dr hab. Andrzej Mazur, prof. UMCS
Department of Genetics and Microbiology
Institute of Biological Sciences
Maria Curie-Skłodowska University
Akademicka 19 St.
20-033 Lublin

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Review of doctoral dissertation of MSc in Engineering Nadella Marchelina entitled

“Experimental studies of microbiologically induced improvement of fine sands” prepared in Faculty of Civil Engineering and Architecture, Civil Engineering, Geodesy and Transport, Lublin University of Technology under the supervision of Prof. Małgorzata Franus, Ph.D., and doctor, engineer Joanna Fronczyk, Ph.D. (assistant supervisor)

Over the past few decades, there has been continuous and intensive development of urban infrastructure, often in areas with unfavorable ground conditions. This trend requires improvements to existing methods or the introduction of new soil stabilization and strengthening solutions prior to constructing new buildings. Traditional ground improvement techniques based on cement materials are not economically efficient or environmentally friendly. Cement production is a highly energy-intensive process associated with significant carbon dioxide emissions, estimated to account for approximately 6% of total anthropogenic CO_2 emissions worldwide. The European Union aims to achieve climate neutrality by 2050. Ongoing climate change and increasing environmental pollution further emphasize the importance of addressing CO_2 emissions, leading to progressively stricter regulations aimed at minimizing the construction industry's negative impact on ecosystems.

The aforementioned challenges motivate researchers to develop materials and technologies with lower environmental impact and reduced emissions. In this context, biocementation has received increased attention. This process involves the use of microorganisms, which are often isolated from natural environments, to produce substrates that can induce the precipitation of carbonates (e.g. calcium carbonate) in soils through their metabolic activity in response to a suitable nutrient supply. These minerals bond soil grains together, transforming loose soil into a more cohesive, compact structure with improved mechanical properties. This approach is a significantly more environmentally friendly alternative to conventional soil stabilization methods.

Within this framework, the research undertaken by the Ph.D. candidate in her dissertation, which focuses on applying the Microbially Induced Carbonate Precipitation (MICP) process to soil biocementation, is highly relevant and justified. The dissertation aimed to investigate the potential of MICP for various geotechnical applications.

Nadella Marchelina's doctoral dissertation, submitted for review, comprises 210 numbered pages and follows the typical structure of a doctoral thesis. It is divided into clearly separated chapters, including an introduction, research hypotheses and objectives, materials and methods, results, and references. The dissertation also includes abstracts in Polish and English, information on research funding, a list of tables and figures, and extensive appendices containing detailed statistical analysis data. A table of contents and a list of abbreviations precede the text. In my opinion, the dissertation's overall structure and proportional distribution of content across chapters are appropriate. Although there is no separate chapter explicitly entitled "Discussion," the PhD candidate continuously compares her experimental results with literature data within the results chapters. This approach is commonly adopted in scientific publications and enhances the clarity and readability of the work, in my view.

In the introduction, the Ph.D. candidate logically and consistently familiarizes the reader with the dissertation's topics. She presents the concept of soil biocementation, along with the methods and pathways of MICP application, the factors affecting MICP efficiency, the mechanisms of soil particle bonding related to mineral precipitation, and the effects of cementation on soil improvement.

She also discusses the potential for large-scale, practical applications of MICP. In my opinion, the emphasis distribution within the introduction is appropriate, and the introduction is generally written correctly.

However, in fulfilling my duties as a reviewer, I must note that I have several critical remarks regarding imprecise or unfortunate formulations, especially with regard to the 'microbiological aspects' of the study.

Major remarks

p. 15 Inconsistent use of the term MICP (calcite vs. carbonate)
In the Introduction, the acronym MICP is expanded interchangeably as Microbially Induced Calcite Precipitation and Microbially Induced Carbonate Precipitation. In my opinion these terms are not synonymous and should not be used interchangeably, particularly in a study that explicitly includes magnesium ions in the cementation solutions. While calcite is a carbonate mineral, not all carbonate precipitates are calcite, and the presence of Mg^{2+} may promote the formation of other carbonate phases. This terminological inconsistency affects the conceptual clarity of the work and should be corrected.

pp. 24–25. Overgeneralization of microbiological mechanisms
Several statements in the Introduction imply direct causal relationships between bacterial presence, urease activity, carbonate precipitation, and soil strengthening, without sufficient distinction between bacterial abundance, metabolic activity, and enzyme expression. For example, the following statements raise concerns:

"*The presence of a higher rate of ureolytic bacteria resulted in larger carbonate crystals, suggesting that bacterial concentration plays a significant role in crystal growth."*

"High concentrations of metal ions in MICP can negatively impact the process, potentially causing it to inhibit the bacteria."

These formulations oversimplify complex microbiological processes and remain ambiguous with respect to the specific mechanisms involved. In particular, it is unclear whether the inhibition refers to bacterial growth, metabolic activity, or enzyme (urease) efficiency. Given the microbiological component of the study, a more precise and mechanistically accurate description would improve the scientific rigor of the Introduction.

Specific/Minor remarks

p. 23. Figure caption clarity (Fig. 2.6)
Figure 2.6 is conceptually valuable; however, its caption does not fully reflect its role in illustrating bacterial transport through soil pores and its implications for MICP efficiency. A more descriptive title would improve the clarity and interpretability of the figure.
p. 24. Precision of terminology related to bacterial "density"
The Introduction frequently refers to "bacterial density" or "bacterial concentration" without specifying whether this refers to optical density, CFU – Colony Forming Units, or another quantitative measure. Although this issue is addressed later in the methodology, a brief clarification in the Introduction would improve terminological consistency.
p. 24 *Sporosarcina pasteurii* is the name of the species not the strain.

The scientific problem addressed in the dissertation is based on three main research hypotheses, which enabled the formulation of four specific research objectives. The research sought to develop a potential workflow that maximizes the mechanical properties of cemented soils by focusing on the optimization of different MICP parameters, such as bacterial densities, cementation solution composition, and injection procedures. In my opinion, the clarity of the research hypotheses does not raise any major concerns, and the planned research activities – described in detail within the specific objectives and subsequently implemented - largely address the questions posed therein.

I only have doubts about the justification of the second main research hypothesis (pp. 48–49). It assumes that soil improvement achieved through biostimulation of non-ureolytic bacteria may exhibit efficiency comparable to bioaugmentation using the ureolytic pathway with non-ureolytic support. While this hypothesis is relevant and scientifically interesting, its theoretical justification in the Introduction appears a little limited to me. A clearer explanation of the assumptions and mechanisms underlying this comparability would strengthen the conceptual framework of the study.

The research plan was divided into four parts corresponding to the specific objectives. The plan was logically structured into successive stages, with each stage comprising a defined sequence of experiments. The research plan follows a hierarchical structure and, in my opinion, is appropriately and coherently organized.

In the next chapter, the Ph.D. candidate describes **the materials and methods** used in her research. This section covers microbiological procedures and methods for evaluating the mechanical properties of soils treated with MICP. The microbiological methodology is basic and limited to the essential procedures required for the study, generally corresponding to the stated research objectives. However, this limited scope imposes constraints on interpretation of some of the results. In my opinion, the range of methods employed to assess the mechanical properties of biocemented soils was appropriately selected and adequate for evaluating the intended effects of the applied MICP treatments. In most cases, the methodological descriptions provide sufficient detail to allow other researchers to reproduce the procedures. The experimental design incorporates control setups and statistical analyses to evaluate the variability of the results obtained, however the selection and presentation of these elements require further clarification in some instances. I have several remarks concerning Materials and Methods section.

Major remarks

pp. 51–53 Lack of chemical characterization of soils
The soils were characterized in terms of physical properties; however, no chemical characterization (e.g. elemental composition, Ca and Mg content, salinity, buffering capacity) was provided. This may limits the interpretation of the MICP process, particularly in the context of carbonate precipitation and Mg/Ca interactions.

pp. 53–55 Lack of microbiological characterization of soils used for biostimulation
In experiments involving biostimulation, no information is provided about the indigenous microbial communities potentially present in the soils. Without at least a basic microbiological characterization, it is difficult to assess which microorganisms were stimulated and how this may have influenced the observed results.

p. 55 Section 4.1.2 Insufficient characterization of bacterial species
The description of the bacteria used in the study is limited. In particular, it was not clearly stated which bacteria are ureolytic. Given the central role of urease activity in MICP, this represents an important methodological limitation. Generally *Bacillus subtilis* and *Sporosarcina pasteuri* used in the study are positive in the test for urease activity and are frequently tested in MICP but the genus of Arthrobacter may be diverse with respect to this activity. It would be optimal if the urease activity was tested prior to the experiments described in the thesis.

p. 57, Fig. 4.4. Ambiguity regarding tested bacterial strains in optimization experiments
From Figure 4.4 and the accompanying text, it is unclear which bacterial species or strains were tested during the optimization of the ureolytic pathway with non-ureolytic support and other pathways. This ambiguity complicates the interpretation and reproducibility of the results. I can imagine that it was mainly *B. subtilis.*

pp. 57–63 Sterilization of sand samples not described consistently

Information regarding sand sterilization appears only in later sections of the methodology. Given the distinction between bioaugmentation and biostimulation approaches, the sterilization status of samples should be clearly described at the beginning of the experimental procedures.

p. 56 Incorrect definition of dissolved inorganic carbon (DIC)
Urea and calcium lactate are described as sources of dissolved inorganic carbon (DIC). By definition, neither urea nor lactate constitutes DIC; rather, they may act as precursors that can indirectly contribute to DIC formation through biochemical transformations. Calcium lactate represents a source of dissolved organic carbon (DOC) rather than dissolved inorganic carbon (DIC), although its biodegradation may indirectly contribute to DIC formation. The use of the term DIC in this context is therefore inaccurate and affects subsequent analyses. It should be rather: calcium lactate and urea served as precursors of dissolved inorganic carbon, because their biochemical transformation can contribute to the formation of DIC in the system.

<u>Specific/Minor remarks</u>
p. 52, Table 4.1. Soil classification and origin of data
The soils were classified in accordance with the ASTM D2487 standard. However, it is not entirely clear whether all parameters used for the soil classification were determined within the scope of this study or obtained from previous investigations or external sources.

p. 54 Terminology related to bacterial strains
The term strains is used inconsistently, although only one strain of Bacillus subtilis (KP17) appears to be investigated. In addition, the term bacterial species would be more appropriate for this work than strains because bacteria are not identified at the strain level and especially, like in the case of Arthrobacter sp., even at the species level.

pp 56–58 Bacterial concentration is expressed in terms of optical density (OD). While OD is a convenient and commonly used proxy, it does not distinguish between viable and non-viable cells and does not reflect metabolic or enzymatic activity. Given that OD is repeatedly used as a key biological parameter throughout the study, explicitly acknowledging this limitation would strengthen the biological interpretation of the results.

p. 59 pH and electrical conductivity measurements
It is not explicitly stated whether pH and electrical conductivity were measured in the solutions, the sand, or the combined sand-solution system. Providing this clarification would help to better interpretation of the reported measurements.

p. 58, Fig. 4.6 - Storage conditions of soil (sand) samples
The storage conditions prior to experimentation are not fully described, which may be relevant for microbial activity in biostimulation experiments.

On page 65 (Table 4.3) and in Appendix 15, clarification of the terminology used for freeze-thaw testing would be helpful. Specifically, it is unclear to me how the term "freeze-thaw cycle" differs from "cycle number," which is reported in the last column of Table 4.3, and whether these terms refer to distinct parameters.
Additionally, the control sample described in Appendix 15 is biocemented soil that was not exposed to freeze-thaw cycles. It can be inferred that the purpose of this control was to evaluate the impact of freeze–thaw cycling on biocemented material. Could the Ph.D. candidate please clarify whether a control consisting of non-biocemented soil exposed to freeze-thaw cycles was considered and how the chosen control setup affects the interpretation of durability under freeze-thaw conditions?

p. 66 Drying of samples after biocementation
The rationale for air-drying the samples for 14 days after treatment is not discussed. This procedure may influence both microbial viability and carbonate precipitation and its relevance to field-scale applications should be clarified.

The **results section** provides an overview of the outcomes of an extensive experimental programme, organized into subsections corresponding to the stages of the research plan. In the first part, the influence of different bacterial species and treatment conditions on carbonate precipitation was examined, with a focus on the effects of bacterial concentration, cementation solution composition, and Mg/Ca ratios. The results demonstrated clear differences in carbonate content and cementation efficiency based on the biochemical and chemical conditions applied.

Next, the mechanical response of the treated soils was analyzed using a range of laboratory tests, including unconfined compressive strength (UCS), shear strength, and shear wave velocity measurements. The results showed varying degrees of improvement in mechanical properties depending on the MICP strategy and treatment parameters applied. This section discusses the relationship between carbonate content and mechanical performance.

The next sections of the Results chapter focused on durability-related aspects of biocemented soils. These sections included freeze–thaw resistance tests and mass loss measurements. These tests were used to evaluate the stability of the treated specimens under cyclic environmental loading. Additionally, erosion resistance tests, such as wind erosion experiments, were presented to evaluate the effectiveness of surface crust formation and the ability of MICP-treated soils to withstand external mechanical degradation. Complementary microstructural and mineralogical analyses, including SEM observations and XRD results, were employed to support the interpretation of macroscopic mechanical behavior. These analyses provide insight into the morphology, distribution, and mineralogical composition of the precipitated carbonate phases, linking micro-scale features to the observed changes in mechanical performance and durability.

In my opinion, the results obtained by the Ph.D. candidate are original and scientifically interesting. The Results chapter provides a broad and valuable experimental dataset that establishes a solid foundation for evaluating the effects of various MICP strategies on soil improvement. The results obtained address the vast majority of the questions formulated within the specific research objectives and potentially open new opportunities for further investigation. It is also worth emphasizing that the manner in which the Ph.D. candidate formulates her conclusions demonstrates her ability to critically analyze her own experimental results. I agree with most of the conclusions in the summary, though some require further clarification, as discussed below.

Major remarks

pp. 84–90; Figs. 5.4, 5.6 Definition and calculation of dissolved inorganic carbon (DIC)
The interpretation of several key results relies on the parameter referred to as dissolved inorganic carbon (DIC); however, DIC is neither directly measured nor clearly defined in the dissertation. Based on the description provided, DIC appears to be inferred from the molar concentrations of urea and calcium lactate, but the method used to calculate DIC is not presented in the form of an explicit equation, in contrast to approaches reported in the literature (e.g. Fronczyk et al. 2023). This lack of definition and transparency complicates the interpretation of trends linking "DIC" to carbonate precipitation efficiency and mechanical performance.

pp. 84–88 Overinterpretation of microbial and biochemical mechanisms
Several mechanistic explanations proposed in the Results and Discussion appear to go beyond the experimental evidence provided. Statements suggesting disrupted bacterial metabolism, reduced urease efficiency, or enhanced ureolytic activity are not supported by direct measurements such as urease activity, ammonium production, or pH changes. While the observed correlations are valuable, the inferred causal relationships between reagent composition, microbial activity, and carbonate precipitation should be expressed more cautiously.

Example:
p. 87 "This mechanism was likely due to higher DIC concentration to increase the precipitation rate of $CaCO_3$ through its biocompatibility and its capacity to support bacterial growth". This shows only the potential correlation but does not prove specific mechanism and metabolic pathway. Thus again the next sentence: "It can be concluded that using calcium lactate as a calcium source may enhance microbial activity by providing an additional organic carbon source" is overstatement. It is should be rather: Previous studies have suggested that calcium lactate may support microbial activity in MICP

systems; however, its influence on urease production and carbonate generation is likely indirect and dependent on various environmental conditions.

pp. 96–99 Conceptual ambiguity of the “non-ureolytic pathway”
Experiments described as addressing a “non-ureolytic pathway” involve bacterial species that are known to be ureolytic (e.g. Sporosarcina pasteurii and Bacillus subtilis), which raises concerns regarding the conceptual consistency of this classification. This ambiguity complicates the interpretation of the results and slightly weakens conclusions related to pathway-specific effects.

pp. 82–84 Use of optical density as an indicator of biological activity
As already noted in the Methods section, bacterial concentration expressed as optical density (OD) does not provide information on cell viability, metabolic activity, or enzyme production. In the Results section (pp. 82–84), OD is repeatedly used to explain differences in MICP efficiency and is directly linked to urease availability. Therefore, interpretations implying a direct relationship between OD and ureolytic activity should be treated with caution.

Specific/Minor comments

On page 101 (Figure 5.14), samples treated with *Arthrobacter* sp., *Bacillus subtilis* and *Sporosarcina pasteurii* are reported to exhibit no signs of carbonate precipitation. However, on page 85 (Figure 5.5), carbonate precipitation (including calcite, magnesite–calcite, and magnesite) is reported for *B. subtilis* under identical chemical conditions (OD = 1, 0.25 M $CaCl_2$, 0.2 M calcium lactate, 0.25 M $MgCl_2$, 0 M urea).

Could the PhD candidate clarify the reasons for this apparent discrepancy and indicate whether differences in experimental setup, treatment or interpretation criteria may account for the contrasting observations?

p 112, Fig. 6.1 During the first three applications, when only the growth medium was introduced, a sharp increase in pH value up to approximately 9.0 was observed and attributed to ureolytic activity. However, since urea was not present in the growth medium and was only introduced later with the cementation solution, clarification would be helpful regarding the mechanism responsible for this early pH increase?

p 129, Fig. 7.2 Could the PhD candidate clarify whether the interpretation of uneven cementation caused by pore clogging applies equally to both bioaugmentation and biostimulation approaches? In the case of biostimulation, where bacterial distribution is expected to be relatively uniform, could alternative mechanisms such as nutrient or cementation solution transport limitations play a more significant role?

p 133 As noted earlier in the dissertation, optical density (OD) provides only a limited proxy for bacterial concentration and does not reflect metabolic activity or functional behavior. In this context, the discussion on page 133 regarding the effect of bacterial density on the homogeneity of cemented columns would benefit from additional caution.

Higher bacterial densities do not necessarily lead to improved cementation homogeneity, particularly in bioaugmentation-based approaches. Many bacteria, including Bacillus subtilis, produce extracellular polymeric substances (EPS), which can influence OD measurements and may play a dual role in MICP processes. While EPS can enhance bacterial adhesion to soil particles, provide nucleation sites for $CaCO_3$ precipitation, and stabilize biofilms within the pore space, they can also strongly bind Ca^{2+} and other cations, modify local transport conditions, and promote pore clogging, potentially resulting in uneven cementation. Consequently, bacterial density alone - especially when inferred solely from optical density - may not adequately capture the mechanisms governing cementation homogeneity

p 135 The terminology used to describe the experimental approaches could be clarified for consistency. In particular, the term “biostimulation with ureolytic bacteria” would be more precisely formulated as “biostimulation of ureolytic bacteria”.

p 146 The comparison of surface strength values with those reported by Maleki et al. (2016) (p. 146), particularly with respect to the definition of “high bacterial mix concentration samples”, would benefit from clarification regarding the comparability of experimental conditions and measurement methods.

In my opinion, despite numerous remarks and comments, the Ph.D. candidate successfully achieved the research objectives of the dissertation. The results clearly demonstrate that the ureolytic pathway, supported by non-ureolytic components, significantly improves soil stability. This improvement extends beyond surface layers to deeper soil zones. A wide range of complementary measurements consistently support this finding, including shear wave velocity (Vs), unconfined compressive strength (UCS), surface strength, crust thickness, wind erosion resistance, and durability-related tests. Together, these results provide a coherent and convincing assessment of the mechanical performance of MICP-treated soils.

The conclusions and future research directions presented on pages 160 and beyond are well-justified. In particular, the emphasis on optimizing the MICP procedure, especially the injection strategies, underscores the fact that a fully standardized approach is unlikely to be applicable to all soil types and site conditions. This underscores the need for case-specific optimization. While this poses challenges for large-scale implementation, it reflects the practical complexity of real-world geotechnical applications

Given that non-ureolytic MICP pathways were not comprehensively explored in this study, future research should focus on investigating bacterial species that are strictly non-ureolytic and possess alternative metabolic mechanisms for carbonate precipitation. Such studies would be essential for fully assessing the potential and limitations of non-ureolytic approaches to soil biocementation.

Moreover, I believe that future research on MICP-based soil stabilization could benefit from incorporating soil microbiome analyses. Assessing the composition and metabolic potential of indigenous microbial communities would help determine the feasibility of biostimulation at a given site. Integrating these analyses with metabolic profiling tools, such as Biolog assays, could provide valuable insight into which MICP pathways are most likely to be effective under specific environmental conditions.

Final conclusion

In conclusion, I consider Nadella Marchelina's doctoral dissertation to be an original solution to a scientific problem. As such, it fulfills the requirements specified in Article 187 of the July 20, 2018 Act on Higher Education and Science, in terms of both its scientific content and methodological approach. Therefore, I respectfully request that the Scientific Discipline Council of Civil Engineering, Geodesy and Transport at Lublin University of Technology allow Nadella Marchelina to proceed to the next stage of the doctoral degree-awarding process.

Dr hab. Andrzej Mazur, prof. UMCS

Andrzej Mazur