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Microplastic extraction from water, sediment, soil, and atmospheric deposition samples

Introduction

This document is the Ecohydrology Research Group (ERG) standard operating procedure (SOP) for separating microplastics (MPs) from sediment, soil, atmospheric deposition, and water samples. It applies to aqueous samples, such as atmospheric bulk deposition, surface water grab samples, and pore water, wet sediment or soil, and dry sediment or soil. The procedure includes the following major steps (**Figure 1a & 1b**): spiking and preliminary separation (Steps 1 and 2), density separation (Step 3), organic matter removal (Step 4), manual counting (Step 5), and particle identification (Step 6). While not strictly required, we recommend spiking samples with known amounts of MP standards to assess recovery efficiencies. The sample, spiked or not, is sieved through a 500 µm sieve and collected on a 20 µm sieve. The next step consists of a density separation using a 60% $ZnCl_2$ solution, a broadly accepted technique to separate MPs from sediment by flotation (Prata et al., 2019). The Fenton oxidation reaction is then used to remove organic matter from the MP surfaces, before characterising and counting the MP particles with Laser Direct Infrared (LDIR) spectroscopy. The steps described in this SOP were optimized by extensive laboratory testing at ERG, University of Waterloo, as part of the "Microplastics fingerprinting at the watershed scale: from sources to receivers" project supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) under the program "Plastics science for a cleaner future" (Alliance grant ALLRP 558435 – 20).

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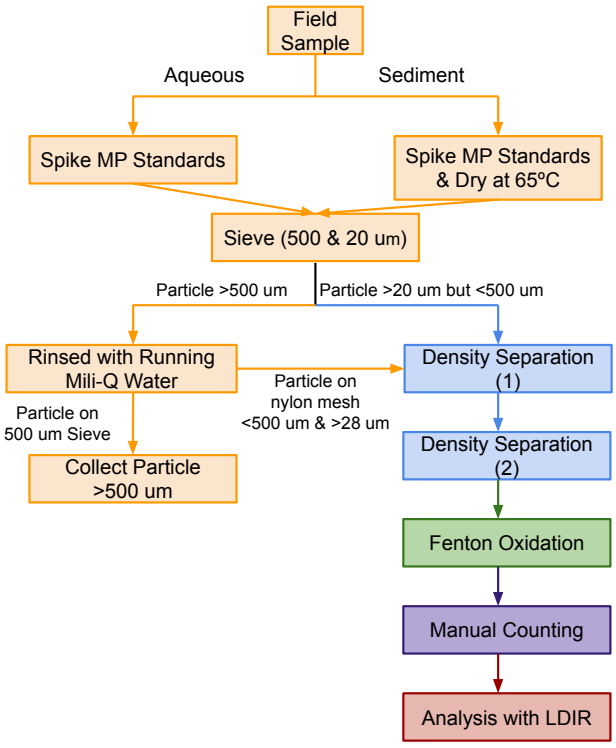
A



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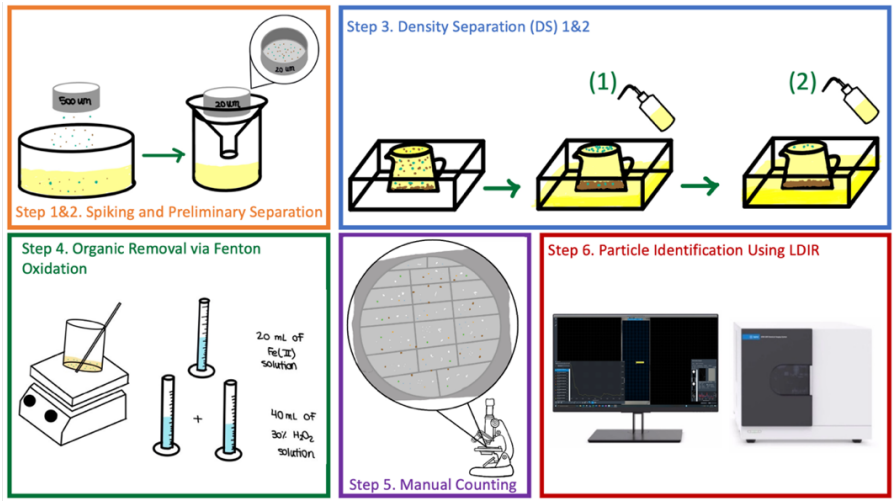

Figure 1. Extraction procedure for microplastic (MP) particles from water and sediment matrices: flow chart (panel A) and schematic overview with green dots showing lighter particles including MPs and brown dots representing heavier particles, such as suspended or deposited sediment or soil particles (panel B).

General Lab Practices

- All activities should be performed inside the designated clean fumehood. Because the health risks associated with MPs (including nanoplastics) are largely unknown, a risk assessment must be completed **before** extracting MPs.
- In general, the MPs are separated (i.e., suspended into solution) inside the designated fumehood. Avoid using plastic materials (vials, stirrers, etc.) whenever possible; if this is not possible, the plastic materials used must be documented.
- All chemical solutions – H_2O_2 (Fisher Chemical H325-4, 30%, certified ACS grade), $FeSO_4$ (pH adjusted with concentrated H_2SO_4 and NaOH), $ZnCl_2$ (Sigma Aldrich 793523, anhydrous, reagent grade, ≥ 98%), alcohol, and Tween solution (Sigma Aldrich 655204, Tween® 20 detergent, molecular biology grade) – should be filtered through 0.7 µm pore size GF filters (Whatman® glass microfiber filter, grade GF/F) to remove possible particulate contaminants prior to every use. All filtrations are carried out with a vacuum filtration system.
- Prevent air contamination by covering any openings (like beaker, funnel, dishes, reagent bottles and so on) as quick as you can with aluminum foil, carton, or secondary glassware.
- Cotton laboratory coats are worn to reduce contamination from synthetic textiles. However, latex gloves, plastic goggles, and surgical masks are worn for safety at work.
- A proper waste disposal protocol for all chemicals used must be established before proceeding with the separation.

Main Steps

Steps 1 and 2: Spiking and Preliminary Separation (500 µm)

- Whenever handling $ZnCl_2$, you MUST use double gloves to protect yourself!
- To prepare 60% $ZnCl_2$ solution and check/correct the solution density, refer to **Appendix III: Preparation and Density Correction of 60% $ZnCl_2$**.

Please note that Steps 1 and 2 may vary depending on the type of sample, including aqueous samples, and wet or dry sediment samples.

Spiking MP standards

For MP standards and spiking procedure, refer to **Appendix I: MP Standards Preparation and Generation** and **Appendix II: Spiking and QA/QC Procedures**

Aqueous Samples

1. Place a funnel into a 600 mL beaker. Stack a 500 µm stainless steel sieve on top of a 20 µm sieve, and then place them into the funnel.
2. Spike the MP standards into the original sample.
3. Slowly pour the aqueous sample into the sieve, making sure the 20 µm sieve is not overflowing.
4. Continue to pour in intervals and recording the total volume of the sample.
5. Rinse the particles (with both sieves still stacked) using a Mili-Q (MQ) water squeeze bottle.
6. Rinse the particles on top of the 500 µm sieve, **following step 8 in the section "Clean & store particles ≥ 500 µm" below**.
7. Transfer the particles on the 20 µm sieve into a glass pitcher using the $ZnCl_2$ squeeze bottle.

Wet Sediments

1. Place a 100 mL beaker on the analytical balance and add ~2 g of wet sample into a 100 mL beaker.
2. Cover the beaker with aluminum foil to avoid contamination. Once done, bring the sample to the oven and dry it at 65ºC.
3. Dry until a constant dry weight of the sediment is achieved and log down the weight.
4. Continue the separation process as a dry sediment sample.

Dry Sediments

1. Using $ZnCl_2$, fill the beaker containing dry sediment halfway to loosen up any big clumps.
2. Set up a small crystallizing dish (diameter 100 mm) with the 500 µm sieve inside. Transfer the wet sample to sieve and shake to allow smaller particles to fall through.
3. Using $ZnCl_2$, fill the sieve halfway and then gently shake the sieve to allow the small particles (< 500 µm) to go through and collect in the small crystallizing dish.

4. Stop shaking when there is no $ZnCl_2$ solution remaining in the sieve; then rinse down the inner/bottom edges of the sieve and place on a clean glass petri dish.
5. Repeat steps Error! Reference source not found. & **4** until the $ZnCl_2$ is clear when exiting the 500 µm sieve.
6. Only for training: Using a UV flashlight, shine over all equipment (i.e., crystallizing dish, beaker containing sample, and 500 µm sieve) to check no MP particles are left behind. If not, continue to rinse until clear.

*Transferring **targeted particles (i.e., <500 µm)** from the small crystallizing dish to the density separation container:*

7. Place a 20 µm stainless steel sieve into a glass funnel, ensuring that the funnel's stem is in an "unfiltered $ZnCl_2$" bottle.
8. Pour the mixture of $ZnCl_2$ and sample particles (<500 µm) from the small crystalizing dish into the 20 µm sieve. Using $ZnCl_2$ solution, rinse down the particles collected on the sieve into a 350 mL glass pitcher for subsequent density separation (DS).
    a. The sample particle sizes are now in the 20-500 µm range. The 350mL glass pitcher will be referred as the DS container from here onward.
    b. It is important that the DS container is only ¾th filled with $ZnCl_2$.
    c. $ZnCl_2$ collected in the "unfiltered $ZnCl_2$" bottle is to be recycled.

*Clean & store **particles ≥ 500 µm***:

9. Rinse off the 500 µm sieve using MQ water squeeze bottle to collect particles ≥ 500 µm,
10. Attach a pre-cut nylon mesh (pore size 28 µm; polypropylene; Mc-Master Carr 9318T51) onto the bottom and outside of the 500 µm sieve using a rubber band (**Figure 2**).
    a. Perform the running MQ water rinse following the **Appendix IV: Particles Cleaning by Running MQ water.** Note that the nylon mesh is attached to the bottom of the sieve to prevent loss of particles < 500 µm that remained stuck on the sieve.
    b. The particles collected on the mesh now have sizes < 500 µm and > 28 µm.

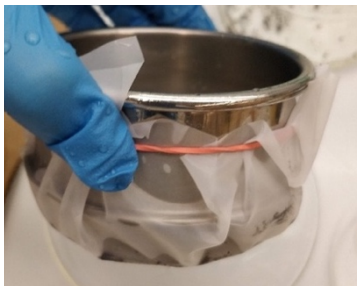

Figure 2. Attaching a 28 µm pore size nylon mesh to the bottom of the 500 µm sieve to catch particles smaller than 500µm during the running MQ rinse step.

11. Once the rinse is done, place the sieve with the attached mesh on a clean petri dish. Separate the sieve from the mesh, flip the sieve upside down into a 600 mL beaker and flush the sample using pre-filtered ethanol.
    a. Transfer the sample from the 600 mL beaker into a proper container for storage.
12. Allow the mesh to dry on the petri dish until damp. Line the edge of the nylon mesh around the DS container and rinse the collected particles into the DS container using $ZnCl_2$ solution.
13. Clean the stainless-steel sieves (i.e., the 500 µm and 20 µm sieves) with MQ water very carefully to avoid contamination and damage (that would cause enlarged pore sizes) in future use of the sieves.

Step 3: Density Separation (DS)

Starting now, the remaining steps are identical for all three sample types (aqueous samples, wet sediments, and dry sediments).

Density separation 1 (Overflow Technique)

1. Place a 300 mL glass tea pitcher into the center of a large crystallizing dish or rectangular glassware (i.e., a food storage container).
2. Fill the pitcher ¾th with $ZnCl_2$ solution.

Re-suspend the particles with air bubbling:

3. Attach a tube with a filter and a disposable glass pipette to the "air" valve.
    a. Test the air flow using a beaker filled with MQ water. If too strong, air flow will cause big bubbles and splashing can happen.
    b. The right flow of air should only cause an indentation of the solution surface when the pipette is hovering above the solution.
4. Once you have found the right air flow, stick the pipette into the solution and pass air through for 10 minutes while carefully moving the pipette.
5. With the air flow ON, take out the glass pipette, rinse it down into the DS container to collect any particles that might got stuck on the pipette.

Particle settling:

6. Fill the DS container (i.e., the glass pitcher) with more $ZnCl_2$ almost to the rim.
7. Let the suspended sediment settle overnight or longer if needed.

Collect the particles by overflow:

8. Once settled, overflow the DS container with $ZnCl_2$ solution.
    a. Be careful not to disturb the sediment deposited at the bottom!
        i. Aim squeeze bottle tip to the back of the glass pitcher's rim and let the solution start overflowing.
        ii. Once most floating particles have flown into the receiving crystallizing dish (or rectangular glass container), rinse around the glass pitcher's rim.
    b. Try to use the minimum but necessary amount of $ZnCl_2$ to ensure all the floating particles have overflown into the receiving dish. DO NOT overfill the receiving dish as it is used in BOTH density separation steps.
9. Using personal judgment, once you think the majority of floating particles have flown out, pour out extra ~ ¼ of the top layer solution into the crystallizing dish/rectangular container.
    a. Tip over the DS container carefully and rinse the outer area of the DS container to prevent particles sticking to its outer wall when drying.

Density separation 2 (overflow technique)

1. Repeat steps 3 to 7 of density separation 1 above.
2. After settling, repeat the overflow procedure with $ZnCl_2$ again.
3. Remove the DS container, but making sure the outer surface and bottom of the container are rinsed off well to prevent MP loss.
4. Place a 20 µm sieve in a funnel above the un-filtered $ZnCl_2$ container; transfer the solution in the crystallizing dish or rectangular glassware into the sieve.
5. Use a petri dish to cover the 20 µm sieve, rinse the particles collected on the sieve by performing a running MQ water rinse following the **Appendix IV: Particles Cleaning by Running MQ Water**
    a. You do not need a nylon mesh for this step.
6. Once rinsing is finished, transfer particles from the 20 µm sieve into a 600 mL beaker using pre-filtered ethanol and let it evaporate in the incubator at 50 °C until dry.
    a. Cover the beaker with a glass evaporating dish to prevent particle loss and contamination.
7. Carefully clean the 20 µm sieves with MQ water to avoid contamination or damage that would cause enlarged pore sizes.

Step 4: Organic removal via Fenton oxidation

Prepare Fe(II) 0.05 M solution following Rivoira et al. (2020)

- For every 500 mL of water, use 7.5 g of $FeSO_4 \bullet 7H_2O$ and 3 mL of concentrated H_2SO_4.

Organic removal

Timing of 30 minutes start when the H_2O_2 is added to Fe(II) solution.

1. Add 20 mL each of Fe(II) (0.05 M) solution to the dried particles collected in Step 3.
2. Add stir bar and glass thermometer; place beaker on the stir plate at 190 rpm and temperature set to 75 °C
    a. Measure out 20 mL of H_2O_2 solution and add it into the Fe(II) solution: **start the 30 minute timer now!**
3. Cover by placing an evaporating dish on top of the beaker (**Figure 3**)
    a. TEMPERATURE CONTROL IS VERY IMPORTANT - TEMPERATURE OVER 75°C WILL DAMAGE THE MP PARTICLES. ALSO AT THIS STEP, AGGRESSIVE BUBBLING CAN OCCUR.
4. Add a little MQ water when the temperature reaches 72 °C to slightly reduce the temperature. Continue to add until the temperature stabilizes (this should not take more than 30 mL).
5. Measure out 20 mL of H_2O_2 again when the timer indicates 5 minutes are left.
6. Once 30 minutes are up, reset the timer and add H_2O_2. Continue stirring at the same speed (190 rpm).
    a. Use MQ water if the temperature reaches 72 °C again (this will not take more than 30 mL)
7. Once the 30 minutes time is up, remove from heat and let it cool to 30–25 °C.

8. While the beaker with the sample particles is cooling, prepare a nylon mesh, porcelain funnel, and another 600 mL beaker (**Figure 4**)
    a. Rinse nylon mesh by soaking it in 4% Extran for a few minutes and rinsing with MQ water.
    b. View the mesh under microscope and note down any potential MP contaminants (i.e., fragments, fibers, beads)
    c. Make sure the rinsed side is facing up (i.e., the side that will be collecting the sample particles)
    d. Holding the nylon mesh over the porcelain funnel, push it down along the interior sides so that the nylon mesh is stuck against the interior side of the funnel.
9. Once cooled, pour the sample solution on the nylon mesh – pour the solution incrementally so it doesn't overflow.
10. When all the sample solution has been poured through, rinse the beaker with MQ water for ~2 minutes.
11. Once rinsed, make the mesh as "flat" as possible, which will help with counting.

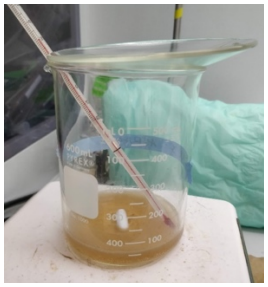

Figure 3. Set up for Fenton oxidation: a 600 mL beaker containing 20 mL Fe(II) solution and 20 mL H_2O_2, a magnetic stir bar, thermometer, and an evaporating dish as a cover. The hotplate is set to 75 °C and the stir plate to 190 rpm.

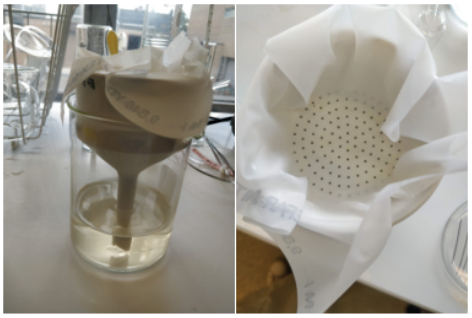

Figure 4. Set up for filtering and rinsing of chemical reagents after the Fenton oxidation (left) and how the nylon mesh should look from above (i.e., flat along the sides and bottom of the funnel) (right).

Step 5: Manual Counting

- This step is carried out under the Leica EZ4 microscope. Please refer to the microscope's manual for operation and calibration. MAKE SURE THE MICROSCOPE IS CALIBRATED BEFORE COUNTING.
- It is important to keep the nylon mesh wet. If it appears to be drying, add some MQ water using a squeeze bottle to keep the sample and nylon mesh moist.

1. Lift the nylon mesh from the funnel and into a large petri dish. Cover the sample with another petri dish.
2. Count:
    a. Spiked MPs standards are identified by colour and shape.

i. Record particle numbers from (at least) 3 separate countings.
ii. Note that the length of fibers are measured with the microscope measurement tool (**Figure 5**).

b. Fragments
   i. Any shiny/transparent/irregular-shaped particles are counted as fragments. Black particles are NOT counted as fragment (these may be mineral or black carbon particles instead).
   ii. Record using a ">" as it is impossible to count all MPs (especially the small ones).
   iii. Record the average number of the 3 counts or, if numbers differ by more than 50%, record the smallest value.

c. Fibers & beads
   i. Record the numbers of 3 separate countings.

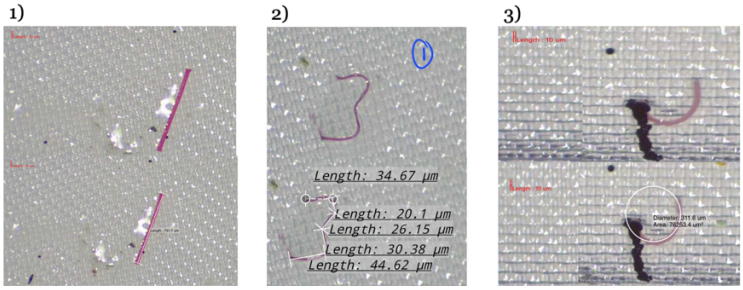

Figure 5. Length measurements of fibers in three configurations: 1) linear, 2) irregular shaped, and 3) semicircular. For 2 and 3, line segments are added together to represent the total length of the fiber.

3. After counting, carefully lift the nylon mesh and rinse the particle down into a proper container for storage.

Step 6: Particle Identification

1. The collected particles are transferred to the Laser Direct Infrared Chemical Imaging (Agilent 8700) (LDIR) system following the user's manual.
2. The ERG's LDIR SOP can be requested – it will be accessible via a DOI soon.

Appendices

Appendix I: MP standards stock preparation

- Prepare a filtered 0.1% (w/w) Tween solution to store MP standards.
- Mixing MP standard (beads, fiber, and fragment) into 0.1% (w/w) Tween solution:
  - Based on **Table 1**, take out the number of scoops needed for the corresponding standard and place into a 20 mL glass vial.
  - After adding the appropriate number of scoops, pipette 10 mL of filtered 0.1% Tween solution.
  - Cap and manually invert the vial 50 times to suspend the standard.
- For details on the in-house generation of the fiber standard, please refer to the relevant publication (TBA soon).

Table 1. Number of scoops needed per standard stock being used.

MP standard	Vendor: Product ID	Recommended number of scoops	Tween solution needed (mL)
PE Blue (38-45 µm)	Cospheric: UVPMS-BB-1.13 38-45 µm – 5 g	2	10 mL
PE Orange (53-63 µm)	Cospheric: UVPMS-BO-1.03 53-63 µm – 10 g	1	10 mL
PE Blue (75-90 µm)	Cospheric: UVPMS-BB-1.13 75-90 µm – 10 g	2	10 mL
PE Green (180-212 µm)	Cospheric: UVPMS-BG-1.025 180-212 µm – 10 g	3	10 mL
PE White (300-355 um)	Cospheric: WPMS-1.25 300-355 µm – 10 g	3	10 mL
PE Yellow (355-425 µm)	Cospheric: UVPMS-BY2-1.00 355-425 µm – 10 g	2	10 mL
PA Red Fiber (20 – 500 µm)	Duufin: B07Z92Z3XD	1	10 mL
PET Green Fragment (200 µm)	VViViD: B07BWMQH93	1	10 mL

Appendix II: Spike and QA/QC Procedure

Spike Procedure

To estimate particle losses in the separation process, various known amounts of MP standards are manually added into the sample (i.e., **Spike**) at the beginning of the process, following the suggested volumes of the MP standards stock solutions given in **Table 2**. The particle number differences of these standards before and after the extraction protocol then provide estimates of the potential losses.

Table 2. Number of microplastic standards used for spiking

MP Standard	ERG Suggested Volume (µL)	Expected Particle Number
PE Blue (38-45µm)	2.5	~100
PE Orange (53-63 µm)	10	~60
PE Blue (75-90 µm)	10	~50
PE Green (180-212 µm)	100	~30
PE White (300-355 um)	100	~10
PE Yellow (355-425 µm)	100	~8
PA Red Fiber (20 – 500 µm)	25	~40
PET Green Fragment (200 µm)	50	~30

MP beads spike:

- Manually invert the standard solution vial 50 successive times
- Pipette the beads standards onto a glass slide (~10 µL for standards < 300 µm; 100 µL for standards > 300 µm)
- Count particles on the slide under a microscope and record the number
- Ensure/verify under microscope that no particles are remaining on slide and/or its back side
    - Actual amount spiked is given by:

        Actual spiked MPs = counted MPs – remaining MPs on slides
- Use either MQ water (for wet samples) or 60% $ZnCl_2$ (for dried sediment samples) to immediately rinse off the slide into the sample container

MP fiber spike: Use the same spiking procedure as with the beads; pipette 25 µL of the standards solution and aim for 30-40 fibers per sample.

MP fragment spike: Use the same spiking procedure as with the beads. pipette 50µL of standards solution and aim to 25-30 fragments per sample.

Manually count MPs under microscope to confirm the spike recovery rate at the end of the separation.

QA/QC Procedure

To account for process contaminations in the evaluation of the samples, a procedural blank is run. For this purpose, in parallel to the extraction of the MPs from the samples, initiate a run with an empty clean beaker (i.e., without sample) – all the following procedures are performed in the same way as for the other samples.

Appendix III: Preparation and Density Correction of 60% $ZnCl_2$

- The density of 60% $ZnCl_2$ at 20 ^oC is 1.749 g/mL (Perry & Green, 2008)

Preparation

1. Weigh out 600 g of solid powder $ZnCl_2$ (Sigma Aldrich 793523, anhydrous, reagent grade, ≥ 98%) in a clean 600 mL beaker.
2. Add 400 mL MQ water and manually stir with a glass rod until all the $ZnCl_2$ is dissolved. Note that the reaction is exothermic!
3. Filter before use.

Density Correction

4. Weigh 10 mL of the used/recycled $ZnCl_2$ and calculate the actual density (=m/V).
5. After obtaining the actual density of the current $ZnCl_2$, find the difference in density of the actual and target density.
6. Multiply the difference in density by the actual volume of the current $ZnCl_2$ (i.e., 10 mL in step 4) to find the mass of $ZnCl_2$ needed to make a density of 1.8 g/mL (i.e., back to 60%)
7. Weigh out the amount of solid $ZnCl_2$ that needs to be added and add it to the current $ZnCl_2$ solution. Mix until the solid $ZnCl_2$ is dissolved and filter before use.

Appendix IV: Particles Cleaning by Using Running MQ water

1. Shallow glass dish placed at an angle using bottle caps as in **Figure 7**
2. Place the particles (staying on top of the sieve) in the tray and cover with a glass Petri dish.
3. Carefully turn on the MQ water and let it continuously flow to wash away the unwanted chemicals/reagents from the particle sample.
4. DO NOT let the water directly from MQ water tap run into the sieve, it will damage the sieve and create ununiformed pore sizes!
5. DO NOT let the water level in the sieve go higher than 1/3 of the sieve height, as your particles might be overflown out of the sieve!
6. Gently shake the sieve for ~10 minutes

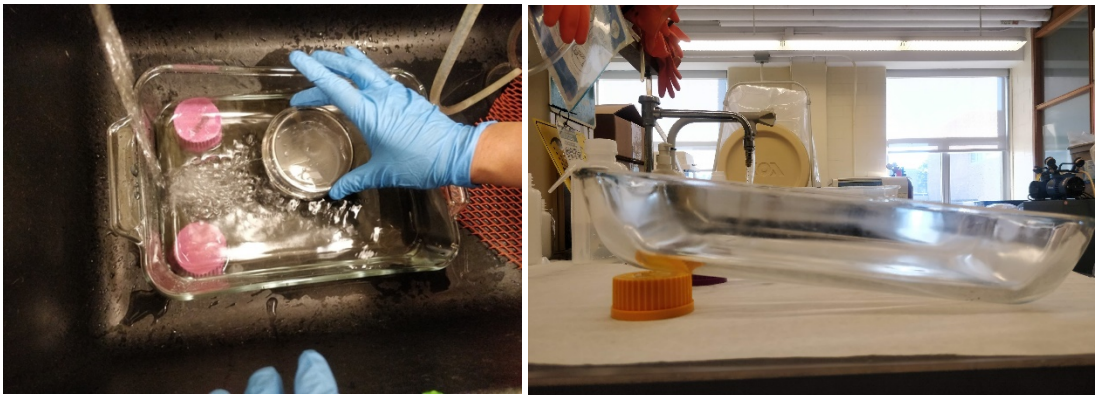

Figure 7: Birds eye view and side view of rinsing contraption

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