Biological Aspects of Microbial-Induced Calcite Precipitation

S H Vamsi Krishna[1*], B H S Sai Prasanth[2], R Sai Chandu[3]

Abstract

Microbially-induced calcite precipitation (MICP) is an emerging ground-modification technique. This paper presents the results of laboratory experiments that elucidate some biological factors affecting bioaugmentation and biostimulation strategies of MICP. Co-culture experiments suggest that ureolytic bacterium Sporosarcina pasteurii might release the enzyme urease once introduced into a medium containing non-ureolytic bacterium Bacillus subtilis due to lysis by the latter, resulting in uncontrolled calcite precipitation. This suggests that exogenous bacteria introduced into a native soil might not survive due to adverse action by indigenous bacteria. It is shown that effective biostimulation of indigenous ureolytic bacteria in low-nutrient sand can be achieved using a stimulation medium containing 200 mM urea, complemented with a simple carbon source (molasses). Changes in microbial population following stimulation were quantified, using genetic enumeration, to show that (a) the net increase in urease activity is not accompanied by increases in the relative abundance of ureolytic bacteria, (b) nitrifying bacteria are part of the enriched indigenous population and (c) nitrifying bacteria can be stimulated by the addition of ammonium only. The use of the lowest effective urea concentration and simple carbon is advocated for sustainable biostimulated MICP, yielding lower ammonium emissions and reduced post-treatment recovery overheads

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Introduction

Traditionally, geotechnical engineers regarded soil as an inorganic multiphase system comprising solids, fluids and gases. This rational approach stemmed from the early works of Coulomb, Rankine and Darcy, and their twentieth-century followers, such as Terzaghi and Skempton. However, soil is also a living system. Soil is one of the largest terrestrial carbon pools, constituting about 33% of the total terrestrial carbon (Lal, 2008). The organic carbon in the top 1 m constitutes more than 50% of the total soil carbon. Prokaryotes comprise up to 17% of the soil organic carbon (Whitman et al., 1998). These unicellular organisms, mostly bacteria (0·5–3·0) × 10-6 m in size, are about three orders of magnitude smaller than the pore throat size of sand and about the D10 size of kaolinite (Mitchell and Santamarina, 2005). As these bacteria are either motile or fixed to mineral surfaces (grains), their metabolism may change the chemical and physical properties of their surroundings.

Many bacteria are capable of inducing mineral precipitation through various metabolic paths, in both oxic and anoxic environments. Boquet et al. (1973) showed that most heterotrophic bacteria can induce precipitation of calcium carbonate (CaCO3), a common natural cementing agent, by various metabolic paths. Hydrolysis of urea (CO(NH2)2), catalyzed by the microbial enzyme urease (Reaction I), is considered to be the most efficient microbial pathway for microbially-induced calcite precipitation (MICP) (Reaction II) (De Muynck et al., 2010).

Depending on the quality and quantity of calcium carbonate precipitation, the stiffness and strength of the soil can be increased, the hydraulic conductivity can be reduced (van Paassen et al., 2010)

Corresponding author: S H Vamsi Krishna
Address: [1]Assistant Professor, Department of Civil Engineering, GMRIT Rajam, Andhra Pradesh, India, [2]Assistant Professor, Department of Civil Engineering, SITAM, Vizianagaram, Andhra Pradesh, India, [3]Assistant Professor, Department of Civil

Engineering, ANITS, Visakhapatnam, Andhra Pradesh, India
E-mail: vamsikrishna.sh@gmrit.edu.in1, prasanthbhs262@gmail.com2, ronankisaichandu@gmail.com3

and the undrained shear response can be modified (DeJong et al., 2006; Montoya and DeJong, 2015; Montoya et al., 2013).

Following the hydrolysis of urea, ammonium ($NH4^+$) accumulates in the soil, possibly leading to stimulation of ammonia-oxidising bacteria (AOB) and nitrifying bacteria (Swensen and Bakken, 1998). Ammonia oxidation (Reaction III) results in a decrease in pH and in carbonate concentration, which can lead to dissolution of the precipitated calcium carbonate.

Moreover, the end product of nitrification, nitrate, is a highly soluble contaminant that is likely to infiltrate into the groundwater of the treated site. Various studies have shown that enrichment of soil, or aquifer, with urea results in urea hydrolysis, which in turn is followed by nitrification (e.g. Reed et al., 2010; Swensen and Bakken, 1998).

Harnessing natural biogeochemical processes to modify the hydromechanical properties of soils has only recently received attention in the geotechnical community (DeJong et al., 2010). MICP is an emerging ground-improvement technique aimed at various geotechnical, structural and environmental applications, including mitigation of liquefaction (Montoya et al., 2013), soil strengthening (van Paassen et al., 2010), concrete healing (Jonkers et al., 2010) and contaminant sequestration (Fujita et al., 2008), among others.

MICP strategies

To date, most geotechnically related MICP research has been carried out on the laboratory scale by using model bacteria (typically Sporosarcina pasteurii). Two notable studies applied MICP, as a geotechnical measure, on the field scale: those of van Paassen et al. (2010), who used the model bacteria S. pasteurii, and Burbank et al. (2013), who stimulated on-site native soil bacteria. These studies represent two different strategies for onsite MICP: bioaugmentation, in which a specific ureolytic bacterial strain is added to the treated site along with urea, nutrients and calcium; and biostimulation, in which a specific mixture of nutrients is added to stimulate and enhance the ureolytic activity of indigenous bacteria.

The advantages of bioaugmentation include usage of bacteria with high ureolytic activity and their introduction into the ground at a known activity stage. However, population sizes of exogenous bacteria decline rapidly following introduction into a natural soil, and growth of introduced populations in microbiologically undisturbed soils is a rare phenomenon (van Veen et al., 1997). This has been attributed to the scarcity of available nutrient sources in soil and the hostility of the soil environment to incoming microbes due to a myriad of adverse abiotic and biotic factors. Population decline has thus been observed for a wide variety of newly introduced bacteria irrespective of the source of their original isolation. The survival of exogenous bacterium can be somewhat overcome by injecting the bacterium anew with every cementation cycle. However, growing monoclonal cultures in the quantities needed to change effectively the engineering properties in large volumes of soil is challenging and expensive. Moreover, the introduction of exogenous bacteria into soil may often result in an uneven distribution of bacteria and increased calcite precipitation near the injection well (e.g. van Paassen et al., 2010).

This paper presents results from liquid media batch experiments. Each of the experiments was designed to study different aspects of bioaugmentation and biostimulation strategies and to the elucidate advantages and limitations of each approach. The first experiment consisted of a co-culture of urea-hydrolysing bacteria and non-hydrolysing bacteria at different initial cell density ratios. The main goal of this experiment was to study the interaction of two bacterial species in a bioaugmentation setting. The second experiment was aimed at stimulating native bacteria in sand. This experiment also tested the potential stimulation of AOB as a result of the accumulation of ammonium due the hydrolysis of urea. The third experiment consisted of optimization of urea concentration for effective hydrolysis and minimization of the ammonium by-product by using an enrichment culture of sand bacteria.

Materials and methods

The base solution used for all the experiments described in this paper was artificial groundwater solution (AGW), with the following composition: magnesium chloride (MgCl2), 1 mM; magnesium sulfate (MgSO4), 1 mM; sodium bicarbonate (NaHCO3), 2·56 mM; sodium chloride (NaCl), 14·35 mM; potassium chloride (KCl), 0·32 mM; and calcium chloride (CaCl2) at various concentrations, depending on the specific experiment. AGW was sterilised by filtration using 0·22 mm filters. All reagents used for this research were of analytical grade.

Precipitation in co-culture of ureolytic and non-ureolytic bacteria

For the co-culture of ureolytic and non-ureolytic bacteria, two model bacteria were used: the ureolytic, alkaliphilic soil bacteria, S. pasteurii, and the non-ureolytic B. subtilis. Prior to inoculation, a single colony of each bacterial strain was inoculated in separate nutrient broth (NB)-based media (HiMedia), according to the manufacturer's instructions. For S. pasteurii, 20 g/l of urea was added to the medium. Growth took place at 30°C in an orbital shaker (200 revolutions per minute (rpm)) and lasted 36 h, until the bacteria reached an optical absorbance at 600 nm of approximately 1. The bacterial cultures were then transferred to a filter-sterilised precipitation medium, based on AGW, as specified earlier. The medium was amended with 5 mM calcium chloride and 25 mM urea. To prevent premature calcite precipitation, the pH of the AGW (7·7) was adjusted to 7·0 prior to the filtration in 0·22 μm sterile filter units; however, some precipitation did take place, resulting in a decreased initial concentration of dissolved calcium (3·4 mM). No organic carbon was added to the precipitation medium, thus preventing bacterial growth and limiting bacterial activity mainly to urea hydrolysis. Four different treatments were prepared (in duplicates). All treatments were inoculated with the model ureolytic bacteria, Spasteurii, at a density of 104 cells/ml, but differed one from another in the densities of the non-ureolytic model bacteria, B. subtilis, as follows: (a) control (no B. subtilis), (b) 106 cells/ml, (c) 107 cells/ml and (d) 108 cells/ml. To achieve the different bacterial densities, different volumes of bacterial cultures were harvested as follows: 8 ml of S. pasteurii culture were used to inoculate all four treatments (2 ml each), and volumes of 200, 20 and 2 ml of B. subtilis were used for the different co-culture treatments. The growth cultures were centrifuged at 16 000g for 3 min (for volumes of several millilitres) and at 3600g for 10 min (for culture volumes of tens to hundreds of millilitres). Following centrifugation, the supernatant was discarded and the pellet was resuspended in the precipitation medium; this procedure was repeated three times in order to ensure that all growth media were washed prior to inoculation. Each treatment was initially prepared at a volume of 200 ml; after inoculation with the appropriate bacterial pellet, the treatments were divided into duplicates of 100 ml each, in order to ensure similar bacterial density in each replicate. Incubation was static at ambient temperature for 24 h. pH, concentrations of ammonium and calcium ions (Ca2+) and colony-forming units (CFUs) were counted at predetermined time intervals. Prior to each sampling, each flask was manually stirred for several seconds to ensure a homogeneous sampling. CFUs were counted on two different growth media: NB agar (HiMedia) and NB agar supplemented with 20 g/l urea (333 mM), referred to as NBU agar plates. Each sample was serially diluted before plate spreading; three or four dilutions for each sample were spread in four or five repetitions per each dilution. The colonies were counted after 24–36 h of incubation at 30°C.

Biostimulation of ureolytic bacteria and AOB

Enrichment of sand in ureolytic bacteria and deoxyribonucleic acid (DNA) extraction methods are described by Gat et al. (2016). Briefly, sand from Ziqim beach (31·611° north, 34·503° east), a semi-arid zone (BSh in Köppen climate classification) located in the southern part of the Israeli coastal plain, was collected (60 m from shoreline, 0·4 m depth). The site was selected for its soil characteristics – a mineral soil low in organic matter and subject to relatively dry conditions – and was therefore assumed to have relatively low bacterial biomass. Sand, 10 g, from a single homogenised sample, was added to each flask (250 ml Erlenmeyer glass flasks) containing 100 ml liquid medium based on AGW. Three different enrichment treatments (in duplicates) were prepared, all containing 20 g/l (333 mM) urea: (a) control (AGW + urea), (b) 3 g/l yeast extract (YE) and (c) 1 g/l molasses (MLS). Additional treatment (AGW + 10 g of sand) contained 100 mM of ammonium chloride (NH4Cl), with the purpose of studying whether nitrifying bacteria were present and viable in the native sand. The flasks were corked using a cellulose cork covered in aluminium foil. All treatments were incubated at ambient temperature with shaking (100 rpm). The experiment lasted for more than 2 months; however, certain treatments were terminated earlier (see description in 'Results' section). DNA was extracted from the native sand shortly after its collection, using the PowerMax (Mo Bio) kit, and from each treatment upon the termination of incubation by using the PowerSoil kit (Mo Bio).

Urea concentration optimization

Bacterial extract from the stimulation treatment MLS (as previously described) was used to optimize the concentration of urea required for sufficient hydrolysis. The liquid medium was based on AGW with 2·43 mM of calcium chloride (following the concentration in natural groundwater). Four different treatments were prepared in duplicates, all amended with 1 g/l of MLS and with different concentrations of urea: (a) 3 g/l (50 mM), (b) 6 g/l (100 mM), (c) 12 g/l (200 mM) and (d) 20 g/l (333 mM). The experiment lasted 7 d (168 h).

Chemical analyses

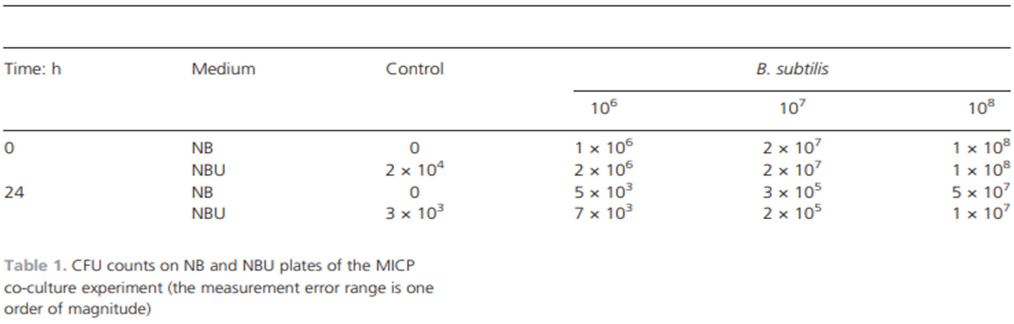
For all measurements of the concentrations of chemical compounds, the samples were filtered (0·22 mm) upon sampling and kept in sterile containers at 4°C until measurements took place. pH measurements were taken immediately upon sampling, before filtration. The pH was measured using various instruments that were calibrated periodically using two or three buffer solutions of 4·01, 7·00 and 10·00, according to the measured pH range.

Urea hydrolysis was inferred from ammonium measurements by using Spectroquant kit number 1.00683.0001 on an Spectroquant Pharo 100 spectrophotometer (Merck KGaA, Darmstadt, Germany) with a standard error of measurement of ±0·07 mM. Calcium concentrations were measured using Spectroquant kit number 1.14815.0001 (Merck KGaA, Darmstadt, Germany) with a minimal detection limit of 0·25 mM and a standard error ± of 0·04 mM.

Results

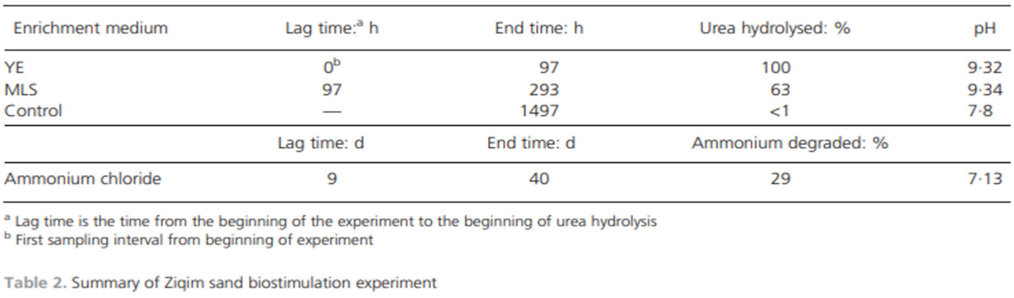
MICP experiment in a co-culture of model bacteria

Figure 1 presents the results of the MICP co-culture experiment. The rate of urea hydrolysis and calcium carbonate precipitation (as inferred from the concentration of calcium ions) increased with increasing densities of B. subtilis. For B. subtilis concentration of 108 cells/ml, all of the urea was hydrolysed within 5 h (Figure 1(a)) and, within this time, all the dissolved calcium was removed from the medium (Figure 1(c)). For a B. subtilis concentration of 107 cells/ml, the urea was fully hydrolysed in 24 h, while the calcium depleted in the first 10 h of the experiment. In the control treatment (not containing B. subtilis), urea hydrolysis proceeded at a much slower rate, with 38% of the urea hydrolysed in 24 h. Calcium depletion in the control medium also proceeded at a slower rate when compared with the 108 and 107 mediums (Figure 1(c)). The pH of all treatments (Figure 1(b)) showed similar trends, finally reaching the value of 9·1. The control and 106 treatments exhibited the similar time history of constant increase and final plateau value. The 107 and 108 treatments showed an initial increase in pH, followed by a decrease and again an increase in pH. The decrease in pH can be attributed to enhanced calcium carbonate precipitation, which for the 108 treatment was simultaneous.



Time: h	Medium	Control	*B. subtilis*		
			10^6	10^7	10^8
0	NB	0	1×10^6	2×10^7	1×10^8
	NBU	2×10^4	2×10^6	2×10^7	1×10^8
24	NB	0	5×10^3	3×10^5	5×10^7
	NBU	3×10^3	7×10^3	2×10^5	1×10^7

Table 1. CFU counts on NB and NBU plates of the MICP co-culture experiment (the measurement error range is one order of magnitude)



Enrichment medium	Lag time:[a] h	End time: h	Urea hydrolysed: %	pH
YE	0[b]	97	100	9·32
MLS	97	293	63	9·34
Control	—	1497	<1	7·8
	Lag time: d	End time: d	Ammonium degraded: %	
Ammonium chloride	9	40	29	7·13

[a] Lag time is the time from the beginning of the experiment to the beginning of urea hydrolysis
[b] First sampling interval from beginning of experiment

Table 2. Summary of Ziqim sand biostimulation experiment

Biostimulation of native bacteria in sand

The results of the Ziqim sand stimulation experiment are presented in Table 2. The control medium showed no significant urea hydrolysis (~1%) over 2 months. The YE treatment showed a nearly immediate response, and full hydrolysis of urea took place within 97 h. MLS treatment reached maximum urea hydrolysis of 63% after 293 h, after which the treatment was terminated and the culture was suspended in glycerol (final concentration of 25% v/v) and kept at −80°C for further experimentation. It should be noted that, while in YE treatment the hydrolysis started once sand was introduced into the enrichment medium, the MLS treatment showed a protracted urea hydrolysis with significant values (>3%) starting after 97 h. The rate of urea hydrolysis accelerated between the 97th and the 216th hours (end of experiment), resulting in conversion of 52% of initial urea. The pH values of the control and the YE and MLS treatments followed the general trend of hydrolysis described earlier: the control treatment reached a low plateau value of 7·8, whereas YE and MLS reached a plateau of 9·3, the pKa value of ammonia/ammonium at the end of the experiment.

An important observation is that a single cell could contain several copies of different genes. Hence, the amount of genes enumerated might represent the total count of genes rather than population. For the 16S gene, the number of copies per cell ranges from 1 to 14 (Klappenbach et al., 2001), whereas for ureC gene, the number of copies is typically 1 and in some cases 2 (Gresham et al., 2007). The initial bacterial population of the sand (Ziqim sand) was relatively small, 105 copies per gram of soil, as inferred from copies of the 16S gene. This is considerably lower than the typical values reported for soil (109 cells per gram of soil); however, this sand in its location represents an inhospitable environment for soil bacteria. The ureolytic population, as inferred from ureC copies, was initially extremely small, with only 101 copies per gram of soil; the ammonium-oxidising population was also small, with 103 copies per gram soil.

Conclusions

This paper explored biological aspects associated with the bioaugmentation and biostimulation strategies of MICP. A coculture experiment using model ureolytic and non-ureolytic bacteria showed that exogenous bacteria introduced into the subsurface might be vulnerable to predation- or starvation-related enzyme release. The application of bioaugmented MICP will require injection of large volumes of cultured bacteria, to ensure survival, complemented with high-urea concentration fluids. This may hinder field-scale applications due to high costs of culturing, injection fluids and post-cementation recovery treatment.

The authors have shown that biostimulation of ureolytic native bacteria is feasible in low-nutrient soils such as coastal sand. The addition of a carbon source was required for significant ureolysis to take place, probably due to the low initial concentration of ureolytic bacteria. The addition of a simple carbon source – that is, MLS – yielded an enrichment factor of four orders of magnitude, similar to the one attained by using a complex carbon source – that is, YE. The main difference between the two carbon sources was the time lag to efficient ureolysis observed in MLSamended enrichment. Once ureolysis started, both carbon sources supported similar ureolysis rates.

The enrichment of the soil with ureolytic bacteria resulted in enrichment in AOB, as observed by an increase in the copy numbers of a genetic marker for AOB. Ammonia oxidation could result in pH decrease that might induce dissolution of calcium carbonate and should thus be taken into consideration when applying MICP in natural environments.

Successful enrichment and ureolysis results in the accumulation of ammonium in the soil. The authors have shown that, for an enriched bacterial extract, significant ureolysis can be sustained with a urea concentration of 200 mM. Low urea concentrations will facilitate more effective post-enrichment and cementation recovery treatments.

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