Hofmockel Lab Protocol
DENITRIFICATION ENZYME ACTIVITY (DEA) OF SOILS

Procedure Overview

The DEA method is a measure of the potential denitrifying enzyme activity in a soil sample, given optimal conditions for denitrification. This assay employs the acetylene inhibition technique, which results in the accumulation of N2O because acetylene blocks the activity of nitrous oxide reductase. Procedure outlined below follows Philippot et al. (2009).

Briefly, anaerobic soil slurries are made by mixing 25 mL nutrient with 25 g soil in 250 mL Wheaton bottles and caps fitted with septa. Bottles are then purged and flushed four (4) times with He to evacuate ambient air and brought to atmospheric pressure after the last He flush. An overpressure of 25 mL purified acetylene is the added and samples are placed on a shaker. Gas samples are then collected at 30, 60, and 90 minutes in evacuated vials purged of ambient air. Immediately after sampling, a volume of He equal to that sampled is replaced to maintain adequate overpressure for sampling.

Samples are run on a GC in batches by statistical block. Standards are made with every batch by diluting a known concentration of N2O gas in a He + 10% acetylene matrix. Samples are run within one week of data collection and calibrated against the corresponding 5-point standard curve. N2O concentrations are corrected with consideration for N2O in the aqueous phase using the Bunsen absorption coefficient (Groffman et al. 1999). Rates of N2O production are evaluated for linearity. If rates of N2O production is non-linear, a product equation that takes exponential growth into consideration is used to calculate rate (Pell et al. 1996).

Note on chloramphenicol:
Chloramphenicol is usually added to prolong the linear range of N2O production by inhibiting synthesis of new enzymes. However, it is also known to decrease the activity of existing enzymes (Pell et al. 1996).

Equipment

- Incubation chambers, e.g. 250 mL Wheaton bottles (Fisher # 06408) + caps (Fisher #0645074) (replacement septa from Wheaton)
- Evacuation manifold
- Helium
- Labeling tape in various colors
- Parafilm
- Small diameter Tygon tubing
- Reciprocal or rotary shaker
- Potassium nitrate (KNO_3)
- Glucose
- Ultrapure water
- 30 mL, 10 mL, and 1 mL gas tight syringes
- 26 G 3/8 Intradermal Bevel for manifold and syringes to make standards and add acetylene, VWR# BD305110

- 22 G 1 ½ for GC, VWR# BD305176
- Purified acetylene gas (see SOP – Purified acetylene (C_2H_2) gas production for use in denitrification experiments)
- 20 mL gas sampling vials
- Septa and caps
- Timers
- Box for bottles + bungee cords

Blanks
Blank = everything but soil (optional)

Spiked samples
Everything + soil + spiked amount of N_2O (optional)

Standards
When running samples with acetylene on the GC, the standards should be made with similar C_2H_2 concentrations as the samples. Acetylene poisons the ECD and changes its response to N_2O (personal correspondence with Tim Parkin; email 15 July 2010). See below for details on making standards

Protocol
Check manifold

1. Check all ports on the manifold using snoop: place septa-capped vials on the needs, flush with He and open valves one at a time while you squirt Snoop on the connection between the needle and the manifold. If any bubbles form, re-attached needle and try again. If bubbles persist, discard needle in Sharps container and try a new needle. Do not use a port until it passes the Snoop test with no bubbles forming!

Evacuate vials

2. Evacuate and label enough sampling vials
    a. Check He tank, turn on valves, turn on pump
    b. Evacuate for 30 sec
    c. He flush for ~ 20 sec, or until gauge stops moving
    d. Evacuate for equal time
    e. x 3
    f. End on evacuate for 1 min 30 sec

Prepare acetylene

3. Prepare acetylene (see Acetylene SOP)

Prepare samples

4. Weigh and label 25 g (+/- 0.05 g) of sieved soil into 250 mL bottles. Seal the flasks with caps and septa and keep refrigerated until ready to use.
5. Prepare nutrient solution – make fresh daily
    0.2525 g KNO_3 (1mM)
    0.45 g glucose (1mM)
    Dissolve in 2.5 L ultrapure water
    Seal flask with Parafilm.

6. Before each run, add 25 mL nutrient solution to each sample. Seal tightly.
7. Prepare assay blanks = empty bottle with no soil but with broth (optional)
8. Place tygon tubing on a port of manifold and stage a beaker with water for bubbling off overpressure.
9. Evacuate each of the flasks on the evacuation manifold with the cycle vacuum-helium **4** times. Leave an over-pressure of helium in the flasks the last time and bubble off the excess helium so the headspace in each flask is approximately that of atmospheric pressure.
    a. Check He tank, **turn on valves**, turn on pump
    b. Evacuate for 2 mins
    c. He flush for 1 min 30 secs
    d. x 4, end with He flush when gauge has stopped moving (overpressure)
    e. Turn vacuum and He off; keep samples valves up
    f. Place tygon tubing in water and on manifold and turn valve on
    g. He should bubble from samples and into water
    h. Turn off **ALL** values once He has completely stopped bubbling
    i. **Remove tubing from water and manifold**
10. **For total denitrification ($N_2O + N_2$)**: **in fume hood**, add 25 ml of acetylene gas (technically, 10% of headspace, i.e. volume of bottle less soil and solution volume) to each flask. Note: incubating slightly pressurized flasks prevents contamination with laboratory air during sampling and the development of negative pressure in the flasks from sample removal.
11. **For N_2O production only (no acetylene block)**: add 25 ml He to each flask
12. Put the flasks on a shaker, and turn the shaker on low.
13. At 30 minutes, 90, and 120 minutes sample a full 10cc syringe of headspace and plunge into pre-labeled evacuated vial. Note: taking a "full" syringe as opposed to 10 cc ensures greater consistency of volume sampled.
14. After sampling, add a full 10cc syringe of He (or He + 10% acetylene).
15. Record time it takes to sample all bottles.
16. Analyze gas samples for N_2O content on gas chromatograph within 48 hours.

Notes on running samples on GC
Make sure acetylene is not captured by sensor.

Standards
1. Evacuate and label vials, as with samples
2. Flush regulator and syringes before use of standard
3. Add matrix first, then standard gas
4. Flush syringe and add gas
    a. Pump syringe up and down in order to consistently add dead space in needle tip
    b. Volume of dead space in needle tip must be accounted for in calculations
5. If acetylene was added to samples, add 1 mL acetylene to standards (in fume hood).
6. If acetylene was used in some samples only, make two sets of standards: One with 1mL acetylene added on top of standard + He and another with 1 mL He added on top of standard + He.

Calculations

1. Calculate standard curve
    a. Plot low (0-10 ppm) and high (10-100 ppm) standards separately
    b. Force low curve through zero
    c. Remove outliers, if necessary (should not be any)
    d. Determine linear equation
    e. Apply appropriate curve (low, high), depending on concentration of sample
2. Convert sample Area to ppm using linear curve = nL/mL = uL N2O/L headspace
3. To convert to gram basis: (uL N2O/L headspace) / 24.465 L N2O/mol at 25C, 1 atm * 44 g/mol = ug N2O / L headspace
4. ug N2O / L headspace * headspace = ug N2O
    a. Headspace = (volume gas phase + (volume liquid phase x Bunsen coefficient))
        i. Volume gas phase = headspace = total volume flask – soil volume – media volume + C2H2 volume +/- volume sampled and not replaced
            1. Soil volume = (FW DEA soil / (FW/DW)) / bulk density / 1000 cm3/L = L dry soil
            2. Bunsen coefficient at 20C = 0.632
5. Divide ug N2O / g dry soil = ug N2O / g dry soil
6. Calculate slope and r2 of ug N2O / g dry soil

<u>From Standard Soil Methods for Long-Term Ecological Research, Robertson et al.</u>:

Denitrification Rate = $[(C_{90} \times H) – (C_{30} \times H)]/(D \times T)$

where:
C_t = N_2O concentration at t minutes in ug N_2O-N/L headspace; see equation below
H = flask volume headspace (it is necessary to account for the removal of air by sampling Volume (L) can be calculated as total flask volume less added media volume less soil volume. Soil volume can be calculated based on bulk density. SEE BELOW FOR TOTAL GAS VOLUME.
D = soil dry weight
T = time (duration) of incubation in hours

<u>To calculate total gas volume using Bunsen coefficient:</u>
It is necessary to account for N_2O dissolved in solution using Bunsen coefficients that predict the amount of gas dissolved in the liquid phase from the concentration in the gas phase:

$$M = C_g \times (V_g + V_l \times B)$$

where:
M = total amount of N_2O in the water plus gas phase
C_g = concentration of N_2O in the gas phase
V_g = volume of gas phase
V_l = volume of liquid phase
B = Bunsen coefficient (0.632 at 20C, 0.544 at 25C)

In a shaken assay, it is safe to assume that liquid- and gas-phase N_2O are in equilibrium (i.e. that the Bunsen coefficients are accurate). Total N_2O production values can be converted to an area basis using bulk density values.

Converting ppm to ug N_2O-N/L:
All measured concentrations should be converted to mass units and corrected to incubation conditions through application of the Ideal Gas Law:

$C_m = (C_v \times M \times P)/(R \times T)$

where:
C_m = the mass/volume concentration, ug N_2O-N/L = mg N_2O-N/m^3 headspace
C_v = the volume/volume concentration as ppm or uL N_2O /L headspace (also called mixing ratio)
M = the molecular weight of the trace species, 28 ug N_2O-N/umol N_2O
P = barometric pressure in atmospheres
T = air temperature in K (K = C + 273.15)
R = the universal gas constant (0.0820575 L atm . K . mole)

Health and safety

Acetylene is a flammable gas. Do not use near heat sources or open flames.

Please read MSDS sheets for all chemicals involved in the analysis.

You are working with vessels undergoing positive and negative pressure. Please take care with the flasks on the evacuation manifold, and do not use any chipped or cracked flasks.

Personal protective equipment (PPE) needed

Gloves, safety goggles, and a lab coat should be worn at all times.

Decontamination, waste disposal, and clean-up procedures

- Syringe needles must go into an approved sharps container.
- Excess nutrient solution should be disposed of via Environmental Health and Safety.

References

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