Biology 331 - General Microbiology
The Nitrogen Cycle

Nitrogen Fixation (17.28)
- N2 fixed to NH3 and NH2 groups - COSTS ATP
- ONLY done by Prokaryotes - Bacteria and Archaea
- Conserved O2-intolerant Nitrogenase/Fe-Mo cofactor
- Assayed via acetylene reduction assay - understand

Acetylene and N2 both share triple bond substrates.

Microbial Diversity & O2 Strategies - Table 17.10
- Azotobacter: fastest aerobic ETC uses O2, capsule
- Cyanobacteria: O2-resistant heterocysts
- Methanogens & anoxygenic phototrophs: live anoxic
- Symbionts: co-produce O2-binding "leghemoglobin"

You will view both phototrophs during that lab.

Symbiotic Nitrogen Fixation (19.22)
- Legumes: root hairs secrete inducer molecules
- Symbionts: aerobic soil Rhizobium (Proteobacteria)
- Separate - no fixation; together - leghemoglobin
- Bacteroids: misshapen Rhizobium in nodules

Non-leguminous Azolla/Anabaena, Alnus/Frankia; fine line between bacteroids and organelles…

Rhizobium Enrichment
- Dissect, clean, surface-sterilize nodule, comparing
- Antiseptic: tissue-safe alcohol, H2O2 (Tables 20.3-4)
- Disinfectant: not tissue-safe, bleach (Tables 20.3-4)
- Media HAS nitrogen and simple sugar - capsule

Hospital antiseptics/disinfectants first used by Lister, 1976.

Azotobacter Enrichment (12.9, 17.28, and 18.1)
- Understand Beijerink's enrichment, late 1800's
- Media lacks nitrogen, has simple sugar - capsule

Nitrification and Denitrification
Prominent in soil, water, sewage/feces, and GI tract

Nitrification, Aerobic Chemolithotrophy (17.12)
- Two-step process - ammonia then nitrite oxidation
- NH3 + O2 yields NO2 + H2O + ATP, Nitrosomonas
- NO2 + O2 yields NO3 + ATP, Nitrobacter
- Both are Proteobacteria (12.3); packed with lamellae

Soil enrichment using NH3 media with aeration; measure either NH3 depletion or NO3 appearance.

Anaerobic Nitrate Reduction (17.14)
- EITHER denitrifying (to N2) or nitrifying (to fixed N)
- NO3 + CH2O yields N2 + CO2 + ATP, Pseudomonas
- NO3 + CH2O yields NH3 + CO2 + ATP, Enterics

Review river lab; many other living things also nitrify.

Nitrate Reduction Enrichment/Testing - Table 24.3
- Soil and water inoculation - nitrate reduction tube
- 1 - bubble in Durham = N2 (denitrification)
- 2 - A/B turns red = NO2 present (nitrification)
- 3 (if 2 clear) - Zn turns red = no NO3 reduction

ACTIVITIES

Azotobacter Inoculation and Enrichment

Add 1 g dry N-poor soil (e.g. outside west entrance) to 100 ml Azotobacter broth in flask
Incubate loosely covered on countertop for 1 week before streaking onto 2 Azotobacter plates
Incubate plates on countertop 2-4 days before looking for slimy 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. Agar should be added to Solution B. This high-sugar enrichment medium is designed to enhance capsule production, an Azotobacter trait. This medium is also selective because it contains no nitrogen.

Rhizobium Inoculation and Enrichment

Carefully remove root nodules from target clover legume - surface sterilize two nodules…

One in disinfectant, another in antiseptic - soak 2 minutes - KEEP TRACK of treatment
Place each nodule in 1 drop sterile water in empty dish/lid - crush using a sterilized forceps
Streak loopfuls of each crushed Rhizobium preparation onto 2 Rhizobium plates
Incubate on countertop for 2-4 days before looking for slimy 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. These plates are not particularly selective and must be used in conjunction with surface-sterilization to obtain the best results.

Nitrification Inoculation and Enrichment
Gather 1 g of organic soil (e.g. by NSM Pond) and dilute in 9 ml water
Aseptically add 75 ml Nitrification BASE to sterile 250 flask - LABEL carefully
Calculate how much 0.1 g/ml NH4SO4 stock is added to 75 ml BASE for concentration = 0.5 g/L
Confirm math and add (NH4)2SO4 to flask ; add 1 ml well-mixed soil dilution (save rest for later)
Perform NH3 and/or NO3 tests (will be provided in lab) before and after incubation
For incubation, make sure flasks are placed somewhere well-aerated at room temperature

Nitrification BASE	
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	Prepared as a 1 L liquid stock bottle. Care should be taken to use distilled water. For Ammonia-Oxidizers, aseptically add (NH4)2SO4 solution stock to a final concentration of 0.5 g/L

Nitrate Reduction Inoculation and Enrichment
Obtain 4 nitrate reduction tubes - LABEL carefully, indicating the following contents…
2 replicates should each be inoculated with 1 ml organic soil dilution (collected/prepared above)
Gather N-poor soil and dilute 1 g in 10 ml water - inoculate 2 replicates with 1 ml dilution each
After 1 week on countertop, complete all portions of the nitrate reduction test

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 = NO3 to N2); (2) Add 10 drops A & B. Red? (yes = NO3 to NO2); (3) If not, add 10 grains Zn and wait 2-3 minutes. Red? (yes = NO3 present/no NO3 reduction) If not, NH3, NH2-compounds.

Biology 331 - General Microbiology
Nitrogen Cycle - Web Template

Team Names:

AZOTOBACTER ENRICHMENT

Soil Sample Description	Picture of Soil Sample Pre-Incubation
Flask Post-Incubation Description	Picture of Flask Post-Incubation
Plate Subculture 1	Picture of Plate Subculture 1
Plate Subculture 2	Picture of Plate Subculture 2

Azotobacter Discussion

Did you retrieve Azotobacter? Explain your reasoning fully.

Based on your understanding of the nitrogenase assay, would your Azotobacter isolates - as they exist on the plate - to be positive? Explain.

RHIZOBIUM ENRICHMENT

Sample Description	Picture of Root Nodules
Description of Nodule Disinfection Treatment	Picture of Nodule Disinfection Treatment
Description of Nodule Antiseptic Treatment	Picture of Nodule Antiseptic Treatment
Plate Subculture 1 - Disinfection	Picture of Disinfection Plate Subculture
Plate Subculture 2 - Disinfection	Picture of Disinfection Plate Subculture
Plate Subculture 1 - Antiseptic	Picture of Antiseptic Plate Subculture
Plate Subculture 1 - Antiseptic	Picture of Antiseptic Plate Subculture

Rhizobium Discussion

Compare surface-sterilization results in terms of observed plate data.

Did you retrieve Rhizobium? Explain your reasoning fully.

Based on your understanding of the nitrogenase assay (from above), would your Rhizobium isolates - as they exists on the plate - to be positive? Explain.

NITRIFICATION ENRICHMENT

Soil Sample Description	Picture of Soil Sample
Ammonia Test - Pre-Incubation/Description	Picture of Ammonia Test/Pre-Incubation
Nitrate Test - Pre-Incubation /Description	Picture of Nitrate Test/Pre-Incubation
Ammonia Test - Post-Incubation /Description	Picture of Ammonia Test/ Post-Incubation
Nitrate Test - Post-Incubation /Description	Picture of Nitrite Test/ Post-Incubation
Enrichment Flask/Describe Cloudiness etc.	Enrichment Flask/Detailed Picture of Media

NITRATE-REDUCER TESTING

All samples Pre-Growth/Description	Picture of all samples Pre-Growth
All samples Post-Growth/Description	Picture of all samples - Bubble Detail?
All samples - A/B Testing/Description	Picture of all samples - A/B Detail?
All samples - Zinc Testing (if carried out)	Picture of all samples - Zn Detail?

Nitrification and Nitrate-Reducer Experiments

For each term below, explain whether this process was or could have been occurring in these enrichments, and (if yes) name a bacteria that does it. SOME answers really are a simple "no."

AEROBIC CHEMOLITHOTROPHY

ANAEROBIC CHEMOLITHOTROPHY

AEROBIC CHEMOHETEROTROPHY

ANAEROBIC CHEMOHETEROTROPHY

Methods Extension

In addition to culture-based approaches, microbiologists rely on 2 more expensive culture-independent metabolic assays for detecting microbial activities in natural environments: microelectrodes and radioisotopic label uptake (both described in your text). Describe how would you use these 2 different approaches in a field situation/scenario to detect Nitrosomonas and Nitrobacter, respectively. Your answers should be creative and detailed, naming specific equipment and chemicals you would use/test for, and predicting the observed data (assuming each community was positive).

If the class does well on this question, I won't test you over the material. If not, you may get asked questions about these assays on the mid-term. Historically, students have not enjoyed being asked to do this in an exam setting.