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MICP via denitrification pathway under aerobic conditions

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ABSTRACT

Microbially induced calcite precipitation (MICP) through the denitrification pathway is achieved when bacteria use nitrate as the electron acceptor to oxidize organic matter and generate ATP for growth. Most denitrifying bacteria are facultative anaerobes and in aerobic conditions, they use oxygen as the electron acceptor instead of nitrate. In other words, these bacteria can grow under aerobic conditions but they do not denitrify unless under anoxic conditions. This is because using oxygen yields more energy compared to denitrification. Recently, however, it has been shown that many bacteria (e.g., Paracoccus denitrificans) can denitrify under aerobic conditions even though it might result in lower levels of cell reproduction. These aerobic denitrifiers are commonly found in soil and aqueous environments including activated sludge used in wastewater treatment. In this study, batch experiments were conducted to confirm the occurrence of denitrification-MICP under aerobic conditions. Furthermore, samples from easily erodable sand dunes of Lake Superior were treated via denitrification-MICP under aerobic conditions. Activated sludge from a local wastewater treatment facility was used as the source of denitrifiers. Carbonate content tests, Scanning Electron Microscopy, Energy Dispersive Spectroscopy, Transmission Electron Microscopy, and X-ray diffraction analysis were conducted on treated soils. The results confirmed that different calcium carbonate polymorphs including calcite were precipitated and also showed that some salts and extracellular polymeric substances (EPS) were precipitated at the soil surface.

INTRODUCTION

In recent decades, Microbially Induced Calcite Precipitation (MICP) has garnered attention because of the ability to reduce carbon emissions, be more environmentally sustainable, and offer a more economical alternative to current methods (El Mountassir et al. 2018). This method, which begins with a biological process and ultimately precipitates calcite, can be conducted through various pathways such as urea hydrolysis, nitrate reduction (denitrification), sulfate reduction, photosynthesis, methane oxidation, and amino acid deamination (van Paassen et al. 2010; Castro-Alonso et al. 2019; Liu et al. 2021; Xue et al. 2023). Recently, MICP via denitrification has gained attention due to its advantages over other pathways, such as its non-harmful by-products compared to the more common urea hydrolysis method that produces a harmful by-product, i.e., ammonia. The denitrification-MICP method not only does not produce harmful by-products (Gebru et al. 2021) but also diverse groups of bacteria with higher tolerance to unfavorable environmental conditions can be used which makes it more convenient in field applications.

Denitrification relies on denitrifying bacteria respiration, which utilizes a source of organic carbon, such as acetate, as an electron donor and reduces nitrate, which acts as an electron acceptor. This reduction is a sequential process (Eq. 1) that reduces nitrate to nitrogen gas in the case of a complete reaction (Kornaros et al. 1996).

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \quad (1)$$

In the presence of calcium in the environment, the carbonate produced due to metabolic reactions can react with calcium and precipitate calcite (Eq. 2).

$$x\ Ca(C_2H_3O_2)_2 + x\ Ca(NO_3)_2 \rightarrow xCaCO_3 + xN_2 + x\ CO_2 \quad (2)$$

The actual stoichiometry of equation 2 depends on the bacteria growth rate (Pham et al. 2018), and therefore the stoichiometric coefficients are replaced with x to represent the general reaction. In the geotechnical engineering literature, MICP via denitrification is mostly known as an anaerobic process (Goswami et al. 2019; Jain et al. 2021; Wei et al. 2022; Feng et al. 2024), possibly because many types of microorganism strains prefer oxygen over nitrate, as oxygen acts as the preferred terminal electron acceptor in microbial respiration. When oxygen is present, microorganisms will use it instead of nitrate, thereby limiting denitrification (Rohe et al. 2021; Wei et al. 2022). Considering denitrification as an anoxic process may pose challenges such as selecting anaerobic bacteria, their cultivation conditions, the required equipment, and challenges in the field especially for their application in aerobic environments such as shallow soils. It should be noted, however, that a vast number of microorganisms are capable of denitrification in oxic conditions (Ji et al. 2015), even at high oxygen concentrations up to air saturation levels (Lloyd et al. 1987). These aerobic denitrifying bacteria can be found in activated sludge (Ji et al. 2015; Xia et al. 2020), which is designed to remove nitrogen from wastewater through nitrification and denitrification in periodic aerations (Ji et al. 2015).

Utilizing activated sludge for denitrification purposes provides aerobic and anaerobic denitrifying bacteria allowing the calcite precipitation to be achieved in both oxic and anoxic environments (both shallow and deep soil stabilization). It also eliminates the need for growing a specific bacteria strain and reduces the challenges and costs associated with the inoculation process of a single strain that requires very restrictive conditions for the bacteria to grow. Additionally, since the denitrifiers can be essentially inoculated in a non-sterile environment, it is easily applicable with minimal concern about bacterial culture medium contamination.

In this study, activated sludge from a local wastewater treatment facility was used to examine the possibility of performing denitrification MICP under aerobic conditions. Liquid batch experiments as well as soil experiments were used to perform the aerobic denitrification MICP experiments.

MATERIAL AND METHODS

First, denitrification-MICP batch experiments were conducted under aerobic conditions using activated sludge and a calcium source. Activated sludge obtained from a wastewater treatment facility near Houghton, MI, USA, was used to provide the denitrifying microorganisms. To cultivate the bacteria, a substrate, a nutrient broth, and a trace element solution were used. The substrate consisted of 50 mM calcium nitrate ($Ca(NO_3)_2$) and 60 mM calcium acetate

($Ca(C_2H_3O_2)_2$). The nutrient broth contained 0.003 mM ammonium sulfate ($(NH_4)_2SO_4$), 0.0024 mM magnesium sulfate ($MgSO_4$), 0.006 mM monopotassium phosphate (KH_2PO_4), 0.014 mM dipotassium phosphate (K_2HPO_4). A 1 ml/L trace element solution SL12B was used which includes (EDTA-$Na_2.2H_2O$, 3000 mg/L; $FeSO_4.7H_2O$, 1100 mg/L; H_3BO_3, 300 mg/L; $CoCl_2.6H_2O$, 190 mg/L; $MnCl_2.4H_2O$, 50 mg/L; $ZnCl_2$, 42 mg/L; $NiCl_2.6H_2O$, 24 mg/L; $Na_2MoO_4.2H_2O$, 18 mg/L; $CuCl_2.2H_2O$, 2 mg/L; pH adjusted to 7.2-7.3). The substrate, nutrient broth, and trace element solution were added to the activated sludge which was then allowed to settle for 6 days. The solid sludge that formed on top of the cultivated activated sludge, as well as the solid sediments, were discarded, and the supernatant was utilized as the source of bacteria (Pham et al. 2018). For the batch experiments, a fresh culture medium containing 50 mM calcium nitrate and 80 mM calcium acetate, along with the same amount of nutrients used in the previous step, was added to distilled water (The pH of the treatment solution was neutral and about 7). This solution was mixed with the bacteria source (supernatant mentioned before) under aerobic conditions, and the specimen was allowed to air-dry completely. The sediments formed in the container were used for further analysis. It is worth mentioning that no attempt was made to make the inoculation process completely aerobic, but the water was not de-aired and the whole system was in contact with the air throughout the inoculation. It is worth mentioning that dissolved oxygen (DO) in water at room temperature typically ranges from 6.5 to 8 mg/L. This value can provide an aerobic condition to perform aerobic denitrification. Although its worth mentioning that optimal value of the oxygen depends on many factors such as type of the bacteria, for instance, Kim et al. (2008) mentions that Pseudomonas putida AD-21 tolerated oxygen levels of 5.0-6.0 mg/L, while Zhu et al. (2012) reports that Pseudomonas mendocina 3-7 could perform aerobic denitrification at DO levels of 3-10 mg/L.

A second batch experiment was conducted following the same steps in which nitrate and nitrite concentrations were measured over 5 days to ensure denitrification occurred under the aerobic condition. During these five days, 30 ml (three 10 ml specimens for repetition) of the batch experiment solution was collected each day. The samples were then diluted and transferred into vials with filter caps to prevent any probable contamination. Ion Chromatography (IC) was used to measure the concentration of remaining nitrate (and produced nitrite) in the diluted solution.

To investigate the aerobic denitrification-MICP in the soil environment, a poorly graded sand sample taken from the sand dunes of the southern beaches of Lake Superior, MI, was prepared in a loose condition as shown in Figure 1. Gravel was used as the base layer of the specimens to allow for free vertical drainage.

Figure 1. Sand dune samples prepared for the denitrification-MICP experiments (spray test) in the soil environment

A cultivated bacteria suspension prepared as explained before was sprayed on the soil specimen. The total volume of sprayed bacteria suspension was equal to the void volume of the

top 3 cm of the sample specimen ensuring most of the denitrification-MICP occurred under near oxygen saturation conditions. Fresh culture medium (substrate, nutrients, and trace elements as explained before) was sprayed on the sample once a week for 6 weeks. The acetate-to-nitrate concentration ratio of 1.6 was used for the first three weeks to encourage more bacterial growth (Pham et al. 2018). The ratio was changed to 1.2 during the last 3 weeks which corresponds to the stoichiometry of maximum growth condition (Pham et al. 2018). The total volume of the sprayed culture medium for each cycle was equal to the void volume of the top 3 cm of the specimen.

Finally, two sand column experiments were conducted using the molds shown in Figure 2. The height of the samples in these experiments was 18 cm and the diameter was 14.5 cm. A sponge was used at both the top and the bottom of the samples to provide uniform solution flow. One pore volume of distilled water was first flushed through the sample. Then, bacteria suspension and culture medium (prepared as explained before) were flushed into the soil from the top of the mold using a constant head. Fresh culture medium was flushed into the soil from the top using the constant head every week for nine weeks. At the end of each cycle, one pore volume of distilled water was flushed through the sample right before starting the next cycle to remove the remaining substrates from the previous cycle. After flushing the fresh culture medium for each cycle, the lid of the mold was removed from one of the samples until the next flushing to create a more oxic condition (relatively aerobic) while the other sample's lid was kept closed throughout the entire nine cycles which would provide more of an anoxic condition. It is worth mentioning that the culture medium and the substrate solutions were not de-aired, therefore the second experiment is not considered completely anaerobic. However, as many denitrifiers are facultative anaerobic bacteria, it is very likely that these bacteria will use up the limited available oxygen in the environment causing the experiment to approach an anoxic condition over time (at least on microlevel around the bacteria).

Figure 2. Sand column test specimens, (left: relatively aerobic, right: relatively anaerobic)

In this study, the main focus was to maintain a process as close as possible to field conditions. No attempt was made to deair the treatment solution or soil matrix. Oxygen availability was similar to that in many studies on ureolysis under aerobic conditions, where the sprayed or injected material targeted the upper portion (3-5 cm) of soil in sprayed samples and the entire chamber length in sand column tests. These volumes were considered to be fully saturated, with oxygen supplied through dissolved oxygen and air exposure, replicating field conditions.

To investigate the amount of precipitated calcite in the treated samples, the rapid carbonate content measurement method was used ASTM D D4373-22 (Small et al. 2022). In addition, a

control (untreated) sample was tested to study any pre-exsisting calcium carbonate in the untreated soil. For the batch experiment test, two grams of residual compounds from the batch experiment were oven-dried at 110°C, ground, and placed into the rapid carbonate content measurement chamber and tested for the amount of precipitated calcium carbonates. For the spray test, multiple samples (each about 2 grams of soil) were taken from the top of the treated specimen, oven-dried, and tested in the chamber. For both aerobic and anaerobic sand column experiments, soil samples were taken from different depths of the column (each about 10 grams of soil), oven-dried, and tested in the chamber.

Finally, Scanning Electron Microscopy (SEM) characterization, Energy Dispersive X-ray Spectroscopy (EDS), and X-ray Diffraction (XRD) tests were conducted on the residual compounds from the batch experiment as well as denitrification-MICP treated soil samples.

RESULTS AND DISCUSSION

The results of the rapid carbonate content measurement test revealed that 20% by dry weight of calcium carbonate ($CaCO_3$) was precipitated at the end of the batch experiments. It is worth mentioning that in the rapid carbonate content measurement test, the pressure of the produced CO_2 gas during the reaction between carbonates and HCl acid is measured. As HCL is not an effective reducing agent for breaking down organic materials (Hu and Qi 2013), the pressure measurement in this test accurately represents the amount of calcium carbonate precipitated during the denitrification MICP. It is worth noting that the calcite content test on the untreated soil revealed zero percent calcium carbonate, confirming that any calcium carbonate present in the treated samples is a result of MICP reactions.

An average of 1.5 % by dry weight of calcium carbonate ($CaCO_3$) was precipitated at the surface of the sand dunes after six cycles of treatment using the spray method. The stoichiometry calculation reveals that in case of a complete reaction, in one treatment cycle about 3 grams of calcite will be precipitated. If this amount of calcite is evenly distributed within the whole treated area (i.e., the top three cm of the soil which is about 763 grams of soil), the average calcite content would be around 0.4 % for each cycle or about 2.4 % after six cycles of treatment. The amount of calcite measured in the treated samples (1.5 % after six cycles) is less than this amount which suggests that the substrates might not have been used efficiently.

Figure 3 shows a portion of the sand dune treated via aerobic denitrification MICP during the spray test. This sample was taken from the top of the spray-treated sand dune which illustrates that the most effective stabilization happened in the top 1-1.5 centimeters of the sample. Therefore, more than 2.4 % calcite should have been measured at the end of the six-cycle treatment. One possible explanation for this inefficient precipitation could be that the infiltration of bacteria and substrates into the soil decreased as the treatment cycles increased due to the precipitated calcite in previous cycles and clogging of the pores. The clogging could result in the accumulation of the substrate solution in the top portion of the soil. The substrate water evaporates as the substrate cannot penetrate into the soil leading to the precipitation of substrate salts on the surface. Therefore, the bacteria do not have access to the precipitated substrates which in turn reduces the amount of precipitated calcite.

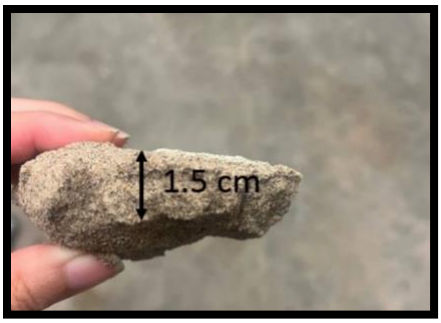

Figure 3. A portion of the sand dune stabilized by the spray method

For the aerobic sand column test, the amount of precipitated calcium carbonate at the top, middle, and bottom of the sand column was 2.5%, 1.5%, and 0.4%, respectively. In the anaerobic sand column test, the amount of precipitated calcium carbonate at the top, middle, and bottom of the sand column was 1%, 0.25%, and 0.15%, respectively. These correspond to an average calcite precipitation in aerobic and anaerobic samples of 1.4% and 0.47%, respectively.

It is worth mentioning that, the range of calcite precipitation via denitrification MICP in sand column experiments conducted by other researchers under anaerobic conditions ranges from 0.5% to 1.1 % after 6 to 9 weeks of treatment (O'Donnell et al. 2017; Pham et al. 2018), Comparing the results of the experiments in this study with the mentioned previous studies (O'Donnell et al. 2017; Pham et al. 2018), it appears that the amount of precipitated calcite in the anaerobic experiments of this study is almost well close to the expected range. Additionally, the amount of precipitated calcite in aerobic and anaerobic experiments conducted in this study shows that the aerobic denitrification MICP in this study was about two to three times more effective than the anaerobic denitrification MICP. One possible explanation for this observation could be that the denitrifiers grow better and faster under aerobic inoculation which leads to a higher concentration of denitrifying microorganisms and eventually faster denitrification and higher precipitated carbonates. It is also possible that the higher number of bacteria use the available oxygen faster and create a local anoxic microenvironment around the bacteria where anaerobic denitrification occurs increasing the amount of precipitated calcite. Another possible scenario could be the consumption oxygen by the aerobic microorganism present in the environment which can create an anoxic condition and then denitrifiers conducted denitrification in an anaerobic condition. Also a combination of these senarios could be possible in the MICP treatment process. The amount of residual salt in the initial cycles showed zero percent (which is in contrast by the significat remained salt in the anaerobic condition studied by Pham et al. (2018)). This could be attributed to the consumption of part of the substrates by the aerobic bacteria presentin the environment. An other possibility is the higher activity and efficiency of aerobic denitrifiers in higher acetate to nitrate ratio compared to anaerobic denitrification (Pelaz et al. 2018; Chen et al. 2024). In addition, the facultative behavior of denitrifier microorganisms could consume the oxygen available in the environment and then switch to their anaerobic behavior which use nitrate for respration.

Figure 4 shows the concentrations of nitrate and nitrite during the first 5 days of the batch experiment (day 1 is the day that the bacteria and the fresh culture medium were mixed, and days 2 to 5 represent the next four days). As can be seen in this figure, the concentration of nitrate is reducing and the concentration of nitrite is increasing which indicates nitrate is being reduced to nitrite, i.e., denitrification is occurring under aerobic conditions. Over this period, denitrification proceeded with a reduction of 19 mmol of nitrate, decreasing from 47 mmol to 28 mmol. During

this process, 5 mmol of nitrite accumulated, while 14 mmol of nitrogen gas was produced, assuming complete reaction. This indicates that 40.43% of the nitrate was reduced. Of this reduced amount, 73.68% was converted to nitrogen gas (under complete reaction conditions), while 26.32% remained as nitrite accumulation. Further studies are necessary to evaluate the long-term progress of the reaction. It is also crucial to measure other intermediates, such as N_2O, to ensure that the reaction proceeds fully without producing hazardous byproducts.

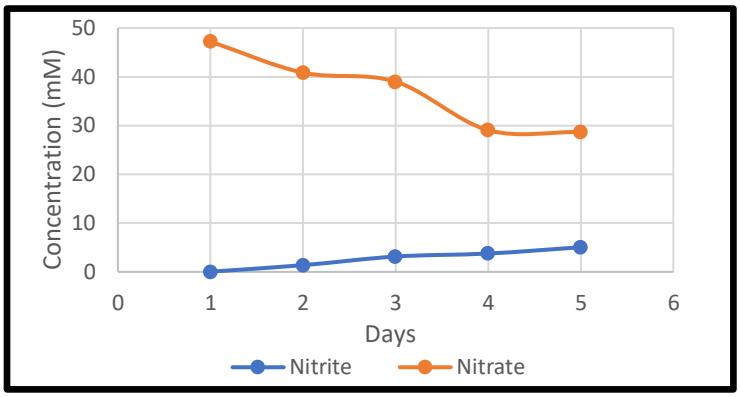

Figure 4. Nitrite and Nitrate concentrations during the first 5 days of the batch experiment

Figure 5 shows the results of the Scanning Electron Microscopy (SEM) analysis on the batch samples, which confirms the presence of calcium carbonate, specifically in the form of calcite, characterized by its hexagonal crystal form. The Energy Dispersive X-ray Spectroscopy (EDS) analysis, as shown in Figure 6, also indicates the presence of calcium (Ca), carbon (C), and oxygen (O), which are indicative of calcium carbonate components.

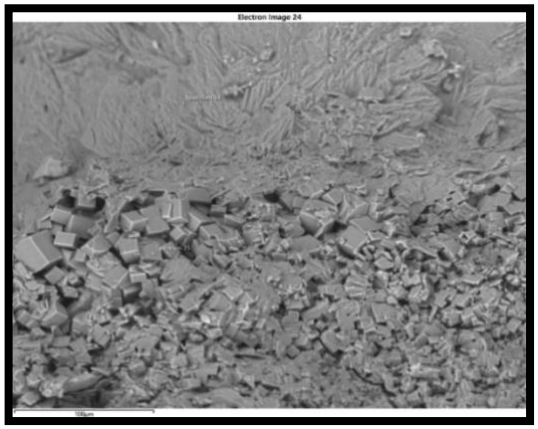

Figure 5. SEM image of the batch experiment sample

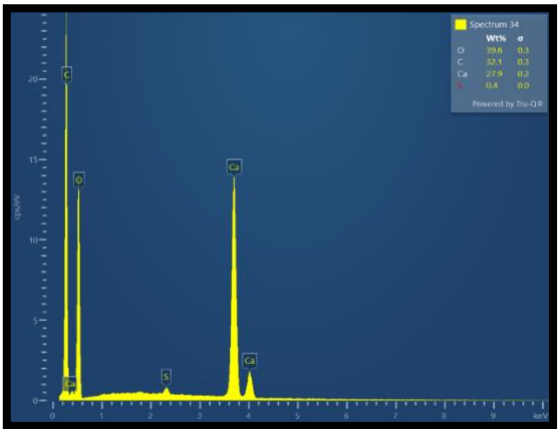

Figure 6. EDS peaks of batch experiment sample

The X-ray Diffraction (XRD) results depicted in Figure 7, also confirmed the precipitation of calcium carbonate in the batch experiments with the distinct peak corresponding to calcite. The XRD results of the sand column tests (aerobic and anaerobic denitrifications), and spray tests showed very small and negligible peaks associated with calcium carbonate which is probably due to the low percentage (below the detection range of XRD) of calcite precipitation.

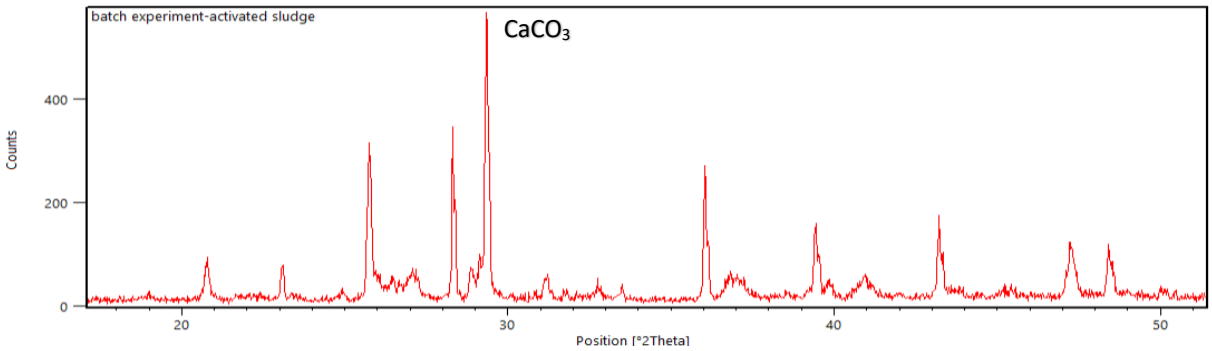

Figure 7. XRD result of the batch experiment sample

SEM analysis of the treated soil sample, illustrated in Figure 8 a, shows the precipitation of calcite on sprayed sand particles. The SEM analyses of spray tests additionally revealed the presence of needle-shaped crystals (see Figure 8b), which could possibly be aragonite, another polymorph of calcium carbonate. Needle-shaped calcium carbonate crystals are typically associated with the aragonite polymorph, which forms elongated, acicular structures (Loste et al. 2003; Wang et al. 2006) It is also worth mentioning that organic molecules can significantly influence the morphology of calcite crystals during precipitation, often leading to the formation of needle-shaped or rod-like structures (Liu and Yates, 2006).

SEM analyses of the sand column tests (not shown here) also confirmed the precipitation of calcium carbonate polymorphs after treatment under both aerobic and anaerobic conditions. The SEM image shown in Figure 8 c, captures the EPS precipitated between soil particles of the spray sample. As can be seen in the figure, the thread-like EPS materials form a bridge between soil particles. It is also worth mentioning that some salts precipitated on the surface of the spray samples as well as the open-lid sand column sample (the left image in Figure 2). These salts were not observed on the closed-lid sand column samples.

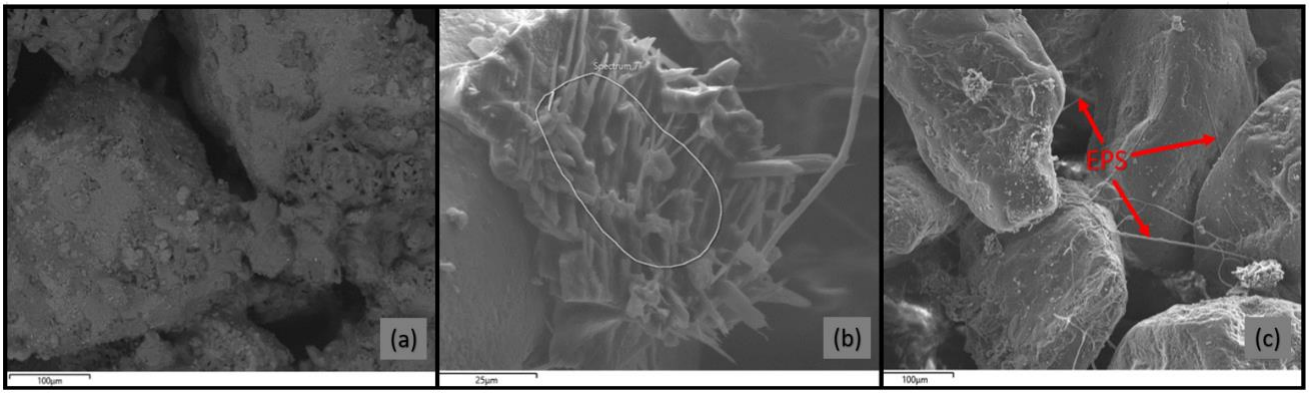

Figure 8. SEM imane on sand sample a) based on morphology probably calcite b) based on morphology probably aragonite, c) EPS

The EDS image analysis of the treated sand dune samples, presented in Figures 9, demonstrates the presence of silicon (Si) and aluminum (Al), elements typically associated with quartz sand particles as well as oxygen (O), carbon (C), and calcium (Ca) which demonstrate the presence of calcium carbonate within the treated sand matrix in sprayed samples. Similar results were observed for sand column tests after treatment under both aerobic and anaerobic conditions.

Figures 10 and 11 present the EDS test results on the treated samples which confirms the precipitation of calcium carbonate in the treated sand samples.

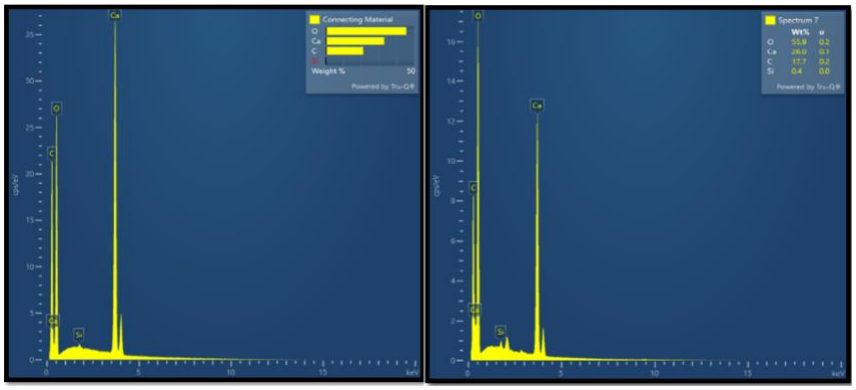

Figure 9. EDS peaks on precipitated agent in sand sample; Left: probably calcite, Right: probably aragonite

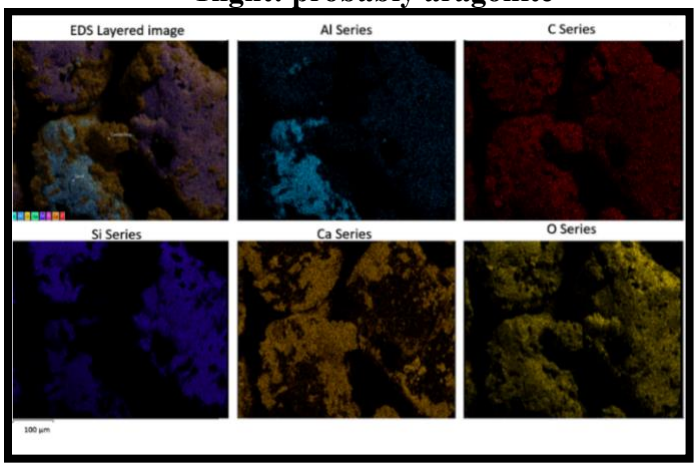

Figure 10. EDS image on sand sample-probably calcite

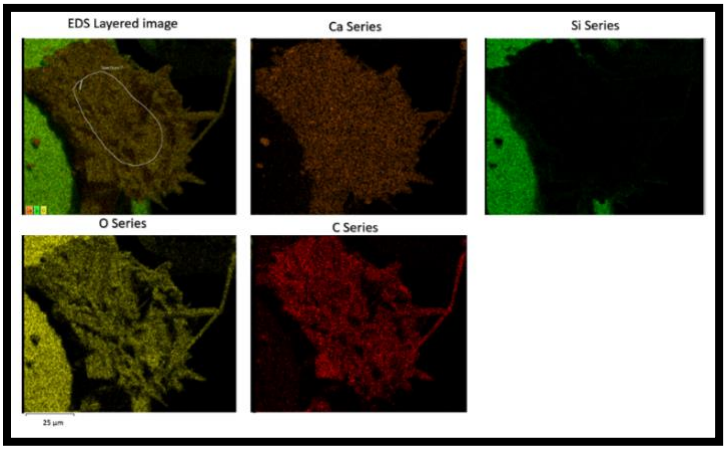

Figure 11. EDS image on sand sample-probably aragonite

CONCLUSION

In this study, a series of denitrification MICP experiments, i.e., batch experiments, surface treatment using spray tests, and sand column tests, were conducted in the presence of oxygen. The experimental data, including rapid calcite content tests, IC (Nitrite and Nitrate concentrations), SEM, XRD, and EDS analyses collectively confirmed the occurrence of denitrification under oxic conditions and successful formation and precipitation of calcium carbonate in both batch and soil environments. Around 20%, 1.5%, 1.4% by weight of calcite was precipitated in the batch experiments, sand treated using spray method, and sand column experiments under oxic conditions, respectively. Furthermore, the sand column tests conducted in the presence of oxygen precipitated 2 to 3 times more calcium carbonate compared to sand column tests conducted under restricted oxygen presence (relatively anaerobic). These findings highlight the potential of utilizing MICP via denitrification without the need for strictly maintaining an anaerobic environment or limiting oxygen accessibility. This approach could facilitate more practical and efficient field applications of the method.

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