Bacterial Denitrifier Method

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INTRODUCTION

The denitrifier method uses *Pseudomonas aureofaciens* or *Pseudomonas chlororaphis* bacteria to denitrify nitrate (NO_3) to nitrous oxide (N_2O). *P. aureofaciens* is used when the user would like both $\delta^{15}N$ and $\delta^{18}O$ data while *P. chlororaphis* is used when the user is only interested in $\delta^{15}N$. *P. aureofaciens* has markedly reduced exchange rates of the oxygen in water with that of NO_3 during denitrification in contrast to *P. chlororaphis* and thus, $\delta^{18}O$ is more reliable when using *P. aureofaciens*. However, *P. aureofaciens* can yield reduced precision for $\delta^{15}N$ relative to *P. chlororaphis* and thus, if the user is interested in $\delta^{18}O$ and $\delta^{15}N$, consider using both stains for analysis.

Currently the isotope ratio mass spectrometer (Brave Irene) Finnigan DeltaPlus can be configured to run N_2O as N_2O (mass 44, 45, and 46) or as O_2 and N_2 (32, 33, 34, and 28, 29) via hot gold tube pyrolysis of N_2O. This document describes everything from bacteria maintenance to running the mass spectrometer and is rather long. Here are some quick links to get you where you may want to go: Bacterial Growth, Isotope Analysis, Cleaning up, Troubleshooting, References.

BACTERIAL METHOD SUPPLIES

This section outlines how to make everything needed for the denitrifier method. Ideally, everything in this section has been done weeks in advance.

PETRI PLATE PREPARATION:

Petri plates are used to grow colonies from freezer stocks and to ensure you are working with a pure batch without contaminant species.

1. Add 16 g Tryptic Soy Agar and 0.4 g KNO_3 (potassium nitrate) into a 500 mL media bottle, add about 390 mL deionized water, and 5 drops of Antifoam B.
2. Place a plastic ring on top of the threads, cap the bottle and shake. Make sure everything has dissolved completely.
3. Autoclave with indicating tape on bottle (see autoclaving section below)
4. Use autoclave gloves to handle bottle.

5. Pour plates (swirling the bottle periodically so that the solution does not settle while pouring)
6. Let plates sit until cooled, ideally in constant volume hood in 302C, for solidification (~30 minutes).
7. Wrap with parafilm, label with the date plate was created, and store upside-down in refrigerator in room 303B.

GROWTH MEDIA PREPARATION:

The growth media is used, along with the nutrient buffer stock described below, to grow up a batch of bacteria to be harvested for a run of samples.

1. Weigh 60 g TSB (Trypic Soy Broth) into a 2.5 L bottle.
2. Add 2 L DI water (within 3-4 inches of bottle neck) and stir or shake until completely dissolved.
3. Distribute media into desired bottles. Pour in 400 mL each and use a graduated cylinder to top off the last 80 mL. There should be 480 mL of media poured into 500 mL bottles.
4. Put on pour rings. USE PURPLE CAPS. Keep caps loose on media bottles. Use purple media cap.
5. Autoclave with indicating tape on bottles (see autoclaving section below)
6. Tighten all caps very soon after autoclave run is complete.
7. When cool, label with ingredients and prep date and store in bacteria cabinet in 303B.

NUTRIENT BUFFER STOCK PREPARATION:

The nutrient buffer stock provides supplemental nutrients to the bacteria in addition to the media that is outlined above. The original method used by IsoLab and Sigman et al. 2001 and Casciotti et al. 2002 had the growth media and nutrient buffer stock together as one growth media. Also, Casciotti et al. 2002 specifies Potassium phosphate monobasic (KH2PO4). Meredith Hastings (post doc at the time who, with Julia Jarvis set up the denitrifier method) chose to use K2HPO4 thinking it might be a better buffer when bacterial stocks kept dying off (Meredith, personal communication). The Casciotti method also specifies ammonium chloride where we use ammonium sulfate. This was a choice early in the setup of the method in IsoLab based on what was available at the time (Meredith Hastings, personal communication). The goal is to make nitrogen other than nitrate available for building. Its not clear to me if the sulfate is affecting things.

1. Weigh 50 g KNO_3 (Potassium nitrate), 250 g K_2HPO_4 (Potassium phosphate) and 100 g $(NH_4)_2SO_4$ (ammonium sulfate) into a 2.5 L bottle.
2. Add 2 L deionized water (within 3-4 inches of bottle neck) and stir or shake until completely

dissolved.
3. Distribute 500 mL into 500 mL bottles.
4. Put on pour rings. USE PURPLE CAPS. Keep caps loose on bottles.
5. Autoclave with indicating tape on bottles (see autoclaving section below)
6. Tighten all caps very soon after autoclave run is complete.
7. When cool, label with ingredients and prep date and store in bacteria lab cabinet in room 303B.

NITRATE-FREE MEDIA PREPARATION:

The nitrate-free media is used during a harvest to keep the bacteria growing, but depriving them of nitrate so they must use nitrate from samples.

1. Weigh 60 g Trypic Soy Broth, 10 g K_2HPO_4 (Potassium phosphate), and 4 g $(NH_4)_2SO_4$ (ammonium sulfate) into a 2.5 L bottle.
2. Add 2 L deionized water (within 3-4 inches of bottle neck) and stir or shake until completely dissolved.
3. Distribute Nitrate-Free Media into desired bottles (400-500 mL into 500 mL bottles.)
4. Put on pour rings. USE ORANGE CAPS. Keep caps loose on media bottles.
5. Autoclave with indicating tape on bottles (see autoclaving section below)
6. Tighten all caps soon after autoclave run is complete.
7. When cool, label with ingredients and prep date and store in bacteria lab cabinet in room 302C. Media should be transparent.

AUTOCLAVING:

The autoclave is a large unit the uses steam under pressure to sterilize solids and liquids.

1. Autoclave as part of larger batch for efficiency.
2. Use autoclave indicating tape to determine successful Autoclave run. The transparent stripes on the indicating tape turn black if the autoclave reached a proper temperature for a proper time.
3. The autoclave is located in Johnson Hall room 227. IsoLab office has a key.
4. Select the appropriate program for your autoclaving purposes. "Liquid 60" for all liquids. "Grav 20" for petri plates and other solids.
    - Liquid autoclaving: Keep caps on bottles loose, the autoclave should be set to "Liquid 60" 121 ˚C for 1 hour 30 minutes.
    - Dry items (such as pipette tips, centrifuge tubes, used bacterial agar plates): Used bacterial agar plates should be placed in autoclave bag which can be found in the

cabinets beneath the fume hoods in room 303B, the autoclave should be set to "Grav 20" 121 ˚C for ~45 minutes

5. Sign-in on autoclave log with name, room number, budget number (06-9146), cycle used, and whether or not a test strip is present.

MAKING CONCENTRATED SALT SOLUTION FOR STANDARDS:

1. Calculate the amount of the salt to weigh out to make 500 mL of 4mM salt solution. Approximately 170 mg of $NaNO_3$ salt is needed to make a final volume of 500 mL at 4 mM. Refer to BacterialDenitrifierCalculations.xlsx located in the Methods folder of the lab server. You can also weigh appropriate amounts of salt to go straight to a 20 uM or 100 uM solution.
2. Rinse out the plastic nalgene bottle thoroughly with 18 MΩ water.
3. Use the graduated cylinder to measure out 500 mL of 18 MΩ water and pour into the thoroughly cleaned plastic nalgene bottle.
4. Measure out the needed amount of salt on the scale and add it the clean nalgene bottle.
5. Cap the bottle and shake until the salt has dissolved.
6. Label the bottle with standard name, concentration, date created, and name of the creator.
7. If the standard is not going to put to immediate use, wrap with parafilm and store in the freezer, otherwise put in the refrigerator.

DILUTING STANDARDS:

1. Calculate the amount of 18 MΩ water and concentrated standard mixture you need to make the desired standard.
   - ex: For 500 mL of 100 uM of desired standard:
     - Use 12.5 mL of 4mM desired standard
     - Use 487.5 mL of 18 MΩ water
     - (Desired Concentration / (4mM of desired standard × 1000)) × total volume of desired standard
       (100uM / (4mM × 1000)) × 500 mL = **12.5 mL of desired standard is needed**
       500 mL – 12.5 mL = **487.5 mL of 18 MΩ water is needed**
     - Refer to BacterialDenitrifierCalculations.xlsx located in the Methods folder of the lab server for more help if needed.
2. Rinse out the plastic nalgene bottle thoroughly with 18 MΩ water.
3. Use the graduated cylinder to measure out the correct amount of 18 MΩ water and desired standard water and pour into the thoroughly rinsed nalgene bottles.
4. Label the bottle with standard name, concentration, date created, and name of the creator.

5. If the standard is not going to put to immediate use, wrap with parafilm and store in the freezer, otherwise put in the refrigerator.

BACTERIAL STRAIN PRESERVATION

The section describes is to preserve bacteria strains of Pseudomonas aureofaciens and Pseudomonas chlororaphis in 15% glycerol solution for long-time storage in a -80˚C freezer. The purpose of storing strains of bacteria in a -80˚C freezer is to have archives of newly acquired or created strains.

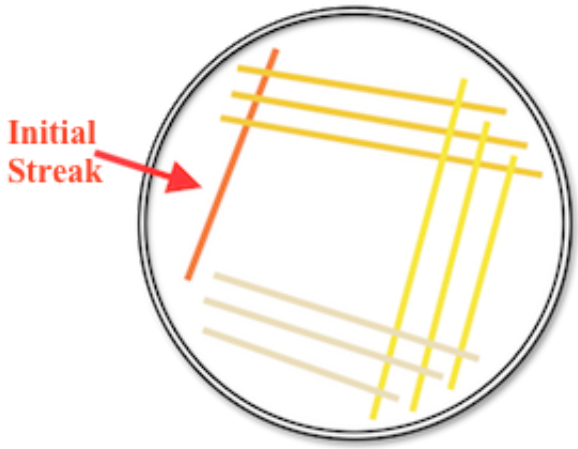
1. Making 60% Glycerol Solution
    - The solution is stored in the refrigerator.
    - If solution is low, make more by using a purple screw cap 100 ml media stock bottle to store the solution.
    - Using a graduated cylinder, measure 30 ml of Glycerol. Pour into the media bottle.
    - Rinse the graduated cylinder with DI water and then fill to 20 ml of DI water. Pour into the media bottle. The total volume of solution should be 50 ml.
    - Vortex or just swish the solution well and have it autoclaved.
    - Store solution in the refrigerator.
2. Making Fresh Liquid Bacteria Culture (Inoculation) P. aureofaciens + P. Chlororaphis
    - Use flamed loop to transfer a single colony of P. aureofaciens from plate #2 or #3 to 5 ml of nutrient broth in a sterilized culture tube.
    - Leave on shaker overnight
3. Autoclaving
    - Autoclave as part of a larger batch for efficiency at 121˚C for 30 minutes.
    - Located in Johnson Hall room 227
    - Autoclave should be set to 'liquid 60'.
    - Supplies to autoclave include: Micro centrifuge tubes, Pipette tips, 60% glycerol solution, Include other supplies related to method for efficiency (ie: Petri plates, etc)
4. Making the Bacteria Archive
    - Label a sterile micro centrifuge tube with the date and name of bacteria
    - Pipette using a sterile pipette tip 0.1 ml of sterile 60% glycerol solution into the tube.
    - Using a new pipette tip, add 0.3 ml of the bacterial culture (frozen stock will be 15% glycerol). Use a new pipette tip for each bacteria strain.
    - Cap the tube and shake to mix the bacteria culture and the glycerol solution together.
    - Using a cooler filled with liquid nitrogen, immediately place the tubes inside to flash freeze the bacteria stock.
    - Bring cooler to Oceanography building (Marine Sciences) room 207 where the -80˚C

freezer is located and store tube in the freezer.
    - Remember to bring back the cooler.
5. References for bacterial preservation
    - Long Term Storage of Bacterial Strains, Nov. 14, 1990. Donis Keller Lab, C. Helms.
    - Bacterial Glycerol Stocks, University of North Carolina.
    - Glycerol Stocks of Transformed Cells, South African Structural Biology Initiative.

BACTERIAL GROWTH

REVIVING BACTERIA CULTURES FROM FREEZER STOCK:

1. Freezer stocks of *P. aureofaciens* and *P. chlororaphis* are stored in the -80 *C freezer in Johnson Hall Room 211. Enter through 202A or 203A.
2. Label plate with bacteria species, date and plate #.
3. Use a cooled flamed loop to remove a dollop of frozen cells and spread on the plate.
4. Flame loop again and streak as in Figure 1, flaming between each new streak direction.



Ideal streak pattern

5. Wrap the plate using the parafilm.
6. Incubate the plate at room temperature overnight.
7. When single colonies are visible, transfer plate to the refrigerator.
8. Three to four days after streaking plate #1, transfer a single colony by flamed loop from plate #1 to new plate and streak as in Figure 1 and label as plate #2.
9. Using plate #2, repeat step 8 to make a plate #3. In this way, you are ensuring you have a pure, single species, culture.

STARTER CREATION:

We try to inoculate media bottles with a large number of viable cells by using these "starters".

1. Add 20 mL of the Buffer Stock Solution into 480 mL of media. Inoculate from a single colony from plate 2 or 3. If you inoculate from an existing starter or from left-overs of a harvest, make a plate from this same source so we can verify its purity. Refer to section on Inoculation of Media Bottles for inoculation steps.

2. When ready, it is time to harvest the bacteria. Equally distribute media to 16 centrifuge tubes and centrifuge (18 °C, 10 min, 7500 g).
3. Turn on centrifuge in 302B, use SS-34 rotor, use arrows to find rotor type, set "rcf" to 7500, set temperature to 18 °C, adjust time to 10 minutes...you can also use "recent" settings if this spin was used recently.
4. The bacteria plug should be dime-sized and pink when finished.
5. It is very important that the plug be pink and dime-sized as anything otherwise it will produce poor results.
6. Pour off supernatant into media bottle.
7. Make new media bottle as in step 1 and use it to re-suspend dime-sized plugs remaining in the centrifuge tubes. Transfer each resuspension back into the media bottle you just created.
8. Distribute ~10 mL of media into 50 VWR sterilized centrifuge, conical bottom tubes with blue caps.
9. Label starter solution with starter label and date. Store in the fridge until needed for inoculating other media.

INOCULATION OF MEDIA BOTTLES:

1. Add 20 mL of the Buffer Stock Solution into the 480 mL of media. Shake well.
2. Remove one centrifuge starter tube **of appropriate species** from the fridge and re-suspend using the vortex.
3. Once re-suspended and mixed, pour starter into 500 mL media bottle to inoculate the 480 mL of media and 20 mL Buffer Stock Solution mixed earlier.
4. Label bottle(s) with species, and inoculation date.
5. Cap tightly and put on orbital shaker in room 303B. Keep the orbital shaker set to 4.
6. Two days after inoculating, test for nitrite and nitrate using the fish tank test kits in the bacteria lab drawer. Nitrate and nitrite should test 0-5 ppm. If this is not the case, leave on the orbital shaker for additional time and retest later.
7. Store in the fridge until the you are ready to harvest.
8. Bacteria are ready for harvest 2 days after inoculation. Ideally, the bacteria are harvested slightly before finishing up with all the nitrate in the media bottle. In this way, you are reasonably certain that all appropriate enzymes are active. The time until they are ready varies and is largely influenced by the number of active cells you inoculate with. It might be that P. aur. and P. chlor. are ready at different times as well.
9. You can use the nitrate aquarium test to coarsely measure nitrate concentrations before harvesting. Tests so far suggest that its ok to harvest even if this test yields a concentration of 40-80 ppm nitrate.

HARVESTING BACTERIA:

1. Equally distribute media to 16 centrifuge tubes and centrifuge (**18 °C, 10 min, 7500 RCF**). Turn on centrifuge in 302B, use SS-34 rotor, use arrows to find rotor type, set "rcf" to 7500, set temperature to 18 °C, adjust time to 10 minutes. You can also use "recent" settings if this spin was used recently. The bacteria plug should be dime-sized and pink when finished.
   White pellets are either the result of P. aur being at a different life stage than desirable or the presence of a different species. The pink color originates from the enzyme responsible for converting NO2 to N2O. This enzyme is red. P. chlor. has a slightly different enzyme with a different metal center and one should not expect such a pink pellet. The distinction is ambiguous if your pellet is somewhere between white and pink. If it is most certainly white, you should consider abandoning that bottle. Don't mix one bottle that has white pellets with one that has pink pellets.
2. Pour supernatant back into media bottle.
3. Using an autoclaved pipette tip, reconstitute the bacteria plug in **2.8 mL of nitrate free media** and vortex until plug is completely dissolved.
4. If harvesting two bottles, fill centrifuge tubes to the neck and use **3.4 mL of nitrate free media.**
5. Remaining nitrate free media from the opened container should be stored in the refrigerator.
6. Add the 2.8 mL aliquots together into a 100 mL media bottle, then add a few drops of well mixed Antifoam B, mix well (Antifoam B must be mixed thoroughly to be effective).
7. Repeat steps 1-4 for a second 500 mL inoculated media bottle.
8. Using an autoclave pipette tip, add **2 ml of media to each of 40 sample vials.**
9. Crimp the aluminum seals and gray septa onto the vials with the crimper. Make sure the crimper head is tight. When crimping you should NOT be able to squeeze both handles all the way.
10. Put a long blue needle through each septum in each vial for venting.
11. Turn on nitrogen cylinder. Output pressure should be 10 psi. Flow rate through each vial will be approximately 7 mL / min.
12. Invert vials and push brown needle through septum. It must begin bubbling immediately. If it does not, ensure nitrogen is on, check needles for clogs.
13. Purge for 2 hours (1 hour is enough, 2 hours is safe, 3-4 hours is excessive).
14. Record details into the Bacteria Notebook (or personal notebook).
    - Date of harvest, who is harvesting, the samples to be run, plate dates, inoculation dates, nitrogen pressure used on the manifold, start and end purge times and any other information you feel is necessary.
15. When sufficient time has passed, remove each vial immediately followed by the blue needle.

If you poke yourself with a needle immediately wipe the punctured area with Iodine wipes found in the First Aid Kits. Blue needles are discarded into the red sharps container.

16. Label vials 1-40 on the bottom with sharpie.
17. Add designated sample to each vial, taking care to rinse syringe and needle thoroughly with 18 MΩ water between each sample. Using three beakers of 18 MΩ, rinse syringes 3 times and 1 full mL through needle. NEVER touch needle to sample. Always pull up sample and 18 MΩ with syringe only.
18. Vent vial if injecting more than 2 mL of total liquid by using a brown needle as a vent.
19. The maximum sample volume that can be injected into a vial is **13 mL** (total volume then is 15 mL - sample + media).
20. The injection amount can be calculated using the spreadsheet BacterialDenitrifierCalculations.xlsx.
    - The Target NO3 amount for N2O method is 20 nmoles
    - The Target NO3 amount for O2N2 method is 100 nmoles (350 nmoles is maximum)
21. If possible, rinse needle and syringe with 1 mL of sample water before injecting each sample into their vial.
22. Put sample vials in Styrofoam container upside-down and put on shaker set to low overnight.

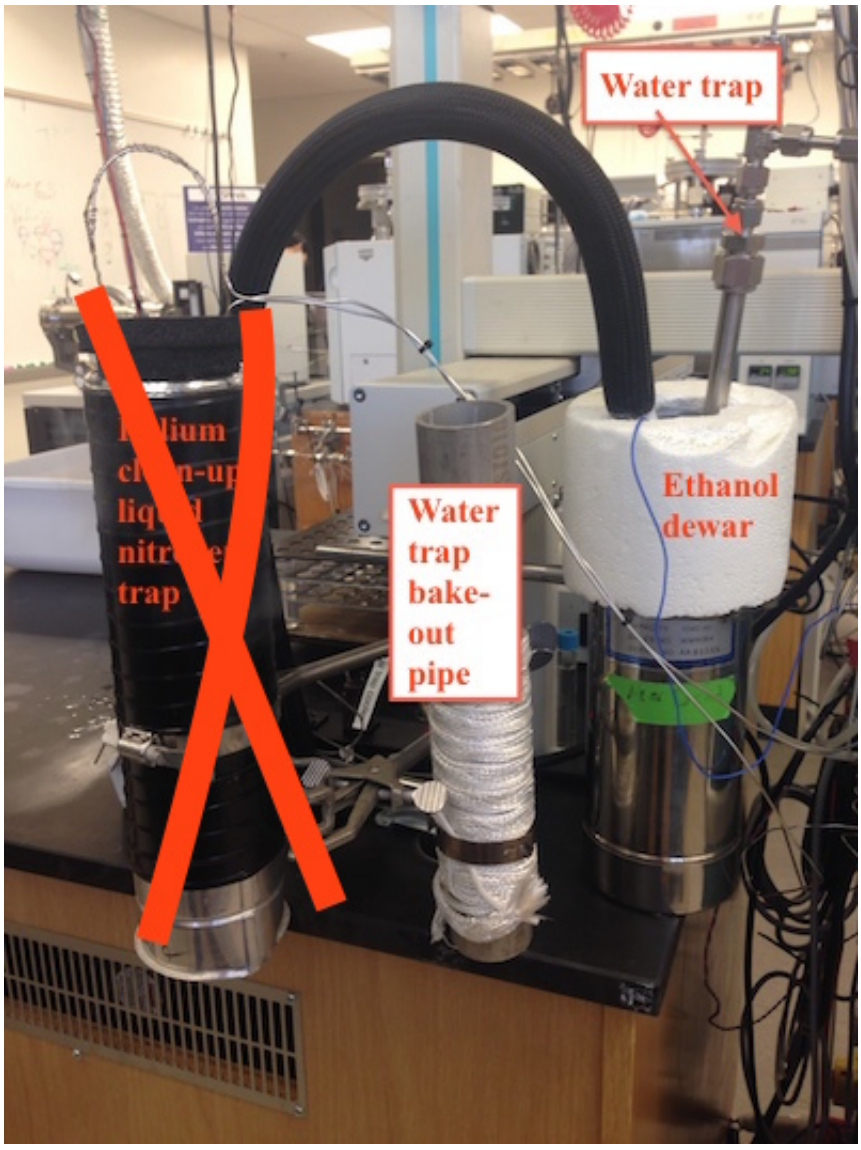
ISOTOPE ANALYSIS ON BRAVE IRENE

INSTRUMENT PREPARATION:

1. **Bake-out Complete** - Make sure everything associated with the baking out procedure (see Baking Out the Instrument) is ready for samples.
2. **-60 °C chiller is on AND cold** - Plug in the chiller using the brown extension cord coming up out of the cabinet below. Use the digital thermometer to verify the temperature is

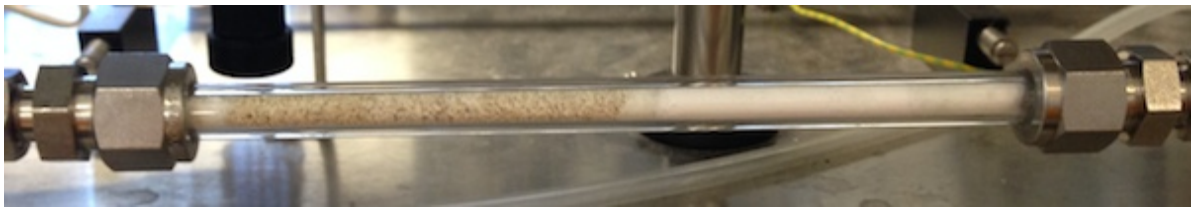
below -40 ˚C before running. If the ethanol appears solid, it needs to be replaced with fresh ethanol. Remove the trap, loosen the clamp on the dewar, dump the ethanol into a white dish pan until its room temperature, add fresh ethanol.

3. **Bake the water trap between runs -** See baking out the water trap below in Baking Out the Instrument.
4. **Helium clean-up liquid nitrogen trap** - This is now a loop that is within the large blue precon liquid nitrogen dewar. Be careful when removing it to allow it the thaw.



Helium clean-up and water traps

5. **GasBench GC temperature** - The GasBench GC should be at lab temperature. The set point is 0 ˚C and the measured temperature should be ~ 22 ˚C.
6. **PreCon liquid nitrogen dewar** - Fill the liquid nitrogen dewar on the PreCon. This dewar often collects water at the rim. This ice should be removed before each run by putting the liquid nitrogen back into a capped handled dewar and then allowing the PreCon dewar to warm up (you can fill it with hot water, dump the water, wipe out the dewar to dry it).
7. **PreCon Reagents** - The reagents should be checked weekly and changed when necessary. Pack a 17.5 cm long 3/8" OD pyrex tube with half ascarite and half magnesium perchlorate separated by glass wool and glass wool at either end. Use the vortex to pack down the



Precon ascarite / magnesium perchlorate reagents

ascarite and magnesium perchlorate. Remove existing trap, install the new trap, tighten with wrenches, leak check. All waste goes into a waste container.

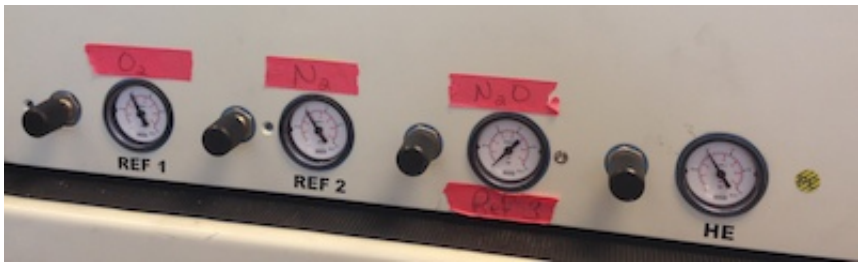
8. **Measuring N_2O or O_2 / N_2** -
   - N_2O - If you are measuring your samples as N_2O to obtain $\delta^{15}N$ and $\delta^{18}O$, you need to make sure the N_2O GC is selected. On the computer, in the program IsoDat Acquisition, make sure the GasBench Valco valve is in "Load" position. If it is in "Inject" click on it once.
   - O_2 / N_2 - If you are measuring your samples as O_2 and N_2 by hot gold tube pyrolysis of N_2O to obtain $\delta^{15}N$, $\delta^{17}O$, and $\delta^{18}O$, you need to make sure the O_2 / N_2 GC is selected. On the computer, in the program Isodat Acquisition, make sure the Gasbench valco valve is in the "Inject" position. If it is in "Load", click on it once. You also need to make sure the gold tube furnace is set to 800 ˚C.

SAMPLE PREPARATION:

1. **Lyse bacteria** - All bacteria must be dead to prevent any further activity that might affect your samples. Lyse the bacteria with 0.2 mL 10M NaOH and shake well. Wipe off all NaOH from tops of septa.
2. **Antifoam** - Add 5-10 drops of well mixed antifoam-B into media solution and 5-10 drops to the side-walls of the vial. Shake vial ONCE. Anitfoam-B must be well mixed to be effective...make sure you shake the syringe before using it. Wipe off all antifoam from tops of septa.
3. **Fill rack with vials** - The first and last position vials should be an N_2OinHe. These are not essential for data interpretation and are only useful if you are struggling with no samples peaks. They allow you to decide if the reason you are not see sample peaks is a bacterial reason or a mass spec reason. Flush an empty, freshly capped vial with N2OinHe for 30 seconds using the cylinder on the wall in the mass spec lab. If you must make an N_2OinHe using pure N_2O, inject at most 0.5 uL into a 20 mL vial.
4. **Oil** - Put 5 drops of vacuum pump oil on the top of each septum. The oil acts to lubricate the needle as it pierces the septum. The autosampler does not have the strength to push a dry needle through a dry septum.

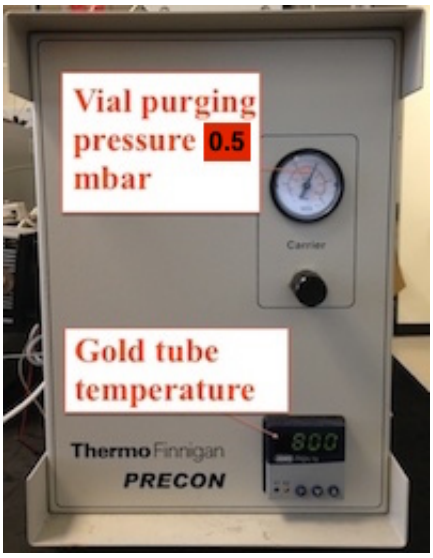
DAILY LOG:

1. The daily log is Irene_DailyLog.xlsx.
2. Turn all reference gases off in the Gas Bench window on the left side of Isodat Acquisition



Gasbench pressure gauges

3. Record in the date and your name, source settings and gas pressures
4. Enter backgrounds for water (m/z 18), argon (m/z 40), nitrogen (m/z 28, 29, 30), oxygen (m/z 32, 33, 34), and N2O / CO2 (m/z 44, 45, 46) by selecting the appropriate gas from the little menu located in the lower left corner of Isodat Acquisition. If any of the values are unreasonably high relative to historical measurements, see troubleshooting below.
5. If you are measuring your samples as N2O, turn on N_2O reference gas and once it is stable, enter these values into the daily log
6. Enter the number of samples (excluding standards) and the species of bacteria used
7. Enter notes related to the dataset itself



Helium pressure and gold tube controller

SAMPLES SEQUENCE:

1. **Samples sequence** - Open "N2O_Samples.seq" or "O2N2_Samples.seq" sequence from the left side of the screen within the "File Browser" window under the "Sequences" tab.
   - Make sure every row in the peak center column is checked
   - Make sure the "AS Sample" column increments by 1 for each row from one to the number of vials you have
   - Make sure the "AS Method" column has ">Internal No 9" selected
   - Identifier 1 – Sample / Standard name
   - Identifier 2 – Nitrate concentration of the sample/standard in µM
   - Comment – Sample / Standard injection volume in mL
   - Preparation - volume of 18 MΩ injected in mL
   - Make sure the Method has "N2O_samples.met" or "O2N2_samples.met" selected.
   - highlight the rows containing the vials you wish to run
2. **Start the run** - Highlight rows if not running the entire sequence; click start; enter your

dataset name, making sure that the dominant folder starts with "yymmdd".

DATA:

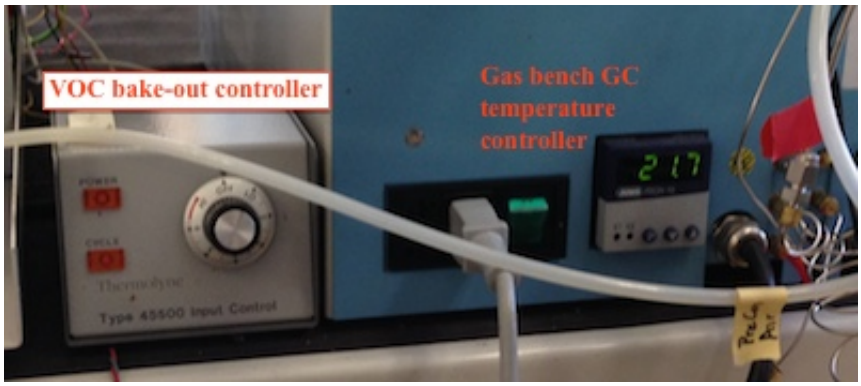
After your run is finished, use matlab to reduce the data.

1. Here is the path to you data file C:\Thermo\Isodat NT\Global\User\Gas Bench\Results\[YOUR FOLDER STARTING WITH DATE]\Excel\[YOUR .CSV FILE]
2. You can use the "Results" shortcut on the desktop to get you most of the way.
3. **You only need the .csv file**. You do not need the entire folder that largely contains irene specific data files.
4. Copy this file to the server S:\Data\projects\[YOUR PROJECT FOLDER]\raw
5. go to either the "North Going Zax" or the "South Going Zax" computers and open matlab
6. type 'irene' and hit enter
7. follow prompts
8. (more is needed here...I know)

BAKING OUT THE INSTRUMENT:

While the water trap needs to be baked out between each run, it is probably sufficient to bake out the GC, VOC, and IRMS weekly or bimonthly.

1. **Water trap** - The water trap is held at -60 °C during analysis and acts to trap any water vapor that is carried away from the sample vial. It also acts to prevent liquid media from traveling into the PreCon in the event of excessive foaming while purging. This traps needs to be thawed and baked out between each run. You can leave the immersion cooler on if you are going to run again the same day. Remove the water trap out of the ethanol dewar, taking care not to tangle the tubing with anything. Place the trap into the heat-tape-wrapped pipe and plug in the heat tape into the power strip. Allow it to heat for as long as possible (30 minutes ok, 1 hour good, overnight is great). Helium is flushing out both holes of the needle, so any water should evaporate and exit out the needle. If you are recovering from no peaks due to a clogged trap, allow the trap to become very hot, use gloves, pick up the trap while hot and CAREFULLY invert it such that any liquid inside will flow out the exit arm and then out the needle (look at the needle to see if its dripping).
2. **GC columns** - The GasBench GC box has two GC columns in it. Once for N_2O and one for O_2/N_2. Both



VOC trap temperature controller and GasBench GC controller

always have helium flowing through them and can be baked out at 200 °C. If you are baking out the GCs, make sure to follow the next step and turn the mass spec heaters on. At the north side of the GasBench, press the "P" button on the "Jumo iTron 16". Hold the up arrow down until the set point temperature reads 200 °C, press the "P" button again to apply this selected temperature. Repeat this sequence when you are finished baking out the GCs except the set point will be 0 °C (no heat at all, the GC box can not be cooled, this is lab temperature).

3. **IRMS** - If you are baking out the GCs, you should turn on the mass spec heaters. Within Acquisition, under the "MS State" window, click the bottom 4 gray buttons to turn them green. This heats up the inlet, source, and flight tube.
4. **VOC Trap** - The VOC (Volatile Organic Compound) trap retains any organics that are carried away from the sample vial. If these compounds make it to the gold tube, the organics will react and harvest all oxygen from your sample. Turn the Thermolyne controller located between the Precon and the autosampler to 4.
5. **Running Samples** - NOTE, to run samples, the water trap should be at -60 °C, the GC should be set to 0 °C (room temperature), the IRMS heaters should be off, and the VOC trap should be at room temperature.

CLEAN UP AND WASTE DISPOSAL

1. **Supernatant waste media from harvest** - This is the media you discard after a centrifuge run. While you managed to grab most of the bacterial cells in the pellet, some cells remain in the media and thus, this media must be autoclaved. Autoclaving as part of normal Liquid 60 batch. Once the liquid is cool enough to handle, pour the, now sterile media, down the drain.
2. **Sample vial media with NaOH** - This media, while sterile (because of the NaOH) is very basic and must be neutralized. Use the crimp cap removal tool to pull off the caps. Discard the caps into the trash and pour the media into a 1 L glass medial bottle which needs to be labeled "High pH Waste". When this bottle is full, neutralize the liquid with Hydrochloric acid (HCl) waste (this is a 10-20% HCl solution that has previously been used to acidify soil or rock samples). Use pH paper to verify it is neutral. If you overshoot and make it acidic, use the

carbonate that is in the hood to bring it back to neutral. Pour the neutral pH liquid down the drain and flush with water. Enter the appropriate information into the drain log.

3. **Waste solids and plates** - Place solid waste materials (e.g. petri plates but NOT NEEDLES) into an autoclave bag with indicator tape and autoclave as a "Grav 20" cycle (gravity, ~45 min) cycle. Once the bag is cool enough to handle, put in garbage.
4. **Used pipette tips** - The pipette tips are reusable and are autoclaved with waste solids under "Grav 20" cycle and put on shelf for reuse. You can also successfully put the container in with a liquid 60 cycle if you have space. Use autoclave tape on the outside of the tip box.
5. **All glassware** - Wash all glassware as per the Cleaning Laboratory Glassware method.
6. **Centrifuge tubes** - Rinse caps and centrifuge tubes with alcohol. Rinse caps and vials 3x with DI water. Loosely cover caps in a beaker with foil. Loosely cover right-side-up vials with foil in tube tray. Put both caps and vials in drying oven.
7. **Syringe Needles** - All syringe needles go in the red plastic sharps container.

TROUBLESHOOTING

1. No sample peaks during a run
   - Make sure the liquid nitrogen dewar has liquid nitrogen in it otherwise the mass spectrometer cannot produce sample peaks and the sample cannot be analyzed.
   - Did the needle insert completely into the sample vial? If you did not oil the septum (or did not wipe all NaOH and antifoam off the septum before oiling) the needle may have jammed half way in.
   - Are you getting samples peaks for N2O in helium but not bacteria? Could be the batch.
2. Low Peaks
   - Look to see that the vials are bubbling when the needle is injected, if it is not, the needle is clogged
   - Check for leaks using the helium leak detector
   - Does N_2OinHe produce peaks.
3. Plating
   - If the plates are growing as lawns instead of streaks, the agar is too diluted. Either there is too much water or not enough Tryptic Soy Agar in the solution.
4. High Backgrounds
   - May indicate there is a leak. Use the helium leak detector and check for leaks.
   - Pay particular attention to the valves as they have a history of leaking.

REFERENCES

- Casciotti, K.L. 2002. *Measurement of the Oxygen Isotopic Composition of Nitrate in Seawater and Freswater Using the Denitrifier Method.* Analytial Chemistry, Vol. 74, No. 19.
- Kaiser, Jan, 2007. *Triple oxygen Isotope Analysis of Nitrate using the Denitrifier Method and Thermal Decomposition of N_2O.* Analytical Chemistry, Vol. 79, No. 2.
- McIlvin MR and Casciotte KL. 2011. Technical Updates to the Bacterial Method for Nitrate Isotopic Analyses. Analytical Chemistry, vol 83, 1850-1856.
- Sigman et al. 2001. *A Bacterial Method for the Nitrogen Isotopic Analysis of Nitrate in Seawater and Freshwater.* Analytical Chemistry Vol. 73, No. 17.

Sydney Clark PERMALINK

Hey Andy,

These are minor things, but some details that might be helpful to add at some point. I have organized these by section.
Inoculation:
1) For P.chlororaphis - the inoculation process seemed to take less than 2 days. Without more testing, it might be hard to determine a precise amount of time, but it might be good to look into this more. If we decide to continue using P.chlor, I will probably do some more rigorous testing to figure it out.

Harvest:
1) Maybe this is dumb, but maybe clarify the color of pink you're looking for based on the species of bacteria. We have seen that when P.aur is really happy the pellets are pepto bismal pink, but when I used P.chlor they seemed to be much lighter (I wasn't sure if that was normal or due to the unknown appropriate inoculation time). In any case, it might be helpful to have some kind of a reference.
2) For steps 14 and 15, I think you're trying to say standardize the total sample volume using 18 mega ohm water after injecting the sample, but it's slightly unclear. I think I would swap 14 and 15 and clarify that you want people to add water to standardize the volume. Swapping these steps might be clear enough actually.

Post Analysis Section:
1) Maybe include a MatLab section...Example, Open MatLab, type Irene, select Standards in the following order: USGS32, USGS34, IAEA, etc ....I don't know maybe this isn't necessary, but it might be helpful.

OK - If I think of anything else, I'll write to you. Thanks for putting all of this together. It's very helpful.

Sydney

about 1 year ago 0

Andrew Schauer > Sydney Clark PERMALINK

Thank you, Sydney. I have tried to address each of your points. The matlab addition will come shortly. For today, these changes were made:
- Caps are tight after inoculating. No more loose caps for one day.
- The time to readiness is a function of many things and I don't want to lead anyone to think that two days is needed for P.aur and 16 days is needed

anyone to think that two days is needed for P. aur. and 1.6 days is needed for P. chlor. I have added text in the inoculation section in an attempt to explain this.
- This is completely arbitrary. White pellets are bad. Pink pellets are good. Anything in between is up to the user and experience.
- I tried to add text to clarify. I don't want to swap those two steps because one should add 18 M ohm water first.

Thank you for the comments. More updates coming...

about 1 year ago 0

Andrew Schauer PERMALINK

Updates that need to be included in method:
- 2 hour purge time instead of 3-4 hour (blanks are no different after 1 hour purge time)
- aggressive antifoam use
- make sure crimper is tight and crimping all the way
- make sure caps are tight from the beginning of inoculation
- fish tank nitrate test is marginally helpful (its acceptable to use a bottle measuring 40-80 ppm NO3)

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about 1 year ago 0

Andrew Schauer › Andrew Schauer PERMALINK

and...wipe off septum after NaOH and after antifoam...to ensure the only thing on the septum when running is oil

about 1 year ago 0

Andrew Schauer PERMALINK

Comments from Feb 2015 have been incorporated as of today 150301. -andy

about 1 year ago 0

Andrew Schauer

PERMALINK

Note to users of Irene - the helium cleanup trap has been combined with the large liquid nitrogen dewar. Be careful when removing it between runs. Also, the precon helium pressure should now be set to 0.5 bar (not 1.5 bar) when running. Lastly, if the precon will not be used for more than one day, set the helium pressure to just above the zero peg.

about 4 months ago from Johnson Hall (JHN), Seattle, WA 98195, USA

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