AN ABSTRACT OF THE DISSERTATION OF

Carla Ribeiro Machado e Portugal for the degree of Doctor of Philosophy in Environmental Sciences presented on November 24, 2021.

Title: Evaluation of *Serratia Ureilytica* as a Greener Unsealed Road Stabilization Method.

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Dr. Carolyn Fonyo

Covering half of South America, Brazil is one of the largest agricultural and commercial forest producers globally, with a correspondingly complex road network necessary to support the production and marketing processes of such goods. Contrary to global statistics, almost 90% of the country's road network is classified as unsealed or dirt roads (CNT, 2020). This dissertation briefly reviews the history of such roads and explores the construction standards of unsealed roads in Brazil, showing how such standards can affect adjacent water bodies through hydric erosion and sedimentation. Specifically, this dissertation analyzes *Serratia ureilytica* as an innovative biocement formed through the process of Microbiologically Induced Calcium Carbonate Precipitation (MICCP) and serving as a potential new alternative to stabilize unsealed roads, thus reducing hydric erosion and minimizing sedimentation. To test the effectiveness of this method, we compared our novel MICCP protocol to three classic methods of unsealed road stabilization: granulometric stabilization, cement, and hydrated lime, as adopted by Brazilian's road construction standards. We simulated and compared the effects of these four stabilization methods on 1) surface erosion due to percolation effects from piping erosion and 2) water quality impacts from sedimentation due to traffic resistance of the unsealed roads.

This study tested the biocementation effects of *S. ureilytica* over unsealed road grades (URG) determined as A, C and F by the Brazilian standards for unsealed road

construction. To contribute to the global efforts to achieve a homogeneous biocement layer, this research tested fixed volumes of biocementation solution with variable granulometric distributions, in addition to testing the traditional stabilization methods of compaction, cementation, and hydrated lime addition. The greatest rate reduction in permeability was achieved for unsealed road grade (URG) F, which has the highest content of fine particles. A permeability rate reduction of 98.25% was achieved with the application of biocement when complared to granulometric stabilization with no sample compaction, and 95.64% reduction when compared to granulometric stabilization with sample compaction. URG A and URG C samples showed similar behavior after the biocementation treatments. Cement treatments were 100 % impervious for all samples, and hydrated lime treatments were less effective at reducing permeability rates than biocementation treatments. Our main findings lead to the conclusion that *S. ureilytica* is a strong candidate as a potential alternative method for unsealed road stabilization through the biocementation process. Our results gave enough evidence and data to expand the research to field scale, where the granulometric distribution proposed adaptations can be tested under all variables involved in unsealed road construction and use.

Our secondary study was designed to test potential markers to evaluate the potential environmental impacts from erosion of chemically stabilized unsealed roads, focusing on how the stabilization methods may impact water quality. Cement and hydrated lime are the two most commonly used stabilizers to improve unsealed roads. Calcium, magnesium, and silicon were selected as potential markers since they are the most abundant ions in cement, hydrated lime and biocement, and low-cost tests are available to determine their concentrations in samples. The levels of calcium and magnesium found in percolated water from the test samples in this study could serve as indicators of increased sediment from road erosion which carries other toxic chemicals, heavy metals, and petrochemicals due to road traffic, contributing to the pollution of water bodies and potential public health concerns. Further research in the field is needed to test for the presence of specific road pollutants in adjacent waterways under variable conditions of traffic flow and weatherization. The silicon lixiviated from the unsealed roads can be recycled and reused as a valuable source for agriculture and the regeneration of aquatic systems. Future research should include the research to field scale, adding all variables that may affect road weatherization and its erosion process, plus complete water quality assessment, including heavy metals, dissolved oxygen, turbidity, and sediment levels to test the connections and correlational parameters that would support the use of calcium, magnesium, and silicon as potential indicators of water quality pollution from unsealed roads. Silicon extraction and reuse costs should also be addressed.

Evaluation of *Serratia Ureilytica* as a Greener Unsealed Road Stabilization Method

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Major Professor, representing Environmental Sciences

Director of the Environmental Sciences Graduate Program

Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Carla Ribeiro Machado e Portugal, Author

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Nevertheless, she persisted!

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DEDICATION

I dedicate this work to my family, relatives, friends, and the master force of the universe that supported me in, in ways seen and unseen, on this intense and unique journey. To my spectacular committee, in special Dr. Fonyo and Dr. Meganck, for all the extra miles and guidance: you were my compass in the middle of the storm. To Dr. Carlos Machado for all the guidance and support that made this research possible. To Dr. Leslie Blair and MSc Mark Lavery for the fantastic journey as a graduate student assistant at the Bio 10X series and Dr. Mary Santelmann as a GTA at the Biogeography class. To the Graduate Writing Center, most notably Chris Nelson, for countless hours of support and guidance. I appreciate the support and trust of the Graduate School and all its champions like Dr. Brenda McComb and Dr. Jennifer Brown. To the Office of Equal Opportunity and Access for the eternal work to guarantee students' support. To the Coalition of Graduate Employees for all the dedication, the good fight, and support. Each colleague from different programs and countries has left a special mark on this work along the way.

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Chapter 1: General Introduction

1 - Introduction

Brazil covers nearly half of the land area of South America and while it leads the continent's markets for mining, agriculture, and manufacturing (Britannica, 2021), it still suffers considerable losses due to the precarious conditions of its road network which is the main form of production flow to national, regional, and global markets. As the world's third largest agricultural producing country, 41% of its total land is occupied by agriculture (351 million out of 849.8 million hectares), 19% of which is dedicated to soybean production (FAO, 2021). Brazil is the global leading exporter of oranges, sugarcane, and coffee, second in global production of oilseeds and cellulose, and third largest producer of meats, fruits, and grains. Their agribusiness is responsible for US$120.42 billion annually, one-twelfth of the Country's GDP (Gross Domestic Product) of US$ 1.445 trillion in 2020 (World Bank, 2021). This solid agribusiness system must rely on a robust logistics network to guarantee the optimal freight tonnage hauled, reducing the loss and costs on its way to various markets.

Brazil has a logistics system ruled by freight, where road transportation delivers 85% of the agribusiness sector production, which on average generates 250 million tons of grains per year (CONAB, 2021). However, contrary to global statistics for other leading producers, almost 80% (1,349,938 kilometers) of the Country's 1,720,700 km of road network are unsealed or dirt roads (CNT, 2020). Apart from other issues, unsealed roads are crucial for a region's development since they also bring social, economic, and commercial benefits to the interconnected communities (Rammelt & Leung, 2017). Still, any road implementation program is potentially a source of negative environmental impacts, including impacts to local water bodies via erosion and sedimentation (Thomaz & Peretto, 2016). In addition to road surfaces limiting infiltration, the surface material is often disturbed by traffic activities, increasing the sedimentation rates transported to water bodies via the hydrological connectivity of roads (Fu; Newham; Ramos-Scharrón, 2010). Figure 1 details the transportation modes in Brazil; the red lines on the main map show the extension of the unsealed roads network.

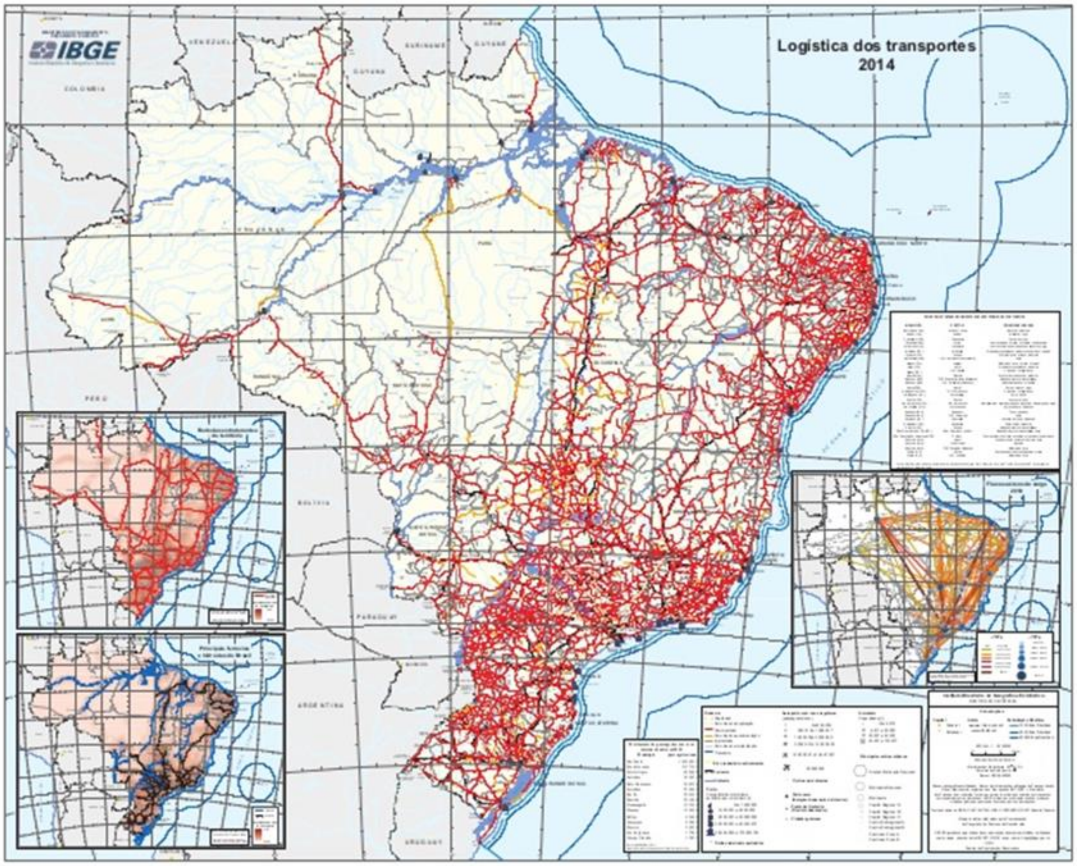

Figure 1: Map of transportation infrastructure of Brazil. The main map shows all the roads (unsealed and sealed). The map on the right highlights the domestic airline routes. The map on the upper left shows the details of railroads, and on the bottom left, details of waterways and pipelines. Source: IBGE, 2014.

The main factors affecting road surface erosion rates include the intensity and duration of rain, snowfall, temperature variation and weatherization, surface material characteristics, hydraulic characteristics of the road surface, the highway, traffic volume and loads, construction and maintenance programs, and the adjacent land uses (Fu; Newham; Ramos-Scharrón, 2010). The soil properties that most influence erosive processes are water content, mineralogy, clay proportion, density, porosity, soil structure, organic matter content, and iron and aluminum oxides content. These properties directly affect the mechanical strength of the soil, with the increase in the mechanical strength of the soil being the most common way to decrease soil erodibility, thus increasing the stabilization of the road (Machado et. al.., 2017).

In this sense, biocementation appears to be an alternative for soil stabilization via calcium carbonate precipitation or Microbiologically Induced Calcium Carbonate Precipitation (MICCP) by stimulating the bacterial population which form a biocement that potentially aggregates and stabilizes soil particles. Furthermore, some studies show that the process improves shear strength and decreases the permeability of sandy soils (Whiffin et. al.., 2007; Ivanov and Chu, 2008; Ng et. al.., 2012). Furthermore, authors such as Shahrokhi-Shahraki et. al.. (2015), Canakci et. al.. (2015), Zhu and Dittrich (2016), and A'la et. al.. (2020) indicate that biologically induced carbonate precipitation mimics natural calcium carbonate deposition processes (in the form of calcite, aragonite or vaterite) binding the soil grains, which increases the rigidity and mechanical strength and reduces erodibility.

Even though biocementation is presented here as a new alternative to chemical stabilization for unsealed roads, MICCP was first described for its use as a protective coating to buildings facades by Adolphe et. al. (1990). Rivadeneyra et. al. (1994) evaluated the MICCP of Vibrio spp. in its natural habitat, opening the path to explore alternative uses for MICP. Whiffin et. al. (2007) were one of the first authors to suggest MICP as a sand improvement technique. They proposed a model called "Whiffin's conductivity method" used to predict the urea hydrolysis changes during the MICP process by measuring the MICP solution electrical conductivity.

Specifically, this dissertation analyzes the *Serratia ureilytica* bacterium species as an innovative MICCP source, tested as a new alternative to stabilize unsealed roads via biocementation, aiming to reduce hydric erosion and minimize sediment loading to nearby water bodies. To test the effectiveness of this proposed method, we explored a novel technique to advance the protocol to reach the biocementation homogeneity necessary to sustain unsealed road traffic. We also explored potential environmental impacts from three classic methods of unsealed road stabilization: granulometric stabilization by compaction and chemical stabilization by adding cement or lime, as adopted by Brazilian road construction standards. Finally, the tests evaluated the percolation effects from piping erosion on water quality by selecting potential and traceable markers in percolated water, and the direct shear strength to traffic resistance of the unsealed roads. The end of this chapter summarizes details of these experiments.

In summary, this research briefly reviews the history of unsealed roads, and explores the construction standards parameters for unsealed roads in Brazil, showing how these standards can potentially lead to undesired environmental outcomes, such as hydric erosion and sedimentation of adjacent water bodies. This research also explores MICCP as a "green novelty" to increase the mechanical strength of the soil mix as an alternative to reduce the erodibility of unsealed road soils.

2 - The importance of unsealed roads to Brazil

Brazil has 8,515,767 km^2 of land surface, and it is ranked as the fifth most populous country globally – estimated in 2017 at more than 207 million people. Of this total some 86.2% reside in urban areas (IBGE, 2018). With a diamond shape, Brazil stretches 4,350 km from north to south and includes large expanses of both tropical and subtropical ecosystems. The landscape diversity includes wetlands, savannas, plateaus, and low mountains, sustained by geographic and environmental conditions extremely favorable to maintain one of the most extraordinarily biodiverse areas of our planet. “Blessed by God and beautiful by nature” (famous Brazilian song: “Abençoado por Deus e bonito por natureza” - País Tropical by Ben Jor, 1969), the absence of high-deserts, high-mountains or arctic environments complete the unique scenario that favors the Country with conditions ripe for agribusiness, the Country’s main economic focus.

Like many developing countries, Brazil struggles with social inequalities, environmental degradation, intermittent economic and political crises. However, hope still exists for a brighter future. IBGE (2018) reported that more than 91% of the population above 15 years old is literate - 91.3% of the male and 91.6% of the female population. The country invests substantial financial, scientific, and human resources to reduce deforestation and increase agribusiness yields under strict environmental standards and laws to promote sustainability. These efforts aim to meet the Organization for Economic Co-operation and Development (OECD) and the UN Food and Agriculture Organization’s (FAO) 2015 projections, when by 2025 Brazil will become the world leader in food production, and by 2026 will overtake the USA as the largest soybean producer (FAO, 2017). The trade surplus in 2015 was $75 billion, representing 46% of total export volume and 21.5% of GDP (Gross Domestic Product) (MAPA, 2015; FAO & OECD, 2015). The 2015-2016 crop year harvested 58 million hectares of grains and seeds (MAPA, 2016; OECD & FAO, 2015; Conab, 2017), 330 million tons of sugar cane, 32 million tons of citrus fruits, and 1,8 million tons of coffee (MAPA, 2019) and had more than 10 million hectares of commercial forest under management (Ibá, 2016). Additionally, hydroelectric energy plants, industrial complexes, the most significant mining sector, and one of the biggest commercial forest sectors in the world contribute to the vitality and viability of the country’s economy and its future prospects as a world leader in many sectors.

As highlighted in the map (Figure 1), Brazil’s national transportation system for the production and movement of goods and services includes some 62,8% by roads, 21% by railroads, 12,6% by waterways, and 3,6% by air and pipeline (Machado et. al.., 2014 (a); CNT, 2020; Lobo, 2017, World Economic Forum, 2017). The percentage of the road transportation system is relatively high when compared to the average of 40% in developing countries and an average of 30% in developed countries (32% in the USA and 43% in Canada) (Machado et. al.., 2014 (a)).

In every country, road transportation comprises both sealed and unsealed roads. Sealed roads include an engineered base (surface or rolling surface) covered with asphalt, concrete, or another rigid pavement over the sub-base layer. Unsealed roads have the sub-base layer as the rolling surface shaped with natural roadbed material and not enhanced by any bituminous layers, concrete, or sealant coverage. The sub-base layer of any road is often enhanced (stabilized) by chemical additives such as cement, lime, or gravel plus mechanical compaction to improve traffic performance. In Brazil, both road types are considered all-season roads, supporting traffic throughout the year (DNIT, 2005). The National Department of Infrastructure and Transport (DNIT, acronym in Portuguese for Departamento Nacional de Infraestrutura e Transporte) defines unsealed roads (also unpaved or dirt roads) as a road on its "natural bed." The road lane is natural soil/terrain, a mixture without any pavement and only under granulometric stabilization. Granulometric stabilization, discussed in Section 4, is mandatory according to the DNIT as a minimal requirement for road construction to support an all season-traffic regime (MTPA, 2018).

The Brazilian national and extensive unsealed road network has 1,349,938 km of potential sedimentation and erosion point sources that can impact adjacent lands, human settlements, and local watersheds. Brazil recognizes the potential impact of unsealed road erosion by supporting scientific studies, including new techniques to minimize the erosion process and its related consequences (Machado, 2013; CNT, 2020; MTPA, 2018).

In summary, Brazil has an agribusiness-based economy that demands a complex and efficient infrastructure, including a consistent logistics structure and a reliable transportation system (modal) to connect production sites with national distribution centers and international markets. However, all those activities also play intensive roles as environmental impact sources.

3 - Economic, political, social and environmental impacts of roads

Roads are vital routes that open new economic horizons to develop and facilitate the movement of goods and services to and from isolated or hard-to-reach communities and national and international destinations. Roads, which connect production and commercial marketing activities, consolidate the development and strength of regional economies. Other significant economic benefits of a well-built and maintained road system are related to the tourism sector and reducing traffic congestion and accidents (Machado, 2012).

New roads are fundamental to expanding internal frontiers and opening new settlements which may develop into new communities and cities over time. This trajectory contributes directly to national development goals. However, the larger picture must also include a solid political, economic, social, and environmental foundation to minimize the negative impacts of unpredicted developments and related issues, such as lack of educational opportunities or health system infrastructure for new communities.

Washington Luis, Brazil's Thirteenth president (1926-1930), also known as "o estradeiro" or "the roadster," considered the development of a national system of roads a national security factor and one of his administration's priorities. As a result, Decree 5.141 (January 5th, 1927) established the Special Fund for the National Road Network Construction and Maintenance (Pinto, n.d.). As a result, the first major Brazilian road, the BR-040, built by Washington Luis in 1928, marked the Country's new era of road construction. That road received asphalt coverage by 1931, and the construction was of such high quality that some critics believed that the Americans or Europeans constructed it (O Globo, 2013). As a result, the travel time from Brasília to Rio de Janeiro was reduced from 33 days to 14 hours. The positive economic and social impacts from this success brought credibility to the National Road Plan (enhanced in the 1950s), which, in turn, justified having the road modal at the present scale: 62.8% of overall transportation modal (O Globo, 2013).

An ecological perspective of road construction implies reducing or mitigating the negative environmental impacts to a minimum, understanding that in addition to removing the original vegetative cover and regarding the original topography, erosion and subsequent sedimentation loads and composition will increase unless corrective measures form part of the planning process. Therefore, EIA (Environmental Impact Assessment) development must address actions to minimize the negative impacts of road construction, maitaining an ecological equilibrium while aligning with the social and economic development priorities of the impacted area. Furthermore, alternative scenarios should be considered in the planning phase, including options such as green corridors, changes in road trajectory, and examine all aspects of construction and long-term maintenance while meeting the need for road access (Machado, 2012).

Bartholomeu and Caixeta Filho (2008) demonstrated that roads not meeting construction and maintenance standards have a more significant negative economic impact and generate economic losses, such as increased road and vehicle maintenance costs and downstream environmental impacts and costs. Furthermore, DNER (National Department of Roadways) studies show that a degraded road increases fuel consumption by 58%, vehicle maintenance costs by approximately 38%, accidents by 50%, and up to 100% in travel time (CNT, 2017), contributing to greenhouse gas emissions, increased public health costs, and reduced productivity.

As noted in Section 1, investment up-front in developing the road modal is vital for Brazil, justifying the investment in research to develop and test new alternatives to reduce the costs of construction and maintenance and of environmental damage mitigation. In addition, improvements in vehicle energy efficiency may also lead to greater competitiveness of Brazilian products by reducing the fuel costs associated with getting products to market (Bartholomeu and Caixeta Filho, 2008). In summary, roads that comply with stricter standards generally imply a range of secondary economic and environmental benefits (Bartholomeu and Caixeta Filho, 2008; Machado, 2012).

4 - Unsealed roads in Brazil

Unsealed roads are necessary for developing any region, particularly in developing countries, since they provide a range of social, economic, and productive benefits while keeping initial investment costs down (Rammelt; Leung, 2017). Many unsealed roads in Brazil started as informal foot trails connecting population settlements or commercial centers. The indigenous trail Peabiru is 1,200 km and was consolidated in 1524 as one of the first Brazilian roads and constructed similarly to many existing unsealed roads (Machado et. al.., 2013). The first paved road, Rio-Petrópolis, was built in 1928, marking the beginning of road policy in Brazil (DNIT, 2006). The main goal of a road project is to ensure the best allocation of resources while minimizing earthmoving and utilizing the terrain contour lines and considering watershed limits (DNIT, 2005).

Unsealed road construction in Brazil follows the DNIT standards (2006), optimal for construction quality, safety, and traffic standards but still needs improvement in the best environmental practices and reducing social impacts. The DNIT's standards request granulometric soil stabilization aimed toward greater shear strength and bearing capacity than the original soil composition. Granulometric stabilization implies limiting the percentage of fine particles (source of sediments), resulting in a predetermined and homogenized grading mixture of particles at different sizes, followed by compaction under Proctor energy parameters (Vizcarra, 2010). Soil compaction under a specific energy implies a determined compaction effort that it is applied to the soil per unit of volume. This energy is determined by the weight, height of drop, and number of blows of a hammer inside a mold to compact the soil sample for the desired tests (Vizcarra, 2010). The standard Proctor energy test requires a 2.5 kg hammer and height of 305 mm, and determines the optimal moisture content (OMC), dry density or void ratio of the compacted sample (Day, 2001). In summary, granulometric stabilization alters soil properties by adding particles of varying sizes, reducing fine particles, and focusing on an adequate final composite material for its proposed use. However, even after granulometric stabilization and compaction, unsealed roads are more susceptible to erosion than sealed roads, especially to hydric erosion and sedimentation. For this reason, the DNIT standard suggests the use of additional stabilization methods for unsealed roads, like cement or hydrated lime, as chemical stabilizers.

DNIT developed the Brazilian soil classification system for road construction (DNER-ES-P 10/1971, based on AASHTO (American Association of State Highway and Transportation Officials) standards, which tolerate the presence of the finest soil material common in Brazil. DNIT standards describe the parameters to achieve road subgrade stability with optimal mechanical resistance but do not consider the potential environmental impact from the erosion processes. These standards imply that roads will receive an extra asphalt layer or similar sealant process. However, as previously cited, about 80% of Brazil's road network is classified as unsealed, i.e., do not receive any cap layer after granular stabilization and mechanical stabilization (i.e., compaction), leading to potentially extensive environmental impact sources via erosion and sedimentation.

Unsealed roads combine soil components, like coarse and fine rocks, sand, silt, and clay, at appropriate particle size distribution (PSD) per desired soil mixture, with subsequent compaction. The objective is to obtain a final product with greater stability than the original soils.

The DNIT standard (2006) describes the construction methods for sub-pavement (or subgrade) layer construction using granular stabilized soil. The unsealed road classification defines the soil mixture types from A to F, where soil mixtures A and B are subject to heavy traffic, soil mixtures C and D for medium traffic, and soil mixtures E and F to low traffic volumes. The soil mixture types A through F are granular stabilized soils that constitute the subsurface of any road but are the only structure on unsealed roads. Therefore, the DNIT regulations are optimal for sealed (or paved) and unsealed road construction standards since they guarantee good quality of construction and use but still need improvement in environmental standards, including erosion and sedimentation impacts on water quality.

This dissertation analyzed Brazilian unsealed road surfaces as soil mixture types A, C, and F, according to the DNIT 141/2010-ES standard, meaning granular stabilization defined by the designed traffic flow for the planned road. This granular stabilization must conform to the DNIT standards: ABNT, 1984 (a, b, c, d); ABNT, 1986 (a, b), ABNT, 1988; ABNT, 1990; ABNT, 1995; ABNT, 1996; ABNT, 1998; ABNT, 2000; and ABNT, 2004 (a, b, c). Per these standards, the material used for unsealed road construction needs to comply with the granular correction distribution followed by compaction under Proctor's energy parameters (mechanical stabilization), and when needed or desired, can receive additional chemical treatment (commercial and non-commercial products, including industrial residues and products for soil correction) to enhance the road stabilization (Machado, 2012). The granular and chemical stabilizations can enhance the mechanical support capacity of the subgrade, thus enhancing the road quality and life span.

5 - Hydric erosion as a significant environmental impact of unsealed roads

Water supply, erosion control, and sediment retention are natural ecosystem services responsible for water storage and soil retention within an ecosystem (Constanza et. al.., 1997). Dutton et. al.. (2005), Machado et. al.. (2014, b), and Reid et. al.. (2016) demonstrated that unsealed roads represent a significant source of fine sediment erosion that contributes to diminishing water quality through rapid runoff pathways and a source of fine erodible material. Sediments and chemical residuals concentrations are the primary sources of negative impact on water quality (Dunne & Leopold. 1978; Hammer & MacKichan, 1981). Surfaces like unsealed roads have a high concentration of loose sediments, especially after long dry periods, washed to the nearest stream within the first rain/storm (Hammer & MacKichan, 1981; Machado, 2012). The discharge carries the loosened sediments and pollutants over the surface or drains them through percolation (Hammer & MacKichan, 1981). Sedimentation from soil erosion directly affects water turbidity and ecosystem quality, which, in turn, affects the fishing activity of local communities (Hammer & MacKichan, 1981). In addition, the fine sediments stay suspended in the water column, influencing plant life by shading it, affecting food production and the consumer's chain above it (Hammer & MacKichan, 1981). Like silt and sand deposition, coarse sediments tend to settle out and, on the riverbed, and damage spawning sites by changing the availability of dissolved oxygen in the water (Hammer & MacKichan, 1981). The knowledge about the effects of unsealed roads on water quality are relatively recent and appears to be intuitive since most of the published material refers to how forest management practices can affect the environment, though very few publications focus on how fine sediments derived from unsealed roads can affect water quality (Dutton et. al.., 2005; Zemke, 2016).

Figure 2 – Conditions of unsealed road in Brazil. (A) demonstrate a state-of-the-art unsealed road properly built and maintained. This specific road was built with cement as the chemical stabilizer (Source: personal archive from Dr. Carlos Machado). (B) unsealed road with poor maintenance conditions, highlighting the erosion at advanced stages. The loose material will be caried to the closest water body by rain oof or as dust emissions by traffic (Source: https://capacitacao.ana.gov.br/conhecerh/bitstream/ana/62/8/Unidade_4.pdf).

Road construction negatively impacts the local ecosystem and adjacent and downstream river basins since it is the primary source of soil degradation caused by hydric erosion affecting the water resources directly (Thomaz et. al.., 2013; Thomaz and Peretto, 2016). The location and construction of a new road is a complex environmental and engineering decision process, and according to Brazilian law (CONAMA 237, 19/12/1997), it must consider the alternative with less environmental impact. Building unsealed roads based on quality background investigation is generally straightforward but still characterizes roads susceptible to climate influences. As such, they require careful planning with constant maintenance (Machado, 2012). In this context, classic chemical stabilization methods like cement and hydrated lime for unsealed roads based on economic, social, and technical criteria can reduce the overall construction and maintenance costs, which justifies their use. However, the environmental impact analysis of how chemical stabilization can impact water quality still needs more study (Machado, 2012).

The traffic intensity on unsealed roads and its use characteristics (commercial, private, public, trail, or other) directly affect sedimentation rates into adjacent water bodies. Roads with heavy traffic, like commercial roads with heavy trucks, produce an estimated 7.5 times more sediments than roads with low traffic, like trails or private roads with light vehicle uses (Reid & Dunne, 1984). However, stabilized soil that can support heavier road stress is not naturally common. The most common options to stabilize unsealed roads are granular stabilization (mechanical stabilization through particle size distribution (PSD) and compaction) and chemical additives (including industrial residues and products for soil correction, like cement and lime) (Machado, 2012). The global concern about environmental health has led to the search for greener solutions to stabilize soil and reduce erosion (Van Passen, 2011; Morajev et. al.., 2018). Among these initiatives, Microbiological Induced Calcium Carbonate Precipitation (MICCP) is a novelty; this is where the targeted stimulus to precipitate calcium carbonate as a product of microorganisms' lifecycle, bonds the soil particles, increasing the soil strength that reduces its erodibility rates. This process is called biostabilization or biocementation since the bond between calcium carbonate and soil particles results in a cement-like structure (Whiffin, 2004; van Paassen, 2011, Moravej, 2018). Biocement is a thriving, innovative soil stabilizer technique on unsealed roads but still requires more study to understand better the stabilization process and its impact on water quality (Portugal et. al.., 2020; Ivanov & Chu, 2008; Paul & Meyer, 2001).

6 - Justification for the study

Roose & FAO (1996) connect the consequences of environmental degradation with population intensity and development processes as part of an interdisciplinary effort. Society is in constant flux, and the resulting environmental impact varies according to economic, social, and educational levels, political commitment, and the profile of *in situ* natural resources relative to its production and marketing profile. Developed countries have more advanced assets at their disposal to better define strategies for new developments such as settlements and new transportation corridors and their maintenance over time. These strategies generally involve long-term planning and budget projections based on experience and forecasts for the future. However, developing countries face a very different planning context with distinct parameters including scarce financial resources, a dearth of trained professionals with extensive experience, corruption affecting the quality of the outcome, and high and unregulated population growth rates and settlement patterns, all of which are barriers to better define comprehensive development strategies including, ecosystem health. Brazil offers a somewhat different profile from most developing or transition countries: rich in biodiversity and other natural resources such as water, profitable economic activities at the global level, potential high per capita income, a network of world-class universities and research institutions, an advanced and extensive agribusiness sector, a well-developed heavy industrial base (steel, cement, aluminum, automobiles, among others), energy independence, and a global powerhouse in exporting food and bio-products. Few would argue against the concept that these attributes put Brazil in a position to join the ranks of a fully developed nation by the end of this century. However, completing the national road infrastructure plan may be one of the keys to realizing this ambition.

Studies of the unsealed road network and its environmental impacts are critical to countries like Brazil since the road construction methods commonly used must allow low to heavy traffic volumes the entire year, even during the rainy season (Machado, 2012). In 2014, Brazil entered a hydric stress crisis that affected the entire country and every production sector. The Northeast and Southeast regions declared a state of emergency in terms of water supply. Many reservoirs and natural watercourses reached such low levels that the government allowed the use of stagnated water by public water distribution systems. The United Nations (UN) issued an alert on the possibility of hydric stress potentially affecting 18 million people until 2020. Moreover, the problem is not only affecting Brazil but rather the entire world. The United Nations World Water Development report for 2018 warned that almost 6 billion people are likely to live in areas that suffer water shortages for at least one month a year by 2050, up from 3.6 billion in 2016 (UN Water, 2018).

Personal communications with the Brazilian mining and the forest sector[1] in 2016 related to hydric stress validated a significant water crisis but a paradox on water use. To minimize the dust and its social impacts, especially health issues and meet environmental regulations (including EIA/EIR (Environmental Impact Assessment/Environment Impact Report)), most companies in these sectors must keep the unsealed roads moist during the dry season.

On average, a mining company in Brazil uses 4,000 liters of water/day/km^2 to reduce dust emissions to an acceptable level. The paradox of facing a severe drought while complying with the law becomes clear in cases where the forest or mining companies are neighbors of communities living under water rationing. An impactful Brazilian forest company, representing the central reality of the sector, uses water trucks wetting roads 2-4 times a day, with an average of 1 liter/m^2, to reduce dust from traffic. In summary, a company maintaining 10,000 meters of unsealed roads uses 80,000 liters of water, wetting the road two times a day, representing 160 m^3 of water per day. A small residence uses 1m^3 of water per day, which means that a company wetting roads twice a day during the dry season consumes the same amount of water as 160 small residences. Most of this water will likely return directly to the local watershed, carrying sediments and any chemicals present on the road surface. Reid et. al.. (2016) disagrees and state that research is not conclusive about the connection between sediment production on road surfaces and its transport to watercourses. The authors agree that more studies are necessary to fully understand the complete environmental impacts from unsealed road erosion.

Political crisis, the absence of enforceable policies, and competing political interests, among other factors are under constant investigation by the Brazilian federal bureau of investigation, the Policia Federal (PF) as published by Brazilian press in many decades, have led to diverting funds and investments destined for road construction and maintenance, decreasing the overall quality of the constructed roads. However, Brazil is a young country still learning how to overcome the consequences of political and economic crisis and challenges. Younger generations are fighting against corruption while working for a brighter future. Despite this trend, road development will likely continue to be focused on unsealed roads with only incremental progress in minimizing environmental impacts. This dissertation is a component of a larger project searching for green alternatives that could minimize the erosion process of unsealed roads while improving several related road quality parameters such as drive lane life span, driver safety and

[1] The companies requested to stay non-identified due to internal regulations.

comfort, shear strength of traffic lanes, ditches, and slope stabilization, infiltration and percolation rates and parameters.

7 - Biocement of *Serratia ureilytica* as a new green solution for unsealed road erosion

DNIT requires compliance with its standards for the construction of any road, but its standards do not address the problem of erosion. This gap is where our research aims to fill part of the environmental impacts gap of a new unsealed road stabilization method to reduce the erosive processes and its potential to reduce the negative impact on the water quality of local water bodies.

Our research focused on the MICCP process, where the bioprecipitated $CaCO_3$ (calcium carbonate) biocemented soil particles and reduced its hydraulic conductivity (Portugal et. al.., 2020; Umar; Kassim; Ping Chiet, 2016; Van Paassen, 2011). Calcium carbonate is an attractive element for the biostabilization process because the precipitation of $CaCO_3$ is a natural process. Bessler and Rodrigues (2008) summarized that calcium carbonate minerals are found in nature under one of its three crystalline forms: vaterite, aragonite, or calcite. Vaterite is the most unstable form and crystallizes in the hexagonal system. Because of its unstable condition, it is a rare calcium carbonate to form naturally. They are classified as aragonite when the organization of the crystal is in orthorhombic form. The aragonite shape is a more common crystal form via the enzymatic MICCP. When they are under the trigonal or rhombohedral crystalline structure, they are called calcite, the most stable form of calcium carbonate, thus being the target of our research.

This research is part of an umbrella project in development by Forest Road and Transportation Research Center (CETEFLOR), located at the Federal University of Viçosa (UFV)-Brazil. The umbrella project title is **Estabilização de solo com biocimento para pavimento de Estrada Florestal** (Soil stabilization with biocement for forest road pavement). This dissertation describes the biostabilization of unsealed roads and its contribution to maintaining water quality by reducing the hydric erosion processes. Enriquez (2017), also part of our umbrella project, describes how *Serratia ureilytica*, urease-positive bacteria, is a promising biocementation agent on sandy soils and has excellent potential for biostabilization and erosion control. Their results state that the *S. ureilytica* has a biocementation rate higher than *Sporosarcina* (former *Bacillus*) *pasteurii*, the common gram-positive anaerobic soil bacterium used as commercial biocement. *S.*

ureilytica as an alternative of MICCP is essential to the Brazilian scenario since it is a local soil bacterium representing lower cost as road stabilizers versus imported versions, like Biogrout® or *Sporosarcina pasteurii*, among others. Another crucial environmental advantage is that as native soil bacterium, the environmental risk of contamination is potentially low, especially considering that the bacterium become locked inside the calcium carbonate precipitated and dies at the end of the process.

Our results on *S. ureilytica* biocementation demonstrated its viability as a potential unsealed road stabilization method due to being an abundant soil native urease-positive bacterium, meaning a great MICCP agent. However, further research is necessary to evaluate the optimal biocementation rate to promote a solid and homogeneous biocementation for unsealed road systems and a detailed description evaluation of its environmental impacts from the construction, maintenance, and runoff from unsealed roads.

The results of our research can also provide inputs to extend the research about the potential positive impacts on the health condition of the communities that may adopt the *S. ureilytica* biocementation as a new method for unsealed road construction and management since it also significantly reduces dust production from traffic. Dust reduction may also benefit local fauna and flora especially in areas adjacent to the road surface – the so-called ecological border effect. Preliminary tests (Enriquez, 2017) indicate that *S. ureilytica* may represent zero or near-zero environmental impact. However, the MICCP process has ammonium (NH_4) as by-product, that according to Lee et.al. (2019), has its concentration ruled by site-specific enzyme requirements and require further investigations. Further developing knowledge about *S. ureilytica* behavior and its optimal concentration and application method justifies this research on a laboratory scale. Undertaking a field-scale pilot is the next step, considering the outcomes and support to expand this research.

8 - Research hypothesis

The present research contributes to a better understanding of the characterization and behavior of unsealed road soils; to the identification of the MICCP *Serratia ureilytica* calcium carbonate production rate to be used for unsealed road stabilization; and to analyzing adjacent water quality conditions of an unsealed road stabilized with MICCP *Serratia ureilytica*. Our research theorized that it is possible to enhance unsealed road stabilization with the application of *S. ureilytica* biocement, thereby reducing erosion impacts from sedimentation.

The objectives were to:

1. Determine the optimal concentration of *S. ureilytica* application and biocementation solution to ensure the optimal biocementation of the unsealed road sub-base.
2. Compare the *S. ureilytica* biocement results with cement and lime stabilizations to evaluate the biocementation behavior.

To test our hypotheses, the laboratory tests used were:

1. Constant Head test for permeability of granular soils and percolation rate.
2. FAAS (Flame Atomic Absorption Spectrometer) for water quality analysis from percolation tests.
3. SEM (Scanning Electron Microscopy) to qualify the calcium carbonate precipitation.

Comparing the test results with the control (mechanical stabilization method), our hypotheses were:

1. *S. ureilytica* biocement is a strong candidate for unsealed road stabilization with a lower to null environmental impact when compared with cement and hydrated lime.
2. Cement and hydrated lime have a higher environmental impact on water quality over mechanical stabilization.

9 – Dissertation structure

This present research investigated the novel use of biocementation via MICCP (Microbiologically Induced Calcium Carbonate Precipitation) to promote unsealed road stabilization. A novel bacterium was selected: *S. ureilytica*, which has a high biocementation ratio in the sand, as Enriquez (2017) described. The biocement as an unsealed road stabilization method was compared with two classic chemical stabilization methods – cement and hydrated lime. Despite the well-known evidence about cement and lime behavior as effective chemical additives for soil stabilization, more information on runoff and percolation and how they affect water quality is needed. Our research selected calcium, magnesium, and silicon as potential markers to trace the effects of cement and hydrated lime stabilization on water quality since they are common factors from cement and hydrated lime.

Chapter 2 describes the critical literature review about biocementation and the state-of-art of MICCP as an alternative for soil stabilization. The manuscript was published in 2020 by the Journal of Cleaner Production (Elsevier system), cited ten times and 70 captures according to the PlumX metrics (Elsevier system), and has 9.2 research interests, 174 reads, and 11 citations according to the Research Gate system. This manuscript's results suggested that most of the current investigations worldwide are at a similar point of limitation: guaranteeing uniform distribution of biocementation into organic soil and at large extensions. The discussion provided insights and information that can enrich and support future research and improve the understanding of the biocementation process in organic soils and for unsealed road stabilization.

Chapters 3 and 4 focus on our methods, results, and discussions. Chapter 3 discussed the internal erosion (percolation) from chemically stabilized roads. It proposed to use calcium, magnesium, and silicon as potential markers for cement and hydrated lime stabilization, promoting the evaluation of the possible environmental impacts from chemical stabilization. The manuscript was submitted and accepted by the Journal of Transportation Research Part D: Transport and Environment (Elsevier system), and it is under the Journal's requested review by the authors for final submission.

Chapter 4 described the *Serratia ureilytica* biocementation and its behavior over adjusted unsealed road layers. The manuscript proposed the biocementation protocol to reach the desired biocementation and tested some variations to reach the potential optimal biocementation rate. Publication will be submitted for journal review during the first quarter of 2022. Journals in consideration: Frontiers in Microbiology, Frontiers in Materials, Journal of Cleaner Production.

Chapter 5 concludes this research project and proposes further developments and future actions to define a standard protocol to test biocementation as a green alternative for unsealed road construction.

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Chapter 2: Microbiologically Induced Calcite Precipitation Biocementation, green alternative for roads – is this the breakthrough? A critical review

Carla Ribeiro Machado e Portugal[1], Carolyn Fonyo[2], Carlos Cardoso Machado[3], Richard Meganck[4], Todd Jarvis[5]

1 - Author for correspondence. Ph.D. candidate at Environmental Sciences Graduate Program. Oregon State University. Graduate School - Heckart Lodge, 2900 SW Jefferson Way. Corvallis, OR – USA. 97331-1102. Telephone: +1 (541) 9797-8741. Carla.portugal@oregonstate.edu. ORCID: https://orcid.org/0000-0001-9508-8831

2 – Director of Environmental Sciences Graduate Program. Oregon State University. Graduate School - Heckart Lodge, 2900 SW Jefferson Way. Corvallis, OR – USA. 97331-1102. Carolyn.fonyo@oregonstate.edu. ORCID: https://orcid.org/0000-0002-8919-6585

3 – Professor, Federal University of Viçosa, College of Forest Engineering. Avenida Peter Henry Rolfs, S/N, Viçosa, MG – Brasil. Zipcode 36570-900. Machado@ufv.br. ORCID: https://orcid.org/0000-0002-2386-9791

4 - Institute for Water and Watersheds, part of the UNESCO-ICIWaRM category II center, Oregon State University, USA. rameganck@gmail.com. ORCID: https://orcid.org/0000-0002-1523-9992

5 – Director, Institute for Water and Watersheds. Strand Agriculture Hall 230, 170 SW Waldo Place. Corvallis, OR – USA. 97331. Todd.jarvis@oregonstate.edu. ORCID: https://orcid.org/0000-0002-7020-8048

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Abstract

The past fifteen years have been a rich developing field for Microbiologically Induced Calcite Precipitation (MICP) as an alternative to decrease the hydraulic conductivity of sandy soils or fractured rocks through biocementation formation. While significant development happened, the field still lacks a viable answer for the use of MICP for organic soil stabilization. Sandy soils or fractured rocks have a completely different structure and mechanical behavior than organic soils, which can impact the behavior of biological components and MICP microorganisms. This factor per se changes all the research perspective and demand adaptations different than the ones used for sandy soil or fractured rocks. In the search for answers, this article compiles an extensive and systematic literature review with papers and books from platforms like Academic Search Premier, Web of Science, Google Scholar, 1Search and Gale Virtual -Reference Library, having the selection based on defined parameters and criteria. Focusing on how MICP is a potential solution for unsealed road stabilization, this article discussed the main gaps and constraints that could explain why biocementation still needs extensive research under different perspectives and scenarios. The results suggested that the majority of investigations are at a similar stage of limitation: how to guarantee an evenly spread of biocementation into organic soil at large extensions. The discussion here provided some insights and pieces that can enrich future researches, support the expansion of the development, and improve the understanding of the biocementation process into organic soils and its use for unsealed road stabilization.

Keywords

Microbiologically Induced Calcite Precipitation (MICP); ureolytic bacteria; unsealed roads; biocement; organic soil; soil stabilization.

1 – Introduction - From biomineralization to biocementation to improve the soil for roads

Biomineralization is the precipitation of crystals (organized shape mineral) in the cellular or extracellular matrix of a living organism (Lowenstam & Weiner, 1989). It is a widely known process wherein microbes move different metals to form minerals (Dhami et. al.., 2013; Dhami et. al.., 2016; Phillips et. al.., 2013; Zhu & Dittrich, 2016). This multistep process is natural for many microorganisms and animals that precipitated various minerals, like iron oxides in magneto bacteria, magnesium silicates in crustaceans, and calcium carbonates and calcium phosphates in invertebrate shells and vertebrate skeletons (Boskey, 1998). The resulting biominerals deposits in elaborated shapes and hierarchical structures based on the organic-inorganic interface. The rate of crystal formation controls the microenvironment and the mineralization. One of the most studied biomineralization processes results in calcium carbonate ($CaCO_3$), as Calcite, one of the most stable forms (Fernandez et. al.., 2018).

New awareness in the role of microbial processes in biomineralized geological formations has created the interest of researchers worldwide (Banks et. al.., 2010; Ronholm et. al.., 2014; Rusznyak et. al.., 2012). There has been a growing interest in the exploration of novel microbes and routes which have the similar ability to precipitate desired compounds as calcium carbonates to fix or mobilize metals, sequester atmospheric CO_2 for applications in different engineering areas (Dhami et. al.., 2013, Dhami et. al.., 2014).

Meldrum (2003) classifies biomineralization as “biologically induced” mineralization or "organic matrix mediated mineralization". "Biologically induced" mineralization is the interaction between an organism and its environment under low biological control of the mineralization process, resulting in precipitation of a biomineral. "Organic matrix mediated mineralization" has the live organism directly controlling the biomineralization process. This article focuses on the "biologically induced" mineralization branch, limiting on Microbiological Induced Calcite ($CaCO_3$) Precipitation (MICP) process.

Several biomineralization research projects and methodologies are under development and evaluation, including biostabilization or biocementation, which is a robust green alternative for soil stabilization processes, including unsealed road surfaces and dams. Biostabilization consists of improving the geotechnical properties of soils by Microbially-Induced Calcite Precipitation (MICP), the precipitation of calcium carbonate from ureolytic bacteria activity (Whiffin, 2004; van Paassen, 2009; DeJong et. al.., 2013). The calcium carbonate precipitation (or biostabilization process) is the result of a microbial metabolic process such as photosynthesis, and hydrolysis of urea and reduction of sulfates, among others (Valencia, 2009; DeJong et. al.., 2011; Valencia et. al.., 2014). This article focuses on review the MICP process, where the precipitate of $CaCO_3$ can biocement soil particles and reduce its hydraulic conductivity (Whiffin, 2004; Umar et. al.., 2016; van Paassen et. al.., 2011). Calcium Carbonate is an attractive element for the biostabilization process since the calcite formation is a natural process. Several authors such as Stepkowska et. al.. (2003), Bessler & Rodrigues (2008), Yang et. al.. (2009), Zhang et. al.. (2010), Wei et. al.. (2015), Özen and Simsek (2015); Piekarska et. al.. (2017); Donnelly et. al.. (2017); Jaji et. al.. (2017) summarize that Calcium Carbonate Minerals (CCM) are present in nature under one of its three crystalline forms - vaterite, aragonite or calcite. When the CCM's are under the trigonal or rhombohedral crystalline structure, they are called Calcite. Having Calcite as the most stable form of calcium carbonates, thus being the target of majority MICP's research.

The most studied MICP microorganisms are urease-positive bacteria. Since MICP bacteria are common in the soil environment, many studies indicate the probability of a successful in situ biostabilization treatment of sand or sandy soil with ureolytic bacteria (Whiffin, 2004; Umar et.

al.., 2016; Whiffin et. al.., 2007; van Paassen et. al.., 2011; Mujah et. al.., 2017; Bibi et. al.., 2018). The majority of the research, including the cited researchers above, focused on Bacillus spp and Sporosarcina spp, which can be exotic bacteria depending on the location. The bacterium death reduces the risk of gene escape due to physical isolation and lack of nutrients. However, Hokkanen & Lynch (1995) reported that predicting the behavior of exotic bacteria introduced into the soil must be analyzed on a case-by-case basis, as it involves specific kinetics principles for each species. The same authors stated that the general trend is the decline in the population density of bacteria introduced in a short time (15 to 25 days for common ones). They conclude by stating that competition against bacteria already in the soil and predation by other microorganisms represent the most significant potential impacts on the new bacterial population.

In summary, the authors stated that the establishment of a bacterial population depends on the soil-bacterial interaction, with significant weight in the bacterial sepa, determining a highly specific interaction, classified as soil-dependent interaction. Even though the use of native bacteria can potentially reduce the cost of using imported and patented products, the authors could not find any analyses about the risk-evaluation of the potential biohazard contamination from exogenous bacteria proposed for soil stabilization. This lack of information indicates the first gap of the MICP field, evaluated in this paper.

This manuscript presents a detailed review of significant publications related to MICP as a soil stabilization technique. It discusses the main critical gaps and constraints for MICP as a green alternative for soil stabilization: how to ensure homogeneous and widespread biocementation without compromising the shear strength or durability of the soil surface and its extension for road stabilization. Many publications demonstrate the MICP process on the sand and sandy soils, but this review focused on organic soils, which is a topic in early development at MICP technology.

These factors, allied with the urgent need of a green solution for unsealed road erosion, guided the research questions of this article: a) Which is the current state of the art of MICP on organic soils?; b) What are the possible gaps and restrictions in the behavior of ureolytic bacteria, agents of MICP, for organic soils?; and c) What are the gaps and constraints for the use of indigenous bacteria in the MICP process on unsealed roads? This review brings more information about MICP and its intrinsic and unique interaction with organic soils, promoting a focused debate about the gaps and constraints of the homogeneous application and resistant biocementation. The MICP process can not compromise the shear strength or durability of the unsealed road (granulometric stabilized soil, a composite of gravel, sand, silt, and clay).

2 – Research Methodology

This review article compiled an extensive range of papers that collect details and information about the MICP process, soil stabilization, unsealed roads, and above all, why the ureolytic bacteria can be a potential solution for biocementation of unsealed roads. This systematic literature review, conducted under a holistic approach, sought to condense information about the possible gaps and constraints in the development of the protocol for the use of biocementation via ureolytic bacteria. The protocol will target construction and management of unsealed roads in tropical areas, indirect benefit the conservation of water resources, which at least would no longer receive sediment from the erosion process of unsealed roads.

2.1 – Literature research

Many authors published reviews about MICP, having the most cited ones from Whiffin (2004), Whiffin et. al.. (2007), van Paassen et. al.. (2009 and 2011), Anbu et. al.. (2016), Seifan & Berenjian (2019). However, the literature lacks its applicability at large scale, or organic soils left gaps that need answers. Some few articles describe the MICP procedure for organic soils but do not have compiled information, nor clear described protocols. Whiffin (2004), Whiffin et. al.. (2007), and Soon et. al.. (2013) helped shape this review. The first authors were one of the pioneers on research MICP as biocement for soil improvement, and the second authors brought some of the first insights about how to improve the technique with organic soil.

This present review adopted a two-stage search for literature research. The first stage was an extensive search of five databases approved by Oregon State University: Academic Search Premier, Web of Science, Google Scholar, 1Search, and Gale Virtual Reference Library. The relevant literature was selected based on specific keywords including *MICP*, *Microbiologically Induced Calcite Precipitation, Microbially-induced calcite precipitation, calcite, Calcium carbonate, biostabilization, biocementation, biocalcification, biogrout, urea, urease, ureolytic bacteria, native bacteria, nucleation site, shear strength, permeability, porosity, soil stabilization for roads, unsealed roads, and MICP, unsealed roads and biocementation, biological stabilization for unsealed roads*. The selection and combination of this set of keywords were defined to allow the identification of meaningful literature and avoid biased research. The combination of keywords was determined to cover the topics discussed here. The second stage of the study was the cross-reference search in the articles with the highest citation index selected in phase 1, to complement the search with more recent papers. Also, we retrieved the non-detected relevant papers from the bibliographic references from the first stage of the research. The initial selection process identified 2232 articles as potentially appropriate to the topics selected for discussion in this article.

2.2 – Screening and papers selection

The papers search process based on several citations and renowned researchers in the areas of MICP, microbiology related to the behavior of ureolytic bacteria, and unsealed roads. Table 1 shows the criteria for the selection of articles used in this review. Please note that had no limitation on publication date as we seek to consolidate understanding of the entire MICP process to identify the insights that can support new research and ideas in the search for solutions to MICP restrictions and gaps in road stabilization unsealed.

Table 1 *- Inclusion and Exclusion Criteria per parameter for proper paper selection for this study.*

Parameter	Criteria	
	Inclusion	Exclusion
Publication type	Journal paper, thesis, dissertations, book chapters, and conference paper	Editorial and comments
Language	English or Portuguese	Other languages
Accessibility to the full text	Available	Not available
Topics	1. MICP process for diverse materials, except those with the biological and medical purpose 2. MICP as a source for biocement 3. Biocement for soil stabilization 4. Soil stabilization for Unsealed road construction	1. MICP process for biological/medical purposes 2. MICP for other purposes 3. Biocement for construction and art repair in details 4. Soil stabilization for sealed road construction

The first selection evaluated only the titles and abstracts, generating a total of 1124 selected articles. Although this is a large volume, the next stage evaluated the discussion and conclusion of the papers, reducing it to a final selection of 158 papers.

3 – Results & Discussions

3. 1 - Soil bacteria as the biocementation agent

Soil microbe communities form one of the most abundant microbial ecosystems on Earth, led by bacteria species (Mitchell & Santamarina, 2005; Wang et. al.., 2020; Coleman-Derr et. al.., 2016; Wu et. al.., 2005; Umar et. al.., 2016). Some of these bacteria species biomass is present in large numbers than others since the biotic and abiotic factors that affect the fitness of these microorganisms varies across the depth of the lithosphere (Hokkanen & Lynch, 1995; Umar et. al.., 2016).

Madigan et. al.. (2018) reported that bacteria could survive in environments with the most varied rates of acidity, salinity, temperature, and atmospheric pressure. Most bacterial species survive in places with pH values between 5 and 7, which is typical of groundwater and soils close to the surface; and the pH decreases with the increase of the concentration and valence of ions found in the fluids present in the soil (Madigan et. al.., 2018; Chapelle, 2001).

Ureolytic bacteria produce the urease enzyme, which indicates its potential use in the biomedical soil improvement technique - MICP (Kucharski et. al.., 2006). The genera of bacteria most commonly used in MICP techniques are Bacillus, Sporosarcina, Spoloactobacillus, Clostridium, and Desulfotomaculum (Kucharski et. al.., 2012) and, more recently, the genus Serratia, a bacterium native to Brazilian soil with research under development (Enriquez, 2017).

Morales et. al.. (2015) tested a biocementation scenario with a low amount of biocementing solution, simulating the levels naturally produced by a bacterium (undeclared) from the *Bacillaceae* family in its original condition of development in silty-clayey-sandy soil. The objective was to limit the production of ammonia and its oxidized forms to a low level to minimize the potential environmental impact and the possible dissolution of precipitated calcium carbonate due to acidification of the medium by oxidizing ammonia. The results showed a low level of precipitation, less resistance to compression, and the most moderate shear strength when compared with other tests for soil stabilization. The authors concluded that induced biocementação the soil with a low incidence of only precipitation can only be used for purposes of soil filling that do not require high shear strength.

3.2 – Biocementation & MICP

Microbial induced carbonate precipitation (MICP) is a natural process, controlled by different mechanisms (Banks et. al.. 2010; Cacchio et. al.. 2003; Wright and Oren 2005). One of these mechanisms is the production of calcite by ureolytic bacteria in porous soil, when in the presence of urea and calcium ions (Stocks-Fisher et. al.., 1999; Frankel and Bazylinski, 2003; Whiffin, 2004; Mitchell and Santamarina, 2005; Ivanov and Chu, 2008; DeJong et. al.. 2010; De Muynck et. al.., 2010; Ivanov, 2010). The complete understanding of this mechanism is important to understand the other biologically induced natural $CaCO_3$ precipitation mechanisms (Banks et. al.. 2010; Cacchio et. al.. 2003; Fukue et. al.. 2003; Wright and Oren, 2005) and how to transfer this knowledge to commercial-scale production of the MICP in its various uses.

The MICP process depends on six main factors: (1) concentration of calcium, (2) level of dissolved inorganic carbon (DIC), (3) pH of the medium, (4) availability of nucleation sites, (5) urease activity; and (6) carbonic anhydrase activity (Hammes & Verstraete, 2002; Hammes et. al.., 2003; Achal et. al.. 2015). MICP involves a series of complex biochemical reactions that can be affected mainly by ambient temperature, pH, water content, urea concentration, species and concentration of bacteria, pore sizes, and soil void rates (McConnaughey and Whelan 1997; Stocks-Fischer et. al.., 1999; Whiffin, 2004; Whiffin et. al.., 2007; van Paassen et. al.. 2010; Achal et. al.. 2009a; Harkes et. al.. 2010; Achal and Pan 2011; Sharma and Ramkrishnan, 2016; Castro et. al.., 2016).

The MICP process is highly dependent on active ureolytic bacteria, sources of calcium chloride and carbon, source of urea, level of urease that increases the pH, promoting rapid precipitation of calcium carbonate (Ferris et. al.., 2003). The main lines of research for the MICP focus on improving the strength and stiffness of porous media while maintaining permeability (Whiffin et. al.., 2007; van Paassen, 2009; DeJong et. al.., 2010); reduction of permeability in porous media (Tobler et. al.., 2012; Handley-Sidhu et. al.., 2013; Mitchell et. al.., 2013); pollutant compound immobilizers (Mitchell and Ferris, 2005; Fujita et. al.., 2008), concrete self-healing (Jonkers et. al.., 2010) and potential CO2 sequestration (Mitcivanovhell et. al.., 2010; Cunningham et. al.., 2014; Phillips et. al.., 2016), and more recently, the innovative proposal for stabilizing unsealed roads, with biocementation as a potential substitute for chemical stabilization with cement or lime (Enriquez, 2017) and improvement of soil engineering properties (Minto et. al.., 2017; Osinubi et. al.., 2020). It has been showed that biocementation could successfully enhance the strength and stiffness of pure sand in a relatively large area of extension (van Paassen et. al.. 2010, Ivanov and Chu 2008; DeJong et. al.. 2010, 2013; Dhami et. al.. 2013; Gao et. al.. 2019, He et. al.., 2020). However, it was not possible to replicate this result on silty sandy, silt, or clay soil (He et. al.., 2020).

3.3 - Gaps, constraints, and the uses for MICP - a brief microbiology perspective

The MICP or biocementation technology is on the verge of a breakthrough, which is a short period could lead to the expansion of biocement application in a variety of environmental scenarios. Many researchers worldwide like Santamarina, Chou, DeJong, Montoya, and van

Paassen in the USA; Whiffin, Cheng, Premkumar, Al-Thawadi and Ismail in Australia; Al Qabany and Soga in the UK; Valencia e Enriquez in Brazil, (just to cite some) are devoted to solve some significant gaps and constraints of the MICP for soil stabilization. Among the gaps, we highlight: (i) How to obtain and sustain a homogeneous biocementation layer through the treated area; (ii) How to get a homogeneous biocement production despite the media (sand, soil, or any soil mix), (iii) How to maintain homogeneous distribution of the nucleation sites to allow precipitation volume that can lead to a strong biocementation? Until questions remain open, it is not possible any advancement in economic analysis and environmental assessments due to few studies at field scale that can support the conclusions.

All the MICP papers consulted have similar constraints that support our central question: Does the current level of the MICP homogeneity for soil stabilization, independent of the bacteria selected, justify or provide robust parameters and protocols to sustain the MICP at a commercial scale? Tests on MICP for soil stabilization at laboratory scale is still highly used, which can be supported by all these factors cited above and rationale of authors like Al-Thawadi (2008), De Muynck et. al.. (2010), DeJong et. al.. (2013), Dhami et. al.. (2016), Umar et. al.. (2016) among others. This scale can reduce the costs, potential environmental impact, and simplify research efforts promoting a substantial advancement to the field.

Even though the biocementation homogeneity is the most significant gap, we have to consider two main constraints for any MICP research. First, potential biohazard from the proposed use of exogenous bacteria. Second, ammonium concentration (limiting factor) and ammonia production (undesired byproduct) that can lead to potential environmental impact, including overall water quality at the adjacent streams and water bodies.

3.3.1 - Understanding MICP

In summary, the MICP starts with urease activity, where (stage 1) 1 mol of intracellularly hydrolyzed urea ($CO(NH_2)_2$) results in 1 mol of ammonia (NH_3) plus 1 mol of carbonate (NH_2COOH), followed by (stage 2) spontaneous hydrolysis that form 1 additional mol of ammonia (NH_3) and 1 mol of carbonic acid (H_2CO_3). In aqueous media (stage 3), the carbonic acid and ammonia will reach equilibrium, forming 1 mol of bicarbonate ions (HCO_3^-), 1 mol of hydrogen ions (H^+). Then, (stage 4) each 2 mol of ammonia will combine with 2 mol of water, resulting on 2 mol of ammonium (NH_4^-) and 2 mol of hydroxide ions (OH^-), (stage 5) increasing the pH and shifting the balance of bicarbonate into carbonate ($HCO_3^- \rightarrow CO_3^{2-}$). This balance promotes (stage 6) a high influx of calcium ions and other protons expulsion, driving the bacteria to (Stage 7) release calcium outside the cell to survive. At this stage (8), the presence of dissolved inorganic carbon (DIC) as carbonate ions outside the cell and the expelled calcium ions triggers the reaction that culminates (stage 9) with the Calcium Carbonate precipitation outside the cell.

3.3.2 - Urease (UE): is this a limiting factor for the MICP?

Urease (EU), a member of the hydrolases group, is a nickel-containing metalloenzyme that catalyzes the hydrolysis of urea into ammonia and carbonate, initiating the reaction chain of the calcium carbonate precipitation described above (Castro et. al.., 2016). Figure 1 shows a schematic illustration of the MICP of a ureolytic bacterium, highlighting the reactions that occur on the surface of the bacteria in addition to a summary of the internal cell balances that promote calcite precipitation. The carbonic anhydrase can limit the MICP, as discussed in the next section. The most studied ureolytic bacteria present in the soil are *Sporosarcina pasteurii* (formerly *Bacillus pasteurii*), *Bacillus sphaericus*, and *Bacillus cereus*. However, Enriquez et. al.. (2017) found that

Serratia ureilytica sp has higher precipitation rates of $CaCO_3$ (calcite) when compared to *Sporosarcina pasteurii* in sandy soils under the same parameters and the same protocol, based on Whiffin (2004). Allied to the fact that it is a native species, *Serratia ureilytica* sp is a strong candidate for investigations of MICP as a soil stabilizer in Brazil.

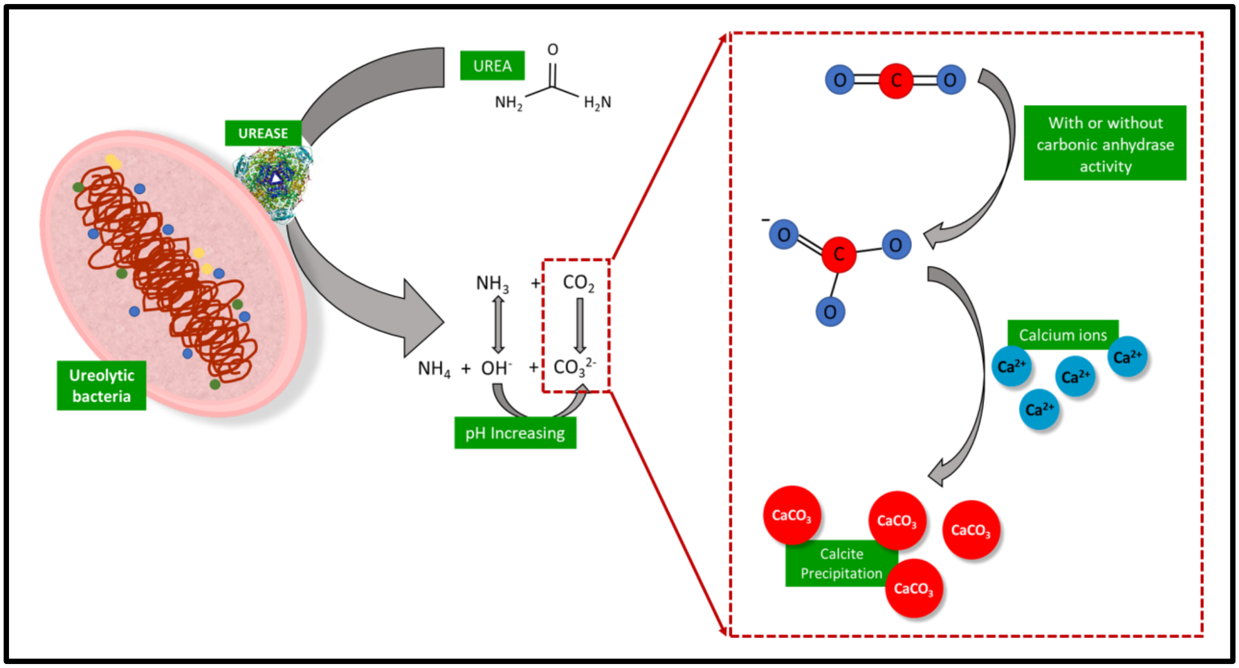

Figure 1 - Schematic illustration of the MICCP process by urease activity and ureilytica bacteria. (Adapted from Castro et. al., 2016)

3.3.3 - Carbonic anhydrase (CA): is this another limiting factor?

Carbonic anhydrase (CA), like urease, plays a vital role in the MICP process, but it still needs more detailed studies on how it works at MICP. CA is also a metalloenzyme, but during stage 9 uses zinc in the catalytic nodes with carbon dioxide (CO_2) and bicarbonate (HCO_3^-), replacing stage 4 in the MICP at the urease pathway. In the CA pathway, after the production of bicarbonate, hydrogen ions (H^+) promote the precipitation of calcium carbonate in the form of calcite (stage 10) plus the output of water and carbon dioxide (Castro et. al.., 2016).

Hwang et. al.. (2013) reported the action of CA in biomineralization and the fact that the morphology of calcium carbonate (calcite) depends on the constant pressure of CO_2 and the addition of polymers that affect the growth and nucleation of the precipitate. Figure 2 is a schematic illustration of $CaCO_3$ biomineralization in the presence and absence of carbonic anhydrase. The $CaCO_3$ precipitated in the presence of CA can take three forms: ellipsoidal, polygonal, or rhombohedron. The same process in the absence of CA only precipitates $CaCO_3$ in the form of a rhombohedron, a less rigid morphology.

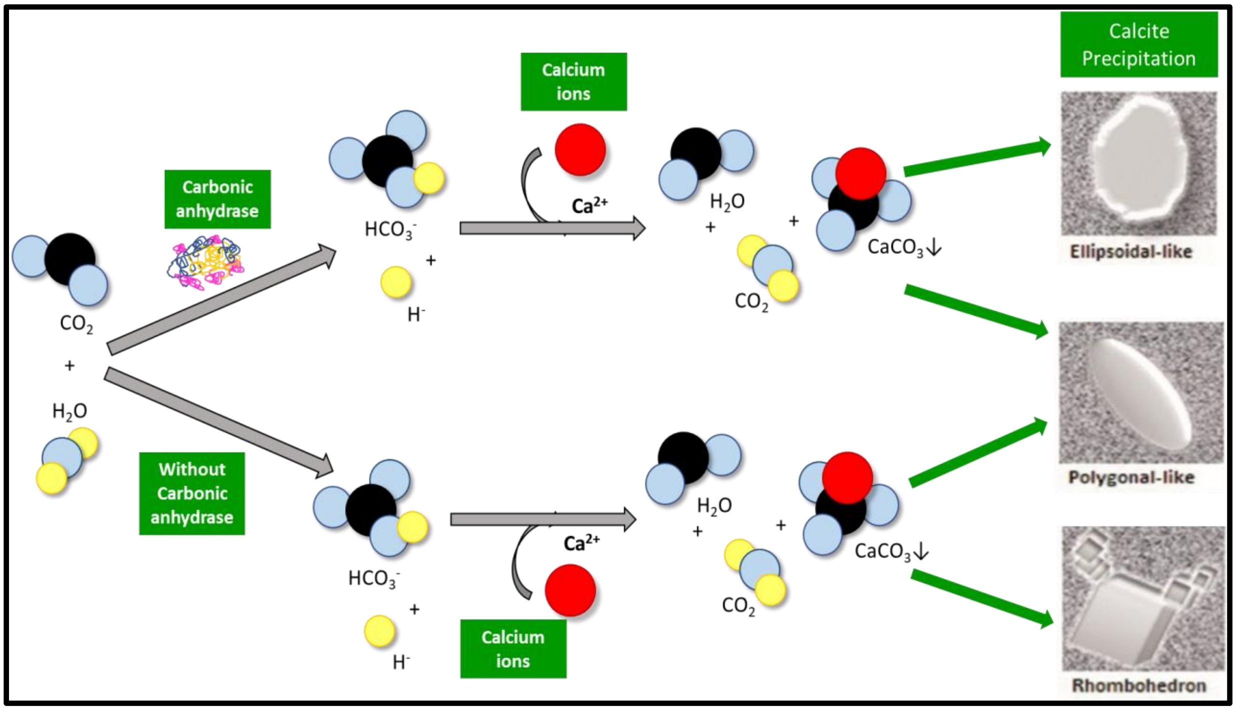

Figure 2 - Schematic illustration of MICCP by carbonic anhydrase activity (compiled and adapted from Castro et. al.., 2016 and Müller et. al.., 2014).

The activities of urease and carbonic anhydrase are interdependent and guide the stages of Calcite precipitation. The activity of urease depends on the incorporation of nickel in its nucleation site, which is regulated by the chemical reaction between carbon dioxide and bicarbonate catalyzed by CA (Jimenez-Lopez et. al.., 2007; Wong, 2015).

3.3.4 - The nucleation sites and their impacts on precipitation

Bosak and Newman (2003) demonstrated that the nucleation processes drove the formation of microbial carbonate on our planet for millennia. However, Bontognali et. al.. (2008) state that the nucleation process and its paleontological significance are sources of much controversy among scientists and require further studies. Aloisi et. al.. (2006), corroborated by Bontognali et. al.. (2008), Obst et. al.. (2009) and Achal & Pan (2011), summarized that the nucleation sites are where the cell surface of microbes secretes Extracellular Polymeric Substances (EPS) and that they have a negative electrical charge and the ability to bind to Ca_2^+ ions. The authors also stated that EPS is vital for the biomineralization process, as they are the key to the initial stage of carbonate nucleation processes, enhanced by the progressive development of large granules of calcium carbonate with a granular texture.

The research by Aloisi et. al.. (2006) contributed to fill the gap of more detailed knowledge about the processes and activities of the nucleation site. This study is relevant, as an acceleration and intensive nucleation of calcium carbonate on the cell surface can lead to the confinement of the organism and, eventually, premature death. The characteristics of the nucleation site define the morphology of the calcium carbonate precipitates: Calcite, Vaterite, or other metastable polymorphs. Studying *Desulfonatronum lacustre*, a gram-negative sulfate-reducing and carbonate precipitating bacterium, Aloisi et. al.. (2006) described a new pattern of nanoscale microbial carbonate nucleation. They found that the calcium carbonate precipitation occurs in blood cells in regions close to the microbial cell wall, and the calcification increases during the release of the blood cells into the aquatic environment around microbes. This model can bring more information and tools to explain the formation of nanospheres and other steps in the MICP process. However, Bontognali et. al.. (2008) described that in sulfate-reducing bacteria, the EPS is external, which promotes precipitation and, consequently, agglomeration in the external medium of $CaCO_3$, which leaves the bacteria mobile and rarely being "buried" by the mineralization process (low biocalcification). Achal & Pan (2011) corroborate Tsuneda et. al.. (2003), Achal et. al.. (2009a) and Achal et. al.. (2009b), stating that EPS is essential in the biofilm biocalcification process, cell adhesion and even in the capture of precipitated calcium carbonate, leading to a potential homogeneous layer of biocement once we have a complete understanding of the process.

We highlight the need for more research on the formation of nucleation sites and their relationship with ureolysis and precipitation of carbonates (calcite), which may contribute to elucidate the gaps around the homogeneity and resistance of biocement aimed at stabilizing unsealed roads. Besides, Warren et. al.. (2001) showed that bacterial surfaces could act as models of mineral nucleation, promoting a reduction in the cost of precipitation activation energy. They demonstrated that $CaCO_3$ precipitation does not occur in the absence of bacteria, indicating that the amount of energy for activation and homogeneous precipitation can be a significant barrier. We understand that this barrier is a potential source of process control. Warren et. al.. (2001) also indicated that the bacteria increase the precipitation of calcium carbonate, promoting supersaturated conditions.

3.3.5 - Nitrogen cycle and Ammonium concentration

MICP can occur via urea hydrolysis, aerobic oxidation, denitrification, sulfate reduction, and other pathways. However, van Paassen et. al.. (2010) and Achal & Pan (2011) stated that MICP via urea hydrolysis (activated by Urease) is the most controllable carbonate precipitation pathway, and according to Achal & Pan (2011), it reaches the highest level of $CaCO_3$ precipitation with a shorter curing period of biocement.

Ureolytic bacteria can be classified into two groups based on their response to the ammonium concentration in the environment (Gat et. al.., 2014). In the first group, the high concentration of ammonium suppresses the activity of urease in bacteria, which is of low interest for MICP purposes. This group includes Bacillus megaterium. In the second group, the high concentration of ammonium does not limit the activity of urease, defining this as the group of interest for MICP. This group includes *Sporosarcina pasteurii* (formerly *Bacillus pasteurii*) - the most studied MICP bacterium and the biological agent of Biogrout® (Umar et. al.., 2016). Whiffin (2004) established that the bacteria in the second group are a wise choice for soil improvement because the high concentrations of urea are hydrolyzed in the process, reducing the potential release of urea in the soil and groundwater. However, the process releases ammonium (NH_4^+) into the environment, which can be detected very easily as the natural odor of ammonium at the site of the experiment. None of the papers evaluated expressed concern about the release of ammonium into the environment. We believe that is more research about the released ammonium level before any claim that the MICP is an environmentally sustainable alternative.

3.3.6 - Precipitation of calcium carbonate crystals and their relevance

Studied by many authors and summarized by Warren et. al.. (2001), calcium carbonate has many polymorphs with different crystalline structures, with calcite, aragonite, and vaterite as the most common and stable. The authors stated that every polymorph has different stability due to its micromorphology, resulting in different reactivities with the surface of its environment. Calcite is thermodynamically stabilized and has a hexagonal-rhombohedral crystal structure; aragonite is metastable and has an orthorhombic crystalline structure, usually in the form of a needle; and vaterite (precursor to calcite and aragonite) is a metastable hexagonal crystalline structure in spherulitic or disc-like form. These authors concluded that identifying the formation of structures and the level of reactivity of precipitated calcium carbonate is vital to define whether this procedure is a potential bioremediation technique for aquifer contamination. Li et. al.. (2013) showed that indigenous ureolytic bacteria can sequester soluble heavy metals present in soil and groundwater and still survive during the MICP process. The authors studied the MICP process via urea hydrolysis in *Sporosarcina pasteurii* and *Terrabacter*. They concluded that both are successful in sequestering Ni, Cu, Pb, Co, Zn, and Cd from the soil, precipitating them under the resistant carbonate composition to the acid attack of a level similar to that of acid rain, facilitating the removal of these heavy metals.

3.3.7 - The potential biohazard of exogenous bacteria for soil stabilization: biological or economic concern?

Fritzges (2005) stated that bacteria are native to the Earth and therefore have little chance of causing any environmental risk in the future. Umar et. al.. (2016) use this assumption as a validation for the potential use of any bacterium anywhere on Earth. Ecosystems (micro to macro) are unique and have a delicate balance, where any change can change the balance and transform the local environment. However, in the face of biological risk concerns, Umar et. al.. (2016) mentions that the MICP bacterium must be selected based on its environmental safety during and after the treatment process, avoiding the use of genetically modified organisms (GMOs), or pathogens or the inclusion of any exchangeable toxic element that may affect local microbial pathogenicity (indigenous bacteria). Have to consider this statement in cases like the Biogrout® or other exotic bacteria species that lack data behavior in the environment of each study. In order to minimize the potential environmental impact of exotic species and reduce costs, we suggest the development of studies with native (indigenous) bacteria.

3.3.8 - MICP as a potential source of carbon sequestration: adding value on unpaved roads

Degens et. al.. (2000), Nannipieri et. al.. (2003), and Murugan et. al.. (2014) studied the role of soil microbial community in the maintenance of soil ecosystem function, such as C sequestration. Millo et. al.. (2012) proposed biocementation via ureolytic bacteria as a potential source for carbon sequestration (carbon dioxide). This model suggests the MICP promotes carbon capture and storage through the use of soluble CO2 as a carbon source in the carbonate precipitation in the biocementation process. The authors summarized recent research that stated that MICP species with alkalinizing metabolism may play an active role in CO2 sequestration and how to act as a potential long-term carbon sink. Castanier et. al.. (1999) described in detail the variety of reactions mediated by bacteria through an increase in pH and carbonate alkalinity, resulting in carbonate precipitation. Dupraz et. al.. (2009a) and Dupraz et. al.. (2009b) referred to this as the "alkalinity engine" that promotes the concentration of HCO_3 and CO_2 by reducing dissolved CO_2 (CO_2 (aq.)). The consumption of CO_2 (aq.) Favors the capture of gaseous CO_2 (CO_2 (g)) in solution, promoting an increase in pH and rapid conversion into HCO_3 and CO_3^{2-}. These events culminate in mineral trapping through the precipitation of carbonates. Millo et. al.. (2012) described the interconnection between the final increase in pH and the decay of the precipitation rate due to the HCO_3 deprotonation and carbonate precipitation. This interconnection indicates a recovery of the alkalinity mechanism since the consumption of available calcium promotes a decrease in $CaCO_3$ precipitation.

Warren et. al.. (2001) stated that calcium carbonate precipitation could interfere with biogeochemical cycles, and therefore, have a potential impact on CO_2 concentration at the atmosphere or even with reactive transport of radionuclides and trace metals in contaminated aquifers. For these reasons, we understand that acknowledge the MICP process in its deep complexity and mechanistic level can bring more information about the potential carbon sequestration by biocementation of unpaved roads.

3.4 - Main soil parameters for a successful biocementation process

Majority of published articles reviewed for this article stated in their titles that the research focused on how to improve the "soil" strength. However, the results reflect the biocementation applications on sand or sandy soil. The analysis of sand, sandy soil, and granulometric stabilized soil demonstrated that the expected MICP behaviors were very different depending on the used soil matrix (distribution of particle sizes). This analysis indicates that it is necessary a considerable diversity of studies and techniques to successfully obtain a range of resistant biocementation applications for unsealed road stabilization.

The technique needs to guarantee reliable and replicable results on the evaluation of geotechnical engineering properties like permeability, porosity, stiffness, shear strength, unconfined compressive strength, and a homogeneous microstructure (or soil uniformity), parameters of a reliable soil cementation procedure (Machado et. al.., 2009). The next subsections discuss the main differences in the studied factors and how they can potentially affect the biocementation process.

3.4.1 - Permeability (Hydraulic Conductivity)

Caputo (1999) defines soil permeability as the capacity of water percolation through its particles under different rates, based on its composition. The permeability coefficient (*k*), derived from Darcy's law, correlates discharge and viscosity, where discharge is directly proportional to soil (media) hydraulic gradient. Permeability evaluation is relevant since the water content of any soil void connects directly with stabilization due to the interconnection between effective soil tension (that drives soil resistance) and neutral pressure, dependable on promoted tension from water percolation (França et. al.., 2009; Rufino et. al.., 2011).

Permeability coefficient is the property of the porous media only and is dependent on temperature and porosity (void rate). Higher temperature drives to lower water viscosity (η), guiding to enhanced percolation rate, increasing the coefficient of permeability (***K***) (Caputo, 1999; França et. al.., 2009; Rufino et. al.., 2011). This well-known factor is commonly neglected on biocementation publications since the majority of papers do not state the temperature of the environment either of the laboratory tests. This factor alone can drive a test replication to a considerably different result, even misinterpretation of results, which can generate misleading publications.

3.4.2 - Soil stratification & Permeability

Complementary to temperature, soil stratification also interferes with permeability rate. Casagrande & Fadum (1940) described the coefficient of permeability (***K***) per soil type, were coarse material like pure gravel has high permeability, leading to fast percolation rate (10^{-2} centimeters/second); and fine particles like clay has low permeability, reaching impervious level, with almost null percolation rate (10^{-11} cm/s). Fine sand to sandy soils, including gravel mixtures, have a coefficient of permeability between 10^{-2} and 10^{-6} cm/s. Organic and inorganic silts range from 10^{-5} to 10^{-9} cm/s, varying from a moderate to a slow percolation rate.

Soil stratification is a relevant constraint on the biocementation process since most published papers tested biocementation on the sand and only a few on sandy soils. Whiffin (2004) and van Paassen (2009), confirmed by many authors like DeJong et. al.. (2010), Cheng & Cord-Ruwisch (2012), Achal & Kawasaki (2016), showed that in sand or sandy soils, the calcium carbonate precipitate does not have a homogeneous distribution over the sample length. Also, the biocementation concentrates near the local of cementation injections, since the biocementation process starts during the injection phase, limiting the homogeneous distribution of cementation solution. In theory, and as stated by van Paassen (2009), soil with a rapid flow rate facilitates faster percolation of cementation solution into the sample column, potentially reducing the bioclogging near the injection point. This rapid flow is feasible (in theory) because a higher percolation rate allows a broader spread of cementation solution before the end of the biocementation process, potentially decreasing the bioclogging. However, van Paassen, Soon, DeJong among other authors tested soils with similar permeability rate (sandy soil), and the same bacteria are still searching for the answer of the central question: why does biocementation of *Sporosarcina pasteurii* in sandy soil does not reach homogeneous distribution along the sample length, independent of its scale? This answer is another threshold that demands a better understanding that can lead to more predictable outcomes on the biocementation process.

3.4.3 - Porosity (Void fraction) and pore size distribution

Nimmo (2004) and Brady & Weil (2008) define porosity (Φ) (void fraction) as the ratio between total void volume (occupied by air or fluid) divided by the total volume of soil, with values between 0 and 1, volume or in percentage. Porosity depends on many factors, such as packing density, distribution and particle size (polydisperse versus monodisperse), particle shape, and cementation rate (or particle welding). Soil with irregular particles tends to form larger voids, increasing its porosity. Sandy soils tend to have more spherical particles and less cementation, promoting larger voids between the particles and a porosity range between 0.30 to 0.35. The clay soils, clay, and organic materials have natural cementation of the particles, creating large volumes of aggregates, which individually support a porosity of 0.35, but as a soil profile, they have a porosity greater than 0.5. Soils with a high organic matter content can reach very low porosity of 0.8-0.9. However, Nimmo (2004) emphasizes that the soil-water-air system has an intrinsic behavior and soil particles do not have a unique shape or size, making it almost impossible to delineate the size and shape of the pores without subjective assumptions. The author also stated that the measurement of pore size is a vital step to assess the structure of the soil, directly impacting on how to improve its geotechnical properties. In our reviews, we note that these assumptions are not often clearly stated, except the porosity values that were calculated, measured, or assumed.

The main objective of biocementation is to promote cementation between soil particles to increase their stiffness and shear resistance, maintaining their porosity until the cementation solution spreads evenly before the completion of the biocementation process (Whiffin, 2004; van Paassen, 2009; van Paassen et. al. 2010; van Paassen et. al., 2011; Cheng et. al., 2013; Cheng et. al., 2014). However, a brief analysis of the porosity concept clarifies the main bottlenecks of biocementation: How to maintain a constant and low rate of biocementation speed to allow the continuity of soil porosity, allowing the percolation of the cementation solution by the sample without restricting the process of biocementation or compromise its reinforcement of shear strength? The answer to this question is another threshold of the biocementation technique, independent of the selected bacteria.

3.3.4 - Shear strength

Roads are susceptible to constant shear stresses from vehicles and heavy machinery. To guarantee the quality of the road, the construction material used needs to meet the minimum requirement of the shear strength parameter for the planned use (Bakhsh & Zollinger, 2014). If the material is not homogeneous or does not have shear resistance, the road suffers damage, and the collapse is inevitable over time (Machado, 2008). Shear strength is directly related to shear stress and porosity (Terzaghi, 1942; Casal et. al., 2001; Vallejo & Mawby, 2000; Costa et. al., 2001).

Soil cohesion (c) and friction angle (ϕ) are the main parameters that define the shear strength of any material. After extensive analysis, we agree with the summary of Mujah et. al.. (2017) on some results of the research by Duraisamy and Airey (2012), Chou et. al.. (2011), Ng et. al.. (2012), Montoya and DeJong (2015), Cheng et. al.. (2013), Soon et. al.. (2013) and Chu et. al.. (2012), where they concluded that the biocemented sandy soils have their cohesion and friction angle increased due to the higher concentration of CaCO3 (calcite precipitation) filling the spaces porous soil. Nimo (2004) stated that pore size is a crucial component to improve the geotechnical properties of the soil since cohesion directly affects shear strength and that cohesion is directly affected by pore sizes. The concepts of the shear strength and porosity presented here back up our hypothesis that another threshold of the biocementation process depends on understanding the interconnection between shear strength and biocementation rate and how they affect each other.

3.3.5 - Soil microstructure

DeJong et. al.. (2010) stated that bacterial behavior and the soil filtration process determine the spatial distribution of CaCO3 precipitation. The same authors stated that the bacteria act in particle-to-particle contact due to the lower shear stress and the availability of nutrients at a granular level, promoting the concentration of bacteria in places where resources are abundant. Also, $CaCO_3$ precipitation reduces porous spaces, replacing the existing fluid (and potential full pore filling), which directly affects the filtration process, as well as percolation and permeability rates. This pore space reduction forces the precipitated $CaCO_3$ to stick close to soil particles as the porous fluid flows through the pore throat, reducing the pore space and potentially bioclogging (pore-clogging due to $CaCO_3$ deposition) (DeJong, 2010).

The soil microstructure is another constraint for a successful biocementation process, regardless of the type of soil or the selected bacteria. Scanning electron microscopy (SEM) images are the tools to evaluate the biocementation process, assessing the amount and distribution of $CaCO_3$ bonds with soil particles, allied with resistance tests to assess the stability of biocemented soil (Whiffin, 2004). DeJong et. al.. (2010) found that only precipitated $CaCO_3$ crystals that form an effective bond between sand particles contribute to increasing the shear strength of biocemented sand. These calcite-particle bonds in the soil occur at the level of interparticle, replacing the fluid and must have a uniform distribution to promote soil resistance. However, $CaCO_3$ precipitation tends to follow a unique pattern of distribution, concentrating in the vicinity of the bacteria's nucleation site.

3.4 - MICP on unsealed roads

Unsealed roads are necessary for the development of any region since they provide social-economic and accessibility benefits (Rammelt & Leung, 2017), and have a much lower cost than paved roads. Unsealed roads in Brazil (a similar situation for the majority of the developing countries) used for temporary purposes such as mining industry access, and as permanent roads in rural areas, reach a considerable percentage of total constructed roads (Ministerio de Obras

Publicas, 2018). The majority of these unsealed roads have massive traffic demand, which increases the demand for maintenance to sustain the performance and security of the roads. Besides, many soils are not suitable to carry loads (be used as road subgrade) without a high cost of maintenance in the long-term (Cabezas & Cataldo, 2019).

Stabilization methods applied to in-situ soils are necessary for improving mechanical behavior via improving bearing capacity or decreasing permeability (or both) and reducing maintenance costs over time (Parsons & Milburn, 2003). The standards for unsealed road construction in the US and Brazil demands granulometric stabilization of the soil to improve its stability to support the designed road. In countries like Brazil, where only 213,453 kilometers (12.4%) from the 1.720 million kilometers of the total road network has asphalt or similar coverage, chemical stabilization is widely used (CNT, 2018). Biocementation presents itself as a potential green alternative since it has the potential to substitute the use of cement or lime on road construction in Brazil.

3.4.1- Granulometric stabilized soil - the foundation for unsealed road construction

Granulometric stabilization is the alteration of soil properties through the mixture of different sizes and concentrations of gravel, sand, silt, and clay particles resulting in a homogenized mixture followed by compaction under Proctor energy parameters (Vizcarra, 2010). Machado et. al.. (2009) adds that granulometric stabilization of road subgrade focuses on a final composite of materials that attend the designed road cargo demand. IPR/DNIT (2006) and IPR/DNIT (2010) determine that the granulometric distribution is the percentage of each particle size present on soil mixture and its distribution. Then, the granulometric soil mixture receives a mechanical stabilization through Proctor compaction, finalizing the granulometric stabilization of the soil. This stage is the granulometric stabilized soil or unsealed road, which is also the subgrade for the paved roads. Even after granulometric stabilization, unsealed roads are highly susceptible to erosion processes, especially hydric erosion. For this reason, the Brazilian unsealed road standards (and also the US) promote the additional chemical stabilization methods, like the classic addition of cement or lime.

3.4.2 - Soils stiffness and stabilization

As stated at the 3.4.1 subtopic, chemical stabilization of the soil is a requirement from road construction standards. Chemical stabilization by cement or lime is the most used stabilization technique and promotes alteration in bearing capacity, shear strength and permeability (Teng et. al.., 2007; Ismail et. al.., 2002; Machado, 2008; Bakhsh & Zollinger, 2014), culminating in high stiffness and brittle behavior (Wang et. al.., 2003; Basha et. al.., 2005; Bakhsh & Zollinger, 2014). A high stiffness soil means soil with the high elastic modulus (E), defined by a high ratio of stress over strain, translating the bonding strength into loose soil grains (Mujah et. al.., 2017).

Yang and Gu (2013) stated that confining stress and void ratio are the main factors affecting shear stiffness (G0). They cited the Wichtmann & Triantafyllidis (2009) research with sand, which confirmed that the shear stiffness (G0) decreases considerably as the coefficient of uniformity (Cu) increases. This ratio means that soil with a higher concentration of particles within uniform sizes leads to a natural decrease in shear stiffness. For this same reason, chemical stabilization is the method to reduce the erosion of subgrades of pavements (Machado et. al.., 2009). In Brazil, unsealed roads cannot use sand or sandy soils, and the soil must be granulometric stabilized, which contains a low volume of sand (IPR/DNIT, 2006). Granulometric stabilized soils have an increased shear stiffness. However, this enhanced shear stiffness is not strong enough to support traffic, which demands extra stabilization, often chemical stabilization with cement or lime (Machado et. al.., 2009; Bakhsh & Zollinger, 2014). Lee et. al.. (2013) tested biocementation on residual soil (43% of silt, 38% of sand, 19 % of clay, and 0% of gravel) and found a similar stiffness behavior than on biocemented natural sand. Rebata-Landa (2007) suggested a grain size ranging between 50 and 400µm to attend the bacterial activity requirement of a low concentration of very fine soil particles (clay). Also, coarse materials demand high production of calcite to fulfill the high number of voids, biocementing the soil particles without compromise the stiffness. This review shows that any study about soil stabilization and unsealed road construction using biocementation procedure should include soil stiffness as it is a critical limit factor.

3.4.3 - Erosion on unsealed roads: chemical stabilization methods and MICP

The erosion of unsealed roads begins when the runoff is concentrated along the road drainage channel, staggering the shear stress until it surpasses the critical shear stress of the road surface. From this moment on, the runoff will carry out the soil particles, contributing to the local watershed sedimentation process. The well-described impacts include sedimentation, water quality degradation, which directly affects the aquatic life of watercourses (Ziegler et. al.., 2000 & 2001; Zhang et. al.., 2009; Liu et. al.., 2009; Corrêa & Cruz, 2010, Bakhsh & Zollinger, 2014).

Terzaghi (1943) and Terzaghi et. al.. (1996) proved that shear stress in soil mechanics is the main force responsible for ruptures in slopes, valleys, dams, and other geomechanical forces over a sedimentary soil. The authors also stated that clay soils demand exhaustive analysis and studies of its mechanics based on intended use, since micro-clays (or clay-minerals) have a larger buffer of water around the particles, meaning that the fluctuation of the water content of soil directly affects road stabilization. Citing Umesh et. al.. (2011), Vakili et. al.. (2012) and Edgar (1991), Premkumar et. al.. (2016) stated that Brazil and the United States are two of the top nine countries that have dispersive soils, meaning that the clay particles are dispersible and behave as a single-grained particle. Premkumar et. al.. (2016), citing Sherard & Decker (1977); Bell (2003); Biggs & Mahony (2004), summarized that dispersive soils lose the interparticle forces and suffer from erosion failure when exposed to water, meaning that these soils have low wet bearing strength.

3.4.4 - Natural calcite precipitation in soils - a prediction model

The soil and agricultural sciences established that calcite precipitation occurs naturally near the root surface once the root quickly absorbs nitrate and release bicarbonate to the soil to maintain the charge balance at the root-soil boundary. This precipitation led to a pH increase, usually enough to facilitate natural CaCO3 precipitation near the root surface (Nye, 1981; Kirk and Nye, 1991; Neumann and Romheld, 2012). This process called microbially-enhanced urea hydrolysis is also known as MICP, having Stocks-Fisher et. al.. (1999) as one of the first research to propose its use to stabilize soils. Urea hydrolysis processes driven by microbial enhanced or induced calcite precipitation have some points in common: reactants promote local CaCO3 precipitation; the rate and distribution of precipitation are directly dependent on the in-out reactant transportation rates at the precipitation zone, and mainly by precipitation kinetics at the nucleation sites per se. Most studies reviewed in this article do not state clear information about transport and precipitation kinetics or do not even mention it at all. Kirk et. al.. (2015) summarize three significant factors that can affect transport and precipitation kinetics. First, the CaCO3 kinetics in simple solutions is well described but still lack information about its interaction with soil systems and other porous media. Second, it is common to have soil solutions supersaturated with CaCO3, which directly affects precipitation due to its sensitive reaction to CaCO3 and other fluids catalysis and inhibition by organic and inorganic ligands present at the soil solution. Third, the fact that the soil transport rate usually is lower than simple solution systems due to most solutes being sorbed on soil surface becoming mostly immobile in the sorbed state.

Kirk et. al.. (2015) proposed a simple reactive-transport model to evaluate the calcite precipitation in soils and concluded that it is vital to evaluate the initial soil pH, the soil pH buffer power, and the CO_2 pressure to promote a long-spread zone of precipitation from its origin. It is necessary for a small pH buffer power for sandy soils or sub-strata and desired a high CO_2 pressure for clayey soils to sustain a high biological activity. The model proved the importance of the geometric system and the spacing between macro-pores where the precipitation is happening. By analyzing the model, we can extend the following question to any biocementation process: How to predict and guarantee the desired conditions of soil pH, soil pH buffer, CO_2 pressure, reactant transport rate, and its movement of in-and-out precipitation zone to promote homogeneous $CaCO_3$ precipitation?

4 - Conclusions & considerations

Studies by Soon et. al.. (2013) have shown the effectiveness of MICP by an improvement of 96% on soil shear strength and reduced soil permeability. The successful research on MICP for soil stabilization should focus on: the identification of microorganisms with higher calcium carbonate precipitation; soil optimal conditions to increase the microorganism growth rate (ureolytic activity); analysis of the calcium carbonate production rate under field conditions; and on differences between indigenous and exotic microorganism growth rates (Whiffin et. al.., 2007;

van Paassen et. al.., 2011; Cheng & Cord-Ruwisch, 2012; Yasuhara et. al.., 2011; Shahrokhi-Shahraki et. al.., 2015). Field studies of soil biostabilization at a large scale are still scarce given the complexity of the process, the long-term research cycle, and the high cost of the field-scale research projects. However, the personal/professional desire to publish before any other researcher has catalyzed several scientists to repeat very similar experiments and rush publication in this field. Studies and tests are necessary to obtain the best results of the MICP for each environment and road conditions to continue to improve field techniques and applicable outcomes. Summarizing the papers evaluated in this review, the ideal biocementation model should consider three central factors, at least. First, guarantee a uniform and balanced environment for bacteria, including the bacteria-specific process, to calculate the necessary concentration of the bacteria. Second, define the relevant porosity combined with the correct concentration of the cementation solution to ensure homogeneity of the $CaCO_3$ precipitation. Third, use the first and second factors to eliminate the need for multiple injection points of the cementation solution or successive applications in the same location, thus reducing operating costs.

Morales et. al.. (2015) research sustain our rationale that it is necessary to develop an optimum biocementation solution that must be available during the MICP process to facilitate the $CaCO_3$ precipitation. This rationale also applies to the research aiming to promote a sustainable unpaved road biostabilization. Since no research has developed to elucidate the optimum concentration of bacteria, bacteria species selection (indigenous species to minimize potential hazardous impact and cost and with a high rate of CaCo3 precipitation), cementation solutions and soil mixture, our primary research continues to pursue the goal of developing an experimental study to develop this innovative field further.

In summary, this review showed that MICP is feasible as a soil stabilization process but still needs considerable development. The protocol development should anchor on a considerable breakthrough that addresses the intricacies of MICP stages and its six main limiting factors: calcium concentration, dissolved inorganic carbon (DIC) concentration, pH of the media, availability of nucleation sites, urease activity; and carbonic anhydrase activity. More exploratory studies are necessary to evaluate the behavior of each proposed bacterial isolates facing the limiting factors and the improvement of the mechanical properties of granular soils (including unsealed roads). This review also delineated another gap for any MICP soil stabilization: which is the best injection method to achieve a homogeneous biocementation for the proposed use of the soil?

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Chapter 3: Assessment of calcium, magnesium, and silicon percolated from unsealed roads chemically stabilized

Ph.D. candidate Carla Ribeiro Machado e Portugal [1], Dr. Carolyn Fonyo [2], Dr. Carlos Cardoso Machado [3], Dr. Richard Meganck [4], MSc José Maurício Machado Pires[5]

1 - Author for correspondence. Ph.D. candidate at Environmental Sciences Graduate Program. Oregon State University. Graduate School - Heckart Lodge, 2900 SW Jefferson Way. Corvallis, OR – USA. 97331-1102. Telephone: +1 (541) 9797-8741. Carla.portugal@oregonstate.edu or carlamacport@hotmail.com. ORCID: https://orcid.org/0000-0001-9508-8831

2 – Director of Environmental Sciences Graduate Program. Oregon State University. Graduate School - Heckart Lodge, 2900 SW Jefferson Way. Corvallis, OR – USA. 97331-1102. Carolyn.fonyo@oregonstate.edu. ORCID: https://orcid.org/0000-0002-8919-6585

3 – Professor, Federal University of Viçosa, College of Forest Engineering. Avenida Peter Henry Rolfs, S/N, Viçosa, MG – Brasil. Zip code 36570-900. Machado@ufv.br. ORCID: https://orcid.org/0000-0002-2386-9791

4 - Institute for Water and Watersheds, part of the UNESCO-ICIWaRM category 2 center, Oregon State University, USA. rameganck@gmail.com. ORCID: https://orcid.org/0000-0002-1523-9992

5 – Geochemistry Laboratory Technician, Federal University of Viçosa, College of Agronomy. Avenida Peter Henry Rolfs, S/N – Edifício Sylvio Starling Brandao, Viçosa, MG – Brasil. Zip code 36570-900. jose.m.pires@ufv.br. ORCID: https://orcid.org/0000-0002-6366-2410

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Highlights

- Unsealed road stabilization as a source of percolated contaminants in water bodies.
- Percolation impacts on water bodies by unsealed roads chemically stabilized.
- The initial investigation showed that cement at 3% promotes impervious stabilization.
- Calcium, magnesium, and silicon from stabilization as indicators of water contamination.

Abstract

Unsealed roads are the primary access route in Brazil, sustaining business efficiency (supply chain), social connectedness, and community safety at lower construction and maintenance costs than paved roads. The water quality assessments quantified calcium (*Ca*), magnesium (*Mg*), and silicon (*Si*) as the primary potential pollutant sources due to the chemical stabilization of unsealed roads. Our data show that 3% cement stabilization proved to be efficient in rendering unsealed road surface impervious. Lime stabilization had higher calcium and silicon percolation rates, and mechanical stabilization contributed to higher magnesium percolation. The percolation rates of *Ca*, *Mg*, and *Si* are above the environmental standards, indicating the need for additional studies to evaluate the potential environmental impact on adjacent or nearby streams receiving runoff. The authors also recommend expanding the study to determine the impacts of variables like time, seasonality, traffic variability on-road life expectancy, and chemical runoff concentration and rates.

Keywords (max of 6 words/terms): unsealed roads, Flame Atomic Absorption Spectrophotometry (FAAS), water quality, percolation, erosion, environmental impact.

1. Introduction

Anthropogenic activities such as infrastructure development, including roads, are the leading cause of global environmental challenges due to biogeophysical impacts. (Zhou *et. al.*., 2019; Deligianni, 2020). Unsealed roads are particularly crucial since they deliver direct access and sustain business efficiency (supply chain), social connectedness, and community safety (van Wijk, Williams, and Serati, 2019) at a lower construction cost compared to paved roads (Deligianni, 2020). Therefore, studies about unsealed road network interactions with environmental impacts are critical, especially for tropical countries like Brazil, due to the constant traffic flow and tropical rain regime (Machado, 2013), plus a lower installation cost than paved surfaces. Exposure to dust emission, surface and internal erosion, sedimentation to recipient streams, and construction and maintenance impacts are the main environmental challenges from unsealed roads (Camarena, 2013; Bluett *et. al.*., 2017).

Erosion creates a downstream effect carrying pollutants and soil particles, resulting in an aquatic ecosystem disruption, water bodies impairment, drinking water contamination, and increased downstream flooding (Dunne and Leopold, 1978; Machado, 2013; Mitchell, 2018). This process can be escalated by combining the kinetic energy intensity from the road surface with reduced infiltration rates, leading to higher erosion, including internal erosion by intensive percolation (Ward and Jackson, 2004). Even though the unsealed road construction methods are well defined, the techniques still leave the unsealed roads susceptible to weatherization and seasonality influences, demanding careful planning with constant maintenance (Machado, 2013).

The Brazilian Paving Manual (DNIT, 2006) allows the addition of a chemical stabilizer to unsealed roads (granulometric stabilization) as a recommended alternative based on economic, social, and technical criteria. However, the same Manual and the Brazilian standards (DNER, 1994a; DNIT, 2019, 2010a) are not explicitly assessing the environmental impact from chemically stabilized unsealed roads (Arrivabeni, Machado and Sant'Anna, 2016; Arrivabeni, 2017; Arrivabeni *et. al.*., 2018). The knowledge about the hydrologic response to unsealed road impacts increased over the last fifty years but is still frequently considered intuitive. Most publications in this field refer to forest management practices and the related environmental impacts but have gaps about how fine sediments (including chemical and mineral characterization) from the unsealed roads can affect water quality (Lane and Sheridan, 2002; Dutton, Loague and Wemple, 2005; Zemke, 2016).

Water quality assessment brings a new environmental perspective into classical unsealed road stabilization methods and practices. The literature (Ola, 1978; Townsend, 1985; Suer, Wik, and Erlandsson, 2014; Arrivabeni, 2017; dos Santos Ferreira *et. al.*., 2018) has repeatedly substantiated that chemical stabilization by cement and hydrated lime increases the impermeability of the soil, and when combined with mechanical stabilization, can support road lanes on unsealed roads. However, runoff and percolation, mainly due to diverted and heavy traffic, weatherization, and the absence of proper maintenance, are the leading causes of intensive erosion on unsealed roads. Many authors (Fu, Newham, and Ramos-Scharrón, 2010; Anderson and Lockaby, 2011; Orndorff *et. al.*., 2017; van Steenbergen, Perez, and Woldearegay, 2018; Silliman and Toman, 2019) evaluated sedimentation from unsealed roads into streams, but it is necessary to investigate the potential impact generated by the percolation of cement and lime elements through the unsealed roads via internal erosion.

This preliminary study assessed the essential elements calcium (Ca), magnesium (Mg), and silicon (Si) via the Flame Atomic Absorption Spectrometer (FAAS) test. These elements were selected since they are present in cement and hydrated lime and have a lower cost of analysis, increasing the potential to expand this chemical assessment to more extensive regions. Summing that, the literature rarely mentions the environmental impacts from unsealed roads chemically stabilized with cement or hydrated lime, enhancing the motivation for this study. This research can also guide further studies on environmental impact assessment of unsealed road stabilization in Brazil and other locations where these practices are commonly employed, like the United States, Europe, and Africa.

Studying *Ca, Mg,* and *Si* concentrations in the water column of unsealed roads and related erosion rates become more relevant, considering that 1.35 million kilometers (almost 80%) of roads in Brazil are unsealed roads (CNT, 2019). Nearly 1.6 million transport and heavy cargo vehicles (CNT, 2019) contribute to hazardous water quality impacts from soil erosion and sedimentation of unsealed roads into local watersheds. The wide distribution of the unsealed road network in Brazil illustrates the high relevance of such environmental impact studies. These studies should aim to fully understand the interconnections among road quality (construction and maintenance), traffic intensity, weather and rain regime, geographic parameters, political and policy factors, plus socio-economic and environmental constraints (Croke and Hairsine, 2006; Machado, 2013; van Steenbergen, Perez, and Woldearegay, 2018; Silliman and Toman, 2019).

A thorough environmental assessment requires a detailed evaluation of all the unsealed roads impacts on water bodies and existing social structures. Therefore, this research investigated the concentration of calcium (*Ca*), magnesium (*Mg*), and silicon (*Si*) as the main chemical components of cement and hydrated lime, two of the most used methods for unsealed road stabilization. The goal is to contribute to building a database focused on tracing the complete environmental impact of unsealed road use in Brazil, including internal erosion effects.

Rapant et. al.. (2017) summarized the risk of classic contaminants found in drinking water, stating that those toxic elements and compounds are well known, documented, and strictly limited by WHO (2017) guidelines. Rapant et. al.. (2017) focused on demonstrating the need to expand the studies about how essential elements like calcium, magnesium, and potassium can affect human health. Those essential elements are not limited by WHO drinking water guidelines (2017). However, Rapant et. al.. (2017) summarized many studies documenting how drinking water's *Ca* and *Mg* deficit can increase incidence and mortality rates for cardiovascular diseases.

The authors understand that the water quality assessment goes beyond the factors evaluated in this preliminary study. Focusing on water quality and flow, the authors recommend expanding this research to field scale, including parameters such as the size of receiving water body and complete infiltration process to downward to underlying soil layers until reach groundwater. The authors present this research as an exploratory analysis to pave the expansion of the research and demonstrate the need for more detailed assessments.

Our general hypothesis is that Ca, Mg, and Si concentrations can affect water quality near unsealed roads through internal erosion. Our results reinforce the need for further investigations on the environmental impacts of unsealed roads, promoting increased management of the erosive process and providing support for evaluating and developing new alternatives to unsealed road construction and maintenance. Furthermore, a more in-depth understanding of the erosion process can enhance control over environmental impacts from anthropogenic activities supports the reduction and prevention of land and water resources degradation (Ziegler, Sutherland and Giambelluca, 2000; van Steenbergen, Perez, and Woldearegay, 2018; Cochand *et. al.*., 2020).

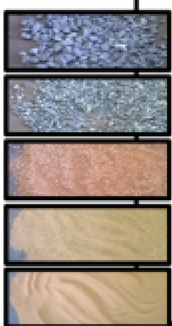
2. Research Methodology and Experimental design

Brazil's primary soil source for road construction is denominated “natural” or “original” soil. According to the Brazilian Pavement Manual (DNIT, 2006) and DNIT 141/2010 (DNIT, 2010a, similar to AASHTO M 147-65, 2008), the base and sub-base layers for any roads have to be granulometric stabilized (implying mechanical compaction) to ensure a resistant base and sub-base, used to build an unsealed or sealed road. Unsealed roads (*UR*) consist of a base or a sub-base layer of granularly stabilized (DNIT, 2006; Machado, 2013), which can be treated only with mechanical compaction or additional chemical stabilization. The most common chemical stabilization additives are cement or hydrated lime.

This research focused on granularly stabilized soils, defined by DNIT 141/2010 (DNIT, 2010a), standardizing mechanical soil conditions to achieve erosion resistance on road construction. This research investigated three different soil stabilization methods (treatments): (1) mechanical compaction, (2) mechanical compaction plus cement at 3%, and (3) mechanical compaction plus hydrated lime at 6%. Even though the literature has a considerable volume of published evidence about cement and hydrated lime as an effective additive to soil stabilization methods for road construction, the field still needs more information about the impact of the percolated concentration levels of these chemical additives (cement and hydrated lime) on water quality, particularly to further erosion over time.

The Brazilian Paving Manual (DNIT, 2006) classifies unsealed road grading (*URG*), meaning the base or sub-base, into six different types (A to F), where each type has specific grading requirements of soil particles, as shown in *Figure 1*. Each *URG* has a specific grading distribution resulting in a different load-bearing capacity. Heavy traffic requires *URG A* or *URG B*, intermediate traffic requires *URG C* or *URG D*, and low traffic requires *URG E* or *URG F* (DNIT, 2006). The present research selected one *URG* from each traffic level (*A*, *C*, and *F*) to assess the potential environmental impacts from internal erosion of unsealed roads to water quality. The *URG A* and *URG F* selection represented the extreme ends of the traffic range, and

URG C was a random selection between C and D. The outlined red boxes in *Figure 1* highlight the grading requirements for each *URG* selected.



Sieves		Granular Distribution for URG % of retained material through each sieve					
		Heavy traffic		Intermediate traffic		Low traffic	
		A	B	C	D	E	F
2"	50.8 mm	0.00	0.00	0.00	0.00	0.00	0.00
1"	25.4 mm	0.00	17.50	0.00	0.00	0.00	0.00
3/8"	9.5 mm	52.50	25.00	32.50	20.00	0.00	0.00
N. 4	4.8 mm	7.50	12.50	17.50	12.50	22.50	15.00
N.10	2.09 mm	12.50	12.50	12.50	12.50	7.50	7.50
N. 40	0.42 mm	13.50	10.00	15.00	20.00	35.00	27.50
N. 200	0.075 mm	14.00	22.50	22.50	35.00	35.00	50
Total (%)		100	100	100	100	100	100

Figure 1 - Grading requirements for soil-aggregate materials per each subbase of unsealed road construction. (adapted from Paving Manual (DNIT, 2006)*).*

The experimental design combined the three road types selected (*URG A, C,* and *F*) with the three most common stabilization (called Treatment) methods: (1) mechanical stabilization (called *Control* or *Co*) only, (2) mechanical stabilization plus chemical stabilization with 3% of cement (called *Cement* or *Ce*) with 7-days cure period, or (3) mechanical stabilization plus chemical stabilization with 6% of hydrated lime (called *Lime* or *Li*) with 14-days cure period. The Brazilian Paving Manual (DNIT, 2006) recommends the cement ratio between 2 – 4% and the hydrated lime ratio between 4 – 8%. Therefore, this research selected 3% cement and 6% hydrated lime ratio since both levels represent the average percentage allowed by the Brazilian Paving Manual.

3. - Materials

3.1. - Soil

Under the coordinates 20º47’29.38” S and 42º49’19.26” W, the Gomide gravel pit (Cascalheira Gomide) was the natural soil source for this study, located at Cajuri, State of Minas Gerais, Brazil. The location was selected because it is the regional material source for the base and sub-base layers of unsealed and sealed road construction (Arrivabeni *et. al.*., 2018). The geographic coordinates for the Gomide gravel pit and the country level are on the map in Appendix A, built using Google Earth Pro software.

3.2 - Cement

This research adopted the Cauê cement CPII E-32 for general use, according to the recommendations of the Brazilian Pavement Manual (DNIT, 2006) and DNIT 143/2010 – ES (DNIT, 2010b, similar to ASTM D3282-09, 2009). The CPII E-32 cement is a composite of pure Portland cement and granulated slag from a blast furnace, satisfying the resistance levels required for the chemical stabilization of unsealed roads defined by the abovementioned standards.

3.3 – Hydrated Lime

This research adopted the Dical hydrated lime type CH III. The CH III follows the Brazilian Pavement Manual and DNER - ME 180/1994 (DNER, 1994a; DNIT, 2006, similar to ASTM D5102-09, 2009) and DNIT 422/2019 standards (DNIT, 2019) resistance levels required for chemical stabilization of unsealed road construction, as required by the standards mentioned on this topic.

3.4. – Distilled water

This research used distilled water (DW) to shape the samples and run all the tests (Head, 1992; Sandoval *et. al.*., 2017).

4. – Methods

4.1 - Soil characterization

The natural soil was manually collected from May to October 2018 according to DNER-ME 213/94 standard (DNER, 1994b, similar to ASTM D2216-19, 2019) and measured for soil moisture content and physical characterization. The soil was stored in sealed plastic bags, and the tests were conducted at the Civil Engineering Laboratory at the Federal University of Viçosa, located in Viçosa, Minas Gerais, Brazil.

The soil characterization followed the standardized tests as follows: (i) Grading Analysis via ABNT NBR 7181 (ABNT, 2016a, similar to ASTM D6913 / D6913M-17,) to define the soil aggregates granulometry and to segregate the soil per grading; (ii) Soil properties like bulk specific gravity, apparent specific gravity, and water absorption via ABNT NBR 6508:2016 (ABNT, 2017); (iii) Atterberg limits via ABNT NBR 6459 (ABNT, 2016); and (iv) liquid limits and plasticity index via ABNT NBR 7180 (ABNT, 2016). The tests were conducted at the Civil Engineering Laboratory at the Federal University of Viçosa, located in Viçosa, Minas Gerais, Brazil.

4.2. – Testing the mechanical behavior of the specimens

The soil samples were sieved to achieve the granulometric distribution using the set of seven sieves as shown in *Figure 1* and following the NBR 7181/2016 (ABNT, 2016a). The retained material on each sieve was measured and stored in closed and identified plastic bags to avoid moisture content changes. The specimen followed the required grading distribution of each *URG* selected (*Figure 1*), followed by each soil sample homogenization, creating the desired composition per *URG A, C,* or *F*. This mixture reflects the control treatment specimens and as the base to receive the other treatments tested here.

For specimens with cement or hydrated lime, the soil samples receive the proper amount of either chemical stabilizer, followed by the addition of water and new homogenization. Before shaping the specimens for mechanical behavior tests, the specimens treated with cement had a 1-hour rest period, and the specimens treated with hydrated lime had a 2-hour rest period.

The research tested mechanical behavior for each *URG* and treatments (control, cement, or lime) combination (*Table 1*) for (i) compaction (Optimal Moisture Content (OMC), $\omega opt\ \%$), (ii) Dry Density ($\rho Dmax\ g/m^3$), (iii) and Unconfined Compressive Strength (UCS, $q_u\ MPa$). As established by DNER-ME 202/1994 (DNER, 1994c, similar to ASTM D1633-00, 2000), all the specimens were stored in a moisture level-controlled chamber under 23 ± 2oC ambient air moisture at 95% for the required cure time.

*Table 1 – Matrix of performed tests per URG*Treatment*

	URG A			URG C			URG F			Destiled Water (DW)
	Co	Ce 3%	Li 6%	Co	Ce 3%	Li 6%	Co	Ce 3%	Li 6%	
Cure Period (days)	0	7	14	0	7	14	0	7	14	N/A
Compaction (OMC (ωopt %) and $\rho Dmax$ (g/cm³))	3	3	3	3	3	3	3	3	3	N/A
UCS (MPa)	3	3	3	3	3	3	3	3	3	N/A
Permeability (k_{20}, cm/s)	3	3	3	3	3	3	3	3	3	N/A
Total	9	9	9	9	9	9	9	9	9	N/A
FAAS (mg/L)	3	3	3	3	3	3	3	3	3	1
Total		9			9			9		1

4.2.1 – Compaction test

Following the NBR 7182/2016 (ABNT, 2016b, similar to ASTM D1557-12e1, 2012), the compaction test evaluated the optimal moisture content (OMC) to determine the compaction curve, allowing the determination of the maximum dry density (*ρDmax*). Each specimen was shaped inside the California Bare ratio cylinder using a 4.5 kg hammer, where each one of the five layers received 26 blows under the Intermediate Proctor compaction energy.

4.2.2 – Unconfined Compressive Strength (UCS)

The Unconfined Compressive Strength test measured undrained shear strength and the stress-strain of the specimens (Head, 1992). The *UCS* tests specimens were built according to NBR 12025/2012 (similar to ASTM D1633-00, 2000) and DNER-ME180/94 standards (ABNT, 2012; DNER, 1994a; Head, 1992), under the OMC and maximum dry density determined during compaction test. The process used the Intermediate Proctor compaction energy to shape the specimens. It was built three specimens for each *URG* and treatment combination (*Table 1*), on a total of 27 units. The same cure period was used for this test. The UCS tests used a steadily increasing uniaxial load with a constant speed of 1.27 mm/minute until failure to determine the compressive stress at the failure point.

4.3 –Percolation and water assessment

The soil characterization and mechanical behavior assessment results validated the specimens' construction as average characteristics of the local unsealed roads. In addition, these steps optimized the number of permeability tests and the calcium, magnesium, and silicon content on the percolated water.

4.3.1 - Constant Head Permeability Test (Permeability test)

The Constant Head Permeability Test (Permeability test) (Head, 1992) calculated the coefficient of permeability (*k*). The permeability test simulated the water percolation on unsealed roads, mimicking the internal erosion allowing the percolation rate evaluation.

Constant Head Permeability tests followed the standard described by ASTM D 2434-68 (ASTM, 2006) using a triaxial cell with two back-pressure systems to define the samples' coefficient of permeability (*k*). The permeability test accessed the *k* for each combination on the *URG* and treatments matrix (*Table 1*), with three replicates, resulting in a total of 27 specimens evaluated. The specimen was molded into permeameter cylinders (internal diameter of 100mm, a height of 127.3mm, and a volume capacity of 1000ml), and the tests ran under a controlled temperature of 20^{o}C.

4.3.2. - Flame Atomic Absorption Spectroscopy (FAAS)

The percolated water was tested with the Flame Atomic Absorption Spectroscopy (FAAS) test (Walsh, 1955; Pires, 2010), providing the percolated content of *Ca, Mg,* and *Si*.

The sample preparation for Flame Atomic Absorption Spectroscopy (FAAS) requires a chemical extraction. The atomization process transformed the Ca^{+2}, $Mg^{+2,}$ and Si^{+4} cations into *Ca, Mg,* and *Si* atoms. A total of 27 samples were collected from the percolated water in the permeability tests. Another sample from the distilled water (DW) used at the permeability tests was also tested. The chemical solution used (i) acidification by concentrated nitric acid, (ii) followed by extraction with Strontium Chloride at 16,000 mg/L (or ppm), (iii) and dilution of the extracted sample by a ratio of 1:10 for Ca and Mg. Silicon does not require extraction solution treatment, having a direct evaluation (Pires, 2010).

5. Results and Discussion

5.1. Soil Characterization

The natural soil was characterized as A-2-6 or fine clay soil with medium plasticity (Transportation Research Board (TRB) by AASHTO), as presented in *Table 2*. According to DNIT-ES 141/2010 (DNIT, 2010a), the soil was classified as having good performance for unsealed road construction, presenting 24% fine particles (clay and silt), 63% sand, and 13% of gravel. The high percentage of fine particles (grains) in the soil explains kaolinite presence, a layered silicate clay mineral easily broken and shaped. Kaolinite at high levels promotes a lower soil expansion, increasing its natural compaction. In addition, the expected 13% gravel justifies using this original soil as the graded material for local road construction. These data matched the findings obtained by Arrivabeni et. al.. (2018).

Table 2 – Results for natural soil characterization

Physical Properties			
Liquid Limit (%)	Plastic Limit (%)	Plasticity Index (%)	Bulk density (kN/m^3)
37	23	14	26.62

Granulometric Distribution (%)					
Clay (<0.002mm)	Silt (0.002 – 0.06mm)	Fine sand (0.06 – 0.2mm)	Medium Sand (0.2 – 0.6mm)	Coarse sand (0.6- 2.0mm)	Gravel (2.00 – 60mm)
13	11	17	22	24	13

5.2. Mechanical Behavior: Compaction and Unconfined Compressive Strength (UCS)

The Compaction test results presented in *Figure 2* describe the compaction capacity via the Optimal Moisture Content (*OMC* (ω_{opt} *%)*) and the Dried Bulk Density (ρ_{Dmax} *(g/cm^3)*).

*Figure 2 - Compaction via OMC and Dried Bulk Density for URG*Treatment*

The Compaction tests showed that the cement (3%) and lime (6%) did not vary significantly at the OMC than the control test results for any *URG**Treatment combinations. The OMC. (%) range for *URG A* had the lowest mean and standard deviation (6.88±0.11), followed by *URG C* (7.94 ±0.24) and by *URG F* (11.32 ±0.52). The Dried Bulk Density (ρDmax (g/cm3)) mean was higher for *URG C*, followed by *URG A* and *URG F*, with a standard deviation very similar for all

(2.2 ±0.05; 2.19 ±0.051; and 1.99 ±0.057 respectively). These variations are due to the higher concentration of fine particles on *URG F* versus *URG A*. The higher presence of fine particles (silt and clay) increases the water-holding capacity due to the larger surface area than soils with a higher concentration of coarse particles (sand and gravel). Our results are compatible with Arrivabeni et. al.. (2018). Studies like dos Santos Ferreira et. al.. (2018) and Kogbara and Al-Tabbaa (2011) stated that slight variations at the OMC and ρ_{Dmax} within different road stabilization methods for the same road structure indicates that the road has good mechanical properties to fulfill its objective. Some of these properties include UCS, permeability, and percolation rates.

Figure 3 shows the improvement of sample resistance promoted by cement or lime as chemical stabilizers compared to the control (UCS - q_u (MPa)). The UCS increase (%) per *URG*Ce* and *URG*Li* showed an improved percentage higher than the *URG*Co*. As expected, it is evident that cement achieved a higher stabilization, followed by lime and control, respectively. The cement stabilization had its highest increment on *URG C* (increment of 1.169 MPa, 1658.6%), followed by *URG A* (increment of 1.075MPa, 1533.3%), and the lowest on URG F (increment of 1.263MPa, 842.9%). These numbers can be justified by the almost homogeneous granulometric distribution (see *Figure 1*) on *URG C*, meaning a uniform distribution among coarse, medium, and fine soil particles.

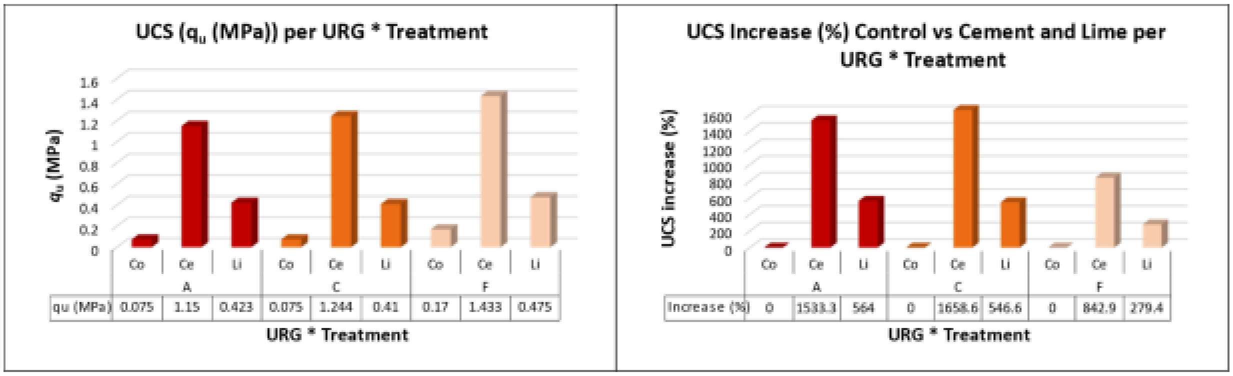


*Figure 3 - UCS results per URG*Treatment*

As expected, the *URG*Ce* specimens showed the highest improvement in the strength (resistance) against stress that can promote erosion process, followed by *URG*Li*, which is also a considerable improvement compared with *URG*Co* (mechanical) stabilization (DNIT, 2006; Machado, 2013). Ingles and Metcalf (1972) proposed that the cement or lime physicochemical process creates a bond between the soil particles and the soil matrix responsible for strength development, increasing compressibility, and reducing permeability. The results from soil characterization (*Figure 1*), compaction (*Figure 2*), and UCS (*Figure 3*) verified the conformity of the specimens with the unsealed road construction standards, allowing the continuity of the experiment.

5.3. Percolation and water assessment

5.3.1 - Constant Head Permeability Test (Permeability test)

Table 3 shows the permeability coefficient (k_{20}) for each test performed. Again, the data matched the expected results (Borio and Peila, 2010; Neptune and Putman, 2010; Ibrahim *et. al.*., 2014; Chandrappa and Biligiri, 2016; Sandoval *et. al.*., 2017).

*Table 3 - Coefficient of Permeability per URG * Treatment*

	URG A			URG C			URG F		
	Control	Cement	Lime	Control	Cement	Lime	Control	Cement	Lime
K_{20} (cm/s)	2.990E-05	0.000E+00	1.904E-05	3.149E-05	0.000E+00	2.180E-05	3.457E-06	0.000E+00	5.084E-06

All *URG*Ce* tests achieved the maximum k_{20} = 0 (cm/s), allowing us to state that our specimens treated with 3% cement became impervious. The expected outcome matches the published literature since cement is commonly used to reduce the porosity and permeability to zero, increasing the compressive strength (UCS, see *Figure 2*). URG F has a high concentration (50%) of fine particles (see *Figure 1*), meaning an expected lower porosity, leading to a lower permeability than *URG A* and *URG C*. The *URG A*Li* and *URG C*Li* achieved a lower permeability coefficient than *URG A*Co* and *URG C*Co*. These results were expected since lime reduces porosity and permeability, increasing the compressive strength (UCS, see *Figure 2*). However, *URG F*Li* achieved a slightly higher permeability coefficient than *URG F*Co*.

The *URG C*Li* had the lowest decrease in k_{20} from 3.149 e^{-05} to 2.180 e^{-05} cm/s (30.77%), followed by *URG A*Li* with the decrease from 2.99 e^{-05} to 1.904e^{-05} cm/s (36.32%). URG F*Li presented an increase in k_{20} from 3.457 e^{-06} to 5.084 e^{-06} cm /s (47%). de Brito Galvão et. al.. (2004) stated that lime's addition promotes the immediate aggregation of fine particles (case of *URG F*), inducing the increase in k_{20} value due to the formation of bigger aggregates, expanding porosity and permeability.

5.4. Flame Atomic Absorption Spectrometry (FAAS) for *Ca, Mg,* and *Si* content

As presented by the FAAS test results below, the percolation rates of *Ca, Mg,* and *Si* varied depending on the road stabilizer agent (mechanical or chemical) and the URG type. The

*URG*Ce* created a waterproof sealant in all mixtures and therefore had no percolation. Since the roads treated with cement at 3% do not allow any infiltration, the runoff runs over the road surface, enhancing the erosion pressure and surface erosion. We provide further considerations about cement stabilization and potential erosion in the conclusion section. Therefore, we discuss the *Ca, Mg*, and *Si* percolated levels for *URG*Co* and *URG*Li* only from this point on.

The permeability test (described in section 5.3.1) simulated the potential seepage on an unsealed road. Therefore, we assume that the volume of water percolated in the permeability test (simulating internal erosion) carried the potential contaminants from the stabilization methods studied here. Hence, all tests carried out up to this point served as a basis for estimating the content of calcium, magnesium, and silicon carried by the internal erosion on unsealed roads, granulometric stabilized, with or without chemical stabilization by cement or hydrated lime.

5.4.1. Calcium percolation

Calcium is a vital element and nutrient to sustain most organisms and is not toxic (Caritat *et. al..*, 2018). Even though calcium (*Ca*) has high mobility in soil, the significant geochemical barrier is its incorporation into organic matter, adsorption, and pH reduction (Caritat *et. al..*, 2018). The same authors also stated that high *Ca* levels in the soil could inhibit the availability of iron due to chemical reactions, impacting the production costs of agriculture. Ayers and Westcot (1985) stated that *Ca* needs to stay below 801.56 mg L^{-1} to minimize soil salinization. FAO defines soil salinization as the intensive increase of water-soluble salts concentration like calcium, carbonates, and magnesium. The soil salinization represents a negative environmental impact since it reduces plant growth, crop yields, turning soils inefficient (FAO, 2015).

Figure 4 shows the lixiviated calcium concentration per URG*Treatment. The *URG*Co* and *URG*Li* combinations had a higher concentration of lixiviated calcium (mg L^{-1} of *Ca*) when compared with the distilled water (DW) (24.63 ± 1.23 mg L^{-1} of *Ca*).

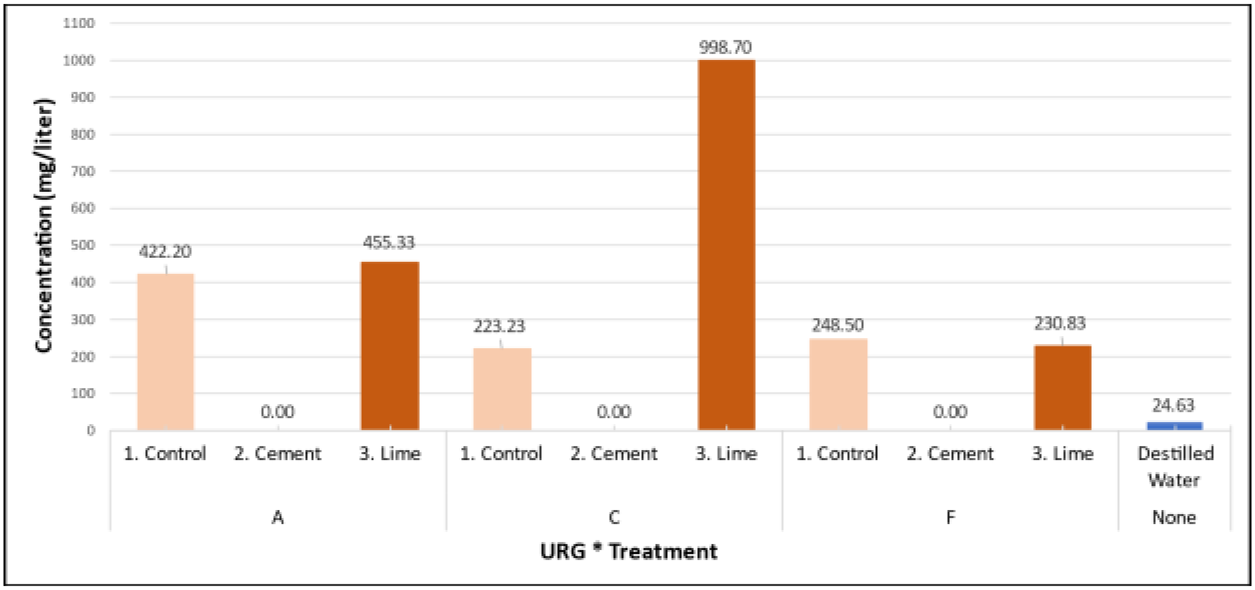


Figure 4 - Concentration of percolated calcium (Ca, mg L^{-1}) per URG Treatment*

*URG A*Co* and *URG A*Li* had a similar percolation rate, 422.2 ± 33.95 mg L^{-1}, and 455.33 ± 62.01 mg L^{-1}, respectively. Compared with distilled water, *URG A*Co* presented an increase of 397.57 mg L^{-1}, and *URG A*Li* had an increase of 430.7 mg L^{-1} on *Ca* percolated on residual water. Even though the lime stabilization improved the impermeability by 36.32% (see *Table 3*), it increased the Ca concentration of the percolated water by 7.85% (33.13 mg L^{-1}) when compared with *URG A*Co*. Knowing that the added lime was 6% of the sample weight and the mean of *Ca* concentration for *URG A Control and Lime*, our data suggests that both stabilizers could have a similar *Ca* percolated contribution to the nearest water body.

*URG C*Co* and *URG C*Li* had the highest discrepancy on percolation rate, reaching 223.23 ± 38.51 mg L^{-1} and 998.70 ± 44.07 mg L^{-1}, respectively. *URG C*Co* increased 198.6 mg L^{-1} (806.33%), the lowest increment on *Ca* concentration compared with distilled water. *URG C*Li* increased 974.07 mg L^{-1} (3954.81%) of Ca, representing the highest percolation rate than distilled water. *URG C**Li* had a low permeability rate improvement, increased the impermeability by 30.77% (*Table 3*), and a considerable increase of the *Ca* concentration compared with *URG C*Co* – 347.39%. Therefore, *URG C*Li* can represent a high impact on the contribution of *Ca* percolated to the nearest water body.

*URG F*Co* and *URG F*Li* also had a low difference in percolation rate, reaching 248.5 ± 33.37 mg L^{-1} and 230.83 ± 22.79 mg L^{-1}, respectively. Compared with distilled water, *URG F*Co* presented an increase of 223.87 mg L^{-1} (908.93%), and *URG F*Li* had an increase of 206.2 mg L^{-1} (837.19%) of *Ca* compared with distilled water. *URG F*Li* decreased permeability rate, meaning it became more permeable by 40.76% (see Table 3) than *URG F*Co*. However, *URG F*Li* had 7.11% less Ca percolation than URG F*Co because the bond between lime and the fine particles promotes a higher aggregation of the particulates, leading to increased porosity and permeability. This process mobilizes more calcium within the soil particles, reducing calcium loss on *URG F*Li* stabilization.

According to CONAMA (2005), hard water has 150 – 300 mg L^{-1} $CaCO_3$, and 'very hard' water has ≥ 300 mg L^{-1} $CaCO_3$. Hard to very hard percolated water combined with the extensive national unsealed road system (1.35 million kilometers – 79% of the total extension) plus 103.363 million registered vehicles in Brazil (CNT, 2019) could potentially lead to an increase in salinization (calcium, *Ca* or calcium carbonate, $CaCO_3$) levels in nearby water bodies. This research did not directly test water hardness, but based on that numbers, our data indicate that *URG A*Co, URG A*Li,* and *URG C*Li* could potentially contribute to a moderate to a significant impact on water salinity and harden levels. *URG C*Co, URG F*Co,* and *URG F*Li* could bring a light to moderate impact on water salinity. According to Aguilar Piratoba et. al..

(2017) and USEPA (2015), soft to moderately hard water bodies can increase the biota susceptibility to toxicity since the harder the water is, the lower the toxicity levels by heavy water metals like copper and zinc, and lead. UNEP (2008) stated that fresh water in the Americas has an average of 22 mg L^{-1} of *Ca*, and Pires et. al.. (2007) found an average of 1.5 mg L^{-1} of *Ca* on rivers inside Brazilian natural reserves. The same authors cited an average of 0.4 – 30 mg L^{-1} of *Ca* in rivers, receiving the most wastewater from cities and industrial plants. Our results indicated that calcium's high content from unsealed roads lixiviate (range of 223.23 – 998.7 mg L^{-1} of *Ca*) could promote the biota toxicity levels by boosting the water to 'hard – very hard' levels. If this hypothesis could be confirmed, unsealed roads would add potential negative impacts from anthropogenic activities such as human settlements and agriculture and their related infrastructure (Matschullat *et. al.*., 2012; Aguilar Piratoba *et. al.*., 2017).

5.4.2. Magnesium percolation

Alongside calcium, magnesium is one of the main factors for hardening water and is a primary component of chlorophyll and other biological molecules. In soil, its deficiency may cause chlorosis in the leaves, and high levels can leave a sour taste to drinking water (Pires, Vaistman, and Dutra, 2007). In addition, magnesium contributes to soil salinization at a high level, affecting germination rates, reducing yield capacity, or in extreme cases, even compromising the entire harvest (Andrade Júnior *et. al.*., 2006).

The magnesium concentrations presented in *Figure 5* detail the concentration lixiviated per URG*Treatment combination. As expected, the *URG*Co* and *URG*Li* combinations had a higher concentration of magnesium (mg L^{-1} of *Mg*) compared with the distilled water (8.63 ± 0.45 mg L^{-1} of *Mg*), except for *URG A*.

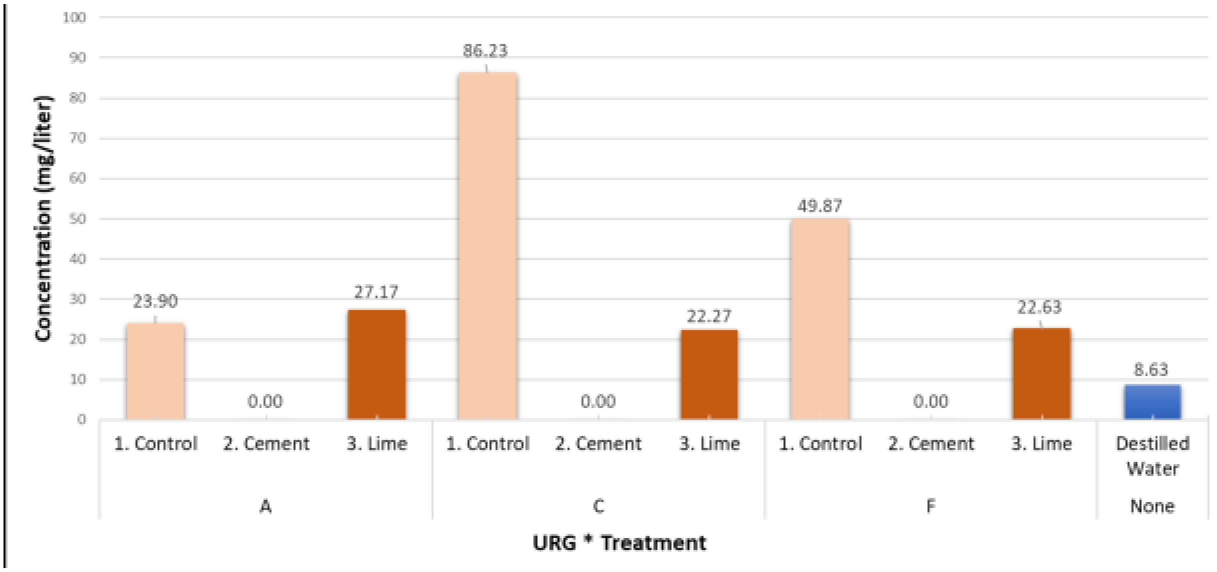


*Figure 5 - Concentration of percolated magnesium (Mg, mg L^{-1}) per URG*Treatment*

*URG A*Co* and *URG A*Li* had a similar percolation rate, 23.9 ± 2.56 mg L^{-1} and 27.17 ± 1.97 mg L^{-1}, respectively. Compared with distilled water, *URG A*Co* presented an increase of 15.27 mg L^{-1}, and *URG A*Li* had an increase of 18.54 mg L^{-1} on *Mg* percolated on residual water. As stated in section 5.4.1 (Calcium percolation), hydrated lime reduced the *URG A* permeability by 36.32% (see *Table 3*), and similar to calcium, magnesium had a considerable increment in concentration (3.27 mg L^{-1}, 13.68%) in comparison with *URG A*Co*. Based on the calcium percolation analysis principles, our data suggest that both stabilizers could also have a similar Mg contribution percolated to the nearest water body.

Contrary to *URG A*, *URG C*Co* and *URG F*Co* had a higher percolation rate than their lime-stabilized version. *URG C*Co* had the highest Mg rate - 86.23 ± 7.1 mg L^{-1} versus a rate of 22.27 ± 2.91 mg L^{-1} on *URG C*Li*, representing a difference of 74.17 % or 63.96 mg L^{-1} of *Mg* concentration between them. *URG C*Co* increased the *Mg* percolation rate by 77.60 mg L^{-1} (899.19%), and URG C*Li increased by 13.64 mg L-1 (Compared to distilled water 158.05%). This result indicates that URG C*Li can potentially reduce 74.17% of Mg percolation than URG

C*Co. It also shows that hydrated lime stabilization is an improvement over mechanical stabilization for magnesium percolation.

URG F*Co had an Mg rate of 49.87 ± 3.08 mg L^{-1} versus 22.63 ± 0.81 mg L^{-1} on URG F*Li, representing a difference of 54.62 % or 27.24 mg L^{-}1 of Mg concentration between them. URG F*Co increased the Mg percolation rate by 41.24 mg L-1 (477.87%), and URG F*Li increased by 14.0 mg L-1 (Compared to distilled water 162.22%). These results show that, like URG C*Li, the URG F*Li can also potentially reduce 54.62% of Mg percolation over URG F*Co.

Magnesium can also contribute to hardening water and is typically presented together with the maximum amounts of $CaCO_3$. The Mg percolated from unsealed roads chemically stabilized could potentially increase the water body salinity level by precipitating $MgCO_3$ and the water alkalinity level. Once more, this study did not measure the hardening water effect by the Mg percolation, but our data indicate that URG C*Co and URG F*Co could bring a moderate to a significant impact on water salinity and hardening levels. The other combinations could bring a light to moderate impact on water salinity. The water quality analysis of artesian wells from the Lajeado Erval Novo watershed, located in RS, Brazil, showed a range of 0.1 – 10.0 mg L^{-1} of Mg (Luiz *et. al.*., 2017). Ayers and Westcot (1985) defined 121.525 mg L^{-1} as the maximum Mg level to minimize soil salinization. Considering only this information, none of our tested URG*Treatment combinations (range 22.27 – 86.23 mg L^{-1} Mg) could potentially bring any considerable negative impact from magnesium percolation.

5.4.3. Silicon Percolation

In silicon dioxide (SiO2), silicon is the second most abundant element in the Earth's crust and essential to animal and plant constitution. However, highly weathered tropical soils have a low natural concentration of primary minerals that contain silicon. In other words, they have low availability of silicon for plants in superficial horizons (Trevizam *et. al.*., 2005; Marafon and Endres, 2011; Tubana, Babu, and Datnoff, 2016).

Figure 6 illustrates the silicon concentration lixiviated per URG*Treatment. Overall, URG*Li had higher Si lixiviated, similar to calcium behavior. Unsurprisingly, the URG*Co and URG*Li combinations had a higher concentration of silicon (mg L^{-1} of Si) than distilled water (33.07 ± 2.63 mg L^{-1} of Si).

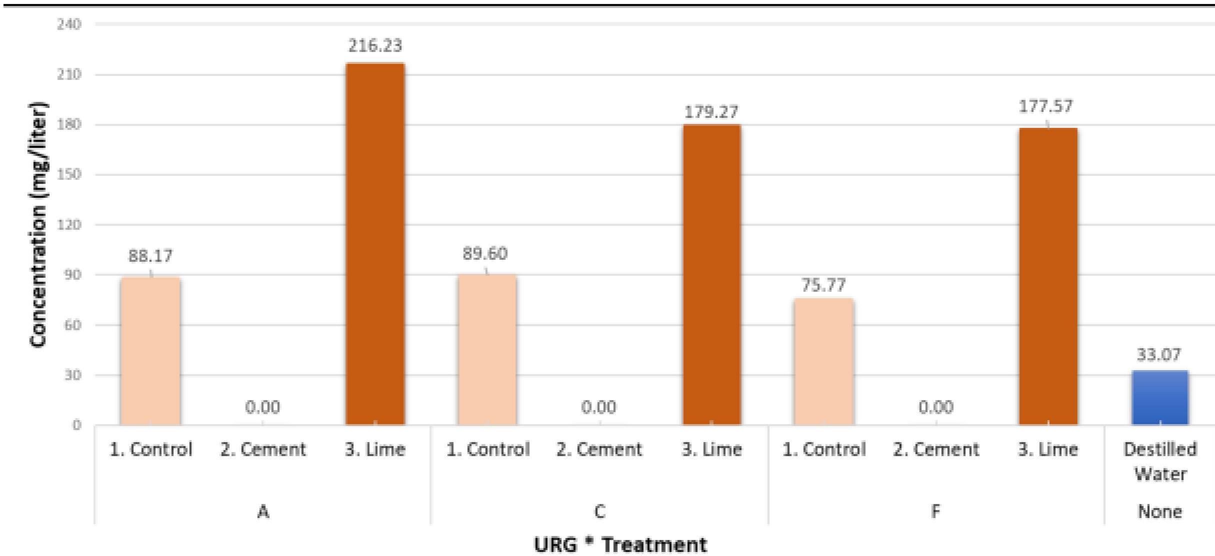


*Figure 6 - Concentration of percolated silicon (Si mg L^{-1}) per URG * Treatment*

*URG A*Co* and *URG A*Li* substantially differed at the percolation rate, 88.16 ± 7.96 mg L^{-1} and 216.23 ± 32.96 mg L^{-1}, an increase of 145.27 % (128.07 mg L^{-1}) between the two treatments. Compared with distilled water, *URG A*Co* presented an increase of 55.1 mg L^{-1} (166.67%), and *URG A*Li* had a significant increase of 183.17 mg L^{-1} (554.05%) of percolated *Si* on residual water. Thus, even though the lime stabilization reduced the URG A permeability by 36.32% (see *Table 3*), the URG A*Li had the highest relative increment of Si concentration than URG*Co.

URG C*Co had the highest Si percolation rate – 89.6 ± 6.7 mg L^{-1} versus the URG C*Li percolation rate of 179.3 ± 33.81 mg L^{-1}, a difference of 100.11 % or 89.7 mg L^{-1} of Mg concentration between them. As a result, URG C*Co increased 56.54 mg L^{-1} (171.02%) on the Si percolation rate than distilled water, and URG C*Li increased by 146.24 mg L^{-1} (442.35%).

The Si percolation rate of URG F*Co was 75.76 ± 2.35 mg L^{-1}, and the URG F*Li was 177.57 ± 25.81 mg L^{-1}, representing a difference of 134.38% or 101.81 mg L^{-1} of Si concentration between them. Compared to distilled water, URG F*Co had an increase of 42.7 mg L^{-1} (129.16%), and the URG F*Li had an increase of 144.51 mg L^{-1} (437.11%) on the Si percolation rate.

Camargo et. al.. (2013) and Korndörfer et. al.. (1999) classified the soil silicon levels for agriculture as low (< 6 mg L^{-1}), medium (6 – 24 mg L^{-1}), and high (> 24 mg L^{-1}). Epstein (1999) stated that the content of Si (in the form of H4SiO4 dissolved in soils) ranges between 2.8 – 16.8 mg L^{-1}, but its dynamic equilibrium depends on the soil pH. Tubana et. al.. (2016) cited the main seven crops worldwide (including sugarcane, maize, rice, wheat, potatoes, sugar beet, and cassava) as the principal Si accumulators, which can absorb around 210 - 224 million tons $year^{-1}$ Si worldwide. Based on these references, our data suggest that all tested URG*Co and URG*Li could significantly increase Si concentration on water bodies near unsealed roads. This impact could be positive, especially for agronomic practice in regions with a lack of plant-available Si. However, this potential impact needs to be measured.

The Ca^{2+}, Mg^{2+}, and Si^{4+} cations concentrations in our data are elevated, which may influence the soil ability to retain essential nutrients that provides a buffer against soil acidification (Brown and Lemon, 2021). This process is called Cation Exchange Capacity (CEC), and it influences the soil structure stability, nutrient availability, soil pH and effects of fertilizers or other high charge of cations in the soil (Hazelton and Murphy, 2016). These cations elevated concentration can also impact the plants development since the nutrients are not in available format to the plants.

These cations mobility is directly related to its hydrated radius, meaning that more water molecules the cations can attract to near its radius, bigger it will be its hydrated radius. Recalculating the electronic distribution, the calcium cation (atomic number of 20 and cation of 18) will have 3 layers of electrons distribution. The magnesium cation (atomic number of 12 and cation of 10) and silicon cation (atomic number of 14 and cation of 10) will have 2 layers each. Even though the silicon has a higher atomic number compared to magnesium, silicon cation lost 4 electrons and will have a smaller atomic radius compared to calcium and magnesium (each one lost 2 electrons), leading Si^{4+} to have the biggest hydrated radius among the three cations, followed by Mg^{2+}, and then by Ca^{2+}. In other words, *Si4+* has the highest mobility and higher percolation rate, followed by Mg^{2+} and then by the Ca^{2+} (Benites, 2010).

6. Conclusions and Future work

This research explored the *Ca, Mg,* and *Si* percolation on laboratory conditions to evaluate their potential as keystones parameters (markers) as environmental impacts indicators from unsealed road erosion. These three chemical components were selected due to their presence in all unsealed road chemical stabilization methods and the inexpensive traceability methods already available in the market and tested here: permeability test and FAAS.

Our data allow us to conclude that *URG*Ce* (3%) has no percolation rate at a short period of unsealed road use. This result validates the in-situ experiment, allowing the extrapolation of our results to guide the development of a large-scale experiment. Furthermore, the exploratory research presented here is highly effective since the costs of building the experimental road tracks are considerably high, which can derail the research or funding proposals in Brazil.

The calcium content for all URG*Treatment combinations suggested that the unsealed roads can elevate the freshwater to hard water status. Hard and very-hard water ($CaCO_3$ and $MgCO_3$ content above 150 mg L^{-1}) reduces the soap cleaning rate significantly, which increases their consumption and phosphates release rates, contributing to the eutrophication of water bodies. Our data also showed that *Ca* and *Mg* percolation from unsealed roads can also contribute to the eutrophication of water bodies. *URG*Li* enhanced the stabilization by chemical additive over mechanical stabilization, which indicates that these combinations could have the least negative environmental impact between *URG*Li* and *URG*Co* combinations for *Ca* percolation.

Our data shows that lime demonstrated its potential improvement over mechanical stabilization for magnesium percolation. Even though our data indicated that magnesium percolation might not represent potential environmental impact, we strongly encourage the inclusion of Mg in the next assessment steps. This research could not find concrete information about the interaction between Mg and soil particles and how they dislocate through soil layers during percolation. Therefore, we suggest studies about how Mg interacts and dislocates along with the soil layers until they reach the water bodies, assessing the potential Mg accumulation in soil and water.

Bastos (2014) and Tubana et. al.. (2016) presented evidence that changes in the Si supply chain caused by dams, agriculture, and other anthropogenic activities directly impact phytoplankton communities, altering the quality of the entire aquatic ecosystem. Facing these factors, we recognize that Si percolated from the unsealed roads erosion process may become a valuable source to be recycled and reused for crop maintenance and the regeneration of impacted aquatic ecosystems.

Based on all the presented facts, we suggest extending the calcium, magnesium, and silicon content assessment by expanding the study to a field scale, adding variables such as seasonality, weather variation, road parameters (design, construction, use, and location), and cation exchange capacity. In addition, we suggest a complete water quality assessment (including heavy metals, dissolved oxygen., turbidity, and sediments) of the percolated water to trace how each element can be accumulated, generating potential environmental impacts. Data collection should be near the unsealed road boundaries, the closest water bodies, and the closest water table to map all the potential interaction layers. These future studies can support a complete understanding of chemical contamination factors originating from unsealed roads and accurate water body accumulation. Collecting enough data can allow the proposal to include assessing *Ca*, *Mg*, and *Si* percolation rates on unsealed road construction and maintenance to measure potential environmental impacts on environmental impact assessments.

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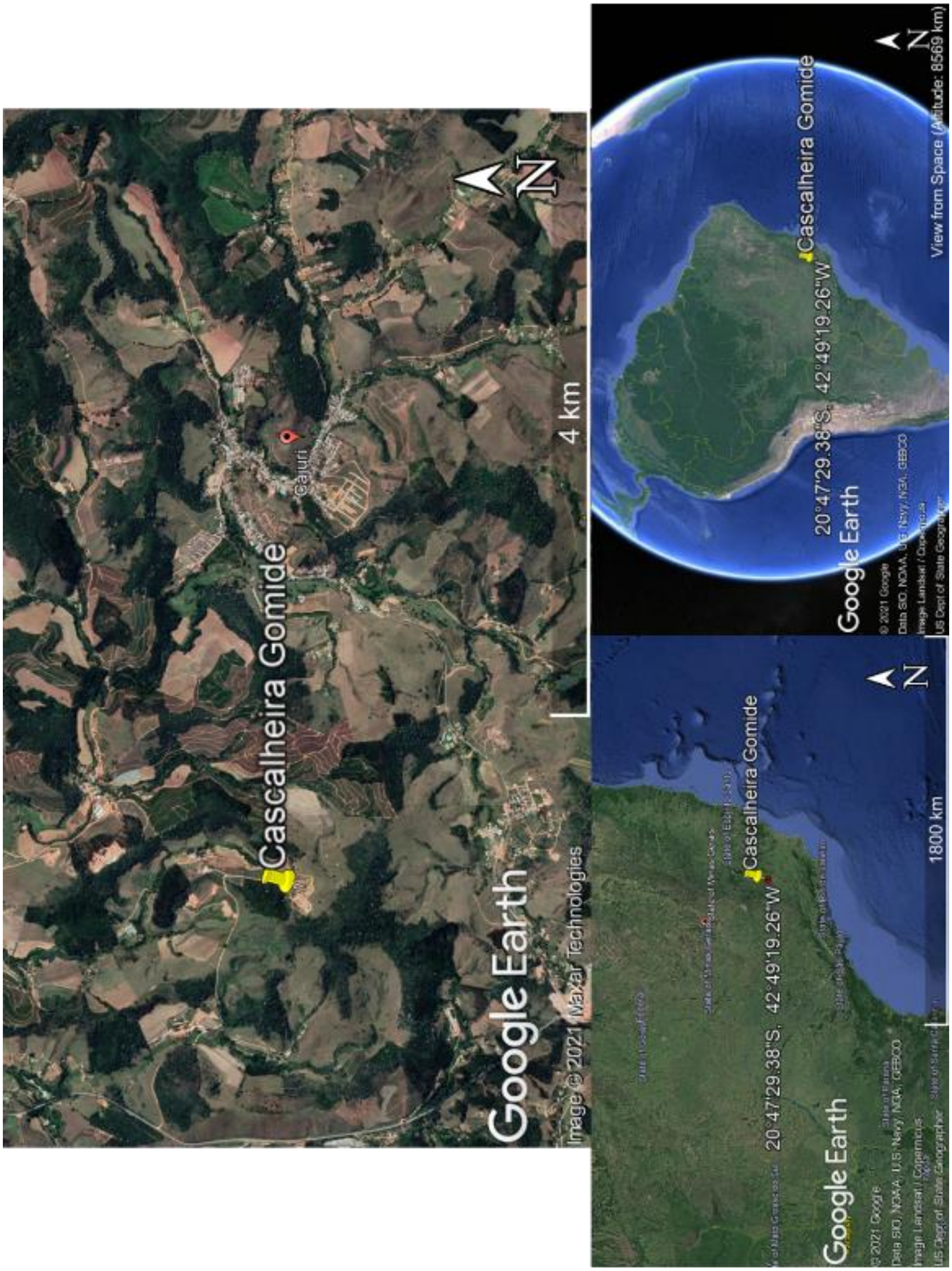
Appendix A

Location of the gravel pit (cascalheira) Gomide. Location of Gomide gravel pit at the city, state, and country levels. Maps built on Google Earth Pro software by Carla Portugal



Chapter 4: Assessment of *Serratia ureilytica* as a sustainable alternative for unsealed road biocementation

Ph.D. candidate Carla Ribeiro Machado e Portugal [1], Dr. Carolyn Fonyo[2], Dr. Carlos Cardoso Machado[3], Dr. Richard Meganck[4]

1 - Author for correspondence. Ph.D. candidate at Environmental Sciences Graduate Program. Oregon State University. Graduate School - Heckart Lodge, 2900 SW Jefferson Way. Corvallis, OR – USA. 97331-1102. Telephone: +1 (541) 9797-8741. Carla.portugal@oregonstate.edu. ORCID: https://orcid.org/0000-0001-9508-8831

2 – Director of Environmental Sciences Graduate Program. Oregon State University. Graduate School - Heckart Lodge, 2900 SW Jefferson Way. Corvallis, OR – USA. 97331-1102. Carolyn.fonyo@oregonstate.edu. ORCID: https://orcid.org/0000-0002-8919-6585

3 – Distinguished Professor, Federal University of Viçosa, College of Forest Engineering. Avenida Peter Henry Rolfs, S/N, Viçosa, MG – Brasil. Zip code 36570-900. Machado@ufv.br. ORCID: https://orcid.org/0000-0002-2386-9791

4 - Institute for Water and Watersheds, part of the UNESCO-ICIWaRM category 2 center, Oregon State University, USA. rameganck@gmail.com. ORCID: https://orcid.org/0000-0002-1523-9992

Abstract

The biocementation by microbiologically induced calcium carbonate precipitation (MICCP) in clayey soil was studied in three different granulometric stabilizations (*URG*) for unsealed road construction and compared with cement and hydrated lime stabilization for unsealed roads. The selection of an indigenous gram-negative ureolytic bacteria allowed the assessment of a low environmental impact species and potentially lower cost compared with commercially available products. This experiment tested variation on the granulometric distribution to minimize bioclogging (biocementation treatments). The biocementation promoted a permeability rate decrease by up to 10^2-fold, reaching as low as 2.44E-06 cm/s for biocementation. These results demonstrated an improvement over the lowest permeability rate of 5.084E-06 cm/s for hydrated lime and a promising alternative to substitute cement, which reached a permeability rate of 0.00E+00 cm/s. The highest permeability rate reduction occurred on URG samples treated with complete granulometric distribution and no compaction (BioFull), ranging from 98.25% to 72.24% of reduction, followed by the URG samples treated with granulometric distribution without fine particles with compaction (BioCoarse), ranging from 95.64% to 42.47% of permeability reduction. Hydrated lime presented an average of 47.06% to 36.20% in permeability reduction, and cement presented a 100% permeability reduction. The scanning electron microscopy (SEM) images showed the $CaCO_3$ precipitation in the early stages, concluding that the biocementation protocol can be enhanced. More studies are logically required, but the results suggest that biocementation by *Serratia ureilytica* may be a sustainable alternative for unsealed roads stabilization compared to the environmental impact of cement and hydrated lime.

Keywords: Biocementation, nucleation site, ureolytic MICCP, unsealed road, cement, lime, road stabilization

1 - Introduction

Unsealed roads are necessary for developing a region, considering that they provide a social, economic, and political benefit for the surroundings (Rammelt; Leung, 2017). However, the implementation and use of these roads generate negative impacts on the environment, including the primary source of soil degradation caused by water erosion (Thomaz; Peretto, 2016). Unsealed roads are also susceptible to erosive processes, mainly due to soil characteristics and hydrological conditions of its location, which in some cases, can increase the sedimentation volume even higher than those produced in agricultural areas (Cao; Zhang; Zhang, 2009). Additionally, roads directly affect the hydrology of its basin water by changing the natural water flow and flooding patterns, concentrating runoff, interfering with the subsurface flow, all contributing to road damage over time. These interactions create a cycle that demands constant maintenance, adding another layer of environmental impacts such as intensifying erosion and sedimentation (Deligianni, 2020; Machado et. al.., 2014).

The main factors that impact surface erosion of unsealed roads are the intensity and duration of rain, snowfall, the characteristics of surface materials, the hydraulic characteristics of the road surface, highway, traffic flow, construction and maintenance, and the runoff rates near and over the road lanes (Fu et. al.., 2010; Machado et. al.., 2014). Therefore, increasing soil mechanical strength is an alternative to decrease soil erodibility. In this sense, biomineralization is an environmentally innocuous technology under extensive development around the globe, particularly in developing countries (A'la et. al.., 2020; Cheng et. al.., 2017, 2017; Enriquez, 2017; Hoang et. al.., 2020; Portugal et. al.., 2020; Zhao et. al.., 2014).

2 - Background

Biomineralization is a natural process induced by bacteria (in soil, seawater, and even in the human body), having the microbiologically induced calcium carbonate ($CaCO_3$) precipitation (MICCP) as its common manifestation. Bacteria are heavily present in soils and vary in size, shape, and metabolic activity. Since they are only microns wide, bacteria can move freely through the soil voids, and when introduced in the proper environment with careful management, can start the MICCP process. The MICCP is led by bacterial ureolysis, leading to selecting species with intense urease enzyme activity. The urease catalyzes the urea to carbonate, which increases the pH, ammonium, and carbonate concentrations. When in the presence of calcium ions, the calcium carbonate precipitates as one of its polymorphic forms: calcite, vaterite, or aragonite (A'la et. al.., 2020; Gomez et. al.., 2018; Hoang et. al.., 2020; Omoregie et. al.., 2017). The calcium carbonate precipitation naturally leads to encapsulating the bacteria due to the precipitation around the cell, but the bacteria end up dying in an augmented process like MICCP. The MICCP can be catalyzed by urease (*UA*) or by carbonic anhydrase (*CA*), having the *UA* process resulting in higher $CaCO_3$ precipitation rates (Zhu and Dittrich, 2016). Portugal et. al.. (2020) did an extensive review on the MICCP process and its interaction with *UA* and *CA*, but in summary, the equations below describe the MICCP via ureilytica bacteria (hydrolysis of urea and $CaCO_3$ precipitation).

$$CO\,(NH_2)_2 \,+\, H_2O \xrightarrow{urease} NH_2COOH \,+\, NH_3 \qquad \text{Equation 1}$$

$$NH_2COOH \,+\, H_2O \,\Rightarrow\, NH_3 \,+\, H_2CO_3 \qquad \text{Equation 2}$$

$$H_2CO_3 \,\Leftrightarrow\, H^+ \,+\, HCO_3^- \qquad \text{Equation 3}$$

$$2NH_3 \,+ 2H_2O \,\Leftrightarrow 2NH_4^{1+}(g) \,+\, 2OH^- \qquad \text{Equation 4}$$

$$HCO_3^- \,+ H^+ \,+ 2OH^- \,\Leftrightarrow CO_3^{2-} \,+ 2H_2O \qquad \text{Equation 5}$$

$$Ca^{2+} \,+\, Bacteria\ cell \,\rightarrow Bacteria\ cell - Ca^{2+} \qquad \text{Equation 6}$$

$$Bacteria\ cell - Ca^{2+} \,+\, CO_3^{2-} \,\Leftrightarrow Bacteria\ cell - \,CaCO_3 \qquad \text{Equation 7}$$

The urease hydrolyzes the urea, resulting in carbamic acid and ammonia (Equation 1). Then, the carbamic acid hydrolyses to ammonia and carbonic acid (Equation 2). Finally, the ammonia and bicarbonate reach the equilibrium in this aqueous solution, forming hydrogen carbonate, ammonium, and hydroxide ions (Equations 3, 4, and 5), which increases the pH. In this alkaline environment, the hydrogen carbonate ion will reach equilibrium by precipitating carbonate ions, which in the presence of calcium ions, culminates in calcium carbonate precipitation at the external bacterial cell wall (Equations 6 and 7) (Portugal et. al.., 2020; Stocks-Fischer et. al.., 1999).

The calcium carbonate ($CaCO_3$) precipitated is considered a cohesive material that is an additive to sandy soils. This cohesion improves soil engineering properties, such as impermeability and shear strength (A'la et. al.., 2020; Gomez et. al.., 2019; Sidik et. al.., 2014a). The MICCP is a potential candidate for soil stabilization since it generates a biocement with reduced environmental impact than Portland cement, lime, and gravel extraction processes. Some studies show that the MICCP improves shear resistance and decreases soil permeability (Ivanov and Chu, 2008; Ng et. al.., 2012; van Paassen et. al.., 2010; Whiffin et. al.., 2007). Shahrokhi-Shahraki et. al.. (2015) indicated that the MICCP precipitation mimics natural processes by depositing calcium carbonate ($CaCO_3$ polymorphic as calcite, aragonite, or vaterite) on the grains of the soil, thus increasing shear strength and resistance, leading to a reduction in permeability and erodibility. Calcium carbonate is a common molecule in nature, making it an attractive element for research on biocementation. In addition, urease bacteria are common in the environment, potentially eliminating non-indigenous bacteria for the in-situ soil treatment.

Several biomineralization methodologies are currently being evaluated, including biostabilization, classified as a potential alternative for soil stabilization processes. Biostabilization consists of improving the geotechnical properties of soils by precipitation of calcium carbonate ($CaCO_3$). The precipitation may result from a microbial metabolic process such as photosynthesis, urea hydrolysis, and sulfate reduction, among others (Valencia et. al.., 2014). The process is also known as microbiologically induced calcium carbonate precipitation (MICCP), where the precipitated *CaCO3* biocement combines the soil particles and reduces the soil hydraulic conductivity (Umar et. al.., 2016).

Studies conducted by Soon et. al.. (2013) revealed the efficacy of $CaCO_3$ precipitation induced by microorganisms, improving shear strength up to 96% while reducing soil permeability. Furthermore, given the significant contribution of biomineralization in soil stability, several studies have been developed, including isolation and identification of microorganisms with higher

$CaCO_3$ precipitation rates, identification of limiting factors and environmental parameters to promote maximum ureolytic activity, evaluation of *$CaCO_3$* precipitation rates in non-sterile conditions, and in-situ bacterial growth, among (Cheng et. al.., 2017; Cheng et. al.., 2013; Shahrokhi-Shahraki et. al.., 2015; van Paassen et. al.., 2010; Whiffin et. al.., 2007; Yasuhara et. al.., 2011).

Studies conducted on biocementation for soil biostabilization on unsealed roads are still scarce, given the complexity of the process and homogeneity of the biocementation capable of supporting the load-bearing capacity of the roads. Our research group, the CETEFLOR of the Federal University of Viçosa-BR, hosts the development team on soil biostabilization for unsealed roads research and the potential reduction of erosive processes. The research advances include isolating indigenous soil MICCP bacterium species (*Serratia ureilytica* sp), identifying the bacterium as an efficient MICCP microorganism, and defining the optimal conditions for bacterial growth in the sand. Furthermore, our MICCP microorganism (*Serratia ureilytica*) produces higher rates of ureolytic activity when compared with *Sporosarcina pasteurii* (Enriquez, 2017).

3 – Material & Methods

3.1 – Soil characterization & unsealed roads classification

The Gomide gravel pit (Cascalheira Gomide), located at Cajuri, State of Minas Gerais, Brazil, (coordinates 20º47’29.38” S and 42º49’19.26” W) was the location of soil sample collection used to construct the regional base and sub-base layers of unsealed and sealed road construction (Arrivabeni *et. al.*., 2018). Appendix A.1 shows the geographic coordinates for the Gomide gravel pit. Appendix A.2 provides a zoom at the Gomide gravel pit (A), and the photo shows a close-up of the soil collected for this research (B) (Photo credit: Altair Carrasco de Souza, 2016). The maps were built using Google Earth Pro software (Jones, 2021).

The gravel pit soil was classified as A-2-6 or fine clay soil with medium plasticity, as presented in Table 1 (ASTM D2487-17, 2017). Therefore, the soil categorization defined it as good performance for unsealed road construction, presenting 24% fine particles (clay and silt), 63% sand, and 13% gravel, according to DNIT-ES 141/2010 (DNIT, 2010a). Furthermore, the gravel content at 13% allows using this soil as a granulometric material for unsealed road construction.

Table 1 - Soil characterization, Gomide gravel pit.

Physical Properties			
Liquid Limit (%)	Plastic Limit (%)	Plasticity Index (%)	Bulk density (kN/m^3)
37	23	14	26.62

Granulometric Distribution (%)					
Clay (<0.002mm)	Silt (0.002 – 0.06mm)	Fine sand (0.06 – 0.2mm)	Medium Sand (0.2 – 0.6mm)	Coarse sand (0.6- 2.0mm)	Gravel (2.00 – 60mm)
13	11	17	22	24	13

The soil characterization followed the standardized tests presented in Portugal et. al.. (2021, to be submitted) as follows: Granulometric Analysis ABNT NBR 7181 (ABNT, 2016) to define the granulometric soil distribution; ABNT NBR 6508:2016 (ABNT, ABNT NBR 6508:2016 - Grãos de pedregulho retidos na peneira de abertura 4,8 mm - Determinação da massa específica, da massa específica aparente e da absorção de água) for bulk specific gravity, apparent specific gravity, and water absorption; ABNT NBR 6459 (ABNT, NBR 6459: Solo – Determinação do limite de liquidez) for Atterberg limits; and ABNT NBR 7180 (ABNT, NBR 7180: Solo – Determinação do limite de plasticidade) to test liquid and plasticity limit, and to calculate the soil plasticity index. The tests were conducted at the Civil Engineering Laboratory at the Federal University of Viçosa, located in Viçosa, Minas Gerais, Brazil.

The Brazilian Paving Manual (DNIT, 2006) classifies unsealed road grading (*URG*) into six different types (A to F). Each *URG* has a specific granulometric distribution, which allows road lanes with different load-bearing capacities. For example, *URG A* and *B* can sustain heavy traffic loads and volumes, *URG C* and *D* can sustain intermediate traffic, and *URG E* and *F* can sustain low traffic (DNIT, 2006). Our research selected *URG A, C,* and *F* to assess the unsealed road permeability behavior stabilized with biocement by *Serratia ureilytica* versus the traditional chemical stabilization via cement or hydrated lime. Furthermore, the *URG A, C,* and *F* selection allows for an extension of the research conducted by Portugal et. al.. (2021, to be submitted).

3.2 – Biological stabilization by biocement

3.2.1 - Microorganism

The *Serratia ureilytica* was the aerobic, gram-negative, urea-dissolving, non-spore-forming bacteria selected with biocementation via Microbiological Induced Calcite Precipitation (MICCP). The inoculum was isolated from local soil samples at Environmental Biotechnology and Biodiversity Laboratory of Federal University of Viçosa, Brazil. The *S.* ureilytica culture was extracted and cultivated following the protocol developed by Enriquez (2017), an adaption of Whiffin (2004).

Serratia ureilytica is an ureolytic bacteria, hydro-anaerobic, meaning that they thrive in low-oxygen conditions. The *S. ureilytica* has a rod shape (bacillus), measuring 0.9 um by 0.85um, and its membrane is anionic, resulting in a high surface area per volume (ratio). The nucleation site formation happens when the anionic membrane attracts positive ions, including Ca^{2+}, and its sedimentation over the membrane surface (Alghamri et. al.., 2016; Mostavi et. al.., 2015).

3.2.2 - Bacterial replication and biocementation solution

The cultivation media selected for the isolated colonies was the solid media type TSA (Trypticase Soy Agar) + Urea at BOD (Bio-Oxygen Demand) incubator temperature under 30^{o}C for 96 hours. The solid media had 40g/L of TSA, 5g/L of agar, and 1.33M of Urea. Then, followed the colonies application in feeding liquid media (M1) to grow for 24 hours. The M1 media consisted of 20g/L of yeast extract, 0.1 M of nickel chloride (NiCl2), and 0.17M of Urea, under 10.5 pH per liter of deionized water. The bacterial solution was incubated in a shaker under 200 rpm at 30^{o}C. The bacteria were grown for 24 hours until they reached optical density (OD_{600}) of 2.5 – 2.9 or ~10^9 cells/L, designated the ‘stationary stage.’ The urease activity was 30 mM of hydrolyzed urea/min. The bacterial solution was stored at 4^{o}C until used (Mortensen et. al.., 2011).

3.3 – Chemical stabilization treatments

3.3.1 – Stabilization by cement

This study selected the Cauê cement CPII E-32 for general use, a composite of pure Portland cement and granulated slag from a blast furnace. The CPII E-32 is the recommendation of the Brazilian Pavement Manual, DNIT ES 143/2010, and DNIT 422/2019 standards (DNIT, 2006, 2010b, 2019) for chemical stabilization of unsealed roads.

3.3.2. – Stabilization by hydrated lime

This study selected the Dical hydrated lime type CH III, which complies with the Brazilian Pavement Manual and DNER 180/1994 (DNER, 1994a; DNIT, 2006) resistance levels required for chemical stabilization unsealed road construction.

3.4. – Distilled water

This study used distilled water to shape the specimens and run all the tests (Head, 1992; Sandoval *et. al.*., 2017).

3.5 – Specimen preparation and treatments

The specimen preparation followed the same protocols adopted in Portugal et. al.. (2021, submitted) but with adjustments. First, to attend the granulometric distribution of the soil, samples were passed through a series of sieves (Table 2), according to the NBR 7181/2016 (ABNT, 2016). Second, the segregated materials were measured and stored in sealed and tagged plastic bags to avoid moisture content changes. Finally, the specimens molding had the same weight following the granulometric distribution of each *URG*, as presented in Table 2.

The bioclogging is one of the main constraints to the MICCP process, and it means that the bacteria and the cementation solution reach the maturation process much faster than the percolation rate, significantly reducing the biocementation distribution and resistance homogeneity. Therefore, this study adapted the granulometric distribution of the *URG A*, *URG C*, and *URG F* to test alternatives that can sustain the traffic flow and meet the Brazilian standards for unsealed roads. Since our preliminary tests demonstrated that the *S. ureilytica* biocementation via MICCP presented bioclogging near the injection areas, this study selected two alternative treatments to test the variation on granulometric distribution and compaction (Table 2), denominated Biocementation Full Particles No Compaction (*BioF*) and Biocementation Coarse Particles Compacted (*BioC*), which both received the biocementation treatment. The Control Full Particles No Compaction (*CF*) was the control for the *BioF* treatment, and the Control Coarse Particles Compacted (*CC*) was the control for *BioC* treatment. Table 2 presents the granulometric distribution per combination between *URG* profile and treatment, defining the granulometric distribution of each specimen tested in this study.

Table 2 - URG granulometric distribution of specimens per treatment.

Sieve	CF & BioF (Standard URG)						CC & BioC (Granulometric Adjusted URG)						Control & Cement & Lime (Standard URG)					
	URG A		URG C		URG F		URG A		URG C		URG F		URG A		URG C		URG F	
	Weight (g)	%	Weight (g)	%	Weight (g)	%	Weight (g)	%	Weight (g)	%	Weight (g)	%	Weight (g)	%	Weight (g)	%	Weight (g)	%
# 4 (4.76 mm)	473.7	15.79	777.6	25.92	450	15	1124.9	37.5	1750.2	58.4	2000	66.7	473.7	15.79	777.6	25.92	450	15
# 10 (2.00 mm)	789.6	26.32	555.3	18.51	225	7.5	1875.1	62.5	1249.8	41.6	1000	33.3	789.6	26.32	555.3	18.51	225	7.5
# 40 (0.42 MM)	852.6	28.42	666.6	22.22	825	27.5	0	0	0	0	0	0	852.6	28.42	666.6	22.22	825	27.5
# 200 + residual (≤ 0.074 MM)	884.1	29.47	1000.5	33.35	1500	50	0	0	0	0	0	0	884.1	29.47	1000.5	33.35	1500	50
Total	**3000**	**100**	**3000**	**100**	**3000**	**100**	**3000**	**100**	**3000**	**100**	**3000**	**100**	**3000**	**100**	**3000**	**100**	**3000**	**100**

Each combination between *URG (A*, *C, or F*) and treatment (*BioF, BioC*, cement, or hydrated lime) had three replications, as detailed in Table 3. The soil mixed with cement at 3% rested for 1 hour, and the soil mixed with hydrated lime at 6% rested for 2 hours before shaping the specimens. The specimens treated with biological solution did not require a rest period before shaping. All treatments also had a cure period before testing for permeability rates and scanning via scanning electron microscopy (SEM), as presented in Table 3.

Table 3 - Details of specimen preparation.

Treatment	Characteristic	Tests	Replications per URG (A, C and F)	Proctor's compaction energy	Number of layers	Blows per layer - Proctor's hammer	Cure period after treatment (Days)
Control (Co)	Standard granulometric distribution, compacted, no stabilizer added	Permeability	1	Normal	3	26	7
Control-Full-Particles-Not-Compacted (CF)	Standard granulometric distribution, not compacted, no stabilizer added	Permeability	3	N/A	N/A	N/A	7
Biocementation-Full-Particles-Not-Compated (BioF)	Standard granulometric distribution, not compacted, treated with biological stabilizer	SEM Permeability	1 with 3 subsamples 3	N/A	N/A	N/A	7
Control-Coarse-Particles-Compacted (CC)	Adjusted granulometric distribution, compacted, no stabilizer added	Permeability	3	Normal	3	26	7
Biocementation-Coarse-Particles-Compacted (BioC)	Adjusted granulometric distribution, compacted, treated with biological stabilizer	SEM Permeability	1 with 3 subsamples 3	Normal	3	26	7
Cement (Ce)	Standard granulometric distribution, compacted, treated with 3% of cement (chemical stabilizer)	SEM Permeability	1 with 2 subsamples 1	Normal	3	26	7
Hydrated lime (Li)	Standard granulometric distribution, compacted, treated with 6% of hydrated lime (chemical stabilizer)	SEM Permeability	1 with 2 subsamples 1	Normal	3	26	14

This experiment built two sets of 3 replicates per URG & biocementation treatment combination, with one set for permeability rate tests and the second set for SEM sample collection. The decision to use two sets was to avoid the potential wash off of the biocement during the permeability tests since the experiment aimed to test the biocementation content and distribution. The permeameter cylinder was the mold used to build all the specimens, according to Enriquez (2017), an adaptation of (Whiffin et. al.., 2007).

3.5.1 – Biocementation treatment (*BioFull* & *BioCoarse*)

The specimens that received the Biocementation Full Particles No Compaction (*BioFull)* treatment were built considering the standard full granulometric distribution of each *URG* and no compaction. The goal was to test whether the granulometric distribution would allow constant permeability to avoid reducing permeability and consequent bioclogging. The absence of compaction was to test the effect of average void volume and potential bioclogging. The specimens that received the Biocementation Coarse Particles Compacted (*BioCoarse*) treatment were built considering only the coarse particles of each URG and compaction. The goal was to test the *S. ureylitica* behavior over coarse particles used on URG and compare these results with BioFull. This idea came from the success rate of *S. ureilytica* biocementation in the sand (Enriquez, 2017). These results will provide evidence about the bacteria behavior over unsealed road soil (granulometric stabilized) since the previous results for *S. ureilytica* at Enriquez (2017) only considered sandy soil specimens. The performed compaction guaranteed the apparent uniform density of each URG, which reduced the void spaces among the coarse particles but not enough to reduce the permeability rate significantly. The specimens were shaped inside the permeameter cylinder, layered as shown in Figure 1, and according to parameters presented in Table 4.

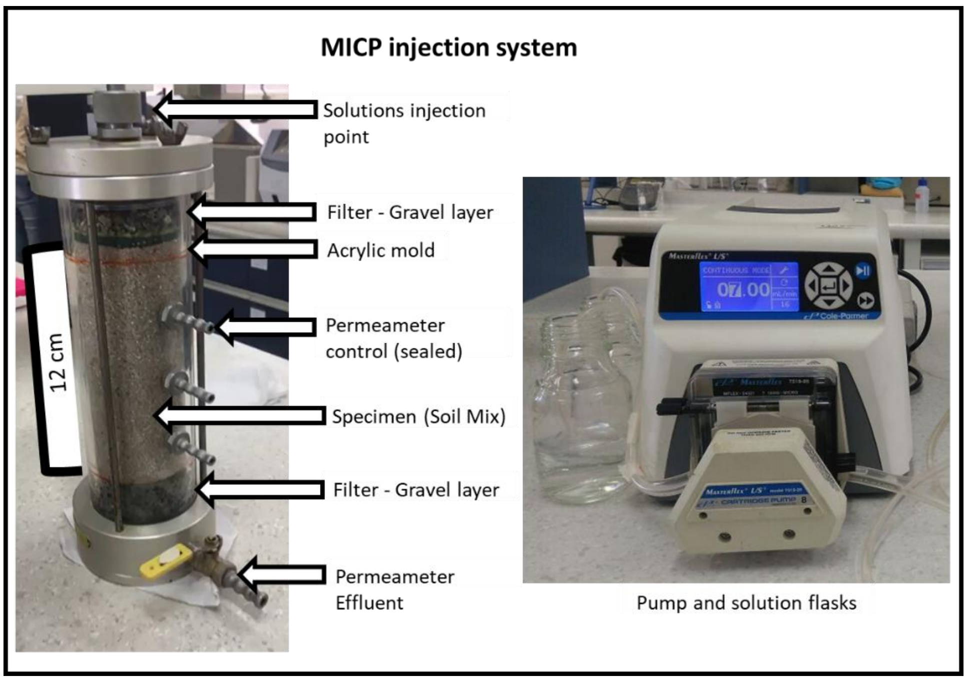


Figure 1 – MICCP solutions injection system. On the left, the distribution of the layers on each specimen settled inside the permeameter cylinders. On the right, the pump model used on the MICCP injection system. All the specimens had the exact dimensions: 6.5 cm of diameter per 12 cm of height—images from the author's archive.

Each specimen assembled vertically promoted the descendent flux, having the biocementation treatment solutions applied at the top via the peristaltic pump Masterflex L/S, model 7519-20 at a constant flow of 24 mL/h. The volume of each solution per specimen type was as defined in Table 4. All the experiments were performed at the ambient temperature of 20±2 °C.

Table 4 - Specimens and solutions parameters for biocementation.

Specimen Parameter	Unit	Cont Coarse or BioCoarse URG A	Cont Coarse or BioCoarse URG C	Cont Coarse or BioCoarse URG F	Cont Full or BioFull URG A	Cont Full or BioFull URG C	Cont Full or BioFull URG F
Specimen volume	cm^3	931.67	931.67	931.67	773.13	773.13	773.13
Mass of Mold and Soil	grams	3914	3834	3928	3601	3534	3612
Mass of Mold	grams	2074	2074	2074	2074	2074	2074
Mass of Wet Soil	grams	1840	1760	1854	1527	1460	1538
Water content	%	10.6	10.4	10.5	10.6	10.4	10.5
Volumetric Water content	$grams/cm^3$	195.0	183.0	194.7	161.9	151.8	161.5
Mass of Dryed soil	grams	1645.0	1577.0	1659.3	1041.0	1022.0	1015.0
Soil Bulk density	$grams/cm^3$	1.76	1.69	1.78	1.35	1.32	1.31
Biocementation Solution Parameters	**Unit**						
Particle density at 20° C	$grams/cm^3$	2.69	2.71	2.70	2.69	2.71	2.70
Soild Space ((Bulk density / particle density)*100)	%	65.32	62.47	65.88	50.19	48.71	48.52
Porosity (100 - Solid Space)	%	34.68	37.53	34.12	49.81	51.29	51.48
Porosity volume	cm^3	323.07	349.70	317.87	385.13	396.55	398.02
Liquid Media flow rate	cm^3 or ml/ min	5.4	5.8	5.3	6.4	6.6	6.6
Bacterial solution applied	cm^3 or ml	40	44	40	48	50	50
Fixation solution applied	cm^3 or ml	485	525	477	578	595	597
Cementation solution applied	cm^3 or ml	323	350	318	385	397	398

Each specimen received the bacterial solution application after the bacterial solution reached the required OD_{600} [2] with a bacterial growth rate at 2.5 – 2.9 or ~10^9 cells/L. The bacterial solution volume was defined based on its porosity (%) and flow rate at one porosity volume (cm^3 or ml/min), as shown in Table 4. The first application was the bacterial solution at 0.125 soil

[2] OD refers to the Optical Density (OD) measurements of microbial and cell growth, common method in microbiology laboratory. The OD_{600} defines the measuring of optical density at 600 nm, meaning that our bacterial culture was harvested at an optimun point measured at 600 nm.

porosity volume (cm^3) of the specimen column, followed by fixation solution (50 mM $CaCl_2$, cm^3) at 1.5 porosity volume. After the fixation solution, the cementation solution (1M of $CaCl_2$ and 1M of urea, cm^3) was applied at one porosity volume and left to rest for 2 hours. After the rest period, it followed a second application of the cementation solution and then a rest period of 24 hours. After the second resting period, the third application of cement solution was followed by a resting period of 7-days to reach mechanical resistance (full curing time).

3.5.2. – Chemical stabilization with cement or with hydrated lime

For specimens with cement or with hydrated lime, the soil samples receive the proper amount of either chemical stabilizer (cement = 3% or hydrated lime = 6%), followed by the addition of water and homogenization. Before shaping the specimens for percolation tests, the specimens treated with cement had a 1-hour rest period, and the specimens treated with hydrated lime had a 2-hour rest period. The specimens were then shaped directly on the percolation cylinders with details presented in Table 5. The specimens from *URG A* and *C* did not reach the calculated optimum moisture content (water volume calculated), which required the addition of water (water volume applied) until the specimen reached the optimal moisture level (OMC) under the normal Proctor compaction energy.

Table 5 – Mass of the components of specimens treated with cement or with hydrated lime.

	URG A		URG C		URG F	
	Cement (Ce) - 3%	Hydrated Lime (Li) - 6%	Cement (Ce) - 3%	Hydrated Lime (Li) - 6%	Cement (Ce) - 3%	Hydrated Lime (Li) - 6%
Wet Soil weight (g)	2750	2668	2850	2850	2700	2850
Chemical Stabilizer weight (g)	77.34	150.12	79.89	159.78	79.78	159.56
Optimum Moisture Content (OMC, %)	7.1	7.1	8.21	8.21	11	11
Water volume calculated (g)	18.6	23.61	38.13	44.88	110.66	119.52
Water volume applied (g)	130.2	165.27	152.52	134.64	110.66	119.52

3.6 – Specimen assessment – tests

3.6.1 – Constant head permeability tests

The constant head permeability test assessed the permeability of the specimens following NBR 13292/1995. The test evaluated the effect of the *BioFull* and *BioCoarse* biocementation treatment on the permeability of each *URG*. In addition, the biocementation effect was compared with cement and with hydrated lime permeability of each *URG* to verify the potential reduction in permeability by biocementation.

The layers inside the permeameter cylinders followed the same structure for permeability tests as presented in Figure 1Figure 2 shows the constant head permeability system. Figure 2A presents a specimen after the permeability test. Note how some soil crossed the black line on the cylinder body and merged into the gravel filter layer. Figure 2B shows the water system used to maintain the constant head pressure of the water column. The *BioFull* and *BioCoarse* specimens first received the MICCP treatment via MICCP injection system (Figure 1), including the final cure period of seven days, and then were assessed by the constant head permeability tests (Figure 2).

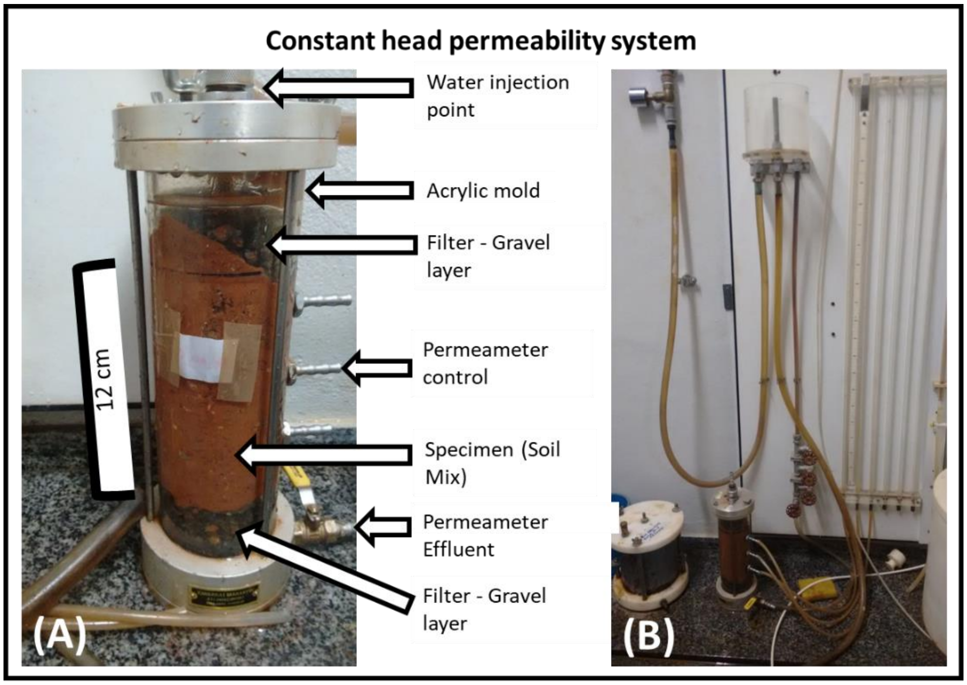


Figure 2 -Permeameter system via constant head flow. (A) Details on the specimen after the permeability test. (B) The constant head system, used for the permeability tests of all specimens. All the specimens had exact dimensions: 6.5 cm of diameter per 12 cm of height—images from the author's archive.

3.6.2 – Scanning electron microscopy (SEM) assessments

The scanning electron microscopy (SEM) evaluated the presence of the calcite precipitation on the specimens treated with biocementation (*BioFull* and *BioCoarse*). Due to the high cost of each assessment, one specimen per combination between treatment (*BioFull* or *BioCoarse*) and *URG* (*A*, *C*, or *F*) provided three samples under different depths. The first sample was collected at the range of 0-6 cm from the injection point, the second sample at 6-12 cm, and the third one at 12-18 cm. These ranges allowed the verification of how the bioclogging could be affecting each treatment.

The cement and hydrated lime are conventional chemical treatments for unsealed road stabilization and are considered uniform across road lane depth. Therefore, the samples for SEM analysis were collected in two layers: 0-6 cm and 6 -12 cm.

All the samples were prepared for SEM assessment as described by Enriquez (2017). The samples were dried in a drying oven with circular airflow, under 60^{o}C, for 72 hours. The samples were gold coated with a sputter coating Quorum Q150R before SEM analysis. The SEM microscope was the LEO 1430VP.

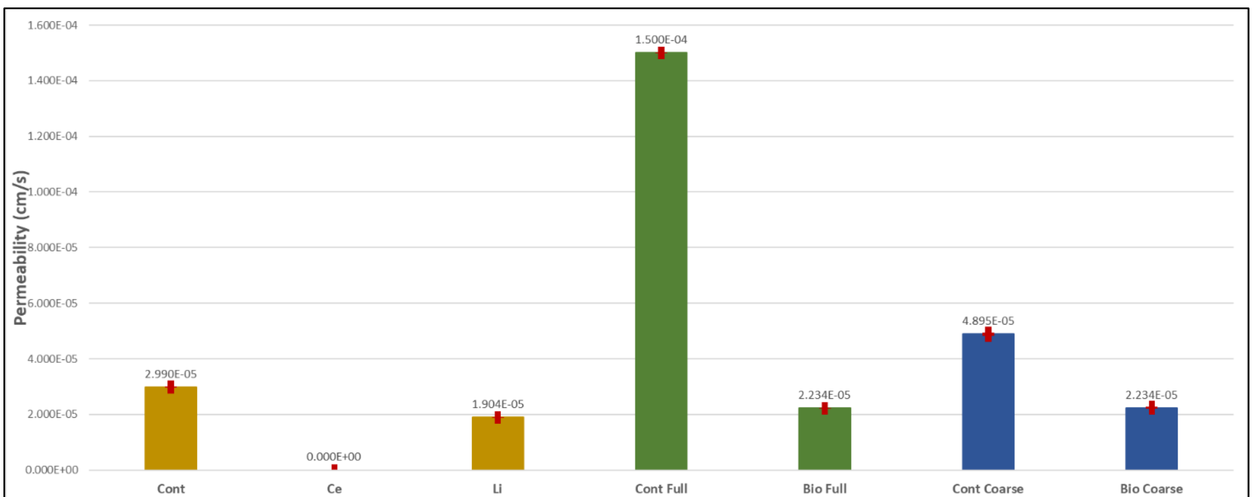
4. – Results and Discussion

4.1 – Effect of biocementation versus classical treatments on permeability

Soil treatments targeting road enhancements can improve soil properties, with permeability reduction as a primary improvement measurement. Figure 3, Figure 4, and Figure 5 detail the changes in treatment permeability levels (*BioFull*, *BioCoarse*, *Ce*, and *Li*) versus the controls (*Cont*, *Cont Full*, and *Cont Coarse*) in clayey soil samples granulometric stabilized for unsealed roads. The DNIT control (*Cont*), cement (*Ce*), and lime (*Li*) samples followed the classic standards defined by DNIT (2010a, 2019, 2010b, 2010c). However, the samples built under DNIT standards for granulometric stabilized roadbed did not accept the biological treatment since the MICCP injection system did not generate any effluent, and the biological treatment did not permeate through the samples. The authors concluded that the compaction demanded by DNIT standards reduced the air voids so that the formation of nucleation sites was impossible. According to (Gollapudi et. al.., 1995 and Sidik et. al.., 2014), the MICCP process is more efficient in the presence of pores, ensuring enough space for the bacterial nucleation sites. Ezzat and Ewida (2021) demonstrated that it is possible to reach MICCP on samples with fine particles, but silt and clay in high quantity can compromise bacterial activity. Moreover, Ezzat and Ewida (2021) demonstrated that soils poorly graded with medium to fine particles could allow the MICCP process. Since this is an exploratory study focused on finding if the biological treatment can substitute for the classic and chemical unsealed roads stabilization methods, we tested some variations on granulometric distribution to test the bacterial behavior and its potential permeability rate reduction to allow its use for unsealed road stabilization, as implied by (Portugal et. al.., 2020).

The samples marked by *Full (Cont Full and Bio Full)* means that the specimens shaping followed the full *URG* granulometric distribution (Table 2) but suppressed the compaction required by the standards. The goal was to test the granulometric distribution defined by the standards isolated from the compaction factor, generating the samples denominated Control Full Granulometric, No Compaction (*Cont Full*), and Biologically Treated Full Granulometric, No Compaction (*Bio Full*). The samples marked by *Cont Coarse* (No Fine Particles with Compaction) were adapted and did not have the fine particles but had compaction demanded by the standards (Table 2). The goal was to test if the fine particles would impact the results via bioclogging and if the compaction without fine particles would allow void spaces large enough to support the nucleation sites and avoid bioclogging. Therefore, the samples were denominated Control No Fine Particles, with Compaction (*Cont Coarse*), and Biologically Treated No Fine Particles, with Compaction (*Bio Coarse*). Overall, cement presented with the lowest permeability, followed by *Bio Full, Bio Coarse*, *Li*, and controls.

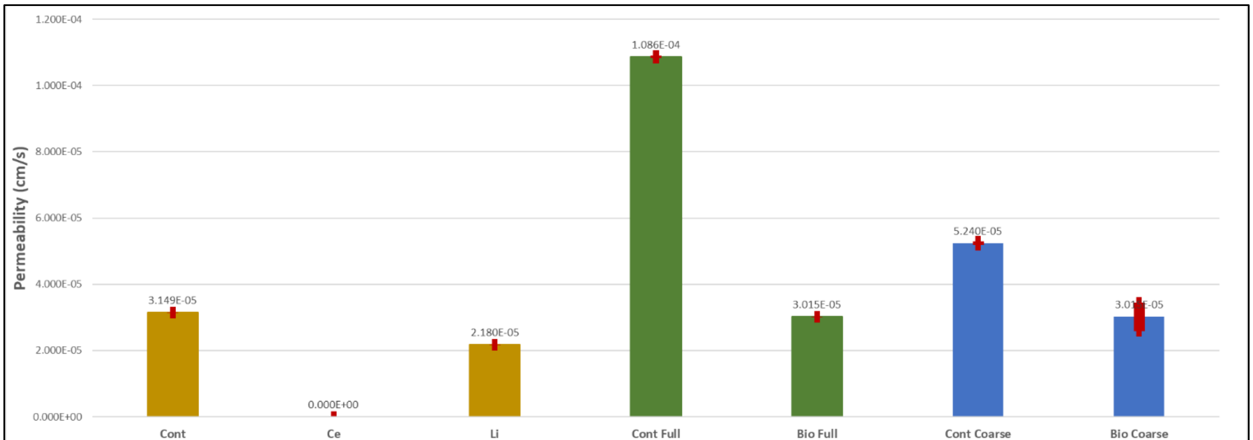
Figure 3 shows the summary for *URG A* treatments. The data corroborate with literature (Arrivabeni et. al.., 2018; Ibrahim et. al.., 2014), where cement turns the specimen impervious, and the hydrated lime reduces the permeability by 47% (from *Cont URG A* mean at 2.990E-05, and st. dev. ± 2.00E-07, to *Li URG A* mean at 1.904E-05, and st. dev. ± 2.000E-08) but stays on the same 10^{-5}-fold scale. This result was the lowest permeability reduction for *URG A*. The *Cont Full* treatment had the second overall most significant permeability reduction when comparing treatments within its control group, and the biological treatment had the greatest reduction in permeability, presenting a significant 10-fold reduction. Decreasing by 85.10% from mean at 1.500E-04 (st. dev. ± 2.000E-07) at control *Cont Full URGA* to mean at 2.234E-05 (st. dev. ± 1.528E-08) at *Bio Full URGA* treatment, it is evident that biological treatment with full granulometric distribution and no compaction reduces the permeability of the soil. The *Cont Coarse* treatment had similar behavior as *Li* versus *Cont*, with an intermediate reduction of 54.36%, from mean at 4.895E-05 (st. dev. ± 5.900E-07) for *Cont Coarse URG A* to mean at 2.234E-05 (st. dev. ± 3.394E-07) for *Bio Coarse URG A*.



	Cont URG A	*Ce URG A*	*Li URG A*	*Cont Full URG A*	*BioFull URG A*	*Cont Coarse URG A*	*Bio Coarse URG A*
Mean	2.990E-05	0.000E+00	1.904E-05	1.500E-04	2.234E-05	4.895E-05	2.234E-05
Standard Deviation	2.000E-07	0.000E+00	2.000E-08	2.000E-07	1.528E-08	5.900E-07	3.394E-07

Figure 3 - Variation in the permeability on URG A per treatment. Cont = DNIT protocol control; Ce = cement; Li= lime; Cont Full = control at full granulometric and no compaction; Bio Full = biological treatment at full granulometric and no compaction; Cont Coarse = control with no fine particles and with compaction; Bio Coarse = biological treatment with no fine particles and compaction. Standard deviation marked as red bars.

Figure 4 shows the summary for *URG C* treatments, which follows the same trend as *URG A*. The data also corroborates with literature (Arrivabeni et. al.., 2018; Ibrahim et. al.., 2014), with the cement specimen reaching an impervious level. The hydrated lime had the lowest permeability reduction, decreasing only 30.76%, from *Cont URG C* with mean at 3.149E-05 (st. dev. ± 3.606E-08) to *Li URG C* mean at 2.180E-05 (st. dev. ± 2.517E-08), staying on the same 10-fold scale for permeability rate. The *Cont Coarse URG C* had the second overall highest reduction, decreasing by 42.47%, from *Cont Coarse URG C* mean at 5.240E-05 (st. dev. ± 6.012E-07) to *Bio Coarse URG C* mean at 3.015E-05 (st. dev. ±4.286E-06). The treatment *Bio Full* had the highest permeability rate reduction for *URG C*, reaching 72.24%, from *Cont Full URG C* mean at 1.086E-04 (st. dev. ±3.215E-07) to *Bio Full URG C* mean at 3.015E-05 (st. dev. ± 1.155E-08). Note that for URG C, the biocementation procedure significantly reduced the permeability rate by a 10-fold scale, meaning a drastic reduction in the specimen porosity.



	Cont URG C	*Ce URG C*	*Li URG C*	*Cont Full URG C*	*BioFull URG C*	*Cont Coarse URG C*	*Bio Coarse URG C*
Mean	3.149E-05	0.000E+00	2.180E-05	1.086E-04	3.015E-05	5.240E-05	3.015E-05
Standard Deviation	3.606E-08	0.000E+00	2.517E-08	3.215E-07	1.155E-08	6.012E-07	4.286E-06

Figure 4 - Variation in the permeability on URG C per treatment. Cont = DNIT protocol control; Ce = cement; Li= lime; Cont Full = control at full granulometric and no compaction; Bio Full = biological treatment at full granulometric and no compaction; Cont Coarse = control with no fine particles and with compaction; Bio Coarse = biological treatment with no fine particles and with compaction. Standard deviation marked as red bars.

Figure 5 shows the summary for *URG F* treatments. As it happened on *URG A* and *URG C*, cement also made the *URG F* specimens impervious, corroborating with (Arrivabeni et. al.., 2018; Ibrahim et. al.., 2014). Hydrated lime had a permeability increase by 47.06%, from *Cont URG F* mean at 3.457E-06 (st. dev. ±2.517E-09) to *Li URG F* mean at 5.084E-06 (st. dev. ±2.517E-09). Since the URG F specimens are rich in fine particles, the hydrated lime trend went on a different direction as compared with *URG A* and *URG C*. The *Bio Coarse* and *Bio Full* had an excellent performance, reaching impressive permeability reduction rates. While the *Bio Coarse* treatment reduced permeability by 95.64%, from *Cont Coarse URG F* mean at 5.602E-05 (st. dev ±8.038E-07) to *Bio Coarse URG F* mean at 2.442E-6 (st. dev. ± 5.282E-07), decreasing by a 10-fold scale, the *Bio Full* treatment reduced the *URG F* permeability rate by 98.25%, from *Cont Full URG F* mean at 1.139E-4 (st. dev. ± 2.517E-07) to *Bio Full URG F* mean at 2.442E-6 (st. dev ± 1.528E-09), decreasing an impressive 10^2-fold scale at permeability rate. It is evident that the biological treatment reduces soil permeability to lower levels than the control treatment (*Cont URG F* = 3.457E-06). The biocementation treatment for *URG* F specimens had the highest permeability reduction compared with *URG A* (Figure 3) and *URG C* (Figure 4), plus reached a lower permeability rate than lime treatment. Considering the *URG F* specimens had a higher concentration of fine particles, the porosity and presence or absence of compaction on the specimens could have affected the bacteria nucleation site or its biocementation ratio.

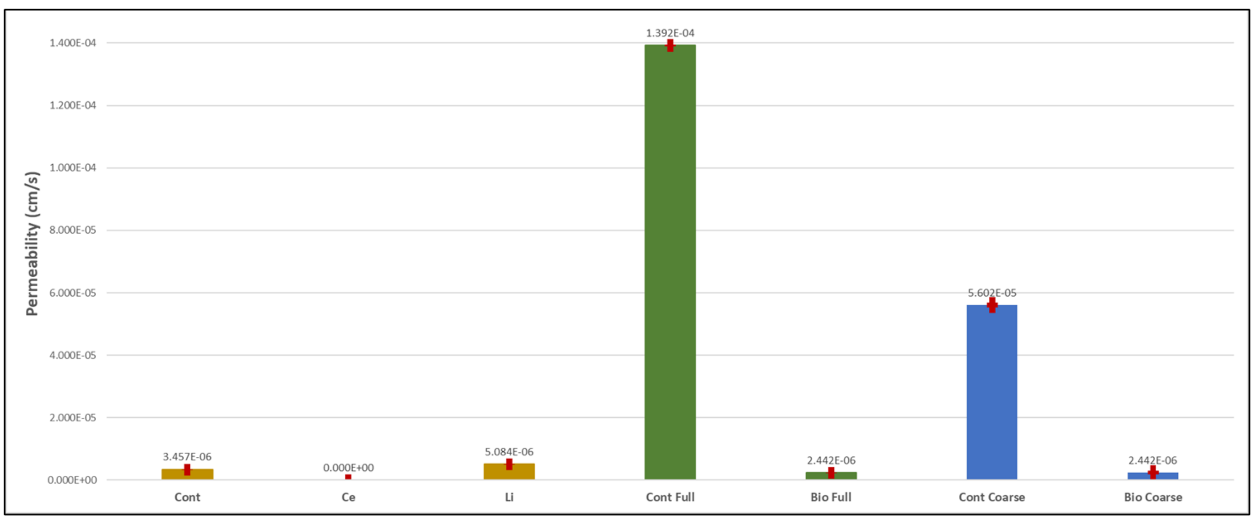


Figure 5 - Variation in the permeability on URG F per treatment. Cont = DNIT protocol control; Ce = cement; Li= lime; ContFull = control at full granulometric and no compaction; BioFull = biological treatment at full granulometric and no compaction; ContCoarse = control with no fine particles and with compaction; BioCoarse = biological treatment with no fine particles and with compaction. Standard deviation marked as red bars.

Overall, all samples returned a very clustered standard deviation, meaning that the permeability rate of each sample was very close to the mean, which was also influenced by the small sample size of the experiment.

We did not compare the controls vs. treatments for the different granulometric distributions and compactions, but we compared each treatment with its control. Even so, the percentage of permeability reduction per each treatment can bring extra insights. For example, cement represents a 100% reduction in permeability despite the *URG* tested, explaining why its use is widely accepted. However, cement production has a high environmental cost (Anastasiou et. al.., 2015; Balaguera et. al.., 2019; Trigaux et. al.., 2017), supporting the search for potential sustainable alternatives such as presented in this exploratory research. Overall, the reduction in the permeability by *BioFull* and *BioCoarse* treatments via MICCP by *Serratia ureilytica* sp versus their controls indicate that this biocementation procedure is a potential permeability solution for lime replacement.

Cement resulted in a 100% reduction in permeability in all *URG*s. Besides cement, *BioFull* presented a permeability reduction higher than *BioCoarse*. Though the preliminary results are favorable, the MICCP approach still demands more studies to consider other variables such as seasonal variability and impacts, traffic load bearing and traffic intensity as presented by Machado et. al.. (2006), and durability of the treatment over time. *URG F* had the highest permeability rate reduction compared to each control for both MICCP treatments, reducing the permeability rate by 98.25% with *BioFull* and 95.64% with *BioCoarse*. In contrast, the lime treatment increased permeability by 47.06% compared with its control (*Cont Coarse URG F*), which means that the lime treatment was less effective than the control treatment. *URG A* had the second-highest permeability rate reductions compared to each control for MICCP treatments.

The *BioFull* reached a permeability rate reduction of 85.10%, and *BioCoarse* reduced permeability by 54.36%. The lime treatment also reduced the permeability rate by 36.32% when compared with its control. The *URG C* had the lowest permeability reduction. The *BioFull* reduced permeability by 72.24%, and the *BioCoarse* reduced permeability by 42.27%. The *URG C* lime treatment resulted in a permeability rate reduction of 30.76% when compared with its control. Considering that unsealed roads treated with lime have lower durability when compared with cement (Arrivabeni et. al.., 2016), the MICCP treatments also presents itself as a potential alternative over lime stabilization. However, it still demand more research on the durability of biocement and a complete life cycle assessment of the unsealed road stabilizers tested here, allowing a complete comparison and definition of sustainability.

4.2 – Scanning electron microscopy (SEM) assessments

The SEM images of the *URG* soil samples after biocementation treatments were captured at various sample depths. The images indicated that $CaCO_3$ (calcium carbonate, possible calcite, or aragonite) crystals precipitated on the surfaces of the particles at all depths sampled, and some also show the bonding bridge of $CaCO_3$ crystals between soil particles. The $CaCO_3$ crystals prove that the bacteria are nucleation sites for the biomineralization process (Achal et. al.., 2009; Sidik et. al.., 2014a).

Figures 6 to 11 show that our ureolytic bacteria induced the $CaCO_3$ precipitation, and the crystals created bonds among the particles, enhancing the soil impermeability compared with the control treatment (Appendix B.1 and Appendix B.2 bring enlarged images). Figure 6 to 8 show the treatment Biocementation Full Particles No Compaction (*BioFull*) per *URG* (*A*, *C*, and *F*) at three different sample depths. The goal was to check for $CaCO_3$ precipitated crystals and the bonding bridge of crystals between the soil particles. Each figure shows the details of the *$CaCO_3$* crystals at their early stage of precipitation with a spheric-like shape. The early stages indicate that the *S. ureilytica* may support additional applications of biocementation solutions to enhance precipitation and uniformity (Ezzat and Ewida, 2021; Sidik et. al.., 2014b). Figure 8B shows the extension of the bonding bridge of *$CaCO_3$* crystals connecting soil particles.

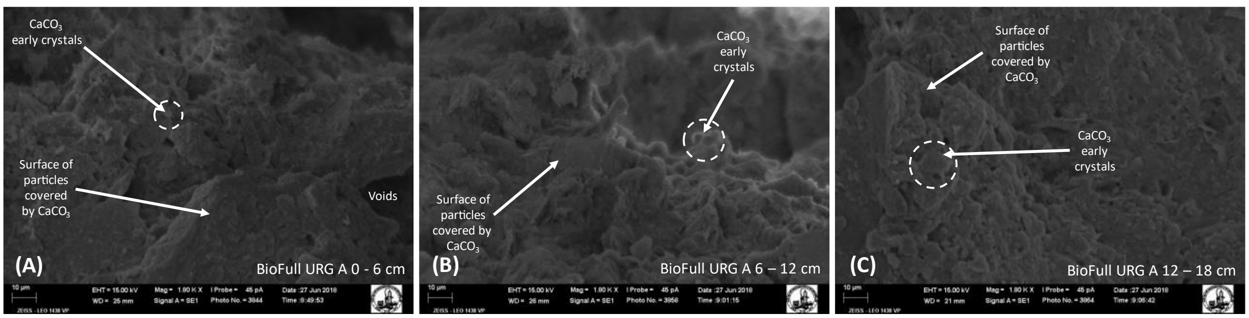


Figure 6 - BioFull on URG A at three different sample depths (A, B, and C). All images are under 1800 X TM.

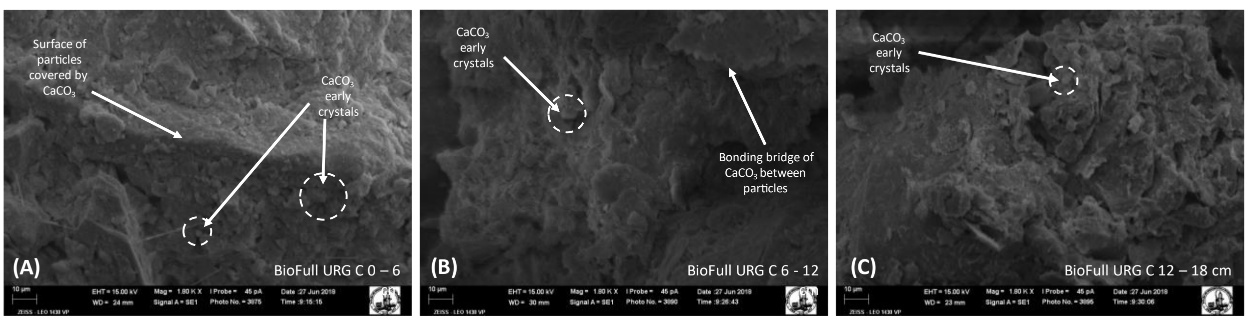


Figure 7 - BioFull on URG C at three different sample depths (A, B, and C). All images are under 1800 X TM.

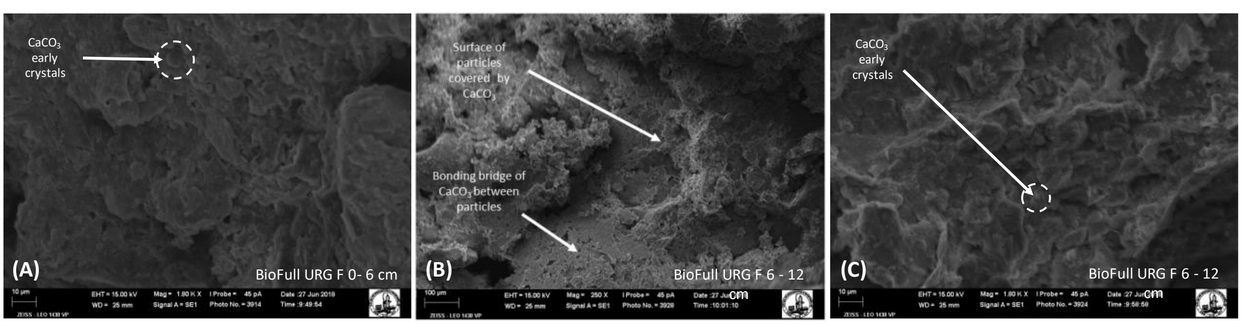


Figure 8 - BioFull on URG F at two different sample depths (A or B and C). All images are under 1800 X TM. B and C were taken from the same sample depth, different locations.

Figure 9 to 11 show the treatment Biocementation Coarse Particles Compacted *(BioCoarse*) per *URG* (*A*, *C*, and *F*) at two different sample depths, scanning for the $CaCO_3$ precipitated crystals and the bonding bridge of crystals among the soil particles. Each figure also shows the details of the $CaCO_3$ crystals at their early stage of precipitation, with a spheric-like shape. In addition, Figures 9 to 11 show that the bonding bridge of calcium carbonate crystals connecting soil particles was less extended, less common, and even not present on *BioCoarse* treatment, different from the *BioFull* treatment, which supports the lower impermeability rates for *BioCoarse* versus *BioFull* (see Figure 9, Figure 10, and Figure 11).

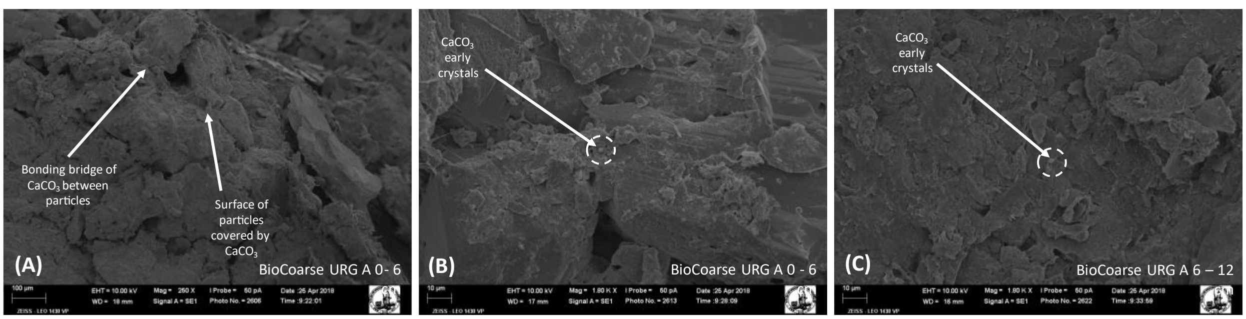


Figure 9 - BioCoarse on URG A at two different sample depths. Image A is under 250 XTM, and B and C are under 1800 X TM. A and B were taken at 0 – 6 cm depth in the sample.

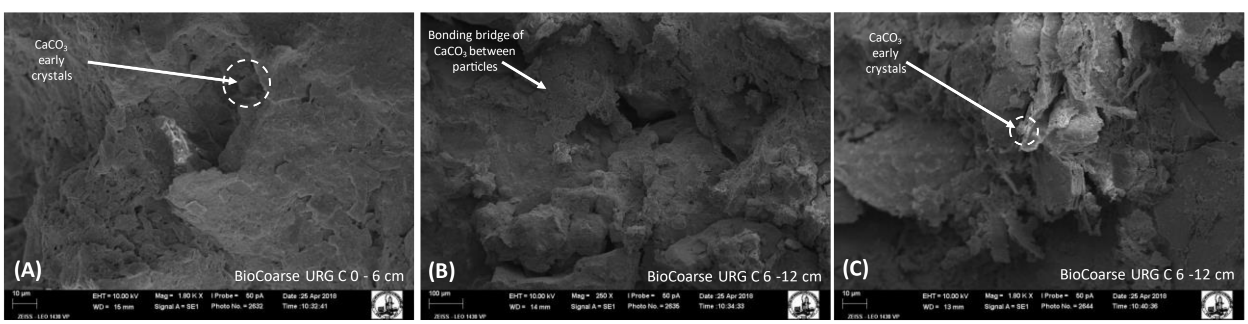


Figure 10 - BioCoarse on URG C at two different sample depths. Image A and C are under 1800 XTM, and B is under 250 X TM. B and C were taken at 6 - 12 cm depth in the sample.

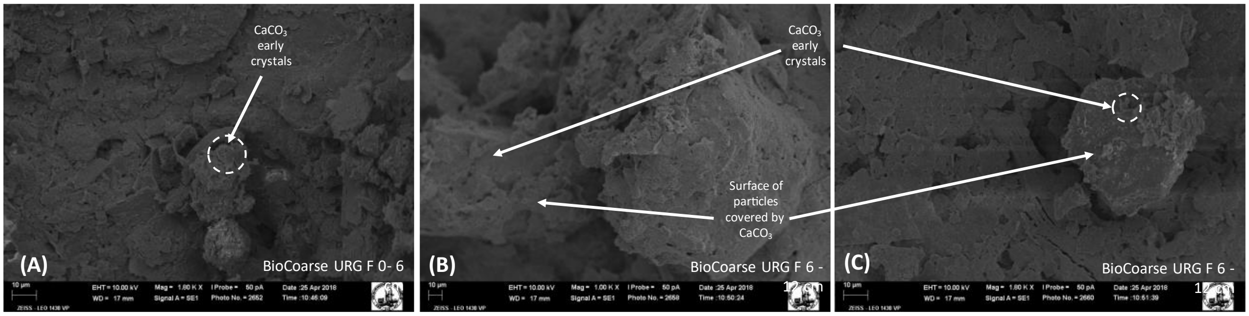


Figure 11 - BioCoarse on URG F at two different sample depths. Image A and C are under 1800 XTM, and B is under 250 X TM. B and C were taken at 6 - 12 cm depth in the sample.

The precipitation of the $CaCO_3$ crystals was not uniform, as shown in Figure 9, Figure 10 and Figure 11, independent of the treatment and URG. Our results can be supported by (Zhao et. al.., 2017, 2014). However, the authors suggested that the injection system may have disturbed the formation of the crystals, and they suggested sample immersion into the biocementation solution. This suggestion is not possible for road construction, eliminating this test from our pool. The immersion system allowed a more uniform crystal distribution, including the bonding formation among the soil particles. Since our images showed the bonding formation, we understand that our bacterium can fulfill the biocementation and uniformity desired for road construction.

The SEM images do not show the imprint of the bacterial cells (which should be spotted as a black/dark void of 0.9 x 0.85 µm) on any $CaCO_3$ crystals, which indicates that crystal precipitation may be due to a pure chemical reaction. Alternatively, this chemical reaction may happen via diffusion of CO_3^{-2} ions from inside the bacterial cells (site of urea hydrolysis) to the biocementation solution in the environment, leading to precipitate rhombohedral crystals under the calcite conformation. The precipitation happens once the Ca^{+2} and CO_3^{2-} reach critical supersaturation (Al-Thawadi and Cord-Ruwisch, 2012; Cheng and Shahin, 2016; Portugal et. al.., 2020).

4.3 – *$CaCO_3$* precipitation in organic soils – further discussions

Sidik et. al.. (2014) demonstrated that $CaCO_3$ precipitation occurs in organic soils but is lower than in sandy soils. In addition, Lebron and Suarez (1998) stated that organic soils contain soluble organic ligands and other organic matter that inhibit $CaCO_3$ precipitation and crystal growth. Many inhibition mechanisms are under evaluation, and scientists (Canakci et. al.., 2015; Inskeep and Bloom, 1986; Lebrón and Suárez, 1998; Lin et. al.., 2005; Lin and Singer, 2006; Sidik et. al.., 2014b) have highlighted the absorption of the organic molecules onto a mineral surface as one of the inhibiting factors which, depending on the saturation condition, can induce crystal dissolution or impair its growth. Another factor cited was the organic matter content preventing the $CaCO_3$ precipitation since it coats the existing $CaCO_3$ crystal surfaces, blocking the nucleation site and inhibiting homogeneous crystal growth.

The soil structure regulates the biochemical reactions in the biocementation process via the available pore network. The pore network regulates the diffusion of the reactants to the nucleation sites, directly influencing the kinetics of the biochemical reactions. This pore network also functions as the reaction surface. *S. ureilytica* has an average size of 0.85 µm by 0.9 µm, meaning that this bacterium strain can move freely among the soil particles, including the granular distribution of URG, tested in this research. However, once the bacterium gets in contact with the cementation solution, the biochemical process starts forming the nucleation sites, and the bacterium distribution moves to bioclogging stages. We recommend expanding research on the nucleation site and other factors that affect the biocementation process to understand how to reach a homogeneous biocementation layer. Some authors reached homogeneous layers on sand material (Cheng et. al.., 2013; Hoang et. al.., 2020; Ivanov and Chu, 2008; Shahrokhi-Shahraki et. al.., 2015; van Paassen et. al.., 2010; Whiffin et. al.., 2007), which is not feasible material for road construction. Therefore, there is a need for more studies on the mechanisms required to reach a uniform biocementation layer in organic-clayey soils.

5 – Conclusions

Preliminary results indicate that *Serratia ureilytica* is a strong candidate as a sustainable alternative for unsealed road stabilization and is worthy of further investigation. In the *URG F* granulometric distribution profile, *BioFull* had the most significant permeability reduction, a total of 98.25% compared to its control, followed by *BioCoarse* with a 95.64% permeability reduction, and in contrast, hydrated lime resulted in a 47.06% increase in permeability. *BioFull* had the greatest permeability reduction for the *URG A* profile, or 85.10% compared to its control, followed by *BioCoarse* with a 54.36% permeability reduction and 36.20% permeability reduction for hydrated lime. Finally, in the *URG C* profile, *BioFull* achieved a permeability reduction of 72.24% compared to its control, followed by *BioCoarse* with a 42.47% permeability reduction and 30.76% permeability reduction for hydrated lime.

The results presented in Figures 3 through 5 show that even though biocementation presents a slightly higher permeability rate than lime and is still significantly more significant when compared to cement, the authors recommend the continuity of this research. This recommendation aligns with the fact that the *BioFull* and *BioCoarse* controls were adjustments from the *DC* control, meaning that we still need more data to fully understand the biocementation process on granulometric stabilized soils under variable environmental conditions. Furthermore, the researchers understand that any effort to reduce cement and lime is vital to natural resources conservation (Arrivabeni et. al.., 2018; Balaguera et. al.., 2019; Fernandes et. al.., 2019). Just in Europe, the cement kilns production per year emits 1.5456 million tons of CO_2, up to 11125 tons of SO_2, and 0.62-522 tons of dust, among other emissions, resulting from the balance of 1.52 tons of raw material to produce 1 ton of cement kilns, releasing greenhouse gases and dust (Dunuweera and Rajapakse, 2018). (Habert, 2013) highlighted the importance of continuing the environmental impact studies of cement production, recommending searching for new binders to be added to the cement kilns. This research is another solid path towards developing new alternatives to preserve natural resources and potentially promote gains to greenhouse effects since the biocementation process can work as a carbon sink, but this is a topic for another research paper.

6 – Future work recommendations

It is necessary to continue this study of biocementation to develop a protocol for unsealed roads, focusing on evaluating mechanical resistance properties of unsealed roads treated with MICCP via *Serratia ureilytica*. Therefore, the researchers strongly recommend the extension of this pilot project, with the following suggestions for topics to be considered:

- Expand the research to assess the factors that can affect MICCP performance on soil treatment: temperature (of soil, of bacteria multiplication, and cementation solution); amount, size, and distribution of nucleation sites (an enzymatic process that enhances the bacterial activity); pH level (soil, cementation solution, bacterial solution); concentration and saturation of cementation solution; multiple applications of the solution; and temporal resistance to degradation.

- The soil food web and interactions among its trophic levels are very susceptible to eternal stimuli and interference. Most of the soil sites used as a supply source for unsealed roads construction are heavily disturbed, meaning that the soil food web structure is also out of balance. The fixation and cementation solutions are a source of calcium and urea, and both work as a food source for an extensive range of aerobic bacteria present in the soil. Based on the logic, our goal is to expand our studies to test the *S. ureilytica* behavior on unsterilized soil samples, which the standard procedures of permeability testing require. In addition, we want to test the interactions and reactions between *S. ureilytica*, and the main bacteria species present in soil sources for unsealed road construction (Cheng et. al.., 2017; Cheng and Shahin, 2016; Hoang et. al.., 2020; Ingham et. al.., 2000; Kiss and Simihăian, 2002).

- We acknowledge that pH has a crucial role in *CaCO3* precipitation, where the rise in pH turns the bacterium into the nucleation site for crystallization (Hammes and Verstraete*, 2002; Sidik et. al.., 2014a). Pondering this fact, the manipulation of pH can potentially guide the bacterial reaction into its nucleation site role. Therefore, we propose testing for the pH range most conducive to enzymatic behavior of the *S. ureilytica*, which enhances its biocementation rate, its homogeneity, and its range of distribution.

- We recommend an extensive environmental impact assessment of the biocementation process by *Serratia ureilytica* after reaching optimal unsealed road stabilization. It should be followed by a Life Cycle Assessment (LCA) study and compared with LCA studies of cement and hydrated lime as chemical stabilizers. The LCA studies must use the same parameters to compare the techniques for unsealed road stabilization to determine the environmental impacts of the stabilizers and production processes.

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Appendix A - Maps

Location of Gomide gravel pit (cascalheira) at the city, state, and country levels. Maps built on Google Earth Pro software by Carla Portugal

Gomide gravel pit: (A) zoom at the gravel pit site, and (B) photo of the soil collected for this experiment. Map credit: Carla Portugal via Google Earth Pro. Photo credit: Altair Carrasco de Souza, 2016.

Appendix B – SEM images

Figure 6 - BioFull on URG A at three different sample depths (A, B, and C). All images are under 1800 X TM.

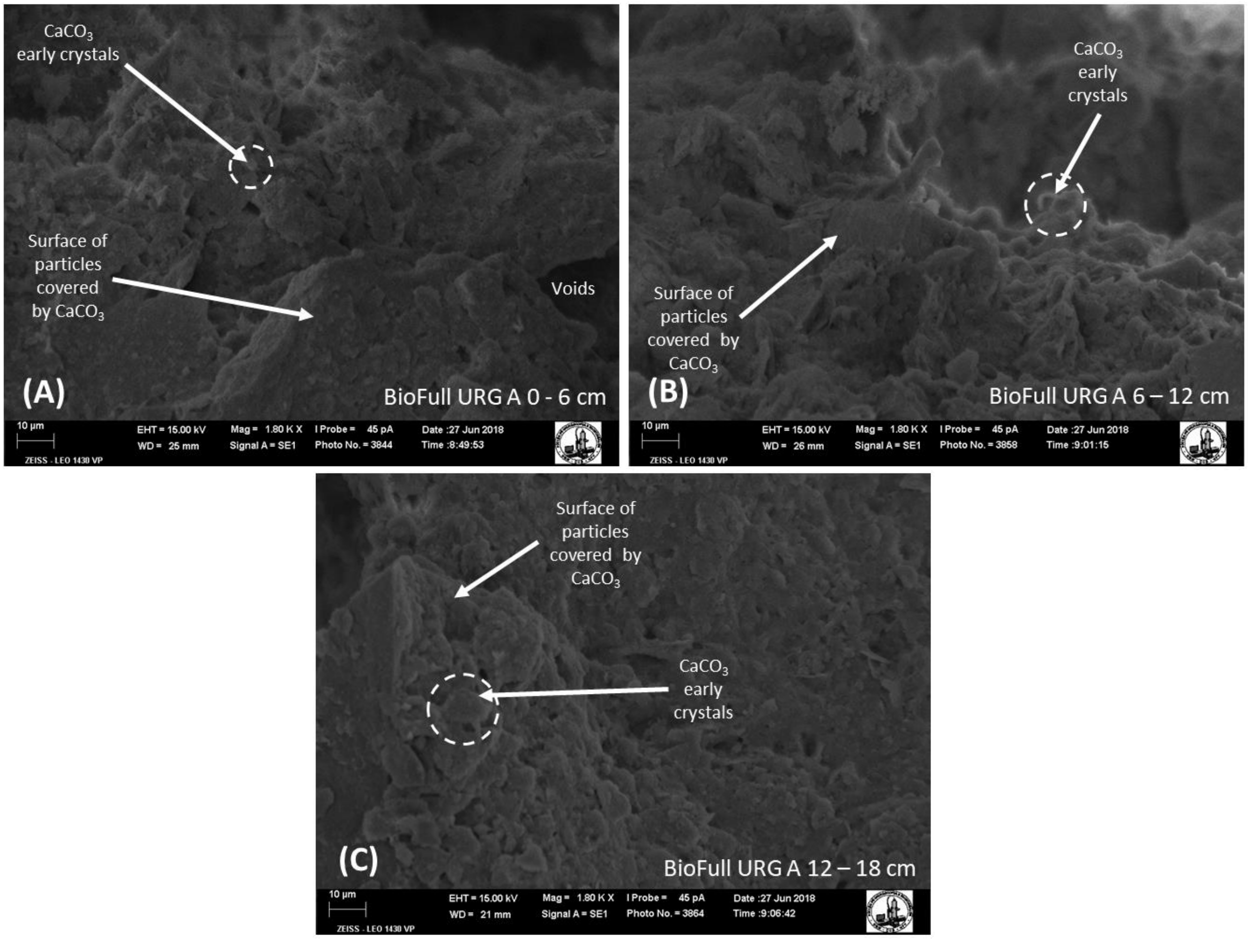
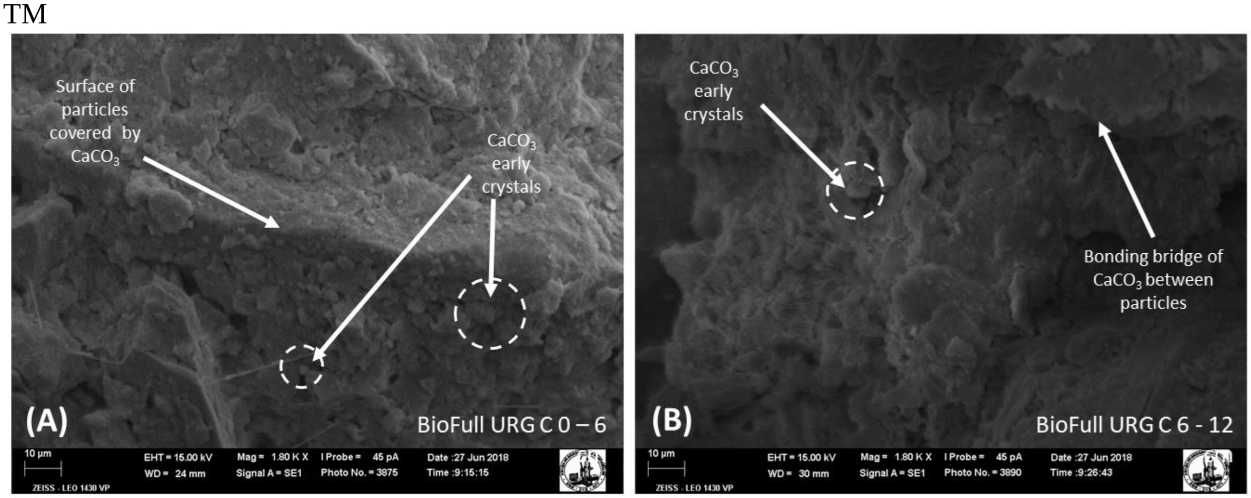


Figure 7 - BioFull on URG C at three different sample depths (A, B, and C). All images are under 1800 X TM



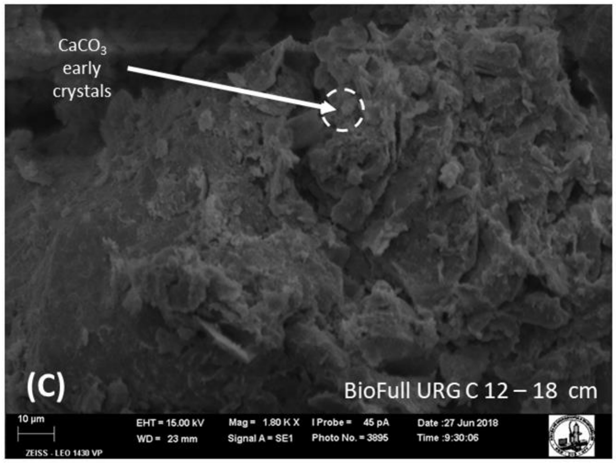


Figure 8 - BioFull on URG F at two different sample depths (A or B and C). All images are under 1800 X TM. B and C were taken from the same sample depth, different locations.

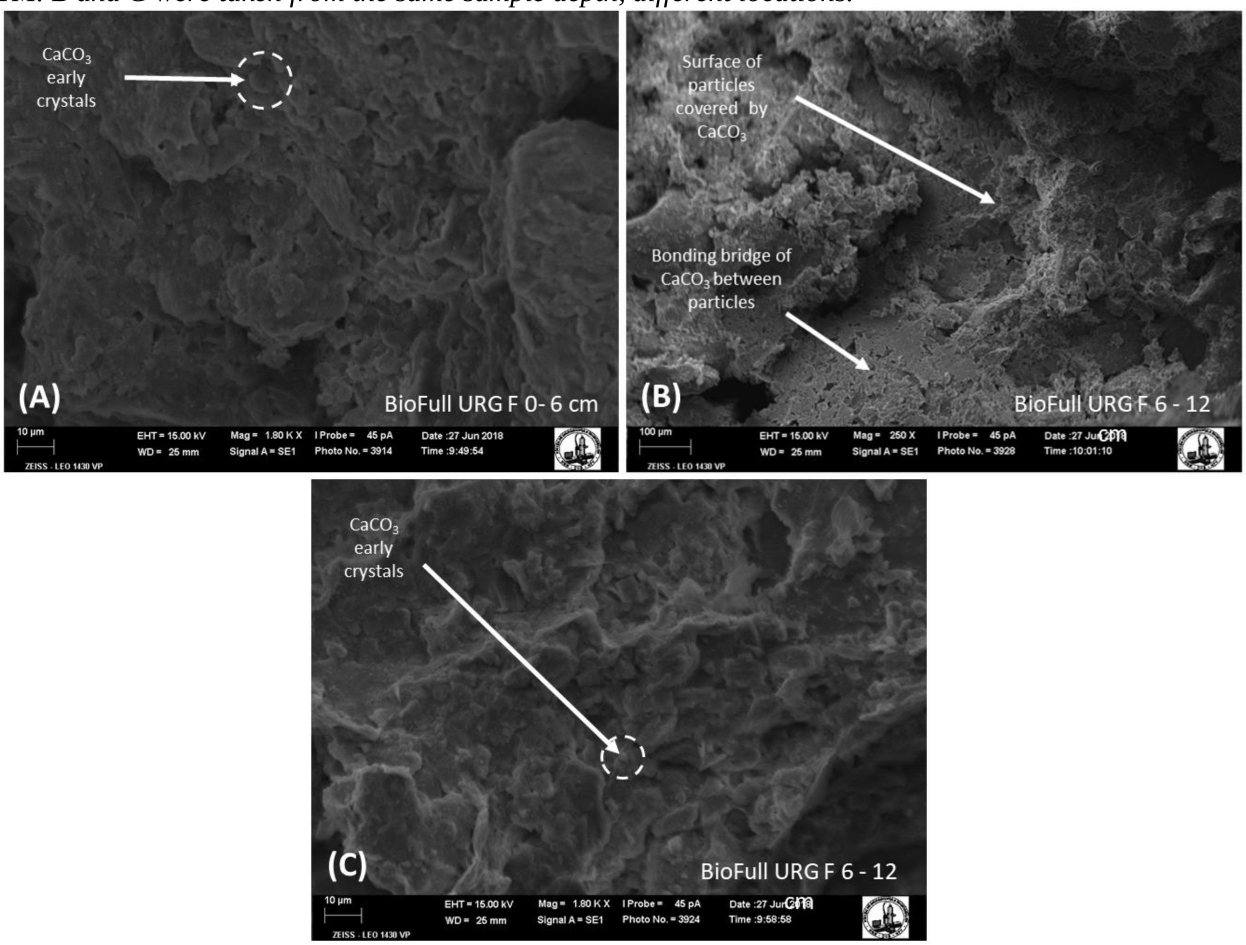


Figure 9 - BioCoarse on URG A at two different sample depths. Image A is under 250 XTM, and B and C are under 1800 X TM. A and B were taken at 0 – 6 cm depth in the sample.

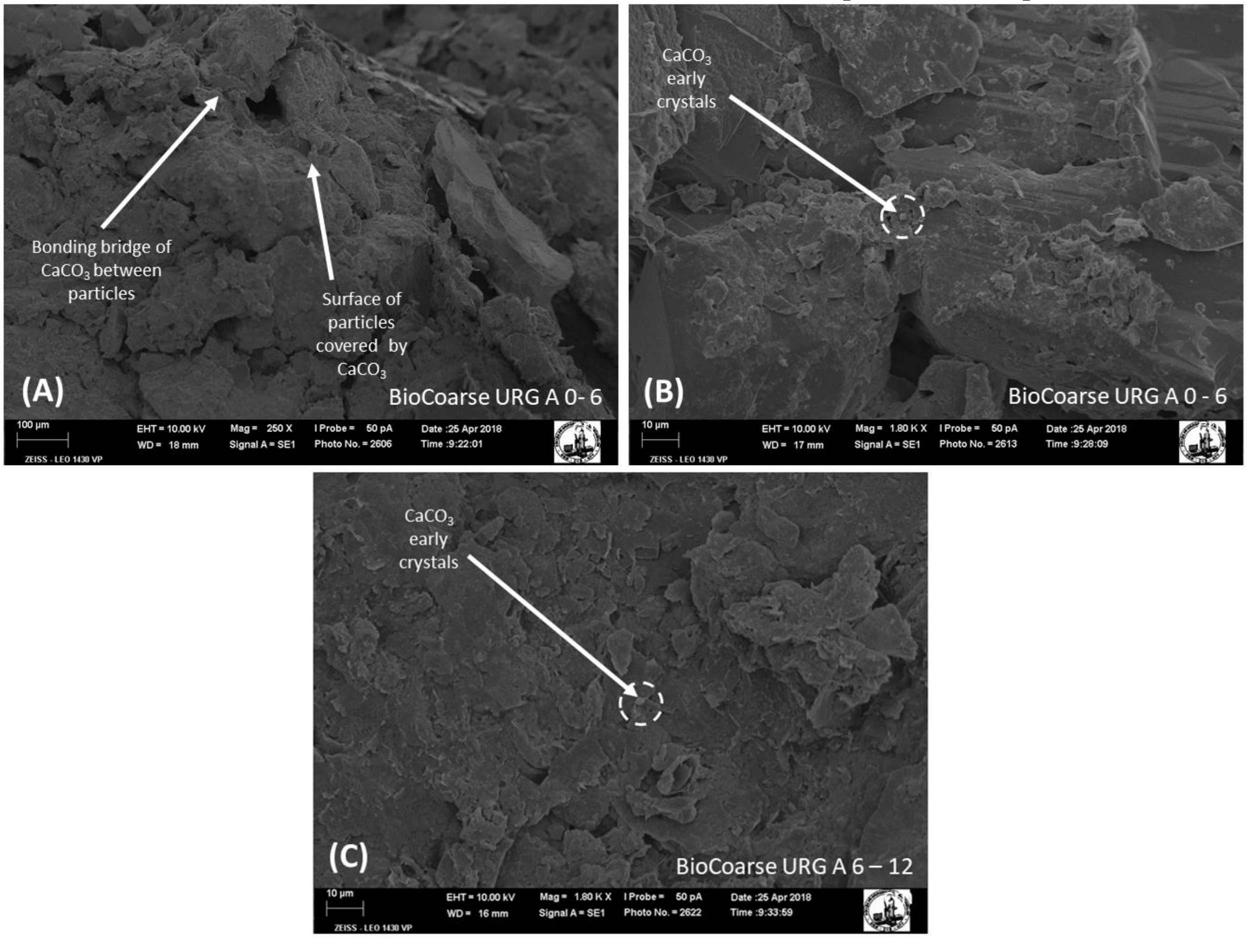
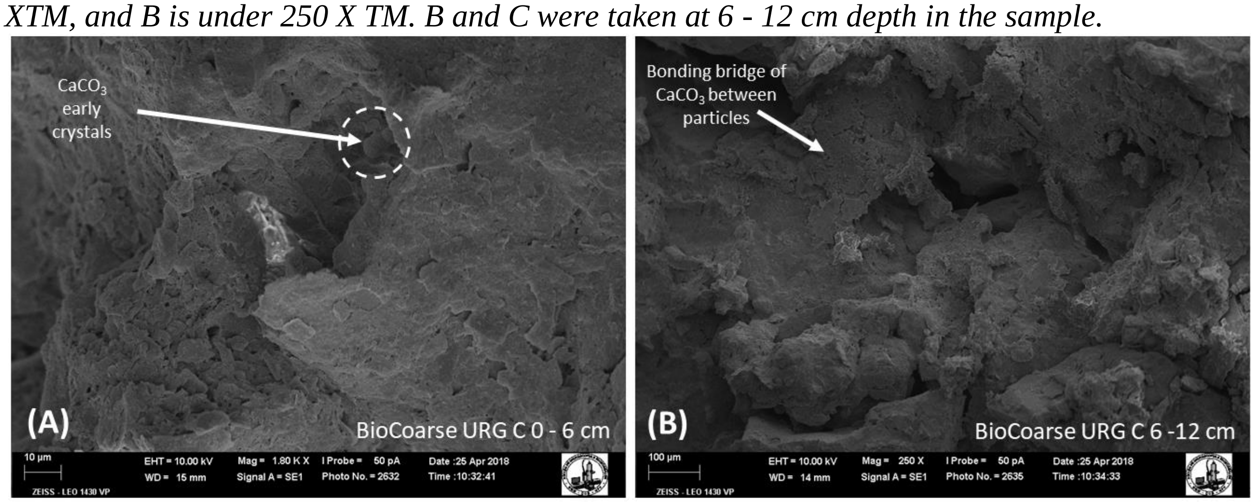


Figure 1 - BioCoarse on URG C at two different sample depths. Image A and C are under 1800 XTM, and B is under 250 X TM. B and C were taken at 6 - 12 cm depth in the sample.



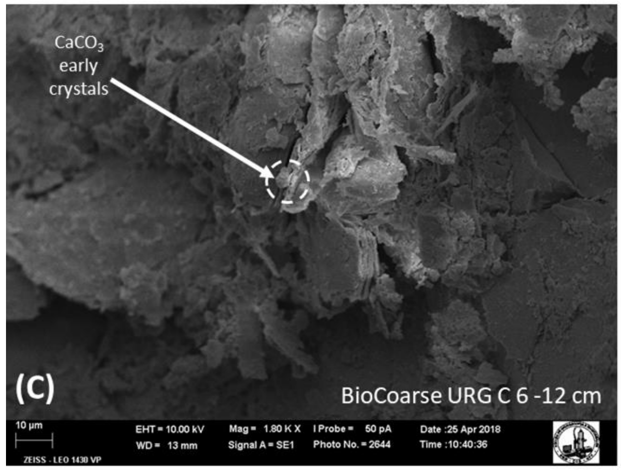
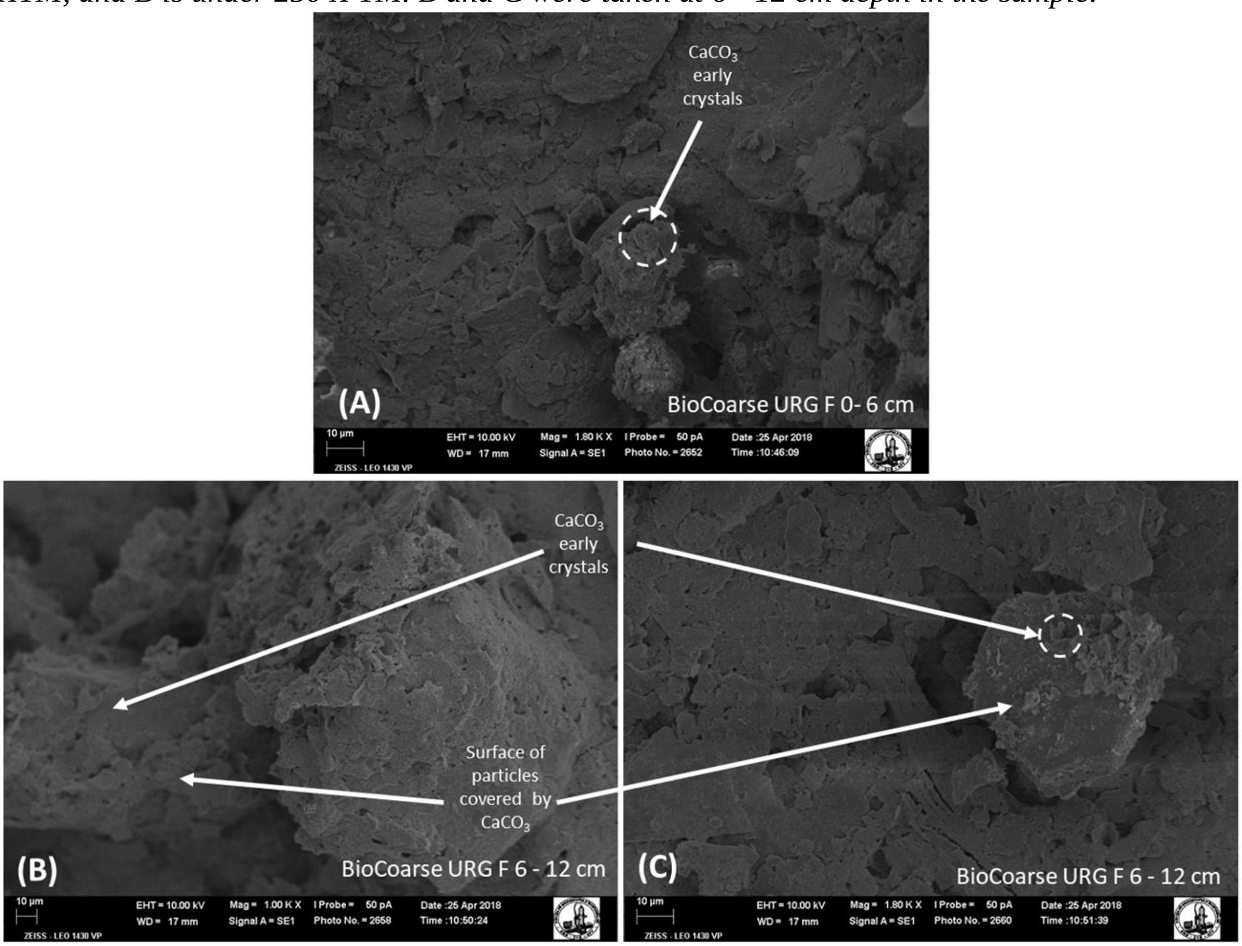


Figure 2 - BioCoarse on URG F at two different sample depths. Image A and C are under 1800 XTM, and B is under 250 X TM. B and C were taken at 6 - 12 cm depth in the sample.



Chapter 5: General Conclusions

1 - Introduction

This chapter will conclude this research by summarizing the key findings related to the assessment of cement and hydrated lime for potential environmental impacts on water quality due to unsealed road stabilization and the assessment of *S. ureilytica*, a novel bacterium, as a green alternative to cement and hydrated lime chemical stabilization of unsealed roads. This chapter also summarizes and reflects on the value and contribution of our advancements. Finally, we review the limitations of this exploratory study and propose further opportunities to continue the research.

Throughout this study, the authors have argued that biocementation using *Serratia ureilytica* represents a strong candidate for unsealed road stabilization over cement and hydrated lime because of the calcium carbonate precipitation, even in early stages, significantly reduced the permeability rate of the tested specimens. Our research demonstrated that it is possible to enhance unsealed road stabilization with *S. ureilytica* biocement, leading to a potential reduction in erosion and other environmental impacts, as suggested by Castro-Alonso et. al. (2019).

This research had its conceptual basis as defined by the sociotechnical-ecological-economic framework diagrammed in Figure 1 which shows the main interactions of three subsystems: (i) ecologic-technical, (ii) socio-technical, and (iii) techno-economic. The ecologic-technical subsystem is defined by the natural MICCP process which uses locally adapted, native *S. ureilytica* bacterium and is associated with minimal environmental impacts throughout the entire process. The socio-technical system is delineated by knowledge, practices, and networks associated with unsealed road stabilization and social sustainability principles, considering the minimization of social impacts. Finally, the techno-economic system is defined by the unsealed road planning and management best practices, influenced by social, political, and economic perspectives. According to Cherp et. al. (2018), these three systems co-evolve along three paths, having distinct boundaries, elements, and connections. This study presented a basic framework to introduce the interconnections among the sociotechnical-ecological-economic context for unsealed road systems construction.

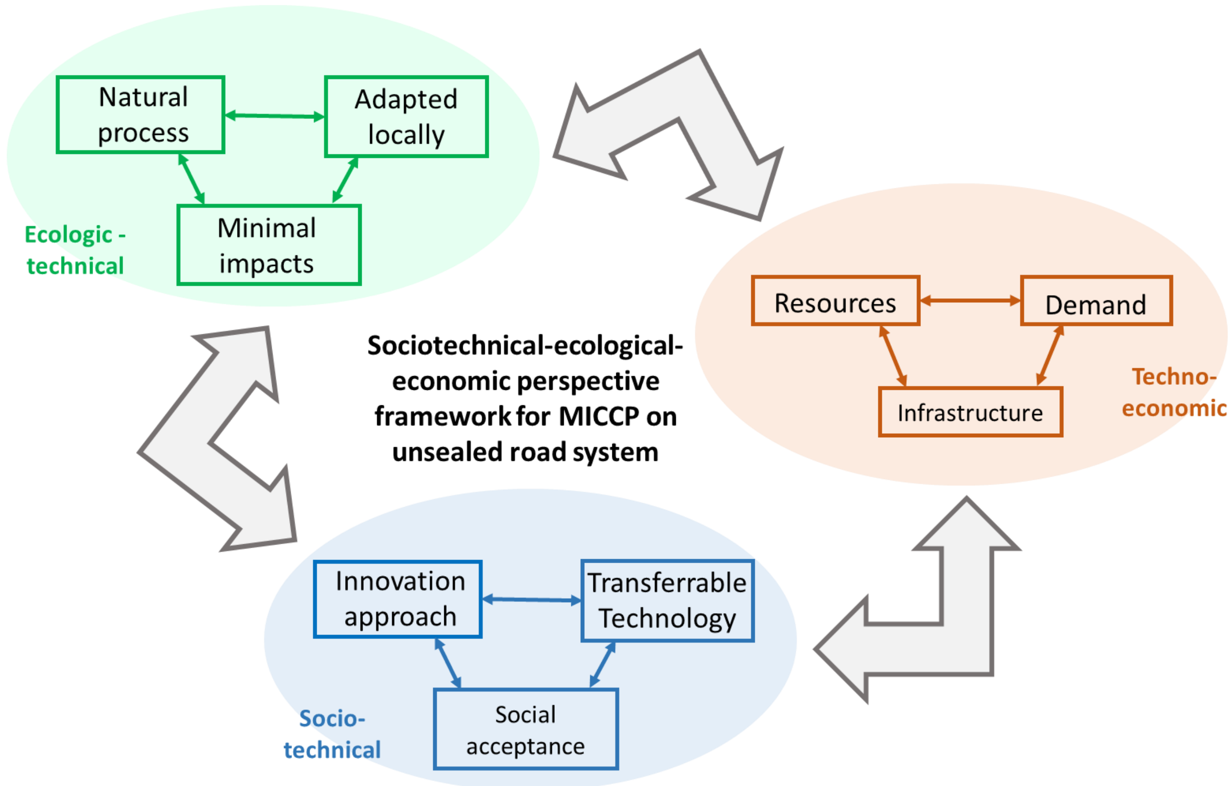


Figure 1 – Diagram of the sociotechnical-ecological-economic perspective framework for MICCP on the unsealed road system.

The ecologic-technical system consists of three defining variables: (i) the solution is nature-based (native bacteria, natural occurrence in soil, and low-impact resources for biocementation solutions), (ii) environmental impacts are minimal (non-exotic bacteria, fewer exogenous resources as compared with chemical road stabilizers, and reduced erosion and percolation rates), and (iii) the solution is locally adaptable (natural soil characterization, adjustments to the protocol accounting for local soil characteristic and environmental variables, MICCP solution material locally sourced). The socio-technical system consists of three defining variables: (i) the approach is innovative (new technology, sustainable alternative over chemical stabilization, and usage in new and pre-existing unsealed road), (ii) technology is transferrable (location on core technology, possibilities for technology export) (iii) the approach is socially acceptable (reduction of air emissions and pollution, road trafficability enhanced, and sustainable solution over chemical road stabilization). The three defining variables for the techno-economic system are: (i) resources are sustainable (goods and services transportation, soil material for road construction, and potential of renewable resources for MICCP procedures), (ii) infrastructure is green (new and existing road system transition to biocementation technology, biocement life-span extended, and policy amendments in place), and (iii) demand supports technology transition (factors driving demand of population and economic growth, costs of operation and construction, EHS (environmental health and safety) benefits are achievable). Integration of sustainability transitions create new challenges for social science. Therefore, we recommend a deeper analysis and adjustment to the sociotechnical-ecological-economic perspective framework for MICCP implementation for the unsealed road system and its variables after the field-scale pilot study (Cherp et. al., 2018).

All three systems would interactively affect any future decisions or adjustments to unsealed road construction and management standards. However, the actual Brazilian standards do not consider all aspects to minimize road construction and maintenance environmental impacts. Therefore, this research expansion can bring more data and information to discuss further how to include sustainability variables and aspects to the unsealed road standards. For this, we recommend the complete studies on the life cycle assessment (LCA), the environmental impact assessment (EIA), and the carbon footprint analysis of unsealed road alternatives.

LCA is a valuable tool to assess the potential environmental impacts of products and services focusing on prevention, input substitution including new alternatives for unsealed road stabilization, and reduced pollution output such as toxic emissions (Vignisdottir et. al., 2019). Many authors published LCA for: urban roads (Trigaux et. al., 2017); paved (asphalt or concrete) roads (Anastasiou et. al. 2015; Cantisani et. al., 2018; Giani et. al., 2015; dos Santos et. al., 2017); paved road maintenance (Vignisdottir et. al., 2019); paved road construction and maintenance (Grael et. al., 2021; Santos et. al., 2015); and road construction alternatives (Balaguera et. al., 2018; Saadé-Sbeih et. al., 2019). Related studies include: a combined analysis of LCA with life cycle costing (LCC) (Antunes et. al., 2016; Trigaux et. al., 2017; Nascimento et. al., 2020; Okte and Al-Qadi, 2020; Riekstins et. al., 2020); carbon footprint of urban roads using LCA (Mao et. al. 2017); and new methods such as organizational life cycle assessment (O-LCA) (Martinez-Blanco et. al., 2020; Forin et. al., 2019). However, few are published about LCA, LCC or both for unsealed roads (Larrea-Gallegos et. al., 2017), or even in Brazil (Grael et. al., 2021; Nascimento et. al., 2020).

LCA has a solid set of methods, each focused on one parameter, meaning that each parameter of a new technology must be assessed independently. The method ReCiPe (hierarchy) was adopted by Vignisdottir et. al. (2019) to identify and categorize the emissions from winter road maintenance, showing how different factors affect the system and highlighting hidden emissions hotspots. Grael et. al. (2021) stated that there are no life cycle data available on road infrastructure in Brazil. Even though their study focused on asphalt-paved highways in Brazil, their data can serve as a base to develop the LCA and LCC for unsealed roads and stabilization methods in Brazil, including biocementation. The extensive systematic literature review done by Hoxha et. al. (2021) showed that 90% of the LCA studies of road design parameters lack basic information on road design and that 82% of those studies are not transparent nor reproducible. The authors recommended a harmonized LCA application, which we will consider in future LCA studies about road impacts. Hoxha et. al. (2021) does not report any studies done in Brazil, which is an opportunity to establish this road design analysis in consonance with robust LCA and LCC studies.

Allied with LCA and LCC studies, a nature-based solutions (NbS) approach is an excellent framework to evaluate alternatives to reduce unsealed road impacts. The International Union for Conversation of Nature (IUCN) (2021) defines NbS as:

> *"actions to protect, sustainably manage, and restore natural or modified ecosystems, that address societal challenges effectively and adaptively, simultaneously providing human well-being and biodiversity benefits"*.

A NbS approach has eight principles that guide ecosystem-related approaches as illustrated in Figure 2 (https://www.iucn.org/commissions/commission-ecosystem-management/our-work/nature-based-solutions):

1. embrace nature conservation norms (and principles);
2. can be implemented alone or in an integrated manner with other solutions to societal challenges (e.g. technological and engineering solutions);
3. are determined by site-specific natural and cultural contexts that include traditional, local and scientific knowledge;
4. produce societal benefits in a fair and equitable way, in a manner that promotes transparency and broad participation;
5. maintain biological and cultural diversity and the ability of ecosystems to evolve over time;
6. are applied at a landscape scale;

7. recognise and address the trade-offs between the production of a few immediate economic benefits for development, and future options for the production of the full range of ecosystems services; and

8. are an integral part of the overall design of policies, and measures or actions, to address a specific challenge.

Figure 2. IUCN schematic of the principles of nature-based solutions (https://www.iucn.org/commissions/commission-ecosystem-management/our-work/nature-based-solutions).

NbS forms the basis from which infrastructure-related approaches (green infrastructure) and ecosystem-based management approaches (integrated water resources management) can be applied to road construction and maintenance. The NbS framework generally starts with ecosystem-based approaches that incorporate restoration, infrastructure, management, and protection, focusing on solving societal challenges, promoting human well-being, and biodiversity benefits.

Authors like Apollonio et. al. (2021) propose using perennial plants to sustain the soil and minimize erosion in hillslopes. However, Brazilian scientists studied this approach for years but it was not labeled as NbS. NbS is a new term for a well-established concept in environmental sciences. Tabalipa and Fiori (2008) demonstrated that vegetal coverage on hillslopes and dams drastically increases soil stability and avoids any unstable region in locations with vegetative coverage. Commercial products like BioMac® deliver a built-in coconut fiber bio-coverage that turns into organic matter/topsoil for native vegetation growth after slow degradation. Portocarrero et. al. (2006) published a report demonstrating the relevance of vegetative coverage to minimize soil surface erosion in slopes, hillslopes, and dams. Holanda et. al. (2008) and Passarinho (2014) evaluated the use of Brachiaria grass as a bioengineering technique for riverbank stabilization. In this sense, we can conclude that biocementation has vast potential to be considered an NbS approach.

5 – Main findings and research contributions

The first main objective of this dissertation research was to search for the optimal concentration of *S. ureilytica* and volume of biocementation solution to reach a homogeneous resistance to erosion when applied on unsealed roads. Here we faced our major challenge: bioclogging, which concentrates the precipitant near the biocementation solution application points, reducing the homogeneity of the biocement layer. Most biocementation research focuses on finding the optimal MICCP bacteria concentration and volume of biocementation solution, plus the best application method for each case to avoid bioclogging (Ivanov and Chu, 2008; Omoregie et. al., 2017; Yasuhara et. al., 2011; Zhao et. al., 2017; Zhu and Dittrich, 2016; Chu et. al., 2015) (Figure 3).

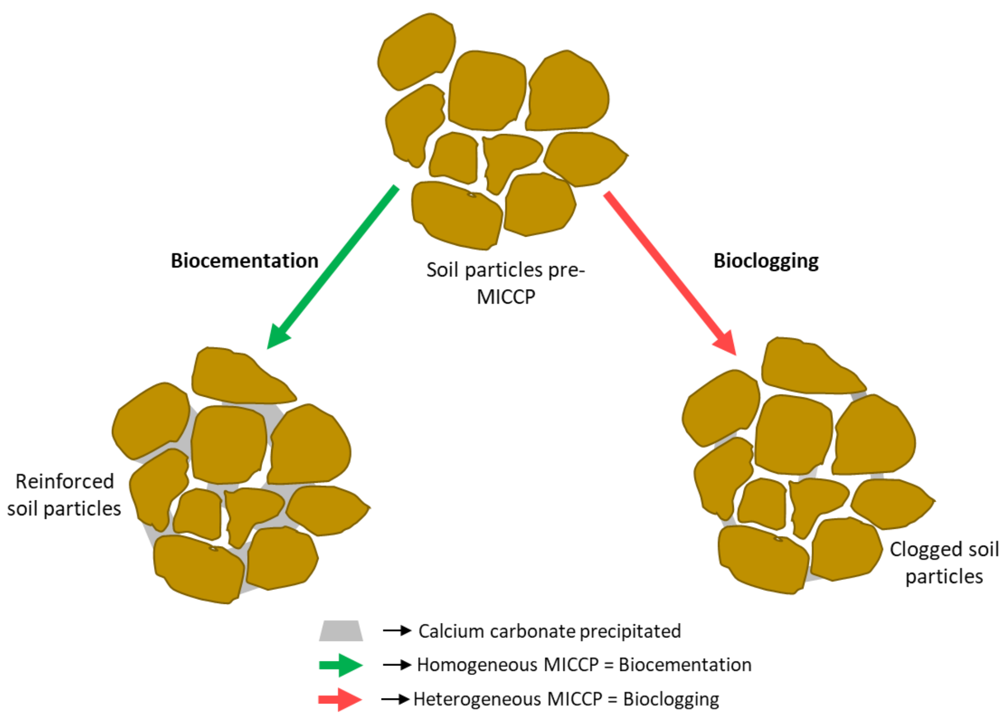


Figure 3. Schematic illustration of biocementation versus bioclogging in soil particles (Adapted from Chu et. al., 2015)

The goal of the biocementation procedure is to achieve a homogeneous distribution along the sample column, representing at the bench scale an unsealed road profile built to specific criteria, to achieve the maximum shear strength and biocementation durability. Bioclogging is the biggest challenge to overcome (Enriquez, 2017; Ivanov and Chu, 2008; Portugal et. al., 2020; Pusat et. al.., 2018). It is marked by the fast MICCP activity and self-trapping calcium carbonate precipitation around the bacteria, limiting its access to biocementation solution and its nucleation size, two of the most important triggers for $CaCO_3$ precipitation (Achal et. al.., 2009; Cheng and Shahin, 2016; Hammes and Verstraete*, 2002; Portugal et. al.., 2020).

Our main target was finding the optimal balance between the bacterial solution, biocementation solution, and application method. Our research tested two variations on the granulometric distribution and mechanical compaction for unsealed roads, proposing potential adaptation to the Brazilian unsealed road standards (DNIT, 2006) to allow a homogeneous and resistant biocementation. The first one had the full granulometric distribution but no compaction, called *BioFull* treatment, and the second one had selected only the coarse particles and had mechanical compaction, called *BioCoarse* treatment. Overall, for all unsealed road grades (URG) tested, the *BioFull* treatment presented the most significant reduction in permeability rates, but the *BioCoarse* had a considerable result, with both performing better than hydrated lime stabilization. The *URG F*, which has the highest fine particles concentration, had the most significant decrease in permeability, reaching an impressive 98.25% reduction with *BioFull* and 95.64% reduction with *BioCoarse* treatment. Considering that cement reaches a 100% impervious state and lime only decreases permeability by 47.06%, the two biocement treatments are a solid alternative to achieve the required low permeability rate for *URG F* road construction. *URG A* and *URG C* had similar behavior to *URG F*. *URG A* had the second-best performance, where *BioFull* reduced the permeability rate by 85.10%, *BioCoarse* reduced permeability by 54.36%, and hydrated lime reduced permeability by 36.20%. Once more, the biocementation procedures proved to be strong candidates over hydrated lime stabilization. Finally, *URG C* followed the pattern found for *URG A* and *URG F*, where *BioFull* had a 72.24% permeability rate reduction, followed by *BioCoarse* reaching 42.47% permeability reduction, and hydrated lime reaching 30.76% permeability reduction. These data allow the conclusion that *S. ureilytica* is a strong candidate as an alternative method for unsealed road stabilization. Furthermore, our results gave enough evidence and data to expand the research to field scale, where the granulometric distribution proposed adaptations can be tested under all variables involved in unsealed road construction and use.

Our second objective was to propose markers to evaluate the potential environmental impact from erosion from the chemically stabilized unsealed road, aiming to test how the stabilization methods can affect water quality. Cement and hydrated lime are two solid components used to improve unsealed road stabilization (Arrivabeni et. al.., 2018, 2016; DNIT, 2019, 2010b; Machado, 2013; Machado et. al.., 2014; Machado and e Portugal, 2020). Even though many articles of research describe the methods of cement and hydrated lime as stabilizers (Camarena, 2013; Dutton et. al.., 2005; Little, 1995; Orndorff et. al.., 2017; Zhao et. al.., 2017), the field still demands more detailed information about cement and lime chemical leachates and how they can potentially impact the environment and water bodies. This research selected calcium, magnesium, and silicon as potential markers since these are the most abundant ions in cement and lime, plus the tests to determine their concentrations are less costly and are more accessible. Our data corroborate the significant publications that demonstrated that cement at 3% is impervious during short, controlled laboratory conditions (Arrivabeni et. al.., 2018, 2016; Machado, 2013; Machado et. al.., 2014, 2006). Our data showed that the traces of calcium and magnesium found in percolated water from the samples could contribute to the eutrophication of adjacent water bodies via the elevation of calcium carbonate and magnesium carbonate levels in the water, which sustain the need for further research with expanded variables such as traffic flow variation and weatherization. The silicon lixiviated from the unsealed roads can be recycled and reused as a valuable source for agriculture and the regeneration of aquatic systems. Further development should include expanding the research to field scale, including all variables affecting road weatherization and erosion processes. Furthermore, we suggest a complete water quality assessment, including heavy metals, dissolved oxygen, turbidity, and sediment levels to test the connections and potential extrapolation of parameters that would allow the use of calcium, magnesium, and silicon as potential markers to preliminary tests for water quality assessment from unsealed roads.

5 – Study limitations

Time and available funding were the major limitations of this research. Therefore, as part of an umbrella project (detailed in Chapter 1), we evaluated two aspects of the researchable question: can *Serratia ureilytica*, a native bacterium, enhance the biocementation process to achieve the desired unsealed road stabilization?

This research focused on testing the adjustments necessary to optimize the biocementation process to meet the standards of unsealed roads. The primary limitations were:

1. The limited budget constrained the tests to two alternatives for granulometric stabilization that could potentially reach the required stabilization and load-bearing capacity for unsealed roads.
2. Shared use of expensive equipment such as SEM microscope, direct shear strength molds, and equipment, sharing laboratory equipment necessary to curate bacterial colonies and biocementation solution, limited access for additional experiments.
3. Reduced sample size due to limited access to the laboratory facilities and dependency on technicians authorized to perform the tests.
4. Lack of time and budget to repeat some tests or expand other variables, providing extensive data for significant statistical testing and analysis.

4 – Future research recommendations

Future research based on the actions taken to acquire more funding to continue the project at field scale, incorporating more variables and constraints, should be highest priority. The main recommendations are:

1. Develop field-scale pilot projects to test the biocementation protocol developed in this project, including more biocementation solution applications and variations in the volume of bacterial solutions applied to each URG. Then, test the road tracks over the weather year, weatherization, and traffic flow, looking for rupture sources and points that can undermine the road stabilization and integrity.
2. After defining the rupture sources and variables affecting road integrity, isolate each variable to search and test potential improvements to minimize their impact. Those

data will result in the definition of the biocemented unsealed road life cycle, bringing valuable information to adjust further applications and enhance the technology.

3. Constant reassessment and adjustment of the biocementation protocol versus the required granulometric distribution are proposed to reach the optimal combination for each unsealed road type. The outcomes of this research will contribute to the development of an official standard normalizing biocementation as a green alternative for unsealed road stabilization.
4. Dubey et. al. (2021) stated that much research is under development to address the potential strategies to minimize ammonia concentration as a byproduct, reduce bioclogging, improve precipitation homogeneity, and enhance the field data about MICCP solution transportation to the application site. Therefore, we intend to contribute to this portion of the field study by testing potential mobile bioreactors to generate the MICP solution on site.
5. Test the potential impact of wood ashes over the biocemented unsealed road. Wood ashes contain potassium hydroxide and potassium carbonate, which can generate lye in contact with rainwater. Lye is a strong alkali solution that is highly soluble in water and produces a caustic basic solution. No research on this topic related to biocementation has been published yet, so the authors recommends adding this variable in the pilot studies.
6. Continue testing the potential use of calcium, magnesium, and silicon as markers to trace the potential impacts of unsealed road erosion. Cross these markers with others like heavy metals, turbidity, dissolved oxygen to trace a parallel extrapolation formula that would allow the adoption of those markers as a more economic monitoring action. These more affordable monitoring markers would allow application in more remote and endangered areas, speeding up preventive actions before unsealed road collapse and more significant environmental impacts occur.
7. Undertake LCA studies. Once the biocement protocol is adjusted, we recommend the ReCiPe (hierarchy) 2016 method (Huijbregts et. al., 2017) to analyze the environmental impacts of biocementation on unsealed roads. We reinforce Grael et. al. (2021) and Balaguera et. al. (2018) recommendations about the need to develop studies for developing countries. We recommend addressing such factors as global

warming potential (GWP), terrestrial acidification potential (TAP), ozone depletion potential (ODP), freshwater eutrophication (FWEP), marine eutrophication (MEP), and freshwater ecotoxicity (FWECP) to identify how factors such as extraction and processing of sand, gravel, and biocement; transportation of these inputs; and machinery use and operation, including direct and indirect emissions, raw materials, and electricity/fuel, could be affecting the LCA of the unsealed road and the biocementation process. ReCipE can also help identify the unknown hidden emissions hotspots to enhance the complete LCA study. For these studies, we recommend the use of the software SimaPro 8.4 (https://simapro.com/) used for modeling and analysis and the database EcoInvent database 3.3 (Wernet et. al.. 2016), which contains robust life cycle inventory data about emissions for products worldwide. Other modeling software such as GaBi (https://gabi.sphera.com/158tilizi/index/) and OpenLCA (https://www.openlca.org/) will be assessed.

8. Incorporate a nature-based solutions (NbS) framework for analysis. The author considers that biocementation has vast potential as an NbS approach, and recommends more studies according to the IUCN (2020) standard (WCC-2016-Res-069) for NbS, which is a user-friendly framework for verification, design, and scale-up of NbS. With this approach, biocementation can be assessed according to the eight NbS criteria: societal challenges, design to scale, biodiversity net-gain, economic feasibility, inclusive governance, balance trade-offs, adaptive management, and mainstreaming and sustainability. Furthermore, Safdar et. al. (2021) stated that biocementation is a nature-based solution since it is inspired by biomineralization, a natural process of microbiologically induced calcium carbonate precipitation (MICCP). The author supports this statement and recommends NbS studies of biocementation for unsealed road stabilization.

5 – Closing summary and final thoughts

Overall, this research demonstrated that *Serratia ureilytica* biocementation is a strong candidate as a green alternative to the chemical stabilization of unsealed roads. However, more research is necessary to test the technology on a field scale, as proposed here. This research also discussed using markers for environmental impact assessment focused on lower-cost, reliable results, and preventive measures.

Discussions with potential stakeholders and grant applications are in development, demonstrating the great potential this research can develop to help solve Brazil's unsealed roads erosion problems. Furthermore, after advanced protocol definition, the researchers intend to propose expanding and adapting this protocol to other developing countries facing similar situations as Brazil but who do not have the same access to research or funding.

We believe in science as a frontier expander and should be accessible to all. Therefore, we hope that our research can inspire others to join the international task to achieve a robust biocementation protocol that would allow this potentially nature-based solution and greener alternative for unsealed road stabilization and a potential substitute for any other alternative cement or lime use. Our research focused on roads, but many projects in development are testing biocementation for construction materials (DeJong et. al., 2014; Iqbal et. al., 2021; Umar et. al., 2016), stabilization of dams (Gowthaman et. al., 2019; Ivanov et. al., 2020), civil construction in the space exploration (Kumar et. al., 2020), and public health and alternative medical explorations such as dental fillers (Seifan et. al., 2020), material for bone reconstruction (Jia et. al., 2010), and even prosthetics to reduce cost (Anderson, 2021). The future is bright for nature-based solutions to reduce environmental impact, and the novel bacterium, *S. ureilytica*, holds promise as an alternative green material across many industries and diverse applications.

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