# **Standard Operating Procedure**

Chlorophyll *a* Analysis

by EPA Method 445 with the Welschmeyer modification

Prepared by:

Watershed Planning Program

Division of Watershed Management, Bureau of Water Resources

Massachusetts Department of Environmental Protection

**Commonwealth of Massachusetts**

**Executive Office of Energy and Environmental Affairs**

Rebecca L. Tepper, Secretary

**Massachusetts Department of Environmental Protection**

Bonnie Heiple, Commissioner

**Bureau of Water Resources**

Kathleen M. Baskin, Assistant Commissioner

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| Prepared by: |  | Date: |  |
|  | Suzanne Flint, QA Analyst |  |  |
| Reviewed by: |  | Date: |  |
|  | Joan Beskenis, Aquatic Ecologist |  |  |
| Approved by: |  | Date: |  |
|  | Shervon De Leon, Monitoring Coordinator |  |  |

Massachusetts Department of Environmental Protection

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Watershed Planning Program

The Watershed Planning Program is a statewide program in the Division of Watershed Management, Bureau of Water Resources, at MassDEP. We are stewards of the water resources of Massachusetts. Together with other state environmental agencies, we share in the duty and responsibility to protect, enhance, and restore the quality and value of the waters of the Commonwealth. We are guided by the federal Clean Water Act and work to secure the environmental, recreational, and public health benefits of clean water for the residents of Massachusetts. The Watershed Planning Program is organized into five Sections that each have a different technical focus under the Clean Water Act: (1) Surface Water Quality Standards; (2) Surface Water Quality Monitoring; (3) Data Management and Water Quality Assessment; (4) Total Maximum Daily Load; and (5) Nonpoint Source Pollution.

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Contact Information

Watershed Planning Program, Division of Watershed Management, Bureau of Water Resources

Massachusetts Department of Environmental Protection

8 New Bond Street, Worcester, MA 01606

Website: <https://www.mass.gov/guides/watershed-planning-program>

Email address: [dep.wpp@mass.gov](mailto:dep.wpp@mass.gov)

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This report is available by contacting: [dep.wpp@mass.gov](mailto:dep.wpp@mass.gov)

1. 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.

1. 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).

1. SAFETY CONSIDERATIONS

General lab safety procedures should be followed (CN 000.35 – SOP Laboratory Safety at 8 New Bond). 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.

1. 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 35.0 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).

1. 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)

1. 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.

1. 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 is in place in the top of the Trilogy
* On the home screen touch “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 informaiton 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.

1. 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.
   1. The first sample should be a QC reading of the solid standard.
   2. 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).
   3. 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

1. Shake the field sample gently 25-30 times to mix completely.
2. 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.
3. 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.
4. Pour approx. 15 mL of measured sample into the filtration apparatus and turn on the vacuum. Continue pouring sample until all is filtered.
5. 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.
6. 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.
7. 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.
8. 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

1. 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.
2. 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.
3. Clean the graduated cylinder with three tap water rinses followed by a DI rinse.
4. 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.
5. 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

1. 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.
2. 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.
3. 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.
4. Rinse the graduated sample cylinder and filter holder several times in de-ionized water.
5. 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

1. 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.
2. 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 change from procedure. A decision will be made by the project manager whether to accept the data.

1. 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.
2. Allow the tubes to come to room temperature (approximately 30 minutes).

Reading the Samples

1. 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).
2. Click on the Trilogy icon on the computer desktop and then 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.
3. 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.
4. 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.
5. 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.)
6. Measure the temperature of the remaining sample in the centrifuge tube.
7. Record the chlorophyll reading, reading time, and sample temperature on the data sheet.
8. Save the Trilogy Excel spreadsheet to designated file.
9. Copy data from the bench sheet to the computer file. The spreadsheet name for each date should be: CHL\_year\_batch#.
10. Record the amount of acetone waste produced that day on the waste generated sheet attached to the post opposite the flammable cabinet.

Data Verification:

1. 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.
2. If any edits are made they should be initialed and dated. The bench sheet should be kept in the chlorophyll lab binder.
3. 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 Flammable 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 Flammable 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.

1. 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%.

RPD= (sample- duplicate)/(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 MDL for the method is 1 ug/l. Readings less than 1 are reported as <1 ug/L.

1. 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).

1. 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.

1. 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.

1. 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.

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1. 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)