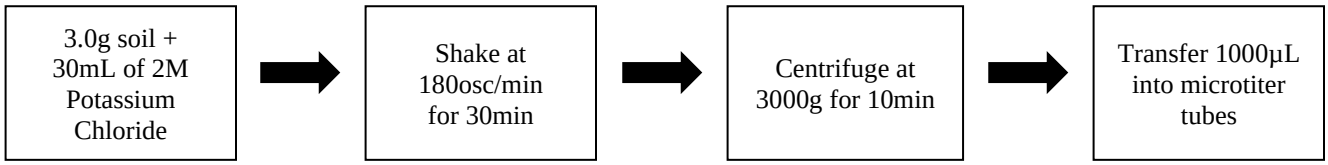
Procedure for Soil Inorganic Nitrogen

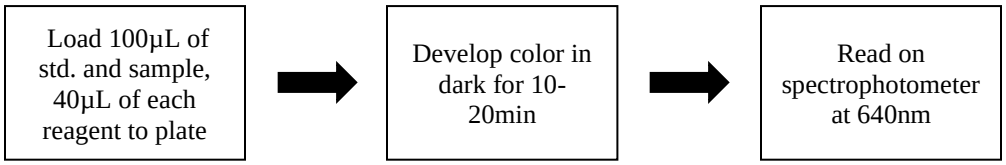
Procedure Overview

This document describes a procedure for the determination of Ammonium Nitrogen (NH_4^+-N) and Nitrate Nitrogen (NO_3^--N) in soil. A single extraction is run on soil and then NH_4^+-N and NO_3^--N are quantified in separate steps. Soils that are air-dried and ground to <2 mm are typically used.

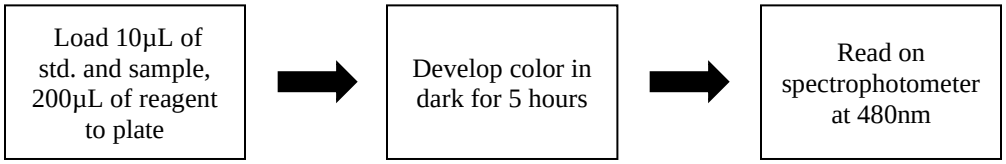
Extraction/Clarification



Ammonium-N Quantification



Nitrate-N Quantification



Instrumentation and Materials:

Potassium Chloride (KCl) Stock Solution Preparation

- Reagent grade Potassium Chloride (KCl: f.w. 74.55g mol^{-1})
- Stir bar and stir plate
- Laboratory glassware for reagent preparation and waste collection
- Glass bottle for reagent storage

Sample Extraction and Centrifugation

- Analytical balance capable of weighing to two decimal places
- Soil checks (pulverized, homogenous soil as internal lab reference samples)
- 50mL polypropylene centrifuge tubes and caps
- Adjustable bottle-top dispenser fitted to a bottle of Potassium Chloride (KCl) and calibrated to deliver 30 mL
- Horizontal shaker
- Centrifuge

- Adjustable 100-1000µL pipettor and tips
- Microtiter tubes (1.1mL open top tubes in strips of 8) and caps
- Freezer for long term sample storage

Ammonium-N Quantification

- Reagent grade Ammonium Sulfate
- Glassware for reagent preparation and storage
- Polyethylene and glass scintillation vials
- Ammonia Salicylate
- Ammonia Cyanurate
- 50ml polypropylene tubes
- Reagent reservoir
- 96-well microplate with lid
- 5-50µL and 30-300µL multichannel pipette and tips
- Spectrophotometer capable of reading at 630nm

Nitrate-N Quantification

- Reagent grade potassium nitrate
- Glassware for reagent preparation and storage
- Polyethylene and glass scintillation vials
- *N* Hydrochloric Acid
- *N*-(1-Naphthyl)Ethylenediamine Dihydrochloride (NED)
- Sulfanilamide
- Vanadium Chloride
- Reagent reservoir
- 96-well microplate with lid
- 1-10µL and 30-300µL multichannel pipette and tips
- Spectrophotometer capable of reading at 480nm

Detailed Procedure:

I. 2*M* Potassium Chloride (KCl) Stock Solution Preparation

1. Weigh 149.1g of Potassium Chloride and add to a 1L volumetric flask, place on stir plate.
2. Add deionized water, bring to final volume of 1L.
3. Add magnetic stir bar, stir until completely dissolved.
4. To make 5L: dissolve 745.5g of KCl in 4712.5mL of DI water.

II. Soil Sample Extraction and Centrifugation

1. Label one 50mL polypropylene centrifuge tube for each sample. Weigh 3.00 grams (± 0.05g) of air-dried soil into the tube (may be done in advance).
2. Soil checks should be prepared in the same manner as the unknown soils and serve as laboratory reference samples. It is recommended to pulverize and homogenize a large batch of air-dried soil for long-term use. The soil checks allow for a quality control check across

inorganic N analyses performed on different batches, over multiple days, and with different reagents.

3. Using the bottle-top dispenser, add 30mL of the prepared 2*M* Potassium Chloride stock solution to each tube containing soil. Cap tightly.
4. Transfer tubes to Styrofoam rack and place on horizontal shaker.
5. Shake at 180 oscillations per minute for 30 minutes ("low" setting on Eberbach reciprocal shaker).
6. After 30 minutes, remove samples from shaker and invert several times to consolidate soil.
7. Transfer tubes to centrifuge, distribute evenly. Centrifuge at 3000g for 10 minutes.
8. Transfer 1mL of clarified extract to microtiter tube in 96-well format rack with a 100-1000µL pipette, using a new, clean tip between samples. Avoid organic matter or any debris when transferring. Cap tubes tightly to avoid ammonia volatilization
    a. Alternatively, decant the supernatant through grade 1 filter paper. Filter paper should be rinsed with 2*M* KCl before use. Collect filtrate in a 20mL plastic scintillation vial.
9. If samples cannot be quantified within 48 hours of extraction, labeled sample tubes/vials can be stored in a freezer until analysis, up to 6 months.

III. Ammonium-N Standard Preparation

Note: stock solution and working standards may be prepared and refrigerated ahead of time. Bring to room temperature prior to plating.

1. Prepare 100ppm Ammonium-N standard stock solution.
    a. Weigh 0.236g of Ammonium Sulfate ($(NH_4)_2SO_4$: f.w. 132.14g mol^{-1}).
    b. Dissolve in approximately 250mL of 2*M* KCl, bring to final volume of 500mL.
    c. Transfer to labeled media bottle.
2. Prepare working standards.
    a. Dilute standard stock solution with 2*M* KCl in labeled 20mL glass scintillation vials according to the table below. Transfer 1mL of each working standard into microtiter tubes for plating.

Standard Concentration	**2*M* KCl**	**Ammonium-N Standard Stock Solution**
(NH_4-N ppm)	(mL to add)	(mL to add)
0	20.0	0.0
1	19.8	0.2
2	19.6	0.4
3	19.4	0.6
4	19.2	0.8
6	18.8	1.2
8	18.4	1.6
10	18.0	2.0

IV. Ammonium-N Quantification

1. Prepare Ammonia Salicylate and Ammonia Cyanurate reagents.
    Note: both reagents must be prepared fresh, in fume hood, prior to plating.

a. Carefully pour one pre-weighed packet of Ammonia Salicylate into a clean 15 mL tube. Add 5mL of DI water and shake for 30 seconds or until completely dissolved.
b. Carefully pour one pre-weighed packet of Ammonia Cyanurate into a separate, clean 15mL tube. Add 5mL of DI water and shake for 30 seconds or until completely dissolved.

2. Ready a 96-well plate. Ensure bottom is free of scratches or debris.
3. Using a multichannel pipette and tips, load 100µL of each sample into the 96-well plate according to plating template. Samples should be loaded in two separate wells as analytical duplicates. Load standards and soil checks into plate as well, following the template.
4. Pour the pre-mixed Ammonia Salicylate reagent into a clean, dry pipetting reservoir. Using a multichannel pipette, transfer 40µL of reagent to each well, wait three minutes.
5. After three minutes, pour the pre-mixed Ammonia Cyanurate reagent into a clean, dry pipetting reservoir. Using a multichannel pipette, transfer 40µL of reagent to each well.
6. Cover microplate with lid and allow colorimetric reaction to develop in the dark for 10-20 minutes.
7. Read on spectrophotometer at 630nm.

V. Nitrate-N Standard Preparation

Note: stock solution, working standards, and Vanadium reagent may be prepared ahead of time. Stock solution and working standards should be refrigerated while Vanadium reagent should be frozen. Bring all to room temperature prior to plating. Keep Vanadium reagent away from light whenever possible to avoid photodegradation. If Vanadium reagent turns a pinkish hue, it is no longer viable and should be discarded.

1. Prepare 100ppm Nitrate-N standard stock solution.
   a. Weigh 0.361g of Potassium Nitrate (KNO_3: f.w. 101.10g mol^{-1}).
   b. Dissolve in approximately 250mL of 2*M* KCl, bring to final volume of 500mL.
   c. Transfer to labeled media bottle.
2. Prepare working standards.
   a. Dilute standard stock solution with 2*M* KCl in labeled 20mL glass scintillation vials according to the table below. Transfer 1mL of each working standard into microtiter tubes for plating.

Standard Concentration	**2*M* KCl**	**Nitrate-N Standard Stock Solution**
(NO_3-N ppm)	(mL to add)	(mL to add)
0	20.0	0.0
1	19.8	0.2
2	19.6	0.4
3	19.4	0.6
5	19.0	1.0
10	18.0	2.0
15	17.0	3.0
20	16.0	4.0

VI. Nitrate-N Quantification

3. Prepare Vanadium reagent.

Note: Vanadium Chloride produces a gas upon opening, therefore reagent prep must be carried out under fume hood.

a. Add 200mL of *N* Hydrochloric Acid to 200 mL of DI water.
b. Add 0.02g of *N*-(1-Naphthyl)Ethylenediamine Dihydrochloride to solution and swirl to dissolve.
c. Add 0.4g of Sulfanilamide to solution and swirl to dissolve.
d. Add 1g ampule of Vanadium Chloride to solution. Cap and mix thoroughly.
e. Aliquot to labeled 100mL media bottles. Reagent can be frozen for up to 6 months.

4. Ready a 96-well plate. Ensure bottom is free of scratches or debris.
5. Using a multichannel pipette and tips, load 10µL of each sample into the 96-well plate according to plating template. Samples should be loaded in two separate wells as analytical duplicates. Load standards and soil checks into plate as well, following the template.
6. Transfer ~21mL of vanadium reagent into a clean, dry pipetting reservoir. Using a multichannel pipette, transfer 200µL of reagent to each well.
7. Cover microplate with lid and allow colorimetric reaction to develop away from light for 5 hours.
8. Read on spectrophotometer at 480nm.

VII. Calculating Mass of Ammonium-N for Unknown Soil Samples

1. Use the following equation to determine Ammonium-N:

Ammonium Nitrogen (mg kg^{-1} soil) =

$$[a(Abs_u - Abs_b)^2 + b(Abs_u - Abs_b) + c] \times 30\ mL / Wt$$

Where:
a = coefficient (a) of standard curve
b = coefficient (b) of standard curve
c = intercept (c) of standard curve
Abs_u = average absorbance of unknown soil sample
Abs_b = average absorbance of blank
Wt = weight of air-dried soil sample in grams

This equation provides a total mass of extracted Ammonium Nitrogen (µg) per unit of soil (g), which is equivalent to and reported as mg NH_4-N kg^{-1} soil.

Example Calculation

Construct standard curve with the following values:

Y-axis Standard Concentrations (ppm)	**0**	**1**	**2**	**3**	**4**	**6**	**8**	**10**
X-axis Average Adjusted Absorbance	0.000	0.177	0.327	0.461	0.547	0.734	0.890	1.009

This produces the quadratic equation: $y = 5.819x^2 + 3.880x + 0.059$; $R^2 = 0.9994$

Average absorbance of unknown sample: 0.531
Average absorbance of Blank: 0.056
Unknown sample weight: 2.99g

Ammonium Nitrogen (mg kg^{-1} soil) =

$$[5.819(0.531-0.056)^2 + 3.880(0.531-0.056) + 0.059] \times 30 / 2.99$$

= 32.26 mg NH_4-N kg^{-1} soil

VIII. Calculating Mass of Nitrate-N for Unknown Soil Samples

Extensive empirical testing in our lab has shown that nitrate-N values can be more precisely quantified using two separate linear equations for the standard curve. The "low" standard curve should be constructed using the first five standards (0-5ppm) and their respective average adjusted absorbances, the "high" standard curve should be constructed using the last four standards (5-20ppm) and their respective average adjusted absorbances. Unknown samples with an average adjusted absorbance that falls below the 5ppm standard absorbance should be calculated using the "low" standard curve, unknown samples with an average adjusted absorbance that falls above the 5ppm standard average adjusted absorbance should be calculated using the "high" standard curve.

1. Use the following equation to determine Nitrate-N:

   Nitrate Nitrogen (mg kg^{-1} soil) =

   $$[m(Abs_u - Abs_b) + b] \times 30 \text{ mL} / Wt$$

   Where: m = slope (m) of standard curve
   b = intercept (b) of standard curve
   Abs_u = average absorbance of unknown soil sample
   Abs_b = average absorbance of blank
   Wt = weight of air-dried soil sample in grams

This equation provides a total mass of extracted Nitrate Nitrogen (µg) per unit of soil (g), which is equivalent to and reported as mg NO_3-N kg^{-1} soil.

Example Calculation

Construct two standard curves (low and high) with the following values:

Y-axis Standard Concentrations (ppm)	**0**	**1**	**2**	**3**	**5**	**10**	**15**	**20**
X-axis Average Adjusted Absorbance	0.000	0.026	0.055	0.082	0.142	0.286	0.455	0.623

This produces two linear equations: LOW (Y-axis 0-5 ppm): $y = 35.130x + 0.057$; $R^2 = 0.9993$
HIGH (Y-axis 5-20 ppm): $y = 30.975x + 0.838$; $R^2 = 0.9986$
Average absorbance of unknown sample: 0.181
Average absorbance of blank: 0.057
Unknown sample weight: 3.00g

Note: In this example, the adjusted absorbance of the unknown sample is 0.124 (0.181 – 0.057) which is lower than the 5ppm standard adjusted absorbance. Therefore, the low standard curve is used to calculate Nitrate Nitrogen.

Nitrate Nitrogen (mg kg^{-1} soil) =

$[35.130(0.181-0.057) + 0.057] \times 30 / 3.00$

=44.1 mg NO_3-N kg^{-1} soil

Clean-up and Disposal

1. Place reaction plates and reagent reservoirs in fume hood or let dry on counter. These can be thrown away once completely evaporated.
2. Using a phosphate free detergent, clean all surfaces of tubes and caps including tube that was used to prepare the reagent. Final rinses should be done with DI water.

References

Doane, T. A., & Horwáth, W. R. (2003). Spectrophotometric determination of nitrate with a single reagent. *Analytical Letters*, *36*(12), 2713–2722. https://doi.org/10.1081/AL-120024647

Sinsabaugh, R. L., Reynolds, H., & Long, T. M. (2000). Rapid assay for amidohydrolase (urease) activity in environmental samples. *Soil Biology and Biochemistry*, *32*(14), 2095–2097. https://doi.org/10.1016/S0038-0717(00)00102-4

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Method Notes

Ammonium

- **NH_4-N Standard Calculation Details**
  - Ammonium Sulfate (AMS): f.w. 132.14 g mol^{-1}; $(NH_4)_2SO_4$
  - The atomic mass of N in ammonium sulfate is 28 g mol^{-1}, which is equivalent to a mass ratio of 0.212 (N:AMS)
  - 100ppm of AMS = 0.1 g AMS per L
  - 100ppm of AMS-N = (0.1 g AMS per L) / 0.212 = 0.4717 g per L = 0.236 g per 500 mL

- **Standard Curve.** Similar to nitrate, standards can be spilt into two ranges for improved precision. However, testing with known standard concentrations showed good relationships up to 10ppm using a single quadratic standard curve. Results returned at 20ppm were unreliable.
- **Time of incubation.** Testing showed that CVs are much greater before 10 min, but that between 10 min and 20 min, there is no difference in CVs or precision.

Nitrate

- **NO_3-N Standard Calculation Details**
    - Potassium Nitrate (KNO_3): f.w. 101.10 g mol^{-1}
    - The atomic mass of N in potassium nitrate is 14 g mol^{-1}, which is equivalent to a mass ratio of 0.139 (N:KNO_3).
    - 100ppm of KNO_3 = 0.1 g KNO_3 per L
    - 100ppm of KNO_3-N = (0.1 g KNO_3 per L)/ 0.139 = 0.722 g per L = 0.361 g per 500 mL
- **Wavelength of Spectrophotometer**. Internal testing with known standard concentrations of nitrate has shown that 450 and 480nm provided better precision and accuracy than wavelengths over 500nm. Results showed that at 480 nm the average percent error was 3.6%, was 5.5% at 450 nm.
- **Time of incubation.** 5-hour incubation time had lower CVs (1.17%) relative to 20-hour (1.53%) using known standards at 480nm (n=28). In over 90% of samples 20 hours predicted 5% lower nitrate values.
- **Two Standard Curves.** Using two standard curves (0-5ppm or 5-20ppm), the quantified concentration is more accurate than using a singular standard curve (0-20ppm). Using a plate with 1ppm nitrate standards, we determined that the average ppm was 1.05 using the low standard curve (0-5ppm) and 2.54 using the high standard curve (0-20ppm), which is a percent error of 5% using the low standard curve and 154% using the high standard curve.
- **Good linearity up to 40ppm.** Nitrate standard curve showed good linearity up to 40 ppm, but not at 60 ppm. Samples with potentially high nitrate values can include a 40 ppm standard if desired. All samples over 40 ppm are unreliable and should be diluted 1:1 with 2M KCl and rerun.