



Review Article

Denitrification processes, inhibitors, and their implications in ground improvement

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ABSTRACT

Ureolysis and denitrification are the two major microbial metabolic pathways commonly used in Microbially induced calcite precipitation (MICP) for geotechnical applications. Although ureolysis is generally the more efficient pathway, the denitrification pathway has gained more attention recently because a diverse group of bacteria can precipitate calcite via denitrification, and no harmful byproduct is generated provided that the reduction of nitrate to nitrogen gas is complete. There are, however, many environmental factors that could inhibit or reduce the efficiency of the denitrification process in soil. Some examples of these factors include salinity, pH, temperature, biodiversity (abundance and species of denitrifiers and competitors), water stress (extreme wet-dry conditions), degree of saturation (anaerobic vs. aerobic conditions), high heavy metal content (e.g., mine tailings), and shortage of dissolved carbon sources. In this paper, the denitrification process, the denitrification inhibitors, and the mechanisms involved in their inhibition of the denitrification process are discussed in detail. This investigation indicates that although general optimum conditions can be formulated for MICP through denitrification, significant adjustments may be necessary if inhibitory conditions are anticipated. It was also shown that when inhibitors are expected, it is crucial to investigate not only the amount of precipitated calcium carbonate but also the N_2O/N_2 gas ratio to ensure the complete reduction of nitrate to nitrogen gas and prevent the release of byproducts (especially N_2O) into the environment. Finally, the implications of the inhibitory factors on the field application of denitrification MICP treatment for different geotechnical projects are discussed.

1. Introduction

Many construction projects encounter undesirable subsurface conditions that require ground improvement before the project objectives can be achieved. Chemical stabilization has been one of the most common ground improvement methods where grouts and other types of cement-/lime-based binders are used to stabilize soft and/or loose soils (Akiyama & Kawasaki, 2012; Cuisinier et al., 2011; Gross & Adaska, 2020; Jiang et al., 2017; Pigeot et al., 2024; Puppala et al., 2013; Small et al., 2022; Wei et al., 2019; Ying et al., 2022). Production of cement and lime is responsible for about 6–8 percent of total global anthropogenic CO_2 emissions (Abdolvand & Sadeghiamirshahidi, 2024; Bing et al., 2023; Castro-Alonso et al., 2019; Spaulding et al., 2008). To reduce the carbon footprint of the construction industry, therefore, researchers have been trying alternative methods such as microbially induced calcite precipitation (MICP) for ground improvement projects (Ali et al., 2021; Fang & Achal, 2024a, 2024b; He et al., 2024; Pakbaz

et al., 2022; Zhang et al., 2024). In MICP, carbonate precipitation is typically induced when bacteria's metabolic byproducts are secreted or passively diffused out of their cells and react with ions present in the environment (Castro-Alonso et al., 2019; Zhu & Dittrich, 2016). Although many metabolic pathways can lead to carbonate precipitation, ureolysis (urea hydrolysis, Fig. 1A) and denitrification (nitrate reduction, Fig. 1B) are the two most commonly used pathways in ground improvement studies.

In MICP via ureolysis (U-MICP), urease-producing bacteria break down (hydrolyze) urea, $CO(NH_2)_2$, to produce ammonium (NH_4^+), bicarbonate (HCO_3^-), and hydroxide (OH^-) ions (reaction 1). This reaction raises the pH and creates an alkaline environment that favors the precipitation of carbonates over bicarbonates. If calcium ions (Ca^{2+}) are present in the environment (or added to the environment), they react with the bicarbonate ions (reaction 2) to precipitate calcium carbonate, $CaCO_3$ (Castro-Alonso et al., 2019; DeJong et al., 2010; Sreekala et al., 2024).

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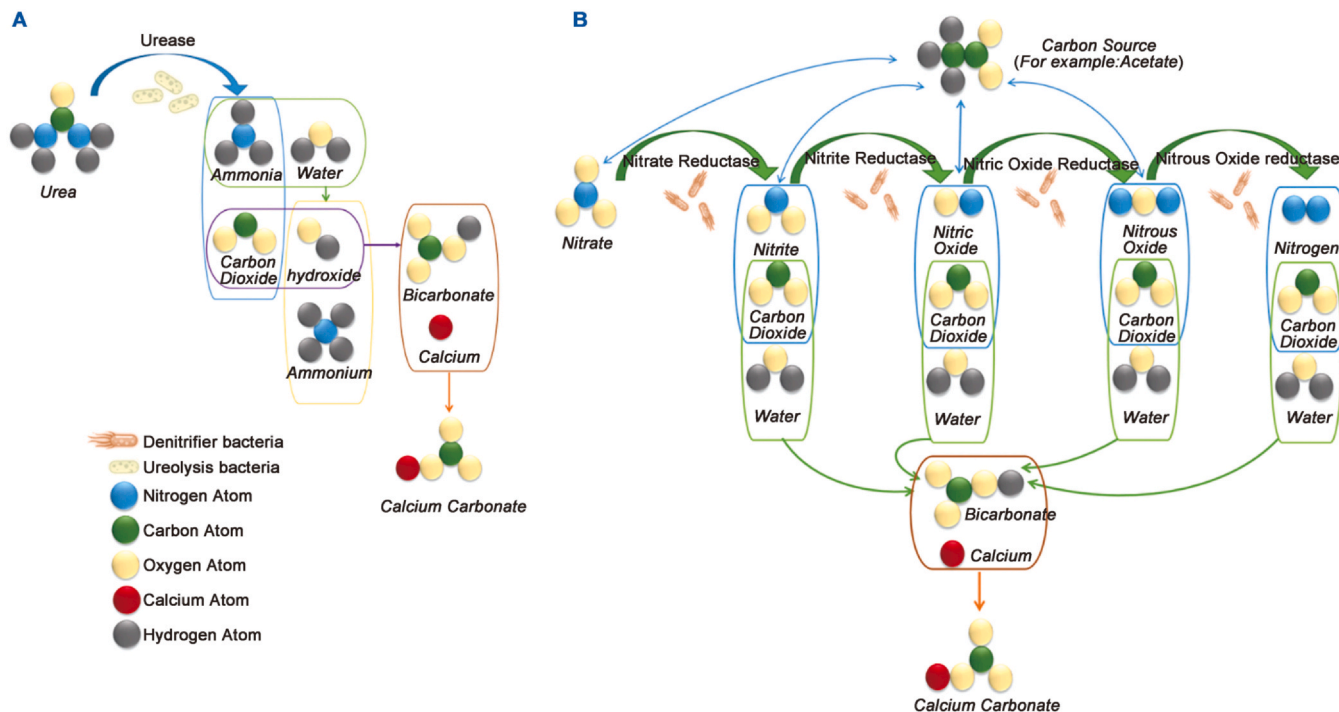
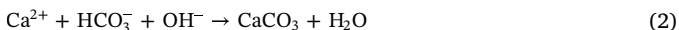
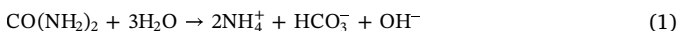


Fig. 1. The two most common pathways used for MICP: (A) U-MICP; (B) D-MICP.



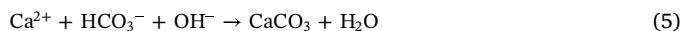
U-MICP is about fifteen times faster than MICP via denitrification (D-MICP) and therefore is the most commonly used method in ground improvement projects. U-MICP is not only faster than D-MICP but, in just a few days, U-MICP can precipitate 2–5 times the amount of calcium carbonate that D-MICP can precipitate in weeks/months (Burbank et al., 2013; Montoya et al., 2013; O’Donnell et al., 2017a, 2017b, Pham et al., 2018a, 2018b). However, the ammonia (concentrated ammonium chloride solution, NH_4Cl) that is produced as a byproduct of the U-MICP needs to be removed after the completion of the carbonate precipitation. Furthermore, the practical application of the ureolysis pathway typically requires cultivating a specific ureolytic bacteria strain. The denitrification pathway, on the other hand, can be achieved with denitrifying bacteria communities (communities with a diverse group of bacteria, including many native soil bacteria) without the need for cultivating a specific bacteria strain. Furthermore, the end byproduct of this pathway is nitrogen (N_2) gas, which is a relatively inert and unharmed gas (provided that the reduction of nitrate to nitrogen gas is complete). Additionally, the produced N_2 gas desaturates and improves the geotechnical properties of the soil (as it is relatively insoluble in water), partially compensating for the lower amount of precipitated calcite compared to the ureolysis pathway. D-MICP also results in biomass growth and the resulting biofilms increase the strength and stiffness of treated soils (Pham et al., 2018a). These advantages have convinced many researchers to focus on the denitrification pathway (Hou et al., 2025; Huang et al., 2024; Li et al., 2024; Liu et al., 2022b; Ren et al., 2023; Wang et al., 2024; Xiang et al., 2022; Yang et al., 2022).

Many factors, however, could inhibit or reduce the efficiency of the denitrification process in different geoenvironments. The key factors include oxygen availability and degree of saturation (oxic vs. anoxic conditions), pH, salinity, temperature, water stress (extreme wet-dry conditions), biodiversity, high heavy metal content (e.g., mine tailings), carbon-to-nitrogen ratio, and shortage of dissolved carbon sources. In this paper, the denitrification process, inhibitors, inhibition mechanisms, and their implications in geotechnical projects are reviewed, the

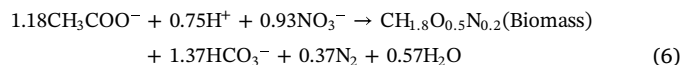
research gaps are identified, and required future research directions are proposed.

2. Overview of the denitrification process

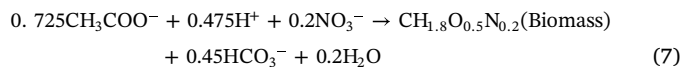
In the denitrification pathway, the electron donor (e.g., acetate: CH_3COO^- or $\text{C}_2\text{H}_3\text{O}_2^-$ in heterotrophic denitrification) reduces the nitrate (terminal electron acceptor, NO_3^-) to N_2 while increasing the alkalinity which in the presence of calcium cation leads to precipitation of calcium carbonate (reactions 3 – 5):



Reactions 3 and 4 can also be written as the combined metabolic reaction 6 (Nakano, 2018):



The metabolic reaction 6 can be broken down into an anabolic reaction that produces new biomass (reaction 7), and a catabolic reaction, which generates the energy required to produce and sustain biomass (reaction 8) (Pham et al., 2018a):



The ratio between reactions 7 and 8, i.e., the ratio of catabolic to anabolic reactions, depends on the bacteria growth rate, and environmental conditions including the availability of substrates, nutrients, and the presence of inhibitors (Pham et al., 2018a).

Reaction 3, involves four intermedialy dissimilatory microbial nitrogen reduction steps, each driven by a specific enzyme as shown in reactions 9 – 12 (O’Donnell et al., 2019) and Fig. 2 (Ye et al., 1994):

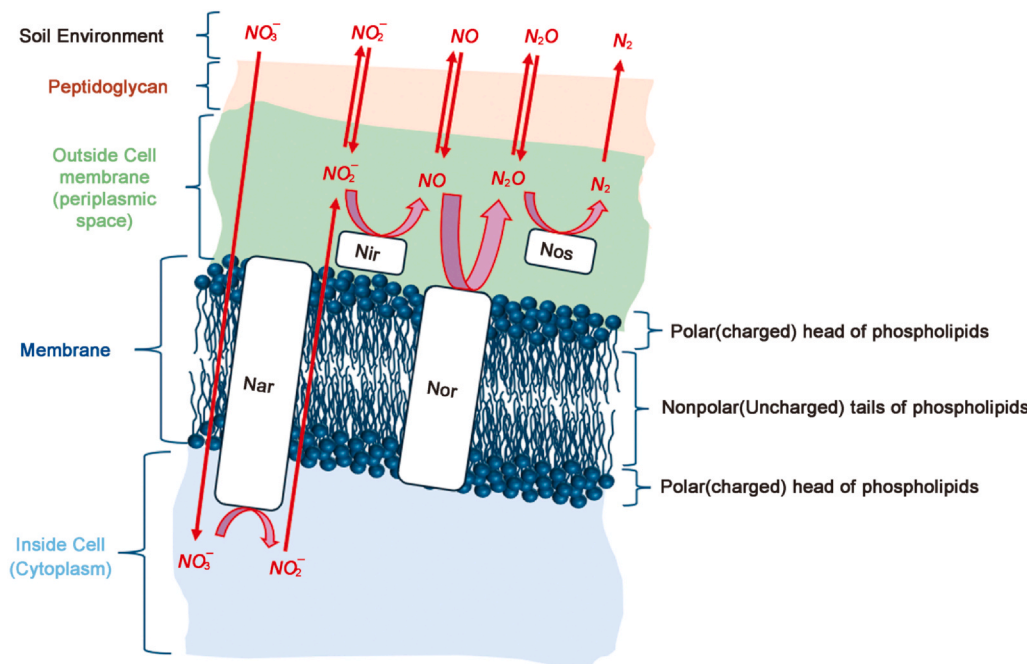
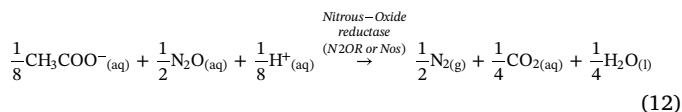
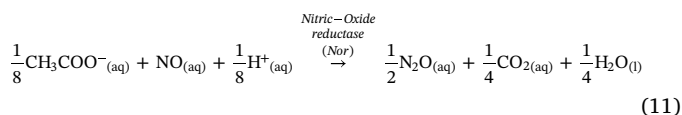
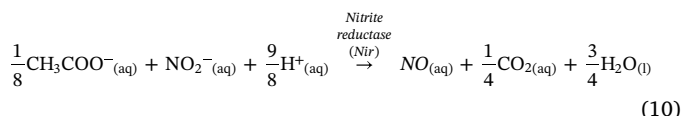
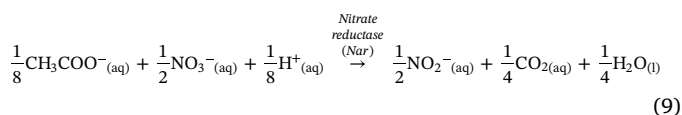


Fig. 2. Stepwise denitrification pathway illustrating the location of enzymes with respect to the membrane.

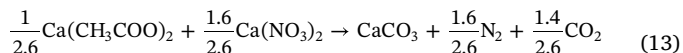


Nitrite reduction is irreversible once NO is formed (Martens, 2005). The reduction of nitrate is facilitated by membrane-bound (Nar) and periplasmic-bound (Nap) enzymes. The nitrite reduction is facilitated by a copper nitrite reductase (NirK) or a cytochrome cd1 nitrite reductase (NirS). The nitric oxide reduction is completed through nitric oxide reductase (sometimes detected using the norB gene code). Nitrous oxide reductase (NosZ) is responsible for reducing Nitrous oxide to nitrogen gas as the final step of denitrification. Due to the extremely diverse bacterial species and groups that are capable of denitrification, common rRNA gene targeting is not practical for investigating denitrification communities. Therefore, the encoding genes for the Nar and Nap enzymes (except for narG and napA that are found in non-denitrifiers such as nitrate respirers that reduce nitrate to ammonia), as well as nirS, nirK, norB, and nosZ are commonly used in molecular methods to characterize the diversity of denitrifying bacteria and to study denitrification rates and inhibition (Wallenstein et al., 2006).

In this stepwise reduction system, electrons are transferred from the electron donor to the electron acceptor via the prokaryotic electron transport chain (ETC) as shown in Fig. 3 (Shapleigh, 2006). Typically, the electron flow begins with organic molecules such as acetate donating electrons to nicotinamide adenine dinucleotide (NAD⁺) molecules forming NADH molecules inside the cytoplasm. The NADH

dehydrogenase (complex I) enzyme catalyzes the transfer of electrons from NADH to the quinone pool. The transfer of electrons releases energy that complex I can use to pump protons across the plasma membrane of prokaryotic cells (pumps to the outside of the cytoplasm). Electrons are then carried by the quinone pool to complex III (also known as cytochrome c-oxidoreductase, or cytochrome bc₁ complex) where the ubiquinol-cytochrome-c reductase enzyme catalyzes the reduction of cytochrome c by oxidation of coenzyme Q. Complex III also pumps protons across the plasma membrane. The cytochrome c carries the electrons to the final destination in ETC, i.e., complex IV, where the Cytochrome c oxidase transduces the electrons from cytochrome c or quinones to the terminal electron acceptor and pumps more protons across the plasma membrane (Chen & Strous, 2013; Wan et al., 2016). At this point, there is a large proton gradient across the plasma membrane of prokaryotic cells, with higher proton concentrations outside the cytoplasm. The ATP synthase uses this proton gradient through chemiosmosis for ATP synthesis (Borrero-de Acuña et al., 2017). The ETC coupled with the synthesis of ATP via chemiosmosis is known as oxidative phosphorylation (Cardol et al., 2009).

All the steps in the reduction series in reactions 9 – 12 accept their electrons from cytochrome c or the quinol pool through the ETC (Chen & Strous, 2013). The substantial consumption of H⁺ during the reduction of nitrite to nitric oxide (reaction 10) increases the alkalinity and the pH of the solution. An alkaline environment favors the precipitation of carbonate over bicarbonate (Castanier et al., 1999), which is necessary to precipitate calcium carbonate. In soil stabilization with D-MICP, typically calcium acetate and calcium nitrate are used as the electron donor and acceptor, respectively, and the complete reaction can be written as (van Paassen et al., 2010):



3. Microorganisms and inoculations for D-MICP

Many denitrifying bacteria genera, including *Alcaligenes*, *Achromobacter*, *Bacillus*, *Denitrobacillus*, *Flavobacterium*, *Micrococcus*, *Paracoccus*, *Pseudomonas*, *Spirillum*, and *Thiobacillus*, have been identified, which are widely distributed in natural and engineered environments (Jain et al., 2021; Jørgensen & Pauli, 1995; Karatas, 2008). The

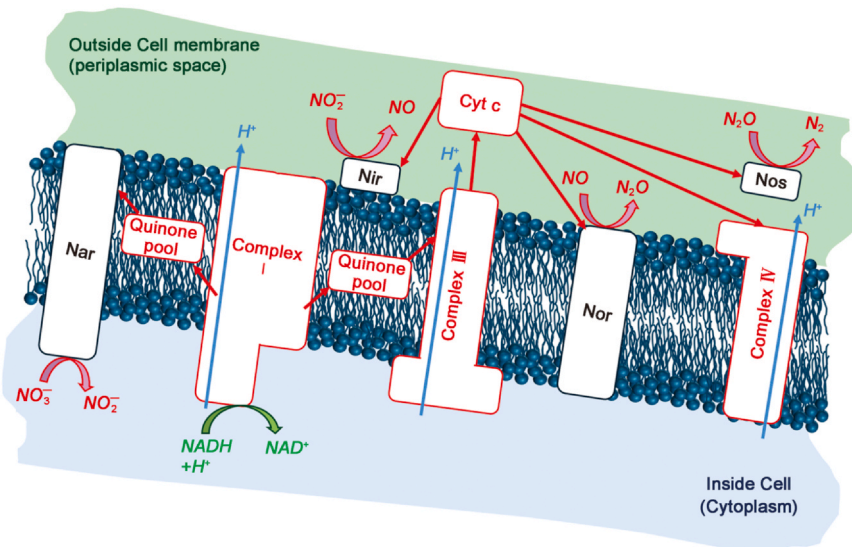


Fig. 3. Stepwise denitrification pathway along with its ETC.

microorganisms successfully used for D-MICP and their inoculation processes are discussed in this section.

3.1. Identified microorganisms for denitrification

Numerous sources have been used to inoculate the denitrifying microorganisms needed for D-MICP. Specific strains that have been successfully used for D-MICP include: *Halomonas halodenitrificans* (NCIMB 700) (Martin et al., 2013), *Pseudomonas denitrificans*, (ATCC 13867) (Erşan et al., 2015; Karatas, 2008), *Castellaniella* y (van Paassen et al., 2010), *Paracoccus denitrificans* PD1222, *Pseudomonas stutzeri* A1501, *Diaphorobacter nitroreducens* TPSY, *Pseudomonas aeruginosa* PAO1, *Diaphorobacter* (Erşan et al., 2015) and *Pseudomonas* sp. Erşan et al. (2015) isolated more than nine denitrifying strains from soil and investigated their tolerance to water stress and nutrient deficiency. They concluded that the best-performing strains are *D. nitroreducens* and *P. aeruginosa* as they were the fastest growing strains and more tolerant to environmental stresses. According to Martin et al. (2013), *H. halodenitrificans* is the best-performing halophile species under high salinity conditions. Denitrifying bacteria communities from different sources, as opposed to a single strain of denitrifier, have also been used for D-MICP applications. For example, topsoils (Pham et al., 2018a, 2018b; van Paassen et al., 2010), sediments (Nakano, 2023), beach sands (O'Donnell et al., 2017a, 2017b, 2019), and activated sludge from municipal wastewater (Nakano, 2018; Pham et al., 2018a; Zhang et al., 2017) all have been shown to have sufficient denitrifying bacteria for ex-situ D-MICP. Typically between 10^5 and 10^{10} denitrifying bacteria can be found in one gram of dry freshwater sediments (Johnston et al., 1974; Jones, 1979). Finally, indigenous denitrifying microorganisms, i.e., in-situ soil bacteria, have also been used for D-MICP tests (Moug et al., 2022; Pham et al., 2013; Wang et al., 2021; Zeng et al., 2022).

3.2. Inoculation

Denitrifiers use nitrate as the terminal electron acceptor and can use a variety of organic and inorganic compounds as their source of carbon and energy (Ambus & Zechmeister-Boltenstern, 2007). The concentrations and the carbon-to-nitrogen ratio used in the inoculation and the carbonate precipitation stages of D-MICP play an important role in the efficiency of the biotreatment process. Even a slight change in the chemical properties of the substrates used for the inoculation or the precipitation stage of the D-MICP can significantly affect the denitrification rate. For example, Pham et al. (2018a) showed that when sodium acetate is used as the source of carbon in liquid batch

experiments, most of the nitrate is reduced in around 9 days, compared to about 20 days when calcium acetate is used. However, using sodium-based carbon sources may lead to nitrite accumulation and inefficient D-MICP. Therefore, an overview of some of the substrates, nutrients, and buffers that have been successfully used for bacterial growth and D-MICP experiments by different researchers is presented in Table 1.

All the studies presented in Table 1 successfully precipitated calcium carbonate and achieved their respective desired soil properties. However, some of these studies did not investigate the efficiency of their D-MICP experiments and whether the stepwise reduction of nitrate to nitrogen gas was complete. One of the major advantages of using the denitrification pathway over the urea hydrolysis pathway for MICP application is the assumption that denitrification does not produce any harmful byproducts. This holds true only when a complete reduction of nitrate to nitrogen gas is achieved. As mentioned earlier, in the denitrification process, nitrate is reduced by denitrifying microorganisms to nitrogen (N_2) gas through the intermediate products of nitrite (NO_2^-), nitric oxide (NO), and nitrous oxide (N_2O) (Pham et al., 2018a; Robertson & Groffman, 2015). N_2O is a major greenhouse gas with a climate change impact that is 265 times greater than that of CO_2 impact (IPCC, 2014) (Mosier, 1998). Although calcium carbonate can still precipitate even if the reduction of nitrate to nitrogen gas is incomplete, the incomplete reduction can result in the accumulation and release of N_2O into the environment. This would negate the environmental advantage of D-MICP over the U-MICP. Accumulation of N_2O also reduces calcium carbonate precipitation and bacterial growth as discussed in later sections. The chemical composition and concentrations of the substrates used in inoculation and D-MICP experiments could significantly affect the efficiency of the denitrification process and prevent the completion of the stepwise nitrate reduction. The optimum concentrations also depend on the type of denitrifying bacteria strain or community as well as the environmental factors such as the oxygen level, pH, and temperature of the environment. Therefore, the complete reduction of nitrate to nitrogen gas should never be assumed and must be investigated for each project. The effects of some of the environmental factors on the stepwise reduction of nitrate to nitrogen gas and the overall produced N_2O/N_2 gas ratio is discussed in the next sections.

4. Key inhibitors to MICP via denitrification pathway

Many factors inhibit or reduce the productivity of D-MICP (Bremner & Shaw, 1958; Bremner & Yeomans, 1986). The main 12 factors with the most significant effects on D-MICP are discussed separately in this section.

Table 1

Summary of substrates, nutrients, and buffers successfully used in inoculation and carbonate precipitation stages of D-MICP experiments.

Denitrifier strain or source of Denitrifier community	Carbon, Nitrogen, and Calcium sources and concentrations During the inoculation Carbon, Nitrogen, and calcium sources and concentrations During the precipitation stage of D-MICP if different	Acetate to nitrate molar ratio (representing Carbon source to nitrogen source ratio) During the inoculation Acetate to nitrate molar ratio (representing Carbon source to nitrogen source ratio) During the precipitation stage of D-MICP if different	pH control	Nutrients and trace element sources During the inoculation Nutrients and trace element sources During the precipitation stage of D-MICP if different	Reference
<i>Halomonas halodentrificans</i>	Calcium acetate: 12.2 mmol/L Potassium nitrate: 10 mmol/L Magnesium acetate: 126.5 mmol/L Sodium acetate: 74.6 mmol/L Potassium nitrate: 50 mmol/L Calcium chloride dihydrate: 100 mmol/L	27.74 1.5	1 mol/L NaOH solution	Yeast extract: 10 g/L Peptone: 5 g/L Glucose: 5.55 mmol/L Sodium chloride: 1283 mmol/L Yeast extract: 2.5 g/L Peptone: 1.25 g/L Glucose: 5.55 mmol/L Sodium chloride: 1283 mmol/L	Martin et al. (2013)
<i>Pseudomonas denitrificans</i> , <i>Paracoccus denitrificans</i> , <i>P. stutzeri</i> , <i>Diaphorobacter nitroreducens</i> , <i>P. aeruginosa</i> , <i>Pseudomonas sp</i>	Sodium formate: 88.24 mmol/L or Methanol: 125 mmol/L Potassium Nitrate: 7.13 mmol/L Sodium formate: 88.2 mmol/L Calcium nitrate: 5 mmol/L Calcium chloride: 57 mmol/L	Formate to nitrate ratio: 12.38 Methyl (from Methanol) to nitrate ratio: 17.53 17.64	^a Buffer	^b M9 solution Magnesium sulfate: 2 mmol/L Sodium phosphate: 31.72 mmol/L Magnesium sulfate: 2 mmol/L Sodium phosphate: 0.064 mmol/L	Erşan et al. (2015)
<i>Castellaniella denitrificans</i> Isolated from soil samples taken from the Botanical Garden of the Delft University of Technology	Calcium acetate: 50 mmol/L Calcium nitrate: 60 mmol/L Calcium acetate: 100 mmol/L Calcium nitrate: 120 mmol/L	0.8		Ammonium sulfate: 0.003 mmol/L Magnesium sulfate: 0.0024 mmol/L Monopotassium phosphate: 0.006 mmol/L Dipotassium phosphate: 0.014 mmol/L Trace element solution ^c SL12B: 1 mL/L	van Paassen et al. (2010)
Soil sample was taken from the Botanical Garden of the Delft University of Technology	First to fourth inoculation: Calcium acetate: 30 mmol/L Calcium nitrate: 25 mmol/L Fifth inoculation: Calcium acetate: 30 mmol/L Calcium nitrate: 25 mmol/L Fifth inoculation: Sodium acetate: 60 mmol/L Sodium nitrate: 50 mmol/L Sixth inoculation: Calcium acetate: 30 mmol/L, Calcium nitrate: 25 mmol/L Calcium acetate: 20 mmol/L, Calcium nitrate: 25 mmol/L Calcium acetate: 15 mmol/L, Calcium nitrate: 25 mmol/L	First to fifth inoculation: 1.2 Sixth inoculation: 1.2 0.8 0.6 1.2		Ammonium sulfate: 0.003 mmol/L Magnesium sulfate: 0.0024 mmol/L Monopotassium phosphate: 0.006 mmol/L Dipotassium phosphate: 0.014 mmol/L Trace element solution ^c SL12B: 1 mL/L	Pham et al. (2018a)
Activated sludge	Calcium acetate: 30 mmol/L Calcium nitrate: 25 mmol/L Calcium acetate: 60 mmol/L Calcium nitrate: 50 mmol/L First to third batch: Calcium acetate: 60 mmol/L Calcium nitrate: 50 mmol/L Fourth to ninth batch: Calcium acetate: 80 mmol/L Calcium nitrate: 50 mmol/L	1.2 1.2 1.2		Ammonium sulfate: 0.003 mmol/L Magnesium sulfate: 0.0024 mmol/L Monopotassium phosphate: 0.006 mmol/L Dipotassium phosphate: 0.014 mmol/L Trace element solution ^c SL12B: 1 mL/L	Pham et al. (2018a)

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Table 1 (continued)

Denitrifier strain or source of Denitrifier community	Carbon, Nitrogen, and Calcium sources and concentrations During the inoculation Carbon, Nitrogen, and calcium sources and concentrations During the precipitation stage of D-MICP if different	Acetate to nitrate molar ratio (representing Carbon source to nitrogen source ratio) During the inoculation Acetate to nitrate molar ratio (representing Carbon source to nitrogen source ratio) During the precipitation stage of D-MICP if different	pH control	Nutrients and trace element sources During the inoculation Nutrients and trace element sources During the precipitation stage of D-MICP if different	Reference
Mixture of Huntington Beach sand and Huntington Beach water	Calcium acetate: 12.5 mmol/L Calcium nitrate: 12.5 mmol/L Calcium acetate: 25 mmol/L Calcium nitrate: 25 mmol/L Anhydrous calcium chloride: 125 mmol/L	1	1 mol/L NaOH	Beef extract and peptones as nutrient broth: 20 g/L Magnesium sulfate: 2 mmol/L ^d Trace metal solution: 0.5 mL/L Potassium hydrogen phosphate	O'Donnell et al. (2017b)
Field study without adding endogenous bacteria (unidentified native soil denitrifying bacteria)	Calcium acetate: 75 mmol/L Calcium Nitrate: 37.5 mmol/L	2		Tryptic Soy Broth (TSB): 0.75 g/L Trace metal solution: 0.5 mL/L	O'Donnell et al., 2017a
Natural sediments	Calcium acetate Sodium acetate Calcium nitrate				Nakano (2023)
Wetted garden soils	Calcium acetate: 25 mmol/L Calcium nitrate: 25 mmol/L	1		Ammonium chloride: 2.24 mmol/L Magnesium sulfate heptahydrate: 0.40 mmol/L Ferrous sulfate heptahydrate: 0.0036 mmol/L Potassium dihydrogen phosphate: 0.066 mmol/L Dipotassium phosphate: 0.18 mmol/L Calcium chloride dihydrate: 0.10 mmol/L Trace element solution ^e : 1 mL/L The concentrations of nutrients and trace elements were reduced to one-tenth of the concentrations used in the enrichment cultivation.	Gao et al. (2022b)
Bottom sludge	Calcium acetate: 14 mmol/L Sodium acetate: 48 mmol/L Calcium nitrate: 30 mmol/L				Nakano (2018)

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Table 1 (continued)

Denitrifier strain or source of Denitrifier community	Carbon, Nitrogen, and Calcium sources and concentrations During the inoculation Carbon, Nitrogen, and calcium sources and concentrations During the precipitation stage of D-MICP if different	Acetate to nitrate molar ratio (representing Carbon source to nitrogen source ratio) During the inoculation Acetate to nitrate molar ratio (representing Carbon source to nitrogen source ratio) During the precipitation stage of D-MICP if different	pH control	Nutrients and trace element sources During the inoculation Nutrients and trace element sources During the precipitation stage of D-MICP if different	Reference
In situ denitrifiers	Calcium acetate Cycle 1–5: 160 mmol/L Cycle 6: 150 mmol/L Cycle 7: 200 mmol/L Cycle 8: 230 mmol/L Cycle 9: 300 mmol/L Cycle 10–12: 340 mmol/L Calcium nitrate Cycle 1–5: 120 mmol/L Cycle 6: 160 mmol/L Cycle 7: 180 mmol/L Cycle 8: 220 mmol/L Cycle 9: 250 mmol/L Cycle 10–12: 290 mmol/L	Cycle 1–5: 1.3 Cycle 6: 0.9 Cycle 7: 1.1 Cycle 8: 1 Cycle 9–12: 1.2		(Magnesium sulfate: 0.4 mmol/L Dipotassium phosphate: 0.5 mmol/L Iron(II) sulfate: 0.4 mmol/L Yeast extract: 15 mg/L Trace elements)	Zeng et al. (2022)
Native bacteria	Calcium acetate: 63 mmol/L later increased to 126 mmol/L Calcium nitrate: 60–120 mmol/L	1.05		Commercial all-purpose plant food: 0.25 mg/L Magnesium sulfate: 4.99×10^{-4} mmol/L	Moug et al. (2022)
denitrifiers within the sediments collected from Richmond, British Columbia, Canada	Calcium acetate: 25 mmol/L Calcium nitrate: 25 mmol/L	1		Ammonium chloride: 2.24 mmol/L Magnesium sulfate heptahydrate: 0.406 mmol/L Iron(II) sulfate heptahydrate: 0.0036 mmol/L Potassium dihydrogen phosphate: 0.0662 mmol/L Dipotassium phosphate: 0.184 mmol/L Calcium chloride dihydrate: 0.102 mmol/L, Trace element solution ^c : 1 mL/L	Wang et al. (2021)

- Some of the concentrations were converted from g/L (reported in the original research) to mmol/L in this table

^a Buffer solution: (8.5 g/L of $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ and 3 g/L of KH_2PO_4)

^b M9 salt solution: (0.5 g/L of NaCl, 0.24 g/L of MgSO_4 , and 0.011 g/L of CaCl_2)

^c SL12B solution: (3000 mg/L of EDTA- $\text{Na}_2 \cdot 2\text{H}_2\text{O}$, 1100 mg/L of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 300 mg/L of H_3BO_3 , 190 mg/L of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 50 mg/L of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 42 mg/L of ZnCl_2 , 24 g/L of $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 18 mg/L of $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$, and 2 mg/L of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (Overmann et al., 1992)).

^d Trace metals solution: 0.5% (w/v) of CuSO_4 , FeCl_3 , MnCl_2 , and $\text{Na}_2\text{MoO}_4 \cdot \text{H}_2\text{O}$

^e Trace element solution: 10 mg/L of EDTA- $\text{Na}_2 \cdot 2\text{H}_2\text{O}$, 0.12 mg/L of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.12 mg/L of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.03 mg/L of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.05 mg/L of $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 0.1 mg/L of $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 0.1 mg/L of $\text{CoCl}_3 \cdot 6\text{H}_2\text{O}$, 0.05 mg/L of $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$, and 0.05 mg/L of H_3BO_3 .

4.1. Oxygen availability and degree of saturation (anaerobic vs. aerobic conditions)

The effects of dissolved oxygen (DO) on denitrifiers, their enzymatic activities, and denitrification ability are still not well understood (Hong et al., 2019; Matsuya et al., 2018). It is generally believed, that oxygen (O_2) represses the bacterial reductase enzymes but these enzymes are reactivated within 40 min to 3 h after oxygen depletion (Payne, 1973; Payne et al., 1971). What is known as “traditional denitrification” typically happens when the DO concentration is less than 0.2 mg/L (Seitzinger et al., 2006). The efficiency of the denitrification process, e.g., the denitrification rate, decreases as the oxygen level increases above this level. When the concentration of dissolved oxygen reaches above 0.5 mg/L, denitrification is inhibited (Sun et al., 2013; Wei et al., 2022; Zhang et al., 2014). Considering this traditional definition, denitrification will be inhibited above the groundwater table (in both the dry zone and the vadose zones) due to the oxic conditions (Berlin et al., 2014). The DO concentration decreases with depth in groundwater due to increased hydrostatic pressure and temperature. Therefore, depending on the groundwater table depth, the DO concentration in the saturated soils near the shallow groundwater tables could also be too high for an efficient denitrification process. Denitrification is inhibited under oxic conditions because oxygen and nitrate compete for the limited available electrons. Since oxygen has a higher reduction potential than nitrate, electron donors are mostly oxidized by aerobic bacteria, consuming all (or most) of the electrons leaving no (or very little) electrons for denitrification to occur (Wei et al., 2022). However, in some environments such as sediments of aquatic systems with an abundance of electron donors (e.g., organic matter and sulfides), denitrification can occur under oxic conditions (Wei et al., 2022). More importantly, there are microbial strains with reductase enzymes that are capable of reducing nitrogen oxides under aerobic conditions. Aerobic denitrification has been studied since the 1980s (Robertson & Kuenen, 1988) and many bacterial strains have been identified that are capable of denitrifying in the presence of oxygen. Some examples of such strains include *Stutzerimonas stutzeri* TR2 and ZoBell, *Paracoccus denitrificans*, *P. aeruginosa*, *Pseudomonas nautica*, *Pseudomonas putida*, *Azospirillum brasilense*, *Pannonibacter phragmitetus*, *Enterobacter cloacae* and *Gemmatimonas aurantiaca* (Bai et al., 2019; Bonin et al., 2011; Davies et al., 1989; Guo et al., 2016; Kim et al., 2008; Wang et al., 2023). For example, Kim et al. (2008) isolated a *P. putida* strain from topsoil that was capable of performing denitrifications at dissolved oxygen concentrations of up to 5–6 mg/L, Guo et al. (2016) isolated another aerobic denitrifying bacterium, i.e., *E. cloacae*, from activated sludge, and Lloyd et al. (1987) isolated eight aerobic denitrifiers including three aerobic pseudomonads from marine sediments. This implies that, despite the common belief that D-MICP can only occur under anaerobic conditions in deeper parts of the ground, D-MICP could potentially be used in shallow stabilization applications such as erosion control projects in dry and unsaturated soils where oxic conditions prevail. To achieve this goal, a higher number of electron donors or bacterial strains that are capable of denitrifying in the presence of oxygen should be used. D-MICP under oxic conditions, however, has not been well investigated and needs to be studied in more detail. To investigate the D-MICP under oxic conditions, it is important to consider that various reductase enzymes in different denitrifiers respond differently to the presence of oxygen. This is true for both anaerobic and aerobic denitrifiers. In the case of *Pseudomonas nautica* which can denitrify in the presence of oxygen, for example, the nitrate reductase is less sensitive to oxygen content whereas nitrite and nitrous reductase enzymes are more sensitive (Bonin et al., 2011). This could have practical implications for D-MICP applications in geotechnical engineering. In slightly deeper soil layers where limited oxygen is present, it is possible for the denitrification process to start by reduction of the nitrate (Schlüter et al., 2018) as the nitrate reductase enzymes are less sensitive to oxygen. At the same time, as other facultative anaerobic

denitrifiers in the environment consume the limited oxygen in the soil without denitrifying, the oxygen level is reduced in the immediate environment surrounding the bacteria. In this local anoxic condition, the nitrite and nitrous reductase enzymes are derepressed and start to complete the denitrification process. This, however, needs to be investigated in more detail because if the nitrous reductase is not derepressed completely or is derepressed with a time lag, the incomplete stepwise reduction of nitrate could result in the accumulation and the release of N_2O gas into the environment, i.e., increased ratio of the denitrification produced N_2O/N_2 gases.

Oxygen concentration not only affects the efficiency but also (maybe even more importantly) the ratio of the produced N_2O/N_2 gases at the end of the denitrification process. The effect of oxygen on different enzymes and the ratio of the produced N_2O/N_2 gases at the end of the denitrification process also depend on the type of the denitrifying bacteria and their community structure. For example, in the presence of oxygen, *Paracoccus denitrificans* and *P. stutzeri* produce more N_2O and less N_2 gases compared to anaerobic conditions (Davies et al., 1989; Lloyd et al., 1987). In the case of *P. aeruginosa*, on the other hand, increased oxygen levels increase the concentration of both N_2O and N_2 gases (Davies et al., 1989; Lloyd et al., 1987). Therefore, the effect of available oxygen on the produced N_2O/N_2 gas ratio during D-MICP application should also be investigated for every project. This is especially crucial if stabilization is to be performed in shallow depths and a plethora of denitrifiers from the soil or ex-situ sources such as activated sludge are to be used. As very limited data is available, more research is required to investigate the applicability, optimization, and produced N_2O/N_2 gas ratio of D-MICP in shallow to relatively deep stabilization projects with different concentrations of available oxygen.

4.2. pH

Another major factor affecting the efficiency of D-MICP is the pH of the environment. Generally, the optimum range of pH for denitrification is between 7 and 8. However, denitrification in wastes having a pH of up to 11 and acid pits with pH values as low as 3.5 have been reported (Knowles, 1982). Low pH conditions lead to progressive inhibition of nitrogen oxide reduction enzymes, especially the nitrous oxide reductase (N_2OR), until at a pH of around 4 where the N_2O will be the major product instead of the N_2 gas (Firestone et al., 1980; Knowles, 1982; Koskinen & Keeney, 1982; Nömmik, 1956; Parkin et al., 1985; Wijler & Delwiche, 1954). For example, it has been shown that *Pseudomonas mandelii* gene expression can be 539-fold and 6190-fold lower at pH 5 as compared to pH levels between 6 and 8 (Saleh-Lakha et al., 2009). This is believed to be due to the accumulation of nitrous oxide (N_2O) at pH < 7 conditions. It should be mentioned that, in addition to N_2O being a major greenhouse gas with adverse environmental effects, the accumulation of N_2O is toxic to the bacteria and inhibits the bacteria's growth and activity (Almeida et al., 1995; Pham et al., 2018a). Similarly, Liu et al. (2010) investigated the controlling role of pH on denitrification in soils under anoxic conditions using liming experiments. Their results revealed that the pH of soil does not affect the denitrification rate except for very acidic soil with a pH of 4 where low denitrification rates were observed. However, the produced N_2O/N_2 gas ratio decreased significantly as the pH increased. Interestingly, their results also showed a maximum gene copy numbers ratio of the (*nosZ*)/(*nirS* + *nirK*) in the unlimed soil (pH 4) and the ratio was practically unaffected by pH during the liming (pH between 5.5 and 8.0). This is interesting because it contradicts the general conception that low pH conditions lead to the selection of organisms lacking the *nosZ* (which is the gene coding for N_2OR) and subsequently reduced N_2OR activity. They instead hypothesized that higher amounts of N_2O are produced at low pH conditions because the activity and the translation or protein assembly of the N_2OR is negatively affected by the low pH (not that organisms lacking the *nosZ* are dominant in low pH conditions). They further observed similar amounts of *nirK* or *nirS* gene copies in their

experiments, contrary to previous studies where the dominant denitrification genes were either nirK or nirS. However, only nirS transcripts were detected which could indicate denitrifiers with nirS and nirK were equally present, but only nirS-carrying denitrifiers were activated under their experimental conditions. Although the nirK primers used in their study could have a role in the observed results, their finding suggests the possibility that the gene expression (not the gene types) is strongly selected under different pH conditions (Liu et al., 2010). It is worth noting that denitrification in wastewater samples containing high nitrate content has been reported to be significantly inhibited at pH values of 7 or below 7 (Glass and Silverstein, 1998).

On the other hand, Lee and Rittmann (2003) showed that higher than optimum pH could also lead to a reduced nitrate removal rate and a substantial increase in nitrite accumulation during autotrophic denitrification with H₂ as the inorganic electron donor (Lee & Rittmann, 2003). Accumulation of nitrite (NO₂⁻) has two major negative effects on D-MICP. First, it limits the increase in alkalinity which is needed to precipitate carbonates. Second, it results in the accumulation of nitrous acid, HNO₂, (Saleh-Lakha et al., 2009) which reduces the proton electrochemical gradient (also known as proton motive force) across the cell membrane. As discussed before, this gradient is key in the energy (ATP) production of bacteria, and therefore, the reduced gradient could lead to slow growth and a lower amount of precipitated calcite (Almeida et al., 1995; Lee & Rittmann, 2003).

It is, therefore, vital to ensure optimal pH conditions are maintained during inoculation and application of D-MICP. Assuring Šimek and Cooper (2002) reviewed over 50 years of research on the effect of pH on denitrification rate in soils. They revealed that different denitrifying communities in soils have different optimal pH ranges depending on the original soil condition and the incubation processes. They concluded that a general optimum pH for denitrification has little or no meaning without a reference to the bacteria community, soil condition, and the attributes of the incubation process. An example of the effects of the incubation process has been reported by Pham et al., 2018a. Their investigations showed that under the same conditions, using sodium-based substrates in their D-MICP experiments resulted in high pH conditions and nitrite accumulation, while calcium-based substrates resulted in complete nitrate reduction despite the less desired pH values of below 7.

Based on the discussion above, it can be concluded that a general optimum pH range for D-MICP applications is meaningless and the optimum pH should be determined on a project-specific basis depending on the soil conditions, substrates used, and the denitrifier strain/community employed.

4.3. Salinity

Increased salinity is considered a major physiological stress for the bacterial community. This stress can cause a significant change in the environment's biota (including denitrifiers) and their ecological functions (Herbert et al., 2015). Soil's microbial ecology, community structure, denitrifiers' diversity, enzyme activity, and the abundance of Nitrite Reductase (nirK and nosZ) and Nitrous Oxide Reductase (nosZ) genes are greatly affected by the salt concentration in soils (Braker et al., 2000; Franklin et al., 2017; Hu et al., 2014; Pan et al., 2023; Wang et al., 2018). Pan et al. (2023) investigated the effects of salt and salt-alkali addition on the denitrification rate in soils and reported that increasing salinity decreases denitrification rates. They concluded that increasing salt and salt-alkali concentration reduces the abundance of nosZ (Clade I) in all saline conditions, while decreasing nirK and nosZ was only observed at very high salt concentrations (8%–15% of soil dry mass). This was also observed by Menyailo et al. (1998) who concluded that the high ionic strength of the salt solution is the key factor inhibiting the activity of Nitrous Oxide Reductase, not the osmotic pressure (Menyailo et al., 1998). They also concluded that in the presence of salts, i.e., CaCl₂, Na₂SO₄, and Na₂CO₃, the final product of

denitrification is N₂O rather than N₂. Marks et al. (2016) investigated the effects of the change in salt concentration on denitrification rates in marsh and bayou soils of fresh and salt marshes. Their results showed that denitrification enzyme activity was higher in fresh marsh soils compared to salt marsh soils. Interestingly, their results showed that when simulating the long-term change from salt to fresh conditions (e.g., during river inflows), the pulses of fresh water with zero salt decreased the denitrification rate of the salt marsh soils. Similar experiments with pulses of water with an intermediate salinity of 15 ppt increased the denitrification rate while pulses of water with a high salinity of 35 ppt (seawater salinity) significantly decreased the denitrification rate. They concluded that the changes in denitrifying enzyme activities (and consequently the denitrification rates) depend on the history of salinity fluctuations of the soil. For example, if saline soils are frequently flooded with fresh water, the microbial community is better adapted to salinity fluctuations and the change in denitrification rates will be less noticeable (Marks et al., 2016). In a similar study, Bai et al. (2017) investigated the effects of flooding frequency on the denitrification rate of salt marsh sediments. They reported higher denitrification rates in salt marshes with higher flooding frequencies. Generally, the hydrological fluctuations affect the denitrification rate by changing the availability of oxygen, and nutrients which greatly changes the dynamics of the soil ecosystems (J. Bai et al., 2017; Day et al., 1988; W. Rassam et al., 2006).

It should be noted that there is a minimum salt concentration for the soil salinity level, under which, the denitrification rate increases with salinity. The exact value of this threshold, however, depends on many factors including the soil type, organic content, pH, and hydrological fluctuation patterns/frequencies. (Zeng et al., 2013). From a practical point of view, this means that even when a specific strain of bacteria is used for denitrification, the optimum D-nitrification conditions depend on several factors including the salt content and salinity fluctuation history. Therefore, whether imported or indigenous bacteria are used, the optimum conditions should be fine-tuned for each specific project.

4.4. Temperature

Similar to pH and salinity, temperature controls the denitrification rate by affecting the denitrifiers' growth, metabolism, and denitrification gene expression (Saleh-Lakha et al., 2009). Most denitrifiers are mesophiles with an optimal growth temperature between 25 and 30 °C. However, there are denitrifying bacteria that belong to other categories. For example, *Bacillus simplex H-b* is a psychrophilic bacterium (Yang et al., 2023) that can grow and even thrive at low temperatures while *Thermothrix thioparus* and some denitrifying *Bacillus* species are thermophilic (Hollocher & Kristjansson, 1992) and can thrive at relatively high temperatures. Denitrifying communities are typically comprised of many microorganisms with varying optimal growth temperatures. Therefore the effects of temperature on the denitrification process depend on the type of the denitrifying bacteria, their community structure, and the diversity of denitrifying bacteria in the community.

Both the long-term average (yearly or over a few years) and the amount of change in the temperature (in the long term) are important in controlling the composition and diversity of denitrifiers and in turn the denitrification process in soils (Wallenstein et al., 2006). This is important especially if the indigenous soil bacteria are to be used for D-MICP. The short-term temperature (average or change in temperature during the D-MICP application period) is also important as it controls the instantaneous denitrification rates (Wallenstein et al., 2006). Stanford et al. (1975), for example, studied the effect of temperature on nine different soils with extensively different physicochemical and organic contents and showed that from 11 °C to 45 °C, the denitrification rate increased approximately by twofold for every 10 °C increase in temperature while the denitrification rate declined abruptly when temperature was lowered from 10 °C to 5 °C. Similar results are also

reported for agricultural soils fertilized with liquid lagoonal swine waste (Fischer & Whalen, 2005). Very low temperatures (0 °C) apply significant stress on denitrifier communities resulting in inhibition or very limited reduction of N₂O (Holtan-Hartwig et al., 2002; Stres et al., 2007). This is because low temperatures have the greatest effect on nosZ gene expression. As the nosZ gene expression is responsible for the reduction of N₂O to N₂, hindering this expression results in N₂O accumulation. Liao et al. (2018), for example, reported the incomplete denitrification and accumulation of N₂O at 10 °C, while complete denitrification was observed at higher temperatures even up to 50 °C. They also reported that temperature variations caused significant shifts in the denitrifier's community structure, diversity, and consequently the abundance of nirK, nirS, narG, and nosZ denitrification functional genes (Liao et al., 2018). Saleh-Lakha et al. (2009), on the other hand, proposed that temperature mostly affects the denitrification rate by changing the time to reach the maximum denitrifying genes' (nirS and cnorB) expression. In other words, low temperatures do not inhibit denitrification, and the expression of maximum gene transcripts can be expected even in very low temperatures but with a significant delay. This is the reason denitrification is ubiquitous in relatively low-temperature conditions (5 °C to 23 °C) of lake sediments. This is in agreement with the notion that the denitrifying activity is limited by the concentration and diffusion of nitrate, not the biochemical reactions (Andersen, 1977).

The effects of temperature could have significant implications for the field application of D-MICP in geotechnical projects. Due to low precipitated calcium carbonate content via D-MICP (especially compared to U-MICP), D-MICP treatment of soil is usually done over several cycles to achieve the desired properties in the treated soils. At the start of each cycle, substrates and nutrients needed to maintain bacteria and calcium carbonate precipitation are introduced into the soil (if ex-situ bacteria are used, the grown inoculum is also added to the soil during the first cycle). The substrate and nutrients are left in the soil for a certain period until the electron donors in the substrate (e.g., acetate) completely reduce the available nitrate in the substrate. At this point, the next cycle begins by introducing fresh substrates and nutrients. These cycles continue until the desired amount of calcium carbonate is precipitated. The delay in reaching the maximum expression in cold regions, therefore, implies that longer time intervals are required between successive cycles for D-MICP projects in cold environments. This is especially important in shallow treatment projects (such as improving wind erosion resistance of soils) where temperature variations are more pronounced. In such conditions, it is recommended that the time intervals between cycles be designed based on the worst-case scenario to make sure the effect of delayed gene expression does not lead to the accumulation of intermediate products and inhibition. In general, it is recommended that the D-MICP process including the time interval between the cycles should be finetuned for each project based on the environmental conditions including the temperature average and fluctuation in the field.

4.5. Microbial communities

In the past, it was generally presumed that the diversity and composition of the denitrifying communities were not controlling factors in the denitrification rates because denitrifying genes are ubiquitous among many microorganisms (Linne von Berg & Bothe, 1992). However, this is not true because the presence of denitrifying genes does not guarantee the expression of the genes (Cavigelli et al., 2000; Cavigelli & Robertson, 2001; Chèneby et al., 1998; Holtan-Hartwig et al., 2000; Rich et al., 2003; Wallenstein et al., 2006). Additionally, many denitrifiers do not have all the enzymes required to complete the four steps of the denitrification process and sometimes multiple microorganisms must work together to finish the process (Zumft, 1997). Additionally, different denitrifier communities have significantly different tolerances to changes in environmental conditions and environmental stresses

(Cavigelli et al., 2000; Holtan-Hartwig et al., 2000). This tolerance difference is in fact the reason that dissimilar denitrifying community compositions and activities are reported during different seasons in a single area (Boyle et al., 2006; Mergel et al., 2001; Wolsing & Priemé, 2004). These seasonal changes in the communities could change the denitrification rate or even inhibit the denitrification process in some seasons. For example, the change in the community diversity and structure could hinder the denitrification process if some of the denitrifiers that must work together to finish the process are eliminated due to environmental stresses.

The competition between different bacterial species could also result in a reduced denitrification rate or inhibition. For example, nitrate ammonifiers compete with denitrifiers for the available nitrate in the environment (Arsyadi et al., 2023; van den Berg et al., 2017a; Chen et al., 2017; Kessler et al., 2018; Kraft et al., 2011; Li et al., 2022; Li et al., 2022; Saghāi & Hallin, 2024). In dissimilatory nitrate reduction to ammonium (DNRA), also known as nitrate ammonification, nitrate ammonifiers use nitrate as the electron acceptor in their anaerobic respiration (Kraft et al., 2011; Lam & Kuypers, 2011). These bacteria first reduce nitrate to nitrite (similar to denitrifiers) using periplasmic (napAB) or membrane-bound (NarGHI) nitrate reductase and then reduce the nitrite directly to ammonium using cytochrome c nitrite reductase enzyme (nrfA/onr) (Kraft et al., 2011; Simon et al., 2000). Similar to denitrifiers, some nitrate ammonifiers are facultative anaerobes while others are obligate anaerobes (Saghāi & Hallin, 2024). Despite their significant diversity and presence in many ecosystems, including sediments of coastal waters and freshwater environments (An & Gardner, 2002; Babbín et al., 2017; Bourceau et al., 2023; Fuchsmann et al., 2017; Kessler et al., 2018; Li et al., 2022; Pandey et al., 2021; Pang & Ji, 2019; Zhao, et al., 2020), terrestrial ecosystems (soils) (Cheng et al., 2022; Nelson et al., 2016; Putz et al., 2018; Saghāi et al., 2023), and even activated sludge (Chutivisut et al., 2018; van den Berg et al., 2017b; Wang et al., 2016a; Wang et al., 2020), they are extremely underinvestigated even in nitrogen cycle studies (Kuypers et al., 2018). From the MICP application point of view, the reduction of nitrate to nitrite in the DNRA still produces the CO₂ required to precipitate calcium carbonate (although probably in much lower amounts). Therefore, even if the presence of nitrate ammonifiers reduces or inhibits the denitrification process, calcium carbonate might still precipitate in lower concentrations. This potential precipitation of calcium carbonates in this situation, however, depends on the pH of the environment. In addition to the production of CO₂, an alkaline environment is required to precipitate carbonate over bicarbonate. In denitrification, as explained earlier, the substantial consumption of H⁺ during the reduction of nitrite to nitric oxide (reaction 10) increases the alkalinity and the pH of the solution. In the DNRA process, on the other hand, such an alkaline environment is not facilitated by the bacteria's metabolic activity and the carbonate could only precipitate if the pH of the environment is already high (Stevens et al., 1998). This process and the effect of nitrate ammonifiers on the D-MICP applications, however, have not been well studied. As it could have important implications especially when D-MICP using in-situ or ex-situ communities (e.g., from activated sludge) is desired, the effects of the presence of nitrate ammonifiers in the communities used for D-MICP on the efficiency and amount of precipitated calcium carbonates need to be further investigated.

Anaerobic ammonium oxidation (anammox) bacteria is another ubiquitous bacteria that could interrupt the completion of the stepwise denitrification process by using the denitrification intermediate products to oxidize ammonium (as an electron donor) to nitrogen gas (Gao et al., 2022a; Kuenen, 2008; Medinets et al., 2015; Pell & Wörman, 2008; Poffenbarger et al., 2018; van den Berg et al., 2017a; Zuo et al., 2023). Again, by interrupting the stepwise denitrification reductions, these bacteria could reduce the amount of produced CO₂ which is required to precipitate calcium carbonate. The reduced CO₂ can, in turn, decrease the efficiency of the D-MICP. On the other hand, the existence

of anammox bacteria could have some potential advantages that could be utilized in MICP applications. As mentioned before, U-MICP is significantly more efficient than D-MICP but it produces ammonium chloride (as a byproduct of the metabolic pathway) that needs to be removed from the environment. Conducting D-MICP using bacterial communities that have both denitrifiers and anammox bacteria after conducting U-MICP (combined MICP) could improve the desired properties of the soils and potentially remove the undesirable ammonia produced by U-MICP. Although it seems possible theoretically, the optimum substrate concentrations and number of cycles, among other details of the application, need to be carefully studied in future research.

Plants and diatoms also compete with soil microorganisms for inorganic nitrogen sources including nitrate (Arsyadi et al., 2023; Diner et al., 2016; Kuzyakov & Xu, 2013; Trinh et al., 2016). Although not as important, these competitions could have some implications for D-MICP applications in certain areas such as shallow wind/water erosion prevention projects where plants and diatoms can compete with denitrifiers. The effects of such competition on D-MICP efficiency have not been well-researched and require some attention in future investigations.

It is also worth noting that, the nitrate-reducing bacteria (denitrifiers) competitively inhibit sulfate-reducing bacteria (SRB) (Martin et al., 2013). Sulfate reduction is mostly undesirable, except maybe in a few special cases (Ontiveros-Valencia et al., 2012), and the inhibition of SBR could be used as an additional benefit of D-MICP. For example, D-MICP can be used on the soils surrounding steel structures to prevent the SBR from corroding steel pipes and foundations (Chen et al., 2021; Gu et al., 2019; Kalajahi et al., 2021; Kuzyakov & Xu, 2013; Liu & Cheng, 2020; Li et al., 2019; Wei et al., 2021a).

Although organisms competing for nitrogen sources could have very important impacts on the D-MICP efficiency and applications, this topic has not been studied systematically and require more attention in future research.

4.6. Water stress (extreme wet-dry conditions)

According to the Intergovernmental Panel on Climate Change, IPCC (Intergovernmental Panel on Climate Change IPCC, 2014), major shifts in precipitation patterns including prolonged droughts are anticipated in the next 20 years. Drought and decreased water potentials in soils have been identified as significant stress factors for bacteria (Austin et al., 2004; Gordon et al., 2008; Pinto et al., 2021; Schimel et al., 2007; Stark & Firestone, 1995). Even seasonal changes in soil moisture can affect the composition of microbial communities in soils (Waldrop & Firestone, 2006). The changes in soil microbial communities are expected to be more drastic due to the anticipated changes in precipitation patterns. It has been shown that different denitrifying genes have different sensitivities to droughts (Hammerl et al., 2019). For example, the nirS gene has been shown to be more sensitive to droughts compared to nirK and nosZ genes (Hammerl et al., 2019). On the other hand, some studies have reported complete inhibition of nirK activity due to increased water stress (Chen & Sung, 1983). It also has been shown that denitrification could resume shortly after the rewetting of dry desert soils (Stres et al., 2007). It is important to keep in mind that the soil type, more specifically the fine content of the soil, the ability of soil to retain water, and soil water tension, play a very important role in how the microbial community responds to water stress and droughts/rewettings (Hammerl et al., 2019; Wollersheim et al., 1987). Overall, the tolerance of denitrifying species and the response of their community composition/dynamic to water stress is not well understood (Stres et al., 2007) and needs to be investigated in more detail. Moreover, the effect of water stress on the D-MICP performance and efficiency has not been investigated. Some projects such as shallow wind erosion/dust prevention projects or D-MICP stabilized low-volume roads, might require periodic reapplication of D-MICP treatment to maintain the

desired properties of the treated soils. Therefore, with the anticipated shift in the precipitation patterns and potential increase in severe droughts and intensive rewetting events, the effects of such extreme actions on the denitrifying bacteria community and their ability to perform D-MICP need to be studied.

4.7. Heavy metals

Low amounts of some heavy metals, e.g., Copper, Zinc, Iron, and Nickel, enhance denitrification by promoting denitrifying enzyme activity and providing multiple electron donors (Liu et al., 2022a). Some studies have even shown a substantial positive correlation between heavy metal concentrations (especially Zinc and Cadmium) and the abundance of napA genes (Hongxia et al., 2024). This finding suggests that heavy metals at environmental concentrations (in the ranges approximately between 0.1 µg/g to 70 µg/g depending on the heavy metal) may enhance aerobic denitrification in soils and reduce the unfavorable effects of high oxygen levels (Hongxia et al., 2024). Soil stabilization via D-MICP could take advantage of this effect in shallow applications (Ahmadzadeh et al., 2024). However, the concentration of heavy metals in natural sediments and their pore water could change significantly during different seasons (Tu et al., 2023) which must be taken into account in the design of such D-MICP treatment projects (Behzadipour & Sadrekarimi, 2024). These changes could be even more pronounced in the near future due to the anticipated shift in the precipitation patterns and potential increase in severe droughts and intensive rewetting events.

On the other hand, higher amounts of heavy metals have a significant role in inhibiting the growth or reducing the metabolic activity of microorganisms including denitrifiers (Abbas et al., 2020; An et al., 2020; de Oliveira et al., 2021; Gui et al., 2017; Gupta & Nirwan, 2014; He et al., 2015; Liu et al., 2022a; Lu et al., 2019; Moon et al., 2006; Ochoa-Herrera et al., 2011; Su et al., 2019; Wang et al., 2015; Wei et al., 2021b; Wu et al., 2021; Yang et al., 2019; Zhang et al., 2019; Zhao et al., 2020; Zhu et al., 2013). Some heavy metals, e.g., Cadmium, Chromium, and Vanadium, at high concentrations directly poison/inactivate the bacteria (Lai et al., 2023), while others affect the expression of functional genes (Liu et al., 2022a). The minimum inhibitory concentration (MIC) of each heavy metal, i.e., the minimum concentration that causes inhibition or a significant decrease in activity, varies for different bacteria. Most of the studies investigating the inhibitory effects of heavy metals on denitrifiers have focused on specific strains of denitrifiers and the effects of these metals on the composition and activity of denitrifying communities have received less attention. As using in-situ denitrifying bacteria or exogenous bacteria communities (instead of single strains) is one of the attractions of using D-MICP over U-MICP, understanding the effects of heavy metals on the denitrification process of denitrifying communities is of great importance and needs to be investigated.

It should also be mentioned that some species of denitrifiers, e.g., some *Pseudomonas* strains, have been shown to be heavy metal-tolerant (Roosa et al., 2014). Employing such bacteria, therefore, has the potential to improve the application of D-MICP in stabilizing geomaterial with high concentrations of heavy metals, e.g., polluted soils or mine tailings. However, the application of heavy metal-tolerant denitrifiers for D-MICP applications has not been sufficiently investigated and requires further attention in future research.

Furthermore, some bacteria can adapt to environmental stresses through bacteria acclimatization and evolution. For example, de Oliveira et al. (2021) successfully acclimated *S. pasteurii* (which is a ureolytic strain) to high concentrations of heavy metals by gradually increasing heavy metal concentrations in the substrates of successive inoculations. They then used the acclimated bacteria for U-MICP treatment of mine tailings with high copper concentrations. Bacteria acclimatization potentially has better chances of success in D-MICP because bacterial communities have a better chance of acclimating to

harsh environments compared to single strains. This is especially the case if a denitrifying community includes heavy metal-resistance denitrifiers (Roosa et al., 2014). Again, the acclimation of denitrifying communities for D-MICP has not been sufficiently investigated and more research is required in this area.

4.8. Carbon dioxide

Contradicting results have been reported on the effects of CO₂ on the denitrification process (Barnard et al., 2004; Barnard et al., 2004; Kanerva et al., 2006; Robinson & Conroy, 1998; Wan et al., 2016). Some studies have reported a significant reduction in denitrification rate with increased carbon dioxide (Kanerva et al., 2006). Other studies have suggested that the presence of carbon dioxide decreases dissolved oxygen and increases soil moisture content which results in an increased denitrification rate (Robinson & Conroy, 1998). The presence of carbon dioxide changes the soil's pH, oxygen content, moisture content, and labile carbon sources, all of which are key factors affecting the denitrification process. Therefore, the initial difference in these environmental conditions could contribute to the contradictory results reported in different studies (Wan et al., 2016). However, carbon dioxide can also directly affect the denitrification process. A study by Wan et al. (2016) showed that carbon dioxide at high concentrations damages the bacterial membrane and inhibits the transport and consumption of intracellular electrons. The inhibition of electron transport and consumption could prevent the denitrification process or significantly decrease the denitrification rate and increase the generation and accumulation of N₂O (Wan et al., 2016).

In addition to the initial environmental conditions mentioned above, the ecosystem itself (presence of plants, microbes, etc.) can also influence the response of denitrifiers to increased CO₂ concentration. Comparing the results presented by Usyskin-Tonne et al. (2020) and Wan et al. (2018), for example, shows the significant effect of plants on the response of the denitrifying bacterial community to CO₂ concentrations. Usyskin-Tonne et al. (2020), investigated the effects of CO₂ on the denitrifying community of wheat root microbiome. Their results showed that elevated CO₂ increased the abundance of denitrifying genes in roots. They also concluded that elevated CO₂ affects N₂O-reducing (nosZ-based) bacterial community more than total bacterial community. Wan et al. (2018), on the other hand, investigated the direct effects of CO₂ on denitrifying bacterial communities in sediments by excluding the potential influences from other factors in the ecosystems such as plants or microbes. They concluded that both short-term and long-term exposure to an increased concentration of CO₂ directly suppressed denitrification and increased nitrite accumulation and N₂O emission in soils. Their results showed that CO₂ inhibits the activity of denitrifying enzymes and reduces the abundance of functional denitrifying genes.

The CO₂ concentration in soil profiles depends on many factors, including soil/rock types in the area, water content, temperature, and depth. In noncarbonate areas, for example, the CO₂ concentration increases with depth. In carbonate areas, especially karst regions, the CO₂ concentration decreases in deep areas, possibly due to geological carbonate corrosion that absorbs some of the CO₂ in deep areas (Chen, 2019). Agricultural and farming activities also significantly affect the CO₂ concentration of the soil (Ray et al., 2020). High CO₂ concentrations, exceeding 40 vol percent, have also been reported above the capillary rise zone of stagnant groundwater (Enoch & Dasberg, 1971). The CO₂ concentration in soils also fluctuates significantly, especially in shallow vegetated areas (Buyanovsky & Wagner, 1983). As can be seen, the project site conditions, groundwater table location, stratigraphy of the area, karstification in the area, the type of land use nearby, and many other factors could affect the CO₂ concentration of soils. At the same time, the complex effects of CO₂ concentrations on the denitrification process are not fully understood. Furthermore, the effects of CO₂ concentrations on the efficiency of D-MICP have not been well

studied. Hence, the effects of CO₂ on D-MICP treatment, especially when denitrifying communities are used, needs to be thoroughly investigated in future research.

4.9. Concentration of nitrate, nitrite, and carbon sources

Although nitrate is essential as the nitrogen source for new biomass (cell) production and as the terminal electron acceptor in the absence of oxygen, excessive amounts of nitrate can cause the intermediate compounds to accumulate which inhibits the bacteria's growth (Almeida et al., 1995; Pham et al., 2018a; Saleh-Lakha et al., 2009). For example, increased anthropogenic nitrogen deposition (caused by climate change, fertilization, and other pollutions) has been reported to increase the nitrate concentrations in soils leading to an increased ratio of N₂O/N₂ gases produced during the denitrification (Aerts, 1997; Barnard et al., 2005; Erisman & Vries, 2011; Fenn et al., 1998; Firestone et al., 1979; Gundersen, 1991; Mohn et al., 2000; Monaghan & Barraclough, 1993). Furthermore, long-term frequent nitrogen fertilization of soil has been shown to lower the abundance of denitrifying microorganisms, nirK, and nirS genes despite the availability of nitrate (Wallenstein & Vilgalys, 2005; Wolsing & Priemé, 2004). Nitrate content is also important in the optimization of the substrates used in the D-MICP inoculation and treatment cycles. For example, Pham et al. (2018a) showed that an acetate-nitrate molar ratio of 0.6 in the growth medium resulted in the accumulation of nitrite while an acetate-nitrate molar ratio of 0.8 resulted in the most efficient denitrification in their liquid batch experiments. Their study also showed using sodium-based substrates in D-MICP experiments results in high pH conditions which favors the reduction of nitrate to nitrite but may lead to nitrite accumulation. On the other hand, when calcium-based substrates were used in their D-MICP experiments, the nitrate reduction was complete despite less desired pH values of below 7. However, denitrification in wastewater samples containing high nitrate content has been reported to be significantly inhibited at pH values of 7 or below 7 (Glass & Silverstein, 1998). It is also important to note that nitrate concentration is very important in the short-term (during the D-MICP) but has less controlling effect on the long-term composition and diversity of denitrifying communities (Wallenstein et al., 2006).

Low nitrate/nitrite concentrations could hypothetically result in unbalanced growth and subsequently, the production of mucilage, i.e., extracellular polymeric substances (EPS), that are either loosely attached to bacteria cells or completely detached from them (Stal, 2010). In denitrification, under low nitrate concentrations (nitrogen limitation) but sufficient energy and carbon source (acetate), the denitrifying bacteria can potentially continue the assimilation of the carbon source but due to nitrogen limitation, they cannot synthesize amino acids and protein. The assimilated carbon is therefore converted to carbohydrates, some of which are secreted as mucilage (loose or detached EPS). From the D-MICP application perspective, however, mucilage (and EPS in general) can help stabilize the soil by binding (gluing) soil particles together (Stal, 2010). Nonetheless, the effect of low nitrate/nitrite concentrations (or excessive carbon/energy source) on the EPS production of the denitrifiers and its subsequent effect on the stabilization of soils has not been investigated and warrants more attention.

Denitrifiers can use organic and inorganic compounds as their source of carbon and energy. The type of carbon source not only influences the denitrification rate but also has the potential to alter the structure of the denitrifier community (Cherchi et al., 2009; Dlamini et al., 2020; Elefsiniotis & Li, 2006; Güven & Güven, 2009; Xu et al., 2018). Most D-MICP experiments have used liquid organic substrates (low-molecular-weight organic substances such as acetate or saccharides such as glucose) as the carbon source for the denitrifiers. However, solid organic substances that have been proven to be great carbon sources and electron donors for denitrifiers have received less attention. Some of these biodegradable polymers include polyhydroxyalkanoates (PHAs), polycaprolactone (PCL), starch/PCL, polybutylene succinate

(PBS), polyvinyl alcohol (PVA), starch/PVA, poly(3-hydroxybutyrate-co-3-hydroxyvalerate)/poly(lactic acid) (PHBV/PLA), and PHBV/starch (Chu & Wang, 2011, 2016; Gutierrez-Wing et al., 2012; Li et al., 2013; Marušincová et al., 2013; Shen et al., 2015; Wu et al., 2012, 2013; Zhang et al., 2016). While using liquid organic substrates for D-MICP can lead to overdosing and elevated dissolved organic carbon in groundwater, using biodegradable polymers reduces such risks because they are insoluble (Wu et al., 2013). Therefore, it is worth investigating the potential for using such carbon sources for D-MICP treatment of soils in areas with shallow groundwater tables or coastal areas.

Limited carbon sources can hinder the denitrification process and result in nitrite or N_2O accumulation (Bernat et al., 2008; Ge et al., 2012; Kornaros et al., 1996; Nakano et al., 2010; Ribera-Guardia et al., 2014; Warneke et al., 2011). It is worth noting that the effects of limited carbon sources on the denitrification process depend on the type of carbon source. Ribera-Guardia et al. (2014), for example, showed that during similar denitrification experiments, nitrate reduction practically stopped after around 40 min when acetate was used as the carbon source. In contrast, nitrate reduction was significantly reduced and nitrous oxide started to accumulate after only 20 min when ethanol was used as the carbon source. According to their study, N_2O reduction rate was 323% higher than the nitrite reduction when ethanol was the carbon source, which resulted in N_2O accumulation. Conversely, when acetate was the carbon source, N_2O reduction rate was only 31% higher than the nitrite reduction rate, and therefore, N_2O did not accumulate. It should be mentioned that the studies investigating the influence of limited carbon sources on the denitrification process are restricted to liquid organic substrates. The effects of limited carbon source conditions on the denitrification process when biodegradable polymers are used have not been investigated. Regardless of the carbon source type, avoiding a limited carbon source condition is an important factor when determining the optimum carbon-to-nitrogen ratio (e.g., acetate-nitrate ratio) in the substrates for the inoculation and the cycles of D-MICP treatment. Denitrification kinetics, such as those used by Kornaros et al. (1996) or Pham et al. (2018a), can be used to determine an appropriate starting carbon-to-nitrogen ratio. For example, the optimum carbon-to-nitrogen ratio for the cases where acetate is the carbon source is approximately between 0.6 and 1.25 (Pham et al., 2018a). This starting ratio can be further finetuned for each specific project taking into account other environmental factors.

It is also crucial to understand that the optimum carbon-to-nitrogen ratio depends not only on the stoichiometry of the metabolic reactions but also on the ratio of catabolic to anabolic reactions which itself depends on the bacteria growth rate. For example, Pham et al. (2018a), showed that when acetate is used as the carbon source, the theoretical acetate-to-nitrate ratio at maximum growth is about 1.25 which corresponds to a carbon-to-nitrogen ratio of 2.5. The theoretical acetate-to-nitrate ratio at zero growth is about 0.6 which corresponds to a carbon-to-nitrogen ratio of 1.2. Past research has focused on making sure that the maximum growth rate conditions are met when optimizing the carbon-to-nitrogen ratio for their laboratory experiments. Although this does not cause any problems in the laboratory D-MICP sand column experiments, using the carbon-to-nitrogen ratio corresponding to maximum growth can be problematic in the field unless the time intervals between cycles are adjusted. This issue can be best demonstrated by using the following example. Pham et al., 2018a conducted liquid batch experiments and showed that when using a carbon-to-nitrogen ratio of 2.5 (corresponding to maximum growth), a complete reduction of nitrate to nitrogen gas was achieved in around 23 days. However, when a carbon-to-nitrogen ratio of 1.6 (acetate-to-nitrate ratio of 0.8) was used, a complete reduction of nitrate to nitrogen gas was achieved in approximately 9 days. In their sand column experiments, they used the carbon-to-nitrogen ratio of 2.5 to ensure a maximum growth condition but the time interval between their consecutive cycles was between 7 and 9 days. At the carbon-to-nitrogen ratio of 2.5, according to their test data, only about half of the initial nitrate was consumed

before they started the second cycle with fresh substrates of the same carbon-to-nitrogen ratio of 2.5. They did not encounter any problems because, between the cycles, the sand column was flushed with distilled water to remove the remaining solutes before applying the substrates for the next cycle. In field applications, however, soil cannot feasibly be flushed with water between cycles. Therefore adding fresh substrate with a carbon-to-nitrogen ratio of 2.5 after 7–9 days would result in excessive nitrate concentration (because after 9 days half the nitrate from the previous cycle is probably still in the soil) and inhibition. Therefore, for field application of D-MICP, either the time interval between the cycles should be adjusted or the carbon-to-nitrogen ratio should be chosen to ensure all the substrate is consumed before adding new substrate to avoid the accumulation of intermediate products and inhibition.

4.10. Sulfur compounds

There are three major sources of sulfur in soils, i.e., weathering of minerals such as iron, nickel, and copper sulfides, decomposition of organic matter, and atmospheric deposition (Service & Edwards, 1998). Sulfur compounds have complicated effects on the diversity and activity of denitrifying microorganisms (Wang et al., 2016b). Many researchers have reported that sulfides inhibit NOR and N_2OR enzymes and prevent the reduction of NO and N_2O resulting in the accumulation of these gases in soils and other environments (Knowles, 1982; Kowalenko, 1979; Myers, 1972; Sorensen et al., 1980; Tam & Knowles, 2011; Wang et al., 2023). For example, the inhibition of denitrification and accumulation of NO and N_2O in sulfidic marine sediments has been reported by Sorensen (1978). As explained earlier, the accumulation of NO and N_2O is toxic to the bacteria and inhibits the bacteria's growth and activity (Almeida et al., 1995; Pham et al., 2018a). Therefore, soil stabilization using the D-MICP method in these sulfidic soils could be challenging and should be considered in the design stages.

Most denitrifying bacteria are facultatively anaerobic and can use different electron acceptors with the order of preference being O_2 , NO_3^- , SO_4^{2-} , and CO_2 (Kowalenko, 1979). Based on this order of preference, when both nitrate and sulfate are simultaneously present in the environment, the denitrifying bacteria use nitrate, and therefore, sulfate will not inhibit the denitrification process. However, if excessive amounts of sulfate exist in the environment, a proportion of the denitrifying microorganisms will use SO_4^{2-} instead of NO_3^- , which could lead to the accumulation of nitrate and other intermediate compounds and reduced efficiency of denitrification (Kowalenko, 1979). This could be an important factor in stabilizing sulfate-rich soils (e.g., acid mine drainage contaminated soils, gypseous soils, and high organic content soils) and mine tailings using D-MICP. This issue can probably be resolved by reducing the concentration of nitrate in the inoculation/MICP treatment substrates. The appropriate amount of reduction in nitrate concentration and the optimum substrate concentrations, however, depend on the concentration of sulfur compounds in the soil and should be determined using project-specific optimization experiments. Furthermore, it is worth mentioning that although organic sulfur in soils is largely immobile, rapid mobilization can occur due to depolymerization and the soil's bacterial activity (Ghani et al., 1993; Scherer, 2009; Service & Edwards, 1998) which could affect the D-MICP in soils with high organic contents.

Unlike heterotrophic denitrifiers that use organic compounds such as acetate as their electron donor, autotrophic denitrifiers use inorganic compounds, e.g., reductive sulfur compounds such as sulfite (SO_3^{2-}), thiosulfate ($S_2O_3^{2-}$), tetrathionate ($S_4O_6^{2-}$), and elemental sulfur (S^0) as electron donors (Enrich-Prast et al., 2014; Pang & Wang, 2020, 2021; Xue et al., 2022). There are also mixotrophic bacteria that could use both organic and inorganic substrates as their electron donors (Pang & Wang, 2020; Shao et al., 2010; Wu et al., 2018). The effects of the presence of such sulfur compounds on the denitrification process, especially when denitrifier communities are used, largely depend on the

diversity and structure of the community. In other words, if autotrophic or mixotrophic denitrifiers exist in the community, the presence of these sulfur compounds can improve the denitrification rate (depending on the nitrate concentration in the environment/substrate). However, if only heterotrophic denitrifiers are present, these sulfur compounds could have an inhibitory effect. Other factors could also affect the response of the denitrification process to these sulfur compounds, but the mechanisms involved are not fully understood. For example, Kowalenko (1979), investigated the effects of sulfite and thiosulfate on nitrate reduction by native soil bacteria. They reported that the presence of sulfite increased the nitrate reduction rate while the presence of thiosulfate in the same soil resulted in a lower rate of nitrate reduction. Not many researchers have investigated the effects of such compounds on denitrification especially for the D-MICP process, and more research is required in this area. It should be noted, however, that these compounds (except native sulfur) are less stable and they are usually found in low transient concentrations in unpolluted natural soils (Service & Edwards, 1998). Higher concentrations could be found near agricultural sites (due to the continuous use of pesticides, insecticides, fungicides, and fertilizers (Jordan & Ensminger, 1959; Wind & Conrad, 1995)) and areas exposed to heavy atmospheric pollution (in which case, the concentrations are only high in the top few centimeters of the soil). On the other hand, large concentrations of native sulfur in soils and even large native sulfur deposits have been reported in lake sediments, shallow marine sediments, and volcanic areas (Ferraris & Vila, 1990; Labrado et al., 2019; Lindtke et al., 2011; Philip et al., 1994; Schulz & Schulz, 2005). Finally, it should also be taken into account that the effects of these reductive sulfur compounds on the D-MICP treatment projects will depend on the acetate-nitrate ratio of substrates used in the process. If the optimum acetate-nitrate ratio is determined without considering the existence of autotrophs or mixotrophs, the results could be the accumulation of intermediate compounds and inhibition of denitrification or leaving excess amounts of acetate in the soil.

4.11. Nutrients

In addition to electron donors and acceptors, microorganisms including denitrifiers need nutrients, e.g., trace elements, vitamins, and yeast, for their growth and activity during engineering applications such as D-MICP. Among the trace elements, iron and molybdenum play a key role in the catalytic activity of nitrate reductase (Nar), while iron and copper enhance the catalytic activity of nitrite reductase (Nir) (H. Liu et al., 2021). The lack of or limited availability of these trace elements and other nutrients could result in a lower rate or complete inhibition of the denitrification process (Chénier et al., 2006; Cyplik et al., 2007; Erşan et al., 2015; Liu et al., 2021; Wang et al., 2019). Most of the laboratory experiments are first optimized by adjusting the amounts of nutrients to achieve maximum efficiency. These optimized nutrient conditions are then used to investigate the effects of other factors. However, in some field applications such as ground improvement, self-healing (crack repair) concrete, and calcium ion removal from waste streams, denitrifiers often experience nutrient deficiencies which in turn affects the denitrification rate, the overall efficiency of the project, and the response of the bacteria to other environmental factors (Erşan et al., 2015). The response of different bacteria to nutrient deficiency and its effects on the denitrification rates varies drastically. Erşan et al. (2015), for example, showed that denitrifier strains *P. aeruginosa* PAO1 and *D. nitroreducens* are resilient to nutrient deficiencies. They showed that more than half of the D-MICP's maximum theoretical calcium carbonate precipitation yield can be achieved with these strains under minimal-nutrient conditions, i.e., substrates lacking trace elements, yeast, and vitamins. They emphasized, however, that the interval between D-MICP cycles must be optimized to avoid inhibition. High concentrations of trace elements could also result in the inhibition of denitrification. For example, Fu and Tabatabai (1989), showed that

trace elements Ag(I), Cd(II), Hg(II), Se(IV), As(V), and W(VI) at a concentration of 2.5 ($\mu\text{mol/g}$ soil) result in more than 75% inhibition of nitrate reductase activity. The effects of nutrient deficiency or high trace element concentrations on bacterial activity also depend on the soil type and environmental conditions (Fu & Tabatabai, 1989; Liang & Tabatabai, 1978). For instance, Fu and Tabatabai (1989), showed that while B(III) inhibited the activity of nitrate reductase in an acidic soil, no inhibition was observed in a calcareous soil, and the activity was even enhanced in a neutral soil.

While many studies have investigated the effects of nutrient deficiency or surplus on different denitrifying strains, these effects on denitrifier community structure and composition and denitrification rates of these communities are less understood. Especially the combined effects of nutrients and other environmental factors including soil types on denitrifying communities and its implications for D-MICP applications need to be investigated in detail.

4.12. Phosphorous/phosphate

Although phosphorous can promote bacterial activity, excessive phosphorous can lead to the precipitation of $\text{Ca}_3(\text{PO}_4)_2$, reducing the concentration of dissolved Ca^{2+} available for calcite precipitation, i.e., prevent carbonate precipitation in D-MICP (Erşan et al., 2015). Martin et al. (2013), using a growth media for *H. halodenitrificans* consisting of yeast extract and peptone, showed that the phosphate present in the media reduced the calcium carbonate precipitation during the D-MICP. They found that reducing the phosphate content by 75% increased the microbially precipitated calcium carbonate by more than three folds. They concluded that high phosphorous content leads to the oversaturation of phosphate in the environment causing calcium phosphate to precipitate instead of calcium carbonate (Martin et al., 2013). It is worth mentioning that some denitrifiers, e.g., some species of *Pseudomonas* such as *P. aeruginosa* and *P. fluorescens*, have the ability to convert excess orthophosphates to polyphosphates and store them (polyphosphate accumulation) (Jørgensen & Pauli, 1995). Typically, this accumulation happens under aerobic conditions and the stored polyphosphate is broken down by the bacteria under anaerobic conditions as the source of energy to store carbon. While typically polyphosphate accumulation and denitrification happen in alternating aerobic-anaerobic conditions, recent research indicates that simultaneous polyphosphate accumulation and denitrification under anaerobic conditions might be possible (Shukla et al., 2020). Simultaneous polyphosphate accumulation and denitrification under anaerobic conditions, the consequent removal of phosphate from the environment, and its effects on calcium carbonate precipitation during the D-MICP need further investigation. Furthermore, simultaneous polyphosphate accumulation and aerobic denitrification have not been well studied and need to be investigated as a potential approach to improve D-MICP using phosphate phosphate-rich growth medium. Additionally, caution must be exercised in applying D-MICP in soils with high phosphorous content. For example, due to the formation of phosphorites in marine sediments, these sediments typically contain high concentrations of phosphorus and phosphate-containing minerals (Schulz & Schulz, 2005). The design of D-MICP treatments in such sediments, therefore, must take into account the effects of these compounds on the efficiency of the D-MICP.

4.13. Other factors

Other than the major factors mentioned above, there are many other factors that could influence the denitrification process and consequently the D-MICP projects. Some of these factors are discussed briefly in this section.

It has been shown that Ca^{2+} complexation with dissolved organic carbon released from metabolism products and/or bacterial EPS can reduce calcium ion activity. This reduced activity can influence the calcium carbonate morphology and polymorphism and can even reduce

the amount of CaCO_3 precipitation (Braissant et al., 2007; Martin et al., 2013; Tournay & Ngwenya, 2009). Pressure has also been shown to inhibit the growth of some denitrifying species such as *H. halodenitrificans*, after around 20 MPa (Martens DA, 2005). Assuming other species have a minimum inhibitory pressure in the same range as the *H. halodenitrificans*, this will not cause any concern for typical geotechnical projects (the working geostatic and hydrostatic stresses in most projects shallower than 250 m deep are less than 5 Mpa). The clay content of the soil could also affect the concentration and accumulation of the denitrification intermediate material (Henault et al., 1998; Khalifah & Foltz, 2024; Skiba & Ball, 2002). Khalifah and Foltz (2024), for example, showed through a series of laboratory experiments, that the clay content of soils has a positive linear correlation with the $\text{N}_2\text{O}/\text{N}_2$ gas ratio produced at the end of the denitrification process in soils. Similar observations have also been reported from field data (Henault et al., 1998; Skiba & Ball, 2002). These observations, however, are based on limited data, and the potential mechanisms involved in the inhibitory role of clays and their effects on the produced $\text{N}_2\text{O}/\text{N}_2$ gas ratio are not well understood. More research is therefore required to confirm these effects are in fact directly related to the different clay contents of the soil and not due to other environmental factors indirectly affected by the clay content such as limited access to nutrients and substrate due to the lower permeability of clayey soils. Furthermore, as the low permeability of clays hinders the delivery of bacteria (if ex-situ bacteria is used) and nutrients/substrates to different sections of the soil, less efficient treatment and potentially uneven distribution of the precipitated calcium carbonate are expected as the fine content increases. The effects of fine content (the percentage of fine-grained particles, especially clay) on the field application of D-MICP and the subsequent spatial variability of the precipitated calcium carbonate have not been sufficiently studied and warrant more investigations.

Acetylene (C_2H_2) has also been reported to inhibit the reduction of N_2O (Balderston, Sherr, & Payne, 1976; Fedorova, Milekhina, & Il'Yukhina, 1973; Yoshinari & Knowles, 1976) at very low concentrations (Acetylene gas pressure of 10^{-3} atm (Knowles, 1982)). However, the presence of sulfide (which itself is an inhibitor) reduces (or eliminates) the inhibitory effects of acetylene. Additionally, acetylene inhibition is not always complete in soils, water sediments, and marine systems due to the utilization of acetylene by Nocardia (Kanner & Bartha, 1979), or oxidation by aerobic and anaerobic soil/sediment bacteria (Culbertson et al., 1981; Terry & Duxbury, 1985; Watanabe & de Guzman, 1980). Another potential denitrification inhibitor is Azide (N_3^-) which inhibits the establishment of adaptive enzymes (Spiegelman, 1947). According to Sacks and Barker (1952), azide does not inhibit nitrite reduction but does inhibit N_2O reduction. They, however, determined that azide (and dinitrophenol) may not inhibit the formation of the enzymes but could directly block the enzyme after it has formed. They also concluded that N_2O is not a mandatory intermediate in the N_2 production during bacterial denitrification of *Pseudomonas denitrificans* (Sacks & Barker, 1952). For biogeotechnology projects, therefore, any inhibitor that prevents the reduction of N_2O might not necessarily inhibit the D-MICP process. Whether or not N_2O is a precursor of N_2 formation depends on the microorganisms, as suggested by Matsubara and Mori (1968). They investigated the stepwise process of bacterial denitrification and proposed that while the first product of nitrite reduction is NO, there could be several different mechanisms for the following reduction of NO to N_2 depending on the denitrifying microorganism. They also concluded that azide, cyanide, and DNP (2,4-Dinitrophenol) in low concentrations inhibit the reduction of N_2O to N_2 and subsequently, inhibit the entire denitrification process when *P. denitrificans* are used.

Contradicting results have been reported on the effects of Nitrapyrin [N-serve; 2-chloro-6-(trichloromethyl)pyridine] on the denitrification process. Nitrapyrin is a well-known nitrification inhibitor and some studies have reported that it also inhibits denitrification (McElhannon & Mills, 1981a, 1981b; Meng et al., 2020) at concentrations as low as $0.5 \mu\text{g}\cdot\text{g}^{-1}$ soil (Bremner & Yeomans, 1986). Other researchers have

reported that no inhibition occurs at Nitrapyrin concentrations up to $10 \mu\text{g}\cdot\text{g}^{-1}$ soil (Bremner & Yeomans, 1986). Bremner and Yeomans (1986) even reported denitrification enhancement by adding $50 \mu\text{g}\cdot\text{g}^{-1}$ soil of Nitrapyrin. The effectiveness of Nitrapyrin can be influenced by many factors including clay minerals, soil organic matter, and soil microorganisms (Zhang et al., 2020).

Many pesticides (Vapam, Dalapon, Fungicides Captan, Toluidine derivatives, etc), especially after long-term continued application, have also been reported to inhibit denitrification (Bollag & Henninger, 1976; Grant & Payne, 1982; Knowles, 1982; Michel et al., 2020). Some of these pesticides remain in the soil for years after application. Continuous application of pesticides reduces bacterial count, diversity, and enzyme and biological activities (Al-Ani et al., 2019; Arora & Sahni, 2016; Kumar et al., 2021; Schäffer, 2021; Wołejko et al., 2020). Rainwater percolation, runoff, fast transport of water through cracks (preferential water flow), and other similar hydrogeological features transfer these pesticides laterally and vertically to wider and deeper areas of the ground, increasing the affected areas (Holbak et al., 2022; Karim et al., 2023; Schlögl et al., 2022; Zhu et al., 2019). Therefore, the use of D-MICP for ground improvement in new developments near agricultural activities or on agricultural/forestry lands, especially if the plan is to use in-situ bacteria, could be challenging and needs to be investigated on a project-specific basis.

Finally, it is crucial to note that some of the published literature on denitrification rates and factors affecting the efficiency of the process do not consider the complete reduction of nitrate to nitrogen gas (N_2). For example, Saleh-Lakha et al. (2009) investigated the effects of pH and temperature on the denitrification process where they determined the accumulation of nitrous oxide (N_2O) as a measure of denitrification rate/efficiency. This would be acceptable if the main goal of the denitrification process is removing nitrate and nitrite from the environment. In D-MICP, however, the complete reduction of nitrate to nitrogen gas is essential to avoid the emission of N_2O . Furthermore, as discussed above, the accumulation of N_2O and other intermediate products are inhibitory factors. Therefore, if a denitrification process is optimized for purposes other than D-MICP without confirming the complete reduction of nitrate to nitrogen gas, the optimization might not work for D-MICP applications. Hence, caution must be exercised when referring to the published data, especially the literature on denitrification for purposes other than D-MICP, for designing and optimizing the denitrification process for D-MICP treatment projects.

5. The order of significance of the inhibitory factors in ground improvement

The order of significance of the inhibitory factors described above depends on the nature of the ground improvement project. For example, the oxygen level would be one of the most important factors if the ground improvement is to be conducted in shallow or intermediate depths while the effects of the temperature would be a major factor in D-MICP applications in cold regions. Similarly, the effects of pH would be the key factor when treating acidic soils, heavy metals would be the important factor when treating mine tailings, water stress would become the major factor in arid areas, and the sulfur compounds would be very important in treating gypseous soils. In some cases, more than one of the inhibitory factors discussed above will be of primary concern and should be considered during the design of the ground improvement project. For example, if the goal of the ground improvement project is to treat mine tailings for dust control in cold regions, oxygen level, temperature, and heavy metals are key factors that should be prioritized.

6. Outlook on future research

Broadly speaking, future research should focus on finetuning and understanding the interplay between different factors affecting the

optimum condition for D-MICP. Some of the key research gaps and recommended future works include:

- One of the major conclusions of this research is that, despite the common belief, anaerobic conditions are not necessarily required for successful D-MICP soil stabilization. Many denitrifiers are capable of denitrifying in the presence of oxygen and D-MICP can possibly be used for shallow ground improvement projects where aerobic conditions prevail. D-MICP under aerobic conditions, however, has not been investigated and requires further research.
- Effects of biodiversity and denitrifier competitors on denitrification process efficiency require more research. Furthermore, despite their inhibitory consequences, the existence of some competitors, such as anammox bacteria, could have some potential advantages (e.g., using denitrifiers and anammox bacteria for combined U-MICP and D-MICP) that should be investigated. The effects of the anticipated water stresses caused by climate change, i.e., increased frequency and severity of droughts and intensive rewetting/flooding events, on denitrifying bacteria community and their ability to perform D-MICP, require further investigations.
- Using heavy metal-tolerant denitrifiers and/or bacteria acclimatization has the potential to improve D-MICP treatment of heavy metal-rich geomaterial such as mine tailings. This potential has not been comprehensively investigated and should be studied in detail.
- The complicated effects of the CO₂ concentrations in soil's air on the denitrification process and D-MICP applications are not fully understood. The CO₂ concentrations could have significant implications in D-MICP treatment projects especially in karst regions. Therefore, these effects require more detailed investigations.
- Effects of temperature, salinity, water stress, limited nutrients, nitrogen, and carbon sources, and CO₂ concentrations have been studied in some detail for specific denitrifying strains. However, their effects on the composition, diversity, and structure of denitrifying communities and consequently on denitrification process efficiency for D-MICP application using denitrifying communities (as opposed to specific strains) are less understood and require further research.

7. Concluding remarks

Optimal conditions for D-MICP vary due to multiple influencing factors, but the following general guidelines can serve as a starting point. The substrate should have a carbon-to-nitrogen ratio of roughly 2.5 and 1.6 for the inoculation period and the D-MICP cycles, respectively. The pH of the substrate should be around 7–8 with the time interval between D-MICP cycles of 7–9 days. The optimum temperature is between 25°C to 45°C which could only be approximately controlled in the field by choosing an appropriate time to conduct the field work. The main environmental and inhibitory factors that could change these optimum conditions include soil types, type of bacteria strain or community used, source of carbon used, depth of applications and groundwater table (availability of oxygen), pH, salinity, temperature, biodiversity of the in-situ soils (or bacterial community used), heavy metal concentrations, previous or expected severe droughts and intensive rewetting events, carbon dioxide concentration, nutrient availability, and sulfur/phosphorous compounds concentrations. Site-specific optimization is recommended when one or more of the inhibitory conditions are expected. During the site-specific optimization, it is crucial to investigate not only the amount of precipitated calcium carbonate but also the ratio of the N₂O/N₂ gases to make sure that the stepwise reduction of nitrate to nitrogen gas is complete and no harmful byproducts (N₂O) is released into the environment during the D-MICP ground improvement.

Some key considerations to avoid inhibition and improve the D-MICP effectiveness include:

- If yeast extract and peptone were used as the nutrient and trace element sources, lower amounts should be used during the D-MICP cycles compared to the inoculation period to reduce the phosphorus in the environment and improve the carbonate precipitation.
- The carbon-to-nitrate ratio should be adjusted in areas/applications prone to nutrient deficiency, limited or excessive carbon and nitrate sources, and excessive amounts of sulfur and phosphorous compounds.
- Either heavy metal-tolerant denitrifiers or bacteria acclimatization methods should be used in the D-MICP stabilization of geomaterial with high heavy metal concentrations.
- Longer time intervals are required between the D-MICP cycles in cold environments, especially in temperatures below 10°C. Additionally, longer time intervals between the D-MICP cycles are required if carbon-to-nitrate ratios higher than 1.6 prevail during the D-MICP cycles in the field.
- The same optimized processes used in the lab do not always automatically transfer to the field applications sometimes due to differences in steps involved in the lab compared to the field. For example, the optimum carbon-to-nitrogen ratio corresponding to maximum growth used in the lab experiments cannot be directly used in the field unless the time intervals between the cycles are adjusted to make sure all the substrates are completely consumed before introducing new substrates.

CRedit authorship contribution statement

Yasaman Abdolvand: Writing – review & editing.
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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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