Biogeosciences Discuss., https://doi.org/10.5194/bg-2017-517
Manuscript under review for journal Biogeosciences
Discussion started: 7 February 2018

Improving the Strength of Sandy Soils via Ureolytic $CaCO_3$ Solidification by *Sporosarcina ureae*

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Key words: Urease, calcite precipitation, MICP, *Sporosarcina*, *Bacillus*, biomineralization, biofilm

Abstract

'Microbial induced carbonate precipitation' (MICP) is a biogeochemical process that can be applied to strengthen materials. The hydrolysis of urea by microbial catalysis to form carbonate is a commonly studied example of MICP. In this study, *Sporosarcina ureae*, a ureolytic organism, was compared to other ureolytic and non-ureolytic organisms of *Bacillus* and *Sporosarcina* in the assessment of its ability to produce carbonates by ureolytic MICP for ground reinforcement. It was found that *S. ureae* grew optimally in alkaline (pH ~9.0) conditions which favoured MICP and could degrade urea (30.28 U/mL) at levels similar to *S. pasteurii* (32.76 U/mL), the model ureolytic MICP organism. When cells of S. ureae were concentrated (OD_{600} ~15-20) and mixed with cementation medium containing 0.5 M calcium chloride ($CaCl_2$) and urea into a model sand, repeated treatments (3 x 24 h) were able to improve the confined direct shear strength of samples from 15.77 kPa to as much as 135.8 kPa. This was more than any other organism observed in the study. Imaging of the reinforced samples with scanning electron microscopy and energy dispersive spectroscopy confirmed the successful precipitation of calcium carbonate ($CaCO_3$), organized as calcite, across sand particles by *S. ureae*. Treated samples were also tested experimentally according to model North American climatic conditions to understand the environmental durability of MICP. No significant ($p < 0.05$) change in strength was observed for samples that underwent freeze-thaw cycling or flood-like simulations. However, samples fell to 29.2 % of untreated controls following acid-rain simulations. Overall, the species *S. ureae* was found to be an excellent organism for MICP by ureolysis to achieve ground strengthening. However, the feasibility of MICP as a durable reinforcement technique is limited by specific climate conditions (i.e. acid rain).

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1 Introduction

Biomediated calcium carbonate ($CaCO_3$) production is the process by which organisms induce the precipitation of calcium carbonate. With reference to bacterial $CaCO_3$ precipitation, also known as, 'microbial induced carbonate precipitation', 'microbial induced calcite precipitation' (MICP) and 'microbial induced calcium carbonate precipitation' (MICCP), the phenomenon is well documented (Stocks-Fischer et al., 1999; Dejong et al., 2006; Whiffin et al., 2007; van Paassen et al., 2010). For example, cyanobacteria precipitate $CaCO_3$ in microbial processes related to the shedding of the S-layer, forming the stalagmites and stalactites in limestone caves and adding to the rocky sediments of coral reefs (Southam 2000). Crystal aggregation of $CaCO_3$ in the kidney, urinary tract or gallbladder have been shown to be induced by microorganisms such as *Proteus mirabilis*, a urease positive organism due to secondary infection (Worcester and Coe 2008). Ureolytic soil organisms of the species *Sporosarcina* or *Bacillus*, can also induce $CaCO_3$. For example, in their cycling of nitrogen with a urease enzyme (Hammes et al., 2003; Gower 2008; Worcester and Coe 2008). This last group of MICP producers has peeked recent engineering interests to apply them in a bioengineering and repair context.

MICP biotechnology utilizing ureolytic soil organisms, most notably *Sporosarcina pasteurii*, has been shown to directly reinforce or restore engineered or natural structures, such as the repair of historical monuments (Le Métayer-Levrela et al., 1999; see also Webster and May 2006), marble slabs (Li and Qu 2011) and stone heritage sites (Rodriquez-Navaro et al., 2012) and reduce weathering of soil embankments (Chu et al., 2012). The enzyme urease (urea amidohydrolase, E.C. 3.5.1.5) initiates the process, catalyzing the breakdown of urea to raise local pH and produce $CaCO_3$ in a solution of calcium ions often supplied as calcium chloride ($CaCl_2$), as summarized in equations 1 and 2 (eq. [1, 2]). The produced $CaCO_3$ fills structural gaps or bridges materials (i.e., soils grains, etc.) to form a cemented product with unconfined strengths of up to 20 MPa (Whiffin et al., 2007).

$$[1]\ CO(NH_3)_2 + 2\ H_2O \text{ <--> } 2\ NH_4^+ + CO_3^{2-} \text{ (Urea Hydrolysis)}$$
$$[2]\ 2\ NH_4^+ + CO_3^{2-} + CaCl_2 \text{ <--> } CaCO_3 + 2\ NH_4Cl \text{ ($CaCO_3$ Formation)}$$

Bacterial species such as *Bacillus sphaericus* (van Tittelboom et al., 2010) and *Bacillus megaterium* (Krishnapriya et al., 2015) have also been applied in material or volume strengthening. The aforementioned ureolytic soil organisms are attractive for MICP as they are, 'generally regarded as safe', (GRAS) bacteria with accessible substrates (i.e., urea) and an aerobic metabolism applicable to most engineering and terrestrial environments (DeJong et al., 2006). These gram positive organisms offer other attractive features such as spore forming capability allowing for long term capsule storage in cements (Jonkers 2011) and exopolysaccharide (EPS) secretion for improved material bonding (Bergdale 2012).

The application of MICP in industry as a biotechnology is proposed to help reduce the need for current structure repair practices such as chemical grouting, which have been found to be environmentally detrimental in its permanence (DeJong et al., 2010) and, in some cases, posing serious human health risks (Karol 2003). That said, ureolytic MICP does produce excess ammonia which can be harmful (van Paassen et al., 2010). The use of

nitrifying and denitrifying bacteria could help solve this issue by oxidizing ammonia to nitrate and later nitrogen gas without affecting MICP. In fact, the work of Gat et al. (2014) has shown co-cultures of ureolytic and non-ureolytic bacteria can actually be beneficial to MICP. Alternatively, denitrifying bacteria can be used to directly induce MICP to avoid ammonia toxicity, though the level of $CaCO_3$ is comparatively less to ureolytic MICP and harmful nitrites can build up in solution (van Paassen et al. 2010). Other pathways to achieve MICP have also been explored with *B. megaterium* and *B. spahericus* (Li et al., 2015; see also Kang et al., 2015).

Problems on large scale application of the MICP technology have occurred too and remain unsolved. Research by van Paassen et al. (2009) found poor sample homogeneity of MICP as well as decreasing biomass and urease-inducing $CaCO_3$ activity over time and increasing soil depth in a pilot 100 m^3 sand study using *Sporosarcina pasteurii*, attributing these heterogeneities mostly to the application process. Alternative metabolisms and bacteria for large scale applications in biomineralization of $CaCO_3$ have also been investigated by the group (van Paassen et al., 2010). Indeed, it has been commented that the type of bacteria utilized is one of the major considerations and potential limitations in large scale geotechnical operations (Mitchell and Santamarina, 2005).

Therefore, the search for new bacteria by which to achieve viable levels of MICP is important for optimizing the protocol best suited (in terms of performance, economics and environmental impact) for marketing in green industry (Cheng and Cord-Ruwisch 2012; Patel 2015; van Paassen et al., 2010). Following a literature review of the nine documented species of *Sporosarcina* (Claus and Fahmy, 1986), seven species were found to be urease positive and distinct from *Sporosarcina pasteurii* as alterative ureolytic MICP sources. While no candidate improves on some of the short comings of ureolytic MICP (i.e., ammonia toxicity), each candidate was found to be poorly investigated in the current MICP technology, despite fitting the ureolytic model for MICP. One candidate, *Sporosarcina ureae* was selected at random for investigation as it was deemed appropriate to explore the feasibility of a single candidate species in thorough comparison to other, already published species applied in ureolytic MICP.

Thus, the primary goal of this study was to investigate the suitability of *S. ureae* as a MICP organism in material improvement by testing it experimentally against the previously investigated species of *Sporosarcina pasteurii*, *Bacillus megaterium* and *Bacillus sphaericus*. In its assessment, a parallel investigation was also performed to assess how the MICP technology, utilizing *S. ureae* as the candidate MICP organism, can perform under various environmental conditions including acid rain, flooding and freeze-thaw cycling concurrent with colder North American climates.

2 Materials and methods

2.1 Bacteria strains, media, culture and stock conditions

Strains of *Sporosarcina ureae* (BGSC 70A1), *Bacillus megaterium* (BGSC 7A16), *Lysinibacillus sphaericus* (BGSC 13A4) and *Bacillus subtilis* (BGSC 3A1^T) were obtained from the Bacillus Genetic Stock Centre (BGSC). *Sporosarcina pasteurii* (ATCC 11859) was kindly donated by the group of Rodrigues *et al.* (University of Houston, USA). *Escherichia coli* DH5a^{TM} was obtained from ThermoFisher. *S. ureae* and *S. pasteurii* strains were grown at 30 ^oC in a modified ATCC 1832 medium as follows: 5 g/L yeast extract (YE) (BD Bacto^{TM}), Tris-Base (Trizma^{TM}), 5 g/L ammonium sulfate (Molecular biology grade, Sigma-Aldrich), 10 g/L urea (Molecular biology grade, Sigma-Aldrich), pH 8.6 . The

culture broth, ATCC Medium 3 (3 g/L Beef extract [BD Bacto™] and 5 g/L peptone [BD Bacto™]) was used for *B. megaterium*, *L. sphaericus* and *B. subtilis*. and grown at 30 °C, unless otherwise specified. Colonies of *Bacillus* and *Sporosarcina* were maintained on plates prepared as described supplemented with 15 g/L agar [BD Difco™] . *E. coli* was grown in Luria-Bertani (LB) broth (10 g/L tryptone [Molecular biology grade, Sigma-Aldrich], 5 g/L yeast extract [BD Bacto™], 10 g/L NaCl [Molecular biology grade, Sigma-Aldrich], pH 7.5) and maintained on LB plates at 37 °C supplemented with 15 g/L agar (BD Difco™). Long term stocks of all cultures were prepared as described (Moore and Rene, 1975) but using dry ice as the freezing agent.

2.2 Chemical and Biological Analysis

2.2.1 Culturing

Single colonies were lifted and grown overnight at 200 RPM in 5mL of respective strain culture medium in a 15 mL Corning Falcon© tube. The overnight stock was combined with 200 mL of appropriate culture medium in a 500 mL Erlenmeyer flask and cultured at 175 RPM. When OD_{600} reached ~ 0.5, the culture was spun down at 5000 RPM for 5 minutes followed by a pellet re-suspension in 50 mL Tris buffered saline (TBS; 50 mM Tris-base [Trizma©, Sigma-Aldrich], 150 mM NaCl [Molecular biology grade, Sigma-Aldrich], pH 7.5). The process was repeated with final re-suspension in 200 mL of a urea broth (UB) medium in a 500 mL Corning PYREX© round glass media storage bottle containing a modified Stuart's Broth (Stuart et al., 1945) as follows: 20 g/L Urea (BioReagent, Sigma-Aldrich), 5 g/L Tris-Base (Trizma©, Sigma-Aldrich), 1 g/L glucose (Reagent grade, Sigma-Aldrich), pH 8.0, with (UB-1) or without (UB-2) 10 g/L yeast extract (YE) (BD Difco™). A negative control included a medium only condition. All steps were performed aseptically with preparations run at 200 RPM at 30 °C in triplicate for each medium condition: UB-1 and UB-2. Each culture for a medium condition was staggered 10 min apart and observed for 12 h, with duplicate 2.5 mL aliquots aseptically withdrawn every 1hr, beginning at time zero (t = 0 h). The entire protocol was performed twice for a total of 6 data sets (n = 6), measured in duplicate, per culture in a single medium condition.

2.2.2 Total Ammonia (NH_3-NH_4^+), pH and growth (OD-600) aliquots

To evaluate different cell parameters efficiently, duplicate aliquots (2.5mL) were taken for pH tracking, OD_{600} absorbance and sample storage for NH_3-NH_4^+ analysis. In brief, first, whole aliquot volume pH was taken with a SB20 symphony pH probe (VWR). Next, a 1mL volume was removed for OD_{600} reading using a BioMate 3 UV-Vis Spectrophotometer (Thermoscientific). Finally, a 500 uL sample for NH_3-NH_4^+ analysis was retrieved and diluted in 500 uL of ddH_2O and stored as described by HACH Inc. (Hach Co. 2015) with the following modifications: -20 °C storage, 1 drop 5 N H_2SO_4. To avoid errors in volume delivery by micropipette, measurements were taken as mass over an analytical balance, and volumes calculated assuming a density of 1 g/mL.

2.2.3 Spectrophotometric analysis of NH_3-NH_4^+

Samples were thawed and neutralized with 5 N NaOH as described by HACH Inc. (Hach Co. 2015). NH_3-NH_4^+measurements were then performed as outlined (HACH Co. 2015) using a portable DR2700 HACH spectrophotometer after samples were brought to a measureable range (0.01 to 0.50 mg/L NH_3-N). All measurements for appropriate dilutions were made by mass and corrected to volume as described above. Final values were reported as units (U = mol of NH_3-NH_4^+ produced per minute) per mL of culture.

2.3 Microbial cementation

2.3.1 Model sand

Industrial quality, pure coarse silica sand (Unimin Canada Limited) was examined with the following grain distribution where D_{10}, D_{5o}, D_{60} are 10 %, 50 % and 60 % of the cumulative mass: D_{10} = 0.62 mm, D_{50} = 0.88 mm, D_{60} = 0.96 mm. The uniformity coefficient, C_u was 1.55 indicating a uniform, poorly graded sand as designated by the Unified Soil Classification System (USCS) (ASTM 2011). A poorly graded soil was used as a model due to its undesirable geotechnical characteristics in construction (i.e., settling) and tendency for instability in nature (i.e., liquefaction) (Nakata et al., 2001; Scott 1991).

2.3.2 Cementation medium (CM) and culture

Cells of each strain were grown in 1L of their respective medium split into two 1 L Erlenmeyer flasks containing 500 mL medium each at 175 RPM to an OD_{600} of ~ 1.5 - 2.0. Cells were then harvested and successively concentrated over three runs to 50 mL. Runs involved a spin down at 5000 RPM for 5 min followed by a pellet re-suspension in Tris buffered saline (TBS; 50 mM Tris-base [Trizma™, Sigma-Aldrich], 150 mM NaCl [Molecular biology grade, Sigma-Aldrich], pH 7.5)). Prior to sand inoculation, 50 mL of a two-times (2X) concentrated cementation (CM) medium (2X CM; 0.5 M $CaCl_2$ [Anhydrous granular, Sigma-Aldrich], 0.5 M urea [BioReagent, Sigma-Aldrich], 5 g/L yeast extract [YE] [BD Difco™], 50 mM Tris-Base [Trizma©, Sigma-Aldrich], pH 8) was added to the final suspension. Negative controls were 1:1 mixes of ddH_2O and 2X CM as well as the non-ureolytic strain (BGSC 3A1^T) *B. subtilis* (Cruz-Ramos et al., 1997). A positive control with *S. pasteurii* (ATCC 11859), a ureolytic organism capable of ureolytic MICP, (van Paassen et al., 2009) was also run. The procedure was repeated every 24 h to provide fresh sample inoculate for injection during cementation trials.

2.3.3 Sample preparation and cementation trial

Triplicate test units were constructed from aluminum (Fig. 1), each housing a triplicate set of sample moulds measuring 60 x 60 x15mm. Moulds were sized according to the sample intake for the direct shear apparatus (Model: ELE-26-2112/02) utilized in confined shear tests. Each mould had equipped to it a drainage valve for media replacement. Filter paper was placed over the drainage valve holes during sand packing to prevent material loss. Silica (autoclaved; dry cycle, 120 ^oC, 15 min) was packed to a dry density of 2.50 - 2.55 g/cm^3 and injected

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with 25 mL CM suspension containing bacteria. Volumes were drained and replaced 3 times, each at 24 h periods. Thereafter, at the beginning and end of each 24 h incubation period, 1 mL of solution was reserved and serially diluted using TBS (50 mM Tris-base [Trizma©, Sigma-Aldrich], 150 mM NaCl [Molecular biology grade, Sigma-Aldrich], pH 7.5) onto appropriate agar plates (as described above) laced with 0.1 mg/L Ampicillin (Sigma-Aldrich) to measure biomass as colony forming units (CFU). Many species of *Bacillus* were found to be resistant at these Ampicillin concentrations (Environment Canada 2015), but otherwise lethal to most contaminant bacteria. In-lab tests observed more than 95 % survival rates for all considered *Bacillus* and *Sporsosarcina* strains compared to a less than 0.1 % survival rate among a model *E. coli* (DH5α™ , Thermofisher). Ambient temperatures of treated sands were maintained at 22 °C, reflective of average sub-surface soil temperatures of central North American climate in the summer (Mesinger et al., 2006).

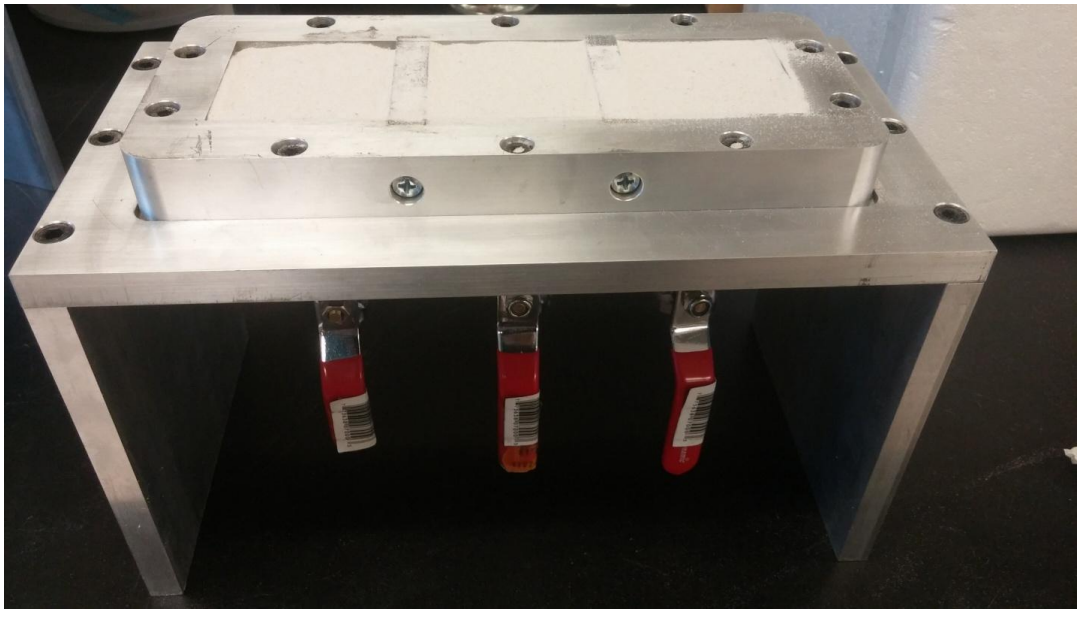

Fig. 1. Aluminum model constructed for cementation testing

2.3.4 Confined direct shear tests

Treated samples were drained, flushed twice with 50 mL of ddH_2O and dried in an oven at 65 °C for 48 h prior to removal from the moulds. The shear strength tests were performed in a direct shear machine as detailed above. Unless otherwise specified, shear tests were performed on samples with an applied normal stress of 25 kPa. Shear stress was then applied to failure at a rate of 2.5 mm/min under dry and drained conditions. Stress-strain curves were acquired via LabView data acquisition software.

2.4 Scanning electron microscopy (SEM) observation

Visualization of silica grains from the surface layer of treated sands was carried out to confirm the crystalline nature of the resulting precipitates using a JEOL6610LV scanning electron microscope (5 kV). Elemental composition of surface structures was analyzed, in parallel, by energy dispersive x-ray spectroscopy (EDS). Prior to microscopy analysis, samples were dried at 65 ^{o}C for 48 h.

2.5 Environmental simulation tests

2.5.1 Water flushing

The ability for cured samples to perform following long-term saturation was tested over a one month trial. Treated sands were incubated with ddH_2O over 6 periods of incubation. Each period involved injection of 25 mL of ddH_2O followed by a 5 day treatment under ambient temperature of 22 ^{o}C. Volumes were replaced at the end of each period. No aliquots for colony counts were taken.

2.5.2 Ice-water cycling

To understand the degree to which cemented trials could withstand ice cycling, a selected number of samples were treated over 6 periods of ddH_2O incubation as described immediately above. However, each period began with a freezing at -20 ^{o}C for 24 h, holding for 3 days at -20 ^{o}C, followed by a thawing for 24 h at 22 ^{o}C. The selected maximum and minimum temperatures reflect those capable of being reached in Ontario winters and summer (Canada), respectively, according to Environment Canada (Climatic station: Ottawa CDA) (Government of Canada 2017).

2.5.3 Acid erosion

Formulation of an acid rain model was made according to average pH values (pH ~ 4.4) of rainfalls reported for North-Eastern regions of North America (Environment Canada, 2013). The final pH was adjusted using concentrated sulfuric acid (H_2SO_4). One delivery volume of acid rain was equivalent to the average monthly precipitation of a North American region (April, Ottawa, Canada), calculated from records of Environment Canada (Climatic station: Ottawa CDA) (Government of Canada 2017). Rain was delivered as described for 'Water Flushing' with ddH_2O but for a single incubation period. Following incubation, the treated volumes were flushed with 25 mL of ddH_2O.

2.6 Statistical processing

All statistical manipulations were performed in Excel (2007). Sample means were reported alongside the standard

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error of the mean (SE) or standard deviation (SD). Normality of all data sets were confirmed with the Anderson-Darling test ($\alpha = 0.05$). The Student's t-test (unpaired, two-tailed; $\alpha = 0.05$) was utilized to compare sample means of experimental conditions for statistical significance. Prior to each t-test, homogeneity of variances for data sets were determined using a F-test ($\alpha = 0.05$). Where variances were statistically observed as unequal, a Welch's t-test was adapted to test statistical significance between two sample means.

3 Results

3.1 NH_3-NH_4^+ production

Among the different bacterial strains considered, *S. pasteurii* (32.50 U/mL [UB-1]; 32.76 U/mL [UB-2]) *and S. ureae* (29.00 U/mL [UB-1]; 30.28 U/mL [UB-2]) were capable of producing the first and second highest levels of NH_3-NH_4^+, respectively, per unit of time, in both UB-1 and UB-2 medium (Fig. 2). Isolates of *B. subtilis* (2.91 U/mL), *B. megaterium* (4.87 U/mL) and *L. sphaericus* (5.89 U/mL) displayed a lower peak of NH_3-NH_4^+ production in both media types. When urea in medium moved from the sole (i.e., UB-2) to a co-contributor (i.e., UB-1) for nitrogen provision, NH_3-NH_4^+ production dropped to near zero values (Fig. 2) for *B. subtilis* (0.44 U/mL), *B. megaterium* (0.56 U/mL) and *L. sphaericus* (1.20 U/mL) ($p < 0.05$). However, isolates of *S. ureae* and *S. pasteurii* observed no significant ($p > 0.05$) decrease; a rise in production (t = 0-5 h) followed by a levelling off in value (t = 6-12 h) as the general trend observed in UB-1 and in UB-2 (Fig. 2).

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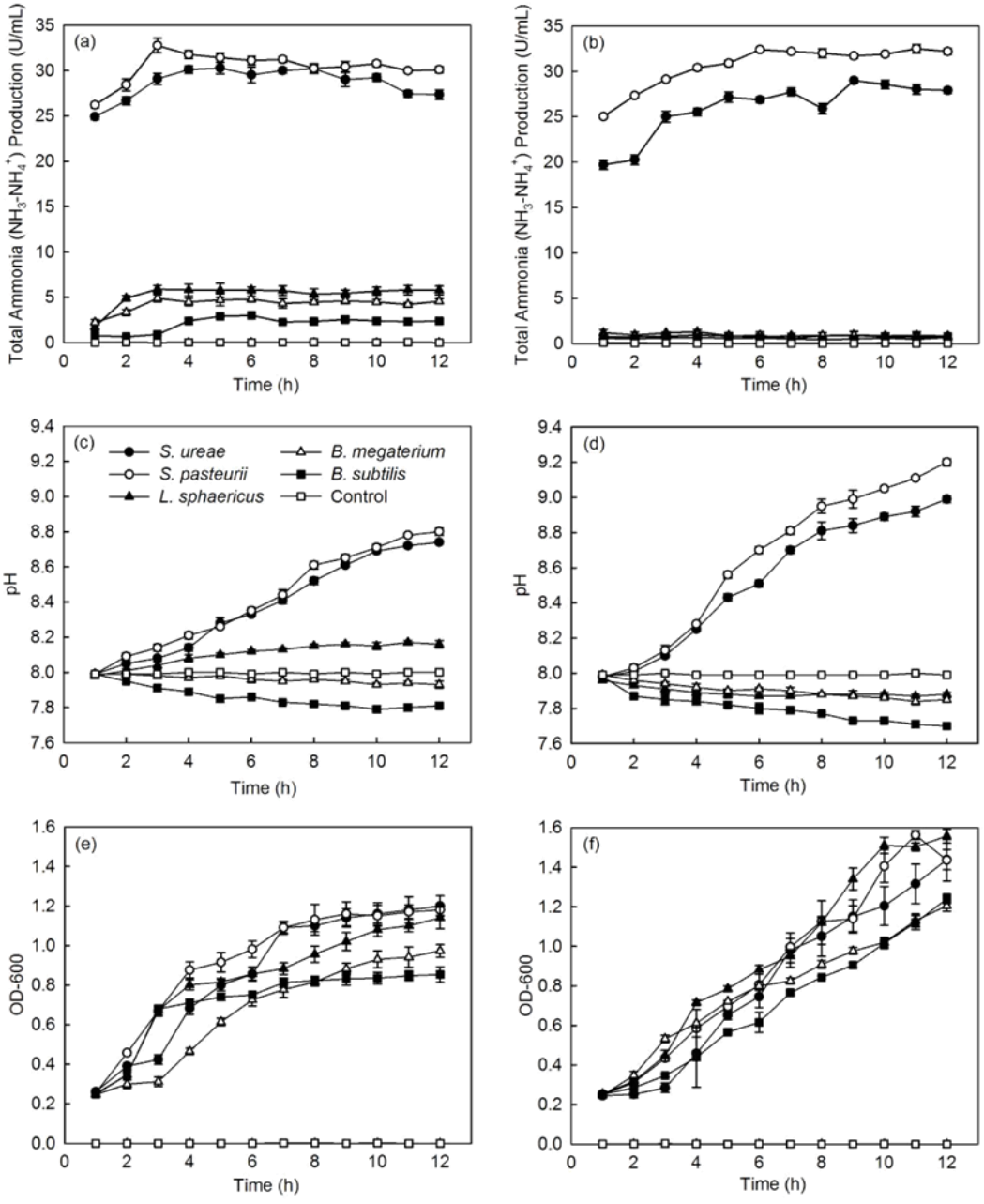

Fig. 2. **(a) , (b)** NH_3-NH_4^+ production ; **(c), (d)** pH ; and **(e), (f)** growth of selected bacteria types in **(a) , (c) , (e)** UB-2 (*No YE*) and **(b) , (d) , (f)** UB-1 (*10 g/L YE*) nutrient conditions (*SD, n = 6).*

3.2 Examination of colony growth in culture

All strains showed a decline in growth progression when medium was restricted (i.e., UB-2) to urea as nitrogen and glucose as carbon, sources, respectively (Fig. 2). Growth repression was greatest in the cases of *B. subtilis* (-33.9 %), *L. sphaericus* (-26.8 %) and *B. megaterium* (-23.6 %) compared to *S. pasteurii* (-17.8 %) and *S. ureae* (-16.6 %). Additionally, the final OD600 (t = 12 h) achieved for all strains in UB-2 medium was significantly decreased ($p < 0.05$) compared to UB-1 medium values. In UB-2 medium, bacterial communities of *L. sphaericus*, *B. megaterium* and *B. subtilis* had sessile growth patterns observed as early as 10 h ($p > 0.05$, *L. sphaericus*); however, continual and significant ($p < 0.05$) increases in optical density were observed when comparing identical times for these cultures in UB-1 medium. Growth cessation occurred for *S. ureae* and *S. pasteurii* in both conditions but later in UB-1 (t = 11 h) compared to UB-2 (t = 9-10 h) medium (Fig. 2).

3.3 Changes in pH

The alkalinity increased with the increase in time for the strains of *S. ureae* and *S. pasteurii* studied, in both UB-1 (8.99, 9.2) and UB-2 (8.74, 8.8) medium. The lowest final pH values were observed in *L. sphaericus* (7.88; 8.16), *B. megaterium* (7.85 ; 7.93) and *B. subtilis* (7.70 ; 7.81) in UB-1 and UB-2 medium, at the end of 12 h (Fig. 2). While pH continued to rise ($p < 0.05$) for *S. pasteurii* and *S. ureae* in either UB-1 or UB-2 medium, it was constant ($p > 0.05$) for *L. sphaericus*, *B. megaterium* and *B. subtilis* after time in UB-1 medium as early as 6 h (*L. sphaericus*) and 7 h (*B. subtilis*) in UB-2 medium. While final pH values for *L. sphaericus*, *B. megaterium* and *B. subtilis* reached significantly ($p < 0.05$) higher final (t = 12 h) values in UB-2 medium compared to UB-1 the opposite was true for *S. pasteurii* and *S. ureae*; values in UB-2 were significantly ($p < 0.05$) lower compared to UB-1. In general, acidity increased with the increase in time for *L. sphaericus*, *B. megaterium* and *B. subtilis* in UB-1 medium to a critical value. This was also true in UB-2 medium except for *L. sphaericus* which showed an increase in pH over time.

3.4 Mechanical and biological behaviour in MICP reinforced sands

Experiments of sand consolidation with triplicate holding vessels (Fig. 1) mixed with *S. ureae* (135.77 kPa) or *S. pasteurii* (135.5kPa) and fed MICP media (i.e., CM-1) were significantly ($p < 0.05$) improved in shear strength compared to controls vessels (15.77 kPa) with MICP media only. Mixtures of non-ureolytic *B. subtilis* (28.1 kPa) showed no significant improvement ($p > 0.05$) compared to control (Fig. 3). While pre-injection (21.9×10^7 CFU/mL) and post incubation (3.2×10^7 CFU/mL) colony numbers were highest in the case of *B. subtilis*, (Fig. 4) all bacterial isolates showed a significant decrease ($p < 0.05$) in the percent of viable colonies (CFU), with non-significant differences between percent values ($p > 0.05$), at the end of incubation in sands (-77.7 % [*S. ureae*], -75.4 % [*S. pasteurii*], -77.7 % [*B. subtilis*]). Of note, the medium-only control observed no colony growth before and also after incubation.

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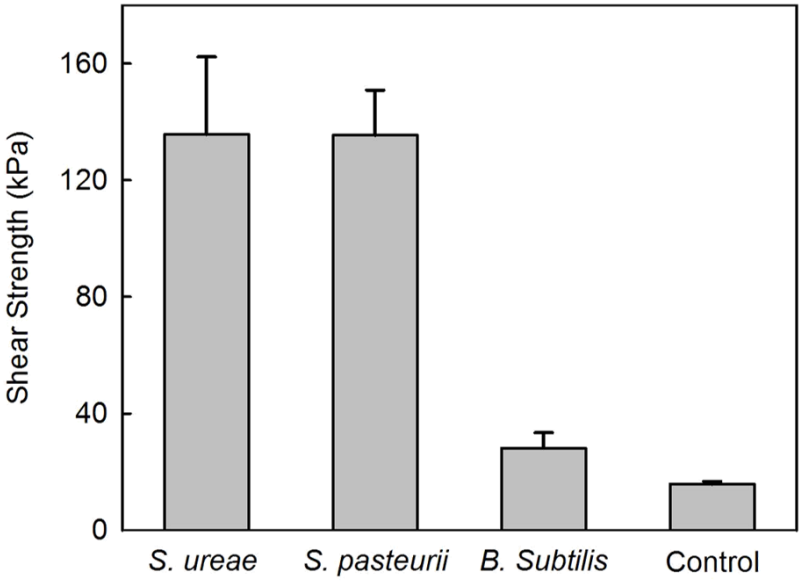

Fig. 3. Direct shear strengths (*τ, kPa*) of treated sands (*SE, n = 3*).

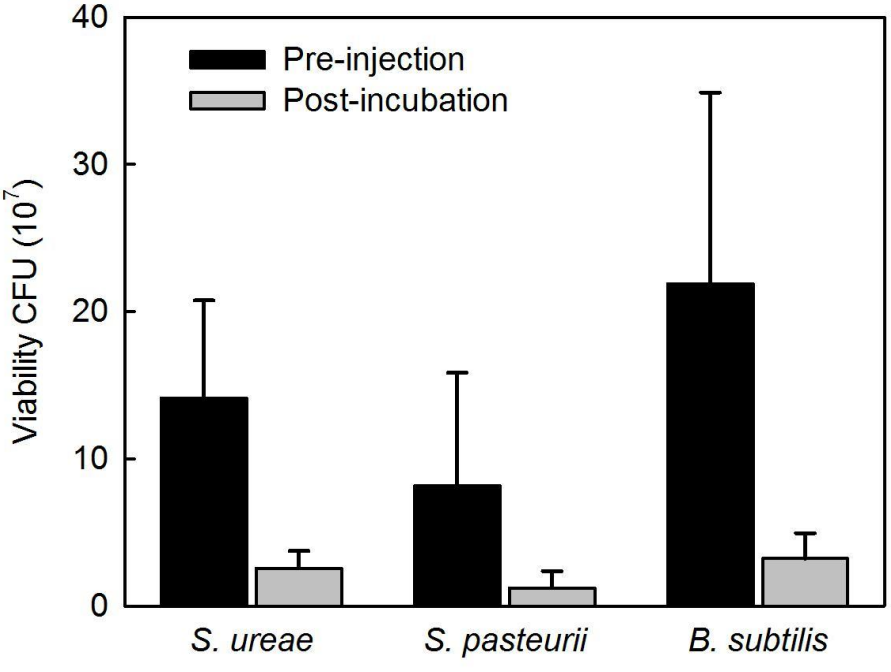

Fig. 4. Microbial viability of treated sands before injection (*black bars*) and after incubation (*gray bars*) (*SD, n = 9*).

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3.5 Microstructure investigation

The precipitation of calcium as $CaCO_3$ via MICP was visualized. Sand granules from approximately the first 1cm of sands treated with MICP solution (i.e., CM-1) combined with *S. ureae* are shown (Fig. 5) where rhombohedra shaped crystals arranged in rosette peaks (20-40 μm) can be seen across the surface of a sand grain (Fig. 5). Rod-shaped structures (40-80 μm) can also be visualized, though less commonly, across grain surfaces (Fig. 5). Calcium, carbon and oxygen peaks captured by EDS analysis for the rhombohedra crystals suggest $CaCO_3$ precipitation whereas only calcium peaks present in the rod-shaped formations support amorphous calcium precipitation.

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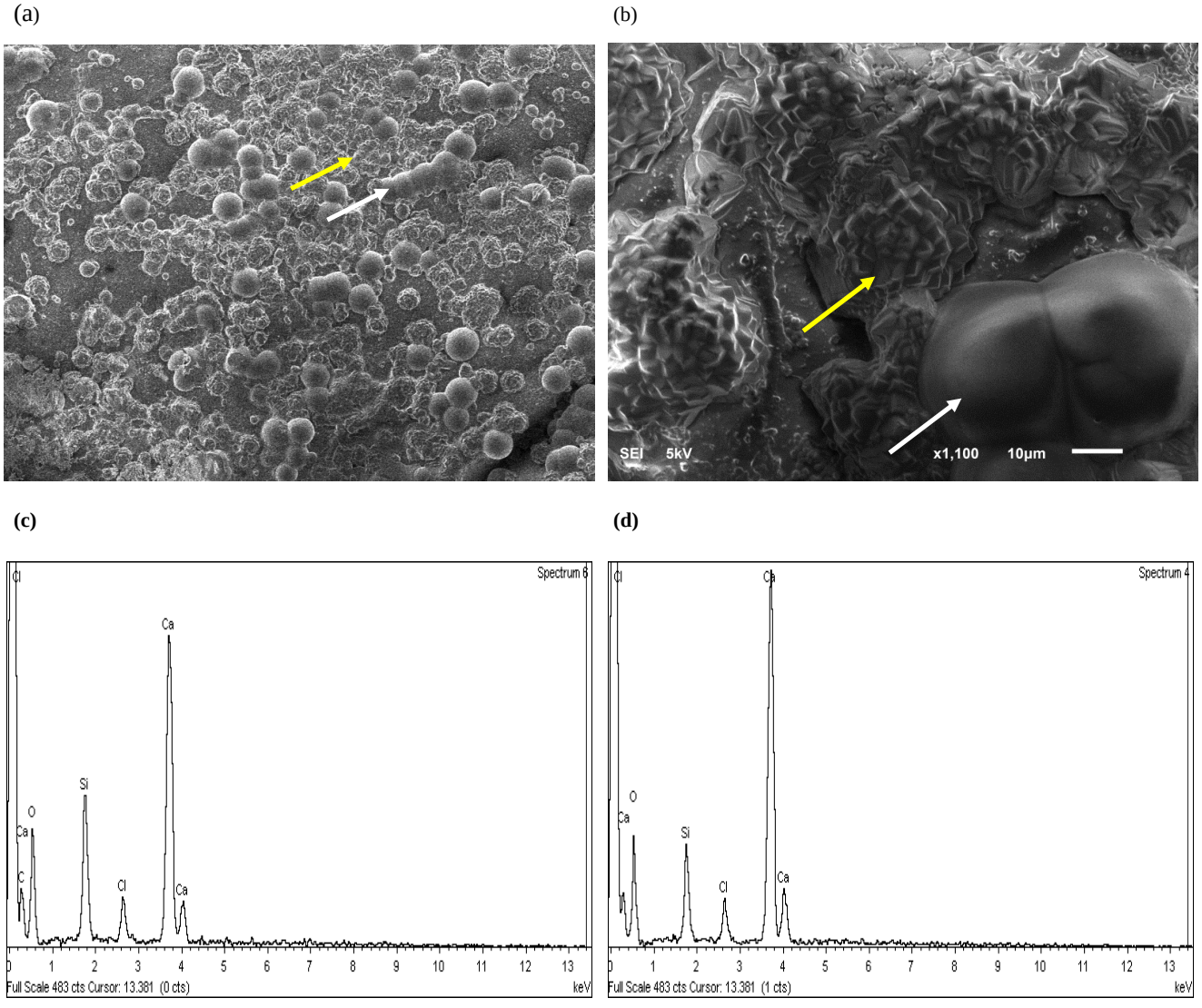

Fig 5. SEM image of the (a) whole surface (*bar, 100 µm*) and (b) magnified (*bar, 10 µm*) silica granule with crystalline (*yellow arrow*) and amorphous (*white arrow*) calcium structures following bacterial treatment. EDS analysis shows the chemical composition of (c) crystalline and (d) amorphous precipitates.

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3.6 Environmental durability of MICP

Destruction of MICP sands with *S. ureae* inoculations was evident following exposure to acid rain as direct shear strengths reduced to 39.7 kPa (Fig. 6) or 29.2 % compared to those with no such treatment (Fig. 3). There was a significant increase ($p > 0.05$) in durability (i.e., strength retention) of treated sands under flooding (111.7 kPa) or freeze thaw (93.5 kPa) rounds compared to acidified states. In fact, no severe mechanical damage was significantly ($p > 0.05$) incurred by samples treated under simulated flooding or freeze-thaw cycles (Fig. 6) compared to sands tested under ideal (i.e., non-environmental) conditions (Fig. 3).

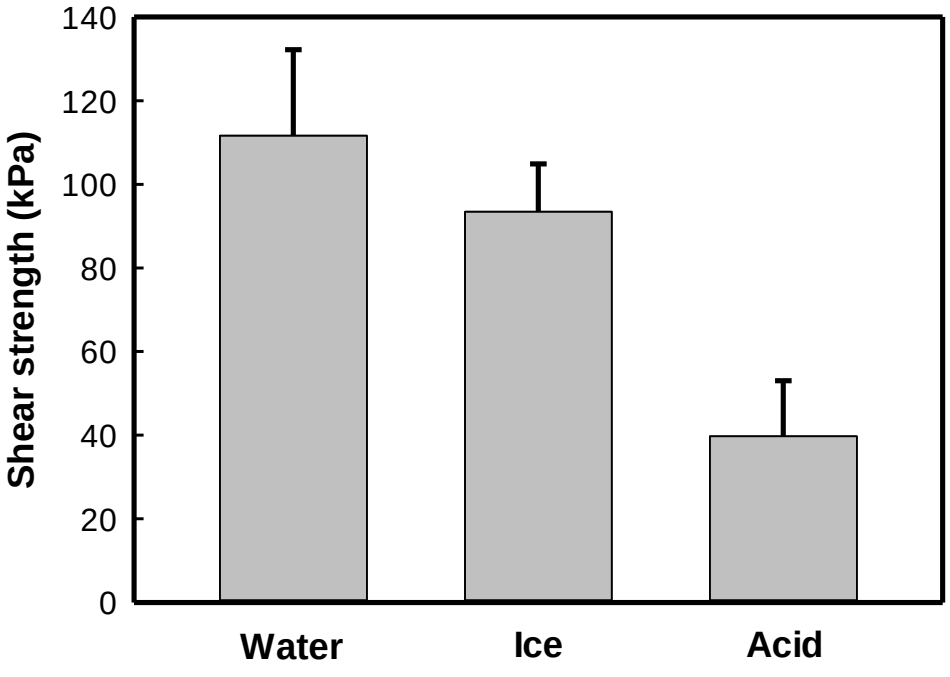

Fig 6. Direct shear strengths (*τ, kPa*) of treated sands with *Sporosarcina ureae* in flood (*water*), freeze-thaw (*ice*) and acid rain (*acid*) simulations (*SE, n = 3*).

Biogeosciences Discuss., https://doi.org/10.5194/bg-2017-517
Manuscript under review for journal Biogeosciences
Discussion started: 7 February 2018

4 Discussion

In characterizing *S. ureae* as a ureolytic organism in MICP, it was chief to understand: (1) its ability to degrade urea over time relative to other commonly applied MICP bacterial isolates and (2) its preference for urea as a nitrogen source. The strain (BGSC 70A1) was consistent in its total nitrogen (NH_3-NH_4^+) production ($p > 0.05$) regardless of source nitrogen availability as yeast extract or urea. This can be attributed to mostly urea catabolism in UB-1 medium and entirely so in UB-2 medium as urea was the sole source of nitrogen. It is important to note that minor mineralization of the yeast extract components in UB-1 medium would likely have contributed ammonium (Gat et al., 2014) in this medium condition. This is supported by data recorded for the negative control (medium-only) in UB-1 medium with production as high as 0.12 U/mL (Fig. 2). Also, degradation of amino acids from bacterial metabolism, such as ornithine, particularly supplied in UB-1 medium via yeast extract, could also contribute to total nitrogen in solution for this condition (Cruz-Ramos et al., 1997). For both mediums (UB-1 and UB-2) dissolution of ammonium as ammonia into the atmosphere would have reduced available nitrogen for measurement, over time. Thus, a quantitative urea hydroylsis rate cannot be determined from the data collected, as nitrogen production over extended periods of time is a complex collection of some or all of these processes. However, overall, the total nitrogen production over time draws support for *S. ureae* as a promising MICP candidate in biocement as over the time period measured it was able to produce a consistent amount of nitrogen as ammonia-ammonium in UB-1 or UB-2 medium and ammonia production has been found to be directly proportional to $CaCO_3$ production (Reddy et al., 2010) and soil stabilization (Park et al., 2012). As mentioned, the production of nitrogen by *S. ureae* in medium is due mostly, or completely, to urea catabolism and this process is likely driven chiefly by its urease enzyme (Mobley and Hausinger, 1989; see also Gruninger and Goldman, 1988). Alternatively, an unknown urea-degrading enzyme other than urease could produce or contribute to the result. Notably, all *Bacillus* strains observed a significant decrease in total ammonia production ($p < 0.05$), when yeast extract was available (i.e., UB-1). This was not observed for *S. ureae* ($p > 0.05$) much like *S. pasteurii*. Urea is a nitrogen source for bacterial growth, often catabolised by urease, (Lin et al., 2012) which has been found to be controlled by nitrogen levels and pH as well as other factors which can differ between bacterial species (Mobley et al., 1995; see also Mobley et al., 2001). Our observations indicate that *S. ureae* selects for urea in a metabolic pattern potentially similar to *S. pasteurii* and quite differently from the *Bacillus* strains investigated here, which appear to have medium-dependent metabolism of urea. This is particularly interesting for *B. subtilis* as it has been applied as a non-ureolytic control organism in previous literature (Stocks-Fischer et al., 1999; Gat et al., 2014). However, it had significant ($p < 0.05$), non-zero total ammonia activity especially in UB-2 medium. These observations are consistent with previously published literature linking total ammonia production to urea breakdown from urease, when urea is the sole source of nitrogen and urease is the assumed main catabolic enzyme; the enzyme expressed constitutively in species of *Sporosarcina* (Mobley et al., 1995) but in a repressible manner (i.e., activated in the absence of NH_4^+ and other forms of nitrogen [i.e., NO_3^-] and urea being the sole nitrogen source) in strains such as *B. megaterium* (Mobley and Hausinger, 1989) and *B. subtilis* (Cruz-Ramos et al.,1997; see also Atkinson and Fisher, 1991). This is indeed suggested by our data as it was observed for *B. subtilis*, *B. megaterium* and *L. sphaericus* that increased total ammonia production reached significantly ($p<0.05$) higher values in UB-2 media compared to near

zero values in UB-1 with yeast extract as a co-nitrogen source. In fact, in UB-2 medium peaks were reached within 3-6 h from near zero values (t = 0-1 h) for all *Bacillus* species, further suggesting an increase in processes related to urea hydrolysis, such as urease expression, overtime following a reduction in genetic repression. This also corroborates well with growth patterns. Biphasic growth of a comparatively slow (t = 6-12 h) rate following a brief plateau (t = 5-6 h) from a comparatively fast (t = 0-5 h) rate of growth was observed for these strains, in general (Fig. 2). An increase in urease, or other urea hydrolysis processes, may account for an ability to grow still further following plateau as nitrogen was made available by increased urea degradation, though slower as glucose carbon was more depleted. Alternatively, the decreased growth could be due to decreased oxygen content for respiration and/or an increase in harmful metabolites in solution over time; each *Bacillus* species switching to a slower, anaerobic growth pattern. Taken together, this has significance as while *B. megaterium* and *L. sphaericus* have been investigated as candidates in ureolytic MICP, this has not been extensively the case for *B. subtilis* which in this study shows ureolytic capability under specific conditions. This may guide future research on ureolytic MICP with *B. subtilis*, particularly where cementation media do not contain nutrient rich additives such as yeast extract. This has been the case in some literature solutions for inducing ureolytic MICP (Cheng et al., 2013; van Paassen et al., 2010). In this study *B. subtilis* was included in sand solidification as a non-ureolytic strain control as cementation media contained yeast extract, intended for maximum biomass support and $CaCO_3$ production rates (van Paassen et al., 2010). Returning to *S. ureae*, it is very clear that it prefers an alkaline environment, like *S. pasteurii* and quite different from other isolates in trials, as in all growth conditions samples grew not only exponentially but towards an increased pH. Urea hydrolysis, driven potentially by urease, in this species, may maintain high levels for production of the highly alkaline environment to which it is suited for growth as an alkalophile and for its role as a nitrogen cycler (Gruninger and Goldman 1988). This is also found important for $CaCO_3$ production (Whiffen et al., 2007) (Fig. 2). The species *S. ureae* may use the proton gradient for energy production (Jahns 1996) to support growth energetically (i.e., ATP) as well as materially (i.e., nitrogen source). This may partly account for *S. ureae* and *S. pasteurii* having the smallest change in growth between UB-1 and UB-2 medium by having the material but also energetic means to multiply. This is extremely promising as van Paassen et al. (2010) determined the $CaCO_3$ precipitation rate is positively correlated to the number of viable micro-organisms in solution. Thus, taken together, the ureolytic, pH and growth data of this study support *S. ureae* as superior in ureolytic action to every *Bacillus* strain considered except *S. pasteurii*. Instead, *S. ureae* and *S. pasteurii* may be considered co-capable MICP candidates. This should prompt interest for further differential investigations between the two strains on such parameters as protease activity, exopolysaccharide production and biofilm levels, also connected to MICP capability, so as to identify the superior candidate. Some work in this direction has already been done (Achal et al., 2009).

To understand the macroscopic engineering aspects of *S. ureae* in MICP application, efforts of this study were instead focused on measuring and assessing its ability to strengthen model sands via urea hydrolysis to form $CaCO_3$. In experiments with a model silica sand featuring poor geotechnical characteristics (i.e., uniform sand profile) for high susceptibility to settling and static strength decreases (Conforth 2005), it was clearly shown that the *S. ureae* treatment led to consolidation of the medium in only 48 h with a significant improvement in strength ($p < 0.05$) approximately eight times that for the treated (135.77 kPa) versus the control (15.76 kPa) samples (Fig. 3). In addition, while average consolidation strengths showed no significant change ($p < 0.05$) between species of *S. ureae*

and *S. pasteurii*, the peak sample strength recorded for an *S. ureae* mould (175.8 kPa) exceeded the maximum sample strength recorded for *S. pasteurii* (165.7 kPa), the typical model ureolytic organism in MICP soil strengthening. It was also well above peak average strength recorded for *B. subtilis* (28.1 kPa) (Fig. 3). This is as expected; *B. subtilis* is a non-ureolytic organism in the 'good nitrogen' (Atkinson and Fisher, 1991) nutrient conditions supplied by the yeast extract of CM-1 medium. Other *Bacillus* sp. were not tested under the assumption that they too would experience repressive urea hydrolysis expression in CM-1 medium and would produce similar observations as a result. This is supported by data provided by the groups of Al-Qabany et al. (2012) and van Paassen et al. (2010) that found $CaCO_3$ precipitation, and by inference soil strength, improved with more suitable micro-organisms in MICP. Taken together, *S. ureae* shows significant capability in soil improvement and a competitor in ureolytic MICP similar to *S. pasteurii*.

The presence of crystals as rhombohedra was observed (Fig. 5) along sand granules treated with *S. ureae* providing credence to the idea that it is capable of inducing prevalent organized formation of secondary minerals (Fig. 5). The crystals were analyzed by EDS and the results provided strong evidence for $CaCO_3$ formation. Rhombehedra organization also dictates calcite crystallization (Anthony et al., 2003). Media and *B. subtilis* treated sands gave no discernible crystal $CaCO_3$ formation. This provides evidence of superficial strengthening in shear tests for these treatments based on natural biofilm excretion (*B. subtilis*) or sporadic mineral crystallization. Of note, amorphous calcium deposits were widespread among *S. ureae* treated sand granules (Fig. 4). These large amorphous precipitates likely indicate inefficiencies in conversion of calcium to crystalline $CaCO_3$ perhaps due to high calcium concentrations which have been found to hinder crystal formation (Al Qabany et al., 2012). Investigators may be prompted to test alternative calcium concentrations to increase calcium to $CaCO_3$ conversion efficiency among *S. ureae* inoculates applied to MICP.

Interestingly, in analyzing cell viability of inoculates before and after incubation in treated sands, it was found that *S. ureae* maintained significantly higher ($p < 0.05$) post-incubation (2.56×10^7 CFU) colony numbers compared to *S. pasteurii* (1.21×10^7 CFU) (Fig. 5) and that both species' counts were significantly lower than those of *B. subtilis* (3.2×10^7 CFU). This could be due to the dilution medium (TBS) utilized favouring survival of more neutrophilic (i.e., *B. subtilis*) as opposed to alkaliphilic species. Thus, a deflated value for *S. pasteurii* and *S. ureae* would result. Also, moulds become mostly anaerobic overtime below the subsurface and within the microenvironments of sand grains as oxygen is depleted by bacterial respiration (van Paassen et al,. 2010). *B. subtilis* may have survived anaerobically (Clements et al., 2002) as opposed to the obligate aerobic *S. ureae* and *S. pasteurii* leading to higher post incubation cell counts. However, percent survival rates were non-significantly ($p > 0.05$) different between all three species indicating neither outperforms the other in colony retention while in the high salt, high urea CM-1 medium with incubation in treated sands. That said, total numbers in *S. ureae* are higher, suggesting additional cellular nucleation points available for $CaCO_3$ formation compared to *S. pasteurii* (Fig. 4). Indeed, the literature reports designate that strength enhancement by ureolytic MICP is driven by urea hydroysis activity but also by the presence of bacteria acting as nucleation sites (Stocks-Fischer et al., 1999; see also Gat et al., 2014). The group of Cheng and Cord Ruwisch (2013) has modeled a novel idea that plentiful cell nucleation could provide best soil strengths. Although the current study's findings suggest higher cell number, having theoretically more nucleation sites, did not factor into meaningful strength increases as *S. ureae* gave rise to non-significantly (p

> 0.05) different strengths in sands versus *S. pasteurii*, which both had approximately the same total NH_3-NH_4^+ production, the microbial ureolytic MICP may have been driven by nucleation in a non-linear fashion based on a number of factors. For example, the ability for individual cells to precipitate $CaCO_3$, which would include their ability to act as effective nucleation sites for crystal formation, can be hindered when an abundance of cells injected into porous material (i.e., sands) lead to pore plugging from the organic matter (i.e., cells) based on a sup-optimal spreading process. This has been seen to lead to a varied amount of $CaCO_3$ precipitation throughout the volume of a mould (van Paassen et al., 2009). Where cells are distributed more evenly to prevent clogging of this nature, nucleation may be beneficial for MICP (Hommel et al., 2015) and reveal a positive, linear relation between cell count and $CaCO_3$ precipitation. Collectively, this may work to explain why *S. ureae* with a near identical NH_3-NH_4^+ activity to *S. pasteurii* did not outperform it on average in undrained, direct shear strength tests despite a higher colony total on average ($p < 0.05$). It may also explain the broader range of strengths achieved in *S. ureae* (Fig. 3). For example, a sub-optimal spreading mechanism could have hindered strength achievement in some moulds of *S. ureae* treatment where pore plugging by organic matter (i.e., cells) occurred. Optimization of treatment protocols may prove *S. ureae* to be the superior candidate compared to *S. pasteurii* more consistently as it has increased total cell numbers (Fig. 3) to support more regular nucleation of $CaCO_3$ overtime, in tandem with a NH_3-NH_4^+ production, proximal to that of the highly ureolytic strain *S. pasteurii*. However, it is important to note that *S. ureae* cells are significantly smaller than cells of *S. pasteurii* (Claus and Fahmy, 1986) and that, therefore, the total cellular surface area available for nucleation of $CaCO_3$ would be quite proximal. This provides a ready explanation for why no significant differences in strength are expected if total cellular surface area for nucleation, regardless of whether it is spread over a relatively high number of smaller cells (i.e., *S. ureae*) or fewer number of larger cells (i.e., *S. pasteurii*) was most important for indicating $CaCO_3$ nucleation and thus strength enhancement potential, where NH_3-NH_4^+ production is similar.

It was the current authors' focus to also apply tests in conditions reflective of a Canadian environment and with a novel bacterial isolate (*S. ureae*). Sands treated with *S. ureae* and which underwent short-term flooding (111.67 kPa) or freeze-thaw cycling (93.47 kPa) showed non-significant ($p > 0.05$) strength decreases compared to in-lab (135.77 kPa) conditions (Fig. 6). It has been shown that MICP treated sands remain some porosity in materials (Cheng and Cord-Ruwisch 2012; Chu et al., 2012) and that good strength maintenance in seasonal water saturation and freeze-thaw is possible with porous materials (Cornforth 2005). Further studies may wish to investigate the permeability of hardened sands via *S. ureae* at various levels of $CaCO_3$ precipitation to strike a balance between porosity, peak strength and endurance overtime in weather simulations.

Predictably, it was seen that the acid rain model, reflective or a Northern Ontario rain pH (4.4), eroded the shear strength of sands (Fig. 6) to 35.5 % of originally observed values (Fig. 3). This is a result of the reaction of acid with $CaCO_3$ producing units of H_2O, CO_2 and salt, known as weathering. A study by Cheng and Cord-Ruwisch (2013) reported similar results with a *Bacillus sphaericus* model. This prompts the idea that a MICP strength model, regardless of the bacteria treatment selected (*S. ureae*, *S. pasteurii*, etc.) for strength enhancement, would require a time-based repair of treated volumes. This realistically limits its geotechnical and economical practicality in the industry. However, it does prompt interest to test the ability of natural buffers, such as limes and sodas, to increase the life-span of MICP induced strength enhancement by reducing acid rain degradation.

5 Conclusions

This study has worked to verify that *S. ureae* is a suitable organism to be applied in the soil hardening technology currently being developed via ureolytic MICP. The authors designate it a close ureolytic MICP candidate, in performance, to the well studied *S. pasteurii* and a superior one to several other *Bacillus* strains. As larger scale simulations are employed, it is strongly encouraged by the authors that further optimization in the treatment procedure, regardless of the MICP organism selected, be undergone including ideal soil buffering to reduce certain climatic effects (i.e., acid rain) and optimum volume porosity in the space to be treated to assure an economical application in industry.

6 Compliance with Ethical Standards

Funding: The study was funded by NSERC (Discovery grant number 2016-2021 ; Discovery grant number 2015-2020) and the University of Ottawa (UROP grant 2012 ; USRA grant 2014)

7 Competing Interests

The authors state they have no conflict of interest.

8 Acknowledgements

The authors would like to acknowledge the University of Ottawa (UROP Grant) and the National Sciences and Engineering Research Council of Canada (NSERC USRA and NSERC Discovery grants to D. Fortin and S. Vanapalli) for financial provisioning in support of this project. Thanks are also given to Mr. Jean Celestin, Mr. Yunlong (Harry) Lui, Mr. Penghai (Peter) Yin, Dr. Nimal De Silva, Dr. Erika Revesz and Mr. George Mrazek; each providing assistance in shear measurements, microscopy and/or data analysis.

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