



MassDEP

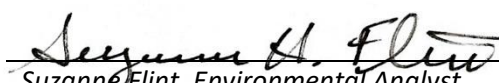

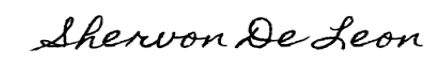
**Massachusetts Department of Environmental Protection
Bureau of Water Resources
Watershed Planning Program**

**STANDARD OPERATING PROCEDURE
Chlorophyll a Analysis
by EPA Method 445 with the Welschmeyer modification**

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CN 003.44

Prepared by:	 Suzanne H. Flint, Environmental Analyst	Date:	7/15/25
Approved by:	 Jasper Sha, QA Officer	Date:	7/15/25
Reviewed by:	/s/ Joan Beskenis Joan Beskenis, Aquatic Biologist	Date:	7/15/25
Approved by:	 Shervon De Leon, Monitoring Section Chief	Date:	7/15/25

List of Revisions

Revision Date	Revision	Pages #s	CN/ (Old CN if applicable)	Initials
2004	Original Document		CN 003.42	
2023	Revisions throughout; addition of Quick Guide	All	CN 003.43	SF
2025	Addition of List of Revisions	2	CN 003.44	SF

Chlorophyll a Analysis by EPA Method 445 with the Welschmeyer modification

1.0 SCOPE AND APPLICATION

Chlorophyll is a pigment found in plants that allows the organism to use radiant energy for converting carbon dioxide into organic compounds in a process called photosynthesis. Several types of chlorophyll exist and these and other pigments are used to characterize algae. One type, chlorophyll *a*, is most widely used for biomass estimates because of its presence in all algae and because it constitutes approximately 1-2% of the dry weight of organic material. A knowledge of chlorophyll *a* concentrations can provide an estimate of biomass of the phytoplankton and the periphyton that can be used for comparative assessments of geographical, spatial and temporal variations (American Public Health Assoc., 1981). Chlorophyll *a* measurements are made from both phytoplankton samples and periphyton samples from lakes, streams, rivers, and estuarine waters. These measurements are used in several indices to determine the trophic status of lakes and rivers.

2.0 SUMMARY

A modification of the EPA Method 445.0 for chlorophyll *a* analysis is being used with the Turner Design Trilogy Fluorometer. The modification requires no acidification and has EPA approval. The method was developed by Nicholas Welschmeyer (1994). According to Turner Design product information, the filters used with the Trilogy as well as the special blue lamp eliminate the interference to chlorophyll *a* from chlorophyll *b* and phaeopigments. Background fluorescence is reportedly reduced with this method from interfering compounds including chlorophyll *b*, *c*, pheophytin and dissolved organic matter (Arar and Collins 1997).

3.0 SAFETY CONSIDERATIONS

General lab safety procedures should be followed (CN 000.36 – SOP Laboratory Safety). In the laboratory, care should be taken in the use of acetone which is inflammable and can irritate skin as well as other health effects. No smoking or lighting of matches or any sparking agents should be used in the lab. When possible, use the fume hood to make all transfers involving acetone. If work cannot be done under the hood because of the height considerations or logistics, then the fan located to the right of the sink should be used at low velocity pointed away from the source of the acetone. Nitrile gloves should be used when handling the acetone. Goggles or safety glasses are also available for use.

4.0 SAMPLE COLLECTION, PRESERVATION AND HANDLING

Grab and integrated samples are the most common types of sample collection used for chlorophyll analysis from phytoplankton per field SOPs (CN 03.5 SOP for Chlorophyll and CN 35.1 and CN 60.0 – SOPs for Periphyton). Samples should be kept in the dark and on ice until they are brought back to the lab. At the lab, the sample should be stored at 4°C and in complete darkness in the refrigerator. The samples should be processed within 24 hours of their collection. If samples cannot be analyzed within this time, then the samples should be filtered and the filters frozen for future analysis (within 28 days of their collection).

5.0 APPARATUS, EQUIPMENT, MATERIALS

- Chlorophyll *a* Calibration Standard: Turner Design, 10-850
- Secondary Standard-Turner Design
- Fluorometer – Turner Design (Trilogy)
- Fluorescence module for chlorophyll *a* (non-acidification method)-minimum detection limit 0.025 µg/L
- Tissue grinder and tube – Thomas Tissue Grinder
- Side arm vacuum flask and pump
- Millipore filter holder
- Glass fiber filter: Whatman GF/F, 2.1 cm
- Centrifuge - International Equipment Co., model, Centra CL2
- 15-mL graduated conical end polypropylene centrifuge tubes with screw caps
- Test tube racks
- Disposable borosilicate cuvettes –12 mm by 75 mm cuvettes
- Aluminum foil
- Test tube brushes – conical end
- Parafilm
- Disposable glass 1 ml pipettes
- Calibrated glass pipette (TD) 1-mL, 1/100 markings
- Glass rods
- Volumetric flasks and pipettes, 4-mL, 6-mL
- Computer with serial port connection to the Trilogy fluorometer (see Turner Designs manual for set up details)

6.0 REAGENTS

- The EPA Modified 445.0-requires no acidification.
- HPLC grade acetone is used to make up the 90 % solution: 90 mLs of acetone is added to 10.0 mLs of de-ionized water.
- De-ionized water is prepared at WPP using a Barnstead E-pure Filtration System.

7.0 CALIBRATION

The trilogy fluorometer (turner design) is calibrated using pure Chlorophyll *a* concentrations purchased from Turner Design and used to set the solid standard (Turner Design) concentration. Calibration is done at the start of the field season and then re-checked with each batch with the solid standard. The calibration procedure is outlined in the Turner Design Trilogy Operating Manual. The general calibration steps are as follows:

For Direct Concentration Calibration a blank solution and at least one standard solution are required; two chlorophyll a concentrations are recommended (one low and one high).

To calibrate the Trilogy:

- Turn on the Trilogy using the toggle switch in the back
- Insert (or check) that the non-acidification module chlorophyll (marked “CHL A NA”) is in place in the top of the Trilogy
- Select Chl a – NA and from the home screen select “Calibrate”
- Select “Run New Calibration”
- Select µg/L as the unit of measurement
- Insert the calibration ‘blank’ (90% acetone reagent) and touch ‘OK’
- Going from the lowest concentration to the highest, enter the concentration of the first standard (the concentration information will be supplied with the purchased standard)
- Insert the glass borosilicate cuvette containing the standard and touch ‘OK’
- Select ‘Enter More Standards’
- Insert the cuvette with the standard and touch ‘OK’
- Save the calibration and give it a name with the calibration date
- Re-measure the first standard to see if value is the same or <5% difference.
- When asked for volume of standard sample and of solvent enter 1 mL for sample and 1 for solvent respectively.
- Insert the Solid Secondary Standard* in the Optical Module and use the Allen wrench included with the solid standard to change the adjustment screw to get the same reading as a Standard that was previously measured. Turning the screw clockwise will produce a lower signal. Record the reading in the front of the lab binder and in the season’s results files.
- Re-measure the standard and see if the standard reading and the solid standard reading are the same or close. Repeat until they are.

* The main benefits of using the Solid Secondary Standard are that it can be used in place of a primary standard once the relationship between a primary standard and the solid standard has been established, and that it can be used to check the fluorometer stability, and/or check for loss in sensitivity resulting from instrument/optical module problems.

8.0 SAMPLE ANALYSIS

Samples are generally processed as soon as they are received at the WPP laboratory but must be processed within 24 +/- hours of the time of collection. Samples that cannot be analyzed within 24 +/- 2 hours of collection must be filtered and the filters frozen for future analysis. Samples must be analyzed within 28 days of first filtering and freezing.

Data Management - Chlorophyll-a sample information and results are recorded on the printed bench sheets and in Excel worksheets (on OneDrive) by batch number. Batches are designated “CHLyy-xx” where yy is the year and xx is the consecutive batch number for the year. Individual samples are given lab sample #s appended to the batch number (e.g. CHL23-01-01 is the first sample of batch 1 in 2023).

Metadata recorded for each sample includes: lab sample#, OWMID #, project name, sample collection date and time. Sample analysis data includes: date/time of filtration and freezing, date/time of grinding, date/time of

reading, volume filtered, dilution, temperature, and result. It is critical that the amount of sample filtered be recorded for each sample because it is used to calculate the chlorophyll concentrations.

Setup (Day One)

1. Use the Chain of Custody to transfer sample custody from the field crew (or refrigerator) to the analyst.
2. Retrieve the samples. Compare the numbers on the samples to the chain of custody form for any missing samples or sample-specific notes. Check that the samples containers are the amber colored round bottles for chlorophyll.
3. Line up the samples by time collected-earliest time should be run first.
4. Fill out bench sheet with the information listed on the chain of custody form and assign lab sample #s.
 - a. The first sample should be a QC reading of the solid standard.
 - b. Every tenth sample should be a duplicate (or at least once per analysis batch). This sample is given a consecutive lab number like the other samples, but it is denoted duplicate (LD).
 - c. The last sample in the batch is a lab blank. This sample is given a consecutive lab number like the other samples, but it is denoted blank (LB).
5. Label the plastic 2-inch Whatman petri dishes and aluminum foil squares with the lab number and collection date. Aluminum foil cut into ~4x4 inch squares is used to cover the petri dish to protect the samples from light.
6. Set up spreadsheet from the chlorophyll template file. Save a copy of the worksheet ("save as") with the batch number as the file name.

Filtering Samples

7. Shake the field sample gently 25-30 times to mix completely.
8. Using tweezers, take a 2.1 cm Whatman GC/F, glass fiber filter and place it on the Millipore filtering flask screen. Attach the glass tube to the filter flask using the metal clamp.
9. Measure 50 mLs of sample in a graduated cylinder. To accurately measure 50 mLs the final level can be reached using a 1 mL pipet-disposable.
10. Pour approx. 15 mL of measured sample into the filtration apparatus and turn on the vacuum. Continue pouring sample until all is filtered.
11. If the sample is not filtering through – either because too much sediment is present or the algal concentration is too high – then less than 50mL's can be filtered or a dilution made. Record the amount of sample filtered.
12. Wash down the graduated cylinder and filter: fill the sample cylinder with 50 mL DI water; with the filter still in place, run the 50 mLs of de-ionized water through the filter column, using the designated graduated cylinder, to wash loose phytoplankton cells off the sides of the column. Use the spray bottle to wash any remaining cells from the filter funnel. Turn off pump in between filling the cylinder to also aide in washing phytoplankton off the sides of the column.

13. After all water has been filtered, transfer the filter to the previously marked petri dish. Cover the petri dish and wrap it in the labeled aluminum foil to keep out the light.
14. Keep the petri dishes out of the light until you have finished preparing all samples, then transfer them all to the freezer.

Cleaning Between Samples

15. 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.
16. Put a new filter on the filter apparatus, re-attach the funnel and rinse the filter by filtering at least 100 mL DI water. Discard filter disk.
17. Clean the graduated cylinder with three tap water rinses followed by a DI rinse.
18. If at any time the graduated cylinder or other glassware looks dirty after rinsing, a small amount of washing detergent should be added to the cylinder and then scrubbed with the brush to remove any film or dirt that might have built up.
19. Check the level of the water in the filter flask from time to time! Make sure that it is not overflowing. To empty the filtering apparatus, disconnect the tube leading from the pump. The sample plug will need to be removed in order for the collected water to be poured out into the 'algae' bucket located under the sink.

Lab QC

20. After the last sample, a batch blank should be run. The blank is denoted LB on the data entry sheet. After the yellow graduated cylinder has been cleaned, as in step 17 filter 50 mL of DI for the blank.
21. Every tenth sample a duplicate should be run, this is denoted LD. This sample is given a new number and is denoted as a duplicate in the computer file.
22. If the sample is not needed further then pour it in the sink, rinse the container out with tap water three times and put it in the recycling bin.
23. Rinse the graduated sample cylinder and filter holder several times in de-ionized water.
24. Transfer all sample filters to the freezer.

Grinding the Samples (Day Two)

Caution: Acetone and acetone waste must be stored in the yellow "Flammables" cabinet. When acetone is being used, turn on the fume hood and the fan by the grinding apparatus. 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. The oscillating fan should be used when running the chlorophyll samples. It should be operated at low velocity between the analyst and the acetone source but pointing away from the analysts' face.

1. Samples must be analyzed within 28 days of first filtering and freezing.
2. Retrieve petri dishes containing the filters from the freezer.
3. Remove the filter from the petri dish using the tweezers and add to grinding tube.

4. Push the frozen filter to the bottom of tissue grinding tube using a glass rod.
5. Using a 4-mL volumetric pipette, add 90% aqueous acetone solution to the grinding tube. Volumetric pipettes are marked TD for 'to deliver'. This means that the pipette is designed to deliver the volume stated on the pipette. Do not force or blow the last drop out, there will always be a small amount of liquid inside the tip after pipetting. If you do force it out your volume will be slightly over the design volume.
6. 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.
7. The contents of the grinding tube are 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.
8. Write the sample # on the top of the cap and on the side of the centrifuge tube.
9. Cap the centrifuge tubes, shake vigorously, and wrap with aluminum foil to eliminate any exposure to light. The wrapped tubes must be stored at 4°C for 24 hours plus and minus 2 hours before reading.

Cleaning between Samples

10. Between samples the grinder tube and the grinder pestle should be wiped with a Kimwipe and then washed with 90 % acetone to remove any remaining chlorophyll. Use the acetone wash bottle to clean the grinder tube and then pour the gathered material into the waste acetone jar. Both the acetone wash bottle and the waste acetone jar are found in the box for inflammable items.
11. Finally, the grinder tube is cleaned with a brush and then washed three times with tap water, one time with DI and then one time 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.

Analysis (Day Three)

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 deviation from the procedure. A decision will be made by the project manager whether to accept the data.

12. After 24 hours, centrifuge tubes are then taken out of the refrigerator and centrifuged for 10 minutes at 1000 rpm. Note: the centrifuge must have a balanced load to work.
13. Allow the tubes to come to room temperature (approximately 30 minutes).

Reading the Samples

14. Turn on the lab computer and the Trilogy fluorometer. Connect the Trilogy to the computer (see the QuickGuide or Turner Designs, 2010, for details; see section 7.0 above for details on instrument calibration).
15. Click on the Trilogy icon on the computer desktop, select the "comm" setting, and click connect to Excel. Name the Excel file by the date you are currently reading the sample concentrations (e.g., 25May2022) and save it to the season's results folder.

16. For the first reading, use the solid standard for the Trilogy to check for drift in the readings. To use it, remove the sample adaptor from the Trilogy and insert the solid standard with the tab located towards the back. When prompted, enter 1 for the sample volume and enter 1 for the volume of solvent used. Acceptable drift is 10% from the expected value (set during calibration). If the reading is >10% different from the expected value, check with the Field and Laboratory Operations Coordinator to recalibrate.
17. When samples have come to room temperature (21-24 °C), read the samples one at a time: pour approximately 4 mL of supernatant into a clean, disposable 12x75-mm borosilicate culture tube.
18. Take sample readings: Put the tube in the fluorometer and select “measure”. When prompted, enter the sample and solvent volumes and sample name. For phytoplankton samples enter 50 mL for the sample volume and 10 mL for the solvent volume unless other quantities were used. (For periphyton samples enter 1 ml for both the sample volume and the solvent volume.)
19. Measure the temperature of the remaining sample in the centrifuge tube.
20. Record the chlorophyll reading, reading time, and sample temperature on the data sheet.
21. Save the Trilogy Excel spreadsheet to designated file.
22. Copy data from the bench sheet to the computer file. The spreadsheet name for each date should be: CHL_year_batch#.
23. Record the amount of acetone waste produced that day on the waste generated sheet attached to the post opposite the flammable cabinet.

Data Verification:

24. After completing the readings, a second person should check that the data from the bench sheet and Trilogy file are correctly copied to the computer worksheet.
25. If any edits are made they should be initialed and dated. The bench sheet should be kept in the chlorophyll lab binder.

9.0 CLEANING AND WASTE HANDLING

Typically, disposable borosilicate cuvettes are now used. If these are unavailable then regular borosilicate cuvettes are cleaned by emptying the contents into the waste jar held in the Flammables Storage Cabinet, using the cuvette brush to wash out the interior and rinsing the interior with de-ionized water. Following this the cuvettes are rinsed with acetone and then the de-ionized water again.

Other reusable glassware is soaked for four hours in laboratory grade detergent and water, rinsed with tap water and then with acetone followed by two more de-ionized water rinses.

The waste acetone container should be emptied every night into the disposal bottle that is kept in the Flammables Storage Cabinet. The waste containers, both the transfer and larger storage containers, must be labeled with proper waste labels which include the following information: “hazardous waste”, the date that the disposal jar was first used, the contents must be listed and the word “ignitable” included on the label. The labels should be facing forward in the storage cabinet.

10.0 QUALITY CONTROL

A laboratory duplicate is run on every tenth sample. The precision for these samples will be determined by calculating their relative percent difference (RPD). The RPD should not exceed 20%.

$$\text{RPD} = (\text{sample} - \text{duplicate}) / (\text{average of sample and duplicate}) * 100.$$

The accuracy of the sample measurements will be determined by comparing the concentration of chlorophyll check samples (Turner Design, Inc.) to the calculated concentration. The check sample values should fall within plus and minus 20% of the standard calibration readings. If this level of accuracy is not met then the source of the error must be identified before further analyses are done. If the project data objectives allow it, a larger percent error may be acceptable.

A laboratory blank is the last filter extracted of a sample set. It is used to check for contamination of the reagents or apparatus. This laboratory blank filter is extracted and analyzed similar to a sample filter. If the sample value is greater than 1 ug/L then the samples for that day must be re-run or the data flagged and judgment made as to whether the data objectives are still met.

The computer data file entries will be 100% checked against entries the Trilogy Excel file from the fluorometer and with the hand-written lab bench sheet.

Chlorophyll-a results are reported as mg/m3 or ug/L. The chlorophyll values are recorded to the nearest tenth if the results are below 50 ug/L and those greater than 50 ug/L are reported as whole numbers. The method detection limit (MDL) for the method is 1 ug/l. Readings less than 1 are reported as <1 ug/L.

11.0 INTERFERENCES

High concentrations of humic acids can cause interference because they fluoresce at the same wavelengths as the chlorophyll a. Other substances that fluoresce in the red region of the light spectrum may interfere with chlorophyll a measurement.

Pheophytin a (a degradation product of chlorophyll a) and chlorophyll a are measured at similar wavelengths. This can result in an overestimation of chlorophyll a measurement if the acidification method for chlorophyll a analysis is used. A correction formula is used to adjust for this problem. These formulas are provided in Standard Methods (American Public Health Assoc., 1981).

12.0 PREVENTIVE MAINTENANCE

The oil level on the water pump used for filtering must be checked daily and oil added if necessary. The oil is kept in the bottom cabinet to the right of the fume hood.

When filtering always check that the volume of water in the 'side-arm' flask is not more than half full. When it gets to about that level it should be emptied out and the water disposed of in the sink.

The waste acetone is recycled at WES. It must be transported there in an iced cooler and packed in such a manner that it cannot tip over. The cooler should be tied down with a bungee cord and labeled "waste acetone" on the outside.

13.0 CORRECTIVE ACTIONS

If the calibration, laboratory duplicate or laboratory blank fail to meet the criteria in section 10.0, then the analyst must re-examine the collection and analysis techniques and inspect the fluorometer for contamination or malfunction. If the problem persists the analyst must initiate a corrective action following the directions in

the standard operating procedure CN 5.0 Corrective Actions. The survey and project coordinators must be notified.

14.0 WASTE AND POLLUTION PREVENTION

The use of disposable borosilicate cuvettes has resulted in a dramatic decrease in the use of acetone. The use of the “no-acid” method has resulted in the elimination of the use of concentrated sulfuric acid and of the waste acid. While not immediately harmful to the environment the use of disposable cuvettes increases the waste returned to the landfills.

15.0 REFERENCES

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16.0 DEFINITIONS/ACRONYMS

Phytoplankton-algae that is not attached and is usually semi-buoyant so that it floats within the water column

Periphyton-attached algae

Phaeopigments-breakdown pigments from chlorophyll a

HPLC-high pressure liquid chromatography (grade of acetone)

17.0 APPENDIX A: QuickGuide

Chlorophyll Analysis QuickGuide

MassDEP Watershed Planning Program

(updated 3/17/25)

GENERAL INFORMATION

Samples must be processed (filtered/frozen) within 24 +/- 2 hours of the time of collection. Frozen filters must be 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 test tubes
Solid Standard

DAY ONE

PREPARATION

- 1) Save a **copy** ("save as") of the [Chlorophyll Worksheet Template.xlsm](#) with the new batch number as the file name in the "Chlorophyll Results" subfolder. 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., CHL25-01 is the first batch of 2025.
- 2) Get samples from the WPP fridge (the *round* amber bottles for chlorophyll with OWMID suffix "_I"). Sign the Chain of Custody (in pocket of fridge) transferring sample custody from field crew (or fridge) to analyst.
- 3) Line up the samples by time collected. Compare the sample IDs to the COC 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). See the example in the binder.
- 5) 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 plastic 2-inch Whatman petri dishes using a Sharpie. Aluminum foil cut into ~4x4 inch squares is used to completely cover the petri dish. Label the petri dish and foil cover with the lab sample number.

SAMPLE FILTRATION

- 7) 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.

- 8) **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.
- 9) **Put a filter** (2.1-cm Whatman GC/F) on the filtering flask screen with the tweezers. Do not touch the filter. Attach the glass top to the filter flask using the metal clamp.
- 10) **Mix the sample:** gently shake the sample bottle 25-30 times.
- 11) Measure **50 mLs** of sample 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. The meniscus is read at the bottom of the liquid curvature. Do not re-use these pipets.
- 12) Pour ~ 15 mL of measured sample into the filtration apparatus and turn on the vacuum. Continue pouring sample until all is filtered.
 - a. 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.
 - b. **Record** the amount of sample filtered in the workbook (column I).
- 13) Rinse the cylinder and the filter with 50 mLs DI water:
 - a. Fill the sample cylinder (yellow collar) with 50 mL DI water and, with the filter still in place, run the 50 mLs of de-ionized water through the filter.
 - b. Use the spray bottle with DI water to wash any remaining cells from the filter funnel.
- 14) Take the filter off: 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.
- 15) Cover the petri dish and wrap it in aluminum foil. Make sure outside of the foil is labeled. Temporarily store the petri dish in the drawer until you have finished with all samples, then transfer 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 the samples went into the freezer.



CLEANING BETWEEN SAMPLES

- 16) Wash the funnel: 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.
- 17) Rinse the filter apparatus with 100 mLs DI water: Put a new filter on the filter apparatus, re-attach the funnel and rinse the filter by filtering at least 100 mL DI water. Discard that filter disk.
- 18) Clean the **graduated cylinders** with three tap water rinses followed by a DI rinse.
- 19) Scrub if necessary: 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).
- 20) Check the level of the water in the filter flask! After about 4-5 samples the flask will need to be emptied out otherwise it could overflow and ruin the pump.
 - a. 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.

- b. Use the bucket (under the sink) or a large beaker, carry it over to the countertop and pour the wastewater out there. Empty the bucket into the sink and rinsed out with tap water or if needed detergent.

21) QC SAMPLES:

- a. Every tenth sample (or at least once per batch) make a duplicate (filter a second 50 ml of one of the field samples). Mark the sample "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).
- b. 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).

22) 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.

23) Check that the bench sheet is filled in. Copy the information to the Excel worksheet.

24) Check that the COC has been signed and return it to the pocket on the staging room fridge.

25) Return the sample bottles to the refrigerator in case they are needed for resampling. When the sample is not needed further (or after 24 hours), pour it in the sink. Rinse the container out and put it in the recycling bag underneath the sink in the 'staging room'.

26) Rinse the equipment: rinse the graduated cylinders with 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.

DAY TWO: GRINDING

CAUTIONS! Acetone is highly flammable, don't allow fumes to build up in the room. Avoid contact with acetone - gloves and protective goggles are recommended 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.*

GRINDING

26. 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.
27. Turn on the oscillating fan.
28. For each sample, remove the filter using the tweezers and add to grinding tube. Push the frozen filter to the bottom of grinding tube using a glass rod.
29. Add exactly 4 mLs of 90% aqueous acetone solution to the grinding tube using a volumetric pipette.
30. Set clock/alarm for two minutes. Grind contents in grinding tube for 2 minutes or until the sample has been sufficiently macerated. Take care not to overheat the sample; heat degrades the chlorophyll.
31. Carefully wash the contents of the grinding tube into a 15-mL graduated centrifuge tube using exactly 6-mLs of 90% aqueous acetone. Use the 6-mL volumetric pipette to measure the acetone. (This will be a total of 10-mLs of acetone solvent for the sample.)
32. Write the sample # on the top of the cap and on the side of the centrifuge tube using a permanent marker pen.
33. Cap the centrifuge tube, shake vigorously and wrapped in aluminum foil to eliminate any exposure to light.
34. While working, store the finished test tubes temporarily in the cabinet below bench to keep them out of the light.
35. Fill out the worksheet after each sample is completed with the date and time they were ground.
36. 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.

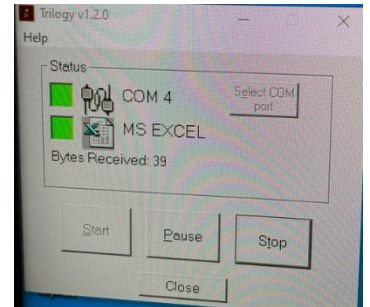


CLEANING BETWEEN SAMPLES

37. 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 stored in the safety box for inflammable items.
38. 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) Log 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 ([Trilogy Readings 2024](#)) in Trilogy readings folder.
- 4) Name 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

- 6) On the fluorometer screen, select **Chl-NA** and click OK which will bring you to the home screen.
- 7) Click **calibrate** and then select the **most recent calibration**.
- 8) Check the calibration against the Trilogy solid standard labeled with this year's date (small blue box kept in the drawer).
 - a. 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.
 - b. Select **Sample ID** and enter "QC" and date.
 - c. Select **measure fluorescence** and enter 1 for the sample volume and 1 for the solvent.
 - d. 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 & Lab Operations Coordinator before proceeding.



READING SAMPLES

- 9) 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 test tube. Insert the tube in the Trilogy and close the lid.
- 10) Select **Sample ID** and enter the lab sample number you are analyzing. Save.
- 11) 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).
 - a. Check bench sheet to see if other amounts were used - particularly for the sample volume.
 - b. If it's a periphyton sample enter 1 for sample volume and 1 for solvent.
 - c. 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.
- 12) Put the thermometer in the remaining sample in the centrifuge tube while you wait for the reading on the Trilogy.
- 13) Record the chlorophyll reading on the data sheet.
- 14) Record the temperature of the remaining sample on chlorophyll a data sheet (column O).

- 15) Copy the bench sheet information to the computer file.
- 16) Record the amount of acetone waste produced that day on the waste generated sheet attached to the post opposite the flammable cabinet.
- 17) 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 test tube 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 test tube. Fill it about $\frac{3}{4}$ full or about 4-5 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 centrifuge tube 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

2023 Chlorophyll-a Lab Book			CHL23-01		Analyst (Filter)	D. Tympanick		Analyst (Grinding)	J. Beskenis		Analyst (Reading)	D. Davis						
Project / Basin	Lab QC (LB, LD)	Lab ID Example: CHL23-##-##	OWMID Example: 71-####	Collection Date	Collection Time	Filter Freeze Date	Filter Freeze Time	Volume Filtered (mL)	Grinding Date	Grinding Time	Reading Date	Reading Time	Dilution (if none enter ""1")	Temp (°C)	Reading Result (ug/L)	Final Result (ug/L)	Comments	
TAM B1-4 (2023)	QC	CHL23-01-01	NA								07/03/23	9:00	1	NA	23.00	23.00	Example QC check Data Entry	
TAM B1-4 (2023)		CHL23-01-02	23-3210	07/01/23	11:16	07/01/23	9:45	50.0	07/02/23	8:45	07/03/23	9:09	1	24.0	0.01	0.01	Example Data Entry	
TAM B1-4 (2023)		CHL23-01-03	23-3211	07/01/23	11:30	07/01/23	9:45	50.0	07/02/23	8:55	07/03/23	9:12	1	23.8	4.48	4.48	Example Data Entry	
TAM B1-4 (2023)		CHL23-01-04	23-3212	07/01/23	11:36	07/01/23	9:45	50.0	07/02/23	9:08	07/03/23	9:13	1	23.5	3.90	3.90	Example Data Entry	
TAM B1-4 (2023)		CHL23-01-05	23-3213	07/01/23	12:24	07/01/23	9:45	50.0	07/02/23	9:15	07/03/23	9:15	1	23.9	3.50	3.50	Example Data Entry	
TAM B1-4 (2023)	LD	CHL23-01-06	23-3213	07/01/23	12:24	07/01/23	9:45	50.0	07/02/23	9:21	07/03/23	9:17	1	23.9	2.67	2.67	Example Lab Dup Data Entry	
TAM B1-4 (2023)	LB	CHL23-01-07	NA			07/01/23	9:45	50.0	07/02/23	9:30	07/03/23	9:19	1	24.1	0.03	0.03	Example Lab Blank Data Entry	