

Laboratory Activity to Effectively Teach Introductory Geomicrobiology Concepts to Non-Geology Majors †

Massimiliano Marvasi^{1*}, Yarely C. Davila-Vazquez², and Lilliam Casillas Martinez²

¹Soil and Water Science Department, University of Florida, Gainesville, FL 32610-3610,

²Biology Department, University of Puerto Rico-Humacao, Humacao, PR 00791

We have designed a three-week experiment that can complement any microbiology course, to teach main geomicrobiology concepts for non-geology majors. One of the most difficult concepts for non-geology majors to comprehend is how bacteria serve as a platform for different mineralization reactions. In our three-week laboratory practice, students learn the main principles and conditions required for an induced bacterial mineralization. Upon completion of the laboratory experience, students will: 1) learn how microbial-induced mineralization (such as calcium carbonate formation) is affected by differential media and growth conditions; 2) understand how bacterial physiology affects any induced *in situ* or *in vitro* mineralization; 3) comprehend how growing conditions and bacterial physiologies interrelate, resulting in differential crystal formation. The teaching-learning process was assessed using a pre-/posttest with an increase from 26% to 76% in the number of positive answers from the students. We also measured the students' proficiency while conducting specific technical tasks, revealing no major difficulties while conducting the experiments. A final questionnaire was provided with satisfactory evaluations from the students regarding the organization and content of the practices. 84–86% of the students agreed that the exercises improved their knowledge in geomicrobiology and would like to attend similar laboratories in the future. Such response is the best indicator that the laboratory practice can be implemented in any undergraduate/graduate microbiology course to effectively teach basic geomicrobiology concepts to non-geology majors.

INTRODUCTION

Geomicrobiology is a branch of microbiology that studies the interaction of microorganisms with minerals at the interface of Earth's biosphere and lithosphere in both space and time (5). The fascinating implications of geomicrobiology have resulted in the design of novel pilot courses and discussions in lectures offered in courses like Microbial Ecology or Environmental Microbiology (6, 14). We have designed a three-week experiment that can complement these microbiology courses and teach main techniques and geomicrobiology concepts for non-geology majors.

Indeed, a recurrent topic in all these courses is the study of bacterial-induced mineralizations (BIM), which focuses on how bacteria foster mineral precipitations. Interesting examples of BIM are the recently discovered marine sediments with 3.5 billion year old rocks, and the calcium carbonate formations in modern microbial mats (3, 8). Bacteria,

with some exceptions, induce the carbonate precipitation (or dissolution) of minerals such as CaCO_3 through their metabolic activities. Induced mineralization is regulated by physiological activities and carried out *in situ* via metabolic pathways like photosynthesis, urea hydrolysis, and sulfate reduction (1, 3, 4, 7, 13).

Recently, we have developed an *in vitro* system to study how carbonate precipitation takes place during biofilm formation using environmental strains (9, 10). This methodology, used in our research laboratory, was incorporated into a three-week laboratory practice offered to undergraduate and graduate students attending a microbiology course. The practices in this laboratory were designed to appeal to undergraduate and graduate students, as both populations will discover the relevance of bacterial mineralization, a phenomenon that is frequently completely ignored by students, particularly those with no geology background. Our main lab theme contemplated the interactions and impact of microorganisms in environments with special focus on how microbes transform such ecosystems. Even though they work with bacterial biofilms using *in vitro* settings, through this experience, students learn concepts that can be easily translated into any ecosystem (e.g., carbonate precipitations for cementations of coral reefs). Furthermore, these exercises help students comprehend the relationship

*Corresponding author. Mailing address: Soil and Water Science Department, University of Florida, 2033 Mowry Road – Room 330E, PO Box 103610, Gainesville, FL 32610-3610. Phone: 352-273-8195. Fax: 352-273-5645. E-mail: mmarvasi@ufl.edu.

†Supplemental materials available at <http://jmbe.asm.org>

between specific bacterial metabolisms and their surrounding environments, and how these elements might foster (or delay) specific mineral precipitations.

Intended audience

This activity was designed for microbiology and biology majors with limited knowledge in geology. This laboratory was offered to undergraduate students at the final part of the semester as a complement to the Microbial Ecology Laboratory, to avoid interference with the regular class schedule. Students received a 10-point bonus to attend to the extra laboratories, which were added to their final examination grade. For Master's students attending the Environmental Microbiology class, the activity was a stand-alone module, complementing the course lectures with geomicrobiology concepts.

Learning time

Three laboratory practices, each consisting of three hours on a weekly basis, were sufficient to effectively teach and demonstrate the concepts. As seen in Figure 1, during the first week, students learn to characterize soil microorganisms (provided by the instructor) and inoculate them into different precipitating media. During the following week, students compare the mineralization capacities of such microorganisms, and, finally, in the third week they are able to analyze the crystals formed by the isolates. At the beginning of each laboratory, the professor can reinforce main concepts to teach through a short discussion of the results and/or with the help of a power point presentation.

Prerequisite knowledge

As prerequisites for the course, the students require a general microbiology and general chemistry course. Some of the required skills include: basic culturing and aseptic techniques, classification of bacteria by colony morphology, pigment production and Gram staining, and finally, proper management of a light microscope and a stereomicroscope. Regarding the concepts required for comprehension of the exercise, students need to know about: basic concepts for bacterial precipitation and dissolution of minerals, main bacterial physiologies, acid/base reactions, and the chemistry of redox dyes. All these concepts were reinforced during the course as part of their regular syllabus. General safety guidelines, regularly discussed in all microbiology or chemistry labs, are required to conduct the experiments.

Learning objectives

Upon completion of the three-week laboratory in Introductory Geomicrobiology, students will be able to:

1. Learn about the pH conditions required for a microbial-induced mineralization to take place.
2. Visualize how bacterial metabolism produces changes in pH on the biofilms that consequently alter crystal formation.
3. Contrast how mineral formation is affected (and impaired) by changes in the pH of the growing media.
4. Observe how, in most cases, biogenic crystal formation takes place on an associated matrix.

PROCEDURE

Materials and equipment

To accomplish the three-week laboratory each student will require the following materials (a complete and detailed list is provided in Appendix 1):

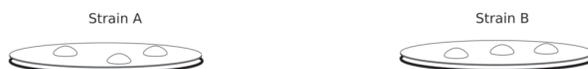
- Five three-compartment Petri dish plates containing standard, alkaline and acidic B4 media
- Five axenic cultures of wild type strains, previously isolated from soil or marine environments (or alternatively soil bacteria such as *Bacillus sp.* strains)
- 10 ml of 0.1 N HCl
- Any commercially available Gram staining kit
- 5 ml Crystal violet 0.1%
- Ethanol
- Gloves
- Chemical goggles
- Inoculation loops
- 1 Beaker (200 ml)
- Glasses and cover-glasses for the microscope

The following equipment is also required: a Biological Safety Hood (BSL level 2), a Stereo (40 X) and Optical Microscope (400 X) and an incubator at 39°C.

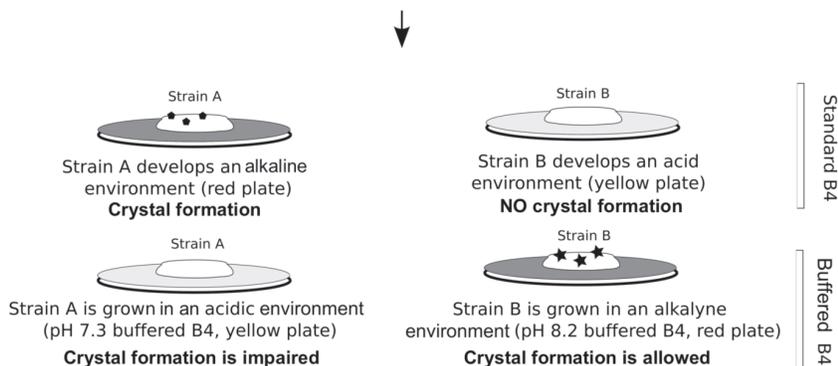
Student instructions

After an introduction to the safety rules, students received a laboratory handout with a table to register their findings (please refer to Appendices 2 and 3 for a complete description). Once students read their protocols, each one received five wild-type strains isolated from different soil samples. Students worked alone. Each student was responsible for characterizing the colonies based on morphology and pigmentation using the stereomicroscope (Leica ES2). Students were encouraged to stain and classify each isolate after Gram staining (or alternative procedure (12)). Cellular morphologies were classified after Gram staining. After reporting their findings in the card (Appendix 3), each student inoculated the identified bacterial isolates on the three-compartment Petri dish plates (Fig. 2 (A) and (B)). Students were provided with three different types of B4 precipitation media (standard, alkaline, and acidic pH)

WEEK 1: Characterization of the different morphotypes.



WEEK 2: Comparison of color development and association with crystal precipitation among the different isolated morphotypes.



WEEK 3: Comparison of different crystal morphology among isolates. Observation of the matrix embedded into the crystals.



FIGURE 1. Example of workflow of the experiments conducted over the three-week period to introduce main geomicrobiology concepts. **Week 1.** Characterization of soil microorganisms based on Gram type, morphology and pigmentation. Unique morphotypes were streaked on different B4 media. **Week 2.** An example is provided of possible results: strain A is grown on both Standard B4 and Buffered B4. When strain A is grown on Standard B4 it can modify the pH by itself, inducing alkalization and fostering precipitation. Below, when the same strain is grown on a buffered acidic environment (Buffered B4), the precipitation does not occur. A similar but opposite example is shown for strain B. Students discriminated between acid/alkaline conditions and presence or absence of crystal precipitation. **Week 3.** Crystals and their matrices were collected from the biofilms and analyzed using the optical microscope.

that were previously prepared and supplemented with a pH indicator (see Appendix 1 for the Instructor's user guide). Plates were then incubated for one week at 39°C inside a plastic bag to prevent dehydration.

During the second week, students were introduced to microbial metabolism and how the production of organic acids results in changes of pH that can be visualized using indicators. Once the concept was understood, students analyzed the formation of crystals and color development on the B4 plates. The development of an alkaline environment in standard B4 plates (visualized by a red color due to the pH indicator) coincided with crystal formation, whereas acidification (yellow-colored plates by a 6.4 or lower pH) was always associated with lack of crystal formation (Fig. 2 (A) and (B)) (10). Crystal formation was completely inhibited in the biofilms grown under acidic B4 media (pH = 7.3). When the standard medium was buffered to pH 8.2, the majority of the strains were able to form crystals (alkaline B4). Students were encouraged to observe the biofilms under the stereomicroscope to see the crystals' formation (Fig. 2 (C) and (D)), and to compare their development using the same strain and media, but grown under different pH conditions.

Students compared their results particularly when a strain was able to precipitate carbonates in an alkaline environment (alkaline B4), but unable to form them under acid conditions (acidic B4). Color development and crystal formation were also reported in the card (Appendix 3).

During the third week, students were asked to study the crystals according to the morphology and the associated matrix, if any. To accomplish these tasks, students collected their crystals from the biofilms with forceps and boiled them in distilled water for 15 minutes. Once biofilm aggregates were removed from the crystals after boiling, they were collected by filtration and dried for 20 minutes at 70°C. Once crystals were isolated, students used an optical microscope with 100X magnification (Fig. 2 (E) and (F)) to observe the grown crystals and their different morphologies. Students were encouraged to compare their crystal morphologies to those described in the literature and those obtained from classmates (for suggested references see Appendix 1).

The last part of the laboratory was devoted to identifying the matrix where the crystals were embedded. Recent studies have shown that carbonate morphology could be

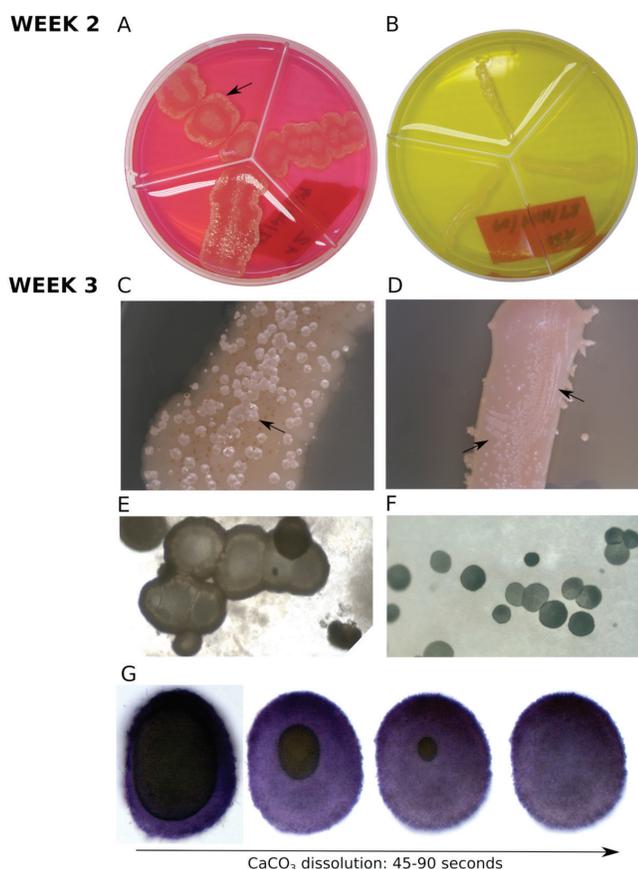


FIGURE 2. Main findings reported after the three-week laboratory experience to introduce geomicrobiology concepts. On panels A and B, two different morphotypes were grown on standard B4 plates with Phenol Red as indicator and incubated for 7 days at 40°C. Biofilms in panel A were alkaline (see red coloration, indicative of pH \geq 8.2 with an arrow indicating the crystals). In panel B, we show a strain with a more acidic metabolism and, consequently, no crystals were formed (yellow, indicative of pH \leq 6.4). Panels C and D demonstrate the observation of CaCO₃ crystals (see arrows) with the stereomicroscope (magnification 4X). Panels E and F show examples of CaCO₃ crystals observed with the optical microscope (magnification 100X). Panel G shows the matrix stained with crystal violet associated with the carbonate crystals that are progressively dissolved after 0.1 N HCl treatments.

dictated by the extracellular polymeric substances (EPS) composing the matrix (1, 10, 11). Indeed, an amorphous matrix was reported in 90% of the examined crystals generated on B4 medium by our environmental strains (10). To observe the matrix, the isolated crystals were stained with crystal violet for one minute followed by 15 seconds in ethanol (70%), and then washed with abundant distilled water. Crystals were then placed on a glass with a micro cover glass, and observed with a Nikon Eclipse E400 optical microscope at 40–100X magnification. Mineral dissolution was tested by adding one drop of 0.1 N HCl between the glass and the cover slip. The students were able to appreciate the gradual dissolution of the crystals by using 0.1 N HCl. This was possible through the formation of CO₂ bubbles

caused by dissolving carbonate (Fig. 2 (G) and Appendix 2). All students reported their findings, including matrix observations, on the card provided (Appendix 3).

Faculty instructions

This experiment requires two to three hours of preparatory activities the day before the first week, and 30 minutes for the other two weeks. The most time-consuming parts are the preparation of the B4 media plates and the isolation of the environmental strains as recommended in Appendix 1. In some courses, students will isolate soil microorganisms as part of a prior microbiology experience. If that is the case, such plates should be preserved. Details for media preparation, time required, storage and safety tips are described in Appendix 1 (2, 10).

During the activities, students are allowed to proceed at their own pace, with the instructor passing through the benches verifying the students' progress. As a summary of the main activities, during the first week students characterize the strains by Gram staining and morphological assessment. If time is limited, as an alternative to the Gram staining, students can use the KOH test using one drop of 0.3 M KOH for quicker identification (12). Approximately three hours are required to accomplish the experiments proposed in the first week. During the second week, students will observe the plates, determine if crystals are present, and fill out the reporting card provided. Approximately one hour and half are required to accomplish the experiments proposed in the second week. The third week is mainly focused on using the optical microscope to identify crystal morphology and the EPS matrix. During this last week, the instructor may arrange different microscope stations in order to have students rotating in a logistical and organized manner, thus making this an easier process. If possible, in one of the stations, the instructor can place a slide with chemically produced carbonate crystals to compare them with biogenic crystals in terms of morphology. Three hours are required to accomplish the experiments in the third week.

Suggestion for determining student learning

To assess student learning of introductory geomicrobiology concepts, we designed a pre- and posttest that consisted of five questions in the following formats: multiple choice, true or false, and fill in the blanks (Fig. 3 and Appendix 4). Each set of questions was designed to address the three main learning objectives. We also designed a checklist to determine the proficiency of the students while conducting different laboratory practices (Fig. 4). At the end of the laboratory experiment, a rubric was provided to the students to evaluate the activities (Appendix 4). In addition, the adoption of a student handout is strongly encouraged (Appendix 2) and it is recommended that it be provided one week in advance, for students to familiarize themselves with the materials and the workflow of the laboratory.

Sample data

In Figure 2 we have provided illustrations of how the three-compartment Petri dishes (each compartment was used as a replica) will look after inoculation of the wild-type strains, and visualization of the crystals on top of the biofilms. In Appendix 3, we have also included an example of a completed student reporting card.

Safety issues

Students wore standard laboratory protection (i.e., lab coat, closed shoes, and gloves at all times). For the manipulation of the environmental isolates, students were requested to work always under a biosafety hood (BSL 2). In addition, students wore safety goggles when handling 0.1 N HCl. At the end of the experiments students were taught proper disposal procedures for the biohazardous material.

DISCUSSION

Field-testing and evidence of student learning

The data presented were collected from undergraduates ($n = 45$) enrolled in the Microbial Ecology laboratories in the Biology Department of the University of Puerto Rico-Humacao (UPRH), and master students ($n = 8$) from the Environmental Science program at the Biology Department of the Pontifical Catholic University of Puerto Rico (PCUPR). The geomicrobiology laboratory sections were organized into these courses during the fall semester in academic years 2011 and 2012. To evaluate learning gains in the field of geomicrobiology, students were given a pre-/posttest (Appendix 4). Results from the test showed a significant increase in student learning, from 26% correct answers in the pretest to 76% correct answers in the posttest (Fig. 3).

In addition, laboratory skills were evaluated during the laboratory exercises. For this evaluation, the instructor used a checklist of seven practices (Fig. 4) to evaluate the students while conducting their tasks. Prior to the laboratory, the instructor prepared one checklist per student and evaluated their performance based on a rubric (see below) while the students were working on the assigned tasks. The scale of the rubric used was as follows: Very Good (5 points): Every time the student was requested to conduct the task he/she was proficient; Good (3 points): the student was able to conduct the task most of the time (failing only on 1 or 2 occasions); Poor (1 point): the student was not able to conduct the assigned task. No significant differences among the averages were reported (significant probabilities at 0.05%), revealing no major difficulties detected while conducting the experiments. Minor difficulties were reported in the color discrimination (Fig. 4, bar C). Petri dish plates that did not develop clear differences were incubated for longer periods for proper classification. Another difficulty

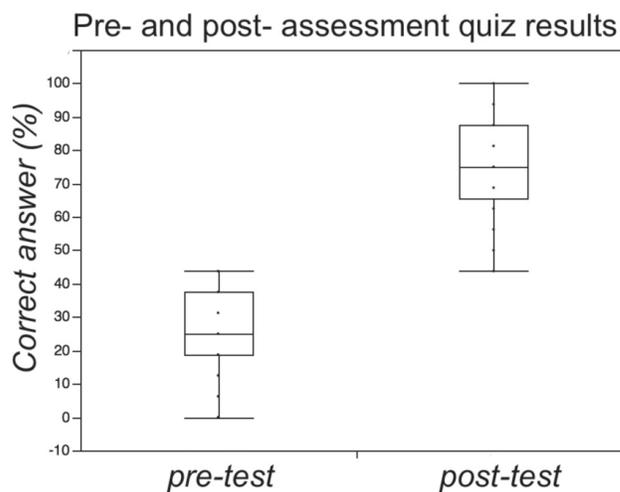


FIGURE 3. Box-and-whisker plot showing the median of 25th to 75th percentile of positive responses in the pre- and posttests to assess student learning in geomicrobiology after the three-week laboratory experiment.

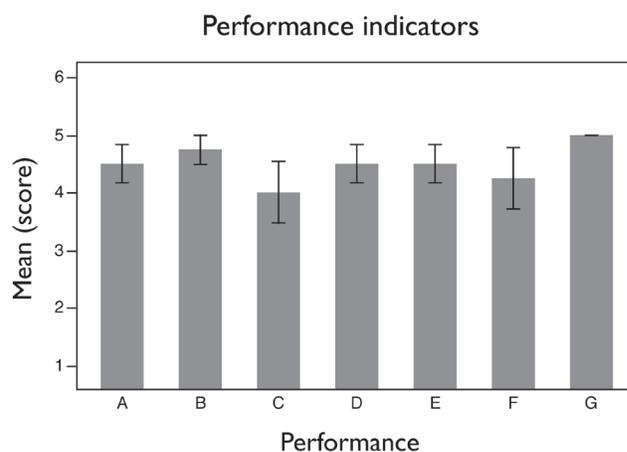


FIGURE 4. Assessment for student performance. The scale ranges from very good (5), to good (3), to poor (1). Tasks assigned: (A) The student is able to prepare the B4 medium as instructed*. (B) The student uses aseptic techniques to streak the isolates on B4 media plates. (C) The student can discriminate among alkaline (red) and acidic (yellow) conditions in the B4 media plates. (D) The student is able to associate acidic conditions on B4 plates with impairment of crystal formation. (E) The student discriminates among different crystal morphologies using the microscope. (F) The student is able to visualize the EPS matrix using the microscope. (G) The student properly identifies the crystals on the biofilm.

* During our experiment we trained students in making Standard B4. The experience was successful but it was very time consuming. Consequently, during the preparation of the manuscript we decided to exclude this part, suggesting the instructor prepare the media for the students.

arose during the observation of the embedded matrix with the optical microscope. In such technique, crystals treated with HCl dissolve in 45–90 seconds (Fig. 4, bar F). Consequently, students were advised to look at the microscope

immediately after HCl addition, otherwise they would not be able to see the reaction.

Students' evaluation of the laboratory activities.

At the end of the three weeks, students were asked to evaluate the three-week laboratory on a scale from 1 to 5, where 1 indicated complete disagreement and 5 indicated complete agreement (Table 1). Eighty-four to 100% of the students completely agreed that the exercises were well explained, organized, and easy to perform. Eighty-six percent of the students improved their knowledge in geomicrobiology and would like to attend similar laboratories in the future. Such responses are the best indicator that the laboratory practice can be implemented in any undergraduate/graduate microbiology course to effectively teach basic geomicrobiology concepts to non-geology majors.

Possible modification

The laboratory experiment can be easily proposed for other settings, for example a one-day workshop to undergraduates and possibly advanced high school students and their teachers. In such a case, the plates will need to be previously inoculated and the crystals must be already formed. Besides the pH, instructors may be interested in testing carbonate formation on standard B4 enriched with different amounts of calcium acetate or using different incubation temperatures. Other acetates work pretty well, such as magnesium acetate and strontium acetate. Interestingly, precipitation can be specific for some ions but not for others: for example students can obtain precipitation with calcium acetate but not with magnesium acetate. This can be another interesting aspect to be explored during the laboratory experience. The calcium acetate recipe can be used for the preparation of other acetates.

SUPPLEMENTAL MATERIALS

Appendix 1: Procedures for B4 media preparation and inoculation of the strains for the Geomicrobiology laboratory practice

Appendix 2: Student laboratory handout and answer key

Appendix 3: Reporting card

Appendix 4: Pre- and post-activity assessment and answer key

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TABLE 1.
Student evaluation of the laboratory activities.

Question	Disagree		Neutral		Agree
	1	2	3	4	5
I enjoyed the exercises	•	2.2%	•	8.9%	88.9%
The experiments were well explained	•	•	2.2%	13.3%	84.4%
The experiments were well organized	•	•	2.2%	11.1%	86.7%
The experiments were easy to perform	•	•	•	•	100%
The experiments were well designed to improve my knowledge in geomicrobiology	•	•	4.4%	8.9%	86.7%
I would attend additional geomicrobiology laboratories in the future	•	•	4.6%	11.2%	84.2%

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