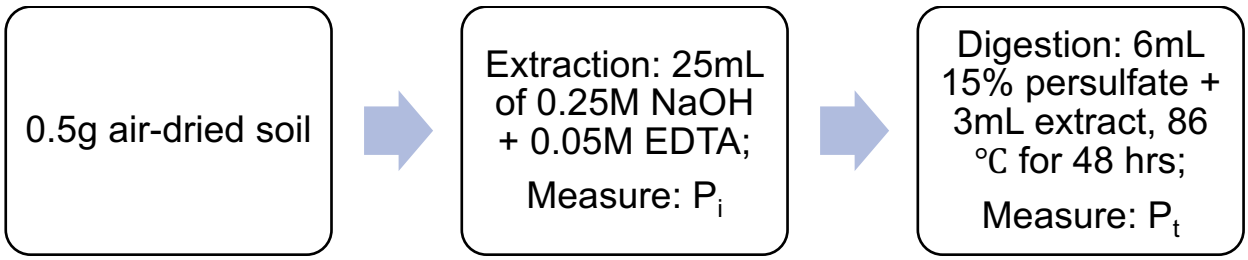
SOP: **Total organic P**

Overview:

This standard operating procedure (SOP) describes a protocol for extraction of soil P to estimate the total organic phosphorus (P_o) in soil. This method may not extract all P_o, and thus may be underestimating total P_o in soil. This protocol is modified from the method developed by Turner (2008) and Bowman and Moir (1993). Air-dried soil that is ground to pass a 2 mm sieve is typically used or finely ground powdered soil.



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: Nitrile Gloves
Particularly hazardous substances: Concentrated sulfuric 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 centrifuge tube (Falcon brand is preferred for avoiding leak)

Reagent preparation

- Sodium hydroxide and Ethylenediaminetetraacetic (EDTA) disodium salt dihydrate (MW: 372.24 g mol^{-1}) (0.25M NaOH + 0.05M EDTA)
- Sodium persulfate
- Sulfuric acid (1.2M)

- Commercial P standard (1000 mg P/L)
- Ammonium molybdate
- Antimony potassium tartrate
- Concentrated sulfuric acid
- Ascorbic acid
- Sodium Hydroxide (5%)

Extraction

- Horizontal shaker
- Centrifuge
- Microcentrifuge
- Dispensette
- 15 mL centrifuge tubes
- 2 mL microcentrifuge tubes
- Pipette and tips (20-200 µL and 1000-5000 µL)

Digestion

- 15 mL centrifuge tubes (use Falcon brand (material: PP) to avoid leak)
- 15% persulfate in 1.2 M H_2SO_4
- Oven (able to maintain temperature at 86 °C overnight)

Colorimetry

- 96-well microplates
- Microplate UV spectrophotometer capable of reading at 882 nm
- Pipette and tips (20-1000 µL)

Detailed Procedures:

I. Sample preparation

1. Measure 00.50 ± 0.02 g of air-dried soil into 50 mL centrifuge tube with proper labeling. Record exact weight of soil to at least 1/100th of one gram (1.XX g). Falcon tube is recommended for avoiding leak during extraction. Note: for soils with low P content, the soil mass can be adjusted (ranged from 0.5-2 grams) until the P_t concentration can be measured through colorimetric method.
2. Prepare empty 50 mL centrifuge tube for blanks (no soil, but treated the same way as samples to account for background P throughout the extraction)

II. Reagent preparation

1. Extractants
    i. 0.25M NaOH + 0.05M EDTA

a. To make 1 L solution, fully dissolve 10 g of sodium hydroxide in approximately 500 mL nanopure water, add 18.61 g of EDTA, and use nanopure water to bring volume to 1 L.

ii. 1.2 M H_2SO_4 (Molarity Calculator by Sigma-Aldrich can be helpful)

iii. 15% persulfate salt in 1.2M H_2SO_4

2. Standards

i. Dilute commercial standard (1000 mg P/L) in the 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.

a. It is essential to use the same extracting solution (0.25M NaOH and 0.05M EDTA) because molybdate colorimetry of P is sensitive to pH (color development and intensity, precipitation).

b. Standards can be prepared using the reagent volumes provided in the following table (total volume 40mL)
Note: to make 200 mL of 0.5M NaOH + 0.1M EDTA solution, fully dissolve 4 g of sodium hydroxide in approximately 100 mL nanopure water, add 7.44 g of EDTA, and use nanopure water to bring volume to 200 mL.

Standard [P] (mg P L^{-1})	0	0.5	1	2	5	10	15	20
50 mg/L P standard solution (mL)	0	0.4	0.8	1.6	4	8	12	16
0.5M NaOH + 0.1M EDTA (mL)	20	20	20	20	20	20	20	20
Nanopure water (mL)	20	19.6	19.2	18.4	16	12	8	4

Note: the 0.5M NaOH + 0.1M EDTA used here is to create the background 0.25M NaOH + 0.05M EDTA in the standards. After dilution, all standards will contain 0.25M NaOH + 0.05M EDTA in the background.

ii. All standard solutions should be treated the same as the soil sample.

a. For P_i measurement, add 360 µL of 1.2M H_2SO_4 to each 1mL of the standard.

b. For P_t measurement, use the same digestion method for the standards and the samples.

3. Colorimetry reagents

i. Murphy-Riley Solution A (stable for 4 weeks at 4°C)

a. Dissolve 4.3 g ammonium molybdate in 400 mL of nanopure water in a 1L beaker.

b. Dissolve 0.40 g antimony potassium tartrate in 400 mL nanopure 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.

ii. Murphy-Riley Solution B (stable for 24 hours at 4°C)
a. 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 (stable for 24 hours at 4°C): combine 56 mL of solution B + 44 mL of solution A, and mix (should turn to light yellow color)
Note: smaller amount with the same ratio of reagent A and B can be mixed with the same A to B ratio (e.g., 28 mL reagent B + 22 mL reagent A)

iv. 5% NaOH (for adjusting pH for colorimetry)

III. Extraction and colorimetry

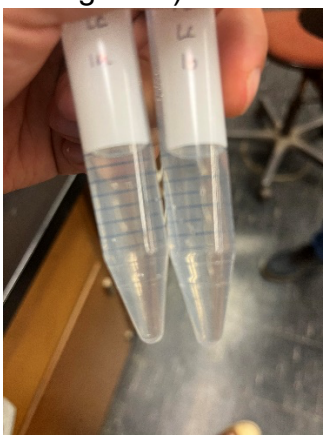
1. Procedures
i. NaOH-EDTA-P_i
a. Extraction:
1) Extract the pre-weighed soil in the 50 mL centrifuge tube with 25 mL of 0.25M NaOH + 0.05M EDTA solution, using a horizontal shaker (120 rpm; “low” for Eberbach E6010.00) for 16 hrs. Wrapping parafilm around cap can help preventing spills while shaking
2) Centrifuge each tube at 4000 rpm for 20 min until clear supernatant

b. Precipitation of SOM
1) 1000 µL NaOH-EDTA extract + 360 µL 1.2 M H_2SO_4 in a microcentrifuge tube
2) Spin down at 15,000 rpm for 3 min (should see dark pellet of precipitated SOM)
3) Use clear aliquot for colorimetry
Notes: remember to perform this step for P standards (made up in NaOH-EDTA extraction solution)

c. Colorimetry: 42 µL 5% NaOH + 50 µL acidified NaOH-EDTA extract + 200 µL MR
Notes: The addition of NaOH helps to maintain a neutral pH. Reaction is complete when the color development of standards has stopped increasing. This process takes approximately 30-60 min. The absorbance can be measured several times within an hour until the absorbance does not increase.

ii. NaOH-EDTA-P_t

a. Digestion: 3 mL of NaOH-EDTA extract + 6 mL 15% persulfate (in 1.2 M H_2SO_4), digest at 86 °C for 48 h
   Note1: For soil samples with high SOM, increase the volume of acidic persulfate solution or decrease the volume of sample extract. If the sample to persulfate ratio is changed, do the same for standards. The digestion time can also be longer (i.e., 72 hrs) to ensure full digestion.
   Note2: During the digestion, if dark precipitates are observed (figure below, left), tubes should be shaken several times by hand to suspend SOM periodicallyperiodically, which helps with full digestion. If the dark SOM precipitates remained after the digestion, this means that the digestion has not completed yet, and the shaking and digesting procedures should be continued until no dark precipitates can be observed (figure below, right).
b. Colorimetry: 52 µL 5% NaOH + 21 µL NaOH-EDTA digestate + 42 µL nanopure water + 200 µL MR
   Note: This colorimetry method already contains the dilution of samples. Generally, dilution is needed to avoid the bleaching effect of unreacted persulfate. If soil sample contains a low content of P, using a greater soil mass in the beginning of the NaOH-EDTA extraction is recommended instead of adjusting the dilution. If the P content is high, a greater dilution factor can also be used. P standard with a higher P concentration (i.e., 40 mg L^{-1} P) can also be used.



2. Colorimetry is performed directly in the microplate, which can hold up to 350 µL
3. Absorbance is measured at 882 nm. The blanks should be run for each microplate and the absorbance for blanks should be recorded.
4. Digestions are performed to convert P_o to P_i, and P_t is quantified in the digestate as P_i.

5. 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 each 1mL of NaOH-EDTA-P_i should be made in 0.25M NaOH + 0.05M EDTA background solution with the addition of 360 µL of 1.2 M H_2SO_4. If colorimetry reactions are diluted to bring absorbance into linear range, do the same for standards. Also, be sure to only use a standard curve with the R^2 higher than 0.99 for analytical chemical method.

IV. Clean up

1. Make sure to clean up dispensette (rinse with dilute sulfuric or hydrochloric acid, followed by water), shaker (especially if tubes leak), and centrifuge (especially if tubes leak).
2. 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.

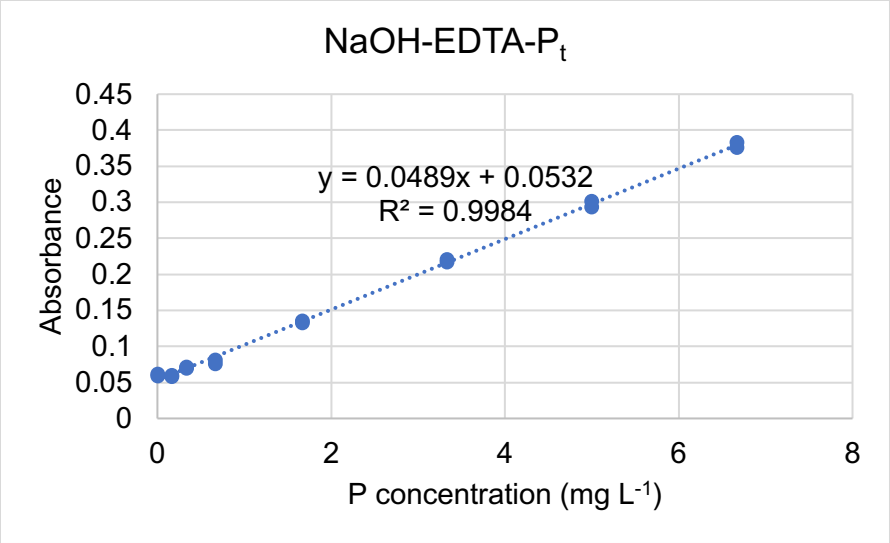
V. Calculations

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

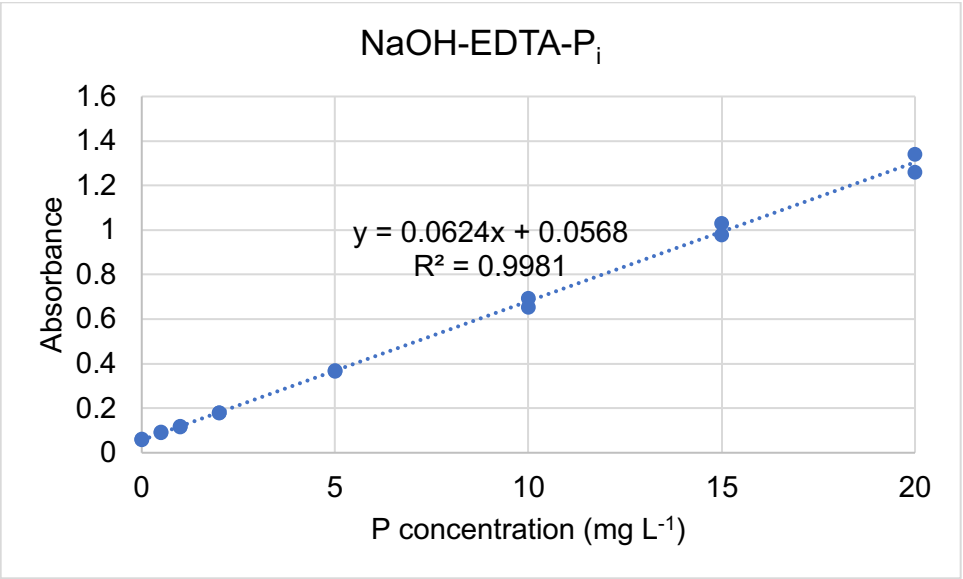
1. Convert raw absorbance to concentration (mg P L^{-1}) using calibration curve constructed specifically for each fraction (as noted previously, calibration standards should be treated exactly the same way as samples, so the absorbance can be directly converted to concentrations in the extract). Multiply the concentration by dilution factor if diluted.
2. Multiply the concentration by the extract volume (0.025 L) and divide by soil mass (0.5 g = 0.0005 kg) to yield concentration in mg P kg^{-1} soil.
3. Soil total organic P concentration can be calculated by subtracting P_i from P_t: $P_o = P_t - P_i$

Example calculation:

Given, for NaOH-EDTA-P_t
Absorbance = 0.185
Dilution = 3
Calibration curve: y = 0.0489x + 0.0532
Extraction volume = 0.025 L
Soil mass = 0.0005 kg
Concentration in extract = (0.185-0.0532) / 0.0489 × 3 = 8.09 mg L^{-1}
Concentration in soil basis = 8.09 mg L^{-1} × 0.025 L / 0.0005 kg = 404.3 mg P kg^{-1} soil



For NaOH-EDTA-P_i
Absorbance = 0.534
Dilution = 1
Calibration curve: y = 0.0624x + 0.0568
Extraction volume = 0.025 L
Soil mass = 0.0005 kg
Concentration in extract = (0.534-0.0568) / 0.0624 × 1 = 7.65 mg L^{-1}
Concentration in soil basis = 7.65 mg L^{-1} × 0.025 L / 0.0005 kg = 382.4 mg P kg^{-1} soil



For NaOH-EDTA-P_o,
$P_o = P_t - P_i$ = 404.3 – 382.4 = 21.9 mg P kg^{-1} soil

References:

Bowman, R. A., & Moir, J. O. (1993). Basic Edta as an Extractant for Soil Organic Phosphorus. *Soil Science Society of America Journal, 57*(6), 1516-1518. doi:10.2136/sssaj1993.03615995005700060020x

Turner, B. L. (2008). Soil organic phosphorus in tropical forests: an assessment of the NaOH–EDTA extraction procedure for quantitative analysis by solution31P NMR spectroscopy. *European Journal of Soil Science, 59*(3), 453-466. doi:10.1111/j.1365-2389.2007.00994.x

Turner, B. L., Cade-Menun, B. J., Condron, L. M., & Newman, S. (2005). Extraction of soil organic phosphorus. *Talanta, 66*(2), 294-306. doi:10.1016/j.talanta.2004.11.012

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