

# ***Interactive comment on “Calcite and vaterite biosynthesis by nitrate dissimilating bacteria in carbonatogenesis process under aerobic and anaerobic conditions” by Marwa Eltarahony et al.***

**Marwa Eltarahony et al.**

saharzaki@yahoo.com

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Response to peer-reviewers Firstly, we appreciate the reviewers' feedbacks and their careful reading of our manuscript, interest in our study and thoughtful comments that greatly improve the quality of the paper. Secondly, we did our best to respond to the points raised. The Referees have brought up some constructive suggestions and we appreciate the opportunity to clarify our research objectives and results. As indicated below, we have checked all the general and specific comments pointed out and have made the necessary changes accordingly to their indications.

The reviewer's comments are in Bold and underlined

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## Reviewer #1: General comments

The article deals with an interesting topic that deserves more attention from the scientific community. The manuscript has a potential to be acceptable due to the increasing interest on bioformation of carbonates, both from basic science and technological application point of view. However, (1) in the section "Results and discussion" the authors often refer to articles that are not strictly related to the interpretation of the results obtained, leading to a confused and sometimes inaccurate dissertation. Often, the bibliographic references quoted are nothing but references reported by others authors. Despite the interesting topic dealt with and some results worthwhile to be circulated among the scientific community, (2) the article is extremely confused, badly written, the results and the discussion are often disorganised and difficult to follow. The discussion is not always coherent with the results reported. (3) I strongly suggest a complete revision by a native English speaker. The manuscript is still far from be ready.

A1: Thanks for your notice and deep revision. We have carefully revised the manuscript and the references cited. In the revised manuscript the references were more fit to the discussion and interpretation.

A2: Thanks for your comments. We have carefully revised the manuscript and we have taken special care to clarify our results in narrative way and with convenient interpretations. We would clarify that each point studied in this research was essential to make the research complete work and free from defect or shortage, as much as we can. Any details or discussion in each result was employed to interpret the result concisely, precisely and without redundancy. Thorough revision and total reorganization for the manuscript were performed.

Where, it began by selection of the most potent strains in both NR activity and also in CaCO<sub>3</sub> precipitation. The selected isolates were identified and phylogenetic tree for them was constructed, interpreted and compared with other studies briefly (Page 10). Then NR activity of selected strains and their CaCO<sub>3</sub> deposition capability were deter-

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mined aerobically and anaerobically in a comparative way (Page 11). Thereafter, the complete process for CaCO<sub>3</sub> deposition under oxic/anoxic nitrate utilization conditions was studied in details and also in comparative way which was not studied before, till our knowledge (Page 11-16). The variation in the size, morphology and the identity of crystals was revealed by mineralogical analysis EDX, SEM and XRD. The reasons for different polymorph (calcite/vaterite) (Page 17-18), different size and morphology (Page 20-21) were elucidated. Finally, the comparison with other literatures was performed in a way that served the manuscript.

A3: We appreciate your suggestion. We do our best in this concern. Correction of grammatical errors and improvement for English quality were carried out by a native English-speaking colleague as suggested and additionally by expert website. The suggested corrections have been made.

#### Specific comments

In order to help authors to improve their text, I suggest a complete rewrite of the article according to the comments below:

C1) Abstract - From line 25 onwards, replace the strain codes (71A, VIP, EM4) with the names of the bacterial species (*Lysinibacillus sphaericus*, *Raoultella planticola*, *Streptomyces pluricolororescens*).

A1: The correction was performed according to your suggestion.

C2) Keywords - Choose keywords not listed in the title and more relevant to the topic: *Lysinibacillus sphaericus*, *Streptomyces pluricolororescens*, *Raoultella planticola*, CaCO<sub>3</sub> bioformation, ...; delete "biocementation"..

A2: Appreciating your recommendation. Your suggestions were considered Page 2).

3) Lines 45-49 - The different biomineralization processes described in lines 45-49 and 69-77 are reported in a confusing way. Please be clear about BCM, BIM, autotrophic, eterotrophic, SRB, etc. mechanisms that are randomly referred to in the text.

A3: we are grateful for your comment. Your suggestions were considered and we manifested the detailed difference between BIM and BCM clearly in the revised manuscript (Line 50-60, page 3). For autotrophic, heterotrophic, SRB, etc., they are the pathways by which microbes deposit CaCO<sub>3</sub> in microbial induced calcium carbonate precipitation process (MICCP). Generally, BIM was mediated by those mechanisms (Line 86, page 4).

4) Lines 47-49 - References are not strictly related to the statement.

A4: Thanks for your deep revision. We deleted (Ghosh et al., 2019) only. With deep reviewing, we confirmed that the other references mentioned both types of biomineralization.

5) Line 51 - I suggest adding the adjective "microbial" to the term "carbonatogenesis".

A5: We followed your recommendation (Line 63, page 3).

6) Lines 54-55 - It is not clear how MICCP can participate in the solution of the water crisis. I suggest deleting this sentence.

A6: We followed your recommendation and deleted it.

7) Lines 82-84 - Ureolytic bacteria does not cause the "calcite disintegration", but the "decay of the calcite formation". Thirumalai states: "The use of aerobic bacteria in urea hydrolysis unable to grow in situ due to lack of oxygen, which will results in decay of the calcite formation in time (Van Passen et al., 2010)".

A7- The sentence was corrected (line 103-106, Page 5).

C8) Lines 91-92 - Report the increase in carbonate precipitation.

A8- The value was added (Line 115, page 6).

C9) Lines 97-98 - In the manuscript there is no experimental evidence about the suitability of CaCO<sub>3</sub> crystals for the potential applications listed in the section "Results and

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discussion”

A9- We would point out that the current study is the basic stone for several applications. Through such study, the characteristic features of bioformed CaCO<sub>3</sub> such as their shape, purity (single or mixed polymorphic phase), size and time frame, at which they formed, were recognized. All such features determined which strain and under which condition could be employed in which application.

In addition, the general characteristic features of calcite and vaterite were known previously from other literatures (Tas, 2009; Trushina et al., 2014; Dizaj et al., 2015; Svenskaya et al., 2016). Where, calcite is the most stable form, potent and the least solubility. So, it could be harnessed in applications required stabilization and low dissolution, such as soil consolidation and sequestration of pollutants (e.g. heavy metals). While, vaterite is metastable and rapidly dissolves at acidic pH; thus, it can undergo degradation both in vitro and in vivo solutions like body fluid which contains a number of acidic metabolites, such as citrate, lactate and acid hydrolysis enzymes. So, the spherical shaped vaterite formed biologically by our strains *Raoultella planticola* and *Streptomysetes pluricolorescens* could be utilized in medical applications.

As indicated by mineralogical analysis, the biosynthesized calcite by *Lysinibacillus sphaericus* (fine and large particles) would be harnessed in strengthening of soil/sand (according to particles size), crack healing, and the reduction of the permeability of geological formations. Currently, in our lab, there is an ongoing study for remediation of heavy metals using *Raoultella planticola* under aerobic & anaerobic nitrate utilization. Additionally, the vaterite bioformed by *Streptomysetes pluricolorescens* is invested nowadays, in our lab, medically in drug delivery.

âĀĀ Svenskaya, Y.I., Fattah, H., Inozemtseva, O.A., Ivanova, A.G., Shtykov, S.N., Gorin, D.A., and Parakhonskiy, B. V.: Key Parameters for Size- and Shape-Controlled Synthesis of Vaterite Particles, *Crystal Growth Design*, 1, 331-337

âĀĀ Trushina D., Tatiana V. Bukreeva, Mikhail V. Kovalchuk, Maria N. Antipina, CaCO<sub>3</sub>

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vaterite microparticles for biomedical and personal care applications, Materials Science & Engineering C (2014),

âĀĀ Dizaj S., Mohammad Barzegar-Jalali, Mohammad Hossein Zarrintan, Khosro Adibkia, Farzaneh Lotfipour, 2015. Calcium Carbonate Nanoparticles; Potential in Bone and Tooth Disorders. Pharmaceutical Sciences, March 2015, 20, 175-182.

âĀĀ Tas A., 2009. Monodisperse Calcium Carbonate Microtablets Forming at 701C in Prerrefrigerated CaCl<sub>2</sub>-Gelatin-Urea Solutions. Int. J. Appl. Ceram. Technol., 6 [1] 53-59.

C10) Line 130 - How was the inoculum standardized?

A10: We use fixed amounts of inoculum according to Mcfarland standard, in association to bacterial count, to ensure uniformity of the inoculum. As reported at Material & methods section (2.3) line 161 (page 7), About 250  $\mu$ L of bacterial cultures ( $1.8 \times 10^6$  CFU/mL) was used as inoculum for precipitation test.

C11) Lines 130-137 - How many flasks have been inoculated to carry out what is reported on lines 156-157? Describe the inoculum set more clearly. The flasks analyzed in section "2.5. Study of the parameters associated with CaCO<sub>3</sub> precipitation" are the same described in section" 2.3. CaCO<sub>3</sub> precipitation and crystals collection"?

A11: We inoculated 10 flasks for each strain under each condition to carry out what reported in point 2.3 (lines 159-186 (page 7- 9), revised manuscript and lines 130-137 in previous manuscript). According to your suggestion, we added the details about inoculum set and experiment design (Line 176-186, page 8-9).

For the points (2.3 & 2.5 in old manuscript), the exact media, inoculation, incubation conditions and incubation time were applied exactly, but the studied items were different. So, according to your note and also Reviewer's #3 suggestion, we merged both sections and clarified the studied items.

12) Line 158: How long were the plates incubated?

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A12: The plates were incubated for 24 h (line 188, page 9 (revised manuscript; Line 158 in old manuscript)).

13) Line 160 - Report drying times and temperatures of the crystals before being weighed.

A13): We are grateful for your notice. The required data were added (Line 192, page 9).

14) Line 166 - Have the selected strains been isolated from the same soil?

A14: The samples were collected from different non-calcareous Egyptian sites (different governates), as reported (line 131-133, Page 6). Additionally, according to your notice and suggestion of Reviewer #3, we mentioned the isolation site behind each isolate (Line 214-216, page 10).

15) From line 172 onwards - Replace the strain codes with their species names.

A15: Your recommendation was performed.

16) Lines 172-174: Firmicutes, Bacillaceae, Proteobacteria, Enterobacteriaceae, Actinobacteria, Actinomycetaceae are written in italics.

A16: The corrections were performed (Line 223-225, page 10).

C17) Lines 176-179 - The discussion is not strictly related to the results obtained.

A17: We referred to different bacterial groups (phylum Firmicutes, family Bacillaceae; phylum Proteobacteria, family Enterobacteriaceae and phylum Actinobacteria, family Actinomycetaceae) that related to the exact classification of our isolates and had the same scope (CaCO<sub>3</sub> production with various applications). So, we thought the importance for mentioning other studies that are similar to our results. However, according to your notice, we stated such information in convenient way in the revised manuscript (line 227-229, page 10), particularly that there were different publications stated this point and dealt with their data with the same sort of discussion.

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C18) Line 131 – please make clear the full composition of M9 media. Without such an information, it is impossible to verify the accuracy in the evaluation of precipitated CaCO<sub>3</sub> (lines 238-239)

A18: Although we pointed out to the reference that mentioned the composition of M9 media, we followed your recommendation and reported the composition in the revised manuscript (Line 162-164, page 7-8). Additionally, the accuracy in the evaluation of precipitated CaCO<sub>3</sub> (lines 238-239) was verified through abiotic control, without bacterial inoculum, (i.e. there was no chance for precipitation of media components even in absence of bacteria).

C19) Lines 189-190 – please consider the result obtained by Maciejewska et al. (2017) “Assessment of the Potential Role of Streptomyces in Cave Moonmilk Formation”. Front. Microbiol. 8:1181. doi: 10.3389/fmicb.2017.01181

A19: We are grateful for your deep recommendation. We took the results of recommended publication by Maciejewska et al. (2017) in consideration. We would clarify that such study investigated all possible mechanisms that lead to CaCO<sub>3</sub> precipitation in Moonmilk Cave. Where they studied ammonification of proteins, nitrate/ nitrite reduction, ureolysis and oxidative glucose breakdown but the actual application of such mechanisms in CaCO<sub>3</sub> precipitation was only performed through ureolysis and protein ammonification. For more clarification, they detected only the previous five mechanisms to ensure their presence (without estimation of corresponding enzyme activity U/ml), but for CaCO<sub>3</sub> precipitation, it was performed by ureolysis and protein ammonification only and not examined, characterized or monitored through nitrate reduction process, as we studied. Such might support our sentence in old manuscript. (Line 239-241, page 11).

20) From line 208 onward: results are presented and discussed in a very confusing manner preventing the comprehension of the text.

A20: Thanks for your comment. We took your valuable opinion in our consideration.

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We revised and rewrote such part in simple, narrative and comprehensible way in the revised manuscript.

C21) The figures are so small and blurry that it is impossible to read them

A21: You are right. We apologize for such unintended mistake. Respect your opinion. All figures were adjusted and subjected to improve the image quality using photo editing software. All figures become clear enough to read and perceive.

C22) 237-244: the units of measure of the precipitated CaCO<sub>3</sub> need to conformed to an unique standard.

A22: Thanks for your critical observation. We followed your comment and made the required changes to confirm uniformity of unites according to standards (gm/ 100 mL) (Line 336-340, page 15).

C23) 247-252: the assumptions made by the authors seem to be of speculative nature. Are they any bibliographic references confirming their interpretation of the results?

A23: The lines 343-348 (Page 15, revised manuscript) described the obtained result and its proposed interpretation. As observed in Figure 3, there was increasing in the values of E.C, which could be attributed to the presence of ions such as NO<sub>2</sub><sup>-</sup>, N<sub>2</sub>O<sup>-</sup>, NO<sup>-</sup>, Ca<sub>2</sub><sup>+</sup> and CO<sub>3</sub><sup>-</sup> that were generated by the microbial activity and utilization of C/N substrates (sodium acetate and Ca(NO<sub>3</sub>)<sub>2</sub>·4H<sub>2</sub>O). Generally, E.C of any solution increases with increasing of ions. In MICP studies, the ions in the media generated as a result of microbial activity on the substrates as indicated by several references. The references were added according to your recommendation (line 350, Page 16).

C24) 283-286: could the detected P derive from the ingredients used to make the culture broth?

A24: We thought that it isn't derived from media components, especially it presented only in vaterite samples and in considerable percentage, other than Na and Cl which were present in both vaterite and calcite samples in small percentage (0.5-0.66 %).

We recommended that it is biologically driven from bacterial cells which were incrustated by CaCO<sub>3</sub> stones. It represents essential constituent of bacterial biomolecules such as phospholipids, nucleic acids, proteins and/or polysaccharides. In addition, SEM images could confirm our suggestion. Where, the presence of calcified hyphae of Streptomyces and bacterial imprints of Raoultella planticola on vaterite spheres implied its biological nature.

C25) Please carefully check all the bibliographic references, that are not always compliant with statement made by the authors.

A25: Thanks for your deep and thorough notice. All citations in the manuscript were revised in regards to their suitability and fitness to the mentioned interpretations and statements.

C 26) Wei et al. (2015), Wu et al (2011) and APHA, (1999) are not reported in the References section.

A26: The missed references were added.

C 27) Lines 241, 436 and 300 and reference section. Please replace “Kaur D.N.” con “Dhami N.K.”

A27: We followed your comment and all replacements were performed.

C28) All what was referred to Figures 3, 4, 5 and 6 are not verifiable. The Figures are small, blurry and illegible.

A28: We apologize for this unintended mistake. The amendments and adjustments for such figures were performed in new revised manuscript.

C29) 297-298 and 303: how the authors can state that on Figure 6C and 6E a mucous matrix and mucous substance are evident?

A29: As refereed by arrows, there was slime like matrix or mucoid, curved string-like that link between calcified particles. Such mucoid strings were also calcified, so ap-

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peared thick. Microbiologically, it is known characteristic shape of exopolysaccharides that surround bacterial cells. Several literatures reported such shape:

â€” Dawson L., Valiente E., Faulds-Pain A., et al. 2012. Characterisation of *Clostridium difficile* Biofilm Formation, a Role for Spo0A. *Plosone*, 7, 12.

â€” J. Lam, r. Chan, k. Lam, and j. W. Costerton. 1980. Production of mucoid micro-colonies by *pseudomonas aeruginosa* within infected lungs in cystic fibrosis. *Infection and immunity*, may 1980, 28, 2, 546-556.

â€” El Abed S., Ibensouda S., Latrache H. and Hamadi F., 2012. Scanning Electron Microscopy (SEM) and Environmental SEM: Suitable Tools for Study of Adhesion Stage and Biofilm Formation. In: *Scanning Electron Microscopy*. V. Kazmiruk (ed). New York: INTECH Open Access Publisher.

â€” Wille, G., Hellal, J., Ollivier, P., Richard, A., Burel, A., Jolly, L., et al. (2017). Cryo-scanning electron microscopy (SEM) and scanning transmission electron microscopy (STEM)-in-SEM for bio- and organo-mineral interface characterization in the environment. *Microsc. Microanal.* 23, 1159–1172.

â€” <https://en.wikipedia.org/wiki/Biofilm>

â€” <https://phys.org/news/2018-09-key-bacterial-molecule-antibiotics.html>

C30) 305-309: spores are notoriously inactive and cannot participate to Ca precipitation.

A30: In our study, it is probable that strain *L. sphaericus* entered into sporulation stage upon complete depletion of nutrients, which means that  $\text{CaCO}_3$  were formed by the vegetative cells before/during sporulation. As known, the vegetative cells transform to the spores under harsh conditions and return back again to vegetative upon removal of such conditions. Additionally, Jonkers et al., 2010 reported the application of *Bacillus pseudofirmus* DSM 8715 and *B. cohnii* DSM 6307 spores directly on the cement, proving that they remain viable for four months. Different literatures, listed below, reported

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the applications of spores in soil solidification, biopugging and biocementation, which were harsh and stress conditions.

âĀĀ Jonkers, H. M., Thijssen, A., Muyzer, G., Copuroglu, O., & Schlangen, E. (2010). Application of bacteria as self-healing agent for the development of sustainable concrete. *Ecol Eng*, 36(2), 230–235.

âĀĀ Anbu P., Kang C., Shin Y. and So J., 2016. Formations of calcium carbonate minerals by bacteria and its multiple applications.

âĀĀ Ghosh T, Bhaduri S, Montemagno C, Kumar A. 2019. Sporosarcina pasteurii can form nanoscale crystals on cell surface. *Plosone*, 30, 14.

âĀĀ MontanoâĀŚSalazar S., LizarazoâĀŚMarriaga J., Brandăo P., 2017. Isolation and Potential Biocementation of Calcite Precipitation Inducing Bacteria from Colombian Buildings. *Curr Microbiol*.

C31) Lines 304-309. The statements do not make any sense. The spores in harsh condition remain spores and do not evolve in vegetative form, the only one able to contribute to the Ca precipitation.

A31: As we referred to the previous studies (direct above comment) dealing with the same issue, the spore suspension of different bacterial genera was applied in the harsh conditions and all of them proved the viability of the spores even after long time reached to months. So, we thought the importance of mentioning such sentence. Such characteristic property (sporulation) seemed to be advantageous, especially in the applications of soil stabilization and concrete healing (ongoing investigation in our lab).

Additionally, cells could be applied in the form of spores with its media and so could be transformed to vegetative cells that perform its function (e.g. CaCO<sub>3</sub> deposition). Upon depletion of nutrients and entrance of relative harsh conditions (starvation), the cells don't lose their viability, but instead they transformed to spores till removal of such conditions.

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C32) 313-316: the reference Hou et al. (2011) is not coherent with the discussion and it has been quoted in wrong way.

A32: From our point of view, this reference stated that the size of the precipitated calcite by *Alternaria* sp. was ranged from less than 1- and not exceeded 10  $\mu\text{m}$ , which was agreed with the size of calcite crystals formed by strain *Raoultella planticola* (VIP), and under the same nitrate utilization conditions. Despite that, we followed your opinion and deleted it in the revised manuscript.

C33) 321: Rodriguez-Navarro et Al. (2012) obtained calcite and vaterite precipitation under experimental conditions widely differing from those described in this manuscript

A33: Totally agreed with your point of view. But, till our knowledge, there was no previous publication studied aerobic and anaerobic incubation's effect on  $\text{CaCO}_3$  deposition, in a comparative way, which we could take as a reference. So, we pointed out to the final results which was "variation in experimental conditions could result in variation in polymorph". Where, Rodriguez-Navarro et Al. (2012) mentioned that "at least in the systems studied, polymorph selection in bacterial calcium carbonate mineralization by heterotrophic bacteria is not bacterium or strain specific. Rather, under equal culture conditions, the nature of the substrate strongly influences which polymorph is formed".

We would manifest that there were recently published studies on aerobic and anaerobic MICP process, but the results indicated that calcite was only formed aerobically. While, the precipitation was neglected anaerobically so, its polymorph wasn't determined (Surabhi Jain & D. N. Arnepalli (2019); Lee et al., 2017). Besides, other recent studies just compare the kinetics of growth and precipitation between two conditions (Mitchell et al., 2019). Additionally, other studies reported formation of  $\text{CaCO}_3$  under only one condition of aeration (anaerobic) without testing it aerobically. Surabhi Jain & D. N. Arnepalli (2019): Biochemically Induced Carbonate Precipitation in Aerobic and Anaerobic Environments by *Sporosarcina pasteurii*, *Geomicrobiology Journal* Yun Suk Lee, Hyun Jung Kim, and Woojun Park. Non-ureolytic calcium carbonate

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precipitation by *Lysinibacillus* sp. YS11 isolated from the rhizosphere of *Miscanthus sacchariflorus*

Andrew C. Mitchell<sup>1</sup>, Erika J. Espinosa-Ortiz, Stacy L. Parks, Adrienne J. Phillips, Alfred B. Cunningham and Robin Gerlach. Kinetics of calcite precipitation by ureolytic bacteria under aerobic and anaerobic conditions. *Biogeosciences*, 16, 2147–2161, 2019.

Bin Sun, Hui Zhao, Yanhong Zhao, Maurice E. Tucker, Zuozhen Han and Huaxiao Yan. 2020. Bio-Precipitation of Carbonate and Phosphate Minerals Induced by the Bacterium *Citrobacter freundii* ZW123 in an Anaerobic Environment. *Minerals*.

34) 327-328: please drop out. The sentence is useless in this context

A34: The sentence was deleted according to your recommendation

35) 335-483: the discussion is vague and superficial and carried out in a confusing way. In addition, the references quoted refer to article that used experimental protocols widely differing from those described in this manuscript .

A35: Thanks for your comment. We thoroughly revised the manuscript, reformatted and reorganized it to avoid such critical points. Additionally, the references quoted were also revised.

36) Figure 6: The pictures are too small and difficult to read. Please consider a reduction of the photos by eliminating the redundant ones.

A36: Your observations were taken in consideration and adjusted in the revised manuscript to be readable and informative. Some pictures were deleted to reduce redundancy, while other contained some informative details.

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Interactive comment on *Biogeosciences Discuss.*, <https://doi.org/10.5194/bg-2019-444>, 2019.

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