Last modified by Kristen Veum 4/16/2018

Potentially Mineralizable Nitrogen (PMN) – Anaerobic Incubation

This protocol measures the amount of extractable ammonium in soil before and after an anaerobic incubation. The initial nitrate is assumed to be lost through denitrification early on, and presumably, no nitrate is produced under the anaerobic conditions (**but system may not be completely anaerobic). Common incubation times include 7 days (T_7), 14 days (T_{14}) or 28 days (T_{28}). Alternatives to this protocol include using 1M KCl vs. 2M KCl, using the salicylate reaction instead of the phenolic reaction (less hazardous), and centrifuging vs. filtering samples.

Reference for ammonium analysis: Rhine, E.D., R.L. Mulvaney, E.J. Pratt and G.K. Sims. 1998. *Improving the Berthelot reaction for determining ammonium in soil extracts and water*. Soil Sci. Soc. Am. J. 62: 473-480.

PMN is calculated by difference:

T_7-T_0 = 7 day PMN
T_{14}-T_0 = 14 day PMN
T_{28}-T_0 = 28 day PMN

Initial Ammonium Flush (T_0)

1) Weigh 4.0g air-dried soil into a clean (acid-washed) Falcon tube. Weigh all samples before proceeding to the next step.
2) Add 40ml **1M KCl** to each sample using the dispensette and cap tightly.
3) Shake side-to-side for 30 min at ~200 rpm.
4) Decant filtrate into a clean syringe with a washed 0.45um syringe filter disc ***OR*** centrifuge at 5,000 rpm for 15 min (choice depends on equipment and soil).
5) Collect filtrate in clean glass vial or microtube (label with sharpie not adhesive labels).
6) Refrigerate glass vials (< 1 week) or freeze plastic microtubes in the -80 until analysis.

Incubation (T_7, T_{14}, or T_{28} day) and Ammonium Extraction

1) Weigh 4.0g air-dried soil into a clean (acid-washed) Falcon tube. Weigh all samples before proceeding to the next step.
2) Add 20 ml 'fresh' ultrapure water to each tube using the dispensette and cap tightly.
3) Label the rack of tubes with project name, date/time in, and date/time out.
4) Incubate at 40C. Record the time and date of incubation start and finish in lab book.
7) After incubation, add 20 ml of **2M KCl,** cap tightly, and vortex briefly.
8) Shake side-to-side for 30 min at ~200 rpm.
9) Decant filtrate into a clean syringe with a washed 0.45um syringe filter disc ***OR*** centrifuge at 5,000 rpm for 15 min (choice depends on equipment and soil).

10) Collect filtrate in clean glass vial or microtube (label with sharpie not adhesive labels).
11) Refrigerate glass vials (< 1 week) or freeze plastic microtubes in the -80 until analysis.

Colorimetric Reaction

1) Fill out a 96-well microplate log sheet including 2 reps each of blanks (filtered **1M KCl**), standards, and samples.
2) Allow samples and reagents to warm to room temperature.
3) Pipette 70µl of each standard or sample into the designated wells. Use a fresh pipette tip for each sample.
4) Pipette 50µl of citrate reagent into each well using a multichannel pipette.
5) React for 1 minute before proceeding.
6) Pipette 50µl of PPN reagent to each well using a multichannel pipette
7) Pipette 25µl of buffered NaOCl solution to each well using a multichannel pipette.
8) Pipette 50µl of ultrapure water to each well using a multichannel pipette.
9) Swirl plate by hand on counter for 30s (do not use VortexGenie --- too vigorous!!).
10) Allow to react for 2 hours in a dark, room temperature chamber for color development.
11) Measure plate absorbance on GloMax plate reader at 660nm (PMN 660 full plate method).
12) Export data file to USB drive and ***record GloMax file name on log sheet***.

Reagents

1M KCl for flush (T_0) extraction
Weigh 150g KCl in 2L volumetric flask. QS with ultrapure water. Dissolution reaction is endothermic and volume will decline as dissolution progresses. After KCl is thoroughly dissolved, transfer to 1M KCl carboy.

2M KCl for post-incubation T_7, T_{14}, or T_{28} extractions
Weigh 450g KCl into prep bottle and QS to 3L with ultrapure water. ~~Takes at least a day to dissolve, so make well in advance!!! *Switching to 1M KCl extraction would solve this problem.*~~ Do NOT put on shaker. Dissolution reaction is endothermic and volume will decline as dissolution progresses

Citrate Reagent: need 50ul per well, **Good for 6 weeks. Refrigerate.** Dissolve 5g trisodium citrate in 80ml ultrapure water in a 100ml volumetric flask. Adjust pH to 7.0 using 0.1M HCl (usually takes about 0.5-2ml). QS to 100ml with ultrapure water. Store in designated opaque bottle and refrigerate.

PPN Reagent: need 50ul per well, **Good for 6 weeks but may turn brown after 2 weeks! So replace as needed**. **Refrigerate.** Dissolve 3.22g 2-phenylphenol Na-salt (*NOT* 2-phenylphenol as indicated in the reference!!) and 0.015g sodium nitroprusside in 80ml of ultrapure water in a 100ml volumetric flask. QS to 100ml with ultrapure water. Color should be dark yellow but not brown. Store in opaque bottle and refrigerate.

Buffered NaOCl: need 25µl per well, **Good for 2 weeks. Refrigerate.** Dissove 2.32g Na_3PO_4 in 80ml ultrapure water ***in brown plastic bottle.*** Add 10ml 0.7M 'fresh' NaOCl (original bleach). Add 10ml 2M NaOH. Allow to mix and react before use. Store in brown plastic bottle and refrigerate.

2M NaOH (for buffered NaOCl): 16g NaOH in 200ml ultrapure water, or 20g in 250ml ultrapure water.

Calibration Standards

2000ppm Standard Stock Solution (2000ppm NH_4-N **make fresh monthly**) Dissolve 4.673g $(NH_4)SO_4$ in 400ml ultrapure water in 500 mL volumetric flask. QS with ultrapure water. Store in amber bottle. Refrigerate.

200ppm Working Standard Stock Solution (200ppm NH_4-N **make fresh weekly**): 10ml of 2000ppm stock with **1M KCl** in 100ml volumetric flask. Refrigerate.

20ppm Working Standard Solution (20ppm NH_4-N **make fresh daily**): 10ml of 200ppm stock with **1M KCl** in 100ml volumetric flask. Refrigerate.

Calibration Standards (make in 10ml tubes/vials with **1M KCl**; **make daily**)

Final Cal STD (ppm)	ml 20ppm STD	ml 1M KCl
0.0	0	10
0.5	0.25	9.75
1.0	0.5	9.5
2.0	1.0	9.0
4.0	2.0	8.0
6.0	3.0	7.0
8.0	4.0	6.0
10.0	5.0	5.0

Above 10 ppm is not linear.

QC Check Standards

1000ppm Check Standard Stock Solution (1000ppm NH_4Cl-N, **make fresh monthly**): 1.9095g NH_4Cl in 500ml volumetric, QS with ultrapure water. Store in amber bottle. Refrigerate.

100ppm Working Check Standard Stock Solution (100ppm NH_4Cl-N, make fresh weekly): 10ml of 1000ppm solution in 100ml volumetric flask, QS with **1M KCl. Make fresh weekly**. Refrigerate.

10ppm Working QC Check Standard: 10ml of 100ppm working check solution in 100ml volumetric, QS with **1M KCl. Make fresh daily.**

QC Check Standards

Final Check STD (ppm)	ml 10ppm Check STD	ml 1M KCl
10.0	10.0	0.0
5.0	5.0	5.0
2.0	2.0	8.0
1.0	1.0	9.0

Blanks

Make at least one "blank" by passing 1M KCl through a clean 0.45µm filter. Soil sample blanks are not necessary, as T_0 represents any soil-specific interference (extractable compounds) in the sample as well as initial ammonium.