Orthophosphate Protocol (PO_4^{-3})

•Ellen Esch, May 2019, adapted from Ringuet, S., L. Sassano, and Z. I. Johnson. 2011. A suite of microplate reader-based colorimetric methods to quantify ammonium, nitrate, orthophosphate and silicate concentrations for aquatic nutrient monitoring. Journal of Environmental Monitoring 13:370-376. **There is a correction for this paper linked on the journal website, that is where the molarity comes from, make sure to read. Also from Murphy, J., and J. P. Riley. 1986. A Modified Single Solution Method for the Determination of Phosphate in Natural-Waters. Current Contents/Agriculture Biology & Environmental Sciences:16-16.

•If you need to extract from soils, you can do that, usually you need to create resin strips and then extract P from the soil with the resin strips. Then you would strip the extracted P from the resins using a salt, and then analyze the resulting salt for P. For more information, see: 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.

Make solutions:

32 mM ammonium molybdate ($(NH_4)_6 \cdot Mo_7O_{24} \cdot 4H_2O$)

- Look in fridge to see if any is already made
- Add 9.89 g $(NH_4)_6 \cdot Mo_7O_{24} \cdot 4H_2O$ into a 250 ml volumetric with nanopure.
  - (1235.86 g / mol) * (32 mM / L) * (1 M / 1000 mM) * (250 ml) * (1 L / 1000 ml)
  - (molecular weight) * (desired molarity) * (conversion) * (desired amount) * (conversion)
- Toxic!!!! Wear PPE, don't inhale/eat. Store, but if must dispose, it is HAZARDOUS WASTE.

4.9 N sulfuric acid (H_2SO_4)

- Look under hood (acid storage) to see if any is already made
- Add 133 ml concentrated H_2SO_4into 1 L volumetric with nanopore (mix acid into water!!).
  - (98 g H_2SO_4 / 100 g acid) * (1.84 g acid / mL) * (1 mol H_2SO_4 / 98.072 g) * (1000 ml / L) = 18.39 M (or 36.8 N)
  - (acid purity by weight) * (density of acid) * (molecular weight) * (unit conversion) = molarity (or covert to N)
  - Normality = molar concentration of acid component; N = M * (# of hydrogen ions); 4.9 N = xM * 2 → 2.45 M
- Store, but if needed neutralize with sodium bicarbonate and dispose down drain

100 mM ascorbic acid ($C_6H_8O_6$)

- Make fresh daily!! ascorbic acid oxidizes quickly
- Add 0.886 g ascorbic acid into a 50 mL volumetric with nanopure
  - (176.12 g $C_6H_8O_6$ / mol) * (100 mM / L) * (1 M/ 1000 mM) * (50 mL) * (1 L / 1000 mL)
  - (molecular weight) * (desired molarity) * (conversion) * (desired amount) * (conversion)
- Neutralize with sodium bicarbonate and dispose down drain

4.5 mM antimony potassium tartrate ($K_2Sb_2(C_4H_2O_6)_2 \cdot 3H_2O$) (look in fridge to see if any is already made)

- Add 0.3005 g antimony potassium tartrate to a 100 mL volumetric with nanopure
  - (667.87 g $K_2Sb_2(C_4H_2O_6)_2) \cdot 3H_2O$ / mol) * (4.5 mM / L) * (1 M/ 1000 mM) * (10 mL) * (1 L / 1000 mL)
- Toxic!!!! Wear PPE, don't inhale/eat. Store, but if must dispose, it is HAZARDOUS WASTE.

Reagent solution

- make fresh daily in 100 ml beaker or volumetric (good for ~4 hrs)! and mix well after each addition, and order is important)
  1. 15 ml ammonium molybdate solution
  2. 50 ml sulfuric acid solution
  3. 30 ml ascorbic acid solution
  4. 5 ml antimony potassium tartrate solution
- HAZARDOUS WASTE (look in hood for marked container)

100 ppm stock P solution

- Add 0.2197 g oven dry potassium phosphate monobasic (KH_2PO_4) to a 100 mL volumetric with nanopure
  - (0.1 g P / 1 L) * (136.084 g KH_2PO_4 / 30.974 g P) * (0.5 L) = 0.2197 g KH_2PO_4
  - (desired ppm) * (percent P) * (desired volume) = g KH_2PO_4 to add
  - 100 ppm P = (100 µg P / 1 mL) = (0.1 g P / 1 L)
- Add 0.2197 g oven dry potassium phosphate monobasic (KH_2PO_4) to a 100 mL volumetric with nanopore
- Store, but if must dispose, it is HAZARDOUS WASTE.

Determine how many plates you need, and create a set-up:

1. If running triplicates = each plate will have a standard curve (6 levels * 3 reps = 18 wells), so you can fit in 26 samples (78 / 3).
2. If running quadruplicates = each plate will have a standard curve (6 levels * 4 reps = 24 wells), so you can fit in 18 samples (72 / 4).

Make standard curve:

**High is crazy high for water samples, probably want to run low, but run both curves if unsure.

1. Dilute the 100 ppm stock solution to either:
   •(low) 1 ppm, add 1 ml of 100 ppm stock solution to 100 ml volumetric flask
   •(high) 10 ppm; add 10 ml of 100 ppm stock solution to 100 ml volumetric flask
2. Create the following standard curves in 1.5 ml centrifuge tubes.

	Low	
[Std]	µl 1 ppm	µl matrix
0 ppm	0	1000
0.05 ppm	50	950
0.10 ppm	100	900
0.20 ppm	200	800
0.50 ppm	500	500
1.00 ppm	1000	0

	High	
[Std]	µl 10 ppm	µl matrix
0 ppm	0	1000
0.5 ppm	50	950
1.0 ppm	100	900
2.0 ppm	200	800
5.0 ppm	500	500
10.0 ppm	1000	0

Run analysis:

NOTE: run samples in triplicate (or quadruplicate!!) and add the standard curve to one plate. Add the samples to the wells first, and then use the multichannel pipette to add the reagent. Make sure to label plates and create a diagram for your sample lay-out.

Add the following to each well:

1. 200 µl sample
2. 50 µl reagent solution (use multichannel pipet)

Tap corner of plate to mix well, cover with foil and incubate for 30 min. Read plate at 880 nm. **Note, if your P concentration is low, the standard curve still has excellent fit for 2 hours (maybe even more??!). The standard curves are indistinguishable for 30 min, 45 min, 60 min, and 120 min. If your P concentration is high, the 30 min is important or the colors start to saturate.

Reporting results:

"Concentration of orthophosphate was determined colorimetrically using an Epoch microplate reader (BioTek, VT, USA) using a microplate adaptation (Ringuet et al. 2011) based on the molybdenum blue method of Murphy and Riley (1962). Samples were run in triplicate (or quadruplicate), and outliers were determined by xyz."

Waste disposal:

Most reagents will get stored for future use. Look in hood for labeled hazardous waste containers (might need to create a new one). Package plates in plastic bag (in hood). If things are getting full (they cannot be >75% full!!!), please fill out this form from EHS and email it to ehs@uoguelph.ca to request pickup.