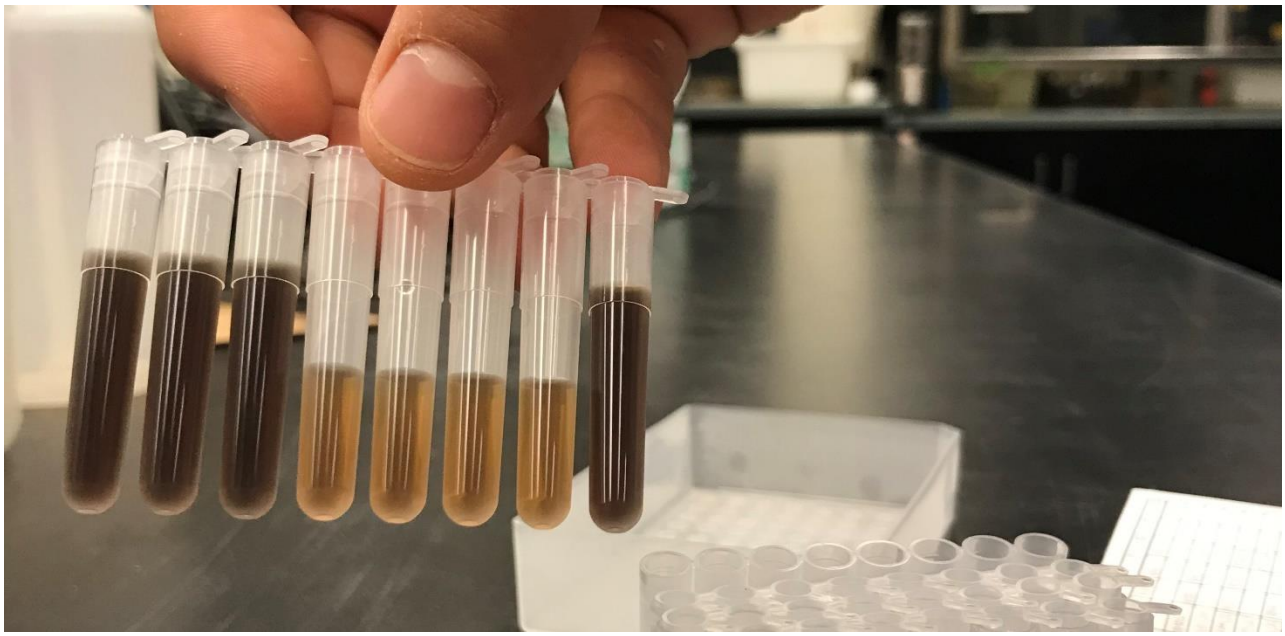

Protein Protocol

For extraction and Quantification of Autoclaved-Citrate Extractable (ACE) Soil Proteins
Cornell Soil Health Assessment
Daniel J. Moebius-Clune, PhD

Summary of Purpose

The purpose of measuring soil proteins is essentially twofold. Firstly, as an index of the size of a pool of organically-bound N in the soil, consisting of the major compound class of soil organic matter derived from expected biomass inputs that is likely to contribute to N mineralization and subsequent plant uptake. Secondly, as a general indicator of the (re-)coupling of C and N cycling in the soil ecosystem. Of the major compound classes that make up the biomass contributing to the soil organic matter, protein is likely to contribute to the immobilization storage and remineralization of plant-available N, as it is present in abundance, is relatively high molecular weight and stability, enzymatically degradable by a wide variety of microbes, with a C:N ratio that is low relative to numerous other compound classes in the same mixed materials. N cycling in systems with a healthy active soil biological community is strongly coupled to C cycling. The microbial activity in these systems is fueled by energy stored in organic compounds, which, when broken down by microbial activity, release N if the C:N ratio is sufficiently low. The organic N stored in such systems is to a large extent stored in the microbial biomass, much of it as protein. The presence of a substantial fraction of the soil N in protein form indicates that the processes involved in the storage and release of N in organic form are functioning.

Objective:

The objective of this procedure is to extract protein from the organic matter in soil samples using a neutral sodium citrate buffer to disaggregate soil and dissolve soil protein with high heat and pressure in an autoclave, and to quantify the protein content of such an extract using a bicinchoninic acid protein assay. The extraction procedure used is a modification of an approach used to extract proteins from fungi and from soil (Keen & Legrand 1980, Wright & Upadhyaya 1996) which has been shown to extract proteins of numerous sources. The quantification assay used is a well-established procedure and chemistry, run at high temperature for an extended time to increase protein sensitivity and decrease variation by protein type (Walker 2002).

Summary of Approach

In this procedure, proteins are extracted from soil samples in a sodium citrate solution adjusted to neutral pH with citric acid.
The sodium and citrate ions contribute to soil dispersal, which is

further achieved through mechanical agitation by shaking of the mixture. Soil is disaggregated or slaked and the soil organic matter particles are exposed to the extractant solution. The mixture is exposed to high heat and temperature by holding in an autoclave at 121 °C to further solubilize proteins. After cooling, aliquots of the mixture are clarified by centrifugation, to settle soil particles. The concentration of dissolved proteins in the clarified extract is determined by reaction in a bicinchoninic acid assay at 60 °C, and quantified, against a bovine serum albumin (BSA) standard curve, by colorimetry using a 96-well spectrophotometric plate reader.

References:

Keen N.T. and Legrand M. 1980. Surface glycoproteins - evidence that they may function as the race specific phytoalexin elicitors of *Phytophthora megasperma* f. sp. *glycinea*. Physiological Plant Pathology 17: 175-192.

Walker J.M. 2002. The bicinchonic acid (BCA) assay for protein quantitation. In: Walker J. M. (ed), The Protein Protocols Handbook. Humana Press, Totowa, NJ.

Wright S.F. and Upadhyaya A. 1996. Extraction of an abundant and unusual protein from soil and comparison with hyphal protein of arbuscular mycorrhizal fungi. Soil Science 161: 575-586.

Procedure for protein extraction and quantification:

NOTE: ***For quarantined soils, see labeled procedures in italics at the bottom of each section.***

Materials:
Soil (air-dried, sieved to 8mm)
Glass extraction tubes with caps
Extraction buffer (20 mM sodium citrate, pH 7.0)
Microcentrifuge tubes
Storage tubes in racks
Transfer pipettes
Pipettors and Tips
(1000 ul ClipTip pipettor, large and small volume 8 channel ClipTip Pipettors)
(1000, 200, 20 ul tips in boxes)
Pipetting reservoir
96-well clear flat bottom chimney well polystyrene plate
Tape seal for plate
BCA reagents A and B
Standards set
50 ml tube for mixing working reagent

Sterilization solutions approved for use with Quarantined soil:
Bleach- 10% bleach solution within a labeled spray bottle must be left on contaminated equipment for 30 minutes before rinsing.

Ethanol solution (70%) within a labeled spray bottle must be left on contaminated equipment for 30 minutes before rinsing.

Additional Quarantine Protocols:

1. ***In the event of spilling quarantine soil, the soil should be swept up using a hand broom and dustpan and disposed of in an autoclave bag. The hand broom and dustpan as well as any surfaces contaminated with Q soil should be heavily sprayed down with 70% ethanol. The alcohol should be left on surfaces for 30 minutes.***
2. ***In the event of spilling bleach water, the water should be mopped or sponged up and bleached again with a 10% bleach solution for two hours. The mop head or sponge should be disposed of in an autoclave bag and autoclaved. The surface contaminated should be sprayed down with 70% alcohol. After two hours the bleach water can be disposed of down the drain.***

Additional Safety Considerations and Protocols:

1. When working with dry soil a mask should always be worn.
2. When working with bleach a mask, gloves and eye protection should always be worn.
3. Always work within a secondary container when working with quarantined soil.

4. Be aware of locations of PPE, first-aid kit and contact info for EHS and Lab management.

Prior to performing ACE Protein analysis please make sure the following safety measures have been taken:

1. Proper training on ACE Protein methodology provided in the following SOP
2. Completion of EHS 2555-Laboratory Safety
3. Completion of EHS 1475- Chemical Storage and Segregation
4. Completion of EHS 3355-Fume Hood Safety and Energy Conservation
5. Wear appropriate PPE (latex gloves, safety glasses, lab coat & Respirator when indicated)- All PPE can be found in the supply cabinet in 822

Method

Part 1: Extraction

Wear gloves and lab coat throughout, wear a mask while weighing soil:

- Weigh soil into tubes
    - Label tubes in advance. Each set of protein consists of 38 samples and 2 QC's. Weigh out one rep for each sample. Use labeling tape rather than marking straight on the glass tube.
    - Weigh 3.00g (2.97-3.03) air-dried soil onto clean weigh paper
    - Transfer to glass extraction tube

        Curling the paper into a funnel, and tapping on the back of it with a fingernail several times should leave no measurable mass of soil behind on the paper. Use a clean weighing paper for each new sample.

- Add extractant- When using sodium citrate take proper precautions. This is not a hazardous substance or mixture. If contact with eyes occurs, flush eyes at the eye flushing station located in the sink of 822 Bradfield. If spilled, clean with water.
    - Add 24.00 ml extractant (20 mM sodium citrate, pH 7.0), using the bottle top dispenser
    - Dispense two or more times into a waste beaker to prime the dispenser. Make sure no bubbles are in the dispensing tube, as these will impact volume dispensed. Dispense additional aliquots into the waste beaker if there are bubbles or other volume-impacting issues with the dispenser. Waste an aliquot or two rather than dispense a poorly measured aliquot into a tube with a sample.
    - Always have the next tube under the spout when drawing the dispenser plunger up, as a small part of the volume comes out when you hit the top. This is factored into the volume setting for the dispenser. Make the draw up and the dispensing push down smooth and not stuttered as this will affect the volume dispensed. Likewise ensure that your fingers are not in a position to be trapped under the plunger as it is pushed down. Drawing up with thumb and middle finger on the sides of the plunger cap, and gently keeping pressure on the plunger as it descends with your index finger works best.
    - Cap tubes tightly after adding extractant
- Shake to slake
    - Place an inverted empty rack over the filled rack of tubes, and fill the small gap above the filled, capped tubes with a rectangle of corrugated cardboard. Hold the racks to each other with several rubber bands.
    - Place the double rack on the shaker rack assembled to hold them on the shaker bed. This rack has threaded rod sides, and can accommodate two racks of 24 extraction tubes. Use additional rubber bands to hold the threaded rods snugly against tube racks. (Alternately, fit tubes and racks onto whatever shaker is available in your lab space).
    - Shake at 180 rpm for 5 min
    - Remove from shaker, and swirl mixture to consolidate solids
        - Extractant and soil may be left on the sides and around the collar of the tubes following shaking. With the caps on the tubes, and holding the rack upright, swirl once or twice rapidly, with a slight lift to part of the swirling motion. This should wash these trailing amounts of soil back down into the extractant in the bottom of the tube, consolidating the contents.
        - Remove the rubber bands and upper rack.
    - Loosen caps so they are not airtight, but still placed on tube to protect contents
        - Unscrew the caps while holding down on the top with one finger, until the cap snaps down into place level after climbing the tube threads. This should ensure that the caps are *fully* disengaged (needed to avoid any pressure differential inside and outside of the tubes in the following, autoclave, step). Leave disengaged caps on top of tubes.
        - Autoclave 30 min

- Preheat autoclave while samples are shaking
  - Add dH_2O to autoclave chamber, to marked line, making sure that the drain valve is off
  - *Leaving autoclave open*, turn on the heathers by turning the timer knob past the 5 minute mark. Do not preheat autoclave with door shut, or it will have to cool and vent before the door can be reopened.
- When ready to autoclave extraction tubes, put on oven gloves located in the drawer below the autoclave and place two racks of tubes into autoclave, on metal tray, not touching sides. Tray should be well supported by rails that are built into the autoclave. If the tray seems poorly supported by rails, adjust it prior to placing racks of tubes on it.
- Close autoclave door securely, paying attention to the gasket seating correctly, and by pivoting lever, rather than pushing, to avoid moving autoclave on bench.
- When door is securely closed, turn knob to the 35 minute mark (timing empirically determined to expose to full temp for just 30 min)
- Be certain that the vent/exhaust switch is in the slow/liquid position, as fast exhaust would cause severe boil-over. This switch should remain taped in this position, as the autoclave we use is dedicated to this extraction, and is not used routinely for dry materials sterilization.
- When the pressure gauge reads zero, and the temperature gauge reads 100, open the autoclave door, taking care to avoid the steam which will come out. Wear oven gloves, as autoclave surfaces are very hot and the steam can burn exposed skin quite badly.
- Remove racks gently and slowly, taking care to not jostle caps loose or bump sides of autoclave.
- Set aside to cool to room temperature before clarification

Sterilize work station with sterilization solution after using. Discard any soil not used for test into autoclave bags and autoclave.
Note: After soil samples have been autoclaved for 35 min per protocol, quarantine procedure is no longer necessary.

Part 2: Clarification

- Label, ahead of time, a set of 2.2 ml microcentrifuge tubes, and one or more racks of sample strip tubes (1.1 ml open top tubes, in strips of 8, racked in 96-place format) to accommodate the sample range to be clarified.
- Close caps on glass extraction tubes again, and re-suspend all the soil at the bottom of the centrifuge tube.
- Withdraw approximately 1.75 ml of mixture using a disposable plastic transfer pipet, and place this in a clean, *labeled* 2 ml microcentrifuge tube. Close microcentrifuge tube cap. Note: Avoid the lipid surface of the liquid; as well as near the soil.
- Use fully labeled tubes because of high likelihood of sample order mix-up in these steps, moving from one rack format to another!
- Place labeled microcentrifuge tubes in microcentrifuge rotor slots, distributing to ensure rotor balance if less than a full rotor
- Place lid on microcentrifuge rotor, and close centrifuge cover.
- Centrifuge at 10,000 xg for 3 min (make sure that the settings are for 10k gravities, not 10k rpm – these are quite different)

- Gently remove tubes one at a time, and transfer 0.8ml of the cleared extract liquid layer to a storage tube (microtiter tube) in a 96-well format rack, using a 1000 ul pipettor with a new, clean, tip for each sample. Avoid dislodging the pellet of solids at the bottom of the tube.
    - See additional notes about pipetting
- Be certain to keep good track of placement of tubes in this rack, and orientation of 8-tube strips. Label the first tube of the strip to ensure this (Top of each strip tube). Note: read from top to bottom, and left to right.
- Place clean cap strip on tubes, keeping the orientation of the tab on the cap strip facing the same direction each time.
- Set aside rack with tubes in refrigerator overnight if not quantifying on the same day.

Part 3: Quantification- Reagents are located in chemical cabinet in 822 Bradfield. When working with Reagent's, wear latex gloves, safety glasses and lab coat. Reagent A is not hazardous. However, Reagent B has severe toxicity to aquatic life with long lasting effects.

- Remove sample tubes in 96-well format microtiter tube racks from refrigerator.
- Allow tubes to equilibrate with room temperature before quantifying.
- Ready a microplate plate, inspecting bottom to avoid scratches, and avoiding getting plate dusty. Dust or fibers or hairs will interfere with quantification
- Preheat the heat block to 61.5 degrees, but without turning on the timer. Check to be sure there is no debris in the heating block that could scratch the bottom of the plate or prevent it seating well. This should remain covered generally.
- Prepare the BCA working reagent in a 50 ml 'Falcon' tube. Make enough for 200 microliters per well, plus enough extra 'for the reservoir' and to avoid pipetting bubbles. 20 ml is just barely enough if you're quite steady with the pipettors, 25.5 ml is a safe and convenient amount.
- The working reagent is a 50:1 mixture of two parts: Reagent A, a clear mixture in a larger bottle, and B, a blue-green copper sulfate solution. For 25.5 ml put 0.5 ml (500 microliters) of the blue-green reagent B into a falcon tube or clean small beaker, and then add 25 ml of reagent A to it, and stir or swirl to mix. A cloudiness that appears initially and then dissipates is normal. Make sure the parts are well mixed, and avoid getting dust or fibers into the liquid. Set this aside, covered, while preparing the plate with samples and standards
- Remove strip caps from tubes with standards (0, 125, 250, 500, 750, 1000, 1500, and 2000 micrograms per milliliter BSA), being careful not to splatter any. A small droplet of carryover would have a very large effect on all further use of these standards. This bears repeating – remove caps gently, and with a kimwipe or other protection, to avoid any splatter or droplet carryover between tubes of the standards
- Using the 8 channel, small volume, multichannel pipettor and the ClipTip size 20 tips, pipette 10 microliters of the standards into the first column of the reaction plate. Using a new set of tips, draw another 10 microliters and place in the 7th column (just past the middle) of the plate. Dispense this droplet slowly, and carefully, onto the bottom of the wells, at the edge of the sidewall, maintaining contact with the side of the plate. DISPENSE SLOWLY! (see additional notes on pipetting) This is a small volume to pipette precisely, and precision is important here, so practice with water and a practice plate if necessary before doing this part. Recap standards with new cap strips and set aside.

- Uncap samples with care, and pipet samples into the available columns of the plate. Keep careful track of which samples are where on the plate, and go in order.
- Pipet two replicate columns of each strip of 8 sample tubes into the plate wells. The best arrangement for this is to have the plate's left and right halves be copies of each other. Columns 1 and 7 should be standards, columns 2 and 8 should be the same, columns 3 and 9 the same, and so forth (Figure 1 below).

Figure 1. Suggested layout for standard locations and their corresponding columns.

	1 (µg/mL)	2	3	4	5	6	7 (µg/mL)	8	9	10	11	12
1	0	1a					0	1a				
2	125	2a					125	2a				
3	250	3a					250	3a				
4	500	4a					500	4a				
5	750						750					
6	1000						1000					
7	1500						1500					
8	2000						2000					

- When all samples (and standards) have been placed in the appropriate wells of the reaction plate, recap the samples and set aside
- Retrieve the pre-mixed working reagent, and transfer it to a clean, dry, multichannel pipettor reservoir
- Using the larger volume multichannel pipette and the 200 microliter tips, add 200 microliters of working reagent to each well of the reaction plate. Pipette onto the sidewalls of the well, sufficiently below the top edge as to not spill, but up away from the filling liquid level in the well. Keep the tip of the tips gently in contact with the sidewall of the wells, and pipette gently and smoothly and SLOWLY. The swirling action should mix the liquids in the wells, but if you pipette too vigorously, it will splash, making the plate unusable. Further mixing will happen naturally while the reaction is heating, due to convective motion in the wells.
- When the plate is filled, seal with a tape seal, using the roller to press the sealing tape to the well tops as well as upper surface of the plate. After this use your thumbs to make sure the seal is sound. Don't let the plate skid around on the surface of the bench as you do this or other steps, as this could scratch the bottom, interfering with the optical clarity.
- When plate is sealed, place gently in heat block and cover.
- Start timer, which should be pre-set for 60 min.
- Carefully pour leftover reagent mixture into glass storage container with secondary containment in the fume hood. When full, contact EHS for chemical removal.

To Read:

- When plate has incubated for 60 min, gently remove from heat block and place on benchtop to cool for at least ten minutes undisturbed.
- Turn on plate reader and computer, make sure plate reader is plugged into computer with USB cable. Start the plate reader program (Gen5) on the computer, and see that it is detecting the plate reader attached (this should happen automatically).
- Select the appropriate protocol from the Gen5 menu, to read the plate at 562nm wavelength without any shaking or other special effects.
- When plate has cooled, ensure that the sealing tape is well in place still, using you thumb or the roller or both. Invert and re-right the plate to incorporate the droplets which will have collected on the tape seal. Be gentle.
- Carefully remove the tape seal without letting the plate be jarred by the motion. Hold it securely by the plate edges or skirt and smoothly peel back the tape without letting droplets cross contaminate wells.
- Place plate in tray of plate reader
- Click the read plate button. The tray will automatically retract.
- When plate is done being read, save the file generated by the plate reader software, and say yes to the question about exporting to excel. Save the excel document newly created with an informative name including the sample range quantified on the plate.
- Remove plate from tray of reader, and set aside to dry in hood. If this is the last plate for the day, press the small black button to close the tray, and power down the reader.
- *Do not close the reader and power it down with a plate in the tray!*
- Open the protein quantification workbook template, answer the questions relating to sample range and replicates and standards, and copy and paste (paste values only) readings from the auto-generated excel sheet into the appropriate location in the calculation workbook.
- Save a copy of the calculation workbook.
- Copy and paste (values only) into the protein data repository. Save.
- Note any samples for which the concentration was too high to calculate (these will need to be re-quantified after an aliquot is diluted to quarter strength), or those for which extraction reps deviate by too much (these will need to be re-extracted). Don't include values in the protein data repository for these 'redo' samples. Make a note of these and include them with the next batch of extractions.

Calculation

Calculation of protein concentrations from A_{562} readings in 8x12 (RxC) format from the plate reader output (or for values input individually for readings from single channel spec with flow-through cuvette), is handled automatically using the auto-calculation workbook on the computer in 822 Bradfield, including handling of variable number of reaction reps, standard sets, and in-run check samples, 2nd order standard curve calculation, replicate averaging and deviation

checks.
To calculate protein concentrations manually from absorbance (A_{562}) readings, follow the process outlined below:
Separate out standards readings from samples, placing them in different columns in an excel worksheet.
Arrange standards concentrations in the left most column of a block of columns, stacked into a single column (concn), regardless of the number of replicate sets. In the column to the right of this, calculate the square of the concentration value ((concn)^2). In the third column, copy the appropriate absorbance values.
Use an array formula (linest) to obtain coefficients for the parabolic (second order) regression line of best fit. The "LINEST" formula will do this if entered the correct way. Label three empty consecutive columns a, b, and c. Drag to select a 3 x 3 block of cells below these labels, and while these are selected (they should be highlighted in grey) type in the formula =linest(While typing, excel should allow click-and-drag selection of Ys and Xs for the formula. Select the absorbance values for Ys, as a column of values only (leave the header unselected), type a comma, and the prompt should now ask for 'known Xs'. Select as a block both the 'concn' and 'concn^2' columns' values (values only, leaves the headers unselected) for Xs. The last two arguments to LINEST() should be TRUE and TRUE. After typing the closing parenthesis, hold down CTRL and SHIFT while pressing ENTER. This enters LINEST as an array formula, rather than as a regular formula, allowing the output to span an array of cells rather than a single cell.

The a, b, and c values for the parabolic best fit line should now show up in the first row of the array output. These are the coefficients for the line y=ax^2+bx+c, where y=A_{562} and x=concn. The r^2 value should be displayed in the 1st column of the 3rd row of the output array. If this r^2 value is greater than or equal to a set threshold for standard curve quality (say, 0.99), use this equation to calculate protein concentrations from observed absorbance values, otherwise, re-do the reaction plate (after checking that data were input correctly).
Provided that the standard curve is acceptable, use the a, b, and c coefficients in an excel formula to calculate protein concentration from absorbance, using the quadratic formula.
The excel formula below is one way:
=IF(H2="","",(((-1*K3)+(SQRT((K3^2)-(4*K2*(K4-H2)))))/(2*K2)))
In the above formula, column H contains Absorbance values, and the focus is currently on row 2 (hence the reference reads H2). Cells K2, K3, and K4 contain coefficients a, b, and c, respectively.
We average absorbance values for multiple reaction reps of the same extract, prior to calculating protein concentration, and average concentration values across replicate extracts of the same soil sample. If the relative average deviation of replicates from their mean exceeds 5% the sample is flagged for re-running.

Reagent preparation-Wearing latex gloves
We mix 20 L at a time of 20 mM sodium citrate pH 7.0
Prepare the 20 L carboy by emptying it and rinsing it thoroughly. Make sure the final rinses are

with distilled deionized water, and that the spigot as well as the inside of the carboy are well rinsed.
In a clean, dry, glass 1 L beaker, place an appropriately sized stir bar.
Add 115.19 g Tribasic sodium citrate dihydrate (m.w. 294.10), and 1.603 g citric acid (located in the chemical cabinet in 822 Bradfield). Gently add ~ 500 ml ddH_2O, stirring at moderate speed.
When salts are fully dissolved, pour solution into carboy, being sure to retain the stir bar with the beaker (use another magnet held outside the beaker to hold back the stir bar.
Rinse the beaker three times, with ddH_2O to full, with the stir bar in it, into the carboy, to ensure complete transfer of the citrate salts
Add ~10 L more ddH_2O, then cover and agitate the carboy to mix contents. Fill the rest of the way (to 20 L total), using ddH_2O, until the water level just reaches the line at the top of the raised panel of graduation marks on the front of the carboy. Cover again and agitate to mix.
Draw off a gallon through the spigot into a clean jug, and pour back into top of carboy, repeat several times
An alternative approach, which would be appropriate if using different water or preparing smaller quantities is to make a solution of strictly 0.020 M sodium citrate, and another of 0.020 M citric acid, and adjust the citrate solution pH to 7.0 using the citric acid solution. The masses above have been empirically determined, given our water purification system, to yield a final pH of 7.0.

When filling 4 L bottles with citrate, or when moving the repeating dispenser between bottles, take extra care to be sure that the glass pieces are well supported, and avoid disturbing the set screw that sets the volume to be dispensed. This has been set carefully by water mass dispensed to ensure accurate delivery of the right volume of reagent.

Expanded Materials List with Notes and Catalog Numbers

- Soil
  - Air-dried to constant mass
  - Sieved to 8 mm
- Weighing papers
  - VWR PAPER WEIGHING 4X4IN PK500, Cat # 12578-165
- Glass extraction tubes
  - "Disposable" Plain Centrifuge Tubes, Glass, Kimble Chase, 50 mL, 29 x 137 mm, VWR Cat # 21020-695, Supplier Part # 73785-50

  Caps for glass extraction tubes:
  - Black Phenolic Screw caps, with White Rubber Liner, Kimble Chase, size 24-400, VWR Cat # 89046-840, Supplier Part # 75204G-24400
- Racks for glass tubes
  - VWR SCIENCEWARE Poxygrid 50mL Centrifuge Tube Rack, Bel-Art Cat #30617-644
- Labeling tape
  - VWR General Purpose Laboratory Labeling Tape, 1/2 IN, Cat # 89097-920
- Sodium citrate
  - Sodium Citrate Tribasic Dihydrate, Sigma Cat # C8532-1KG
- Citric acid
  - Citric Acid, Sigma Cat # 251275-500G
- Rubber bands
- BCA reagents A and B
  - Pierce BCA Protein Assay Kits, Thermo Scientific, VWR Cat # PI23225
- Transfer pipettes
  - VWR Disposable Transfer Pipettes, Cat # 414004-001
- Microcentrifuge tubes
  - 2.2ml tube, flat cap, 500/UN, LPS Cat # L250803
- Pipette tips
  - Clip-Tip 20 Reload Stacks (PK960), VWR Cat # 89348-030
  - Clip-Tip 200 Reload Stacks (PK960), VWR Cat # 89348-034
  - Clip-Tip 1000 Reload Stacks (PK768), VWR Cat # 89348-038
- 96-well plates
  - 96 Well Non-binding Microplate, Greiner Bio-One, Polystyrene, Flat Bottom, Chimney Style Wells, VWR Cat # 89131-676
- Sealing films for plates
  - Qiagen Tape Pads, Cat # 27112600
  - Roller for sealing tape: VWR Cat # 60941-118
- 'Falcon' tubes
  - Polypropylene flat capped centrifuge tube, VWR Cat # 89004-364
- Multichannel reservoirs
  - BTX RESERVOIR 25ML PS WHT ST, VWR Cat # 89511-196

        - Can order individual parts (Reagents A and B) separately if needed, on the lookup page under the above part number. Fisher also supplies this, filed under the same part number, if needed
- Protein standards
    - Pierce Pre-Diluted Protein Assay Standard Set, Thermo Scientific, VWR Cat # PI23208
        - Bovine Serum Albumin (BSA)
        - Each set comes with 3 mL each of the following seven dilutions: 125, 250, 500, 750, 1000, 1500, and 2000 µg/mL

- Microcentrifuge tube racks for bench
    - VWR 96-Place Microtube Racks, Cat #82024-494
- Microtiter tubes with racks and caps
    - 1.1 ml polypropylene tubes in 96 well format boxes, VWR Cat #89005-584
    - Caps in 8-strips, VWR Cat # 89005-724
- Block Heater
    - VWR Block Heater, Cat # 12621-088
    - Block for 96-well Plates: VWR Cat # 13259-295

Additional Supplementary Notes and Commentary on Purpose and Approach

Purpose

The purpose of measuring soil proteins is essentially twofold. Firstly, as a direct measurement of a desirable compound class, and secondly, as an indicator of important processes. Organic matter in the soil is made up of a numerous compounds, derived from a number of sources. Plant residues which are left behind after harvest, such as roots or stover, cover crops, green manures and mulches which are either grown in place or brought in as amendments, and manure, composted, and other organic materials added primarily for their nutrient content, all contribute to the accumulation of organic matter in the soil.

Much of this material is plant biomass, with additional contribution from fungal and bacterial biomass, and in the case of manures, digestive residues from animals. In addition, soil microbes and soil dwelling micro- and meso-fauna contribute biomass to the pool of organic matter in the soil when they die, leaving their bodies or cellular debris behind.

A large fraction of the dry matter in these residues is cellulose, which may contribute to the accumulation of soil organic matter and the storage of carbon in soils, but does not contain N and therefor does not contribute directly to N cycling. Similarly, hemicelluloses and lignin, also found in cell walls, do not contain N or contribute directly to its cycling. However, all cells contain protein, and protein can make up a significant fraction of the organic matter found in soil. Proteins are fairly high in N, relative to other large (high molecular weight) compounds contributing to biomass and to the organic matter in soil. Proteins, when broken down by soil

biotic activity, can contribute to the available (free, or mineral) N pool in the soil through mineralization. Mineralization in this case happens as organisms consume organic compounds (through extracellular digestion and absorption, or in the case of those higher in the 'food chain', by consuming smaller organisms) as both an energy and nutrient supply, and release soluble nitrogenous compounds as a waste product. It is both the relative contribution of C and N in an organic material, as well as the relative quantities of compounds like cellulose and lignin, that serve to determine whether the net effect of incorporation of these materials into the soil organic matter pool will result in net mineralization or immobilization. It is the growing understanding that in natural systems, the N cycling rate is limited by the depolymerization (or breakdown) of high molecular weight N containing biopolymers (primarily protein). We can manage agroecosystems to rely to a greater or lesser degree on nutrient storage in soil organic matter (in contrast with application in soluble low molecular weight form and storage only on the soil's exchange complex). Much of the sustainable agricultural paradigm rests at least in part on this management objective.

This brings us to the second major purpose in measuring soil proteins, as an index of the coupling of carbon and nitrogen cycling in the system. The storage and release of nutrients in the organic matter and the soil microbial biomass is an important functional process in soil. Where nutrients, particularly N, are stored in the biomass and organic matter, they are present in the system, but are not as readily subject to loss as when they are in a more soluble mineral form. As plants grow in the agricultural system, they can access nutrients from the smaller available pool, over the course of their growth, with this pool being replenished by further turnover of nutrient containing organic matter, which the plants can in fact stimulate through the secretion of root exudates, which encourage the growth and metabolic activity of rhizosphere microorganisms that accomplish the breakdown of the organic matter. This is in contrast with management of nutrients by application of larger amounts of nutrients in the early part of the season in soluble form, and the reliance on sufficient quantities of these nutrients to remain available throughout the plants' growth. Growing concern for the vulnerability of the latter approach to losses of nutrients through leaching, runoff, and denitrification, along with the understanding that the combined presence and activities of the microbes and organic matter heavily involved and presumably increased by the former approach contributes substantially to not only improved nutrient storage and release functionalities but also to other important soil processes such as aggregation and aggregate stabilization, plant growth support and promotion, and carbon sequestration, argues in favor of management that relies to a greater extent on the former.

If we wish to manage systems with a greater reliance on N incorporation into organic matter and subsequent release from that organic matter, then we are seeking to couple N cycling with C cycling in that system to a greater degree. We describe elemental cycles in ecosystems through stocks and flows, or pools and fluxes. Where N is stored in organic form, it is (by definition) bound to C, and the compounds that represent the N stock also are a C stock. The fluxes into and out of this pool are biotically mediated, as both the incorporation of N into biomass by plants and microbes and its release through mineralization are metabolically driven activities, necessarily

using stored energy. The energy use in these fluxes utilize energy stored in and released from reduced C compounds. The C cycle, as driving force, and the N cycle, are here coupled to each other. This tight coupling of C cycling and the cycling of nutrients that can take an organic form, is a general feature of natural ecosystems, but are de-coupled to a large extent in systems that rely on nutrient storage in mineral form. As we seek to design and manage agroecosystems to better take forms that are modeled after natural systems, we seek to recouple these C and N cycles.

The presence of a substantial pool of organic N in a system materially indicates the process of coupled C and N cycling in that system. As we consider a soil system to be in a healthier state if the process of N storage and turnover in organic matter is functioning well, and as the activity of the microbial community, that is essential for the cycling of N into and out of the biomass pool and soil organic matter, accomplishes numerous functional processes that are necessary for optimal soil functioning, we use the pool of soil proteins as an indicator of soil biological health.

Approach

The approach we use to extracting and quantifying soil proteins is extraction in a citrate buffer assisted by shaking and autoclaving, followed by quantification using a standard, widely used chemical assay for protein content, modified to better quantify mixtures of various kinds of protein. The basic extraction approach is a modified form of an approach that has been used by various soil researchers since the mind-1990s. A number of studies have indicated that the amount of protein retrieved in such a way is related to management, and is generally higher where tillage intensity is lower, aggregate stability is higher, and biological activity is high. These studies generally grew out of work tracking the presence and activity of particular fungal groups in the soil, including antibody driven approaches that appeared to preferentially identify biomass of these fungi. While the antibody-driven approach sought specific binding of a monoclonal antibody to antigenic material extracted from these fungi, and from soil in which they presumably were active, a more rapid and straightforward quantification of total proteins in a crude citrate extract was used in most of these studies wither in addition to or instead of the antibody-based quantification. As this extraction process retrieved a substantial quantity of material that reacted with the antibody in use, the quantification of total proteins extracted was frequently taken to be synonymous with or nearly synonymous with (or at least a sufficient proxy for) quantification of the immunoreactive material, and the same terminology was subsequently applied to both pool quantities. However, the quantification of protein concentration in a crude citrate extraction is not specific to any one protein component of the protein mixture extracted. The autoclaved citrate extraction likely works through the enhanced solubilization at highly elevated solvent temperature, with sodium ion enhanced disaggregation of soil particles and citrate ion chelation of cationic species that could otherwise counter this. Some improved retrieval of proteins from soil samples using sodium pyrophosphate solution instead has been reported, but for consistency with the lager body of information regarding soil protein pool size and the relationship of this with soil quality, we retain the citrate buffer approach. We found that this extraction protocol retrieves proteins from organic matter sources

that are unrelated to the fungal groups these earliest studies were predominantly focused on, and that quantification of total protein in the extracts should be taken as a measure of proteins from numerous sources. The numbers reported in this earlier soil protein work for the total protein on citrate extracts remain comparable to proteins quantified with our approach, but the source of extracted proteins should be considered to be any proteinaceous material in the soil sample, rather than any particular microbial group.

As the protein pool extracted is of a mixed nature, we use a quantification chemistry that has a reduced sensitivity to differences in protein type or amino acid composition. There are numerous standard and widely used protein quantification approaches, and the bicinchoninic acid assay we employ is fairly rapid, and when carried out at elevated temperature quantifies protein by reaction of copper ion with the protein peptide backbone, reaction of the products of this with an indicator compound, and subsequent light absorbance wavelength shift in the reaction solution. This is in contrast with approaches such as the Bradford assay, which quantifies proteins by direct reaction of a dye compound with amino acid side groups, and differentially so with proteins of differing amino acid composition. Other approaches require one or more distillation steps or brief, carefully timed sequential reagent additions, which are less appropriate for the needs of a higher throughput service-lab protocol.