# $^{15}N$ Diffusion Technique for Soil KCl Extracts.

Last update 10/05/01 includes the recommendations from Herman et al. (1995)

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## Introduction:

This method describes the procedure for analyzing the $NH_4^+$ and $NO_3^-$ in soil KCl extracts for atom % $^{15}N$. It is designed to prepare aliquots in the 20-60 mL range containing 20-400 μg inorganic N at <30 atom % , using a diffusion procedure and subsequent analysis on a ANCA mass spectrometer. Potential users should read the following method fully before attempting any analysis.

The filtering of samples can be a significant source of error with this type of analysis as the filter paper and reagents can contain significant inorganic N. Users are referred to Sparro and Masiak, 1986, Qasim and Flowers, 1989, and Scharf and Alley, 1987, to determine ways of reducing the blank in their filter papers.

In organic soil extracts some differences have been noted between distillation and diffusion for $^{15}N$ in $NH_4^+$ (Liu and Mulvaney 1991, Sorensen and Jensen, 1991). This may cause up to a 11% error in the isotope ratio for $NH_4^+$. This is not usually significant in pool dilution experiments, but should be considered where high precision is needed.

Some types of automated $NH_4^+$ analyzers may increase the apparent amount of ammonia in a solution high in organic matter, apparently by using a high pH and temperature which breaks down some of the soluble organic matter. Users should check their auto analyzer and make sure the pH of the final color solution is pH 12 or less, and that the temperature bath is less than 60 degrees C, as recommended by White and Gosz (1981).

Theory: From Brooks et al. 1989 and Conway 1950. The soil extract is placed in a closed container, Devarda's alloy added to reduce the $NO_3^-$ to $NH_4^+$, and MgO added to buffer the solution to approx. pH 10.5. The $NH_3$ volatilizes and is trapped on paper acidified with $KHSO_4$. After a usual six day incubation, the paper is removed, dried, wrapped in a tin capsule, and analyzed by the ANCA mass spectrometer. Blanks are always prepared with the samples, using KCl that has been poured through filter paper like the samples. To diffuse the $NO_3^-$ alone the $NH_4^+$ must be removed first, followed by the addition of Devarda's alloy. To diffuse $NH_4^+$ alone the sample is diffused with only MgO, no Devarda's alloy.

Before analyzing actual samples with the mass spectrometer, the diffusion method must be evaluated to ensure that more that 95 % of the NO3 and NH4 in the sample is being trapped on the filter paper and wire. This is covered in the procedures section under Evaluation.

Blank refers to KCl that has been treated the same as the samples. Generally this means it should have been shaken and passed through filter paper, in the same proportions as the sample.

To get the best results the samples should be:-

1) Adjusted so that the blank is the same for each batch.
2) Calibrated with standards bracketing the expected N content and enrichment of the unknowns.
3) Organized in groups of similar N content and isotope ratio.

To achieve these objectives requires that the samples be analyzed prior to diffusion for $NO_3^-$ and $NH_4^+$ concentration, usually by using an autoanalyzer.

MATERIALS

1) 104 mL specimen containers (cat. 14-375-148, Fisher Scientific)

2) 2*M* KCl. Dissolve 1184 g of KCl in 6 L of distilled water; bring to 8 L volume.

3) Paper disks. Leach Whatman #3 paper by washing 3 times with 100 mL 2*M* KCl, then three times with 100 mL $H_2O$, and dry in a 55 - 80$^oC$ oven. Cut 6 mm disks from the Whatman #3 filter paper using a paper punch.

4) Stainless steel wire (0.030' diam. type 308, #PA766-2044, Thermacote Welco, Hwy 161, York Rd., Kings Mtn, NC 28086).

5) Acid washed 6 mm glass beads. Wash by soaking in approx. 1 *M* HCl; rinse thoroughly with deionized water. Alternative: 3 x 10 mm acid-washed stir bars (cat 14-511-69, Fisher Scientific).

6) 2.5 *M* $KHSO_4$. Carefully add 7 mL of conc. $H_2SO_4$ to 50 mL deionized water; add 22 g $K_2SO_4$, make to 100 mL with deionized water, and stir until dissolved (a long time).

7) Powdered MgO, heavy.

8) Devarda's alloy, finely ground (cat DX0125-2, EM Science, Gibbstown, NJ 08027, available from VWR).

9) Tin capsules 8 x 5 mm, 250/pk, p/n SC0009, $23.85, Sercon, Inc., 7791 Beech Run Rd., Waynesville, OH 45068, 937-885-9912.

10) ELISA plates and lids (Fisher Scientific catalog numbers 07-200-103 and 07-200-598)

11) Desiccator with drierite and a small beaker with 30 - 50 mL concentrated $H_2SO_4$.

12) 30% Brij-35. Dissolve 30 g of Brij-35 in water (may need to stir overnight). Bring to 100 mL final volume.

## PROCEDURES

### Evaluation

Prepare a solution of 2 *M* KCl containing the same concentrations of $NH_4^+$ and $NO_3^-$ that are expected in the sample. Fill a minimum of five specimen containers with the correct volume to contain 100 μg N as $NH_4^+$-N and $NO_3^-$-N. Follow the instructions below "A:Preparation for every sample", and then B, C, D or E depending on the analysis that will be used for $^{15}N$.

After the six day incubation carefully remove the wires and diffusion papers with tweezers, being careful not to loose any drops of liquid off the paper, and put THE WIRE AND PAPER into a specimen container containing at least 40 mL 2*M* KCl. Shake the containers for half an hour then analyze for $NH_4^+$. Recovery should be better than 95%. The wire usually has some droplets and $NH_4^+$ on it, and can affect recovery if only the paper is measured.

If recovery is not better than 95%, carefully check standards and analyze the original solution and make sure the solution were the correct concentration. Then try again! Experience has shown that this method requires careful labwork and that the procedure must be followed closely to get the best results. Many users get poor results the first time.

### Organizing the samples

Sort the samples by ascending N content. Determine a volume of sample that will put all samples in the batch in a range of 20 - 400 μg of N, and use that sample volume for the entire batch. Note that as atom% $^{15}N$ increases, the maximum sample size decreases. At natural abundance, you can go as high as 400 μg N, but at 30 atom% $^{15}N$, you should not exceed 100 μg N.

### Standards and Blanks

Blanks should be handled exactly as samples, right down to filtering and storing them. Preferably, the same batch of KCl used to extract samples should be used for blanks. Prepare 3 blanks for each batch. Whatever volume of extract is chosen for the batch (as above), measure that volume into each of three specimen cups and diffuse along with the standards and unknowns.

Standards should be included at the beginning of the batch and after, at most, every eight unknowns, and should bracket the N content of the unknowns. (Each batch should begin with a “dummy” of the same makeup as the first standard.) Suppose samples having 20, 22, 25, 26, 30, 37, 40, 45, 48, 55, 61, 69, 79, 85, 116 μg N with an average enrichment of 4 atom% $^{15}N$ are to be analyzed. You would prepare 2 standards at 20μg N, and one standard each at 46 μg N and 116 μg N, all at 4 atom% $^{15}N$. The batch would be ordered:

1. dummy (20 μg N)
2. STD 20 μg N
3. SAM1 (20 μg N)

4. SAM2 (22 μg N)
5. SAM3 (25 μg N)
6. SAM4 (26 μg N)
7. SAM5 (30 μg N)
8. SAM6 (37 μg N)
9. SAM7 (40 μg N)
10. SAM8 (45 μg N)
11. STD 46 μg N
12. SAM9 (48 μg N)
13. SAM10 (55 μg N)
14. SAM11 (61 μg N)
15. SAM12 (69 μg N)
16. SAM13 (79 μg N)
17. SAM14 (85 μg N)
18. SAM15 (116 μg N)
19. STD 116 μg N

This serves three purposes of calibrating the mass spec for enrichment, sample size, and instrument drift. Note that to choose an appropriate enrichment for the standards, an expected enrichment of the soil extracts should be calculated. You should know the $NH_4^+$ or $NO_3^-$ content of the unlabeled soil, the $NH_4^+$ or $NO_3^-$ concentration of the added label, and the atom% $^{15}N$ of the added label. So you can solve a mixing equation:

$$E = [(\mu g\ S)(aS) + (\mu g\ L)(aL)\}/(\mu g\ S + \mu g\ L)$$

where

E = expected sample enrichment
μg S = mass of $NH_4^+$- or $NO_3^-$-N in the unlabeled soil extract
aS = atom% $^{15}N$ in the unlabeled soil extract (assumed to be 0.3663 atom% $^{15}N$)
μg L = mass of $NH_4^+$- or $NO_3^-$-N in the added label
aL = atom% $^{15}N$ in the added label

To prepare a standard, measure the batch volume (selected above) of 2*M* KCl into a specimen cup. Immediately prior to adding MgO (described below) pipette the appropriate volume of the appropriate standard into the KCl. Then finish setup as described below and diffuse along with samples.

The complication in this ordering scheme comes with $NO_3^-$ diffusions. There is a small amount of $NH_4^+$ carried over into the $NO_3^-$ diffusion. Our preferred way of dealing with this is to prepare an entirely separate set of standards for $NO_3^-$ diffusions, based in part on the $NH_4^+$ content of the samples. First, order the samples, based on $NO_3^-$ content, as described above. Then, for every standard, get the range of $NH_4^+$ content of the samples which that standard is calibrating. In the above example, for STD 20 μg N, you would want the average $NH_4^+$ (not $NO_3^-$) mass of SAM1 - SAM8; for STD 47 μg N, you would want the and the average $NH_4^+$ mass of SAM1 - SAM15; and for STD 116 μg N, you would want the average $NH_4^+$ mass of SAM9 - SAM 15. Pipette the corresponding average $NH_4^+$ mass of a natural abundance $NH_4^+$ standard into a cup with 2*M* KCl and diffuse along with the samples being diffused for $NH_4^+$. At the conclusion of the $NH_4^+$ diffusion, discard the filter disks, add the appropriate amount of standard for calibrating the $NO_3^-$ samples, and diffuse along with the samples being diffused for $NO_3^-$.

Sample Preparation

1. Measure the chosen volume of sample or blank into a specimen cup and add an acid-washed glass bead or micro stir bar. Standards are to be diffused along with samples, and if extracts will be diffused for $NO_3^-$, prepare an extra set of standards as described in the preceding paragraph.

2. Place a filter paper on a wire and pipette 10 µL of 2.5*M* $KHSO_4$ onto the filter paper.

3. Add a small scoop of MgO (approx. 0.2 g) to the specimen cup; insert the filter paper unit so that the filter paper is suspended above the solution, and cap container tightly.

4. Being careful not to splash KCl onto the filter paper, mix the contents so that a cloud of MgO develops in the KCl.

5. Let the container stand at room temperature for 6 d.

6. Carefully remove the cap. Grip one end of the wire with a pair of tweezers. **IMPORTANT:** From now on, handle the wire only from that end. Push the side of the wire you are holding into the drying rack. Carefully note the number on the hole and the sample placed in it.

7. Dry the filter disks in the desiccator overnight.

8. Take a bunch of paper clips and straighten out one end of each. Spread out some clean paper to work on. Lay a tin disk in front of you, remove a wire from the drying rack by grabbing it with tweezers at the end **nearest the rack**, point the other end down on the tin disk, and work the filter paper off the wire with an unbent paper clip. Then discard the paper clip and use a new one for each filter disk. Wrap the tin over the filter disk into a nice tight ball, being careful not to tear the tin. If you tear the tin, double-wrap it. Store wrapped samples in a microtiter plate in a desiccator.

9. For $NH_4^+$ diffusions, you are done.

10. If you are only going to analyze $NO_3^-$ diffusions, you must still carry out steps 1 - 6 to get rid of the $NH_4^+$, except that in steps 6, all you need to do is remove the wire and filter and throw them away. And you don't need to do steps 7 - 8. But if you are doing both $NO_3^-$ and $NH_4^+$ as sequential diffusions, you have to do steps 1 - 9, and 11 - 18.

11. Add 0.1 mL of 30% Brij-35 to the specimen cup.

12. Place a filter paper disk on the wire and pipette 10 μL of 2.5*M* $KHSO_4$ onto the filter paper.

13. Add a scoop of Devarda's alloy (approx. 0.4 g) to the specimen cup; insert the filter paper unit so that the filter paper is suspended above the solution, and cap container tightly.

14. Being careful not to splash KCl onto the filter paper, mix the contents so that a cloud of MgO-Devarda's alloy develops in the KCl.

15. Let the container stand at room temperature for 6 d.

16. Carefully remove the cap. Grip one end of the wire with a pair of tweezers. **IMPORTANT:** From now on, handle the wire only from that end. Push the side of the wire you are holding into the drying rack. Carefully note the number on the hole and the sample placed in it.

17. Dry the filter disks in the desiccator overnight.

18. Take a bunch of paper clips and straighten out one end of each. Spread out some clean paper to work on. Lay a tin disk in front of you, remove a wire from the drying rack by grabbing it with tweezers at the end **nearest the rack**, point the other end down on the tin disk, and work the filter paper off the wire with an unbent paper clip. Then discard the paper clip and use a new one for each filter disk. Wrap the tin over the filter disk into a nice tight ball, being careful not to tear the tin. If you tear the tin, double-wrap it. Store wrapped samples in a microtiter plate in a desiccator.

19. You are done.

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