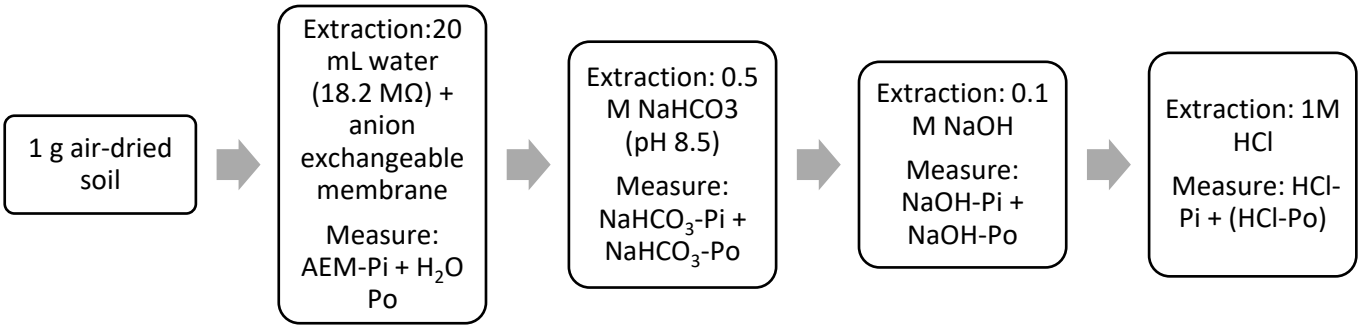
SOP: **Hedley fractionation**

Overview

This standard operating procedure (SOP) describes a protocol for commonly used sequential extraction of soils to estimate pools of soil phosphorus (P) hypothesized to vary by availability and forms. The method was originally reported by Hedley et al. (1982) and modified by Tiessen and Moir (2007). Soils that are ground to pass a <2 mm sieve are typically used. Plan ahead of time given this extraction procedure will require at least 5 straight days of lab work. A sample size of less than 50 should be reasonable for someone new to the procedure (4-8 hours per day for a week). Once experienced, a sample size of 80-100 per week should be doable with similar amount of time.

Note: Interpretations of extracted P fractions as certain P pools based on hypothesized difference in availability and speciation vary and potential artifacts of the extractions are reported by Cross and Schlesinger (1995), Klotzbücher et al., (2019) and Gu et al., (2020).



Safety

All standard safety protocols and online safety training via UIUC Division of Research Safety (DRS) are required.

Personal protection (PPE) for this procedure include:

Eye Protection: Safety goggles

Body Protection: Lab coat

Hand Protection: Gloves

Particularly hazardous substances: Concentrated sulfuric acid and hydrochloric acid should be handled in the fume hood. Persulfate is an oxidizer, which can cause fire when in contact with combustibles, and exposure to high temperature should also be avoided. Make sure to check Safety Data Sheet if unsure about how to handle these chemicals.

Specific details on these substances are incorporated in the **Detailed Procedure** below.

Instrumentation & Consumables

Sample Preparation

- Analytical balance (two decimal places sensitivity)
- 50 mL tubes (Falcon brand is preferred for avoiding leak)

Reagent Preparation

- pH meter
- Anion exchangeable membrane (AEM, also called resin) strip (1x4 cm, VWR International, West Chester, PA)
- $NaHCO_3$ (0.5 M at pH 8.5)
- NaOH (0.1 M; 5%; 10%)
- HCl (1 M)
- H_2SO_4 (0.2 M; 1.2 M)
- Sodium persulfate
- Commercial P standard (1000 mg P/L)
- Ammonium molybdate
- Antimony potassium tartrate
- Ascorbic acid

Sequential Extraction

- Horizontal shaker
- Centrifuge (4000 rpm)
- Microcentrifuge (15000 rpm)
- Dispensette
- 15 mL centrifuge tubes
- Pipette and tips (100-1000 µL)

Digestion

- 15 mL centrifuge tubes
- 10% and 15% persulfate in 1.2 M H_2SO_4
- Oven (able to maintain temperature at 90 °C overnight)

Colorimetry

- 96 well microplates
- Spectrophotometer capable of reading at 882 nm (or microplate spectrophotometer)
  - ❖ Note: this SOP's colorimetry procedure is based on microplate, but can be scaled up for cuvette while keeping same ratio of reagents and extracts
- Pipette and tips (100-1000 µL)

Detailed Procedures

Reagent Preparation

1. **Extractants and digestant**
   - AEM strip
     - Shake AEM strip in 0.5 M $NaHCO_3$ for > 3 hours on horizontal shaker (120 rpm; “low” for Eberbach E6010.00), and then decant the $NaHCO_3$ (careful not to drop any strip; 0.5 M $NaHCO_3$ can go down the drain with copious amount of water running) and then shake the AEM strip in 18.2 MΩ water for 5 min. Repeat this procedure three times and this will ensure that AEM is pre-loaded with bicarbonate.
     - AEM strip can be reused until they deform, but make sure to pre-load them before every use.
     - For dirty strips, shake the strips in 0.25 M H_2SO_4 for 1 h, then wash 2 times in 18.2 MΩ water (shake for 5 min each time), then $NaHCO_3$ wash, 18.2 MΩ water wash.
   - 0.5 M $NaHCO_3$ at pH 8.5
     - Dissolve 42 g sodium bicarbonate ($NaHCO_3$) and 0.72 g sodium hydroxide (NaOH) pellets in 900 ml of de-ionized water. Adjust the pH to 8.5 using a NaOH (e.g., 10% NaOH) or acid (e.g., 10% HCl) and make up to 1 liter with 18.2 MΩ water.
   - 0.1 M NaOH
     - Take dry and clean 1000 mL volumetric flask.
     - Add 4.0 g of sodium hydroxide pellets.
     - Add 100 ml of 18.2 MΩ water and shake the flask to dissolve the sodium hydroxide pellets.
     - Allow to cool the solution at room temperature.
     - Make-up the volume up to 1000 mL with 18.2 MΩ water and mix the solution thoroughly.
     - Keep the solution for an hour to cool at room temperature.
   - 1 M HCl, 1.2 M H_2SO_4, and 0.2 M H_2SO_4 (Molarity Calculator by Sigma-Aldrich can be helpful for making solutions); ADD WATER BEFORE ACID
   - 10% persulfate (sodium persulfate) in 1.2 M H_2SO_4
     - Weigh 10 g sodium persulfate, and then add 100 mL 1.2 M H_2SO_4 solution.
   - 15% persulfate (sodium persulfate) in 1.2 M H_2SO_4
     - Weigh 15 g sodium persulfate, and then add 100 mL 1.2 M H_2SO_4 solution.
   - 5% NaOH: dissolve 5 g of NaOH in 100 mL of 18.2 MΩ water.

2. **Standards**
   - Calibration standards (ranging from 0 – 20 mg P/L) need to be made in each extracting solution: 18.2 MΩ water, 0.2 M H_2SO_4, 0.5 M $NaHCO_3$, 0.1 M NaOH,

and 1M HCl. Dilute commercial standard (1000 mg P/L) in each extracting solution and sequentially dilute them to make calibration standards that are within the linear range and cover the concentration you are expecting for your soil samples. The Dilution Calculator can be helpful for sequential dilution.

Note: concentrations of the standards should be on ***P, not PO_4^{3-}, basis***

a. It is essential to use the same extracting solution because molybdate colorimetry of P is sensitive to pH (color development and intensity, precipitation).
b. Example standards: 20 ppm, 10 ppm, 5 ppm, 2.5 ppm, 1.25 ppm, 0.625 ppm, 0 ppm (details in the table below).
c. **Treat the standards the same way as the samples during the digestion processes**!

Standard concentration (mg/L)	Amount of 0.5 M $NaHCO_3$ (µL)	Serial dilution of standards Amount of P standard (µL)
100	900	100 of 1000 mg/L
20	800	200 of 100 mg/L
10	500	500 of 20 mg/L
5	500	500 of 10 mg/L
2.5	500	500 of 5 mg/L
1.25	500	500 of 2.5 mg/L
0.625	500	500 of 1.25 mg/L
0	500	-

3. Colorimetry reagents

i. Murphy-Riley Solution A
   a. Dissolve 4.3 g ammonium molybdate in 400 mL of deionized water in a 1-litre beaker.
   b. Dissolve 0.40 g antimony potassium tartrate in 400 mL deionized water, then add to the ammonium molybdate solution in the beaker.
   c. Slowly and carefully, with stirring and while cooling in an ice bath (may be unnecessary but the beaker does heat up when you add sulfuric acid), add 54 mL conc. H_2SO_4.
   d. Allow to cool and make to 1000 mL with deionized water. Mix well and store in a dark bottle in a refrigerator. *The reagent is stable for at least 4-6 weeks at 4°C.

ii. Murphy-Riley Solution B

Ascorbic acid, 1%: Dissolve 1.00 g of ascorbic acid in 100 mL deionized water. Make a fresh solution daily as needed.

iii. Final Murphy-Riley (MR) reagent: combine 56 mL of solution B + 44 mL of solution A, and mix (should turn to light yellow color). This solution only lasts up to a few days, thus best to make this solution fresh on the same day you perform colorimetry.
iv. 5% NaOH (for adjusting pH for colorimetry)

Sequential Extraction and Colorimetry

AEM-Pi and H_2O-Pt Extraction

1. Measure 2.00 g ± 0.02 of air-dried soil into 50 mL Falcon centrifuge tube. Falcon tube is recommended for avoiding leak during extraction. Record exact weight of soil to at least 1/100th of one gram (1.XX g).
   Note: 2 g is our current default, but extraction ratio may vary among studies
2. Prepare 2 empty 50 mL Falcon centrifuge tube for blanks (no soil but treated the same way as samples to account for background P throughout the fractionation).
3. Add 20 mL 18.2 MΩ water and 1 piece of AEM strip to each tube.
4. Put the tubes of samples on horizontal shaker (120 rpm; "low" for Eberbach E6010.00) for 16 hrs. Wrapping parafilm around cap can help prevent spills while shaking.
5. With tweezers, fish out AEM strip, and attempt to remove as much sediment by shaking strip in the extraction tube.
6. Using a squeeze bottle with 18.2 MΩ water, use a thin but powerful stream of water to wash sediment off AEM strip (**do not** wash into extraction tube, as this will dilute the H_2O-P_t).
7. Immerse washed AEM strip in 12 mL of 0.2 M H_2SO_4 (20-25 mL for soils expected to have high AEM-Pi) and shake for 3 h. Note: 15 mL Falcon centrifuge tubes are used. The concentration of H_2SO_4 used for elution is different from 0.25 M used in microbial biomass phosphorus (hexanol) protocol. Concentration may be changed as long as full elution can be achieved.
8. Centrifuge the 50 mL tubes containing soil and water at 4000 rpm for 20 min (until clear supernatant).
9. AEM-P_i colorimetry: pipette 21.4 µL 5% NaOH + 128.6 µL 1.2 M H_2SO_4 eluent+ 150 µL MR solution into microplates with two replicates.
   Notes: this colorimetry reaction is the slowest of them, due to low pH of reaction. The addition of NaOH helps. Reaction is complete when standards have stopped increasing and the standard curve is linear. Colorimetry may take 45-60 min (but can be as short as 15 min if pH is adjusted well).
   Volume before scaled down from cuvette: 100 µL 5% NaOH + 600 µL 1.2 M H_2SO_4 eluent+ 700 µL MR.
10. Pipette 1.5 mL of H_2O extract + 1 mL 10% persulfate (in 1.2 M H_2SO_4) into 15 mL Falcon tubes, digest in the oven at 90°C for 16 h, or autoclave for 30 min.

Note: as true for all other digestion, make sure standards for H2O-Pt fractions are also digested the same way as samples!

11. H_2O-P_t colorimetry: pipette 50 µL 5% NaOH + 60 µL digestate+ 150 µL MR solution into microplates. 15 min should be sufficient but check for color development of standards and linearity of resulting standard curve when in doubt.
    Note: volume before scaled down from cuvette: 250 µL 5% NaOH + 300 µL digestate + 700 µL MR
    Note on colorimetry on digested samples (applies to digested samples in subsequent extractions): sometimes the color may not show due to the excessive persulfate in the solution (especially for samples with low P concentration). One way to solve this is to use 18.2 MΩ water to dilute the digested samples before colorimetric measurement. If we dilute the samples, we should also do the same thing for the standard curve.
    Note on H_2O-P_t interpretation: this fraction is operationally referred to as H_2O-P_t in this SOP, but this fraction may be interpreted as H_2O-P_o assuming AEM adsorbed all of the P_i from the water extract.

$NaHCO_3$-P_i and $NaHCO_3$-P_t Extraction

1. After step 10 of the AEM-Pi and H_2O-P_t Extraction, decant the rest of the extract while avoiding loss of soil samples, add 40 mL 0.5 M $NaHCO_3$ into the tubes and shake for 16 h.
2. Centrifuge each tube at 4000 rpm for 15 min until clear.
3. $NaHCO_3$-P_i colorimetry: pipette 60 µL $NaHCO_3$ extract + 140 µL MR in microplates with two replicates. 15 min waiting time should be sufficient but check for color development of standards and linearity of resulting standard curve when in doubt.
   Notes: Add MR to extract slowly to avoid spills, as strong effervescence results from acid being added to base. If there is substantial organic P in extracts, mineralization of organic P into Pi will result in slow upward creep of absorbance. In rare cases may need to add 18.2 MΩ water to bring absorbance into linear range.

4. Pipette 1 mL of $NaHCO_3$ extract + 1 mL 10% persulfate (in 1.2 M H_2SO_4) into 15 mL Falcon tubes, digest in the oven at 90 °C for 16 h.
   Notes: Adding acid to $NaHCO_3$ can cause strong effervescence, thus adding slowly to avoid spills. Wait approximately 10 min before capping the tube to allow effervescence to go away.
   Note: as true for all other digestion, make sure standards for $NaHCO_3$-P_t fractions are also digested the same way as samples!
5. $NaHCO_3$-P_t colorimetry: pipette 50 µL 5% NaOH + 60 µL $NaHCO_3$ digestate + 140 µL MR solution into microplates.

*Volume before scaled down from cuvette: 2*50 µL 5% NaOH + 300 µL $NaHCO_3$ digestate + 700 µL MR solution.

Notes: add in this order, as it ensures mixing of base and acidic digestate

NaOH-P_i and NaOH-P_t Extraction

1. After step 4 of the $NaHCO_3$-P_i and $NaHCO_3$-P_t Extraction, carefully remove the remaining $NaHCO_3$ extract from the tubes, add 40 mL 0.1 M NaOH and shake for 16 h.
2. Centrifuge each tube at 4000 rpm for 15 min until clear supernatant.
3. Pipette 1000 µL NaOH extract + 120 µL 1.2 M H_2SO_4 in a microcentrifuge tube and spin down at 15,000 rpm for 3 min (should see dark pellet of precipitated SOM). Use clear aliquot for colorimetry.

   Notes: remember to perform this step for NaOH-P_i standards (made up in NaOH extraction solution)
4. NaOH-P_i colorimetry: 28.5 µL 5% NaOH + 34.3 µL acidified and micro-centrifuged NaOH eluent + 137.2 µL MR solution into microplates.

Notes: addition of 18.2 MΩ water may be needed to bring absorbance into linear range

5. Pipette 1 mL of NaOH extract + 1 mL 15% persulfate (in 1.2 M H_2SO_4) into 15 mL Falcon tubes, digest in the oven at 90°C for 16 h.

   Notes: as true for all digestion, make sure standards for NaOH-P_t fractions are also digested the same way as samples! for high SOM soils, may need to increase volume of acidic persulfate solution. Repeat digestion (also for standard) if you see incomplete digestion of SOM in the tube
6. NaOH-P_t colorimetry: pipette 44.9 µL 5% NaOH + 54 µL NaOH digestate + 151.1 µL MR solution into microplates.

Notes: addition of 18.2 MΩ water may be needed to bring absorbance into linear range

HCl-P_i and HCl-P_t^* Extraction

1. After step 5 of the NaOH-Pi and NaOH-P_t Extraction, carefully remove the rest of the liquid from the 50 mL tubes, add 40 mL 1 M HCl to the tubes and shake for 16 h.
2. Centrifuge each tube at 4000 rpm for 15 min until clear.
3. HCl-P_i colorimetry: pipette 50 µL 5% NaOH + 60 µL HCl extract µL + 140 µL MR solution into microplates.

   Notes: Add MR to extract slowly, as effervescence results from acid being added to base. If there is substantial organic P in extracts, mineralization of organic P into Pi will result in slow upward creep of absorbance
4. Pipette 1.5 mL of HCl extract + 1 mL 10% persulfate (in 1.2 M H_2SO_4) into 15 mL Falcon tubes, digest in the oven at 90 °C for 16 h.

Notes: as true for all digestion, make sure standards for HCl-P_t fractions are also digested the same way as samples!

5. HCl-P_t colorimetry: pipette 93.1 µL 5% NaOH + 62.1 µL HCl digestate + 144.8 µL MR solution into microplates.
   Notes: add in this order, as it ensures mixing of base and acidic digestate. Before scaled down, the amount is: 450 µL 5% NaOH + 300 µL digestate + 700 µL MR

**Not included in standard Hedley, but evidence suggests it may be a small portion of soil organic P in HCl extract*

Supplementary Instructions

- Colorimetry is performed directly in 96-well microplates, which can hold up to 300 µL.
- Absorbance is measured at 882 nm. Use 0 ppm standard as blank reading.
- For a given extraction, I prefer to start the P_i colorimetry, and then set up the digestion for P_t of that extract. P_i concentration is more likely to change with time due to the conversion of P_o to P_i in each extract solution, thus running P_i samples for colorimetry should be the priority (except AEM-Pi). P_t in digested solution should be relatively more stable compared to P_i because there should be no more P_o to be converted to P_i once fully digested. Extracts that do not need to be prioritized for colorimetry specifically are AEM Pi, NaHCO3-P_t, NaOH-P_t, and HCl-P_t, although they should still be analyzed within a few days. HCl-Pi could also be waited if HCl-P_o is negligible.
- Digestions are performed to convert P_o to P_i, and P_t is quantified in the digestate as P_t. Then P_o is calculated as difference between P_t and P_i. The exception is AEM-Pi and H_2O-P_t, given H_2O-P_t measured P_t after removal of P_i by AEM, so typically no subtraction is performed and H_2O-P_t is directly interpreted as H_2O-P_o.
- **Golden Rule: Treat standards the same as samples**. This goes for the working standards being made up in the same solution as extracts. For example, working standards for $NaHCO_3$ extraction should be made in 0.5 M $NaHCO_3$ (pH 8.5) and used as such for $NaHCO_3$-Pi. For P standards for $NaHCO_3$-P_t, digestate the working standards made up in 0.5 M $NaHCO_3$ (pH 8.5) in the same manner as samples. If colorimetry reactions are diluted to bring absorbance into linear range, do the same for standards. Etc.

Clean up

- Make sure to clean up dispensette (**rinse with dilute sulfuric or hydrochloric acid, followed by water, especially after pumping $NaHCO_3$ after use**), shaker (especially if tubes leak), and centrifuge (especially if tubes leak).

- Collect solutions (except those that can go into drain with copious amount of water; check DRS for further information) into chemical waste bottle, **clearly labelled with contents and their concentrations**. Request pick up when the bottle is almost full. Clean up any spills with absorptive tissues and soap water immediately as needed. For spilled dilute acids, immediately **neutralize with sodium bicarbonate,** and then clean up.

Calculations

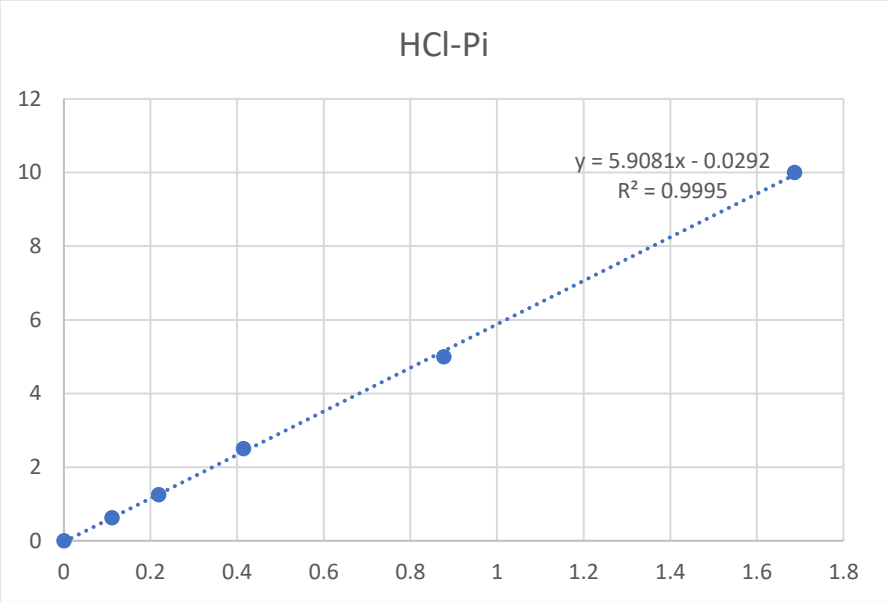
Measurement of P fractions is usually expressed in units of mg P kg^{-1} soil. P_i and P_t are converted to the final unit separately and then P_o is calculated by difference at the end.

1. Convert raw absorbance to concentration (mg P L^{-1}) using calibration curve constructed specifically for each fraction (as noted previously, calibration standards should be treated exactly the same way as samples, so the absorbance can be directly converted to concentrations in the extract, before dilution if samples were separately diluted). Multiply the concentration by dilution factor if diluted.
2. Multiply the concentration by the extract volume (e.g., 0.02 L for H_2O-P_t and 0.04 L for HCl-Pi) and divide by soil mass (2 g = 0.002 kg) to yield concentration in mg P kg^{-1} soil

Example calculation:

Given for HCl-Pi
Absorbance = 0.760
Dilution = 1
Calibration curve: y = 5.9081x - 0.0292
Extraction volume = 0.04 L
Soil mass = 0.002 kg

Concentration in extract = (5.9081*0.760 – 0.0292) * 1 = 4.46 mg L^{-1}
Concentration in soil basis = 4.46 mg L^{-1} * 0.04 L / 0.002 kg = 89.2 mg P kg^{-1} soil



Note: this calibration curve only goes up to 10 mg P/L, but typical range is 0-20 mg P/L. This can vary, but what you always need to make sure is that your sample absorbance is below the absorbance of your highest standard.

References

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Suggested reading:

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