Carbonate Mineral Precipitation for Soil Improvement

through Microbial Denitrification

by

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ABSTRACT

Microbially induced calcium carbonate precipitation (MICP) is attracting increasing attention as a sustainable means of soil improvement. While there are several possible MICP mechanisms, microbial denitrification has the potential to become one of the preferred methods for MICP because complete denitrification does not produce toxic byproducts, readily occurs under anoxic conditions, and potentially has a greater carbonate yield per mole of organic electron donor than other MICP processes. Denitrification may be preferable to ureolytic hydrolysis, the MICP process explored most extensively to date, as the byproduct of denitrification is benign nitrogen gas, while the chemical pathways involved in hydrolytic ureolysis processes produce undesirable and potentially toxic byproducts such as ammonium (NH_4^+). This thesis focuses on bacterial denitrification and presents preliminary results of bench-scale laboratory experiments on denitrification as a candidate calcium carbonate precipitation mechanism.

The bench-scale bioreactor and column tests, conducted using the facultative anaerobic bacterium *Pseudomonas denitrificans*, show that calcite can be precipitated from calcium-rich pore water using denitrification. Experiments also explore the potential for reducing environmental impacts and lowering costs associated with denitrification by reducing the total dissolved solids in the reactors and columns, optimizing the chemical matrix, and addressing the loss of free calcium in the form of calcium phosphate precipitate from the pore fluid.

The potential for using MICP to sequester radionuclides and metal contaminants that are migrating in groundwater is also investigated. In the sequestration process, divalent cations and radionuclides are incorporated into the calcite structure via substitution, forming low-strontium calcium carbonate minerals that resist dissolution at a level similar to that of calcite. Work by others using the bacterium *Sporosarcina pasteurii* has suggested that *in-situ* sequestration of radionuclides and metal contaminants can be achieved through MICP via hydrolytic ureolysis. MICP through bacterial denitrification seems particularly promising as a means for sequestering radionuclides and metal contaminants in anoxic environments due to the anaerobic nature of the process and the ubiquity of denitrifying bacteria in the subsurface.

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CHAPTER 1

INTRODUCTION

1.1 Background

Human ingenuity has been said to be driven by need. The need to accommodate an ever-growing global population has taken center stage in many societies and has pushed human inventiveness to new levels. One area of need that has relied on evolving resourcefulness is the development of civil infrastructure needed to accommodate expanding populations; in particular, civil infrastructure should be built on and within suitable ground that must reliably support it. The idea of suitable ground has taken new meaning as expanding populations move into previously undeveloped areas and into areas previously bypassed due to poor ground conditions. As a consequence of movement into these areas, engineers face new challenges in defining suitable ground. Areas where seismic activity, geologic hazards, rising sea levels and declining water tables affect ground conditions are of particular concern. Various techniques for ground improvement have been developed over the years to meet these challenges. Recently, efforts to develop new ground improvement techniques have focused on searching for sustainable, cost-effective methods to either supplement or replace traditional techniques.

Efforts to develop cost-effective ground improvement solutions to meet ground improvement challenges have become increasingly complex due to the varying nature and broad scope of problematic soils. These efforts are further complicated by sustainable considerations on local and global scales. Building

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materials, designs, and methods of previous generations often need to be either replaced or supplemented by innovative materials and sustainable practices to limit environmental impacts while simultaneously meeting design considerations. One prime example of a nearly indispensible building material that poses significant sustainability concerns is Portland cement and, by extension, its use in making concrete and mortars. Direct treatment with Portland cement is widely used in ground improvement applications where existing soils require strengthening through soil binding. Unfortunately, Portland cement production is extremely energy intensive and a major source of carbon dioxide (CO_2) pollution, as well as emissions of sulfur and nitrogen oxides. Cement production, most commonly Portland cement, accounts for the second largest source of global greenhouse gas emissions (18%) within the industry sector (World Resources Institute 2005). It is estimated that the cement industry is one of the top two manufacturing industries responsible for global CO_2 emissions (van Oss & Padovani, 2003). Cement will mostly likely always be required for many construction projects. However, reductions in widespread use of Portland cement through either substitution when possible or complementary use of environmentally friendly methods and materials would be a considerable contribution in meeting long-term sustainability goals.

As discussed above, sustainable construction practices rely on the use of environmentally friendly alternative building methods and materials where possible. One possible alternative method for ground improvement is microbially induced carbonate precipitation (MICP), a biologically mediated subsurface

process. Recent research has demonstrated the potential for soil improvement through biologically mediated subsurface processes. In particular, emerging research in the field bio-geotechnical engineering suggests soil cementation through MICP may be a promising method for mitigating a number of geotechnical problems in granular soils.

Successful development and implementation of microbial mineral precipitation for soil improvement would have wide application to a variety of important geotechnical problems including: the stabilization of slopes; controlling soil erosion and scour; reducing under-seepage of levees and cut-off walls; increasing the bearing capacity of shallow foundations; facilitating excavation and tunneling in cohesionless soils; and remediating the potential for seismic settlement and liquefaction (Burbank et al. 2012; Chou et al. 2011; DeJong et al. 2006; Dejong et al. 2010; Harkes et al. 2010; Karatas 2008; Kavazanjian and Karatas 2008; van Paassen et al. 2008; van Paassen et al. 2010; Whiffin 2004). The utility of MICP extends beyond its use as a cementing agent, as it may be especially useful near or beneath existing structures, where the application of traditional soil improvement techniques is limited because of ground deformations and/or high cost associated with alternative techniques. Indeed, a directed cementation process making use of soil microbes through bio-stimulation or bio-augmentation could have a broad range of applications for ground improvement, and possibly groundwater remediation, while simultaneously reducing the need for traditional energy intensive materials.

The biological basis for microbial mineral precipitation is well established. Microorganisms, bacteria in particular, are associated with the formation of carbonate minerals and are thought to play a fundamental role in carbon cycling on the geologic timescale (Ehrlich 2002; Fredrickson and Fletcher 2001; Shock 2009; Warthmann et al. 2000). Estimates suggest that nearly half of the Earth's biomass is comprised of microorganisms found in the subsurface and oceanic subsurface (Whitman et al. 1998). The complex interactions between microorganisms and minerals have been well documented through the efforts of researchers attempting to understand the formation, dissolution, and alteration of minerals by microorganisms on geologic and engineering timescales (Ehrlich 2002; Fredrickson and Fletcher 2001; Karatas 2008; Phoenix and Konhauser 2008; Shock 2009). Many microbial processes are capable of producing relatively strong soils through carbonate mineral precipitation. For example, a predominately calcium carbonate rock such as calcrete can have an average uniaxial compressive strength of 12 MPa with an associated modulus of elasticity between 29-65 MPa and an allowable bearing capacity between 1.5-2.0 MPa (Zorlu and Kasapoglu 2009). Although there are several pathways by which caliche can be formed, bio-mediated processes is one pathway that can explain the formation of calcrete (Dixon and Mclaren 2009).

Ground improvement via carbonate precipitation is only one of the many applications of bioremediated subsurface processes. For example, microbes have long been the workhorses for many modern-day engineering processes such as wastewater treatment. Reclamation and treatment of wastewater has taken on a

new sense of urgency in recent years as the ready availability of freshwater appears to be threatened in most parts of the world either due to increased demand, drought, climate change, contamination, or any combination thereof. In the face of such threats, many concerned authorities have recognized that any reasonable notion of sustainability must incorporate preservation of freshwater resources. A major source of water that has seen increased environmental and human-induced stress is groundwater. Groundwater has been and continues to be an important source of freshwater on earth, as well as an integral component of the hydrologic cycle. Recent estimates place approximately 22% (8,400,000 km^3) of Earth's freshwater in the subsurface where a large portion of this total figure can be readily accessed via aquifers (Christopherson 2009). Unfortunately, groundwater is susceptible to contamination through wells, unlined waste storage units, run-off, and surface waterways. The contamination of aquifers is a growing concern in many areas and, therefore, has been the focus of recent research efforts to develop novel and effective remediation methods.

One such novel approach is microbial carbonate precipitation of radionuclides and metal contaminants through *in-situ* remediation of contaminated aquifers (Mitchell and Ferris 2005; Colwell et al. 2003; Fujita et al. 2000; Smith et al. 2004). Poor waste-disposal practice has resulted in the release of low-level radioactive waste and metal contaminants (e.g., $^{90}Sr^{2+}$, $^{60}Co^{2+}$, Cd^{2+}) into the vadose zone and groundwater at many U.S. Department of Energy (DOE) weapons-production sites. These toxic waste products are a legacy of DOE chemical synthesis and nuclear waste facilities in locations such as Hanford, WA

(100-N area) and the Idaho Nuclear Technology and Engineering Center (INTEC) at the Idaho National Engineering and Environmental Laboratory.

Microbial sequestration of metals is a microbially mediated mineral precipitation process that results in the formation of mineral deposits found in the natural environment, including calcium-carbonate ($CaCO_3$) minerals such as calcite. In principle, the geochemical conditions conducive to carbonate precipitation are not unique to any specific microorganism; rather, carbonate precipitation can occur when carbonate (CO_3^{2-}) forms in the vicinity of suitable cations under alkaline conditions. Microbial sequestration of radionuclides and contaminant metals into calcite is essentially a co-precipitation reaction, governed by both thermodynamic and kinetic factors, in which suitable divalent cations are incorporated into the calcite lattice. Incorporation of divalent ions into the calcite structure appears to slow their transport and possibly immobilize them within the calcite structure (Mitchell and Ferris 2005; Colwell et al. 2003; Fujita et al. 2004; Fujita et al. 2000; Smith et al. 2004). If proven effective, immobilization of these contaminants through MICP may provide a sustainable and cost-effective *in-situ* remediation scheme for radionuclide and metal contaminated sites.

1.2 Scope and Organization

Research on microbial induced carbonate precipitation (MICP) at Arizona State University is comprised of several components including:

- proof-of-concept for MICP in a concentrated chemical matrix
- conducting bench-scale experiments in well-mixed batch reactors
- carrying out static sand column experiments

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- designing and performing flow-through sand column experiments.

In addition, unintended mineral precipitation in the reservoir section of flow-through sand column experiments led to work to investigate MICP via bacterial denitrification as a potential groundwater remediation tool for sequestration of radionuclides and metal contaminants.

The scope of this thesis is limited to presenting the results of bench-scale experiments in batch reactors and sand columns, as well as outlining potential applications based on these experiments. In addition, chapters 2, 3 and 4 provide a review of relevant literature in order to introduce the reader to the application in that particular chapter and provide background information that is most relevant to that chapter. It should be noted that there is some overlap among chapters, as this thesis is rooted in the potential applications of MICP through bacterial denitrification. Chapter 5 presents the results obtained in static and flow-through sand columns. This thesis closes with a summary and conclusion in Chapter 6. In summary, the goals of the work presented herein are to:

(a) Investigate the applicability of MICP for soil improvement through denitrification, *Chapter 2*

(b) Investigate and outline the potential for sequestration of radionuclides and metal contaminants through MICP, *Chapter 3*

(c) Perform proof-of-concept experiments in a concentrated chemical matrix and present the results of bench-scale experiments of MICP using *Pseudomonas denitrificans, Chapter 4*

(d) Present the results of MICP using *Pseudomonas denitrificans* in flow-

through and static sand columns, *Chapter 5*

(e) Summarize and provide a conclusion that may guide future work,

Chapter 6

CHAPTER 2

CARBONATE MINERAL PRECIPITATION FOR SOIL IMPROVEMENT

THROUGH MICROBIAL DENITRIFICATION

2.1 Introduction

Recent research has demonstrated the potential for soil improvement through biologically mediated subsurface processes. In particular, new and exciting research suggests soil cementation through microbial mineral precipitation as a promising method for mitigating a host of geotechnical problems in granular soils. Successful development and implementation of microbial mineral precipitation mechanisms for soil improvement would have wide application to a variety of important geotechnical problems including: the stabilization of slopes; controlling soil erosion and scour; reducing under-seepage of levees and cut-off walls; increasing the bearing capacity of shallow foundations; facilitating excavation and tunneling in cohesionless soils; and remediating the potential for seismic settlement and liquefaction (DeJong et al. 2006; Dejong et al. 2010; Karatas, 2008; Kavazanjian and Karatas 2008; van Paassen et al. 2008; van Paassen et al. 2010; Whiffin 2004). Microbially induced mineral precipitation may be especially useful near or beneath existing structures, where the application of traditional soil improvement techniques is limited because of ground deformations and/or high cost associated with alternative techniques.

2.2 Geochemical Basis for Carbonate Mineral Precipitation

MICP is a mineral precipitation process that results in the formation of calcium-carbonate ($CaCO_3$) mineral deposits found in the natural environment, including calcite. Calcite is the most thermodynamically stable polymorph of $CaCO_3$ and the primary product in many microbially induced ground surface calcium carbonate rocks and cementing agents. Aragonite and vaterite are the other less thermodynamically stable forms of $CaCO_3$ that may occur during precipitation. The formation of aragonite is typically associated with marine environments where kinetic factors may favor the incorporation of Mg^{2+} ions in place of Ca^{2+} (calcium ions) in the calcite lattice. Aragonite precipitation is also associated with biologically controlled processes such as those found in nearly all mollusk shells. In general, biologically *controlled* processes are distinctly different than microbially mediated (or microbially induced) precipitation; whereas biologically controlled processes are tailored to form a particular polymorph, microbially mediated precipitation is an inorganic process in which microorganisms create the environment conducive to $CaCO_3$ precipitation without regard to a specific polymorph. The thermodynamic stability of $CaCO_3$ polymorphs from most to least are: calcite, aragonite, vaterite.

Formation of calcite, the primary mineral precipitate formed through denitrification in a calcium-rich environment, relies on microbial metabolism to increase the total carbonate (CO_3^{2-}) content and pH to the point of super-saturation with respect to calcite, thereby inducing calcite precipitation. Biologically mediated precipitation of $CaCO_3$ minerals can occur when carbonate

ions (CO_3^{2-}) are formed in the presence of calcium ions under alkaline conditions, as illustrated by Eq. 1.

$$Ca^{2+} + HCO_3^- + OH^- = CaCO_3(s) + H_2O \qquad (1)$$
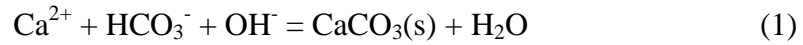
Equation 1 shows that bicarbonate ions (HCO_3^-) and some form of alkalinity (OH^- in this case) are critical to the precipitation of $CaCO_3$. Note that the presence of OH^- (hydroxyl) ions is an indication of increased pH, whereas in natural systems alkalinity is a general description of negatively charged aqueous species capable of acting as hydrogen ion (H^+) acceptors to the equivalence point of carbonate or bicarbonate ions.

The specific mode by which alkalinity is produced (or pH is increased) and HCO_3^- is formed depends on the microbial process in question. In general, a given heterotrophic microorganism metabolizes an organic carbon source (e.g., acetate or glucose) and produces metabolic end product in the form of $CO_{2(g)}$ (Note: the subscript "(g)" indicates a gaseous form while the subscript "(aq)" indicates an aqueous form). In the presence of water (e.g., pore fluid), the oxidized end product ($CO_{2(g)}$) hydrolyzes to form carbonic acid as illustrated in Eq. 2.

$$CO_{2(g)} + H_2O \leftrightarrow HCO_3^-{}_{(aq)} + H^+{}_{(aq)} \qquad (2)$$
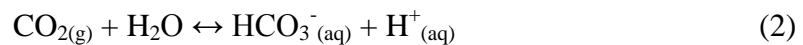
Under sustained basic conditions, further speciation can occur to form CO_3^{2-} per Eq. 3, which then precipitates to form $CaCO_{3(s)}$ in the presence of Ca^{2+} ions.

$$HCO_3^-{}_{(aq)} + OH^-{}_{(aq)} \leftrightarrow H_2O + CO_3^{2-}{}_{(aq)} \tag{3}$$

2.3 Candidate Processes for MICP

In principle, the geochemical conditions conducive to carbonate precipitation are not unique to any specific microorganism. Rather, carbonate precipitation can occur when carbonate forms in the vicinity of suitable cations under alkaline conditions, i.e., when a solution becomes supersaturated with respect to $CaCO_3$. MICP relies on the byproducts of bacterial metabolism (e.g., CO_2, alkalinity) to facilitate the formation of carbonate ions, which precipitate in the presence of divalent cations (e.g., Ca^{2+}).

Candidate processes that can induce MICP include bacterial ureolysis, sulfate reduction, fermentation of fatty acids, and denitrification. Each of these processes produces a by-product. Table 1 summarizes the by-products and side effects of these four processes. In three of four cases, the by-product is undesirable. Only denitrification produces an end product that does not have an undesirable side effect. Bacterial ureolysis, as the MICP process that has been studied most extensively for ground improvement, and denitrification, as the candidate process that is the subject of this thesis, are discussed in more detail below.

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Table 1 Microbially Induced Carbonate Precipitation Mechanisms

Microbial Process	End Products	Undesirable Side-Effect
Bacterial Ureolysis	NH_3 (ammonia) NH_4^+ (ammonium)	Toxic gas Readily forms toxic salts
Sulfate Reduction	H_2S (hydrogen sulfide)	Toxic, odorous gas
Fermentation of Fatty Acids	CH_4 (methane)	Combustible gas
Denitrification	N_2 (nitrogen)	None

2.3.1 Bacterial Ureolysis

A widely studied candidate MICP process for use in ground improvement is bacterial ureolysis using *Sporosarcina pasteurii* (DeJong et al. 2006; van Paassen et al. 2008; Whiffin 2004). Ureolysis, or a form of ammonification (transformation of organic nitrogen into ammonia), produces ammonium (NH_4^+), dissolved organic carbon in the form of bicarbonate (HCO_3^-), and base (OH^-) through the metabolism of urea (NH_2CONH_2), as illustrated in Eq. 4.

$$NH_2CONH_{2(s)} + 3H_2O = 2NH_4^+_{(aq)} + HCO_3^-_{(aq)} + OH^-_{(aq)} \qquad (4)$$
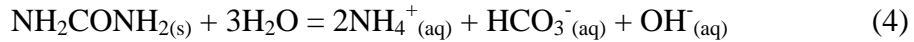
Upon formation of a sufficiently saturated solution with respect to calcite, precipitation ensues and calcite is formed per Eq. 1.

Since ammonium speciation is highly pH dependant in the elevated pH environment characteristic of bacterial ureolysis, and in MICP reactions in general, the dominant nitrogen form may be $NH_{3(g)}$, as illustrated in Eq. 5.

$$H_2O + NH_{3(g)} \leftrightarrow NH_4^+_{(aq)} + OH^-_{(aq)} \qquad (5)$$

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The chemical reactions involved in bacterial ureolysis produce undesirable and potentially toxic end products: ammonia ($NH_{3(g)}$) and ammonium (NH_4^+). A given fraction of ammonium may be converted to NO_3^- (nitrate) through bacterial nitrification, which may then be reduced to nitrogen gas (N_2) via bacterial denitrification in the presence of denitrifying bacteria with a suitable source of organic electron donor. But, it is unclear whether or not a substantial portion of ammonium can be converted to nitrate in comparison to the amount produced, since nitrifying bacteria are rate limited by the lack of subterranean dissolved oxygen and may also experience severe inhibition at elevated ammonia concentrations (Anthonisen et al. 1976; Antoniou et al. 1990). MICP via bacterial ureolysis will be a suitable technique for microbially mediated soil improvement (i.e. MICP) only if the ammonia/ammonium can be properly treated.

2.3.2 Denitrification

An alternative MICP mechanism to bacterial ureolysis is denitrification. Denitrifying organisms are ubiquitous in the subsurface. One common denitrifying organism is *Pseudomonas denitrificans*, a Gram-negative facultative anaerobe capable of inducing calcite precipitation without the production of toxic byproducts (Ehrlich 2002; Karatas 2008; van Paassen et al. 2010). The initial steps of calcite precipitation involve denitrification to produce alkalinity and carbon dioxide (CO_2), where nitrate (NO_3^-) serves as an electron acceptor and is ultimately reduced to nitrogen gas (N_2) in a process driven by heterotrophic bacterial metabolism. Equation 6 describes this process.

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$$2.6H^+_{(aq)} + 1.6NO_3^-{}_{(aq)} + CH_3COO^-{}_{(aq)} \rightarrow 0.8\ N_{2(g)} + 2CO_{2(g)} + 2.8\ H_2O \qquad (6)$$
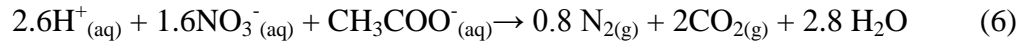
The remaining reactions required for MICP, described in Eqs. 7 and 8, involve the speciation of chemical constituents that facilitate calcite precipitation similar in form to those described above for ureolytic hydrolysis:

$$CO_{2(g)} + H_2O \leftrightarrow HCO_3^-{}_{(aq)} + H^+_{(aq)} \qquad (7)$$

$$Ca^{2+}_{(aq)} + HCO_3^-{}_{(aq)} + OH^-_{(aq)} = CaCO_3(s) + H_2O \qquad (8)$$
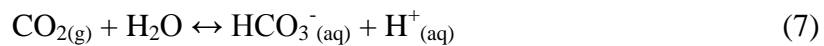
Nitrate is used as an electron acceptor under anaerobic conditions by *Pseudomonas denitrificans* (and many other bacteria) in dissimilatory reduction to nitrogen gas (N_2) through several intermediates. This biologically mediated chemical process occurs through intermediate steps carried out by different enzymes and synthesis of these enzymes is dependent on various factors, including O_2 content, pH, and temperature. The rate of heterotrophic denitrification is affected by pH. The optimum pH for most strains of denitrifying bacteria appears to be between 7 and 8, although heterotrophic denitrification can occur in environments with pH as high as 11. However, intermediates may accumulate outside the optimal pH range (Lee and Rittmann 2003).

2.4 Advantages of Denitrification

As noted above, denitrification has a distinct advantage over ureolysis for MICP in that the major end products of denitrification are chemically inert and non-toxic. In contrast to ureolysis, which can produce potentially hazardous ammonia or ammonium, MICP via denitrification is free of toxic by-products, producing nitrogen gas (N_2) and possibly small amounts of unused carbon dioxide (CO_2).

Denitrification using *Pseudomonas denitrificans* (or other denitrifying microorganisms) offers several other advantages over bacterial ureolysis for MICP ground improvement. Denitrification is thermodynamically more favorable than ureolysis and, therefore, is expected to be the dominant microbial process under typical subsurface conditions. The change in standard Gibbs energy for denitrification is more than an order of magnitude greater than for ureolysis, -785 kJ/mol acetate and -27 kJ/mol acetate, respectively (DeJong et al. 2010).

Pseudomonas are highly ubiquitous in subterranean and aquatic environments (Ehrlich 2002; Fredrickson and Fletcher 2001; Fujita et al. 2000; Karatas 2008). As a facultative anaerobe, *Pseudomonas denitrificans* activity in the presence of an electron acceptor (e.g., NO_3^-) occurs readily in oxygen deficient subsurface environments, whereas activity of ureolytic bacteria is restricted under low-oxygen conditions. Assuming an appropriate native strain is present, it seems perfectly plausible that denitrification may be employed for soil improvement through MICP with minor soil amendment; perhaps only an exogenous electron donor is required.

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Denitrification can occur in a relatively broad range of low NO_3^- concentrations such as may be found in some contaminated ground water. Strains of *Pseudomonas* have been identified in aquifers with nitrate concentrations as low as 0.080mM, possibly indicating that very low concentrations of NO_3^- may be sufficient for denitrification (Ehrlich 2002; Fujita et al. 2000). However, high NO_3^- concentrations (>25mM) have been observed to inhibit denitrification in bench-scale experiments (Karatas 2008). The prospect of MICP using *Pseudomonas denitrificans* without the addition of exogenous NO_3^- (i.e., utilizing native NO_3^- concentrations) may further enhance its allure as a preferred soil improvement method.

Denitrification produces 2 moles of $CO_{2(g)}$ per mole of acetate consumed, resulting in greater production of HCO_3^- (Eqs. 6 and 7) than in ureolysis, where only 1 mole of HCO_3^- is evolved per mole of urea (Eq. 4). The benefit derived from greater production of HCO_3^- is that it promotes more precipitation of $CaCO_3$ on a per molar basis of reagent (Eq. 8).

2.5 Improving Denitrification

The introduction of chemical or biological substances into the environment is always a matter of serious concern. Denitrifying bacteria are ubiquitous in the subsurface and, as such, eliminate the need to introduce non-native microorganisms into the subsurface for denitrification processes. Preventing the unnecessary addition of certain chemical constituents necessary for denitrification is also helpful in minimizing the potential environmental impacts of denitrification. Recent efforts at Arizona State University (ASU) to develop

MICP via denitrification for soil improvement purposes have focused on minimizing the quantity of reactants in the reaction medium required for denitrification. The ASU team has conducted several experiments to assess the potential for reducing potential environmental impacts and lowering the costs associated with MICP by denitrification by reducing the total dissolved solids (TDS) in the reaction medium. For example, recently conducted batch reactor experiments that show it is possible to significantly lower the concentration of certain chemical constituents over previous work done at ASU while still inducing carbonate precipitation.

In experiments by others (van Paassen et al. 2010), one reagent was employed to perform two functions. Calcium acetate ($Ca(CH_3CO_2)_2$) was used to provide calcium ions and to serve as the electron donor. This eliminated the unnecessary addition of chloride ions (Cl^-) in the form of $CaCl_2$ to the medium, in addition to sodium acetate or acetic acid. Furthermore, if the organic donor is more oxidized (i.e., has a more positive oxidation state), the ratio of inorganic carbon released to NO_3^- reduced increases since each mole of C can donate less electrons to biomass. Therefore, the use of calcium acetate over other more reduced electron donors, such as glutamic acid, yields more inorganic carbon and less bacterial biomass per mole carbonaceous electron donor.

Current work at ASU also seeks to address the loss of free Ca^{2+} from the pore fluid. In the presence of phosphate ions (PO_4^{3-}), free calcium can produce a strong precipitate in various forms of calcium phosphate (e.g. $Ca_3(PO_4)_2$), a highly pH-sensitive process. The availability of free Ca^{2+} and PO_4^{3-} is important to the

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successful formation of calcium carbonate precipitate via denitrification. It is not clear to what extent, if any, Ca^{2+} or PO_4^{3-} is bio-available in the reaction medium once the $Ca_3(PO_4)_2$ precipitate is formed. In the event that the ionic species are bio-available in the $Ca_3(PO_4)_2$ form, another problem that may arise is the lack of mobility of $Ca_3(PO_4)_2$ within the soil medium due to its insolubility. A possible solution to this problem may be the use of short-lived biodegradable chelating agents such as SS-ethylenediaminedisuccinic acid (EDDS) to temporarily bind calcium ions.

Chelating agents are chemicals that form soluble complexes with certain metal cations, which prevent the cations from reacting with other elements to form precipitates. EDDS is a chelating agent similar to EDTA (ethylenediaminetetraacetic acid), but with far lower toxicity. In addition, EDDS has a weaker affinity for metal cations and readily biodegradable within engineering time frames with an expected half-life of 4-6 days (Tandy et al. 2006). In theory, the chelating agent should prevent the formation of $Ca_3(PO_4)_2$ precipitate by temporarily binding to Ca^{2+}. As the chelating agent degrades over a relatively short time, Ca^{2+} can remain soluble and mobile, while PO_4^{3-} continues to be bio-available.

2.6 Conclusion

Recent research has shown that MICP may be employed to improve the physical properties of granular soils for engineering applications. MICP has a variety of potentially beneficial applications in geotechnical engineering, including enhancing soil stability, improving foundation performance, mitigating soil

liquefaction and controlling groundwater. Cementation by MICP may allow us to realize the modern engineering goals of effective and sustainable engineering ground improvement in granular sediments.

The benefits of MICP through denitrification are summarized in Table 2. As denitrifying bacteria are ubiquitous in the subsurface, denitrification offers the potential for bio-stimulation of indigenous microorganisms. Furthermore, in comparison to other processes, denitrification does not produce toxic end products, may be cost effective since nearly 100% utilization of electron donor is possible, does not require the addition of potentially harmful exogenous organic materials such as urea, is thermodynamically more favorable, readily occurs in anoxic conditions typical of subsurface environments, and has a potentially greater carbonate yield per mole of electron donor than ureolysis based on reaction stoichiometry. However, the ultimate success of MICP via denitrification for ground improvement will depend on the interaction among the microbes present in the subsurface, temperature, soil characteristics and composition, pH, and specific soil chemistry.

Table 2 Advantages of Denitrification for MICP

Denitrifying bacteria are ubiquitous in the subsurface
Readily occurs in anoxic environments
Does not produce toxic end products
Is thermodynamically more favorable than ureolysis
Has a greater carbonate yield than ureolysis
Allows nearly 100% utilization of electron donor
Does not require potentially harmful exogenous organic material

Understanding carbonate rock formation may also aid in developing novel ways to make use of Earth's biological resources and possibly provide efficient and sustainable pathways for the development and improvement of current geotechnical engineering practices. Additional interdisciplinary research by microbiologists, chemists, geologists and geotechnical engineers, collaboratively, is required to realize the potential of this and other microbiological soil improvement technologies.

CHAPTER 3

SEQUESTRATION OF RADIONUCLIDES AND METAL CONTAMINANTS THROUGH MICROBIALLY-INDUCED CARBONATE PRECIPITATION

3.1 Background

As discussed in the introduction, groundwater is an important source of freshwater and is an essential component of the hydrologic cycle. A large quantity of this water is contained in sub-surface aquifers making this groundwater susceptible to contamination from a variety of sources including wells, unlined waste storage units, and surface waterways. Contamination of aquifers is a concern in many areas and, consequently, has been the focus of recent research efforts to develop novel and effective remediation methods. These methods include MICP of radionuclides and metal contaminants through *in-situ* remediation of contaminated aquifers (Mitchell and Ferris 2005; Colwell et al. 2003; Fujita et al. 2000; Smith et al. 2004).

3.2 Carbonate Precipitation for Contaminant Sequestration

Microorganisms, bacteria in particular, have long been associated with carbonate minerals and are thought to play a fundamental role in carbon cycling on the geologic timescale (Shock 2009; Vasconcelos et al. 1995). The complex interactions between microorganisms and minerals have been well-documented through the efforts of researchers attempting to understand the formation, dissolution, and alteration of minerals by microorganisms on geologic and engineering timescales (Ehrlich 2002; Drever 2008; Karatas 2008; Phoenix and

Konhauser 2008; Shock 2009). Indeed, the geologic record is replete with examples of carbonate deposits, such as the White Cliffs in Dover, England and the Bahamas Banks that are associated with microbially driven processes. Microbial carbonate precipitation is a microbially mediated mineral precipitation process that results in the formation of mineral deposits found in the natural environment, including calcium-carbonate ($CaCO_3$) minerals such as calcite. Calcite is the most thermodynamically stable polymorph of $CaCO_3$ and the primary product in many microbially induced calcium carbonates. Biologically mediated precipitation of $CaCO_3$ minerals can occur when carbonate ions (CO_3^{2-}) are formed in the presence of calcium ions (Ca^{2+}) under alkaline conditions (Eq.1).

$$Ca^{2+} + HCO_3^- + OH^- = CaCO_3(s) + H_2O \qquad (1)$$

In principle, the geochemical conditions conducive to carbonate precipitation are not unique to any specific microorganism; rather, carbonate precipitation can occur when carbonate (CO_3^{2-}) forms in the vicinity of suitable cations under alkaline conditions. MICP relies on the byproducts of bacterial metabolism (e.g., CO_2, alkalinity) to facilitate the formation of carbonate ions, which precipitate in the presence of divalent cations. Precipitation of radionuclide and contaminant metals into calcite is essentially a competitive co-precipitation reaction in which suitable divalent cations are incorporated into the calcite lattice (Eq. 2).

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$$^{90}Sr^{2+} + OH^- + HCO_3^- = SrCO_3(s) + H_2O \qquad (2)$$
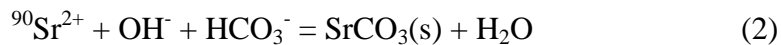
These cations and radionuclides are thought to be integrated into the calcite structure via substitution of calcium ions in the microenvironment of the mineral precipitate, forming low-strontium carbonate minerals. In theory, these minerals should similarly resist dissolution since strontium carbonate ($SrCO_3$) is at least as equally insoluble as $CaCO_3$ ($K_{sp\text{-calcite}} \approx 10^{-9}$, $K_{sp\text{-strontianite}} \approx 10^{-10}$, at STP). Incorporation of divalent ions into the calcite structure appears to slow their transport and possibly immobilize them within the calcite structure (Mitchell and Ferris 2005; Colwell et al. 2003; Fujita et al. 2004; Fujita et al. 2000; Smith et al. 2004). If proven effective, immobilization of these contaminants through MICP may provide a sustainable and cost-effective *in-situ* remediation scheme for radionuclide and metal contaminated sites.

3.3 Microbially Induced Carbonate Precipitation

3.3.1 Contaminant Sequestration via Bacterial Ureolysis

Recent work conducted by Smith et al. (2004), Fujita et al. (2004) and others on artificial groundwater designed to mimic contaminated groundwater beneath a DOE research facility provides some insight into the feasibility of MICP for radionuclide and metal contaminant sequestration. The artificial groundwater samples were formulated to simulate those found in contaminated areas of the Snake River Plain Aquifer (SRPA), a highly productive aquifer with a storage capacity of nearly 1.23×10^{11} m^3 located in south-eastern Idaho. The SRPA

underlies an area of interest near the Idaho National Engineering and Environmental Laboratory (INEEL), where radionuclides and contaminant metals have been detected in the groundwater and vadose zone. Although the researchers' area of interest focused on the SRPA, the overall context and applicability of their studies presumably apply to other similarly contaminated DOE sites.

The primary contaminant of interest in these studies was the divalent radionuclide $^{90}Sr^{2+}$. Incorporation of $^{90}Sr^{2+}$ into calcite through bacterial ureolysis was carried out using *Sporosarcina pasteurii*, a heterotrophic bacterium capable of ureolytic hydrolysis. Ureolysis, the ammonification (transformation of organic nitrogen into ammonia) of urea, results in the production of ammonium (NH_4^+), dissolved organic carbon in the form of bicarbonate (HCO_3^-), and an increase in alkalinity (shown by ^-OH in Eq. 3).

$$NH_2CONH_2 + 3H_2O = 2NH_4^+ + HCO_3^- + OH^- \qquad (3)$$
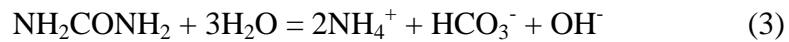
This process is associated with high rates of carbonate precipitation, but may be limited in low-oxygen environments typical of subsurface aqueous environments, since *Sporosarcina pasteurii* carries out aerobic respiration during ureolysis. Upon formation of a sufficiently saturated solution with respect to calcite, a precipitation reaction incorporates $^{90}Sr^{2+}$ into the calcite structure (Eq. 4).

$$Sr^{2+} + HCO_3^- + OH^- = SrCO_3(s) + H_2O \qquad (4)$$
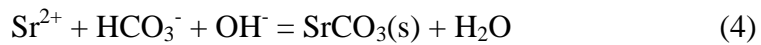
In carefully controlled bench-scale experiments, researchers successfully demonstrated the co-precipitation of Sr^{2+} into calcite through bacterial ureolysis (Mitchell and Ferris 2005; Colwell et al. 2003; Fujita et al. 2004; Fujita et al. 2000; Smith et al. 2004). Although the total amounts of Sr incorporated into calcite varied considerably, the overall trend of these experiments indicate that a predominately Ca-calcite product is formed with Sr-ions partitioned between calcite and artificial groundwater. As such, treatment of radionuclide-contaminated groundwater using MICP will likely require considerable ureolysis and precipitation of Ca-calcite to immobilize the contaminants in sufficiently large quantities.

Large-scale application of MICP through bacterial ureolysis may pose significant environmental hazards since one of the primary byproducts is ammonium (NH_4^+). Ammonium is considered a toxic substance with exceptionally high water solubility (31% w/w as NH_3 at 25°C) and, therefore, has the potential to profoundly impair groundwater quality if left unmitigated. Although the U.S. Environmental Protection Agency (EPA) has not established a maximum contaminant level (MCL) for ammonium in drinking water, the EPA has issued a lifetime exposure advisory of 30 mg/L for ammonia. Furthermore, the 1998 European Union Council Directive 98/83/EC regarding water quality for human consumption recommends a drinking water standard of 0.5 mg/L, a measure adopted by several European nations.

Several studies have suggested that ammonium ions produced by the ureolysis reaction may exchange with radionuclides and metal contaminants sorbed to subsurface minerals, and this may enhance the availability of these contaminants for co-precipitation into calcite (Colwell et al. 2003; Fujita et al. 2004; Fujita et al. 2000; Mitchell and Ferris 2005; Smith et al. 2004). Although the ion-exchange concept may be theoretically feasible, none of the studies offered either bench-scale or field-scale data supporting the idea that ammonium, a water-soluble polyatomic ion, may sorb to minerals in exchange for radionuclides and metal contaminants. Furthermore, the inherent geochemical variability of natural systems may introduce many uncertainties in the potential sorption behavior of water soluble contaminants such as ammonium. For example, it has been reported that different radionuclides exhibit varying retention (and release) times and transport behavior in similar soil or rock-deposits (Beasley et al. 1998). Often times, some simplifying assumptions are required regarding transport and soil/rock retention of contaminants, but these factors may be secondary to highly pH-dependant mechanisms governing chemical equilibria of ammonium speciation. For example, it is likely that, at the higher pH values characteristic of bacterial ureolysis, a sizable fraction of ammonium may shift to ammonia and volatilize, thereby limiting the possibility of ion exchange phenomenon for this fraction. Furthermore, the generation of ammonium will have a range of negative environmental effects that include: the potential for large nitrogenous oxygen demand, reactions with chlorine, and the potential for

eutrophication of surface waters. Thus, ureolysis introduces a new contaminant while displacing another.

3.3.2 Denitrification: An Alternative to Bacterial Ureolysis

A more environmentally acceptable bioremediation alternative to bacterial ureolysis may be bacterial denitrification, which can be carried out by many types of bacteria, including the well-studied *Pseudomonas denitrificans*. *P. denitrificans* is a gram-negative facultative anaerobe capable of inducing calcite precipitation without the production of toxic byproducts (Ehrlich 2002; Karatas et al. 2008; van Paassen et al. 2010). Recent developments in bio-soils research suggest soil cementation through denitrification is gaining ground as an alternative candidate process due to environmental concerns associated with bacterial ureolysis (DeJong et al. 2008; Karatas 2008; van Paassen et al. 2008; van Paassen et al. 2010).

As noted above, the geochemical conditions conducive to carbonate precipitation are not unique to any specific microorganism. The initial steps of calcite precipitation by bacterial denitrification involve the production of alkalinity and carbon dioxide (CO_2) when nitrate (NO_3^-) serves as an electron acceptor. The nitrate is ultimately reduced to nitrogen gas (N_2), driven by bacterial metabolism of an electron donor (e.g., acetate, CH_3COO^-). In terms of the amount of carbonate yield per mole of electron donor consumed, denitrification produces 2 moles of CO_3^{2-} per mole of acetate (and 1.6 mole NO_3^-), whereas ureolysis produces only 1 mole of CO_3^{2-} per mole of urea (Eqs. 5 & 3, respectively).

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$$-\,NO_3^- + CH_3COO^- \rightarrow -\,N_2 + 2\,CO_2 + -\,HO^- + -\,H_2O \qquad (5)$$

As illustrated in Eq. 5, denitrification has a distinct advantage over ureolysis in that the major byproduct, nitrogen gas, is completely inert and non-toxic. The remaining chemical processes in bacterial denitrification (Eqs. 6 & 7) involve the speciation of chemical constituents conducive to calcite precipitation similar to that described above for ureolytic hydrolysis under alkaline conditions in the presence of calcium ions. The rate of carbonate precipitation through denitrification is not limited by the lack of oxygen normally observed in the subsurface since the denitrifiers use nitrate as their electron acceptor instead of oxygen.

$$CO_2 + H_2O = HCO_3^- + H^+ \qquad (6)$$

$$Ca^{2+} + HCO_3^- + OH^- = CaCO_3(s) + H_2O \qquad (7)$$
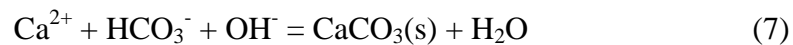
The highly ubiquitous bacteria of genera *Pseudomonas* have been identified in the SRPA (Fujita et al. 2000; Ehrlich 2002). Assuming an appropriate native strain, it seems perfectly plausible that denitrification may be employed for the effective treatment of aquifer contaminants through MICP with minor solution amendment; perhaps only an exogenous electron donor is required if nitrate already is present. Denitrification can occur in a broad range of low NO_3^-

concentrations. For example, Lee and Rittmann (2003) conducted autotrophic denitrification experiments using a hollow-fiber, membrane-biofilm reactor (MBfR) in which denitrification readily occurred at input NO_3^- concentrations as low as 12.5 mg/L (0.201 mM), while concentrations in the MBfR were as low as 0.1 mg-N/L. It should also be noted that high nitrate concentrations (>25mM) have been observed to inhibit denitrification in bench-scale experiments (Karatas 2008).

Based on the reported composition of artificial groundwater designed to mimic the contaminated SRPA, existing nitrate concentrations (~0.080 mM NO_3^-) may be minimally sufficient for denitrification. MICP without the addition of exogenous NO_3^- would be desirable, as it avoids costs and the constraints associated with a regulated water pollutant.

As previously noted, autotrophic denitrification also may be achieved by using an MBfR. In the presence of suitable divalent cations and sufficiently high levels of aqueous CO_2, an MBfR promotes calcite precipitation by producing the necessary alkalinity and raising the pH (Lee and Rittman 2003; Tang et al. 2010). Furthermore the MBfR has the potential for almost 100% utilization of the electron donor, hydrogen gas (Lee and Rittmann 2003).

3.4 Conclusion and Suggestions for Future Work

Co-precipitation of strontium along with calcium in calcite appears to be a viable option for *in-situ* remediation of radionuclide-contaminated aquifers. Co-precipitation via denitrification has the potential to be a preferred method for *in-situ* remediation of radionuclide and metal contaminants since: (1) denitrification

is cost-effective, particularly when nearly 100% utilization of electron donor is possible; (2) denitrification does not produce toxic byproducts, such as ammonium from urea; (3) denitrification has a greater CO_3^{2-} yield per mole of electron donor than ureolysis; (4) denitrifiers readily grow and actively function under anoxic conditions typical of subsurface environments; (5) denitrification does not require the addition of potentially toxic organics such as urea; and (6) denitrification does not require the addition of a water-soluble, organic electron donor to a freshwater aquifer if hydrogen gas is used.

Exploratory work could be undertaken to determine/confirm the feasibility of denitrification for *in-situ* co-precipitation of radionuclides and metal contaminants in artificial SRPA groundwater. If preliminary results prove promising, further efforts could involve direct comparisons between hydrolytic ureolysis and denitrification for contaminant sequestration via MICP, where parameters of interest may include relative rates of co-precipitation, cost per unit(s) of contaminant removed and net environmental impact of proposed remediation.

CHAPTER 4

MICROBIALLY INDUCED PRECIPITATION OF CALCIUM

CARBONATE USING *PSEUDOMONAS DENITRIFICANS*

4.1 Background

Bacterial denitrification has potential applications in soil improvement and groundwater remediation. One of the first steps in assessing this potential is to understand the geochemical changes induced by the microbial process of denitrification. This chapter focuses on proof-of-concept experiments and initial stage results in very simple bench-scale denitrification systems. This chapter also introduces the geotechnical aspects of soil improvement via cementation in the greater context of MICP, such as the effects of cementation on the strength and stiffness of granular soils. The chapter concludes with preliminary results from laboratory experiments focusing on denitrification as a candidate calcium carbonate precipitation mechanism.

A portion of the work in this chapter includes methods and techniques used in early experiments conducted by a previous graduate student at Arizona State University, Dr. Ismail Karatas. In addition, although updated with a few recent developments in the emerging field of bio-geotechnical engineering, much of the background information presented in here is attributable to Dr. Karatas since very little changed since he completed his dissertation; furthermore, this section is also a technical journal paper on which he is a co-author. Some of the test results presented here are very similar to those obtained by Dr. Karatas in his

dissertation (Karatas 2008) where similar proof-of-concept experiments were conducted. The experimental repeatability shown by these tests is reassuring.

4.2 Soil Improvement via Microbially Induced Mineral Precipitation

Microbially induced precipitation is recognized as a source of a wide variety of minerals in soils, including carbonates, oxides, phosphates, sulfides, and silicates (Fortin et al., 1997). Carbonate precipitation is perhaps the most widely studied of this phenomenon. Many metabolic mechanisms can induce carbonate precipitation by increasing either the total carbonate content, the pH, or both. For instance, anaerobic and aerobic oxidation of an organic compound results in production of carbon dioxide (CO_2) for the carbon fraction that is not incorporated into biomass. If the medium is a well-buffered neutral or alkaline environment, CO_2 speciation forms carbonate (CO_3^{2-}) and then precipitates carbonate minerals out of solution if there is an adequate amount of suitable cations, such as Ca^{2+}. The precipitation is enhanced if the pH increases due to microbial production of alkalinity, which can occur in several ways. For instance, organic nitrogen released from organic compounds in the form of ammonia (NH_3) can enhance precipitation. This mechanism includes the release of organic nitrogen in urea, which releases NH_3 by ureolysis (i.e., hydrolysis of urea). Protonation of NH_3 generates OH^- and leads to an increase in pH: $H_2O + NH_3 \rightarrow NH_4^+ + OH^-$ (Bavendamm 1932; Fortin et al. 1997; Stocks-Fischer et al. 1999; Fujita et al. 2000; Ehrlich 2002; Hammes et al. 2003; Whiffin 2004; Whiffin et al. 2007).

A basic or alkaline environment is essential to the formation of geochemical conditions necessary for carbonate mineral precipitation. In some

cases, certain metabolic pathways can produce conditions favorable for mineral precipitation. Under anaerobic conditions, nitrate (NO_3^-) can be used as an electron acceptor by many bacteria (i.e., denitrification), producing N_2 gas (g), CO_2, and alkalinity: $NO_3^- + 1.25CH_2O \rightarrow 0.5N_2 + 1.25CO_2 + 0.75H_2O + OH^-$. Sulfate ($SO_4^{2-}$) also can be used as an electron acceptor by microorganisms (i.e., sulfate reduction). In sulfate reduction, sulfate-reducing bacteria such as *Desulfovibrio spp. and Desulfotomaculum spp.* oxidize organic compounds while reducing sulfate to produce H_2S, CO_2, and alkalinity: $SO_4^{2-} + 2CH_2O \rightarrow H_2S + 2CO_2 + 2OH^-$ (Abd-el-Malik and Rizk 1963a; Abd-el-Malik and Rizk 1963b). Another precipitation mechanism involves methane formation from acetic acid, which removes the acidity of acetic acid and adds CO_2: $CH_3COOH \rightarrow CH_4 + CO_2$ (Brune et al. 1991; Fleming et al. 1999; Cooke et al. 2001; Rowe et al. 2002).

In addition to these mechanisms, Ehrlich (2002) lists "removal of CO_2 from bicarbonate containing solutions" as one of the microbial mechanisms that may lead to calcium carbonate precipitation. The most well-known process that may result in carbonate precipitation through this mechanism is oxygenic photosynthesis. In principle, all autotrophs (organisms that consume CO_2 as source of carbon, e.g., sulfide oxidizing bacteria, nitrifying bacteria) may precipitate carbonate unless they generate acids. Moore (1983) reported that cyanobacteria and algae deposited calcareous nodules and crusts on subaqueous levees in the Flathead Lake delta in Montana, USA. However, anoxygenic and oxygenic photosynthesis depend on light as the source of energy, which limits the depth at which these microorganisms live. Because sunlight can only penetrate up

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to couple of millimeters below ground surface, oxygenic photosynthesis is limited to the soil crust. A team of researchers discovered obligate photosynthetic green sulfur bacteria which live off the dim light coming from hydrothermal vents at a depth of nearly 2,400 m in the ocean (Beatty et al. 2005).

In summary, there are a variety of mineral precipitation mechanisms that can change the mechanical properties of soil. For instance, carbonate precipitation can result in cementation within soil with a potential increase in shear strength and stiffness. Furthermore, based upon geologic evidence, microbially induced carbonate precipitation is expected to be long lasting, suggesting that soil improvement due to this mechanism may be assumed to be permanent (i.e. irreversible or very slowly reversible) for engineering purposes. Ideally, based on the site characteristics, the optimal microbial mineral precipitation mechanism would be identified through a screening process and then applied in the field.

4.3 Microbial Precipitation of Calcium Carbonate via Hydrolytic Ureolysis

Work carried out by Whiffin (2004) studied the effects of microbial precipitation of calcium carbonate through hydrolysis of urea (ureolysis) on the physical properties of sands. An aerated solution of urea, calcium and ureolytic bacteria was injected into sand specimens to induce calcium carbonate precipitation (Figure 1). The change in physical properties due to microbial calcium carbonate precipitation was initially evaluated using compressional wave (P-wave) velocity measurements that were assumed to be correlated with uniaxial compressive strength. The P-wave measurements indicated an increase in cementation and shear strength with increasing concentration of hydrolyzed urea (Whiffin 2004).

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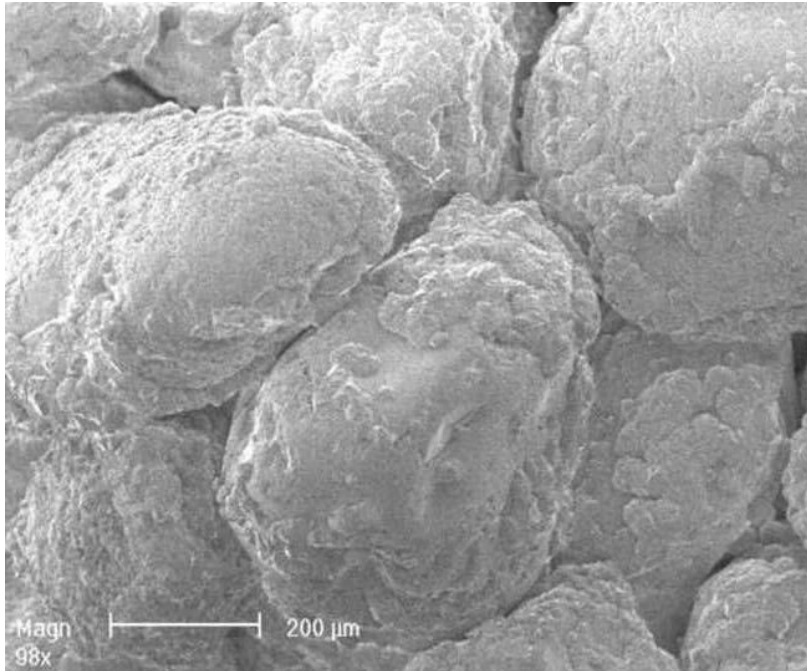
Figure 1– Scanning electron micrograph of <600μm silica sand with $CaCO_3$ coating on surface and at inter-particle contacts (Whiffin 2004).

Whiffin (2004) subsequently performed triaxial shear strength tests on Dutch Koolschhijn sand injected with urea, calcium, and urea-hydrolyzing (ureolytic) bacteria and reported that shear strength increased by a factor of 8 and stiffness (secant modulus at 50 percent of peak shear stress) increased by a factor of 3, without a significant change in pore volume. However, several important details of the triaxial tests, such as the confining pressures and drainage conditions, were not reported.

Research work initially carried out by Whiffin (2004) at Murdoch University in Western Australia led to development of the GeoDelft biogrout testing program at the Delft University of Technology (TU Delft) and the

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independent GeoDelft Institute (GeoDelft) in the Netherlands. The objective of the Delft programs is to study the improvement of soil properties using various microbiological processes. In 2003, the SmartSoils® program was created by GeoDelft to study further study and develop ground improvement technologies for commercial applications. Two of several processes that are concurrently being studied and applied commercially are Biogrouting and Bio-Sealing. Both the Biogrout and Bio-Seal processes are in the advanced stages of study and, as such, have been developed into patented commercial processes. Biogrouting is an in-situ cementation process controlled by ureolytic microorganisms that can form carbonate mineral precipitates at inter-particle contacts resulting in cemented granular soils. Bio-Sealing is a sealing process that relies primarily on growth of microbial biomass and other associated products that alter native geochemical conditions in order seek out and seal leaks in water retaining soil/fractured rock layers.

GeoDelft's Bio-Sealing process involves a consortium of microorganisms that form a biofilm and an insoluble iron sulfide (FeS) precipitate that accumulate around a leak. Bio-Sealing technology was field tested 2004 and was used on a major infrastructure project in 2005, construction of the Aquaduct Ringvaart Haarlemmermeer, a part of a high-speed rail link (GeoDelft 2012). Biosealing has been used in at least two other major infrastructure projects: (1) to reduce seepage through a hydropower dam in Greifenstein, Austria and (2) to reduce salt water seepage into groundwater near the town of Haarlemmermeer, Netherlands, in 2008 and 2009 respectively (GeoDelft 2012).

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DeJong et al. (2006) evaluated the effects of calcium carbonate precipitation induced microbially through ureolysis on the shearing properties of loose sands. Shear wave velocity measurements were employed by DeJong et al. (2006) to monitor the development of cementation during microbial treatment (over a period of approximately 24 hours). Consolidated undrained triaxial shear tests were performed at the end of the treatment period. DeJong et al. (2006) observed an increase in shear wave velocity from approximately 200 m/s to 540 m/s due to microbial treatment of sands and reported that the microbially cemented soils displayed a similar shearing response to gypsum cemented soils under undrained conditions (Fig. 2). DeJong et al. (2006) concluded that pH, oxygen supply, calcium concentration, and the metabolic status and concentration of microorganisms are all important factors for the success of this application. It should be noted that although oxygen supply is important for ureolytic bacteria, it may not necessarily be a limiting factor once a sufficiently large microbial population is established.
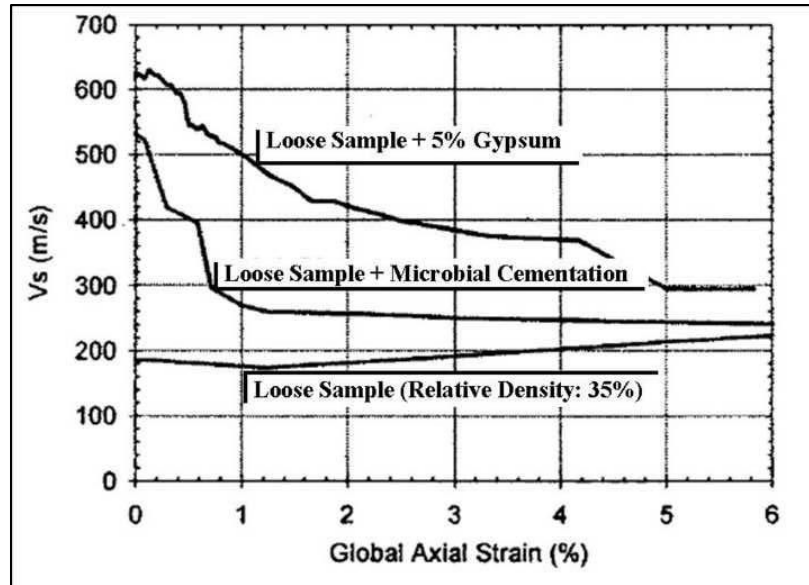
Figure 2– Shear wave velocity measured during monotonic triaxial tests (DeJong et al. 2006)

Whiffin et al. (2007) performed a series of drained triaxial tests on samples (25 cm high, 6.6 cm dia) from a sand column cemented with calcium carbonate precipitated through ureolysis under a confining pressure of 50 kPa. These investigators observed improvement in the peak compressive strength for samples with calcium carbonate concentrations greater than 60 kg/m^3 (Fig. 3). Based on the dry unit weight reported for the sand used in these experiments (1.65 g/cm^3), this "threshold" calcium carbonate content of 60 kg/m^3 corresponds to 3.6 percent cement by weight. However, contrary to this threshold for biologically mediated precipitation, increases in cohesion and friction angle with as low as 0.5 percent cement by weight have been for Portland cement mixed ex-situ with sand (Rad 1983). Whiffin et al. (2007) reported that the drained peak compressive strength of the sand samples increased from 167 kPa to 570 kPa for a calcium

carbonate content of approximately 110 kg/m^3 (6.7 percent cement by weight). Whiffin et al. (2007) also reported that microbially induced calcium carbonate cementation did not seem to affect either the residual compressive shear strength or the hydraulic conductivity of their samples.
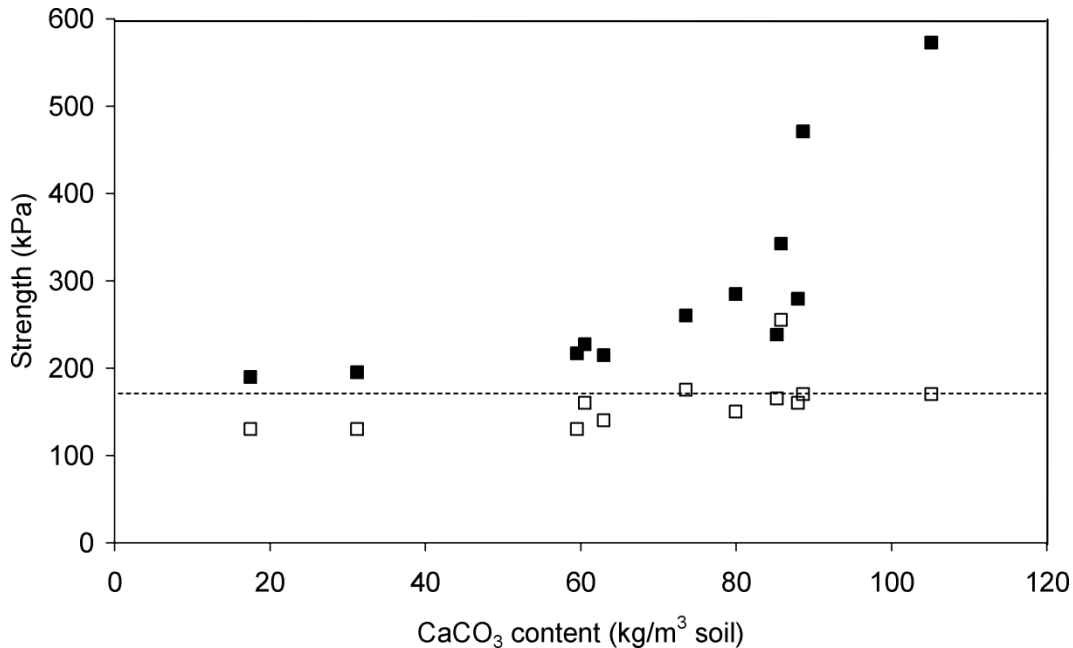
Figure 3– Change in Peak and Residual Compressive Strength of Sand
(d_{50}=165μm) with CaCO$_3$ (Whiffin et al. 2007)

4.4 Denitrification and Calcium Carbonate Precipitation

The chemical reactions involved in bacterial ureolysis produce undesirable and potentially toxic end products: ammonia ($NH_{3(g)}$) and ammonium (NH_4^+). Microbial denitrification is an alternative mechanism to ureolysis for carbonate precipitation without toxic or undesirable end-products. The terms denitrification and nitrate reduction have sometimes been used interchangeably in the literature. In this thesis, the term "denitrification" refers to dissimilatory reduction of nitrate

(NO_3-) to nitrogen gas (N_2) through intermediates, including nitrite (NO_2^-), nitric

oxide (NO) and nitrous oxide (N_2O). Although some microorganisms perform

dissimilatory nitrate ammonification (reduction of NO_3^- to NH_3) or one-step

nitrate reduction (reduction of NO_3^- to NO_2^-), these processes are not included

under the term denitrification in this work.

Each one of the intermediate steps in denitrification is carried out by a

different enzyme, and synthesis of these enzymes depends on various factors,

including O_2 content, pH, and temperature. The rate of heterotrophic

denitrification is affected by pH, and the optimum pH for most of the strains

appears to be between 7 and 8 (Lee and Rittmann 2003). Lee and Rittmann (2003)

reported that heterotrophic denitrification can take place at environments with pH

as high as 11, but intermediates may accumulate outside the optimal pH range. In

addition, accumulation of intermediates at low pH has been shown to severely

inhibit denitrification (Abeling & Seyfried 1992; Kornaros et al. 1996; Glass et al.

1997; Glass & Silverstein 1998). Accumulation of intermediates is an important

factor in application of denitrification for carbonate precipitation because only the

step that reduces NO_2^- to NO generates alkalinity.

Denitrification of organic compounds results in production of CO_2 and

OH^-. The net change in the pH and the alkalinity of the denitrifying environment

depends on a number of factors, including relative production of OH^- ions

compared to the production of CO_2 (Drtil et al. 1995) and precipitation of

carbonate complexes. Drtil et al. (1995) performed a series of experiments using

different electron donors (glycerine, glucose, acetate and ethanol) to investigate the decrease in pH observed in some denitrification experiments.

Stoichiometric analysis of denitrification of the above mentioned electron donors and the results from laboratory experiments presented by Drtil et al. (1995) indicate that the net change in pH and alkalinity during denitrification is influenced not only by dissimilative reactions but also by assimilative reactions. If the organic donor is more reduced (more negative oxidation state), then the ratio of CO_2 released to NO_3^- reduced goes down because each mole of C can donate more electrons, and the alkalinity goes up in higher proportion causing a stronger pH impact. Thus, Drtil et al. (1995) concluded that the oxidation state of carbon in the electron donor can be used to assess whether or not the pH increases as a result of the growth of denitrifying microorganism.

Other factors that influence pH and alkalinity during denitrification are the biomass yield and nitrogen in the organic donor. An organic donor that gives a higher yield puts more of its electrons and C into biomass, instead of into reducing nitrate. Therefore, normalized to the amount of organic donor consumed, a higher-yield system should show a smaller pH impact. On the other hand, if the oxidized organic compound contains nitrogen, the released NH_3 is a base that increases the alkalinity and pH.

In addition to the oxidation state of carbon and the nitrogen content of the electron donor, precipitation of carbonate complexes affects the acid-base chemistry of the denitrifying environment by consuming alkalinity and causing pH to decrease. Thus, the microbially induced precipitation of calcium carbonate

through denitrification is a complex phenomenon due to the various factors affecting microbial growth and precipitation-dissolution reactions, including, but not limited to, electron donor composition, relative amount of electron donor to acceptor, accumulation of intermediates and nutrient limitations (N, P, K, Na, Mg, Ca, Fe, etc.). In any case, an increase in pH and alkalinity due to denitrification results in increased carbonate (CO_3^-) content and, in the presence of suitable cations and nucleation sites, carbonate mineral precipitation may occur if the solubility product is reached.

Researchers have reported precipitation as a result of denitrification in the laboratory and in the field. MacCallum and Guhathakurta (1970) attribute the origins of carbonate sediments on the Bahama Bank to denitrification, though it is not clear whether the sediments resulted from chemically or microbially induced precipitation. Lee and Rittmann (2003) observed precipitation of calcium carbonate as a result of autohydrogenotrophic denitrification within hollow fiber membrane biofilm reactors. Calcium carbonate precipitation was also reported by Castanier et al. (2000) for denitrification experiments with *Bacillus cereus*. These laboratory findings as well as the bioclogging and mineral precipitation problems reported for in-situ permeable reactive barriers that employ denitrification (Moon et al. 2008) suggest that denitrification is a promising mechanism for calcium carbonate precipitation for soil improvement purposes in anaerobic conditions below the groundwater table.

4.5 Denitrification Experiments at Arizona State University

Bench-scale experiments conducted at Arizona State University explored the potential for microbially induced calcium carbonate precipitation through denitrification for improvement of engineering properties of granular soils. These laboratory experiments were conducted in two stages. The first stage experiments are discussed in this chapter and consist of bench-scale, closed system batch reactors inoculated with a pure culture of a bacterium capable of denitrification. These first stage experiments serve as a proof-of-concept and were used to investigate the effect of various factors on the production of alkalinity and change in pH in the reactor, including electron donor type, accumulation of intermediates, nutrient limitations, concentration of salts and initial pH. The first stage experiments also included batch reactors with calcium added to the bacteria-growth medium to investigate the effect of calcium carbonate precipitation on the geochemistry of the medium and the growth of bacteria.

The results from the first stage experiments were used to develop a better understanding of the denitrification-precipitation system to optimize the contents of the growth medium for the second stage experiments. The second stage experiments, discussed in the next chapter, detail the techniques used to precipitate calcium carbonate in a bench scale soil column apparatus.

4.6 First Stage Experiments – Methods

The first stage denitrification-precipitation experiments were performed with a strain of *Pseudomonas denitrificans*, American Type Culture Collection (ATCC) designation 13867. *P. denitrificans* is one of the most widely studied

microorganisms in denitrification experiments (Radcliffe 1969; Koike and Hattori 1975a; Koike and Hattori 1975b; Wang et al. 1995; Kornaros et al. 1996) and is a Gram negative, facultative anaerobe. Kornaros et al. (1996) reported that *P. denitrificans* (ATCC 13867) lacks the capability of fermentation, which eliminates one of the mechanisms that may complicate interpretation of the results. Studies on the effect of pH on the nitrate and nitrite reduction by *P. denitrificans* (ATCC 13867) have indicated that the optimal pH range is approximately 7.4 to 7.6 and 7.2 to 7.3, respectively, at $30^{\circ}C$ (Wang et al. 1995). However, other studies indicate that at high concentrations of nitrate and nitrite (approximately 100 mM), the optimal pH for the reduction of nitrate and nitrite to N_2 is approximately 7.5-9.0 (Constantin & Fick 1997; Glass & Silverstein 1998).

A freeze-dried pellet received from the ATCC was revived by aerobically growing it in a sterilized nutrient broth (Difco brand) medium and incubating it overnight at $30^{\circ}C$. Samples of 10 mL of this nutrient broth-bacteria solution were frozen at $-85^{\circ}C$ with 5 percent glycerol to store the bacteria for subsequent use in batch reactor experiments. For each batch reactor or acrylic column test (described below), one of the frozen samples was grown anoxically in a 150-mL glass serum bottle containing 2.5 g/L (29 mM) of $NaNO_3$ and 10 g/L of nutrient broth to reduce the lag time (i.e., time necessary for a bacteria to synthesize and activate enzymes necessary when switching between different electron donors or acceptors) and to minimize the possibility of cross-contamination.

The denitrification-carbonate precipitation experiments were performed in two systems: (1) 2.0L glass bottle batch reactors and (2) clear acrylic columns

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filled with sand and tested under static (non-flow) conditions. The 2.0-L glass bottles were capped with a rubber stopper and placed in between two plywood sheets connected through the corners by threaded steel rods. The steel rods were screwed tightly to prevent the displacement of the rubber stopper upon generation of any positive gas pressure inside the glass bottle. The 2.0 L glass bottles had a cylindrical sampling port at the bottom, which was capped with a rubber stopper and an aluminum cap. For each glass bottle experiment, approximately 1.5 L of liquid medium was used. A reactor size of 2.0 L was chosen to minimize the effect of sample size on the results, as observed in initial trials with 150 mL glass serum bottle reactors.

The rigid acrylic columns were approximately 7-inches in length with a 3 inch (nominal) inside diameter and were capped at both ends with specially designed solid acrylic blocks (Fig. 4). The square acrylic blocks were approximately 6 inches in length and 2 inches thick and had o-ring seals to prevent fluid and gas escape/intrusion. The square blocks were connected by threaded steel rods through the corners. Wing nuts on the steel rods were screwed down tightly to prevent the displacement of the acrylic tube and block assembly upon generation of any positive gas pressure inside the unit. Sampling ports located on both the top and bottom blocks, protected by an internal mesh layer to prevent sand escape, were used extract fluid and gas samples.
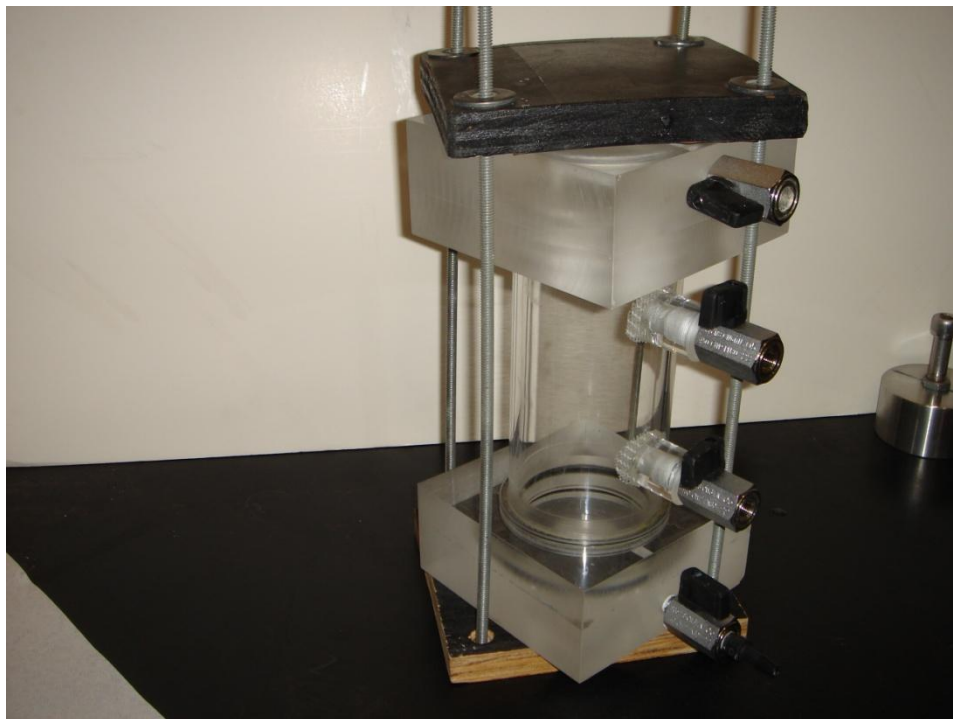
Figure 4-Acrylic columns approximately 7"x 3" inside diameter with sampling ports, capped at both ends via o-ring sealed solid acrylic blocks connected by threaded steel rods through the corners.

The components of the liquid medium for each experiment (i.e., electron donor, electron acceptor, and inorganic salts) were autoclaved separately to prevent any chemical reaction during sterilization. Next, in a biological safety cabinet, the liquid medium was either (1) poured into sterilized 2.0-L glass bottles for immediate use or (2) 500-mL bottles for later use in the acrylic sand-filled column assemblies. After the pH of the initial medium was modified to be approximately 7.0 using 1 M NaOH or 1 M HCl, the rubber stopper was placed on the bottle and the top and bottom plywood sheets were screwed on tightly.

The 2.0-L reactors were filled liquid medium then removed from the biological safety cabinet and purged with filtered $N_2(g)$ for 1 hr using sterile

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needles through the sampling port at the bottom to minimize the $O_2(g)$ within the reactor. At the end of each N_2 purging episode, the gas in the headspace of the 2.0-L glass reactors was analyzed using a gas chromatography device (Shimadzu GC-2010 with Thermal Conductivity Detector) to verify that O_2 levels within the reactor were reduced to minimum levels.

After confirming that the $O_2(g)$ levels within the glass reactor were minimal, a 5-mL inoculum of anoxically grown *P. denitrificans* (ATCC 13867) was injected into each reactor. The inoculated 2.0-L glass batch reactors were then placed on a shaker at 120 rpm in a dark room at constant temperature, and the acrylic sand column reactors were covered with aluminum foil and placed on a warming plate. Unless otherwise noted, the temperature was kept at $30^{o}C$ throughout each denitrification-precipitation experiment. Approximately 10-mL samples were collected daily through the sampling port for the 2.0-L glass bottles using sterilized needles and syringes.

The acrylic components of the sand columns were alcohol sterilized (70%v/v ethanol), while the sand and other heat tolerant components were autoclaved. Ottawa 20-30 sand (US Silica Company), a uniform quartz sand with a mean grain size of 0.6 mm, was poured into the columns in four lifts with gentle tapping between lifts (approximately 10 taps via flathead screwdriver) along the circumference of the acrylic tube. Soil weights were not recorded during these initial experiments; therefore, soil specimen index properties (e.g. density, void space, and moisture content) could not be determined.

The acrylic sand column reactors were filled with approximately 290 mL of liquid medium on an open bench following aseptic techniques. The acrylic sand columns were closed and purged with $N_2(g)$ for 1-hr without testing. Upon completion of $N_2(g)$ purging, a 5-mL inoculum of anoxically grown *P. denitrificans* (ATCC 13867) was injected into each acrylic reactor through the bottom port. The columns were then wrapped with aluminum foil and placed on a warming plate.

Only two samples were collected from the acrylic columns over the 7-day experiment period. The first sample was collected at the start of experiment and the second sample at the end of the experiment. Samples were collected from the bottom port of each column. These samples were first analyzed for pH and then filtered through a 0.2-µm filter to remove all the biomass from the sample. Although the pH of the sample after exposure to the atmosphere may not be equal to the pH of the medium within the closed batch reactor due to the difference in the partial pressure of $CO_2(g)$ and $NH_3(g)$, if present, the measured pH is an indicator of the relative changes in the chemistry of the liquid medium.

The filtered samples were used in the analysis of alkalinity, calcium hardness, NO_3^-, and NO_2^- concentration. The alkalinity measurements were performed using a digital titrator and Method 8203 proposed by HACH® (2006). The calcium hardness was evaluated by titration of diluted samples using 0.01 M EDTA and calmagite indicator. The NO_3 and NO_2 concentrations were analyzed using a HACH® DR/4000 Spectrophotometer. The chromotropic acid method (Method 10020) and the ferrous sulfate method (Method 8153) proposed by

HACH® (2003) were followed for evaluation of nitrate and nitrite concentrations, respectively. Based on the evaluated nitrite concentrations, if necessary, the samples were treated with urea before assessment of nitrate concentrations to prevent interference of nitrite as suggested by HACH® (2003). Gas chromatography analyses were periodically performed on samples from the headspace during each experiment to monitor the $CO_2(g)$ and $O_2(g)$ levels within the reactor.

Control reactors for the 2.0-L glass bottles and acrylic sand columns also were prepared with the liquid medium used for each experiment (no bacteria added) and kept under the same conditions as the corresponding denitrification experiment. Samples from the control reactors were periodically assayed for pH, alkalinity, calcium hardness, NO_3^-, and NO_2^- concentration, and the reactors were periodically monitored for $CO_2(g)$ and $O_2(g)$ levels. One important distinction between the 2.0 L glass bottle experiments and acrylic column experiments was the difference in total volume of liquid medium added: the 2.0 L glass bottles contained 1.5 L of liquid medium while the acrylic sand-columns contained approximately 290 mL.

4.7 Results

The first experiments, conducted using 2.0-L glass bottles, were performed using a medium consisting of 20 g/L nutrient broth (Difco brand), 1.50 g/L (17.6mM) of $NaNO_3$, 0.27 g/L KH_2PO_4 (2mM), and 0.24 g/L $MgSO_4 \bullet 7H_2O$ (1mM) to evaluate the effects of denitrification by *P. denitrificans* (ATCC 13867) on the geochemistry of the medium over a 7-day period. The nitrate and nitrite

concentrations were closely monitored over the 7 days. In order to minimize potential effects of sample volume disturbance, approximately 35 ml of nitrate only with a similar concentration to the initial amount (17mM) was periodically injected into the reactor after sampling (days 3 and 5 of the experiment). The results from the assays of the samples presented in Figure 5 indicate that the pH increased from 7.0 to 8.2 over approximately 7 days through the reduction of approximately 29 mM of NO_3^-. Total alkalinity also increased during this experiment (from 520 mg/L to 3800 mg/L as $CaCO_3$). However, a decrease in the rate of increase in pH and alkalinity was also observed. The decreasing rate may be due to a decrease in the rate of enzyme activities or to accumulation of organic complexes or other intermediates in the medium. The increase in pH and alkalinity observed in this experiment promote precipitation of carbonate minerals when the amount of CO_3^{2-} added to the medium and the concentration of Ca^{2+} exceed the solubility product of calcium carbonate. The assays on samples from the control reactors indicated that the pH, alkalinity, and NO_3 concentrations stayed the same for the duration of the experiment.
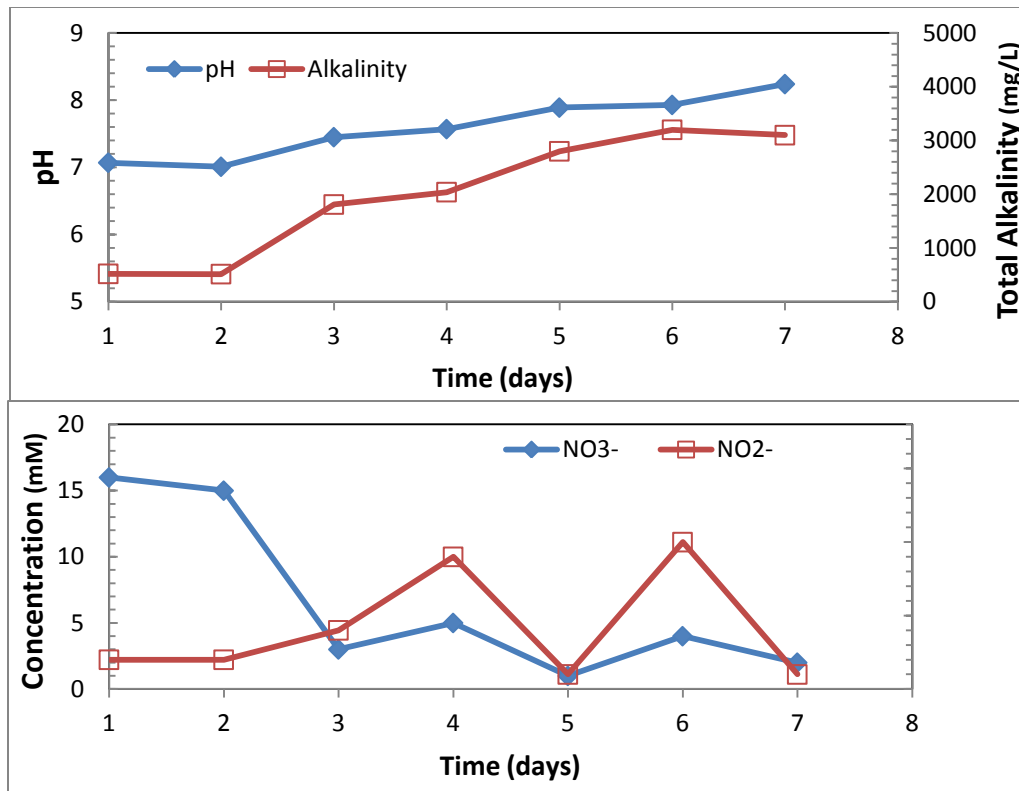
Figure 5– Denitrification experiments with *Pseudomonas denitrificans* (ATCC 13867) in 2.0-L glass bottles under anoxic conditions.

The second set of experiments, performed in the sand-filled acrylic columns described above, contained calcium chloride in a solution that was otherwise identical to the liquid medium used in the 2.0 L glass bottles. The addition of calcium to the liquid medium in the form of 2.9 g/L (20 mM) CaCl$_2$•2H$_2$O resulted in a loose, white precipitate before the start of the experiment that visually increased in quantity during pH increase (i.e. neutralization to pH≈7.0). It was later determined through precipitation experiments that the white precipitate was a form of calcium phosphate (e.g., Ca$_3$(PO$_4$)$_2$) that formed with increasing pH, starting at around pH = 5.0, and was easily reversed upon acidification. The experiment was allowed to run for

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approximately 7 days. The pH, nitrate, and nitrite concentrations were determined only at the start of the experiment and at the end. The reasons for why only initial and final concentrations were determined in this experiment were: (1) to minimize potential effects of sample volume disturbance, an effect that could have acute consequences on the comparatively small starting volume (290 mL vs. 1500 mL); (2) to allow for more complete chemical diffusion with the column as it was determined from previous practice experiments that spatial variations developed in the geochemistry of fluid drawn from the top port vs. the bottom port of the sand column shortly after the start of the experiment; and (3) to prevent the potential geochemical impact that fresh liquid medium may have at the point of injection in a static, soil-filled system. Since sampling only at the beginning and the end of the experiment resulted in minimal withdrawal of fluid, no additional medium was added after the start of the column experiments.

The results from the samples collected through the bottom port, presented in Table 2, indicate that the pH decreased from 7.0 at the beginning of the experiment to 6.5 over the 7 day duration of the experiment. Nitrate and nitrite concentrations at the start of the experiment were approximately 16 mM NO_3^- and 1 mM NO_2^- as determined from the samples taken at the beginning of the experiment, which closely matched the calculated starting liquid medium concentrations of 17.6 mM (1.50 g/L) NO_3^- and 0 mM NO_2^- (NO_2^- was never added at any point during these experiments). Nitrate concentration at the end of the experiment was <1 mM and nitrite was approximately 3mM. Assuming that only 14.6 mM of the total available nitrate (17.6 mM) in the 290 mL of medium

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was completely reduced to nitrogen gas, the total amount of NO_3^- reduced in this experiment was 4.23 mmol (0.290L • 0.0146 moles/L = 4.23 mmol). Alkalinity was not monitored during this experiment.

Sampling	pH	Nitrate (mM)	Nitrite (mM)
Initial	7.1	16.3	0.92
Final	6.5	0.71	2.86

Table 3- Results from sand-filled acrylic columns containing $CaCl_2 \cdot 2H_2O$ in an otherwise identical medium to that used in the 2.0 L glass bottles.

Upon termination of the experiment, several small chunks of weakly cemented sand grains were observed (no images available). A carbonate mineral precipitate (presumably $CaCO_3$) formed through the denitrification of NO_3^-, and appeared to be the agent holding the dried sand grains together. The mineral precipitate formed during these experiments was qualitatively identified as a carbonate mineral via acid digestion tests. No effort was made to quantify the mineral precipitate, nor was any analysis conducted to definitively identify the mineral phase present (e.g. x-ray diffraction).

Others electron donors reported to have been used in denitrification experiments with *P. denitrificans* include acetate, L-glutamic acid, methanol, aspartate, alanine, formate and peptone-yeast extract (Koike and Hattori 1975a; Koike and Hattori 1975b; Nishimura et al. 1980; Wang et al. 1995; Kornaros et al. 1996; van Paassen et al. 2010). Preliminary acrylic sand column experiments with the same liquid medium used in acrylic sand columns described above containing 75 mM L-glutamic acid as an electron donor, rather than nutrient broth, resulted

in the small pieces of loosely cemented sand grains, shown in Figure 6, presumably cemented via precipitation of a carbonate mineral (presumably $CaCO_3$) through denitrification of NO_3^-. As described above, the mineral precipitate formed during these experiments was qualitatively identified as a carbonate mineral via acid digestion tests, but no further effort was made to quantify the mineral precipitate or identify the mineral phase present.

Figure 6-Small pieces of loosely cemented sand grains from static sand column experiments containing $CaCl_2$ with L-glutamic acid as an electron donor.

4.8 Summary

Cementation of granular sediments in nature by microbially induced mineral precipitation (in particular, precipitation of calcium carbonate), along with observations of certain "adverse" effects associated with microbially induced mineral precipitation at engineered facilities (e.g., clogging of water treatment plant filters), suggest that in the proper context microorganisms can be used to improve the mechanical properties of soils for engineering purposes. Microbially induced mineral precipitation has a variety of potentially beneficial engineering

applications in geotechnical engineering, including enhancing soil stability, improving foundation performance, mitigation of soil liquefaction, and control of groundwater.

The applicability of microbiological processes to soil improvement will likely depend on a variety of factors, including the type of microbial metabolism desired, interactions with other microbes present in the environment, soil type, available nutrients, depth below ground surface, pH, temperature, pressure, concentration of ions, and the availability of oxygen and other oxidants. Current research at Arizona State University includes performing bench-scale experiments to establish candidate mechanisms for microbially induced calcium carbonate precipitation and ultimately conducting field tests for mechanisms that look promising based upon the bench scale experiments. Initial experiments focusing on denitrification as a candidate mechanism resulted in change in geochemistry of the medium favorable to calcium carbonate precipitation through an increase in pH and alkalinity of the environment. Subsequent trials resulted in precipitation of calcium carbonate when calcium cations were present in the medium, suggesting that this mechanism may be appropriate for field application. Additional interdisciplinary research by microbiologists, chemists, geologists, and geotechnical engineers, collaboratively, is required to realize the potential of this and other microbiological soil improvement technologies.

CHAPTER 5

MICROBIALLY INDUCED PRECIPITATION OF CALCIUM

CARBONATE USING *PSEUDOMONAS DENITRIFICANS* IN STATIC

AND FLOW-THROUGH SAND COLUMNS

5.1 Introduction

This chapter describes design and construction of flow-through and static sand columns for bench-scale testing of MICP via denitrification and a set of experiments conducted in these columns. Background information pertaining to MICP was discussed at length in the previous chapters. Bench-scale MICP testing in homogenous batch reactors and un-sampled sand columns established proof-of-concept and set baseline parameters for the work in this chapter. This chapter builds on what was learned in the previous experiments and describes a set of sand column experiments designed to provide a deeper understanding of MICP in sand via denitrification. Experiments with concentrated liquid media described in this chapter lead to exploration of different chemical formulations and denitrifying conditions. The experimental set-ups were constructed in a manner that facilitates continuous sampling in both static and dynamic sand columns inoculated with denitrifying bacteria. The ultimate benefit of the work described in this chapter is that it leads to suggestions for methods and techniques that will help advance the MICP process in future research.

5.2 Methods

5.2.1 Sand Column Test 1

A bench-scale testing apparatus was constructed for microbial precipitation of calcium carbonate via denitrification in a flow-through sand column. The flow-through system, shown in Figure 7, primarily consisted of a 2.5-L fluid reservoir, peristaltic pump, stir plate and heater, pressure gauges on the reservoir and sand column, a jacketed sand column set-up, and a nylon base plate for the sand column. The jacketed sand column was suitable for triaxial testing for stiffness and strength at the conclusion of the experiment (upon removal of the jacket).

The 2.5-L fluid reservoir was constructed from 102 mm (4") ID clear PVC (Schedule 40) glued to a gray PVC base. The cap was threaded and made of gray PVC with a recessed o-ring seal. The cap had a 32 mm (1.25") diameter port to accommodate a $CO_2(g)$ probe inserted into the reservoir for the purpose of monitoring headspace gas. The outer body of the $CO_2(g)$ probe was modified with rubber tubing to prevent gas intrusion into and/or escape from the reservoir. The probe began to malfunction within 48 hours of initiation of the first column test and was removed and the port was sealed for the duration of the experiment. It was later determined that the probe failed due to moisture collecting on the internal circuitry.
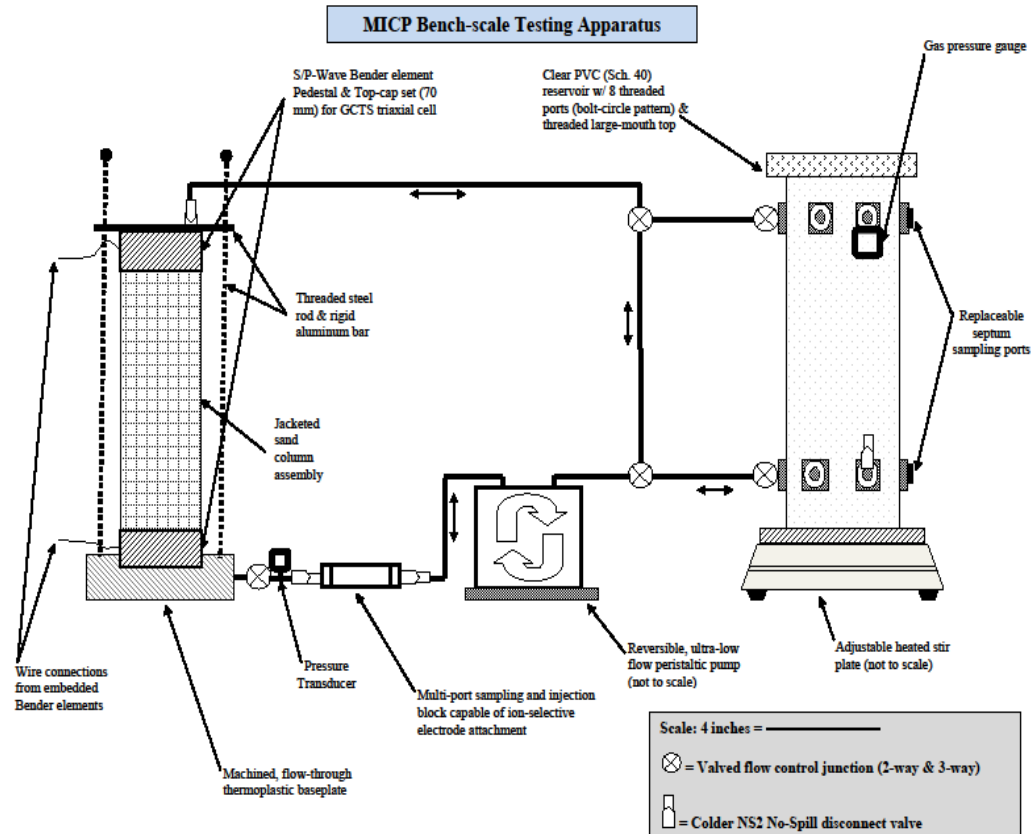
Figure 7- Bench-scale apparatus for flow-through testing of MICP in a sand column. Note that reservoir and sand column circuits can be isolated from each other while permitting flow in the sand column.

The reservoir has six sampling ports arranged in a circular pattern around the circumference of the reservoir and configured to allow for liquid sampling below the fluid line and gas sampling above the fluid line. The reservoir was placed on the heating and stir plate and maintained at a constant temperature (30°C) and stirred at least once week and during any event where reservoir fluid was circulated through the sand column. Several ball valves connected by clear 6.3 mm (¼") ID Nalgene 180 PVC tubing were used to control flow direction and isolate the sand column from the reservoir when desired. Clear polypropylene

fittings were used for most of the system. Chemically inert perfluoroalkoxy (PFA) fittings were used in locations where excessive bacterial attachment and/or mineral precipitation were a concern (PFA is a fluoropolymer very similar to PTFE (polytetrafluoroethylene)). Pressure gauges were installed on the sand column base plate and above the fluid line in the reservoir. Pressure measurements collected from the base plate gauge were corrected to account for elevation head during the experiment.

The peristaltic pump was connected via reducer bushings to the main system tubing (6.3 mm Nalgene 180 PVC) using a short section of thick-walled Tygon R-1000 tubing (0.79 mm ID). A three-port polypropylene manifold was connected in-line for real-time monitoring of pH, NO_3^-, and Ca^{2+} via pH electrode and ion selective electrodes (ISEs). The electrodes were sealed with o-rings and secured to the manifold via polypropylene compression fittings. The manifold was mounted at an angle of approximately 20° from horizontal to prevent entrapment of gas bubbles. Temperature probes were attached to the outside of the sand column and reservoir. Threaded fittings were sealed with Teflon tape and all non-threaded connections were secured with polypropylene hose camps. Low fluid/gas inclusion quick disconnect fittings (Colder Products NS2 & NS4) were used in several strategic places to allow for removal of the reservoir, peristaltic pump, sand column or ISE manifold for service or other reasons. The material types and thicknesses used for all components of the flow-through apparatus were chosen to (1) minimize gas permeability, (2) tolerate environments conducive to mineral precipitation and (3) allow for visual observations where possible.

The rigid nylon base plate for the sand column, presented in Figure 8, has a 6.3 mm (¼") circular channel to permit fluid flow through the pedestal (bottom) and into the sand column. The top cap and pedestal for the column are made from 316 stainless steel with 6.3 mm (¼") machined ports to allow for fluid flow through the column. The upward face of the top cap has a 6.3 mm (¼")-NPT threaded port for a quick disconnect fitting to allow for drained triaxial testing. The soil-facing ends of the top cap and pedestal are channeled, as shown in Figure 9, to permit flow along the surface for a more even distribution of flow between the soil-pedestal (or soil – top cap) interface. Two sheets of nylon woven mesh with 400-μm opening size (250-μm thread diameter) were placed over the soil facing side of the pedestal and top cap to further diffuse fluid flow.
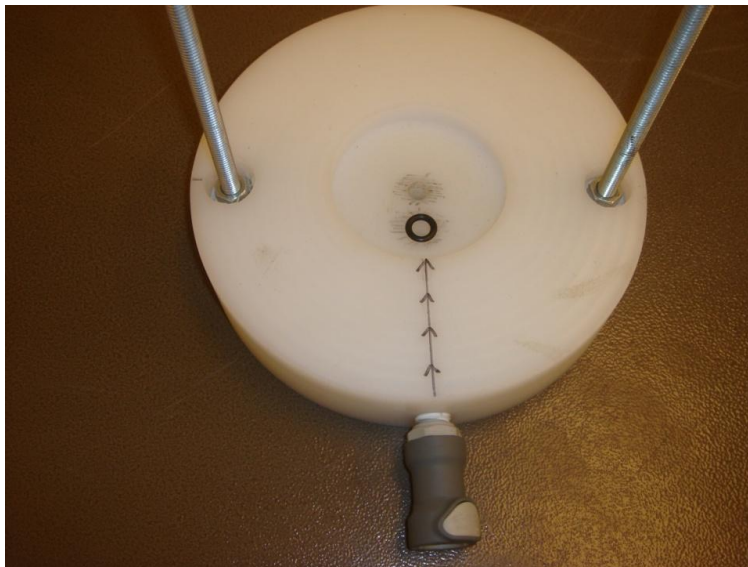
Figure 8- Base plate without sand column. Arrows on face illustrate the orientation of the flow channel.
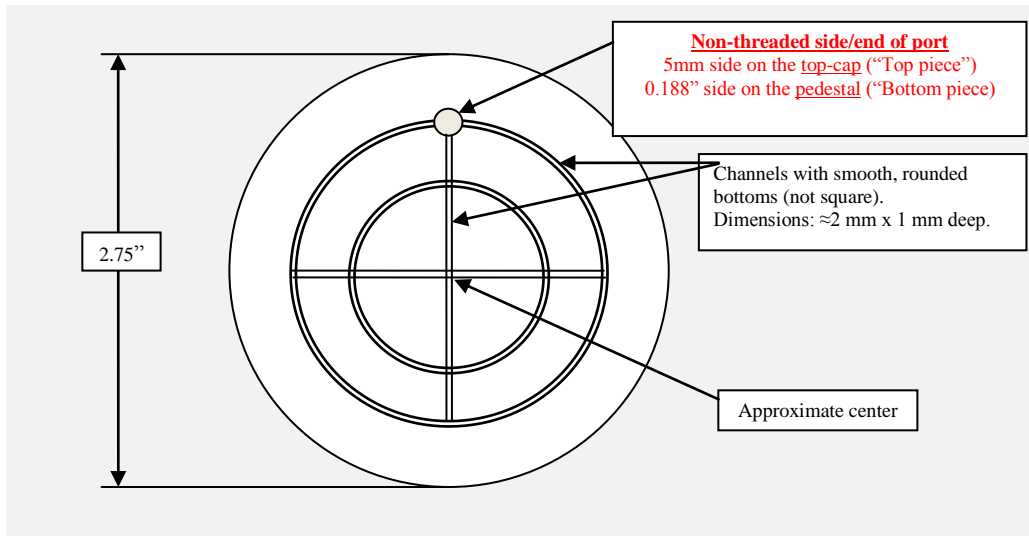
Figure 9- Plan view of pedestal/top cap illustrating small flow channels along the soil interface to promote even fluid flow. Nylon mesh (400 μm opening) was placed over the grooved faces to diffuse flow.

The pedestal and top cap were machined to the same dimensions as specialized units containing bender elements capable of measuring shear wave and compressional wave velocity through a soil specimen that may be used in future testing. Shear wave velocity is a function of elastic modulus or stiffness of a material and therefore could serve as proxy for extent of cementation. Compressional wave velocity can serve as an indicator of desaturation of the pore water due to gas generation. These specialized units, manufactured by GDS Instruments, were not used here since they were still in production at the time of this work.

The sand-filled triaxial column used a 71 mm (2.8") ID x 0.6 mm (0.025") thick membrane attached to a pedestal and top cap and supported by an aluminum jacket for the duration of the MICP experiment. The membrane was sealed against the top cap and pedestal with two conventional (round/oval) o-rings and a

square-edge o-ring that were compressed with a stainless steel hose clamp at each end. Vertical displacement of the top cap due to gas pressure generation was prevented by a rubber-coated rigid aluminum crossbar connected to threaded steel rods mounted to the base plate. A cloth insulated heating tape (Cole-Palmer, 51 mm x 13 mm, 156 watts) was loosely wrapped around the column and used intermittently when column temperature dropped below 25$^{\text{o}}$C. The fully assembled sand column bolted to the base plate is shown in Figure 10.

Figure 10- The fully assembled sand column bolted to the base plate.

5.2.2 Chemical Analysis and Characterization of Sand Column

Chemical analysis of ions in solutions drawn from the column and the reservoir was performed through ion chromatography using a Dionex ICS-3000. Standard

ICS-3000 operating procedures were followed for all ion analyses. Anion and cation calibration standards were made using Dionex 7-Anion and Dionex 6-Cation IC standards. Nanopure (18.2 MΩ·cm) deionized water was used in all dilutions and any case where water was required for testing. A typical batch run included the following: 2 DI water blanks to make sure the column was clean before starting; 5 standards (cation or anion) from lowest to highest concentration; test samples; test sample of known concentration (check standard); and an additional DI water blank every 3-4 test samples.

Determination of acetate content in the solutions drawn from the columns and the reservoir was performed using a Shimadzu Prominence High-Performance Liquid Chromatograph (HPLC) with diode array and refractive index detectors. The HPLC was operated following standard operating procedures. Acetate standards were formulated from reagent grade sodium acetate trihydrate (Sigma Aldrich, ≥99%). A typical batch run included the following: 2 DI water blanks to make sure the column was clean before starting; 5 standards from lowest to highest concentration; test samples; test sample of known concentration (check standard); and a DI water blank every 2 test samples.

Wet laboratory techniques were used to confirm the presence of precipitated carbonate minerals in the sand columns, lines, and reservoir at the end of testing. Mineral precipitates ranging from large pieces found in the reservoir to small grains in the lines and valves were collected for testing. Acidification of carbonate minerals with warm 1 M HCl acid resulted in strong effervescence and complete dissolution. Electron microscopy was used for

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analysis and characterization of the large pieces of precipitate recovered from the reservoir. A Rigaku D/Max II-B Powder X-Ray Diffractometer (XRD) was used to determine mineral phase. Samples were ground in an agate mortar & pestle and powdered coated onto a standard glass slide. Jade 9.0 software was used for post-run analysis of XRD results.

A JEOL JSM-6701F field emission high-voltage scanning electron microscope (SEM) was used to investigate morphological features on coated (gold-palladium, 50-50) pieces of the precipitate. Later stages of SEM analysis were performed with an Agilent 8500 Low-Voltage SEM (LV-SEM). A LV-SEM is a field emission scanning electron microscope (FE-SEM) capable of imaging insulating materials, such as organic and biological substances, without the need for a metal coating and without causing radiation damage to samples. In this SEM method, original samples are simply adhered via carbon tape to aluminum stubs and loaded onto the microscope stage for imaging.

Analysis through isotopic ratio mass spectrometry (IR-MS) was used to determine the origin of the carbon source in the carbonate mineral precipitate. A method known as GasBench IR-MS analyzes carbon in carbonate minerals by sampling the gas (CO_2) evolved upon acidification with 100% phosphoric acid. The gas is analyzed for $\delta^{13}C$ (carbon-13 enrichment) and final isotope ratios are reported with respect to Vienna Pee Dee Belemnite (VPDB). The reagents used in the denitrification system were analyzed using an elemental analyzer-IRMS where the solids are combusted at high temperatures. The $\delta^{13}C$ results from the analyses were then compared with known $\delta^{13}C$ values (with respect to VPDB).

Forston Labs ion selective electrodes (ISEs) were evaluated for use in sand column denitrification experiments but failed to properly measure concentrated ion solutions under flowing test conditions. ISEs were inserted into a flow-through polypropylene manifold to monitor NO_3^- and Ca^{2+} concentrations in real-time. Forston Labs PVC membrane type ISEs were used and were calibrated following the manufacturer's specifications in comparatively dilute (10 to 1000 mg/L) single-ion standards. The ISEs failed immediately upon starting the denitrification experiment. Concentration values varied tremendously and failed to stabilize after several days of operation. The ISEs were removed from the denitrification system and evaluated for defects or calibration errors. In every case tested, the ISEs failed to correctly measure ion concentrations in mixed solutions and solutions with ion concentrations greater 1000 mg/L. Errors ranged from 10x to 100x the actual standard values. Forston Labs technical support was contacted for trouble-shooting assistance; after many attempts to solve the ISE problems through warranty repairs/rebuilds/calibrations, the ISEs failed to operate properly in the high ion concentration ranges of the denitrification matrix. Further analysis of these ISEs in dilute single ion systems (Ca^{2+} or NO_3^-) indicated that relatively reliable (approximately ±10%) results could be obtained. This observation was also confirmed using a different set of Forston Labs ISEs borrowed from another researcher in the ASU Biodesign Institute. In any case, based upon these results the ISE's were considered unreliable under these test conditions and, therefore, were not used.

5.3 Sand Column Testing

5.3.1 Sand Column 1

Autoclaved Ottawa 20-30 sand was poured into the membrane lined column with a specified drop height in order to obtain a target relative density of 80% (D_r = 80%). The height of the sand column between the pedestal and top cap was ≈162 mm (6.37"). The fully assembled bench-scale apparatus was pressure tested to 124 kPa (18 psi) with nitrogen gas prior to experimentation and monitored for leaks. The system remained charged at 124 kPa (18 psi) for approximately 90 hours, indicating that the system was sufficiently air-tight for preventing non-diffusive gas exchange. All bench-scale apparatus components that were not sterile upon receipt were alcohol sterilized (70% v/v) prior to assembly.

Upon depressurization, the reservoir was disconnected from the system, filled with approximately 1700-mL of liquid medium, and then immediately evacuated for approximately 10 minutes. After evacuation, the reservoir was purged with nitrogen gas for 30 minutes and held to a slight overpressure (≈13 kPa) until the experiment was begun. The headspace in the reservoir after filling was approximately 800-mL (2500-mL capacity minus 1700-mL of fluid). Approximately 150-mL of liquid medium was drawn from a reservoir sampling port, transferred to a 250-mL glass bottle, and refrigerated for future testing. The liquid medium was formulated in nanopure (18.2 MΩ·cm) deionized water as follows: 24.7 g/L (140 mM) calcium acetate monohydrate, 11.8 g/L (50 mM) calcium nitrate tetrahydrate, 0.25 g/L (1 mM) magnesium sulfate heptahydrate and 3 drops/L of trace metal solution. The contents of the liquid medium are

summarized in Table 4. Calcium acetate was autoclaved separately from the other reagents. The remaining components were autoclaved in solution. Upon cooling, the components were moved to an anaerobic chamber where they were mixed in a sterile 2.0-L glass bottle. The pH was adjusted to 7.7 and the bottle was left loosely capped in the anaerobic chamber for 5 days to de-gas prior to use.

Immediately prior to the start of the experiment, the system was depressurized then evacuated and left under a slight vacuum. A 65-mL inoculum (OD_{600} = 0.572) of anaerobically grown *Pseudomonas denitrificans* (ATCC 13867) was injected into the sand column through the base plate port. The inoculum was quickly drawn into the column due to the slight vacuum. Next, a 250-ml mixed nutrient liquid medium with an ionic strength of 39 mM was introduced into the base plate. The purpose of the mixed-nutrient medium was to facilitate bacterial adsorption onto sand particles and promote acclimation to high ionic strength solutions. The contents of the 250-mL mixed nutrient liquid medium are as follows: 2.72 g/L (20 mM) sodium acetate trihydrate (nutrient), 1.27 g/L (15 mM) sodium nitrate, 0.40 g/L (3 mM) monopotassium phosphate, 2 drops of trace metals solution and 1.6 g/L nutrient broth (Difco). The contents of the mixed nutrient liquid medium are summarized in Table 4.

Table 4- Composition of reservoir liquid medium and mixed-nutrient medium

Medium	Chemical Component	Brand	Concentration (mM)	Ionic Strength (M)	Total Ionic Strength (mM)
Reservoir Liquid Medium	$Ca(CH_3COO)_2$	Sigma Aldrich	140	0.420	574
	$Ca(NO_3)_2$	BDH	50	0.150	
	$MgSO_4 \cdot 7H_2O$	Mallinckrodt	1	0.004	
250 mL Mixed Nutrient Medium	$Na(CH_3COO) \cdot 3H_2O$	BDH	20	0.02	39
	$NaNO_3$	J.T. Baker	15	0.015	
	KH_2PO_4	J.T. Baker	3	0.003	
	Nutrient Broth	Difco	1	0.001	

Trace metals solution consists of 0.5% (w/v) of $CuSO_4$, $FeCl_3$, $MnCl_2$, $Na_2MoO_4 \cdot 2H_2O$

The different pressures between the column and the reservoir were slowly equilibrated during the addition of the 250-mL mixed nutrient medium. The 65-mL inoculum and 250-mL mixed-nutrient medium did not appear to fill the sand column, as evidenced by the lack of fluid in the clear PVC tubing at the top of the column. Therefore, liquid medium from the reservoir was allowed into the column from the bottom port until fluid appeared in the clear PVC tubing. Based on the change in fluid height in the reservoir *after* the lines and valves were filled, approximately 40-mL of additional liquid medium was required to fill the column. The total liquid volume in the sand column was approximately 355-mL. The valves between the reservoir circuit and the column were closed after filling the sand column with liquid medium to isolate the sand column from the reservoir.

Fluid samples were taken at irregular intervals over the course of the experiment and labeled as either a sand column or reservoir sample. Column

samples were drawn from the base plate port (bottom) and reservoir samples were taken from approximately mid-depth of the reservoir as dictated by the fixed sampling ports. Several attempts to collect liquid samples from the top of the sand column failed due to excessive gas inclusion. Gas generation in the column occasionally produced pressures exceeding 96 kPa (14 psi) that required venting into the reservoir. Sampling intervals were sometimes based on a venting event simply as matter of convenience. Nine samples between 10-12 mL each were collected over the 62 day duration of the first column experiment. Sampling was conducted without replacement of the sampled fluid. Gas pressures dropped upon fluid sampling, presumably due to the volume change induced by fluid withdrawal. The pH of the withdrawn fluid was determined immediately upon sampling using a Beckman Coulter meter that was 3-point calibrated before every sampling event. Samples were then filtered through a 0.2-µm syringe filter (32 mm Pall Acrodisc) into sterile 15-mL polypropylene vials and refrigerated until further testing.

5.3.2 Sand Column 2

The bench-scale testing system developed for flow-through testing and used for the first sand column experiment was simplified for the second sand column experiment, a static sand column test (i.e. the denitrification medium was not continuously circulated through the column in this experiment). The modifications were developed to approximate spatial geochemical variations in a static sand column undergoing microbially induced calcium carbonate precipitation (MICP). The static system, shown in Figure 11, primarily consisted

of the 2.5-L fluid reservoir, a 1000-mL medical grade PVC bag for gas collection, a low-inclusion (0.009 cc/connect) quick disconnect (NS2, Colder Products) between the column and the reservoir, the stir plate and heater, the jacketed column set-up, and the nylon base plate for the sand column.
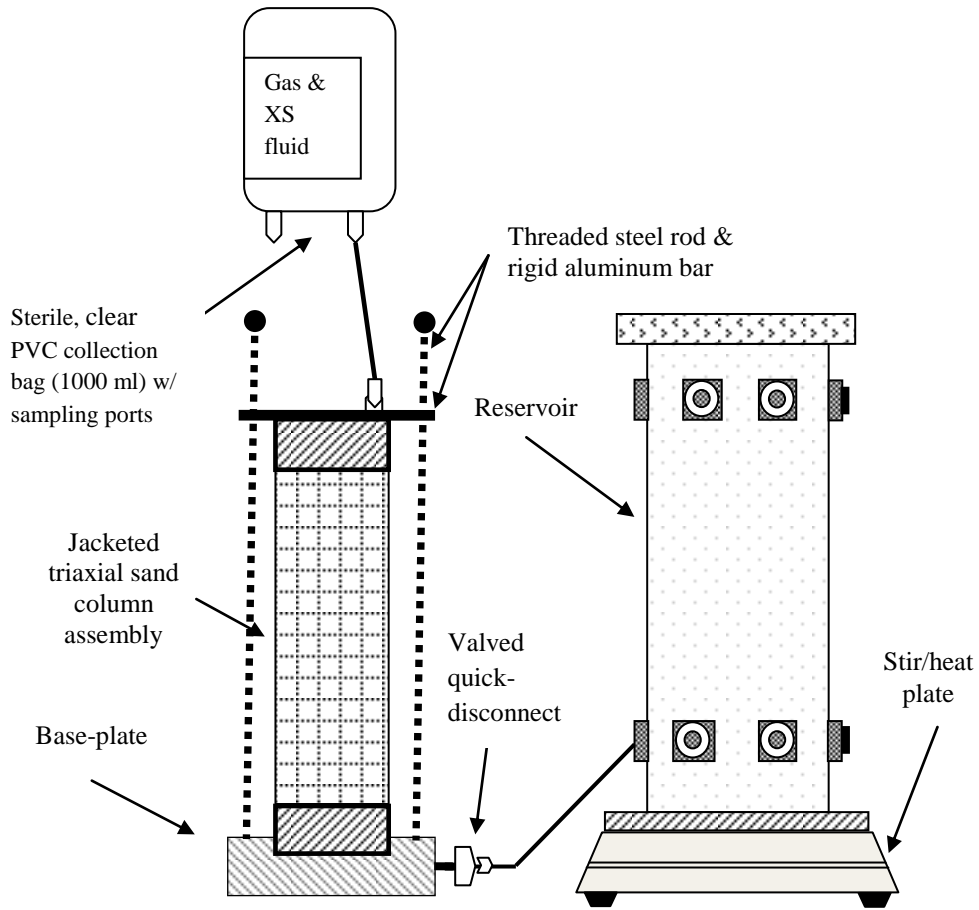
Figure 11- A static bench-scale sand column system for analyzing spatial geochemical variations during MICP.

Ottawa 20-30 silica sand was poured into the membrane lined column in a manner designed to obtain a target relative density of 80% ($D_r = 80\%$). The height of the sand column between the pedestal and top cap was 175 mm (≈ 6.9"). All bench-scale testing apparatus components that were not sterile upon receiving

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were alcohol sterilized (70% v/v) prior to assembly. The fully assembled bench-scale apparatus was pressure tested to 13 kPa (2 psi) nitrogen gas prior to experimentation and monitored for leaks. After the system remained charged at 13 kPa (2 psi) for approximately 10 hours, it was determined that the system was sufficiently air-tight for purposes of preventing non-diffusive gas exchange.

The bench-scale apparatus was depressurized and then the reservoir was disconnected from the system and filled with approximately 1700-mL of liquid medium and immediately evacuated for approximately 5 minutes. After evacuation, the reservoir was purged with nitrogen gas for 10 minutes and held to a slight overpressure (≈13 kPa). The reservoir remained pressurized with nitrogen gas throughout the experiment in order to allow the liquid medium to flow into the sand column when needed (i.e. to prevent a vacuum in reservoir upon fluid loss). The reservoir was only connected to the base plate when the sand column required replenishment with liquid medium. The headspace in the reservoir after filling was approximately 800-mL (2500-mL capacity minus 1700-mL of liquid). Approximately 150-mL of liquid medium was drawn from a reservoir sampling port and transferred to a 250-mL glass bottle and refrigerated for future testing. The liquid medium was prepared in same manner as described for the Reservoir Liquid Medium in Section 5.2.2 and the same chemical composition as described in Table 4. The pH was adjusted to 7.3 by addition of NaOH or HCl and the bottle was left loosely capped in the anaerobic chamber for 5 days to degas prior to use. The pH was rechecked after 5 days in the anaerobic chamber and readjusted to 7.3 and then sealed until use. It is assumed that the pH of the liquid medium was

stable and remained so for the duration of the experiment since the only substance added to the reservoir was nitrogen gas.

At the start of the experiment, the 350-mL medical grade PVC collection bag was connected to the top port of the sand column. Next, a 55-mL inoculum (OD_{600} = 0.058, optical density at 600 nm) of anaerobically grown *Pseudomonas denitrificans* (ATCC 13867) was injected into the sand column through the base plate port followed by 2-ml of 36 mM monopotassium phosphate (KH_2PO_4). The phosphate was added to facilitate microbial growth since the optical density measurement indicated that the inoculum was taken from a relatively dilute microbial culture. The reservoir was then immediately connected to the base plate and liquid medium was slowly allowed into the column from the bottom port. The flow rate into the column was controlled by a screw adjustable tubing clamp. The sand column was assumed to be completely filled when fluid appeared in the clear PVC tubing connected to the top of the column (top cap). A small amount of diffuse white precipitate appeared in the top PVC tube upon filling. Based on prior experience, the contents of the liquid medium, and the introduction of phosphate at near neutral pH, the white precipitate was most likely calcium phosphate.

Based on the change in fluid height in the reservoir, approximately 295-mL of liquid medium was required to fill the column. The total liquid volume (bacterial inoculum, phosphate and liquid medium) introduced into the sand column was approximately 350-mL (295-mL of liquid medium plus 55-mL inoculum plus 2-mL phosphate solution). This is consistent with the amount of

liquid required to fill Sand Column 1 and with the theoretical pore volume of the specimen. After filling the column, the 350-mL PVC collection bag (containing displaced column gas) was replaced with a new (empty) 1000-mL bag and the reservoir was disconnected from the base plate to isolate the sand column from the reservoir.

Sand column liquid samples were taken from the base plate port (bottom) via 3.1 mm (ID) Tygon lab tubing (R-3603) and allowed to gravity flow out of the column. Samples were taken over the 49 day duration of the second sand column experiment at approximately two-week intervals. During each sampling event, fluid was collected in a sequential manner in approximately four equal volumes. Because no additional fluid drained by gravity from the column after the fourth draw, each of the four sequentially collected fluid volumes was assumed to represent a unique vertical section of the sand column; e.g. the first fluid sample was assumed to be drawn entirely from the bottom quarter of the column and the last sample was assumed to be drawn from the top quarter of the column. The total volume of the four samples extracted in any single sampling event was approximately 150-mL and thus was always less than the total initial pore volume of the sand column of approximately 350-mL (295-mL of liquid medium plus 55-mL inoculum plus 2-mL phosphate solution). Sampled fluids were replaced with fresh liquid medium from the reservoir. Relatively long sampling intervals were chosen in order to minimize system disturbance, allow for sufficient reaction time, and minimize chemical gradients within the static column via diffusion. Gas samples were not collected during this experiment.

The pH was determined immediately upon sampling using a Beckman Coulter meter that was 3-point calibrated before every sampling event. Depending on the level of difficulty during filtering, each sequentially collected sample was filtered through a either sterile 0.2-μm or 0.2/0.8-μm two-stage syringe filter (32 mm Pall Acrodisc or Cole-Palmer brand). The samples were stored in sterile 15-mL polypropylene vials and refrigerated until further testing.

5.3.3 Sand Columns 3 through 7

Following the second sand column test, the test apparatus was modified again to enable the testing of multiple columns simultaneously in a static mode. The modified bench-scale testing system was employed to test five sand columns, labeled Columns 3 through 7. The testing protocol for these five columns was developed to investigate the effects of concentration, organic carbon donor type, and inoculum size on denitrification in static sand columns undergoing microbially induced calcium carbonate precipitation (MICP) via denitrification. Each of the five columns consisted of a 76 mm x 152 mm (3"x 6") clear acrylic tube with glued rubber end caps, a 1000-mL medical grade PVC bag for liquid medium delivery or gas storage, two upper and one lower inlet/outlet lines, adjustable polypropylene pinch clamps, and low-inclusion quick disconnects (0.009 cc/connect, NS2 Colder Products). Columns 6 and 7 were connected to a common gas collection reservoir, and Columns 3 through 5 were connected to different common gas collection reservoir. The column setup is shown in Figure 12. The columns were placed on a specially design PVC platform with metal hangers for the PVC bags.
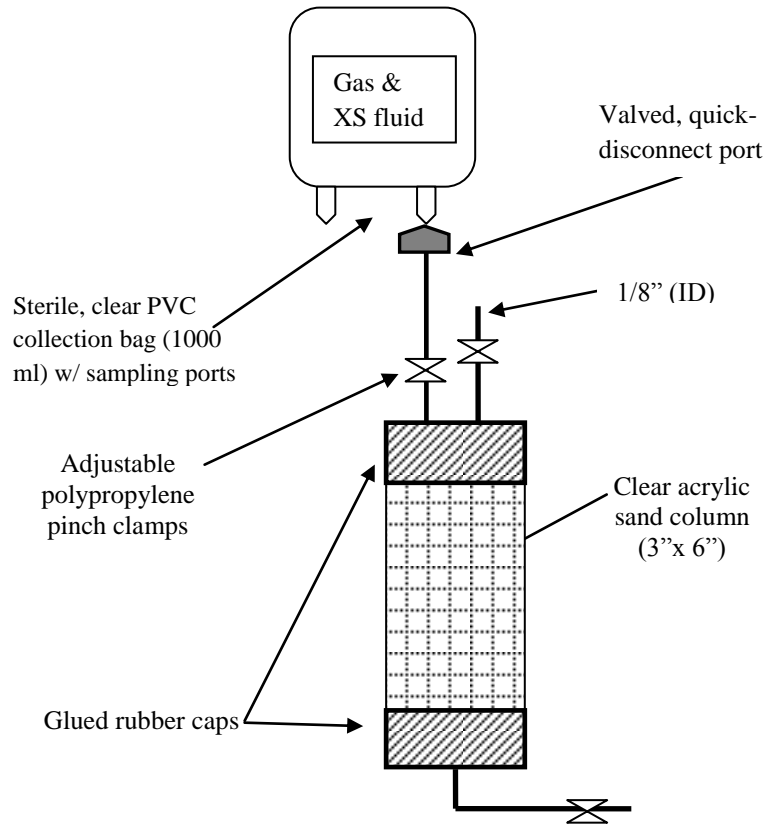
Figure 12- Static bench-scale column design for examining the effects of concentration, organic carbon donor type, and inoculum size on denitrification in sand columns undergoing microbially induced calcium carbonate precipitation.

The five sand columns were individually constructed by silicone gluing the flexible rubber bottom caps to the clear acrylic tubes. Each cap had one barbed polyethylene (polytube) fitting pressed into the cap prior to gluing. Scotch-Brite pads approximately 5 mm thick were placed at the bottom of each acrylic tubes for filtration. The tubes were then filled with 20-30 Ottawa silica sand that was dry pluviated with a funnel at an approximately 76 mm (3") drop height. The acrylic tubes were capped with silicone glued rubber caps with two barbed fittings per top cap. Barbed fittings were connected to 3.1 mm (ID) Tygon lab tubing (R-

3603) equipped with adjustable polypropylene pinch clamps to control gas and fluid flow. Each tube had approximately 25 mm (1") of head space after filling with sand. The thin-walled acrylic columns used in this experiment are susceptible to rupture under pressure. To mitigate potential for rupture, flexible rubber caps were used to accommodate bio-gas generation. The sealed columns were then placed on the PVC platform and columns containing similar carbon donors (e.g. acetate only or acetate-glutamate mix) were connected to similarly treated columns via barbed ploytube T-fittings. All components that were not already sterile were alcohol sterilized (70% v/v) prior to assembly. The columns were then simultaneously flushed with nitrogen gas for approximately 1 hour through the top port and vented from the bottom port to reduce column oxygen gas levels. Based on prior experience using silicone sealant on acrylic columns with rubber caps, it was assumed that the system was sufficiently air-tight and pressure testing was not performed.

Two liters of liquid medium was prepared in same manner as described for the Reservoir Liquid Medium in Section 5.2.2 and with the same chemical composition as described in Table 4 except for the following: (A) 0.5 g/L (2 mM) magnesium sulfate heptahydrate rather than 0.25 g/L (1 mM), and (B) 10 drops/L of trace metal solution rather than 3 drops/L. The pH was adjusted to 7.5 and allowed to de-gas as described in Section 5.2.3 followed by a recheck of pH. The liquid medium was then transferred into two 1000-mL sterile PVC bags in an anaerobic chamber and sealed. A 160 mM solution of glutamate was prepared by combining 500 mL of 18.2-MΩ DI water with 24 g/L L-glutamic acid (Acros,

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reagent grade FW = 147.13 g). The glutamate solution was autoclaved then allowed to de-gas in an anaerobic chamber. The solution was adjusted to pH = 7.5 and transferred to a sterile 1000-mL PVC bag and sealed. Approximately 100-mL of liquid medium and glutamic acid were transferred to separate glass bottles and refrigerated for later testing. A 250-mL of anaerobically grown *Pseudomonas denitrificans* (ATCC 13867) was prepared in a 500-mL glass bottle, and then transferred to a 350-mL sterile PVC bag immediately prior to use in an anaerobic chamber and sealed.

The five sand columns varied in their (1) liquid medium strength, (2) bacterial inoculum size, and (3) organic carbon donor type as shown in Table 5 and summarized as follows:

(1) Liquid medium strength with acetate as the sole organic carbon donor:

Columns #3 and #4 received the same concentration of liquid medium constituents (full strength/high), while column #5 was at approximately ½-strength (low). (Columns #6 and #7 did not use acetate)

(2) Bacterial inoculum size:

Columns #3, #5, #6 and #7 each received approximately 40-mL of bacterial inoculum, while column #5 received approximately 80-mL of bacterial inoculum (OD_{600} = 0.707).

(3) Organic carbon donor type:

Columns #3 through #5 used acetate as the sole organic carbon donor, while columns #6 and #7 used an organic carbon mixture

composed of approximately 70% acetate to 30% glutamic acid. Glutamic acid ($C_5H_9NO_4$) is nitrogen bearing compound that may increase pH of a reaction medium upon formation of NH_3 through microbial oxidation.

Table 5- Column conditions varied in their (1) liquid medium strength, (2) bacterial inoculum size, and (3) organic carbon donor type.

Column #	Organic carbon donor type	Liquid Medium Concentration	Inoculum Size (mL)
3	Acetate	Full strength	40
4	Acetate	Full strength	80
5	Acetate	1/2 strength	40
6	Ac-Glutamate Mix	n/a	40
7	Ac-Glutamate Mix	n/a	40

See Table 4 for liquid medium composition and concentrations. "Ac-Glutamate Mix" is 70% acetate and 30% glutamic (v/v) acid mixture. Inoculum OD_{600} = 0.707

Target inoculum sizes and liquid medium concentrations were achieved by measuring the depth of the fluid in the clear acrylic sand column from the outside using a ruler. Target volumes of each component were determined before the start of the experiment and converted to equivalent millimeters of column length. Liquid medium dilutions were accomplished by adding appropriate volumes of 18.2-MΩ DI water or glutamate (pH = 7.50) based on millimeters of column depth. The basis for the column length (i.e. height, h) to volume conversion is as follows:

- The columns are 3" in diameter (76.2 mm), so column volume as function of height is $\pi(3.81 \text{ cm})^2 \cdot h = 45.6 \text{ cm}^2 \cdot h$

- Every 0.1 cm (1 mm) of column height equals 4.56 cm^3

- 1-cubic centimeter = 1-mL, therefore every 2.2 cm (22 mm) of column height equates to approximately 10-mL of liquid.

The top ports of the columns were interconnected via a manifold to one empty PVC bag (1000-mL) to allow for gas displacement within the column during fluid filling of the five columns. The PVC bags were replaced several times during initial filling of the columns. The experiment was started by inoculating the sand columns from the lower inlet with the appropriate amount of anaerobically grown *Pseudomonas denitrificans* (volumes and concentrations are shown in Table 5) based on fluid height in the column. The bacterial medium was continuously mixed during inoculation by gently shaking the 350-mL PVC bag that contained the medium. In addition, to mitigate against erroneous fluid height measurements caused by capillary action, the bacterial medium was continuously weighed during inoculation to help maintain correct proportions between columns. Next, each column was filled with the appropriate amount of liquid medium, glutamate and DI water from the bottom port. The fluid level after filing was approximately 6.3 mm (¼") above the soil line in all five columns. Unlike the inoculation phase, continuous weighing of PVC bags containing liquid medium, glutamate, and DI water was not done. However, fluid height measurements appeared to be less influenced by capillarity than by the inoculum as additional medium was added to the sand columns. In any case, there is some uncertainty in

the dilutions presented in Table 5. The filled columns, shown in Figure 12a, were covered with aluminum foil after filling.

Figure 12a- Five acrylic sand columns are filled and sitting on a PVC platform. Some of the columns were interconnected depending on the column treatment.

The experiment was conducted for 44 days, and only three samples were collected from each column to allow for sufficient reaction time and minimize disturbance. The sampling events were at t = 0, t = 39 days, and t = 44 days. Samples were collected by gravity draining a column from the bottom port into an empty and sterile 350-mL PVC bag and then extracting 8-mL through the bag's sampling port with a sterile syringe. Each column was drained and sampled with a separate sterile PVC bag and syringe. The total sample volume drained from any single column was always less than the total fluid volume of the sand column, which was approximately 330-mL (average). Gas samples were not collected during this experiment. The pH was determined immediately upon sampling using

a Beckman Coulter meter that was 3-point calibrated before every sampling event (t = 0, t = 44 days, t = 52 days). Each column sample was then filtered through a sterile 0.2-μm or 0.2/0.8-μm two-stage syringe filter (32 mm Pall Acrodisc or Cole-Palmer brand) into sterile 15-mL polypropylene vials and refrigerated until further testing. The remaining fluids in the PVC bags were returned to their respective columns through the bottom port.

During the second sampling event (t = 44 days), 2-mL of NaOH (pH = 12.9) was injected into each PVC bag immediately prior to returning the fluids to the sand columns. The addition of 2-mL of NaOH equates to 1.58E-4 moles of hydroxide ion (OH^-) as follows:

- pH + pOH = 14

- Using pH=12.9 NaOH, 14-12.9 = pOH = 1.1

- Then, $-\log[OH^-] = 1.1$ → $[OH^-] = 10^{-1.1} = 0.079M$

- So, $\rule{2cm}{0.4pt}\ \rule{1.5cm}{0.4pt}$ moles OH^-.

5.4 Results and Discussion

5.4.1 Column 1

The flow-through denitrification system was designed to precipitate calcium carbonate in the sand column. Upon disassembly, the sand column did not appear to have any carbonate precipitation based on visual inspection. Yet, a white precipitate (later verified to be calcite) was clearly evident in the lines, valves, quick disconnects, on the pedestal and top cap, and in the reservoir. Large platy pieces of calcite were found loosely cemented to the bottom of the reservoir. In

general, calcite of varying quality, ranging from fine-grained to cementitious platy pieces, was found in nearly part of the system except the sand, as shown in Figure 13. Fine-grained and large platy samples were determined to be carbonate bearing minerals through acid testing. XRD analysis, illustrated in Figure 14, showed that the samples were calcite phase calcium carbonate.
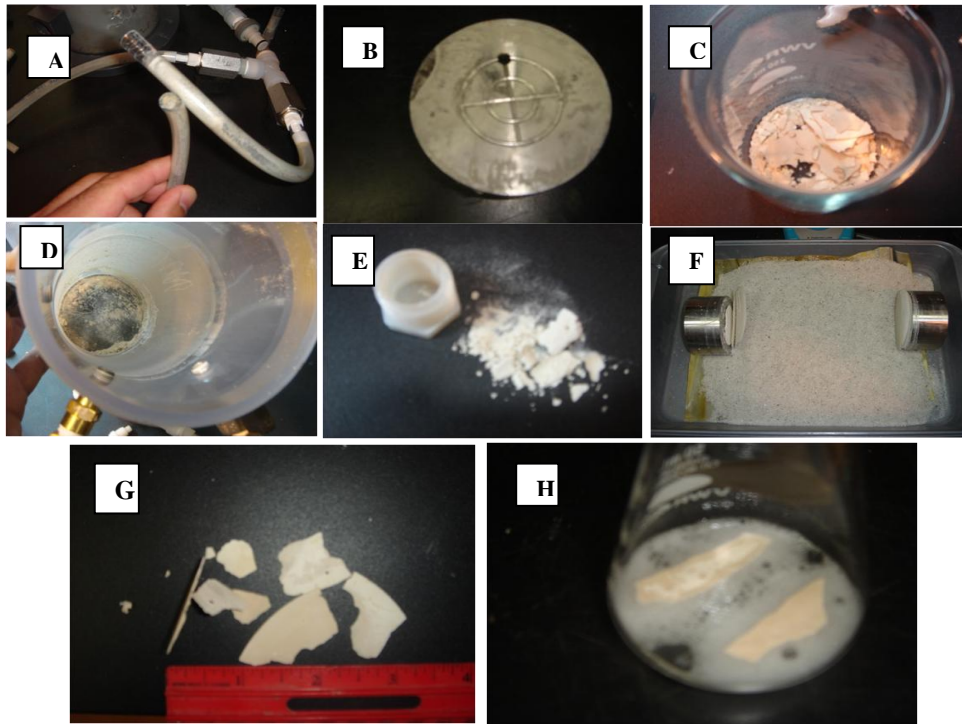
Figure 13- Images of calcite precipitation a.) in lines, b.) on pedestal, c.) platy pieces collected in a beaker, d.) inside reservoir walls, e.) in fittings, f.) no calcite observed in sand column, g.) calcite pieces and h.) acid digestion of calcite.
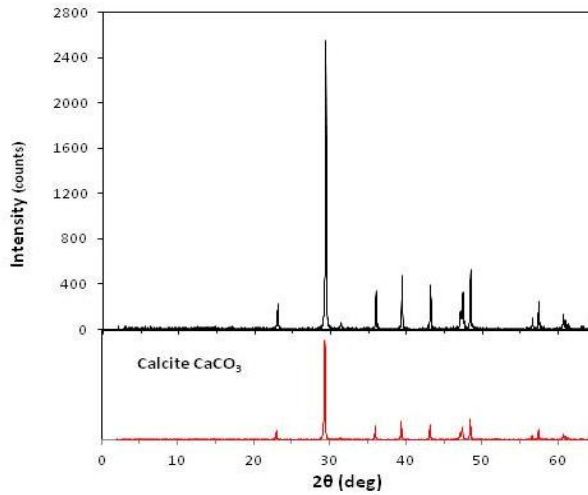
Figure 14- XRD results showing that calcite is the mineral phase present in precipitate from Column Test 1.

GasBench isotopic ratio-mass spectroscopy (IRMS) results, summarized in Table 6, show the ratio of carbon-13 to carbon-12 isotopes in units per mil with respect to the Vienna Pee Dee Belemnite standard ($^0/_{00}$ VPDB). The relative abundance of ^{12}C and ^{13}C is approximately 98.9% and 1.1% (Coplen et al. 2002). The ratio of ^{13}C to ^{12}C indicates the degree of depletion (δ^{13}C) of the carbon-13 isotope in a given sample; depletion can vary by several orders of magnitude due to fractionation effects that are driven by several mechanisms including microbial processes (Coplen et al. 2002; Meckenstock et al. 2004). Certain fractionation processes, particularly in biological systems, tend to favor lighter isotopes (^{12}C) and are consequently more depleted in ^{13}C (Meckenstock et al. 2004). Variations in the ratio of ^{13}C to ^{12}C among carbon bearing substances yields a unique "signature" for these substances that can be correlated to their origin(s).

Table 6 shows that the CO_3^{2-} carbon released from platy reservoir samples have a δ^{13}C $\approx$ -24 ($^0/_{00}$ VPDB). Atmospheric CO_2 has a narrow δ^{13}C range

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between -10 to -8 ($^0/_{00}$ VPDB) and the reagents used in the sand columns have a

δ^{13}C ratio of approximately -12 ($^0/_{00}$ VPDB). Thus, the platy samples are more

depleted in ^{13}C than atmospheric CO_2, indicating that atmospheric carbon was an

unlikely source for the CO_2 in the platy samples. If atmospheric CO_2 had been the

primary source of carbon, the carbonate carbon δ^{13}C signature in the platy

samples would reflect values at or very near atmospheric carbon since this form

(isotope) of CO_2 is the precursor to CO_3^{2-} formation. The depleted ^{13}C values in

platy samples indicate fractionation processes favored lighter isotopes of carbon

derived from sources with mixed carbon isotopes such as found in the organic

carbon donor (acetate). These observations--coupled with N_2 sparging and

purging of the liquid medium and reservoir (respectively) prior to denitrification

and the chemical environment of the denitrification experiment--indicate that the

carbon source for the precipitated calcite was most likely CO_2 derived from

microbial denitrification, which should have favored the lighter carbon isotope.

Table 6- GasBench IRMS test results. Calcium acetate was used in this experiment.

Sample	Weight (mg)	Measured δ13C VPDB (0/00)	σ (0/00)
Reservoir #1	0.477	-24.4	0.4
Reservoir #2	0.856	-24.8	0.4
Na-Acetate	1.145	-11.85	-
Ca-Acetate	1.519	-12.23	-

Platy calcite samples were analyzed for morphological features using a

LV-SEM. The LV-SEM images presented in Figure 15 show many well-

developed calcite crystals covered with bacteria. In some cases, it appears that calcite crystals are growing on bacteria, indicating that the microbes may have served as nucleation sites.
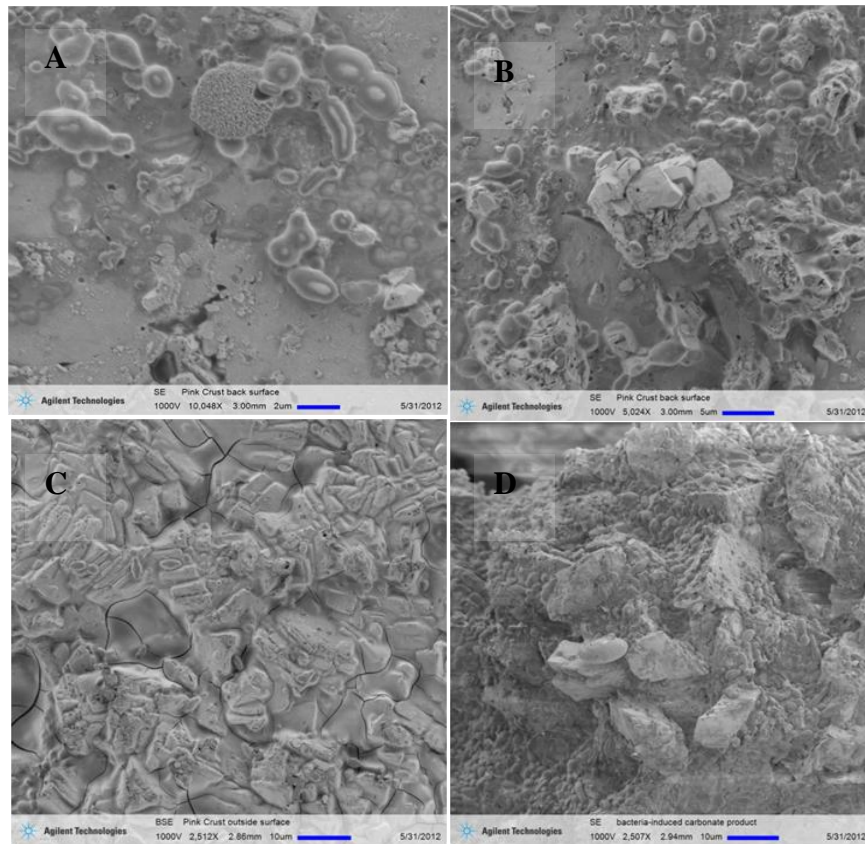
Figure 15- LV-SEM Images from platy reservoir sample a.) microbes/microbial material—top face of platy sample, b.) growing calcite crystals (center)—top face, c.) calcite and microbes—bottom face and d.) calcite crystals growing in a microbial matrix—top face.

Ion analyses of reservoir and column samples, presented in Figures 16 and 17, showed large decreases in Ca^{2+} and NO_3^- as well production of transient concentrations of NO_2^-. HPLC analyses of reservoir and column samples, presented in Figure 18, show a large decrease in acetate concentrations. The

association between the decrease in NO_3^- and the production of NO_2^-, followed by a rapid decline in NO_2^- is consistent with a denitrifying system.
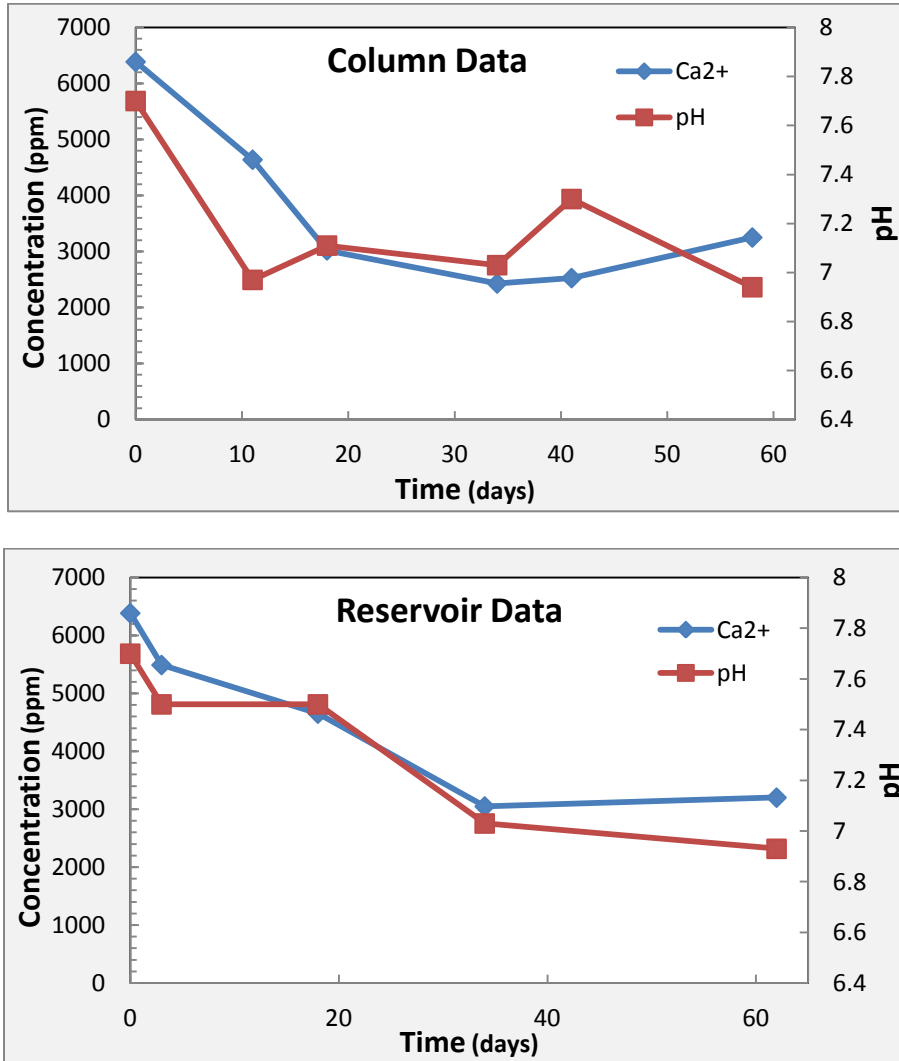
Figure 16- Changes in Ca^{2+} concentration and pH– Column Test 1.

The decline in acetate concentrations (electron donor) correlates well with the decrease in NO_3^- (electron acceptor), as the acetate concentration ceases changing once NO_3^- is depleted.
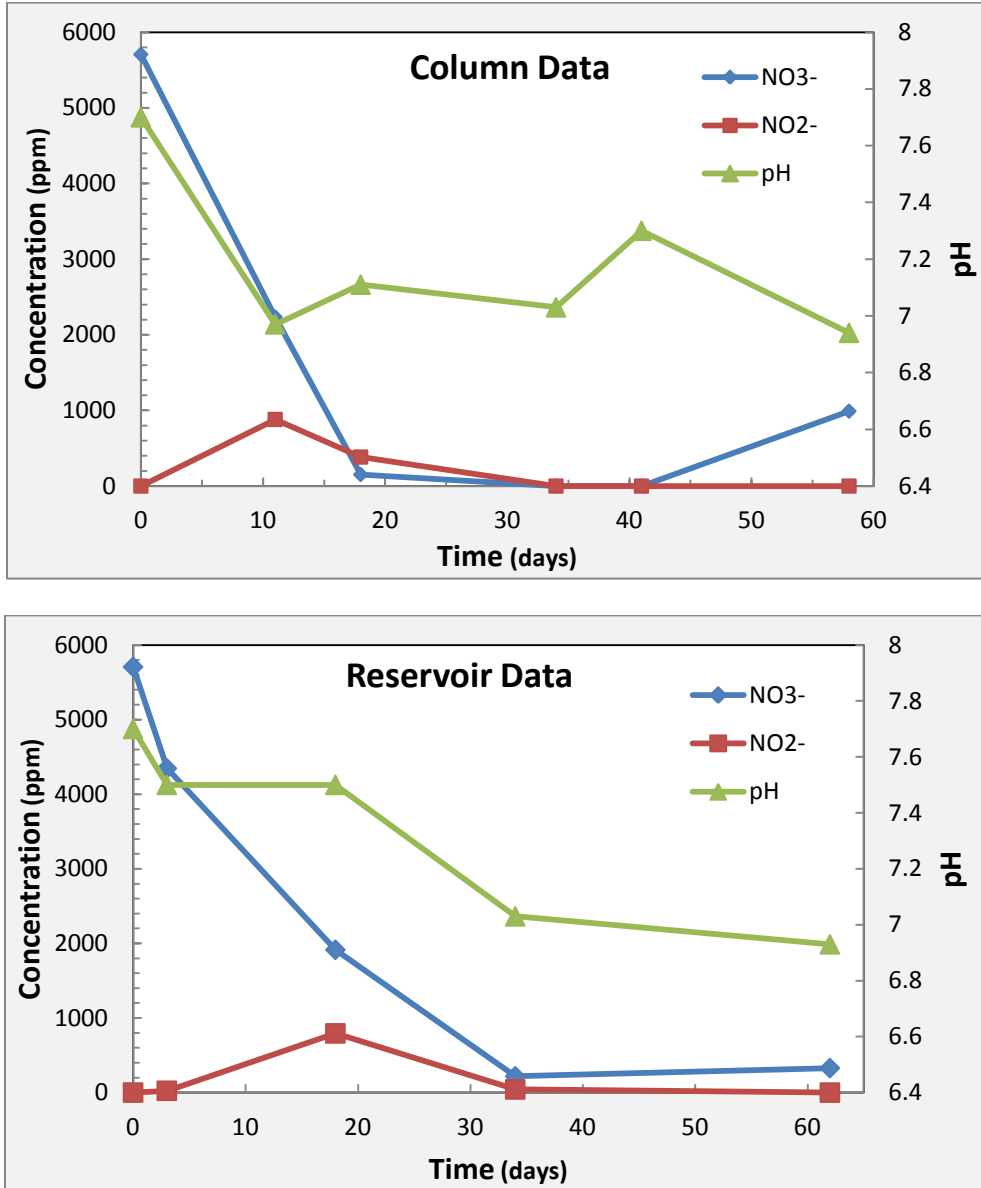
Figure 17- Changes in NO_3^-, NO_2^-, and pH Column Test 1.

The pH of a denitrifying MICP system is critical to precipitation of calcium carbonate. A general decline in pH is observed in all comparisons of pH and concentration in column and reservoir samples. At lower pH, NO_2^- can form

nitrous acid (HNO_2) which can be a strong inhibitor of denitrification (as previously discussed). On the other hand, if NO_2^- is ultimately reduced to N_2 (g), as indicated in these results, NO_2^- probably has only a transient inhibitory effect on denitrification. But, low pH can and does inhibit microbial denitrification. The drop in pH can be correlated to both CO_2 production and Ca^{2+} precipitation. The long-term decline in pH is probably not due to CO_2 production alone, since the drop in pH does not appear to follow the long-term trend in acetate oxidation. In the plots shown in Figures 17 and 18 scaled to the same time frames, acetate concentrations drop to near final lowest levels within 20 days, while pH shows a moderate drop over that period, but generally continues to decline for the next 40 days even as acetate concentrations have essentially stabilized. On the other hand, pH drop appears to be well-correlated with Ca^{2+} concentrations in both the immediate and long term, as shown in Figure 16. The precipitation of calcium carbonate is expected to result in a drop in pH, because it removes the most basic species of the carbonate system from solution. While the initial drop in pH may be due to the production of CO_2, the long term change in pH is mostly influenced by the precipitation of Ca^{2+}. However, the drop in pH is counter-acted by the reduction of NO_2^- to N_2 gas, which consumes H^+ (or acidity).
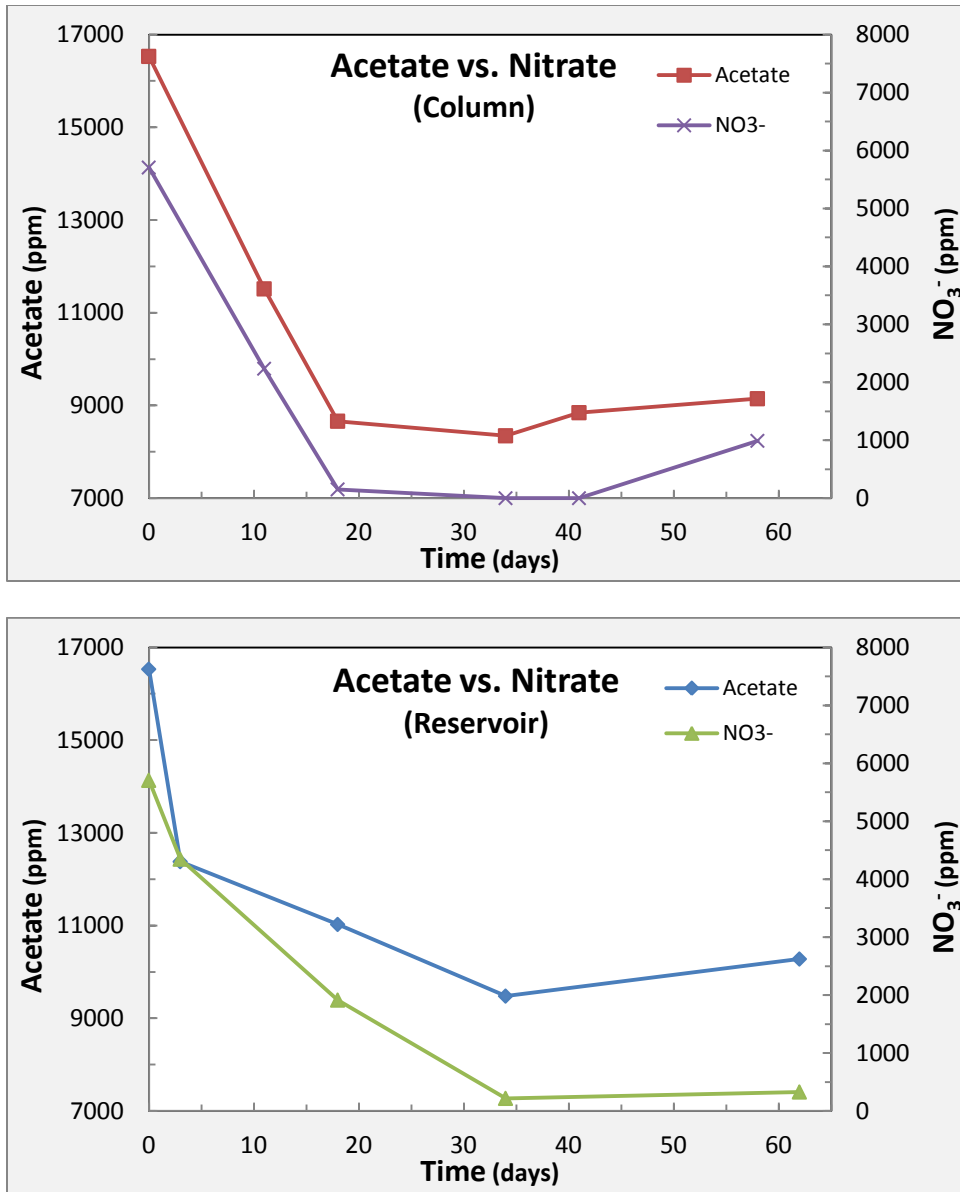
Figure 18- Acetate and NO_3^- concentrations Column Test 1.

Another finding from the first column experiment is that the consistent generation of biogas, presumably CO_2 and N_2, caused gas pressures in the sand column to reach 97.9 kPa (14.2 psi). Although the bench scale apparatus was designed to tolerate pressures in this range, the long-term effects (more than

several days) of maintaining such a pressure were unknown. As such, gas was occasionally vented into the reservoir head headspace. Gas pressures typically regenerated within a few days after venting, but typically did not exceed 48-68 kPa (7-10 psi) after venting from the highpoint of 97.9 kPa (14.2 psi). It is worth pointing out that the head space in the sand column was very small (on the order of 1-2 mL), so generation of small amounts of biogas could produce large pressure changes in the sand column. The reservoir had a comparatively large headspace (approximately 800 mL), as such, gas pressures in the headspace of the sand column typically ranged from 13-34 kPa (2-5 psi) after the column was vented.

Finally, after venting gas into the reservoir, the gas pressures in the reservoir appeared to slowly dissipate over several days, but never completely reach zero. There are at least three possible explanations for this: (1) the reservoir had a small leak, (2) the reservoir pressure gauge was faulty, or (3) the CO_2 was forming CO_3^- and precipitating into the mineral phase in the presence of calcium. The possibility of a leak cannot be ruled out, but the system was designed for and tested at much higher pressures. The reservoir pressure gauge was tested against the column gauge prior to experimentation and both gauges produced the same values. Even if the reservoir gauge was faulty in measuring correct values, the fact that gauge pressure increased when column biogas was introduced into the reservoir indicates that the gauge was at least capable of measuring relative pressure changes. The possibility that reservoir gas pressures slowly dropped due to carbonate precipitation is supported by the large presence of calcite in the

reservoir. Indeed, much of the calcite precipitated in the bench-scale apparatus was found in the reservoir.

5.4.2 Sand Column 2

The objective of the second sand column experiment was to run a simpler and less disturbed system then in the first sand column test in a manner that would (1) induce $CaCO_3$ precipitation in the sand column and (2) provide for a better understanding of the geochemical environment within the column (by sampling the column in layers). Upon termination of the experiment, white fine-grained precipitate was found in or on the tubing, pedestal and top cap, quick-disconnects, and the lower portion of the PVC bag. The sand column did not show signs of cementation, but did show signs of some sort of precipitation, i.e., an off-white grainy material was observed to have partially filled the pores. Furthermore, upon its disassembly, the sand column material exhibited a viscous behavior unlike typical wet sand. Wet laboratory testing indicated that the white fine-grained precipitate was a carbonate mineral, presumably calcium carbonate. Quantification of carbonate content of the sand column via acid digestion indicated that approximately 6 grams of $CaCO_3$ was precipitated in the column. Biogas was generated throughout the experiment as evidenced by the rise in fluid in tubing above the sand column and the occasional burst of gas bubbles into the PVC bag. The fluid rise in the tubing reached the PVC bag (approximately 254 mm (10") above the column) on at least one occasion. Several times during the experiment, accidental jarring of the sand column during sampling resulted in small but sudden gas releases into the PVC bag. The 1000-mL PVC bag was

slightly inflated by the end of the experiment, but no effort was made to quantify the amount or type of gas in the PVC bag or the sand column.

Figure 19 presents the averages of the ion and HPLC analysis results for the four fluid samples withdrawn from Column 2 during each sampling event. These data show large decreases in average concentrations of Ca^{2+}, NO_3^-, and acetate among the fluid samples collected during each sampling event.
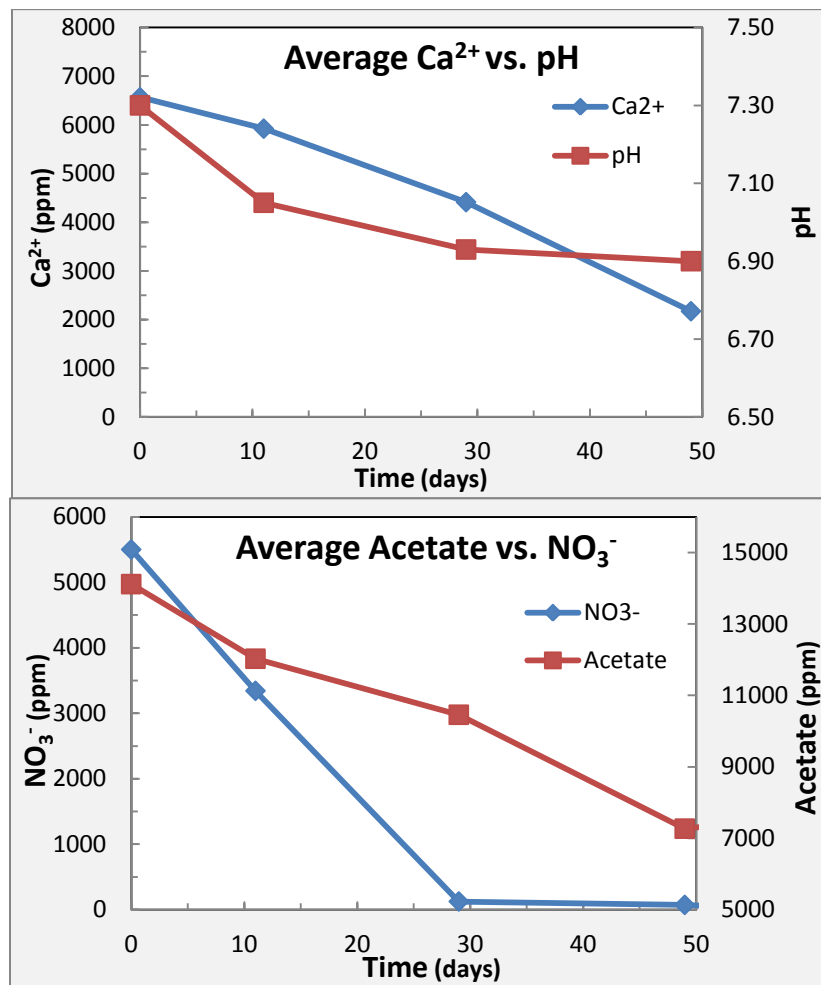
Figure 19- Average concentrations of Ca^{2+}, NO_3, and acetate during each sampling event.

The correlation between electron acceptor (NO_3^-) and donor (acetate) is consistent with expected results, as previously discussed. Again, the long-term decline in pH is probably not due to CO_2 production alone but is also related to Ca^{+2} content. The average pH reaches a minimum by the 29th day and remains at that value for the rest of the experiment, while acetate concentrations continue to decline.

Spatial variations in chemical concentrations and pH were observed in the static sand column as measured by the four liquid samples collected sequentially in each sampling event (the chemical composition of the columns is assumed to be approximately homogenous at the start of the experiment). The results from the first sampling event (t = 11 days), presented in Figures 20 and 21, indicate that large spatial variations in column geochemistry had developed. NO_3^- concentrations ranged from 274 mg/L at the top of the column (i.e. in the last of the four liquid samples obtained from the column) to over 5000 mg/L at the bottom (i.e. in the first liquid sample).

Calcium concentrations ranged from 2500 mg/L to over 7000 mg/L from top to bottom of the column, respectively. Spatial variations in pH were also observed across the column, ranging from 6.9 at the bottom of the column to over 7.4 at the top of the column. However, pH did not vary with calcium ion concentrations as previously suggested (where higher Ca^{2+} concentrations equated with higher pH).
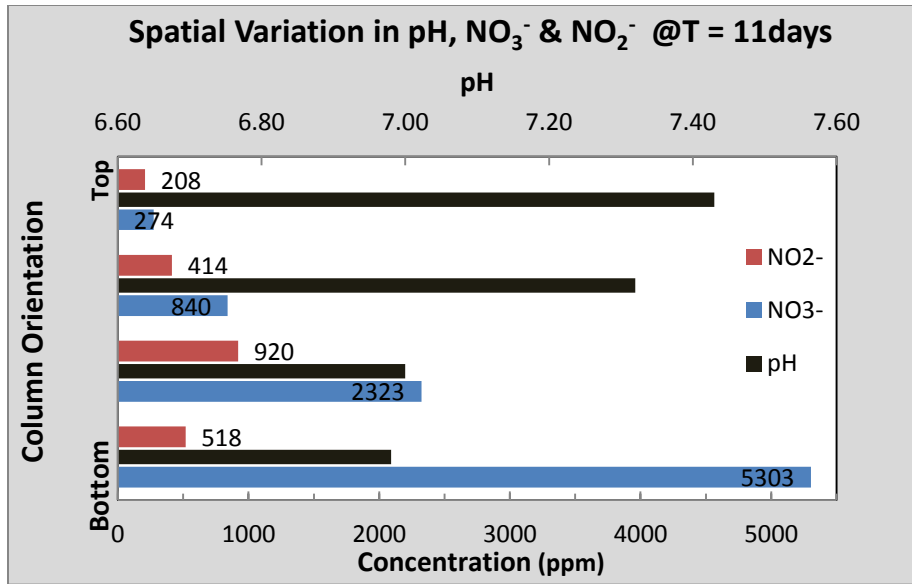
Figure 20- Spatial variations in NO_3^-, NO_2^-, and pH in Sand Column 2 at t = 11 days.
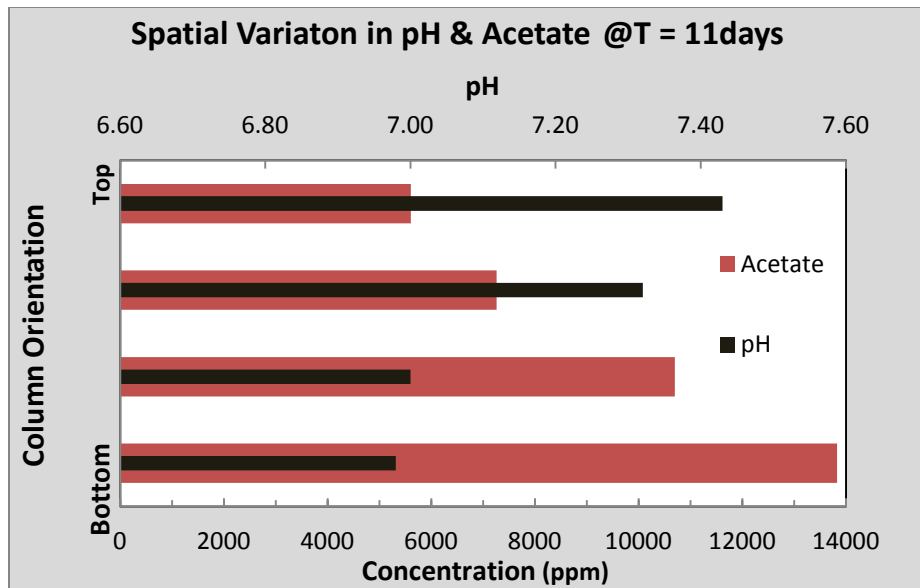
Figure 21- Spatial variations in acetate and pH at t = 11 days in Sand Column 2

The absence of a correlation between pH and calcium content may be related to the 2-ml of 36-mM monopotassium phosphate (KH_2PO_4) that was added to the

liquid medium to facilitate microbial growth. The addition of KH_2PO_4 followed by the addition of liquid medium resulted in a loose white precipitate that was observed in the clear tubing above the column upon first filling. It is possible that some of the Ca^{2+} may have been precipitated as calcium phosphate at the start of experiment and then pushed toward the top of the column upon filling. Calcium phosphate precipitation does not directly impact pH (i.e. lower) the way calcium carbonate precipitation does, but would lead to lower free Ca^{2+} concentration.

Testing was also conducted to evaluate variations in acetate concentrations in the sand column. The results of testing for acetate content at t = 11 days are shown in Figure 22. Acetate concentrations varied in the column from approximately 5,000 mg/L to nearly 14,000 mg/L from top to bottom.
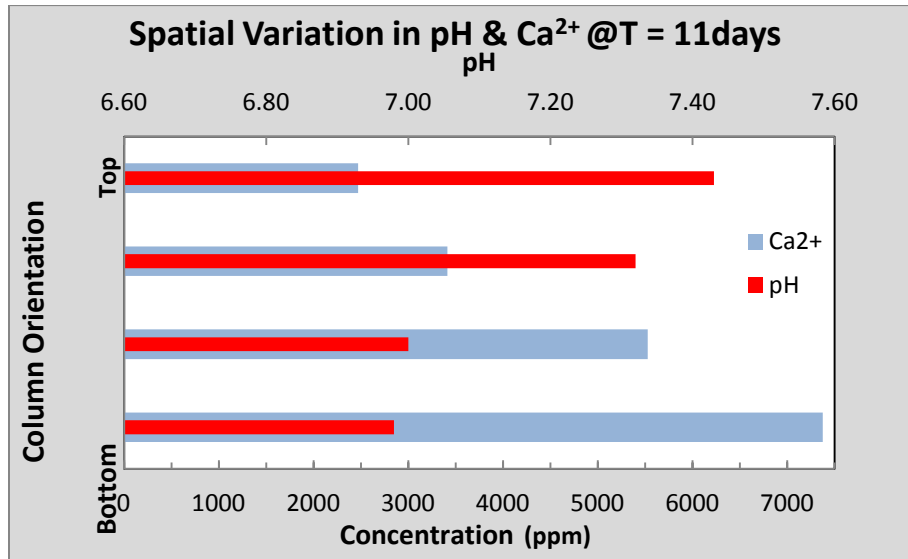
Figure 22- Spatial variations in Ca^{2+} and pH in Sand Column 2 at t = 11days.

The data in Figure 22 also show that pH remained high even as acetate was consumed. As previously suggested, assuming that organic carbon is converted to inorganic carbon (CO_2) rather than biomass, it appears that oxidation of acetate may not be the dominant factor in pH control.

Spatial variations in NO_3^-, Ca^{2+}, pH, and acetate for Column 2 were also plotted for t = 49 days. These variations are shown in Figures 23 to 25. The general trends observed for NO_3^-, NO_2^-, pH, Ca^{2+} and acetate at t = 11 days are also seen at t = 49 days, but the relative differences between the top and bottom of the sand column are much smaller at t=49 days. For example, comparing Figures 20 and 23 (t = 11 days vs. t = 49 day, respectively), NO_3^- concentrations ranged from 274 to 5300 mg/L at t = 11days, while at 49 days the concentrations ranged from 53 to 103 mg/L. A similar pattern is observed for the other measured values as well.
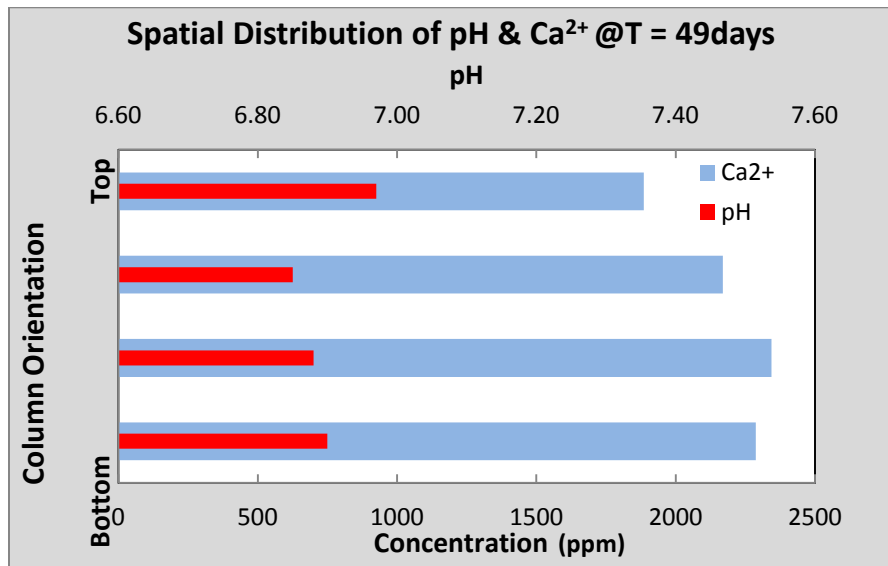
Figure 23- Spatial variations in Ca^{2+} and pH in Sand Column 2 at t = 49 days.
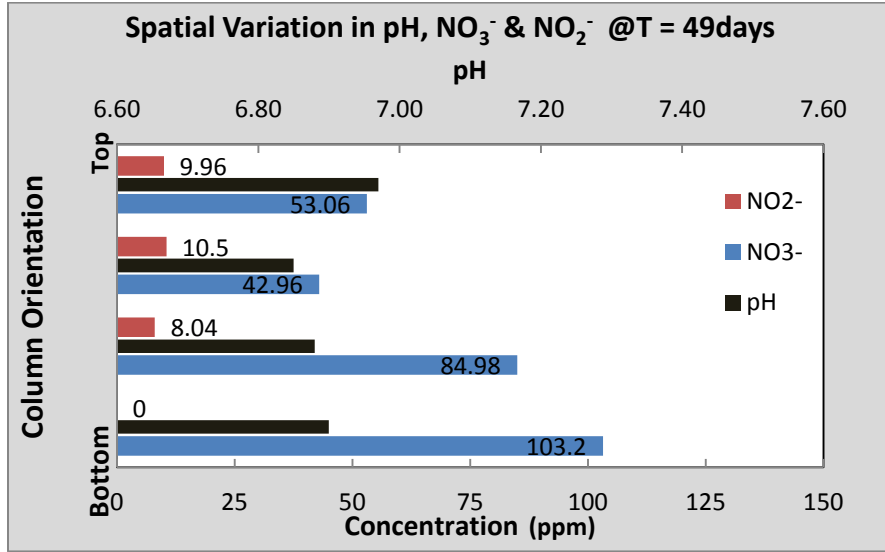
Figure 24- Spatial variations in NO_3^-, NO_2^-, and pH in Sand Column 2 at t = 49 days.
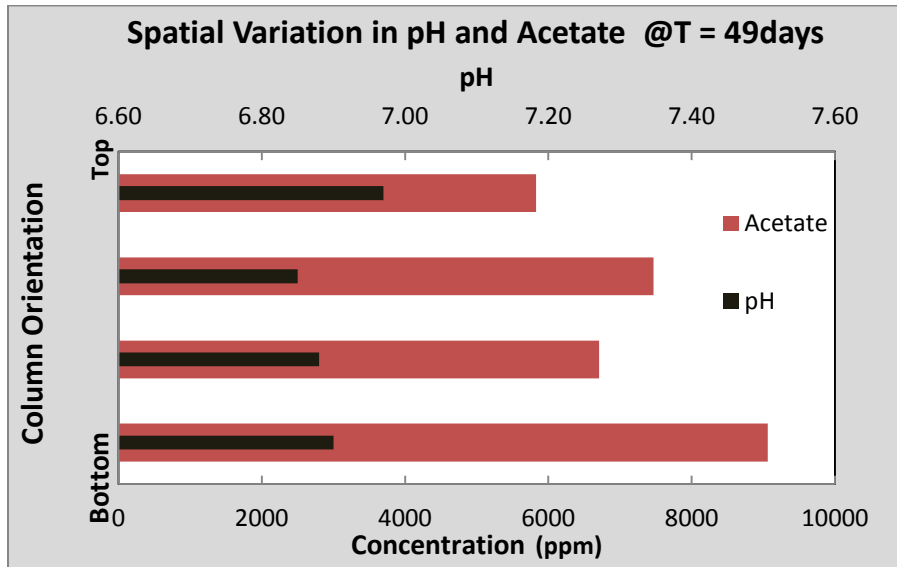
Figure 25- Spatial variations in acetate and pH in Sand Column 2 at t = 49 days

5.4.3 Sand Columns 3 through 7

The objective of the tests on Sand Columns 3 through 7 was to examine the effects of concentration, organic carbon donor type, and inoculum size on denitrification in static sand columns undergoing microbially induced calcium carbonate precipitation. The results of these experiments vary from one column to the next depending on the specific effect tested. A summary table of test conditions and general results for Sand Columns 3 through 7 are presented in Table 7.

Table 7- Summary of test conditions and general results for effects tested in Sand Columns 3 through 7.

Column #	Organic Carbon Donor Type	Inoculum Size (mL)	Carbonate Precipitate in Column (g)	Approx. Ionic Strength (mM)	Biogas	Effect Tested
3	Acetate	40	4	530	Yes	High ionic strength/concentration
4	Acetate	80	4	440	Yes	Large inoculum size
5	Acetate	40	5	350	Yes	Low ionic strength/concentration
6	Ac-Glu Mix	40	6	380	Yes	Organic carbon donor type
7	Ac-Glu Mix	40	-	380	Yes	Organic carbon donor type

See Table 4 for liquid medium composition and concentrations. "Ac-Glutamate Mix" is 70% acetate and 30% glutamic acid mixture. Inoculum $OD_{600} = 0.707$. Sand column #5 could not be quantified due to accidental sample loss. Ionic strength (I) adjusted for inoculum size, water dilution and glutamte substitution (I=80 mM). Liquid medium I=578 mM (adjusted up for magnesium sulfate heptahydrate).

Table 8- Summary of approximate initial concentrations and pH in Sand Columns 3 through 6.

Column #	NO$_3^-$ (ppm)	NO$_2^-$ (ppm)	Ca^{2+} (ppm)	Acetate (ppm)	pH
3	5800	150	6600	14600	7.50
4	4900	130	5500	12100	7.50
5	4000	100	4500	9900	7.50
6	4000	100	4500	9900	7.50

All of the columns in this test series appeared to have substantial amounts of predominantly white, fine-grained precipitate deposited in the lines and PVC bags. Although the presence of a carbonate mineral in this precipitate was verified through acid digestion, no effort was made to determine the mineral phase of the white precipitate material. All columns generated biogas as evidenced by prominent rounding/expansion of the flexible rubber caps and, in some cases, bulging of the bottom rubber cap. Biogas was allowed to vent into 1000-mL PVC bags once during the first two weeks due to excessive bulging. Although no effort was made to quantify the biogas produced, gas generation appeared to rapidly decline by the fourth week. Upon disassembly, the sand columns were almost entirely un-cemented except for the few small weakly cemented fragments shown in Figure 26 from Column 5. But, the columns were not expected to be cemented since the maximum possible amount of carbonate that could have been formed in the most calcium concentrated column, Column 3 (containing approximately 330 mL of liquid medium at 7400 mg-Ca/L before inoculation), is between 5 and 6 grams. The test columns contained 1000 grams of sand on average; so this carbonate content corresponds to approximately 0.5-0.6%, which is well-below

the threshold minimum for strength improvement (3.6%) identified by Whiffin et al. (2007) and discussed in Section 4.3.

Figure 26- Example of weakly cemented chunks of sand observed in Sand Columns 3 through 6 after rinsing and drying (Sand Column 5 shown).

Figures 27 through 33 show normalized plots based on *different* initial concentrations. Precise quantitative interpretations of these figures, as well as other normalized plots in this chapter, require knowledge of the initial concentrations presented in Table 8. Information derived from these normalized plots is based on initial concentrations. The results from tests conducted to assess the potential effect of inoculum size on Columns 3 through 7 are presented in Figures 27 through 29.
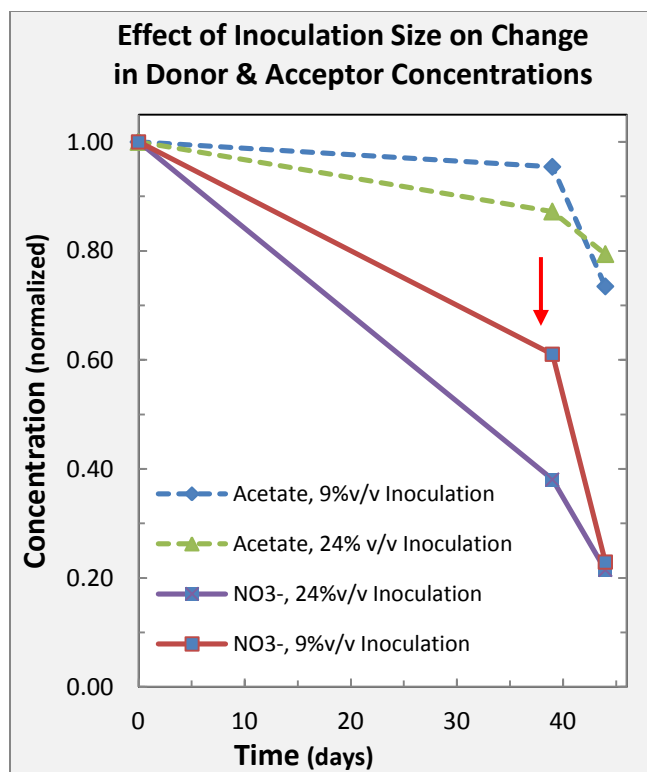
Figure 27- Effect of an inoculation size on electron donor (acetate) and acceptor (nitrate) concentrations in Sand Column Tests 3 and 4. Arrow indicates addition of 2-mL NaOH (1.58E-4 moles OH⁻) after sampling was completed.

The trends in NO_3^- reduction, NO_2^- production, and acetate oxidation agree with those observed in previous experiments, indicating that denitrification was occurring in the sand columns. At an inoculum size of 24%, NO_3^- and NO_2^- concentrations were approximately one-half of those at an inoculum size of 9%. Inoculum size appeared to have small effect on organic carbon donor concentration, suggesting that carbon donor concentration was not a limiting factor in these experiments. In addition, it appears that pH is not closely linked to the oxidation of organic carbon regardless of inoculum size since acetate concentrations, shown in Figure 27, decrease to only about 90-95% of the initial

values by the day 39, but pH values, shown in Figure 29, have reached their lowest point at this time. The red arrows in Figures 27 through 29 indicate the addition of 2-mL NaOH (1.6×10^{-4} moles) *after* sampling on day 39. The addition of NaOH had a dramatic effect on all measured chemical constituents in the sand columns. In terms of percentage change over time, it appears that the effect caused by NaOH produced a greater change in 9 days than the previous 40 days of uninterrupted denitrification. This implies that pH is a major factor affecting denitrification in the highly concentrated solutions required for effective MICP.
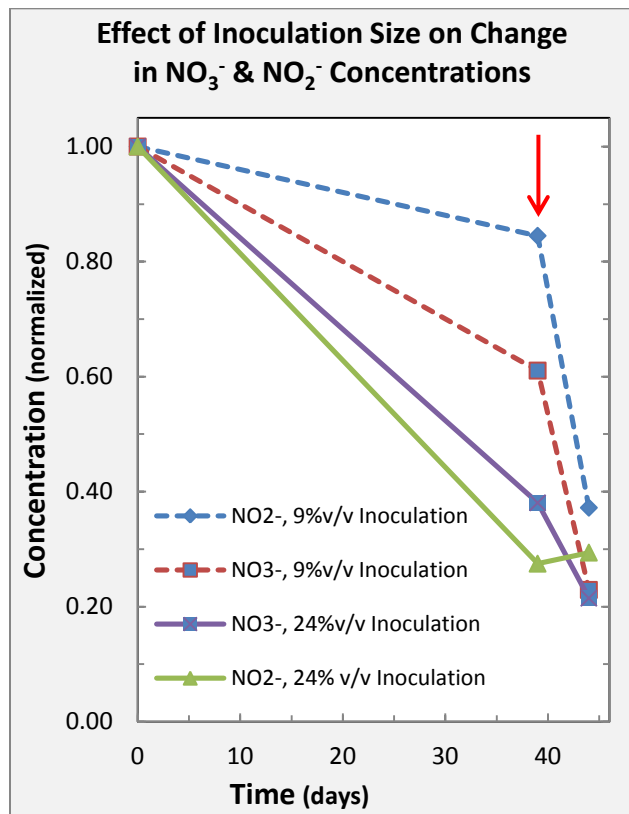
Figure 28- Effect of an inoculation size on the change in NO_3^- and NO_2^- concentrations in Sand Column Tests 3 and 4. Arrow indicates addition of 2-mL NaOH (1.58E-4 moles OH$^-$) after sampling was completed.
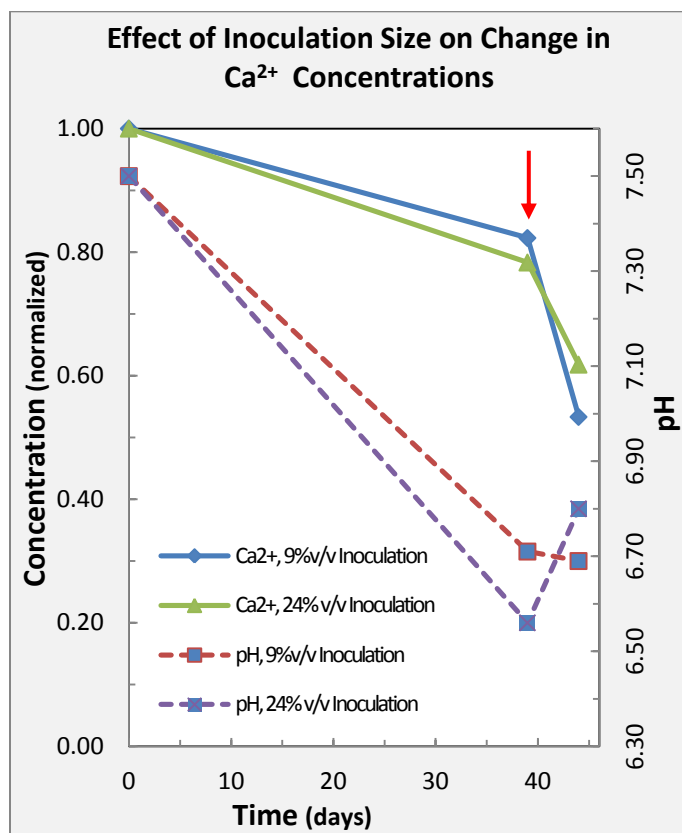
Figure 29- Effect of an inoculation size on the change in Ca^{2+} concentrations and pH in Sand Column Tests 3 and 4. Arrow indicates addition of 2-mL NaOH (1.6x10^{-4} moles OH$^-$) after sampling was completed.

The results from tests conducted to assess the potential effect of initial concentration of liquid medium are presented in Figures 30 through 32. At a lower initial concentration (i.e., lower ionic strength), NO$_3^-$ concentrations were nearly zero by the first sampling event, as illustrated by the data in Figures 30 and 32, and NO$_2^-$ appeared to be reduced immediately after it was generated. A lesser but substantial effect was observed for the organic carbon donor, as shown in Figure 30. Calcium precipitation was also affected by initial concentration, as shown in Figure 31, while pH changes did not appear to be directly impacted by

initial medium concentration. The calcium precipitation rate was substantially greater in the low strength medium than the concentrated liquid medium. Based upon these results, initial medium concentration, or ionic strength, appears to be a significant factor affecting denitrification for MICP.
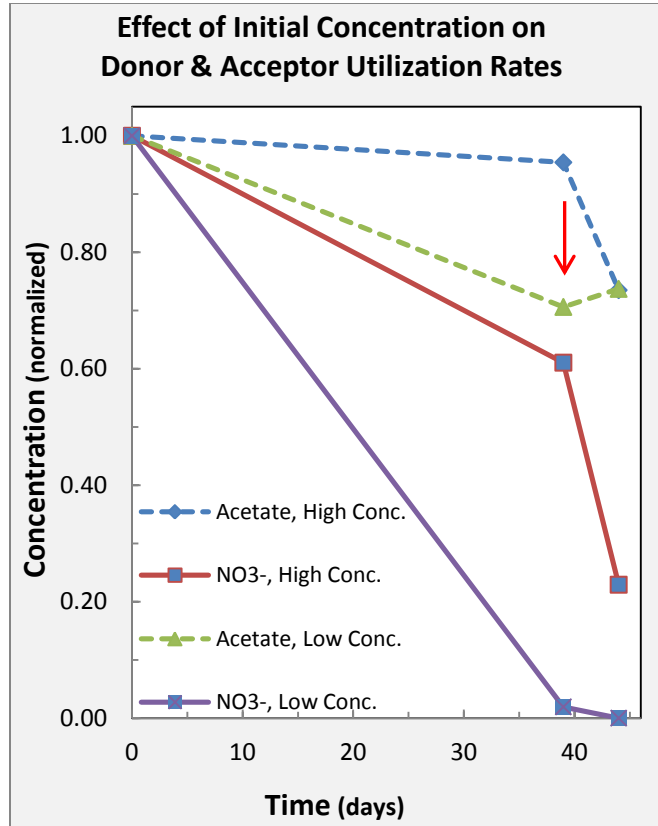
Figure 30- Effect of initial concentration of liquid medium on the change in organic carbon and NO_3^- in Sand Columns 3 and 5. Arrow indicates addition of 2-mL NaOH ($1.6x10^{-4}$ moles OH^-) after sampling was completed.

The red arrows in Figures 30 through 32 indicate the addition of 2-mL NaOH ($1.6x10^{-4}$ moles) *after* sampling on day 39. The addition of NaOH had a dramatic effect on all measured chemical constituents in the concentrated

medium, but little effect on the low strength medium since denitrification was

complete or nearly complete.
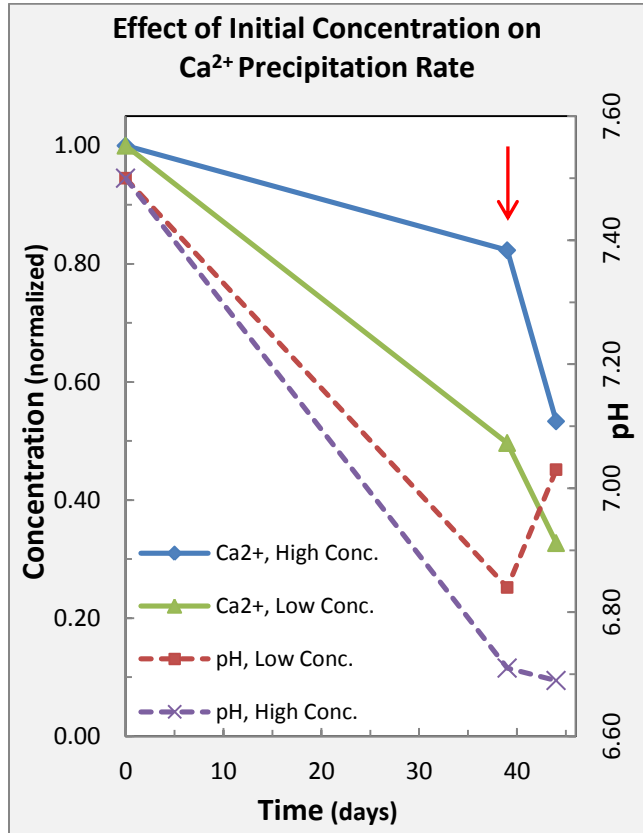
Figure 31- Effect of initial concentration of liquid medium on the change in Ca^{2+} precipitation rate and pH in Sand Columns 3 and 5. Arrow indicates addition of 2-mL NaOH (1.6×10^{-4} moles OH^-) after sampling was completed.
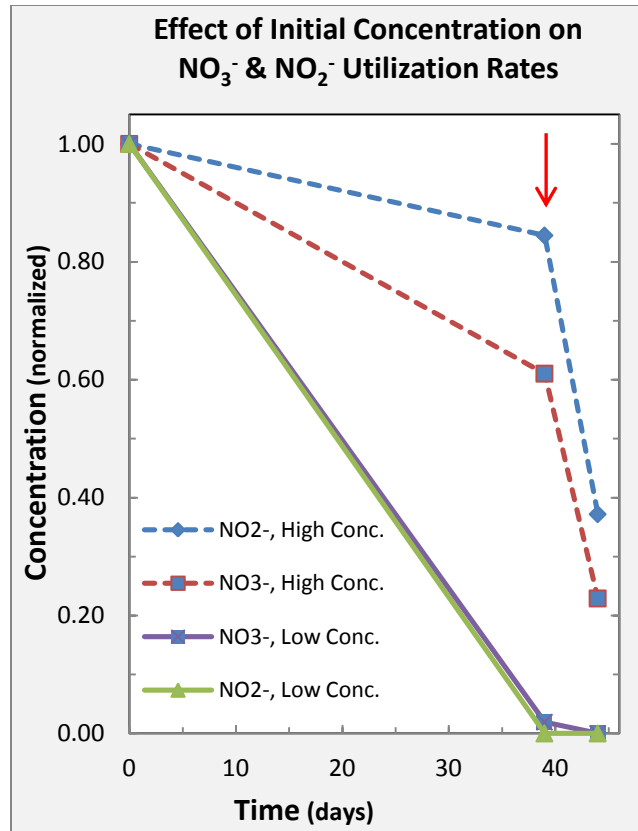
Figure 32- Effect of initial concentration of liquid medium on NO_3^- and NO_2^- utilization rates in Sand Column Tests 3 and 5. Arrow indicates addition of 2-mL NaOH (1.6×10^{-4} moles OH^-) after sampling was completed.

The results from tests conducted to assess the potential effects of organic carbon donor type are presented in Figure 33. There were no significant effects of carbon donor type observed on NO_3^-, NO_2^-, and Ca^{2+} concentrations or on pH. It is possible that a glutamate-acetate organic carbon medium simply has no effect on denitrification and MICP. An alternative explanation for the results could be the ionic strength of the liquid medium. The ionic strength of the glutamate-acetate liquid medium was 380 mM (Table 7), compared to 350 mM for the "low strength" liquid medium used in the previous experiment and 530 mM used for

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the full strength medium (Table 7). Recall from above that the effect of a low concentration/strength medium had the greatest positive impact on denitrification (i.e. increases denitrification rate), therefore the results obtained in the glutamate-acetate experiment may be a result of ionic strength effects rather than just organic carbon donor type.
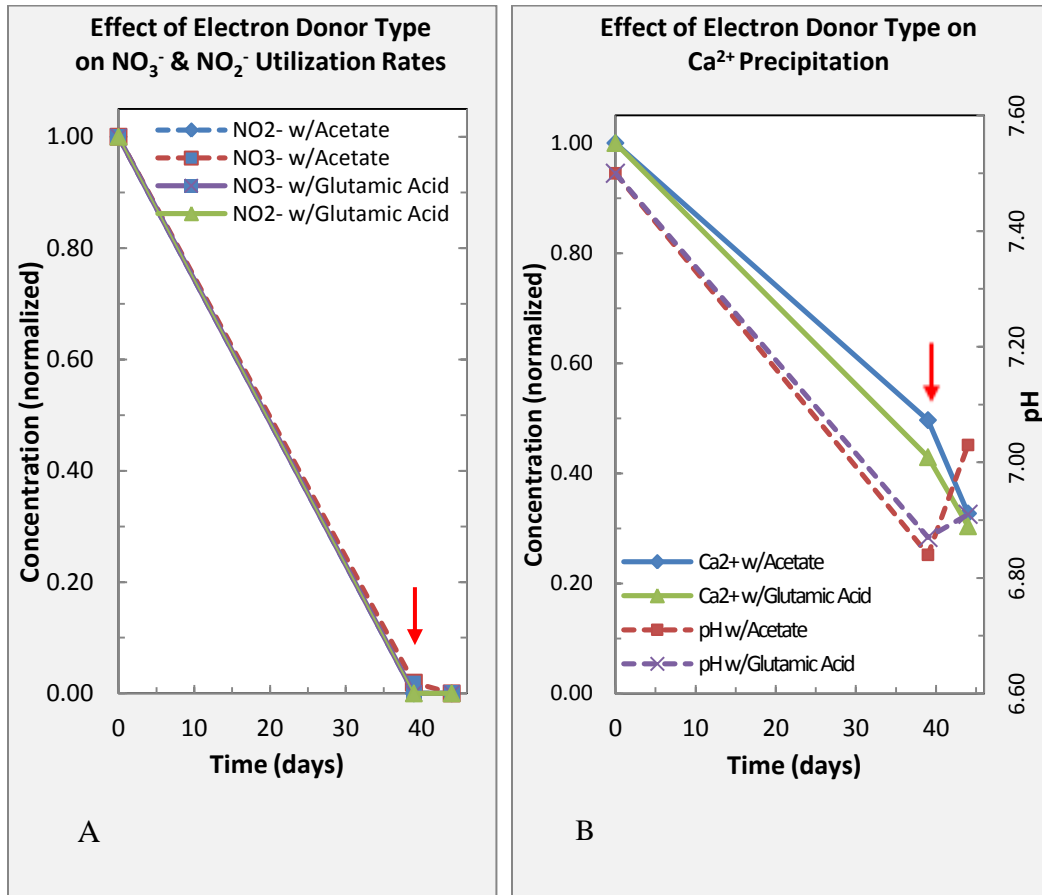
Figure 33- Effect of organic carbon (electron donor) type on (a) NO_3^- and NO_2^- utilization rates and (b) Ca^{2+} and pH in Sand Column Tests 6 and 7. Arrow indicates addition of 2-mL NaOH (1.6×10^{-4} moles OH^-) after sampling was completed.

The addition of NaOH had a dramatic effect on all measured chemical constituents in the concentrated medium, but little effect on the low strength medium, since denitrification was complete or nearly complete in the low strength medium columns. Standard plots showing changes in NO_3^- and Ca^{2+} concentrations, rather than normalized values, are presented in Figure 34. A summary of the test conditions was shown above in Table 7.
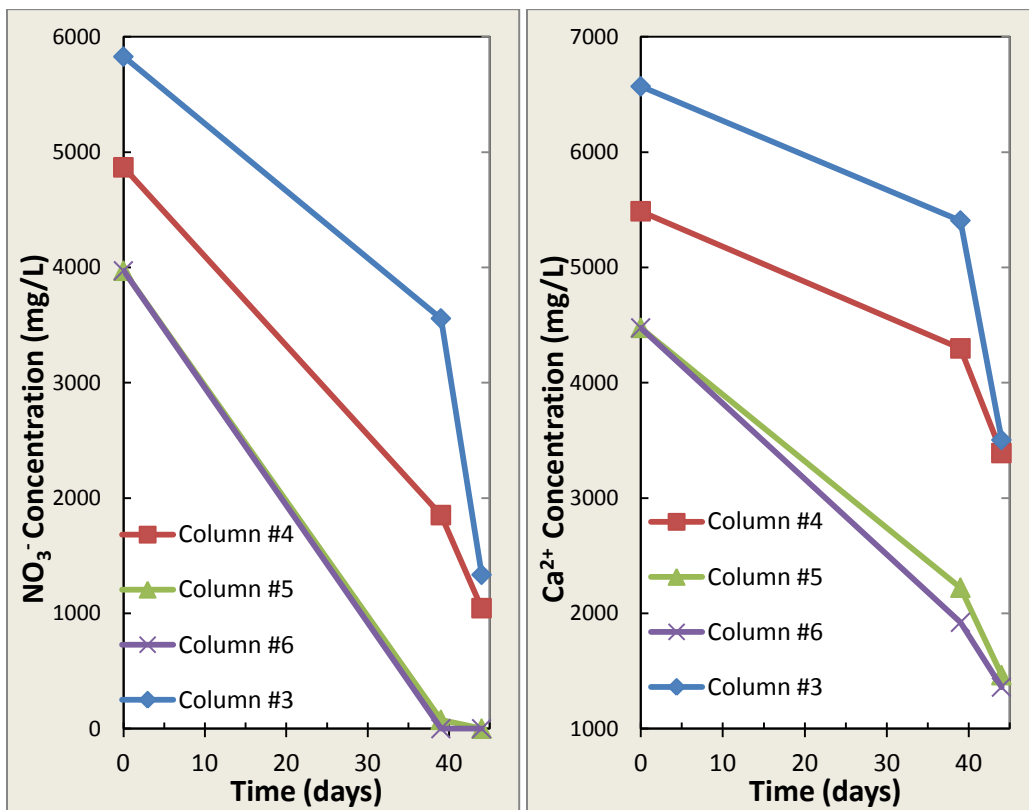
Figure 34- Changes in NO_3^- and Ca^{2+-} concentrations due to effects of concentration, organic carbon donor type, and inoculum size on denitrification. A summary of test conditions was presented in Table 7.

Columns #5 and #6 had the lowest concentrations of chemical constituents, while Column #3 had the highest concentration. Recall that Column #6 contained

glutamic acid in addition to acetate since it tested the effect of organic donor type, while all other columns used acetate as the sole organic carbon donor. Column #4 had an intermediate concentration in addition to a large inoculum size of 80-mL, versus 40-mL for all other columns. Columns #5 and #6 showed the greatest rates of NO_3^- reduction. Column #3 had the lowest rate of NO_3^- reduction, and Column #4 showed an intermediate rate. Changes in Ca^{2+} concentrations follow a similar pattern to that observed for NO_3^-. Overall, it appears that NO_3^- reduction and Ca^{2+} precipitation proceeded quicker in less concentrated liquid mediums.

The organic carbon (acetate) utilization rates for Columns #3 through #6 are shown in Figure 35. The oxidation rate of acetate is greatest in columns containing the least concentrated mediums (#5 and #6), and slowest in the highest concentrated medium (Column #3). Note that the acetate concentration in Column #6 is low (i.e., equally dilute as Column #5), yet appears to be consumed at a much lower rate than the nearly equally dilute Column #5. This anomalous behavior may be explained by the fact that Column #6 also contained glutamate, a mixed organic donor medium, and therefore may have undergone either simultaneous or preferential oxidation of glutamate in the presence of acetate. Similar to the other main chemical constituents NO_3^- and Ca^{2+}, organic carbon utilization appears to proceed quicker in less concentrated liquid mediums.
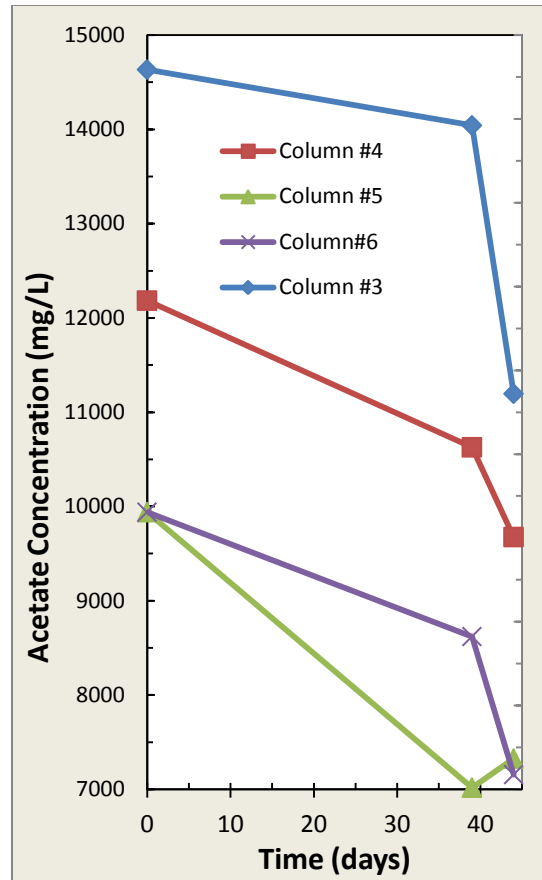
Figure 35- Acetate utilization rates for Columns #3 through #6. A summary of test conditions was presented in Table 7.

Mass balance and alkalinity analyses were also conducted for Columns #3 through #6. A mass balance on carbon through acetate oxidation is presented in Table 9. Sand Column #6 contained a mixture acetate and glutamate as the organic carbon donors. Only acetate was quantified in sand Column #6; therefore, the carbon mass balance is a low estimate, and the amount of $CaCO_3$ estimated on carbon should be a minimum value as glutamate may contribute inorganic carbon to this system. "Estimated" amounts of $CaCO_3$ are based on carbon mass balance, and "digested" amounts are based on actual acid digestion of mineral precipitates

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as described in Section 5.2.2 on Methods. The results of the mass balance evaluation on carbon show a lower than expected amount of $CaCO_3$ in Columns #4 and #6 than determined through acid digestion. Since the carbon balance is based only on acetate, rather than both acetate and glutamate, the expected (calculated) amount of $CaCO_3$ in sand Column #6 is lower. All columns received a bacterial inoculum that was grown in a carbon rich nutrient broth. Columns #4 received a large inoculum ($\approx$80 mL) that corresponded to nearly 24% of the total liquid volume in the column. It is possible that the nutrient broth contributed substantial amounts of organic carbon that was utilized by the microbes, but not accounted for in this analysis. Column #5 received a smaller inoculum ($\approx$ 40 mL) that corresponded to approximately 12% of the total liquid volume.

Table 9- Mass balance on carbon via acetate oxidation in Sand Columns 3 through 6.

Balance on Carbon (acetate)						
Column	Δ Acetate (mg/L)	Total Carbon (mg)	% Δ Acetate (of initial conc.)	Max. CO_2 Formed (mg)	Estimated $CaCO_3$ (g)	Digested $CaCO_3$ (g)
#3	3868	519	27	1901	4.32	**4**
#4	2508	337	21	1235	2.80	**4**
#5	2613	351	26	1286	2.92	**5**
#6	2784	374	28	1370	3.11*	**6**

Asterisk indicates that this sand column contained both acetate and glutamate, but that only acetate was quantified here.

A mass balance on carbon through acetate and glutamate oxidation is presented in Table 10. The actual amount of glutamate degraded in sand Column #6 is unknown. As such, hypothetical amounts of 25% and 75% of the initial concentration (160 mM glutamate) were chosen to illustrate the impact of glutamate oxidation.

Table 10- Mass balance on carbon via acetate and glutamate oxidation in Sand Column #6. Actual acetate oxidation and hypothetical amounts of glutamate (25% and 75%) were chosen to illustrate the impact of glutamate oxidation.

Balance on Carbon w/Acetate & Glutamate (hypothetical values)							
Column	Δ Acetate (mg/L)	Δ Glutamate (mmoles)	Total Carbon (mg)	Percent Δ (of initial concentration)		Max. CO$_2$ Formed (g)	Estimated CaCO$_3$ (g)	Digested CaCO$_3$ (g)
				Acetate	Glutamate			
#6	2784	3.5	584	28	25	2.14	4.86	6
		10.5	1004		75	3.69	8.36	
Column #6 is a 70% acetate to 30% glutamate (v/v) mixture. Correcting for inoculation volume, ≈ 87 mL glutamate was added at 160 mM = 0.014 moles glutamate (FW=147.13g/mol, C$_5$H$_9$NO$_4$). The changes in glutamate of 25% and 75% are hypothetical values.								

Mass balances on calcium and nitrogen are presented in Tables 11 and Table 12, respectively. Carbon mass balance values in Tables 11 and Table 12 were taken from Table 9. The results of mass balance on calcium show a lower than expected amount CaCO$_3$ in Columns #3 and #6 than determined through acid digestion. It is unclear why this discrepancy is particularly large in sand Columns #5 and #6. One possibility is the potential loss of calcium through insoluble, non-mineral precipitates such as calcium phosphate that solubilized upon digestion (i.e., acidification).

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Table 11- Mass balance on calcium for Columns #3 through #6. Asterisk indicates that this sand column contained both acetate and glutamate, but that only acetate was quantified here. See Table 10 for carbon balance results incorporating hypothetical values of glutamate oxidation.

			Balance on Calcium			
Column	ΔCa^{2+} (mg/L)	**Calcium** (mg)	$\%\Delta Ca^{2+}$ (of initial conc.)	**Estimated CaCO$_3$** (g)	**Estimated Inorganic Carbon** (mg)	**Digested CaCO$_3$** (g)
#3	4493	1483	77	3.71	73.4	**4**
#4	3823	1262	78	3.15	41.3	**4**
#5	3971	1310	100	3.28	42.9	**5**
#6	3971	1310	100	3.28	19.9*	**6**

Estimated inorganic carbon (C_T) = Carbon Released via Acetate Oxidation - Carbon Precipitated in CaCO$_3$; C_T = CO$_2$ and speciation products; Carbon released via acetate oxidation taken from "Total Carbon" column in carbon balance table.

Table 12- Mass balance on nitrogen for Columns #3 through #6.

	Balance on Nitrogen		
Column	$\Delta NO_3^-+NO_2^-$ (mg N/L)	**N$_2$ Formed** (mg)	$\%\Delta NO_3^-+NO_2^-$ (of initial conc.)
#3	4590	342	23
#4	3913	292	29
#5	4076	304	100
#6	4075	303	100

In addition, another possible reason for the general inconsistencies between expected and the digested amounts $CaCO_3$ observed in any of the mass balance calculations may be that the fluid samples that were analyzed were taken from the bottom port of the sand columns and may not adequately represent the overall concentration.

The mass balance results presented above in Tables 9, 11, and 12 were used to estimate alkalinity in Table 13. The results indicate that alkalinity decreased in all sand columns, as well as pH, and are consistent with expected values in a system actively precipitating $CaCO_3$.

Table 13- Alkalinity estimates in Columns #3 through #6.

Column	Calcium Precipitated (mg)	N$_2$ Formed (mg)	N$_2$ (mole/L)	Ca^{2+} (mole/L)	Δ Alkalinity (eq/L)	Initial pH	Final pH
#3	1483	342	0.037	0.112	-0.187	7.50	6.69
#4	1262	292	0.032	0.095	-0.159	7.50	6.80
#5	1310	304	0.033	0.099	-0.165	7.50	7.03
#6	1310	303	0.033	0.099	-0.165	7.50	6.91
Calcium precipitated and N$_2$ formed in 330 mL, quantities adjusted to liters. $\Delta Alk\ (eq/L) = \Delta NO_3^- - 2\Delta Ca^{2+}(mol/L); NO_3^- \rightarrow N_2\ \&\ Ca^{2+} \rightarrow CaCO_3$							

5.5 Conclusion

This chapter investigated microbial denitrification in static and flow-through sand columns. The microbial denitrification experiments in static and flow-through sand columns presented in this chapter provide a greater understanding of MICP through denitrification for ground improvement applications. These sand column experiments have illustrated a variety of factors that affect microbial

denitrification for MICP, including microbial biomass, the geochemical environment (both microenvironments and bulk solution), the organic carbon source, and flow conditions (i.e. static vs. flowing).

In the first experiment (Sand Column 1), which employed a flow through column, calcium carbonate precipitation was induced in the reservoir and overall apparatus. It was concluded based upon visual inspection that no carbonate had precipitated in this column, though carbonate could have potentially formed in the column at quantities and/or in a manner not visually observable. As visual inspection is unreliable for detecting small amounts of mineral precipitate in a comparatively large soil sample, this method of detection was not used in subsequent column tests. The results from this first experiment led to the design of static sand columns to (1) estimate micro and bulk geochemical environments in sand columns undergoing denitrification and (2) to examine the potential effects of initial biomass, chemical reaction medium, and organic carbon type on denitrification in sand columns.

The second experiment (Sand Column 2) employed a static sand column. Estimates of the micro and bulk geochemical environments in this test suggest that large vertical chemical gradients develop from initially homogenous solutions. Results from this test also indicate that chemical gradients diminish over time. Nitrate NO_2^- concentrations were consistently lower at the top of the sand column than at the bottom and pH was always higher at the top of the column than at the bottom (based upon sequential extraction of liquid from the specimen). Based on the differences in NO_3^- and NO_2^- concentrations and pH in

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the sand column in these experiments, it appears that denitrification may be more active in certain areas over others in the short term.

In the third experiment, in which a set of five columns were tested simultaneously (Sand Columns 3 through 7), the rate of denitrification was affected by the amount of microbial biomass injected into the sand columns. A sand column with a larger inoculum size exhibited a much greater rate of NO_3^- and NO_2^- reduction than similar columns that received less inoculum. Inoculum size did not appear to affect pH or Ca^{2+} concentrations. The mixed organic carbon donors used in two of the columns (Sand Columns 6 and 7) also did not appear to have an effect on denitrification. Although a liquid medium containing more denitrifiers is expected to reduce total NO_3^- in a shorter time, the medium also exhibited less NO_2^- accumulation (a known denitrification inhibitor). Controlling microbial inhibition may be especially important in static/non-flowing denitrifying environments where accumulation of intermediates could be difficult to mitigate.

The results presented in this chapter suggest that denitrification can and does proceed in sand columns filled with concentrated liquid mediums containing organic carbon, nitrate, and calcium, albeit at a slower rate than in dilute liquid mediums. Sand columns using low concentration liquid mediums showed higher rates of denitrification than columns using high concentration liquid mediums. For example, in two sand columns comparing the effects of initial liquid medium concentration (Columns 3 and 5), NO_3^- and NO_2^- were nearly depleted within 39 days in a low concentration liquid medium , while more than 60% of the initial

concentration of NO_3^- remained in the high concentration medium with persistent

levels of NO_2^-. Rapid NO_3^- reduction in the low strength liquid medium was

coupled with the highest rate of acetate (organic carbon) oxidation observed in the

sand column tests. Calcium precipitation rate, potentially a particularly important

factor in $CaCO_3$ precipitation and the application of MICP for ground

improvement, was greatest in the low strength medium. Furthermore, the high

Ca^{2+} precipitation rate observed in the low strength medium had a small but

consistently greater pH than the high strength medium. It was observed in Sand

Columns 1 and 3-7 that precipitation of Ca^{2+} is a significant factor affecting pH,

as a decrease in pH was typically associated with a decrease in Ca^{2+}

concentrations. Nitrate and NO_2^- reduction also plays an important role in Ca^{2+}

precipitation and pH control, since denitrification, the reduction NO_2^- in

particular, consumes H^+ and thereby increases pH. This would imply that Ca^{2+}

precipitation should be greatest in systems with highest NO_3^- reduction rates and

least NO_2^- inhibition.

The pH of a denitrifying environment appears to be an especially

important factor in determining the amount of Ca^{2+} that precipitates from solution

(presumably as $CaCO_3$) and the extent of denitrification (i.e. complete vs.

inhibited). The effect of pH on denitrification and Ca^{2+} concentration was

overwhelmingly evident when a small amount of NaOH was added to Sand

Columns 3-7. The addition of NaOH had a dramatic effect on all measured

chemical constituents in Sand Columns 3-7. In terms of percentage change over

time, it appears that the effect caused by NaOH produced a greater change in all

chemical constituents in 9 days than the previous 40 days of uninterrupted denitrification. This implies that pH is a major factor affecting denitrification and may be critically important in the highly concentrated solutions required for effective MICP in ground improvement applications relative to typical concentrations in water treatment applications.

Finally, another observation in all of the sand column experiments described herein is the consistent generation of biogas, presumably CO_2 and N_2. Biogas pressures in Sand Column 1 reached 97.9 kPa (14.2 psi). As the excessive gas pressures in Sand Column 1 required venting, even greater pressures may have developed over time if the column was left undisturbed assuming the microbes could tolerate these pressures. Gas pressures in Sand Column 1 typically regenerated within a few days after venting, but typically did not exceed 48-68 kPa (7-10 psi) after venting from the highpoint of 97.9 kPa (14.2 psi). In Sand Columns 3-7, biogas was occasionally allowed to vent into gas reservoirs during the first two weeks due to excessive bulging of the top caps for the columns. However, gas generation appeared to rapidly decline by the end of this experiment possibly due to the lack of fresh liquid medium. It should be noted that gas generation is a potentially beneficial effect in the application of MICP for mitigation of earthquake-induced liquefaction, as desaturation of pore water is in and of itself a liquefaction mitigation technique.

In addition to the conclusions discussed above, the results of these experiments (and from Sand Column 1 in particular) appear to suggest that high rates of flow for the pore fluid may be detrimental to MICP via denitrification.

High flow rates may either inhibit precipitation or nucleation in the pores of a sand column or may sweep the carbonate out of the pores after it precipitates. This could have particularly important implications for practical applications of this technology in an environment where groundwater velocities approach those in Sand Column 1. Another potential factor affecting mineral precipitation is the amount of liquid medium/chemical matrix that is in contact with the soil. The specific soil surface area (the surface area of a soil particle type exposed to its surroundings) is limited, therefore fluid-soil contact is also limited.

CHAPTER 6

CONCLUSION

6.1 Summary

The applicability of MICP for soil improvement through denitrification was assessed in *Chapter 2* of this thesis. A theoretical review bolstered by preliminary research conducted at ASU has shown that MICP may be employed to improve the physical properties of granular soils for geotechnical engineering applications. Some of these geotechnical applications include enhancing soil stability, improving foundation performance, mitigating soil liquefaction, and controlling groundwater. These improvements may be realized by carbonate cementation of soil particles and/or carbonate precipitation in soil pore space. Liquefaction mitigation may also be realized by desaturation through biogas production. As outlined in *Chapter 2*, denitrifying bacteria are ubiquitous in the subsurface and therefore denitrification offers the potential for bio-stimulation of indigenous microorganisms. Also, in comparison to other processes, denitrification does not produce toxic end products, may be cost effective since nearly 100% utilization of electron donor is possible, does not require the addition of potentially harmful exogenous organic materials such as urea, is thermodynamically more favorable, readily occurs in anoxic conditions typical of subsurface environments, and has a potentially greater carbonate yield per mole of electron donor than carbonate precipitation by hydrolysis of urea (the most common carbonate precipitation process investigated to date by geotechnical researchers) based on reaction stoichiometry.

Microbial denitrification may also have applicability for sequestration of radionuclides and metal contaminants. *Chapter 3* outlined the potential for sequestration of radionuclides and metal contaminants through microbially induced carbonate precipitation (MICP). Co-precipitation of radionuclides and metal contaminants with carbonate via denitrification has the potential to be a preferred method for *in-situ* remediation of radionuclide and metal contaminants for many of the same reasons described in *Chapter 2* with respect to MICP for ground improvement.

Chapter 4 presented the results of bench-scale proof-of-concept experiments conducted in dilute and concentrated chemical matrices. The experiments show that microbial denitrification using *Pseudomonas denitrificans* resulted in a change in geochemistry of the reaction medium that is favorable to calcium carbonate precipitation through an increase in pH and alkalinity. Subsequent trials containing calcium resulted in precipitation of calcium carbonate when calcium cations were in the reaction medium, suggesting that this mechanism may be appropriate for field applications.

Results of MICP using *Pseudomonas denitrificans* in experiments employing flow-through and static sand columns are presented in *Chapter 5*. These sand column experiments illustrated that several factors affect microbial denitrification for MICP, including microbial biomass, the micro and bulk scale geochemical environment, and flow conditions (i.e. static vs. flowing). In the first experiment (Sand Column #1), which was a flow through column (i.e. a column with continuous flow of the liquid medium), calcium carbonate precipitation was

induced in the reservoir and other parts of the apparatus but was not visually observed in the specimen (though it could have potentially formed in the column at concentrations below the visually detection limit). As visual inspection is unreliable for detecting small amounts of mineral precipitate in a comparatively large soil sample, this method of detection was not used in subsequent column tests.

The second experiment (Sand Column #2) employed a static sand column. Estimates of the micro and bulk geochemical environments in this test suggest that large vertical chemical gradients develop from initially homogenous solutions. Results from this test also indicate that these chemical gradients diminish over time. Differences observed in NO_3^- and NO_2^- concentrations and pH in the sand column in these experiments imply that denitrification may be more active in certain regions of the sand column in the short term and may be affected by inoculation and liquid medium delivery sequence. Carbonate precipitation was observed and quantified in the sand column and showed that denitrification is promoting the conditions conducive to MICP.

In the third experiment, in which a set of five columns were tested simultaneously (Sand Columns #3 through #7), the rate of denitrification was affected by the amount of microbial biomass injected into the sand columns. A sand column with a larger inoculum size exhibited a much greater rate of NO_3^- and NO_2^- reduction than similar columns that received less inoculum and exhibited less NO_2^- accumulation (a known denitrification inhibitor). The mixed organic carbon donors used in Sand Column #6 did not appear to have an effect

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on denitrification. Sand columns using lower concentration liquid mediums showed higher rates of denitrification than columns using higher concentrated liquid mediums. Rapid NO_3^- reduction in a lower strength liquid medium was coupled with the highest rate of acetate oxidation observed in the sand column tests. Calcium precipitation rate was greatest in the lowest strength medium. The precipitation of Ca^{2+} is a significant factor affecting pH, as a decrease in pH was typically associated with a decrease in Ca^{2+} concentrations. The pH of a denitrifying environment appears to be critically important in determining the amount of Ca^{2+} that precipitates from solution (presumably as $CaCO_3$) and the extent of denitrification (i.e. complete vs. inhibited). The effect of pH on denitrification and Ca^{2+} concentration was remarkable as evidenced by the increase in pH achieved by adding a small amount of NaOH to Sand Columns 3-7 towards the end of the experiment. The addition of NaOH had a dramatic effect on all measured chemical constituents in Sand Columns #3-7. The effect caused by NaOH produced a greater change in all chemical constituents in 9 days than the previous 40 days of uninterrupted denitrification. Carbonate precipitation was also observed and quantified in these sand columns. The presence of carbonate precipitation in these sand columns indicates that denitrification is promoting the conditions conducive to MICP.

Another observation seen in all of the sand column experiments is the consistent generation of biogas, presumably CO_2 and N_2. Biogas pressures in Sand Column 1 reached 97.9 kPa (14.2 psi). Gas pressures in Sand Column #1 typically regenerated within a few days after venting, but typically did not exceed

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48-68 kPa (7-10 psi) after venting from the highpoint of 97.9 kPa (14.2 psi). In Sand Columns #3-7, biogas was occasionally allowed to vent into gas reservoirs during the first two weeks due to excessive bulging of the top caps for the columns. However, gas generation appeared to rapidly decline by the end of this experiment, possibly due to the lack of fresh liquid medium.

The results of these experiments (and from Sand Column #1 in particular) also suggest that high rates of flow for the pore fluid may be detrimental to MICP via denitrification. This could have particularly important implications for practical applications of this technology in an environment where groundwater velocities approach those in Sand Column #1, which were approximately 30 mL/hour. Another potential factor affecting mineral precipitation is the amount of liquid medium/chemical matrix that is in contact with soil. Soil pore space is only a fraction of the total soil volume and, as such, provides limited volume for fluid-soil contact.

6.2 Conclusions

In general, denitrification performed as expected by altering the geochemical conditions of the sand columns through the reduction of (1) NO_3^- to N_2 and (2) oxidation of organic carbon to produce CO_2. These geochemical changes caused the precipitation of varying amounts of $CaCO_3$. The denitrifying bacteria were affected by the initial chemical medium, which affected the rate and extent of the observed geochemical changes and the subsequent precipitation of $CaCO_3$. The precipitation of $CaCO_3$ is a dynamic process that is related to rate and degree of microbial metabolism of NO_3^-, NO_2^-, and organic carbon. Nitrite accumulation

and subsequent inhibition, driven mostly by initial NO_3^- concentration, appears to be highly sensitive to pH. In addition, pH and alkalinity is further affected by the precipitation of $CaCO_3$ since precipitation itself causes a decrease in pH and alkalinity. As such, the pH of a denitrifying environment is a major factor affecting denitrification and carbonate precipitation. pH may be critically important in the highly concentrated solutions required for effective MICP in ground improvement applications relative to typical concentrations in water treatment applications of denitrification. In any case, carbonate precipitation was observed and quantified in several sand columns. Spots of weak cementation within sand columns accompanied by what appeared to be unusually viscous behavior of the overall sand mass strongly implies that denitrification can affect changes in soil properties.

Biogas generation, i.e. production of CO_2 and N_2, is a potentially beneficial effect in the application of denitrification for mitigation of earthquake-induced liquefaction, as desaturation of pore water is in and of itself a liquefaction mitigation technique.

Flow rates may affect MICP by either inhibiting precipitation or nucleation in the pores of a sand column. In addition, fluid flow may sweep carbonate out of the pores after it precipitates and into other areas of the denitrification system where flow rate is lower or nonexistent. This could have particularly important implications for practical applications of this technology in an environment where groundwater is flowing.

The ultimate success of MICP via denitrification for ground improvement in granular soils and, potentially, the sequestration of radionuclides and metal contaminants will depend on the interaction among the microbes present in the subsurface, temperature, soil characteristics and composition, pH, and specific soil chemistry. Understanding the conditions necessary for carbonate rock formation may also aid in developing novel ways to make use of Earth's biological resources and possibly provide efficient and sustainable pathways for the development and improvement of current geotechnical engineering practices. Additional interdisciplinary research by microbiologists, chemists, geologists and geotechnical engineers, collaboratively, is required to realize the potential of this and other microbiological soil improvement technologies.

6.3 Recommendations for Future Research

Microbial denitrification has the potential to improve the mechanical properties of soils, mitigate against earthquake induced liquefaction, and may serve as a useful tool in the remediation of radionuclide and metal contaminated aquifers. Tighter controlled experiments focusing on key parameters of interest would be useful in further understanding and successfully developing MICP. One key parameter that requires further investigation is the apparent detrimental effect of concentrated liquid mediums on denitrification and, subsequently, carbonate precipitation. The negative effects seen in concentrated mediums appear to be dominated by the initial amount of NO_3^- in the liquid medium. Therefore, reduction in initial NO_3^- concentration is an important first step to investigate. Also, the initial organic carbon donor (acetate) concentrations can be reduced to approximately one-

quarter to one-third of the high concentrations tested in this thesis. This would (1) dramatically reduce residual values of acetate and (2) decrease the ionic strength of the liquid medium—high ionic strength solutions are known to inhibit calcium carbonate precipitation. Calcium concentration should remain high (possibly higher than used in Columns 1-7) in all future tests until effects of altering organic carbon donor and nitrate in a MICP system are better understood and quantified. Finally, all changes to the chemical medium must be guided by one overarching principle in order to facilitate rapid denitrification and $CaCO_3$ precipitation: maintaining sufficiently high pH.

Biogas production is an important parameter that warrants further investigation. Experiments are needed to precisely quantify biogas generation through stoichiometric calculations and gas volumes collected from venting events. Other valuable information that may be gleaned from biogas experiments include the amount of gas retained in the pore fluid, the length of time gas is retained in the pore fluid, partitioning between free phase gas and dissolved gas in the pore fluid, and the type of gas partitioned between soil and atmosphere (i.e. N_2 and/or CO_2). Ultimately, some method(s) of determining a correlation between the gas generation and the degree of saturation and between degree of saturation and liquefaction mitigation would be extremely useful.

Knowledge gained from this work would also extend to other applications such as sequestration of radionuclide and metal contaminants. Exploratory work could be undertaken to determine/confirm the feasibility of denitrification for *in-situ* co-precipitation of radionuclides and metal contaminants in artificial

groundwater. If preliminary results prove promising, further efforts could involve direct comparisons between hydrolytic ureolysis and denitrification for contaminant sequestration via MICP, where parameters of interest may include relative rates of co-precipitation, cost per unit(s) of contaminant removed and net environmental impact of proposed remediation.

One final area of discussion that may be helpful in future work is related to experimental set-up. One example of the importance of experimental design is in the ostensibly simple matter of non-destructively extracting representative fluid samples from a reacting sand column. In the set-up employed herein, sand specimens are contained in a thin flexible rubber membrane and then further encased in a rigid metal jacket, base, and top cap. Fluid sampling the entire specimen would yield an average/overall snapshot of the chemical environment within the column but would not reveal information pertaining to spatial variations within the column. Adding sampling ports to the side of the column would provide better geochemical information but would require penetrating the rubber membrane and metal jacket and then forming an airtight seal. Once this difficult task is accomplished, this column is no longer viable for triaxial testing and therefore could not be used to provide this important piece of geotechnical information on the sand specimen. In addition, minor sampling would likely greatly affect measurements pertaining to biogas production and subsequent liquefaction testing.

Biogeotechnical engineering is an emerging field that incorporates mostly untested and seemingly incompatible materials and methods in experimentation.

The traditional tools and methods of the geotechnical engineer, chemist, microbiologist, and geologist are all required in biogeotechnical engineering. Ultimately, ways have and continue to be found to incorporate the methods and tools of vastly different disciplines for testing in biogeotechnical engineering. This level interdisciplinary work requires innovative and creative experimental design. Occasionally, the intersection of these vastly different disciplines produces unusual circumstances and unexpected results. But, as with any emerging field, small amounts of trial and error guided by well-planned and executed testing produces meaningful and useful results that informs future work.

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APPENDIX A

SOIL INDEX PROPERTIES FOR SAND COLUMNS 3-7

The liquid above the soil to the top of the column was approximately 1.3-2.0 cm deep. Each centimeter of fluid depth above the soil corresponds to approximately 46 mL. Approximately 20 mL of fluid was also present in the 6 mm filters placed inside each column between the soil and the bottom cap. The total liquid volume within a given column is the sum of fluid below the soil (20mL), fluid above the soil, and the porosity (n) multiplied by the void volume (V_{void}). For example, the fluid volume in Column #4 is determined as follows:

20 mL below the soil + 87 mL above soil + 634cm^3 (0.408) = 366 mL.

Equations used to estimate soil index properties:

Where $G_s = 2.7$, $\gamma_w = 62.4$ lb/ft^3 = 1.0 g/cm^3, and γ_{dry} = Soil Weight/V_T.

Sample computation for Column #4:

Column	Soil Wt. (g)	Soil Height (cm)	V_T (cm^3)
#3	1001	13.2	602
#4	1012	13.9	634
#5	1029	13.2	602
#6	1018	13.7	625
#7	1019	13.7	625
Average	1016	13.5	618

Column	γ_{dry} (g/cm^3)	e	V_{void} (cm^3)	n
#3	1.66	0.62	230	0.382
#4	1.60	0.69	259	0.408
#5	1.71	0.58	221	0.367
#6	1.63	0.66	248	0.397
#7	1.63	0.66	248	0.397
Average	1.65	0.64	241	0.390

Column	Fluid Above Soil (mL)	Fluid Below Soil (mL)	Total Fluid Volume (mL)
#3	93	20	343
#4	87	20	366
#5	93	20	334
#6	70	20	338
#7	70	20	338
Average	83	20	344