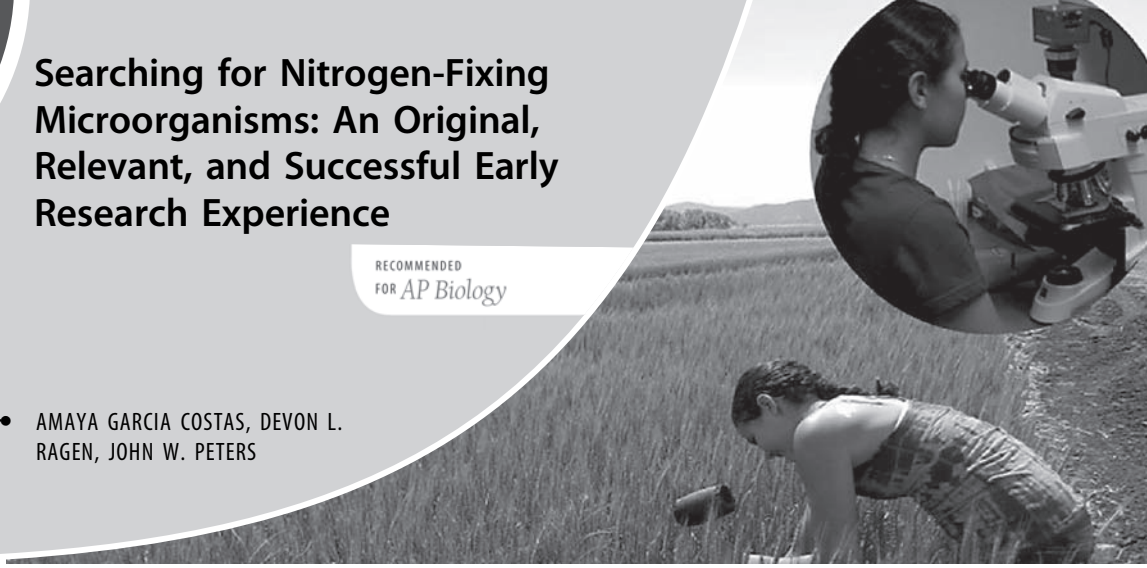
INQUIRY & INVESTIGATION

Searching for Nitrogen-Fixing Microorganisms: An Original, Relevant, and Successful Early Research Experience

RECOMMENDED FOR *AP Biology*



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Abstract

A five-week research project was designed as part of a summer internship for high school students, and could also be used with educators or in introductory undergraduate research courses. This is a guided-inquiry-based project, framed within the significant issue of supplementing fertilizer use in agriculture with nitrogen-fixing microorganisms. This experience exposes students to how scientists are studying real-world problems; it teaches them basic research techniques, and promotes inquiry-based learning in a real research environment. It also fills a current gap in K-12 education that lacks enough microbiology emphasis. Research interns collect soil samples from various fields and use culture-dependent and culture-independent techniques to test whether there are nitrogen-fixing microorganisms that can be isolated and identified in each soil sample. Students work in a research laboratory making nitrogen-free media; culturing, isolating, and identifying microorganisms; extracting soil DNA; and amplifying the 16S rRNA and nifH genes. We administer a pre-test and a post-test, and students present their research both in a short talk and with a poster. By hosting high school students in a research laboratory and immersing them in laboratory science, we hope to inspire them to pursue a STEM-related career.

Key Words: *microbiology; microbial ecology; nitrogen fixation; agriculture; biochemistry; early science research; summer science research; high school science research.*

Introduction

Compared to other groups of organisms, microorganisms play a disproportionately large role in the history of life on Earth, biogeochemical cycles, relationships and interactions with other life forms, and as biotechnology tools, among other topics (Falkowski et al., 2008; Ingraham, 2010). Furthermore, microorganisms are now synthesizing many of our pharmaceutical products and biofuels, are a source of novel medicines, and are predicted to be significant factors in global climate change (Ingraham 2010; Singh et al., 2010). Yet, microorganisms are often ignored or relegated to a side topic in traditional K-12 education. As a result we still have a U.S. citizenry that largely equates microbes with germs.

Research laboratories can be an additional means to narrow the gap between the scientist and the nonscientist view of microorganisms.

A push to increase the role of microbiology in citizen science and in K-12 education is underway (Barberan et al., 2016), with appeals to both educators and researchers to facilitate this process. An increasing number of resources incorporate microbiology-centered lesson plans. In fact, all of the five key life sciences topics outlined in the Next Generation Science Standards can be presented using microorganisms as models (Westenberg, 2016).

Research laboratories can be an additional means to narrow the gap between the scientist and the nonscientist view of microorganisms (Druger & Allen, 1998; National Research Council, 2012). Here K-12 students can observe how scientists do science and can delve in a particular project that involves microbiology. In addition, a guided-inquiry-based research program within a research laboratory is an excellent way to give students the skills and confidence necessary to pursue science careers. This type of outreach experience has been shown to increase the likelihood that students will choose a STEM career, and to boost enthusiasm and understanding of the scientific process (Markowitz, 2004; Roberts & Wassersug, 2009). Furthermore, during a research experience students are able to acquire and use specific laboratory skills and to practice clear and articulate science communication, both of which are necessary to succeed in a career in science.

Here we introduce a five-week-long summer research program that introduces students at the high school level to a research project centered on the relevant problem of feeding an increasingly large human population and how microorganisms will be part of the solution. Specifically, students spend three hours each afternoon

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investigating the nitrogen-fixing bacterial community of various agricultural soils using two microbiological approaches: culture-dependent and culture-independent. Because students are in the lab only in the afternoon, they do not disrupt on-going research activities. This project could be adapted to be carried out by educators wanting to experience inquiry-based science, or to be part of an undergraduate early research experience.

Background on the topic

In the past hundred years, the human population has grown from nearly 2 billion to over 7 billion people, and it is predicted to reach 9 billion by 2050. The challenge of feeding this large population has focused efforts toward increasing crop productivity (Godfray et al., 2010). In the 1960s the so-called Green Revolution made strides toward this goal with the introduction of chemical fertilizers consisting mostly of nitrogen and phosphorous, essential nutrients typically depleted in soils. Currently, about 40–60 percent of crop yields and the feeding of 3 billion people on Earth can be attributed to fertilizer use (Erisman et al., 2008; Curatti & Rubio, 2014). However several problems have surfaced with this practice (Tomich et al., 2016). Fertilizer prices are high, fluctuate, and often represent the largest expense for farmers, diminishing their already small profits. Because of these costs the Green Revolution has not reached or alleviated food scarcity in areas such as Sub-Saharan Africa, where a substantial proportion of the human population resides (Curatti & Rubio, 2014).

Fertilizer use is also responsible for significant ecological damage. Nitrogen leaches into the soil, polluting waterways and leading to algal blooms, loss of dissolved oxygen, and ultimately to the generation of dead zones such as the one in the Gulf of Mexico caused by fertilizer run-off carried down the Mississippi River (Vitousek et al., 1997; Erisman et al., 2013). Nitrogen from fertilizers also pollutes the atmosphere in the form of nitrous oxides; these potent greenhouse gases also damage the ozone layer and contribute to acid rain (Park et al., 2012; de Bruijn, 2015). Finally, fertilizers are energetically expensive to synthesize, constituting 1–2 percent of the U.S. energy budget, and significantly contributing to rising CO_2 atmospheric levels (Vitousek et al., 1997; Erisman et al., 2013).

Prior to fertilizers, biological nitrogen fixation (BNF) was responsible for introducing nitrogen into soils. BNF is the process by which certain members of the Archaea and Bacteria domains, called diazotrophs, are able to reduce inert molecular nitrogen from the atmosphere into reactive (and thus biologically useful) ammonia (Geddes et al., 2015). The two nitrogen atoms in molecular dinitrogen are held by a triple covalent bond and require substantial energy to break. The Haber-Bosch process (industrial nitrogen fixation), developed in the early 1900s, employs high temperature (>450°C) and pressure (>200 atm) to carry out this reaction, hence its high energy budget. On the other hand, nitrogen-fixing organisms, diazotrophs, synthesize the enzyme nitrogenase, which is able to catalyze the 6-electron reduction of N_2 to NH_3 at physiological conditions (Equation 1). This endergonic reaction requires substantial amounts of ATP, as six electrons from a cellular electron carrier such as ferredoxin are combined with six protons to reduce the two nitrogen atoms, three times. A molecule of H_2 is also generated, which requires the input of two additional electrons and protons. Nitrogenase is a complex metalloenzyme, as expected since it catalyzes such a difficult reaction, and consists of an iron-containing homodimer, coded by the gene *nifH*, and a molybdenum- and iron-containing heterodimer coded by *nifDK* (Rees et al., 2005). The iron and molybdenum atoms are responsible for carrying electrons from ferredoxin to N_2 and, therefore, the reductive catalysis of nitrogenase (Rubio & Ludden, 2008; Peters et al., 2011).

Equation 1:

$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi$$

Many plants and diazotrophs have evolved relationships whereby plants provide organic carbon and diazotrophs supply fixed nitrogen (Oldroyd et al., 2011; Geddes et al., 2015). The most relevant of these for agriculture is the legume-rhizobia endosymbiosis that legume crop plants such as lentils, peas, and soybeans have evolved with a group of α and β *Proteobacteria* collectively termed "rhizobia." Because of this relationship, legume crops are not dependent on fertilizer and produce protein-rich foods. Currently there is an active research effort in many laboratories to engineer this symbiotic relationship using different species of bacteria and crop plants such as cereals. This development would reduce the need for fertilizer and increase crop productivity. A critical aspect of this research is to understand the composition of nitrogen-fixing microbial communities in the rhizosphere of crop plants (Geddes et al., 2015; Mus et al., 2016). Our outreach research project contributes to this latter aspect, with students probing various agricultural soils to isolate nitrogen-fixing bacteria.

○ Learning Objectives

Research Project Relevance and Background

1. Explain the segment of the nitrogen cycle that includes the conversion of nitrogen gas to ammonia
2. Distinguish between industrial nitrogen fixation (Haber-Bosch) and biological nitrogen fixation (BNF)
3. Describe various agricultural practices
4. Describe pros and cons of fertilizer use
5. Define symbiosis between legumes and nitrogen-fixing microorganisms
6. Identify nitrogenase as the enzyme responsible for nitrogen fixation

General Microbiology Concepts

7. List biological macromolecules that require nitrogen
8. Distinguish between culture-dependent and culture-independent methods to study microorganisms
9. Define the properties of microbiological media that would be needed to isolate nitrogen-fixing microorganisms
10. List macronutrients
11. List trace elements needed by nitrogenase
12. Define the role of enzymes
13. Distinguish between liquid and solid media
14. Define the outcome of the polymerase chain reaction (PCR)
15. Define the outcome of gel electrophoresis
16. Identify the 16*S rRNA* gene as a common marker for microbial phylogeny

Laboratory Skills

17. Operate the autoclave
18. Prepare microbiological media from supplied powder chemicals
19. Use aseptic technique to distribute media into flasks and to inoculate microorganisms
20. Pour agar plates
21. Isolate DNA from soil using common molecular biology kits
22. Set up PCR reactions
23. Carry out and interpret DNA gel electrophoresis
24. Operate a light microscope
25. Do a BLAST search
26. Follow instructions to construct a 16*S rRNA* gene phylogenetic tree using the Ribosomal Database Project website

Additional Skills

- Understand safety procedures in a laboratory
- Maintain a lab notebook
- Give a 10–15 minute research talk
- Present a poster

○ Procedure

Learning Time and Structure

In our program, students conduct research for three hours each afternoon over a five-week period under the supervision of a laboratory member. See Table 1 for suggested schedule. Most days usually begin with a brief discussion of the previous days' activities, followed by a plan for what should be accomplished during the next few hours. If students will be using a new technique, the discussion will include background and hands-on instruction on that technique.

Student Instruction

First week. During the first day students are given an overview of the project focusing on how it relates to current world problems. This introduction is in the form of a guided talk where students are prompted to be part of the conversation. (For example: What do you think is easier to break, a single covalent bond or a triple covalent bond? What macromolecules contain nitrogen? Are those essential? What do you think are pros of fertilizer use? Do you know any cons?) The students have just completed a pre-research survey, and many of the terms might begin to resonate already. They also watch a short video and are given literature on the topic. At the end of this talk they are introduced to a component of their overall project, isolating nitrogen-fixing bacteria from agricultural soils, and to the first day's activity, making media that does not contain nitrogen. Typically students take the first day and part of the second

Table 1. Suggested schedule.

	Culture dependent	Culture independent	Common
Week 1	• Make and autoclave media • Distribute media into sterile flasks • Inoculate media		• Introduction to relevance of research project • Watch Oxford Sparks, "Give Peas a Chance," https://www.youtube.com/watch?v=R37GuXoLKaQ • Sampling
Week 2	• Monitor liquid enrichments • Make solid media • Spread plating (if enrichments are ready)	• Extract soil DNA (2 samples) • Nanodrop DNA measurement • PCR with 16*S rRNA* primers • Gel electrophoresis	
Week 3	• Spread plating • Streak plates • Make more solid media if needed	• Extract soil DNA (rest of the samples) • Nanodrop DNA measurement • PCR with 16*S rRNA* primers • Gel electrophoresis	
Week 4	• Streak plates • Microscopy of isolates • Colony PCR • Gel electrophoresis • Clean up PCR for sequencing	• PCR with *nifH* primers • Gel electrophoresis	• Prepare poster and talk presentations
Week 5	• Analyze sequences using BLAST • Construct a phylogenetic tree with Ribosomal Database Project website • Clean up flasks, old agar plates		• Finish poster and talk presentation • Give talk and present posters

to make all solutions. During the rest of the week they learn to operate the autoclave and to use an aseptic technique to transfer liquids, and will go sampling. Ideally they will inoculate their flasks containing sterile media with their soil samples by the end of this week.

Second week. Students monitor their cultures most every day by measuring optical density with a spectrophotometer. They also learn how to make solid media. This week students begin the culture-independent component by extracting DNA from their soil samples: they extract DNA from only two samples, as this is their first time using a molecular biology kit. The next day they learn how to use the Nanodrop spectrophotometer to measure DNA concentration, and they set up their first PCR with primers targeting the amplification of the 16*S rRNA* gene to confirm that their DNA is of enough quality to yield amplicons. The third day students learn how to do gel electrophoresis to verify the success of their PCR.

Third week. Students extract DNA from the rest of their soil samples (our students typically have 6 to 12 samples each) and repeat the culture-independent series of experiments from the previous week. Early in the week they take aliquots from their liquid enrichments to spread-plate onto agar plates. Ideally those plates show significant growth by the end of the week, so they can be used as inoculum for streak plates to obtain isolated colonies.

Fourth and fifth weeks. During the fourth week students amplify the *nifH* gene from their soil DNA using universal *nifH* primers; this experiment finalizes the culture-independent component of their project. Students also learn how to operate a microscope and take micrographs of their purified cultures. If isolated colonies are clearly visible, students carry out colony PCR using 16*S rRNA* primers and send those amplicons for sequencing. During the fourth week students also start preparing their presentation and poster. During the fifth week students analyze their sequencing data using BLAST and the Ribosomal Database Project Website, finish preparing their presentation and posters, and make their presentations. Figure 1 shows some of the results students generate throughout their research, and Supplemental Material Appendix 7 shows an example of a student poster.

Faculty Instructions and Supplemental Materials

The supervisor should be familiar with standard microbiology and molecular biology techniques. A handout for every technique introduced in this research project and other supplemental materials are available at https://synthsym.org/news/costas-garcia-et-al-2017-supplementary-information/.

Culture-dependent

- Chemicals (see Supplemental Material Appendix 1), agar, balance, spatula, weigh boats, flasks and bottles, autoclave, petri dishes, rocker or shaker
- Bunsen burner, pipettes and pipette bulb, inoculating loops or sterile toothpicks
- Microscope, microscope slides, and cover slips
- Spectrophotometer, cuvettes
- PCR DNA clean-up kit

Culture-independent

- Soil DNA isolating kit, vortex, vortex adapter/bead beater/homogenizer
- Nanodrop spectrophotometer (optional)

Common

- Thermocycler and PCR reagents
- Gel electrophoresis apparatus, UV trans-illuminator if staining with ethidium bromide
- Micropipettes and tips, Eppendorf tubes and Eppendorf tube trays, centrifuge
- Computers with PowerPoint and Internet access
- Lab notebook
- Diversity of soils, 50 mL-falcon tubes and spatulas for sampling
- Gloves

Suggestions for Determining Student Learning

We conduct a pre- and post-survey with questions that cover general, microbiological, and laboratory concepts (Figure 2; Supplemental Material Appendix 10). During the research program we observe students working in the lab and make sure that they understand and are capable of carrying out each technique. Because most of the techniques must be done several times during the project, students have a chance to practice them and become increasingly comfortable with the procedures. We also informally ask them questions about their project throughout the program. At the end of the five weeks students are required to give a brief talk in which they explain their project and results, and to present a poster at a mini-symposium.

○ Discussion

We have used this curriculum for two years, hosting one and two students, respectively. Each year we have received substantial positive feedback from our students and their outside-of-lab coordinators that indicate that our students are eager to come to the laboratory, engaged and excited about their project, and proud of their laboratory skills. Both our post-survey results (Figure 2) and the quality of the students' poster and oral presentations reveal that students have acquired a clear and sophisticated understanding of their research project and are able to put it in context of a larger problem. Throughout the program we have conversations—often prompted by the students, and often repetitive—regarding the reasoning, hypothesis, and controls behind a particular activity. This opportunity to have ongoing conversations about their project greatly facilitates student understanding and student curiosity.

In addition, all of our students have acquired standard laboratory skills. For example, although many of them had seen an agarose gel in a classroom demonstration prior to this research experience, they had not had the chance to make their own and interpret their own results. Likewise none of the students knew what an autoclave was prior to their time in our lab, nor had any experience in most of the other techniques required for this project, but were confidently carrying out these procedures by the time they finished their research experience. Lastly, students became aware of a pressing societal problem. Although prior to the research experience they had an idea of what fertilizers do, none of them were aware how fertilizers were responsible for nearly 50 percent of the food production in developed countries or of the dramatic ecological footprint of fertilizer use; none had heard of lentils, or of pre-fertilizer agricultural practices such as crop rotation. All

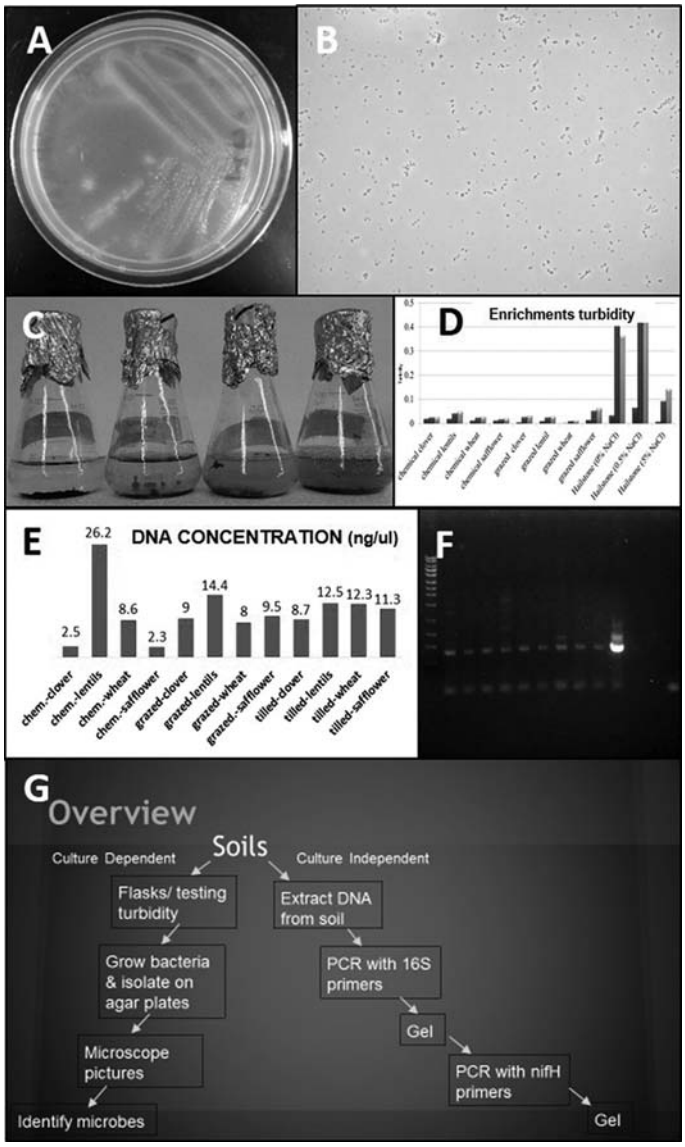

Figure 1. Sample data from student projects. Students are able to obtain isolated colonies **(A)** and micrographs **(B)** from their enrichments **(C)**. They monitor the growth of these enrichments by measuring optical density (turbidity) **(D)**. They are also able to extract DNA from soils **(E)** and carry out PCR and gel electrophoresis; amplicons shown were generated using universal *nifH* primers **(F)**. At the end of the experience, they give an oral presentation; sample slide from presentation **(G)**.

students estimated global human population to be within millions rather than billions at the beginning of the project, even though overpopulation is such a timely topic.

Even though this is a guided inquiry, we strive to let the students be in charge of their projects and activities as much as possible. Accordingly, we keep some aspects of the overall project and of the schedule slightly loose. For example, we take them sampling to an agricultural field station that has four crops cultivated under three different conditions. Although we had imagined that students would set up twelve different enrichments, one for each crop and condition, we find that they develop their own ideas, and might just test one crop under all conditions, or all four crops of one condition, and do that in triplicate. One of our students asked to test an alternative site that consisted of saline soils, which we facilitated. We are also flexible with experiment scheduling: we might have scheduled one afternoon for the students to set up a PCR, but have to adjust when they come with plans to measure their enrichment's optical density and make solid media. Just as with any research experience, experiments can go wrong and might have to be repeated. For example, in one instance the negative control for *nifH* amplification also generated an amplicon. Although disappointing, these errors—and having to repeat experiments to correct the errors—are part of research. The student who had to repeat the *nifH* PCR was visibly elated when her second agarose gel showed a clear positive and negative control.

The presentations, both talk and poster, are critical in getting students to think about the question they are addressing with their project, about how each experiment fits into the larger context, and about the scientific method they are employing. However, they are time consuming, and once students begin preparing their talk and poster, they necessarily spend less time at the bench; during the fourth week and until they finish their presentations, students typically carry out only one or two experiments per afternoon (e.g., setting up a PCR and examining cultures under the microscope) and spend the rest of the time preparing their presentations.

Our most significant challenge is that enrichments take about a week to become dense, and when subcultured into solid media they may take another week to exhibit significant growth. We have had to conduct colony PCR during the last days of their research experience, and the sequencing results have come in during the last day or even a day or two after they have left the lab. In this case, we have instructed students on how to conduct BLAST searches, and we send them the files by email for this post-research experience (in our two years we have not had time to construct a phylogenetic tree with the 16*S rRNA* isolate genes). To avoid this we recommend that sampling be done during the middle of the first week so that plates can be inoculated by the end of the second week and colony PCR can be conducted early or middle of the fourth week. If an incubator is available, cultures could be incubated at 30°C—we leave ours at room temperature (20–25°C)—which would increase the growth rate of the microorganisms. Alternatively, soil particles could be thoroughly mixed with liquid media and then inoculated directly on plates, skipping the liquid culture incubation step (Aquilanti et al., 2004). Lastly, sequences from previous years could be used to construct a phylogenetic tree if sequences will not be available in time.

Possible Modifications

In addition to sampling earlier, we have observed that we are least successful in teaching general microbiology concepts such as the most common elements in living organisms and how those are reflected in our media composition (Figure 2). Although the goal of the research experience is not to teach core concepts, we think it will be useful to the students to see the bridge between the concepts they are learning in the classroom and how they relate to their research project. We will probably add a weekly game on a board that addresses these concepts without being too overwhelming.

This project could be part of an Introduction to Research semester course with the addition of a few experiments in both the culture-dependent and culture-independent components. For

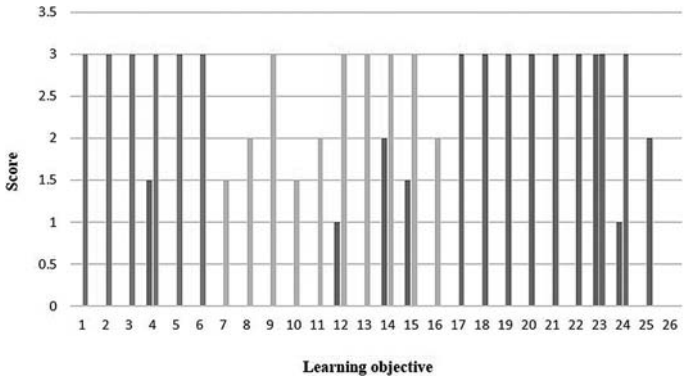

Figure 2. Evaluation of learning objectives. Please see list of learning objectives in the text. Learning objectives are colored as follows: orange = project relevance and background; yellow = general microbiology concepts; red = lab skills. Score is as follows: correct or complete answer by one student = 1 point; partially correct answer by one student = 0.5 points. Three students over two summers were surveyed. In blue are the pre-test or pre-research experience answers. If no bar appears, the score was 0.

example, we use a media composition that is typically used to culture diazotrophs belonging to the *Azotobacter* genus, and students could additionally make media and isolate other diazotrophs such as rhizobia from legume nodules. If agricultural soils are not available, supervisors could plant seeds in small pots ahead of time (lentils, sprouts, etc.), or the question could be rephrased to include any type of soils (e.g., Do we need to fertilize our lawn?). Students could further characterize their isolates by testing their growth with a variety of carbon sources—including lowering the carbon concentration and examine how that affects capsule formation. Quick growing plants such as peas could be grown and watered either with sterile water or with a dilute culture from the nitrogen-fixing isolates to examine the effects of these new isolates on plant growth. For the culture-independent component, students could either characterize the soil community with high throughput 16*S rRNA* sequencing, or they could simply clone some of their *nifH* amplicons into *E. coli* with a TA cloning kit and sequence a few clones from each soil sample. Moreover, there are many bioinformatic resources on the enzyme nitrogenase—for example, available structures in the Protein Data Bank and protein sequences in the National Center for Biotechnical Information (see Supplemental Material Appendix 8)—that could be incorporated into a more biochemically focused examination of enzyme catalysis, including conserved amino acids surrounding the active site, location of the metal cofactors, and protein structure in general.

○ Conclusions

We have designed a research project that guarantees student success, and maximizes student learning and involvement, while exposing students to a research atmosphere. Our research project involves a relevant topic that will be increasingly on the news, and students gain an appreciation for some of the issues that scientists are currently studying while acquiring expertise in routine microbiological and molecular biology techniques. Although this project has been designed as a high school summer research experience, it could be modified to be a research experience for educators, for students beginning their undergraduate career, or as an Introduction to Research semester course.

Supplemental Materials

Handouts and other supplemental materials for this article are available at https://synthsym.org/news/costas-garcia-et-al-2017-supplementary-information/.

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References

Aquilanti, L., Favilli, F., & Clementi, F. (2004). Comparison of different strategies for isolation and preliminary identification of Azotobacter from soil samples. *Soil Biology and Biochemistry, 36,* 1475–1483.

Barberan, A., Hammer, T. J., Madden, A. A., & Fierer, N. (2016). Microbes should be central to ecological education and outreach. *Journal of Microbiology and Biology Education, 17*(1), 23–28.

Curatti, L., & Rubio, L. M. (2014). Challenges to develop nitrogen-fixing cereals by direct *nif*-gene transfer. *Plant Science, 225,* 130–137.

de Bruijn, F. J. (2015). Biological nitrogen fixation. In B. Lugtenberg (Ed.), *Principles of Plant-Microbe Interactions* (pp. 215–224). New York: Springer Publishing Company.

Druger, M., & Allen, G. (1998). A study of the role of research scientists in K-12 science education. *The American Biology Teacher, 60*(5), 344–349.

Erisman J. W., Sutton, M. S., Galloway, J. N., Klimont, Z., & Winiwarter, W. (2008). A century of ammonia synthesis. *Nature Geosciences, 1,* 1–4.

Erisman, J. W, Galloway, J. N., Seitzinger, S., Bleeker, A., Dise, N. B., Petrescu, A. M., Leach, A. M., & de Vries, W. (2013). Consequences of human modification of the global nitrogen cycle. *Philosophical Transactions of the Royal Society London. Series B Biological Sciences, 368*(1621), 20130116.

Falkowski, P. G., Fenchel, T., Delong, E. F. (2008). The microbial engines that drive Earth's biogeochemical cycles. *Science, 320*(5879), 1034–1039.

Geddes, B. A, Ryu, M. H., Mus, F., Garcia Costas, A., Peters, J. W., Voigt, C. A., & Poole, P. (2015). Use of plant colonizing bacteria as chassis for transfer of N(2)-fixation to cereals. *Current Opinion in Biotechnology, 32,* 216–222.

Godfray, H. C., Beddington, J. R., Crute, I. R., Haddad, L., Lawrence, D., Muir, J. F., . . . , & Toulmin, C. (2010). Food security: The challenge of feeding 9 billion people. *Science, 327,* 812–818.

Gu, B., Chang, J., Min, Y., Ge, Y., Zhu Q., Galloway, J. N., & Peng, C. (2013). The role of industrial nitrogen in the global nitrogen biogeochemical cycle. *Scientific Reports, 3,* 2579.

Ingraham, J. L. (2010). *March of the Microbes: Sighting the Unseen.* Cambridge, MA: Belknap Press.

Markowitz, D. G. (2004). Evaluation of the long-term impact of a university high school summer science program on students' interest and perceived abilities in science. *Journal of Science Education and Technology, 13*(3), 395–407.

Mus, F., Crook, M. B., Garcia, K., Garcia Costas, A., Geddes, B. A., Kouri, E.-D., . . . , & Peters, J. W. (2016). Symbiotic nitrogen fixation and challenges to extending it to non-legumes. *Applied and Environmental Microbiology, 82*(13), 3698–3710.

National Research Council. (2012). *A Framework for K-12 Science Education: Practices, Crosscutting Concepts, and Core Ideas*. Committee on a Conceptual Framework for New K-12 Science Education Standards. Board on Science Education, Division of Behavioral and Social Sciences and Education. Washington, DC: The National Academies Press.

Oldroyd, G. E., Murray, J. D., Poole, P. S., & Downie, J. A. (2011). The rules of engagement in the legume-rhizobial symbiosis. *Annual Reviews of Genetics, 45,* 119–144.

Park, S., Croteau, P., Boering, K. A., Etheridge, D. M., Ferretti, D., Frase, P. J., . . . & Trudinger, C. M. (2012). Trends and seasonal cycles in the isotopic composition of nitrous oxide since 1940. *Nature Geoscience, 5,* 261–265.

Peters, J. W., Boyd, E. S., Hamilton, T. L., & Rubio L. M. (2011). Biochemistry of Mo-nitrogenase. In J. W. B. Moir (Ed.), *Nitrogen cycling in bacteria: Molecular analysis* (pp. 59–99). Norfolk, UK: Caister Academic Press.

Rees, D. C., Tezcan, F. A., Haynes, C. A., Walton, M. Y., Andrade, S., Einsle, O., & Howard, J. B. (2005). Structural basis of nitrogen fixation. *Philosophical Transactions of the Royal Society A. Mathematical, Physical and Engineering Sciences, 363,* 971–984.

Richardson, A., Barea, J-M., McNeill, A., & Prigent-Combaret, C. (2009). Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. *Plant and Soil, 321,* 305–339.

Roberts, L. F., & Wassersug, R. J. (2009). Does doing scientific research in high school correlate with students staying in science? A half-century retrospective study. *Research in Science Education, 39*(2), 251–256.

Rubio, L. M., & Ludden, P. W. (2008). Biosynthesis of the iron-molybdenum cofactor of nitrogenase. *Annual Reviews of Microbiology, 62,* 93–111.

Singh, B. K., Bardgett, R. D., Smith, P., & Reay, D. S. (2010). Microorganisms and climate change: Terrestrial feedbacks and mitigation options. *Nature Reviews Microbiology, 8*(11), 779–790.

Tomich, T. P., Brodt, S. B., & Dahlgren, R. A. (2016). *The California Nitrogen Assessment: Challenges and solutions for people, agriculture, and the environment*. Berkeley: University of California Press.

Vitousek, P. M., Aber, J., Howarth, R. W., Likens, G. E., Matson, P.A., Schindler, D. W., Schlesinger, W. H., & Tilmanl, G. D. (1997). Human alteration of the global nitrogen cycle. *Ecological Applications, 7,* 737–750.

Westenberg, D. J. (2016). The engaged microbiologist: Bringing the microbiological sciences to the K-12 community. *Journal of Microbiology and Biology Education, 17*(1), 29–31.

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