

MICROBIALY INDUCED CALCIUM CARBONATE PRECIPITATION:
MESO-SCALE OPTIMIZATION AND MICRO-SCALE CHARACTERIZATION

by

Neerja Milind Zambare

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DEDICATION

This dissertation is dedicated to my wonderful parents, Sarita and Milind Zambare, who have supported me through everything.

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ABSTRACT

Microorganisms have the potential to impact processes on a scale orders of magnitude larger than their size. For example, microbe-mineral interactions at the micro-scale can drive macro-scale processes such as rock formation and weathering. Many bioremediation technologies derive inspiration from microbial mineralization processes. Microbially induced calcium carbonate precipitation (MICP) can produce calcium carbonate (CaCO_3) precipitates which can be utilized as a biological cement to strengthen porous media by reducing fluid permeability in subsurface fractures or as an immobilization matrix to remove metal contaminants dissolved in groundwater. To make MICP a feasible and successful bioremediation technology in the world outside the lab, it is necessary to bridge the gap between the meso-scale research studies and macro-scale applications. This thesis focuses on such meso-scale studies but also contributes to bridging the gap in the other direction, i.e., meso-scale to micro-scale to gain a fundamental understanding of the cellular level processes behind MICP.

The research presented here investigates two applications of MICP with a focus on controlling precipitate distribution and process efficiency in target environments. Subsurface precipitate distribution and metal partitioning during MICP were studied in novel reactive transport systems that mimic application-environment conditions. A radial flow reactor was used to study the spatial distribution of precipitates in conditions similar to subsurface injection well environments. The distribution and degree of metal partitioning during MICP was investigated in batch reactors and porous media flow cells to study kinetics and reactive transport effects on kinetics. In the radial flow environment, more precipitates formed away from the center injection zone. Results showed that longer reactant residence times and an equimolar ratio of calcium to urea were able to maximize precipitation efficiency. Metal partitioning could be maximized at low reactant flow rates and low metal concentrations. The novel flow cell set up used revealed a spatial decoupling between ureolysis and precipitation.

A micro-scale investigation of the fundamental MICP process itself is presented wherein microbe-mineral interactions are observed at the cell level. A semi-correlative approach to investigating individual precipitates in microdroplets is presented, using a multitude of microscopy and microanalysis techniques. The presented studies capture MICP across a range of scales.

CHAPTER ONE

INTRODUCTION

Background

The research presented in this thesis contributes to the understanding of microbially induced calcium carbonate precipitation (MICP), with focus on the reactive transport nature of the process, and micro-scale bacteria-mineral interactions. MICP is a natural phenomenon that results in the precipitation of calcium carbonate (CaCO_3). There are several metabolisms that can promote MICP including photosynthesis, sulfate reduction, and nitrate reduction.¹ The most widely studied MICP pathway is ureolysis, a process through which urea is hydrolyzed into ammonium and carbonate. Hydrolysis is typically slow, but bacteria that have the enzyme urease can increase the rate of ureolysis through the catalytic activity of the enzyme. Ureolysis facilitates increases in local pH and alkalinity, coupled with the presence of calcium cations can increase the saturation state of calcium carbonate, leading to the precipitation of CaCO_3 .

MICP is an attractive technology for a wide range of environmental remediation applications, a majority of which are associated with the subsurface. MICP has been proposed as a technique to strengthen porous media such as soil and sand as well as to remediate leakage pathways such as cracks in cement or bedrock around oil and gas wells. To promote MICP, native ureolytic microorganisms can be enriched by supplying selective growth conditions or known ureolytic strains or enzymes can be introduced into the target environment. Fortunately, the most widely researched bacterium that can efficiently

facilitate MICP, *Sporosarcina pasteurii*, is commonly found in soil and can be introduced into an environment likely without detrimental consequences. This makes MICP an appealing environmental remediation technology. MICP can be deployed in environments that cannot be accessed by cement; pumping cement through the subsurface is difficult due to its viscosity and high pH and cement cannot reach the fine cracks that are accessible to the water-based MICP fluids. Ureolysis driven MICP is the focus of all studies in this thesis.

Chapter 2 provides background information about ureolysis-driven MICP and discusses selected MICP studies performed under flow conditions. The importance of reactive transport and reactive transport modeling is discussed.

For subsurface applications, ureolytic microbes and necessary reactants need to be transported to the appropriate locations where CaCO_3 precipitates are desired. Being able to predict and control the spatio-temporal distribution of precipitates is necessary to maximize the potential of MICP as a sealing and groundwater remediation technology. MICP-promoting fluids are typically injected into the subsurface via injection wells. Around these wells, injected fluids move radially outward into the surrounding porous media or along flow paths formed by fractures. Work exploring reactive transport during MICP in a radial flow regime is presented in Chapter 3.

The nature of the precipitation pathway offers potential for divalent cations to co-precipitate with calcium. With metal co-precipitation, contaminated groundwater can potentially be remediated by sequestering dissolved heavy metals and/or radionuclides (ex. strontium, barium, radium etc.) in CaCO_3 precipitates. Metal co-precipitation can occur by

various pathways but the most prevalent is when the metal cations occupy the lattice structure sites of calcium in calcium carbonate. This mode of metal co-precipitation has potential to remove metal contaminants present at trace concentrations, which are difficult to treat with conventional methods. Co-precipitation of strontium with calcium carbonate was investigated under flowing conditions and a spatio-temporal analysis of the process is presented in Chapter 4. Often, heavy metal or radionuclide contamination exists as co-occurring metals. Chapter 5 assesses the potential of MICP to remove a mixture of strontium and barium.

The success of the MICP technology inherently depends on the stability of the precipitates and the microbe-mineral interactions behind the precipitation process. In general, microbe-mineral interactions have major implications in geomicrobiology as microbes and minerals have reciprocal interactions. MICP studies have traditionally been performed at the meso-scale, which poses a challenge for studying microbe-mineral interactions, which occur on the micro-scale. With the advent of microfluidics, multiple conventional laboratory procedures and protocols can be carried out in a single microfluidic system, reducing the demand for time, energy, and resources. Applied to MICP, droplet-based microfluidics was used in this work to enable the microscopic investigation of the precipitation process. Chapter 6 discusses micro-scale MICP visualization efforts and precipitation characterization by semi-correlative microanalysis.

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Part of the work presented in Chapter 6 was enabled through a limited scope user proposal funded by and carried out at the Environmental Molecular Sciences Laboratory (EMSL), a part of the Pacific Northwest National Laboratory. EMSL is funded by the Department of Energy's Office of Science.

Dissertation Overview

A variety of reactors and microscopy techniques for studying MICP are detailed in this dissertation, all geared towards providing a comprehensive understanding of MICP at various scales. Chapter 2 provides a literature review of reactive transport MICP studies in porous media and mathematical modeling approaches. Chapters 3-5 are based on meso-scale MICP studies and Chapter 6 discusses microbe-mineral interactions during MICP characterized at the micro-scale. Chapter 3 discusses the behavior (extent and distribution) of MICP under radial flow, the type of flow most commonly encountered near wells used to introduce MICP fluids into the subsurface. Chapter 4 presents a spatio-temporally resolved analysis of strontium co-precipitation with MICP in a modified linear flow cell. In Chapter 5, the potential for MICP to remove heavy metals from solution is investigated. Removal by co-precipitation is tested with artificial groundwater containing a mixture of strontium and barium.

Chapter 6 showcases the most recent efforts undertaken to study micro-scale MICP by means of microfluidic techniques, correlative imaging and microanalysis. To close, Chapter 7 presents conclusions drawn from the work and recommendations for future work needed to advance MICP as a biotechnology for larger scale, real world applications.

The literature review in Chapter 2 is titled “A REVIEW OF REACTIVE TRANSPORT DURING MICP-EXPERIMENTAL STUDIES AND MATHEMATICAL MODELING EFFORTS”. The literature review presents a summary on MICP, published flow MICP studies and mathematical modelling efforts for chemically driven and biologically driven precipitation under flow. It then discusses the significance of radial

flow followed by chemical and biological reactive transport models specific to radial flow. Chapter 2 ends with the need for flow studies to inform modeling efforts.

Chapter 3 is an experiments-based publication focused on MICP under radial flow simulated in a novel radial flow reactor designed and constructed at MSU. Sub-surface radial flow conditions were mimicked (in 2-D) in the experiments and MICP was facilitated by the ureolytic bacterium *Sporosarcina pasteurii*. Two controllable parameters – media flow rate and calcium to urea ratio – were varied to test their effects on MICP efficiency and distribution. Along with assessing bacterial attachment under radial flow, the utility of the dimensionless Damköhler number was demonstrated. The Damköhler number is a measure of the reaction-transport relationship. The journal article is titled “CONTROLLING THE DISTRIBUTION OF MICROBIALLY PRECIPITATED CALCIUM CARBONATE IN RADIAL FLOW ENVIRONMENTS” and was published in the peer-reviewed journal Environmental Science and Technology (ES&T), Volume 53, Issue 10, Pages 5916-5925.

Chapter 4 analyzes strontium co-precipitation with calcium carbonate in rectangular porous media flow cell reactors operating under continuous media flow. To study the spatio-temporal behavior of strontium co-precipitation and MICP, a modified flow cell was fabricated that allowed for samples to be drawn from multiple locations within the reactor during the experiments. The efficiency and distribution of strontium incorporated into the calcium carbonate precipitates were analyzed using the dimensionless strontium distribution coefficient. The relationship between precipitation rate and strontium distribution coefficient in the modified flow cell was compared to numerous

published studies. Based on the results presented in Chapter 4, a manuscript titled “SPATIO-TEMPORAL DYNAMICS OF STRONTIUM PARTITIONING WITH MICROBially INDUCED CALCIUM CARBONATE PRECIPITATION IN POROUS MEDIA FLOW CELLS” has been prepared for submission to ES&T. Additional data on the effect of controllable parameters on co-precipitation of strontium are presented in Appendix C.

The simultaneous removal of strontium and barium from a solution undergoing ureolytic MICP is discussed in Chapter 5. Co-precipitation of strontium and barium was studied in flask reactors. The influence of heavy metals and their mixtures on co-precipitation efficiency was assessed and the distribution of strontium and barium in the precipitates was characterized. Chapter 5 is a manuscript titled “CO-PRECIPITATION OF STRONTIUM AND BARIUM” and is being prepared for submission to ES&T.

Chapter 6 describes a non-application study focused on gaining a deeper understanding behind the microbe-mineral interactions involved in MICP. Microdroplets containing single bacterial cells were generated using microfluidic devices. A modified bacterium (*Escherichia coli* MJK2) was used for this project due to its ability of expressing fluorescence (through green fluorescent protein, GFP) in addition to performing ureolysis. The GFP signal facilitated imaging of bacterial cells using Confocal Laser Scanning Microscopy. The droplets, initially containing single cells, were imaged over time to monitor bacterial growth and calcium carbonate formation by MICP. The precipitates were characterized using a number of advanced microscopy and spectroscopy techniques performed at MSU and the Pacific Northwest National Lab. Chapter 6 is a manuscript in

preparation titled “DROPLET BASED INVESTIGATION OF MICROBE-MINERAL INTERACTIONS DURING MICP”.

Chapter 7 summarizes conclusions drawn from the experiments-based chapters and discusses future directions of microbe-mineral interaction research using microscopy and microanalysis.

Appendices A and B provide the supplemental information for the manuscripts in chapters 3 and 4 respectively. In Appendix C, unpublished experiments on strontium co-precipitation under flow using standard flow cells are discussed. Appendix D provides supplemental information for the manuscript in chapter 6. Additional experimental information pertinent to Chapter 6 is provided in Appendix E. Appendix F summarizes anion incorporation efforts using selenate and selenite to explore the potential of selenium sequestration by MICP. Appendix G reports findings from mine tailings leachate studies, including leachate characterization, ureolytic potential in the leachate, extent of MICP and potential for heavy metal sequestration.

CHAPTER TWO

A REVIEW OF REACTIVE TRANSPORT DURING MICP-EXPERIMENTAL STUDIES AND MATHEMATICAL MODELING EFFORTS

Introduction

Microbe-mineral interactions are ubiquitous in nature and have a reciprocal nature. Micro-scale microbial processes bring about macro-scale geomicrobiological processes such as formation and/or weathering of rocks or biotransformation of minerals. In turn, these processes can affect microbial growth and functions. Numerous bioremediation strategies utilize microbe-mineral interactions. In the late 1990s, the field of Microbially Induced Calcium carbonate Precipitation (MICP) started gaining considerable interest for potential bioremediation applications in the subsurface. MICP is a set of biologically driven chemical reactions that increases pH and alkalinity, which, in the presence of dissolved calcium, can facilitate calcium carbonate (CaCO_3) precipitation. MICP was envisioned as a biotechnology capable of strengthening soil, repairing underground fractures, and sealing leakage pathways (especially as in the case of geologically stored carbon dioxide) by manipulating subsurface physical properties with the precipitated CaCO_3 . Co-precipitation of heavy metals and radionuclides via MICP can be used for groundwater remediation.

Fluid flow in biological systems can have a significant impact on the survival and localization of the biological entities. Fluids often supply biological entities with their required nutrients and growth factors, particularly in subsurface environments. Various natural events and anthropogenic activities result in physical and chemical heterogeneities in the porous subsurface, which can alter fluid flow and thus affect biological organisms.

MICP-based bioremediation efforts deliberately use this phenomenon of introducing heterogeneities that change porous media properties by producing CaCO_3 precipitates in the pore space. For example, accumulation of precipitates in the pore space can lead to physical changes in fluid flow such as constriction and blocking of flow paths, resulting in altered macroscopic properties like porosity and permeability. Precipitates that form in the pore space can severely affect solute velocity and dispersion up to several orders of magnitude.² If the precipitates are being formed as a result of biological activity, changes in nutrient transport because of accumulated precipitates can cause a feedback on the precipitation rates.³ These examples serve to highlight the importance of reactive transport during MICP. Understanding reactive transport effects on the efficiency of the precipitation process and on the distribution of the precipitates can inform design strategies for field-deployment of MICP technology.

It is crucial to study MICP under potential application environmental conditions to i.) study the effects of environmental conditions on the process and through that knowledge, ii.) develop controls on the process that can be used during field applications. In this review, mechanisms that can facilitate MICP are discussed along with a summary of studies exploring the effects of fluid flow on MICP. Finally, reactive transport modelling is discussed along with examples of numerical models simulating flow MICP experiments under linear and radial flow regimes.

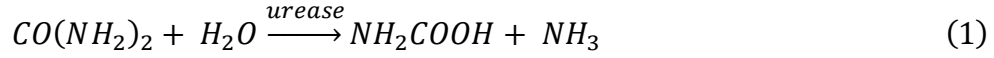
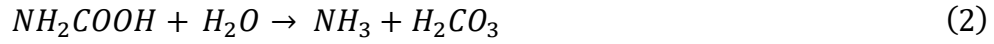
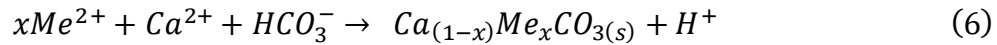
MICP Mechanisms

Precipitates can form i.) through cells actively controlling precipitate nucleation and growth (biologically controlled precipitation), ii.) passively through the presence of

organics (biologically influenced precipitation), iii.) via supersaturation conditions resulting from biological activity (biologically induced precipitation), or a combination of these pathways.^{1, 4} Any biochemical processes that can increase the alkalinity of the microenvironment and therefore increase dissolved inorganic carbon concentrations can create supersaturated conditions for carbonate mineral precipitation. MICP is an example of biologically induced precipitation specific to calcium carbonate precipitation. MICP can occur via a variety of mechanisms that cause calcium carbonate supersaturation such as photosynthesis, sulfate reduction, iron reduction, urea hydrolysis, denitrification and methane oxidation.⁵ It is widely agreed that calcium carbonate precipitation depends primarily on four factors: i.) pH, ii.) dissolved inorganic carbon concentrations, iii.) calcium concentrations and iv.) nucleation sites.⁶ Other factors such as ionic strength, temperature, microbial activity and microbial mode of growth also influence the extent of MICP.⁷

During ureolysis-driven MICP, urea hydrolysis is catalyzed by the intracellular urease enzyme (Equation 1). The resultant carbamic acid spontaneously hydrolyzes to carbonic acid (Equation 2). Both these reactions also produce ammonia which protonates to ammonium around neutral pH conditions (Equation 3). The hydroxide ions that are generated during the protonation step shift the carbonate equilibrium towards bicarbonate and carbonate (Equation 4). When divalent cations like calcium are present at sufficient ionic activity, calcium carbonate can precipitate out of solution (Equation 5). Similarly, heavy metal cations such as strontium, barium and radium also have potential to co-precipitate with calcium carbonate (Equation 6, Me^{2+} is a divalent metal cation). Co-

precipitation is different than the metal cations forming their own carbonate minerals and can occur even when the solution is undersaturated with respect to the metal carbonates.



MICP Flow Studies

Reactive transport has complicated implications for MICP. All the factors mentioned above that influence MICP can change dynamically under flow conditions. As a result, MICP during reactive transport is considerably more complicated to study than MICP without a fluid flow component. Numerous MICP studies have utilized packed columns to study MICP under flow conditions. Flow MICP studies have characterized the amount and distribution of MICP precipitates and the influence of different fluid injection strategies. Among MICP flow studies, the majority have been conducted under linear flow regimes.

Chiung et al. tested combinations of loosely and densely packed sand in PVC columns inoculated with resting or growing cells of *Sporosarcina pasteurii*.⁸ Urea-calcium media was then pumped upwards through the column to promote biomineralization and the California Bearing Ratio (CBR) was determined as a measure of sand strength. Growing

cells in a densely packed sand column showed the highest increase in the CBR indicating the most gain in sand strength via MICP. Cunningham et al. used a vertical quartz sand packed column (61 cm long, 2.54 cm diameter) to study MICP promoted by *S. pasteurii*.⁹ Their injection strategy consisted of flushing the column with calcium-free media between biomineralization periods of calcium-rich media. This protocol of sequential injections resulted in a quasi-uniform precipitate distribution in most of the column except for the region close to the inlet where high precipitate mass was measured.

Bottom-to-top parallel vs sequential injections of bacteria and urea-calcium solutions were tested by Tobler et al. in 10 cm long, 3.7 cm diameter plastic columns packed with dry quartz sand.¹⁰ This study also showed more plugging of the pore-space close to the inlet. A near homogeneous distribution of precipitates was nevertheless observed with sequential injections of bacterial suspensions and mineralizing media.

In addition to sand, glass beads have been a popular choice to represent porous media in packed column studies. Glass beads offer a higher confidence on pore size distribution compared to sand. Eryuruk et al. tested seven glass bead sizes (diameters ranging from 0.01 mm to 3.0 mm) in 10 cm long, 3 cm diameter plastic columns.¹¹ The highest decrease in average pore size was detected in columns containing smaller glass beads. They also showed that the observed decrease in hydraulic conductivity occurred due to the deposition of CaCO_3 and not from bioclogging by bacterial cells. Changes in hydrodynamics within pores due to CaCO_3 deposition was non-invasively studied by Fridjonsson et al. using nuclear magnetic resonance (NMR).¹² They found that after biologically mediated precipitation, there was increased pore-scale mixing.

Jack bean meal, a plant-based urease rich source, was used to promote ureolysis and subsequently CaCO_3 precipitation in a Perspex column filled with borosilicate beads by Handley-Sidhu et al.¹³ Destructive sampling of the column at the end of this microbe-free experiment showed higher precipitate mass close to the inlet, similar to the findings of MICP column studies. Magnetic resonance profiles across the column length showed a decrease in porosity primarily in the first quarter of the column length over 65 hours. Sham et al. found a 14.5% decrease in average porosity of a borosilicate ballotini beadpack after two MICP fouling events.¹⁴ The beads were packed in a 105 mm long, 37 mm diameter Perspex column. The decrease in porosity was quantified by magnetic resonance and it occurred predominantly near the entrance. An additional experiment performed using a sandstone core placed in a high-pressure system yielded a relatively more homogeneous precipitate distribution, evidenced by a uniform decrease in porosity across the column length.

El Mountassir et al. conducted an intricate flow study to test the effects of various hydrodynamic factors on MICP by *S. pasteurii* in simulated rock fractures.¹⁵ Two polycarbonate flow cells (20 cm X 14 cm) were prepared by etching the lower polycarbonate sheet to mimic rock fractures. Flow cell 1 had etchings of varying widths and apertures (depth from top plate) and Flow cell 2 had etchings of varying widths but the same aperture. A digital camera captured images of the precipitates within the simulated fractures through the top clear polycarbonate plate. The formation of flow channels formed by mineral fill (preferential flow paths) was observed, suggesting a feedback mechanism between fluid velocity and precipitation. At higher flow rates, wider channels remained

open. The competition between bacterial floc sedimentation velocity and fluid shear drove the deposition of bacteria (and therefore the spatial distribution of precipitates) in the fractures.

Such meso-scale studies are crucial in identifying the basic parameters that affect MICP. The ultimate goal is to accurately estimate the effects of MICP on bulk properties of the application environments (macro-scale), a key task in optimizing MICP as a bioremediation technology.

Reactive Transport Modeling

In addition to lab investigations of MICP in reactive flow systems, researchers are interested in mathematically modeling this process and estimating its effects on bulk porous media properties. Reactive transport models are beneficial in predicting effects of coupled processes during flow.¹⁶ Mineral precipitation is a good example of a coupled process in this context, involving physical (transport phenomena), chemical (reaction chemistry) and biological (growth) aspects. Deposition of precipitated solid particles in pore spaces of a porous medium can cause drastic changes in flow patterns and significantly alter macro-scale properties of the porous medium such as porosity and permeability, as has been seen in lab studies summarized previously. The thickness of the biofilm and extent of calcium carbonate precipitation contribute to development of preferential flow paths caused due to plugging of the pores in the porous media, affecting diffusion and dispersion.^{12, 17, 18}

Reactive transport models offer the advantage of performing MICP investigations in reduced timeframes and at scales not feasible for experimentation. Model parameters

can be changed more readily and rapidly than conditions in lab experiments, making them time efficient. Moreover, numerical models are capable of coupling various concurrent processes or scenarios, which is challenging to execute in lab experiments. Often, numerical simulations are performed in domains that mimic reactors used in lab studies.

Many reactive transport models are grid-based numerical methods that solve mass, energy and momentum balances (Navier-Stokes (N-S) equations for incompressible fluids) using techniques like finite difference or finite volume element. Other popular numerical modeling methods include the Lattice-Boltzmann Method (LBM) and Smooth Particle Hydrodynamics (SPH). LBMs emerged in the late 80's by introducing probability density functions (PDF) into the Lattice Gas Cellular Automata method (LGCA). LBM simulates statistical particle collision processes and is analogous to solving the N-S equations under certain limitations. It is a uniform lattice method that tracks PDF of particles as opposed to N-S methods that calculate macroscopic variables.¹⁹ In comparison, SPH is a mesh-free method that can discretize N-S equations offering a solution to meshing problems.¹⁹ As particle-based methods, both LBM and SPH might be more applicable for multiphase flow systems.²⁰ For example, in SPH transport models, fluid and solid phases are represented by different sets of particles.²¹ Pore-network modeling (PNM) is a pore-scale modeling approach that represents actual pore geometries (pore throats and pore bodies). This method retains the complete topology of the actual pore space without needing to represent the details of individual pore/throat geometry as required by the other methods.²⁰

In reactive transport modeling, the pore-scale considers micro-scale processes while the continuum-scale is associated with the macro-scale or Darcy-scale which

combines and averages effects of micro-scale processes over the entire system or the representative elementary volume in question. For example, during MICP, the pore-scale microbial activity is affected by chemical gradients in the pore fluid. These pore-scale phenomena must be coupled to Darcy-scale changes in overall porosity and permeability of the media brought about by deposition of the resulting precipitates. Traditionally, pore-scale modeling and continuum-scale modeling have been studied separately because their governing equations and boundary conditions are different.²² Pore-scale models rely on molecular diffusion in the pore-fluid, described by N-S equations. Continuum-scale models include dispersion and focus on the medium's continuous material properties.²³ In recent years, modeling efforts have been focused on the significant challenge of coupling pore-scale processes to the continuum-scale properties because of the fundamental effects micro-scale processes have on the macro-scale.

Chemical

Mineral formation in porous media flow is often modeled by simulating the flow of mineralizing solutions in parallel. When two fluids flow parallel to each other, the Reynolds number can have a large effect on the mixing of the two fluids. In porous media, mixing (and therefore precipitation) preferentially occurs at the common boundary of the two fluids. Such transverse mixing is controlled to a large extent by molecular diffusion in the pores.²⁴

Emmanuel and Berkowitz modeled mixing induced precipitation using a numerical model that coupled equations for mass conservation, solute transport and porosity evolution.²⁵ Two porous media conditions were tested in a 10 cm X 5 cm rectangular

domain with porosities of 26% for a homogeneous medium and a combination of 26% and 50% for a heterogeneous medium. Surface area, a typically less constrained parameter, was considered in their model. Two scenarios were tested: i.) the porous media grains approximated by spheres and ii.) the pore bodies approximated by spheres. For case i.) the specific area increases with decreasing porosity and for case ii.) the specific area decreases with decreasing porosity. A narrower precipitation region near the center of the rectangular domain was predicted for case i.) whereas for case ii.) the precipitation region was considerably wider. The model showed that mixing-induced precipitation can reduce porosity in concentrated regions preferentially rather than result in even precipitation, which could explain instances in nature where high porosity regions are filled where others are not. For example, in interconnected fractures, uneven precipitation might be a result of mixing-induced precipitation in concentrated regions rather than poor connectivity between fractures. The model predicted high inlet precipitation similar to observations in experimental studies. However, in the mixing-induced model, the influent fluid was able to bypass the plugged inlet region which resulted in precipitation downstream.

Katz et al. performed flow-precipitation experiments in 25 cm X 10 cm flow cells with homogeneous and heterogeneous packing which were compared to a reactive transport numerical model.³ The model was based on space-averaged advection dispersion reaction equations (ADRE) for mixing-induced reaction as well as for conservative (non-reactive) transport. For the conservative transport, the model fit the data well for the homogeneously packed domain. The model simulated precipitation but severely underpredicted the clogging process and the resulting changes in concentration

distribution. The authors attributed this to the use of a continuum model in the presence of pore-scale reactions, highlighting the importance of the relationship between pore-scale and continuum-scale. For the conservative model, they found the longitudinal dispersion to only have an influence on the mixing predictions during the early stages while the original fluid in the model was being pushed out. Transverse dispersion played a more important role overall since it controls the mixing process, similar to the findings of the model used by Acharya et al.²⁴

A predictive numerical model based on diffusion of HCO_3^- anions from a HCO_3^- -containing resin towards Ca^{2+} -rich soil was used by Kirk et al. to study the interplay between kinetics and transport.²⁶ Concentration-distance profiles in soil for HCO_3^- , Cl^- and soil acidity were determined by solving diffusion equations. The concentration profiles determined by the model aligned well with soil experiments conducted by the authors. Sensitivity analysis showed that the amount and spread of precipitation was impacted by factors that affect the reactant transport rate and the precipitation rate constant.

Yoon et al. studied transverse mixing-induced precipitation in micromodels and developed a numerical model to simulate micromodel experiments.²⁷ The model consisted of three coupled modules: a LBM based Stokes flow module, a finite volume reactive transport module for multicomponent advection and diffusion, and a precipitation/dissolution pore-scale crystal growth module to determine pore-space filling by precipitate. The effective diffusion coefficient and the reactive surface area were identified as important parameters for prediction of precipitation and dissolution. The model was able to successfully capture the precipitation process (rate and amount) but fell

short in explaining the dissolution extent that is attributed to reduced mixing and pore blockage.

Acharya et al. used LBM to simulate dispersion and reaction during transverse mixing in a 2-dimensional porous medium.²⁴ Their model showed that at larger Peclet numbers (ratio of advection and diffusion rate, Pe), the fluid traveling distance was longer (traverse) for the transverse dispersion coefficient to stabilize. They also showed that transverse dispersion coefficients yield a better estimate of the reaction product than longitudinal dispersion coefficients, as transverse dispersion is driven by molecular diffusion and is therefore weaker than longitudinal dispersion.

Tartakovsky et al. used SPH to study the effects of different Peclet (Pe) and Damköhler (Da) numbers on concentration distributions and precipitate formation in fractured and porous media.²¹ Pe and Da are dimensionless numbers with application in reactive transport. The Da is the ratio of reaction rate to transport rate. Tartakovsky et al. proposed a new transport coefficient (K) for the reaction term in the advection-dispersion equation. K is a function of Pe and Da and varies from 0 to 1. $K \rightarrow 0$ when Pe is small and $Da \rightarrow \infty$, and the solute concentrates in the middle of the pores causing more precipitation in areas where the supersaturates solute is injected and along preferential flow paths. Conversely, $K \rightarrow 1$ when Pe is large and $Da \rightarrow 0$, leading to a more uniform pore-scale distribution of the solute causing more uniform precipitation in the porous media.

PNM was used by Bekri et al. to simulate single phase flow with mineral reaction in sandstone.² The porosity-permeability relationship was characterized at the core-scale in a cubic lattice pore network using the effect of geometrical changes induced by

precipitation on solute velocity, dispersion, mean reaction rate and the porosity-permeability relationship. The dependence of the reactive-transport properties on two dimensionless numbers (Pe and $PeDa$) was evaluated. $PeDa$ is the dimensionless product of Peclet and Damköhler numbers. At high Pe and $PeDa$ values, reaction occurs predominantly along preferential flow paths as the reaction time scale is shorter than the diffusion time scale. Contrastingly, at low values of $PeDa$ (regardless of Pe), the process is reaction limited and faster diffusion (relative to reaction) allows for uniform diffusive spread of solute resulting in uniform reaction. As a result, variations in permeability are low at low Pe and $PeDa$ values.

Li et al. used regularly latticed 3D PNMs that represented consolidated sandstones containing anorthite and kaolinite in varying amounts.²⁸ The different models had different spatial distributions of reactive minerals. In particular, the common scaling problem between pore-scale models and continuum-scale models was explored by comparing pore-scale and continuum-scale model reaction rates to corresponding reaction rates from continuum-scale models that ignore pore-scale effects. This work defined a parameter $\eta = \frac{\text{Continuum-scale reaction rate}}{\text{Pore-scale reaction rate}}$ as a measure of the scaling effect from pore-scale heterogeneities. If η is significantly different than 1, the pore-scale heterogeneities have a significant effect on reactive transport and empirical data (collected as averaged over the porous medium) should not be used directly in the model. The model comparisons showed that in the presence of abundant reactive minerals, scaling effects were very small and differences in spatial orientation of minerals did not significantly affect reaction rates.

Formation of mineral clusters or accumulation of clusters parallel to flow caused large scaling effects, with scaling effects increasing with increasing reactive cluster size.

Biological

In biological reactive transport modeling, biological growth kinetics and effects on transport need to be considered in addition to solute consumption and product formation. Zhang and Klapper presented a mathematical model for the MICP process in a rectangular domain with a slow fluid time scale (biofilms are shown to have long elastic retention times of approximately 18 minutes²⁹), enabling the biofilm to be approximated as a viscous fluid³⁰. Dykaar and Kitadinis developed a model for a reactive compound undergoing biological degradation in porous media, to determine how pore-scale mechanisms affect transport and reaction at the macro scale.³¹ Significant driving factors that were considered for the model included pore water velocity, spatially variable diffusion in the water and biofilm and volumetric reaction rates in the biofilm.

After determining the microscale transport equations, macroscale parameters were estimated using the moment method. Using centered finite differences, the eigenvalue problem for a unit cell was discretized and then solved using a Quasi-Minimal Residuals (QMR) algorithm. The solved eigenfunction served as the normalized solute distribution within the pore geometry as the basis for the effect of microscale processes on long-term mass distribution within the pores which ultimately drives macroscale concentration distributions. The model successfully calculated macro-scale transport coefficients based on microscale input parameters, in the form of the dimensionless Da and Pe . At a given Da , the peak concentration decreases with increasing Pe , suggesting that higher velocities

facilitate bulk fluid mixing which in turn reduces mass transfer limitations to the biofilm. Additionally, at low Pe , the highest solute velocity occurs in the center of the pore chamber with steep gradients towards the edges and pore throats where the biofilms consume the solute. The model predicted that the biofilm in the pore throats is more reactive relative to an equivalent mass of biofilm in the pore chamber, owing to the smaller gradients in solute velocity in the pore throats. At a constant Pe , higher Da correlated to higher reaction rate and poorer bulk fluid mixing leading to development of mass transfer limitations. At the macroscale, velocity has a higher effect on the macroreaction rate as Da increases. This is because at higher Da , the process becomes increasingly diffusion limited and a higher velocity fluid provides the reactive biofilms with more solute. This model successfully linked microscale processes to macroscale fluid properties and showed that relative solute consumption rates differ within a pore. As a result, relatively small changes in biofilm volume could lead to major fluid flow changes leading to significant macroscale effects.

Barkouki et al. modeled bacterially driven calcite precipitation in batch and continuous flow conditions, incorporating geochemistry by using TOUGHREACT (a geochemical transport simulation program) and PHREEQCII (an aqueous geochemistry program). The model was calibrated with experiments using *Sporosarcina pasteurii* in 0.5 m long, 2.5 cm radius columns filled with sand. Inverse modeling was performed using UCODE with pH and calcite abundance data from collected experimental samples. Ureolysis was modeled in TOUGHREACT v2 using a previously published kinetic model by Fidaleo and Lavecchia.³² In the column simulations, flux boundary conditions were applied to both ends of the columns. The calcite growth that the model predicted showed a

close correlation to shear wave velocities obtained from the columns attesting to shear wave velocity being a good measure of soil stiffness. The models also showed that for the given experimental conditions, a barrier of high calcite content can form close to the inlet during continuous flow while with increasing distance, the calcite content decreased. Contrastingly under pulsed flow conditions, calcite distribution was more uniform. Bacterial distribution was concluded to play a major role in controlling calcite precipitation rates. However, characterization of the spatial bacterial distribution was not the focus of these experiments and was thus recommended to achieve better model calibration for future models.

Ebigbo et al. developed a numerical model for calcium carbonate precipitation via bacterial ureolysis consisting of water and carbon dioxide as fluid phases, and porous medium, biofilms, and calcite precipitates as solid phases.³³ The model was calibrated using vertical sand-packed columns as described by Cunningham et al. The model showed higher calcium deposition efficiency when biomineralization periods were balanced with bacterial resuscitation as longer biomineralization periods led to reduced precipitation from reduced ureolysis/bacterial inactivation. This is similar to the injection strategy experimental findings subsequently published by Tobler et al.¹⁰ Hommel et al. expanded upon the model presented by Ebigbo et al. by including updated literature values for model parameters and ureolysis kinetic data.³⁴ Additionally, inverse modeling was implemented instead of the trial-and-error modeling used by Ebigbo et al. Modeled calcite distributions were different than experimental distributions and the need for investigations into biofilm-based parameter values was recognized. Experiments on biofilm-based ureolysis kinetics

of *S. pasteurii* were recommended and were subsequently published but have not been incorporated into improved reactive transport models.³⁵

Planar fracture, pore-scale sand media and field-scale MICP modeling was recently presented by Minto et al.³⁶ Their model, called biogroutFoam, advanced MICP modeling efforts by accounting for bacterial attachment in addition to reactive transport and subsequent precipitation. Bacterial attachment had a major impact on precipitate distribution and was able to reproduce the hydrodynamic effects observed by El Mountassir. Their ability to incorporate real-world subsurface properties by using information from geophysical surveys expands the applicability of MICP as a biotechnology.

During application of MICP, bacterial suspensions and dissolved reactants are often pumped into the subsurface. From the point of injection, the fluids flow out radially into the surrounding pore space. Studying MICP under a radial flow regime can provide additional support to optimizing MICP application as it will eliminate the assumption of identical behavior in all directions in a system as heterogeneous as porous media.

Radial flow

Radial flow can be described as fluid flowing along the spokes of a wheel, either toward or away from the center. Radial flow is more complicated than steady 1- or 2-dimensional flow. In radial flow, the linear velocity of the fluid decreases as the fluid flows radially outward. Under a constant volumetric flow rate (Q) in a system ' h ' deep, the linear

fluid velocity (v) is inversely proportional to the radius (r) given by: $v = \frac{Q}{2\pi r * h}$. Thus,

higher residence times are encountered at larger distances from the inlet.

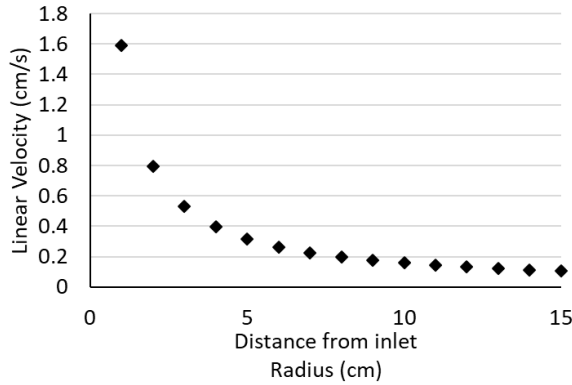


Figure 1: Linear velocity vs radial distance for an example flow rate of 10 mL min^{-1} into a radial flow environment that is 1 cm deep.

This spatially varying residence time makes radial flow a more complex transport problem to be modeled than linear flow. The extent of reaction is affected by the residence time, making the radial coordinate and the corresponding flow velocity important considerations in predicting the reaction.

Radial flow is often encountered in subsurface environments such as around wells⁹. In radial flow through porous media, Darcy's law can often be applied and can be written as:

$$Q = \frac{k}{\mu} * A * \left(\frac{dP}{dr} \right) \quad \text{Equation 1}$$

where

k is the permeability of the medium in L^2

μ is the fluid viscosity in $\text{ML}^{-1}\text{T}^{-1}$

A is the cross-sectional area through which flow is occurring in L^2

$\frac{dP}{dr}$ is the differential pressure along the radius in NL⁻³

General Transport Equation for Ground Water

The groundwater flow equation combined with Darcy's law results in the following partial differential equation:

$$S_s \frac{\partial h}{\partial t} = -\nabla \cdot (-K \nabla h) - G \quad \text{Equation 2}$$

where

S_s is the specific storage

$(-K \nabla h)$ is the flux given by Darcy's law

h is the hydraulic head [L]

t is time [T]

K is the hydraulic conductivity [LT⁻¹]

G is the source/sink term

Specific storage is a physical property of aquifers that describes the capacity to release groundwater. It is the amount of water that is released from storage per unit change in h on a unit mass/volume basis. Storativity (expressed as S), a related property, is the volume of water released per unit change in h and area. For confined, homogeneous aquifers,

$$S = S_s * b \quad \text{Equation 3}$$

where b is the aquifer thickness [L]

Hydraulic diffusivity α is defined as $\frac{K}{S_s}$. Using this and assuming that the hydraulic conductivity is isotropic, Equation 2 can be written as

$$\frac{\partial h}{\partial t} = \alpha \nabla^2 h - G \quad \text{Equation 4}$$

G is now the source/sink term divided by the specific storage

Thus, when considering steady state (no sources or sinks, no time dependence), Equation 4 simplifies to the Laplace equation. Assuming radial symmetry (no deviation with angle) and no flow in the z -direction, the steady state Laplace equation becomes:

$$0 = \left[\frac{\partial^2 h}{\partial r^2} + \frac{1}{r} \frac{\partial h}{\partial r} \right] \quad \text{Equation 5}$$

The Theis model is considered to be the simplest model for fluid transport in a radial geometry.³⁷ It models 2-D flow to a point source from an infinite reservoir based on simplifying assumptions. Some of these assumptions are:

1. Flow in the aquifer is described by Darcy's law
2. The aquifer is homogeneous, isotropic and confined
3. The well is of zero radius such that no water can be stored in the well.
4. Flow of groundwater is perfectly horizontal

The Theis equation is given by

$$s = \frac{Q}{4\pi T} W(u) \quad \text{Equation 6}$$

$$u = \frac{r^2 S}{4Tt} \quad \text{Equation 7}$$

where

s is the change in hydraulic head

Q is the volumetric discharge of ground water [L^3T^{-1}]

T is the transmissivity [L^2T^{-1}]

u is a dimensionless time parameter

r is the radius from the center of the well [L]

$W(u)$ is called the well function in hydrology and is the exponential integral Ei

Transmissivity is given by

$$T = K * b = \frac{k * \rho * g * b}{\mu} \quad \text{Equation 8}$$

where

K is the hydraulic conductivity [LT^{-1}]

b is the aquifer thickness [L]

ρ is the fluid density [ML^{-3}]

g is the gravitational acceleration [LT^{-2}]

Transmissivity and storativity values around a wellbore can be calculated using the Theis equation using observed values.

The Thiem equation for confined aquifers is based on steady state radial flow of groundwater to a pumping well. Since the movement of the water through the ground near wells resembles flow through porous media, Darcy's law applies. It is assumed that flow is steady, i.e., the well is pumped at a constant rate and there is no change in the drawdown with time. Combining Darcy's law with the definition of transmissivity (hydraulic conductivity * aquifer thickness), yields

$$Q = 2 * \pi * r * T * \left(\frac{dh}{dr} \right) \quad \text{Equation 9}$$

where

h is the head at radial distance r [L]

$\left(\frac{dh}{dr} \right)$ is the hydraulic gradient

Manipulation and integration of Equation 9 gives the Thiem equation (Fetter, Applied Hydrology, 3rd edition, pg. 217)

$$T = \frac{Q}{2 * \pi * (h_2 - h_1)} * \ln \left(\frac{r_2}{r_1} \right) \quad \text{Equation 10}$$

Equation 10 shows that during steady state flow to a well, the head varies logarithmically with radius.

In terms of pressure, separating and integrating Equation 4 with $A = 2 * \pi * r * b$ gives

$$Q = \frac{2 * \pi * k * b * (P_2 - P_1)}{\mu * \ln \left(\frac{r_2}{r_1} \right)} \quad \text{Equation 11}$$

The Thiem equation is related to Equation 11 by the definition of liquid pressure ($P = \rho * g * h$).

For radially symmetric 1-D horizontal flow, discretization can be used to study lateral conductance, vertical conductance and storage capacity. (Bennett, Technical Training notes in Ground Water Hydrology - Radial Flow to a Well, USGS)

Chemically driven precipitation in radial reactive transport

Detailed analytical solutions have been presented for radially symmetric reactive transport³⁸ as well as for transient radial flow³⁹. Wang and Zhan developed a semi

analytical-numerical model for radial transport of a reactive solute which contributed to known solutions by including aquitard advection⁴⁰. Jun-qi and Ci-qun obtained an analytical solution to partial differential equations for transport in the radial dimension using Laplace transforms. They developed a solution that can predict change of concentration and concentration distribution in porous media with dual porosity⁴¹. Dual porosity is important to consider while modelling groundwater flow in fractured rock because in most cases the subsurface has a pore matrix as well as fractures. Fluid exchange between the pore system and the fracture system is important. In their solution, they considered solute decay as well as mass exchanges between the pores and fractures without intersystem adsorption. Laplace transforms were also used by Barker to develop a generalized radial flow model⁴². The model solutions can readily be adjusted for dual porosity environments. The Laplace transform solutions to flow equations give relationships between source head, injection rate and fracture head. Barker also considered different cases for flow. Under constant flow rate, the solutions for infinitesimal flow simplified to the Theis equation for infinite flow region and to the Thiem equation for a fixed head boundary. In few parameters, this generalized radial flow model can be used for various flow patterns as well as different geometries. However, the model has some limitations - the dimension is not an intrinsic hydraulic property and the model is unable to be applied to spherical source geometry or when anisotropy is present.

Broyda et al. developed a stochastic model to predict the fate of a solute reacting heterogeneously with a solid phase under a radial flow regime.⁴³ This was done by computing a statistical distribution of the system. Spatial distributions of advective velocity

and the Damköhler number were identified as important factors controlling the probability distribution function of the reacting solute concentration.

Radial flow reactive transport was modeled in terms of radially traveling acid dissolving carbonate rocks and forming wormholes in two studies. The first study, by Kalia and Balakotaiah, used a two-scale continuum model (Darcy-scale and pore-scale physics) to simulate dissolution of carbonate rock by reactive transport of acid in a 2-dimensional radial flow domain.⁴⁴ A power law relationship between reaction penetration depth and injection time was derived based on the simulations. Interestingly, the injection rate did not affect the number of wormholes (tubular cavities formed by dissolution) that developed in the system, but the heterogeneity of the porous media was found to impact the wormhole density. As observed in previous wormholing studies, Kalia and Balakotaiah also noted the patterns of wormholes in their simulations to be of a fractal nature.

The second study by Liu et al.⁴⁵ used fractal geometry theory in their two-scale continuum model for a 3-dimensional radial flow domain. At low acid injection rates, acid was consumed instantaneously whereas at high injection rates, the acid does not have sufficient residence time to react and therefore leads to farther extending dissolution. This is comparable to MICP studies where low fluid injection rates led to inlet plugging whereas a higher transport rate led to precipitation at larger distances.⁴⁶ The model also showed that higher the heterogeneity of the solid medium, more branches the wormholes formed by dissolution, similar to the findings of Kalia and Balakotaiah. The branching of wormholes could be considered analogous to preferential flow path formation due to precipitate deposition.

Biologically driven precipitation in radial reactive transport

There is limited literature on modeling MICP under radial flow conditions. Both the studies outlined here are numerical modeling approaches based on field-scale MICP experiments. Cuthbert *et al.* used data from boreholes drilled 27 m deep into a fractured dacite formation. MICP was promoted in a fracture 25 m below ground level by flowing MICP-promoting fluids through one borehole and abstracting through another.

Reactive transport was modeled in COMSOL using the convection-diffusion differential equation with a reaction term⁴⁷.

$$\frac{\partial c}{\partial t} = \nabla \cdot (D \nabla c) - \nabla \cdot (vc) + R \quad \text{Equation 12}$$

where

$\frac{\partial c}{\partial t}$ is the change in concentration over time

$\nabla \cdot (D \nabla c)$ is the diffusion term

$\nabla \cdot (vc)$ is the convection term

R is the reaction term (mass flux)

The COMSOL model considered not only flow and reactive transport equations but also bacterial accumulation and encapsulation during MICP.

Modeling indicated that bacterial accumulation was strongly centered around the injection borehole. A significant decrease in transmissivity was achieved not only close to the injection borehole (99% decrease) but also at 2 m away from the injection point (35% decrease). A recommendation was made for developing ways to control the bacteria distribution to ensure an evenly distributed transmissivity reduction over the entire region of interest.

An attempt at simulating MICP at the field scale was made by Hommel et al. through modeling MICP fracture sealing under radial flow in a 3-dimensional domain.⁴⁸ A horizontal fracture in a cylindrical sandstone core was sealed using MICP under radial flow,⁴⁹ and the model calibrated by these experiments (with a domain of a 3-dimensional sector of the cylindrical core) was used to simulate field-scale MICP. In their study, the model accurately predicted increased injection pressure with MICP fracture sealing, as observed in a field-scale MICP experiment by Phillips et al.⁵⁰

Outlook and introduction to thesis chapters

Ultimately, during field applications of MICP, control of the process in real time will be necessary. Modeling can inform on-site control through simulations wherein parameters can be changed rapidly to guide operational decision-making in real time. Thus, reactive transport models that can accurately capture MICP and its chemical and physical impact on subsurface properties can make this technology more effective by saving time, money and effort spent on sample collection, expensive instrumentation and data analysis.

A vital step in modeling is comparing modeling results to experiments to calibrate, validate and improve models. To this end, experiments need to be optimized to provide spatial and temporal information. The research presented in this thesis contains meso-scale experimental investigations of MICP under fluid flow conditions. Spatially and temporally resolved measurements and analyses pursued in these research projects lay the foundation for future modeling efforts.

CHAPTER THREE

CONTROLLING THE DISTRIBUTION OF MICROBIALLY PRECIPITATED CALCIUM CARBONATE IN RADIAL FLOW ENVIRONMENTS

Contribution of Authors and Co-Authors

Manuscript in Chapter 3

Author: Neerja Zambare

Contributions: Performed all experiments including reactor set up, operation, and analysis. Envisioned graphics, table design and wrote and revised manuscript.

Co-Author: Ellen Lauchnor

Contributions: Identified major topics for review during development of manuscript and aided in data analysis and interpretation. Contributed to writing and revision of the manuscript in the form of comments and feedback.

Co-author: Robin Gerlach

Contributions: Identified major topics for review during development of manuscript and aided in data analysis and interpretation. Contributed to writing and revision of the manuscript in the form of comments and feedback.

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Controlling the Distribution of Microbially Precipitated Calcium Carbonate in Radial
Flow Environments

Neerja M. Zambare^{a,b}, Ellen G. Lauchnor^{a,c}, Robin Gerlach^{a,b}*

^a Center for Biofilm Engineering, Montana State University, Bozeman, MT 59717

^b Department of Chemical and Biological Engineering, Montana State University,

^c Department of Civil Engineering, Montana State University,

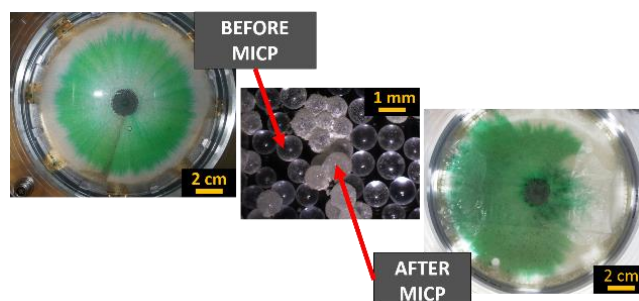
Bozeman, MT 59717

Abstract

Bacterially driven reactions such as ureolysis can induce calcium carbonate precipitation, a well-studied process called microbially induced calcium carbonate precipitation (MICP). MICP is of interest in subsurface applications such as sealing leaks around wells. For effective field deployment, it is important to study MICP under radial flow conditions, which are relevant to near-well environments. In this study, a laboratory-scale radial flow reactor of 23 cm diameter, with a 1 mm glass bead monolayer serving as porous medium, was used to investigate effects of fluid flow rates and calcium concentrations on the mass and distribution of MICP by the ureolytic bacterium *Sporosarcina pasteurii*. Experiments were performed at hydraulic residence times of 14, 7 and 3.5 minutes and calcium-to-urea molar ratios of 0.5:1, 1:1, 2:1. The total amount of CaCO₃ precipitated in the reactor increased with increasing residence time and with decreasing Ca²⁺:Urea molar ratios. Increased bacterial attachment and increased CaCO₃ precipitation were observed with distance from the center inlet of the reactor in all

experiments. More uniform calcium distribution was achieved at lower flow rates. The relationship between reaction and transport rate (i.e. the Damköhler number) is identified as a useful parameter for the prediction of MICP in radial flow environments.

Abstract Art

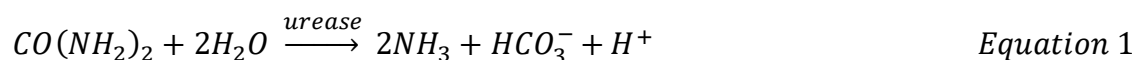


Keywords

Ureolysis, Radial Flow, Reactive Transport, Damköhler number, Biomineralization

Introduction

Microbially induced calcium carbonate precipitation (MICP) is a potentially environmentally friendly technique used for subsurface applications such as repairing fractures in concrete,^{51, 52} strengthening soil,⁵³⁻⁵⁵ and reducing leakage of geologically stored carbon dioxide.^{9, 56-58} Ureolysis-driven MICP is the most commonly studied process in biological calcium carbonate (CaCO₃) precipitation (Equation 1)^{7, 59-61} and is catalyzed by the enzyme urease (urea amidohydrolase).⁶²



In the presence of calcium, ureolytic bacteria are capable of inducing CaCO₃ precipitation via a rise in alkalinity during ureolysis (Equations 2 and 3, and SI).^{10, 63, 64}



Sporosarcina pasteurii is an alkaliphilic soil bacterium^{61, 64, 65} that produces up to 1% cell dry weight of urease,^{10, 63-68} making it a model organism for MICP. In comparison, Jack Beans, one of the most important plant sources of urease, have been reported to contain only between 0.07% and 0.14% of its dry weight as urease⁶⁹.

In porous media, the constant interplay between biomass presence, substrate transport, and microbial reaction determines $CaCO_3$ distribution during MICP. Precipitation/dissolution reactions significantly modify the physical properties of porous media.³ Biomass itself can lead to constriction or clogging of pore throats,^{31, 65, 70} blocking flow and/or leading to the formation of preferential flow paths. Resulting variations in reactant transport can affect the rate and extent of MICP.^{3, 71} Cuthbert et al. demonstrated in a modeling study that the mass of $CaCO_3$ precipitated is strongly dependent on the distribution of attached bacteria.⁷ In a flow system, $CaCO_3$ precipitation would be affected by bacterial distribution and mass transport. This is especially true for radial flow wherein fluid velocity decreases with distance.

Numerous studies have been performed investigating ureolysis and $CaCO_3$ precipitation kinetics,^{59, 62, 63, 72} controls on ureolysis and $CaCO_3$ precipitation,^{7, 73, 74} $CaCO_3$ morphology,^{7, 75, 76} and ureolytic MICP potential of environmental enrichments and isolates.^{72, 77-79} The importance of controlling the spatial distribution of precipitates in a target environment has been recognized.^{9, 10, 33, 65, 80-82} Various strategies have been proposed to control the balance between supply and precipitation of calcium in the

subsurface such as controlling fluid flow rates⁵⁴ and substrate concentrations,^{9,73} injecting MICP substrates and microbial catalysts in sequence,^{10,33} and controlling ureolytic bacteria distribution.⁸⁰ Many flow MICP studies have been conducted in porous media systems with uniform flow regimes, or without considering a radial flow component.^{8, 11,83} The research presented here focuses on MICP in porous media under radial flow. Many MICP applications include delivery of microorganisms and substrates through injection wells, from where fluids may travel radially outward into the surrounding subsurface. This makes porous media radial flow a representative environment for field applications.

Radial flow fields exhibit decreasing velocity with increasing radial distance. Under a constant volumetric flow rate, linear fluid velocity is inversely proportional to the radius, which leads to increased fluid residence time with radial distance. There is less total pore volume available per radial distance increment in the area closest to the injection point compared to areas farther radially outward. For a given amount of bacterial cells and/or CaCO_3 precipitates, the inlet region would plug faster than any other region under a radial flow regime, potentially significantly influencing flow into areas farther from the inlet by altering flow paths. MICP studies have shown inlet region plugging due to precipitates.^{10, 54} A modelling study on reactive transport and mineral precipitation by Tartakovsky et al. showed that as precipitation proceeded, fluid flow was increasingly controlled by the entrance region.²¹ Linear velocity changes in radial flow can exacerbate this effect. Quantifying pore-scale reactions under radial flow can also be challenging due to heterogeneous microbial distribution leading to spatially non-uniform solute consumption and significant macro-scale consequences.³¹

Larger scale MICP applications have shown significant reduction in transmissivity⁴⁷ and increase in granular soil stiffness.⁸⁴ Cuthbert et al. observed in their field study that besides urea and calcium concentrations, bacterial distribution predominantly controlled the spatial distribution of precipitates.⁴⁷ The ability to control precipitate amount and distribution has the potential to make MICP a highly effective biotechnology tool. Under flow conditions, parameters like media injection rate and concentration can be used for such control. The influence of these parameters on MICP under conditions that mimic target environments (i.e. radial flow) has, to our knowledge, not been studied in detail.

The goals of this work were to understand the effects of two controllable parameters: (1) fluid flow rate and (2) influent dissolved calcium concentration, on MICP under radial flow conditions. The focus on these parameters provided insight into strategies to control MICP efficiency under field-relevant conditions. Reactive transport was examined using the dimensionless Damköhler number. The distribution of CaCO_3 precipitates in the reactor was assessed in angular and radial dimensions. A separate bacterial distribution study was conducted to quantify the initial distribution of *S. pasteurii* in the laboratory system prior to MICP.

Experimental Methods

Reactor Set-up

The radial flow reactor (RFR) consisted of a polycarbonate plate with a 23 cm diameter, 1 mm deep circular etching (Figure 1), filled with a tightly-packed monolayer of glass beads (1 mm diameter, Biospec Products, Oklahoma, USA). The glass beads formed

a porous medium in the etched space between the polycarbonate plate on top and a glass plate on the bottom (Figure 1.a).

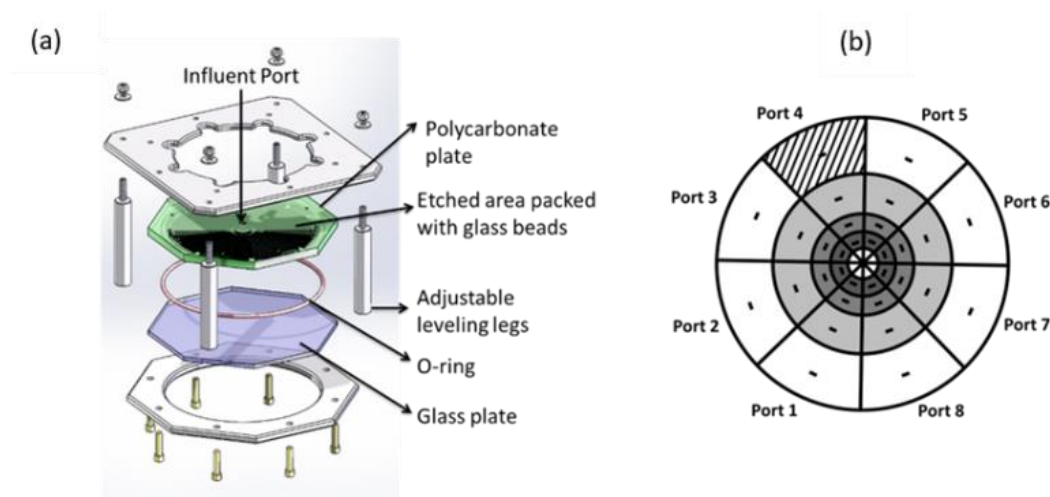


Figure 1: (a) Radial Flow Reactor assembly ‘explosion’ drawing. (b) 2D schematic showing the 32 volume elements for precipitate analysis. Note that there are 4 radial distances (2, 3, 5 and 9 cm from the inlet) and 8 angles. The 32 sampling locations are indicated by dashes (-). As an example, the port 4 volume element at 9 cm from the inlet is the element around 11 o’clock marked with lines.

The influent port (inlet) was located at the center of the polycarbonate plate. An additional port was installed using a ‘Y’ connector in the tubing immediately upstream of the inlet to inject tracer fluid or bacterial inoculum. Gravity effects on fluid transport were minimized using adjustable leveling legs. Eight effluent ports were spaced equally around the circumference. The eight effluent ports were designed to collect fluid traveling in the eight respective sectors to facilitate spatially resolved sampling of the system, which would not have been possible easily for a free-draining system. Although the fluid exiting each sector was pooled into one collection port, the flow within the beadpack was radial (*cf.* Figure A1.a). Tubes connected to effluent ports were fitted with needles (23G, Becton

Dickinson, New Jersey, USA), providing equal backpressure and allowing for the collection of effluent fluids in separate lanes of an 8-lane collection well.

Tracer Studies

Tracer studies were performed before each experiment (flow rates in Table 1) using green food dye (Western Family, Oregon, USA) in de-ionized water to ensure uniformly radial flow within the reactor before injecting bacteria. Absorbance (630 nm) of effluent samples was measured over time using a BIOTEK Synergy HT spectrophotometer to characterize dye transport and identify the peak time of the tracer breakthrough curves. Towards the end of each MICP experiment, an additional tracer study was performed, through which flow patterns were visually compared to those of the clean reactors.

Media Preparation

Three types of liquid media were used in this study: (1) resuscitation medium consisting of Brain Heart Infusion medium (BHI, Fisher Scientific) amended with 0.33 M urea; (2) bacterial growth medium (used during flow experiments) consisting of 3 g L⁻¹ Difco Nutrient Broth (Becton Dickinson, New Jersey, USA) amended with 0.33 M urea and 0.19 M ammonium chloride (Fisher Scientific, New Hampshire, USA) adjusted to pH 6-6.3 prior to filter-sterilizing with 0.45 µm pore size bottle-top filters (ThermoFisher Scientific, Massachusetts, USA); (3) calcium mineralization medium (CMM) was prepared by adding the appropriate mass of calcium chloride dihydrate (CaCl₂•2H₂O, Fisher Scientific, New Hampshire, USA) to pH-adjusted growth medium as done previously by us and others.⁸⁵⁻⁸⁷ Nutrient broth was maintained in the mineralization medium to maintain

bacterial activity during the mineralization phase, according to protocol followed in other successful MICP experiments in literature.^{50, 78, 87}

Inoculum Preparation

A 1 mL aliquot of a frozen stock culture of *S. pasteurii* (ATCC 11859) was added to 100 mL resuscitation medium and incubated on a horizontal shaker at 150 rpm at 30°C for 24 hours. A 1 mL aliquot of this overnight culture was transferred into 100 mL fresh BHI+0.33 M urea medium (to reduce glycerol carry-over from the frozen stock) and incubated for 24 hrs under the same conditions before harvesting the bacteria by centrifuging at 2964-x-g for 10 minutes at 4°C. The supernatant was decanted, the cell pellet re-suspended in bacterial growth medium (as defined above), and the culture was centrifuged again. Re-suspension and centrifugation were repeated once more, after which cells were diluted in fresh bacterial growth medium to a final OD₆₀₀ of 0.4 (using flat-bottom 96-well plates containing 200 µL per well, reference was the blank well OD of 0.044), leading to a cell density of $4\text{--}7 \times 10^8$ CFU*mL⁻¹. Resuspension was performed using growth medium (and not resuscitation medium) to introduce the cells to the medium used for the experiments and thus reduce stress that could develop from sudden changes in chemical environments. The disinfected RFR (protocol in SI) was inoculated with 120 mL of the final culture (equal to slightly more than the system volume) and following inoculation all reactor tubing was clamped for an 18-hour (overnight) no-flow period to promote bacterial attachment.

Reactor Operation

After the no-flow period, bacterial growth medium was pumped through the reactor at 5 mL min^{-1} for 24 hours. This growth phase was maintained constant in all experiments to minimize differences in bacterial distribution and growth. Flow through the effluent ports was checked visually based on the volume collected in the 8-lane collection well. During the latter half of 2F and 0.5Ca, flow from some effluent ports was blocked and these experiments were terminated when this occurred.

For the bacterial distribution study, the reactor was destructively sampled after the growth phase. In MICP experiments, CaCO_3 precipitation was initiated following the growth phase by flowing CMM continuously (flow rates and calcium concentrations in Table 1).

Table 1: Experimental parameters including flow rates, calcium concentrations (Ca^{2+}), urea concentrations, and duration of each experiment. The ‘designed hours’ in Table 1 reflect the calculated time necessary to inject 0.87 moles of calcium. Two experiments (2F and 0.5Ca) were terminated earlier than the designed duration due to plugging by CaCO_3 .

Experiment Description	ID	Flowrate ($\text{mL} \cdot \text{min}^{-1}$)	Ca^{2+} (M)	Ca^{2+} :Urea molar ratio	Duration of Mineralization phase (hours) (actual/designed)
Bacterial Distribution	--	5	--	--	-- *Growth phase only*
Base Case	BC	5	0.33	1:1	9/9
Half Flow	0.5F	2.5	0.33	1:1	18/18
Double Flow	2F	10	0.33	1:1	3/4.5
Half Calcium	0.5Ca	5	0.165	0.5:1	16/18
Double Calcium	2Ca	5	0.66	2:1	4.5/4.5

This mineralization phase was conducted at three flow rates and three calcium concentrations. The mineralization phase duration was chosen to inject an equal amount of calcium in each experiment (0.87 moles), to facilitate direct comparisons between experiments. This led to different calcium-to-urea molar ratios in the CMM for the three calcium concentration variation experiments. Effluent samples were collected at intervals throughout the mineralization phase from all effluent ports.

Bacterial Distribution Study Sampling

At the end of the growth phase, 100 mL of NH_4Cl solution (0.19 M) was pumped through the reactor at 5 mL min^{-1} to remove unattached and loosely attached cells. This was done to mimic the bacterial distribution at the beginning of the mineralization phase, initiated through the injection of CMM, which has a base of 0.19 M ammonium chloride

and also likely led to the wash-out of unattached and loosely attached cells at the beginning of the mineralization phase. The reactor was drained and opened; bead samples (0.1 g) were collected at locations 2, 3, 5 and 9 cm from the inlet in the eight directions of the effluent ports (Figure 1.b). There is no universal method that ensures complete desorption of cells from porous media⁸⁸ but Tween 80 (Polysorbate 80) has been suggested by multiple studies as effective at dislodging attached bacteria.⁸⁹⁻⁹¹ Therefore, all samples were vortexed for 1 minute with 5 mL phosphate buffered saline solution (PBS) amended with 5 g L⁻¹ Tween 80. While it is unclear whether all cells on the beads were desorbed, it was expected that the same fraction of cells was desorbed from each sample when applying the same method to all samples. This likely resulted in a consistent underestimate of the cell count but provided an estimate of the spatial distribution of cells. The cell suspension was serially diluted in PBS and plated onto BHI+0.33 M urea agar to determine viable cell concentrations (Colony Forming Units or CFUs). The beads were washed with de-ionized water and dried at 40°C to a constant weight to determine dry weight; bead-specific cell concentrations were calculated as CFU per gram of dry glass beads. This bacterial distribution study was performed without mineralization; desorbing and quantifying viable cells after mineralization is even more challenging because cells can become entombed in the precipitates. Inoculation and the growth phase were kept constant between all experiments. It was therefore assumed that the bacterial distribution at the beginning of the mineralization experiments was identical to the results from the bacterial distribution study.

Liquid Analyses

Filtered liquid samples from the mineralization phase were analyzed for dissolved ammonium and calcium by ion chromatography using a Metrosep C4 150 mm x 4 mm cation column (Metrohm USA, Inc.) and 20 mM dipicolinic acid at pH 2.7 as the eluent along with conductivity detection (Metrohm 732). A Symphony SB70P pH meter was used for pH measurements on unfiltered samples.

Precipitate sampling

After each mineralization phase, the reactor was drained, opened and the beads were allowed to air-dry overnight. 0.1 g bead samples were collected in triplicate from the 32 locations (Figure 1.b) and precipitates on the beads were dissolved using 10% trace metal grade (TMG) nitric acid (Fisher). After one hour, the acid samples were diluted to 5% using nanopure water (resistivity $\geq 18.2 \text{ M}\Omega \text{ cm}$). Samples were diluted further using 5% TMG-HNO₃ and analyzed using an Inductively Coupled Plasma-Mass Spectrometer (ICP-MS, Agilent 7500ce). The acid-washed bead samples were dried and weighed to calculate bead-specific precipitate mass (mg Ca per gram dry glass beads). Precipitates on the reactor plates and in the effluent tubing were also dissolved using TMG nitric acid and analyzed using the ICP-MS.

Results and Discussion

The RFR porosity was calculated to be 0.43 for the bead pack, excluding the circumferential collection wells. This is lower than 0.476 for ideal packing potentially due to the reactor being slightly bowed out and greater than 1 mm depth in the center and

allowing a slightly denser packing. Tracers provided visual confirmation of uniform radial flow before each experiment (Figure A1.a) and <10% relative standard deviation was observed in the peak time of the 8 breakthrough curves for each tracer study. As an example, the 8 breakthrough curves for a clean reactor tracer study for the base case are provided in Figure A1.d. Stratified flow of tracer fluid in the porous medium was not visually observed and thus transport of tracer was assumed to be uniform in the radial direction. At the end of the experiments, preferential flow paths were observed (Figures A1.b and A1.c). Each experiment showed different patterns due to differences in CaCO_3 distribution between experiments.

Only one colony-type was observed in samples obtained from the reactor effluents. Additionally, the characteristic smell of ammonia and the elevated pH (indicative of ureolysis, see “Aqueous Chemistry” section) were used as indicators that *S. pasteurii* was growing inside the reactor and that the reactor disinfection protocol was successful.

Bacterial Distribution

The bacterial distribution study showed that bacterial cells, reported as CFU/g beads, were not homogeneously distributed. With distance from the RFR inlet, attached cell concentrations increased exponentially (corresponding to the linear increase in log transformed data in Figure 2, $R^2=0.987$). No growth was observed in the PBS + Tween 80 solution used for cell desorption, indicating that this solution was not a source of contamination.

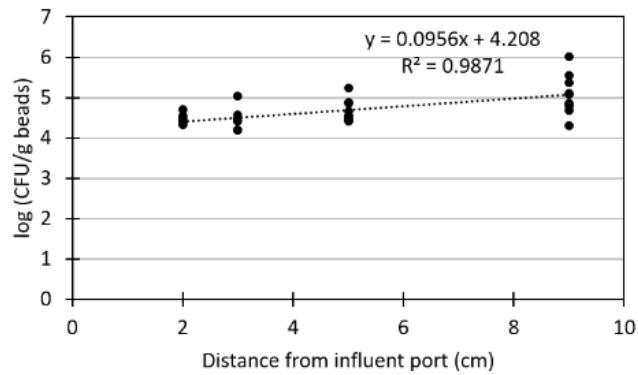


Figure 2: Semi-log plot of CFUs per gram of glass beads at the four radial locations. At each location, data from all 8 angular locations are shown.

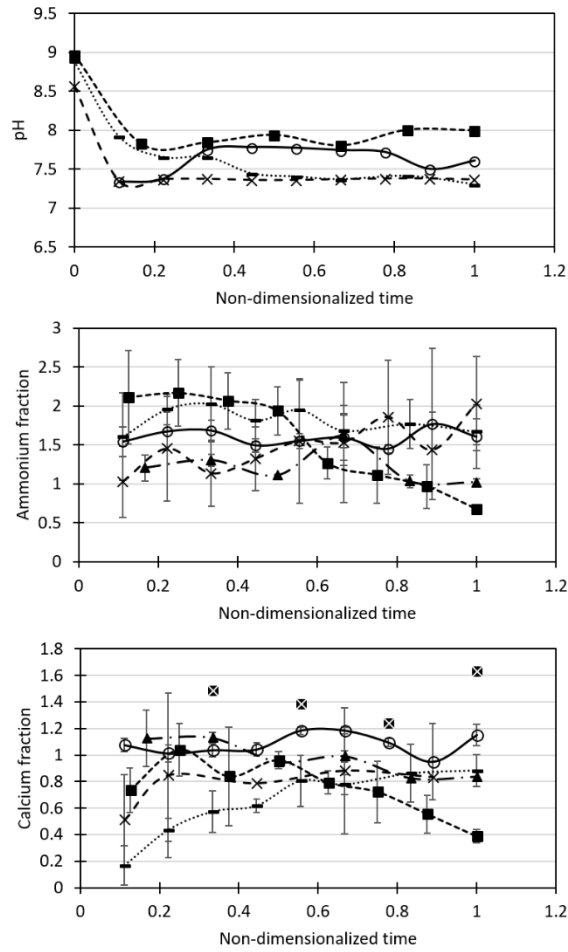
A linear mixed effects model fitted to these data yielded $p < 0.0001$ for distance from the inlet, indicating that with increasing distance, there was significantly greater bacterial attachment. Tukey's pairwise comparison tests showed that only the CFU data at the 9 cm location were significantly greater than those at the other locations.

The angular variance in CFU/gram beads increased with radial distance, indicated by the increased spread of data with increasing distance (Figure 2). There was less angular uniformity in bacterial attachment at larger radial distances. Such radial and angular bacterial distributions can have implications for spatially varying reaction rates.

Shear stress near the injection point is the highest in this radial flow environment, which decreases the ability of cells to attach in this area^{92, 93}, provided that the inoculum injection rate is high enough to avoid inlet plugging by bacteria. A decrease in shear stress with distance will allow for increased attachment (at a given suspended cell concentration and constant injection rate). For larger application environments, it is likely that the number of attached microbes will reach a maximum at a certain distance after which attached cell concentrations will likely decrease due to decreasing suspended cell concentrations.

Aqueous Chemistry

In all experiments, at the end of the growth phase, effluents were spread-plated onto BHI+0.33 M urea agar to verify the presence of *S. pasteurii*. During the growth phase (data not shown), effluent pH increased from approximately 6 to above 8.5 indicating alkalinity production from ureolysis (Equation 2). The pH decreased again during the mineralization phase of each experiment (Figure 3.a), consistent with proton release due to CaCO_3 precipitation (Equation 3). The effluent pH variation between experiments resulted from the extent of ureolysis and precipitation reactions that occurred in the reactor during flow, which varied with both calcium concentration and retention time. Although pH monitoring was not performed during 2F due to short sampling intervals, the effluent pH at the end of the growth phase was 8.9, consistent with ureolysis.



the influent concentration and uncertainties in the calcium concentration measurements. Error bars indicate ± 1 standard deviation between the eight effluent ports.

Ammonium generation, as evident by ammonium fractions greater than 1 in Figure 3.b, also indicated ureolysis (Equation 2). While ammonium volatilization was possible in effluent samples with our methods and would result in underestimation of ammonium production, the increase in effluent ammonium for virtually all samples confirmed that ureolysis occurred, which was the goal of measuring ammonium. One exception is the 0.5Ca data, which decreases in later experimental time, which could be related to the higher pH values and thus increased likelihood of ammonia volatilization. Due to this issue, a

Figure 3: (a) pH, (b) normalized ammonium concentrations and (c) normalized calcium concentrations in the effluent samples during the mineralization phase. BC (O), 0.5F (-), 2F (▲), 0.5Ca (■), 2Ca (×). Due to the variation in total experiment duration, the time is presented in nondimensionalized form. (a) Influent pH was between 6 and 6.3 and effluent pH at the end of the growth phase (and thus beginning of the mineralization phase) had increased to above 8.5; during the mineralization phase the effluent pH was between 7.5-8. At each time point, the pH measurement deviation between the 8 ports was less than 1%. (b) Ammonium fractions above 1 indicate generation of ammonia through the ureolytic activity of the bacteria in each experiment. (c) Fraction of influent calcium close to 1 indicate very low reaction rates. For 2Ca, data points above the quantification limit of the instrument are shown by inverse crosses (white crosses in black boxes) and are likely the result of variations in

quantitative correlation between the extent of ammonium generation and calcium precipitation based on equations 1-3 is not possible. The large error bars are unsurprising because they reflect standard deviations over all effluents and as Figure A1 shows, the flow pattern became non-uniform due to biomass and precipitate accumulation. This likely led to differences in hydraulic residence times, extent of urea hydrolysis and thus ammonium generation. Similarly, effluent calcium concentrations (Figure 3.c) showed high standard deviations caused by fluid residence time changes resulting from preferential flow paths.

Using effluent calcium concentrations from the first sampling time point, initial rates of calcium precipitation were estimated for all experiments (Table A1). The initial precipitation rate was lower at higher flow rates. In fact, the low residence time in 2F was insufficient for any measurable calcium precipitation and an initial rate could not be calculated due to non-statistically significant changes in calcium concentration between influent and effluent samples ($p=0.09$). The initial precipitation rates increased with increasing calcium concentration, with the highest rate observed in 2Ca. This could be due to the higher dissolved calcium concentration in 2Ca leading to a higher saturation index, increasing the likelihood of precipitation relative to the other experiments.

Precipitation Efficiency

Destructive sampling at the end of the experiments revealed that previously loose glass beads were cemented to the glass plate of the reactor and to each other (Figure A2). The calcium recovery in Table 2 reflects the mass balance between the total amount of calcium supplied to the reactor and the calcium detected in the precipitates and the effluent. Lower calcium recovery in 0.5F was attributed to precipitates in the influent tubing which

could not be reliably quantified. The calculations of calcium recovery have some uncertainties ($\pm 20\%$) associated with them, arising from estimating the amount of dissolved calcium exiting the reactor between sampling times. These estimates were based on averaging IC measurements over 8 effluent ports and time intervals which unavoidably resulted in high standard deviations due to changing flow paths. Bead-specific precipitate mass data were used to fit linear mixed effects models with radial distance from inlet and flow rate/concentration as fixed effects. The angular dimension was considered a random effect since the same reactor was used for all experiments, but the eight angular directions were assigned randomly for each experiment. The mass of urea injected in the experiments was also considered a fixed effect because it varied based on the condition tested. ANOVA on the models showed that radial distance from the inlet, flow rate and calcium concentration were all significant factors influencing CaCO_3 precipitation ($p < 0.0001$). The mass of urea injected was found to not be a statistically significant factor for the calcium concentration experiments where urea injected varied by design ($p = 0.9784$).

To analyze flow rate experiments and concentration experiments together, the calcium mass flow rate, $\dot{M}_{Ca}$ (calculated as $Q * [\text{Ca}^{2+}]$) was considered. $\dot{M}_{Ca}$ is thus a parameter that can be manipulated in practice by changing the flow rate or the calcium concentration. The high $\dot{M}_{Ca}$ experiments (2F and 2Ca) resulted in a lower calcium conversion than the BC; the low $\dot{M}_{Ca}$ experiments (0.5F and 0.5Ca) showed a higher calcium conversion. In experiments with the same $\dot{M}_{Ca}$ (0.5F and 0.5Ca or 2F and 2Ca), a Ca^{2+} :Urea ratio of 1:1 (i.e. in 0.5F and 2F) resulted in a lower calcium conversion than a Ca^{2+} :Urea ratio of 0.5:1 (in 0.5F) or 2:1 (in 2F). The tested Ca^{2+} :Urea ratios were achieved

by changing the calcium concentrations (*cf.* Table 1), leading to differences in total urea mass injected and thus potentially different saturation indices.

We propose that the low calcium conversion observed in 2Ca occurred due to the initially rapid calcium precipitation (*cf.* Table A1), which has been shown to result in faster bacterial inactivation and cell encapsulation by CaCO_3 ⁷ as bacteria can serve as nucleation sites for precipitation.^{6, 73, 78, 94, 95} In addition to cell inactivation, the potential for generating carbonate, the second ‘building block’ necessary for CaCO_3 precipitation, was considerably lower in 2Ca because less urea was added in 2Ca than in any other experiment (Table 2).

Table 2: Fraction of calcium precipitated in the reactor relative to total calcium supplied and total calcium recovery for all experiments expressed as a percentage. Spatial analyses were focused on CaCO_3 precipitation only in the pore space (i.e., on and between the beads). The calcium recovery includes the precipitates that formed in the effluent tubing. Calcium conversion was calculated by dividing the calcium mass precipitated inside the reactors by the total aqueous calcium mass injected.

	Experiment	$\dot{M}_{Ca}$ (mmol*min ⁻¹)	Urea mass injected (mol)	Calcium recovery (%)	Calcium conversion (%)
Flowrate Variation	0.5F	0.825	0.89	77	8.1
	BC*	1.65	0.89	112	6.6
	2F	3.3	0.59	104	1
Calcium Concentration Variation	0.5Ca	0.825	1.6	120	21.5
	BC*	1.65	0.89	112	6.6
	2Ca	3.3	0.45	108	3.3

**note that the Base Case (BC) is listed twice to facilitate comparison with the other data provided.*

Recognizing that 2F and 0.5Ca were terminated earlier than planned, we considered the following two extreme cases to estimate calcium conversion, had these experiments lasted their planned durations:

- (a) If no additional precipitates had formed in the remaining duration.
- (b) If during the remaining duration, precipitation had occurred at the same rate as the time-averaged precipitation rate over the elapsed duration.

In either of these cases, 2F would still have had the lowest calcium conversion of all experiments. Instead of 1% (Table 2), the calcium conversion would have been 0.7% for case (a) and 0.9% for case (b). For 0.5Ca, case (a) would be 19% and case (b) would be 21%, thus maintaining this experiment as the highest calcium conversion condition.

Precipitate Distribution

Impact of high flow velocity near the inlet The effect of fluid velocity and shear forces was most evident in the influent region of the high flow rate experiment (2F); beads were pushed outward due to the high shear experienced at high fluid velocities. In 2F, bead-free regions developed near the inlet in six out of the eight angular directions (Figure A3). These regions extended radially outward 1-2 cm from the inlet, and the beads immediately downstream from these regions (up to 4 cm from the inlet) had visibly less precipitates on them compared to beads in the rest of the reactor. At the base case flow rate (BC, 0.5Ca and 2Ca), there was no physical bead displacement, but regions with visibly less precipitate (Figure A3) were observed up to 2-3 cm from the inlet, confirmed by low bead-specific precipitate mass (mg Ca/g beads) measured on these beads. The slowest flow rate experiment (0.5F) did not have such regions near the inlet, and precipitates were observed throughout the bead pack. Accordingly, this experiment had the highest bead-specific precipitate mass near the inlet among all experiments.

Assessing reaction and transport Precipitate distribution patterns seen in the RFR form because of the interplay between reaction and transport. For example, near the influent where the transport rate is high, shear forces are also high, which leads to low bacterial attachment (as shown by the bacterial distribution study). Consequently, the potential for reaction is low which leads to less MICP. To analyze this relationship between the reaction and transport, the Damköhler number is considered. The Damköhler number (Da) is the dimensionless ratio of reaction rate to transport rate. Damköhler numbers were calculated in defined volumes surrounding the 32 sampling locations for each reactor based on initial calcium transport rates and time-averaged reaction (precipitation) rates. For a given volume element 'A' (volume elements shown in Figure 1.b), a time-averaged reaction rate was calculated based on the precipitate mass:

$$Reaction\ rate_A = \frac{m_{Ca,A}}{V_A * t} \quad \text{Equation 4}$$

where V_A = fluid volume in volume element A, $m_{Ca,A}$ = total calcium mass precipitated in A, and t = experiment duration.

The initial (pre-MICP) transport rate was constant across the angular dimension in the RFR at a given distance from the inlet. As volume element A is one of eight at a given radial distance, the transport rate in A is given by:

$$Transport\ rate_A = \frac{\left(\frac{Q}{8}\right) * [Ca_o^{2+}]}{V_A} = \frac{\left(\frac{\dot{M}_{Ca}}{8}\right)}{V_A} = \frac{\dot{M}_{Ca,A}}{V_A} \quad \text{Equation 5}$$

where Q = volumetric flow rate, $[Ca_o^{2+}]$ = influent calcium concentration, $\dot{M}_{Ca}$ the mass flow rate of Ca^{2+} into the reactor, and $\dot{M}_{Ca,A}$ the mass flow rate of Ca^{2+} into volume element A. Da at location A is

$$Da_A = \frac{\text{Reaction Rate}_A}{\text{Transport Rate}_A} = \frac{m_{Ca,A}}{\dot{M}_{Ca,A} * t} \quad \text{Equation 6}$$

The transport rate likely changes over time as precipitates form, calcium concentrations decrease with distance and flow paths change. Such temporal changes in transport rate were not incorporated in Da calculations because spatially resolved flow rate and calcium concentration measurements were not possible with the reactor setup used. Accordingly, the parameter calculated using equations 4-6 will be referred to as the apparent Damköhler number (Da_{app}).

Reaction rates were highest for each experiment in the volume elements 9 cm away from the inlet, consistent with greater bacterial attachment observed at 9 cm (*cf.* Figure 2). Transport rates decreased with distance from the inlet due to decreasing velocity under radial flow. Thus, near the inlet, Da_{app} were relatively small but increased with distance due to decreasing transport rate and increasing reaction rate (Figure 4). Bead-specific precipitate mass also increased with distance, demonstrating that Da_{app} can indeed be an indicator of areas of high/low precipitation (Figure A4).

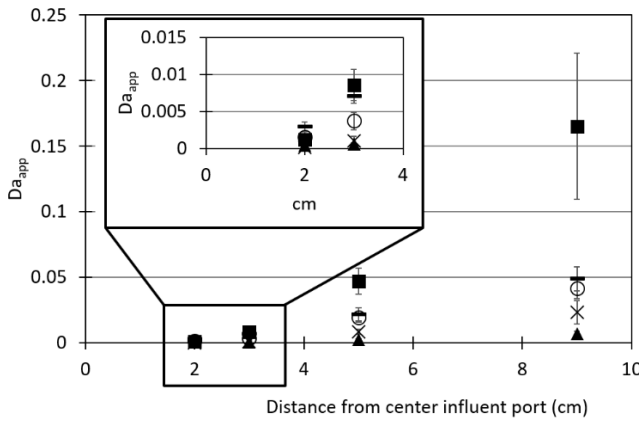


Figure 4: Apparent Damköhler numbers averaged over time and the eight ports for each radial distance. The inset details Da in the influent region (at 2 and 3 cm). At 2 cm, the highest Da_{app} was calculated for 0.5F, which exhibited the highest concentration of CaCO_3 precipitates close to the inlet. BC (O), 0.5F (—), 2F (▲), 0.5Ca (■), 2Ca (×). Error bars indicate ± 1 standard deviation.

To determine which rate (reaction or transport) controlled apparent Da distribution to a higher degree, Da_{app} were re-calculated assuming either a constant average reaction rate (Da_R) or a constant average transport rate (Da_T) over the entire reactor (Figure A5). These were compared to the Da_{app} numbers in Figure 4, which account for the measured reaction rates and changing transport rates. While Da_R were similar to the observed Da in Figure A5, Da_T were different and overestimated the observed Da_{app} . This difference in Da_T and Da indicates that accounting for changing transport rates is critical in these systems. Precipitate distribution, in these systems, appeared to be dominated by transport rate changes (decreasing velocity) rather than by reaction rate changes (caused by increasing cell numbers).

Figure 5.a shows mineralized glass beads at approximately 5 cm from the inlet. Precipitate distribution maps (Figure 5.b) indicate preferential flow path development during the mineralization phase. Areas of more precipitation were generally farthest from the inlet despite the angular heterogeneity. This angular heterogeneity is less evident in the apparent Damköhler number maps (Figure 5.c) because changes in reaction rates and especially in transport rates were more dominant in the radial direction than in the angular dimension.

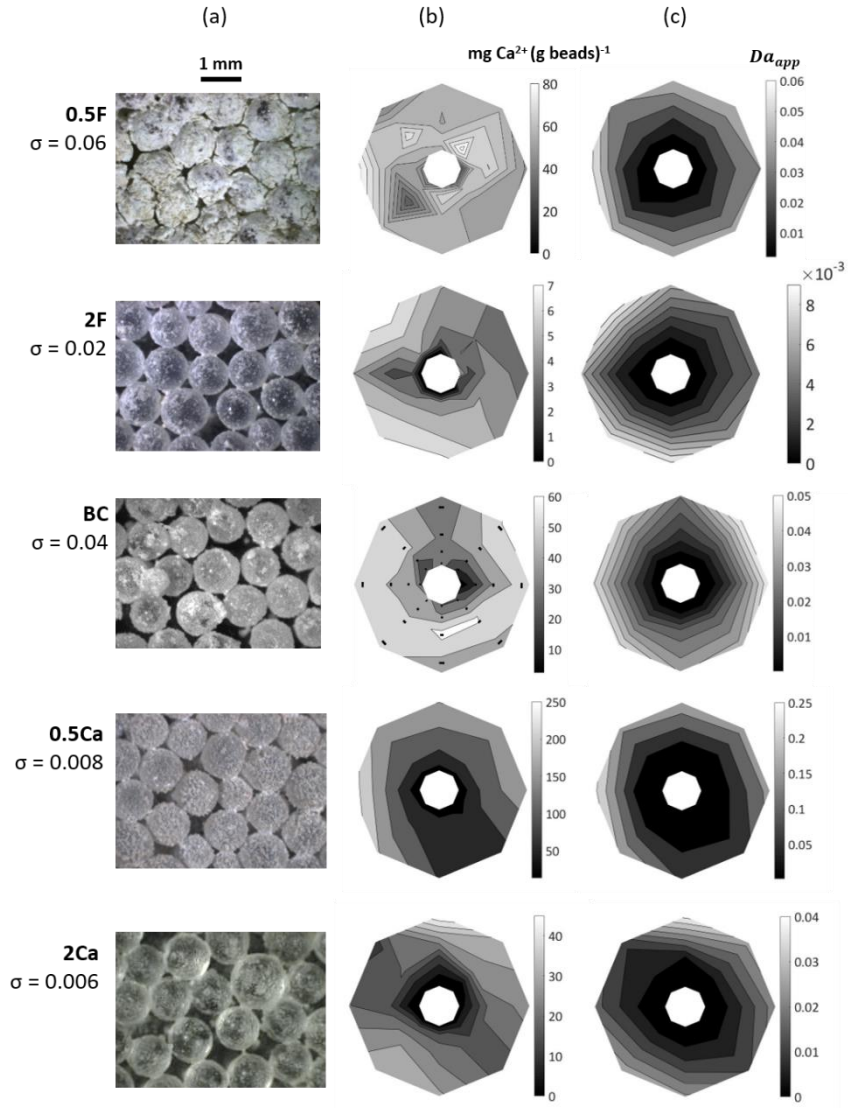


Figure 5: (a) Stereoscopy images of precipitates on glass beads 3-5 cm from the RFR inlet in each experiment. (b) Distribution maps of calcium precipitated, obtained by generating color filled contour plots in MATLAB*, with scale bars in $\text{mg Ca}^{2+} (\text{g beads})^{-1}$. The 32 sampling points used for creating the distribution maps are shown for reference on the BC calcium distribution map and are also indicated in Figure 1.b. (c) Apparent Damköhler number distribution, with scale bars of Da_{app} . It should be noted that the scale bars are different due to the varying maxima of precipitate mass in each experiment. σ values on the left are the distribution coefficients for calcium precipitates defined below (in Eq. 4). *Distribution maps generated as colored parametric surfaces using the 'surf' plotting command in MATLAB 9.0, R2016a.

Spatial distribution of precipitates Calcium distribution was quantified using the distribution coefficient (σ) (Figure 5.a), calculated as an average of Equation 16 in all eight angular directions.

$$\sigma = \frac{\text{mass } Ca^{2+} \text{ at } 2 \text{ cm}}{\text{mass } Ca^{2+} \text{ at } 9 \text{ cm}} \quad \text{Equation 7}$$

In all experiments, σ in all directions was much smaller than 1 indicating that more $CaCO_3$ formed farther away from the inlet. With increasing flow rates, σ decreased, meaning that higher flow rates led to relatively more calcium precipitates at greater radial distance in the reactor. For the range tested here, an equimolar ratio of calcium and urea resulted in a more uniform precipitate distribution than $Ca^{2+}:\text{Urea} \neq 1$.

High variance in precipitate distribution in angular direction was indicated by solids (Figures A1 and 5.b) and aqueous analyses (Figures 3.b and 3.c). Angular variance in precipitate distribution increased with increasing flow rates, reaching 36% at the highest flow rate (Table 3).

Table 3: Angular variation in bead-specific precipitate mass expressed as a percentage of the total variance in each experiment. Variances were estimated using linear mixed effects models for each experiment.

	Experiment	Contribution of angular variance to total variance
Flowrate Variation	0.5F	7.0%
	BC	23%
	2F	36%
Calcium Concentration Variation	0.5Ca	9.3%
	BC	23%
	2Ca	6.3%

Variances were estimated by running linear mixed effects models considering the angular dimension (8 directions) as a random effect. Individual models were run for each experiment to estimate variance due to angle within each experiment.

Even at the same $\dot{M}_{Ca}$, the lower flow rate experiment had lower angular variance. Preferential flow paths are a major factor in increasing angular variance because of the spatial heterogeneities they trigger. The data in Table 3 suggest that preferential flow path formation occurs to a greater degree at higher flow rates.

The results demonstrate that the apparent Damköhler number is positively correlated with the extent of precipitation in this system, which was operated under flowing conditions during MICP. Hence, if the reaction and transport rates in a system can be estimated a priori (e.g. through experiments or mathematical modeling), Damköhler numbers could potentially be used as predictors for calcium carbonate precipitate distribution in field-scale applications. An improved prediction of spatially resolved precipitation would likely be possible if angular heterogeneity in transport and reaction could be estimated. However, capturing spatio-temporal variations of porosity, permeability and changes in flow paths due to precipitate formation was not possible even in these well-defined laboratory experiments.

The research presented here provides insight into ureolysis-driven MICP in porous media under radial flow. In the RFR, more bacteria attached at greater distances into the porous medium, likely due to decreasing shear stress with distance. Additionally, fluid residence time (and therefore time available for attachment) increased with radial distance.

In the radial flow system used here, the extent of precipitation was primarily driven by transport rate changes. Potential reaction rate changes from the observed higher bacterial attachment with radial distance did not seem to affect MICP to the same extent as transport rate changes. It should be noted that reaction rates were averaged over the entire duration of each experiment which does not capture possible temporal changes in reaction rates that likely occurred. Quantifying temporally varying reaction rates at all locations would require spatio-temporal sampling, which the current reactor set up did not permit; however, the relatively stable effluent pH (Figure 3.a) indicates that the reaction rate remained fairly constant over time in these experiments.

For the meso-scale system tested here, decreasing the calcium mass flow rate increased calcium conversion. More precipitates formed at greater radial distance in all experiments due to decreasing transport rates with distance. Less precipitates were observed near the inlet at the higher flow rates, as suggested by Whiffin et. al., because reactants were transported farther into the system before significant precipitation could occur.⁵⁴ To decrease angular heterogeneity in precipitate distribution, lower volumetric flow rates are recommended. However, very low volumetric flow rates or mass transport rates could lead to injection point plugging caused by excessive precipitation near the inlet. Minto et al. demonstrated how flow velocities above a critical threshold can limit bacterial attachment, thus enabling control over CaCO_3 distribution.⁹⁶

Modeling MICP under radial flow and not axial (1-dimensional) flow might be crucial for subsurface injections because it can significantly impact predictions about MICP⁴⁸. Smaller angular heterogeneities in precipitation are somewhat undefined and

unsystematic, but certainly important because they can lead to measurable changes in porous media properties. Studying the effect of angular heterogeneities on porous media hydrodynamics, transport and reaction is challenging. One potential approach is stochastic modeling which could be coupled with time-resolved microscopy of mineral development to study changing hydrodynamics (as shown in Figure 5 in Connolly et al.⁹⁷). However, this type of investigation was beyond the scope of the current study. Additional studies investigating the interplay between Da and MICP dynamics under a wide range of conditions are necessary to provide further insights. Such studies will require the measurement of spatially and temporally resolved reaction and transport rates, and thus Damköhler numbers, to account for the development of and changes in non-homogeneous flow fields caused by precipitate accumulation. This work as well as future work will aid in the design of MICP-based technologies and further explore the utility of the Damköhler number as a design and control parameter.

Supplemental Information (SI): summary of ureolysis-driven MICP, reactor disinfection protocol, tracer and glass bead photos, and additional apparent Damköhler number analyses. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Author Information:

Corresponding Author: Dr. Robin Gerlach, robin_g@montana.edu, (406) 994-1840.

The authors declare no competing financial interest.

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CHAPTER FOUR

SPATIO-TEMPORAL DYNAMICS OF STRONTIUM PARTITIONING WITH
MICROBially INDUCED CALCIUM CARBONATE PRECIPITATION IN
POROUS MEDIA FLOW CELLSContribution of Authors and Co-Authors

Manuscript in Chapter 4

Author: Neerja Zambare

Contributions: Performed all experiments including reactor set up, operation, and analysis. Envisioned graphics, table design and wrote and revised manuscript.

Co-Author: Robin Gerlach

Contributions: Identified major topics for review during development of manuscript and aided in data analysis and interpretation. Contributed to writing and revision of the manuscript in the form of comments and feedback.

Co-author: Ellen Lauchnor

Contributions: Identified major topics for review during development of manuscript and aided in data analysis and interpretation. Contributed to writing and revision of the manuscript in the form of comments and feedback.

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Spatio-temporal dynamics of strontium partitioning with Microbially Induced Calcium carbonate Precipitation in porous media flow cells

Neerja M. Zambare^{a,b}, Robin Gerlach^{a,b}, Ellen G. Lauchnor^{a,c}

^a Center for Biofilm Engineering, Montana State University, Bozeman, MT 59717

^b Department of Chemical and Biological Engineering, Montana State University,

^c Department of Civil Engineering, Montana State University,

Bozeman, MT 59717

Abstract

Microbially induced calcium carbonate precipitation (MICP) is a biomineralization process which can be initiated by various bacterial pathways that increase alkalinity and pH. During MICP, divalent metal cations can co-precipitate with calcium carbonate. Metal co-precipitation is an attractive alternative for groundwater remediation, particularly because it can effectively immobilize metals present even at trace concentrations. Here, we investigate strontium co-precipitation with calcium carbonate during MICP in a porous media flow cell modified to facilitate spatio-temporal sampling of fluid within the pore space. Spatio-temporal measurements revealed that most precipitation occurred downstream despite the highest ureolysis rates measured close to the inlet of the flow cell. Smaller precipitates with higher strontium content relative to calcium (D_{Sr}) were observed closer to the reactor inlet while larger precipitates with lower D_{Sr} were observed further downstream. These spatio-temporal analyses of ureolysis-driven MICP in porous media revealed that strontium partitioning increased with precipitation rate in each experiment,

as well as with formation of smaller precipitates. However, the relationship between calcium precipitation rate and strontium distribution coefficient was found to be impacted by experimental factors (ex. abiotic vs biological precipitation), when compared to published data and across different experiments. Controlling the transport of bacterial catalysts and substrates to influence reaction rates may provide a strategy for optimizing removal of aqueous Sr from contaminated groundwater.

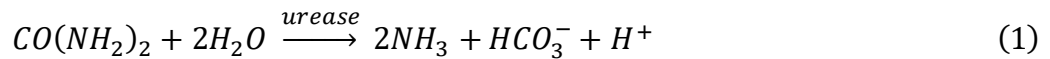
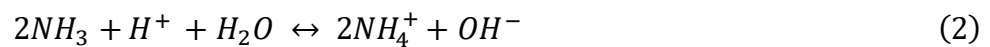
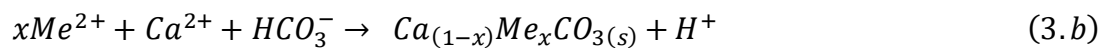
Introduction

Trapping metal contaminants and radionuclides in a mineral phase such as calcium carbonate is a viable option to achieve groundwater remediation by effectively immobilizing the contaminants.^{98, 99} Mineral precipitation and metals sequestration can be achieved through a process called microbially induced calcium carbonate precipitation (MICP). MICP can result from a number of bacterial metabolic processes with ureolysis-driven MICP being the most widely studied for engineering applications because it is rapid⁶³ and growth-independent.

During ureolysis, the bacterial enzyme urease catalyzes the hydrolysis of urea (Equation 1), which leads to a rise in alkalinity (Equation 2) and enables CaCO_3 precipitation (Equation 3).^{10, 63, 64} The alkaliphilic bacterium *Sporosarcina pasteurii* is considered the model ureolytic microorganism as it produces ample amounts (~1% cell dry weight^{64, 65, 68}) of the enzyme urease.^{10, 63, 64, 66, 67} *S. pasteurii* and related species are commonly found in soil^{64, 65} and microbiological precipitation by *S. pasteurii* (formerly *Bacillus pasteurii*) has been shown to precipitate larger quantities of CaCO_3 compared to abiotic precipitation.⁶³ Higher bacterial concentrations can generate larger CaCO_3 crystals

with lower solubility than smaller crystals that have greater surface area-to-volume ratios and can dissolve faster.⁷⁶ Metal contaminants can precipitate to form metal carbonate minerals (Equation 3.a) but some divalent metal cations can co-precipitate by replacing calcium (Ca^{2+}) in the CaCO_3 crystal lattice, leading to partitioning of the metal out of the water phase and long-term, stable sequestration in the solid phase^{87, 100-103} (Equation 3.b). Metal cation co-precipitation in CaCO_3 can occur with metal concentrations lower than those required to supersaturate a mineral phase of that metal, which makes co-precipitation an attractive remediation strategy for radionuclides which can be present at trace concentrations.¹⁰⁰ Being able to use MICP to reduce or prevent the spread of heavy metal and radionuclide contaminants has the potential to be an effective technique for bioremediation.

The reactions that take place during the process of ureolysis-driven MICP and metal co-precipitation are shown through equations 1-3.b:



In Equations 3.a and 3.b, the metal cation (Me^{2+}) can be calcium or another divalent metal or radionuclide. This case demonstrates metal carbonate precipitation while Equation 3.b shows cation co-precipitation into CaCO_3 .

⁹⁰Sr is a groundwater contaminant at numerous U.S. Department of Energy sites in the U.S. as well as locations across the world.⁸⁷ ⁹⁰Sr is a highly toxic and carcinogenic

radionuclide and is introduced into the environment primarily through nuclear waste at weapons facilities¹⁰⁴ or accidental release from nuclear reactors.^{104, 105} Sr can get deposited in bone and marrow, raising the risk of bone cancer and leukemia.¹⁰⁴ Removal of Sr after incorporation into bone is unfeasible, making Sr exposure an irreversible threat.¹⁰⁶ ⁹⁰Sr can also be found in teeth¹⁰⁷ and other calcium biominerals such as kidney stones¹⁰⁸ as it can readily replace Ca.

With the relatively long half-life of ⁹⁰Sr (~29 years), long term sequestration in CaCO₃ allows for decay of radioactivity and mitigation of ⁹⁰Sr toxicity to the environment. The stable metal ion Sr²⁺ is chemically and physically similar to ⁹⁰Sr and can be used as a surrogate for radioactive strontium. Thus, Sr studies have the potential to inform about ⁹⁰Sr partitioning into CaCO₃ and to help design strategies for ⁹⁰Sr removal from nuclear contamination sites. Strontium and calcium are both alkali earth metals and because of similar outer electron shell structures, they exhibit similarities in chemical (reactivity) properties.¹⁰⁹ Replacement of calcium by strontium within calcite can be thermodynamically favorable if both cations are present in solution¹¹⁰ making strontium a good candidate for co-precipitation by MICP.¹¹¹

Previous abiotic co-precipitation studies have investigated the extent of Sr co-precipitation and the kinetics of the process.¹¹²⁻¹¹⁶ Research on removal of Sr with MICP has mostly focused on characterizing the kinetics of the co-precipitation process with Sr partitioning being greater at higher Ca precipitation rates.^{101, 103, 116} Kanget al. showed 80% removal of aqueous Sr by the ureolytic bacterium *Sporosarcina pasteurii* in sand slurry columns.¹⁰² Achal et al. achieved a comparable 80% soluble Sr removal (presumed co-

precipitated into CaCO_3) by urease producing *Halomonas sp.* in contaminated aquifer quartz sand.¹¹¹ Other co-precipitation studies found that the carbonate to calcium ion activity ratio affected Sr partitioning¹¹⁷ and that Sr incorporation led to the formation of smaller crystals.¹¹⁸ Mitchell and Ferris investigated the temperature dependence of Sr partitioning kinetics using *S. pasteurii*.¹⁰³ Precipitation rates were found to be slightly lower in the presence of Sr and at a given precipitation rate, Sr partitioning showed a positive correlation to temperature.

Only a few studies so far have investigated biologically aided Sr co-precipitation under continuous fluid flow through porous media. Lauchnor et al. compared the effects of continuous and pulsed fluid injection strategies on Sr co-precipitation with MICP in porous media flow cells.⁸⁷ They found that pulsed injection of fluids could lead to a higher fraction of Sr co-precipitating with calcium carbonate. Redden et al. tested Sr co-precipitation via ureolysis-driven MICP using Jack Bean urease enzyme immobilized on silica gel in porous media columns.¹¹⁹ Their results suggested that Sr removal was primarily from sorption to CaCO_3 rather than co-precipitating under their tested conditions.

Very few co-precipitation studies are dedicated to bridging the gap between lab-scale co-precipitation and field-scale application. An important aspect of making co-precipitation a successful biotechnology for the field-scale is quantifying and controlling metal co-precipitation over space and time, and especially in field-relevant conditions like porous media flow. The flow cell study by Lauchnor et al. was the first study that investigated co-precipitation by MICP during flow through porous media. However,

spatially resolved strontium partitioning could not be directly measured, presenting a limitation to characterizing co-precipitation in flow systems.

The research presented here eliminates the need for assumptions of concentration gradients and analyzes reactive transport resolved over time and space during Sr co-precipitation with MICP under flow through porous media using a modified flow cell. Additionally, artificial ground water was used in the experiments presented here in an attempt to more closely resemble aquifer environments where this technology might be applied.

Experimental Methods

Media and Bacterial Inoculum

Growth medium for the modified flow cell (“modified FC”) experiments was prepared by adding 1 g L⁻¹ Difco Nutrient Broth (Becton Dickinson) and 10 g L⁻¹ urea (Fisher Scientific) to Artificial Ground Water without calcium (AGW recipe from Ferris et al.⁵⁹, see Supplemental Information). To prepare Co-precipitation medium, 0.45 mM Sr (as SrCl₂·6H₂O (Fisher Scientific)) and 83 mM Ca (as CaCl₂·2H₂O (Fisher Scientific)) were added to fresh growth medium, resulting in an initial Sr:Ca mass ratio of approximately 0.01. Thus, the influent media had a urea-to-calcium molar ratio of 2:1 allowing for excess ureolysis. All media were pH-adjusted to around 6.5 before filter-sterilized using 0.45 µm pore size bottle top filters (ThermoFisher Scientific, Waltham, MA, USA).

For the inoculum in each experiment, 1 mL of *S. pasteurii* (ATCC 11859) stock culture was added to 100 mL Brain Heart Infusion medium (BHI, Fisher Scientific) amended with 0.33 M urea and incubated at 30°C (at 150 RPM). After 24 hours, 1 mL of

the culture was transferred into 100 mL fresh BHI + 0.33 M urea. This transfer culture was incubated under the same conditions before centrifuging at a relative centrifugal force of $2964 \times g$ for 10 minutes at 4°C . The supernatant was decanted, and the cell pellet was re-suspended in the respective growth medium (for each type of flow cell) and centrifuged again. Re-suspension and centrifugation were repeated once more, after which cells were diluted in growth media to a final OD_{600} of 0.4 on a BIOTEK Synergy HT absorbance plate reader in 96-well plates with 100 μL per well. The reactor was disinfected (protocol in Supplemental Information) and inoculated with approximately 10 mL (>2 flow cell pore volumes) of the final culture. Following inoculation, the reactor tubing was clamped for a no-flow attachment phase of 2 hours to promote attachment of bacterial cells.

Flow cell set up and operation

The modified FC had perforations in 45 locations in the bottom polycarbonate plate (Figure 1.b, one perforation location is shown magnified). Below the polycarbonate plate, a rectangular rubber sheet was held tightly using a polycarbonate piece with holes aligning with the 45 perforations enabling pore-space fluid sampling from 45 different locations. The reactor had one inlet and one outlet and was balanced using adjustable stand legs. Media were pumped into the modified FC through the inlet using a peristaltic pump and the bacterial inocula in each experiment were injected through a port installed in the tubing immediately upstream of the inlet. Three spatial ports along the center line of the modified FC (p1, p2 and p3 in Figure 2.b) were utilized for fluid sampling in these experiments, in addition to the inlet and outlet. Disposable needles (23 G, Becton Dickinson, NJ) were inserted through the ports and rubber sheet into the pore space of the modified FC. For ease

of sampling, the needles were left in the reactor and when not in use and capped with rubber septa using luer-lock connectors. At each sampling time, liquid samples were taken one at a time (p1, p2, p3) by taking the rubber septum off the selected port, clamping the effluent and collecting fluid into microcentrifuge tubes. After collecting 1.5 mL of sample, each port was re-capped before opening the next port. After the 3 spatial samples were collected, fluid was collected out of the effluent sampling port.

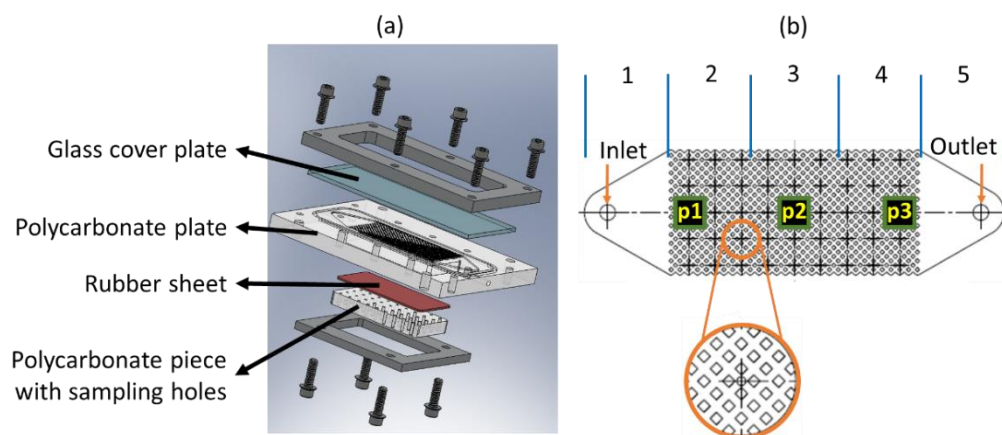


Figure 1: (a) Modified flow cell assembly drawing. (b) Polycarbonate plate drawing showing the etched area (porous media region), inlet and outlet. The circular explosion drawing shows the 1mm³ square pegs and p1, p2 and p3 were three sampling ports chosen for this study. Numbers between lines indicate the 5 sections utilized for precipitate acid digestion and data analysis.

Following the no-flow attachment phase, growth medium was fed to the reactor for 12 hours at 0.1 mL min⁻¹. After this ‘growth phase’, co-precipitation medium (AGW with Sr²⁺ and Ca²⁺) was pumped continuously for 72 hours at the same flow rate. Spatial sampling was only performed during this ‘co-precipitation’ phase. The modified FC experiment was performed in triplicate.

Solution chemistry analysis

Fluid samples (1-1.5 mL) were collected in microcentrifuge tubes. Aliquots filtered with a 0.2 μm syringe filter (VWR, PA) were analyzed for dissolved urea, calcium and strontium concentrations and the unfiltered leftover samples were used to measure pH using a Symphony SB70P pH meter (VWR, PA, USA). Urea concentrations were measured using the colorimetric Jung assay.¹²⁰ Calcium and strontium concentrations were measured using an Inductively Coupled Plasma-Mass Spectrometer (ICP-MS, Agilent 7500ce) after appropriately diluting the samples using 5% trace metal grade nitric acid (TMG-HNO₃). The calcium precipitation rate was calculated as the rate of aqueous calcium removal (R_{aq}) at each sampling time (t) and each section (s) as:

$$R_{aq(s,t)} = \frac{Ca_{in(s,t)} - Ca_{out(s,t)}}{HRT_s} \quad (4)$$

where Ca is the aqueous calcium concentration and HRT_s is the hydraulic retention time in section s . The same formula was used to calculate strontium removal rates and ureolysis rates using dissolved strontium and urea concentrations respectively.

Solids analysis

At the end of the co-precipitation phase, the reactor was carefully drained and taken apart. After air-drying overnight, both plates were imaged using a Leica M 205 FA stereoscope. After stereoscopy, precipitates on both plates were dissolved in 5 sections (Figure 1.b) using 10% TMG-HNO₃. The samples were diluted to 5% strength using nanopure water (0.22 μm filter, resistivity=18.2 M Ω .cm) and then diluted further using 5% TMG-HNO₃ to analyze for calcium and strontium concentrations using ICP-MS. For

modified FC 1, Laser-Ablation ICP-MS (LA-ICP-MS) was performed along the centerline in the direction of flow (Figure B1.a). An NWR193 laser ablation instrument equipped with a TwoVol2 ablation chamber (Elemental Scientific Lasers, Bozeman, MT) was used with an Agilent 7700 ICP-MS for LA-ICP-MS.

Strontium Partitioning

The degree of strontium partitioning was quantified by the strontium distribution coefficient (D_{Sr}):

$$D_{Sr} = \left(\frac{Sr_{ppt}}{Ca_{ppt}} \right) * \left(\frac{Ca_{aq}}{Sr_{aq}} \right) \quad (5)$$

where Sr_{ppt} and Ca_{ppt} are the mass of strontium and calcium in the precipitates and Sr_{aq} and Ca_{aq} are the aqueous concentrations (mol*L⁻¹) of strontium and calcium.⁸⁷

Results and Discussion

Spatio-temporal concentrations within the porespace

Dissolved strontium concentrations in the modified FC showed similar behavior to dissolved calcium concentrations over time and over space (Figure 2.a). Each replicate showed identical spatio-temporal trends for calcium and strontium concentrations (Figure B2.a). Relative standard deviations in Ca and Sr concentrations between the three replicates were calculated at each sampling location and time. The average of these values was 15% for Ca and 3% for Sr. Calcium and strontium concentrations generally decreased with increasing distance into the flow cell. This decrease over distance was statistically significant for both Ca and Sr ($p < 0.0001$). Pairwise comparison tests showed that concentrations were significantly different over all combinations of distances (all pairs of sampling locations $p < 0.001$). With these measurements, the modified FC eliminated the need for assumptions about spatial concentration gradients which were required in previous work.⁸⁷

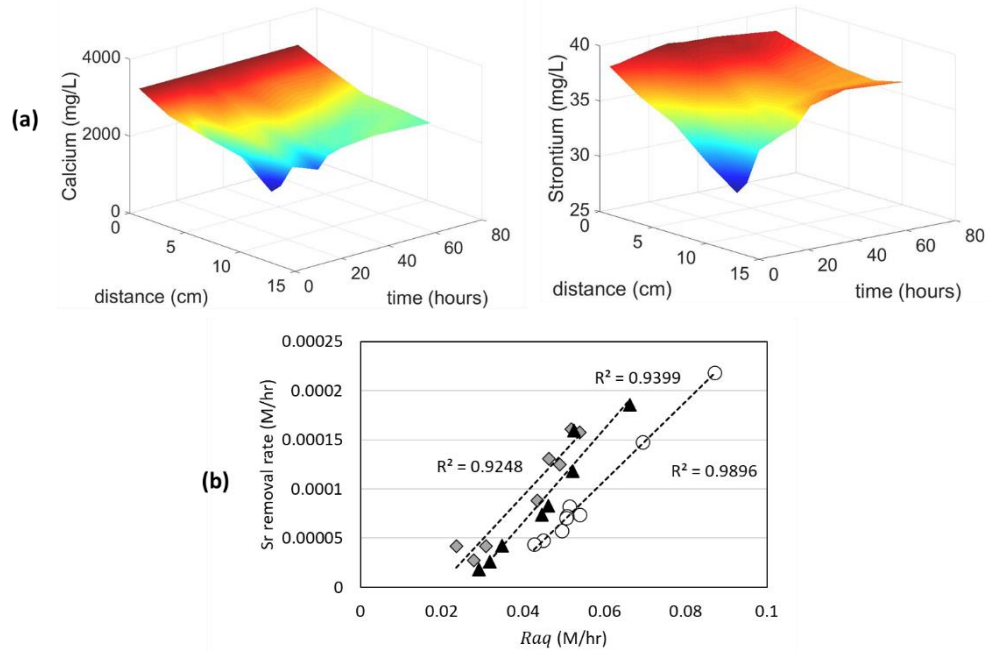


Figure 2: (a) Spatio-temporal distribution of Ca and Sr dissolved concentrations averaged over the three replicates. All replicates showed similar distributions. (b) Strontium removal rates increase linearly with increasing Ca precipitation rates in trial 1 ($\circ$), trial 2 ($\diamond$) and trial 3 ($\blacktriangle$).

The concentration gradients for calcium and strontium over distance show R^2 values of 0.97 and 0.99 respectively (Figure B2.b), which align with the linear gradient assumption made by Lauchnor et al. for the given reactor scale and conditions. Figure 2.b shows a positive linear correlation between strontium removal rates and Ca precipitation rates (R_{aq}) for the three triplicates, indicating that strontium was removed along with calcium.

Spatial decoupling of ureolysis and CaCO_3 precipitation

Spatio-temporally resolved ureolysis rates indicated that the highest ureolysis rates occurred during the first half of the experiments and closer to the inlet (Figure 3.a). It is hypothesized that hydroxyl ions generated upstream (in the high ureolysis rates region)

were transported downstream by the continuous transport through the flow cell. The pH data support this hypothesis as fluid in the downstream regions had a high pH while the inlet region maintained a pH closer to the influent medium pH (Figure 3.b). The highest calcium precipitates rates (R_{aq}) were also encountered downstream from the location of highest ureolysis rates (Figure 3.c). This spatial decoupling between highest ureolysis rates (close to inlet) and highest calcium precipitation rates (close to outlet) likely resulted from a higher concentration of carbonate ions in the downstream regions. Higher carbonate concentrations in the downstream regions could be attributed to a combination of increased carbonate generation in high pH conditions existing in these regions (Figure 3.b) and potential transport of carbonate generated in upstream regions.

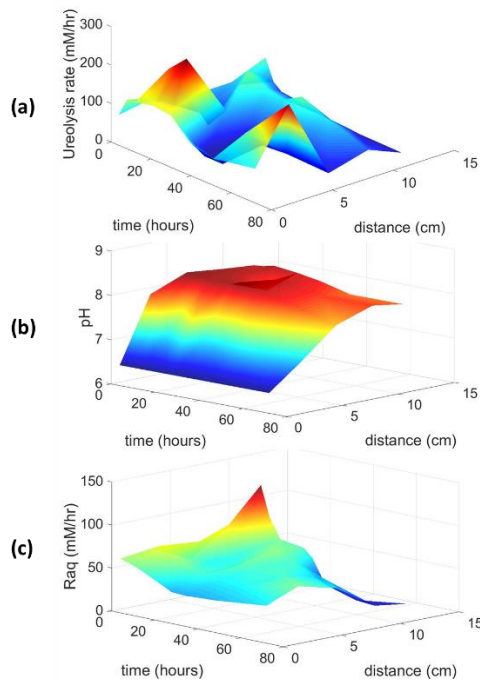


Figure 3: Spatio-temporal maps of (a) ureolysis rates, (b) pH and (c) R_{aq} averaged over the three replicates. It should be noted that the time and distance axes in Figure 3 are switched compared to Figure 2. This orientation provided the most comprehensive view of the spatio-temporal patterns. Ureolysis rates were higher in the first half of the reactors but conditions more conducive to precipitation existed further downstream and precipitation rates were higher towards the outlet.

The modified FC experiments also revealed that the decrease in R_{aq} over distance was different for different regions of the flow cell with the most pronounced decrease over

time occurring in sections 4 and 5 (Figure 3.c). Initially high Ca precipitation rates in sections 4 and 5 might have increased the likelihood of cell encapsulation and inactivation⁷ thus potentially causing ureolysis and Ca precipitation rates to decrease over time in these sections.

Spatial distribution of D_{Sr}

The calculation of D_{Sr} requires strontium-to-calcium ratios (Sr:Ca) in the solid phase and in the liquid phase (Equation 5). For solid phase Sr:Ca, end-point masses of Sr and Ca in each section of the flow cell (from acid digested precipitates) are used. For aqueous Sr:Ca, spatially resolved aqueous concentrations of Sr and Ca are necessary. Lauchnor et al. assumed linear and exponential gradients between measured inlet and outlet concentrations to estimate aqueous concentrations within the flow cell, but concluded that spatial sampling of the reactor was necessary to determine the existing gradient.⁸⁷ The modified FC eliminated the need for concentration gradient assumptions as direct measurements from within the flow cell were used. The modified FC experiments showed that the concentration gradient within the flow cell was linear, providing supporting evidence for the assumption made by Lauchnor et al.⁸⁷ The final strontium-to-calcium (Sr:Ca) molar ratio in the precipitates (measured by ICP-MS of acid-dissolved precipitates) and the resulting D_{Sr} decreased with distance into the flow cell (Figure 4).

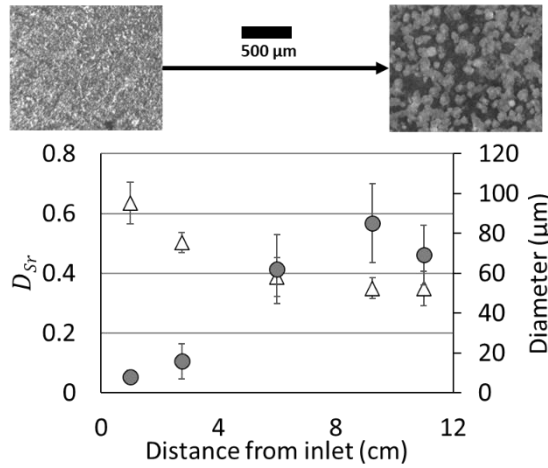


Figure 4: D_{Sr} decreased with distance into the modified FC (triangles, left y-axis). Average particle diameter (●, right y-axis) generally increased over distance from the inlet. Standard deviations are from triplicate experiments. Selected images from the inlet and outlet regions are shown to highlight the difference in crystal size.

Closer to the inlet, precipitates were smaller and had higher strontium partitioning (Figure 4). It has been reported that smaller crystal sizes associated with nucleated calcite may permit more extensive substitution of divalent trace metal ions into calcium sites within calcite.¹⁰¹ In fact, the incorporation of metals into a precipitate might be the reason for repeated termination of precipitate development and subsequent nucleation of new precipitates, resulting in a higher relative rate of nucleation and smaller precipitates. This phenomenon was shown with Sr co-precipitation where Sr contaminants inhibited the growth rate of calcite and modeling showed that strontium inhibits crystal development during the nucleation stage.¹¹⁸ Accordingly, more strontium incorporation in precipitates closer to the inlet in the modified FC could have led to a higher nucleation-to-growth ratio, resulting in smaller precipitates (Figure 4).

D_{Sr} in the modified FC experiments were lower than those reported by Lauchnor et al.⁸⁷, likely because of differences in media composition. Media used by Lauchnor et al. contained more organic carbon (3 g L⁻¹ nutrient broth) and more urea (20 g L⁻¹) compared to the modified FC media (1 g L⁻¹ nutrient broth and 10 g L⁻¹ urea). Enhanced

biofilm/bacterial growth along with more available urea likely led to more MICP in their experiments compared to the modified FC. Additionally, Lauchnor et al. used an order-of-magnitude higher strontium concentration midway through the experiment which could have affected the extent of strontium partitioning.⁸⁷

For the modified FC experiments and those in Lauchnor et al., spatial resolution of D_{Sr} was limited by the precision possible during manual acid digestion of reactor sections. For mapping with higher spatial resolution, LA-ICP-MS analyses were performed using the cover glass on the top of the porous media region of one modified FC triplicate. LA-ICP-MS revealed that the relative abundance of strontium-to-calcium decreased with distance from the inlet along the centerline of the reactor (Figure B1.a). LA-ICP-MS analysis ablates only a few nanograms from the top surface of the precipitates per laser run. Contrastingly, sampling for ICP-MS requires the dissolution of all precipitates in a given section. The Sr:Ca observed by ICP-MS on acid-dissolved precipitates (Figure B1.b) followed a similar trend to Sr:Ca observed by LA-ICP-MS analysis, indicating fairly uniform strontium incorporation with depth of the precipitates, i.e. over time as precipitates grow.

Strontium partitioning and Ca precipitation rate

A positive correlation between strontium partitioning (D_{Sr}) and Ca precipitation rate has been published previously based on batch experimentation.^{101, 103, 116} These batch experiments have relied on aqueous concentration measurements to estimate D_{Sr} and Ca precipitation rates, instead of directly measuring the amounts of Ca and Sr precipitated (Ca_{ppt} and Sr_{ppt} in Equation 5). In order to perform valid comparisons to literature, D_{Sr}

for the MFC experiments were recalculated using only aqueous chemistry measurements. The same positive trend was observed between these aqueous chemistry-based D_{Sr} and R_{aq} for the triplicate modified flow cell experiments (Figure 5, *filled in triangles*). Granted, some differences in D_{Sr} between the triplicate flow cell experiments are apparent in Figure 5 and may be attributed to differences in influent concentrations. For example, in modified FC 1 (black triangles in Figure 5), the influent calcium concentration was slightly lower at 73 mM Ca compared to the other two replicates (83 mM Ca). The strontium concentration was the same in all three replicates and as a result, D_{Sr} for modified FC are lower.

Ca precipitation rates compared to strontium partitioning for abiotic and biotic co-precipitation studies are shown in Figure 5. Studies analyzed can be broadly classified as MICP studies in batch reactors (*diamonds*)^{101, 103, 118, 121}, MICP studies in flow cells (*triangles*)⁸⁷, abiotic batch precipitation studies (*squares*)^{116, 117, 122, 123}, and an enzyme column study (*'plus' symbols*).¹¹⁹ For data sets with more than one data point, a positive correlation between D_{Sr} and Ca precipitation rate is observed (trendlines in Figure 5). However, this trend is likely sensitive to experimental conditions (flow/no-flow,

abiotic/MICP, concentrations, cell densities etc.) as a universal correlation is not observed across all the different data sets.

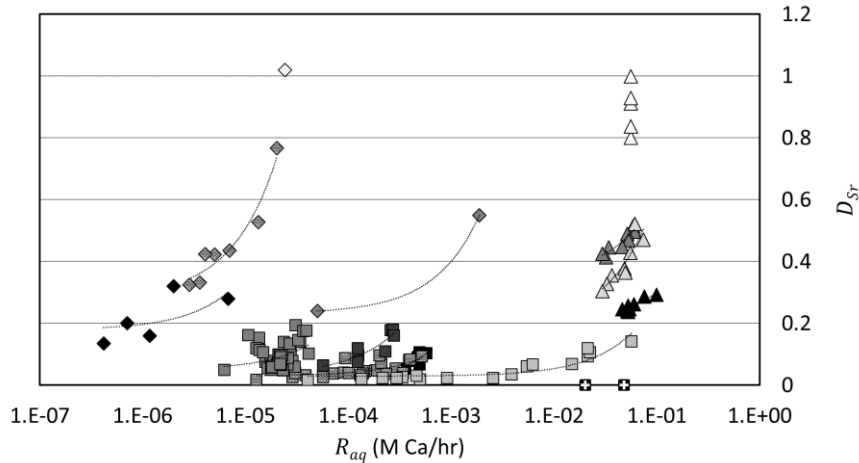


Figure 5: A semi-log plot of strontium distribution coefficients (D_{Sr}) based on aqueous chemistry vs calcium precipitation rates (R_{aq}) compiled from calcium carbonate-mediated strontium co-precipitation literature with linear regression trendlines. The R^2 for the linear fits are given here for all studies except for the ones with only one or two data points. Diamonds show biological precipitation batch studies: Horiike et al., 2017 ($\diamond$), Fujita et al., 2004 ($\blacklozenge$), Mitchell and Ferris, 2005 ($\blacklozenge$, $R^2 = 0.936$) and Mitchell and Ferris, 2006 ($\blacklozenge$, $R^2 = 0.3636$). Squares show abiotic precipitation batch studies: Tesoriero and Pankow, 1996 ($\blacksquare$, $R^2 = 0.8251$), Pingitore and Eastman, 1986 (Data sets A: $\blacksquare$, $R^2 = 0.1476$ and B: $\blacksquare$, $R^2 = 0.8249$), Lorens, 1981 ($\blacksquare$, $R^2 = 0.3983$) and Gebrehiwet et al., 2012 ($\blacksquare$, $R^2 = 0.5083$). Triangles show data from the flow cell experiments: Lauchnor 2013 ($\triangle$), and this work (filled triangles, $R^2 = 0.8567, 0.7774, 0.8232$). The continuous flow cell data from Lauchnor et al. are presented as they most closely relate to the other flow cell data in the figure. Only one reaction rate is reported in Lauchnor et al. and accordingly, all D_{Sr} for Lauchnor et al. appear at the same R_{aq} . The enzyme column study by Redden et al., 2014 is shown by $\blacksquare$. All reaction rates were converted to mol Ca/L*hr or M Ca/hr. For Horiike et al., the reaction rate was calculated based on the aqueous calcium removal data provided. R_{aq} provided in terms of rate per seed crystal mass in Lorens and in Tesoriero and Pankow were converted to M Ca/hr using the seed crystal masses and total reaction volumes reported by the authors. Gebrehiwet et al. reported reaction rates per area, which were converted to M Ca/hr using the specific surface area of seed calcite used. Data sets A and B from Pingitore and Eastman were used as they were appropriate for comparison. For both data sets, an overall reaction rate was calculated using the change in calcium concentration and the duration of the experiments. For Redden et al., the change in aqueous calcium concentration within the enzyme zone was converted to a reaction rate using the linear flow velocity through the column.

Figure 5 shows that for MICP, Ca precipitation rates in flow cells (and columns containing immobilized enzyme) were orders of magnitude higher than in batch experiments. Higher reaction rates in flow cells can be attributed to a continuous supply of reactants with influent medium, higher surface area available for precipitation reaction (porous media elements), or a combination of both.

Strontium partitioning is considerably lower for abiotic calcium carbonate precipitation than for MICP according to the studies considered (Figure 5). Fujita et al. attributed observation of this phenomenon to higher rates of precipitation during MICP compared to abiotic precipitation. Strontium co-precipitation can occur via multiple pathways, including adsorption, occlusion, solid solution formation (Sr^{2+} occupying Ca^{2+} sites) and formation of a discrete phase (e.g. SrCO_3). The differences in D_{Sr} for MICP versus abiotic precipitation could potentially be attributed to the Sr co-precipitation pathway.

Since the goal of the flow cell studies was to characterize the spatio-temporal distribution of ureolysis-driven MICP and strontium co-precipitation, the specific pathway of Sr co-precipitation was not investigated/determined. Biological precipitation might favor one pathway for co-precipitation over others due to the influence of metabolic byproducts or nucleation on bacterial surfaces. Further research is necessary to test this hypothesis.

Flow cells better mimic reactive transport conditions potentially present in contaminated subsurface environments. The insights from the modified FC experiments highlight the importance of studying reactive transport with spatio-temporal resolution and

demonstrate that overall system trends (based on inlet and outlet measurements) do not comprehensively account for variations within the system. For example, the modified FC experiments reveal that the region with higher ureolysis rates did not correspond to the region with the maximum Ca precipitation rates. Ureolysis combined with advective transport in the reactor resulted in increased pH, as well as increased predicted carbonate concentration, toward the effluent. The increasing carbonate concentrations likely resulted in maximum CaCO_3 precipitation downstream from the location of highest ureolysis, which was only observable through the spatially and temporally resolved sampling approach employed here. While the calcium precipitation rates over the entire reactor decreased with time, this decrease over time varied between regions of the reactor.

In the modified FC, strontium was co-precipitated in calcium carbonate via MICP and the extent of strontium incorporation into calcium carbonate was assessed using the dimensionless strontium distribution coefficient (D_{Sr}). A decrease in D_{Sr} was accompanied by an increase in precipitate size indicating that more nucleation relative to growth occurred closer to the inlet (smaller precipitates), which resulted in higher Sr partitioning compared to larger precipitates downstream.

A comparison between published Sr co-precipitation studies and the studies presented here revealed that within a system, D_{Sr} increases with increasing Ca precipitation rate. However, no universal trend is apparent between D_{Sr} and R_{aq} across different systems, which indicates that experimental factors likely have a large impact on this trend. To maximize strontium removal from the aqueous phase, bacterial precipitation might be preferred over abiotic precipitation. The pathway of co-precipitation (adsorption,

occlusion, solid solution formation, discrete phase formation) might influence the extent of strontium partitioning.

The spatio-temporal nature of metal incorporation during MICP in porous media was explored in the research presented here and has implications in bioremediation optimization. For example, precipitation can occur downstream from ureolytically active zones, but the distance between the zones of highest ureolytic activity and maximum calcium carbonate precipitation depends on reactive transport dynamics (such as flow velocity) which require more exploration. The ability to control reactive transport can inform reactor or subsurface injection design to promote precipitation in desired locations. More nucleation (relative to crystal growth) appears to occur closer to the injection zone leading to higher metal partitioning.

Repeated injections of urease-producing microbes would increase ureolysis rates and provide additional nucleation sites for precipitation, thus enhancing strontium incorporation via nucleation-dominated precipitation. Increased extracellular organics such as glycoproteins and other macro molecules produced by microbes could increase crystal aggregation.¹²⁴ More crystal aggregation could enhance the retention of suspended Sr-containing precipitates which otherwise would be transported out of the reactor. Increasing precipitate retention would potentially enable the collection and removal of the sequestered metal.

The presented research highlights reactive transport dynamics of ureolysis and ureolysis-induced calcium carbonate precipitation in a laboratory flow system. The results of this work can contribute to predicting the distribution of precipitates formed by MICP

and the extent of co-precipitation in the precipitates to utilize this process as a bioremediation technology.

Supplemental Information (SI): constituents of artificial ground water, protocol for flow cell disinfection, additional data for laser ablation inductively coupled plasma mass spectrometry and spatially resolved concentrations of calcium and strontium within the flow cell. This material is available free of charge via the Internet at <http://pubs.acs.org>

Author Information:

Corresponding Author: Dr. Ellen Lauchnor, ellen.lauchnor@montana.edu, (406) 994-2134.

The authors declare no competing financial interest.

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CHAPTER FIVE

CO-PRECIPITATION OF STRONTIUM AND BARIUM

Contribution of Authors and Co-Authors

Manuscript in Chapter 5

Author: Neerja Zambare

Contributions: Performed all experiments including reactor set up, operation, and analysis. Envisioned graphics, table design and wrote and revised manuscript.

Co-Author: Robin Gerlach

Contributions: Contributed to data interpretation and presentation. Contributed to writing of the manuscript in the form of comments and feedback.

Co-author: Ellen Lauchnor

Contributions: Identified major topics for review during development of manuscript and aided in data analysis and interpretation. Contributed to writing and revision of the manuscript in the form of comments and feedback.

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Co-precipitation of strontium and barium

Neerja M. Zambare ^{a,b}, Robin Gerlach ^{a,b}, Ellen G. Lauchnor ^{a,c}

^a Center for Biofilm Engineering, Montana State University, Bozeman, MT 59717

^b Department of Chemical and Biological Engineering, Montana State University,

^c Department of Civil Engineering, Montana State University,

Bozeman, MT 59717

Abstract

Groundwater resources at numerous DOE waste sites and abandoned mining sites are contaminated with dissolved radionuclides or heavy metals, which pose a threat to the environment and to human health. Trapping contaminants in biologically induced minerals has the potential to remove radionuclides or heavy metals from contaminated water, even in instances where these contaminants are present at trace concentrations. During microbially induced calcium carbonate precipitation (MICP), contaminants can be sequestered into calcium carbonate precipitates, a phenomenon called co-precipitation. Here, the co-precipitation of strontium and barium mixtures in calcium carbonate formed through ureolytic MICP is investigated in laboratory batch tests. MICP was promoted in batch reactors by the ureolytic bacterium *Sporosarcina pasteurii*. Co-precipitation of strontium and barium was reduced when both metals were present in solution, compared to experiments with a single metal present in solution. Spatial elemental mapping on precipitates from a mixed metals co-precipitation experiment revealed that although barium was removed from solution, it did not co-locate with calcium and was likely

forming a barium sulfate precipitate. These experiments demonstrate the potential of MICP to remove multiple metals simultaneously.

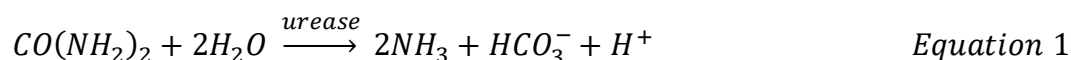
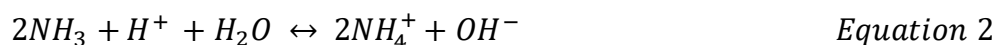
Introduction

Groundwater and soil near nuclear waste disposal sites can contain heavy metals and radionuclides that are released as by-products or waste-products from reactor operations during fuel production, fuel processing and isotope separation.¹²⁵ Strontium-90 (⁹⁰Sr) is a radionuclide produced as a result of uranium and plutonium fissions. It is commonly encountered in spent nuclear fuel, and radioactive wastes from nuclear fuel use and fuel reprocessing plants.¹²⁶ Chernobyl is considered to be the largest contributor of strontium-90 contamination.¹⁰⁴ Strontium has also been found in groundwater at numerous DOE waste sites,^{106, 125} often at concentrations exceeding the federal guidelines for drinking water.¹²⁵ If it enters the food-chain and is ingested, strontium can act as a “bone-seeker” given its similarity to calcium. Strontium deposits in bone and blood-forming tissue and can lead to bone cancer or leukemia.¹⁰⁴

Often, strontium is disposed in a mixture with barium because of similarities in their chemical behavior.¹²⁵ Additionally, Ba is chemically similar to radium¹²⁷, which is a dangerous radionuclide that can co-occur with strontium in nuclear waste. Barium has reportedly been discharged from coal¹²⁸ and uranium¹²⁹ mines. Mine tailings can contain elevated levels of barium¹³⁰, which can leach with other heavy metals into groundwater and surrounding soil/sediment. Steel production, copper smelting and automobile manufacturing can also lead to barium emissions.¹³¹ Barium ingestion can lead to cardiac and renal failure, pulmonary edema, respiratory paralysis, and gastric and intestinal

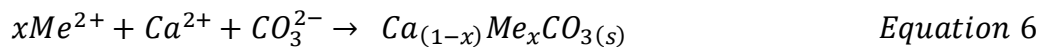
hemorrhages.¹²⁷ Mixed contaminant scenarios such as the co-occurrence of strontium and barium can be better addressed with remediation strategies capable of targeting multiple contaminants simultaneously.

Microbial interactions can be advantageously used to manipulate the environmental fate of heavy metal and radionuclide contaminants. Bio-sorption, -accumulation, -mineralization, and -reduction are some biological pathways for heavy metal/radionuclide remediation. Calcium carbonate precipitation can be efficiently promoted by bacteria while hydrolyzing urea, a well-studied process called microbially induced calcium carbonate precipitation (MICP). The reactions that take place during the process of ureolysis-driven MICP are shown in Equations 1-4:



Strontium and barium are both divalent cations that either co-occur in waste or can serve as surrogates for radionuclide contaminant mixtures. Given their chemical similarity to calcium, co-precipitation with calcium carbonate ($CaCO_3$) has been proposed as a bioremediation strategy to stabilize or remove dissolved strontium and barium from contaminated groundwater.^{111-116, 122} Equations 5 and 6 show possible co-precipitation mechanisms of discrete phase formation (Equation 5) and incorporation into calcium carbonate (Equation 6) for a divalent metal ' Me^{2+} '.





Co-precipitation is an efficient form of remediation because once the metals are co-precipitated out of groundwater and sequestered into the $CaCO_3$, the precipitates are stable over long periods of time if the water remains saturated with respect to $CaCO_3$.^{87, 101-103} Because barium is similar to radium, barium co-precipitation studies can inform radium bioremediation strategies.

The studies presented here focus on the co-precipitation of strontium and barium present as co-contaminants in artificial groundwater. The efficiency of co-precipitation enabled via ureolysis-driven microbially induced calcium carbonate precipitation decreased in the presence of strontium and barium when they were co-existing in solution compared to when they were present as single contaminants. Such studies focusing on more complex mixtures of contaminants are necessary to gain a deeper understanding of the effect of metal co-occurrence on metal incorporation into calcium carbonate. In summary, the work presented here contributes to bioremediation research as it pertains to metal removal by biomineralization.

Experimental Methods

Media and Set-Up

Artificial Ground Water (AGW) was prepared according to the recipe given in Ferris et al.⁵⁹ and amended with 1 g L⁻¹ Difco Nutrient Broth (Becton Dickinson) and 10 g L⁻¹ urea (Fisher Scientific). For single metal batch studies, 0.1 mM strontium ($SrCl_2 \cdot 6H_2O$, Fisher Scientific) or 0.1mM barium ($BaCl_2 \cdot 6H_2O$, Fisher Scientific) was added to the AGW. These ‘single metal’ experiments were named ‘Sr-only’ and ‘Ba-only’

respectively. For the co-contaminant experiments ('Sr+Ba'), both strontium and barium were added to final concentrations of 0.1 mM each. This concentration was chosen as it is an intermediate value in the range of strontium concentrations reported in groundwater at contamination sites.¹⁰⁶ All media were adjusted to a pH of 6.3-6.5 to avoid abiotic precipitation prior to all experiments. Each batch experiment started with 150 mL of the appropriate AGW, inoculated with 1 mL of inoculum. Flasks were covered with aluminum foil and placed on a 150 RPM shaker at ambient temperature for 96 hours. Each condition was tested in triplicate along with a metal-free control condition. The control was set up and inoculated in the same way but the AGW used had no strontium or barium.

Inoculum Preparation

For the inoculum in each experiment, 1 mL frozen stock of *S. pasteurii* (ATCC 11859) was added to 100 mL Brain Heart Infusion medium (BHI, Fisher Scientific) amended with 0.33 M urea and incubated at 30°C (at 150 RPM). After 24 hours, 1 mL of the culture was transferred into 100 mL fresh BHI + 0.33 M urea. This transfer culture was incubated for another 24 hours under the same conditions before centrifuging at a relative centrifugal force of 2964 x g for 10 minutes at 4°C. The supernatant was decanted and the cell pellet was re-suspended in freshly prepared AGW and centrifuged again. Re-suspension and centrifugation were repeated once more, after which cells were diluted in AGW to a final OD₆₀₀ of 0.4 on a BIOTEK Synergy HT absorbance plate reader in 96-well plates with 200 µL per well (reference blank well absorbance of 0.044).

Chemical Analyses

Using sterile syringes, 1 mL samples were drawn from each flask at scheduled time points over the duration of the experiments. Half of each sample was filtered using a 0.2 μm filter (VWR, PA) and the unfiltered aliquot was used for pH measurement using a Symphony SB70P pH meter (VWR, PA, USA). The filtered samples were used to measure dissolved calcium, strontium, and barium using an Inductively Coupled Plasma-Mass Spectrometer (ICP-MS, Agilent 7500ce). Dissolved urea concentrations were measured using the Jung assay.¹²⁰ After the 96-hour sample, all the fluid in the flasks was decanted into a fresh set of flasks. After measuring the volume of the decanted fluid, an equal volume of 20% nitric acid (HNO_3) was added to each flask slowly under a chemical fume hood. The solutions were kept in the fume hood overnight and then the calcium, strontium and barium concentrations were quantified using the ICP-MS. In parallel, the precipitates adhered to the original flasks were digested using 20% HNO_3 , and diluted to 5% strength for analysis using the ICP-MS.

Kinetics and Metal Partitioning Expressions

The ionic strength of the solution was calculated at each sampling time point (according to table 3.3 in Stumm & Morgan¹³²) using the AGW constituent concentrations, measured calcium, strontium and barium concentrations and estimated ammonium concentrations. Urea and pH measurements were used to estimate the concentrations of produced ammonia $[\text{NH}_3]$ and ammonium $[\text{NH}_4^+]$ produced as per the following set of equations:

$$\Delta[\text{urea}] * 2 = [\text{NH}_3] + [\text{NH}_4^+] \quad \text{Equation 7}$$

$$pH = pK_a + \log_{10} \frac{[NH_3]}{[NH_4^+]} \quad \text{Equation 8}$$

$$[NH_4^+] = \frac{(\Delta urea * 2)}{(10^{(pH-9.29)} + 1)} \quad \text{Equation 9}$$

where all concentrations are in mol*L⁻¹, pK_a is the equilibrium constant for ammonia-ammonium equilibrium, and $\Delta[urea]$ is the measured change in urea concentration. Equation 9 is the result of combining Equations 7 and 8. $[NH_4^+]$ estimates calculated by Equation 9 were included in temporally resolved calculations of ionic strength. The pK_a of ammonia/ammonium was adjusted using the Güntelberg approximation (Equation 10) to incorporate changes in ionic strength, resulting in a small change from 9.24 to 9.29 (used in Equation 9).¹³²

$$pK' = pK_a + \frac{0.5(z_{HB}^2 - z_B^2) * \sqrt{I}}{1 + \sqrt{I}} \quad \text{Equation 10}$$

where pK' is the adjusted equilibrium constant, z_{HB} and z_B are charges of acid and base respectively, and I is the ionic strength in units of mol*L⁻¹. The second term on the right side of Equation 10 corrects for non-ideality of the solution. Table 3.5 from Stumm & Morgan is used for values of z_{HB} and z_B for Equation 10.

The measured change in urea concentration was used to calculate the amount of inorganic carbon from all carbonate species (carbonic acid, bicarbonate, carbonate) generated during ureolysis according to stoichiometry ($C_{ureolysis} = \Delta[urea]$). Estimates of inorganic carbon generated by ureolysis ($C_{ureolysis}$) and inorganic carbon consumed during calcium carbonate precipitation ($C_{precipitation}$), were used in the total dissolved inorganic carbon balance, evaluated at each time point, given by Equation 11.

$$\begin{aligned}
C_{total} &= C_{AGW} + C_{ureolysis} - C_{precipitation} \\
&= DIC_{AGW} + \Delta[urea] - \Delta[Ca^{2+}]
\end{aligned}
\tag{Equation 11}$$

where all concentrations are in $\text{mol}\cdot\text{L}^{-1}$, C denotes inorganic carbon, DIC_{AGW} is the dissolved inorganic carbon present in AGW, $\Delta[urea]$ is the measured change in urea concentration, and $\Delta[Ca^{2+}]$ is the measured change in calcium concentration. ($C_{precipitation} = \Delta[Ca^{2+}]$ since the molar ratio of calcium-to-carbon in CaCO_3 is 1:1).

While total $C_{ureolysis}$ can be estimated as equimolar to urea consumed (*cf.* Equations 1-3), the speciation within the carbonate system (i.e., carbonic acid vs. bicarbonate vs. carbonate) is affected by the equilibrium chemistry, which is dependent on pH. To estimate the carbonate (CO_3^{2-}) fraction of the total inorganic carbon in solution, C_{total} from Equation 11 is written in terms of CO_3^{2-} using equilibria reactions between the different carbonate species. The fraction of carbonate ($\alpha_{\text{CO}_3^{2-}}$) can then be estimated as:

$$\frac{[\text{CO}_3^{2-}]}{C_{total}} = \alpha_{\text{CO}_3^{2-}} = \frac{1}{1 + \frac{[H^+]}{K_2} + \frac{[H^+]^2}{K_1 K_2}}
\tag{Equation 12}$$

where K_1 and K_2 are equilibrium constants for carbonic acid/bicarbonate and bicarbonate/carbonate equilibria respectively, and $[H^+]$ is calculated from measured pH. K_1 and K_2 were also adjusted using the Güntelberg approximation to account for ionic strength changes during the process. At each time point, the activity coefficients for calcium, strontium, barium and carbonate were calculated using the extended Debye-Hückel equation provided in Table 3.3 in Stumm & Morgan.¹³²

The saturation state of calcium carbonate (S_{CaCO_3}) was calculated at each time point as:

$$S_{CaCO_3} = \frac{(\gamma_{Ca^{2+}}[Ca^{2+}]) * (\gamma_{CO_3^{2-}}[C_{total} * \alpha_{CO_3^-}])}{K_{sp,CaCO_3}} \quad \text{Equation 13}$$

where $\gamma_{Ca^{2+}}$ and $\gamma_{CO_3^{2-}}$ are activity coefficients for calcium and carbonate respectively, $[Ca^{2+}]$ is the measured calcium, C_{total} is the total inorganic carbon from Equation 11, $\alpha_{CO_3^-}$ is the carbonate ionization fraction from Equation 12 and $K_{sp,CaCO_3}$ is the solubility product for calcium carbonate.

Metal partitioning was assessed in terms of the distribution coefficient calculated as (D_{Me})

$$D_{Me} = \left(\frac{Me_{ppt}}{Ca_{ppt}} \right) * \left(\frac{Ca_{aq}}{Me_{aq}} \right) \quad \text{Equation 14}$$

where the 'Me' denotes the divalent metal cation of choice (here, either Sr or Ba). Me_{ppt} and Ca_{ppt} are the mass (moles) of metal and calcium in the precipitates and Me_{aq} and Ca_{aq} are the aqueous concentrations ($\text{mol} \cdot \text{L}^{-1}$) of the metal and calcium respectively.⁸⁷ Me_{ppt} and Ca_{ppt} are estimated as mass removed from the aqueous phase based on the assumption that all removed metal appears in the precipitate.

Results and Discussion

Influence of Sr and Ba on ureolysis and calcium removal

In these experiments, MICP was promoted in artificial groundwater using *Sporosarcina pasteurii*. Co-precipitation of strontium and barium was investigated when they were present as single contaminants (Sr or Ba present) and co-contaminants (both Sr and Ba present). In all conditions tested, the pH increased from 6.4 ± 0.1 to above 9,

indicative of ureolysis (Figure 1.a). Ureolysis rates in the control experiments (*black markers*) were higher than in metal-inclusive experiments (*gray markers*) (Figure 1.b) and this difference was greater during the first half of the experiments. All conditions tested resulted in complete urea depletion within 96 hours. The urea and pH data show that at the metal concentrations tested (which are representative of trace level contamination in groundwater), ureolysis is not significantly inhibited.

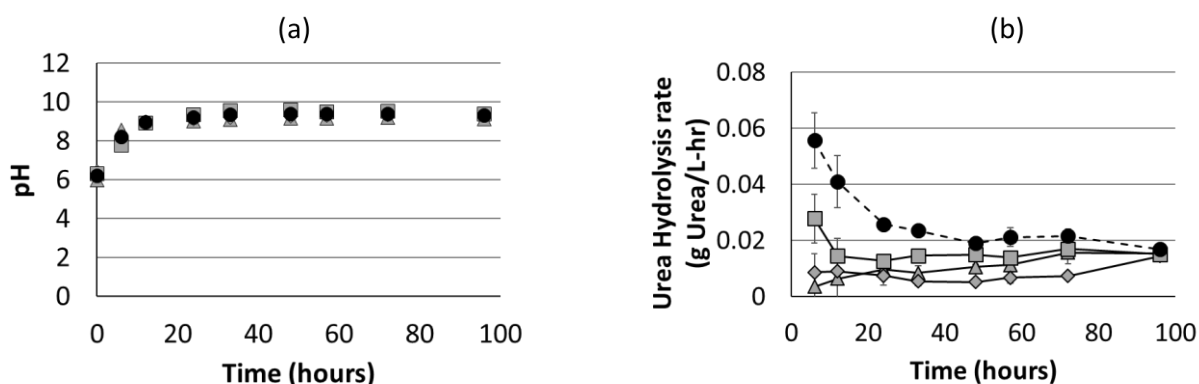


Figure 1: (a) pH over time for Sr-only (▲), Ba-only (◆), Sr+Ba (■), and metal-free controls (●). In all experiments, regardless of metal-inclusive or metal-free, the pH increased to above 9, strongly indicative of ureolysis. (b) Ureolysis rates over time for all conditions. The metal-free controls initially showed higher ureolysis rates. In both (a) and (b), error bars denote ± 1 standard deviation.

During the first half of all experiments, dissolved calcium concentrations did not appear to change significantly ($\Delta Ca < 9\%$) (Figure 2.a). Calcium concentrations appeared to decrease after 57 hours in Sr-only (▲) and in Sr+Ba (■), and after 72 hours in Ba-only (◆). In the metal-free control experiments (●), a decrease in calcium concentrations was observed after 48 hours.

Saturation indices calculated based on aqueous chemistry (Equation 13) indicated that the AGW became supersaturated with respect to calcium carbonate ($SI_{CaCO_3} > 0$) at time points earlier than the observed onset of aqueous calcium removal (Figure 2.a). A similar

“lag” in calcium removal despite solution oversaturation was observed by Ferris et al.⁵⁹, who hypothesized that during MICP, a solution needed to achieve a degree of critical supersaturation prior to onset of nucleation and precipitation. Stable nuclei can form only after passing the free energy barrier for nucleation activation. Thus, unstable CaCO_3 nuclei might exist between supersaturation and critical supersaturation with a very small amount of calcium moving in and out of the solid phase, but a significant decrease in calcium concentrations can only be observed after critical saturation is achieved and when forming nuclei are stable, and precipitates are growing. Minor fluctuations ($< 9\%$) in calcium concentration measurements in the first 48 hours could potentially be attributed to the small amounts of calcium forming unstable CaCO_3 nuclei and re-dissolving, as well as uncontrolled differences in sampling, filtering, acid-diluting and/or ICP-MS performance.

In the studies presented here, concentration measurements were performed on filtered and acidified samples. As a result, any precipitates smaller than the filter size of $0.2\ \mu\text{m}$ could have been measured as part of the aqueous calcium concentration. Thus, precipitation could indeed have initiated earlier than could be detected with our methods. Ferris et al. did not detect aqueous calcium removal until a certain amount of ammonium was detected (i.e. a certain level of ureolysis had occurred). For the conditions tested in our studies, calcium removal was detected after approximately 1.6 mmol urea were hydrolyzed in single contaminant and control experiments. In Sr+Ba, 1.95 mmol urea were hydrolyzed before calcium removal was observed.

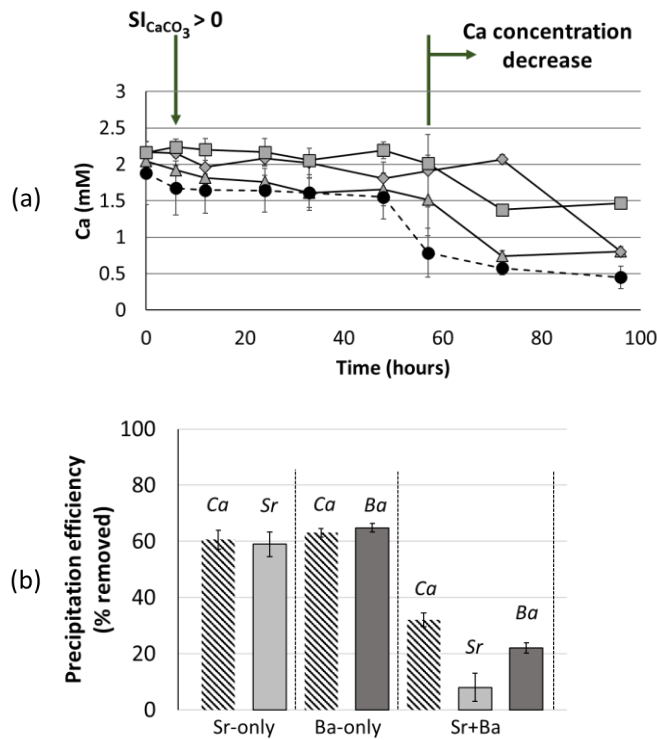


Figure 2: (a) Calcium concentrations over time for Sr-only (Δ), Ba-only ($\diamond$), Sr+Ba ($\square$), and metal-free controls ($\bullet$). (b) Removal efficiencies in all conditions for Ca, Sr, and Ba. Diminished removal of both calcium and contaminant was observed in the co-contaminant condition.

The precipitation efficiency (calculated as percentage of mass removed from the aqueous phase, Figure 2.b) was significantly lower in the co-contaminant experiments compared to the single metal experiments for calcium (hatched , $p < 1 \times 10^{-4}$), strontium (white , $p = 0.0012$) and barium (solid grey , $p < 1 \times 10^{-3}$). As the ureolysis and pH data for Sr+Ba are comparable to the single metal experiments, the lower precipitation efficiency seen in Sr+Ba cannot be attributed to lowered cell (ureolytic) activity in the presence of both metals. However, the higher concentration of total heavy metals in Sr+Ba could have been detrimental to cell growth and/or cell activity, leading to fewer nucleation points leading to fewer precipitates. To investigate this hypothesis, an in-depth study of crystal nucleation and the effect of heavy metals on growth is required. The slightly higher calcium

precipitation efficiency in Ba-only compared to in the Sr-only condition could not be determined as statistically significant ($p=0.486$).

Sr and Ba removal

Concentration ratios of Sr:Ca and Ba:Ca (Figure 3.a) stayed relatively constant over time until significant precipitation started (57 hours onwards, *cf.* Figure 2.a). In single contaminant experiments, Me:Ca ratios stayed constant after the onset of precipitation, suggesting that strontium/barium removal was occurring at the same rate as calcium removal. In the co-contaminant condition Me:Ca ratio in solution increased when precipitation was detected, indicating that strontium and barium were removed from solution to a lesser degree than calcium. However, Ba:Ca was lower than Sr:Ca in solution, indicating that more barium precipitated during the experiment. For the co-contaminant condition, the calcium carbonate saturation state (S_{CaCO_3}) was consistently higher during the entire experiment (Figure 3.b, *squares*) compared to single-metal conditions (Sr: *triangles*, Ba: *diamonds*).

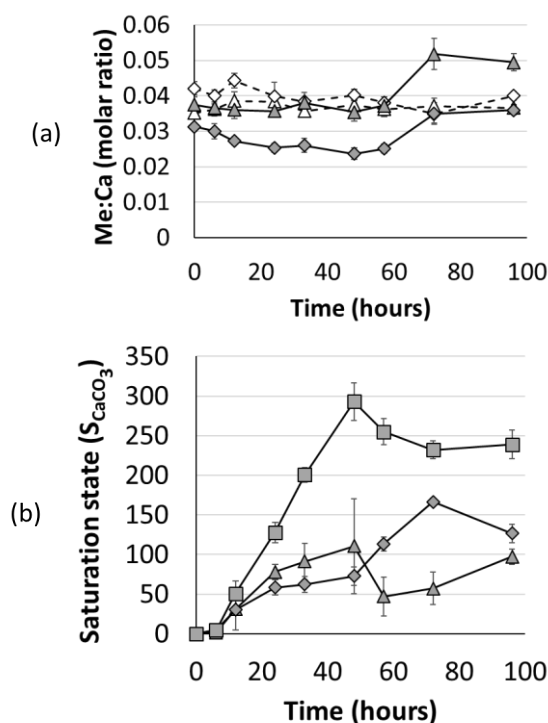


Figure 3: (a) Metal to calcium molar ratio in solution over time for Sr-only ($\triangle$), Ba-only ($\diamond$), Sr in Sr+Ba ($\triangle$), Ba in Sr+Ba ($\diamond$). (b) Saturation state for calcium carbonate over time for Sr-only ($\triangle$), Ba-only ($\diamond$) and Sr+Ba ($\square$).

To determine the potential for precipitation of different calcium, strontium and barium minerals, aqueous geochemical modeling was performed using MINTEQA. A range of carbonate concentrations corresponding to 0-100% urea hydrolyzed (of the initial 10 g L⁻¹ urea present in the AGW) was used for the model. As the number of experimental sampling points was lower than the points used for the model, estimates for solution pH were made by interpolating linearly between adjacent measured pH values where necessary. Initial concentrations for calcium, strontium and barium were used at each model point to determine the potential of the original AGW medium (without accounting for ureolysis or temporal changes in metal concentrations) to precipitate various minerals via ureolysis-driven alkalinity changes.

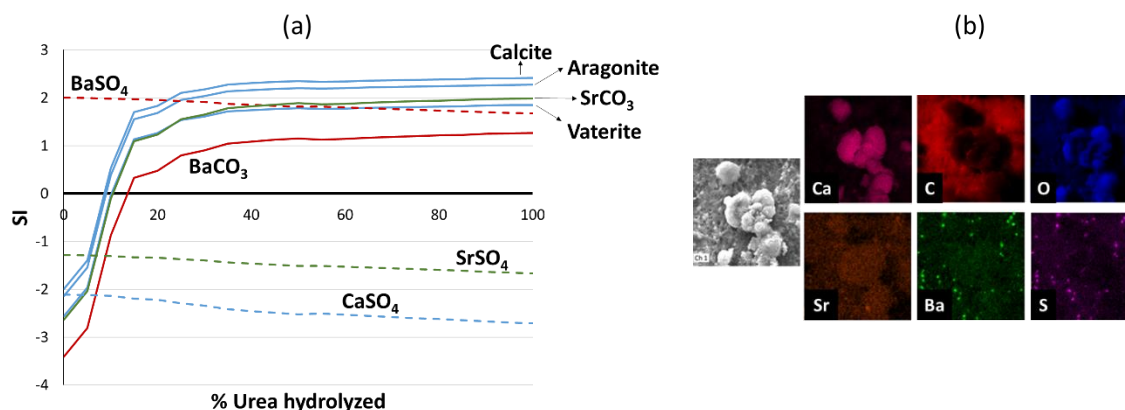


Figure 4: (a) Saturation indices for carbonates (continuous lines) and sulfates (dashed lines) of interest in Sr+Ba. (b) Nano-Auger energy dispersive X-ray spectroscopy maps showing spatial distribution of elemental abundance in a precipitate cluster from a Sr+Ba replicate.

The MINTEQ results (Figure 4) show that the AGW was oversaturated with respect to BaSO_4 , and undersaturated with respect to SrSO_4 and CaSO_4 during the entire duration of the experiments. The AGW was oversaturated with respect to other carbonate minerals of interest (CaCO_3 , SrCO_3 and BaCO_3) after about 8-13% of ureolysis (within the first 24 hours of the experiments). Aragonite, vaterite and calcite crystalline forms of CaCO_3 showed potential to precipitate first (8.5-10% ureolysis) followed by SrCO_3 (10% ureolysis), and then BaCO_3 (13% ureolysis). Elemental spatial distribution maps of calcium, strontium and barium were acquired by Energy Diffraction X-Ray on precipitates from the Sr+Ba experiment using a Scanning Auger Electron Nanoprobe (Physical Electronics 710). Strontium appeared to co-locate with calcium, consistent with the assumption of strontium incorporation into CaCO_3 . However, barium did not co-locate with calcium (Figure 4.b) suggesting that the mechanism of barium removal was not incorporation into CaCO_3 . Barium was found to indeed co-locate with sulfur, consistent with MINTEQ modeling results of BaSO_4 supersaturation.

Traditional methods of quantifying metal partitioning rely on calculating the metal distribution coefficient (Equation 14). For comparison with published studies, distribution coefficients for strontium (D_{Sr}) and barium (D_{Ba}) were calculated and are presented in Figure 5.

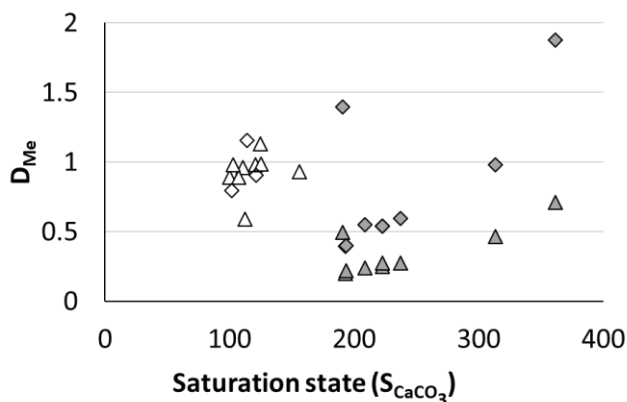


Figure 5: Distribution coefficients for strontium in Sr-only ($\triangle$) and Sr+Ba ($\triangle$), and for barium in Ba-only ($\diamond$) and Sr+Ba ($\diamond$) at the corresponding saturation state for calcium carbonate.

When both metals were present (*grey markers*), a higher $CaCO_3$ saturation state was necessary to achieve metal removal (D_{Me}) to an equivalent degree as in the single metal experiments (*white markers*) (Figure 5 and *cf.* Figure 3.b). D_{Ba} (*diamonds*) were consistently higher over time compared to D_{Sr} (*triangles*) when comparing between single metal experiments and within the co-contaminant experiments. Abnormally high values of D_{Ba} have been reported to appear more readily than for D_{Sr} due to barium having larger influence over the crystal form of $CaCO_3$ compared to strontium.¹¹⁴ Even small amounts of barium have been found to favor calcite precipitation while strontium had little influence on the calcium carbonate polymorph. The calculated D_{Ba} values for the experiments discussed here are high as they likely include barium in $BaSO_4$ in their estimate.

According to the assumption underlying Equation 14, all the metal removed from the aqueous phase is assumed to be sequestered in the calcium carbonate precipitates. The

elemental distribution maps (Figure 4.b) however suggest that barium did not sequester in CaCO_3 to a large extent but rather precipitated as BaSO_4 . The microscale analysis and geochemical modeling therefore inspires avenues for research into other potential minerals that can be used for heavy metal remediation. Sequestration into calcium carbonate has been proposed as a remediation application because it can reduce/remove even trace concentrations of heavy metals¹⁰⁰ and the precipitates are stable over long periods of time.^{87, 101-103} With the results of the presented studies showing the potential for other minerals to form in aqueous geochemical conditions that mimic groundwater, more research into their stability and interactions with carbonate minerals is required.

To accurately test heavy metal incorporation into calcium carbonate as the sole mechanism of metal removal, experiments that are designed to remain undersaturated with respect to discrete heavy metal minerals would be optimal. If the precipitation potential is isolated to calcium carbonate, any reduction in aqueous concentrations of metals could then be attributed to incorporation into CaCO_3 with higher confidence.

In this work, the application of MICP as a remediation tool for mixed contaminants was investigated. Strontium and barium were removed from solution alongside calcium. Removal efficiencies for calcium, strontium and barium were high when strontium and barium were present as single contaminants. Strontium co-precipitation with calcium carbonate occurred regardless of the presence of a second contaminant (barium). However, precipitation of all cations (Ca, Ba and Sr) was significantly reduced when all three species were present in the AGW. This phenomenon was not due to changes in bacterial activity

(ureolysis rate), as the saturation index was higher under the mixed condition prior to the start of precipitation. More investigation into the reason why this occurred is needed.

Although barium removal was observed, it was found to co-locate with sulfur instead of calcium and therefore was considered to not be co-precipitated with calcium to a considerable degree. For future investigations of metal or radionuclide co-precipitation, supplemental microanalytical investigations focused on spatial elemental mapping are encouraged to provide robustness to the conclusion of metal incorporation.

Author Information:

Corresponding Author: Dr. Ellen Lauchnor, ellen.lauchnor@montana.edu, (406) 994-2134.

The authors declare no competing financial interest.

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CHAPTER SIX

VISUALIZING MICROBIALLY INDUCED MINERAL PRECIPITATION FROM SINGLE CELLS USING DROP-BASED MICROFLUIDICS

Contribution of Authors and Co-Authors

Manuscript in Chapter 6

Author: Neerja Zambare

Contributions: Designed and performed all experiments, collected data and analyzed data. Envisioned graphics, table design and wrote and revised manuscript.

Co-Author: Nada Naser

Contributions: Assisted with media preparation, experimentation, and data collection.

Co-author: Robin Gerlach

Contributions: Identified major topics for review during development of manuscript and aided in data analysis and interpretation. Contributed to writing and revision of the manuscript in the form of comments and feedback.

Co-Author: Connie Chang

Contributions: Identified major topics for review during development of manuscript and aided in data analysis and interpretation. Contributed to writing and revision of the manuscript in the form of comments and feedback.

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Visualizing Microbially Induced Mineral Precipitation from Single Cells Using Drop-
Based Microfluidics

Neerja M. Zambare^{a,b}, Nada Naser^{a,b}, Robin Gerlach^{a,b}, Connie B. Chang^{a,b}

^a Center for Biofilm Engineering, Montana State University, Bozeman, MT 59717

^b Department of Chemical and Biological Engineering, Montana State University,
Bozeman, MT 59717

Abstract

Microbe-mineral interactions are ubiquitous and can facilitate major biogeochemical reactions behind dynamic Earth processes. Biomimetic approaches to environmental remediation often involve microbe-mineral interactions (MMI). An example of MMI is Microbially induced calcium carbonate precipitation (MICP), a process by which microbial activity leads to the formation of calcium carbonate precipitates. These precipitates can be utilized as ‘biocement’. A majority of MICP studies are conducted at the mesoscale, with some application studies at the macroscale. However, fundamental questions behind MICP are challenging to answer because this would require studying MICP at the microscale (cellular level). Research on fundamental processes such as precipitate nucleation on bacteria, bacterial interactions with the precipitates, polymorph transitions, and effects of organics on precipitates will benefit from microscale studies. This paper presents a drop-based microfluidic approach to visualizing MICP starting from single ureolytic cells. Bacterial ureolysis is promoted in 25 μm diameter drops and calcium

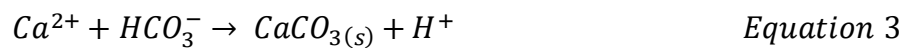
carbonate precipitates are observed in the drops. Multiple visualization and characterization techniques were performed on the precipitates to verify they were calcium carbonate.

Amorphous calcium carbonate (ACC) is observed first in drops and the instances of ACC decrease over time alongside an increase in instances of vaterite. Upon focused ion beam milling, the vaterite precipitates showed a different interior structure compared to the exterior structure. With a micro-level analysis of MICP as presented here, microbe-mineral interactions can be better visualized and understood.

Introduction

Numerous geomicrobiological phenomena in nature involve interactions between bacteria and minerals, such as heavy metal mobility^{98, 133}, pollutant bioavailability^{98, 133} and sediment magnetization.¹³⁴ Interactions between microbes and minerals are a central aspect of geomicrobiology, with many of these interactions having reciprocal consequences. For instance, minerals can affect microbial processes such as nutrient acquisition¹³⁵, energy generation¹³⁵, and biofilm structure¹³⁶; microbial processes can in turn, affect mineral formation, transformation, and dissolution¹³⁵. These microbe-mineral interactions (MMI) play a fundamental role in rock formation and weathering¹³⁵, acid mine drainage¹³⁷, and hydrothermal vent formations.¹³⁸ While geomicrobiological processes can be readily observed at the macroscale, they are driven by cellular and enzymatic reactions that occur at the micro- or even nanoscale.¹³⁵

One example of MMI is Microbially Induced Calcium Carbonate Precipitation (MICP), a biomineralization process that occurs both in nature and in engineered systems as a result of bacterial activity.¹ MICP can occur when a metabolic process (such as sulfate or nitrate reduction, urea hydrolysis, or photosynthesis) results in an increase of pH and alkalinity, raising the saturation state of calcium carbonate (CaCO_3) and causing CaCO_3 precipitation.¹ Equations 1 and 2 show the stoichiometry for ureolysis and the resulting increase in alkalinity. Under such conditions, aqueous calcium present in the microenvironment can precipitate out of solution as CaCO_3 (Equation 3).



MICP is studied most often at the mesoscale in bench-scale reactor systems to investigate the kinetics of ureolysis and precipitation. Visualization of microbe-mineral interactions is typically performed on samples extracted from these reactor systems, which are not necessarily accurate representations of the original state of the microbes or minerals. Such ‘ex-situ’ visualization does not allow for the time-resolved observation of microbes and minerals starting from single cells or small groups of cells. Non-invasive visualization of MICP at the nano- and microscale has the potential to provide statistically sound quantitative comparisons of cell growth, cell motility, mineral nucleation, mineral growth and mineral types that can develop. All these processes are important in linking the biogeochemical reactions occurring at the nano- and microscale to macroscale phenomena.

Drop-based microfluidics allows for the study of complex biochemical processes at the single cell level. Single cells can be isolated in picoliter-sized drops that are tens of micrometers in diameter. These drops are formed at rates of thousands per second by flowing fluids of an aqueous phase and an oil phase into a flow-focusing microfluidic device that creates drops of water-in-oil.¹³⁹ When bacterial cells are isolated within drops,¹⁴⁰ the drops serve as microscopic bioreactors, allowing studies on microbial growth, kinetics and fundamental cellular physiology.^{141, 142} Drop-based microfluidics can be paired with microscopy to perform time-lapse studies of single cell growth and enzyme kinetics occurring within the drops.¹⁴³

Drop-based microfluidics has been used to observe abiotic precipitation^{45, 144, 145} but to our knowledge, has never been used to study MICP starting from single bacterial cells. Drop-based microfluidics can enable high resolution, time-wise observation of biomineralization at the nano- to microscale, providing insight into MMI over time. The ability to study biomineralization in such a non-invasive manner can provide valuable insights into the mechanisms underlying large scale precipitation processes.¹⁴⁶

Here, we visualized ureolysis-driven MICP by single *E. coli* MJK2⁹⁷ cells using drop-based microfluidics. We generated 25 μm diameter drops of media containing single MJK2 cells in media and we observed the growth of cells and CaCO_3 minerals over time using confocal laser scanning microscopy (CLSM). We discovered that a high calcium concentration in the media decreased cell growth and cell motility. We varied calcium ion concentrations in the media and discovered that CaCO_3 precipitates were observed in drops at all concentrations if urea and MJK2 cells were present. In these experiments, precipitates

were also observed in neighboring drops without ureolytic cells and these precipitates formed via diffusion of ureolytic byproducts (carbonate and hydroxyl ions) between drops. Precipitates that formed inside drops were characterized using a range of microscopy and microanalysis techniques. We observed two distinct polymorphs of CaCO_3 precipitates in drops with vibrational spectroscopy signals corresponding to amorphous calcium carbonate (ACC) and vaterite. The ratio of drops containing ACC to drops containing vaterite was dependent on the concentration ratio of dissolved calcium to urea in the initial media and reached a maximum at an equimolar calcium to urea ratio. The structure of the vaterite precipitates themselves exhibited two optically different polymorphs. This work investigates MICP formation starting from single cells, with a focus on differences in cell growth, cell motility and precipitate polymorphs as a function of dissolved calcium available in the media. The methods presented here offer a platform for studying microbe-mineral processes starting from single bacterial cells, including biological precipitate formation and polymorph transitions.

Experimental Methods

Media and Bacterial Cell Culture

Escherichia coli MJK2, a strain of *E. coli* engineered to express GFP and produce urease⁹⁷, was used to promote MICP. ‘Growth medium’ for MJK2 was Luria-Bertani medium (BD Difco™, Fisher Scientific, NH, USA) amended with $10 \mu\text{g mL}^{-1}$ gentamicin, 50 mM L-arabinose and $10 \mu\text{M}$ nickel chloride (Sigma-Aldrich, MO, USA).⁹⁷ The cell culture was prepared by adding 50 μL of MJK2 frozen stock culture to 10 mL of growth medium in a 50 mL centrifuge tube loosely covered with aluminum foil to ensure ample

oxygen availability to the growing cells. The centrifuge tube was placed on a horizontal shaker operating at 150 RPM and the cell culture was allowed to grow for 24 hours at ambient temperature (23-26°C). After 24 hours, the optical density (OD) was measured using a BIOTEK Synergy HT spectrophotometer (600 nm wavelength, 200 µL samples in a 96-well plate, with the blank well OD of 0.044 as reference) and adjusted to 0.4 using growth medium. From this adjusted OD, a further dilution was necessary to generate drops with 1 in 10 drops exhibiting a single cell per drop. Through a series of cell enumeration experiments (see Supplemental Information and Figure D1), a 1:5 dilution was determined to be optimal for this purpose.

Urea and calcium chloride (Fisher Scientific, NH, USA) were added to growth medium at the concentrations listed in Table 1 to prepare media for the six conditions tested in this study. Three conditions (Ca_{Low} , Ca_{Int} and Ca_{High}) had the prerequisites for MICP to occur (dissolved calcium, dissolved urea, and MJK2, cf. equations 1-3); the other three conditions were negative control experiments (marked with a '*' in Table 1), either without calcium in the medium (calcium-free), without urea in the medium (urea-free) or without bacteria (bacteria-free). All media were filter sterilized using Nalgene™ Rapid-Flow™ 0.45 µm pore size bottle-top filters (ThermoFisher Scientific, MA, USA).

Table 1: Experimental conditions for the drop studies. The three experiments marked with (*) are control experiments and the others are mineralization experiments.

Experiment Description	Calcium (M)	Urea (M)	Calcium-to-urea molar ratio (Ca:Urea)	MJK2 cells
Low Ca ('Ca _{Low} ')	0.0825	0.165	1:2	Yes
Intermediate Ca ('Ca _{Int} ')	0.165	0.165	1:1	Yes
High Ca ('Ca _{High} ')	0.33	0.165	2:1	Yes
Calcium-free*	0	0.165	-	Yes
Urea-free*	0.165	0	-	Yes
Bacteria-free*	0.165	0.165	1:1	No

Drop-Generation and work-flow

Co-flow microfluidic devices made of polydimethylsiloxane (PDMS) were fabricated using soft lithography.^{142, 147} Drops of 25 μm diameter were generated by flowing the 1:5 diluted bacterial culture, media and 3MTM NovecTM 7500 engineered fluid (oil) with surfactant¹⁴⁸ (1.5% (w/w)) in a flow-focusing co-flow microfluidic device (Figure 1). The bacterial culture and media formed the inner phase of the water-in-oil emulsion and the oil containing surfactant formed the outer phase. Using calibrated syringe pumps, the bacterial culture and media were set to flow rates of 200 $\mu\text{L h}^{-1}$ each, and the oil was set to a flow rate of 800 $\mu\text{L h}^{-1}$. Drops were collected in 1.5 mL microcentrifuge tubes and stored at ambient temperature for experimentation over the next 4 days (Figure 1).

Bulk experiments

Bulk experiments were performed to compare cell growth in drops to cell growth in larger volumes and to test ureolysis at the different calcium concentrations. For bulk experiments, 10 mL of media were used and the volume of bacterial inoculum added was

designed to simulate the cell-to-media ratio in the drops at the time of drop generation. Bulk experiments were conducted in triplicate in 50 mL centrifuge tubes loosely covered with aluminum foil and placed on a horizontal shaker (150 RPM) at ambient temperature. Sampling times were consistent with drop imaging times with five total samples being drawn from each tube reactor at 24-hour intervals, starting from and including the time of bacterial inoculation. Fluorescence (excitation 485 ± 20 nm, emission 528 ± 10 nm) measurements were performed using a BIOTEK Synergy HT spectrophotometer on 200 μ m samples drawn aseptically from the tube reactors (reference blank well fluorescence reading of 88 fluorescence units). Sample pH was measured using a Symphony SB70P pH meter (VWR, PA, USA). Samples were diluted using 5% nitric acid (Fisher Scientific, NH, USA) for urea concentration measurement using the colorimetric Jung assay.¹²⁰

Microscopy and Microanalysis

Time-resolved analysis in drops From the time of drop generation (Day 0) to the end of the experiment at 96 hours (Day 4), drops were imaged every 24 hours, for a total of five time points in all experiments except in the bacteria-free control, for which images were taken on days 0 and 7.

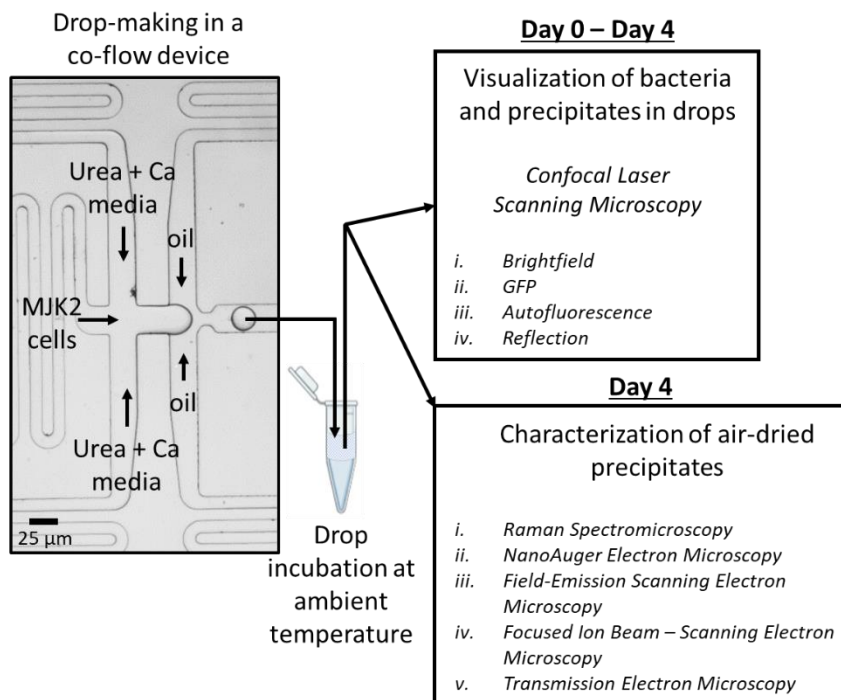


Figure 1: Schematic of experimental work-flow showing drop generation, storage, visualization of drops and characterization of air-dried precipitates.

In all experiments, an aliquot of drops stored in the microcentrifuge tube was injected into a DropSOAC microfluidic device¹⁴² for imaging using an inverted Leica SP5 confocal laser scanning microscope (CLSM). During imaging, the hydrated oil-soaked DropSOAC device was tightly capped under hydrated oil to maximize drop stability.¹⁴² Imaging was performed at ambient temperature. Drops were imaged from randomly-chosen locations in the DropSOAC device using a 20x air objective on using the following illumination channels:

- (i) Brightfield image of the entire drop (transmission images), 168 gain, 60 μm pinhole
- (ii) GFP (Excitation: 488 nm laser, Emission: 490 - 590 nm) to detect MJK2 cells, 612 gain, 200 μm pinhole

- (iii) UV (Excitation: 405 nm laser, Emission: 415 nm - 550 nm) to detect CaCO_3 through its autofluorescence^{50, 149}, 600 gain and 200 μm pinhole and
- (iv) Reflection light (488 nm incident laser, detection between 478 - 498 nm) to detect precipitates (no excited fluorophore), 200 gain, 60 μm pinhole. A reflection signal allows visualization of precipitates as it is generated when the incident laser reflects off solid material.

Image acquisition parameters for CLSM (gain and pinhole aperture) were kept consistent between experiments for each channel. The initial fluorescence of the 0.4 OD-adjusted cultures used for the different experiments was comparable with a low relative standard deviation of <2%. Drops containing cells were detected using the ImageJ TrackMate plug-in¹⁵⁰ by specifying 25 μm as the drop detection diameter, centered around the GFP signals on GFP channel images (visually checked against the corresponding brightfield images). The single-plane images taken with a large 200 μm pinhole aperture captured signal from a greater imaging volume. This method captures signal from out-of-focus planes, allowing rapid imaging of an entire drop while preventing signal overestimation from motile cells appearing in more than one plane during Z-stack acquisition. The fluorescence intensity per drop was measured by TrackMate on the GFP signal images from each day and each experiment. The numbers of drops analyzed are provided in Table D1. For motility measurements, a 30 second acquisition video of approximately 300 drops from each experiment was analyzed for mean track velocity (in $\mu\text{m s}^{-1}$) using TrackMate. Tracks were detected in drops containing cells based on GFP signal detection constrained to 1 μm diameter (numbers of tracks in Table D2).

For drop tally measurements, similar drop detection was performed centered around reflection signals on reflection channel images. Drop diameter detection settings were used to differentiate between drops containing the two observed precipitate morphologies characterized later as amorphous calcium carbonate (ACC) and vaterite (see Figure 5). The number of drops counted at the 25 μm diameter detection setting for the reflection signal provided the total number of drops containing precipitate (either morphology). Drop tally measurements with a diameter detection setting of 5 μm selected for the drops containing the smaller vaterite precipitates. The difference in the total number of precipitate-containing drops (either morphology) and the number of vaterite-containing drops resulted in the number of ACC-containing drops.

Precipitate Characterization On day 4, precipitates from drops were collected for visualization and determination of elemental composition and precipitate mineralogy. A 10 μ L sample of drops was air-dried at ambient temperature on a glass slide overnight, breaking the drop emulsion and exposing the precipitates. The dried sample was transferred onto sticky carbon tape on a metal stub, sputter coated with iridium at 20 mA for 1 minute and imaged using a Zeiss SUPRA 55VP Field Emission Scanning Electron Microscope (SEM). The same sample was analyzed using energy dispersive X-ray spectroscopy to characterize the elemental composition of the precipitates (Scanning Auger Electron Nanoprobe, Nano-Auger, Physical Electronics 710). In addition, air-dried precipitates from each mineralization experiment were analyzed using a Horiba LabRAM HR Evolution NIR Raman microspectroscope. Samples were also analyzed using a Focused Ion Beam-Scanning Electron Microscope (FEI Helios Nanolab dual-beam FIB-SEM) and a high-resolution Scanning Transmission Electron Microscope (aberration-corrected Titan 80-300 STEM) at PNNL, WA. Samples for FIB-SEM were prepared by placing air-dried sample on a metal SEM stub and coating with carbon. For TEM, sterile water was added to air-dried drop samples and precipitate suspensions were transferred onto lacey carbon grids.

Results and Discussion

In these experiments, MICP was promoted starting from single cells in 25 μ m drops. Although the calcium concentration in the drops was varied (Table 1), CaCO_3 precipitates formed in drops at all calcium concentrations when urea and MJK2 cells were present. Representative drop images from all experiments on all days are provided in

Figure 2. The columns titled ‘Overlay’ show overlays of the brightfield, GFP and UV signals (channels (i), (ii), and (iii) listed in methods) and the columns titled ‘Refl’ show the reflection signal images (channel (iv) listed in methods). For the bacteria-free control, day 0 and day 7 images are shown. In Figure 2, the green is the GFP signal from the MJK2 cells and the red is autofluorescence exhibited by the precipitates. The three control experiments confirmed that the precipitates that formed inside the drops in all mineralization experiments were indeed a result of ureolytic MICP. Lack of precipitation in the calcium-free and urea-free controls indicated that it was necessary for both calcium and urea to co-exist with the bacteria to enable MICP. The lack of precipitation in the bacteria-free control ruled out the possibility of abiotic precipitation.

Condition	Controls										Control	
	Urea-free		Ca-free		Ca _{Low}		Ca _{Int}		Ca _{High}		Bacteria-free	
	Ca:Urea 1:1		0:1		0.5:1		1:1		2:1		1:1	
	Overlay	Refl	Overlay	Refl	Overlay	Refl	Overlay	Refl	Overlay	Refl	Overlay	Refl
Day 0											Day 0 ↓ Day 7 	
Day 1												
Day 2												
Day 3												
Day 4												

Figure 2: Images of drops from each experiment listed in Table 1. The column ‘Overlay’ shows overlaid images of channels (i) brightfield, (ii) GFP and (iii) UV listed in methods while column ‘Refl’ shows the corresponding reflection signal (channel (iv) reflection listed in methods). For the bacteria-free condition, the images are from day 0 and day 7. Individual drops shown are 25 μ m in diameter.

Cell growth and motility

Fresh samples were taken from the drops stored in microcentrifuge tubes each day for imaging because imaging the same drops incubating in the DropSOAC over time resulted in little-to-no cell growth. Contrastingly, drops stored in the microcentrifuge tubes showed considerable growth. The apparent difference in cell growth between drops in the DropSOAC device and drops in the microcentrifuge tubes could be due to limited oxygen transfer to cells when drops are in the device, but this phenomenon was not quantified. Additionally, ureolysis generates ammonium which can be toxic to cells if it accumulates. There might be an elevated potential for ammonium to off-gas as ammonia into the headspace of the microcentrifuge tube as compared to the PDMS device, thus leading to greater growth in drops stored in the microcentrifuge tubes. More research is required to quantify oxygen and ammonia transfer in drops to determine their impact on cell growth.

Depending on the calcium concentration in drops, different types of cell aggregates were observed by day 1 (Figure 2). In the calcium-free drops, cells did not aggregate, and the drops appeared full of individual cells. In comparison, cells in Ca_{Low} drops did not appear as individual cells but as clusters and in Ca_{Int} drops cells formed long chains. Cell-chain formation was not further investigated here but has been previously observed for *E.coli* K-12 (MJK2 is a K-12 derivative).¹⁵¹ In Ca_{High} drops, cells formed tight clumps and were only identifiable as bacteria by their GFP signal.

Bacterial growth in the drops was quantified by quantifying the average GFP intensity per drop. Figure 3.a shows that GFP intensity per drop generally increased with time for Ca_{High} (▲), Ca_{Low} (◆) and calcium-free (○) experiments. The calcium-free condition had more growth than the Ca_{Int} and Ca_{High} conditions, but maximum growth was

observed in Ca_{Low} drops (Figure 3.a). Ca_{High} drops showed minimal increase in GFP. In the Ca_{Int} drops, GFP decreased drastically after day 1 and continued to decrease over time (Figure 3.a). To test whether this was a repeatable observation, GFP in drops of three additional biological replicates of Ca_{Int} was measured for 2 days. Figure D2 shows that this decrease in fluorescence was observed in all biological replicates of the Ca_{Int} experiment.

Corresponding bulk experiments for the Ca_{Int} condition showed a sustained increase in GFP signal after day 1 (Figure 3.b), unlike the decrease in GFP signal observed in Ca_{Int} drops after day 1. A potential reason for this discrepancy could be decreased interference of fluorescence measurements by precipitates in the bulk experiments. Precipitates that form in these bulk experiments can settle out more easily and might not affect bulk fluid fluorescence measurements while in drops precipitates indeed would affect fluorescence measurements. The inverted confocal microscope used in this study has its excitation light source and fluorescence detector under the sample stage holding the DropSOAC device and thus, the precipitates could obstruct GFP signal collection if they are located between the cells and the source/detector. This is supported by Video D1, which shows that precipitates are observed at the bottom of the drops.

The Ca_{Int} drops showed the highest number of vaterite precipitates on day 2 and onwards (discussed in section '*Precipitation in drops*'). Accordingly, the spatial interference by the precipitates would be enhanced in this condition compared to the other conditions and could explain the observed decrease in GFP signal.

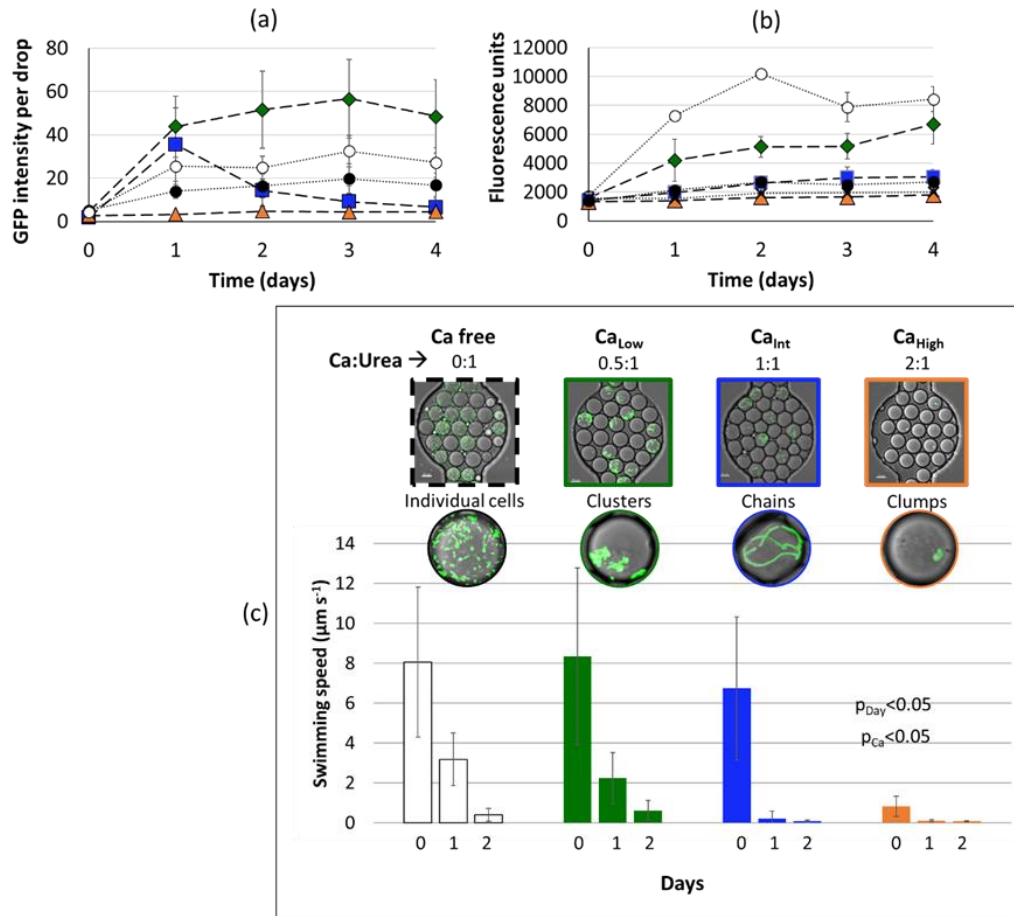


Figure 3: (a) Cell growth in drops, measured as GFP intensity per drop for Calcium-free (○), Ca_{Low} (◆), Ca_{Int} (■), Ca_{High} (▲), and urea-free (●). (b) Cell growth in bulk experiments (10 mL experiments), measured in terms of GFP intensity (c) Cell motility in drops over days for calcium-free (white), Ca_{Low} (green), Ca_{Int} (blue) and Ca_{High} (orange).

The initial cell motility observed in each experiment was different; higher calcium concentrations reduced the average swimming speed (Figure 3.c). Initial motility was lower at higher calcium concentrations. The differences in initial motility across experiments were likely not due to biological differences because drops made simultaneously using the same bacterial culture (calcium-free and Ca_{Int} ; urea-free and Ca_{High} , urea-free data not shown) also showed different initial motility. Cell motility decreased drastically from day

1 to day 2 in all experiments and continued to decrease until the detection of autofluorescence precipitates. After precipitate detection, cell motility could not be reliably quantified because in many cases, moving cells were seen attached to the precipitates and cell motion could not be differentiated from motion conferred by moving precipitates.

Precipitation in drops

In all MICP experiments, precipitates in drops were observed by their reflection signal (Figures 2 and D3). Precipitates detected on day 1 (Ca_{Low} and Ca_{Int}) did not exhibit a very defined morphology, which we call amorphous calcium carbonate (ACC). Starting on day 2, denser precipitates were observed, which we call vaterite. The terms ACC and vaterite are applied here, as Raman microspectroscopy was used to identify these two forms of CaCO_3 (discussed later). The vaterite precipitates had an autofluorescence signal (405 nm excitation) in addition to a reflection signal. As seen in Figure 4.a, both forms of CaCO_3 were observed in the Ca_{Low} , Ca_{Int} , and Ca_{High} drops at the end of the experiments on day 4.

The number of drops containing vaterite and ACC precipitates were quantified over a period of 0 - 4 days (Figure 4.b). Total drops with either precipitate morphology and drops containing GPF were also quantified.

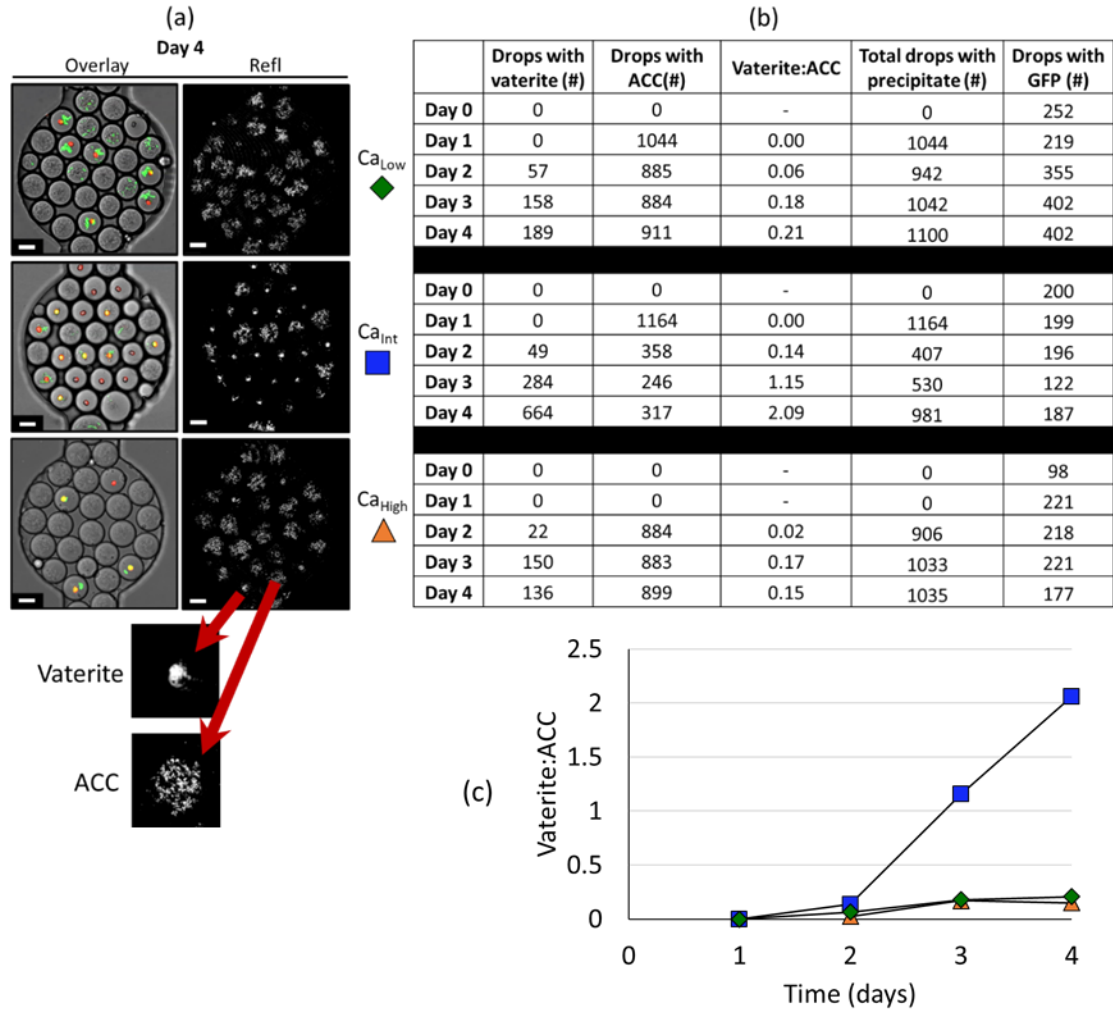


Figure 4: (a) Image overlays of Day 4 drops from Ca_{Low} ($\blacklozenge$), Ca_{Int} ($\blacksquare$) and Ca_{High} ($\blacktriangle$) experiments showing precipitates in drops with cells (green signal) and without cells. Scale bars are 15 μm . (b) Counts of drops containing vaterite, counts of drops ACC, ratio of Vaterite:ACC, total counts of drops containing precipitates and counts of drops showing GFP signal. (c) Vaterite:ACC ratio over time for the mineralization experiments.

The ratio of number of drops containing vaterite to number of drops containing ACC (Vaterite:ACC) increased over time (Figure 4.c). The increase in Vaterite:ACC ratio was most pronounced for Ca_{Int} drops. This condition contained an equimolar ratio of calcium to urea and showed the highest number of vaterite precipitates compared to the

other two Ca:Urea tested (Ca_{Low} and Ca_{High}), which showed a comparable increase in Vaterite:ACC ratio.

Precipitates were detected later in Ca_{High} (day 2) compared to in Ca_{Low} and Ca_{Int} (day 1). This delayed onset of precipitation potentially resulted from lower ureolytic activity due to low cell growth in Ca_{High} (Figure 3). In fact, in bulk experiments, Ca_{High} showed the slowest ureolysis (Figure D4).

The vaterite precipitates were denser than the ACC as they were observed at the bottom of the drops whereas the ACC was predominantly observed in the top half of the drops (Video D1, CLSM z-stack slices from top to bottom of drops). In drops with cells, the cells were closely associated with the either ACC or vaterite (Video D2). Interestingly, in Ca_{Low} , Ca_{Int} and Ca_{High} drops, precipitates (ACC and vaterite) were observed in drops that exhibited no GFP signal and thus likely had no cells (Figure 4.a). This was quantified by the number of drops with GFP signal, which was less than the number of drops with precipitates (Figure 4.b), indicating that precipitates were observed in drops even without any GFP. The three control experiments demonstrated that all three MICP prerequisites were necessary for precipitation to take place. Precipitation in drops without cells suggested that these drops formed precipitates as a result of ureolytic byproducts (carbonate and hydroxyl ions) diffusing into them from drops containing ureolytic cells and undergoing ureolysis. This hypothesis was proved by an additional experiment testing for diffusion between drops (Supplemental Information and Figure D5). In this diffusion test experiment, drops with fluorescent microbeads and dissolved calcium (no urea and no MJK2 cells) were mixed with Ca_{Int} drops. Precipitates were observed in the drops with

fluorescent microbeads, confirming diffusion of ureolytic byproducts from Ca_{Int} drops containing all three MICP prerequisites.

Characterization of precipitates using Raman microspectroscopy, SEM and TEM

The ACC was distributed throughout the drops and its less-defined morphology resembled that of previously published images of amorphous calcium carbonate (ACC).^{152, 153} Raman signal collected from these precipitates showed a weak and broad Raman peak at 1084 cm^{-1} (Figure 5) which is comparable to published Raman wavenumbers (cm^{-1}) for ACC (1084^{153} , 1077^{154} and 1083^{155}).

The autofluorescent precipitates from all MICP condition drops showed a Raman signal similar to that of vaterite (Figure 5), indicated by the characteristic double peak for vaterite at wavenumbers (cm^{-1}) 1078 and 1092, comparable to literature values of 1074, 1090^{156, 157} and 1076, 1092 cm^{-1} .¹⁵³ The vaterite standard shown in Figure 5 was a lab-generated precipitate sample from a flask containing MJK2 and mineralization media, identified as vaterite by XRD using a Scintag X1 Diffraction System (Figure D6); no other minerals were detected using XRD. In drops with precipitates, either ACC or vaterite was observed. No instances were observed where both ACC and vaterite were present in the same drop suggesting that vaterite was forming at the expense of ACC.

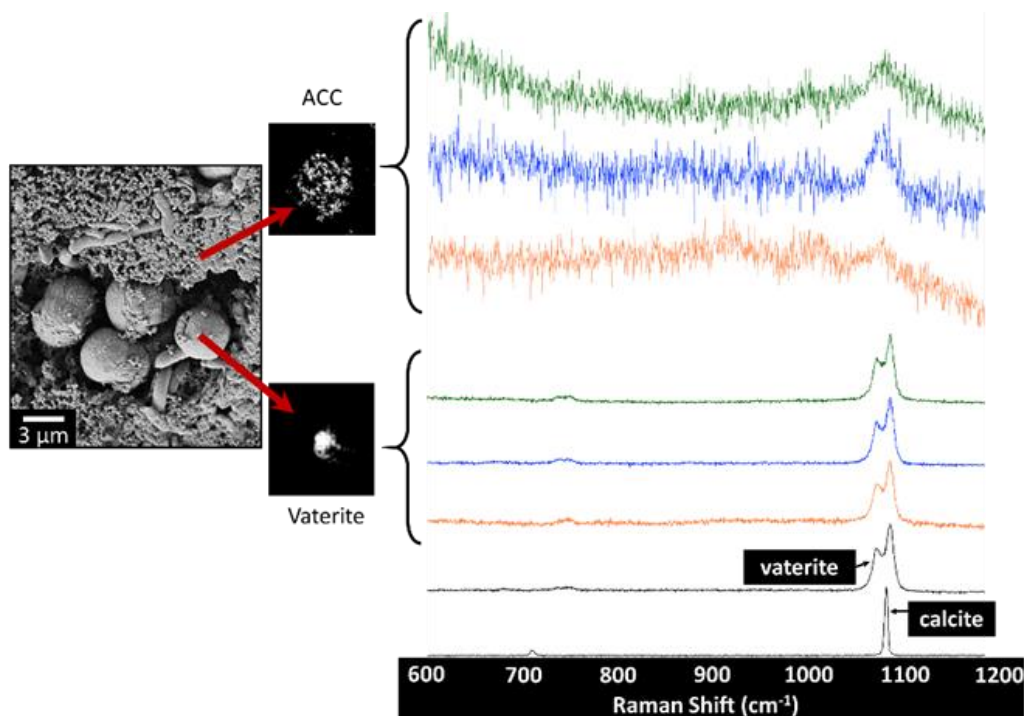


Figure 5: Raman microspectroscopy data collected from both morphologies of precipitates observed in Ca_{Low} (green), Ca_{Int} (blue) and Ca_{High} (orange). An SEM image of material within drops collected by air-drying. Intermediate Ca drops is provided on the left to show the two morphologies of precipitates. Corresponding reflection signals (from when the precipitates were inside drops) are also shown. The less defined morphology precipitates showed a weak Raman signal 1084 cm^{-1} , consistent with published Raman spectra for ACC: 1084^{153} , 1077^{154} and $1083^{155}\text{ cm}^{-1}$. The denser precipitates showed Raman spectra identical to that of the vaterite standard and published data.^{153, 156, 157} An ACC standard is not shown in Figure 5 as it was not available and stable ACC could not be generated via experiments for use as a standard. The vaterite standard shown in Figure 5 was generated experimentally and verified as vaterite using XRD.

One vaterite precipitate from a Ca_{Int} drop was milled layer-by-layer using a Focused Ion Beam and imaged simultaneously with a Scanning Electron Microscope (FIB-SEM). The precipitate had a dense core covered with a grainy rind (Figure 6.a). Transmission Electron Microscopy (TEM) performed on the rind of another vaterite precipitate (from a Ca_{Int} drop) suggested that the rind was crystalline (Figure 6.b) but diffraction patterns were not matched to any known CaCO_3 polymorphs.

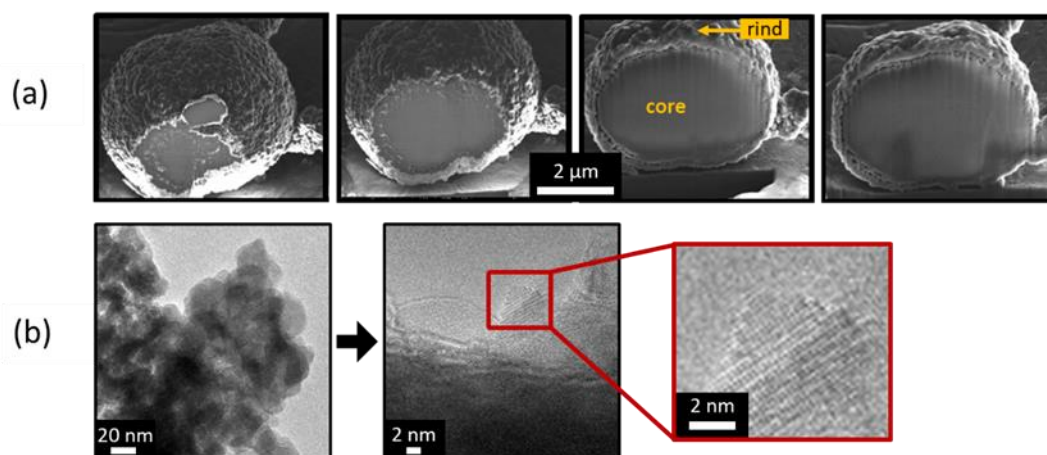


Figure 6: Visualization of a single vaterite precipitate using FIB-SEM and TEM. (a) Left to Right: SEM images showing progression of FIB milling of a single precipitate from a Ca_{Int} drop. The interior of the precipitate appears densely packed with a distinct outer rind. (b) TEM images of a precipitate from a Ca_{Int} drop showing atomic column structures suggestive of crystallinity.

Cell-shaped extensions in various modes of imaging (CLSM, SEM, TEM, FIB-SEM)

Some precipitates had cylindrical extensions ($< 1 \mu\text{m}$ diameter, variable length), observed using various modes of imaging including CLSM, SEM, TEM, FIB-SEM (Figure 7). Under CLSM, these extensions exhibited fluorescence when excited at 405 nm (autofluorescence) and at 488 nm (GFP fluorescence) (Figure 7.a). The shape of the narrow GFP signal (green) resembled the cell-chains seen in Figure 2 and appeared to be in the interior portion of the thicker extension, surrounded by the (red) autofluorescent signal around them (Figure 7.a). Figure 7.b shows an SEM image of a precipitate with two such extensions covered in material visually similar to the precipitate rind shown in Figure 6.

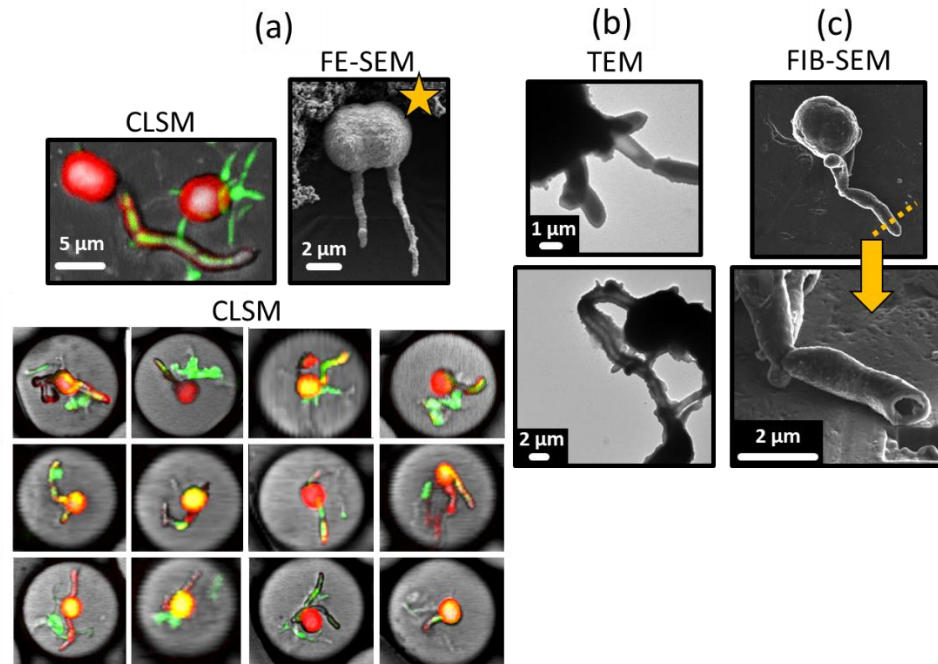


Figure 7: Cylindrical extensions observed using different imaging techniques. (a) CLSM images of drops with autofluorescent extensions showing interior GFP signal resembling the cell-chains displayed in Figure 2. Individual drops shown are approximately 25 μm in diameter. (b) SEM image of a vaterite precipitate with two extensions. (c) TEM micrographs showing cell-shaped structures covered with crystalline material. (d) FIB-SEM slicing of a cell-shaped extension.

One precipitate with cell-shaped extensions was analyzed for its elemental composition using Nano-Auger Energy Dispersive X-ray Spectroscopy. Calcium and oxygen were detected on the precipitate as well as on the extensions (Figure D7). TEM images of cell-shaped extensions showed lower density structures resembling cells covered with precipitate material (Figure 7.c). A cell-shaped extension was cut and imaged using FIB-SEM (Figure 7.d) and the interior appeared hollow, which is consistent with cell material either having degraded or collapsed due to the SEM vacuum conditions. Overall, the microscopic investigations using CLSM, confocal laser scanning microscopy, SEM and TEM indicate that cells were encased in precipitate.

Conclusions

Drop-based microfluidics has been previously utilized to study abiotically-driven calcium carbonate precipitation via mixing of calcium chloride and sodium carbonate solutions inside drops.^{144, 145, 153} Biologically-driven precipitation, which is dependent on bacterial activity, has to our knowledge never been studied in drops before. In this work, drop-based microfluidics was coupled with microscopy to visualize ureolysis-driven MICP starting from single cells.

Bacterial ureolysis was promoted in microscopic droplets starting from single ureolytic cells. Calcium carbonate precipitates formed in the presence of the three prerequisites for MICP (ureolytic cells, dissolved urea, and dissolved calcium). Bacterial aggregation in the drops was impacted by calcium concentrations with cells aggregating as clusters, chains, or clumps with increasing calcium concentration. Cell motility decreased over time in all conditions and increasing calcium concentration led to a decrease in initial cell motility. Precipitates in drops underwent morphological/mineralogical changes over time. The Raman signal from the initial precipitate morphotype indicated the presence of amorphous calcium carbonate. The subsequent denser morphotype that formed in the drops had a Raman signal consistent with that of vaterite but this morphotype also had an autofluorescence signal indicating the presence of calcite. Precipitates were observed in drops not containing the prerequisites for MICP, which was indicative of diffusion of carbonate and hydroxyl ions across drops.

The mineralogy of the vaterite precipitates was challenging to identify and requires further investigation. FIB-SEM performed on a vaterite precipitate showed a denser

interior core covered in a grainy rind. Fluorescence emission scans (405 nm excitation, emission range: 400 nm to 600 nm) performed on 6 randomly chosen vaterite precipitates indicated a peak emission wavelength (for autofluorescence) of $481.5 \text{ nm} \pm 6.6 \text{ nm}$ (Figure D8). Based on published data for calcite fluorescence¹⁴⁹, the measured peak emission wavelength of 481.5 nm coincides with the maximum fluorescence region described for calcite. However, chemical characterization of these precipitates indicated vaterite as the major mineral phase (based on Raman microspectroscopy and XRD). The autofluorescence suggests the existence of a non-vaterite component within or on the surface of the precipitates. Further correlative microscopy studies focused on characterizing the developing precipitates, specifically the changes in morphology and mineralogy of the dense core and grainy rind are necessary. A microscopy approach involving thin-sectioning of the precipitates followed by a spatially resolved fluorescent, as well as diffraction- or vibration-based identification of crystal phases would be advantageous for understanding mineralogical changes in the future.

Though we were not able to observe time-resolved processes of cells acting as nucleation points for mineralization, precipitates observed surrounding the bacterial cells support the hypothesis that the cells can act as nucleation points and become encapsulated with MICP.^{6, 7, 73, 94} Future work will investigate nucleation points for precipitation and the research presented here provides a platform for such single-cell level or single-crystal level studies. Many other microbe-mineral interactions can be studied using similar experimental set-ups as the one described here, such as other biomineralization processes, sequestration of heavy metals or radionuclides, bioleaching and mineral degradation. In

addition, the observed uniformity in size and shape of precipitate aggregates is promising for micro-manufacturing of small particulate minerals or nanoparticles.

Supplemental Information (SI): summary of optimizing bacterial culture to achieve 1 cell per drop, numbers of drops analyzed for growth curves, biological replicates of Ca_{Int}, precipitate morphologies in drops, ureolysis in bulk experiments, and formation of precipitates in drops without bacteria. Also provided are XRD data for the vaterite standard, elemental maps of calcium and oxygen on precipitate extensions, fluorescence emission spectra for precipitates from 6 drops, and videos of cells and precipitates in drops.

Author Information:

Corresponding Author: Dr. Connie Chang, connie.chang@montana.edu, (406) 994-4592.

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CHAPTER SEVEN

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

Conclusions and Impacts

Under reactive transport conditions, microbe-mineral interactions (MMI) can have feedback effects on the kinetics of the system. In porous media, transport limitations can arise from mineral formation in the flow regime or on microbial surfaces, leading to altered flow paths or reduced nutrient access for the microbes, respectively. Empirically gathered data on such processes can be advantageously used for prediction and control of the effects of MMI. The research presented in this thesis used MICP as an example of MMI and applied it to two reactive transport systems. MICP was promoted in a radial flow regime to study the effects of reactive transport in a flow environment representative of near-well subsurface media. In the lab-scale radial flow system, MICP was transport-dominated with lower flow rates resulting in more even distribution of precipitates. The dimensionless Damköhler number was identified as a useful parameter for MICP prediction. Bacterial attachment showed a dependence on distance from the inlet. The velocity of the inlet fluids imparted shear on the inlet porous media elements. To characterize temporal changes in ureolysis and precipitation within the pore-space, a modified flow cell was developed to enable spatio-temporal sampling. A linear flow system was chosen for these studies to eliminate differences in residence time with distance from the inlet that would have been encountered in a radial flow system. This spatio-temporal analysis approach revealed a

spatial decoupling between ureolysis and MICP. The strontium partitioning coefficient decreased with an increase in precipitate size.

In these meso-scale reactors, precipitate distribution was analyzed using mass spectrometry techniques. An imaging-based approach was then adopted to focus on the fundamental MMI behind MICP. Droplet-based microfluidics was used to promote MICP inside microscopic droplets and for the first time, MICP was visualized at the micro-scale in a fully hydrated environment. Semi-correlative microscopy was employed to study the microprecipitates that formed in the droplets. Microanalysis of the precipitates revealed their complex nature, highlighting the need for further correlative microscopy investigations.

Recommendations for future work

The research in this thesis describes multi-scale investigations: from reactive transport at the meso-scale to optical imaging of mineralizing bacteria and chemical imaging of precipitates at the micro-scale. Considering the potential application of MICP for contaminant sequestration, co-contaminant co-precipitation studies under flow conditions are required.

From this micro-scale MICP work, future research can broaden into numerous biogeochemical phenomena that involve microbe-mineral interactions. Multi-modal microscopy approaches integrating chemical and optical imaging can provide a comprehensive understanding of the interactions between minerals and the microbes that bring about their formation.

Microscopy has progressed from mere visualization of samples on a simple microscope (like Antonie van Leeuwenhoek observing insects and bacteria) to *in situ* characterization and quantification of systems in a non-destructive manner (such as measuring localization and expression of specific proteins within single bacteria). Advances in microscopy have enabled single-molecule resolution. Biological imaging can link sub-cellular level information to biofilm-level and systems-level processes, providing insight into how micro-scale processes have a cumulative effect on the macro-scale. Additionally, the plethora of microscopy and microanalysis techniques available today has made correlative microscopy more feasible. However, microscopy techniques that require samples to be removed from their environment pose a challenge to confidently correlate *in situ* and *ex situ* observations. Therefore, there is a need for further work in this field to optimize true correlative microscopy across a wide range of techniques to maximize the information that can be extracted from a system. To this end, microbe-mineral interactions provide an ideal platform for further research into refining correlative microscopy techniques.

Within the field of MICP, the role of bacteria and bacterial functions in controlling the precipitation pathway need further exploration. The observed heterogeneity in precipitates formed by planktonic cells implies that this phenomenon might be considerably more complex in higher order systems such as biofilms. Some important features of the biofilm-mineral relationship have been identified for various microorganisms through microfluidic studies. Biomineralization can alter susceptibility of *Pseudomonas aeruginosa* biofilms to chlorine, enhancing solute penetration leading to

higher chlorine killing when compared to chemically deposited calcite (not biomineralized) particles.¹⁵⁸ Conversely, struvite crystals produced by *Proteus mirabilis* biofilms reduced susceptibility to ciprofloxacin due to transport resistance offered by biomineralized regions of biofilms.¹⁵⁹ This was also shown, more recently, by Oppenheimer-Shaanan et al. who studied biofilm rigidity imparted by mineral scaffolds within *Bacillus subtilis* and *Mycobacterium smegmatis* biofilms grown on agar. They showed that biofilm resistance to antibiotic penetration and shear stresses depends not only on extracellular matrix properties but also on mineral crystals produced within the biofilm.¹⁶⁰ Li et al. developed a noninvasive real-time imaging method to visualize the spatial and temporal distribution of biomineralization in *Pseudomonas aeruginosa* biofilms.¹³⁶ Precipitation was observed starting at the base of the biofilm and then building upward toward the bulk fluid. Such studies recognize the importance of the close association between biofilms and biominerals. However, focused investigations on understanding the precipitate crystal phase transitions during biofilm-level precipitation have not been widely reported.

Organic secretions by microbes in a biofilm might play a key role in determining the final precipitate polymorph, providing potential to control polymorph transitions. Small proteins of different types of EPS secreted by *Schizothrix* sp. could influence the polymorphism of the CaCO_3 precipitates.¹⁶¹ In another study, EPS from certain stromatolites seemed to have an inhibitory effect on biomineralization.¹⁶² Lopez-Moreno et al. found that calcium could influence the quantity of EPS produced by some CaCO_3 precipitating bacterial isolates, which is a key factor in formation, maintenance and spread

of biofilms. The importance of studying the molecular level controls on biomineralization in the presence of organics has been acknowledged.^{163, 164}

Determining the role of organics in microbe-mineral interactions is a valuable and necessary future direction for MMI research as the effect of organics might be more predictable/controllable than the effect of microbes. The natural intersection of microscopy-microanalysis and microbe-mineral interactions is a molecular level investigation of biomineralizing biofilms. An -omics based approach coupled with biofilm microscopy and chemical imaging (e.g. using vibrational spectrometry) has potential to provide insight into the effect of organics on crystal polymorphs.

The resulting improved understanding of microbe-mineral interactions will not only lead to the optimization of current bioremediation strategies but also to the development of novel biomimetic approaches for applications in industry, environment and energy sectors.

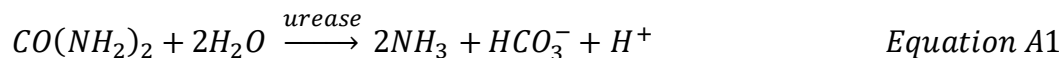
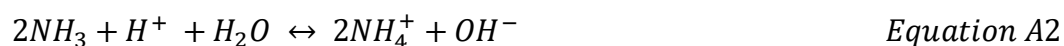
APPENDICES

APPENDIX A

SUPPLEMENTAL INFORMATION FOR THE MANUSCRIPT IN CHAPTER 3

Ureolysis driven MICP

Ureolysis is the hydrolysis of urea to ammonium and inorganic carbon. The series of reactions that take place during the overall process of ureolysis driven MICP is shown by equations A1-A3:



Urea hydrolyzes to generate ammonia, bicarbonate and a proton in the presence of urease (Equation A1). Aqueous ammonia at pH values well below the pK of the ammonium/ammonia equilibrium results in the production of ammonium and hydroxide ions (Equation A2), raising the alkalinity of the fluid. As a result, the dissolved inorganic carbon speciation shifts toward bicarbonate and carbonate. When calcium ions with sufficient ion activity are present under such conditions, calcium carbonate can precipitate (Equation A3) along with the release of a proton. This causes a decrease in pH during precipitate formation. The onset and rate of calcium carbonate precipitation also depend on calcium concentration, dissolved inorganic carbon concentration, pH of the microenvironment, the saturation state and the availability of nucleation sites.^{6, 165}

Reactor Disinfection

Prior to inoculation, the reactor was disinfected by pumping approximately 100 mL of each of the following solutions into the reactor at 5 mL min⁻¹ in the given order: (1) Bleach plus Tween 80 (5 mL and 3.5 g per 500 mL solution, respectively), (2) sodium chloride (10 g L⁻¹), (3) sodium thiosulfate (2.52 g L⁻¹), (4) ethanol (70%), (5) ammonium

chloride (10 g L^{-1}). Each solution remained in the reactor for 30 minutes without flow before the next solution was injected.

Preferential Flow Paths

Uniform radial flow before MICP was verified by dye tracer studies in all experiments. Preferential flow paths were observed after MICP.

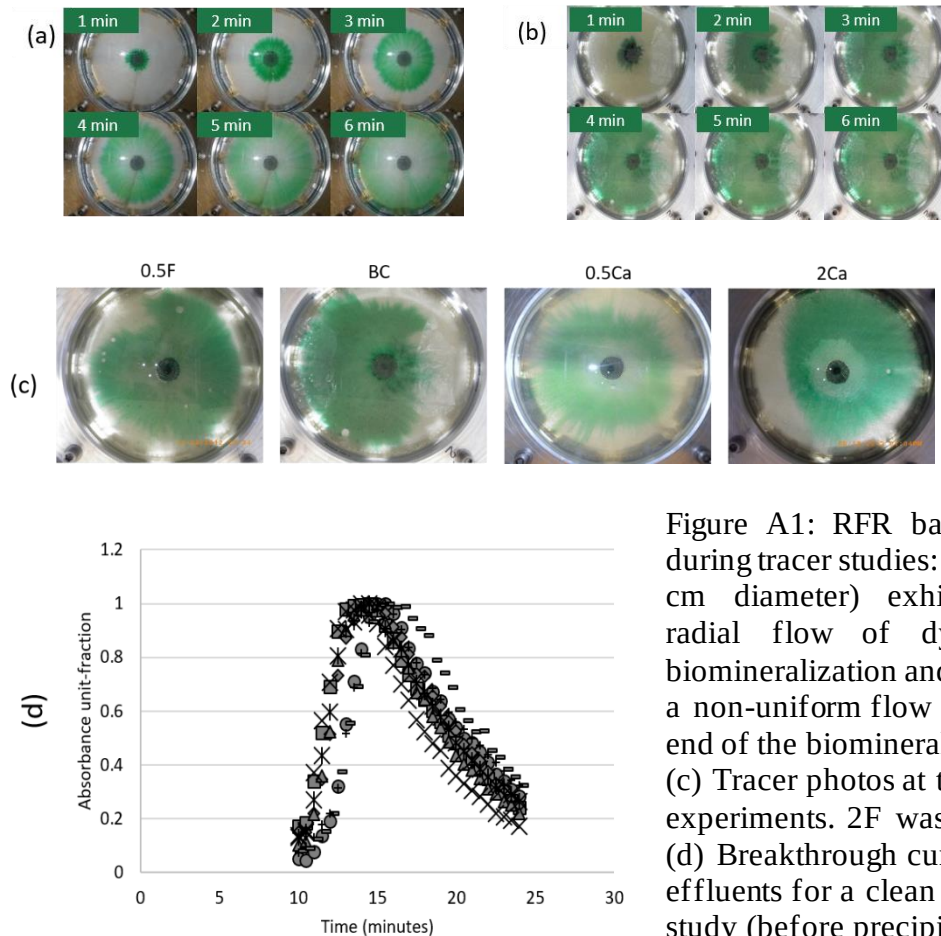


Figure A1: RFR base case (BC) during tracer studies: (a) reactor (23 cm diameter) exhibits uniform radial flow of dye prior to biomineralization and (b) develops a non-uniform flow pattern at the end of the biomineralization phase. (c) Tracer photos at the end of the experiments. 2F was not imaged. (d) Breakthrough curves for the 8 effluents for a clean reactor tracer study (before precipitation) for the base case experiment.

Cementing of glass beads to reactor plate

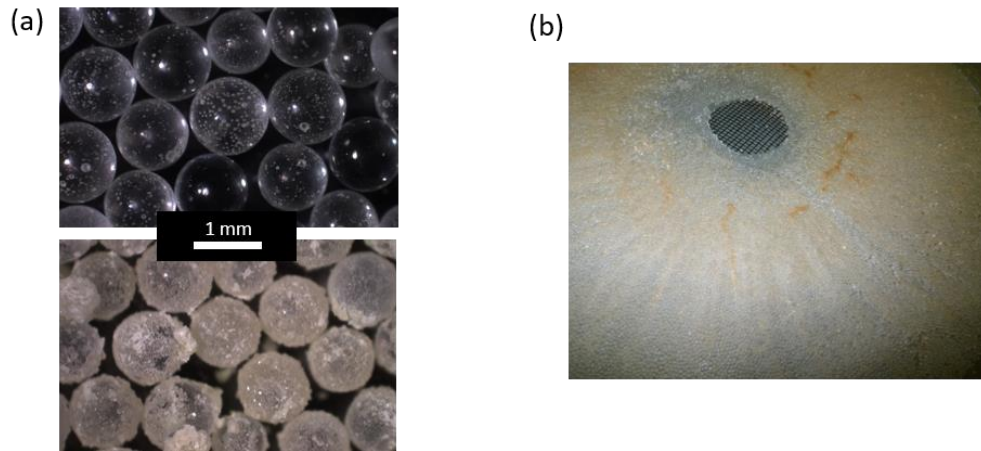


Figure A2: (a) A comparison of loose (top) versus cemented (bottom) glass beads. (b) Glass beads cemented to the bottom glass plate post-MICP in the Base Case experiment.

Influent region beads

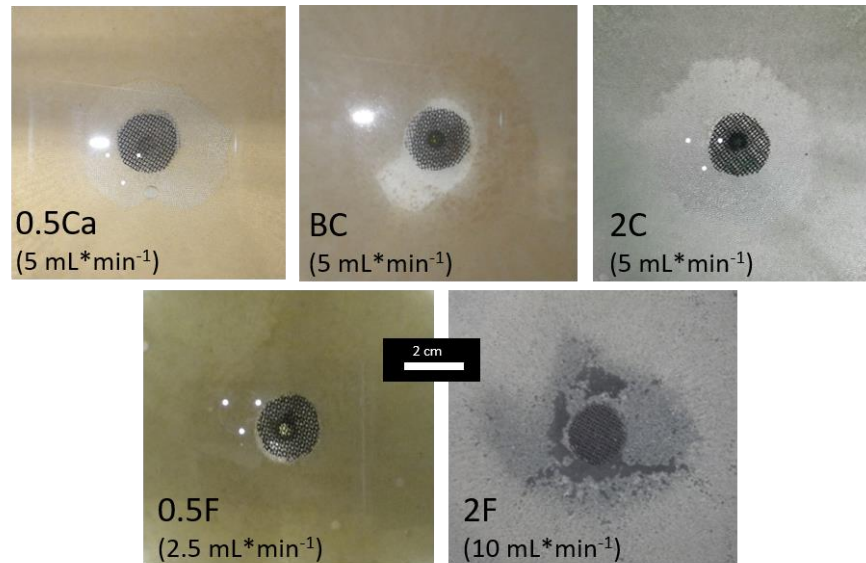


Figure A3: Approximately circular areas with visibly less precipitates observed in the influent region. At the low flow rate tested (0.5F), an area of fewer precipitates was not observed and precipitates were seen right around the influent port as well. Physical displacement of the beads was observed only at the high flow rate (2F).

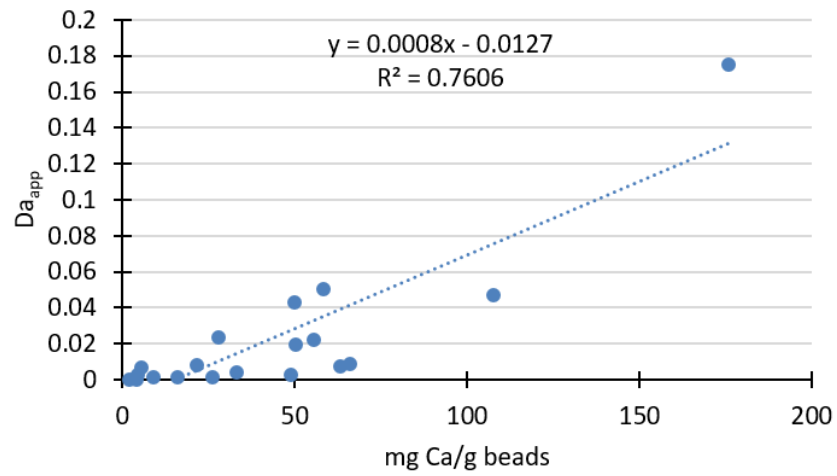
Apparent Damköhler number as an indicator of precipitate mass

Figure A4: Apparent Damköhler numbers showing positive correlation to bead specific precipitate mass.

Constant versus spatially varying reaction and transport rates

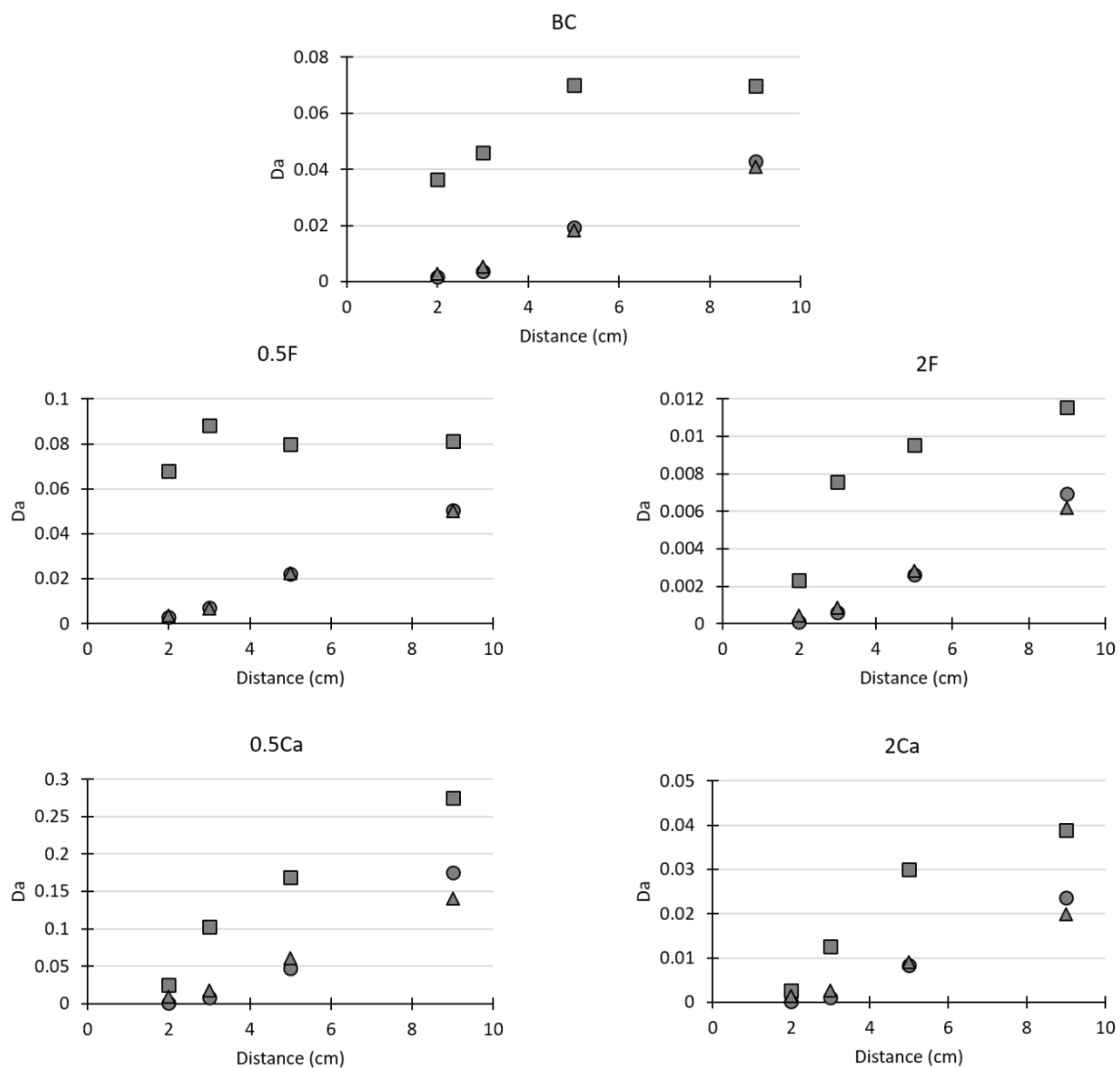


Figure A5: Damköhler numbers versus radial distance. Squares show Da calculated assuming a constant transport rate at all radii. Da assuming constant reaction rates (triangles) resemble the Da_{app} (circles).

Table A1: Initial rates of precipitation or calcium removal based on the dissolved calcium concentration measurements in the effluent samples.

Experiment ID	Initial precipitation rate (M/hr)
0.5F	2.8
BC	1.7
2F	NA
0.5Ca	0.9
BC	1.7
2Ca	6.5

**Initial reaction rates in 2F could not be calculated due to non-statistically significant changes in [Ca] between influent and effluent samples.*

APPENDIX B

SUPPLEMENTAL INFORMATION FOR THE MANUSCRIPT IN CHAPTER 4

Artificial Ground Water recipe

The AGW was prepared according to the recipe published by Ferris et al.⁵⁹ as follows:

KNO₃ (0.0403 mM)
 MgSO₄ (0.448mM)
 NaNO₃ (0.044 mM)
 NaHCO₃ (1.1 mM)
 KHCO₃ (0.0623 mM)

Flow Cell Disinfection

Two pore volumes of each of the following solutions was flowed through the reactors with 30-minute- no-flow intervals between solutions:

1. Bleach plus Tween 80 (5 mL and 3.5 g per 500 mL solution, respectively)
2. Sodium Chloride (10 g/L)
3. Sodium Thiosulfate (2.52 g/L)
4. Ethanol (70%)
5. Ammonium Chloride (10 g/L)

Laser Ablation Inductively Coupled Plasma Mass Spectrometry data

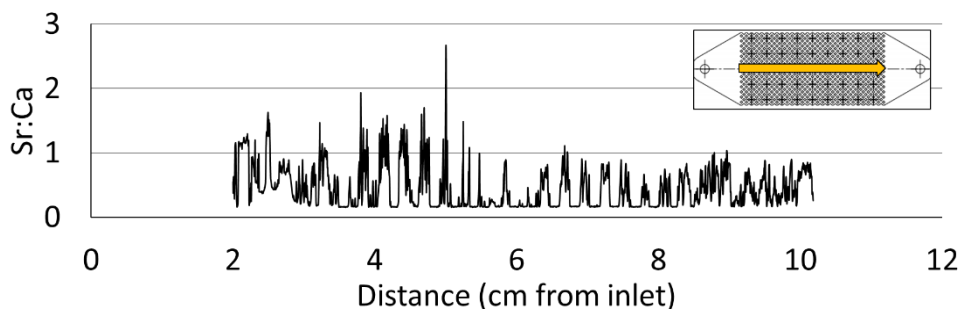
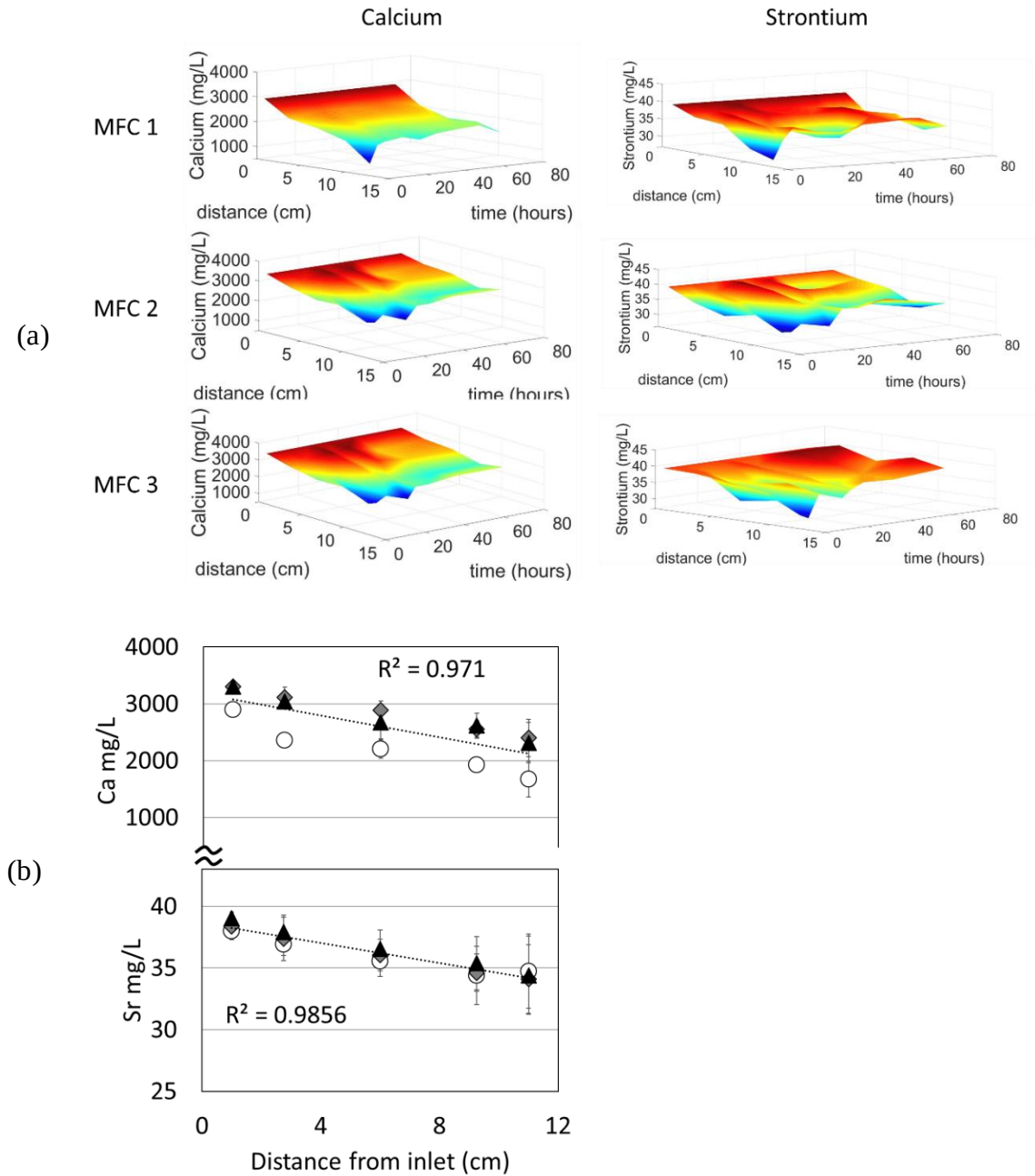


Figure B1: (a) LA-ICP-MS data showing relative Sr:Ca counts along the centerline. Laser path shown in inset. The periodic low Sr:Ca values are from locations where there were almost no precipitates as the polycarbonate posts of the flow cell were flush against the cover plate. (b) Sr:Ca measured via ICP-MS on acid-digested precipitates.

Spatially resolved concentrations of calcium and strontium



APPENDIX C

ADDITIONAL DATA ANALYSES FOR MICP DRIVEN STRONTIUM
PARTITIONING IN POROUS MEDIA FLOW CELLS

Transverse LA-ICP-MS in the modified flow cell

Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICP-MS) was also performed along three transverse lines (d) on the glass plate of modified FC 1. The resulting strontium to calcium counts are shown in Figure C1.

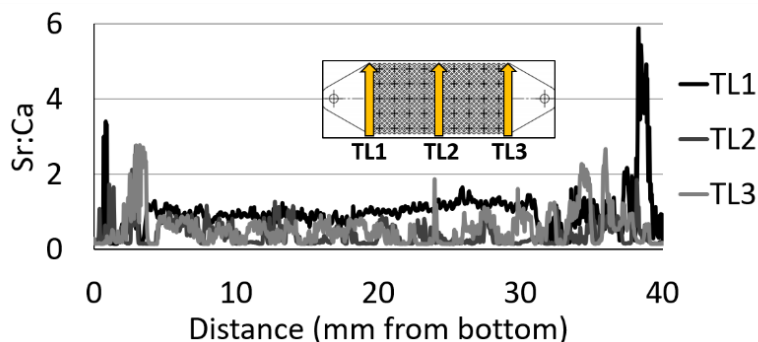


Figure C1: Transverse LA-ICP-MS data for 3 transverse lines ('TL'). Higher counts of strontium relative to calcium were observed close to the side-walls of the flow cell.

The velocity profile in these rectangular duct-type reactors is parabolic with the slowest flow at the walls of the reactor and the fastest flow along the centerline, with minor internal gradients due to the porous media elements. Figure C1 shows that at the walls where flow is slow, strontium partitioning occurs to a greater degree. In this transport limited system, slow flow at the walls would lead to lower precipitation rates thus suggesting that more strontium partitioning occurred at low precipitation rates.

Standard flow cell experiments to test the effects of controllable parameters

Experiments performed previously in standard flow cells (described in Lauchnor et al.⁸⁷) were analyzed for distribution and efficiency of strontium co-precipitation with calcium carbonate precipitation. The effects of controllable parameters (flow rate and concentration) were quantified. All the figures and equations referred to in this appendix are from Chapter 4. These experiments were performed by Dayla Topp.

Methods - Inoculum For the inoculum in each experiment, 1 mL frozen stock of *S. pasteurii* (ATCC 11859) was added to 100 mL Brain Heart Infusion medium (BHI, Fisher Scientific) amended with 0.33 M urea and incubated at 30°C (at 150 RPM). After 24 hours, 1 mL of the culture was transferred into 100 mL fresh BHI + 0.33 M urea. This transfer culture was incubated under the same conditions before centrifuging at a relative centrifugal force of 2964 x g for 10 minutes at 4°C. The supernatant was decanted and the cell pellet was re-suspended in the respective growth medium (for each type of flow cell) and centrifuged again. Re-suspension and centrifugation were repeated once more, after which cells were diluted in growth media to a final OD₆₀₀ of 0.4 on a BIOTEK Synergy HT absorbance plate reader in 96-well plates with 100 µL per well. The reactor was disinfected (protocol in Supplemental Information) and inoculated with approximately 10 mL of the final culture. Following inoculation, the reactor tubing was clamped for a no-flow attachment phase of 1.5 hours to promote attachment of bacterial cells.

Methods - Media Growth medium for standard flow cell (standard FC) experiments was prepared with 3 g L⁻¹ Difco Nutrient Broth (Becton Dickinson), 10 g L⁻¹ urea (Fisher Scientific), and 10 g L⁻¹ ammonium chloride (Fisher Scientific) and adjusted to pH 6 before filtering. Co-precipitation medium was prepared by adding CaCl₂·2H₂O and SrCl₂·6H₂O (Fisher Scientific) to pH-adjusted fresh growth medium. Table 1 shows the calcium and strontium concentrations used. Influent media pH for the standard FC was 5.7-6.1.

Methods - Flow cell set up and operation The standard FC used for the flow rate and concentration variation experiments is described in Lauchnor et al.⁸⁷ Following the no-flow attachment phase, the co-precipitation phase started in which co-precipitation medium was flowed through the standard FC for a duration designed to supply 27 mmol Ca in each experiment, while keeping molar Sr:Ca constant at 0.01. The flow rates and calcium concentrations used for the standard FC experiments are given in Table C1 and their resulting calcium mass flow rates, defined as $\dot{M}_{Ca} = \text{flow rate} * \text{Ca concentration}$, are also provided. High values are denoted by capital letters: Q for high flow rate and C for high concentration. Effluent samples were collected periodically for chemical analyses. A strontium-free control (Sr-) was performed for each of the 4 standard FC experiments (Sr+).

Table C1: Experimental parameters tested and concentrations for the co-precipitation media for the standard FC experiment. q= flow rate, c=calcium concentration, lower case denotes low and upper case denotes high. For example, (QC)=high flow rate, high calcium concentration.

Reactor	Experiment ID	Flow Rate (mL*min ⁻¹)	Calcium (M)
Standard FC (Urea: Ca ²⁺ =1) (Sr ²⁺ : Ca ²⁺ =0.01)	qc	0.15	0.02
	qC	0.15	0.25
	Qc	0.5	0.02
	QC	0.5	0.25

Results - Effects of flow rate and concentration In all standard FC experiments, the effluent pH at the beginning of the co-precipitation phase was high (8.7 ± 0.5) compared to the influent (5.9 ± 0.2), indicative of ureolysis (Equations 1 and 2). The effluent pH stabilized at 7-8 over the course of the experiments due to proton release during CaCO_3 precipitation (Equation 3) (data not shown). The calcium removal rate (R_{aq}) was the highest during the initial time points in each experiment. The first 25% of the total experiment duration showed the most change in R_{aq} , after which, a quasi-constant rate was achieved (Figure C2.a). Higher calcium removal (higher $R_{overall}$) occurred in experiments with higher calcium mass flow rates into the reactor ($\dot{M}_{Ca} = \text{flow rate} * \text{Ca concentration}$) (Figure C2.b). The reaction thus appeared to be limited by calcium supply.

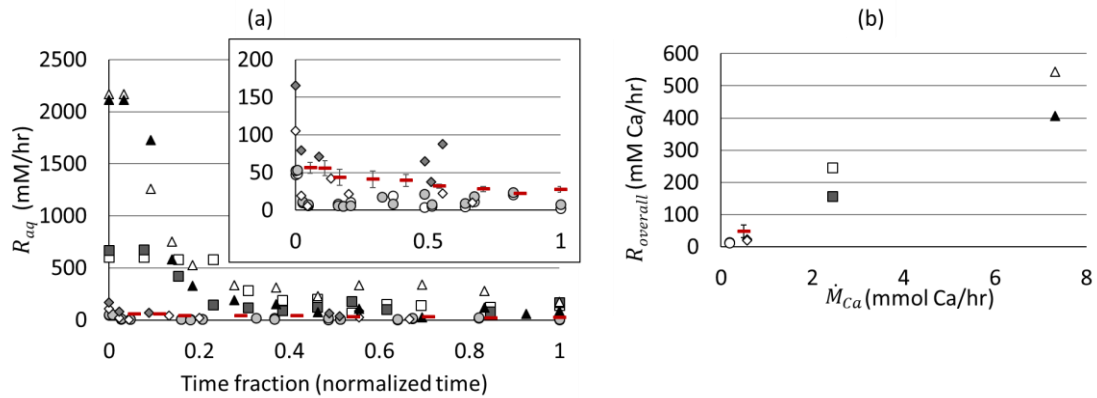


Figure C2: (a) Initially high R_{aq} decreased over time, approaching a steady state value after 25% of the total calcium was injected. Open markers are Sr- and filled markers are Sr+ experiments for qc (○), Qc (◇), qC (■) and QC (▲). Modified FC experiments shown by a dash (—) with error bars showing standard deviation between the triplicates. The inset shows Qc, qc and modified FC data at a scale that enables visualization of the spread of the data points better. (b) Overall calcium precipitation rates increase linearly with calcium supply to the system (Values in Table C2).

The aqueous concentrations measured at the end of the time intervals were applied to the entire time interval to estimate the mass of calcium and strontium being carried out of the system in the aqueous phase (without reacting). The sum of estimated unreacted masses and the solids digested in the system accounted for the total mass added to the system for calcium ($104 \pm 9\%$) and strontium ($109 \pm 9\%$). For QC, there was a high discrepancy between $R_{overall}$ and R_{ppt} because a considerable mass of precipitates was found in the tubing. When the tubing precipitates were included, the R_{ppt} for QC was 445 mM Ca/hr which more closely resembled $R_{overall}$. Table C2 provides the averages and ranges of spatial D_{Sr} values for each reactor, along with percent Ca and Sr precipitated.

Table C2: Comparison of Ca removal rates, percentage of fed Ca and Sr precipitated, and Sr partitioning for standard FC experiments.

Experiment ID	$R_{overall}$ (mM Ca/hr)	$\dot{M}_{Ca}$ (mmol Ca/ hr)	% Ca	% Sr	D_{Sr}	R_{ppt} (mM Ca/hr) Porous media/total system
qc	14	0.19	42	29	0.62	23/24
qC	155	2.4	22	10	0.37	158/186
Qc	21	0.6	12	7.5	0.67	20/23
QC	405	7.3	3.2	1.0	0.45	67/445

Precipitates in qC were not acid digested in sections and thus, spatially resolved D_{Sr} could not be calculated for this experiment. Instead, an overall strontium partitioning coefficient was calculated for qC using total precipitate mass and end point concentrations. Both calcium and strontium precipitated to a greater percent at lower dissolved Ca and Sr concentrations and lower flow rates, with the maximum percent precipitated for both in qc. Even though D_{Sr} was high in QC, there was lower strontium removal because there was

lower calcium precipitation. Thus, to maximize strontium removal, maximizing precipitation was more effective than maximizing the D_{Sr} for the conditions chosen.

APPENDIX D

SUPPLEMENTAL INFORMATION FOR THE MANUSCRIPT IN CHAPTER 6

Optimizing bacterial concentration to achieve 1 cell per drop

Escherichia coli MJK2 cells were grown overnight in growth medium and then diluted to an optical density of 0.4 (OD₆₀₀), measured using a BIOTEK Synergy HT spectrophotometer (200 µL samples in a 96-well plate, with the blank well OD of 0.044 as reference). The adjusted culture was serially diluted using growth medium (recipe in paper) and drop-plated¹⁶⁶ on LB+2% Urea agar. Bacterial growth was assessed in terms of Colony Forming Units per milliliter (CFU/mL) using the number of colonies observed at 24 hours in each drop that was plated. The dilution of 10⁻⁸, which resulted in colony counts of 1-15 per drop was chosen for total bacterial concentration estimates. This protocol was performed on 25 separately grown overnight cultures with a resulting average bacterial concentration of 5.1(± 1.3) *10⁸ CFU/mL. The desired concentration of cells for a single cell in drop was calculated to be 1.2 *10⁸ per mL for 25 µm diameter drops. Accordingly, the OD-adjusted culture was diluted 1:5 using growth medium prior to making drops.

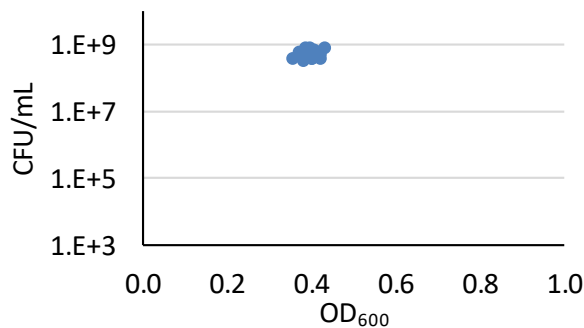


Figure D1: Cell concentrations (as colony forming units per mL, CFU/mL) of 25 biological replicate cultures adjusted to an optical density of 0.4 (600 nm reading).

Numbers of drops analyzed for growth curves (Figure 3)

Table D1: Number of drops analyzed on each day for each condition for growth curves in Figure 3.

	Urea-free	Calcium-free	Ca _{Low}	Ca _{Int}	Ca _{High}
<i>Day 0</i>	238	81	252	200	98
<i>Day 1</i>	200	212	219	199	221
<i>Day 2</i>	342	178	355	196	218
<i>Day 3</i>	447	200	402	122	221
<i>Day 4</i>	390	189	402	187	177

Numbers of motility tracks (Figure 3)

Table D2: Number of motility tracks detected in drops containing cells on each day for each condition. The averages and standard deviations for velocities of tracks for each day and condition are plotted as swimming speeds shown in Figure 3.

	Urea-free	Calcium-free	Ca _{Low}	Ca _{Int}	Ca _{High}
<i>Day 0</i>	157	710	261	220	52
<i>Day 1</i>	228	1326	1079	162	46
<i>Day 2</i>	99	451	460	69	14

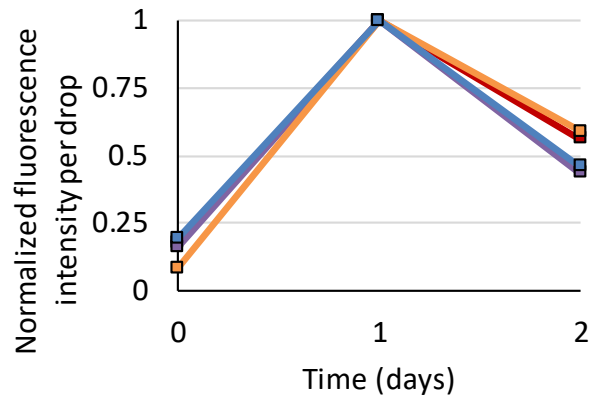
Biological replicates of Ca_{Int} 

Figure D2: Fluorescence intensity per drop normalized to the respective maximum value for each biological replicate of Ca_{Int} . The maximum fluorescence signal is measured on day 1 for all replicates, followed by a decrease in fluorescence signal. The colors correspond to different replicates.

Precipitate morphologies in the drops

Images from Intermediate Ca drops show the progression of precipitates in drops over time (Figure D3). The top row shows brightfield, GFP and autofluorescence signal overlays (channels (i), (ii) and (iii) described in Materials and Methods). The bottom row shows the corresponding reflection signals (channels (iv)). The first precipitates detected had a reflection signal but no corresponding autofluorescence. The autofluorescent precipitates ('particles') formed in drops with cells but were also seen in drops without GFP signal.

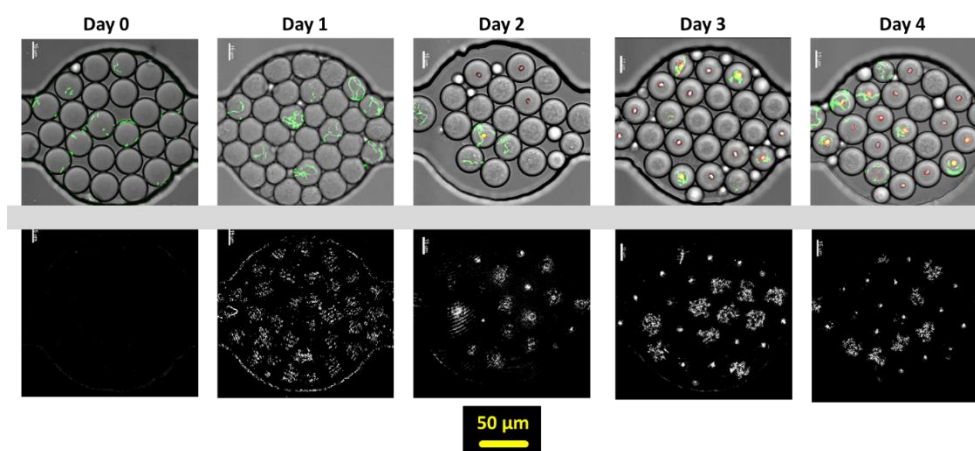


Figure D3: Drops showing cells (GFP, green) or precipitates (autofluorescence, red) for the Ca_{Int} condition. The bottom row shows the corresponding reflection signals from the drops in the top row. Two visually distinct morphologies of precipitates are observed via reflection imaging.

Bulk Ureolysis

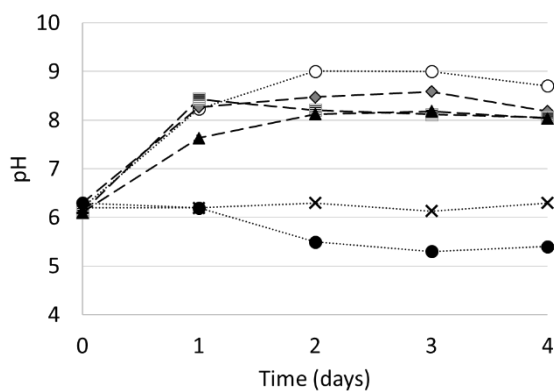


Figure D4: pH measurements in tube reactors over time for Ca free (○), Ca_{Low} (◆), Ca_{Int} (◻), Ca_{High} (▲), urea-free (●) and bacteria-free (×).

An increase in pH is an indicator for ureolysis (Equations 1 and 2). The pH curves show evidence of ureolysis in calcium-free, Ca_{Low}, Ca_{Int}, and Ca_{High}. Urea measurements (data not shown) indicated that most of the urea was consumed in calcium-free, Ca_{Low} and Ca_{Int} bulk experiments whereas Ca_{High} showed no significant ureolysis. The delayed rise in pH for Ca_{High} supports slower ureolysis. In the bacteria-free control, no ureolysis was observed. As expected, the bacteria-free and urea-free controls did not show an increase in pH.

Formation of precipitates in drops without bacteria

In experiments with bacteria, precipitates were even seen in drops that did not contain bacteria (Figures 4.c and D3). Hence, it was hypothesized that diffusion of carbonate and/or hydroxyl-ions occurred from drops with actively urea-hydrolyzing cells to bacteria-free drops. To test this hypothesis, control drops containing only fluorescent microbeads and dissolved calcium were generated. These control drops did not contain bacteria or urea, two of the three prerequisites for MICP (actively urea-hydrolyzing cells

(MJK2), urea and calcium). These control drops were mixed with Ca_{Int} drops (50:50 mixture by volume) and incubated in a microcentrifuge vial. After three days, precipitates were observed in all drops regardless of whether they initially contained ureolytic cells (Figure D5). Since no precipitates had formed in completely bacteria-free control experiments (Figure 2 in manuscript), it was hypothesized that diffusion of ureolysis-generated products (e.g. carbonate and/or hydroxyl-ions) between drops is possible and thus precipitation of calcium carbonate is possible even at a distance from actively urea-hydrolyzing cells.

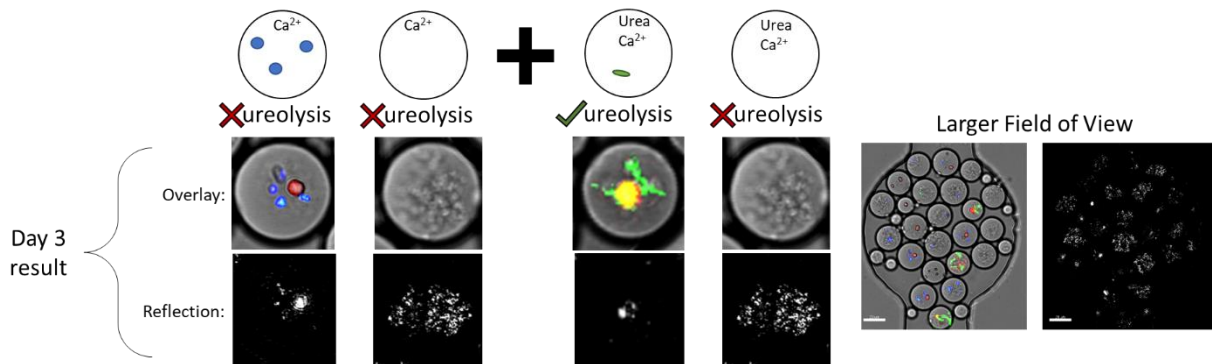


Figure D5: Experimental schematic of drops containing fluorescent microbeads (blue) and dissolved Ca mixed in 50:50 proportion (by volume) with Ca_{Int} drops. The corresponding results of each drop scenario in the schematic is provided below. A larger field of view is provided on the right. Similar to generation of drops with bacteria where only 1 in 10 drops generated contain a single cell, not all control drops contain microbeads. Note that for drops without fluorescent beads but with dissolved calcium, and for drops with dissolved urea and calcium but no bacteria, the Day 3 images shown are the same. This is because it is impossible to differentiate between these drops based on microscopy as they do not have a detectable indicator such as fluorescent microbeads or cells. Precipitates (detected by reflection signal) appeared in all drops, even those not containing bacteria or beads.

X-Ray Diffraction data for vaterite standard in Figure 5

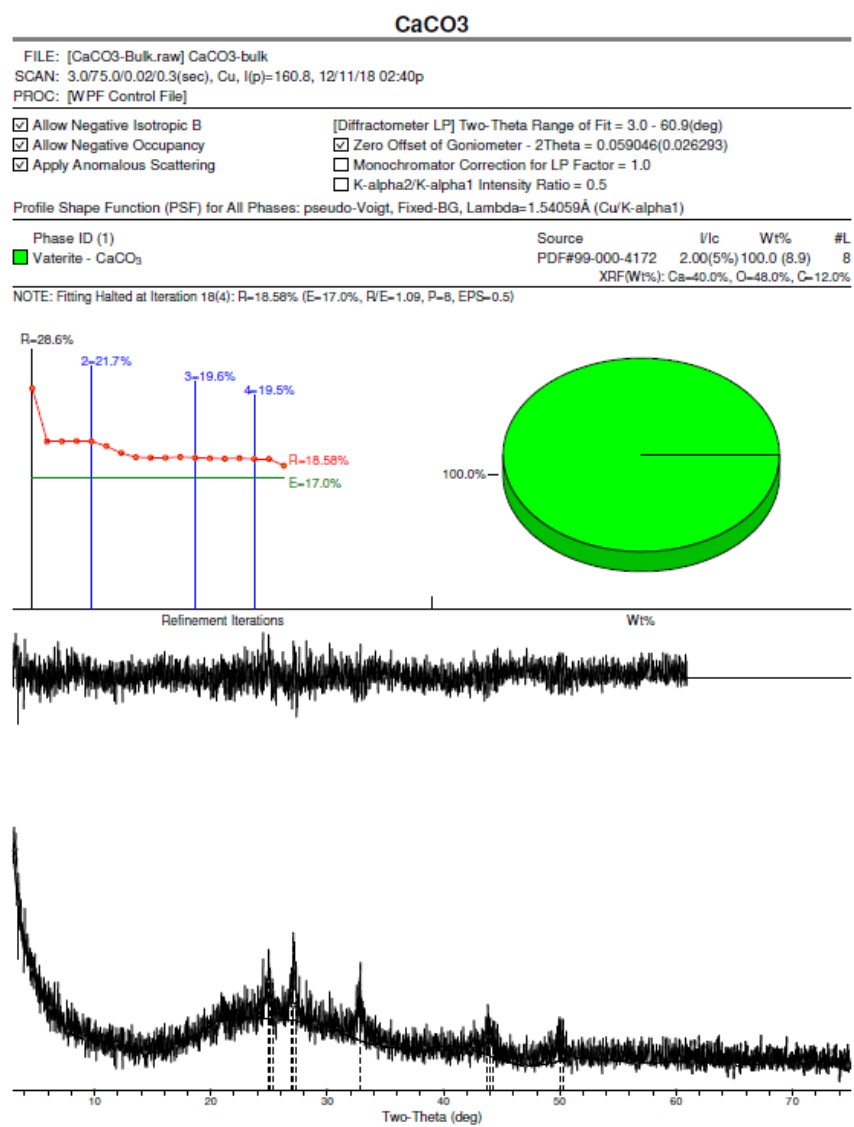


Figure D6: XRD characterization of a lab-generated precipitate sample from a flask containing MJK2 cells and mineralization medium using a Scintag X1 Diffraction System. The precipitates were characterized as vaterite by XRD.

Presence of Ca on the extensions

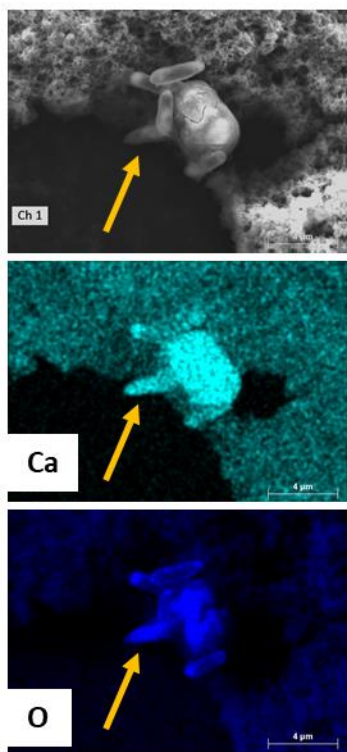


Figure D7: EDX maps for calcium and oxygen taken from an area showing one vaterite precipitate and some cell-type structures. The cell-type extension shows a strong calcium and oxygen signal similar to the precipitate, suggestive of CaCO_3 presence.

Fluorescence emission scans

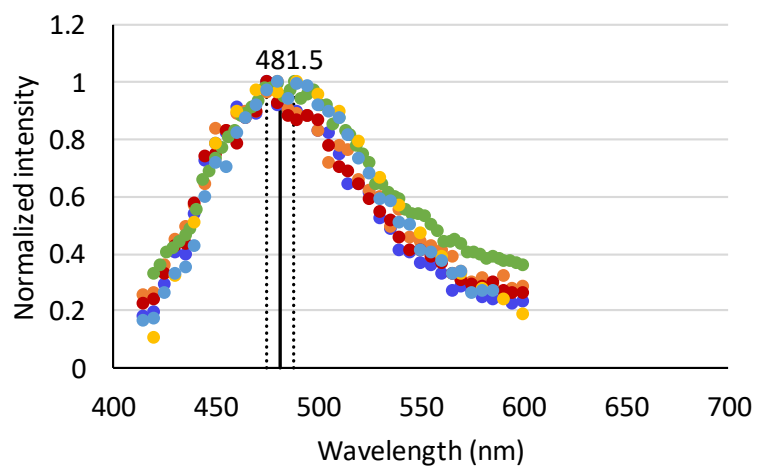


Figure D8: Fluorescence emission scans (405 nm excitation) from six randomly chosen vaterite precipitates. The y-axis is normalized so a value of 1 signifies the maximum fluorescence intensity, which was observed at an average emission wavelength of 481.5 ± 6.6 nm (shown by vertical lines on plot).

Supplemental Videos



Video_S1.avi

Video D1: Frames moving sequentially from the top of the drops (PDMS side) to the bottom (glass coverslip side). The vaterite precipitates appear predominantly at the bottom of the drops. Frames are from a z-stack with z-step resolution of 0.8 μm .



Video_S2.mp4

Video D2: Movie showing all channels overlaid (channels (i), (ii), (iii) and (iv) listed in methods). The movie shows, over 2 minutes of acquisition time, the close spatial association of cells to precipitates.

APPENDIX E

ADDITIONAL ANALYSES FOR MICP IN DROPS

Test for chloride precipitation in drops

Figure E1.a shows precipitates from the Intermediate Ca drops (from Chapter 6) and EDX data showed the presence of calcium, carbon and oxygen. Dominant sodium and chlorine signals were also observed, leading to the hypothesis that chloride-containing precipitates might exist in the drops.

To test this hypothesis, control drops were made with the same chloride concentration as the Intermediate Ca drops. The control drops contained urea and MJK2 but no calcium. The chloride in the medium was added as sodium chloride. The lack of any precipitates in these control drops (Figure E1.b) showed that sodium and chlorine signals observed in Figure E1.a likely resulted from halite (NaCl) precipitating on calcium carbonate during the air-drying process, instead of from a chloride-precipitate.

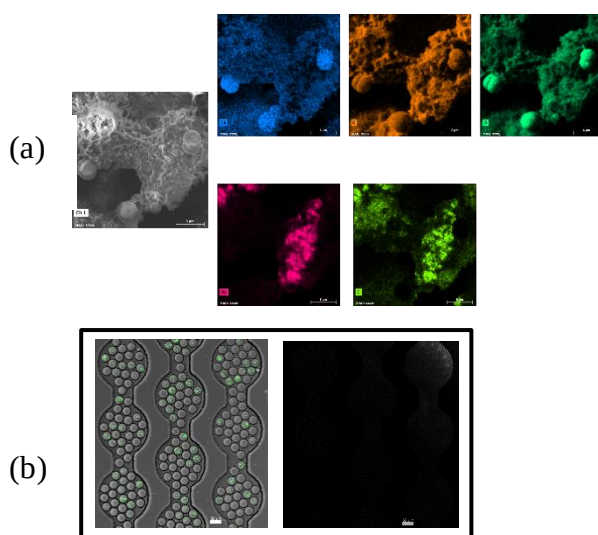


Figure E1: (a) Nano-Auger electron spectroscopy maps showing surface elemental components of precipitates in the center left frame, from Intermediate Ca. Top L to R: calcium, carbon, oxygen. Bottom L to R: sodium, chlorine. (b) Day 3 images from the chloride control experiment showing drops with cells (left) and the corresponding reflection signal (right).

Potential for various minerals to precipitate in drops

Table E1 shows saturation indices for various calcium and chloride minerals. A saturation index of over one indicates that there is potential for the precipitate to form.

Table E1: MINTEQ simulation results of potential mineral precipitation in drops for Intermediate Ca.

Mineral	Saturation Index	Potential to precipitate
CaCO ₃ X H ₂ O	3.03	Yes
Aragonite	4.22	Yes
Vaterite	3.81	Yes
Calcite	4.37	Yes
Halite	-3.04	No
Lime	-12.53	No
Natron	-2.89	No
Portlandite	-2.54	No
Thermonatrite	-4.77	No

A MINTEQ model simulating aqueous geochemistry of the drops indicated that only calcium carbonate showed potential to precipitate (saturation index >0) in the drops. Complete ureolysis was assumed for the model to estimate the potential for any carbonates to form. The media in the drops was not supersaturated with respect to sodium or chloride salts even after all urea had theoretically hydrolyzed.

Thermogravimetric analysis for precipitate mass estimation

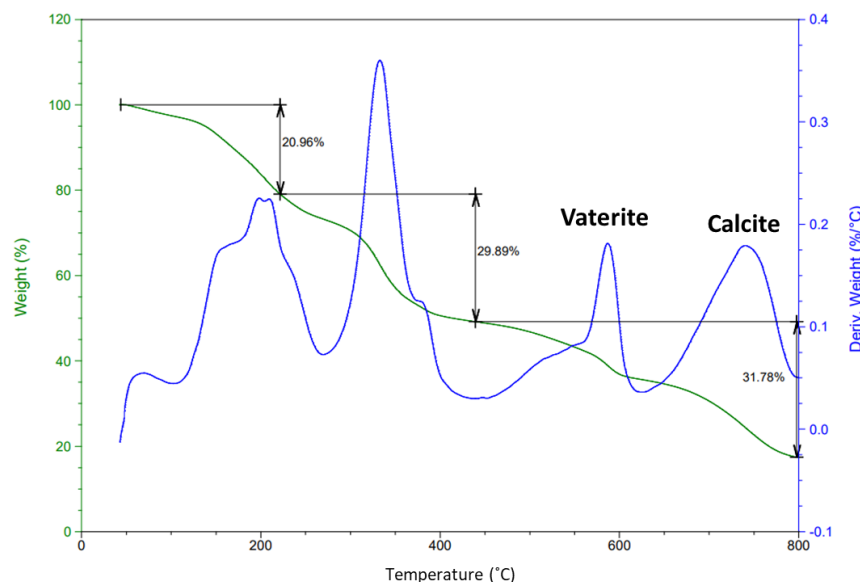


Figure E2: Thermogravimetric measurements from a sample of Ca_{Int} drops.

On day 4, a 40 μL aliquot of drops was pipetted onto a sample platinum pan of a Thermogravimetric analyzer (TA Instruments Q5000 IR). The drop suspension was broken as the drops evaporated on the sample pan. The instrument measured the changing mass as evaporation occurred and the TGA run started only when the sample mass measurement was constant, thus restricting the TGA analysis only to the solids in the drops. The number of drops in the 40 μL aliquot was estimated using the volume of a 25 μm diameter drop. 80% of the drops were assumed to have precipitates (based on day 4 images).

The area under the two TGA curves after 500 $^{\circ}\text{C}$ was pooled as total CaCO_3 mass.^{167, 168} The first peak is hypothesized to indicate vaterite mass and the second peak is hypothesized to be calcite. Vaterite is thermodynamically less stable than calcite and is therefore hypothesized to decompose before calcite, leading to two peaks.¹⁶⁹ Vaterite from within the drops might be converted to calcite during the operation of the TGA. These

hypotheses need further investigation but for the purpose of this project, any peaks above 600 °C were attributed to decomposition of CaCO_3 and a total mass of CaCO_3 was used for further calculations.

The total mass of CaCO_3 and the number of drops were used to estimate a mass of CaCO_3 per drop of 0.14 ng/drop. Assuming the vaterite precipitates in drops to be spherical, the mass of the precipitate was estimated using the density of CaCO_3 . This mass estimate was 0.18 ng/drop, which is close to the TGA mass estimate. The total dissolved calcium present in each drop was 0.05 ng (Ca^{2+}). A theoretical maximum of 0.13 ng of CaCO_3 could form in each drop if all dissolved calcium precipitated as CaCO_3 . The mass estimates for CaCO_3 from TGA (0.14 ng/drop) and image analysis (0.18 ng/drop) suggest that all the calcium present in the drops was precipitated.

APPENDIX F

SELENIUM INCORPORATION INTO MICP

Selenium (Se) is a contaminant that can leach out from coal mining waste when exposed to groundwater under aerobic conditions causing release into surrounding water streams. Selenium is known to bio-accumulate. As it moves up the food chain, it can cause deformities and reproductive abnormalities in fish, while in humans it is known to lead to gastrointestinal disturbances, nail and hair loss and dermatitis, and neurotoxicity.¹⁷⁰ Co-precipitation has been studied abiotically with selenium, where it was shown that selenite can incorporate in the CaCO_3 crystal lattice more readily compared to selenate.¹⁷¹ However, biological co-precipitation has not been tested and the work presented here was based on biological precipitation of calcium carbonate. Ureolytic bacteria can bring about the hydrolysis of urea (ureolysis) which creates chemical conditions conducive to calcium carbonate precipitation when aqueous calcium is present. The most common forms of aqueous Se are selenite and selenate, which are oxyanions.¹⁷² Therefore, co-precipitation of Se with MICP would involve these oxyanions potentially replacing the carbonate anion.

The aim of this study was to investigate whether aqueous selenium can incorporate into the calcium carbonate that is formed via bacterial ureolysis by the ureolytic soil bacterium *Sporosarcina pasteurii*. This was done in two phases, the first phase of experiments looked at the effects of concentrations of calcium and selenium on the removal of aqueous selenium. The second phase investigated the removal behavior of selenite versus selenate, and looked at MICP with selenium co-precipitation in field wastewater samples from the coal-fired power plant in Colstrip, MT. This work did not establish the specific mechanism of removal but focused on investigating whether selenium removal alongside MICP is possible and feasible.

Methods

Batch experiments were set up in artificially prepared groundwater (AGW) with calcium and selenium added at various test concentrations. The AGW was also supplemented with urea to facilitate ureolysis. The batch reactors were inoculated with 1 mL overnight culture of *Sporosarcina pasteurii* which was centrifuged, washed and adjusted to an OD of 0.4 prior to inoculation. Over the 100-hour duration of each experiment, fluid samples were collected at various time points to measure the pH, dissolved urea, calcium and selenium concentrations.

In the first phase of experiments, four combinations of two calcium concentrations (1.75 mM and 100 mM) and two selenium concentrations (2.5 ppm and 5 ppm) were tested to assess the effects of concentrations on selenium removal in addition to investigating the possibility of the process (Table F1). In addition to these MICP experiments, several control experiments were also performed to quantify potential selenium volatilization or abiotic precipitation in the absence of MICP (Table F1).

Table F1. Experimental conditions for phase 1 batch tests for selenium incorporation via MICP.

	#	<i>S. pasteurii</i>	Calcium (mM)	Selenium (ppm)
MICP experiments	1	Yes	1.75	2.5
	2	Yes	1.75	5
	3	Yes	100	2.5
	4	Yes	100	5
Controls	5	No	No	2.5
	6	No	100	5
	7	Yes	No	No
	8	Yes	100	No
	9	Yes	1.75	No
	10	Yes	No	2.5
	11	Yes	No	5

In phase 2, 10 other experiments were performed with field samples from the coal-fired power plant in Colstrip, MT (Table F2). Samples were collected from the wastewater ponds at this power plant. In the published abiotic selenium co-precipitation study, there was evidence showing selenite was able to incorporate into calcite better than was selenate. To test this with MICP, in the AGW experiments selenium added as selenite or selenate (Table F2, #1-4). Colstrip plant water was used instead of AGW in the rest of the experiments, which included a combination of filtered and unfiltered water, and selenium added as selenite and selenate. All conditions tested are given in Table F2.

Table F2. Experimental conditions for phase 2 batch tests for selenium incorporation via MICP.

#	Base water	Selenium form (ppm)	pH ₀
1	AGW	Selenate (2.5)	7.4
2	AGW	Selenate (5)	7.7
3	AGW	Selenite (2.5)	7.7
4	AGW	Selenite (5)	7.8
5	Colstrip	No	3.6
6	Colstrip	No	6.7
7	Colstrip	Selenate (2.5)	7.2
8	Colstrip	Selenite (2.5)	6.9
9	Colstrip filtered	No	7.3
10	Colstrip filtered	Selenate (2.5)	7.3

Results

Phase 1:

Four combinations of calcium and selenium (as selenate) concentrations were tested under batch conditions in phase 1. In all experiments inoculated with *S. pasteurii*, the pH increased to about 9.5 indicating the occurrence of ureolysis (Figure 1)

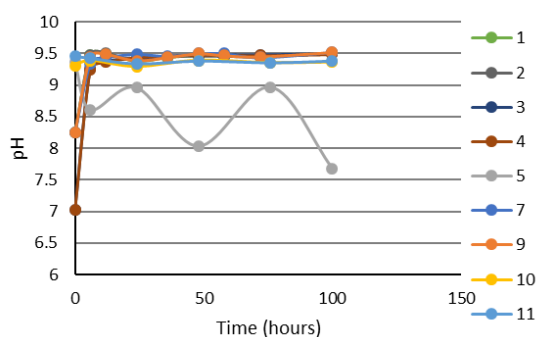


Figure F1: pH increased over time in all experiments where bacteria were present, indicating ureolysis.

Preliminary results showed that selenium removal depends on the initial concentrations of both selenium and calcium. Only 1% of aqueous selenium was removed at low calcium and selenium concentrations (condition #1 in Table 1.a). Increasing either calcium or selenium concentrations (within the design concentration limits) increased

percent selenium removed comparably (2-5%). High concentrations of both selenium and calcium led to the highest selenium removal of 76%. The controls without bacteria and calcium showed no selenium removal indicating that MICP is indeed the mechanism responsible for aqueous selenium removal. Energy Dispersive X-Ray results suggested that the precipitates were calcium carbonate. Figure F2 shows FE-SEM images of precipitates formed in the absence and presence of selenium.

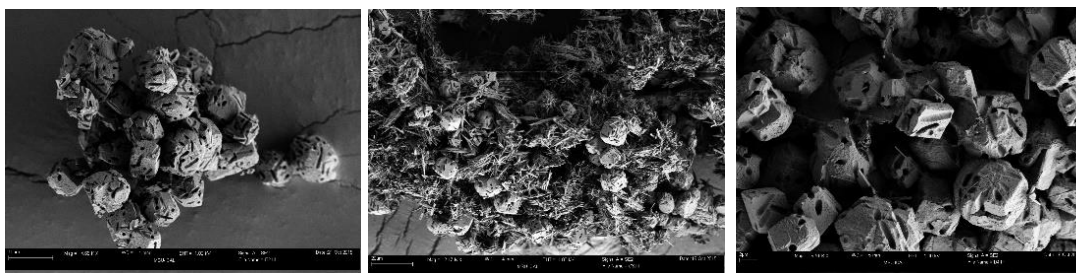


Figure F2: (Left to Right) Scanning Electron Micrographs of crystals formed in the no selenium control (#9), 2.5 ppm Se (#1) and 5 ppm Se (#2) respectively.

XRD results indicated that only calcite formed in the absence of selenium. At 2.5 ppm Se, aragonite (needle-like structures in Figure F2) formed alongside calcite whereas at 5 ppm Se, predominantly calcite was observed.

Phase 2:

In all AGW experiments inoculated with *S. pasteurii*, the pH increased to about 9.5 indicating the occurrence of ureolysis. In the experiment where Colstrip water was not adjusted to a pH of 7, there was no increase. This can be attributed to the inability of cells to survive in the low pH conditions of the untreated waste water (around pH 4) (Figure F3). In the Colstrip field water reactors when the pH was adjusted to 7 prior to inoculation, the pH increased but plateaued at a value of 8.

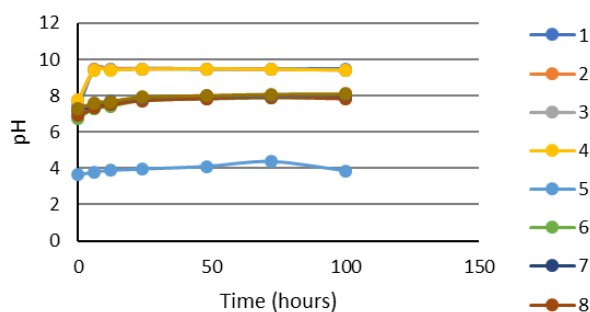


Figure F3: pH increase over time was higher for AGW experiments compared to Colstrip water experiments. A pH increase was not observed in the Colstrip water experiments that were not initially pH-adjusted.

Despite the observed pH increase in some Colstrip water reactors, the urea concentrations did not decrease (Figure F4) suggesting that the pH increase did not result from ureolysis in these experiments.

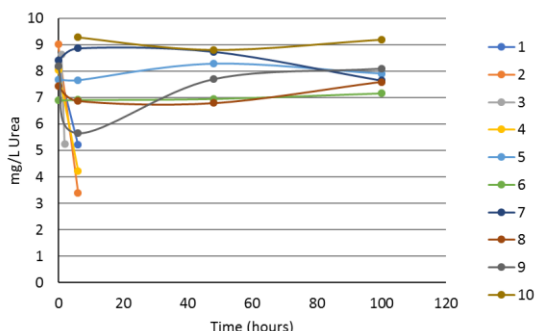


Figure F4: Urea was consumed rapidly in the AGW experiments whereas no urea decrease was measured in the Colstrip water experiments.

Conclusions

Under certain conditions, selenium (as selenate) was removed from solution alongside the occurrence of MICP. Selenium affected the polymorph of calcium carbonate that formed with aragonite and calcite forming in the presence of 2.5 ppm Se. More work is required to characterize the Colstrip plant water. A knowledge of the constituents of the Colstrip water will help determine factors that are inhibiting ureolysis and can provide avenues for making such water samples conducive to MICP.

APPENDIX G

MINE TAILINGS LEACHATE POTENTIAL FOR UREOLYSIS DRIVEN MICP

Mine tailings from a gold mine in Montana were collected to characterize their metal leaching potential. Urea supplemented tailings pond water was inoculated with *S. pasteurii* to test whether ureolysis is possible given the high metal leaching potential. Once it was established that ureolysis is indeed possible, these experiments were repeated with added calcium to study MICP in tailings water.

Leaching Tests

Tailings, either 5 g (*circles*) or 15 g (*squares*), were placed in contact with 50 mL artificially prepared ground water. The water was sampled weekly to determine the concentration of leachates by ICP-MS. Elements that leached out of the tailings were sodium, magnesium, strontium, calcium and molybdenum. Barium concentrations were not seen to change over time.

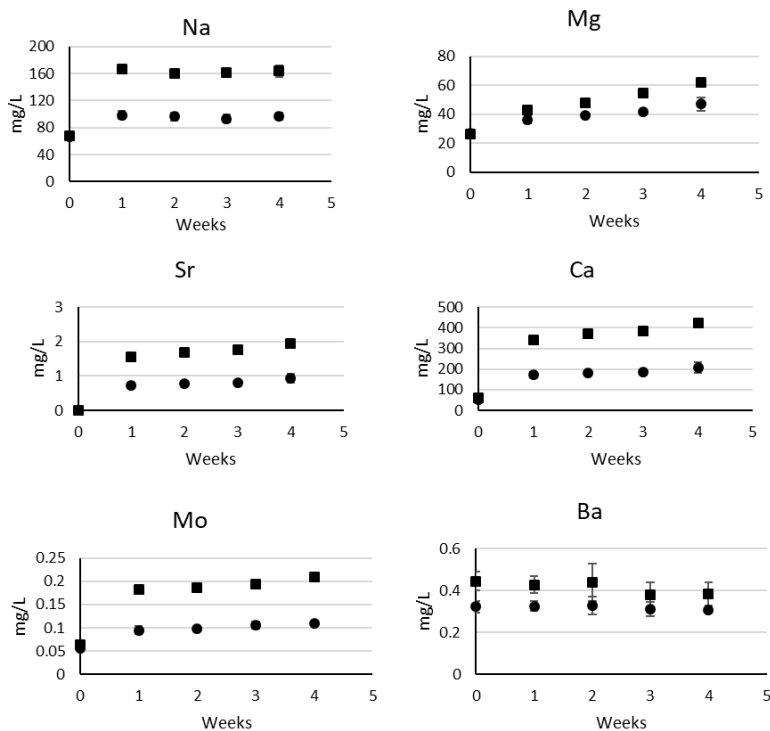


Figure G1: Leaching results for sodium, magnesium, strontium, calcium, molybdenum, and barium over 4 weeks.

Ureolysis in presence of mine tailings (5 g tailings in 100 mL AGW)

In these experiments, AGW was supplemented with urea and inoculated with 1 mL overnight *S. pasteurii* culture ($OD_{600}=0.4$), or 5 g tailings or both. Ureolysis was tested using the Jung assay.¹²⁰ On the graph, the gray triangles with dashed lines are the triplicates with *S. pasteurii*. Two additional controls were tested in this experiment, one without tailings (*white circles with dotted line*) and one without bacteria (*crosses with dotted line*).

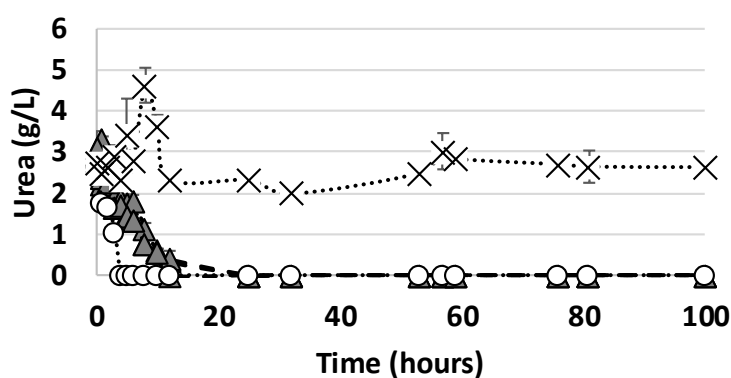


Figure G2: Urea concentrations over time for experiments with *S. pasteurii* and tailings (▲), only *S. pasteurii* (○), and only tailings (×).

The presence of tailings appeared to decrease the rate of ureolysis compared to the tailings-free control. However, the tailings did not prevent ureolysis from occurring. This suggests that ureolysis might initially occur in the pond water of the tailings ponds from which samples were taken. For the effect of tailings leachate on ureolysis potential, these experiments should be repeated using AGW that has been in contact with tailings for at least one week (*cf.* Figure G1).

MICP in presence of mine tailings (5 g tailings in 100 mL AGW)

The experiments discussed in part 2 also had calcium in the AGW. Samples analyzed for dissolved metal concentrations using ICP-MS showed that calcium decreased in the experiments with *S. pasteurii*.

Along with calcium removal, the *S. pasteurii* and tailings experiments also showed some barium removal.

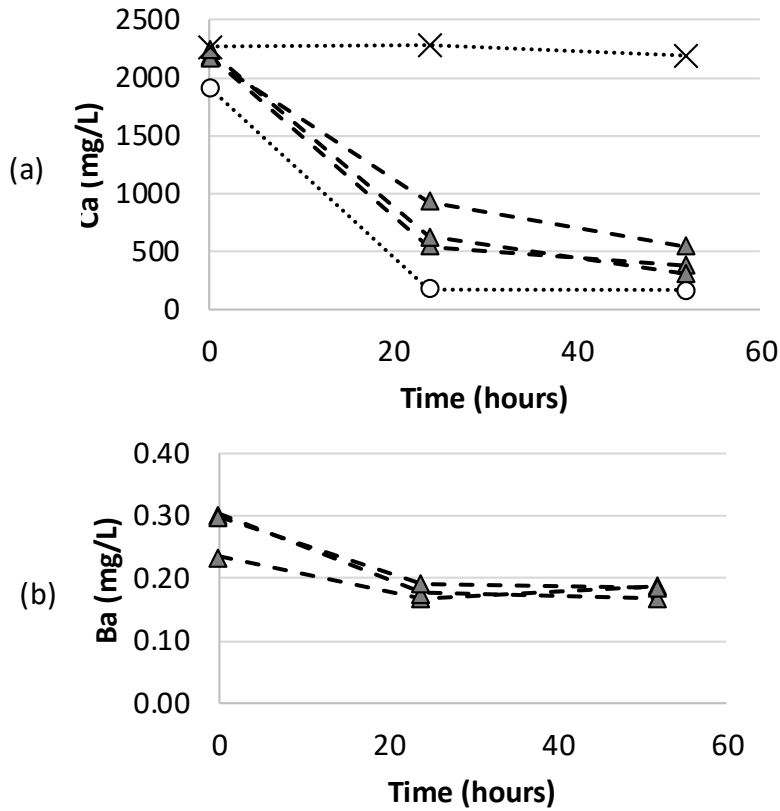


Figure G3: (a) Calcium and (b) barium concentrations decreased over time for experiments with *S. pasteurii* and mine tailings in AGW (▲).

Ureolysis driven MICP was successfully implemented in the presence of mine tailings. However, to test the efficiency of MICP in the presence of tailings leachate, these experiments should be performed after considerable leaching from tailings has taken place (~1 week or more).

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