Standard Operating Procedure for:
Modified Fluorometric Determination of Chlorophyll *a* (Free of Phaeophytin) Using Narrow Bandpass Filters

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1. SCOPE AND APPLICATION

1.1. This method is based on EPA Method 445.0 and is used to determine the amount of chlorophyll *a* in marine and freshwater algae. Chlorophyll *a* is calculated as "free of phaeophytin" by using a special narrow bandpass filter in this modified fluorometric method.

1.2. This procedure is used for samples with LIMS Test ID CHL-MDFL-W.

1.3. Method Detection Limits (MDL)

1.3.1. The chlorophyll *a* MDL is 0.060 µg/L for a filtered sample volume of 1000 mL with an extract volume of 13.5 mL. The practical quantitation limit (PQL) is calculated at 3 times the MDL.

2. SUMMARY OF METHOD

A sample is vacuum filtered onto a glass fiber filter. The filter is then macerated with a bead mill homogenizer or tissue grinder and steeped in 90% acetone to extract chlorophyll from the algal cells. The sample is clarified through centrifugation. The fluorescence of the clarified extract is then measured using a fluorometer with special narrow bandpass filters at an excitation wavelength of 436 nm and an emission wavelength of 680 nm.

2.1. Interferences

2.1.1. Chlorophyll pigment is sensitive to heat and light. To avoid degradation, extraction and fluorometer readings are performed in a dark room using subdued lighting only. The grinding tube, or centrifuge tubes, are kept on ice between samples, or between bead disruption cycle, to minimize heat from friction during extraction. Filters are then wrapped in foil and stored at -20º.

Note: Chlorophyll samples are collected in brown, opaque bottles, stored on ice during shipment, and filtered upon receipt.

2.1.2. Fluorescence is temperature sensitive. Fluorescence decreases as the sample temperature increases. Blanks, standards, and all samples are analyzed at ambient temperature. Using the instrument's Discrete Sample Averaging feature ensures that samples remain inside the unit for the same period of time and are therefore approximately the same temperature when measured. Recalibrate the fluorometer if the ambient temperature fluctuates ± 3 ºC between calibrations. Calibrate bimonthly or if there are any changes to the instrument. If recalibration is not required based on temperature fluctuations, check the solid standard or 200µg/mL working standard to ensure that it is passing. If not, recalibrate the instrument.

2.1.3. Any compound that absorbs light between 630 and 665 nm may interfere with chlorophyll measurement. The spectral overlap of chlorophyll *a*, *b*, *c* and

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phaeophytin can cause over or underestimation of chlorophyll. The amount of chlorophyll *b* and *c* in a sample is dependent on the taxonomic composition of the phytoplankton it contains. Special narrow bandpass filters are used to nearly eliminate the interferences of chlorophyll *b* and phaeophytin *a.* This modified fluorometric method does not provide an estimate of phaeophytin *a.*

2.2. Definitions

2.2.1. Subdued Lighting – Only red bulbs are used in subdued lighting. All other lights are turned off. This helps minimize chlorophyll degradation caused by exposure to light.

2.2.2. Fluorescence – A fluorescent molecule has the ability to absorb light at one wavelength and almost instantly emit light at a new and longer wavelength. Any fluorescent molecule, including chlorophyll *a*, has a unique "fluorescent signature."

2.2.3. Narrow Bandpass Filter – This device passes wavelengths within a certain range and screens out wavelengths outside of that range. The excitation filter specific to chlorophyll *a* allows a small band of light energy, from 431nm to 441nm, to pass through the sample extract. The emission filter for detecting chlorophyll *a* screens out stray light and background material fluorescence allowing only wavelengths from 675nm to 685nm to pass through to the detector.

2.2.4. Modified Fluorometry – By using narrow bandpass filters, this fluorometric method does not include the acidification process. The "Uncorrected" chlorophyll *a* calculations from EPA Method 445 Section 12.1 are used. Since the interference of phaeophytin *a* is selected against by filters, acidification is not needed and the chlorophyll *a* result is considered free of phaeophytin. Therefore, no correction for phaeophytin is necessary.

3. EQUIPMENT AND SUPPLIES

3.1. Filtration

3.1.1. Vacuum filtration system (See Figure 12.1)

3.1.2. Whatman GF/F 47mm Glass Fiber Filters

3.1.3. Small roll of aluminum foil, cut in 3-inch squares

3.1.4. Class A TD (to deliver) 1000 mL Graduated Cylinder

3.1.5. 2 Class A TD 500 mL Graduated Cylinders (for duplicates)

3.1.6. Extra Fine tip black marker

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3.1.7. Rinse bottle

3.1.8. Rubber bands

3.1.9. Plastic zip-top bag

3.1.10. Aluminum foil, cut into approximately 4×6 inch, rectangular pieces

3.2. Extraction

3.2.1. 15 mL centrifuge tubes with screw caps (Omni brand preferred)

3.2.2. Disruption beads, 1.0mm, glass

3.2.3. Omni Bead Ruptor Elite (S/N 00162529)

3.2.4. Bead Ruptor Elite 15 mL Tube Carriage & Finger Plate

3.2.5. Test tube rack

3.2.6. Scissors

3.2.7. Eberbach Tissue Grinder motor

3.2.8. Tissue Grinder with 30 mL tube

3.2.9. Container with ice

3.2.10. Rinse bottle

3.2.11. Fume hood in dark room

3.2.12. Cut resistant gloves

3.2.13. Eppendorf Repeater E3 pipette (I57847H), calibrated per SOP MB-03.3

3.3. Fluorometric Analysis

3.3.1. Megafuge ST4R PLUS-MD, 120V Centrifuge (S/N 00159655)

3.3.2. Turner Designs TD- Trilogy (S/N 721000163)

3.3.3. Fluorescence module CHL-A NA (Module #7200-046)

3.3.4. PC with Turner Designs software

3.3.5. 13mm x 100 mm glass cuvettes

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3.3.6. Eppendorf Research Plus 1-5 mL pipette with tips (K45340J or I21192N), calibrated per SOP MB-03.3, or glass serological disposable pipettes, sterile and individually wrapped, with pipette pump, 10 mL (volume of each new lot is checked per SOP MB-03.3).

3.3.7. Kimwipes

3.3.8. Talosys 24-hour temperature monitoring probe, S/N LA666049770.

3.3.9. Gloves, latex, or nitrile

4. REAGENTS AND STANDARDS

4.1. Deionized Water

4.2. Acetone, HPLC grade

4.3. Aqueous Acetone Solution (90% acetone)

4.3.1. Add 900 mL of Acetone (HPLC grade) to 100 mL of deionized water.

4.3.2. To record preparation, go to **LIMS Laboratory\Technical\Standard Preparation Tracker**. Choose "BIO-MICRO" **Group**. Enter it as a **Prepared** "New Reagent" or "Duplicate Reagent". (Duplicate Reagent can be used to copy an old record. New Reagent will have all of the fields blank.) Print a label and attach to the container. Print date prepared, expiration date, and analyst initials on the label.

4.4. Turner Solid Secondary Standard

4.5. Sigma C5753 Chlorophyll *a* from spinach, 5 mg

4.6. Chlorophyll *a* Stock Standard

4.6.1. Prepare under subdued lighting. Tap the ampoule of chlorophyll *a* spinach until all the powder has settled to the bottom. Break open the tip and empty contents into a Class A 25 mL volumetric flask containing about 10 mL of 90% acetone. If necessary, rinse ampoule thoroughly with 90% acetone into the volumetric flask. Dilute to volume with 90% acetone. Add a small stir bar and mix thoroughly. Transfer standard to a dark glass bottle, label as "Chlorophyll *a* Parent Stock" with the preparer's initials, date prepared and expiration date and store in biology lab freezer.

Note: Be careful when preparing stock solution. The entire contents of the spinach ampoule must be emptied into the flask. Any spill can alter the concentration of the stock solution. Do not touch the top of the broken ampule. The edges can be very sharp.

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4.6.2. To record preparation, go to **LIMS Laboratory\Technical\Standard Preparation Tracker**. Choose BIO-MICRO" **Group**. Enter it as a **Prepared** "New Standard" or "Duplicate Standard". (The Duplicate Standard can be used to copy an old record. A New Standard will have all of the fields blank.) Print a label and attach to the bottle.

4.6.3. Concentration of parent stock standard is 200 mg/L. The expiration date is 9 months from the date of preparation.

4.7. Chlorophyll *a* Working Calibration Standards

4.7.1. 200 µg/L standard (Working Std): Pipette 0.20 mL of chlorophyll parent stock standard to a 200 mL Class A graduated cylinder. Bring up to volume with 90% acetone and mix thoroughly. Transfer to a dark glass bottle, label as "Chlorophyll *a* Working Std" with the concentration, preparers initials, date prepared, and expiration date. Store in biology lab freezer. The expiration date is 3 months from the date of preparation. Note: This standard is also used for SOP BB-29.

4.7.2. 20 µg/L standard: Pipette 2 mL of the 200 µg/L standard into a Class A graduated cylinder and bring up to a volume of 20 mL with 90% aqueous acetone solution. Transfer to a dark glass bottle and label with concentration, preparers initials, date prepared, and expiration date.

4.7.3. 5 µg/L standard: Pipette 5 mL of the 20 µg/L standard into a Class A graduated cylinder and bring up to a volume of 20 mL with 90% aqueous acetone solution. Transfer to a dark glass bottle and label with concentration, preparers initials, date prepared, and expiration date.

4.7.4. 2 µg/L standard: Pipette 8 mL of the 5 µg/L standard into a Class A graduated cylinder and bring up to a volume of 20 mL with 90% aqueous acetone solution. Transfer to a dark glass bottle and label with concentration, preparers initials, date prepared, and expiration date.

4.7.5. 0.2 µg/L standard: Pipette 2 mL of the 2 µg/L standard into a Class A graduated cylinder and bring up to a volume of 20 mL with 90% aqueous acetone solution. Transfer to a dark glass bottle and label with concentration, preparers initials, date prepared, and expiration date.

4.7.6. All standards should be recorded in **Standard Preparation Tracker**. Enter the pipette QC ID into the comments section.

Note: Be as precise as possible when measuring. Rinse the graduated cylinder at least 3 times. Use a new pipette tip for each standard concentration. The fluorometer is only as accurate as the standards used to calibrate it.

5. SAMPLE COLLECTION, PRESERVATION, AND HANDLING

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5.1. See FDEP Field SOP FS 1000 for sample collection and preservation.

6. SAMPLE PREPARATION

Refer to SOP BB-30 for sample custody, labels and worksheet instructions.

6.1. Chlorophyll Filtration

6.1.1. Shake sample bottle to ensure sample is thoroughly mixed. Use a clean, well rinsed, Class A graduated cylinder to measure 1000 mL of sample. Filter through a GF/F glass fiber filter. If sample is highly turbid and difficult to filter, do not filter the entire 1000 mL volume. A smaller volume may be used as long as the filter is sufficiently loaded with algae (ex. filter is visibly green or water flow has slowed or stopped). Do not allow the vacuum to suck the filter dry. Slowly turn off the vacuum as the sample water approaches the filter and turn it off completely when the filter is nearly dry.

6.1.2. Rinse the funnel with deionized water (about 10-20 mL). Turn the vacuum back on, and when the rinse water approaches the filter, slowly turn off the vacuum until the filter is nearly dry. Rinse the funnel and graduated cylinder a total of 3 times. Remove and fold the filter in half with the algae facing in, using the filtrate edge as a guide (not the filter edge). Blot gently with a Kimwipe to remove excess liquid, if necessary. Wrap the folded filter completely in aluminum foil. Place the "A" prep label on the foil and write the filter volume on it.

6.1.3. If there is a "B" label for a sample, repeat steps 6.1.1 and 6.1.2 for the "B" label. Use equal volumes for "A" and "B" aliquots of replicate samples.

6.1.4. Repeat steps 6.1.1 - 6.1.3 for each sample. Filter a laboratory blank **after** every 20 samples and at least one per day and one duplicate for every batch of 20 samples. If less than 20 samples are received in the day, filter a blank and one duplicate. Wrap the blank filter in foil and label as "BLK_WS#-#".

6.1.5. When all samples are complete, record filter volumes, filtration date/time, analyst, blanks, and dups in **LIMS Laboratory\Technical\Prep Worksheet Manager** per SOP BB-30. The LIMS record number for the GF/F filters and grinder (if used) are also added to the worksheet and are found in **LAB_SUPPLIES_LOG**. Use a rubber band to bind the samples together, place the samples in a plastic zip-top bag, squeeze the air out of the bag and seal it. Label the plastic bag with the date & worksheet number and store in the biology lab walk-in freezer at -20°C. Holding time for frozen samples is 28 days from time of filtration until extraction. Record the time that samples are placed in the freezer in **Prep Worksheet Manager**.

6.1.6. Discard unused sample in the sink unless it is suspected of being hazardous waste. If suspected of being hazardous waste, notify the safety officer and supervisor for

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proper disposal of the sample. Place caps on the empty bottles and put them in a garbage bag with the filter date visible on the exterior for discard purposes.

6.2. Chlorophyll Batch Preparation

Using the Prep Worksheet as a guide, create batches of up to 24 samples for analysis. Combine worksheets from different days as necessary. Batches should be labeled sequentially with a number 1-10. After the 10th batch, repeat the sequence again, starting with #1. One batch will have up to 24 samples (including a blank and any duplicates of the 22 samples) for analysis. If performing extraction via grinding, adjust the number of samples that are in a batch.

Note: Samples that are filtered by another facility should be batched with a filtered blank and duplicate per 20 samples provided by the facility sending the samples. Method blanks and duplicates from the DEP lab cannot be used.

If there are priority 1 samples, place them in the first available batch. Otherwise, create batches in the order they appear in prep worksheets.

6.2.1. Copy the prep worksheet batches into the *Chlorophyll Spec Run Log*.

6.2.1.1. Write the batch number and "MDFL" at the top of the Run Log page.

6.2.1.2. Print sample labels and add stickers to the "Sample Label" column for Tube #1- up to 23, or up to 21 for samples processed via grinding.

6.2.1.3. Line # 24 is for the duplicate "dup" sticker. Complete this row after extracting a "B" sample. In the Chlorophyll Spec Run Log for extraction via grinding, the "dup" sample will be in line 22.

6.2.1.4. The blank is added to the Chlorophyll Spec Run Log page in the order it appears on the corresponding Prep Worksheet Manager worksheet. However, the blank cannot be the first sample in the run log. The sample order can be switched to accommodate for the blank.

6.2.1.5. Write "B" volumes at the bottom of the page next to the tally marks. (i.e. B=500). If using the grinder spec run log, write "B" in the "comments" column in the corresponding row or on the label itself.

6.2.2. Using the *Chlorophyll Spec Run Log*, retrieve corresponding samples from the freezer to create packets for extraction.

6.2.2.1. For each batch, place "A" samples and a blank in the order they appear in the run log/worksheet, followed by any "B" samples from that batch. Wrap in foil and label the packet with batch number. Place the batches in ascending numerical order in a plastic bag removing as much air as

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possible. Place the sealed bag in the appropriately labeled plastic bin in the Biology lab freezer until extraction.

7. SAMPLE ANALYSIS

7.1. Extraction with bead homogenizer.

7.1.1. Pour 2 x 0.5 mL scoops (approx. 1.2 – 1.5 g) of disruption beads into each centrifuge tube (24 tubes per batch). Ensure each tube is clearly labeled.

7.1.2. Retrieve the chlorophyll packet associated with the first available batch in the Chlorophyll Spec Run Log from the biology freezer. Record extract date/time/analyst in the Chlorophyll Spec Run Log. Fill in the Run ID. The format is YYYY-MM-DD-A. The last character is an A for the first batch of the day, B for the next batch and so forth.

7.1.3. In subdued lighting, unwrap a sample filter from foil and make sure sample information matches the Chlorophyll Spec Run Log. Fold the filter in half and place the folded glass fiber filter into a centrifuge tube containing disruption beads.

7.1.4. Use Aqueous Acetone Solution (90% acetone) solution to bring the volume of the tube up to 14 mL and cap.

7.1.5. Repeat steps 7.1.3-7.1.4 for each sample until the entire batch is prepped.

7.1.6. Prior to extraction, fill one small sterilizing pan with ice for cooling. Make sure the centrifuge tubes are tightly sealed. In subdued lighting, place the first half of the tubes (12 tubes) in the bead homogenizer, select “Chlorophyll-A” program and homogenize for 20s at 4 m/s in the bead homogenizer.

7.1.7. Remove sample tubes from the homogenizer and place them in the pan with ice to chill for a minimum of 45s. Make sure the centrifuge tubes are tightly sealed.

7.1.8. Load the second half of tubes and homogenize using the same settings as step 7.1.6.

7.1.9. Repeat steps 7.1.6-7.1.8 until each half has had 5 cycles of bead beating. Retighten the caps after each cycle of bead disruption and for each cycle, make a tally on the logbook for each half of tubes until 5 cycles for each half are complete.

7.1.10. Cover the tube rack to avoid light exposure and allow samples to steep at 4 °C for a minimum of 2 hours. Do not exceed 24 hours. Shake tubes at least once and up to three times while steeping.

7.2. Extraction with tissue grinder

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7.2.1. Retrieve the chlorophyll packet associated with the first available batch in the *Chlorophyll Spec Run Log* from the biology lab walk-in freezer. Record extract date/time/analyst and reagent serial numbers in the *Chlorophyll Spec Run Log*. Fill in the Run ID. The format is YYYY-MM-DD-A. The last character is an A for the first batch of the day, B for the next batch and so forth.

7.2.2. In subdued lighting, unwrap sample filter from foil and make sure sample label information matches the *Chlorophyll Sepc Run Log*. If needed, trim the filter above the filtrate line to remove excess filter (See Figure 12.2). To avoid losing sample, do not cut too close to the filtrate line. Place folded filter flush against the inside of the grinding tube. Add 2-3 mL of 90% aqueous acetone solution.

7.2.3. While wearing cut resistant gloves, grind the filter into a slurry solution without allowing the sample to overheat. This may take up to 1 minute. Grooves may be cut into the bottom of the pestle to facilitate grinding. During extraction keep the grinding tube in ice water when not in use to minimize overheating from friction. Rinse the pestle and the walls of the tube with 1-2 mL of 90% aqueous acetone solution collecting the rinse in the tube.

7.2.4. Transfer mixture to the corresponding numbered centrifuge tube. Centrifuge tube numbers can be found in the *Chlorophyll Spec Run Log*. Use aqueous 90% acetone solution to rinse any remaining sample in the grinding tube and on the grinding pestle into the centrifuge tube. Rinse the grinder and tube at least three times. Rinse the centrifuge tube wall, bring the final volume up to 14 mL, and cap the tube. Record extract volume in the *Chlorophyll Spec Run Log*.

7.2.5. Repeat steps 7.2.2-7.2.4 for each sample.

7.2.6. Shake each tube vigorously, and then cover centrifuge tube rack to avoid light exposure. Allow samples to steep at 4 °C for a minimum of 2 hours. Do not exceed 24 hours. Shake tubes vigorously 1-3 times during the steeping period. Record the start and end steep time/date in the *Chlorophyll Spec Run Log*.

7.3. Instrument Set up and Calibration

7.3.1. Perform an instrument calibration bimonthly, when there are any instrument changes or maintenance, or if the room temperature fluctuates ±3° C from the temperature of the previous calibration. Use the primary working standards made in step 4.7. to calibrate the fluorometer. If an instrument calibration is not needed, skip to step 7.4.2.

7.3.2. Turn on the fluorometer by locating the toggle switch on the back of the instrument.

7.3.3. Turn on computer and open the TD Trilogy Excel software.

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7.3.4. Select **CHL-NA** module for the chlorophyll without acidification method and select "**OK**".

7.3.5. Select, "**Calibrate**" on the home screen.

7.3.6. Prior to running a new calibration make sure there is enough space to save it by selecting "**Use Stored Calibration**," and if there aren't any free spaces, select the oldest one and delete.

7.3.7. Go back and select "**Run New Calibration**."

7.3.8. Select **ug/L** for the unit of measurement.

7.3.9. Using a clean pipette tip, pipette 3.0 ml of 90% acetone into a cuvette. Wipe the sides completely removing dust and smudges. Insert the blank. Gently push the cuvette to the bottom. Close the lid and select "**OK**."

7.3.10. After the blank has been measured, remove it and discard into acetone waste. Drain excess liquid out of the cuvette.

7.3.11. Enter **0.2 ug/L** for the first standard and select "**OK**".

7.3.12. Pipette 3.0 ml of the prepared standard from section 4 into the cuvette. Use a Kim Wipe to wipe the cuvette clean and place it into the sample compartment.

7.3.13. Close the lid and select "**OK**" to measure the standard.

7.3.14. Rinse the cuvette with 90% acetone at least 3 times and drain excess liquid from the tube.

7.3.15. Select "**Enter More Standards**" and repeat steps 7.3.9-7.3.12 until all standards have been measured. The maximum number of standards is 5.

7.3.16. Once all standards have been measured, select "**Proceed with Current Calibration**."

7.3.17. Save the calibration with the current date.

7.3.18. Immediately after calibration, measure the secondary solid standard.

7.3.19. Remove the sample adapter and place to the side.

7.3.20. Insert the secondary solid standard. Ensure that the mode is in "**Direct Concentration**" and select "**Measure Fluorescence**"

7.3.21. Enter 1 for both filter and extraction volumes.

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NOTE: There is no way to verify that this information was entered correctly. The screen will briefly show the values entered.

7.3.22. This is the "True" value, and it will be used as a quick reference before running the analysis to ensure that the instrument is working properly.

NOTE: Never adjust the level of the solid standard.

7.3.23. To view calibration details from the home screen, select "**Calibrate**". Then select "**Use stored calibration**". Select the saved calibration and select "**View Calibration Details**".

NOTE: This model does not automatically export the calibration report. You must use the calibration calculator located in folder: \\floridadep\data\LABS\Lab5\Biology\BenchCommon\CHL\Fluorometer\TD Trilogy calibration

7.3.24. Enter the blank value into the corresponding fields for each standard. Enter measurements of each standard in RFU. The calculator will calculate a slope %RSD. This value should be preferably less than 5%; however, less than 25% is considered acceptable. Re-calibrate if greater than 25%.

NOTE: The instrument is only as accurate as the calibration. Do not analyze samples until the instrument is properly calibrated.

7.3.25. In the top right corner of the file, enter the true value of the solid secondary standard, the serial number of the standard, and the current temperature of the room. Also enter the analyst's initials and date.

7.3.26. The temperature of the room at the time of calibration is the new reference temperature.

7.3.27. Save the file as "MM-DD-YY_" followed by the analyst's initials.

7.4. Fluorometric Analysis

7.4.1. Check the temperature of the room to determine if an instrument calibration should be performed. If an instrument calibration is needed, follow the steps in section 7.3. If a calibration is not needed, continue to the next step.

7.4.2. Shake sample tubes vigorously and centrifuge samples for 20 minutes at 3000 rpm. Decant supernatant from each sample into a clean centrifuge tube with matching tube number.

Note: Supernatant of samples extracted with the bead homogenizer get centrifuged right after decanting for an additional 5 minutes at 3000 rpm.

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7.4.3. Open the TD Trilogy Excel file. Select **COM port 3**. Click "**OK**" and start.

7.4.4. Choose the appropriate saved calibration data. The measurement screen will show the name of the file in the top right corner of the screen.

7.4.5. Select mode to ensure that "**Direct Concentration**" is in use. Do not use Raw Fluorescence for measuring samples.

7.4.6. Remove the sample adapter, and place the secondary solid standard into the module. Ensure it is pushed to the bottom of the module.

7.4.7. Select "**Measure Fluorescence**".

7.4.8. Enter **1** for both filtration and extraction volumes.

7.4.9. The value is automatically sent over to the Trilogy Excel file. Calculate the % recovery using this measurement and the measurement taken immediately after calibration. The acceptance limit is 85-115%. If it is outside this range, determine the cause and repeat calibration.

7.4.10. Return the sample adapter to the module, and close the lid.

7.4.11. Ensure that the cuvette has been rinsed 3 times. Remove excess liquid. Then, pipette 3.0 ml of the sample into the cuvette. Remove smudges and dust from the outside of the cuvette with a Kim Wipe. Place the cuvette into the sample adapter and ensure it is pushed down to the bottom. **NOTE:** The cuvette must be pushed all the way down or else the measurements will be erroneous.

7.4.12. Select "**Measure Fluorescence**".

7.4.13. Enter **1** for both extraction and filtration volumes. The screen will show the values entered while reading the sample. This information is not stored anywhere so pay close attention.

7.4.14. If the measurement is greater than 200 ug/L, a dilution must be performed. Start with a 50% dilution by pipetting 3.0 ml of acetone to 3.0 ml of sample in a separate tube. Use the 50% dilution for further dilutions if necessary. Example: If you get a reading of 400 ug/L, perform a 50% dilution. If the measurement is 205 ug/L, for example, then perform another 50% dilution. The final reading must be less than 200 ug/L.

7.4.15. Make a note of dilutions in the comments section of the run log. Ensure filter volumes are adjusted appropriately before uploading data.

7.4.16. Remove the sample. Rinse the cuvette 3 times with 90% acetone, and remove excess liquid.

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7.4.17. Repeat steps 7.4.11 to 7.4.14 until all samples and method blanks have been analyzed.

7.4.18. The following calculation can be used as a quick check to ensure that the method blanks are within passing limit. (Extraction volume * direct concentration)/filter volume. This value should be less than or equal to 0.060 ug/L.

7.4.19. Rerun all failing method blanks. If they are still failing, run 90% acetone to ensure the instrument has not drifted.

7.4.20. If all quality controls (QC) meet the requirements in section 9, empty the centrifuge tubes into the acetone waste container. Do not discard any samples until all QC has been checked and the samples do not need to be re-read due to QC failure.

8. DATA ARCHIVAL

8.1. Save the TD Trilogy Excel file as the Run ID in \\floridadep\data\LABS\Lab3\Biology\Chlorophyll\TD TRilogy.

8.2. Upload data using LIMS **QC Manager**. See SOP BB-031 for verification and upload instructions.

8.3. After verification and upload, refer to SOP BB-032 for data authorization.

8.4. Calculations for Phytoplankton samples:

$$\text{Chlorophyll } a \text{ free of phaeophytin } (\mu g/L) = \frac{[(C_e) \times V_1 \times DF]}{V_2}$$

where: C_e = chlorophyll concentration of extract solution
V_1 = volume of extractant (L)
DF = dilution factor
V_2 = volume of sample filtered (L)

9. QUALITY CONTROL

9.1. Continuing Calibration Verification and positive control (CCV)

9.1.1. Analyze the solid secondary standard at the beginning of every batch to verify calibration. Record the standard number in the appropriate box at the top of the *Chlorophyll Spec Run Log* page.

9.1.2. If solid standard is not available, analyze the 200 ug/L calibration standard along with the positive control as described in Section 9.3.

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9.2. Negative Control

9.2.1. Filter 1000 mL of deionized water for a laboratory blank after every 20 samples filtered, or at least one per day when there are less than 20 samples available.

9.2.2. Analyze the laboratory blank in the order it appears in the *Chlorophyll Spec Run Log*.

9.2.3. The acceptance limit is ≤ 0.060 µg/L (method detection limit).

9.2.4. Corrective Action: If a blank failure occurs, return the blank sample to the centrifuge tube, rinse the cuvette thoroughly with 90% aqueous acetone, and then re-read the blank. If this does not correct the problem, run a 90% acetone sample to help determine whether the problem is caused by instrument malfunction or blank contamination. If there was an instrument malfunction, correct the problem and then re-read the samples.

9.3. Positive Control

9.3.1. If secondary solid standard is not available, as a laboratory control sample (LCS), analyze the 200 ug/L chlorophyll *a* working standard at the beginning of every batch during fluorometric analysis.

9.3.2. Record the working standard serial number in the Standard S# box on the *Chlorophyll Spec Run Log* page.

9.3.3. Calculate percent recovery of LCS as follows:

$$\%\,\mathrm{Rec} = \frac{(C_e)}{TV} \times 100\%$$

where: C_e = chlorophyll concentration of extract solution
TV = True value of the chlorophyll standard

9.3.4. The acceptance limit for recovery is 85-115%.

9.3.5. If the recovery exceeds the limits, re-calibrate the fluorometer.

9.3.6. Corrective Action: If standard has repeated low failures, it is possible that the standard was exposed to heat or light over time and has degraded. Make a new batch of working standard. If standard continues to fail, the instrument may need to be re-calibrated.

9.4. Sample Duplicates

9.4.1. Analyze a laboratory replicate for 5% of samples. The acceptance limit is ≤25% RPD.

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9.4.2. Corrective Action: Calculate the RPD for the duplicates. If they are not within the acceptance limit, re-run both duplicates at the end of the batch. Record the RPD at the bottom of the *Chlorophyll Spec Run Log.*

10. SAFETY/HAZARDOUS WASTE MANAGEMENT

10.1. Do not pour acetone or any reagent/standard containing acetone down the sink. Pour any waste acetone in the waste flask located in the chlorophyll room sink. When this is full, cap it and label it with "Acetone Waste, Hazardous Waste," amount, date generated, and initials. Follow the Health and Safety plan for proper disposal of full waste acetone bottles.

10.2. The extraction process which involves filter grinding should take place under a fume hood since the tissue grinder causes volatilization of acetone.

10.3. The tissue grinder fits tightly inside the grinder tube and can become stuck if not altered before use. This can cause the grinder and tube to heat up and increase the risk of glass breakage. Be sure to reduce the size of new tissue grinders using sandpaper before using for the first time.

10.4. Refer to the Health and Safety Plan.

10.5. Read MSDS information for reagent handling and disposal.

11. REFERENCES

11.1. Standard Methods 10150 B-2022

11.2. EPA Method 445.0 Revision 1.2

11.3. NELAC Quality Systems Manual

11.4. TD-Trilogy Fluorometer Operating Manual

11.5 FDEP Laboratory Quality Manual https://floridadep.gov/dear/florida-dep-laboratory/content/dep-laboratory-quality-assurance-manual-and-sops

11.6. FDEP Laboratory Health and Safety Plan https://www.floridadep.net/resources/health-safety-manual.

11.7. DEP SOP BB-030: *Sample Custody, Preparation Labels and Worksheet Instructions for Bench Biology Samples*

11.8. DEP SOP BB-031: *LIMS QC Manager Data Uploading Instructions for Bench Biology and Microbiology Samples*

11.9. DEP SOP BB-032: *Test Level Authorization of Uploaded Results for Bench Biology Samples*

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11.10. DEP SOP FS1000: *General Sampling Procedures*

11.11 Omni Bead Ruptor Elite Manual \\floridadep\Data\DEAR\Labs\Lab5\Biology\BenchCommon\Equipment.

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12. TABLES, DIAGRAMS

Figure 12.1. Vacuum Filtration System

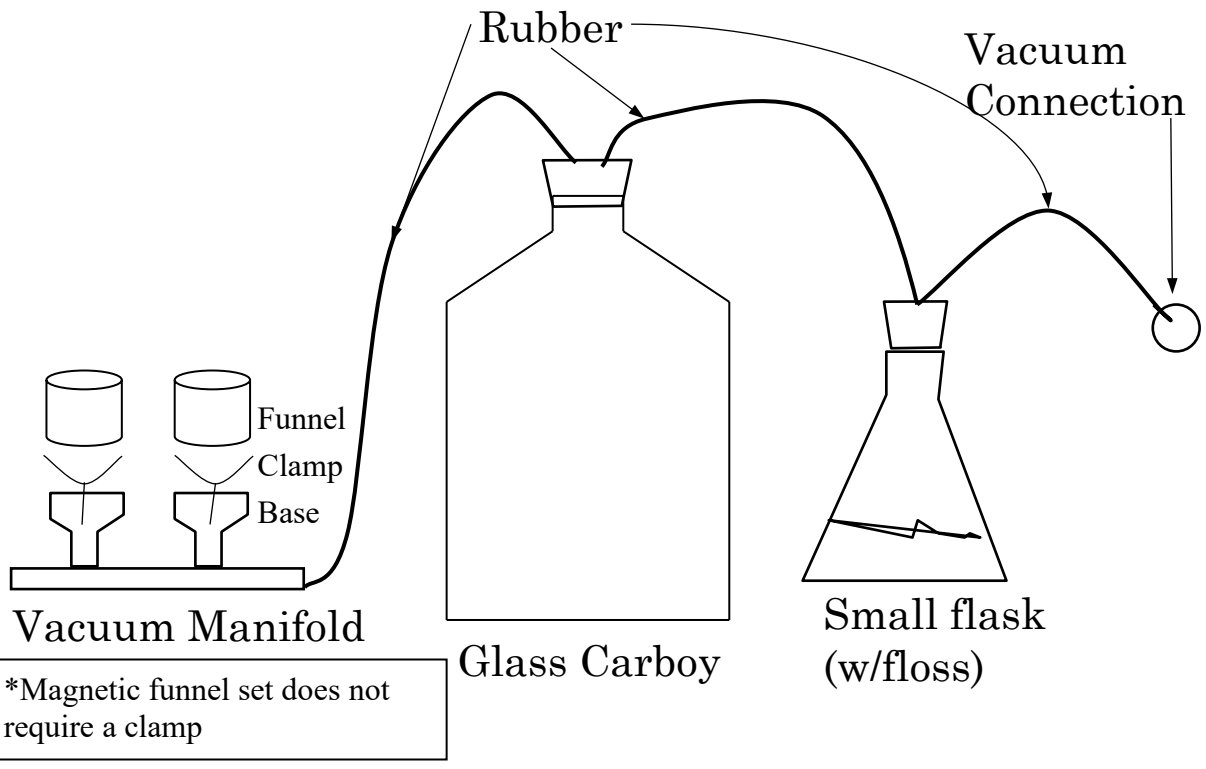
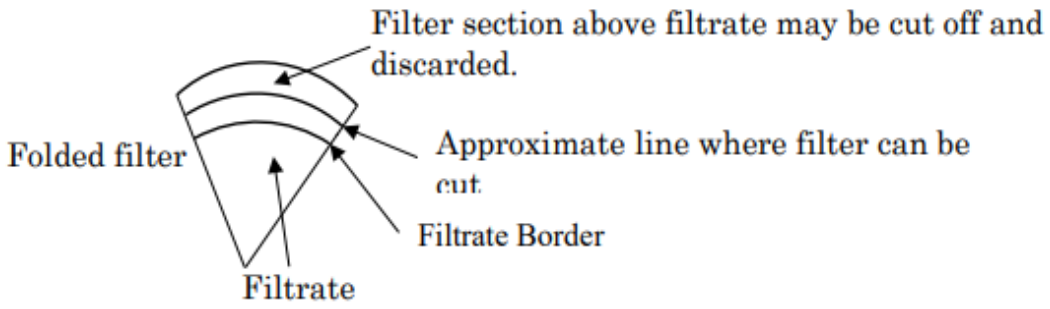


Figure 12.2 Filter trimming for extraction



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Appendix of Significant Changes

01/13/2010	This is a new SOP for fluorometric determination of chlorophyll. There is currently no active SOP for this procedure. I incorporated the three remaining SOPs relating to fluorometry into this document; BB28.2, BB28.3, BB28.4. CF
12/09/2010	Added section for CCV, updated reference to Health and Safety Plan, removed reference to $MgCO_3$ suspension which is not part of EPA Method 445.
12/06/2011	Updated information in Fig 12.1 to reflect new equipment. Updated reference to quality control manual.
12/18/2012	Updated chlorophyll calculation to use correct term for component.
12/12/2013	Updated filtration and extraction volumes. Updated MDL and PQL values.
08/06/2014	Added new LIMS chlorophyll manager data collection instructions.
08/19/2015	Added centrifuge to the equipment list; added LIMS Logbook for recording temperatures and Standard Prep Tracker for recording reagents; updated instructions for filling in the Chlorophyll Run Log; re-worded the Extraction section to more closely match the process in SOP BB-29; reformatted Section 7.3. Instrument Calibration; updated the file link in Sec 7.3.1.3; removed "approximately" from 7.3.3. and replaced with a defined limit; updated the network link in Section 7.3.; corrected the time and speed required for centrifuging the samples.
12/30/2016	Added instructions to include new information to be recorded on updated run log.
12/14/2017	Revised information in sections 6.3, 7.2, 7.3, 8.1, 9.2, and 9.3. Updated links to FDEP Laboratory Quality Manual and FDEP Laboratory Health and Safety Plan in References section.
01/03/2019	Updated new MDL. Revised sections including chlorophyll filtration; chlorophyll batch preparation; and fluorometric analysis. Updated link for FDEP Laboratory Quality Manual.01/03/2020 New instrument added.
01/27/2021	Clarified parent and working stock standard preparation instructions.
11/28/2022	Updated number of rinse bottle types in section 3.1. Removed reference to "Filter vol (mL)" column of Run Log. Updated instructions for handling glass fiber filters in sections 6.2.2 and 7.1.2. Updated link to FDEP Laboratory Health and Safety Plan.
12/12/2023	Updated equipment and supplies. Clarified how often laboratory blanks should be analyzed. Updated how temperature is recorded. Removed instruction to keep the cuvette oriented the same throughout run. Updated centrifuge speed. Clarified how to calculate percent recovery. Edited links in sections 7.2.23 and 8.2. Updated reference to standard method 10150-B 2022.
04/05/2024	Edited summary and equipment list to include the bead disruption method. Added extraction with bead homogenizer to section 7. Added reference and link to the

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	Omni Bead Ruptor Elite Manual. Added note to section 7.4 to mention an additional centrifuge cycle for samples extracted with the bead homogenizer. Updated links to the safety manual and the folder locations for fluorometer calibrations and runs. Clarified instructions to all sections in #2 and #6. Added section 3.3.9.
04/23/2025	Extraction volume updated to 13.5 mL. Revised the list of extraction equipment and supplies to include the Eppendorf Repeater E3 pipette. Updated fluorometer measurement terminology to 'Measure Fluorescence.' Set TD Trilogy Excel file option to COM Port 3.
04/23/2026	Updated section 7 to clarify method procedure. Removed CheckPoint temperature monitoring and room temperature logbook entries. Talosys has been added as the new temperature monitoring system, including probe information.

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