Standard Operating Procedure for:

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

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1. SCOPE AND APPLICATION

1.1. This method is based on Standard Methods 10150 B – 2022 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 *a* 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 CHLSUITE-W and CHL-CORR-P.

1.3. Method Detection Limits (MDL) at 500 mL filter volume with 12 mL extract volume for CHLSUITE-W:

1.3.1. Chlorophyll *a* corrected for phaeophytin: 0.82 µg/L

1.3.2. Uncorrected chlorophyll *a*: 0.60 µg/L

1.3.3. Phaeophytin *a*: 0.90 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 both analyses.

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 aqueous acetone-$MgCO_3$ solution 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.

2.1. Interferences

2.1.1. 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 centrifuge tubes (or grinding glass tube) are kept on ice between bead disruption cycles or between samples in order to minimize heat generated from friction during extraction.

Note: 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.

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

2.1.3. 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.

2.2. 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 from exposure to light.

2.2.2. 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.

2.2.3. 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 DEP Biology Program currently uses a 1 cm path length reference cuvette and a 4-cm path length sample cuvette.

2.2.4. 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.

2.2.5. 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 *a* 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/F 47mm Glass Fiber Filters

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3.1.3. Small roll of aluminum foil, cut in 3-inch squares
3.1.4. Class A TD (to deliver) 500 mL graduated cylinders
3.1.5. Black laboratory water-proof marker
3.1.6. Rinse bottles (small and large)
3.1.7. Gloves, latex or nitrile

3.2. Extraction

3.2.1. 15 mL centrifuge tubes with screw caps (Omni brand preferred)
3.2.2. Disruption beads, 1.0 mm, 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. Wheaton Tissue Grinder with 30 mL tube
3.2.9. Container with ice
3.2.10. Rinse bottle
3.2.11. Fume hood in each dark room
3.2.12. Cut resistant gloves

3.3. Spectrophotometric Analysis

3.3.1. Perkin Elmer Lambda 365 UV/Vis Spectrophotometer (S/N 365K20041702)
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. Megafuge ST4R PLUS-MD, 120V Centrifuge (S/N 00159655)
3.3.5. TX-750 Rotor for Megafuge, radius max/min of 195mm/83mm
3.3.6. Soft glass stirring rods
3.3.7. PC with Perkin Elmer software
3.3.8. Small beaker
3.3.9. Class A 200 mL volumetric flask
3.3.10. Class A 100 mL volumetric flask
3.3.11. Class A 25 mL volumetric flask
3.3.12. Eppendorf Research Plus 1-5 mL pipette with tips (K45340J), calibrated per SOP MB-03.3

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3.3.13. Eppendorf Research Plus 100 µL pipette and tips (L40124J), calibrated per SOP PCR-2.4

3.3.14. Kimwipes

3.3.15. Gloves, latex or nitrile

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 $MgCO_3$ to 1 L of deionized water. Store in a clean screw cap bottle. Shake thoroughly before use.

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”. (The "Duplicate Reagent" option allows you to copy an existing record, while the "New Reagent" will have all fields blank, requiring you to fill them in). Print a label and attach to the bottle.

4.4. Aqueous Acetone Solution with Magnesium Carbonate

4.4.1. Use a Class A graduated cylinder to measure 3600 mL of Acetone (HPLC grade) and pour into a 4 L flask **in the hood, ensuring proper ventilation and safety protocols are followed.** Filter a bit more than 400 mL of the saturated magnesium carbonate solution to account for any loss in the supernatant. Once filtered, measure out 400 mL of the solution and transfer it to the 4L 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.

4.4.3.1. 1 L may be kept in a screw cap volumetric flask at room temperature in the chlorophyll room 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 200 mL of deionized water and pour into a 2 L flask. Measure 1800 mL of Acetone (HPLC grade) **in the hood, ensuring proper ventilation and safety protocols are followed,** 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

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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 magnetic stir plate. Alternatively, a pre-prepared 0.1 N Hydrochloric Acid Standardized Solution (Thermo Scientific Chemicals) can be purchased for convenience.

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. Sigma C6144 Chlorophyll *a*, from *Anacystis nidulans* algae, 5 mg

4.9. Chlorophyll *a* Stock Standard (Parent Stock)

4.9.1. Prepare under subdued lighting in the hood, ensuring proper ventilation and safety protocols are followed. Tap the ampoule of chlorophyll *a* from spinach or *Anacystis nidulans* algae 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. Rinse the ampoule thoroughly with 90% acetone several times and empty 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 preparer's initials, date prepared and expiration date and store in Biology lab freezer.

Note: Be careful when preparing the stock solution. The entire contents of the 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.

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

4.9.3. The concentration of the parent stock standard is **200 mg/L**, with an expiration date of 9 months from the preparation date. After 9 months, the parent stock can be retested to verify its concentration and, if accurate, recertified for an additional 9 months.

4.10. Chlorophyll *a* Working Standard

4.10.1. Prepare under subdued lighting in the hood, ensuring proper ventilation and safety protocols are followed, pipette 0.20 mL of chlorophyll stock standard to a 200 mL Class A TD 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 concentration of the working standard is **200 µg/L**. The expiration date is 3 months from the date of preparation. Note: This standard is also used for SOP BB-28.

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

5. SAMPLE COLLECTION, PRESERVATION, AND HANDLING

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 Class A (TD) graduated cylinder to measure 500 mL of sample. Filter through a GF/F glass fiber filter. If the sample is highly turbid and difficult to filter do not filter the entire volume. A smaller volume may be used if 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. When there is 25-50 mL of sample left to filter, shake the bottle of prepared $MgCO_3$ solution and add approximately 2 mL of $MgCO_3$ solution to the sample. 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 each. 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 (and p# for periphyton samples) on it.

6.1.2.1. 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.

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 duplicate 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#-#".

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6.1.5. When all samples are complete, record filter volumes/p-numbers, filtration date/time, analyst, $MgCO_3$ serial number, 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 in a plastic zip-top bag, squeeze the air out of the bag and seal it. Label the plastic bag with the current date & worksheet number and store in the Biology lab walk-in freezer, or the Fisher Refrigerator located in the micro lab 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**.

6.1.5.1. For CHL-CORR-P samples, the p-numbers 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**.

6.1.6. 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 proper disposal of the sample. Place bottles in a black 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 24 samples for analysis. Combine worksheets from different days as necessary. Batches should be labeled sequentially with a number 1-10. After the 10^{th} batch, repeat the sequence again, starting with #1. One batch will have 24 samples (along with 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.

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 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.

6.2.1.3. Line # 24 is for the duplicate “dup” sticker. Complete this row after extracting a “B” sample.

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.

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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. For periphyton sample preparation, include the p-number calculation ($\frac{\text{volume filtered}}{\text{total volume}} \times$ total # of slides) in the comments or as an annotation at the bottom of the page.

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 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). Seal tube tightly. Examine the cap for integrity and ensure that each tube is clearly labeled.

7.1.2. 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.

7.1.3. In subdued lighting, unwrap a sample filter from foil and make sure sample information matches the Chlorophyll 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-MgCO3 solution to bring the volume of the tube up to 13.5 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 two small buckets with ice for cooling. Make sure the centrifuge tubes are tightly sealed. In subdued lighting, place the first half of tubes (12 tubes) in the bead beater, 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 bucket of ice to chill for a minimum of 45s. Make sure the centrifuge tubes are tightly sealed.

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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.

7.1.11. Place a label in the logbook for the designated batch that includes the record numbers of the centrifuge tubes and disruption beads used.

7.2. Extraction with tissue grinder

7.2.1. 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.

7.2.2. In subdued lighting, unwrap a 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. Place the filter in the grinding tube. Add 2-3 mL of aqueous acetone-$MgCO_3$ solution.

7.2.2.1. Make sure to change the grinder motor oil approximately every 6 months when in use. To do so, remove the grinder wand, unplug the motor, and unscrew it from the stand. Unscrew the top and side fill plugs. Drain the oil into a container through the side. Screw the side fill plug back in and fill the grinder with oil through the top hole until it is level with the side plug. Replace the top plug, wipe up any remaining oil, and place the grinder back on the stand. Record grinder motor maintenance in LIMS Lab Logbook under Maintenance_Log.

7.2.3. While wearing cut resistant gloves, grind the filter into a slurry without allowing the sample to overheat. This may take up to 1 minute. Grooves may be cut into the pestle using a razor blade to facilitate grinding. It is helpful to create grooves going parallel to the long edge of the grinder. 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.

7.2.4. 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. Rinse the grinding pestle

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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; beyond 14 mL the solution should be transferred to a larger volume centrifuge tube). The sample and its duplicate should have the same extraction volume. Record extract volume in the *Chlorophyll 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 while steeping. Record the start and end steep time/date in the *Chlorophyll Spec Run Log*.

7.3. Instrument Setup

7.3.1. Turn on the spectrophotometer.

7.3.2. Turn the computer on and open the Perkin Elmer UV WinLab program. Enter credentials to log in.

7.3.3. Select the “Methods” section and create a new method or open a template.

7.3.4. Fill in the Samples table with the correct Sample ID, Volume Filtered, and Volume Extracted information. The first sample will always be the working standard, labeled as "cstd." The filter volume for the standard will consistently be 500 ml, and the extract volume will remain fixed at 8 ml. 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 for all samples, the standard, and the blank is always 4.

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

7.3.5. In the column labeled “w=1/p=2,” type the number “1” if the test is CHLSUITE-W. Type the number “2” if the test is CHL-CORR-P.

7.3.6. Click the **Start** button.

Note: Wear gloves when handling the cuvettes to avoid getting oils from hands on the optical sides.

7.3.7. 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 Kimwipe™. 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 spectrophotometer compartment throughout the entire analysis.

7.4. Spectrophotometric Analysis

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

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matching tube number. Place the standard on a stir plate and allow it to stir for a few minutes to come to room temperature before pipetting.

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

7.4.2. When the spectrophotometer prompts for "Measurement 1" of the first sample, add 4 mL of the working standard to the cuvette. Place in the spectrophotometer and close the compartment door. Click **OK** to begin reading. See 9.2 for QC information pertaining to the working standard.

7.4.2.1. If the absorbance at 750 nm is greater than 0.007 AU, re-centrifuge the sample for 5 minutes at 3000 rpm and re-read. If the absorbance is still greater than 0.007 AU after one re-centrifugation, no further re-spins are necessary.

7.4.3. 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.

7.4.4. If the absorbance at 664 nm is above 0.007 AU but less than 1.5 AU, add 0.133 mL of 0.1 N HCl and stir with the glass rod for approximately 30 seconds. Start the timer right after acidification. Wait another 60 seconds for a total of 90 seconds then click **OK** in the box prompting for "Measurement 2". Skip to Step 7.4.6.

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.

7.4.5. 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 the 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 7.4.2.).

7.4.5.1. 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.

7.4.5.2. 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.

7.4.5.3. **Note: Check raw absorbance values from Perkin Elmer program before proceeding to next sample.**

7.4.6. 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 carboy. If acidified, rinse the cuvette with 100% acetone.

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7.4.7. Repeat Steps 7.4.2.-7.4.6 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. Do not delete ANY readings even if the sample needs to be re-read. The Perkin Elmer UV WinLab software does not allow duplicate sample IDs. Numerical suffixes are added to sample IDs to allow for multiple readings of the same sample. The reading that will be uploaded should not have any suffix. All other readings should have a suffix in the format of "_#" and be numbered sequentially.

7.4.8. 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 top of the *Chlorophyll Run Log* page and initial and date to confirm that the QC has been checked, and appropriate corrective action has been followed. Check if the Phaeophytin *a* and Corrected *a* values add up to approximately the Uncorrected value. If not, re-run the sample.

7.4.9. After completing a batch, place a label in the logbook indicating the serial numbers for the Perkin Elmer spectrophotometer and mechanical pipettes used.

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.

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 in the following directory: \Labs\Lab3\Biology\Chlorophyll\Perkin Elmer 365. Use extract date/time as analysis time in QCM.

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

9. QUALITY CONTROL

9.1. Negative Control

9.1.1. Filter 500 mL of deionized water as a laboratory blank after every 20 samples filtered and at least one per day.

9.1.2. Analyze a laboratory blank with each batch analyzed.

9.1.3. The acceptance limits are ≤ 0.55 µg/L for chlorophyll *a* corrected, ≤ 0.40 µg/L for uncorrected chlorophyll *a* and ≤ 0.60 µg/L for phaeophytin *a*. The results

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are found in the "Measurements" tab in the "Processing" section of the folder list in Perkin Elmer.

9.1.4. Corrective Action: If a blank failure occurs, re-read the blank. If it fails again, run a 90% acetone sample to help determine whether the problem is caused by a spectrophotometer malfunction or blank contamination. Notify a supervisor.

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 the 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 75-120%. 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 spectrophotometer and re-run the 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 working standard.

9.3. Sample Duplicates

9.3.1. Analyze a laboratory replicate for 5% of samples. Filter one replicate per 20 samples, and no less than one per day. The acceptance limit is ≤ 25% RPD. Record RPDs in the space provided in 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-SUITE-W is 1.2 µg/L for phaeophytin *a,* and uncorrected chlorophyll *a,* and is 1.7 µg/L for corrected chlorophyll *a*. The PQL at 500 mL 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 chlorophyll *a*.

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9.3.2. Corrective Action: If the RPD for sample duplicates are not within the acceptance limit, re-run both duplicates at the end of the batch.

9.4. Instrument Performance Verification (IPV)

9.4.1. The IPV is performed by a certified Perkin Elmer technician as part of the annual preventive maintenance visit.

9.4.2. Retrieve the reports from the printer and have them signed and dated by the technician performing the verification.

9.4.3. File reports in the INSTRUMENT PERFORMANCE VERIFICATION REPORTS (IPV) LAMBDA 365 UV/Vis Spectrophotometer binder.

NOTE: Schedule a visit at least 2 months in advance to ensure the test is completed at the one-year requirement.

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 Hazardous Waste carboy located in the chlorophyll room sink. 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 SM 10150 B – 2022

11.2. EPA Method 446.0, Revision 1.2

11.3. Perkin Elmer Lambda 365 UV/Vis Spectrophotometer Manual

11.4. NELAC Quality Systems Manual

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

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

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

11.8. DEP SOP PCR-2.4- *Calibration and Use of Mechanical Pipettes using the PCS Pipette Calibration System*

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

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

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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)

05/31/2017 Updated Quality Manual and Laboratory Health and Safety Plan hyperlinks, added reference to SOP PCR-2.4, removed reference to CHL-CORR-W analysis, added procedure for checking turbidity of samples, added instructions for re-reading samples, edited negative control procedure and corrective action, and made corrections to sample duplicate corrective action.

01/24/2018 Changed Class A glassware type in section 4.9.1.

09/27/2018 Updated Phaeophytin MDL in sections 1.3.3 and 9.1.3.

09/20/2019 Changed run log instructions to accommodate new run log and clarified some procedures. Added calculations for Periphyton samples.

01/27/2020 Updated criteria and instructions for calculating and recording RPD for manual and software calculations in section 8.4. Added sections 7.1.2.1. and 7.1.2.2 with instructions for performing and recording tissue grinder maintenance.

02/02/2021 Updated links for Quality Manual and Health and Safety Plan in Reference section.

02/09/2022 Corrected reagent used for steeping in Summary of Method section 2.1 to be aqueous acetone-$MgCO_3$ solution, not 90% acetone. Updated spectrophotometer and pipette serial numbers and names in section 3.3, as well as adding the centrifuge and rotor to the list. Updated instructions for making aqueous acetone-magnesium carbonate solution, for folding filtered samples, changing the oil in the grinder, and for extracting and running. In section 7.3.1, a detail was added that you can use stir plate for working std. Directory to stored data was fixed. The reference picture in section 8.4 was updated. RCF formula was added to section 8.

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01/30/2023 Updated reference method from SM 10200 H to SM 10150 B in sections 1.1 and 11. Edited sections 6.2.1, 6.2.2 and 6.2.3. to update SOP in accordance with current procedures. Removed section 8.4 since it is no longer part of data archival, and section 9.4 as the IPV test is no longer performed by analysts in the lab.

03/20/2024 Edited summary and equipment list to include the bead disruption method. Added rinsing of graduated cylinder during filtration to 6.2.2. Edited 6.2.5 to include recording serial/record numbers of additional materials. Added reference and link to the Omni Bead Ruptor Elite Manual. Edited section 7 to include extraction with bead homogenizer in section 7.1, and extraction via tissue grinder in section 7.2. Added a note to section 7.4 to mention an additional centrifuge cycle for samples extracted with the bead homogenizer.

03/25/2025 Updated equipment and supplies in Section 3. Updated instructions in section 4.8 and 4.9 for making stock and working standards. Updated chlorophyll filtration and batch preparation steps in Section 6. Added additional Chlorophyll *a* reagent to Section 4. Checked that the SOPs were clear and aligned with the current procedures.

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