

Soil Sample Preparation Protocol

(Modified from Faegri and Iversen¹; Moore and Webb² protocols)

Materials

- Sediment samples
- Volumetric sampler
- Metal spatula
- Permanent marker
- 15 mL Conical centrifuge tubes
- 1.7 mL Microcentrifuge tubes
- Test tube rack
- 10% hydrochloric acid solution
- 10% potassium hydroxide solution
- 10% sodium pyrophosphate solution
- Glacial acetic acid
- Concentrated sulfuric acid
- Acetic anhydride
- 25 mL Graduated cylinder
- Sodium metatungstate solution
- Heat block with aluminum element for both 1.7 mL and 15 mL tubes
- Microcentrifuge with inserts for 1.7 mL and 15 mL tubes
- Glass stir rods
- Wooden applicator sticks
- Waste beaker
- 200 µm nylon mesh secured with clamp
- 40 µm nylon mesh
- 2 µm glass filter disc
- Vacuum filtration setup
- Small funnel
- Polystyrene microsphere solution
- 20 µm to 200 µm micropipette
- Glycerol
- Safranin
- Pasteur pipette with bulb
- 10 mL Pipetter
- 10 mL Pipette
- Drying oven
- 1,000 mL Beaker
- 500 mL Amber bottle
- Vortex
- DI Water in wash bottle
- 95% ethanol (if needed)
- Stir plate
- Magnetic stir bar

Volumetric Sampling:

1. Using a small metal spatula and precision volumetric sampler (0.25 cm³), remove sediment from the sample bag and press it into the mold of the volumetric sampler until it is level with the rim (see Figure 1A:1B).
2. Remove the 0.25 cm³ pellet from the sampler and gently place it into a 15 mL polypropylene centrifuge tube.
3. Repeat steps 1 and 2 for each sample bag and location.

Note: Samples with large amounts of organic material (greater than 200 µm) will benefit from filtering the samples through a 200 µm screen into the centrifuge tube using deionized water (Figure 1C:1D).

1. Faegri K, Iversen J. 1989. Textbook of pollen analysis 4th Ed. Copenhagen: Munksgaard. 237 p.
2. Moore PD, Webb JA, Collinson ME. 1991. Pollen Analysis 2nd Edition. Massachusetts: Blackwell Scientific Publications, p 10-28.

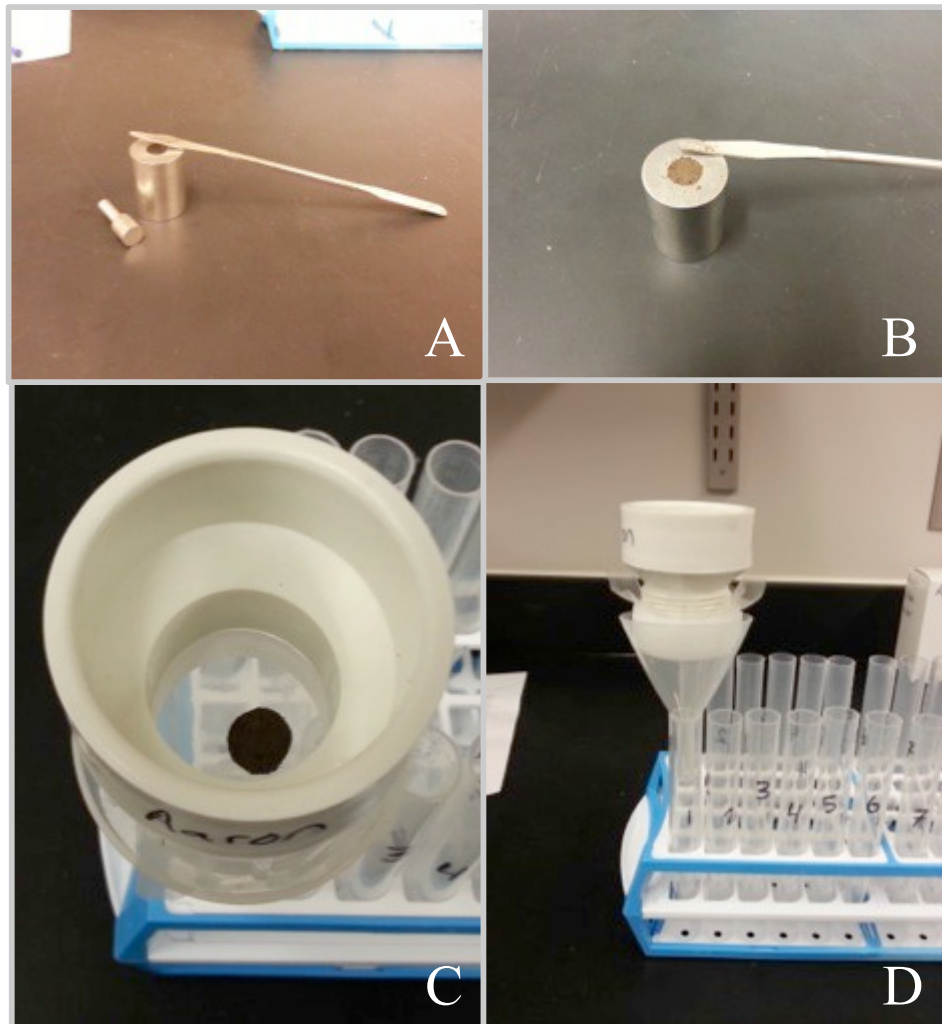


Figure 1: Equipment for soil sample processing. A: Metal spatula, precision volumetric sampler and plug for 0.25 cm³ sampling, B: Soil sample in sampler, C: Pellet on mesh screen above centrifuge tube, D: Sample sieving setup composed of 200 µm mesh in PVC collar and funneled to centrifuge tube

Microsphere Spiking:

To quantify the concentration of pollen types present in a sample, 16 µm polystyrene microspheres are added as a marker to the sediment sample at the start of the pollen processing. This allows the pollen concentrations for the soils from different eco-regions to be determined for the geo-location model.

1. Add the determined volume of solution of the microsphere sample to a flask and label it with the concentration.
2. Place a magnetic stir bar into the flask and allow the solution to mix on a stir plate for at least 2 hours (see Figure 2).
3. Use a micropipette to remove 16 µm of microsphere solution from the flask while it continues to stir. Add it to the centrifuge tube.
4. Repeat step 3 for each sample.

Note: Traditionally, 20,000 marker spores/cm has been utilized as a marker; therefore, a spike of c. 5000 microspheres/0.25 cm³ sample should be used. Divide 5,000 by the volume of solution to be added to determine the concentration of the solution (spheres/mL). This ensures that a homogenous solution is maintained and a consistent microsphere spike is added to the sediment sample every time sediment is prepared. If filtering the soil sample, add the microspheres to the surface of the soil pellet and then filter the sample.



Figure 2: Microspheres stirring with magnetic rod on stir plate

Carbonate Removal:

If the soil is from an area that has carbonate-rich substrate (e.g. limestone, gypsum), an acid wash may be necessary to remove it from the sample. If the sample appears very organic, this step may be skipped.

1. Fill each centrifuge tube to the 5 mL line of the tube with a 10% hydrochloric acid (HCl) solution (Figure 3).
2. Vortex the sample until the soil and acid mix evenly.
3. Place an additional 10% HCl solution into the tube until it reaches the 10 mL line.
4. Place the tubes in a heating block element set on the low temperature setting.
5. Let the samples heat for 10 minutes. If aggressive bubbling or foaming occurs, a second acid wash may need to be conducted to remove the high level of carbonates in the sample.

- Centrifuge the tubes at 2,500 rpm for 5 minutes to pellet the sample after the 10 minute heating period.
- Slowly decant the HCl and dissolved carbonates from the sample pellet.
- Add deionized water to the tubes and vortex for several seconds.
- Centrifuge the samples for 5 minutes at 2,500 rpm to wash the carbonates and HCl from the sample.
- Decant to remove all acid from the sample.

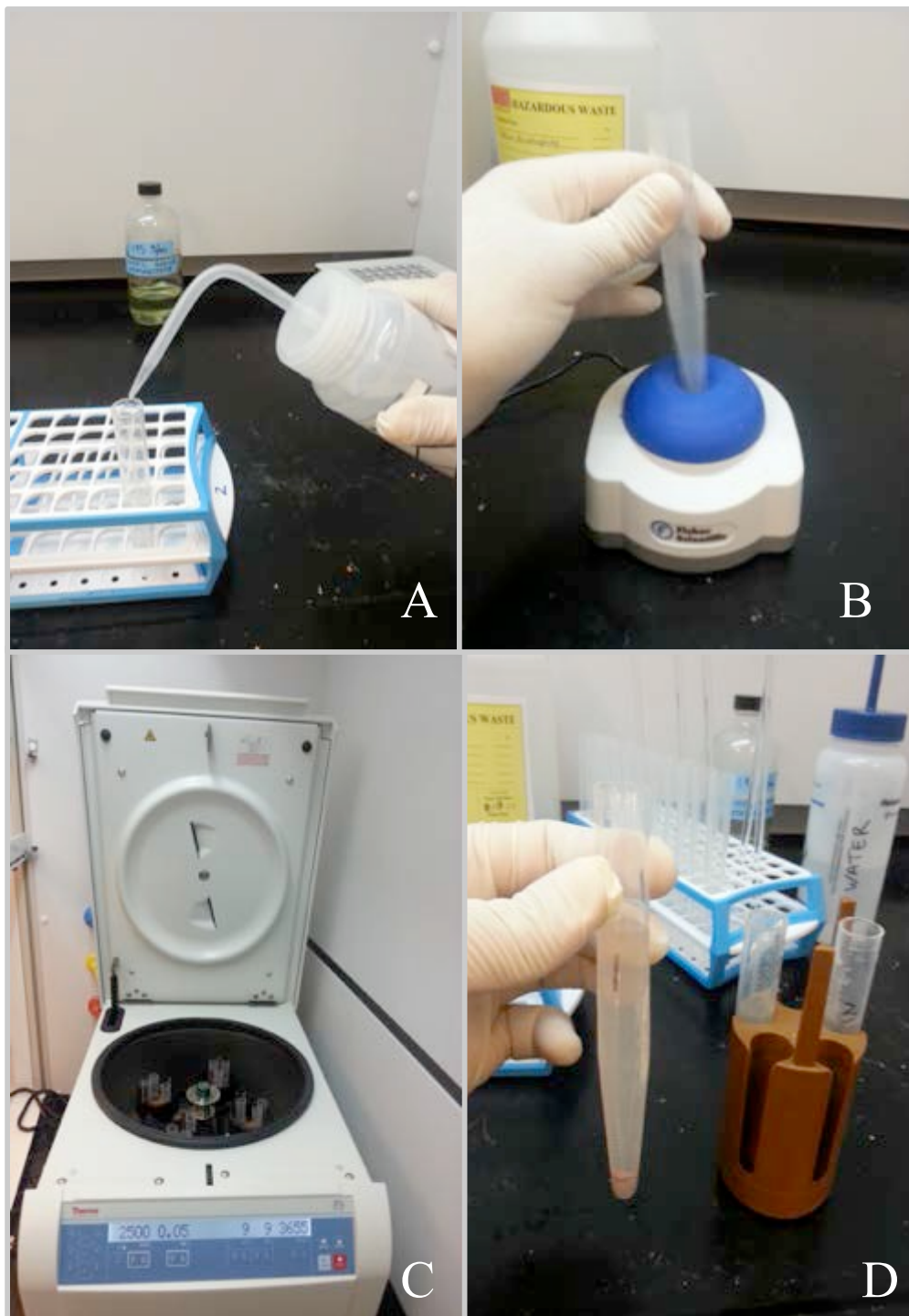


Figure 3: Chemical addition or water washing steps. A: Addition of chemical or water, B: Vortexing sample, C: Centrifuging at 2500 rpms for 5 minutes, D: Pelleted sample ready for decanting of solution from sample

Tannin and Humic Acid Removal:

Tannins and humic acids can cause a sediment sample to stick together and darken pollen residue, making it harder to analyze.

1. Fill the centrifuge tubes to the 5 mL line with a 10% potassium hydroxide (KOH) solution and vortex to mix the sample.
2. Continue filling the tubes with 10% KOH to the 10 mL line.
3. Place the tubes in the heat block on the low setting and leave it for 10 minutes. If tannins and humic acids are present in the sample, the solution will become dark brown or black as the compounds are extracted from the sample.
4. Centrifuge the samples at 2,500 rpm for 5 minutes. The sample should appear lighter gray in color if the tannins and humic acids were removed. Samples containing large amounts of organic debris will remain black due to the material, not necessarily the tannins/humic acid.
5. Add DI water to the tubes. Vortex for several seconds and centrifuge for 5 minutes at 2,500 rpm to wash the tannins, humic acid, and KOH from the sample.
6. Decant to remove the base from the sample.

Clay Removal:

Clays are a major component of soils and should be removed to concentrate the sample and prevent clumping.

1. Fill the centrifuge tubes to the 5 mL line with a 10% sodium pyrophosphate solution and vortex until the mixture is homogenous.
2. Continue to fill the tubes to the 10 mL line with a 10% sodium pyrophosphate solution. Then vortex and mix again.
3. Place the tubes in the heat block on the low setting for at least 45 minutes.
4. Centrifuge the tubes for 5 minutes at 2,500 rpm.
5. Slowly decant the solution.
6. Add DI water to the tubes, vortex for several seconds, and centrifuge the tubes for 5 minutes at 2,500 rpm.
7. Slowly decant the solution.

Density Separation Using Sodium Metatungstate:

Concentrate the pollen from denser soil material using a 1.95 g/mL sodium metatungstate solution. If a 1.95 g/mL solution of sodium metatungstate is not available, one should be made from a powdered or concentrated liquid form of the compound. The solution's density is set at 1.95 g/mL in order to allow for the water trapped in the sediment to decrease the sodium metatungstate solution's density, without the solution becoming less dense than the pollen.

1. Add 2 mL of sodium metatungstate solution to each tube and vortex until the sample is completely homogeneous. This may take 3-5 minutes (Figure 4). Large conglomerates of sample will prevent an efficient density separation and allow other soil material into the pollen residue.
2. Allow the solution to settle. The pollen will settle in a layer on top of the soil material.

3. Add 1 mL of sodium metatungstate along the sides of each tube to wash sample off the inner wall of the tube.
4. Centrifuge at no more than 1,800 rpm for 10 minutes. The rpm rate should be increased very slowly over 3-4 minutes to provide the best separation of pollen from other soil components.

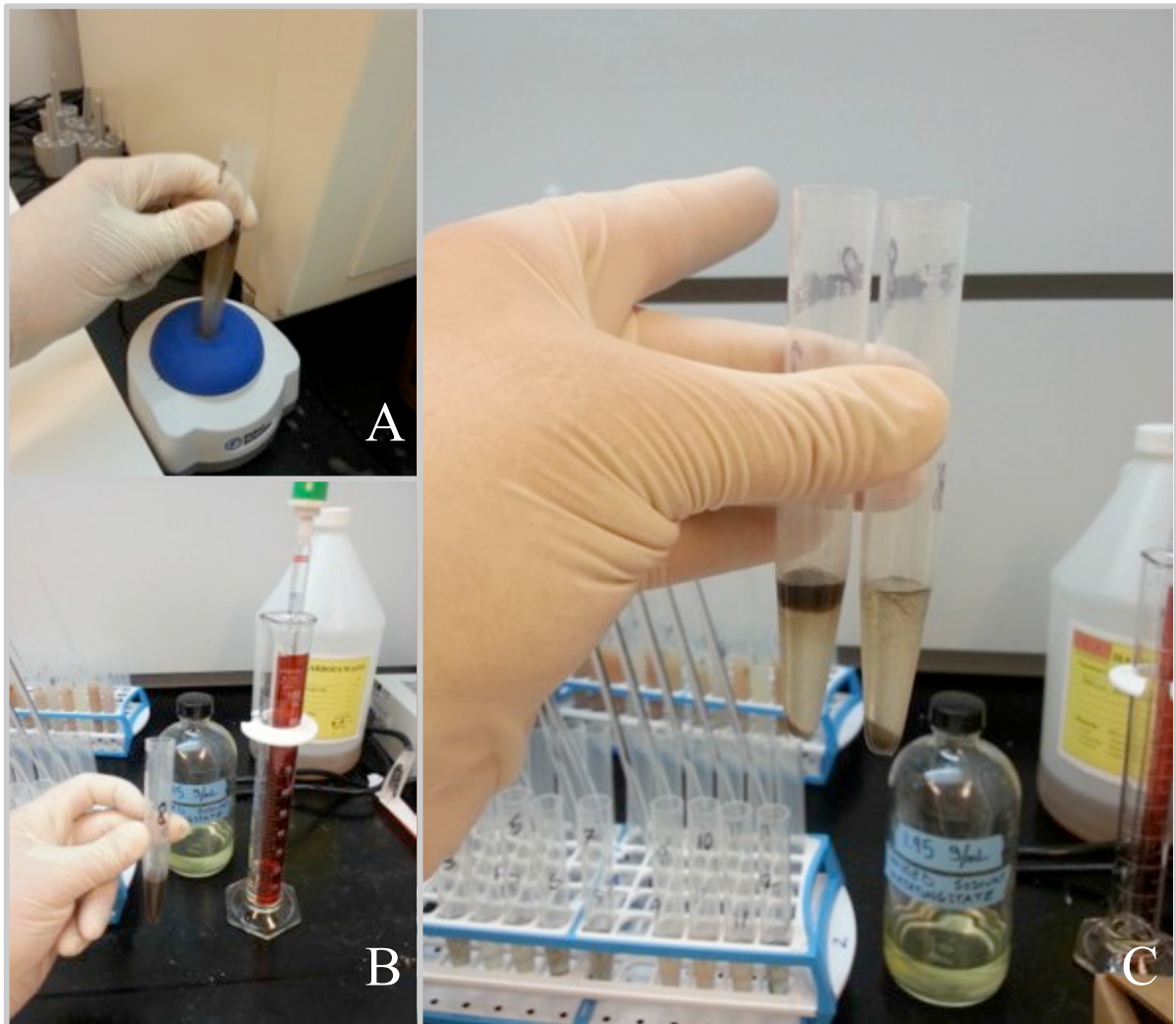


Figure 4: Steps of density separation with sodium metatungstate - A: Vortexing sodium metatungstate, B: Sample ready for centrifuge, C: Samples after centrifuging with the non-organics pelleted at base of the centrifuge tubes (note differences in amount of organic material to be siphoned from the surface of tube)

5. Siphon the top layer of the solution into a clean centrifuge tube with a Pasteur pipette.
6. Pour the bottom layer (sodium metatungstate and non-pollen soil material) into a beaker for sodium metatungstate reclamation.
7. Add DI water to the new tubes to the 5 mL line and vortex.
8. Add more water to the 10 mL line of the tubes. If needed, add a small squirt of ethanol to break any surface tension.
9. Centrifuge at 2,500 rpm for 5 minutes.
10. Decant the sodium metatungstate solution into the reclamation beaker, leaving the pollen pellet in the tube.
11. Repeat steps 7 through 10 until two washes are completed.

Acetolysis:

Acetolysis solution, composed of a 9:1 ratio of acetic anhydride and concentrated sulfuric acid, will digest all organic material, including pollen grains, so this process should be performed with caution. Soil samples should never be acetolyzed for longer than 5 minutes. The Acetolysis solution will turn the sample a dark yellow or light brown color once the reaction is complete. If there is little or no organic material, the sample may never darken to the yellowish brown color. Do not exceed the 5 minute time frame for the Acetolysis process.

Caution: The Acetolysis process must be performed in a water-free environment. Be sure to remove any water containers from the fume hood prior to starting this step.

1. Add a very small amount of glacial acetic acid to the sample. Vortex thoroughly and centrifuge at 2,500 rpm. Decant excess glacial acetic acid, being careful to not disturb the sample.
2. Repeat step 1 three times to ensure all water is removed from the sample.
3. Transfer the sample from the centrifuge tube to a 1.7 mL microcentrifuge tube using a Pasteur pipette. Additional glacial acetic acid can be added to the original tube to ensure that the entire sample is transferred.
4. Add 1 mL of Acetolysis solution to each tube.
5. Stir the sample pellet into the Acetolysis solution with a wooden applicator stick.
6. Place the samples in the heat block on the low setting for 5 minutes or less.
7. Remove the tubes from the heat block.
8. Working quickly, add a small amount of glacial acetic acid to each tube to stop the reaction.
9. Centrifuge the tubes at 2,500 rpm for 5 minutes; then decant.
10. Add DI water to the tubes and mix with a wooden stick.
11. Centrifuge the tubes at 2,500 rpm for 5 minutes.
12. Decant to remove all solution from the sample.
13. Repeat this water wash process. If acid can still be detected, complete a third water wash.

Adding Fluid Media and Drying the Pollen Residue

In order to roll pollen grains on the slide during identification, a fluid media (e.g. glycerol) must be used for mounting.

1. Add the same volume of glycerol as volume of the pollen residue to the sample (e.g. 0.25 mL glycerol added to 0.25 mL of residue). Too small of a glycerol addition makes the sample too concentrated and too dark to easily count. Adding too much glycerol will dilute the sample, increasing the counting time for the sample. If a pollen residue is very small, glycerol should be added to the 0.05 mL line of the centrifuge tube so an adequate volume of sample/glycerol mix will be present to make one slide (see Figure 5).

2. Add safranin to the glycerol to stain the pollen surface, allowing for easier identification. A tiny amount (0.05 or 0.06 g) of safranin can be added to a large volume (50-75 mL) of glycerol. The final color of the glycerol should be a shade or two lighter than strawberry jelly. Too dark of a stain will obscure pollen grain surface structure, and too light of a stain will not stain the surface.

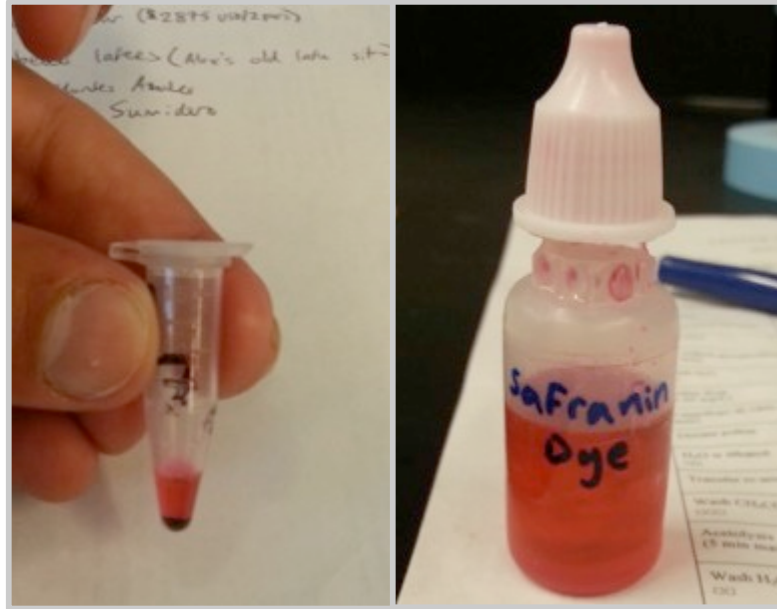


Figure 5: Left: Sample filled with glycerol to 0.05 mL level, Right: Glycerol properly tinted with safranin

3. Dry the samples (with caps open) overnight inside an oven (105° C).
4. The following day, smear the sample on slides for counting or store in a refrigerator/cold room for long-term storage.

Sodium Metatungstate Reclamation

Sodium metatungstate is very expensive, so care should be taken to try and recycle the solution after each pollen float procedure. A vacuum filtration set up makes recycling much faster and more effective (Figure 6).

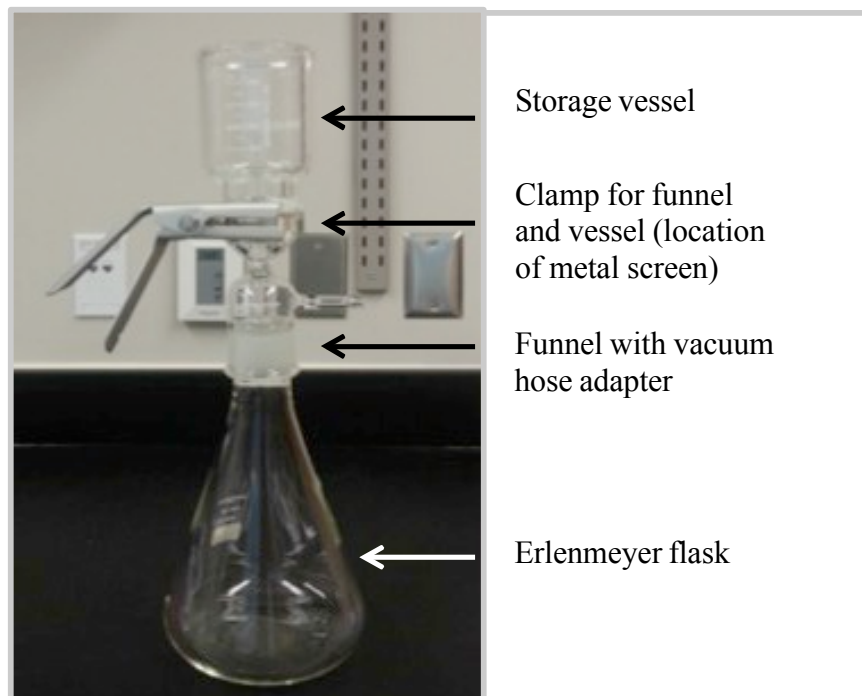


Figure 6: Vacuum filtration set up for sodium metatungstate reclamation

1. Place a 40 μm filter disc on the metal screen that rests on the funnel leading to the Erlenmeyer flask.
2. Clamp the storage vessel onto the funnel and pour the sodium metatungstate reclamation beaker into the vessel.
3. Rinse the beaker into the vessel with DI water.
4. Drain the vessel by gravity and then lightly rinse the sides of the vessel with DI water to remove any sodium metatungstate residue (see Figure 7).

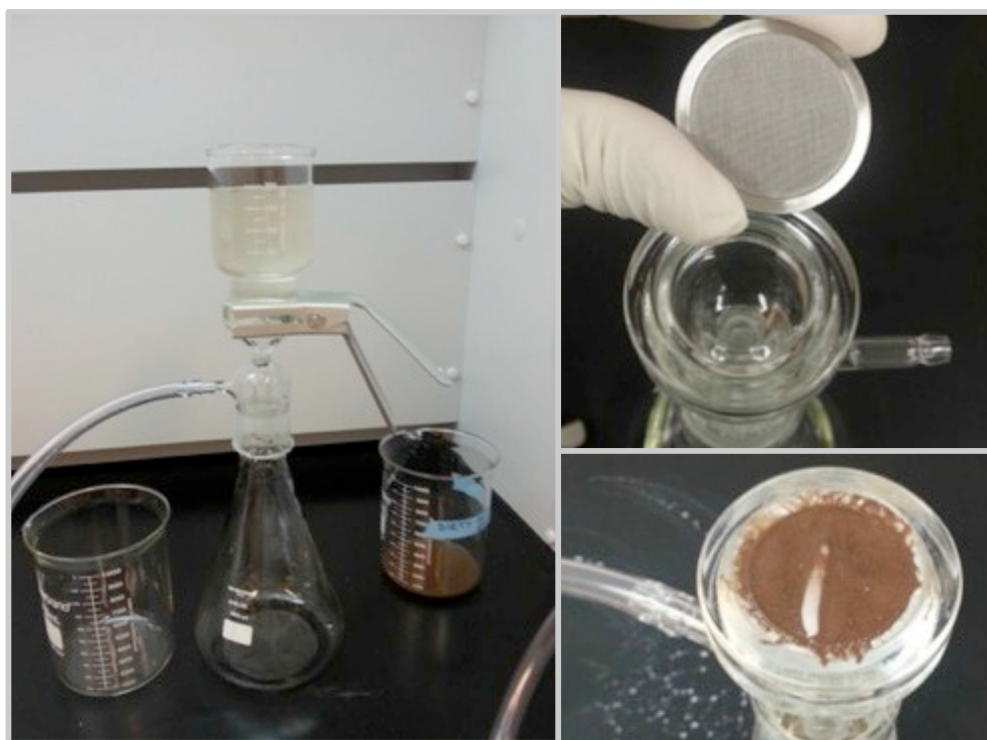


Figure 7: Left: Filtration set up, Right top: Metal screen atop funnel and below storage vessel, Right bottom: Large debris caught by 40 μm filter on metal screen

5. Replace the 40 μm filter with a 2 μm filter disc.
6. Pour the filtered sodium metatungstate back into the waste beaker and rinse the Erlenmeyer flask into the beaker.
7. Re-clamp the vacuum filter set up with the 2 μm filter disc on the metal screen.
8. Pour the beaker into the storage vessel and rinse the beaker into the vessel (see Figure 8). The 2 μm filter will require light negative pressure to filter the dirty sodium metatungstate as it moves from the vessel to the Erlenmeyer flask.
9. Connect the hose from the Erlenmeyer flask to the faucet-style mount on the vacuum unit.
10. Slowly open the vacuum unit so that a slow, steady trickle of sodium metatungstate solution moves from the vessel to the flask. Too strong of a vacuum will pull material that should be filtered through the screen.
11. Rinse the beaker into the vessel and rinse the sides of the vessel to remove sodium metatungstate residue.

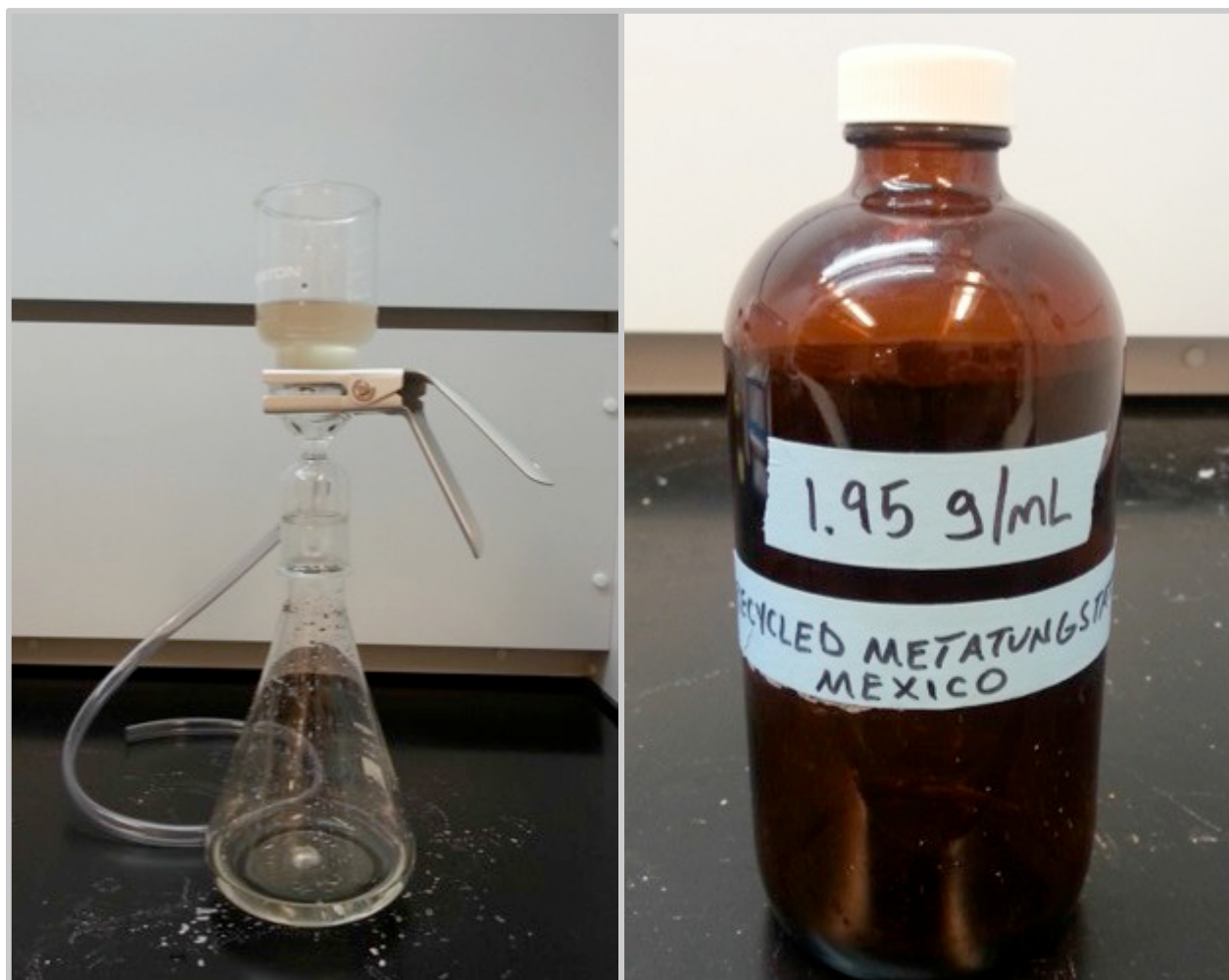


Figure 8: Left: Vacuum filtration of fine debris from sodium metatungstate using 2 μm filter and vacuum pump, Right: Properly labeled recycled sodium metatungstate bottle

12. Repeat steps 5 through 11 two to three more times to ensure all contaminating material (pollen and soil particles) is removed.
13. When the 2 μm filter appears clean, filtering is complete.
14. Transfer this solution from the Erlenmeyer flask to the beaker now labeled "clean sodium metatungstate".
15. Rinse the flask into the beaker and concentrate the filtered sodium metatungstate in a drying oven (105° C) over several days.
16. When the metatungstate is denser than 2.5 g/mL, correct the density to 2.00 g/mL and pour from the beaker into a bottle that is labeled "recycled sodium metatungstate" and include the density, as well as sample country of origin (Figure 8).
17. Rinse the beaker with a small amount of water (5 mL).
18. Mix and retest the recycled solution bottle's density. Note any changes on the bottle. As long as the density is between 1.85 and 2.0 g/mL density correction with water or a denser sodium metatungstate solution is not necessary. To prevent cross contamination, each country sampled should have it's own recycled sodium metatungstate solution to use for later soil sample preparations from that country.