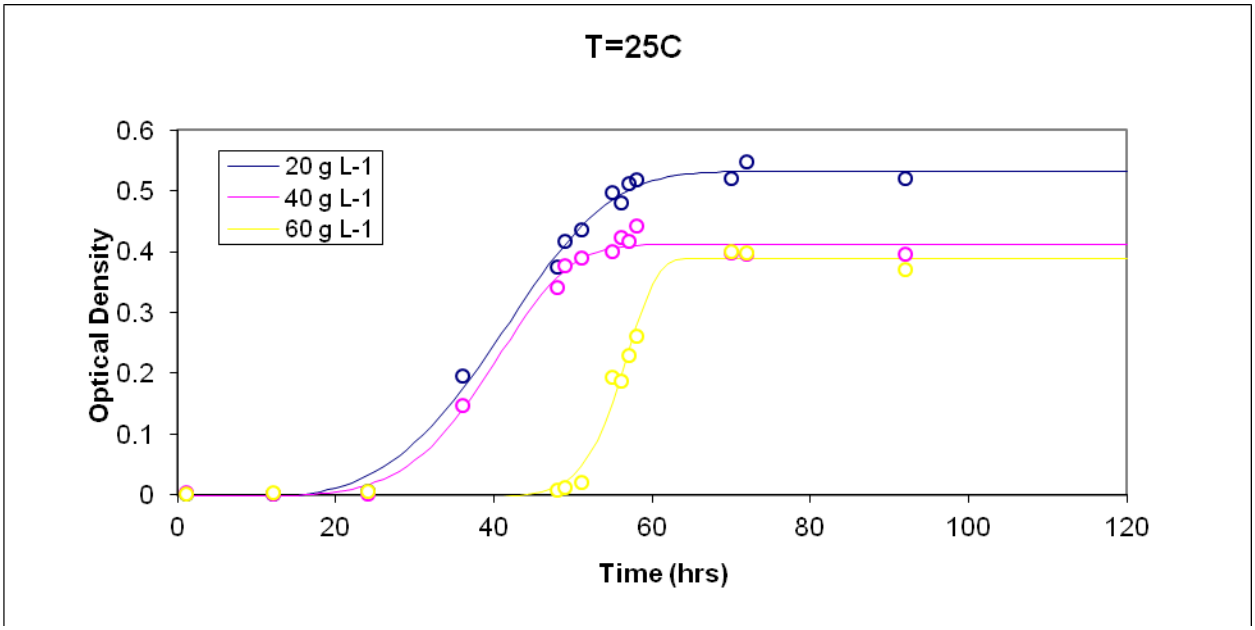

Figure 3 Comparative analysis of *S. pasteurii* at differing initial urea concentrations. Temperature was held constant at 25°C and the Optical Density was measured at 600 nm.

An additional urea experiment at 35 C (Figure 4) shows a strong growth trend. The lag phase is substantially shorter, approximately 3 hours. All of the treatments showed similar growth trends, however the 80 g L^{-1} run showed lower total biomass. The 10 g L^{-1} showed a linear growth trend with little exponential growth phase. The 20 and 40 g L^{-1} runs show similar growth patterns and reach an optical density of 0.7. All experiments were completed using fresh stocks of bacteria. Each run except for the 10 and 80 g L^{-1} showed a 10 to 20-hour exponential phase upon which they entered into stationary phase.

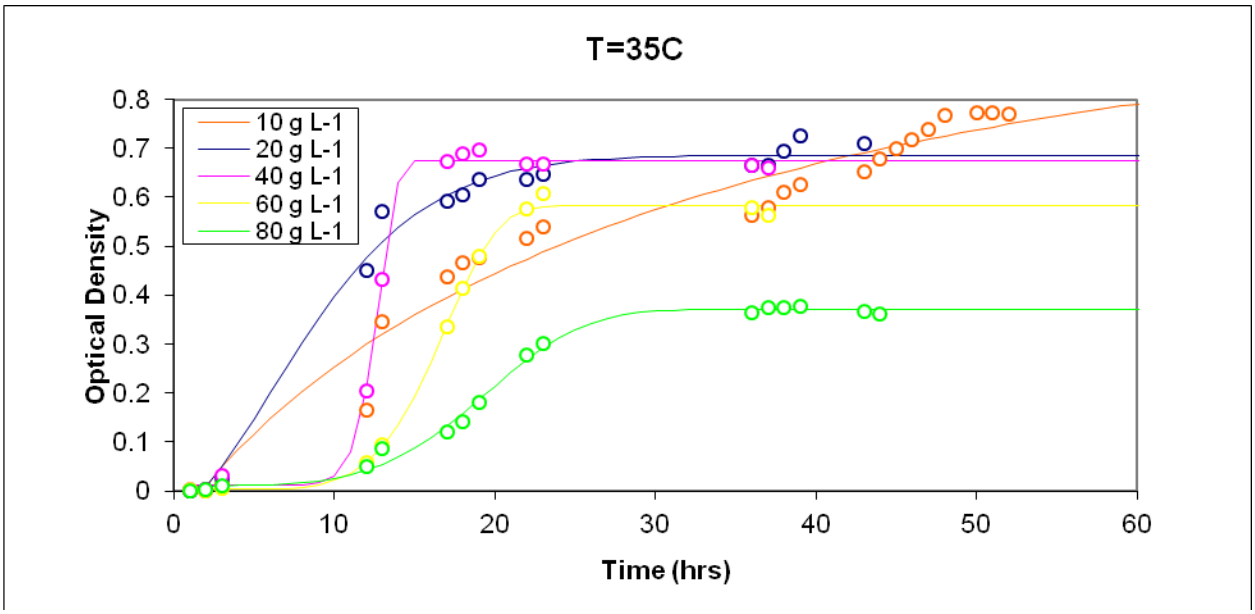

Figure 4 Comparative analysis of *S. pasteurii* at differing initial urea concentrations. Temperature was held constant at 35°C.

3.1.2 Cell Preparation and Distribution

During this experiment bacteria and urea were hand mixed into an alkali soil in two separate media tubs and allowed to grow for 7 days. Soils prepared with each treatment were sampled at two days and seven days to test how the bacteria were distributed after seven days of growth. The results, across two transects, after two days of growth show that there are differences in microbial concentrations based on microbial preparation (Figure 5), but these differences are still within one order of magnitude. The initial period of growth showed some segregation of bacteria into spatially separated locations in the soil. At the completion of this experiment, however, the bacteria showed a fairly consistent distribution throughout the soil medium. Position 2 shows a higher concentration of bacteria in both the freeze-dried and the liquid tests while positions 3 and 4 have a distinctly higher concentration of bacteria from the freeze-dried pellet.

	2-day L	2-day F	7-day L	7-day F
Means	6.965329	7.183081	9.104528	9.039044
Std Dev	0.304000	0.343000	0.032000	0.221000
P-value	0.810000	0.810000	0.000699	0.000699

Table 3 Bartlett's Test showing the Homogeniety of Variance in the 2-day Test and the 7-day Test. Homogeneity is at a P-value of 0.5.

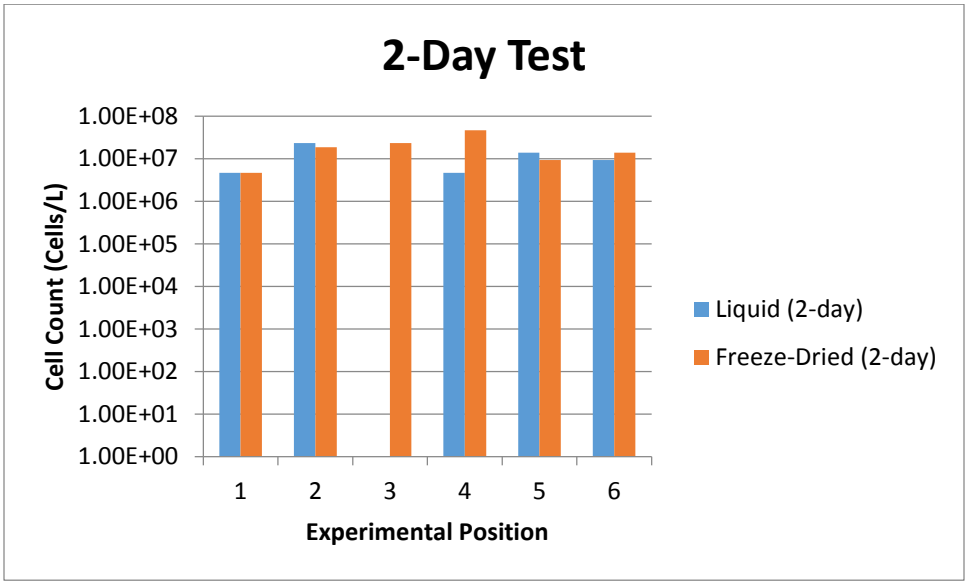

Figure 5 Site by site comparison of *S. pasteurii* at two days of growth. Samples were taken across two transects of an experimental setup to test the distribution of *S. pasteurii* when delivered as a freeze-dried pellet and in a liquid broth.

After seven days of growth the bacteria equilibrate in the soil medium and are well dispersed throughout the testing area for the liquid test. While there is more variability in the freeze-dried treatments, they vary by less than an order of magnitude ($5x10^8$ to 2.5 x 10^9 cell L^{-1}) and these concentrations bracket the concentration of the liquid test (~$1.2x10^9$ cells L^{-1}; Figure 6). After 2-days and 7-days of growth the bacteria equilibrated in the soil to approximately 10^9 cells L^{-1}. However, there is greater variance in the freeze-dried bacteria as shown by the 2-day P-value of 0.81 compared to the 7-day P-value of 0.000699, where 0.5 is the boundary between hetero- and homogeneity.

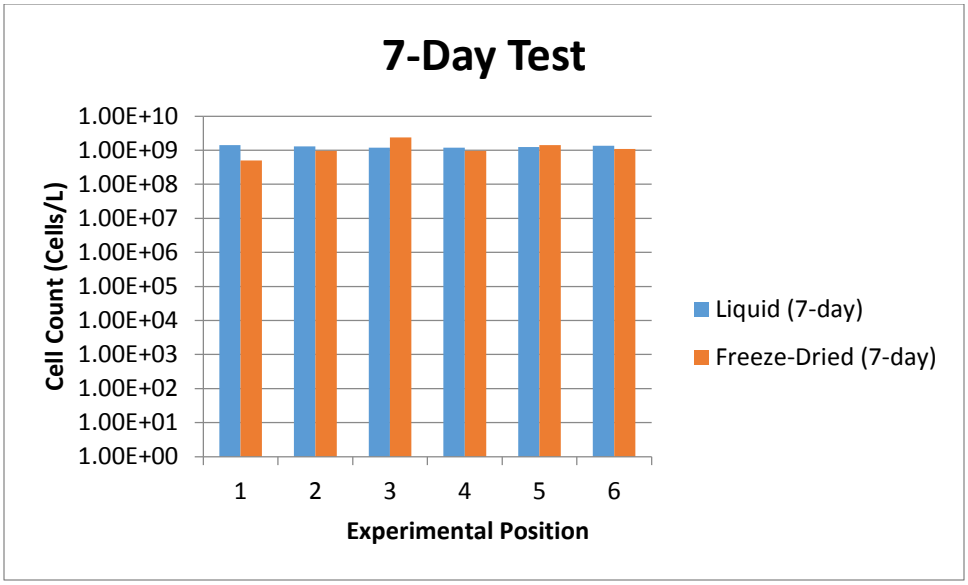

Figure 6 Site by site comparison of *S. pasteurii* at seven days of growth.

3.2 MIRE Experiments

3.2.1 Urea MIRE

MIRE experimental results show an increase in compressive strength from the initial strength tests, as expected. However, control groups had a greater compressive strength than MIRE experiments (Figure 7). The compressive strength of MIRE cylinders increased from 200 psi to 480 psi after 28-days of microbial growth. Alternatively, the control cylinders increased from 300 psi after 7-days to 700 psi after 28 days.

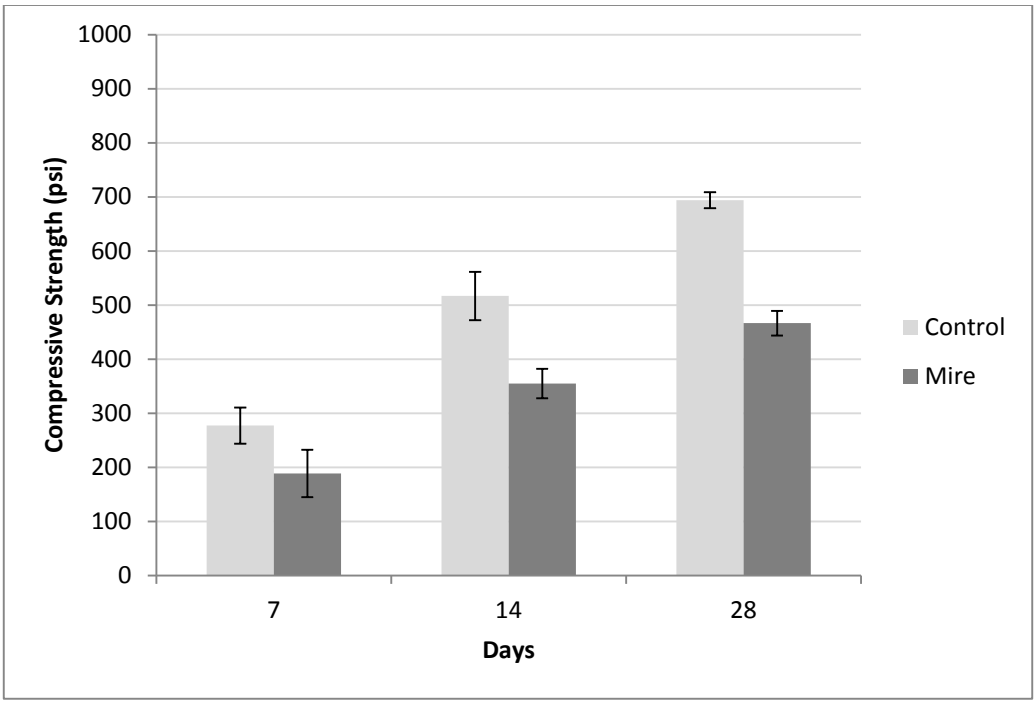

Figure 7 Representative experiments of MIRE cylinders after 7, 14, and 28 days of cylinder curing at 25°C.

3.2.2 Blood MIRE

For MIRE experimental cylinders supplemented with pig's blood as a nitrogen source compressive strength increased by an order of magnitude from 100 psi to 790 psi after 28 days of incubation (Figure 8). To test whether the blood was responsible for the increased strength, in the absence of mineral precipitation, a replicate experiment was performed (Figure 8) with an additional control (blood without bacteria) added to the experimental design. The replicate blood experiment shows an exponential increase in compressive strength in both the blood MIRE and blood controls. The strength increased from 190 psi to 980 psi and 280 psi to 900 psi, respectively.

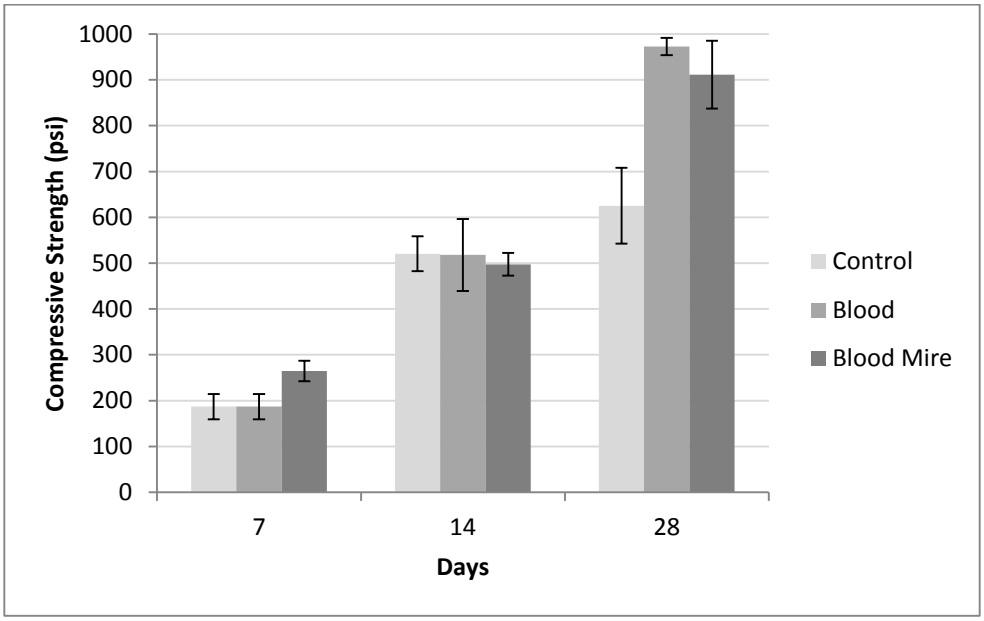

Figure 8 Duplicate of experiment 6.1 with an additional group. The Blood cylinders show that the blood alone was likely responsible for the increase in compressive strength.

3.2.3 MIRE Cylinder Dry Test

Experiments were performed to determine how long it took for MIRE cylinders to dry because bacteria need to have an appropriate length of time to grow and precipitate calcite and can only achieve this with water present. Therefore, a single cylinder was prepared (as per the guidelines above) with no bacteria added, and left out at room temperature (24◦C) for one week. Seven days was chosen because that is the length of time over which standard engineering compressive strength tests are performed. The results of the test show a loss of 65% of the total volume of artificial groundwater added in the first 12 hours (Figure 9). Subsequent loss remained constant until all water

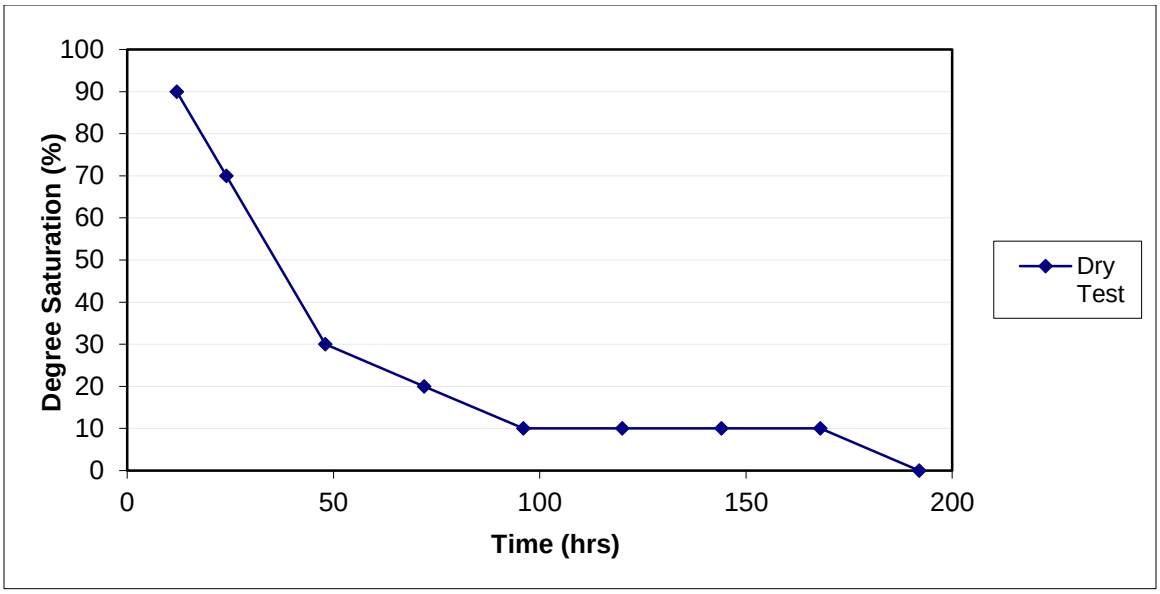

Figure 9 Experimental results showing water loss in a standard engineering cylinder of the course of one week. The cylinder plus the artificial groundwater weighed 3.81kg. The cylinder without the water weighed 3.52kg.

was completely lost after 168 hours. Degree of Saturation is inversely related to pore space. As the degree of saturation decreases to zero the amount of pore space increases.

3.3 Results of pH tests

Results show a steady increase in pH in all MIRE experiments. Experiment 3 increased from pH 8.13 to pH 9.26 and a second cylinder increased from 8.17 to 8.95. Experiment 6.1 increased from, 7.76 to 9.10 and experiment 6.2 increased from 7.93 to 8.89. Geochemical modeling shows that only 1mM of additional alkalinity is needed to achieve supersaturation with respect to calcite at a pH of 9.1. 500mM of urea is available to the microorganisms and they degrade urea to form ammonia and carbonic acid, which speciates to two moles of ammonium, two moles of hydroxyl and one mole of bicarbonate (Eq. 3.1). Therefore, modeling suggests that experiments are well within the

range of calcite precipitation, with ample urea to continue precipitation as long as microbial activity continues.

Eq. 3.1 $2NH_3 + 2H_2O$ <---> $2NH^+_4 + 2OH^-$ (3) H_2CO_3 <---> $HCO^-_3 + H^+$

pH data representative of MIRE experiments		
Experiment	pH initial	pH end
3	8.13	9.26
3	8.17	8.95
6.1	7.76	9.10
6.2	7.93	8.89

Table 4 pH results of the soil from representative MIRE cylinders. Experiment 3 is shown parameters are shown on Table 1. Experiments 6.1 and 6.2 results are shown in Figures 7 and 8, respectively.

Chapter 4: Discussion

4.1 *S. pasteurii* Growth

During the initial phase of this study we investigated the growth conditions for *S. pasteurii* as a first step in understanding best practices for MIRE. We inferred that the maximum amount of calcite would be precipitated by the bacterium at these conditions. These conditions include temperature, moisture (in MIRE experiments), pH, and initial concentrations of both urea and Ca^{2+}. As reported by Stocks-Fischer et al. (1999), calcite precipitation is directly linked to microbial growth, therefore, optimization of microbial growth in the laboratory should provide a metric for applications in MIRE. However, it must be noted that the geochemical character of the fluids and soils will potentially alter MICP due to perturbations in pH and Ca^{2+} during water-rock interaction.

4.1.1 Effect of Urea Concentrations

S. pasteurii is a urease positive bacterium that requires alkaline conditions to thrive. As urea is metabolized the pH increases in the environment surrounding the bacteria promoting further precipitation of calcite as the bacteria grow. Urea concentrations of 500mM (~20 g L^{-1}) have been shown to be an effective target for optimal microbial growth in other studies where urea was used to artificially enhance calcite precipitation rates in the Snake River Plain Aquifer as part of a bioremediation effort (Ferris et al., 2010).

Growth of *S. pasteurii* was investigated using differing concentrations of urea. A concentration of 20 g L^{-1} urea had the highest growth rate, after minimal lag phase. In both the 60- and 80 g L^{-1} there was an increased lag phase. This increased lag time is presumably the time required for the bacteria to overcome the initial pH decrease from the added nitrogen in the system (e.g. Tobler et al., 2011). A lower starting pH has negative growth influences on ureolytic bacteria and therefore any reduction in pH is going to have an inhibitory effect on growth (Gat et al., 2014). *S. pasteurii* shows similar growth trends between 20 and 40 g L^{-1} urea (Figures 3 & 4), therefore a midpoint urea concentration of 20 g L^{-1} was used to ensure minimal lag and maximum cell growth. It has been shown in previous work that 20 g L^{-1} is a good starting concentration of urea to maximize growth in *S. pasteurii* (Tobler et al., 2011). The 20- and 40g L^{-1} had the best growth curves showing a maximization of biomass in the shortest amount of time.

Previous research suggests that *S. pasteurii* grows best at a temperature of 30°C in a growth medium under laboratory conditions (Stocks-Fischer et al., 1999). The results of this study show a greater concentration of bacteria (OD 0.7) and shorter lag time at 35°C compared to 25°C. This suggests that higher temperatures are more conducive to cell growth and the subsequent bioprecipitation of calcite, but slightly lower incubation temperatures as tested in this study still result in abundant growth.

Microbial strains like *S. pasteurii* require the presence of water in order to grow and precipitate calcite; specifically, groundwater rich in compounds containing N_2 and dissolved Ca^{2+} (Fujita et al., 2008). In order to simulate groundwater, an artificial solution, modeled on dilute groundwater, was developed based on Ferris et al. (2004) and was introduced into a limestone soil (Table 3). This artificial groundwater is based on

studies of the Snake River Plain Aquifer (SRPA) where researchers are attempting to bioremediate waters using calcite precipitated by in situ microorganisms. We calculated the AGW content based on optimal saturation, however, a constant input of growth solution over the testing period may be necessary to optimize the precipitation of calcite in MIRE (DeJong et al., 2006).

4.1.2 Distribution of *S. pasteurii* in soils

S. pasteurii was grown in the laboratory and stored as liquid cultures prior to use and also as a freeze-dried pellet. Experiments were conducted to determine the most efficient means of distributing the bacteria throughout the soil medium. In both of the experiments the bacteria became evenly distributed throughout the soil (Figure 6). However, freeze-dried pellets may be preferable to liquid media because bacterial concentrations are stable over time and MIRE production can begin at any time.

After the bacteria were fully distributed throughout the soil material using hand over hand mixing for approximately 10 minutes, the results of the distribution tests show that over time the bacteria equilibrate in concentration throughout the soil. The two-day test shows that the bacteria were unevenly distributed with an outlier population at position 4. After seven days of growth the bacterial abundance began to normalize in the soil with an average cell concentration for the freeze-dried bacteria of 1.225E+09 cells L^{-1} and 1.3E+09 cells L^{-1} when the bacteria were delivered as a fresh liquid stock. This test shows that as long as the bacteria are sufficiently mixed after introduction into the soil, and adequate urea and moisture are provided, there is no benefit to using freeze-dried versus live, active strains of the bacteria. However, it must be noted that freeze-drying the bacteria allows the bacteria to be stored for long periods until required for MIRE

production. This longevity allows large amounts of bacteria to be produced and stored for larger scale production processes.

The variance in the bacterial distribution is shown in Table 3. Based on the results of these data, there is variability between the distributions of freeze-dried bacterial cells in the soil (0.000699), while the freshly grown cells are homogenous (0.81). However, as both preparations of bacteria grew in the soil, after 7 days, they reached a mean concentration of ~10^9 cells g^{-1} at each sample location. Because the error in the method is one order of magnitude (e.g. Yu et al), it is reasonable to conclude that the bacteria were evenly distributed throughout the soil within the margin of error with no practical difference in their concentrations between the two preparation methods.

4.2 MIRE Experimental Analysis

It is apparent that Rammed Earth can be strengthened to a point where its use as a building material is appropriate (NAREBA, 2009). Strengths greater than 300 psi were routinely recorded in the current study. Compressive strengths of 480 psi in the MIRE experiments is enough to build a 1-2 story building anywhere in the United States. Furthermore, Stabilized Rammed Earth (SRE) is continuing to grow in popularity in the United States (Windstorm and Schmidt, 2013). As the main component of SRE is cement, this leads to a reduction in the total sustainability of SRE construction. Therefore, with the interest of sustainability in mind, MIRE is a suitable replacement to cement SRE.

4.2.1 Urea MIRE

MIRE experiments showed a distinct increase in compressive strength for both the experimental groups and the control groups. All soils were autoclaved and any natural consortium of bacteria in the soils, were eliminated. It is possible that the amount of AGW added had a large stabilizing effect due to alignment of the sand and clay fractions (Cho et al., 2006) in the control cylinders during compaction with the pneumatic rammer; however, further investigation is needed to substantiate this claim. It is additionally possible that the experimental groups were initially lubricated by the biofilm secreted by the bacteria. Biofilms are natural exopolymeric substances that encapsulate bacteria. Current research suggests that biofilms may play an active role in the bioprecipitation of calcite (e.g. Cuthbert et al., 2012), but also a higher bacterial cell count may decrease the overall efficacy of strength characteristics in MIRE cylinders (Abo-El-Enein et al., 2012). Our experiments also suggest that the cylinders did not remain saturated through the 14-day compressive strength test, thus the bacteria would not continue to precipitate calcite. The combination of these two factors may explain the lower strength gain in the experimental groups compared to the control groups (Figure 8). Combined, these two reasons potentially explain the disparity between the control and experimental groups in the MIRE study.

4.2.2 Blood MIRE

Blood stabilization of RE is another avenue with promising results. Stabilization of RE using blood show strengths of ~800 psi, more than double the compressive strength needed to satisfy the current United States standards. Indeed, blood has been used as a stabilizer in past RE construction but the scientific basis for strengthening remains unclear (Niroumand et al., 2012). Further study is required to determine exactly what the

blood is doing in the soil matrix and if *S. pasteurii* are using it as a metabolite Blood stabilization of earthen structures dates back to the 1st century AD, and many of these structures remain standing today (Winkler, 1956). Thus, the durability of these structures is not in question; rather, the scientific justification for their longevity is a matter of interest. However, with the current results blood SRE appears to be ready to be scaled up and pilot studies conducted to determine the long-term effects of weather.

In all urea runs the control groups outperformed the experimental groups. In fact, the only group to outperform the control in either MIRE experiment was in the blood MIRE experiment where the blood group had a compressive strength of 975 psi and the blood MIRE group had a compressive strength of 900 suggesting that the bacteria did not add any compressive strength characteristics to the engineering cylinders (Figure 8), nor did they decrease compressive strength as seen in the Urea MIRE (Figure 7). Further work is necessary to deconstruct these complex experiments and shed light on these experimental inconsistencies; however, these encouraging results are a good start to future work in the search for a replacement to cement as a stabilizer in SRE and concrete.

4.2.3 MIRE Physicochemical Conditions Impacting Compressive Strength

It was necessary to determine whether enough AGW was present for the bacteria to precipitate calcite throughout the MIRE experiments, such that the cylinders were strengthened through the 14-day compressive test. Based on the results (Figure 7) it is apparent that an insufficient amount of AGW was left after the cylinders were left out at room temperature for seven days. As the cylinders are tested at 7, 14, and 28 days it is necessary for the moisture content of the MIRE cylinders to remain high enough for MICP to proceed for at least seven days. After seven days, microbial cell growth will

have reached a peak (Figures 2 & 3) and cell counts will begin the regress as the bacteria begin to die from lack of nutrients and encasement in calcite (Douglas and Beveridge, 2006). Upon completion of microbial bioprecipitation, biofilms will begin to dry and harden, further strengthening the soil matrix. However, it may be of benefit to provide a constant input of AGW for the initial week to fully disperse the bacteria throughout the MIRE cylinders to ensure full calcite precipitation in the pore spaces of the aggregate material. Another potential issue is that biofilms may act as clogging agents (Ivanov and Chu, 2008) and inhibit the dispersion of bacteria throughout the MIRE cylinder; however, further research is necessary to fully understand the effect microbial biofilms are having on the compressive strength.

pH buffering occurs in limestone aquifers and soils derived from a limestone protolith, and therefore is an essential control on carbonate mineral saturation state. It was necessary to monitor the pH throughout the experiments to ensure that the limestone soils used in the MIRE cylinders were not buffering pH changes driven by microbial metabolism. We monitored pH values and recorded a rise in pH from an initial value of 8.13 within the first 7 to 10 days. The saturation index in the Cylinders based on the bicarbonate in the system is slightly undersaturated at a pH of 7.1 but at a pH of 8.4 the Cylinders become supersaturated with respect to calcite. The equilibrium state is at a pH of 7.75 for our system. Calcite precipitation is favored at pH conditions above 8.5 (Fujita et al., 2000), therefore our pH results lend further evidence to the formation of calcite in our experiments.

Chapter 5: Conclusions and Future Work

5.1 Conclusions

Microbial Calcite Precipitation (MICP) has a high potential to increase the compressive strength of RE. The increase in compressive strength is a result of the hydrolization of urea by *S. pasteurii* in the presence of 500 mM urea and 500 mM Ca^{2+} in an artificial groundwater. These data suggest that MIRE and blood stabilized RE are in a preliminary stage and that further research is necessary prior to the "scaling-up" of this new building material. Our studies reveal the follow issues related to optimizing MICP in RE materials.

1. *S. pasteurii* grows ~50% faster at 35°C than 25°C.
2. Lower initial urea concentrations (between 20-40g L^{-1}) are better at facilitating *S. pasteurii* growth than higher concentrations.
3. There is little difference in the concentration and spatial distribution of bacteria in soil after a 7-day incubation period, when cells are introduced as a freeze-dried pellet versus living cells.
4. Freeze-drying the bacteria preserves the viability of cells long-term and serves as a means of storing the bacteria until their use in MIRE production.
5. MIRE is a substitute material to cement stabilized building materials. MIRE uses the bioprecipitated calcite as a stabilizing agent, however, results to date suggest that further research is needed to substantiate the claim that calcite increases the compressive strength to a degree that can be utilized in the built environment.

6. Blood appears to stabilize RE to a large extent without any added effects of *S. pasteurii*.

The use of MIRE will reduce waste and reduce the amount of carbon dioxide released into the atmosphere each year. This reduction is an important step to reducing the effects of climate change as the global population increases. Additionally, the reduction of construction waste is in line with regulations in the Waste Reduction Act. Finally, the reduction of carbon dioxide emissions from construction processes will greatly increase our compliance with Clean Air Act.

5.2 Future Work

The parameters tested during these experiments were investigating microbial growth parameters and their spatial distribution in soils. Additionally, we looked at two nitrogen sources for the bacterium, urea and pig's blood. Both nitrogen sources result in increased strength, however the addition of *S. pasteurii* does not increase strength under the conditions tested. Results from the blood experiments suggest that this is a promising approach to stabilizing RE and is ready to be scaled up and used in the building of a pilot structure – A Rammed Earth Pavilion. Further research is necessary to understand why the control groups performed better than the experimental groups during the MIRE experiments. In order to precipitate calcite in a soil medium at room temperature (23°C) a more thorough understanding is necessary. For instance, optimizing microbial growth at slightly lower temperatures is necessary because large scale curing of MIRE cylinders is not feasible at higher temperatures due to the large space requirements to do so. Therefore, an experimental design for MIRE should be much broader in scope. MIRE

experiments should consist of 5 tested variables: soil, water content, bacteria concentration, initial pH, and ramming method. The experimental setup would optimize each variable individually to provide a greater control over the MIRE concept.

Each variable: pH, soil type, temperature, cylinder cure time, urea concentration, mixing technique and initial water content needs to be isolated. A more structured approach to the experimental design that tests each variable separately will help to narrow down any flaws in the experimental design. Additionally, this isolation will provide a more empiric approach to solving the optimal conditions for successful MICP and the successful development of MIRE. Future work should be conducted to expound the successes and failures of phase I experiments documented in this thesis. Additional research will be set up in 2 parts. First, two broad experimental designs should be conducted. The initial approach will be to conduct a more robust experimental design to test the variables affecting microbially induced calcite precipitation in soils used for MIRE. Additionally, it is necessary to expand on the successful results of blood-stabilized rammed earth (BSRE) and work to maximize the compressive strength and optimize the amount of blood added to cylinders. Second, both the MIRE experiments and blood experiments should be combined to produce a stabilized rammed earth composed of MIRE and BSRE to achieve compressive strengths greater than MIRE or BSRE, alone. During this portion of the experiment two pilot structures should be built to elucidate the effects weathering over time on these two new building materials. The first pavilion should be constructed of BSRE alone and the second pavilion will be composed of a combination of BSRE and MIRE.

During the course of this investigation it was discovered that the soil chemistry and mechanics are extremely important. At present it has been the goal to reach the maximum initial strength in the cylinders by ramming with a pneumatic rammer at optimum moisture content determined by Proctor compaction testing. However, the current hypothesis is that this process seems to concentrate the bacteria in distinct regions of the cylinders. The subsequent calcite precipitation is limited to only those regions where capillaries of artificial groundwater (AGW) and bacteria are present, and not throughout the entire cylinder. Pneumatic ramming of the cylinders appears to significantly reduce the overall bioprecipitation of calcite and thus artificially limits the strength gained over time. To solve this problem, future experiments to test the mixing technique by using three techniques: hand over hand mixing, lower psi pneumatic rammer, and a cement mixer.

Microbial concentration should be optimized to conclude if microbial biofilms are keeping the compressive strengths artificially low prior to calcite precipitation. To deduce the effects of biofilms on the compressive strengths of cylinders, experiments should be designed with differing concentrations of urea, and second, experiments should be designed to alter the concentrations of bacteria based on preliminary growth curves. Additionally, it would be necessary to test the effects of initial pH on calcite solubility. We discovered slight variations of final pH depending on the soil used, which affects the speciation of carbonate and ultimately whether calcite will be precipitated.

A second round of experiment would be conducted that would build off of, and scale up the results for Phase I. Phase II experiments would test the effects of various particle size distributions and soil types on the integrity of rammed earth. The assumption of Phase I

was that particle size of soils should have similar characteristics as aggregate sizes used for concrete applications and cement stabilized rammed earth. However, because bacteria are stabilizing MIRE soils the chemistry and mechanics needed to achieve high strengths will likely differ. Therefore, it is of extreme importance that MIRE experiments are provided with conditions that will maximize the bioprecipitation of calcite. Phase II will present the opportunity to test these variables and optimize the conditions necessary for MIRE.

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Appendix

Appendix A: Representative DAPI Stains

-S. pasteurii

Figure 10 DAPI-stained sample (S006) showing bacilli in the Urea Experiment #1 1 μm in length at 60X magnification

Appendix B: Artificial Groundwater

Artificial Groundwater	
Compound	Concentration
indKNO_3	0.0403mM
$MgSO_4$	0.448mM
$CaCl_2$	1.75mM
$NaNO_3$	0.044mM
$NaHCO_3$	1.1mM
$KHCO_3$	0.0623mM

Table 5 Constituents of Artificial Groundwater before the addition of 500mM $CaCl_2$ and 500mM urea.

Appendix C: Initial Blood MIRE Experiment

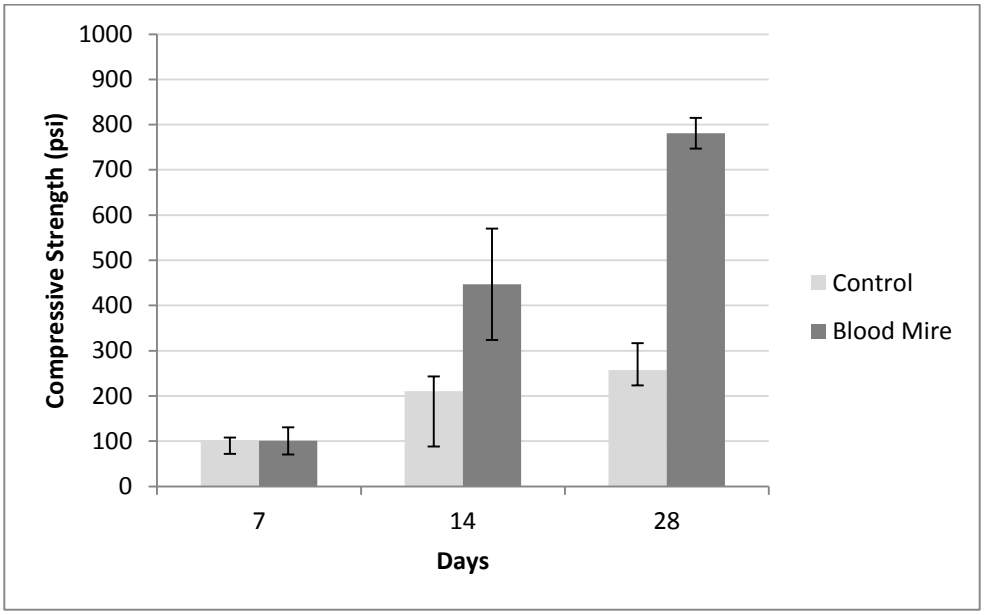

Figure 11 Blood MIRE experimental results showing an increase in Blood MIRE over the Control.

Appendix D: Experimental Parameters using during Urea MIRE Analysis

Experimental Parameters used during Urea MIRE Analysis	
Experiment Number	Either experiment 1 (25˚C) or experiment 2 (35˚C)
Temperature (˚C)	The temperature the bacterial cultures were incubated
Sample Number	The bacterial culture used to inoculate the experiment
Urea Concentration	Urea concentration used in the each experimental run
Optical Density	The OD at each specific time of sampling
Time (hours)	The an Optical Density reading was taken

Table 6 Experimental parameters that were tracked during the progression of the Bacterial Growth experiments

urea	Asym	Drop	lrc	pwr
20	0.53197115	0.53861416	-16.043478	4.22717699
40	0.41160515	0.41392265	-20.574623	5.49967579
60	0.38881851	0.39354639	-68.842561	17.0101602

Table 7 Weibull parameters used in the 25˚C experimental Analysis

urea	Asym	Drop	lrc	pwr
10	0.98811034	1.08678235	-2.8252111	0.82046875
20	0.68536778	0.72284152	-3.8410096	1.632068
40	0.67442857	0.66242857	-34.447061	13.4335426
60	0.58323131	0.58003238	-17.668051	6.18417632
80	0.37039008	0.35965376	-13.025513	4.28790961

Table 8 Weibull parameters used in the 35˚C experimental analysis