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Accelerated microbial-induced $CaCO_3$ precipitation in a defined coculture of ureolytic and non-ureolytic bacteria

D. Gat[1], M. Tsesarsky[1,2], D. Shamir[3], and Z. Ronen[4]

[1]Department of Geological and Environmental Sciences, Ben-Gurion University of the Negev, Beer-Sheva, Israel
[2]Department of Structural Engineering, Ben-Gurion University of the Negev, Beer-Sheva, Israel
[3]Chemistry Department, Nuclear Research Centre Negev, Beer-Sheva, Israel
[4]Department of Environmental Hydrology and Microbiology, The Zuckerberg Institute for Water Research, The Jacob Blaustein Institutes for Desert Research, Ben-Gurion University of the Negev, Sede-Boqer Campus, Israel

Correspondence to: D. Gat (mizdani@post.bgu.ac.il)

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Abstract. Microbial-induced $CaCO_3$ precipitation (MICP) is an innovative technique that harnesses bacterial activity for the modification of the physical properties of soils. Since stimulation of MICP by urea hydrolysis in natural soils is likely to be affected by interactions between ureolytic and non-ureolytic bacteria, we designed an experiment to examine the interactions between ureolytic and non-ureolytic bacteria and the effect of these interactions on MICP. An artificial groundwater-based rich medium was inoculated with two model species of bacteria, the ureolytic species *Sporosarcina pasteurii* and the non-ureolytic species *Bacillus subtilis*. The control treatment was inoculated with a pure culture of *S. pasteurii*. The following parameters were monitored during the course of the experiment: optical density, pH, the evolution of ammonium, dissolved calcium and dissolved inorganic carbon. The results showed that dissolved calcium was precipitated as $CaCO_3$ faster in the mixed culture than in the control, despite less favorable chemical conditions in the mixed culture, i.e., lower pH and lower CO_3^{2-} concentration. *B. subtilis* exhibited a considerably higher growth rate than *S. pasteurii*, resulting in higher density of bacterial cells in the mixed culture. We suggest that the presence of the non-ureolytic bacterial species, *B. subtilis*, accelerated the MICP process, via the supply of nucleation sites in the form of non-ureolytic bacterial cells.

1 Introduction

Prokaryotes comprise the major part of the biomass in all major soil types, with an estimated average of $2.2{\cdot}10^8$ cells per cm^3 of soil in the top 10 m (Whitman et al., 1998). The products of prokaryotes' metabolic activity interact with the different constituents of the soil and may thereby change the soil properties. Bacterial processes may thus be harnessed for modification of soil properties as a sustainable and environmentally responsible methodology for soil amelioration and for certain engineering applications (DeJong et al., 2011). One of the most promising biogeochemical treatments for soil is microbial-induced $CaCO_3$ precipitation (MICP), which effectively precipitates $CaCO_3$ in the soil, thereby increasing its strength and stiffness and reducing water permeability (DeJong et al., 2010; Harkes et al., 2010; Nemati and Voordouw, 2003; Whiffin et al., 2007). The possible applications of MICP are numerous and include, to name but a few, geotechnical engineering (Burbank et al., 2011; DeJong et al., 2006), structural rehabilitation (De Muynck et al., 2008a, b), architecture (Tiano et al., 1999) and environmental protection (Dupraz et al., 2009; Fujita et al., 2008).

Most soil bacteria are capable of inducing $CaCO_3$ precipitation through a variety of metabolic pathways, both autotrophic and heterotrophic (Boquet et al., 1973). The most efficient biogeochemical pathway for MICP involves the microbial hydrolysis of urea, which is catalyzed by the microbial enzyme urease (urea amidohydrolase, EC 3.5.1.5) (De Muynck et al., 2010a; Lloyd and Sheaffe 1973). The

enzymatic hydrolysis of urea is approximately 10^{14} times faster than the spontaneous reaction (Jabri et al., 1995). Urea-hydrolyzing microorganisms, both eukaryotes and prokaryotes, are ubiquitous in natural soils, and urea hydrolysis is a process common to soils worldwide (Bremner and Mulvaney, 1978; Lloyd and Sheaffe, 1973; Mobley and Hausinger, 1989). Urea is initially hydrolyzed to carbamate and ammonia (Reaction R1; Mobley and Hausinger, 1989). Carbamate is then spontaneously hydrolyzed to give carbonic acid and ammonia (Reaction R2), which themselves undergo hydrolysis according to Reactions (R3) and (R4), with equilibrium constants of pK_1 6.3 and pK_a 9.3, respectively. According to these values, it is clear that there will be a net increase in soil pH. In the presence of dissolved calcium, this process could result in the precipitation of $CaCO_3$ (Reaction R5), provided that the medium is oversaturated with respect to $CaCO_3$.

$$CO(NH_2)_2 + H_2O \xrightarrow{\text{urease}} NH_3 + NH_2COOH \quad \text{(R1)}$$

$$NH_2COOH + H_2O \leftrightarrow H_2CO_3 + NH_3 \quad \text{(R2)}$$

$$H_2CO_3 \leftrightarrow H^+ + HCO_3^- \quad \text{(R3)}$$

$$NH_3 + H_2O \leftrightarrow NH_4^+ + OH^- \quad \text{(R4)}$$

$$Ca^{2+} + HCO_3^- + OH^- \leftrightarrow CaCO_3 \downarrow + H_2O \quad \text{(R5)}$$

MICP is thus an intricate process that is delicately balanced by four parameters: (1) dissolved inorganic carbon (DIC), (2) pH, (3) abundance of nucleation sites, and (4) calcium concentration (De Muynck et al., 2010b). The first three parameters are directly affected by urea-hydrolyzing (ureolytic) microbial activity, as described in Reactions (R1) to (R4), and by bacterial cell abundance (with the bacteria providing nucleation sites).

In soil amelioration, two major approaches are applied in the implementation of MICP: (1) bioaugmentation, in which a specific ureolytic bacterial strain is added to the treatment site together with urea, nutrients and calcium; and (2) biostimulation, in which indigenous ureolytic bacteria are provided with a substrate designed to stimulate $CaCO_3$ precipitation. In situ bioaugmentation is not always successful, because it is based on the introduction to the soil of large quantities of monoclonal bacterial cultures, whose survival and proliferation are uncertain (as these bacteria are often exposed to predation by eukaryotes and fail to compete with the indigenous microorganisms; Van Veen et al., 1997). Biostimulation, in contrast, encourages the growth of a particular guild of native soil microfauna through the manipulation of specific growth conditions. However, a possible drawback of this method is that the initial soil concentration of ureolytic bacteria might limit the rate of ureolytic MICP in the site to be treated (Tobler et al., 2011). Another consideration affecting biostimulation is the cellular regulation over urease expression. Three manners of regulation are known: constitutive, in which urease is constantly expressed by the organism; inducible, in which urease is expressed in response to the presence of urea over a certain threshold concentration; and repressible, in which urease expression is inhibited in response to the presence of nitrogen-rich compounds, including high concentrations of urea (Mobley and Hausinger, 1989). Of these three, the constitutive regulation is the most promising for a successful MICP process; however, the more common manner of regulation is the repressible expression (Mobley and Hausinger, 1989).

Interactions within the microbial community affect the geochemistry and the microbial ecology of their environment, and the presence of non-ureolytic bacteria in the soil has been shown to affect the parameters controlling $CaCO_3$ precipitation in different ways: heterotrophic bacterial metabolism, for example, has been shown to induce $CaCO_3$ dissolution under aerobic conditions due to the mineralization of organic carbon and the consumption of ammonium (Bennett et al., 2000; Jacobson and Wu, 2009). In contrast, the electronegativity of the bacterial cell surface encourages complexation of dissolved metals (Schultze-Lam et al., 1996), with the complexes possibly serving as nucleation sites for mineral precipitation, thus accelerating $CaCO_3$ precipitation.

To date, most of the research on MICP has been confined to ureolytic bacteria, with a focus on the catalysis of urea hydrolysis (Ferris et al., 2003), on the efficiency of calcite production (De Muynck et al., 2010b; van Paassen et al., 2010) and on the modification of soil physical properties by model bacteria (Burbank et al., 2011; De Muynck et al., 2010b; DeJong et al., 2011; Whiffin et al., 2007). Although some studies of ureolytic MICP have been conducted using mixed bacterial cultures in the lab (De Muynck et al., 2008a; Tobler et al., 2011) and others have been conducted in situ by stimulation of indigenous ureolytic bacteria (Burbank et al., 2011; Fujita et al., 2008), little attention has been paid to the effect on the system of the non-ureolytic bacteria present in the experimental setting. In this study, the potentially conflicting effects of ureolytic and non-ureolytic bacteria on $CaCO_3$ precipitation were investigated in a simple, two-species batch experiment. An MICP system consisting of two model bacteria, the ureolytic species *Sporosarcina pasteurii* and the non-ureolytic species *Bacillus subtilis*, was used to study the chemical and biological evolution of the $CaCO_3$ precipitation process in soil. This two-species model system was designed so as to reveal some of the possible interactions between bacteria of two different guilds and their effect on MICP.

2 Materials and methods

2.1 Bacteria and growth conditions

Ureolytic bacterium: a pure culture of *Sporosarcina pasteurii* (DSMZ 33) was grown with agitation (100 rpm) at 30 °C in Nutrient Broth (NB; HiMedia™) supplemented with 2 % w/v urea (333 mM) until it reached the exponential phase of growth. The bacteria were then harvested by centrifugation

(16,100 g, 6 min) and resuspended in a sterile $CaCO_3$ precipitation medium (see below). This process was repeated twice to prepare the inoculum of *S. pasteurii*. The final concentration of *S. pasteurii* for all treatments described below was approximately 10^7 bacteria mL^{-1}.

Non-ureolytic bacterium: the inoculum of the model gram-positive bacterium, *Bacillus subtilis* (DSMZ 6397), was prepared as described above. The final concentration of *B. subtilis* in each of the mixed treatments was 10^7 bacteria mL^{-1}. *B. subtilis* is often used as a biotic control for MICP experiments, as it does not induce $CaCO_3$ precipitation (Mitchell and Ferris, 2006; Stocks-Fischer et al., 1999), and according to our preliminary experiments it does not affect dissolved calcium concentrations in our experimental setting.

2.2 $CaCO_3$ precipitation media

The $CaCO_3$ precipitation media were based on an artificial groundwater solution (AGW) representing the composition of Israel's coastal aquifer (Sivan et al., 2005) as follows: $MgCl_2$ (1 mM), $MgSO_4$ (1 mM), $NaHCO_3$ (2.56 mM), NaCl (14.35 mM), $CaCl_2$ (2.43 mM) and KCl (0.32 mM); total ionic strength: 31.5 mM. Two different precipitation media were prepared: (1) a full-strength medium, NBU, in which AGW was supplemented with 7 mM urea and 13 g L^{-1} NB and which provided *B. subtilis* with all the required nutrients but limited the growth of *S. pasteurii* due to the low urea concentration (Jahns et al., 1988); and (2) a one-third-strength medium, 1/3 NBU, which contained AGW supplemented with 7 mM urea and 4.34 g L^{-1} NB which limited the growth of *B. subtilis* due to lower nutrient availability.

To prevent premature $CaCO_3$ precipitation, the pH of the AGW was adjusted to 6.5 using 1 N HCl prior to the addition of urea and NB. Upon the addition of NB to the medium, the pH increased to approximately 7.4 due to the chemical properties of the NB itself. All media were filter-sterilized through 0.2 µm sterile filters (Nalgene®).

2.3 $CaCO_3$ precipitation treatments

To examine the effect of non-ureolytic bacteria on MICP, we inoculated NBU medium and 1/3 NBU medium with both bacterial species, with the treatments being designated NBps and 1/3 NBps, respectively. The biological control treatment comprised NBU medium inoculated only with *S. pasteurii*, designated NBp treatment. Each treatment was prepared in duplicate; the initial volume of each replicate was 200 mL. All treatments were incubated without shaking in corked 250 mL Erlenmeyer flasks at ambient temperatures for 10 days. During the course of the experiment, aliquots from each treatment were taken for analysis at predetermined intervals.

2.4 Chemical analysis

All samples were filtered through 0.22 µm filters (Millex®) upon sampling. Dissolved calcium and ammonium concentrations were determined by ion exchange chromatography (Dionex 500; eluent: 20 mM methanesulfonic acid, flow rate: 1.0 mL min^{-1}; column type: cation separation – IonPac – CS12A, 4 × 250 mm). The standard error of measurement was 0.002 and 0.006 mM for Ca^{2+} and NH_4^+, respectively. The pH was measured upon sampling with a pH meter with a measurement error of 0.01. For DIC measurements, filtered samples were injected into glass vials containing H_3PO_4, which had previously been flushed with helium for 10 min to prevent equilibration with atmospheric CO_2. The DIC content was then determined using an IRMS (isotope ratio mass spectrometer) Delta Plus XP (Thermo Scientific, NY, USA), utilizing Gas Bench II. Ten solutions of $NaHCO_3$ (concentrations ranging from 3.91 to 7.15 mM) were used for calibration. The standard error of the measurements was 0.01 mM.

2.5 Biological analysis

Bacterial growth was determined in terms of optical density (OD) by measuring absorbance at a wavelength of 600 nm. Colony-forming units (CFU) of the two species were counted on two different growth media: NB agar (Himedia®) and NB agar supplemented with 20 g L^{-1} urea (333 mM). Since *S. pasteurii* cannot grow on NB agar in the absence of urea or high concentrations of ammonium salts (Bornside and Kallio, 1956), CFU counts on NB agar plates represent the concentration of *B. subtilis*, whereas CFU counts on NB-urea agar plates represent the total bacterial concentration in the mixed cultures (treatment NBps and 1/3 NBps). Thus, comparing the CFU values between the two plate types enabled us to differentiate between the bacterial species in the mixed culture. CFUs were counted following the inoculation of the media, at the 17th hour and at the 40th hour of the experiment.

2.6 Zeta potential measurements

For measuring the zeta potential, monoclonal cultures of *S. pasteurii* and *B. subtilis* were grown and harvested as described in Sect. 2.1. Each bacterial culture was then resuspended in an AGW-based medium, supplemented with 7 mM of urea, whose pH was then adjusted to one of three different values: 7.40, 7.90 and 8.40, with 3 N NaOH. Each treatment was prepared in triplicate. Zeta potential was measured using the 90Plus particle size analyzer by Brookhaven Instruments (Holtsville, NY). Each measurement was repeated five times; the standard error of measurement was 0.52 mV.

3 Results

The results of our experiments are summarized in Fig. 1. Each point of the measured Ca^{2+}, NH_4^+, pH and OD represents the average of the treatment duplicates. The standard deviations were typically smaller than the symbol size.

3.1 Dissolved Ca^{2+}

The reduction in dissolved calcium concentration observed in this experiment may be attributed to the precipitation of $CaCO_3$. The fastest depletion of dissolved Ca^{2+} was observed for the mixed culture NBps treatment, with 2.40 mM of Ca^{2+} being consumed during the first 80 h of the experiment (Fig. 1a), which is equivalent to nearly 100 % $CaCO_3$ precipitation (Fig. 1b). For the control NBp treatment, calcium depletion was slower and was completed after 123 h. The Ca^{2+} depletion rate for the 1/3 NBps coculture treatment was similar to that for the control treatment NBp, i.e., 100 % $CaCO_3$ precipitation in 123 h.

3.2 NH_4^+ concentration

The increase in ammonium concentration (Fig. 1c) may be attributed mostly to urea hydrolysis (according to a stoichiometry of 2 : 1; refer to Reactions R1 and R2) but also partly to mineralization of NB. The final concentrations of ammonium for the control NBp and coculture NBps treatments were identical (18.43 mM), whereas in the 1/3 NBps treatment the final ammonium concentration was considerably lower, i.e., 14.64 mM.

3.3 pH

Variations in pH over time are presented in Fig. 1d. Initial pH values for all treatments were approximately 7.4, probably due to the pH of the NB that was added after the pH had been adjusted to 6.5. For the control NBp treatment, pH values increased rapidly within the first 100 h of the experiment (approximately by pH 1) to a plateau of about pH 8.40. However, for the coculture NBps treatment, a different trend was observed: pH values increased during the first 10 h of the experiment, reaching a value of 7.74, followed by a decline to a minimum value of 7.39 after 28 h. Thereafter, pH values increased again until the 125th hour, finally oscillating around a value of 8.40. For the 1/3 NBps coculture treatment, pH values increased during the first 18 h of the experiment to a value of 8.06, followed by a slight decrease to a value of 7.98 at the 28th hour and then by an increase until the 123rd hour, finally oscillating around a value of 8.50. For all treatments the final pH values after 123 h were about 8.50.

3.4 DIC concentration

During the first 80 h of the experiment, total DIC concentrations were higher (by 2.6 mM, on average) for the coculture NBps treatment than for the control NBp treatment. For the 1/3 NBps treatment, DIC concentrations resembled those of the NBp treatment (Fig. 1e).

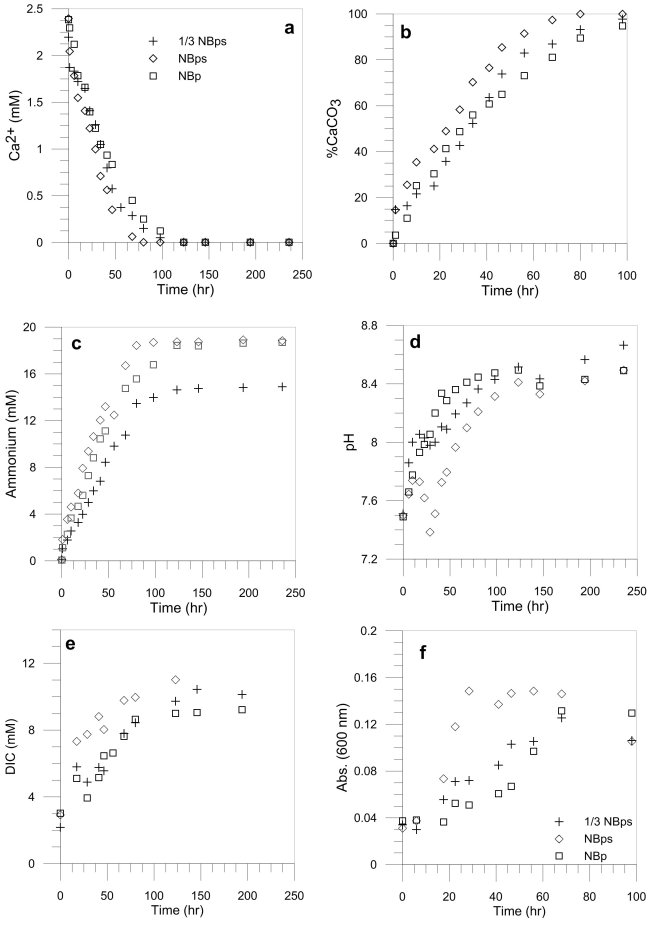

Fig. 1. Changes over time in dissolved calcium concentration **(a)**; percentage of calcium depleted and precipitated as $CaCO_3$ **(b)**; ammonium **(c)**; pH **(d)**; dissolved inorganic carbon (DIC) **(e)**; and OD at 600 nm **(f)**; for treatments NBp (□), NBps (◊) and 1/3 NBps (+). Note the different timescales in panels **(b)** and **(f)**.

3.5 Culture growth patterns

Different bacterial growth patterns were observed for the different treatments (Fig. 1f). For the coculture NBps treatment, the exponential growth phase commenced on or before the 17th hour of the experiment, whereas for the control NBp treatment, the exponential growth phase commenced on or after the 40th hour of the experiment. The increase in OD for treatment 1/3 NBps can be divided into two stages: the first began around the 17th hour of the experiment, as did the OD increase in the full-strength NBps treatment, and the second stage started around the 40th hour, as did the OD increase in the control NBp treatment. In terms of CFUs, the count of *B. subtilis* in the coculture NBps and 1/3 NBps treatments increased by two orders of magnitude within the first 17 h

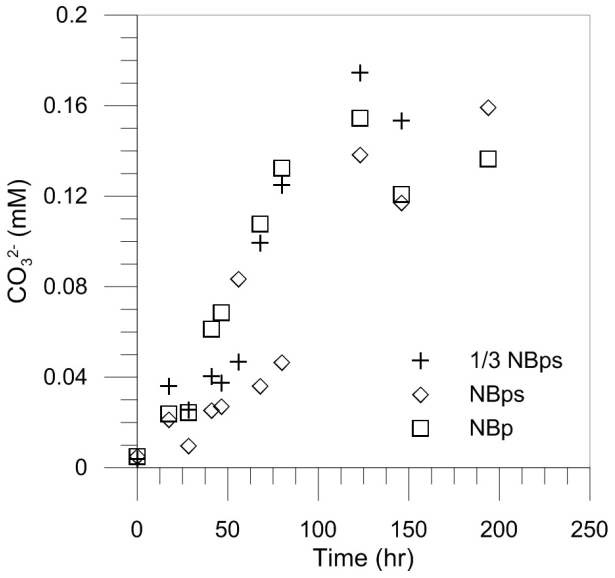

Fig. 2. Calculated changes over time in CO_3^{2-} concentration for treatments NBp (□), NBps (◊) and 1/3 NBps (+).

of the experiment. In the NBp treatment inoculated with *S. pasteurii* alone, the CFU count increased by one order of magnitude by that time. By the 40th hour of the experiment, CFU counts of *B. subtilis* in the 1/3 NBps treatment had not changed significantly, while those in the NBps treatment increased by another order of magnitude, thus confirming the OD measurements.

3.6 Zeta potential

The zeta potentials of both bacterial species decreased with an increase in pH. *S. pasteurii* zeta potentials ranged from −19.51 mV at pH 7.40 to −23.10 mV at pH 8.40. *B. subtilis* zeta potential ranged from −22.28 mV at pH 7.40 to −24.18 mV at pH 8.40.

4 Data analysis

4.1 Calculated CO_3^{2-} concentration

Carbonate ion concentration $[CO_3^{2-}]$ was calculated from the measured DIC concentrations using the following equations (Stumm and Morgan 1996):

$$\left[CO_3^{2-}\right] = C_T \cdot \alpha_2, \quad \text{(R6)}$$

where C_T is the total inorganic carbon concentration, or DIC, and the mole fraction, α_2, is calculated as follows:

$$\alpha_2 = \left(\frac{[H^+]^2}{K_1K_2} + \frac{[H^+]}{K_2} + 1\right)^{-1}. \quad \text{(R7)}$$

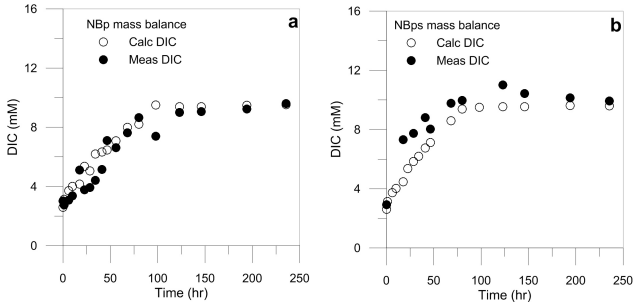

Fig. 3. Calculated (◯) and measured (●) values of DIC for treatments NBp **(a)** and NBps **(b)**.

The acidity constants K_1 and K_2 were obtained from Stumm and Morgan (1996), and hydrogen concentration was determined from pH measurements. For all calculations, it was assumed that equilibrium between carbonate species was maintained in the medium and that no equilibration with atmospheric CO_2 took place (Dupraz et al., 2009).

The calculation showed that CO_3^{2-} concentrations for the control NBp treatment (0.10 mM) were considerably higher than those for the coculture NBps treatment (0.03 mM) during the initial 68 h of the experiment (Fig. 2). However, by the 120th hour of the experiment, CO_3^{2-} concentrations had become similar for the two treatments, i.e., 0.15 and 0.14 mM, respectively. CO_3^{2-} concentrations for the 1/3 NBps treatment resembled those for the NBps treatment up to the 56th hour of the experiment. From then on, the CO_3^{2-} concentration increased to reach values similar to those for the NBp treatment.

4.2 Carbon mass balance

To assess the contribution of the oxidation of organic carbon to the accumulation of DIC, we calculated the amount of total inorganic carbon that originated from urea hydrolysis in the control and mixed-culture (NBp and NBps) treatments and compared the results with those obtained by direct measurement of DIC. The correlation between the calculated and measured DIC concentrations indicates that urea hydrolysis is indeed the main source of DIC production, with differences between calculated and measured DIC concentrations being attributed to the mineralization of the NB. The stoichiometry of the reactions described in Reactions (R1) to (R4) yields the following relationship for the concentrations of urea-derived carbon and ammonium:

$$[C_{urea}] = \frac{\left[NH_4^+\right]}{2}. \quad \text{(R8)}$$

The stoichiometry of the $CaCO_3$ precipitation reaction (Reaction R5) along with direct measurements of dissolved calcium concentrations allowed us to find the amount of

precipitated carbon:

$$[C_{precipitated}] = [CaCO_3] = \Delta\left[Ca^{2+}\right] = \left[Ca^{2+}_{initial}\right] - \left[Ca^{2+}_{t}\right]. \quad (R9)$$

Since precipitation of $CaCO_3$ removes carbonate from solution, the amount of precipitated $CaCO_3$ must be subtracted from the total inorganic carbon produced to give the net increase in DIC (Reaction R10):

$$[C] = \frac{\left[NH_4^+\right]}{2} - \Delta[Ca^{2+}]. \quad (R10)$$

Figure 3a and b present calculated and measured DIC concentrations in the single-species NBp and coculture NBps treatments. For the NBp treatment (Fig. 3a), there was good correlation between measured and calculated DIC values. The root mean square of residuals (rmsr) was 0.9 mM for measurements ranging from 2.6 to 9.5 mM, yielding an estimate error of 13 %. For the coculture NBps treatment, the correlation between the measured and calculated DIC concentrations was weaker (Fig. 3b), with an rmsr of 1.7 mM for measurements ranging from 2.6 mM to 9.8 mM, yielding an estimate error of 26 %. It should be noted that during the first 40 h of the experiment, the highest difference between measured and calculated carbon values was obtained for the NBps treatment (Fig. 3b) and this difference paralleled both the exponential growth of *B. subtilis* and the decrease in pH (refer to Fig. 1f and d, respectively).

5 Discussion

We observed that the precipitation of $CaCO_3$ was enhanced in a coculture of ureolytic and non-ureolytic bacteria (NBps treatment, Fig. 1a and b). To gain a comprehensive understanding of the process, we monitored the following parameters: dissolved calcium concentration, DIC and carbonate ion concentration and pH, which are the principal chemical parameters that control the level of saturation with respect to $CaCO_3$, thus affecting $CaCO_3$ precipitation process. Other parameters, such as ammonium concentration and optical density, were determined as a measure of bacterial growth and activity during the experiment.

5.1 Growth conditions and bacterial growth

The urea concentration used in our experiments was considerably lower than the optimal concentration required for the proliferation of *S. pasteurii*, i.e., 200 mM (Jahns et al., 1988), thus limiting the growth of this species. Similar urea concentration was applied in the work of Ferris et al. (2003), who showed that urea hydrolysis could occur at a concentration as low as 6 mM.

In this experiment, due to its relatively long duration (10 days), it is likely that both bacterial species underwent sporulation to some extent. However, measurements of ammonium concentration, indicating bacterial activity, along with correlation between OD and CFU increase, indicate that both species remained viable during the first 80 h of the experiment.

Bacterial growth in the NBps treatment, containing both bacterial species, was faster than that observed in the NBp treatment, containing only *S. pasteurii*, with the difference presumably representing the growth of *B. subtilis* (Fig. 1f). The two stages of OD increase in the 1/3 NBps treatment may be explained as an initial increase in *B. subtilis* cell numbers followed by an increase in *S. pasteurii* cell numbers. The growth of *B. subtilis* in this treatment was limited – vis-à-vis the full-strength treatment – by the relatively low concentration of NB, i.e., 4.34 g L^{-1} vs. 13 g L^{-1} (Fig. 1f). These conclusions are corroborated by the CFU counts. Thus, the 1/3 NBps treatment represents the superposition of the growth of the two bacterial species. We therefore deduce that there was no competition for resources between *S. pasteurii* and *B. subtilis* in the one-third strength medium and that there was no significant interference between the two species. In addition, the low nutrient concentration in this treatment had a greater effect on *B. subtilis* than on *S. pasteurii*, as shown by bacterial growth, pH and DIC measurements. This conclusion stands in agreement with the findings of previous studies that *S. pasteurii* can hydrolyze urea in the absence of an organic carbon source, although the number of viable cells is likely to decrease significantly under these conditions (Dupraz et al., 2009; Ferris et al., 2003).

The response of non-ureolytic bacteria to an enrichment of the microbial population by adding an organic carbon source could prove to be significant to the propagation of MICP in situ. In this study we showed that the presence of the non-ureolytic bacterium, *B. subtilis*, had no effect on the precipitation process when the concentration of NB (i.e., organic carbon source) was low (1/3 NBps treatment), but increased the rate of $CaCO_3$ precipitation when the NB concentration was high (NBps treatment). Since most studies of MICP in natural soils have used simple organic carbon sources, e.g., molasses, at low concentrations (Fujita et al., 2008; Burbank et al., 2011; Tobler et al., 2011), the results for the 1/3 NBps treatment are particularly relevant to the scaling up of MICP.

5.2 Urea hydrolysis and ammonium concentration

The final ammonium concentration for all the treatments exceeded a value of 14 mM (Fig. 1c), the maximal concentration of ammonium that could have originated from urea hydrolysis. We propose that the surplus ammonium derives from the mineralization of the NB. Therefore, it is possible that the differences in ammonium accumulation between the treatments could have resulted from differences in the bacterial growth rate rather than differences in the urea hydrolysis rate.

5.3 Variation in pH

Urea hydrolysis is expected to lead to an increase in the pH of the medium due to the production of ammonium (Reaction R4), as was indeed found in the single-species NBp treatment (Fig. 1d). However, the coculture NBps treatment displayed a non-characteristic decrease in pH between the 10th and 28th hours of the experiment. This decrease was correlated in time with the exponential growth phase of *B. subtilis* and may therefore be attributed to increased respiration, leading to enrichment in CO_2, thus acidifying the medium. DIC measurements showed an increase in the concentration of inorganic carbon, corroborating this conclusion (Fig. 1e). A similar phenomenon was also described by Tobler et al. (2011) for the induction of urea hydrolysis in a mixed culture of indigenous soil bacteria. Accelerated precipitation of $CaCO_3$ in the coculture NBps treatment might also have contributed to the decrease in pH, but there was no correlation over time between changes in pH and $CaCO_3$ precipitation rate.

A decrease in pH similar to the one observed in the coculture NBps treatment – though to a lesser extent – was noted for 1/3 NBps treatment, further corroborating our observation that this treatment supported a superposition of the growth of the two bacterial species. Despite the decrease in pH observed in both treatments containing *B. subtilis*, the pH remained slightly basic, thereby enabling the continuing precipitation of $CaCO_3$.

5.4 DIC and CO_3^{2-} concentrations

DIC concentrations in our experiments were affected by three processes: (1) hydrolysis of urea to produce bicarbonate, (2) bacterial respiration and mineralization of the NB by ureolytic and non-ureolytic bacteria to produce dissolved CO_2, and (3) precipitation of $CaCO_3$, which led to a reduction in DIC concentration. In order to enable us to differentiate between the two sources of inorganic carbon, we compared calculated and measured DIC concentrations. Since ammonification of amino acids in the growth medium resulted in slightly higher ammonium concentrations than would have been expected from urea hydrolysis, our calculated values of urea-derived carbon are likely to represent an overestimation of the DIC that originated from urea hydrolysis and therefore the differences between calculated and measured DIC values presented here are probably slightly underestimated.

According to our results, more inorganic carbon was produced in the coculture NBps treatment than in the other two treatments (Fig. 1e). The larger amount of inorganic carbon found in this treatment cannot be attributed to the hydrolysis of urea (Fig. 3b) and must thus be attributed to the metabolic activity of the non-ureolytic bacteria. Despite the higher concentration of DIC in this treatment, the calculated CO_3^{2-} concentration was lower than that for the NBp and 1/3 NBps treatments (Fig. 2), due to the lower pH in the coculture NBps treatment.

Table 1. Comparison of chemical and biological conditions between coculture (NBps) and control (NBp) during the first 80 h of the experiment.

Parameter	NBp	NBps
$\left[CO_3^{2-}\right]$	0.005–0.132 mM	0.005–0.087 mM
OD	40 h lag phase	17 h lag phase
$CaCO_3$ precipitation rate	100 % in 123 h	100 % in 80 h

5.5 $CaCO_3$ precipitation

The maximal rate of $CaCO_3$ precipitation was found in the coculture NBps treatment (Fig. 1b). $CaCO_3$ precipitation requires supersaturation, as indicated by a saturation index ($SI = \{Ca^{2+}\} \times \{CO_3^{2-}\}/K_{sp}$) higher than 1. Since the rate of precipitation is affected by the availability of nucleation sites and by the SI, the addition of foreign solids that catalyze the nucleation process, or the increase of the SI of the medium with respect to $CaCO_3$ could increase the precipitation rate (Stumm, 1992). The determination of SI requires a knowledge of the activities of Ca^{2+} and CO_3^{2-}, and it is therefore necessary to know the ionic strength ($I = 0.5 \times \Sigma_i c_i \times Z_i^2$) of the precipitation medium. High ionic strength results in a lower ion activity and therefore lower SI, and vice versa. In our experiment, due to the use of a chemically undefined NB, we could not determine the ionic strength of the precipitation media. When examining the measured concentrations of the major constituents in this experiment (i.e., NH_4^+, Ca^{2+}, pH and the carbonate species), we thus assumed that the ionic strength for the coculture NBps treatment was similar to that for the control NBp treatment, and, therefore, we relied on Ca^{2+} and CO_3^{2-} concentrations as indicators of the SI.

An examination of Table 1 shows a faster precipitation rate for the coculture NBps treatment than for the NBp (control) treatment, despite the higher CO_3^{2-} concentration in the NBp treatment. It is therefore necessary to consider other factors that might have accelerated $CaCO_3$ precipitation in the presence of non-ureolytic bacteria. Previous studies have shown that precipitated $CaCO_3$ encapsulates *S. pasteurii* cells (Castanier et al., 1999; Stocks-Fischer et al., 1999; Mitchell and Ferris, 2006; Dupraz et al., 2009; Cuthbert et al., 2012); it is assumed that the electronegativity of the bacterial cell wall favors the adsorption of cations, such as calcium ions, thus facilitating the $CaCO_3$ precipitation process on the cell wall (Schultze-Lam et al., 1996). In our experiment, the non-ureolytic bacterium, *B. subtilis*, exhibited a significantly higher growth rate (Fig. 1f), resulting in a higher concentration of bacterial cells in the precipitation medium. The similarity between the zeta potentials of *S. pasteurii* and

B. subtilis suggests that the electric charges surrounding the cell envelopes of the two species of bacteria are similar. We therefore suggest that the non-ureolytic bacteria provided additional nucleation sites, thus accelerating $CaCO_3$ precipitation. Our findings are in keeping with the study of Mitchell and Ferris (2006) on the role of *S. pasteurii* cells as nucleation sites for the precipitation of $CaCO_3$, which showed that the presence of bacterial cells in the precipitation medium increased the $CaCO_3$ crystal size and the precipitation rate.

6 Conclusions

The results of our coculture experiment provide insight into the complexity of interactions between different bacteria during ureolytic MICP. We demonstrated that, in our experimental setting, the non-ureolytic bacterial species exhibited a considerably higher growth rate, which resulted in higher bacterial density. This relatively higher growth led to a decrease in pH of the precipitation medium, which resulted in lower carbonate ion concentration despite higher total DIC concentrations. Nonetheless, the presence of non-ureolytic bacteria promoted a higher rate of $CaCO_3$ precipitation. We thus suggest that the non-ureolytic bacterium, *B. subtilis*, facilitated $CaCO_3$ precipitation in our experimental setting by providing additional nucleation sites. The similarity in zeta potentials of the two bacterial species supports this conclusion. We conclude that the presence of non-ureolytic bacteria can have a significant effect on ureolytic MICP and that the scaling up of ureolytic MICP must take into consideration possible interactions between ureolytic bacteria and indigenous non-ureolytic bacteria and their effect on the precipitation process.

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