

Article

Insights into the Precipitation of Biocement Minerals Using Microfluidic Devices and SEM Images

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Abstract

Microbially Induced Carbonate Precipitation (MICP) is a biochemical process that promotes the precipitation of calcium carbonate, mainly in the mineral form of calcite, using urease-producing bacteria. This method has numerous applications, particularly in the field of geotechnical engineering when it is adopted for soil improvement or for the consolidation of porous or cracked construction materials such as stone and concrete. One microfluidic platform made of polymethylmethacrylate (PMMA) was designed with multiple channels, and the minerals precipitated were visualized using an optical microscope. The precipitated mineral observed in all channels analyzed formed spherical mineral structures with a core and multiple external rings. The same spherical mineral structures were observed in the biocement layer precipitated on plates of the same material as that of the microfluidic platform and on limestone, following the same treatment protocol. SEM images of pieces of these layers, complemented with EDS and mineral analysis by XRD, have confirmed the existence of multiple layers of minerals with spherical structures, mainly vaterite, precipitated around a nucleation point. Overlapping minerals in both the confined microfluidic channels and the unconstrained plates indicate that overlap results from repeated injections rather than physical confinement. From the tests with the microfluidic devices, these studies revealed that crystallization depends on different factors, namely the size of the channels and the number of *Sporosarcina pasteurii* cells. The number of injections appeared to affect the number of rings precipitated around the inner core. Substrate effects on spatial distribution or adhesion may still exist but were not detectable in this study and require further investigation. The observation of similar mineralogical structures in both the microfluidic devices and the plates, particularly the limestone, demonstrates that microfluidic systems are effective tools for small-scale visualization of geological processes.



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Keywords: biocementation; calcium carbonate; microfluidics; morphology

1. Introduction

Calcium carbonate is one of the most significant [1] minerals precipitated by biomineralization in nature, which comes mainly in the mineral forms of calcite or vaterite or in various amorphous phases. These minerals are present in carbonate sedimentary rocks,

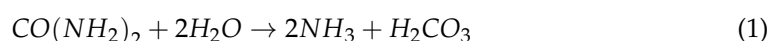
biogenic deposits (components of shells, pearls, and corals), and other earth environments [2–4]. The precipitation of calcium carbonate can occur through different biological or biomineralization processes, such as (i) hydrolysis of urea, (ii) denitrification, (iii) sulphate reduction, and (iv) iron reduction. Enzymatic hydrolysis of urea is the most widely used method due to its high energy efficiency, low cost, and greater control over the reaction process [5,6].

Biomineralization occurring through hydrolysis of urea has been explored in several civil engineering applications, for example, for soil improvement [5–9], consolidation of porous construction materials [10,11], and crack sealing [12–14]. In these cases, the precipitation of calcite is intended to create bonds between particles and fill voids, with the urease enzyme being synthesized by various organisms, including plants (e.g., *Canavalia ensiformis*), bacteria, algae, fungi, and invertebrates [1,5]. When using urease-producing bacteria, the process is named Microbially Induced Carbonate Precipitation (MICP). The microorganisms involved are unicellular structures that integrate enzymes, which are responsible for controlling and regulating the velocity of chemical reactions at specific sites [5]. Besides the intervention of the urease enzyme, the MICP process depends on calcifying components: the source of urea ($\text{CO}(\text{NH}_2)_2$) and the source of calcium (usually calcium chloride, CaCl_2). Both urea and calcium chloride are supplied in the form of a feed solution.

In the context of MICP, the most popular bacterial strain is *Sporosarcina pasteurii* because it is non-pathogenic and has a high ureolytic activity, resulting in a high production rate of precipitate [15] and a strong resistance to high ammonium concentrations. *S. pasteurii* cells do not aggregate, and therefore, the ratio between their surface area and volume is high, providing ideal conditions for the onset of CaCO_3 precipitation [1,16]. *S. pasteurii* bacteria have negatively charged functional groups in their cell walls, attracting Ca^{2+} ions from the environment to their surface, creating nucleation sites for the formed crystals. As the process progresses, the crystals grow by successive stratification, encapsulating the cells [1,6,15].

In the presence of urea and water, the urease enzyme hydrolyzes urea into ammonium and carbonic acid (Equation (1)). The presence of ammonium and the increase in pH lead to the production of OH^- ions, and carbonic acid becomes bicarbonate ions. In the presence of calcium ions (from the dissociation of CaCl_2) and with an increase in pH, calcium carbonate is precipitated (Equation (3)).

The precipitation of calcium carbonate, CaCO_3 , occurs when the product of ionic activity ($[\text{Ca}^{2+}][\text{CO}_3^{2-}]$) exceeds the solubility product, K_{sp} , strongly depending on factors such as the (i) availability of nucleation sites (favors the deposition of calcium ions for precipitation), (ii) concentration of calcium ions, (iii) concentration of carbonate ions, and (iv) pH (the process requires an alkaline pH to change the bicarbonate balance and thus form carbonate ions) (Equation (2)). The K_{sp} is 4.8×10^{-9} for calcite [1,17].



Due to the hydrolysis of urea, the precipitation of CaCO_3 crystals occurs in three phases, as illustrated in Figure 1 (left and center): (i) the development of the supersaturated solution, (ii) nucleation at the point of critical saturation (the phase where precipitation occurs), and (iii) spontaneous growth of crystals in stable nucleation [17,18].

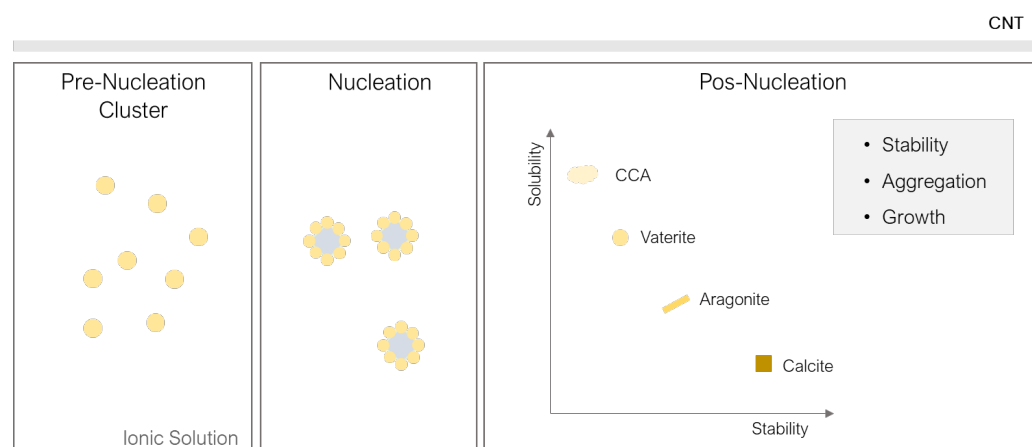


Figure 1. Classical Nucleation Theory (CNT) and Ostwald's step rule applied to CaCO_3 crystallization in the mineral forms of CCA, vaterite, aragonite, and calcite.

Microorganisms can stimulate the achievable saturation and precipitation rate of CaCO_3 by controlling the polymorphs of the CaCO_3 crystals produced [19]. They can occur in different forms, namely from various amorphous phases (calcium carbonate amorphous, CCA), anhydrous polymorphs (calcite, aragonite, and vaterite), and hydrated polymorphs (for example, monohydrocalcite ($\text{CaCO}_3 \cdot \text{H}_2\text{O}$) and ikaite ($\text{CaCO}_3 \cdot 6\text{H}_2\text{O}$)) [20–22]. However, within MICP, the most common crystalline forms range from CCA to anhydrous polymorphs, and their characteristics differ in terms of morphology, stability, and solubility [23]. CCA does not have an ordered crystalline structure, in contrast to the crystalline forms of calcite, aragonite, and vaterite. Vaterite crystals have a hexagonal structure and aragonite crystals have an orthorhombic structure, while calcite crystals have a rhombohedral structure [24]. Calcite is the most stable mineral with the lowest solubility [25].

According to Ostwald's step rule (OSR), as illustrated in Figure 1 (right), the kinetics of CaCO_3 precipitation start with the most unstable phases until it reaches a more stable final state. The CCA phases occur first, transforming into vaterite, a metastable phase, and eventually into calcite, gradually increasing in stability and changing its shape and size [23,24]. The process can be controlled by numerous factors such as temperature, pH, saturation levels, and additive concentration. For example, high saturation levels favor the formation of metastable vaterite and aragonite, while lower saturation levels promote the formation of calcite [26,27]. In addition, when the process is mediated by biological systems, the formation of different types of crystals depends on the organic matrix, the type of urease, and the reaction conditions. These all have a marked effect on crystallization, influencing morphology, structure, size, and appearance [23,28].

For over 100 years, the crystallization process of mineral crystal formation from dissolved ions in aqueous environments has been explained by the CNT (Classical Nucleation Theory) theoretical model, which describes the phases from a homogeneous solution to crystal formation [26,29] (Figure 1). However, this transformation process is not always linear, demonstrating limitations in accurately modeling molecular intermediate phases [29]. It is of great importance to study this phenomenon on a microscale, which can be performed using microfluidic systems. These are devices in which channels with small geometries (micrometers to millimeters) are built into transparent materials. They make it possible to manipulate small quantities of fluid and visualize it on a small scale and in real time, allowing for the analysis of temporal and spatial dynamics of different biochemical reactions [30–32]. Fluid channels are designed to replicate channeled or porous structures (e.g., soil pores, rock fractures, or blood veins) to reproduce the real phenomena that occur in these environments [31–33].

As miniaturized structures, the devices have advantages in the field of research, resulting in a potentially high analytical yield: (i) They allow control of the environmental conditions (pH, temperature, nutrients, injection speeds). (ii) Small amounts of reagents are required. (iii) The experimental time can be reduced. (iv) It is compatible with real-time visualization techniques, including microscopy [31–36].

Microfluidic platforms have only recently been introduced to study the MICP phenomena for geotechnical engineering applications [36–38]. The technique has been used by various researchers to analyze and visualize fluid movements; bacterial action; nucleation sites; precipitation phenomena; and extension of CaCO_3 , crystal growth, and the respective crystal modifications of the polymorphs of CaCO_3 [37–40].

The formation of calcite crystals is preferable due to their stability, binding effect, and low solubility [22,25]. Low solubility, in particular, has a direct impact on the durability of the biocementation treatment. Nevertheless, the presence of vaterite has been reported by many authors when using *S. pasteurii* [41,42], besides the mineral form being affected by the presence of cations [43]. As there is uncertainty associated with the crystallization phenomena of calcium carbonate polymorphs and vaterite, in particular, when using *S. pasteurii*, in this work, an experimental investigation was carried out using microfluidic devices to perform a microscopic analysis of the crystal structures precipitated after multiple injections of bacteria and feeding solution. In addition to the microscopic study, the research was complemented by SEM (scanning electron microscopy) analysis and EDS (energy-dispersive X-ray spectroscopy) analysis to confirm that the mineral form of the precipitate is not dependent on the substrate size (microfluidics) and nature (precipitation in polymethylmethacrylate (PMMA) and limestone plates submerged in the same treatment fluids). This is a novel contribution.

In addition, the morphology of the layer of biocement precipitated both in the microfluidic channels fixed at a selected depth and that of the plates was investigated from a novel point of view: the adhesion of the layer of precipitate to the upper plate of the microfluidic device allowed for the visualization of the contact surface, corresponding to the base of the layers rather than from its top view. This was very useful in comparing calcium carbonate precipitation in the microfluidic device and the plates' surface.

2. Materials and Methods

2.1. Microfluidic Platform

A device was designed consisting of two plates (150×150 mm) in PMMA, inspired by the work of [34]. The thickness of the upper plate was 3 mm. The bottom plate's thickness was 10 mm, which was necessary to install 11 channels of different widths (Table 1). The channels were built with an inlet and an outlet at each end for the fluids. The structures (channels and orifice) were built using a CNC milling machine (Minitch Machinery Corp., Norcross, GA, USA). Only the channels with 3 mm of depth were analyzed in this study, investigating width effect and not depth. The advantage was that this small depth allowed for the visualization of the adhesion of the layer of precipitate to the upper plate, which was seen from the bottom rather than from the top. This can be considered to provide an original comparison between the precipitation in the microfluidic device and the plates' surface.

Table 1. Geometry of the channels of the microfluidic plate.

Channel	1	2	3	4	5	6	7	8	9	10	11
Width (mm)	1.0	2.0	4.0	5.0	5.0	5.0	6.0	7.0	8.0	10.0	10.0
Depth (mm)	2.0	3.0	3.0	2.0	3.0	5.0	5.0	5.0	5.0	2.0	5.0

The plates were bonded by applying an ethanol solution to the surface followed by UV irradiation. The process allows for chemical interaction between the ester carbonyl groups of PMMA and the hydroxyl groups of ethanol [44].

Once the plates were in place, the remaining structures were connected to the system. The connectors were installed in each inlet and outlet using double-sided tape; metal coupler tubes (diameter 0.8 mm; length: 15 mm) were placed in each connector. Polythene tubing (PE-60 tubing, 0.8×1.2 mm) was fitted to each of these metal parts to create the fluid inlets and outlets in the system. Figure 2 presents the final configuration of the plate with the respective elements to be coupled for the functionality of the system.

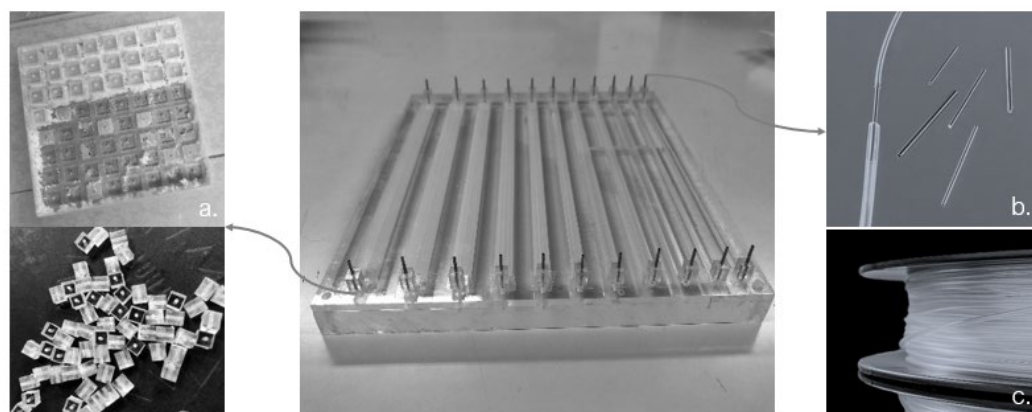


Figure 2. Final configuration of the manufactured board. (a) Connectors; (b) metal coupler tubes; (c) polythene tube.

2.2. PMMA and Limestone Plates

In parallel, 4 mm thick small rectangular plates (50 mm $\times$ 25 mm) of PMMA and limestone were subjected to the same biocementation treatment, with the aim of investigating the precipitation of calcium carbonate in the form of calcite and vaterite (Figure 3). The PMMA was chosen because it is the same material as the microfluidic platform. The comparison between the biocement minerals on this plate and on the stone allowed for the discarding of the influence of the material for bacterial attachment and crystal nucleation.

The limestone selected is beige, roughly calciclastic and abundantly bioclastic. It is a common construction material found in old buildings and monuments. The main mineral present is calcite and dolomite, with quartz and siderite in smaller quantities, as determined through X-ray diffraction (XRD).

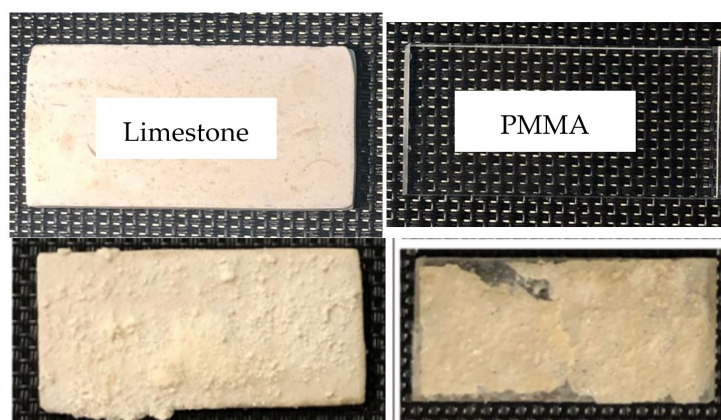


Figure 3. Limestone and PMMA plates before (top) and after (bottom) the treatment.

2.3. Bacterial and Feeding Solutions

The culture for the growth of *S. pasteurii* bacteria was grown in a medium prepared with 20 g/L yeast extract (YE), 10 g/L ammonium sulphate (AS, $(\text{NH}_4)_2\text{SO}_4$), and 0.13 M TRIS base (pH 9). The feeding solution was prepared with 0.5 M urea (30.03 g/L), 0.5 M calcium chloride (CaCl_2 , 73.51 g/L), sodium bicarbonate (NaHCO_3 , 2.12 g/L), ammonium chloride (NH_4Cl , 10 g/L), yeast extract (YE, 2 g/L), and ammonium sulphate ($(\text{NH}_4)_2\text{SO}_4$, 1 g/L).

2.4. Experimental Procedure

Each treatment consisted of applying bacterial and feeding solutions in sequence. For the microfluidics channels, the treatment fluids were injected to completely fill the microfluidic channel. The total volume of the treatment fluids therefore depended on the volume of the channel to be injected. The proportion was $\frac{1}{2}$ channel volume of bacterial solution and $\frac{1}{2}$ channel volume of feeding solution. Injections were performed every 2 days for 8 days for a total of 4 injections.

For the limestone and PMMA plates, they were placed in containers and submerged in the treatment fluids (bacterial solution and feeding solution). The tests began with the addition of 25 mL of bacterial solution and 25 mL of feeding solution. Every two days the treatment fluid was replaced with 25 mL of bacteria solution and 25 mL of feeding solution. The procedure was repeated 4 times to be identical to the treatment in the microfluidics channel.

2.5. Visual Observation and Mineralogical Analysis

Once the test was completed in the microfluidics platform, the mineral precipitated in the channels was visualized with an optical microscope (Nikon eclipse LV 100 NC, Tokyo, Japan). The images were taken in the middle of the channels' cross-section at 1 cm distance from the inlet. At the end of the tests, the microfluidic plate could not be disassembled to extract the precipitate, and therefore, the presence of calcium carbonate was confirmed by acid digestion. This was performed by injecting HCl acid (0.5 M) in the channels and filling them completely. The generation of air bubbles confirmed the presence of calcium carbonate but not the type of polymorph.

For the plates, the minerals precipitated were observed by the naked eye (Figure 3), and the precipitated coat was easily removed with a small knife and taken for mineralogical analysis and visualized with a scanning electron microscope (SEM, Hitachi S2400). SEM associated with EDS (energy-dispersive X-ray spectroscopy) allows for the identification of chemical elements of calcium carbonate such as calcium, carbon, and oxygen. Mineralogical analysis was performed by X-ray diffraction (XRD) analysis. The identification of the crystalline phases was carried out with an X-ray diffractometer (Malvern Panalytical X'PERT PRO model), using $\text{CuK}\alpha$ radiation and the following measurement parameters: 2θ ($5\text{--}70^\circ$); step size: 0.033° 2θ ; scan step equivalent time: 75 s; and generator settings: 35 mA, 40 kV. The analysis was performed using the program High Score Plus and the PDF2 database. Fine-grained powder (1–2 g) was compacted in the bottom of the sampler holder with some manual pressure and final leveling.

3. Results

3.1. Microfluidic Platform

The images at the end of one injection on channel 2 are presented in Figure 4. Circular aggregates of crystals of precipitate are visible, with a core and luminous external ring. Possibly, these crystals are attached to the upper plate because bacteria adhere to these walls and are nucleation sites, therefore facilitating the formation of CaCO_3 crystal aggre-

gates [38,40,45]. For this reason, the cross-section of the precipitated mineral structures is so visible. The spherical geometry of the mineral structures suggests they can be calcium carbonate in the form of vaterite.

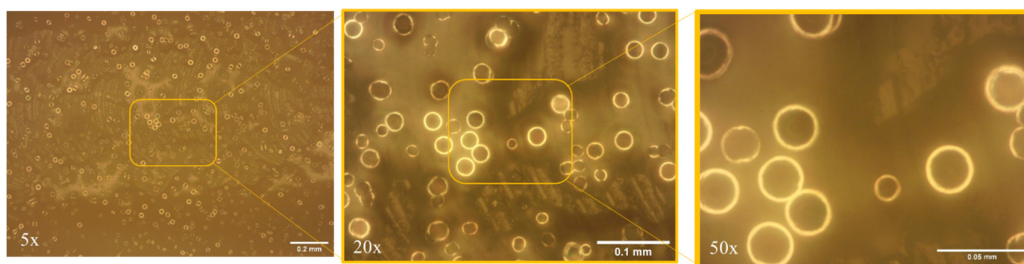


Figure 4. Optical microscope images of the precipitate in channel 2 after 1 injection with the treatment fluids. Magnification: 5×, 20×, and 50× (left to right).

The images collected at the end of the treatment (four injections) are presented in Figure 5 for channels 3 and 5. This figure also includes the images of the channels without any precipitate to discard the images of PMMA material texture and serve as reference.

Some observations can be taken from Figure 5:

- The diameters of the circles are smaller on channel 3 than on channel 5, and the spheres appear to merge and overlap more in this channel than on channel 5. The overlap observed may be caused by using an optical microscope, which does not allow us to focus on depth. Nevertheless, knowing that the same concentration of bacteria and feeding solution were injected in all channels and that the quantities depend on the channel volume, this result suggests that the number of nucleation sites are dependent on the room available;
- The diameter of the spheres is probably reduced for the smallest channel because their growth is restrained by the presence of other spheres. Similar observations were found by others [22,27,38,39], reporting that the dispersion of the precipitate depends on the dispersion methods of bacteria. This is associated with bacterial attachment, as already mentioned, and their cell kinetics and interaction in the liquid medium injected into the channels [1,18,19];
- There are several circles inside the spheres, indicating some internal structure of the crystals precipitated. The precipitation mechanism begins with the predominant presence of CCA, followed by the dissolution of unstable CCA and the recrystallisation into vaterite and calcite. Subsequently, the metastable vaterite may dissolve and the most stable calcite recrystallizes [36,46].

The diameter of the mineral spherical structures has increased with increasing number of injections. Growing appears to be around the central core, with the addition of multiple external rings. This can be seen in Figure 6, showing images after one and four injections. This phenomenon is related to crystal growth, where, after nucleation, successive layers are precipitated. In comparison, in the images on the left, where only one injection was made, these rings are not observed. The diameter of these minerals varies between 0.010 and 0.030 mm in the first injection, appearing to be larger than 0.050 mm after the last one. The number of rings also appears to increase with an increasing number of injections.

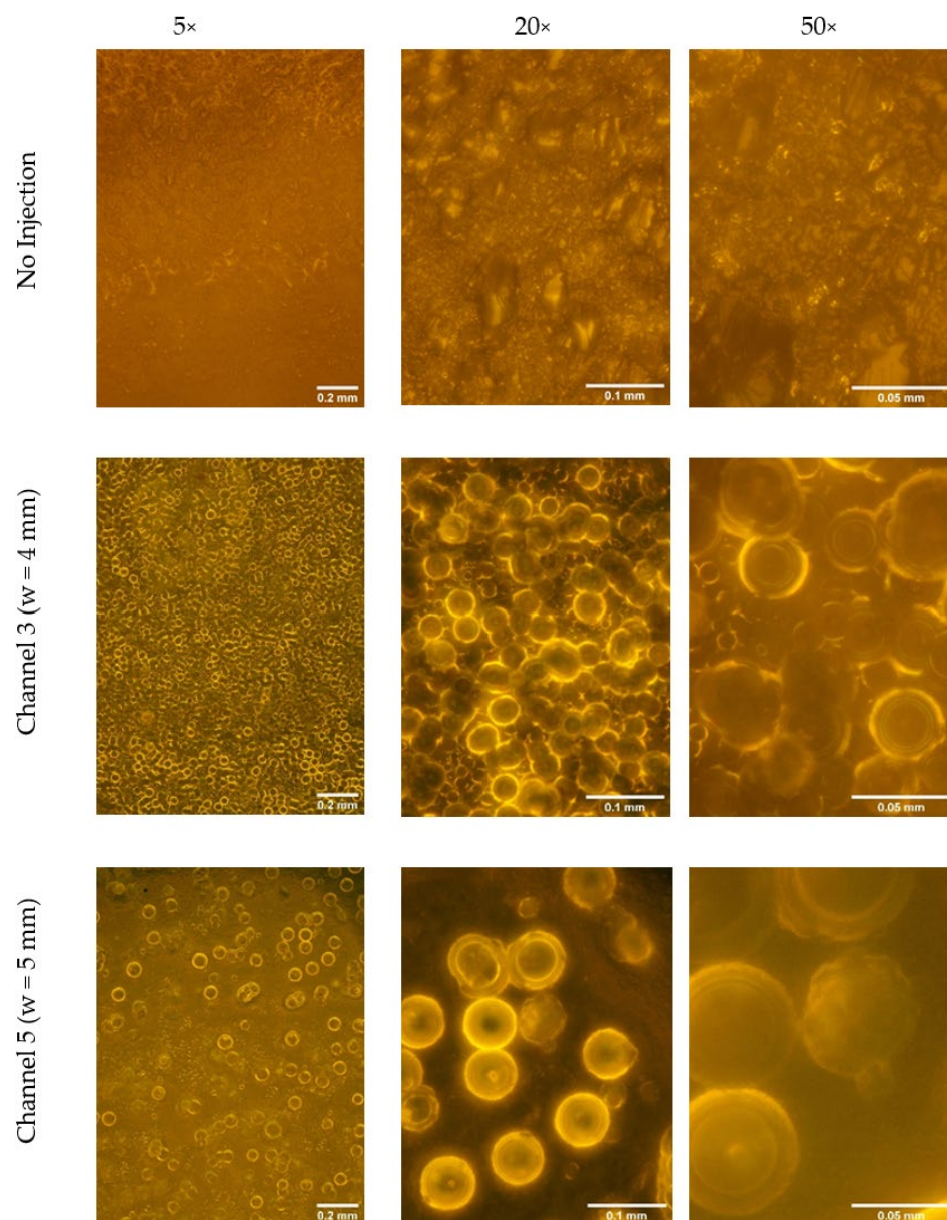


Figure 5. Optical microscope images in channels 3 and 5 after 4 injections (w is the channel width) and a PMMA plate without any precipitate.

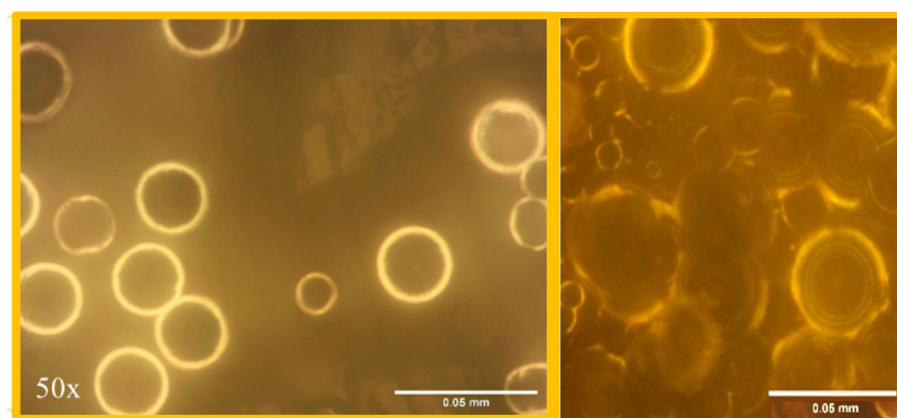


Figure 6. Optical microscope images of CaCO_3 precipitated on channel 2 ($50\times$ magnification) after 1 injection (**left**) and after 4 injections (**right**).

The increasing number of circles with an increasing number of injections, also observed by Wang et al. [37], can also be explained by dissolution and recrystallization of calcium carbonate due to pH changes (not measured). In this case, they are facilitated by refreshing the treatment fluids and biochemical reactions, creating more stable crystalline forms.

Finally, the liberation of gas bubbles during acid injection in the microfluidic platform is presented in Figure 7, confirming the presence of calcium carbonate. The platform was cleaned after washing with distilled water to remove the acid remains.

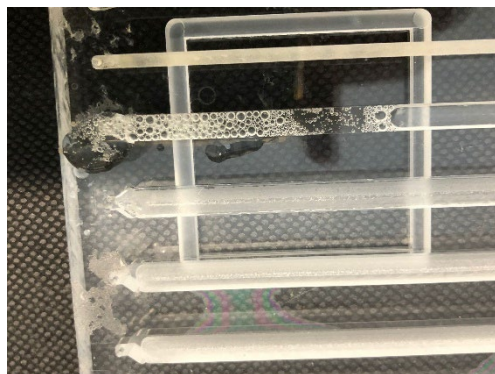


Figure 7. Acid digestion of the calcium carbonate precipitated in the microfluidic channels.

3.2. PMMA and Limestone Plates

Pieces of the biocement crust in the PMMA and limestone plates were visualized in SEM. Aggregates of mineral structures with spherical shape were visible, as well as several bacterial imprints. Figure 8 presents the images of the precipitate on the PMMA plate, as well as XRD spectra. The arrows in this figure point to bacterial imprints, while the square indicates the location where EDS test was carried out, confirming the presence of calcium, oxygen, and carbon.

In Figure 8, one spherical mineral structure appears to be broken, showing a core and an external ring with different textures. This shape resembles that of the crystals observed in the microfluidic device. Multiple bacterial imprints are visible, indicating they serve as nucleation for the precipitate. The spherical form is associated not with the fact that a bacterium is in the center of the sphere but with the biochemical conditions of the solution promoting the crystallization of CaCO_3 [15,16,22]. In this case, the mineral form appears to be vaterite due to the spherical shapes observed. Indeed, the presence of this mineral is confirmed in the XRD spectra, as well as calcite, although calcite was detected in smaller quantities and is not visible in the SEM images presented.

The analysis of the precipitate on the limestone plates is presented in Figures 9 and 10, with respective EDS. The piece of precipitate in Figure 9 is the face in contact with the stone, and the one in Figure 10 is the upper face. The presence of aggregates of spherical mineral structures, like those observed in the PMMA plates, is visible in all SEM images, with calcium, carbon, and oxygen in their composition being detected by EDS.

The minerals visible in Figure 9a are like the patterns observed in the microfluidic devices previously presented in Figure 5 because they have a circular shape with an inner core and an external layer. The diameters of these circles vary between 0.01 and 0.03 mm, which is consistent with the diameters of the crystals observed in the microfluidic device. The circles are joined, forming aggregates, appearing to be covered by an external layer separated from the aggregates with a gap, with irregular interior texture. Overall diameter increases, as observed in the microfluidic device after four injections. Therefore, possibly, the external layer was precipitated after further treatment stages. The precipitation of successive layers was also observed in the microfluidic device, as already discussed.

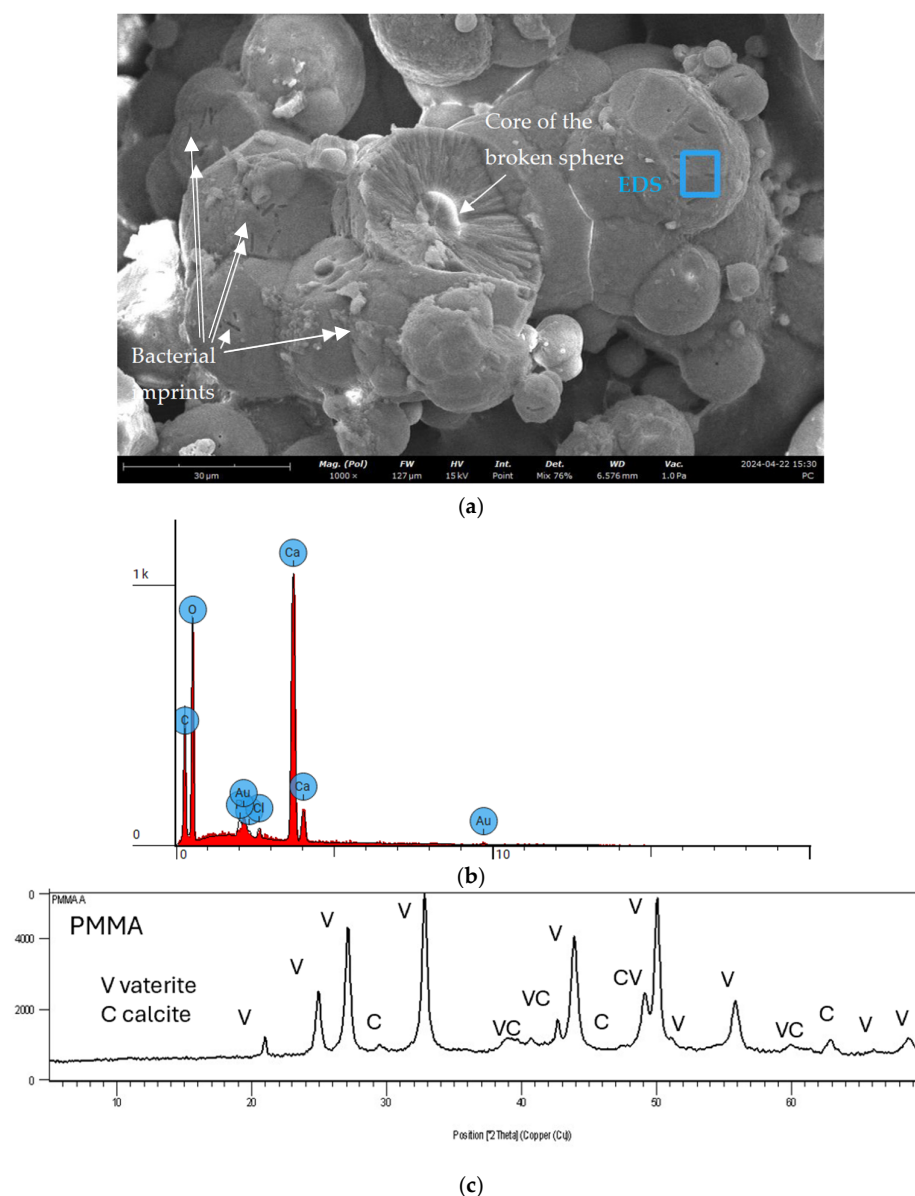


Figure 8. Precipitate on PMMA plate: (a) SEM image; (b) EDS analysis; (c) XRD analysis.

The presence of several layers in the minerals is also visible in Figure 10a, where the aggregation of spherical mineral structures overlapped is obvious. As before, many bacterial imprints are visible, and some were probably injected in further stages, after the precipitation of calcium carbonate crystals that had occurred after the previous injection.

Layers attributed to precipitation occurring during further treatments are visible in Figure 10a on the right (scale bar: 0.050 mm). A piece of material with a flat surface and a broken mineral is visible in Figure 10a on the left (scale bar: 0.030 mm), showing the existence of a core and an external ring with different textures. Similar broken minerals were observed in the PMMA plate in Figure 8a, indicating similar precipitation processes.

EDS was performed, which is presented in Figure 10b and confirms the presence of calcium, carbon, and oxygen, and the presence of vaterite and calcite was confirmed by XRD analysis, as presented in Figure 10c.

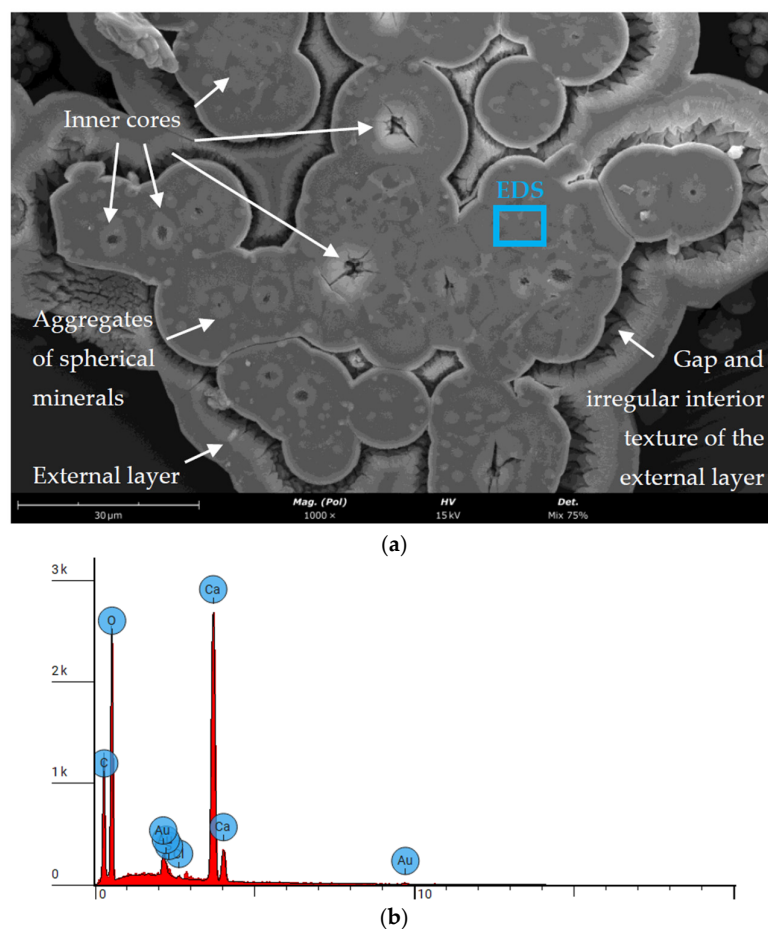


Figure 9. Bottom view of the precipitate on the limestone plate: (a) SEM image; (b) EDS analysis.

Calcite crystals with rhombohedral shapes are not visible in the SEM images presented in Figures 8–10, although they were detected by XRD. Figure 9a may help explain this result if a deep look at the separation wall of the external layer from the core of the sphere is observed. Here, some cubic minerals can be seen, eventually indicating the start of the precipitation of calcite. This may indicate a transition from metastable vaterite to calcite, observed both in solutions and in microfluidic devices [19,26,27,39–41], and should be investigated in the future. Possibly, calcite exists already inside the spheres from the other SEM images, which is not visible but can be detected when the layers are pulverized to perform XRD.

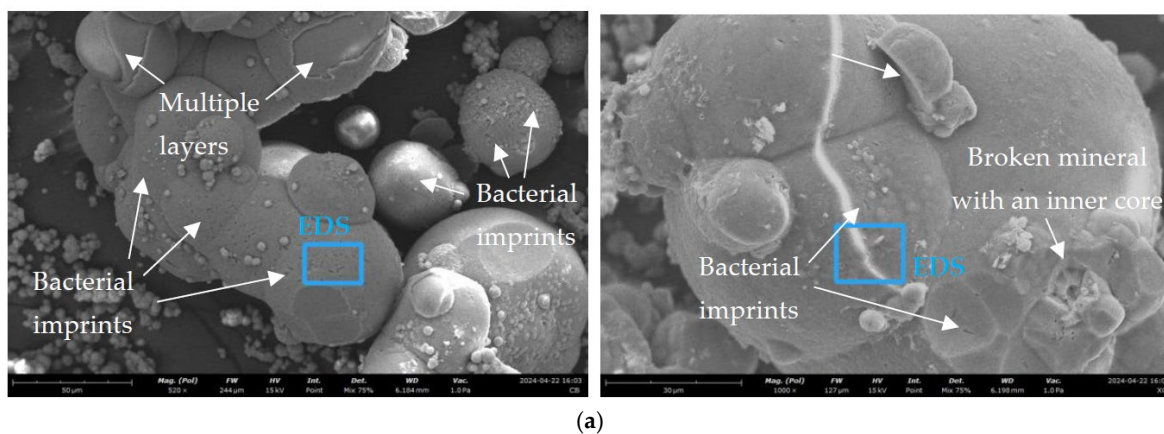


Figure 10. Cont.

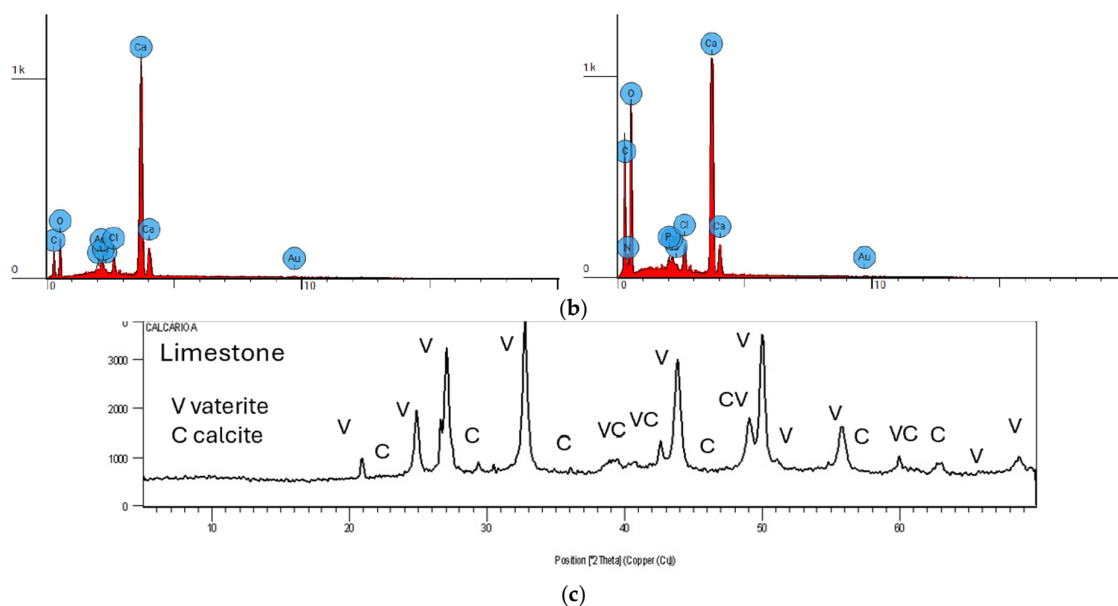


Figure 10. Top view of the precipitate on the limestone plate: (a) SEM image; (b) EDS analysis; (c) XRD analysis.

4. Discussion

The SEM results obtained on both the PMMA and stone plates confirm the presence of the same spherical mineral structures observed under optical microscopy on the microfluidic platform. The precipitation process in the plates is quite visible in Figure 9a and can be compared directly with that of the microfluidic platform upper plate. The nucleation of the mineral appears to be a small hole in the center of some circles/spherical shapes. The geometry (size and shape) of this hole is not consistent with a bacterium, confirming that the nucleation point is not a bacterium but some defect in the substrate. Such defaults were created during the milling of the substrates, both in the microfluidic device and plates. The fact that many bacterial imprints were found in the mineral spherical shapes supports that bacteria are dispersed in the solution.

In general, the diameters of the minerals with spherical structures observed in both types of plates vary between 0.030 and 0.050 mm, consistent with the diameters of the minerals observed in the microfluidic device after four injections. They result from the precipitation of successive layers, which was explained by pH changes caused by successive injections. The smallest spheres precipitated above the largest spheres have a smaller diameter than those below them, suggesting they were precipitated in the following injections.

The reduced size of the samples analyzed, both with the microfluidic device and SEM, makes it difficult to make conclusions about the spatial distribution of the biocement and, in particular, the arrangement of the precipitated minerals and their overlapping. While in the microfluidic channels, the size available was reduced, and for this reason, overlap was expected, and there were no physical constraints in the plates. This suggests that overlap is caused by multiple injections.

Similar mineral shapes were observed in PMMA and limestone. This indicates bacterial survival and not the interference of the substrate, mainly because of the biological origin of the mineral. It appears that the nature of the substrate does not interfere with the process, which validates the use of microfluidic devices to investigate biocementation mechanisms in soils and different construction materials. Possibly, the nature of the substrate may affect the spatial disposition of the spheres, which was not observed in this study because of the small size of the samples investigated. The nature of the substrate may affect the adhesion

of biocement to the surface as well, which was confirmed by Pinto and Cardoso [47] and requires further investigation, which is out of the scope of this paper.

5. Conclusions

The results obtained from optical microscopy were compared with scanning electron microscopy (SEM) images of precipitated material crusts on natural limestone substrates subjected to the same treatment. Similar spherical mineral structures were identified, exhibiting a morphology characterized by a central core and an external ring, confirmed to be mainly vaterite through X-ray diffraction.

Overlapping minerals in both the confined microfluidic channels and the unconstrained plates indicate that overlap results from repeated injections rather than physical confinement. From the tests with the microfluidic devices, these studies revealed that crystallization depends on different factors, namely the size of the channels and the number of *S. pasteurii* cells. The number of injections appeared to affect the number of rings precipitated around the inner core, which increased with the increasing number of injections.

Substrate effects on spatial distribution or adhesion may still exist but were not detectable in this study and require further investigation. The observation of similar mineralogical structures in both microfluidic devices and plates, particularly the limestone, demonstrates that microfluidic systems are effective tools for small-scale visualization of geological processes. The view of the precipitated layers in contact with the surfaces brings novelty and should be explored to study the adhesion of this material, particularly in geological and construction materials. The appearance of evidence of calcite minerals inside the spherical mineral structures of vaterite confirms that the transition from metastable vaterite to calcite is occurring. The link to the use of bacteria is worth investigating in the future.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/min16030253/s1>, Figure S1: SEM images and XRD plots.

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