Aridlands Ecology Laboratory Protocol
2010.05.25, S.Castle

Microplate Nutrient Analysis: NH_4^+, NO_3^-, PO_4^{3+}

Making Solutions:
It is helpful to make the reagents and the concentrated (100 or 50 ppm) stock solution the day before running samples. When you are making the reagents and standards for the microplate always use clean and acid washed dishware. Before using the acid washed dishware, rinse 3 times with nanopure water. Always label solutions with the chemical formula and name, date that it was made, and your initials. Cover solutions with parafilm and store in refrigerator.

For salt solutions, add the salts to a volumetric flask with a clean/dry utensil and then bring the solution up to the desire volume. For salt solutions, use the Denver Instruments balance. In order to weigh out salts, fold a piece of weigh paper diagonally and cut the paper so that a majority of it will fit over the balance tray. It is important that the paper is not touching any other part of the balance. Tare the weigh paper. Using a clean/dry utensil weigh out the correct amount of salt. Dump from paper into volumetric. Fill volumetric to line, where bottom of meniscus is at the line. Cap volumetric or sufficiently cover with parafilm and invert to completely dissolve salts. Solutions should be stored in fridge and tightly covered.

For acid solutions, always add acid to the water and then bring up to volume with additional water. Fill volumetric to line, where bottom of meniscus is at the line. Cap volumetric or sufficiently cover with parafilm and invert to mix.

For stock standards, always use clean and 3x rinsed dishware. Cut a small piece of weigh paper (1in x 1in) and tare on Satorius balance (remember that this measures in mg). Weigh out the appropriate amount of standard and add to volumetric. Fill volumetric to line, where bottom of meniscus is at the line. Cap volumetric or sufficiently cover with parafilm and invert to mix. The amount of standard must be exact!

Preparing Samples:
Remove samples from freezer, up to a day in advance of running them on the microplate. Thawing samples in the fridge or keep them cold after they are thawed. Using a sharpie label the tops of the scint vials in the following fashion (1-1, 1-2…1-25, 1-26), with the first number representing the tray number and the second the sample number on that tray. Each tray will fit 26 samples.

Setting up the Well Plate:
For each well plate it is important to include a standard ladder. Due to the high potential for error with pipetting, it is recommended that you include 3 replicates of each standard as well as 3 replicates for each sample. Follow the template at the end of the protocol by lining up well plate on top of the template.

Pipetting Technique:
Proper pipette technique is crucial for this analysis because you are working with such low volumes of sample. For each sample use a new pipette tip. When adding reagents it is possible to use the same tips over and over, IF you don't contaminate them with sample. When pipetting, make sure that you are getting the exact same volume in the well each time. It is easy to get bubbles in the pipette tip, which will decrease the sample volume.

If you get sample or reagent stuck in the tip that you are unable to shoot out of the tip, get a new tip before proceeding. You are going to use a lot of pipette tips! Generally, the faster you shoot the liquid out of the pipette tips, the better. However, it is important that the sample all goes into the correct well and that there is no carryover from well to well.

Analyzing Samples: Connect computer to microplate with USB. Turn on Microplate. Open the Gen5 1.07 program on the Acer laptop. For each of the analyses, I have made a protocol file already. Go to the Protocol tab and select the appropriate protocol. In this file is where you change the plate layout if you need to do that. If you are not planning to change the layout, and then open a new experiment, select the appropriate method. Add the appropriate number of plates to the experiment using the plus sign plate icon. Name the plates to reflect the tray number (double click on the plate and change the information for that plate). When you are ready to read a plate, click on the plate and push read. All of the information for running the program is included in the microplate user book.

Calculations: When calculating concentrations of samples, you need to subtract out the resin blank and KCl blank values. Sample concentrations also need to be normalized to the amount of soil and the amount of extraction solution.

****Because you are working with such a low sample volume, it is important that you work quickly and efficiently when pipetting to reduce error associated with evaporation. Use lids for 96well plates when you are incubating trays or whenever you are not actively working on a plate****

General Materials:
Sterile 96-well microplate: Fisher #08-772-7
Microplate lids: Fisher # 08-772-2B (can be acid washed and reused check supplies)
Micropipettes
Multichannel pipette
Tips for pipettes (2-200µl) set up in boxes, Fisher # 0540357
Pipetting Basin: Fisher # null 13-681-500
1.5 ml centrifuge tubes: Fisher # 05-408-129

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Ammonium analysis

Adapted from: Allison Lab Protocol

Based on: Weatherburn, M. W. 1967. Phenol-hypochlorite reaction for determination of ammonia. Analytical Chemistry 39:971-974.

Sodium salicylate solution
6.8 g sodium salicylate
5 g sodium citrate
5 g sodium tartrate
0.025 g sodium nitroprusside
(in) 100 ml nanopure water

Sodium hydroxide solution
6 g sodium hydroxide
(in) 100 ml nanopure water

Bleach solution (make fresh each day)
0.2 ml bleach
9.8 ml sodium hydroxide solution (from above)

100 ppm NH_4^+ stock
0.23585 g ammonium sulfate (weigh this on the microbalance)
(in) 500 ml nanopure DI water

2M KCl
149.1 g KCl salt
(in) 1L nanopure DI water

For mixed bed resin bags and soil extractions the matrix is 2M KCl. Extracts from unfertilized plots should follow the protocol for low concentrations and use the low standard curve. Extracts from fertilized plots should be diluted in matrix and run using the high protocol. For the most part, Utah soils are going to fall under the low concentration calibration curve.

Making the Calibration Curve: Create the following standard curves in 1.5 ml centrifuge tubes.
This should be done on the day that you are running samples.
The 100 ppm stock solution will generally last a week or so, but it is recommended that you start over with a new stock solution every week. ***First, dilute the 100 ppm stock solution to 10 ppm or 1ppm depending on if you are running high concentrations or low concentrations.***

10ppm Dilution: In a microfuge 1.5 ml centrifuge tube (150 µl stock:1350 µl matrix) or in a volumetric flask.

1ppm Dilution: In a clean 50mL flask add 500ul of 100ppm stock solution and fill up to the line.

In an additional set of microfuge tubes make the following dilutions. Each tube will provide enough standard for 3 trays.

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Standard curves:

High Conc.	µl 10 ppm	µl matrix
0 ppm	0	1000
0.5	50	950
1.0	100	900
2.0	200	800
5.0	500	500
10.0	1000	0

Low Conc.	µl 1 ppm	µl matrix
0 ppm	0	1000
0.02	20	980
0.05	50	950
0.1	100	900
0.2	200	800
0.5	500	500
1.0	1000	0

Detection limit <0.05 ppm

For low concentrations (0-5 ppm) add the following to each well:
80 µl sample (add using 20-200ul pipette)
60 µl salicylate solution (add using multichannel pipette)
60 µl bleach solution (add using multichannel pipette)

For high concentrations (1-10 ppm) add the following to each well:
20 µl sample (add using 20ul pipettete)
90 µl salicylate solution (add using multichannel pipette)
90 µl bleach solution (add using multichannel pipette)

Pipette up and down or gently tap tray to mix well, incubate 50 min and read plate at 650 nm.

Notes:

*A standard curve should be run with each plate. The standard curves should be added to each plate in the same way as a sample.

*Remember to keep a data sheet recording which sample went into each well. A sample table is attached below.

*The samples will turn a blue green color; however, if the concentration of ammonium is too high, the reaction will go too far and the sample will turn yellow. If this happens the sample should be diluted and ran again (Common dilution for sample: 100 µl sample + 900 µl matrix) or you should run the high conc. samples with a higher calibration curve.

*Remember to run a control (a sample that did not need to be diluted) with your diluted samples.

Nitrate analysis

Adapted from: Allison Lab Protocol

Based on:
Doane, T. A., and W. R. Horwath. 2003. Spectrophotometric determination of nitrate with a single reagent. Analytical Letters 36:2713-2722.

Check freezer for vanadium cocktail reagents prior to making them, reagents will last for 3 months if frozen*

If there is not pre-made reagent, make new solutions and then assemble the reagent. Store aliquots of the reagent in the freezer.

1 M HCl:
Add 500 ml nanopure water to 1 liter volumetric flask
Add 84 ml concentrated HCl to flask and swirl to mix
Bring up to 1 liter with nanopure water

2% sulfanilamide solution:
0.2 g sulfanilamide
(in) 10 ml of 1 M HCl

0.2% NED solution:
0.02 g N-(1-naphthyl)-ethylenediamine dihydrochloride
10 ml ultrapure water

Saturated vanadium chloride solution:
Add 0.35 g vanadium (III) chloride to 50 ml of 1 M HCl; filter if necessary
CAUTION: the vanadium chloride powder is very reactive with air and is corrosive! You are wearing gloves, googles, and a lab coat. Work quickly. This procedure should be done in a hood. You can use the Ohaus balance, weighing the vanadium onto a piece of weigh paper and then quickly add to ml volumetric. Fill up to volume with HCl. Cover immediately with parafilm or a volumetric cap. If stored in dark at 4°C this solution will last a week or so.

Vanadium "Cocktail" Reagent solution:
50 ml saturated vanadium chloride solution
3.3 ml 2 % sulfanilamide solution
3.3 ml 0.2 % NED solution
400 ml DI water

100 ppm NO_3^- stock
0.3609 g potassium nitrate (weigh this on the microbalance)
(in) 500 ml nanopure DI water

Pour reagent solution into acid washed 40mL amber glass vials with rubber/Teflon septa. Cap the vials tightly. Purge 40 ml aliquots for 10seconds with nitrogen or helium using a two syringe method. Label bottles with date and NED/Sulfanilamide and store for up to 3 months frozen.

For resin bag extracts, the matrix is 2M KCl. Extracts from unfertilized plots should follow the protocol for low concentrations and use the low standard curve. Extracts from high nutrient plots

should be diluted in matrix and run using the high protocol. *Utah soils fall into the low nutrient calibration curve.*

Making the Calibration Curve: Create the following standard curves in 1.5 ml centrifuge tubes. *This should be done on the day that you are running samples.*
The 100 ppm stock solution will generally last a week or so, but it is recommended that you start over with a new stock solution every week. ***First, dilute the 100 ppm stock solution to 10 ppm or 1ppm depending on if you are running high concentrations or low concentrations.***

10ppm Dilution: In a microfuge 1.5 ml centrifuge tube (150 µl stock:1350 µl matrix) or in a volumetric flask.

1ppm Dilution: In a clean 50mL flask add 500ul of 100ppm stock solution and fill up to the line.

In an additional set of microfuge tubes make the following dilutions. Each tube will provide enough standard for 3 trays.

Standard curves:

High Conc.	µl 10 ppm	µl matrix
0 ppm	0	1000
0.5	50	950
1.0	100	900
2.0	200	800
5.0	500	500
10.0	1000	0

Low Conc.	µl 1 ppm	µl matrix
0 ppm	0	1000
0.02	20	980
0.05	50	950
0.1	100	900
0.2	200	800
0.5	500	500
1.0	1000	0

Detection limit <0.05 ppm

For low concentrations (0-5 ppm) add the following to each well:
100 µl sample (add using 20-200ul pipette)
100 µl Vanadium cocktail solution (add using multichannel pipette)

For high concentrations (1-10 ppm) add the following to each well:
10 µl sample (add using 20ul pipette)
160 µl Vanadium cocktail solution (add using multichannel pipette)

Tap microplate corner to mix well, incubate at least 5 h or overnight (16-18 hours), and read plate at 540 nm. ***I have found that this tends to work better if you read after 5 hours rather than overnight***

Notes:

*A standard curve should be run with each plate. The standard curves should be added to each plate in the same way as a sample.

*Remember to keep a data sheet recording which sample went into each well. A sample table is attached below.

*Solutions should maintain a pale to bright pink color. If the reaction goes too far or the samples are too concentrated the color will fade and be lost. If samples are too concentrated, either dilute

them or increase the amount of vanadium solution. You will need to keep track of how much is added for the final calculation.

*Remember to run a control (a sample that did not need to be diluted) with your diluted samples.

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Inorganic phosphorus analysis

Adapted from: Allison Lab Protocol

Based on:
Lajtha, K., C. T. Driscoll, W. M. Jarrell, and E. T. Elliott. 1999. Soil phosphorus: characterization and total element analysis. Pages 115-142 in G. P. Robertson, D. C. Coleman, C. S. Bledsoe, and P. Sollins, editors. Standard Soil Methods for Long-Term Ecological Research. Oxford University Press, New York.

AMP solution
Add 250 ml nanopure water to a 500 ml volumetric flask
Add 53 ml concentrated sulfuric acid.
Dissolve 8.775 g ammonium para-molybdate
Fill to line with nanopure DI water

Malachite green solution
Heat 400 ml nanopure water to 80ºC in an Beaker with stir bar
Add 1.75 g polyvinyl alcohol and stir to dissolve
Add 0.175 g malachite green and continue stirring
Cool and pour into a 500mL volumetric flask
Fill to the line with nanopure DI

0.5 M HCl:
Add 100 ml nanopure water to 250mL volumetric flask
Add 10 ml concentrated HCl to flask and swirl to mix
Fill to the line with nanopure DI

100 ppm PO_4^+ stock
0.2195 g oven-dried KH_2PO_4 (weigh this on the microbalance)
(in) 1000 ml nanopure DI water

For resin bag extracts, the matrix is 0.5M HCl. Extracts from unfertilized plots should follow the protocol for low concentrations and use the low standard curve. Extracts from high nutrient plots should be diluted in matrix and run using the high protocol. *Utah soils fall into the low nutrient calibration curve.*

Making the Calibration Curve: Create the following standard curves in 1.5 ml centrifuge tubes.
This should be done on the day that you are running samples.
The 100 ppm stock solution will generally last a week or so, but it is recommended that you start over with a new stock solution every week. ***First, dilute the 100 ppm stock solution to 10 ppm or 1ppm depending on if you are running high concentrations or low concentrations.***

1ppm Dilution: In a microfuge 1.5 ml centrifuge tube (30 µl stock:1470 µl matrix) or in a volumetric flask.

In an additional set of microfuge tubes make the following dilutions. Each tube will provide enough standard for 3 trays.

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Curve for Phosphate		
Concen	µl 1 ppm	µl matrix
0 ppm	0	1000
0.05	50	950
0.10	100	900
0.20	200	800
0.50	500	500
1.00	1000	0

Detection limit <0.02 ppm

For low concentrations (0-5 ppm) add the following to each well:
150 µl sample (add using 20-200ul pipette)
30 µl AMP solution (add using multichannel pipette)
Wait 10minutes and add 30 µl Malachite Green solution (add using multichannel pipette)
Pipette up and down when adding the MG solution this helps get all of the solution out of the pipette tips and mixed in the well. Use new tips for each row

Tap microplate corner to mix well, incubate for 30 minutes, and read plate at 630 nm.