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

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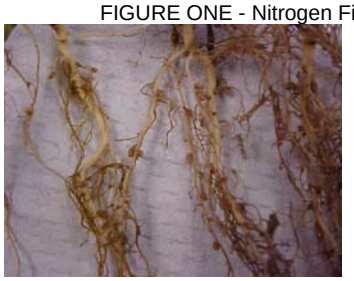
Abstract
In this exercise, students enrich for soil-associated microbes that perform three key nitrogen cycle transformations: nitrogen fixation, aerobic ammonia oxidation, and anaerobic nitrate reduction. In terms of nitrogen fixers, they isolate Azotobacter and Rhizobium using adaptations of Beijerink's 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.

FIGURE ONE - Nitrogen Fixer Enrichment, Rhizobium


Clover sample before general cleaning

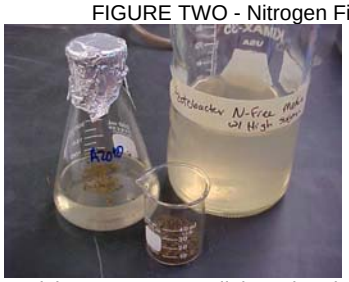

Clover roots and nodules after general cleaning


Crushed nodule in sterile water, after anti-microbial treatment


Rhizobium on high-sugar, primary plate after surface treatment

FIGURE TWO - Nitrogen Fixer Enrichment, Azotobacter


Soil sampling site - dry, little organics/leaf litter


Enrichment set-up: soil, inoculated flask, nitrogen-free media


Primary enrichment flask after two weeks

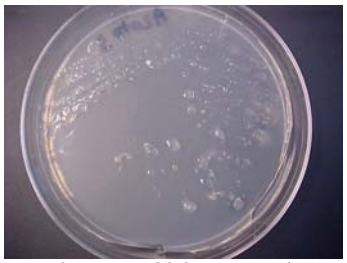

Azotobacter on high-sugar, primary plate after flask enrichment

FIGURE THREE - Lithotrophic Nitrogen Transformations, Ammonia Oxidizers

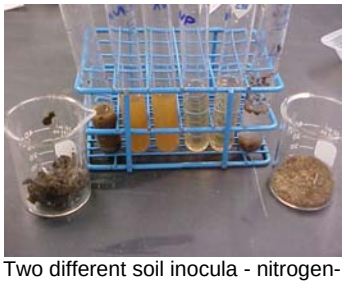

Soil sampling site - wet, high levels of organics/leaf litter

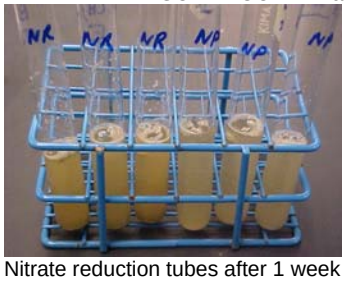

Enrichment set-up: soil, inoculated flask, ammonia media


Primary enrichment flask after two weeks

Nitrate (top) and Nitrite (bottom) testing after two weeks

FIGURE FOUR - Anaerobic Nitrate Reduction


Two different soil inocula - nitrogen-rich/NR (left), -poor/NP (right)



Nitrate reduction tubes after 1 week - NR (3 left); NP (3 right)


Denitification, as evidenced by bubbles


Nitrate reduction reagents and controls

Activity

INTRODUCTION

Learning Objectives.
Upon completion of this activity, students will be able to (1) understand key reactions and microbes in the nitrogen cycle, emphasizing nitrogen fixation, ammonia and nitrite oxidation, and nitrate reduction; (2) 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; (3) compare and contrast enrichment strategies - both in terms of media components and growth conditions - for different groups of nitrogen cycling bacteria; (4) 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 ten-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 instruction materials at the beginning of the term.

PROCEDURE

Materials.
This exercise should be run with students in teams of 2-4. Each team needs:

Session One

Rhizobium Enrichment	Clover plants with root nodules 4-8 Rhizobium Agar Plates (2 per team member; recipe below)* 1 antiseptic (e.g. 70% ethanol), 1 disinfectant (e.g. 10% household bleach)* 1 dropper bottle of sterile water 2-4 empty petri dishes to provide sterile working surfaces **For every additional anti-microbial tested, add 2 plates per team member.*
Azotobacter Enrichment	100 ml Nitrogen-free Azotobacter liquid media in 250 ml flask (recipe below) 2 g dry, nitrogen-poor soil 1 sterile 50 ml beaker and collecting scoop for soil collection Sterile weigh boat, spatula and access to scale
Ammonia & Nitrite Oxidizer	100 ml Ammonia Oxidizer liquid media in 250 ml flask (recipe below) 2 g rich organic soil or mud 1 sterile 50 ml beaker and collecting scoop for soil collection Sterile weigh boat, spatula and access to scale Nitrate/Nitrite Test Strips (Hach, catalog 2745425) 1 sterile water blank for soil dilution (10 ml sterile water in test tube)
Nitrate Reducers	Use leftover dry/nitrogen-poor and organic soil samples collected above 1 sterile water blank for soil dilution (10 ml sterile water in test tube) 6 Nitrate Reduction Tubes (standard test media, commercially available)

Azotobacter N-Free Media (1 L) *Solution A* K2HPO4 : 1.6 g KH2PO4: 0.4 g Add distilled water to make 0.5 L *Solution B* MgSO4: 0.4 g CaSO4: 0.2 g FeSO4/7H2O: 0.006 g MoO3: 0.002 g sucrose: 10 g Add distilled water to make 0.5 L *Aseptically combine 1A:1B after autoclaving; for plate version, add 15 g 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 L) Mannitol : 10 g Yeast Extract: 1.0 g MgSO4/7H2O: 0.2 g NaCl: 0.2 g K2HPO4: 0.5 g FeCl3: 0.005 g Agar: 15 g Add distilled water to make 1 L Ammonia Oxidizer Liquid Media (1 L) Na2HPO4: 13.5 g KH2PO4: 0.7 g MgSO4/7H2O: 0.1 g NaHCO3: 0.5 g FeCl3/6H2O: 0.014 g CaCl2/2H2O: 0.18 g NH4SO4: 0.5 g Add distilled water to make 1 L

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

Session Two and Follow-Up

2-4 Nitrogen-free Azotobacter Agar Plates (1 per team member see below)
Nitrate/Nitrite Test Strips (Hach, catalog 2745425)
Nitrate Reduction Follow-Up Test Reagents - Reagent A and B, Zinc Dust Powder

Student Version.

Lab Procedures - **APPENDIX ONE**

APPENDIX ONE: 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: (1) nitrogen fixation (nitrogen gas to ammonia); (2) ammonia oxidation (ammonia to nitrite); (3) nitrite oxidation (nitrite to nitrate); (4) nitrate reduction to nitrogen gas (denitrification); and (5) nitrate reduction to fixed nitrogen compounds like nitrite, ammonia and amino groups. Of these, the first 4 are carried out exclusively by prokaryotes, and impact agriculture and waste management. Although (5) 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 2 additional advanced methods (radioisotope uptake approaches and microelectrodes) - both of which represent metabolic assays that can be applied to natural populations, bypassing culture/enrichment set-up.

ACTIVITIES - SESSION ONE AND FOLLOW-UP

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

Azotobacter N-Free Media		
Solution A K2HPO4 : 1.6 g KH2PO4: 0.4 g	*Solution B* MgSO4: 0.4 g CaSO4: 0.2 g FeSO4/7H2O: 0.006 g MoO3: 0.002 g sucrose: 10 g	Prepared as liquid or agar (15-20 g/L) plates. Combine 1A:1B after autoclaving. This high-sugar enrichment medium enhances capsule production, an Azotobacter trait.

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

Rhizobium Media	
Mannitol : 10 g Yeast Extract: 1.0 g MgSO4/7H2O: 0.2 g NaCl: 0.2 g K2HPO4: 0.5 g FeCl3: 0.005 g	Prepared as agar plates (15-20 g/L). This high-sugar (enrichment medium is designed to enhance capsule production, a Rhizobium trait.

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

Ammonia Oxidizer Liquid Media	
Na2HPO4: 13.5 g KH2PO4: 0.7 g MgSO4/7H2O: 0.1 g NaHCO3: 0.5 g FeCl3/6H2O: 0.014 g CaCl2/2H2O: 0.18 g NH4SO4: 0.5 g	Prepared as a 1 L 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 4 nitrate reduction tubes - LABEL them carefully, according to the following directions:
2 tubes should each be inoculated with 1 ml organic soil dilution (prepared in Nitrification section)
2 tubes should each be inoculated with 1 ml N-poor soil dilution; prepare by adding 1 g N-poor soil to 10 ml sterile water

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

Nitrate Reduction Media Beef Extract: 3 g Peptone: 5 g KNO3: 1 g	Prepared as 7 ml clear/light gold liquid tubes with Durham tubes. Following growth, read in order: (1) Durham bubble? (yes = N2*); (2) Add 10 drops each of nitrate test reagents A and B. Red? (yes = NO2); (3) If not, add 10 grains powdered Zn and wait 2-3 minutes. Red? (yes = no NO3 reduction) If not, NH3, NH2-compounds. **Gas also could be CO2; additional glucose testing would rule out.*

Nitrogen Cycle Assignment - **APPENDIX TWO**

APPENDIX TWO: Team Worksheet - Nitrogen Cycle Lab Analysis

Names: _____

Azotobacter Sub-Project
1. Is the Azotobacter media 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 nitrogenase/acetylene reduction assay, would your Azotobacter isolates - as they exist on the final plate - be positive? Explain.

Rhizobium Sub-Project
3. Is the Rhizobium media 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 nitrogenase/acetylene reduction assay, would your Rhizobium isolates - as they exist on the final plate - be positive? Explain why or why not.

Lithotroph Sub-Project
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 would you 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/test for and predict observed data - assuming each community was positive.

Nitrate Reduction Sub-Project
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 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.

AEROBIC CHEMOLITHOTROPHY
ANAEROBIC CHEMOLITHOTROPHY
AEROBIC CHEMOORGANOTROPHY
ANAEROBIC CHEMOORGANOTROPHY

Nitrogen Cycle Worksheet - **APPENDIX THREE**

APPENDIX THREE: CONCEPT KEY Team Worksheet - Nitrogen Cycle Lab Analysis

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

1. Azotobacter media is selective because it contains no fixed nitrogen source. It is differential because sugar promotes capsule formation, semi-distinctive trait of Azotobacter. Teams should recognize these traits in justifying Azotobacter retrieval.

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

3. Rhizobium media is considered less selective than Azotobacter media because it contains fixed nitrogen. It is differential for the same reason Azotobacter media is differentia. 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/acetylene reduction.

7. Microelectrodes and Radioisotope Uptake.

Microelectrodes: teams should define/understand 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 define/understand 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 sample - and not the negative control - was radioactive, they would predict that nitrite oxidizers were present.

10. Metabolism Vocabulary Question.

AEROBIC CHEMOLITHOTROPHY - ammonia (Nitrosomonas) and nitrite oxidizers (Nitrobacter).

ANAEROBIC CHEMOLITHOTROPHY - 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 media (e.g. Pseudomonas/denitrification, Escherichia/nitrate to nitrite).

Instructor Version.
This class lab extends over two sessions that are separated by a one-week enrichment period, plus a short follow-up one to two 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 vs. acceptor, lithotrophy vs. organotrophy, and aerobic vs. anaerobic, additional lecture time should be developed to adequately introduce these topics.

Session One
In session one, students are provided with an extensive pre-lab 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 remaining minutes to complete what looks like a daunting set-up. In fact, Azotobacter, ammonia oxidizers, and nitrate reduction set-up time typically require 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 (Figure 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 (Figure 3).

To collect soil samples, student teams are provided with a sterile collecting scoop and 50 ml beaker in order to collect soil samples as aseptically as possible. 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 two 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 One through Four show setup samples and procedures for Rhizobium, Azotobacter, aerobic lithotrophs, and nitrate reducers, respectively. All enrichments are incubated on the benchtop at room temperature (22-25°C).

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 on-line 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 anti-microbial treatments, I cut treatments down because Rhizobium media is somewhat labor-intensive to make. Instructors who want to include a broader array of anti-microbial 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 anti-microbial 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 one week.

Session Two
After one week, teams spend an entire session completing nearly all sub-projects. 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 one to two weeks (Figure Two). For clover nodule preparations, teams examine streak plates to determine whether they have isolated Rhizobium and compare diversity levels following treatment with disinfectant vs. antiseptic. To date, I have never had any team fail to retrieve Rhizobium colonies, which appear as large, viscous, opaque, white to ivory colonies (Figure One). Typically, plates derived from disinfectant-sterilized nodules are more pure/homogeneous than those derived from antiseptic-sterilized nodules. Owing to the fact that Rhizobium media 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/amino groups. While some teams detect both transformations, others observe only one of the possibilities (Figure Four). 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/nitrite production in the lithotroph/ammonia flask, it is preferable to continue this enrichment for at least another week (Figure Three). Even with this extension, however, I have had entire classes fail to observe ammonia or nitrite oxidizers; odds increase if the enrichment proceeds four 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. Sufficed to say, developing pure cultures from enrichment flasks is even more difficult and time-consuming. As an important supplement for this sub-project, 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 non-latex 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 5-10 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.

ASSESSMENT and OUTCOMES

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

Field Testing.
Since being developed in 1997, approximately 160 junior- or senior-level undergraduate Biology Majors have completed this curriculum. Most (50-60%) students 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 nitrogenase/acetylene reduction assay, having only done this experiment as part of advanced graduate course lab. Unfortunately, the low energetics of ammonia/nitrite oxidizers make recommending additional sub-culture or identification extensions extremely difficult; moreover, none of these isolates are readily available in pure culture through commercial vendors. Including any of the following confirmatory tests using pure culture isolates would raise the challenge level of this exercise.

Modifications - Rhizobium

Rhizobium extensions include the following: (1) adding a microscopy component in which students comparatively view bacteroids from nodule crushes vs. free-living Rhizobium from final plates; (2) comparing Rhizobium colonies on high vs. low sugar plates to observe how the environment influences phenotype/gene expression (with connections to medically-relevant microbes that use capsule virulence factors, like Streptococcus); (3) infecting naïve clover seedlings with Rhizobium isolates (alongside negative controls) to observe for growth differences and nodule formation; and (4) 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 - 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 ASM's Manual of Environmental Microbiology (4). Alternatively, students can perform additional phenotypic tests 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.

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