Exploring and Enriching for Nitrogen Cycling Microbes: Nitrogen Fixation, Ammonia Oxidizing Lithotrophy, and Anaerobic Nitrate Reduction

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Abstract

In this exercise, students enrich for soil-associated microbes that perform three key nitrogen cycle transformations: nitrogen fixation, aerobic ammonia oxidation, and anaerobic nitrate reduction. In terms of nitrogen fixers, they isolate *Azotobacter* and *Rhizobium* using adaptations of Beijerink's historic enrichments. In terms of aerobic oxidizers, they use ammonia-based media, indirectly observing for lithotrophs by assaying for the production of nitrate and nitrite. In terms of anaerobic reducers, they inoculate two different soils into standard nitrate tubes and test for denitrification and transformations to fixed nitrogen compounds.

Activity

Invitation for User Feedback. If you have used the activity and would like to provide feedback, please send an e-mail to MicrobeLibrary@asmusa.org. Feedback can include ideas which complement the activity and new approaches for implementing the activity. Your comments will be added to the activity under a separate section labeled "Feedback." Comments may be edited.

INTRODUCTION

Learning Objectives.

Upon completion of this activity, students will be able to
(i) understand key reactions and microbes in the nitrogen cycle, emphasizing nitrogen fixation, ammonia and nitrite oxidation, and nitrate reduction;

(ii) understand diverse nitrogen-fixing bacteria with an emphasis on where they live, how they fix nitrogen, and how they can be studied in the laboratory;

(iii) compare and contrast enrichment strategies—both in terms of media components and growth conditions—for different groups of nitrogen-cycling bacteria; and

(iv) observe and explain real examples of aerobic lithotrophy and anaerobic reductions with an emphasis on electron donors and acceptors.

Background.

This laboratory exercise has been carried out at Western Oregon University. A portion of it was included as one component of our poster entitled "A General Microbiology Lab Curriculum Featuring Culture-Dependent and -Independent Approaches and Computer-Based Project Presentations" at the 2004 American Society for Microbiology General Meeting. This exercise allows students to explore and enrich for bacteria that perform three important and distinct conversions in the nitrogen cycle: nitrogen fixation, lithotrophic ammonia oxidation, and anaerobic reduction of nitrate—either to nitrogen gas (denitrification) or fixed nitrogen compounds. In the process of performing these very different enrichments, students will use adaptations of Beijerink's historic *Azotobacter* and *Rhizobium* enrichment strategies (2), master the medically-relevant nitrate reduction test, and use simple, colorimetric chemical assays to demonstrate lithotrophic processes in action. Finally, the starting inoculum for each of these projects involves soil, mud, or clover—all of which are likely readily available and free to most instructors.

This curriculum was first implemented in 1998 for the laboratory component of General Microbiology (Biology 331), a course that all biology majors are required to take. Students complete this laboratory exercise during the first third of this 10-week course, concurrent with lectures about microbial diversity, ecology, and metabolism. By this point, they have mastered several microbiological skills, including aseptic technique, dilution series, direct counting, Gram staining, and environmental enrichments for soil decomposers and anoxygenic phototrophs (1). The course requires *Brock Biology of Microorganisms* (5), an invaluable resource for bacterial diversity and, particularly for this lab, excellent coverage of the nitrogen cycle, enrichment strategies, and metabolic assays (e.g., nitrogenase, radioisotope uptake, and microelectrodes). The same instructor (Boomer) who has developed and delivered lectures also runs the laboratory (which meets Tuesdays and Thursdays for 2 hours); first-person portions of this report refer to Boomer. Students receive all instructional materials at the beginning of the term.

PROCEDURE

Materials.
This exercise should be run with students in teams of two to four individuals. Each team needs:

Session One

Rhizobium enrichment	Clover plants with root nodules 4 to 8 *Rhizobium* agar plates (Two per team member; recipe below)* 1 antiseptic (e.g., 70% ethanol), 1 disinfectant (e.g., 10% household bleach)* 1 dropper bottle of sterile water 2 to 4 empty petri dishes to provide sterile working surfaces *For every additional antimicrobial tested, add two plates per team member.
Azotobacter enrichment	100 ml of nitrogen-free *Azotobacter* liquid medium in a 250-ml flask (recipe below) 2 g of dry nitrogen-poor soil 1 sterile 50-ml beaker and collecting scoop for soil collection Sterile weigh boat, spatula, and access to a scale
Ammonia and nitrite oxidizer	100 ml of ammonia oxidizer liquid medium in a 250-ml flask (recipe below) 2 g of rich organic soil or mud 1 sterile 50-ml beaker and collecting scoop for soil collection Sterile weigh boat, spatula, and access to a scale Nitrate–nitrite test strips (Hach, catalog 2745425) 1 sterile water blank for soil dilution (10 ml of sterile water in a test tube)
Nitrate reducers	Use leftover dry nitrogen-poor and organic soil samples collected above 1 sterile water blank for soil dilution (10 ml of sterile water in a test tube) 6 nitrate reduction tubes (standard test media, commercially available)

Recipes

Azotobacter nitrogen-free medium (1 liter) Solution A K_2HPO_4: 1.6 g KH_2PO_4: 0.4 g Add distilled water to make 0.5 liter. Solution B $MgSO_4$: 0.4 g $CaSO_4$: 0.2 g $FeSO_4$ $7H_2O$: 0.006 g MoO_3: 0.002 g Sucrose: 10 g Add distilled water to make 0.5 liter. Aseptically combine 1A:1B after autoclaving; for plate version, add 15 g of agar to solution B prior to autoclaving. After autoclaving, media will contain some solid material that should be swirled prior to pouring plates.	*Rhizobium* agar (1 liter) Mannitol : 10 g Yeast extract: 1.0 g $MgSO_4$ $7H_2O$: 0.2 g NaCl: 0.2 g K_2HPO_4: 0.5 g $FeCl_3$: 0.005 g Agar: 15 g Add distilled water to make 1 liter. Ammonia oxidizer liquid medium (1 liter) Na_2HPO_4: 13.5 g KH_2PO_4: 0.7 g $MgSO_4$ $7H_2O$: 0.1 g $NaHCO_3$: 0.5 g $FeCl_3$ $6H_2O$: 0.014 g $CaCl_2$ $2H_2O$: 0.18 g NH_4SO_4: 0.5 g Add distilled water to make 1 liter.

All media should be refrigerated. Supplies can be made 4 to 6 weeks in advance. While bottles of liquid media, if handled aseptically, will last for several months (i.e., 2 to 3 class offerings), plates should be made fresh for each class.

Session Two and Follow-up

- 2 to 4 nitrogen-free *Azotobacter* agar plates (one per team member, see below)
- Nitrate–nitrite test strips (Hach, catalog 2745425)
- Nitrate reduction follow-up test reagents—reagents A and B, zinc dust powder

Student Version.
Appendix 1. Lab procedures
Appendix 2. Nitrogen cycle assignment

Appendix 3. Nitrogen cycle worksheet

Instructor Version.
This class lab extends over two sessions that are separated by a 1-week enrichment period, plus a short follow-up 1 to 2 weeks later (qualitative plate evaluation and nitrate–nitrite testing, estimated time 15 minutes). If students have not been exposed to metabolism concepts such as electron donor versus electron acceptor, lithotrophy versus organotrophy, and aerobic versus anaerobic, additional lecture time should be developed to adequately introduce these topics.

Session One
In session one, students are provided with an extensive prelab lecture about the nitrogen cycle, emphasizing nitrogen fixation, lithotrophic transformations (ammonia oxidation and nitrite oxidation), nitrate reduction, and an overview of how they will be addressing each part of the cycle via enrichment methods in this lab unit. Key nitrogen-fixing prokaryotes that are comparatively described include *Azotobacter, Anabaena*, methanogenic archaea, anoxygenic phototrophs, and plant endosymbionts (*Rhizobium* and *Frankia*). Lithotrophic bacteria that are described include *Nitrosomonas* (ammonia oxidizer) and *Nitrobacter* (nitrite oxidizer). Anaerobic nitrate reducers that are described include *Pseudomonas* (typically involved in denitrification to nitrogen gas) and enteric proteobacteria (typically involved in nitrate reduction to nitrite, ammonia, or amino groups). Lecture materials have not been provided in this presentation as all information and images can be readily derived from the course text (5).

Following lecture, student teams typically have about 75 minutes to complete what looks like a daunting setup. In fact, *Azotobacter*, ammonia oxidizers, and nitrate reduction setup time typically requires less than 15 minutes; I recommend teams complete these procedures first, allowing them to warm up for the more difficult and meticulous *Rhizobium* procedures. After doing these procedures for many years, I have found specific places on campus that provide either dry, nitrogen-poor soils or moist, organic soil. The former is used to inoculate both the *Azotobacter* enrichment flask and half the nitrate reduction tubes. The latter is used to inoculate both the ammonia oxidizer flask and the other half of the nitrate reduction tubes. For nitrogen-poor soils, instructors should search for areas that are typically dry, contain light-colored coarse or sandy soil, and lack substantial vegetation or organic debris (e.g., leaf litter); on my campus, I target areas around physically impacted loading or parking areas that are dry as a result of high trees or building cover (Fig. 2). For organic soil, instructors should search for areas that are typically moist, contain dark soil, support extensive vegetation, and clearly receive regular organic debris (e.g., leaf litter); on my campus, I target muddy areas around a man-made pond that is surrounded by an extensive array of low deciduous trees and shrubs (Fig. 3).

To collect soil samples as aseptically as possible, student teams are provided with a sterile collecting scoop and a 50-ml beaker. It is recommended that they obtain the equivalent of 25 ml of material in each case. For current labs, student teams inoculate soil samples within 30 minutes of collection. If instructors want or need to perform soil collections in advance of the lab, soil samples can be refrigerated a few days in advance of inoculation (I personally have not used samples older than 2 days for these labs). Teams also measure the starting concentrations of nitrate and nitrite in the ammonia flask following inoculation using rapid colorimetric strip tests. After 2 weeks, they will carry out this test again and, if their inoculum contained ammonia or nitrite oxidizers, they will see an increase in nitrite and nitrate products, respectively. Figures 1 through 4 show setup samples and procedures for *Rhizobium*, *Azotobacter*, aerobic lithotrophs, and nitrate reducers, respectively. All enrichments are incubated on the benchtop at room temperature (22 to 25°C).

Fig. 1. Nitrogen-fixer enrichment, *Rhizobium*
Fig. 2. Nitrogen-fixer enrichment, *Azotobacter*
Fig. 3. Lithotrophic nitrogen transformations, ammonia oxidizers
Fig. 4. Anaerobic nitrate reduction

To isolate *Rhizobium*, instructors will need a clover plant with intact roots and root nodules. I obtain these either from my yard, from colleagues, or from areas on or near campus. To date, I have never had a problem finding plants with visible root nodules, regardless of location. If instructors need to dig up plants a few days before lab, they should keep the plants in soil and hydrated. In class, students will have to find and dissect root nodules from, in the case of our area, hard-pack clay soil. I have found that this task is made easier if the clover roots are placed in a large plastic tub that is half-full of water an hour before the lab. Instructors who do not have easy access to clover may want to develop their own growing system; cuttings and seeds are available through Carolina Biological Supply and seeds are readily available through a variety of online sources (e.g., Outsidepride.com and Main Street Seed and Supply Company).

Many students will be surprised at how small the nodules are, and some will have a difficult time properly identifying them; thus, instructors should make some effort to monitor teams during their initial selection. My student teams are required to remove two root nodules, one destined for antiseptic treatment and the other for disinfectant treatment. After cutting nodules from the root, they macroscopically clean all major soil from their surfaces using tap water, and then surface disinfect in either 70% ethanol or 10% household bleach. Although I used to encourage student teams to compare a wider array of antimicrobial treatments, I cut treatments down because *Rhizobium* medium is somewhat labor-intensive to make. Instructors who want to include a broader array of antimicrobial treatments should be aware that iodine readily penetrates the nodules and will likely kill most or all *Rhizobium* endosymbionts, an interesting result in and of itself. After treating the cleaned nodules in respective antimicrobial compounds, students aseptically transfer the nodules to drops of sterile water. Using forceps, they crush each nodule until the water suspension is milky, and then use this material to streak *Rhizobium* plates. At room temperature, these plates should develop growth in 1 week.

Session Two
After 1 week, teams spend an entire session completing nearly all subprojects. For *Azotobacter*, each student prepares two streak plates from enrichment flasks using nitrogen-free media agar plates. To date, I have never had any team fail to retrieve *Azotobacter* colonies, which appear as clear teardrops in 1 to 2 weeks (Fig. 2). For clover nodule preparations, teams examine streak plates to determine whether they have isolated *Rhizobium* and compare diversity levels following treatment with disinfectant versus antiseptic. To date, I have never had any team fail to retrieve *Rhizobium* colonies, which appear as large, viscous, opaque, white to ivory colonies (Fig. 1). Typically, plates derived from disinfectant-sterilized nodules are more pure and homogeneous than those derived from antiseptic-sterilized nodules. Owing to the fact that *Rhizobium* medium contains fixed nitrogen and sugar, retrieved colonies can, in fact, be highly diverse, including fungi and *Streptomyces*. Additional simple and rapid tests student teams could perform on their isolates include microscopic

examination with Gram and/or capsule staining: both *Azotobacter* and *Rhizobium* are gram-negative rods and should, on respective media, express prominent capsules. Additional longer tests are mentioned in the Supplementary Materials section.

Teams should also carefully evaluate nitrate reduction tubes, observing for denitrification (bubble in the Durham tube) and testing for nitrite and/or ammonia and amino groups. While some teams detect both transformations, others observe only one of the possibilities (Fig. 4). In general, teams who observe two products are often perplexed as to how more than one reaction could have occurred, having forgotten they are testing a population—not a single, pure isolate. Given variable results, including standard control microbes is something instructors may want to consider; such procedures are readily described in most standard lab manuals (e.g., 6).

Although it is possible for teams to assess nitrate and nitrite production in the lithotroph or ammonia flask, it is preferable to continue this enrichment for at least another week (Fig. 3). Even with this extension, however, I have had entire classes fail to observe ammonia or nitrite oxidizers; odds increase if the enrichment proceeds 4 or more weeks, with heavy aeration. It should therefore be emphasized, both to instructors and students, that retrieving lithotrophs is challenging, owing to the low energetic yield of these reactions. Suffice it to say, developing pure cultures from enrichment flasks is even more difficult and time consuming. As an important supplement for this subproject, teams use their texts to research two more sensitive and rapid metabolic assays for ammonia and nitrite oxidizers: radioisotope uptake and microelectrodes.

Safety and Handling Issues.

Soil samples may contain unknown pathogens, including fecal enterics, fungi, and *Pseudomonas*. Consequently, students are required to wear gloves (we use nonlatex given allergy issues) and clean hands immediately while handling soil and root nodules. Several chemicals used in this exercise are also moderately toxic. Instructors should carefully research and present these issues with respect to selected antiseptics and disinfectants. Nitrate reagents A and B both contain hazardous ingredients (sulfanilic acid, glacial acetic acid, and N-N-dimethyl-l-naphthylamine) that should not contact skin or be inhaled in significant quantities. Both reagents are provided in 50-ml dropper bottles and gloves are used when adding 5 to10 drops per nitrate reduction tube. Given small volumes and the lack of hood requirements in any safety literature about these compounds, however, students work with these reagents on open benchtops. Safety glasses and lab coats should be worn when working with these reagents.

ML Safety Statement regarding Environmental Isolates

The Curriculum Resources Committee recognizes that isolated organisms can be a powerful learning tool as well as a potential biological hazard. We strongly recommend that:

- Environmental enrichment laboratories should only be performed in classes in which students have been trained to work at a BSL2.
- Direct environmental samples (eg. soil, water) which are known to contain infectious organisms should be handled according to the biosafety level of that infectious agent.
- Cultures of enriched microorganisms, derived from environmental samples, should be handled using Biosafety Level 2 precautions.
- Mixed, enriched or pure cultures of microorganisms from environmental samples with a significant probability of containing infectious agents should be manipulated in a biosafety cabinet if available.
- Where possible, media used for the enrichment of environmental isolates should contain an appropriate anti-fungal agent.
- Instructors should be aware if they are teaching in regions with endemic fungi capable of causing systemic infections, and should avoid environmental isolations.

ASSESSMENT and OUTCOMES

Suggestions for Assessment.
Each team turns in completed copies of the Nitrogen Cycle Lab Analysis worksheet (30 points). Additionally, 15% of the lab midterm exam (15 out of 100 total points) covers this lab exercise. The combined value of this lab unit is 15% of the lab assignment grade (45 out of 300 total points).

Field Testing.
Since being developed in 1997, approximately 160 junior- or senior-level undergraduate biology majors have completed this curriculum. Most students (50 to 60%) were pursuing careers in the health sciences. The remaining students sought careers in secondary education and research (academic, government, or biotechnology).

Student Data. We have completed assessment of lab curricula in General Microbiology, which serves a maximum of 16 students per term. Thirty-eight students rated this curriculum on a 10-point scale in fall 2003, spring 2004, and fall 2005, as summarized in the table below.

Please rate the statement: This lab...	Average rating

Made connections beyond microbiology	7.7
Improved my awareness of microbial diversity	8.7
Enhanced my interest in scientific research	7.9
Exposed me to new technology	7.6
Enhanced my organizational skills	7.6
Enhanced my writing skills	6.9
My overall rating of this lab is	8.6

SUPPLEMENTARY MATERIALS

Possible Modification.
Given that the nitrogen fixation components of this exercise are most amenable to extension, I have described several follow-up activities for *Rhizobium* and *Azotobacter*. Although I have facilitated most of these in a teaching lab setting, I have less experience with the acetylene reduction assay for nitrogenase, having only done this experiment as part of an advanced graduate course lab. Unfortunately, the low energetics of ammonia and nitrite oxidizers make recommending additional subculture or identification extensions extremely difficult; moreover, none of these isolates are readily available through commercial vendors. Including any of the following confirmatory tests using pure culture isolates would raise the challenge level of this exercise.

Modifications for *Rhizobium*
Rhizobium extensions include the following:
(i) adding a microscopy component in which students comparatively view bacteroids from nodule crushes versus free-living *Rhizobium* from final plates;
(ii) comparing *Rhizobium* colonies on high versus low sugar plates to observe how the environment influences phenotype and gene expression (with connections to medically-relevant microbes that use capsule virulence factors, like *Streptococcus*);
(iii) infecting naïve clover seedlings with *Rhizobium* isolates (alongside negative controls) to observe for growth differences and nodule formation; and
(iv) performing plasmid isolations on *Rhizobium* isolates, given that nodulation and nitrogen fixation genes reside on Sym plasmids; class isolates can be compared using restriction enzyme-based fingerprinting and gel electrophoresis methods.

Modifications for *Azotobacter*
If equipment and expertise is available, performing an acetylene reduction assay for nitrogenase is a fantastic supplement to this enrichment; unfortunately, this procedure requires expensive supplies and equipment, the most complicated of which is a gas chromatograph. Adventuresome instructors can easily locate a variety of resources on this procedure, including the Manual of Environmental Microbiology (4). Alternatively, students can perform additional phenotypic determination using an array of different media-based tests and microscopy to better identify their isolates. Instructors should consult the Bergey's Manual (3) for ideas about useful tests.

Acknowledgments.
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References.

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APPENDIX 1. Lab Protocol, All Sessions

Introduction to Nitrogen Cycling Bacteria

In this exercise, you will enrich for and characterize bacteria involved in the nitrogen cycle. The following transformations will be emphasized: (i) nitrogen fixation (nitrogen gas to ammonia), (ii) ammonia oxidation (ammonia to nitrite), (iii) nitrite oxidation (nitrite to nitrate), (iv) nitrate reduction to nitrogen gas (denitrification), and (v) nitrate reduction to fixed nitrogen compounds like nitrite, ammonia, and amino groups. Of these, the first four are carried out exclusively by prokaryotes and impact agriculture and waste management. Although nitrate reduction to fixed nitrogen compounds is carried out by many living organisms, nitrate reduction provides a valuable identification test for some pathogens. Microbes that carry out nitrogen fixation, a process that relies on oxygen-intolerant nitrogenase, have each evolved specific strategies for excluding oxygen, including living in anoxic habitats (e.g., methanogens and many anoxygenic phototrophs), differentiating into heterocysts (e.g., cyanobacteria like *Anabaena*), expressing oxygen-binding proteins like leghemoglobin (e.g., *Rhizobium* in concert with legumes), and metabolically reducing oxygen at extremely high rates (e.g., *Azotobacter*). As you work with all of the following enrichments, it is important to understand what features of the media and/or growth conditions have been designed to enrich for each microbe. Lastly, you will research two additional advanced methods, radioisotope uptake approaches and microelectrodes, both of which represent metabolic assays that can be applied to natural populations, bypassing culture and enrichment set up.

ACTIVITIES - SESSION ONE AND FOLLOW-UP

Azotobacter Inoculation and Enrichment

Using a collecting scoop and sterile beaker, obtain 25 ml of nitrogen-poor soil where directed. Using sterile weigh boats and spatulas, weigh 1 g of nitrogen-poor soil on the class scale. Aseptically add 1 g of soil to 100 ml of *Azotobacter* broth and incubate loosely covered at room temperature. After 1 to 2 weeks, each person streaks two *Azotobacter* plates using top flask material (do not swirl!). The goal will be to streak for the isolation of colonies, so remember to use the proper pattern! Incubate at room temperature for 1 to 2 weeks before looking for slimy, translucent, teardrop colonies.

Azotobacter nitrogen-free medium		
Solution A K_2HPO_4: 1.6 g KH_2PO_4: 0.4 g	Solution B $MgSO_4$: 0.4 g $CaSO_4$: 0.2 g $FeSO_4$ $7H_2O$: 0.006 g MoO_3: 0.002 g Sucrose: 10 g	Prepared as liquid or agar (15 to 20 g/liter) plates. Combine 1A:1B after autoclaving. This high sugar enrichment medium enhances capsule production, an *Azotobacter* trait.

Rhizobium Inoculation and Enrichment

Remove and clean two root nodules using tap water; when finished, place in a sterile petri dish. Sterilize one nodule in disinfectant (10% bleach), and the other in antiseptic (70% ethanol) by soaking for 2 minutes. Move each nodule to one drop of sterile water in a sterile petri lid and crush the nodule using a sterilized forceps. Streak loopfuls of each crushed *Rhizobium* preparation onto two *Rhizobium* plates. The goal will be to streak for the isolation of colonies so remember to use proper pattern! Incubate at room temperature for 1 to 2 weeks before looking for slimy *Rhizobium* colonies.

Rhizobium medium Mannitol: 10 g Yeast extract: 1.0 g $MgSO_4$ $7H_2O$: 0.2 g NaCl: 0.2 g K_2HPO_4: 0.5 g $FeCl_3$: 0.005 g	Prepared as agar plates (15 to 20 g/liter). This high sugar enrichment medium is designed to enhance capsule production, a *Rhizobium* trait.

Nitrification Inoculation and Enrichment
Using a collecting scoop and sterile beaker, obtain 25 ml of moist organic soil or mud where directed.
Using sterile weigh boats and spatulas, weigh 1 g of nitrogen-poor soil on the class scale.
Aseptically add 1 g of soil to 100 ml 10 ml water dilution blank.
Add 1 ml of dilution to 100 ml of ammonia oxidizer broth in a flask and perform the nitrate-nitrite test.
During the 2 to 4 weeks of incubation, make sure flasks are well-aerated at room temperature.
After incubation, observe cultures for increased cloudiness and perform the nitrate-nitrite test.

Ammonia oxidizer liquid medium Na_2HPO_4: 13.5 g KH_2PO_4: 0.7 g $MgSO_4$ $7H_2O$: 0.1 g $NaHCO_3$: 0.5 g $FeCl_3$ $6H_2O$: 0.014 g $CaCl_2$ $2H_2O$: 0.18 g NH_4SO_4: 0.5 g	Prepared as a 1 liter liquid stock bottle. Care should be taken to use distilled water as tap water can contain fixed nitrogen compounds.

Nitrate Reduction Inoculation and Enrichment
Obtain four nitrate reduction tubes. LABEL them carefully, according to the following directions:
Two tubes should each be inoculated with 1 ml of organic soil dilution (prepared in the Nitrification section)
Two tubes should each be inoculated with 1 ml of nitrogen-poor soil dilution; prepare by adding 1 g of nitrogen-poor soil to 10 ml of sterile water.

After 1 week at room temperature, complete all portions of the nitrate reduction test (directions below).

Nitrate reduction medium Beef extract: 3 g Peptone: 5 g KNO_3: 1 g	Prepared as 7-ml clear-light gold liquid tubes with Durham tubes. Following growth, read in order: (A) Durham bubble? (yes = N_2*); (B) Add 10 drops each of nitrate test reagents A and B. Red? (yes = NO_2); (C) If not, add 10 grains of powdered Zn and wait 2 to 3 minutes. Red? (yes = no NO_3 reduction) If not, NH_3, NH_2-compounds. *Gas also could be CO_2; additional glucose testing would rule this out.

APPENDIX 2. Team Worksheet - Nitrogen Cycle Lab Analysis

Names: _____

***Azotobacter* Subproject**

1. Is the *Azotobacter* medium selective and, if so, how? Is it differential and, if so, how? Did you retrieve *Azotobacter*? How do you know?

2. Based on your understanding of the acetylene reduction assay for nitrogenase, would your *Azotobacter* isolates, as they exist on the final plate, be positive? Explain.

***Rhizobium* Subproject**

3. Is the *Rhizobium* medium selective and, if so, how? Is it differential and, if so, how? Did you retrieve *Rhizobium*? How do you know?

4. Describe, discuss, and compare surface sterilization results in terms of your observed plate data.

5. Based on your understanding of the acetylene reduction assay for nitrogenase, would your *Rhizobium* isolates, as they exist on the final plate, be positive? Explain why or why not.

Lithotroph Subproject

6. Record your ammonia oxidation flask nitrate and nitrite test results before and after enrichment. Did you observe evidence for lithotrophic oxidation and, if so, which reactions?

7. Microbiologists can also use two more expensive metabolic assays for detecting microbial activities in natural environments: microelectrodes and radioisotope uptake. Using your text, describe how you would use these two different approaches to test for ammonia oxidation and nitrite oxidation in a field setting. Your answers should name specific equipment and reagents you would use and test for and predict observed data, assuming each community was positive.

Nitrate Reduction Subproject

8. Use the following table to record you nitrate reduction data.

	Denitrification?	Reduction to nitrite?	Reduction to ammonia
Nitrogen-poor 1			
Nitrogen-poor 2			
Nitrogen-rich 1			
Nitrogen-rich 2			

9. Did you notice any differences between the two soil types tested in terms of nitrate reduction? If so, speculate on why or why not.

Comprehensive Question

10. For three of the terms below, explain how this process represents a portion of the nitrogen cycle covered by this exercise and name a specific bacterium that does it. Cross out the term we did not attempt to enrich for.

Aerobic chemolithotrophy
Anaerobic chemolithotrophy
Aerobic chemoorganotrophy
Anaerobic chemoorganotrophy

APPENDIX 3. CONCEPT KEY Team Worksheet - Nitrogen Cycle Lab Analysis

Only concept answers are provided; specific data questions vary and are not included in this key.

1. *Azotobacter* medium is selective because it contains no fixed nitrogen source. It is differential because sugar promotes capsule formation, a semidistinctive trait of *Azotobacter*. Teams should recognize these traits in justifying *Azotobacter* retrieval.

2. *Azotobacter* can fix nitrogen in the presence of oxygen and this medium lacks fixed nitrogen; plate isolates would be positive for nitrogenase and acetylene reduction.

3. *Rhizobium* medium is considered less selective than *Azotobacter* medium because it contains fixed nitrogen. It is differential for the same reason *Azotobacter* medium is differential. Teams should recognize these traits and the fact that they obtained their inoculum from the inside of sterile root nodules, theoretically sterile except for these endosymbionts.

5. Once *Rhizobium* leaves the plant, leghemoglobin is not made, nitrogenase is repressed, and nitrogen fixation does not occur. Isolates would be negative for nitrogenase and acetylene reduction.

7. Microelectrodes and Radioisotope uptake

Microelectrodes: teams should indicate that these tools are pH-meter-like tip sensors that are available to measure a variety of compounds. Thus, if they wanted to look for ammonia oxidizers in a mud sample, they could buy a tip for either ammonia or nitrite and probe the mud, looking for either ammonia depletion or nitrite formation, respectively.

Radioisotope uptake: teams should indicate that this assay method involves feeding radioactive compounds to a sample, washing away unincorporated "food" (usually by filtering to retain bacteria), and measuring filtered cells for radioactivity (i.e., they ate the food). For this assay, it is essential that a killed sample be run as a negative control to ensure that incorporation was via a biological process. Thus, if they wanted to look for nitrite oxidizers in a mud sample, they would remove two mud samples (one for live uptake, one to be heat killed), feed each sample radioactive nitrite, and filter. If the filtered live samplewas radioactive and not the negative control, they would predict that nitrite oxidizers were present.

10. Metabolism vocabulary question

Aerobic chemolithotrophy - ammonia (*Nitrosomonas*) and nitrite oxidizers (*Nitrobacter*).

Anaerobic chemolithotrophy - This term should be crossed out; there are such things in nature and I cover some examples in class lecture (e.g., some deep sea vent microbes).

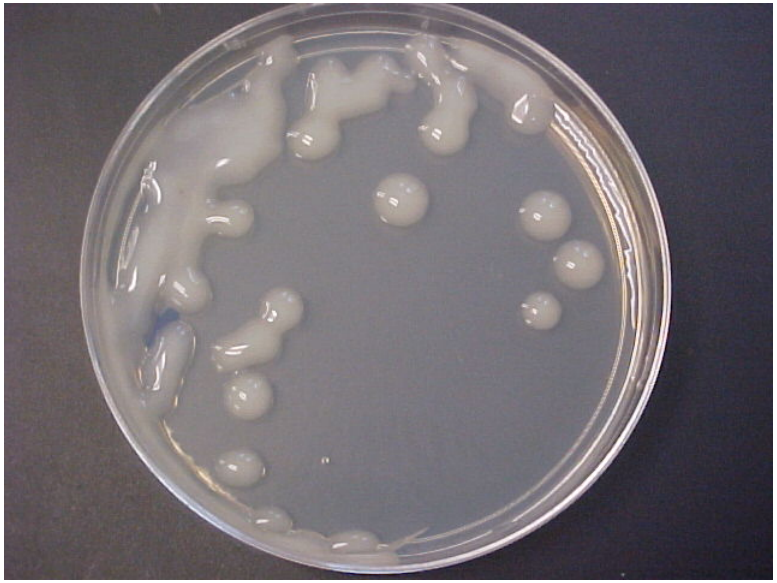
Aerobic chemoorganotrophy - both nitrogen fixers (*Azotobacter* and *Rhizobium*).

Anaerobic chemoorganotrophy - nitrate reducers using this medium (e.g., *Pseudomonas*, denitrification; *Escherichia*, nitrate to nitrite).

A

B

C



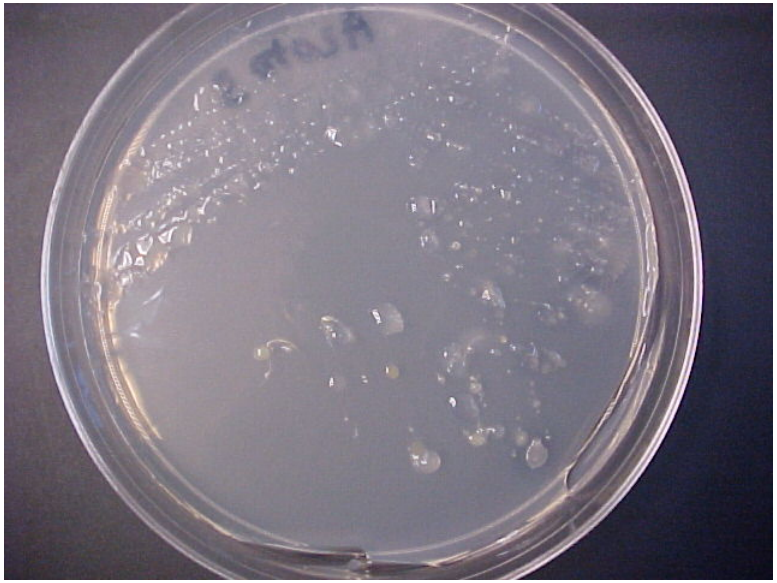
D

FIG. 1. Nitrogen-fixer enrichment, *Rhizobium*. (A) A clover sample before general cleaning. (B) Clover roots and nodules after general cleaning. (C) A crushed nodule in sterile water after antimicrobial treatment. (D) *Rhizobium* on a high sugar primary plate after surface treatment.

A

B

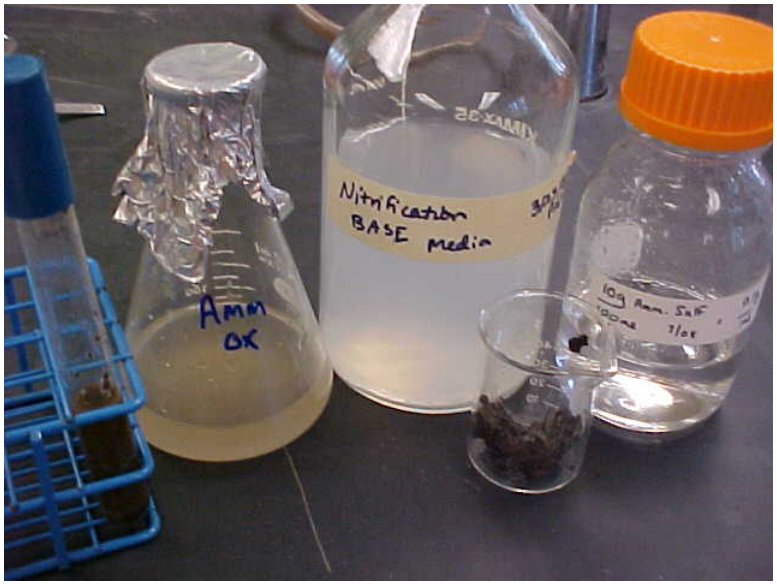
C



D

FIG. 2. Nitrogen-fixer enrichment, *Azotobacter.* (A) The soil sampling site is dry with few organics such as leaf litter. (B) Enrichment setup: soil, inoculated flask, nitrogen-free medium. (C) Primary enrichment flask after 2 weeks. (D) *Azotobacter* on a high sugar primary plate after flask enrichment.

A



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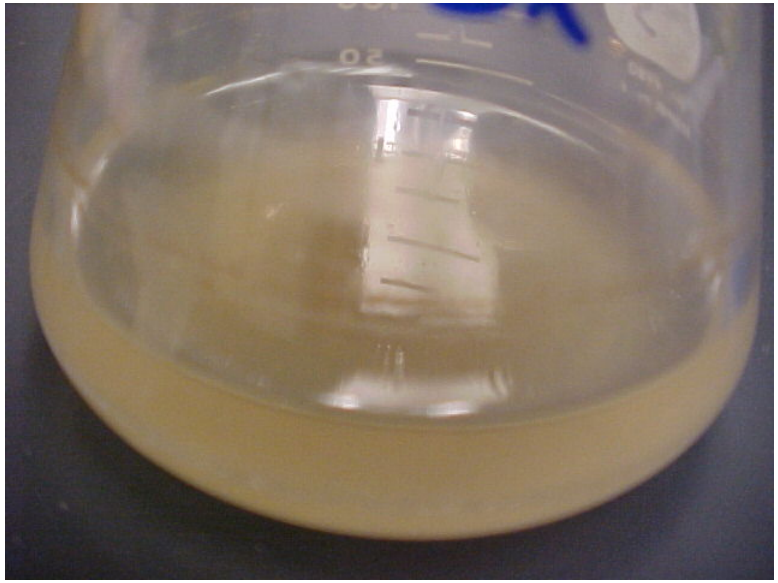

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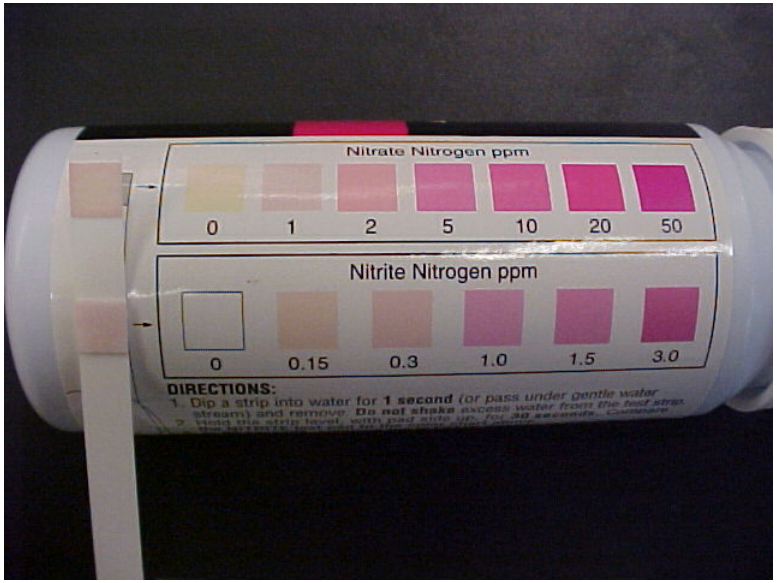
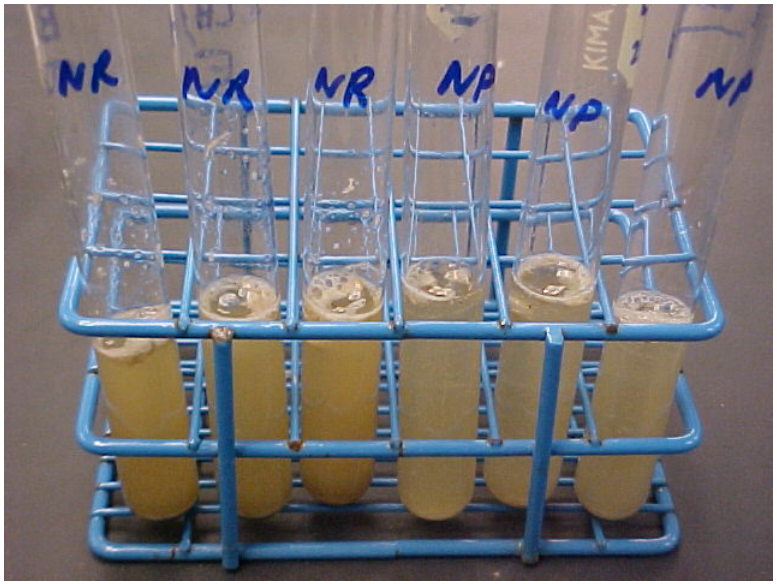


D

FIG. 3. Lithotrophic nitrogen transformations, ammonia oxidizers. (A) The soil sampling site is wet and contains high levels of organics such as leaf litter. (B) Enrichment setup: soil, inoculated flask, ammonia medium. (C) Primary enrichment flask after 2 weeks. (D) Nitrate (top) and nitrite (bottom) testing after 2 weeks.

A



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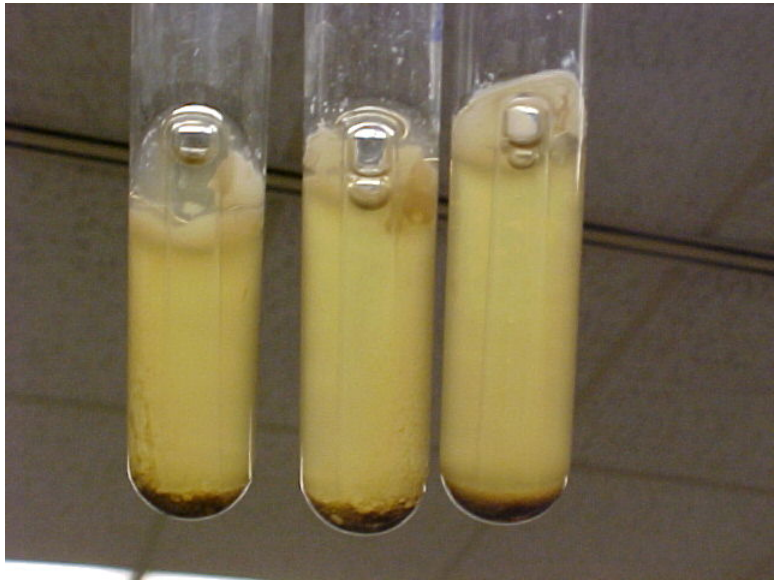

C

D

FIG. 4. Anaerobic nitrate reduction. (A) Two different soil inocula: nitrogen rich (NR; left) and nitrogen poor (NP; right). (B) Nitrate reduction tubes after 1 week: NR (three on the left); NP (three on the right). (C) Denitrification, as evidenced by bubbles. (D) Nitrate reduction reagents and controls.