Standard Operating Procedure for:

Spectrophotometric Determination of Corrected and Uncorrected Chlorophyll *a* and Phaeophytin

DEPARTMENT OF ENVIRONMENTAL PROTECTION
BUREAU OF LABORATORIES
BIOLOGY SECTION
TALLAHASSEE FLORIDA

The OFFICIAL COPY is the on-line version. All other copies are considered unofficial and uncontrolled.

TABLE OF CONTENTS

1. SCOPE AND APPLICATION
2. SUMMARY OF METHOD
    2.1. Interferences
    2.2. Definitions
3. EQUIPMENT AND SUPPLIES
4. REAGENTS AND STANDARDS
5. SAMPLE COLLECTION, PRESERVATION, AND HANDLING
6. SAMPLE PREPARATION
    6.1. Chlorophyll Filtration
    6.2. Chlorophyll Batch Preparation
7. SAMPLE ANALYSIS
    7.1. Extraction
    7.2. Instrument Setup
    7.3. Spectrophotometric Analysis
8. DATA ARCHIVAL
9. QUALITY CONTROL
10. SAFETY/HAZARDOUS WASTE MANAGEMENT
11. REFERENCES
12. TABLES, DIAGRAMS
    Figure 12.1 Filter trimming for extraction

The OFFICIAL COPY is the on-line version. All other copies are considered unofficial and uncontrolled.

1. SCOPE AND APPLICATION

1.1. This method is based on Standard Methods 10200H and EPA Method 446.0. This method is used to determine the amount of chlorophyll *a* and phaeophytin *a* in marine and freshwater algae by visible spectrophotometry. Uncorrected chlorophyll *a* is calculated using the trichromatic equation. Corrected chlorophyll *a* and phaeophytin are calculated using the monochromatic equation. The absorption-peak-ratio (chlorophyll/phaeophytin) is also determined.

1.2. This procedure is used for samples with LIMS test IDs of CHL-CORR-W, CHLSUITE-W and CHL-CORR-P.

1.3. Method Detection Limits (MDL) at 500 mL filter volume with 8 mL extract volume for CHL-CORR-W and CHLSUITE-W:

1.3.1. Chlorophyll *a* corrected for phaeophytin: 0.55 µg/L

1.3.2. Uncorrected chlorophyll *a*: 0.40 µg/L

1.3.3. Phaeophytin *a*: 0.40 µg/L

1.4. Method Detection Limits (MDL) at 500 mL filter volume from 2 slides with 8 mL extract volume for CHL-CORR-P:

1.4.1. Chlorophyll *a* corrected for phaeophytin: 0.14 mg/m^2

1.4.2. Phaeophytin *a*: 0.11 mg/m^2

1.5. The practical quantitation limit (PQL) is calculated at 3 times the MDL for all three analyses.

2. SUMMARY OF METHOD

2.1. A sample is vacuum filtered onto a glass fiber filter. The filter is then macerated with a tissue grinder and steeped in 90% acetone to extract chlorophyll from the algal cells. The sample is clarified through centrifugation. The absorbance of the clarified extract is then measured on a spectrophotometer at 750, 665, 664, 647 and 630 nm wavelengths before and after a 90 second HCl acidification step.

2.2. InterferencesI

2.2.1. Chlorophyll pigment is sensitive to heat and light. To avoid degradation, extraction and spectrophotometer readings are performed in a dark room using subdued lighting only. The grinding tube is kept on ice between samples to minimize heat from friction during extraction. Also, chlorophyll samples are collected in brown, opaque bottles, stored on ice during shipment, filtered upon receipt, and the filters are wrapped in foil and stored in the freezer after filtration.

2.2.2. Any compound that absorbs light between 630 and 665 nm may interfere with chlorophyll measurement. The absorbance measurement at 750 nm is subtracted from the sample's other measured absorbance values (665, 664, 647, and 630 nm) to account for the turbidity of the clarified sample. If the

The OFFICIAL COPY is the on-line version. All other copies are considered unofficial and uncontrolled.

absorbance at 750 nm is greater than 0.007 AU, re-centrifuge the sample to further clarify the extract.

2.2.3. The spectral overlap of chlorophyll *a*, *b*, *c* and phaeophytin can cause over or underestimation of chlorophyll and/or phaeophytin. The amount of chlorophyll *b* and *c* in a sample is dependent on the taxonomic composition of the phytoplankton it contains. In the trichromatic equation, chlorophyll *a* may be overestimated in the presence of phaeophytin. In the monochromatic equation chlorophyll *a* may be slightly overestimated in the presence of chlorophyll *b* and phaeophytin may be overestimated in the presence of carotenoids.

2.3. Definitions

2.3.1. Subdued Lighting – Only red bulbs are used in subdued lighting. All other lights are turned off, and the door to the extraction room is kept closed. This helps minimize chlorophyll degradation from exposure to light.

2.3.2. Absorbance – A measurement of the amount of light at a specific wavelength absorbed by a liquid. Although absorbance has no true units, AU (Absorbance Units) is used for clarity in this SOP.

2.3.3. Path length – The path length is the width of the cuvette cell (length between optical non-frosted sides). For this method, 1 or 4 cm path length cuvettes can be used. The FDEP Biology Section currently uses a 1 cm path length reference cuvette and a 4 cm path length sample cuvette.

2.3.4. Trichromatic equation – Also known as, Jeffrey and Humphrey's Trichromatic Equations, they require absorbance values at 664, 647, and 630 nm to calculate the amount of uncorrected chlorophyll *a* in a sample. Chlorophyll *b* and *c* pigments can also be calculated. No acidification is required and phaeophytin cannot be calculated from this equation.

2.3.5. Monochromatic equation – Also known as Lorenzen's modified monochromatic equation, it requires the absorbance values of 664 and 665 nm before and after a 90 second acidification step to calculate the amount of chlorophyll *a* and phaeophytin in a sample. The chlorophyll *a* is reported as corrected for phaeophytin. Chlorophyll *b* and *c* cannot be calculated from this equation.

3. EQUIPMENT AND SUPPLIES

3.1. Filtration

3.1.1. Vacuum filtration system

3.1.2. Whatman GF/C 47mm Glass Fiber Filters

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

3.1.4. 500 mL graduated cylinder

The OFFICIAL COPY is the on-line version. All other copies are considered unofficial and uncontrolled.

3.1.5. Extra fine tip black marker

3.1.6. 2 Rinse bottles (1 small, 1 large)

3.2. Extraction

3.2.1. BD Falcon 15 mL Centrifuge tubes

3.2.2. Test tube rack

3.2.3. Scissors

3.2.4. Eberbach Tissue Grinder motor

3.2.5. Wheaton Tissue Grinder with 30 mL tube

3.2.6. Small container with ice

3.2.7. Rinse bottle

3.2.8. Fume hood in dark room

3.3. Spectrophotometric Analysis

3.3.1. Perkin Elmer Lambda 35 UV/Vis Spectrophotometer (S/N 101N7032605)

3.3.2. 4 cm path length UV quartz glass cuvette

3.3.3. 1 cm path length UV quartz glass cuvette

3.3.4. Mini cuvette stirrer

3.3.5. PC with Perkin Elmer software

3.3.6. Small beaker

3.3.7. Finpipette 1-5 mL pipette with tips (EH89777), calibrated per SOP MB-03.3

3.3.8. Hamilton 100 µL pipette and tips (49197), calibrated per SOP MB-03.3

4. REAGENTS AND STANDARDS

4.1. Deionized Water

4.2. Acetone, HPLC grade

4.3. Saturated Magnesium Carbonate Solution

4.3.1. Add 10 g of $MgCO_3$ (ACS grade) to 1 L of deionized water. Store in a clean screw cap bottle.

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 bottle.

4.4. Aqueous Acetone Solution with Magnesium Carbonate

The OFFICIAL COPY is the on-line version. All other copies are considered unofficial and uncontrolled.

4.4.1. Use a Class A graduated cylinder to measure 3600 mL of Acetone (HPLC grade) and pour into a 4 L flask. Measure 400 mL of filtered saturated magnesium carbonate solution and add it to the flask.

4.4.2. Record preparation, in **LIMS Laboratory\Technical\Standard Preparation Tracker** as in 4.3.2.

4.4.3. Store in Biology lab refrigerator. May keep 1 L in a screw cap volumetric flask at a time at room temperature in the chlorophyll room for daily use for up to one week. Discard the solution if it becomes cloudy. Use new glassware each time.

4.5. Aqueous Acetone Solution (90% acetone)

4.5.1. Use a Class A graduated cylinder to measure 100 mL of deionized water and pour into a 1 L flask. Measure 900 mL of Acetone (HPLC grade) and add it to the flask.

4.5.2. Record preparation, in **LIMS Laboratory\Technical\Standard Preparation Tracker** as in 4.3.2.

4.6. 0.1 N Hydrochloric Acid

4.6.1. Pipette 0.85 mL of concentrated HCl (ACS grade) into a Class A 100mL volumetric flask containing approximately 50 mL of deionized water then bring up to volume with deionized water. Add stir bar and mix using metal stir plate.

4.6.2. Record preparation, in **LIMS Laboratory\Technical\Standard Preparation Tracker** as in 4.3.2.

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

4.8. Chlorophyll *a* Stock Standard

4.8.1. Prepare under subdued lighting. Tap the ampoule of chlorophyll *a* spinach until all the powder is 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 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.

4.8.2. Record preparation, in **LIMS Laboratory\Technical\Standard Preparation Tracker** as in 4.3.2.

4.8.3. Concentration of stock standard is 200 mg/L. The holding time is 9 months from the date of preparation.

4.9. Chlorophyll *a* Working Standard

The OFFICIAL COPY is the on-line version. All other copies are considered unofficial and uncontrolled.

4.9.1. In subdued lighting, add 0.20 mL of chlorophyll stock standard to a 200 mL Class A graduated cylinder. Bring up to volume with 90% Acetone. Transfer to a dark glass bottle. Store in Biology lab freezer.

4.9.2. Record preparation, in **LIMS Laboratory\Technical\Standard Preparation Tracker** as in 4.3.2.

4.9.3. Concentration of working standard is 0.20 mg/L. The holding time is 3 months from the date of preparation.

5. SAMPLE COLLECTION, PRESERVATION, AND HANDLING

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

6. SAMPLE PREPARATION

6.1. Refer to SOP BB-030 for sample custody, labels and worksheet instructions.

6.2. Chlorophyll Filtration

6.2.1. Shake sample bottle to ensure sample is thoroughly mixed. Use a Class A graduated cylinder to measure 500 mL of sample. Filter through a GF/C glass fiber filter. If sample is highly turbid and difficult to filter do not filter the entire 500 mL volume. A smaller volume may be used as long as the filter is sufficiently loaded with algae. For periphyton samples, (prepared per SOP BB-01), which are more likely to have clumps of algae, make sure to keep the sample well mixed throughout the filtering process to avoid allowing the heavier algae to sink to the bottom of the cylinder. It is unlikely that 500 mL of a periphyton sample can be filtered, so it is important to make sure the aliquot filtered is representative of the entire sample. Add 2 mL of $MgCO_3$ suspension when there is 25-50 mL of sample left to filter through the funnel. 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.

6.2.2. 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. 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. Then, fold the filter in half again while lining up the filtrate edge. Wrap the folded filter in aluminum foil. Place the "A" prep label on the foil and write the filter volume (and p# for periphyton samples) on it.

6.2.3. If there is a "B" label for a sample, repeat steps 6.2.1 and 6.2.2 for the "B" label. Use equal volumes for "A" and "B" aliquots of replicate samples.

6.2.4. Repeat steps 6.2.1.-6.2.3 for each sample. Filter a laboratory blank **after** every 20 samples and two duplicates for every batch of 20 samples (per Section 9).

The OFFICIAL COPY is the on-line version. All other copies are considered unofficial and uncontrolled.

6.2.5. When all samples are complete, record filter volumes/p-numbers (for CHL-CORR-P; calculated per Step 8.4 below), filtration date/time, date/time put in the freezer, analyst, blanks, and dups in **LIMS Laboratory\Technical\Prep Worksheet Manager** per SOP BB-30. Rubber band the samples together, place in a plastic zip-top bag, squeeze the air out of the bag and seal it. Label the plastic bag with the current date and store in the Biology lab freezer at -20°C. Holding time for frozen samples is 28 days from time of filtration until extraction.

6.2.5.1. For CHL-CORR-P samples, the p-numbers (refer to Section 8: Data Archival) should be entered into the **Filtration Volume** column in Prep Worksheet Manager. The filter volume, total volume, and number of slides should be entered in the column labeled **Sample Comments 2**.

6.2.6. 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 proper disposal of the sample. Place bottles in a garbage bag with the filter date visible on the exterior for discard purposes.

6.3. Chlorophyll Batch Preparation

6.3.1. Looking at the prep worksheet, create batches of 20 samples each for analysis. Combine worksheets from different days as necessary. Batches of 20 should be labeled sequentially with a number 1-10. After the 10th batch, repeat the sequence again, starting with #1. In the **Sample Comments** column, fill in the batch number for each sample.

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

6.3.2. Copy the prep worksheet batches into the *Chlorophyll Run Log*.

6.3.2.1. Write the batch number at the top of the Run Log page.

6.3.2.2. Write "BLK" in the column for Field ID for Tube #22.

6.3.2.3. Write the Sample IDs in the Sample # column for Tube #1-20.

6.3.2.4. Line 21 should be labeled "dup". Complete this row after extracting a "B" sample.

6.3.2.5. Enter filter volumes/p #s in the column labeled "Filter Volume (mL)". Fill in the column for Job IDs.

6.3.2.6. Write "B" volumes under Comments in the corresponding row (e.g. B=500) and $\frac{\text{volume filtered}}{\text{total volume}} \times$ total # of slides for p-number calculation.

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

6.3.3.1. For each batch, place "A" samples followed by any "B" samples from that batch, then a laboratory blank at the end. Wrap in foil and label

The OFFICIAL COPY is the on-line version. All other copies are considered unofficial and uncontrolled.

the packet with batch number. Place the batches in a zip-top plastic bag, squeeze the air out of the bag and seal, and return to the freezer until extraction.

7. SAMPLE ANALYSIS

7.1. Extraction

7.1.1. Retrieve the chlorophyll packet associated with the first available batch in the *Chlorophyll Run Log* from the biology lab freezer. Record extract date/time/analyst in the *Chlorophyll 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.

7.1.2. In subdued lighting, unwrap sample filter from foil and make sure sample label information matches the *Chlorophyll Run Log*. If needed, trim the filter above the filtrate line to remove excess filter (See Figure 12.1). To avoid losing sample, do not cut too close to the filtrate line. Roll up filter and place in the grinding tube. Add 2-3 mL of aqueous acetone-$MgCO_3$ solution.

7.1.3. Grind the filter long enough to convert it to a slurry 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 aqueous acetone-$MgCO_3$ solution, collecting the rinse in the tube.

7.1.4. Transfer mixture to the corresponding numbered centrifuge tube. Centrifuge tube numbers can be found in the *Chlorophyll Run Log*. Use aqueous acetone-$MgCO_3$ solution to rinse any remaining sample in the grinding tube and on the grinding pestle into the centrifuge tube. Make sure to rinse the grinding pestle and tube at least three times. Rinse the centrifuge tube wall and bring the final volume up to the next available whole number (a maximum of 14 mL). It is preferable for a sample and its duplicate to have the same extraction volume. Record extract volume in the *Chlorophyll Run Log*.

7.1.5. Repeat steps 7.1.2-7.1.4 for each sample.

7.1.6. Shake tubes vigorously. 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 1-3 times while steeping.

7.2. Instrument Setup

7.2.1. Turn on the spectrophotometer and let warm up for at least 1 hour. After warm up, turn spec off for approximately 30 seconds. Turn the spec on again and allow 3-5 minutes for the spec to re-run initial tests with warm lamps before opening the computer program.

7.2.2. Turn the computer on, open the Perkin Elmer UV WinLab program. Select analyst name from the drop-down menu and click **OK**.

The OFFICIAL COPY is the on-line version. All other copies are considered unofficial and uncontrolled.

7.2.3. Select the "Chlorophyll Suite" method in the shortcut or method list.

7.2.4. Fill in the Samples table with the correct Sample ID, Volume Filtered, and Volume Extracted information. Add "_dup_a" to the end of the first duplicate sample ID and "_dup_b" to the end of the second duplicate sample ID. The cuvette size is always 4.

7.2.4.1. If running CHL-CORR-P, type the p number to two decimal places into the Volume Filtered column.

7.2.5. In the column labeled "w=1/p=2," type the number "1" if the test is either CHL-CORR-W or CHLSUITE-W. Type the number "2" if the test is CHL-CORR-P.

7.2.6. Click the **Start** button.

7.2.7. Fill the reference and sample cuvettes with 90% Acetone. The reference cuvette (1 cm) should be completely filled and capped. The sample cuvette (4 cm) should be at least 2/3 full and does not need to be capped. Wipe the optical sides of the cuvettes with a Kim-wipe. Place in the machine and click **OK** to perform an auto zero. After this, the program will be ready for the first sample. The reference cuvette stays in the spec compartment throughout the entire analysis.

7.3. Spectrophotometric Analysis

7.3.1. Centrifuge samples for 20 minutes at 2500 rpm. Decant supernatant from each sample into a clean centrifuge tube with matching tube number.

7.3.2. When the spectrophotometer prompts for "Measurement 1" of a sample, add 3 mL of the sample to the cuvette. Place in the spectrophotometer and close the compartment door. Click **OK** to begin reading.

7.3.3. If the absorbance at 664 nm is 0.007 AU or less, do not acidify the sample. When the program prompts for "Measurement 2" Click **OK** and skip to Step 7.3.6.

7.3.4. If the absorbance at 664 nm is above 0.007 AU but less than 1.5 AU, add 0.1 mL of 0.1 N HCl and stir with the mini-mixer. Wait 90 seconds then click **OK**. Skip to Step 7.3.6.

Note: Be careful not to touch the optical sides of the cuvette with the stirrer because this will scratch the cell and affect absorbance readings.

7.3.5. If the absorbance at 664 nm is greater than or equal to 1.5 AU, the sample must be diluted. Click **OK**. When the spectrophotometer is done reading, click **Cancel**. Add a "_1" to the end of the sample ID. Number additional readings of the same sample in succession, but leave the reading of sample ID that will be uploaded without a numerical suffix. Highlight the row below the sample and click **Insert**. Fill in the new row with the correct information. Note the dilution in the *Chlorophyll Run Log*. Click the **Start** button and run diluted sample as prompted (Return to Step 7.3.2.).

The OFFICIAL COPY is the on-line version. All other copies are considered unofficial and uncontrolled.

7.3.5.1. If reading is less than or equal to 2.5, make a 50% dilution, by adding 3 mL of sample and 3 mL of 90% aqueous acetone solution. The new volume filtered is 50% of the original sample.

7.3.5.2. If reading is greater than 2.5, make a 25% solution, by adding 1 mL of sample and 3 mL of 90% aqueous acetone. The new volume filtered is 25% of the original sample.

7.3.6. When the spectrophotometer is finished reading, it will prompt for "Measurement 1" of the next sample. Remove the sample cell and empty it into the waste flask. If acidified, rinse the cuvette with 100% Acetone.

7.3.7. Repeat Steps 7.3.2.-7.3.6 until all samples (including the standard and blank) have been read. Do not discard any samples until analysis of all samples is complete and the file has been saved.

7.3.8. Check the percent recovery of the standard, dup RPD, and blank to ensure they all fall within the acceptance limits as described in Section 9. Follow the corrective action in Section 9 if necessary. Record the percent recovery of the standard and dup RPD at the bottom of the *Chlorophyll Run Log* page and initial and date.

8. DATA ARCHIVAL

8.1. Save the file by selecting **File/Save Results/As New Task** from the menu. Collect the sample table printout from the printer. Write the Run ID in the *Chlorophyll Run Log*.

8.1.1. Name the file with the Run ID.

8.1.2. Saving the task will automatically save an output data file in the proper directory and print a copy of the sample table.

8.2. Refer to SOP BB-031 for QC Manager (QCM) data uploading and verification instructions. Output data files are named "Sample Replicates Table.csv" in the Run ID folder and are located in the following directory: \\fldep1\dear\Labs\Lab3\Biology\Chlorophyll\PerkinElmer. Use extract date/time as analysis time in QCM.

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

8.4. Calculations:

Phaeophytin-corrected chlorophyll (µg/L or mg/m^3):

$$\text{Chlorophyll } a \text{ corrected } (\text{mg/m}^3) = \frac{26.7(664_B - 665_A) \times V_1}{V_2 \times L}$$

The OFFICIAL COPY is the on-line version. All other copies are considered unofficial and uncontrolled.

Phaeophytin (µg/L or mg/m^3):

$$\text{Phaeophytin } a \text{ (mg/m}^3\text{)} = \frac{26.7(1.7(665_A) - 664_B) \times V_1}{V_2 \times L}$$

Uncorrected chlorophyll (µg/L or mg/m^3):

$$\text{Chlorophyll } a \text{ uncorrected (mg/m}^3\text{)} = \frac{[(11.85 \times 664_B) - (1.54 \times 647_B) - (0.08 \times 630_B)] \times V_1}{V_2 \times L}$$

Chlorophyll/Phaeophytin Ratio:

$$\text{Absorption peak ratio} = \frac{664_B}{665_A}$$

where:

664_B = Subtract 750 nm value (turbidity correction) from absorbance at 664 nm before acidification

665_A = turbidity-corrected absorbance at 665 nm after acidification

647_B = turbidity corrected absorbance at 647 nm before acidification

630_B = turbidity corrected absorbance at 630 nm before acidification

V_1 = volume of extractant (mL)

V_2 = volume of sample filtered (L)

L = path length (cm)

p number (for CHL-CORR-P analysis)

$$\text{p} = \frac{\text{volume filtered}}{\text{total volume}} \times \text{total \# of slides}$$

If tiles are used instead of slides:

$$\text{Total \# slides} = \frac{\text{surface area of the tile}}{\text{surface area of 1 slide}}$$

Note: Tiles currently in use as of the effective date of this SOP have an average surface area of 375 cm^2. The surface area of 1 slide is 3,950 mm^2. The current tiles calculate to 9.49 slides for use in the p equation above.

9. QUALITY CONTROL

9.1. Negative Control

The OFFICIAL COPY is the on-line version. All other copies are considered unofficial and uncontrolled.

9.1.1. Filter 500 mL of deionized water as a laboratory blank after every 20 samples filtered.

9.1.2. Analyze the laboratory blank at the end of each batch analyzed.

9.1.3. The acceptance limit is ≤ 0.007 AU at all wavelengths.

9.1.4. Corrective Action: If a blank failure occurs, run a 90% acetone sample to help determine whether the problem is caused by a spectrophotometer malfunction or blank contamination.

9.2. Positive Control

9.2.1. As a laboratory control sample (LCS), analyze the chlorophyll *a* working standard at the beginning of every batch during spectrophotometric analysis.

9.2.2. Record the working standard serial number at the top of the *Chlorophyll Run Log* page.

9.2.3. Calculate percent recovery of LCS as follows:

$$\%\, Rec \;=\; \frac{26.7\ (664_B \;-\; 665_A)}{4 \;\times 0.2} \times 100\%$$

9.2.4. The acceptance limit for recovery is 85-115%. Record the percentage in the space provided at the bottom of the *Chlorophyll Run Log*.

9.2.5. As a quick check of the standard during sample analysis, the CORR A result should be 2.72-3.68 for the standard in the "Measurements" tab in the "Processing" section of the folder list in Perkin Elmer. Another calculation of the standard recovery from this tab is:

$$\%\, Rec \;=\; \frac{62.5 \;\times\; CORR\ A}{2}$$

If the standard recovery exceeds the acceptable limits, re-zero the spec and re-run the blank and standard.

9.2.6. 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.

9.3. Sample Duplicates

9.3.1. Analyze a laboratory replicate for 5% of samples. Filter at least two duplicates per 20 samples, and no less than one per day. The acceptance limit is ≤25% RPD. Record RPDs in the space provided at the bottom of the *Chlorophyll Run Log*. If the measurements for the sample and duplicate are below the practical quantitation limit (PQL), "< PQL" can be recorded for the RPDs. The PQL at 500 mL filter volume with 8 mL extraction volume for CHL-CORR-W and CHL-SUITE-W is 1.2 µg/L for phaeophytin-*a* and uncorrected chl-*a* and is 1.7 µg/L for corrected chl-*a*. The PQL at 500 mL

The OFFICIAL COPY is the on-line version. All other copies are considered unofficial and uncontrolled.

filter volume from 2 slides with 8 mL extraction volume for CHL-CORR-P is 0.33 mg/m^2 for phaeophytin-*a* and is 0.42 mg/m^2 for corrected chl-*a*.

9.3.2. Corrective Action: If absorbencies for sample duplicates do not appear similar, re-run both duplicates at the end of the batch.

9.4. Instrument Performance Verification (IPV)

9.4.1. Ensure that there are no cuvettes in the spec compartment.

9.4.2. In Perkin Elmer UV WinLab, select Instruments from the Folder List.

9.4.3. Right click on the appropriate instrument.

9.4.4. Select Instrument Performance Verfication → Perform now…

9.4.5. Select Instrument Performance Verification Tests for CHL.

9.4.6. Click Perform.

9.4.7. The instrument will cycle through three diagnostics:

IPV Wavelength Accuracy D2

IPV Wavelength Repeatability D2

IPV-Wavelength Accuracy Glass (using holmium oxide filter)

9.4.8. Insert the holmium oxide filter when prompted.

9.4.9. Retrieve the reports from the printer and sign and date the summary report.

9.4.10. Put the reports in the Initialization and Instrument Validation of Perkin Elmer Lambda 25 and 35 UV/VIS Spectrophotometers binder.

9.4.11. The IPV should be performed annually on each instrument.

9.4.12. Corrective Action: If any of the IPVs do not pass, re-run all IPVs. If they still do not pass, contact a supervisor.

10. SAFETY/HAZARDOUS WASTE MANAGEMENT

10.1.1. Do not pour acetone or any reagent/standard containing acetone down the sink. Pour any waste acetone into the waste flask located in the chlorophyll room sink. When this is full, empty the flask into an unfilled acetone bottle and label it with “Hazardous Waste”, waste content, amount, date generated and initials. Follow the Laboratory Health and Safety Plan manual to dispose of full waste acetone bottles.

10.1.2. Refer to the Laboratory Health and Safety Plan.

10.1.3. Read MSDS information for reagent handling and disposal.

11. REFERENCES

11.1. Standard Methods Online Edition SM10200H (2001)

The OFFICIAL COPY is the on-line version. All other copies are considered unofficial and uncontrolled.

11.2. EPA Method 446.0, Revision 1.2

11.3. Perkin Elmer Lambda 35 UV/Vis Spectrophotometer Manual

11.4. NELAC Quality Systems Manual

11.5. FDEP Bureau Quality Manual http://www.dep.state.fl.us/labs/library/progplan.htm

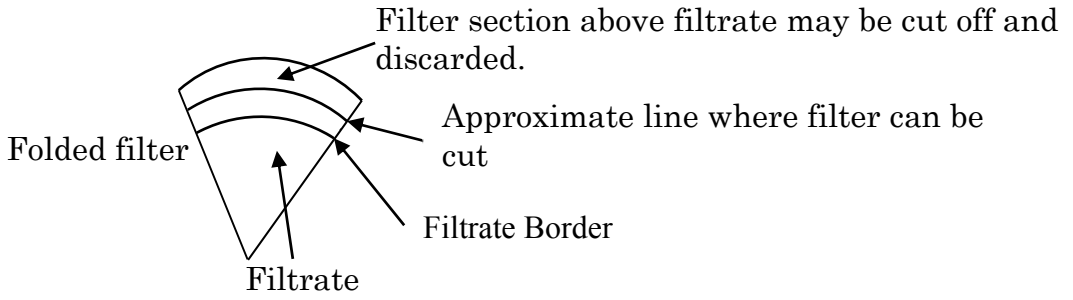
11.6. Laboratory Health and Safety Plan http://depnet/burlabs/safety.htm

11.7. SOP MB-03.3-*Calibration and Use of Mechanical and Disposable Pipettes*

11.8. SOP BB-01-*Periphyton Sample Preparation for Chlorophyll* a *Analysis*

12. TABLES, DIAGRAMS

Figure 12.1 Filter trimming for extraction



APPENDIX OF CHANGES

01/16/2009	Updated SOP by editing sections 6.2.6 and 9.2.2. cf
01/13/2010	Updated MDL section 1.3. Updated laboratory blank information (one blk/20 samples instead of historical 2 blks). Added steps to record filter freeze time and bottle rinsing procedure. cf
12/09/2010	Updated Summary of Method 2.2.2 (changed value), Reagents and Standards 4.6.1(reworded for clarity), and Data Archival 8.1(changed file path). KMS
11/18/2011	Updated information in Fig. 12.1 of Tables/Diagrams to reflect new equipment KMS
12/17/2012	Updated instructions in 7.1.4 to include a rinse step of the grinding pestle KMS
10/09/2013	Updated equipment to reflect new filter pad size. Reworded 6.3.2.4 to indicate new prep worksheet layout. Added extra comment to 7.1.4 KMS
12/03/2013	Added calculation for standard recovery to 9.2.5 & expanded comments in 9.3.1 KMS
06/02/2014	Incorporated CHL-CORR-P analysis into the SOP. (AGR)

The OFFICIAL COPY is the on-line version. All other copies are considered unofficial and uncontrolled.