**DRAFT FOR REVIEW - Chlorophyll Analysis QuickGuide**

MassDEP Watershed Planning Program

CN 003.45 (updated 4/27/23)

# GENERAL INFORMATION

Samples must be processed (filtered/frozen) within 24 +/- 2 hours of the time of collection. Frozen filters must the analyzed within 28 days of first processing. Data are logged in the printed worksheet (“bench sheet”) in the Chlorophyll binder and in Excel files on OneDrive.

EQUIPMENT

DAY ONE

Graduated cylinders (red-collared for DI water, yellow-collared for sample water)

2-inch Whatman petri dishes

4x4-inch squares of aluminum foil

Tweezers

Whatman GC/F, glass fiber filter

Millipore filtration apparatus

1-ml disposable pipets

Squirt bottle with deionized water (DI)

Gloves & lab goggles

DAY TWO

Grinding apparatus (tissue grinder, tube, and pestle)

4-mL and 6-mL volumetric pipettes

Squirt bottle with 90 % aqueous acetone

Timer

15-mL graduated centrifuge tubes

Acetone waste container

DAY THREE

Centrifuge

Trilogy Fluorometer

Thermometer

disposable 75-mm borosilicate cuvettes

**DAY ONE**

PREPARATION

1. Save a copy (“save as”) of the **Chlorophyll\_Lab\_Book\_Template** from OneDrive ([WPP Lab SOPs and Results 2023](https://massgov-my.sharepoint.com/:f:/r/personal/james_meek_mass_gov/Documents/Monitoring/Targeted%20Monitoring%202023/WPP%20Lab%20SOPs%20and%20Results%202023?csf=1&web=1&e=4cxVIn)) with the new batch number as the file name. Check the Chlorophyll binder for the next batch number. Chlorophyll batch numbers are designated “CHLyy-xx” with yy = year and xx = batch number. E.g., CHL23-01 is the first batch of 2023.
2. Get samples from the WPP fridge (the *round* amber bottles for chlorophyll – not the square amber bottles for algal counts). Sign Chain of Custody (in pocket of fridge) transferring sample custody from field crew (or fridge) to analyst.
3. Line up the samples on the benchtop by time collected – earlier should be run first. Compare the numbers on the samples to the chain of custody form to check for any missing samples.
4. Fill out the printed worksheet (from the large binder) with sample information: batch number, lab sample #, OWMID (from the COC), and date/time collected (from the COC).
5. [Skip this step if sending samples to EPA for analysis.] Add lab sample ID#s for a lab duplicate (LD) and lab blank (LB). Duplicates and blanks should be run for every batch; if the batch is more than 10 samples, run a duplicate every tenth sample. The last sample of the batch should be the blank.
6. Organize and label the plastic 2-inch Whatman petri dish using an indelible black marker. Aluminum foil cut into ~4x4 inch squares is used to completely cover the petri dish.
   1. For analysis at WPP: The labels on the petri dish and foil cover should include the lab sample number and collection date.
   2. For analysis at EPA: The labels on the petri dish and the foil cover should include the lab sample number, OWMID#, and collection date. Use pre-printed labels for the outside label.

SAMPLE FILTRATION

1. There are two graduated cylinders to use: the one with the red markings is for DI water only, and the one with the yellow collar is for sample water only.
2. Rinse the yellow collared graduated cylinder 3 times with tap water and once with DI water. Rinse with sample water: gently shake the sample bottle 25-30 times and pour ~40 mls of sample water into the yellow sample graduated cylinder, swirl it around the inside of the graduated cylinder and then pour it out.
3. Using tweezers, take a 2.1 cm Whatman GC/F, glass fiber filter and place it on the Millipore filtering flask screen. Do not touch the filter. Attach the glass top to the filter flask using the metal clamp.
4. Gentle mix the sample: shake the bottle 25-30 times.
5. Measure **50 mLs** of sample or less (if necessary due to presence of an algal bloom or other factors that might clog the filter) into the **yellow-collared graduated cylinder**. To accurately measure 50 mls the final level can be reached using a 1 ml pipet-disposable. Read the level of the meniscus from the side of the graduated cylinder-not looking down. The meniscus is read at the bottom of the liquid curvature. Do not re-use these pipets.
6. A picture containing indoor, automaton

   Description automatically generatedPour ~ 15 mL of measured sample into the filtration apparatus and turn on the vacuum. Continue pouring sample until all is filtered.
   1. If the sample is not filtering through – either because there is too much sediment, or the algal concentration is too high – then less than 50 mLs can be filtered or a dilution made.
   2. Record the amount of sample filtered in the workbook (column I). [If sending samples to EPA, also record the amount of sample filtered in the Comments column of the COC.]
7. Rinse the cylinder and filter:
   1. Fill the sample cylinder (yellow collar) with 50 mL DI water and, with the filter still in place, use that water to wash loose phytoplankton cells off the sides of the funnel by running the 50 mLs of de-ionized water through the filter.
   2. Use the spray bottle with DI water to wash any remaining cells from the filter funnel.
   3. Turn off pump in between filling the cylinder to also aide in washing phytoplankton off the sides of the column.
8. After all water has been filtered, unclamp the filter holder and with tweezers carefully fold the filter, use a glass rod if needed to flatten the fold and place it in the previously marked petri dish.
9. Cover the petri dish with its lid, and then wrap it in aluminum foil to keep out the light. Make sure outside of the foil is labeled. Temporarily transfer the petri dish to the drawer until you have finished preparing all samples, then put them all in the freezer in the Microscopy Lab. (If you can’t finish samples within 1 hour then move the first group of processed samples to the freezer). Record the time.
10. Remove the filter funnel and wash it under the tap water. Use the small brush to loosen any cells on the inside of the funnel or at the bottom of the funnel. Use 3 tap rinses and then a DI rinse. Invert it and put it on a paper towel to drain.

CLEANING BETWEEN SAMPLES

1. Put a new filter on the filter apparatus, re-attach the funnel and rinse the filter by filtering at least 100 mL DI water from red-collared graduated cylinder. Discard that filter disk.
2. Clean the **red-collared graduated cylinder** with three tap water rinses followed by a DI rinse.
3. Next clean the **yellow-collared graduated cylinder** with three tap water rinses followed by a DI rinse.
4. If the graduated cylinder or other glassware looks dirty after rinsing, add a small amount of detergent to the cylinder and then scrub with the brush to remove any film or dirt that might have built up. This should be followed with the rinsing procedure (3x with tap water / 1 x with DI water).
5. Check the level of the water in the filter flask from time to time! After about 4-5 samples the flask will need to be emptied out otherwise it could overflow and ruin the pump.
   1. Take off the rubber plug with the filtering apparatus attached. To safely pour out the water in the flask, the part where the tubing is attached should be facing the ceiling so that water can’t back flow into the tubing and drain into the pump.
   2. Use the ‘algae’ bucket located under the sink, carry it over to the countertop and pour the wastewater out there. The bucket holding the wastewater should be emptied in the sink and rinsed out with tap water or if needed detergent.
6. [Skip this step if sending samples to EPA for analysis] QC SAMPLES:
   1. Every tenth sample (or at least once per batch) make a duplicate (filter a second 50 ml of one of the field samples). This is denoted LD on the data entry sheet (column B), give it a new lab sample number (column C), and record the original sample OWMID (column D).
   2. After the last sample create a blank. After the yellow graduated cylinder has been cleaned, as in step 19, filter 50 mL of DI for a blank sample. The blank will be denoted LB on the data entry sheet (column B).
7. When the last sample in the batch has been filtered (or an hour has gone by since you started filtering), transfer the labeled petri dishes to the freezer in the biology lab. The bench sheet should be filled in with date and time the samples were frozen.
8. Return the sample bottles to the refrigerator if algal counts or identifications are requested. The field sheet should indicate this. If the sample is not needed further then pour it in the sink, rinse the container out with tap water and then put it in the recycling bag underneath the sink in the ‘staging room’.
9. Rinse the graduated sample cylinder yellow collared graduated cylinderwith three tap water rinses followed by a DI rinse. Rinse the filter funnel (top) with tap water, using the brush to remove adhering cells and then with de-ionized water.

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STOP HERE IF SENDING THE FROZEN SAMPLES OUT FOR ANALYSIS

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**DAY TWO**: GRINDING

CAUTIONS! Acetone is highly flammable, don’t allow fumes to build up in the room. Avoid contact with acetone-wear gloves and protective goggles in the lab.

Turn on the fume hood and the oscillating fan by the grinding apparatus to avoid the buildup of acetone fumes in the room. The fan should be operated at low velocity between the analyst and the grinding apparatus but pointing away from the analysts’ face.

Transfers of all chemicals and preparation of the 90% acetone should be done under the hood when possible. *Grinding of the samples; however, should* ***not*** *be done there because of the risk of sparks which could ignite the acetone.*

A picture containing indoor, blender, kitchen appliance

Description automatically generatedGRINDING

1. Retrieve petri dishes from the freezer in the microscopy lab and locate the worksheet for the batch of samples you’re working on. Record the grinding date on the work sheet.
2. Turn on the oscillating fan.
3. For each sample, remove the filter using the tweezers and add to grinding tube. Push the frozen filter to the bottom of tissue grinding tube using a glass rod.
4. Using a 4-mL volumetric pipette add 90% aqueous acetone solution to the grinding tube.
5. Set clock/alarm for two minutes. Grind contents in grinding tube for 2 minutes or until the sample has been sufficiently macerated. Care must be taken not to overheat the sample which degrades the chlorophyll.
6. The contents of the grinding tube are then carefully washed into a 15-mL graduated centrifuge tube using a 6-mL volumetric pipette containing 90 % aqueous acetone to rinse the pestle and the grinding tube. The rinse and the filter slurry are added to the centrifuge tube.
7. Write the sample # on the top of the cap and on the side of the centrifuge tube using a permanent marker pen.
8. Cap the centrifuge tube, shake vigorously and then wrapped in aluminum foil to eliminate any exposure to light.
9. While working, store the finished test tubes temporarily in the cabinet below bench to keep them out of the light.
10. Fill out the worksheet after each sample is completed with the date and time they were ground.
11. After all samples are ground - or before an hour is up – transfer the wrapped tubes to the refrigerator in the Microscopy room at 4°C for 24 hours +/- 2 hours.
12. CLEANING between samples: wipe the grinder tube and the grinder pestle with a Kimwipe and then wash with 90 % acetone to remove any remaining chlorophyll. Use the acetone wash bottle to clean the grinder tube and then pour the waste material into the labeled waste acetone jar. Both the acetone wash bottle and the waste acetone jar are found in the safety box for inflammable items.
13. Clean the grinder tube with a brush and then wash three times with tap water, once with DI and then once with acetone. After wiping the pouring spout on the grinding tube with a Kimwipe, the acetone wash bottle should be used to cleanse the sides of the grinder tube.

**DAY THREE: READING THE SAMPLES**

1. After 24 hours, take the centrifuge tubes out of the refrigerator and put into the centrifuge for 10 minutes at 1000 rpm. Note: the centrifuge must have a balanced load to work! (The centrifuge is set to run 10 minutes at 1000 rpm).
2. Allow the tubes to come to room temperature (approximately 30 minutes). You can leave them in the dark in the centrifuge and take them out one by one as they are to be read.
3. A picture containing text, first-aid kit, scoreboard

   Description automatically generatedLog onto the desktop computer. Turn on the Trilogy fluorometer and check that it is connected to the computer. Click the Trilogy icon on the desktop and then click on connect to Excel, select COM 4 from the popup. Save the Trilogy file to OneDrive ([WPP Lab SOPs and Results 2023](https://massgov-my.sharepoint.com/:f:/r/personal/james_meek_mass_gov/Documents/Monitoring/Targeted%20Monitoring%202023/WPP%20Lab%20SOPs%20and%20Results%202023?csf=1&web=1&e=NvFrZB)) in Trilogy readings folder.
4. Text, whiteboard

   Description automatically generatedName the Excel file by the date with the current date. (The Excel sheet is used because the Trilogy does not store more than 20 measurements at one time. )
5. The chlorophyll readings should be done no more than 24 hours +/- 2 after grinding, if you do go over this holding time still run the samples but annotate this change from procedure.

SET UP THE TRILOGY FLUOROMETER

1. On the fluorometer screen, select **Chl-NA** and click OK which will bring you to the home screen.
2. A picture containing text, indoor, oven, open

   Description automatically generatedClick **calibrate** and then select the **most recent calibration** (recorded by date).
3. Check the calibration against the Trilogy solid standard (small blue box kept in the drawer next to the fume hood).
   1. Take the adaptor out of the top of the Trilogy (DON’T lose it) and insert the solid standard into the holder. The tab should be at the back of the opening.
   2. Select **Sample ID** and enter “QC” and date.
   3. Select **measure fluorescence** and enter 1 for the sample volume and 1 for the solvent.
   4. The reading should be within +/- 10% of the standard it was adjusted to (check the front of the worksheet binder for the expected concentration). If the readings do not meet +/- 10% of the expected value, check with the Field and Lab Operations Coordinator or QA Analyst before proceeding.

READING SAMPLES

1. After samples have come to room temperature (21 – 24 °C), work with one at a time: pour ~ 4 mL of supernatant into a clean, disposable 75-mm borosilicate cuvette. Insert the cuvette in the Trilogy and close the lid.
2. Select **Sample ID** and enter the lab sample number you are analyzing. Save.
3. Select **Measure Fluorescence** and enter the volume of the sample and the volume of the solvent. (E.g., 50-ml sample and 10-ml solvent for most of the phytoplankton chlorophyll samples).
   1. Check bench sheet to see if other amounts were used - particularly for the sample volume.
   2. If it’s a periphyton sample enter 1 for sample volume and 1 for solvent.
   3. Check on the screen to see if you did enter the amount filtered and the amount of solvent correctly. If you made a mistake let the instrument calculate a value, but then read it again.
4. Put the thermometer in the remaining sample in the centrifuge tube while you wait for the reading on the Trilogy.
5. Record the chlorophyll reading on the data sheet.
6. Record the temperature of the remaining sample on chlorophyll a data sheet (column O).
7. Copy the bench sheet information to the computer file.
8. Record the amount of acetone waste produced that day on the waste generated sheet attached to the post opposite the flammable cabinet.
9. Check that the Trilogy readings were recorded properly on the bench sheet and filtered volume was correct. Check the computer entries against the bench sheet. If any edits are made they should be initialed and dated. The bench sheet should be kept in the chlorophyll lab binder.

**WHEN DILUTIONS ARE NECESSARY FOR THE TRILOGY FLUOROMETER**

If your sample in the cuvette looks very green after steeping in acetone overnight and being centrifuged, this is an indication that the fluorometer reading may be off scale and is no longer reading on a linear scale.

To check, make a dilution and if the reading goes down in proportion to your dilution you are still on a linear scale. If you make a 1:1 dilution (1 part sample to 1 part acetone) the reading should be about half of the first reading, if not a dilution and re-reading is needed.

**Steps for making the dilution:**

1. Add 3 mls of acetone to a new centrifuge tube, using a clean 1-mL glass pipet (in the drawer below the centrifuge).
2. Use another clean pipette to add 3 mls of sample from the original centrifuge tube. Do not disturb the sample sediments.
3. Put a cap on the tube with the diluted sample and gently invert the tube several times.
4. Pour the diluted sample into the borosilicate cuvette. Fill it about ¾ full or about 5 mls.
5. Re-read the sample; multiply the reading by 2 for a final result. If the final results are the same, you can choose either reading as the final reading (without a dilution and with a dilution).
6. If the results were not the same, then take another cuvette add 3 mls of acetone and 3 of the diluted sample; make a series of dilutions until a linear response is obtained.
7. The chlorophyll data sheet should indicate that a dilution was made. The dilution value would be 2 for the first one and 4 for the second serial dilution you make.

EXAMPLE WORKSHEET

A screenshot of a computer

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