Denitrifier protocol – HKU

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Summary with material list

Action	**Timing (days before analysis)**	**Materials (to prepare before each step)**
Bacteria medium	≥7 (can be stored in fridge)	1L glass bottle with blue lid x 1 or 2 500mL glass bottles with blue lid x 5 Weighing balance + weighing boat Autoclave tape Chemicals (see details below)
Bacteria culture	7-8	500mL bottles with autoclaved growth medium x2 Bacteria seed vials x2 1mL pipette and 2 pipette tips (SIRMS lab) Shaker Carton box or aluminium foil
Vial preparation		
- Autoclave centrifuge bottles	≥2 (at least one day before the vial preparation)	500mL PP or PC bottles Autoclave tape
- Test bacteria cultures	1	Cryovials x2 100µL pipette with tips (SIRMS lab) 1mL pipette with tips (SIRMS lab) Sulphanilamide NED-solution (from fridge on 6th floor) Beaker for used tips
- Centrifuging	1	Autoclaved 500mL bottles x2/3 Balancing bottle (when using 3 bacteria bottles) x1 1mL pipette to easily balance weight of bottles
- Fill vials	1	Centrifuged 500mL bottles x 2/3 500mL bottle with autoclaved resuspension medium x1 Clean 20 mL sample vials in rack x60 (Used culture bottles to discard the supernatant) Antifoam agent 1mL pipette with tips (SIRMS lab) 10mL pipette with tips (SIRMS lab) Beaker for used tips Rubber stoppers x60 Metal caps x60

		Crimper
- Flushing vials	1	Racks with vials filled with bacteria x60 Flushing system Helium tank Wrench Scissors (to open the seal of the He tank) Long, thick (25G) needles x60 Short, thin (26G) needles x60 *After flushing:* Yellow needle container Wrench Paper towels to clean (inevitable) spills
Sample preparation	1	Flushed bacteria vials x60 Assorted range of syringes with needles Marker to number vials List of samples with required volume Thin needle for venting Yellow needle container Rubbish bin Paper towels Unfrozen samples and standards
Sample analysis	0	Prepared vials x60 NaOH solution 1mL syringe with needle x1 Paper towel Liquid nitrogen and gloves Excel file with sample sequence
Post analysis	-1	Racks to place the vials USB-drive to collect the dataset
Vial cleaning	-1/2	Analyzed vials x60 Container to soak the bottles Acid bath Furnace Aluminium foil Rubbish bin or beaker

Detailed protocol

1. Bacteria medium

Make the growth and resuspension media according to the below recipes. The reagents are stored in the cupboards in the isotope lab. I use the glass bottles with the blue lids (1L bottle to prepare, 0.5L bottle to store). The 2 litres amounts are as reported by Weigand et al. (2016) in the supplements. The second column is what is used for 1 batch (the resuspension medium could even do 2 batches) which can do >60 samples. Note the difference between the phosphates (monobasic vs dibasic) between the media.

After preparation, the glass bottles are sent to the autoclave. The growth medium is distributed over 3 bottles of 0.5L and the resuspension medium is distributed over 2 bottles of 0.5L to make sure the bottles are not too full for autoclaving. The caps need to be loosely on the bottles and secured with autoclave tape. The bottles have to be brought to the autoclave room in 6N for either the morning or afternoon session. Afterwards, they can be poured together and stored in the fridge (for at least 1 month, probably several months).

Reagent		**Weigand et al. (2016)**	**For 60 samples (1 batch)**
***p*H 6.3 *P. aureofaciens* Growth Medium**			
In-house deionized water	H_2O	2 L	1 L
Potassium nitrate, Fisher Chemical, Certified Primary Reference Standard	KNO_3	2.022 g	1.011 g
Ammonium chloride	NH_4Cl	0.802 g	0.401 g
Potassium phosphate monobasic	KH_2PO_4	9.798 g	4.599 g
Bacto™ tryptic soy broth, BD Ref #211825[1]	NA	60 g	30 g
***p*H 7.3 *P. aureofaciens* Resuspension Medium**			
In-house deionized water	H_2O	2 L	0.5 L
Ammonium chloride	NH_4Cl	0.802 g	0.200 g
Potassium phosphate dibasic	K_2HPO_4	10 g	2.5 g
Bacto™ tryptic soy broth, BD Ref #211825[1]	NA	60 g	15 g

2. Bacteria culture

We have seed vials (1.5 ml cryovials with bacteria) to start the bacteria culture, which are stored in the upper shelve of the -80°C freezer. Take as many vials as you have growth medium bottles (usually 2). Let them defrost. Take the growth medium from the fridge. Once the bacteria vials are liquid, pipette 1ml of the liquid into each growth medium. Wrap the bottles in aluminium foil (or put a dark box over them) and place them on the shaker at 70 rpm. Make sure the shaker is at 'time elapsed' in hours, not in countdown or it stops shaking after a while. Put a note on the shaker with your name, the rpm and the time it will be finished. Let it shake for 6-8 days. There will be growing some kind of yellowish slime on the surface.

3. Vial preparation

3.1. Autoclave centrifuge bottles

Prepare at least 1 day before the bacteria are ready bottles for the centrifuge: take 2 or 3 large clear/white Nalgene bottles (500 ml, PP or PC not HDPE) and have them autoclaved (loose lid, autoclave tape) at 6N. Close the bottles completely after autoclaving and cooling down.

3.2. Test bacteria cultures

Prepare the biosafety cabinet (Isotope lab): switch on the UV light and clean the cabinet with alcohol before used. Use the normal light (not UV) when working.

When the bacteria are ready, you can test if the bacteria have grown well. Take as many cryovials as you have medium bottles and fill them with 40µL of sulphanilamide (bottle with liquid in isotope lab) and 40µL of NED-solution (15ml tube in the fridge door in the molecular area, 6S). Add 1 ml of the bacteria bottles to the solution. If it remains light yellow, then the culturing was successful, if it turns brown, then the culturing was not successful and you can't use this bottle.

3.3. Centrifuge

The medium of the good bottles has to be transferred to the centrifuge bottles. When using 2 bottles, make sure the mass in each bottle is equal (use the balance at the Central Facilities and a 1mL pipette) or if you prefer 3 bottles, use an extra dummy bottle filled with water and make sure that you have pairs of equal mass. Put the pairs opposite each other in the large centrifuge in the Central Facilities (if needed, you might have to change the rotor). Fill in the log book. The settings for the centrifuge:

- Speed: 4300 rpm
- Temperature: 20°C
- Time: 30 minutes
- Acceleration: 1
- Deceleration: 1

Make sure to press 'enter' on the last setting to confirm the value. Close the lid and press start. Confirm that it is starting. Fill in the logbook. It will take slightly less than 1 hour (due to the acceleration and deceleration rate). You can use this hour to get your samples out of the freezer so they can start defrosting slowly and prepare the materials for the next step.

3.4. Fill vials

Once the centrifuge is finished, retrieve your bottles and don't forget to fill in the end time in the logbook. Keep the centrifuge lid open so it can ventilate.

In the biosafety cabinet (culture room next to the isotope lab), remove the supernatant (eg return to the growth bottles) so that there are only the bacteria remaining at the bottom. Then add a little amount of the resuspension medium resuspend as much of the bacteria as possible by shaking or rotating the bottle. Repeat this as many times as needed and concentrate the bacteria in 1 bottle. Aim for a growth to resuspension ratio between 1: (3.8-10), but use at least 120 ml of medium if you have a batch of 60 samples. Add 3ml of antifoam agent to the bacteria.

Pipette 2ml of bacteria into each 20-ml glass vial. Leave 1 vial empty for blank analysis. Cover them with a rubber stopper and aluminium cap and use the crimper to seal the values tight (without over tightening).

3.5. Flushing the vials

Connect the gas valve of the flushing system to the He tank with the wrench. Put the regulator valve just before the He flows out (so you don't have to turn it a lot when the vials are on the rack). Be careful not to overpressure the flushing rack.

Take the long, thin needles (high G-value) and stick one in each vial. Take the small, thicker needles (low G-value) and stick them to the flushing system (in the gas cabinet). Then place the vials on the needles in the rack. Once all the vials are in place, open the valve very slowly until you get bubbles in the vials. Check if all the vials are bubbling and reposition if needed. Let it flush for 3 hours. Place the vials upside down in a rack and close the He tank when all samples are removed. Dispose of the needles in the yellow needle container.

4. Sample preparation

Number the bottoms of the vials and prepare a sample list with the concentration and the required volume to inject to add 20 nmol to the vial (or max. 10 ml). For convenience, you can order the samples from low to high concentration, but it is not required. Add the required volume for each sample with a syringe and needle. For volumes >3ml, insert an additional needle (ideally a thin one which is re-used between vials) during the injection to prevent overpressure. Place the vials again upside down.

Inject 1ml, 0.5ml or 0.25ml of the standards (USGS 34 and 35) to achieve 20 nmol, 10 nmol and 5 nmol. The standards (20µM) are stored in the door of the SWIMS freezer in the isotope lab.

Leave the samples overnight.

5. Sample analysis

Next morning, add a 3-5 drops of 10M NaOH (bottle in the reagents cabinet isotope lab) depending on the volume. NaOH is quite sticky so it is better to suck it directly with the syringe (without needle).Remove any droplets on the cap to prevent erosion.

Build your sample sequence (prepare an excel file to link the autosample number, with the name and vial number), including air blank, bacteria blank, standards and samples. Fill the container with liquid nitrogen (±1 cm per hour of analysis). Place the vials in the correct order in the autosampler. Install the needle and check the He pressure which should be at the black marking. Write down the background values of Ar and N_2O in the notebook.

In the sequence, use at auto sampler method 8 and IRMS method denitrifier_extra_info.met.

While it is running, keep an eye on the liquid nitrogen volume and top up if needed. You will also have to replace finished samples in the auto sampler as it can only accommodate 54 samples.

6. Post analysis

When the samples are finished, remove the analysed vials from the auto sampler. Remove the needle and place it back in the storage exetainer. Reduce the He pressure slowly until ± 0.5 bar. Export the data to an Excel file and calibrate the data using the template.

7. Vial cleaning

Remove the caps of the vials with the decapping tool. Open them at an angle to avoid “explosive” caps. Pour the liquid in the sink. Rinse the vials with water for 3 times. Use the bottle brushes to remove any bacteria sticking to the glass. Soak them in water for 1 hr. Then acid wash the bottles for 1 day. Rinse the vials 2 times with DI water. Wrap the clean vials in aluminium foil and put in the furnace at 450°C for 4 hr. Leave them in the aluminium foil until next use.