BIO-TRAPPING UREOLYTIC BACTERIA ON SAND TO IMPROVE THE EFFICIENCY OF BIOCEMENTATION

by

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DEDICATION

This thesis is dedicated to Sky, my son, my greatest source of strength and motivation.
Thank you for always making me smile without even trying.

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ABSTRACT

Microbially induced calcium carbonate precipitation (MICP) has emerged as a novel biocementation technique for its potential solution to sustainable construction. Although current MICP approaches have made significant progress, achieving spatial control over biomineralization is challenging due to its complexity, which is affected by many factors, such as microorganisms, reaction kinetics, and environmental factors. Spatially controlling biomineralization for building or targeted repair of materials can significantly improve efficiency and sustainability while achieving desired outcomes. The purpose of this thesis was to assess whether biomineralization can be enhanced through surface pre-treatment of sand using amino silanes, such as 3-aminopropyl-methyl-diethoxysilane (APMDES), which is one form of spatial control of biomineralization through prescribing the location of the microbes. Moreover, a preliminary study was conducted to assess whether biomineralized sand, with and without the APMDES treatment, can be recycled and reused for biomineralization of subsequent generations. The impact of APMDES treatment on bacterial adhesion on sand, growth, and urease activity was analyzed. Biocementation efficiency was evaluated by comparing compressive strength and calcium gain of APMDES-treated sand with untreated sand.

APMDES treatment promotes abundant and immediate trapping of bacteria on sand surfaces through increased electrostatic interaction that attracts negatively charged walls of bacteria to positively charged amine groups. While APMDES treatment compromises microbial viability, it preserves the urease enzyme for catalyzing urea hydrolysis. APMDES-treated sand achieved comparable strength with fewer bacterial injections compared to untreated sand. APMDES-treated sand biocemented using three injections of bacteria and cementation media gained the same strength as seven injections. Biomineral gain of APMDES-treated sand was similar compared to untreated sand, which shows calcium accrual in the structure may be influenced by additional factors, such as the distribution of calcite, differences in the calcite precipitation patterns, and morphology. Overall, incorporating APMDES treatment can potentially improve the efficiency and sustainability of MICP by spatially controlling biomineralization.

CHAPTER ONE

INTRODUCTION

Background

The research presented in this thesis primarily focuses on improving the spatial control of biomineralization through functionalizing sand surfaces using silane coupling agents, i.e., 3-aminopropylmethyldiethoxysilane (APMDES). After treatment, positively charged amine groups on the sand surface 'bio-traps' negatively charged cells through electrostatic interaction. The hypothesis investigated by this work is that bio-trapping ureolytic cells on sand particles can improve spatial control over biomineralization.

Microbially induced calcite precipitation (MICP) is a process where the metabolisms of microbes are leveraged to induce calcium carbonate precipitation to bind together aggregate as a biocement (Castro-Alonso et al., 2019). MICP is not limited to one bacterial metabolism but frequently relies on urease-producing microorganisms, typically bacteria or fungi, for catalyzing urea hydrolysis and subsequent precipitation of calcium carbonate ($CaCO_3$) (Anbu et al., 2016). MICP-based biocementation technique has gained significant attention due to its potential to provide a sustainable solution for various engineering applications, such as soil improvement and ground stabilization (Phillips, Gerlach, et al., 2013; Whiffin et al., 2007), sealing cracks (Phillips et al., 2016), environmental remediation (Phillips, Lauchnor, et al., 2013), and manufacturing building materials (Bernardi et al., 2014; Cheng et al., 2020; Heveran et al., 2020; Lambert & Randall, 2019; Qiu et al., 2021). MICP is a low-temperature process with the potential for improved sustainability in generating building materials compared with traditional materials like

concrete. However, several facets of MICP currently limit its potential for sustainability and could be improved.

One major challenge is the trade-off between strength gain and the sustainability of biocementation. Achieving desirable strength gain requires a substantial number of microbial solutions and chemical reagents as inputs, which compromises the sustainability of the biocementation process and makes it less eco-friendly for engineering applications. Since biomineralization relies on the urease enzyme activity of bacteria, spatially controlling microbes would potentially allow for control over the distribution of calcium carbonate and provide a new approach for improving the efficiency of strength development. The research presented in this thesis describes investigations that bio-trapping ureolytic bacteria, i.e., *S. pasteurii*, on APMDES-treated sand surfaces may improve the efficiency of the biocementation process while enhancing performance outcomes for engineering applications.

Thesis Overview

The overall objective of this thesis was to improve the efficiency of the biocementation process by altering the surface properties of sand via silane coupling agents, i.e., APMDES treatment, to control bacteria spatially and hence biomineralization. Chapter 1 is a literature review. Chapter 2 discusses changes in surface properties of sand, how the microbial-induced calcium carbonate precipitation (MICP) process is affected by APMDES treatment, the effects of the treatment on the microbes (i.e., bacterial growth, viability, urease activity, microbial localization on surfaces) and on the biocementation process (i.e., compressive strength and calcium gain) were assessed. The results provide evidence that APMDES treatment improves the efficiency of strength gain through MICP-based biocementation. Chapter 3 summarizes the key

results of Chapter 2 while providing recommendations for future work. The appendices contain supplemental work with preliminary considerations for recycling and reusing MICP-based building materials.

Literature Review

Motivation for Using Biocementation for Manufacturing Construction Materials

The demand for construction materials is rising due to increasing population growth and urbanization. As a result of this high demand, concrete is one of the most used materials after water (Gagg, 2014). Production of cement, a primary ingredient of concrete, contributes to 5-8% of the total anthropogenic greenhouse gas emissions globally (Teh et al., 2017). Additionally, construction materials cause resource depletion, such as sand, gravel, and minerals (Teh et al., 2017). If not appropriately addressed, this increase in emissions and resource scarcity can significantly impact our climate and ecosystems. MICP has the potential to make a sustainability impact on construction materials because of its low energy consumption, lower carbon footprint, reversibility, and recyclability (Porter et al., 2021). Biocementation occurs at lower temperatures than conventional cement production, reducing energy consumption and greenhouse gas emissions.

Microbially Induced Calcium Carbonate Precipitation (MICP) has gained significant attention as an innovative and efficient biocementing technique that precipitates calcium carbonate to bind particles together and improves the engineering properties of soil (Achal & Mukherjee, 2015; Mujah et al., 2017; Phillips, Gerlach, et al., 2013; Whiffin, 2004). Various MICP techniques have been developed to improve soil properties (Gomez et al., 2015; Whiffin et

al., 2007), seal cracks (Phillips et al., 2016), and generate building materials (Bernardi et al., 2014; Heveran et al., 2020; Lambert & Randall, 2019).

In MICP, urease enzyme produced by ureolytic microorganisms, such as *S. pasteurii*, catalyzes the hydrolysis of urea ($CO(NH_2)_2$), resulting in the formation of two ammonium ions (NH_4^+) and one carbonate ion (CO_3^{2-}) (Equation 1). Ammonia production upon enzymatic urease activity increases pH levels and alkalinity around the cells and creates a favorable environment for calcium carbonate precipitation in the presence of calcium ions (Ca^{2+}) (Equation 2) (DeJong et al., 2006; Stocks-Fischer et al., 1999)

$$CO(NH_2)_2 + H_2O \xrightarrow{\text{Urease}} 2NH_4^- + CO_3^{2-} \qquad (1)$$

$$Ca^{2+} + CO_3^{2-} \longrightarrow CaCO_3 \qquad (2)$$

The formation of calcium carbonate bridges in MICP allows the biocementation of aggregate particles through an effective bridging at the contact points (Tuller et al., 1999). Aggregates can include sand, crushed rocks, or other components. At this time, there are commercially available MICP-biocemented paving tiles made from biocemented crushed granite, produced by the company BioMason (Dosier, 2016). There is interest in producing larger and stronger structures using MICP, potentially relevant for building new construction and infrastructure materials or repairing these materials. However, since one of the primary motivators to use MICP is sustainability, improving the sustainability of MICP while generating strong materials is important.

Limitations in the Sustainability of MICP

There are several reasons why the sustainability potential of MICP-based biocementation is currently limited. Key among these reasons is that generating strength from biocementation

requires substantial inputs (i.e., microbes, calcium, urea) and produces waste products, including ammonia (Porter et al., 2021). The strength of biocemented structures increases with the amount of calcium carbonate accrued within the structure, which usually requires multiple treatments, requiring additional inputs, and generating additional waste outputs (Mujah et al., 2017; Soon et al., 2014; Whiffin et al., 2007; Xiao et al., 2022). The need for multiple treatments also slows down the process of generating structures.

Previous Work to Increase Strength Gain During Biocementation

Previous researchers have tried to improve the efficiency of strength gain in MICP in several ways, most notably by increasing the number of treatments of microbes and biocementation media. Oftentimes, these treatments are delivered through an injection into a bioreactor. Other methods include choice of soil type, bacteria concentration, chemical concentration, injection method, and addition of other materials.

Soil Type. Researchers assessed the applicability of the MICP technique across various soil types based on their mineralogical composition, particle sizes, gradations, and shapes. DeJong et al. (2006) examined the relationship between particle size, pore throats, and the mobility of bacteria in the MICP process and reported particle size as one of the main factors affecting calcite content and, hence, strength. Their work suggested that for effective MICP treatment, pore throats in the soil should be at least 0.4 μm in size to allow the movement of bacteria. This indicates that a wide range of soils could potentially be treated and strengthened using MICP. However, at the same calcite content, a variation in strength values was observed in coarse-grained soils compared to medium-sized and fine grains due to the heterogeneous distribution of the $CaCO_3$ (Mahawish et al., 2016). Fine and medium-sized grained soils exhibit a

more consistent response to MICP treatment due to their finer particle arrangement, higher density of particle contacts, and potentially more uniform distribution of $CaCO_3$ (Cheng et al., 2013; Xiao et al., 2022).

Bacterial Concentration. Bacteria play an essential role in the MICP process because they affect specific urease activity, calcium carbonate content ($CaCO_3$), and strength performance in biocemented soil. Moreover, bacterial cells have been reported to act as nucleation sites for calcite precipitation (Stocks-Fischer et al., 1999). Increased bacterial concentration leads to more urease production and increases available nucleation sites for calcium carbonate precipitation (Anbu et al., 2016; Okwadha & Li, 2010). Nemati and Voordouw (2003) reported that an increase in urease concentrations resulted in a corresponding rise in urea hydrolysis rate and subsequent $CaCO_3$ production. The results were consistent with the findings of Soon et al. 2014. However, Whiffin (2004) reported no direct correlation between urease activity and bacterial concentration because urease production was not constant and influenced by many factors, resulting in variation in the specific urease activity when bacterial concentration changes. Specific urease activity directly affects the reaction kinetics and the overall time required for mineralization. Because specific urease activity controls the rate of urea hydrolysis, it has been reported to affect the amount of $CaCO_3$ precipitated and the $CaCO_3$ precipitation pattern, and hence strength (Whiffin, 2004).

Chemical Concentration. The concentration of chemical components (e.g., urea, calcium) in the treatment solution has been reported to influence strength development during biocementation. Al Qabany (2013) reported that lower concentrations of urea and $CaCl_2$ resulted in stronger specimens compared to higher concentrations, attributed to the better distribution of

$CaCO_3$ at the interparticle contacts, suggesting that a controlled and uniform distribution of the $CaCO_3$ precipitation enhances biocementation. Cheng et al. (2014) used calcium ions dissolved in seawater as the sole source of $CaCO_3$ precipitation. They found that specimens treated with a low concentration of calcium ions in seawater exhibited higher strength than those treated with a high-concentration cementation solution (Cheng et al., 2014). While high concentrations of urea and calcium solutions can precipitate higher amounts of $CaCO_3$, it reduces the efficiency (Nemati et al., 2005).

Another aspect that affects the strength of biocemented materials is the mineralogical characteristics of the biomineral produced in this process. Mujah et al. (2019) obtained higher strength results when a combination of low-concentration cementation solution and high urease activity were used in biocementation. This combination favored the precipitation of large-sized rhombohedral-shaped crystals at the soil pore throat, enhancing the binding of soil particles and overall strength. Achieving optimal concentrations of calcium ions is crucial for promoting effective biocementation while avoiding excessive precipitation that might hinder uniform distribution.

Injection Method. The methodology used for introducing bacteria and cementation solution is another crucial factor affecting retained bacteria, hence successful biocementation. The retained bacteria can induce $CaCO_3$ precipitation through the supply of a cementation solution. However, improper retention could lead to uneven distribution of the bacteria and non-uniform $CaCO_3$ precipitation, resulting in weak bonding throughout the biocemented soil. Two main methods are commonly used to ensure effective retention: injection and premixing. Injection involves flushing down bacterial solutions and allowing for time for attachment to sand

grains before introducing cementation solution. In contrast, premixing requires the mechanical mixing of bacteria with soil prior to application. Harkes et al. (2010) used a two-phase injection procedure. In this method, the researchers injected a fixation solution with high salt content into sand grains immediately after the initial injection of bacteria. This was done before introducing the cementation media. Their work suggested that the higher ionic strength of the fixation solution is likely to promote bacterial attachment on sand surfaces, enabling the uniform distribution of $CaCO_3$. Zhao et al. (2014a) reported an alternative premixing and soaking method. In their study, bacteria were premixed with sand before being immersed in cementation media within a stirred tank reactor. This method aimed to enhance the homogeneity of $CaCO_3$ precipitation. The mixture of bacteria and sand was packed into geotextile full-contact flexible molds, which allowed the cementation solution to diffuse freely and promote uniform distribution of the precipitated $CaCO_3$. It is essential to control the adsorption and distribution of bacteria to sand to prevent clogging and achieve homogenous $CaCO_3$ precipitation and, hence, strength (Al Qabany et al., 2012; Harkes et al., 2010).

Addition of Other Materials. Another possibility for improving the efficiency of strength gain is adding other components to the biocementation reaction, which may include fibers, polymers, or hydrogels. The addition of fibers has been reported to improve unconfined compressive strength slightly while significantly improving ductility because fiber bridging combined with $CaCO_3$ bridging can enhance tensile strength (Choi et al., 2016; Xiao et al., 2019). Wang and Tao (2018) reported that incorporating polymers like PVA powder into the MICP process can enhance the strength outcomes of biocemented sand. In their work, the polymer-modified MICP approach resulted in higher calcite content and compressive strength

than the traditional method (Wang et al., 2004). The PVA powder's high water retention capacity helps maintain the moist conditions required for MICP. In another research done by Heveran et al. (2020), hydrogels were incorporated into the MICP process, which improved moisture retention in the soil matrix and maintained microbial viability for 30 days.

New Approaches for Improving the Efficiency of Strength Gain in Biocementation

Biomineralized structures in nature, such as bone, tooth, and nacre, are nanocomposites of proteins and minerals with superior mechanical performance (Gao et al., 2003). In nature, biomineralization is spatially controlled through biological processes that control the formation and arrangement of minerals in specific locations (Tang et al., 2021). This work hypothesizes that spatially controlling where microbes perform biomineralization could potentially enhance the efficiency of strength gain in biocementation.

A novel approach would be to capitalize on electrostatic interactions to locate the microorganisms on the surface. This might be achieved by silane functionalization of the aggregate, which covalently bonds amino silane on silicon surfaces via hydroxyl groups on the surface (Carre, 2007). Positively charged amine groups bio-trap bacteria on the surface through increased attraction of negatively charged cell walls of bacteria (Poortinga et al., 2002).

Silane functionalization has been used with success for several prior applications, such as capturing bacteria and water treatment. BiyoTrap ® (Avci, 2016) technology, which functionalizes silicon surfaces with 3-Aminopropylmethyl(diethoxy)silane (APMDES) to trap trace bacteria in liquids and Suo et al. (2008) efficiently immobilized and patterned bacterial cells onto a silane functionalized silicon substrate. In another study conducted by Deliorman et

al. (2014), three-dimensional quartz fiber networks were activated using amino silanes to capture and concentrate bacteria onto surfaces.

Amino silane functionalization using APMDES has not been used on sand or with biocementation. This thesis explores key considerations about applying this process in the context of MICP using ureolytic bacteria, including how APMDES treatment affects microbial activity, biocementation process, and strength outcomes.

CHAPTER TWO

BIO-TRAPPING UREOLYTIC BACTERIA ON SAND TO IMPROVE THE EFFICIENCY OF BIOCEMENTATION

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Abstract

Microbially induced calcium carbonate precipitation (MICP) has emerged as a novel technology with the potential to produce building materials through lower-temperature processes. The formation of calcium carbonate bridges in MICP allows the biocementation of aggregate particles to produce bio-bricks. Current approaches require several pulses of microbes and mineralization media to increase the quantity of calcium carbonate minerals and improve the strength of the material, thus leading to a reduction in sustainability. One potential technique to improve the efficiency of strength development involves localizing the bacteria on the aggregate surfaces using silane coupling agents, such as 3-aminopropyl-methyl-diethoxysilane (APMDES). This treatment localizes bacteria on sand through electrostatic interactions that attract negatively charged walls of bacteria to positively charged amine groups. The APMDES treatment promotes abundant and immediate bacteria association with sand. Though microbial viability is compromised by treatment, urea hydrolysis was not affected. Strength was gained much more rapidly for APMDES-treated sand. Three injections of bacteria and biomineralization media using APMDES-treated sand gained the same strength as seven injections using untreated sand. The higher strength with APMDES treatment was not entirely explained by calcium accrual in the structure and may be influenced by additional factors, such as differences in the microstructure of calcium carbonate bridges between sand particles. Overall, incorporating pre-treatment methods, such as amine silane coupling agents, opens a new avenue in biomineralization research by producing materials with improved efficiency and sustainability.

Introduction

The demand for infrastructure materials is continuously increasing. The impact of this high demand for concrete is that cement, the binding component of concrete, is the second most consumed resource in the world after water (Izumi et al., 2021). The production of portland cement is responsible for generating 5-8% of global anthropogenic greenhouse gas emissions (Teh et al., 2017). Alternative methods of manufacturing construction materials with greater sustainability are needed. Microbially induced calcium carbonate precipitation (MICP) has been the subject of considerable research interest for its potential to strengthen soils, seal cracks, and generate building materials through lower temperature processes (Achal & Mukherjee, 2015; De Muynck et al., 2010; Mujah et al., 2017; Phillips et al., 2016; Phillips, Gerlach, et al., 2013; Zhang et al., 2020). The enzyme urease produced by ureolytic bacteria, such as *Sporosarcina pasteurii* (*S. pasteurii),* catalyzes urea hydrolysis to produce ammonium (NH_4^+) and carbonate ions (CO_3^{2-}). In the presence of (Ca^{+2}), the reaction becomes favorable for $CaCO_3$ precipitation (Equations 1-2).

$$CO(NH_2)_2 + H_2O \xrightarrow{\text{Urease}} 2NH_4^- + CO_3^{2-} \qquad (1)$$

$$Ca^{2+} + CO_3^{2-} \longrightarrow CaCO_3 \qquad (2)$$

Strength development through MICP relies on biocementing calcium carbonate bridges between aggregate particles (Cheng et al., 2013b; Mujah et al., 2017; Phillips, Gerlach, et al., 2013; Whiffin, 2004). Strength gains are achieved through increasing the quantity of calcium carbonate in the material, which is the result of multiple pulses of microbes and biocementation media or higher concentrations of reactants (Cheng et al., 2013b; Whiffin, 2004; Whiffin et al., 2007; Xiao et al., 2022). Therefore, strength gain is usually achieved at the expense of

sustainability (Achal et al., 2015; Achal & Mukherjee, 2015; Rajasekar et al., 2017). Since the MICP reaction occurs locally to bacteria, controlling the location of bacteria could provide a new approach for improving the efficiency of strength development.

One potential method to improve spatial control over MICP is to localize the bacteria on the aggregate surfaces using silane coupling agents. An example of this technology is BiyoTrap ® (Avci, 2016), which functionalizes surfaces with 3-aminopropyl-methyl-diethoxysilane (APMDES). The chemical modification covalently attaches positively charged amine groups on surfaces on which hydroxyl groups are available, thus, electrostatically attracting negatively charged walls of bacteria (Avci, 2016; Metwally & Stachewicz, 2019; Oh et al., 2018; Rzhepishevska et al., 2013). APMDES moieties comply to hydroxyl groups on the sand particle surfaces which can be added to various surfaces, potentially including sand, through ozone treatment. After APMDES treatment, bacteria are localized to the charged area but are not strictly immobilized (Avci, 2016). APMDES and similar treatments have been utilized to rapidly detect trace bacteria in liquids such as drinking water sources and contaminated liquids, as well as isolating sulfate-reducing bacteria from its media (Avci, 2016; Aydın, 2007; Joshua Daniel Martin, 2014). Various surfaces have been successfully functionalized to bio-trap bacteria, including silicon, glass, and glass wool fibers (Avci, 2016; Aydın, 2007; Suo et al., 2008, 2009). APMDES treatment has not yet been explored as a method to functionalize aggregates for the purpose of spatially controlling bacteria for biocementation. The purpose of this study was to test the hypothesis that trapping bacteria on sand surfaces treated with APMDES would increase the efficiency of strength development during biocementation.

Materials and Methods

Materials

Sand. Quikrete™ Commercial Medium Sand (#196251) was used in all the experiments. Sand was soaked in a 4% HCl solution overnight. Afterward, the pH was balanced to 7 by adding sodium bicarbonate, rinsed with tap water, and left to air-dry at room temperature.

Growth and Biocementation Media. The growth medium used for the starter culture contained 37 g L^{-1} Brain Heart Infusion (BHI) (Becton Dickinson, Franklin Lakes, NJ) and 20 g L^{-1} urea (Fisher Scientific, Inc., Pittsburgh, PA) in deionized water. The biocementation medium contained 3 g L^{-1} Difco nutrient broth (BD, Franklin Lakes, NJ), 10 g L^{-1} ammonium chloride (Fisher Scientific, Pittsburgh, PA), 20 g L^{-1} urea (Fisher Scientific, Pittsburgh, PA), and 48 g L^{-1} calcium chloride dihydrate (Fisher Scientific, Pittsburgh, PA) in deionized water. The CMM-medium used for the urea batch study contained 3 g L^{-1} Difco nutrient broth (BD, Franklin Lakes, NJ), 10 g L^{-1} ammonium chloride (Fisher Scientific, Pittsburgh, PA), and 20 g L^{-1} urea (Fisher Scientific, Pittsburgh, PA) in deionized water and was adjusted to pH 6.3. All solutions were filter-sterilized using SteriTop 0.2 μm bottle top filters.

Molds for Generating Biocemented Structures. Interlocking molds for biocementation were designed with OnShape software and printed with a Prusa 3D Printer using Polylactic Acid (PLA) filament. Molds were designed with vertical slits of 0.4 mm width to increase permeability for bacterial suspension and cementation media (Supplementary Figure 1). The inside dimensions of s molds measure 50 x 50 x 50 mm^3. Prism shape molds with dimensions of 90 x 25 x25 mm^3 were also designed with vertical slits of 0.4 mm.

APMDES Treatment of Sand

3-Aminopropyl-methyl-diethoxysilane (APMDES) (Gelest Inc., Morrisville, PA) was used to functionalize the sand surface. Sand was first sonicated in acetone for 15 min and then in ethanol for another 15 min. The sand was dried at 100 °C on a hot plate and then placed in a UV Ozone cleaner (BioForce NanoSciences, Ames, Iowa) chamber for 15 min, and subsequently immersed in a 1 % v/v of APMDES in ethanol for 24 h. Sand was removed from the APMDES solution, rinsed in ethanol for 25 min to remove residual APMDES, and baked in an oven at 120 °C to remove polymerized amine moieties.

X-ray photoelectron spectroscopy (XPS) (Physical Electronics 5600) was used to verify the surface chemistry of APMDES-treated sand. XPS was conducted under ultra-high vacuum conditions ~ 8×10^{-10} Torr using a monochromatized Al Kα X-ray source (1486.6 eV photons) by monitoring the N 1s line and the chemical shifts associated with $-NH_2$ and $-NH_3^+$ species. Zeta potential measurement (Zeta-Meter System 4.0, Staunton, VA 24402) was further used to confirm the surface charge of the sand after functionalization. Zeta potential was measured in neutral pH, and deionized water, and data was collected for 50 sand grains per condition (untreated, APMDES-treated). XPS analyses were conducted to evaluate the quality and consistency of APMDES treatment of the initial batches as a proof of concept. The zeta potential was used again to confirm the functionalization of subsequent batches.

Determining the Effect of APMDES Treatment on Microbial Growth, Viability, and Urea Hydrolysis

A 1 ml cryovial of thawed frozen *S. pasteurii* base stock (ATCC 11859) was added to 100 ml of BHI with 2% urea in an autoclaved 250 ml Erlenmeyer flask and incubated for 24 hours on the orbital shaker at 150 rpm at 30 °C. 1 ml of starter culture was transferred into 100

ml of fresh BHI with 2% urea medium and incubated overnight, 16 hours, on the orbital shaker table at 150 rpm. After the growth, approximately 40 ml of overnight culture was added to two 50 ml centrifuge tubes centrifuged at 6000 rpm for 10 minutes at 4°C. The supernatant of both tubes was decanted into the waste container and resuspended in CMM-. The optical density was adjusted to 0.4 by diluting in sterile growth media. 200µl aliquots were transferred to a 96-well plate, and OD_{600} was measured using a Synergy HT reader (Biotek Instruments, Inc., Winooski, VT). The average OD_{600} of sterile growth media was subtracted from the OD_{600} of bacterial culture to measure the OD_{600} of the culture without influence from the media or the 96 well plates.

A urea batch study was conducted in biotic and abiotic conditions to evaluate the effects of APMDES treatment on urea hydrolysis, microbial growth, and pH. 10 grams of sand, APMDES-treated (n=3) and untreated (n=3), mixed with 100 ml CMM- and inoculated with 2 ml of bacterial suspension with OD of 0.4, and they were incubated at 30 °C on the orbital shaker at 150 rpm. To make APMDES exposed media, 10 grams of APMDES-treated sand was pre-mixed with 100 ml of CMM- media in 250 ml Erlenmeyer flask and incubated overnight on the orbital shaker at 30 °C at 150 rpm, then filter sterilized using SteriTop 0.2 µm bottle top filters and inoculated with 2 ml bacterial suspension to evaluate the toxicity of treatment on microbial growth. 1.5 ml aliquots were collected in microcentrifuge tubes at each time point of 0, 1, 2, 4, 8, 12, 24, and 48 hours to be used to measure urea concentration, optical density, and pH. 60 µl-aliquots from microcentrifuge tubes were diluted in 540 µL of 1.2M sulfuric acid (Fisher Scientific, Inc., Pittsburgh, PA) for a final dilution of 1:10 to use in Jung Assay to determine the urea concentration.

Microbial viability after exposure to APMDES was evaluated by plating cultures. An overnight *S. pasteurii* culture was centrifuged at settings 600G for 10 min at 4°C. The supernatant was removed and resuspended in phosphate buffer saline solution (PBS) to provide bacteria with a stable environment and prevent interference by potential growth in fresh growth media. The optical density was adjusted to 0.06 by adding fresh PBS to the residual pellet. 1.85 grams of sand, APMDES-treated (n=3) and untreated (n=3), was added to 15 ml centrifuge tubes and inoculated with 3 ml of *S. pasteurii* suspended in PBS (OD=0.06). A control that contained no sand was also prepared. 1 ml of initial *S. pasteurii* inoculum (OD 0.06) was added to 9 ml of PBS, and 8-fold serial dilution was performed. 10 µl sample from each dilution series (n=5) was plated on BHI agar plates and incubated at 30°C for 24 hours and colony-forming units were counted. After 1 hour, 1 ml of solution was taken from all groups, and serial dilutions and plating were performed. Following this step, for all centrifuge tubes containing sand, the microbial solution was removed, sand was rinsed with PBS, and then 3 mL of PBS was added back into the tubes. All centrifuge tubes underwent a vortex-sonicate-vortex step (30 seconds for each) to detach any microbes that had sorbed to sand. Serial dilutions and plating were once again conducted after detachment for groups containing *S. pasteurii*. Plates were wrapped with parafilm to avoid contamination and placed upside down in a 30°C incubator for 24 hours, and colony-forming units were recorded.

The influence of APMDES treatment on bacteria cell membrane integrity was evaluated through live/dead staining and confocal laser scanning microscopy imaging. After immersion in bacterial suspension (OD_{600}=0.6), samples were removed at 0h and 1h to investigate the effects of the APMDES treatment on cell membrane integrity. Samples were stained with 200 µL of a

diluted 1:1 mixture of SYTO® (live/green) and Propidium Iodide (red/membrane compromised) (LIVE/DEAD BacLight Bacterial Viability Kit stain) (Invitrogen, catalog #L7012). Samples were rinsed three times with 200 µl Milli-Q water to remove the unreacted dye and stored in the dark until imaging. Images were acquired with an upright Leica SP5 Confocal Laser Scanning Microscope (CLSM) using a 25X water objective. Excitation lasers and emissions were as follows: SYTO® 9 (ex/em 485/498 nm) 488 nm excitation, 495-550 nm emission collection; propidium iodide (ex/em 535/617 nm) 561 nm excitation, 595-650 nm emission collection. A laser beam scanned an area of interest at a frequency of 600 Hz.

Determining the Effect of APMDES Treatment on Microbial Attachment to Sand Grains

Scanning electron microscopy (SEM, Zeiss Supra 55VP) was used to investigate the surface microstructure and morphology of sand grains after immersing in bacterial suspension and biocementation media. Elemental composition was obtained using AZtec EDX (Oxford Instruments) detector. To investigate the microbial attachment on sand grains, 0.6 g of sand was inoculated with 1 ml bacterial suspension (OD_{600}=0.6) in a microcentrifuge tube, then the bacterial suspension was removed at 0.25h, 1h, and 16 h using a pipette, and samples were left in room temperature to air dry. Air-dried samples were mounted on SEM pin stubs using conductive carbon tabs (PELCO®, Ted Pella, Inc, Ca) and sputter coated with gold (Ted Pella 108 Carbon Coater) for 45 seconds to increase conductivity for high-resolution imaging.

Preparation of Biocemented Cubes

A starter culture was prepared by adding a 1 mL cryovial of thawed frozen *S. pasteurii* base stock (ATCC 11859) into a 100 mL growth medium in an autoclaved 250 mL Erlenmeyer

flask. The flask was incubated for 24 h on the orbital shaker at 150 rpm at 30 °C. A new growth culture was made by diluting the starter culture to 1:100 using a flask twice larger than the medium's volume (i.e., 1L media in a 2L flask or 500 ml in a 1L flask). The growth culture was incubated for 24 h on the orbital shaker table at 150 rpm. After 24 h, the optical density (OD_{600}) was adjusted to 0.6 by diluting in sterile growth media. 200µl aliquots were transferred to a 96-well plate, and OD_{600} was measured using a Synergy HT reader (Biotek Instruments, Inc., Winooski, VT). The average OD_{600} of sterile growth media was subtracted from the OD_{600} of bacterial culture to measure the OD_{600} of the culture without influence from the media or the 96 well plates.

Biocemented cubes were manufactured using APMDES-treated sand and untreated sand. First, 3D-printed cube molds were filled with 185 g of sand. Replicates (n=5) were prepared for each condition. Each mold was immersed in 300 mL of bacterial suspension (OD_{600}=0.6) for 16 h and then in biocementation medium for 8 h. To measure urea concentration using the Jung Assay protocol, 60 µL-aliquots were collected from biocementation medium at 0, 1, 2, 4, and 8h and diluted into 540 µL of 1.2 M sulfuric acid (Fisher) for a final dilution of 1:10 (Jung et al., 1975). Biocementation was carried out by repeating the above immersion procedure (16h in bacterial suspension and 8h in biocementation media) for 3 and 7 days at room temperature. Specimens were de-molded, rinsed under tap water, and left to dry at 60 °C for a week. The weight of the cubes was recorded over time, until the equilibrium was reached. Additional specimens were manufactured as 25x25x90 mm^3 prisms (Figure 9C).

Determination of Compressive Strength

Biocemented cubes were subjected to unconfined compression testing in accordance with ASTM D2166/D2166M ('ASTM - 2013 - Standard Test Method for Unconfined Compressive Strength of Cohesive Soil 1', 2013). The specimens were subjected to compression until failure using a constant load rate of 0.003 kips/s on an MTS Criterion Model 64. All replicates (n=5) for each condition were tested, and the height and area of each cube were recorded prior to testing.

Evaluation of Biomineral Content and Mineralogical Characteristics

After compressive strength testing, biocemented cubes were acid digested to estimate calcium carbonate content. Samples were collected from the edge (n=4) and middle (n=3) regions per cube. Two (2) grams of sample were placed in a 15 mL centrifuge tube, and 10 mL of 10% nitric acid was added to each tube and incubated at room temperature for 24 h to digest calcium carbonate. Then, the remaining fluids were removed, and the samples were allowed to dry at 60 °C for 72 hours prior to assessing the final mass. The dry mass difference before and after acid digestion was used to calculate the weight percent of precipitated $CaCO_3$.

Biocemented cubes were ground to fine powder using a pestle and mortar for XRD analyses. Crystalline phases of precipitated calcium carbonate on sand particles were examined using Bruker D8 Advance Powder X-ray Diffractometer with Cu-Kα (λ = 1.54060 Å) at 40kV and 40mA. The angle was set to 5° to 75°, with a step size of 0.2. The NIST Inorganic Crystal Structure Database (ICSD) was used to identify crystal structure data.

Small pieces of 7-day biocemented cubes were embedded in epoxy (Ted Pella INC.) to compare the size distribution of biocemented bridges on untreated and APMDES-treated sand. Embedded biocemented chunks were sectioned using a low-speed diamond saw (Isomet,

Buehler, Lake Bluff, IL) and polished with 600 and 1200 grits of wet silicon carbide papers (Buehler, Lake Bluff, IL), then polished with Rayon fine cloths and different grades of alumina pastes (9, 5, 3, 1 μm). Sections were sonicated in tap water between polishing steps to remove impurities from the surface. Embedded sections were mounted on SEM pin stubs and carbon-coated (108C Auto SE Carbon Coater, Ted Pella INC.) with a thickness of about 16nm to avoid charging artifacts for SEM-EDS analysis. Elemental maps were generated for calcium and silicon at three randomly selected locations, each for three replicates of 7-day treated and untreated cubes (Zeiss Supra55VP, working distance = 8.5, accelerating voltage = 10kV, magnification 200x).

SEM-EDX elemental maps were processed with custom MATLAB code to identify and measure sand grains (i.e., silicon-rich areas) and biomineral bridges (i.e., calcium-rich areas) (Supplemental Figure 2). Images were binarized, thresholded using Otsu's method, subjected to dilation and erosion steps, and filtered to remove objects with areas less than 20 pixels. Measures included biomineral bridge number density, the ratio of biomineral bridge area to sand grain area, and mean and median biomineral bridge area, major axis, and minor axis lengths.

Statistical Analysis

$CaCO_3$ content and compressive strength outcomes were compared between APMDES-treated sand and untreated sand using ANOVA. Three-factor mixed model ANOVA tested whether the weight percent of precipitated $CaCO_3$ depends on APMDES treatment, region, injections, and the interaction of these factors. Two-factor ANOVA tested whether compressive strength depends on APMDES treatment, the number of injections, or their interaction. To test the impact of calcium content on compressive strength, a linear mixed model was used with

random effects of treatment and injections and a covariate of calcium content. The influence of treatment on biomineral bridge characteristics was tested using mixed-model ANOVA. All models were checked for residual normality and equal variance. If necessary, dependent variables were transformed to satisfy these assumptions. The statistical significance was defined *a priori* as $p < 0.05$. In the case of significant interactions, post-hoc tests were performed using a Fisher's least significant difference (LSD) test with family-wise error controlled using the Bonferroni procedure (i.e., critical alpha adjusted to 0.05 / number of comparisons (2) = 0.025). Minitab (ver. 19.2020.1) was used for statistical analyses.

Results and Discussion

APMDES Treatment Changes the Surface Properties of Aggregates and Localizes Bacteria on These Surfaces

Silane coupling agents, including APMDES and other closely related treatments, have been used to achieve bio-trapping on a variety of surfaces, such as silicon and glass wool fibers, for non-biocementation applications (Avci, 2016; Aydın, 2007; Deliorman, 2012; Suo et al., 2008, 2009). This method of 'bio-trapping' bacteria does not cause strict immobilization unless coupled with antibodies but instead localizes (bio-traps) bacteria within regions of treated surfaces (Avci, 2016).

XPS analysis was performed to confirm the elemental composition of aggregates before and after APMDES treatment. The XPS survey spectra of untreated sand showed the presence of Si (2p and 2s), O (1s), and C (1s) signals, and the APMDES-treated sand revealed a peak at 399.6 eV corresponding to N (1s) amine groups, confirming the amine moieties on the APMDES-treated sand (Figure 1 A). The amine groups imparted a positive charge on sand

surfaces, which would be expected to increase electrostatic interactions between bacteria and sand surfaces.

A change in zeta potential also indicated successful functionalization. The zeta potential of untreated sand was -28.18 mV, and APMDES-treated sand was +36.47 mV (Figure 1 B). Since the zeta potential of *S. pasteurii* is -67 mV (Ma et al., 2020), electrostatic interactions between the positively charged APMDES-treated sand surface and the negatively charged wall of *S. pasteurii* likely promote bacterial adhesion.

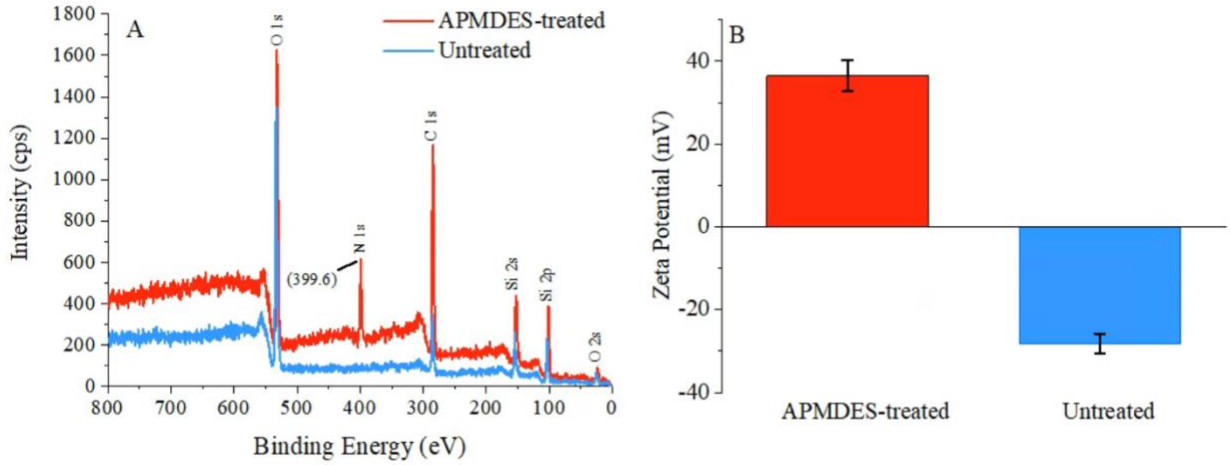

Figure 1. Change in surface properties of sand with APMDES treatment. A) X-Ray Photo Electron Spectroscopy (XPS) of sand grains before (UT) and after (T) APMDES treatment confirming the presence of amine groups at 399.6 eV binding energy for a successful treatment. B) Zeta potential measurements of sand grains in distilled water, a neutral pH of 7, before (UT) and after (T) APMDES treatment, n=50 sand grain per group. Error bars represent 1 standard deviation.

Imaging data confirmed the immediate localization of *S. pasteurii* on sand surfaces, which under scanning electron microscopy, appeared as a thin layer of cells (Figure 2E). Microscopy images reveal that the initial bacterial attachment at 0.25h, 1h, and 16h after immersion in bacterial suspension is greater on the APMDES-treated sand than the untreated

sand (Figure 2). SEM micrographs show a few cells attached to untreated sand at 0.25h, 1h, and 16h of immersion (Figure 2A-C). By contrast, APMDES-treated sand shows nearly instantaneous bacterial adhesion at 15 minutes, abundant bacterial adhesion after 1h of immersion, and a dense layer of microbes at 16h (Figure 2B&D).

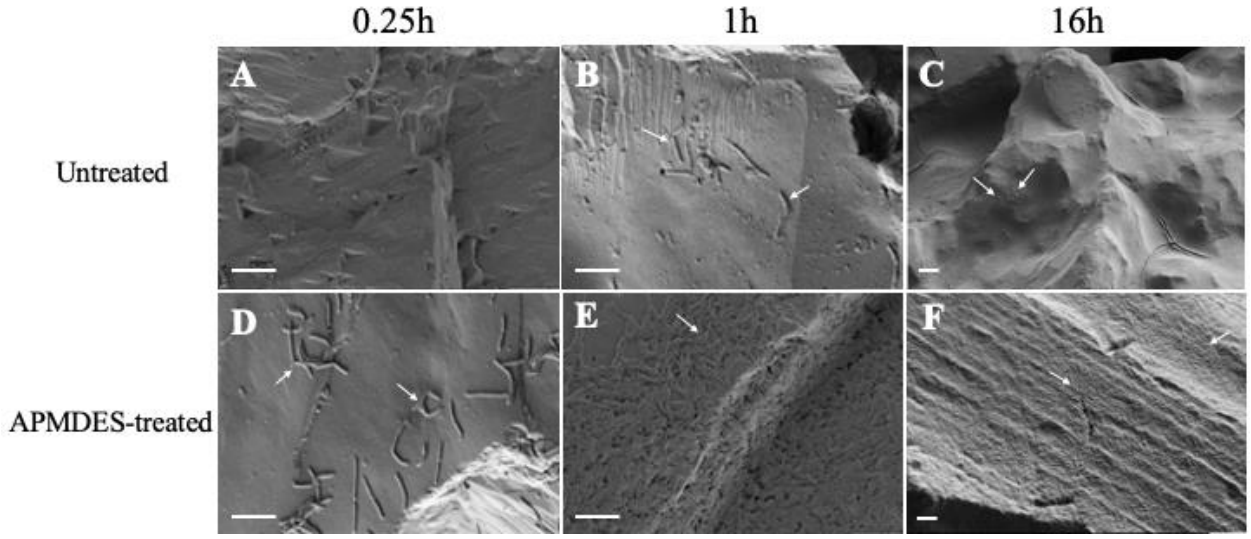

Figure 2. Initial attachment of *S. pasteurii* on APMDES-treated and untreated sand. (A-C) SEM indicates sparse attachment of microbes on untreated sand at 0.25h and 1h and almost no attachment after 16h of immersion. (D-F) APMDES-treated sand shows substantial attachment of microbes to the sand surface at 0.25h, 1h and 16h of immersion. Scale bars are 10 µm. White arrows indicate *S. pasteurii*.

Together, these data confirm that APMDES treatment was successful in bio-trapping bacteria on sand surfaces for time periods relevant to bacterial biocementation. This work is the first demonstration of the effective use of APMDES on sand surfaces. Sand is a common substrate for MICP-based biocementation. Whether APMDES would work as successfully on other types of aggregates was not investigated presently but would be a valuable investigation.

APMDES treatment inhibits bacterial growth and reduces viability but does not impede urea hydrolysis during biocementation

A first batch study was conducted to determine the influence of APMDES treatment on *S. pasteurii* growth and, hydrolysis of urea. The optical density of the initial *S. pasteurii* inoculum was measured as 0.027. The growth curve of *S. pasteurii* in the presence of untreated sand was similar to the planktonic condition without sand, reaching an optical density of 0.3 at 12 hours. The optical density of *S. pasteurii* in the presence of APMDES-treated sand was 0.02 at 12 hours. Bacterial growth was inhibited by the presence of APMDES treatment (Figure 3B), most likely due to the presence of $-NH_3^+$ moieties, expected to be toxic (Jennings et al., 2015; Rzhepishevska et al., 2013). However, even with APMDES treatment, urea hydrolysis and resulting increase in pH in solution still occurred. The 20g/L initial urea concentration was completely hydrolyzed in 48 hours in the presence of APMDES-treated sand, whereas urea was nearly completed hydrolyzed in 8 hours with or without untreated sand. The urea hydrolysis was slower in the presence of APMDES-treated sand because there was no bacterial growth that could produce urease enzyme. The urea hydrolysis was likely limited by the initial number of microbes inoculated. This suggests that despite inhibiting bacterial growth, APMDES does not interfere with the availability of the urease enzyme present within *S. pasteurii* (Figure 3A).

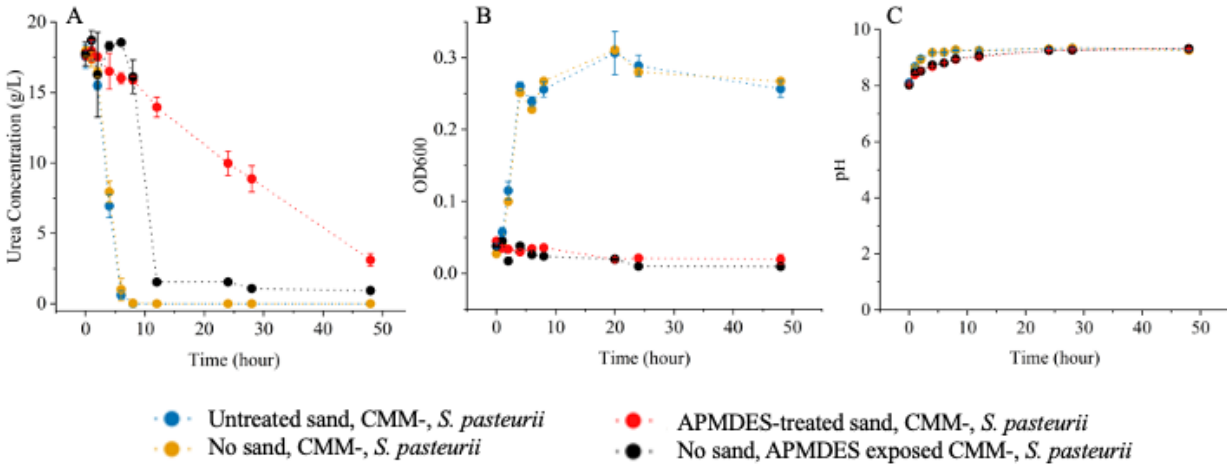

Figure 3. Urea batch study throughout 48 hours. A) Urea concentration decreases rapidly for positive controls and decreases, although less rapidly, for APMDES-treated sand, B) Optical density (OD) increased over time for positive controls but not for APMDES conditions, indicating inhibition of growth, and C) pH increased for microbial cultures, regardless of APMDES treatment.

After establishing that APMDES impedes *S. pasteurii* growth, the impact of the treatment on viability was determined through plate counts. Viability was determined from solutions before, and after, detaching *S. pasteurii* from sand. In the untreated condition, abundant viable *S. pasteurii* was measured before (4.2×10^7 cfu/ml) and after (3.48×10^6 cfu/ml) the vortex-sonicate-vortex procedure used to induce detachment, for both planktonic cultures and for microbes attached to sand (Figure 4). The decrease in viability after vortex-sonicate-vortex steps in the untreated sand condition may indicate that few microbes were attached to sand in this condition, consistent with SEM results (Figure 2). By contrast, there were no viable cells on APMDES-treated sand before and after the vortex-sonicate-vortex procedure, indicating that APMDES treatment renders *S. pasteurii* unculturable under the conditions tested. SEM confirmed that there were microbes attached to sand in the AMPDES-treated condition during this plating study, both before and after the vortex-sonicate-vortex steps (Figure 5).

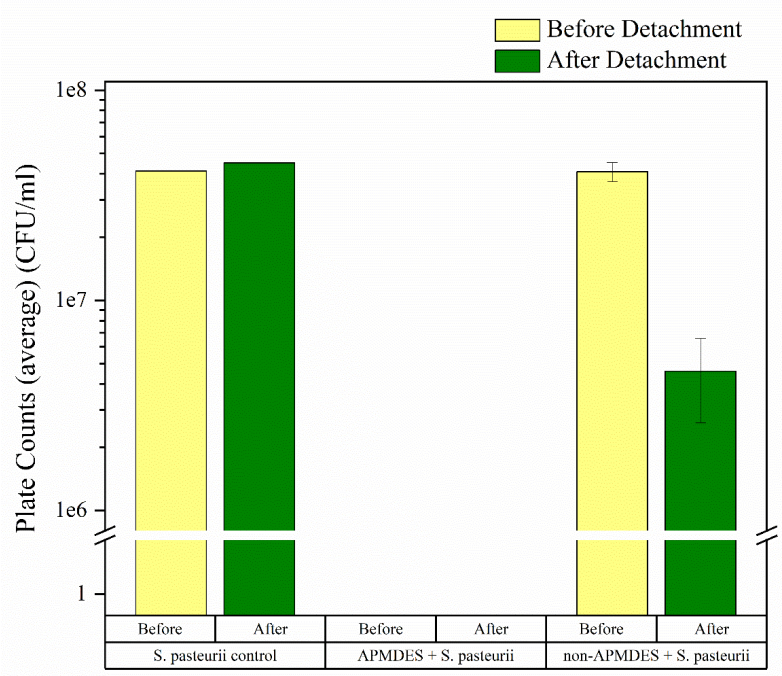

Figure 4. Plate counts of *S. pasteurii* before and after detachment from the sand, showing APMDES treatment causes loss of viability.

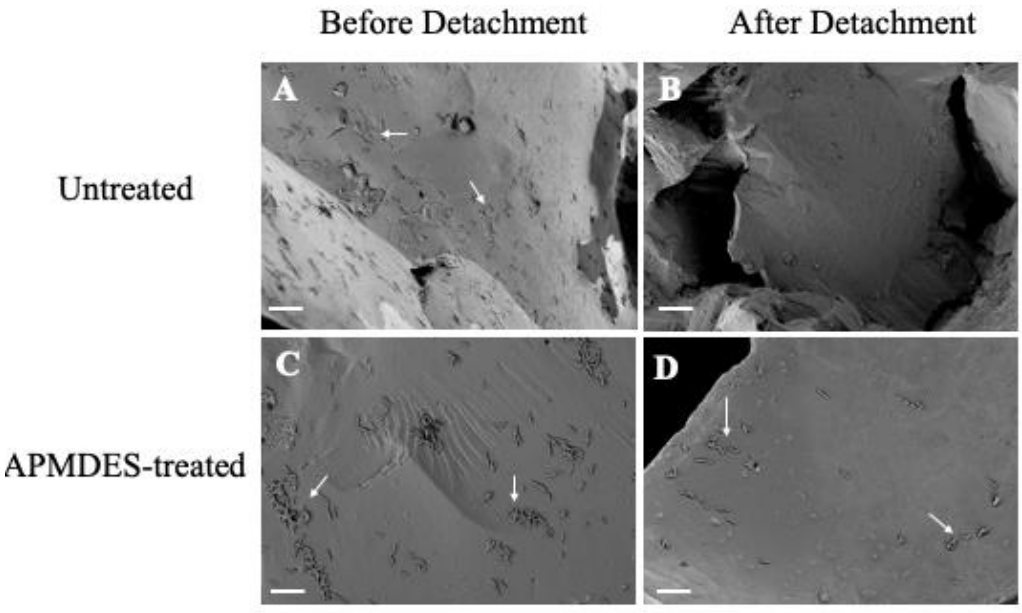

Figure 5. *S. pasteurii* attachment to APMDES-treated and untreated sand, before and after detachment of microbes. SEM images show higher concentration of microbes sorbed to APMDES-treated sand, both before and after detachment. Scale bars are 10 µm. White arrows indicate *S. pasteurii.*

An additional imaging study was performed to visualize the impact of APMDES treated sand on *S. pasteurii* not only at the 1-hour timepoint, but also close to the time of exposure (15 minutes) (Figure 6). CLSM images show red (propidium iodide-stained, membrane-compromised cells) and green (Syto9 stained, live cells) (Figure 6). Untreated sand shows very few cells at 15 minutes and sparse but mostly non-compromised (green) at 1 hour (Figure 6A&B). By contrast, APMDES-treated sand has abundant cells attached to its surface at both time points, with a some viable (green) and mostly membrane-compromised cells (red) (Figure 6C&D). The impaired membrane viability can, but does not always, indicate decreased cell viability (Berney et al., 2007; Stiefel et al., 2015).

Together, these several viability and growth studies indicate that APMDES treatment is detrimental to *S. pasteurii* viability. There are several potential reasons that may explain this effect. The increased electrostatic attraction between the cells and the treated surfaces could cause cell membrane damage. In addition, positively charged surfaces have been reported to have antimicrobial effects due to strong electrostatic interactions disrupting cell membrane integrity (Jennings et al., 2015; Metwally & Stachewicz, 2019; Murata et al., 2007; Nihei, 2017; Rzhepishevska et al., 2013).

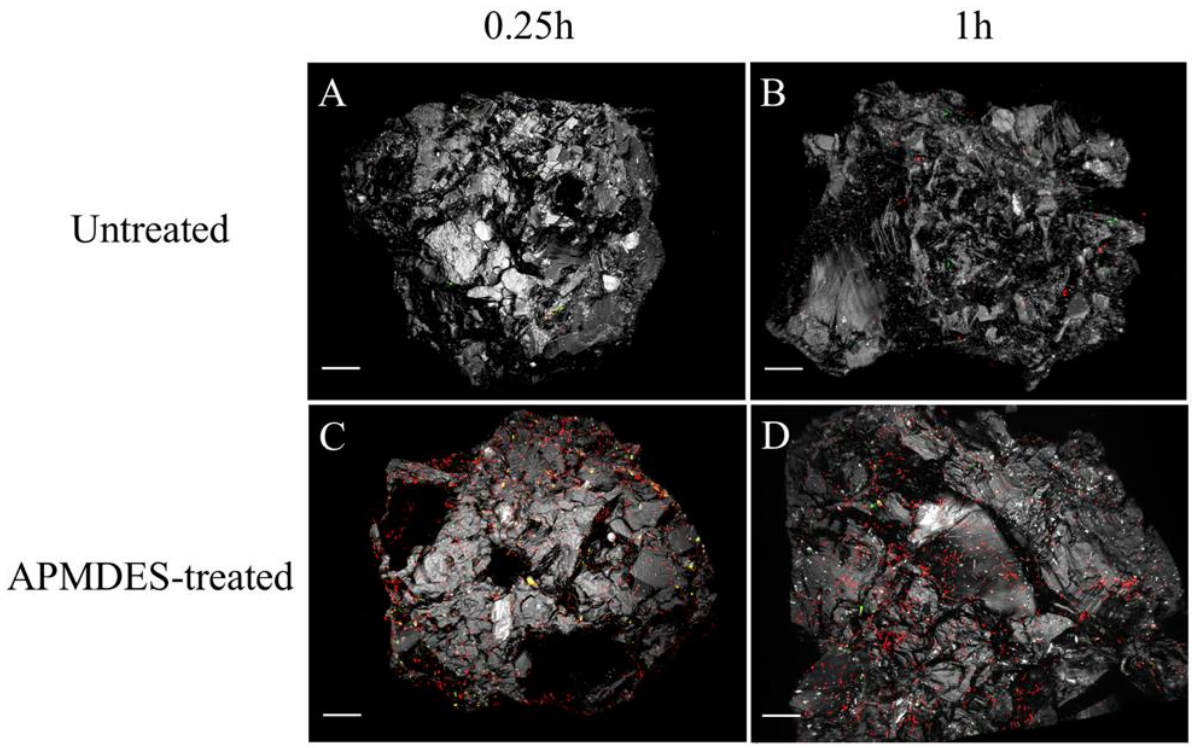

Figure 6. Initial attachment of *S. pasteurii* on APMDES-treated and untreated sand. (A&B) CLSM images demonstrate sparse, but predominantly viable (green) microbes attached to untreated sand at 0.25h and 1h. (C&D) Abundant microbial attachment is apparent on treated sand, with a mixture of viable (green) and membrane-compromised (red) microbes. Scale bars are 30 µm.

Though APMDES treatment decreased the rate of urea hydrolysis in initial batch cultures (Figure 3), urea hydrolysis was not impacted in biocementation conditions. Assessment of the bulk fluid for urea concentration during an 8-hour cementation period of daily injections demonstrated that urea hydrolysis of *S. pasteurii* was equivalent in both APMDES-treated sand and untreated sand conditions (Figure 7), suggesting cell membrane integrity is not necessary to deliver the urease enzyme for biocementation.

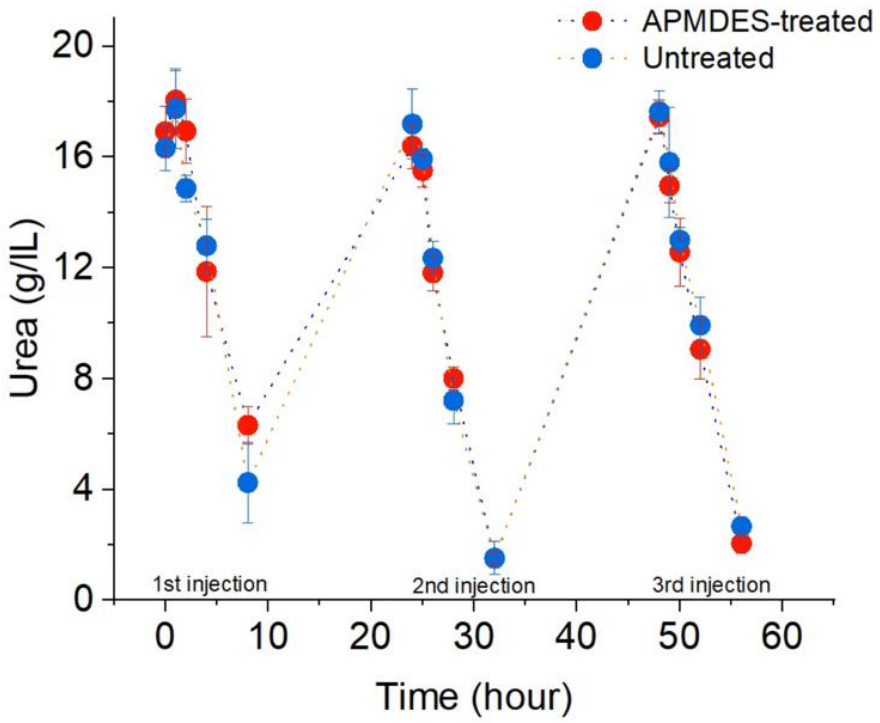

Figure 7. Urea concentrations over time in the presence and absence of APMDES (measured using a modified Jung assay). Initial 20g/L urea hydrolyzed during 8-hour time period

The finding that urea hydrolysis does not depend on *S. pasteurii* viability in APMDES-treated conditions might contribute to the development of more efficient MICP processes, where the focus could be on urease enzyme functionality rather than maintaining high levels of cell viability. There are potential upsides for biocementation treatment protocols that do not preserve microbial viability. For example, the use of microbes in situations (e.g., outdoor usage) where their growth may disrupt the local soil microbiome may benefit from eliminating the viability of those cultures, whether through a treatment like APMDES or through another strategy (e.g., heat treatment, isolating the urease enzyme, etc).

APMDES treatment decreases the time required for strength development via biocementation

After determining that APMDES treatment increases attachment of ureolytic microbes to sand treatment, the next question was whether APMDES would impact the efficiency of strength gain through biocementation. For both APMDES-treated and non-treated conditions, minerals precipitated during biocementation were mostly calcite with a minor phase of vaterite, as confirmed by XRD (Figure 8). However, the appearance of structures was markedly different with APMDES treatment. Cubes and prisms constructed using APMDES-treated sand had much more precise shapes than those constructed using untreated sand (Figure 9).

APMDES improved the compressive strength of cube specimens compared with the untreated condition (+49.7%, $p = 0.007$) (Figure 10, Suppl. Table 2). This effect was seen at early timepoints; after 3 injections, the compressive strength with APMDES treatment was almost double compared with untreated controls (2.31 vs 1.23 MPa). There was not a significant effect of injections ($p = 0.054$) on compressive strength, nor a significant interaction between injections and treatment ($p = 0.147$). It is noted that it is likely that this study was underpowered to detect the expected increase in strength with more injections and that including additional specimens would be likely to increase statistical significance.

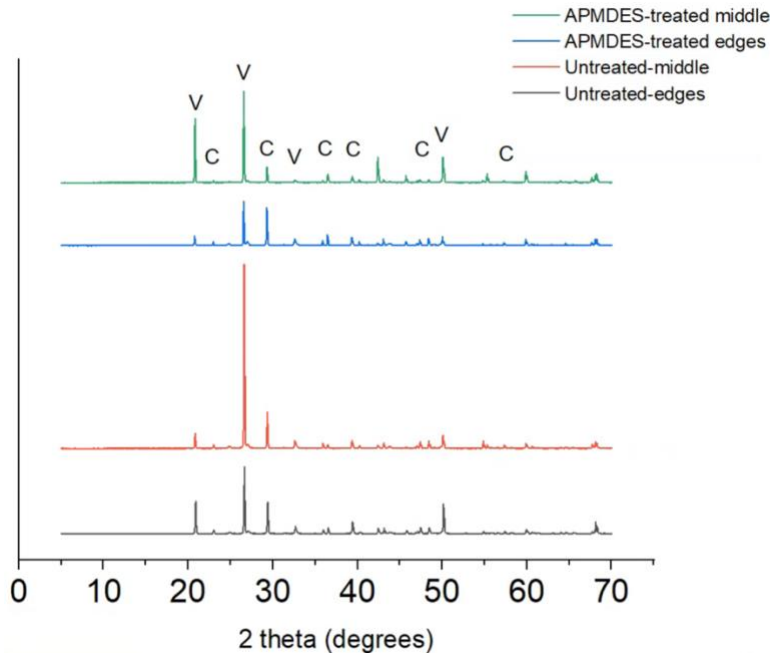

Figure 8. XRD spectroscopy of 7-day biocemented cubes. Results reveal abundant calcite, with minor vaterite phases. Note that the largest vaterite peak overlaps with quartz, which is highly abundant for these samples.

Figure 9. Biocemented specimens after 3 injections. A) 50 x 50 mm cubes prepared using APMDES-treated sand. B) 50 x 50 mm cubes prepared from non-treated sand. C) Prisms (25 x 25 x 90 mm) prepared from APMDES-treated sand (left) and non-treated sand (right).

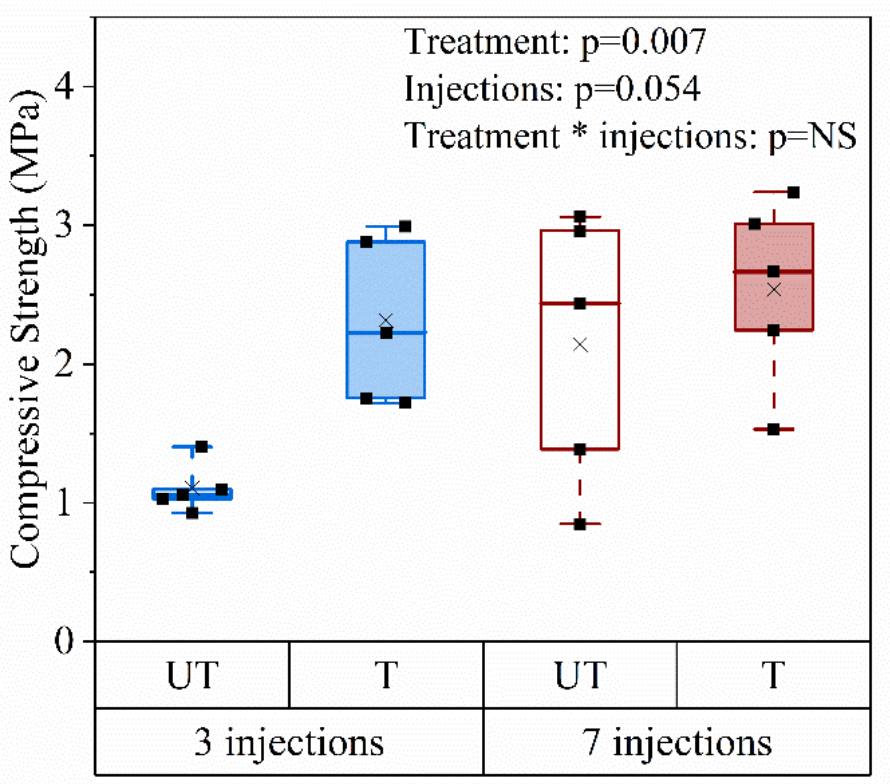

Figure 10. Compressive strength of APMDES-treated (T) and untreated (UT) cubes at A) 3 and B) 7 days injections. Boxplots show the median (line), mean (cross), interquartile range (box), minimum/maximum (whiskers), and symbols all data (dots).

The strength gain seen for APMDES-treated samples was rapid compared with the untreated controls as well as compared with data from other biocementation studies (Figure 11). For example, Bernardi and coworkers used *S. pasteurii* and urea-calcium medium to manufacture bio-bricks with dimensions of 91 mm x 58 mm x 200 mm. After 3 injections per day over 7 days (21 treatments), 14 days (42 treatments), or 28 days (84 treatments), and they achieved average compressive strengths of 0.07 MPa, 0.44 MPa, and 1.65 MPa, respectively [23]. Similarly, Lambert et al., 2019 developed brick-shaped specimens with dimensions of 222 mm x 106 mm x 73 mm using human urine as a urea source, which were biocemented for 8 days (6 injections per day, 48 treatments). This process produced specimens with an average compressive strength

value of 2.7 MPa [24]. Bu et al. manufactured MICP-treated brick specimens with 177 mm x 76 mm x 38 mm dimensions, which had compressive strengths averaging 0.42 MPa after 1 treatment with 7 days of reaction [25]. There are important differences across these studies in the type of microorganism, solution chemistry, size of sand, and other characteristics that can influence compressive strength. Regardless, APMDES treatment results in a very rapid strength development (average 2.1 MPa after 3 injections) compared to the most similarly shaped structures in literature. It is possible that additional injections beyond the endpoint of this study could further increase the strength of APMDES-treated materials, but this possibility requires additional investigation.

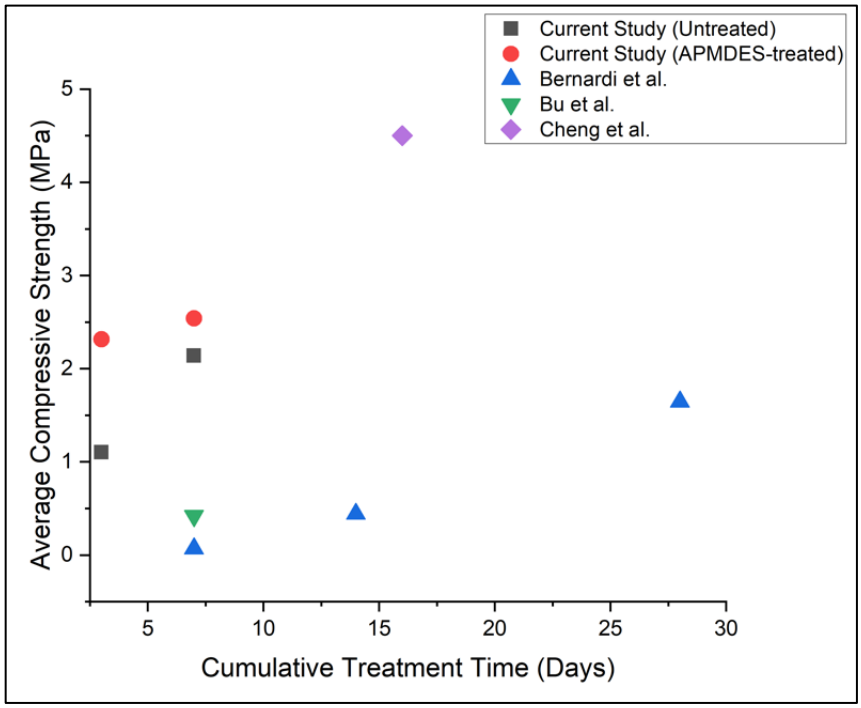

Figure 11. Relationships between cumulative treatment time (days) and unconfined compressive strength. All studies show specimens generated using MICP and sand, with the number of treatments differing between studies.

The strength gain from APMDES treatment is not solely explained by calcium-containing biomineral gain

Because compressive strength has been shown to have a positive correlation with $CaCO_3$ accumulation in prior work [13-15], the relationship between calcium gain and compressive strength was tested for APMDES-treated and non-treated groups. The calcium-containing biomineral gain within biocemented cubes, which would be expected to largely represent $CaCO_3$ accrual (Figure 12), was estimated from the acid digest. Biomineral gain depended on interactions between the number of injections and treatment (AMPDES vs no treatment) or region (i.e., edge or middle) (Figure 12A). Region and injection had an interactive effect (p = 0.003) on the weight percent of calcium-containing mineral. Edges of the cubes had more calcium-containing mineral compared to the middle of the cubes manufactured with 3 injections (4.53% vs 3.54%, respectively, p = 0.006) and 7 injections (7.63% vs 4.06%, respectively p<.001) (Figure 12B, Suppl. Table 2). The effect of APMDES treatment was different between 3-day and 7-day injections (p = 0.015). APMDES-treated sand had a greater percentage of calcium-containing biomineral than untreated sand after 3 injections (4.46% vs 3.60%, respectively, p = 0.019), but calcium-containing biomineral content was not different between APMDES-treated sand and untreated sand after 7 injections (p = 0.280) (Figure 12C, Suppl. Table 3).

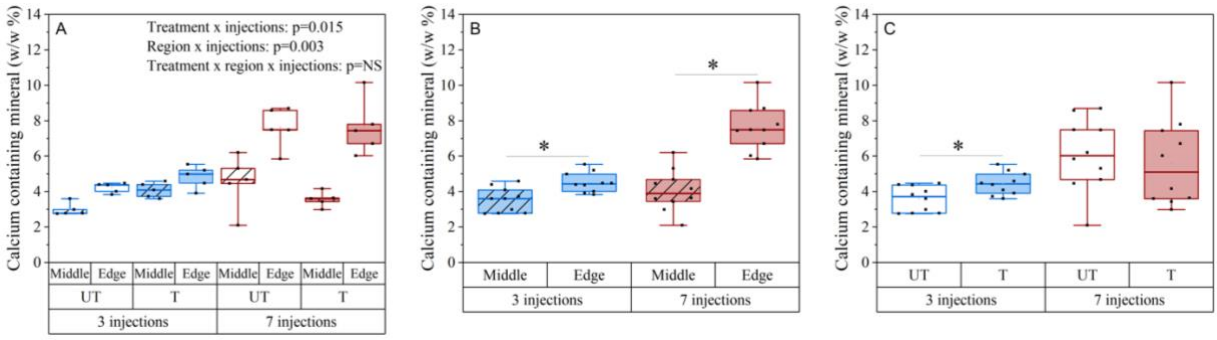

Figure 12. Treatment, region, and number of injections have interactive effects on the weight percent of calcium-containing minerals. A) Biomineral gain estimated from acid digests of edge and middle samples of sand cube specimens prepared with 3 or 7 injections. B) Region-injection interaction effect on % calcium-containing mineral. C) Treatment-injection effect on % calcium-containing mineral. Boxplots show the median (line), interquartile range (box), maximum/minimum (whiskers), and symbols representing all data points (squares). Significant simple effects from post-hoc tests following significant interactions are indicated with asterisks.

Since strength increases with calcium-containing biomineral content, analysis of covariance (ANCOVA) was used to test whether APMDES affects strength after accounting for the relationship between calcium content and strength. After accounting for the linear, positive relationship with calcium content through ANCOVA, there was still a significant effect of APMDES treatment on compressive strength (p = 0.029). Overall, APMDES treatment led to an increase of 49.7% in strength for data pooled across 3 and 7 injections. These data demonstrate that strength gain with APMDES treatment is not solely attributed to improved accumulation of biomineral (Figure 13).

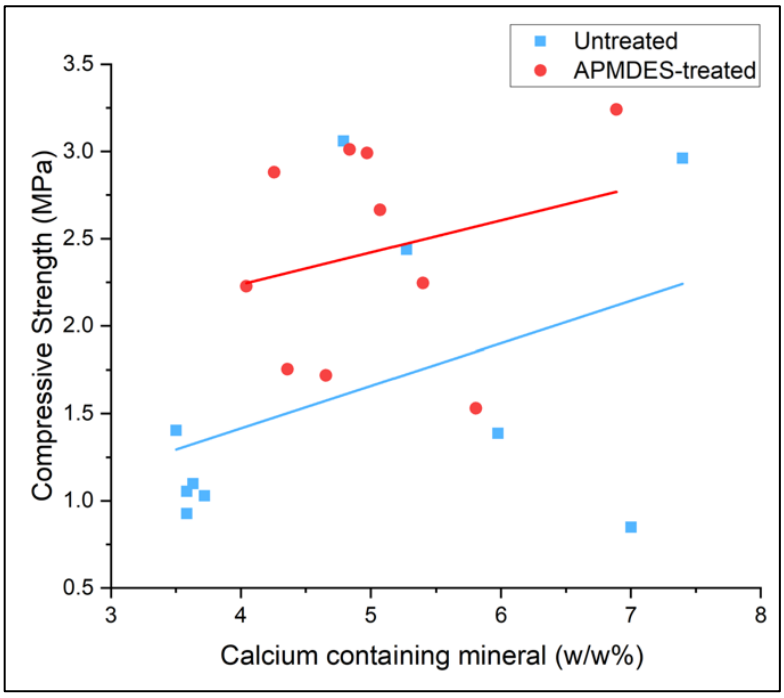

Figure 13. Mean calcium-containing biomineral content versus compressive strength of specimens prepared with untreated (r2 = 0.18) or APMDES-treated (r2 = 0.06) sand combining 3 and 7 injection data. A regression line is shown for each condition.

Since APMDES treatment appears to alter the relationship between biomineral accumulation and strength gain, other characteristics were investigated. The geometry of biomineral bridges was investigated from embedded and polished sections of biocemented cubes. There were several microstructural differences between the APMDES-treated and untreated conditions, such as increased sphericity and decreased size for bridges in APMDES-treated structures (Table 1). This result could indicate a difference in how biomineral bridges nucleate and grow with APMDES treatment. Other measures were not significantly different between treatment groups.

Table 1. Biomineral bridge geometric characteristics from SEM-EDX maps of calcium (biomineral bridges) and silicon (sand grains). Means are calculated from three specimens, which each represent the mean of three randomly selected regions of interest. Data are presented as mean ± standard deviation.

Measure	Untreated	APMDES-treated
Mean total sand area	22.99 ± 1.39 mm^2	24.60 ± 0.88 mm^2
Mean number of sand grains	39.33 ± 2.52	38.56 ± 3.47
Mean total $CaCO_3$ area	2.65 ± 0.91 mm^2	1.89 ± 0.67 mm^2
Mean $CaCO_3$ area / sand area	0.120 ± 0.0490	0.0788 ± 0.0302
Mean number of biomineral bridges	84.22 ± 8.88	86.89 ± 14.26
Mean biomineral bridge number / sand grain number	2.189 ± 0.178	2.256 ± 0.345
Mean biomineral bridge area	0.031 ± 0.012 mm^2	0.021 ± 0.006 mm^2
Mean biomineral bridge major axis	0.29 ± 0.048 mm	0.24 ± 0.032 mm
Mean biomineral bridge minor axis	0.14 ± 0.027 mm	0.11 ± 0.015 mm
Mean biomineral bridge circularity	0.49 ± 0.050	0.55 ± 0.029

Implications for sustainability

APMDES treatment may increase the sustainability of the biocementation process by decreasing the number of injections required to achieve a target compressive strength. Biocementation has garnered considerable attention as a low-temperature process to build load-bearing materials, but sustainability is decreased as the number of injections of bacteria and nutrients increases (Porter et al., 2021). Further, biocementation using ureolytic microbes produces ammonia, which is not desirable in some locations and situations. APMDES treatment has the potential to alter the sustainability of biocementation treatment by decreasing the number of inputs (i.e., injections) and waste outputs. It is important to note that functionalizing the sand surface with silane coupling agents increases the energy input, as the sand undergoes drying and ozone treatment steps, which require energy inputs and the production and disposal of chemicals, including ethanol, acetone, and APMDES. A full comparative life cycle analysis of APMDES

treatment versus conventional biocementation was outside of the scope of the present investigation but would be valuable for comparing the two manufacturing methods.

Limitations

This study has some important limitations. While the APMDES treatment was reported to increase strength development for similar biomineral content, the specific mechanisms contributing to this strength development need further investigation. Additionally, while microscopy images provided qualitative evidence of increased microbial density on APMDES-treated sand surfaces, it is not known if the APMDES treatment and subsequent bacterial distribution were homogenous within all areas of the sand matrix. Additional research is needed to gain a better understanding of how these treatments affect biomineral nucleation and growth and resulting strength development. Furthermore, prior experimentation with $-NH_3^+$ moieties show that these ions are toxic to some bacteria, not to others (Murata et al., 2007). Additional careful study is required to identify combinations of bacteria and surface charges that achieve goals of either viability or non-viability, depending on the intended applications.

Conclusions

This study demonstrated that the use of amino silane coupling agents, such as 3-aminopropyl-methyl-diethoxysilane (APMDES), can be an effective method for improving the efficiency in strength development during biocementation processes. The key findings of this research were that APMDES treatment altered surface properties of sand, which resulted in increased attraction for ureolytic bacteria, and improved strength development over a shorter period. Furthermore, this treatment did not affect biomineral precipitation levels, indicating its effectiveness at enhancing performance outcomes associated with biocementation processes

without compromising other aspects of the process. Overall, these results suggest that aggregate pre-treatment methods such as amino silane coupling agents may offer promising solutions for improving efficiency and effectiveness outcomes related to microbial biomineralization. The method has potential for improvement by reducing the toxicity of charged amine groups on the surface of the sand particles.

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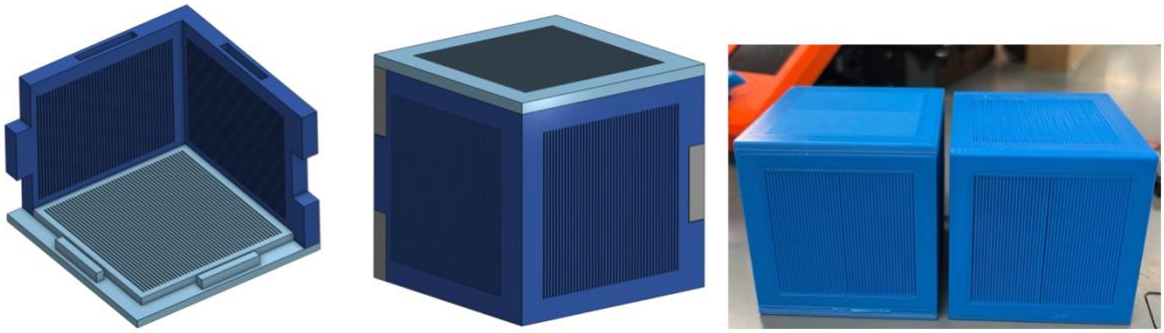
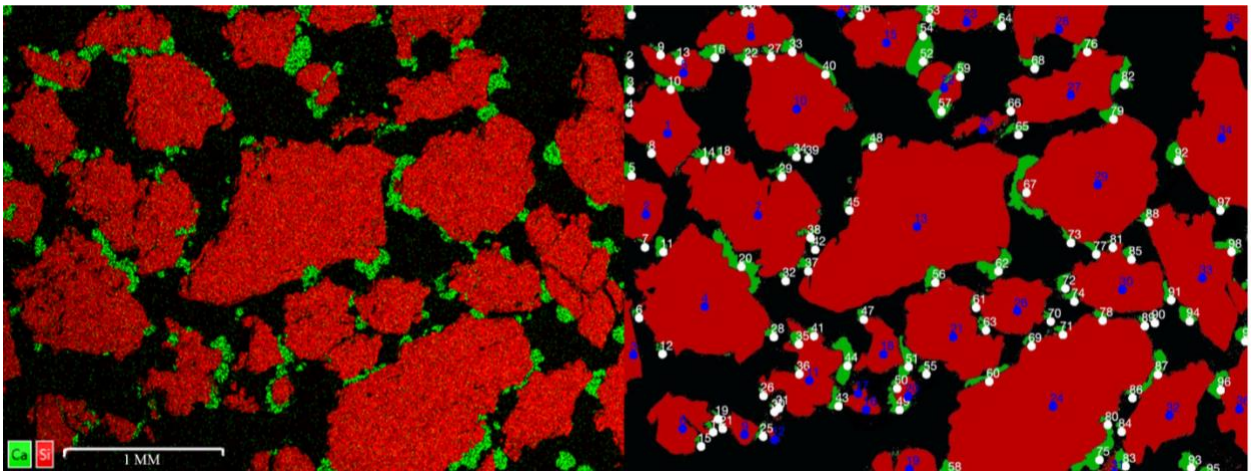
Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary Information



Supplementary Figure 1. 3D model of interlocking permeable molds, 50x50x50mm.



Supplementary Figure 2. Biomineral bridges were assessed through SEM-EDX and Matlab code.

Supplementary Table 1. Effect of APMDES treatment on compressive strength. Data are presented as mean ± standard deviation. N = 5 specimens per testing condition.

Treatment	Compressive strength after 3 injections (MPa)	Compressive strength after 7 injectiosn (MPa)
Untreated	1.12 ± 0.18	2.14 ± 0.98
APMDES-treated	2.31 ± 0.68	2.54 ± 0.68

Supplementary Table 2. Effects of number of injections and region on biomineral accumulation. Data are presented as mean ± standard deviation. # = significant post-hoc test between edge and middle specimens at either 3d or 7d.

Measure	3 injections		7 injections	
	Edges n = 10	Middle n = 10	Edges n = 10	Middle n = 10
$CaCO_3$ (% w/w) region x injection: p=0.003	4.53 ± 0.56	3.54 ± 0.20 # p = 0.006	7.63 ± 1.30	4.06 ± 1.18 # p < .001

Supplementary Table 3. Effects of number of injections and treatment on biomineral accumulation. Data are presented as mean ± standard deviation. * = significant post-hoc test between APMDES-treated and untreated specimens at either 3d or 7d.

Measure	3 injections		7 injections	
	Untreated n = 10	APMDES-treated n = 10	Untreated n = 10	APMDES-treated n = 10
$CaCO_3$ (% w/w) treatment x injection: p = 0.015	3.60 ± 0.72	4.46 ± 0.64 * p = 0.019	6.09 ± 2.05	5.6 ± 2.4

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CHAPTER THREE

CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK

Conclusions

The research presented in this thesis highlights that bio-trapping ureolytic bacteria on sand surfaces via silane coupling agents, specifically APMDES treatment, can potentially increase the efficiency of strength gains through biocementation. Microbial induced calcium carbonate precipitation (MICP)- based biocementation involves injecting ureolytic bacteria into soils or rocks that can induce calcium carbonate precipitates to bind aggregates together to increase soil stability and strength for engineering applications. This study investigated how APMDES treatment influences various aspects of biocementation, including bacterial growth, viability, attachment, urease activity, and material performance outcomes such as strength and calcium gain.

Results show that while APMDES treatment increased concentrations of trapped bacteria on the sand surface, it adversely affected microbial growth and viability. This may be attributed to increased electrostatic interaction between sand surface and bacteria, potentially causing damage to the cell walls of bacteria (Tuson & Weibel, 2013). Despite the adverse outcome to microbial viability, the urease enzyme found in these bacteria was still active to catalyze urea hydrolysis. The results suggest that APMDES treatment could be used to spatially control ureolytic bacteria for biocementation applications, even if the cells are not viable. There could be advantages to this outcome. For some applications where a biocemented structure is being

introduced to an environment interfacing soil with a native microbial community, it may not be desirable to have living cells, for example.

The APMDES-treated sand improved compressive strength development over a shorter period and yielded similar amounts of calcite precipitation compared to untreated sand. This indicates the effectiveness of APMDES treatment at enhancing performance outcomes associated with biocementation processes. Moreover, APMDES treatment improved the overall efficiency of the bicementation process by decreasing the number of injections required to achieve a target compressive strength for engineering applications. While APMDES treatment holds promise to increase the sustainability of the biocementation process, important next questions include identifying the upper limit of strength gain within these structures (i.e., we only investigated 7 injections) and further working to identify why APMDES treatment improves strength gain. This work found that calcium gain was not the only reason that strength was improved for APMDES-treated biocemented structures. Other potential mechanisms include the microstructure of the calcium carbonate bridges between aggregate, the porosity of these bridges, or differences in nucleation and growth.

The study presented in Appendix A primarily aimed to explore whether physically deconstructed MICP-based building materials can be recycled and maintain strength with multiple recycling generations. The data show that recycling and re-biomineralizing physically deconstructed MICP-based building materials is possible. However, compressive strength was not maintained with subsequent generations. Additionally, after biocemented sand was physically crushed, it was subjected to APMDES treatment to investigate whether the treatment would enhance the strength development of subsequent generations. The results showed that

APMDES treatment did not positively affect the strength development of subsequent generations, which may be attributed to surface characteristic changes of sand due to calcium carbonate precipitates from the initial generation. Importantly, as discussed in the future work section, there are differences in study design that will be useful in improving the strength of MICP-reconstructed structures.

Overall, these results suggest that amino silane functionalization of sand can promote the trapping of bacteria on sand surface, and it can be promising for increasing the efficiency and effectiveness of MICP-based biocementation.

Recommendations for Future Work

While the concept of spatially controlling microbes and their resulting biomineralization through APMDES treatment offers a novel approach to enhance the efficiency of the MICP process, further investigation is required to improve the usage of this technology for generating construction and building materials. Although this thesis demonstrated that APMDES treatment improves strength development for similar biomineral content, further investigation should occur to understand specific mechanisms contributing to this strength development. This thesis revealed that $CaCO_3$ bridges in APMDES-treated conditions were smaller with higher sphericity, which may indicate differences in how biomineral bridges nucleate and grow with APMDES treatment. Since biomineralization occurs locally to microbes, APMDES treatment could affect nucleation and crystal growth due to differences in the local density of microbes and urease enzyme. Further, it is possible that APMDES treatment changes the surface properties of the microbes, which may also modify the nucleation and growth of calcium carbonate. The smaller precipitates might have bonded the sand surface more effectively due to their higher surface

area-to-volume ratio, potentially leading to enhanced mechanical properties. These potential mechanisms would benefit from further investigation so that the process can be further optimized.

Real-time brightfield microscopy could be employed to track microbial attachment on sand and calcium carbonate nucleation over time. Another approach can be performing a microfluidic experiment that uses APMDES-treated silicon substrates such as glass to understand how APMDES treatment affects biomineral nucleation and growth. Spatially controlling biomineralization is essential to achieve homogenous calcium carbonate precipitation. Future studies should examine the uniformity of APMDES treatment within the sand matrix, the stability of the treated surfaces, and the longevity of enhanced bacterial adhesion over time.

APMDES treatment can potentially alter the sustainability of biocementation treatment by decreasing the number of inputs (i.e., injections) and waste outputs. However, a full comparative life cycle analysis of APMDES treatment should be examined to compare its environmental effects to conventional biocementation. Further investigations should focus on optimizing APMDES treatment parameters for sustainability. Additionally, further research could investigate the compatibility of APMDES treatment on different substrates, such as fibers, rocks, polymers, etc., and different bacterial strains to improve the efficiency of calcium carbonate precipitation to advance the biocementation process.

The work presented in Appendix A demonstrates that MICP-based materials can possibly be recycled and reused. One of the limitations of this study was that structures were rebiomineralized using large sand particles as opposed to graded sand containing both large and small particles. Graded sand decreases the void space and may promote greater biomineral

bridge formation between particles. A more comprehensive investigation is needed into the influence of graded sand and particle sizes on the recycling and reusing of MICP building materials.

Finally, transitioning laboratory-scale findings to large-scale applications is a critical step. Exploring the feasibility and efficacy of APMDES-treated biocementation in larger-scale scenarios could help determine its applicability and potential challenges in field conditions. Some scale-up challenges include uniformly treating large quantities of sand (which involves vacuum conditions and silane deposition), appropriately containing silane waste, and generating and deploying large amounts of microbes and solutions. Scaling up involves higher resource and infrastructure requirements, potentially impacting the overall cost-effectiveness of the process. Conducting cost analyses and optimizing processes for efficiency is essential.

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APPENDIX

RECYCLING AND REUSING BIOCEMENTED MATERIALS

Introduction

The construction industry produces substantial waste, and demolished concrete ends up in landfills. Annually, The United States generates 534 million tons of construction and demolition waste(Tam et al., 2018). Although 48% of this waste is recycled and used as subbase materials, a relatively small fraction of this recycled aggregate is re-used to produce new concrete (Tam et al., 2018). Furthermore, cement, the binding component of concrete, cannot be separated and reused; thus, a new cement paste is required to generate new concrete. Recycling and reusing building materials is important to increase sustainability in the construction industry because it addresses resource scarcity, energy efficiency, waste reduction, and environmental concerns.

MICP (Microbially Induced Calcium Carbonate Precipitation) based building materials hold a better promise for recycling and re-use. Calcium carbonate produced during MICP can be broken down physically or using acid. The products of the breakdown of calcium carbonate, calcium, and aggregates could potentially be used as inputs for the next generation of MICP-based building materials. The purpose of this study was to determine whether physically broken-down MICP building materials can be recycled and maintain strength with multiple recycling generations.

Materials and Methods

Materials

Refer to the Materials Section of Chapter 2. In this experiment, Yeast Extract was used as a growth-promoting medium instead of Brain Heart Infusion (BHI) due to the high cost of BHI and supply chain issues. The growth-promoting medium for subsequent transfer culture

contained 24 g L^{-1} yeast extract (Fisher Scientific, Inc., Pittsburgh, PA) and 20 g L^{-1} urea (Fisher Scientific, Inc., Pittsburgh, PA) in deionized water.

Preparation of 0th generation biocemented cubes

A starter culture was prepared by adding a 1 mL cryovial of thawed frozen *S. pasteurii* base stock (ATCC 11859) into a 100 mL growth medium in an autoclaved 250 mL Erlenmeyer flask. The flask was incubated for 24 h on the orbital shaker at 150 rpm at 30 °C. A fresh growth culture was made in yeast extract with 2% urea at a 1:100 dilution rate. The growth culture was incubated for 24 h on the orbital shaker table at 150 rpm. After 24 h, the optical density (OD_{600}) was adjusted to 0.6 by diluting in sterile growth media. 200µl aliquots were transferred to a 96-well plate, and OD_{600} was measured using a Synergy HT reader (Biotek Instruments, Inc., Winooski, VT). The average OD_{600} of sterile growth media was subtracted from the OD_{600} of bacterial culture to measure the OD_{600} of the culture without influence from the media or the 96-well plates.

0^{th} generation biocemented cubes were manufactured using APMDES-treated sand and untreated sand. First, 3D-printed cube molds were filled with 185 g of sand. Replicates (n=10) were prepared for each condition. Each mold was immersed in 300 mL of bacterial suspension (OD_{600}=0.6) for 16 h and then in a biocementation medium for 8h. Biocementation was carried out by repeating the above immersion procedure (16h in bacterial suspension and 8h in biocementation media) for 3 and 7 days at room temperature. Specimens were de-molded, rinsed under tap water, and left to dry at 60 °C until the equilibrium was reached.

Compressive Strength Testing

Biocemented cubes were subjected to unconfined compression testing in accordance with ASTM D2166/D2166M [2]. The specimens were subjected to compression until failure using a constant load rate of 0.003 kips/s on an MTS Criterion Model 64. All replicates (n=10) for each condition were tested, and the height and area of each cube were recorded before testing.

Preparation of 1st generation biocemented cubes

After compressive strength, rubble from the 0th generation cubes (n=20) were gently crushed using a roller and passed through a 2 mm sieve followed by a 0.6 mm to ensure uniform particle size. Afterward, half of the crushed 0th generation cubes (n=10) were subject to APMDES treatment (explained in Chapter 2). 170 g of crushed 0th generation cubes were packed into molds and re-biomineralized for 3 (n=4/APMDES-treated, n=4/untreated) and 7 days (n=4/APMDES-treated, n=4/untreated) following the same protocol for biocementation of 0th generation cubes. A compressive strength test was performed on dry specimens.

Results and Discussion

The strength gain for 0th Generation specimens (1.75 MPa) was higher compared to 1st-generation specimens (Figure 1). 1st generation cubes made with untreated sand had an average of 0.21 MPa after 3 injections and 1.08 MPa after 7 injections. Unlike the first study presented in Chapter 2, APMDES treatment did not increase the strength development for 1st generation specimens. 1st generation specimens made with APMDES-treated sand had an average of 0.13 MPa after 3 injections and 0.8 MPa after 7 injections. Strength gain was improved with an increased number of injections. Results from this study support that MICP-based building

materials can be recycled and re-biomineralized. However, these materials lost strength with subsequent generations.

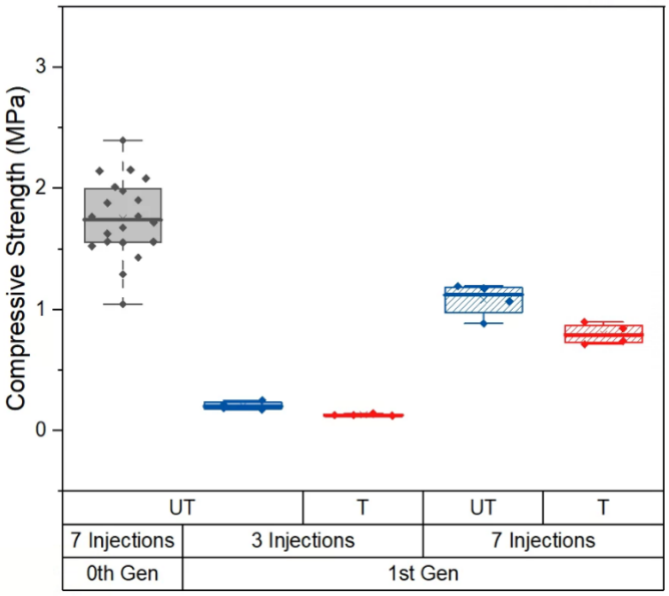

Figure 1. Compressive strength of APMDES-treated (T) and untreated (UT) cubes at A) 3 and B) 7 days injections. Boxplots show the median (line), mean (cross), interquartile range (box), minimum/maximum (whiskers), and symbols of all data (dots).

Conclusion

These findings suggest that while recycling and re-biomineralization of biocemented materials is possible, there are limitations in maintaining strength over multiple recycling generations. Although subsequent generations lost strength compared to the initial generation, their compressive strength was improved with the increased number of injections. Furthermore, APMDES treatment did not have a positive effect on enhancing strength development. Overall, this study addresses an important aspect of sustainable construction and provides valuable insights into the possibilities and limitations of recycling and reusing biocemented materials.

Future Work

Future research could focus on maintaining the strength of rebiomineralized materials through different treatment and injection strategies. Investigating the environmental impact and cost-effectiveness of recycled MICP-based materials is required.