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**SC DEPARTMENT of
ENVIRONMENTAL
SERVICES**

BUREAU OF WATER
AQUATIC SCIENCE DIVISION
AQUATIC BIOLOGY SECTION

STANDARD OPERATING PROCEDURE FOR
CHLOROPHYLL A LABORATORY METHODS
ASDPROC 004

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ASDPROC 004 – Chlorophyll *a* Laboratory Methods

Chlorophyll *a* Laboratory Methods. SCDES, Revision 7

Reference: U.S. Environmental Protection Agency, 1997. Method 445.0 (*In Vitro* Determination of Chlorophyll *a* and Pheophytin *a* in Marine and Freshwater Algae by Fluorescence), Revision 1.2, Methods for Chemical Analysis of Water and Wastes, EPA-600/4-97-020, USEPA Office of Research and Development, Cincinnati, OH

1.0 Summary of Method

This method provides a fluorometric procedure for the low-level determination of chlorophyll *a* in freshwater and marine environments. The method described herein uses instrumentation whereby samples extracted in 90% aqueous acetone are analyzed by a modified (“non-acidification”) method. The instrument employed for this procedure, the Turner Designs Triology Laboratory Fluorometer, is a multifunctional laboratory instrument that can be used for making fluorescence, absorbance, and turbidity measurements using the appropriate snap-in Optical Module. The difference between the modified method and the conventional fluorometric method is that, after proper instrument calibration, chlorophyll *a* concentration is obtained by a direct readout from the fluorometer. In other words, it is not necessary to calculate response factors (F_s) as described in Sections 2.1 and 12.1 of EPA Method 445.0. Direct readouts of the concentrations of both standards and samples are thus possible.

Chlorophyll-containing phytoplankton in a measured volume of sample water are concentrated by filtering at low vacuum through a glass fiber filter. The pigments are extracted from the phytoplankton in 90% acetone with the aid of a tissue grinder and allowed to steep for 2 to 24 h, to ensure thorough extraction of the chlorophyll *a*. The filter slurry is centrifuged at 1000 g for 5 minutes to clarify the solution. An aliquot of the supernatant is transferred to a glass test tube, and the fluorescence is measured. The concentration in the natural water sample is reported in $\mu\text{g/L}$.

Extracted chlorophyll *a* tends to degrade when exposed to heat, light, acid residue, and oxygen. Therefore, to preserve sample integrity, chlorophyll *a* samples are kept cold and in the dark until extraction, and the filtered samples are analyzed within 24

days of collection. “Blanks” (laboratory reagent blanks) are analyzed to check for cross-contamination of samples.

2.0 Apparatus and Equipment

- Fluorometer – The fluorometer used for chlorophyll a analyses is a Turner Designs Triology laboratory fluorometer. It's all solid-state design (no moving parts) utilizes light emitting diodes, sensitive photodiodes, solid glass filters, and electronics to ensure reliable and repeatable readings with no instrument drift. Snap-in modules are configured with the optical components necessary for the application of choice; changing applications is as easy as snapping in a different module. The Chlorophyll a Non-Acidification Module (part # 7200-046-W) is what is used for analysis with the Triology. The LED for this module is at 430 nm, the excitation <500nm, and the emission >665nm. Minimum Detection Limit is 0.25 µg/L and the linear range is 0-300 µg/L.
- Spectrophotometer, with multiwavelength capability (750 nm, 664 nm, 647 nm, and 630 nm) with a spectral bandwidth of 2 nm or less. A Thermo Scientific Genesys 50 UV-VIS Spectrophotometer is used.
- Thermo Scientific SPECTRONIC Spectrophotometer Standards
- Clinical Centrifuge, capable of 675 g up to 1000 g. Current centrifuge used is from Damon/IEC
- Refrigerator, maintained at 4°C, used to store samples.
- Freezer, maintained at -20°C and used for reagent and filter storage.
- Dark room with subdued lighting for working with samples.
- Filtration assembly (Millipore™ multi-sample manifold with hand pump capable of maintaining a vacuum up to 6 in. Hg).
- Mortar and pestle; 65 mL CoorsTek 60313 Porcelain Mortar or similar
- Tissue grind pestle (Kontes® KT885511-0021) on stainless steel shaft with grooves in the Teflon (TFE) tip. Stainless steel shaft is long enough to chuck into a suitable electric drive motor for grinding. Round-bottom glass grinding tubes (Kontes® KT885512-0021) that match pestle.
- Conical plastic centrifuge tubes, 15 mL graduated in 0.5 mL increments with Teflon-lined screw-caps.
- Centrifuge tube racks and cuvette racks.
- Syringe dispenser (Nichiryo Model 8100)
- Graduated disposable syringes (for use with Model 8100 syringe dispenser), 15 mL.

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- Pall type A/E glass fiber filters, 1.0 µm effective pore size, 25 mm diameter.
- Aluminum foil.
- 60 mm x 15 mm sterile, non-vented petri dishes for storing filtered chlorophyll samples in freezer.
- Borosilicate glass cuvettes, 12 mm x 75 mm, disposable.
- Laboratory tissues (Kimwipes®), for cleaning glass cuvettes used in fluorometer.
- Flat-tipped forceps used to handle glass fiber filters.
- Manual pipetter (Brinkmann Pipet Helper).
- Assorted Class A calibrated serological pipets.
- Freezer and refrigerator thermometers.
- Cooler with ice.
- Graduated cylinders, 10 mL, 25 mL, 50 mL, 100 mL, and 1L.
- Glass storage flasks (50 mL, and 100 mL) for storing various dilutions of the standards solutions.
- Polyethylene squirt bottles, for dispensing and storing 90% acetone solution.

3.0 Reagents, Chemicals, and Standards

Safety Data Sheet (SDS) -- Written information provided by vendors regarding a chemical's toxicity, health hazards, physical properties, fire, and reactivity data including storage, spill, and handling precautions. A file of SDS has been made available to all personnel involved in the chemical analysis.

Lot Number Tracking Sheet—Lot numbers, expiration dates, chemical container open date, and chemical container completion date should be tracked in the Lot Number Tracking Sheet which can be found in the Chlorophyll a file in the Aquatic Science Programs on SharePoint.

The following chemicals and/or reagents may be toxic or hazardous: Acetone - This is a volatile reagent and is highly flammable. Acetone can cause irritation to the skin and eyes, and if ingested, could be fatal. To avoid inhaling vapors, the grinding of filters during the extraction step of this method should be conducted in a fume hood.

De-ionized (DI) water -- Obtained from the Sims/Aycock Annex central system.

Acetone (100%), HPLC grade, Honeywell-Burdick&Jackson or similar. Obtained from VWR or similar commercial supplier.

Aqueous acetone solution: 90% acetone/10% DI water. Carefully measure 100 mL DI water into a 1 L graduated cylinder and then bring to volume with 100% acetone. Pour all into the labeled amber 4 L 90% acetone bottle and invert bottle 12-15 times to thoroughly mix.

Chlorophyll *a* free of chlorophyll *b*, 1 mg dry material. May be obtained from a commercial supplier such as Sigma-Aldrich Chemical (St. Louis, MO).

3.1 Stock Standard Solution and Primary Dilution Standard preparations

Chlorophyll Stock Standard Solution (SSS) - A concentrated solution containing chlorophyll *a* prepared in the laboratory using assayed reference materials obtained from a reputable commercial source. Chlorophyll *a* from a commercial supplier is obtained in an amber glass ampoule which has been flame sealed. This dry standard should be stored at -20°C in the dark and the SSS prepared just prior to use.

To prepare the SSS: In subdued light, tap the ampoule until all the dried chlorophyll is in the bottom of the ampoule. Carefully break the tip off the ampoule and transfer the entire contents of the ampoule into a 50 volumetric flask. Rinse the interior of the broken ampoule with 90% acetone with the rinsate going into the 50 mL flask. Dilute to 50 mL with 90% acetone, label the flask and wrap with aluminum foil to protect from light. When stored in a dark, airtight container at freezer temperatures, the SSS is stable for six to nine months. The concentration of the SSS and of all dilutions must be determined spectrophotometrically each time they are made.

Note: The dried chlorophyll *a* from a commercial supplier will not be exactly 1.0 mg. The actual concentration of the SSS must be determined spectrophotometrically and calculations for dilutions made after the actual concentration is determined. For example, a SSS of 14.59 mg/L might be prepared instead of the 20 mg/L listed in the table below. Calculate dilutions accordingly, such as 5 ml of SSS diluted to 73 ml to make a solution of 1 mg/L for PDS 1.

Primary Dilution Standard Solution (PDS) – A solution of chlorophyll *a* prepared in the laboratory from the stock standard solution and diluted as needed to prepare initial demonstration of performance and quality control solutions. Two PDS solutions are to be made and used to create dilutions for Initial Demonstration of Performance

and Quality Control Standards.

To prepare the PDS solutions, follow the table below and prepare according to the concentration of the SSS as determined spectrophotometrically. Using a new pipette each time, transfer the required amount of SSS into a 100 mL or 50 mL graduated cylinder and add 90% acetone to dilute to the calculated volume. Once prepared, transfer the solutions into a 100 mL or 50 mL volumetric flask, wrap with aluminum foil, and label. Rinse graduated cylinders each time. PDS solutions and all other dilutions are stable for six to nine months if kept in a dark, airtight container at freezer temperatures. Verification of the SSS, PDS and QCS standards should be done once a month using the spectrophotometer to ensure that they are within range of their starting concentrations. If the values are not within $\pm 10\%$ of their original starting concentrations, the solutions must be re-made.

The table below provides an example of how to prepare all other solutions from the PDS solution per the actual concentrations of the SSS and PDS. Transfer the recommended amount using new pipette tips and clean graduated cylinders for each solution and dilute to the calculated volume. The solutions marked with an asterisk* are to be transferred to individual volumetric flasks and kept as Quality Control (QCS) checks to be used during bi-monthly calibrations. These solutions are also to be wrapped in foil, labeled, and kept in the freezer.

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For Initial Demonstration of Performance (section 6):

Solution			Concentration
SSS	1 mg Chl a powder diluted to 50ml		20 mg/L
PDS 1	2 mL from SSS diluted to 40ml		1 mg/L
PDS 2	1 mL from SSS diluted to 100ml*		200 µg/L*
From PDS 1:		From PDS 2:	
2.75 mL diluted to 10mL	275 µg/L	10 ml diluted to 100 ml*	20 µg/L*
2.5 mL diluted to 10mL	250 µg/L	↑ 5 ml from (20µg/L) diluted to 20ml = 5 µg/L	
2.25 mL diluted to 10mL	225 µg/L	↑ 10 mL from (20µg/L) to 100mL* = 2 µg/L*	
10 mL diluted to 100mL*	100 µg/L*	↑ 15 mL from (2µg/L) to 30mL = 1 µg/l	
↑ 1 mL from (100µg/L) to 10 mL = 10 µg/l			
		<i>From 1 µg/L:</i>	
		3 mL diluted to 10mL	0.30 µg/L
		10 mL diluted to 50mL*	0.20 µg/L*

For QCS Checks (section 6): Make calculated dilutions per the example below, dependent upon SSS actual concentration, and transfer to volumetric flasks for storage in the freezer.

		Concentration
From SSS	1 ml from SSS diluted to 100ml	200 µg/L
From SSS	0.5 ml from SSS diluted to 100ml	100 µg/L
From 200 µg/L	10 ml from (200µg/L) diluted to 100ml	20 µg/L
From 200 µg/L	1 ml from (200µg/L) diluted to 100ml	2 µg/L
From 2 µg/L	5 ml from (2µg/L) diluted to 50ml	0.2 µg/L

4.0 Chlorophyll *a* Sample Analysis Procedures

4.1 Chlorophyll *a* Filtration

1. The field collection procedures for chlorophyll *a* samples are described under the SCDES Environmental Affairs Standard Operating Procedures **section 8.3** (SCDES, BRLSPROC-400). In the laboratory, chlorophyll *a* samples must be filtered within 36 hours of sample collection and the filters stored in a freezer at -20°C. Record the necessary data in the “Chlorophyll Lab Analysis Logbook” (SCDES form D-1817). This includes: Sample ID or blank, date and time sample collected, date and time sample (or blank) filtered, initials of person receiving and filtering samples.
2. When ready to filter the chlorophyll *a* sample, thoroughly rinse all sample contact surfaces of the filtration manifold with DI water. Excess water should be shaken off the filtration manifold parts before filtering samples.
3. Using forceps, place the appropriate glass fiber filters onto each filter screen. The “rough” side of the glass fiber filter should be facing up, the “smooth” patterned side facing down. Do not touch the filters with hands.
4. Attach the top plate to the filtration assembly and tighten down the top with the hand wheel nut provided with the apparatus. Switch to subdued yellow lighting.
5. Mix the chlorophyll *a* sample by gently inverting the bottle back and forth about 12-15 times.
6. Using a graduated 15 mL disposable syringe attached to the matching syringe dispenser; draw out a 15 mL aliquot of sample.
7. Dispense this sample aliquot into one port of the filtration manifold and repeat, preparing three chlorophyll *a* replicates per sample. Change to a clean syringe between samples from different stations (collections).
8. Prepare sample replicates from other samples by repeating steps 5 – 7.
9. A single “blank” filter is prepared using DI water in place of chlorophyll *a* sample. Prepare a blank with each “filtration series”. A filtration series is defined by a period of time of filtering samples during which not more than 30 minutes has elapsed between filtering activities. The blank samples should be processed last in each filtration series or batch.
10. By using the hand pump, filter the samples under a vacuum not exceeding 6 inches of mercury.

11. Maintain pressure with the hand pump until filtration is complete, but not until the filters are completely dry.
12. Remove the top plate of the filtration manifold. With caution not to touch the filtered material or squeeze the filter, fold each filter in half with forceps so that the filtered material is on the inside of the fold. Transfer each set of 3 filters from the same sample to a petri dish labeled with station number and date collected.
13. Filtered samples in petri dishes are wrapped in aluminum foil and placed immediately in the freezer. A set of up to four petri dishes can be wrapped in one sheet of aluminum foil. Label the foil with initials and date collected.
14. The sample contact surfaces of the filtration manifold is rinsed thoroughly with tap water and DI water between each set of samples (Note: 4 samples x 3 replicates/sample = set of samples). Excess water should be shaken off the filtration manifold parts before filtering more samples.
15. If filtered chlorophyll *a* samples are not extracted immediately, they may be stored in the freezer at -20°C for up to, but not more than, 24 days from date of collection before analysis.

4.2 Chlorophyll *a* Extraction

1. Record the necessary data in the “Chlorophyll Lab Analysis Logbook” (SCDES form D-1817). This includes: Sample ID or blank, date and time sample collected, date sample analyzed, and analyst’s initials.
2. Switch to subdued yellow lighting. Remove filtered chlorophyll *a* samples from freezer and place on ice in a covered cooler.
3. Using forceps, transfer one sample filter from the sample petri dish to a glass grinding tube or mortar and add 4 mL of 90% aqueous acetone.
4. Grind contents for approximately 45 seconds to 1 minute until the filter has been converted to a slurry. Heat generated by excessive grinding may result in loss of chlorophyll, so good judgment will help one decide when the filter is sufficiently macerated. Pestle should be in good condition to ensure thorough maceration of algal cells and glass fiber filter.
5. Transfer contents of the grinding tube or mortar to a 15 mL graduated centrifuge tube with screw-cap. Rinse grinding tube or mortar pestle with

a few ml of 90% aqueous acetone and transfer contents to centrifuge tube.

6. With 90% aqueous acetone, bring volume of extract to 12.5 mL. If volume of extract is other than 12.5 mL, a notation must be made in the "Chlorophyll Lab Analysis Logbook" (SCDES form D-1817) which includes the amount of end volume.
7. As each sample is ground and brought to 12.5 mL, cap the samples with the Teflon-lined screw-caps provided and shake thoroughly. Immediately transfer each sample to a test tube rack in a cooler of ice.
8. When finished grinding a set of samples, cover the rack of centrifuge tubes containing extracted chlorophyll *a* with aluminum foil and place in the refrigerator to steep for at least 2 but not more than 24 h at 4°C in the dark.
9. The tubes of extracted chlorophyll *a* should be shaken at least once during the steeping process.

4.3 Chlorophyll a Measurement

1. Under subdued lighting, remove samples from refrigerator and allow them to warm to room temperature in the dark. This should take approximately one hour. Note: A fan may be directed on the samples to speed warming to room temperature.
2. Turn on or press the soft reset button on the fluorometer. The on/off switch is located on the back of the Triology. The triology has no warmup time, thus once on it is ready for analysis. It is very important that you select the correct module that is being used on the module selection screen. In this case, we want to select "Chl-NA". To see what module is selected at the time of turning on the fluorometer, look in the upper right-hand corner.
 - a. Select "calibrate" at the bottom of the screen.
 - b. Select "used store calibration" if you want to use the most recent calibration saved for the fluorometer.
 - c. Select the most recent calibration from the list of stored calibration.
 - i. Each time the machine is calibrated, the calibration can be named and stored for future use.
 - ii. Store calibration names as the month and date of the calibration- such as Mar26
 - d. Confirm top right is showing the correct calibration name (in this case- Mar26) and the units are ug/L.

3. Vigorously shake each tube to mix the sample and centrifuge for 5 minutes at 1000 g. The RPM required to get to 1000 g with the current centrifuge (with a radius of 17 cm) is 2290, which is setting '5' on the centrifuge.
4. Fill a glass test tube 3/4 full with 90% aqueous acetone. Wipe the outside of the test tube clean with a lab wipe and place the blank inside the fluorometer and close the lid. Touch "Measure Fluorescence" (in top righthand corner) to make a measurement. Enter "1" for the volume of filter sample and "1" for volume of the solvent. The Triology screen will say "measuring" and then in approximately 5 seconds will display the average reading on the screen. Record the reading in the "Chlorophyll Lab Analysis Logbook" (SCDES form D-1817) in the START column at the top of the page for Blank. Take reading of the solid standard and record in the START column at the top of the page.
5. The centrifuged chlorophyll *a* samples can now be analyzed. Without disturbing the filter residue in the bottom of the centrifuge tubes, carefully decant the sample solution into a glass test tube. The sample solution should fill about 3/4 of the test tube. Wipe the outside of the test tube with a lab wipe and place into the fluorometer.
6. When fluorescence reading stabilizes press "Measure Fluorescence", record the final average reading in the "Chlorophyll Lab Analysis Logbook" (SCDES form D-1817). Chlorophyll *a* concentration is read to three significant digits.
7. Remaining samples are read by carefully filling test tubes about 3/4 full with supernatant from the other centrifuge tubes. Record all readings - samples and blanks - in the appropriate place in the Analysis Logbook.
8. Some samples may be too concentrated to be read for the current calibration and linear range of the fluorometer ($\geq 300 \mu\text{g/L}$). Dilute these samples to 50% of their original concentration, make a notation in the logbook, and multiply the reading by 2 to get the actual concentration.
9. After all samples have been completed, take readings of the blank and secondary solid standard, and record in the "Chlorophyll Lab Analysis Logbook" (SCDES form D-1817) in the END column at the bottom of the page.
10. Wash labware:
 - A. Soak in detergent and hot water for 4 hours.
 - B. Scrub with brush or sponge if necessary.
 - C. Rinse thoroughly with hot tap water.
 - D. Rinse 2x with DI water.
 - E. Rinse once with acetone.

4.4 Chlorophyll *a* Data Analysis

Means are calculated by averaging the 3 replicates from each sample together. Calculations for mean values and to account for sample concentration or dilution are performed using a Microsoft Excel® spreadsheet. Calculations for chlorophyll *a* are as follows: Chlorophyll *a* µg/l = 0.8 x (direct chlorophyll *a* concentration readout from the fluorometer).

The 0.8 factor by which the chlorophyll *a* reading is multiplied accounts for concentration of the original sample volume during the extraction process. 15 ml of raw water sample was filtered through a glass fiber filter. The glass fiber filter was then macerated and brought to a volume of 12.5 mL with 90% acetone. The volume of macerated glass fiber filter is 0.5 mL. Therefore, the volume of actual extracted sample has been concentrated to 12 mL from 15 mL. Each chlorophyll *a* reading should be multiplied, then, by 12 mL ÷ 15 mL or 0.8 to get the actual concentration.

Chlorophyll *a* data are entered into a Microsoft Excel® spreadsheet and imported to an in-house Microsoft Access® database within the Bureau of Water. For quality assurance, all of the data in the spreadsheet are reconciled with the "Chlorophyll Lab Analysis Logbook" (SCDES form D-1817). Visual examination of calculated values is also done, with extreme values scrutinized. Any corrections are made prior to conversion into the database.

These data are used to assess the degree of eutrophication in the water bodies of interest and should always be interpreted in conjunction with other concurrently collected physical, chemical, and biological data when making water quality assessments. To evaluate the effects of eutrophication upon water quality, numeric criteria for chlorophyll *a* concentrations in lakes have been adopted by the State of South Carolina. The criteria are based on an ecoregional approach that considers the geographic location of the lakes within the State. These criteria are explained in Water Classifications & Standards (R. 61-68) and are applicable to lakes of 40 acres or more. Lakes of less than 40 acres are protected by narrative criteria contained in the same document.

5.0 Quality Control and Quality Assurance

All photosynthetic pigments are light and temperature sensitive. Work must be performed in subdued light and all standards, QC materials, and filter samples must

be stored in the dark at -20°C to prevent degradation. In addition to sensitivity of light by chlorophyll *a*, fluorescence is temperature dependent with higher sensitivity occurring at lower temperatures. Samples, standards, LRBs, CVSSs, and QCSs must be at the same temperature to prevent errors and/or low precision. Samples are analyzed at ambient room temperature in this method. Ambient temperature should not fluctuate more than $\pm 3^{\circ}\text{C}$ between calibrations or recalibration of the fluorometer will be necessary.

Field duplicates are two separate samples collected at the same time and placed under identical circumstances and treated exactly the same throughout field and laboratory procedures. Analyses of field duplicates give a measure of the precision associated with sample collection, preservation and storage, as well as with laboratory procedures. Approximately 10% of samples should have field duplicates. One duplicate for every ten stations sampled for chlorophyll *a* are taken by field personnel and labeled with station identification, date, time, and FD.

Lab duplicates are two separate samples taken from the same bottle under laboratory conditions and processed and analyzed independently. Analysis of lab duplicates gives a measure of instrument stability and consistency in the laboratory sample preparation and analysis. Approximately 10% of samples should have lab duplicates. One duplicate for every ten samples received for chlorophyll *a* are taken by laboratory personnel and labeled with station identification, date, time, and LD.

Chain-of-custody procedures have been adopted to ensure accurate tracking and accountability for samples from the field to final analysis. Chlorophyll *a* samples collected by field personnel are properly preserved and forwarded within sample holding times to the Aquatic Biology Section. Field personnel should label samples to identify station or site of collection, date sample was taken, and the time of collection. Upon arrival at the Aquatic Biology Section, the samples are logged into the "Chlorophyll Lab Analysis Logbook" (SCDES form D-1817), with the receiver recording initials and date of receipt. Each sample is uniquely identified in the logbook by its station number, date, and time collected. SC DES form 2186 serves as the chain-of-custody form and must be properly completed for all samples received.

The "Chlorophyll Lab Analysis Logbook" (SCDES form D-1817) is kept at a designated area in the Aquatic Biology Section. Any other information accompanying samples will be kept on permanent file.

Any mistakes are to be crossed out with one line and initialed by the recorder. No white-out, erasing, or any other form of covering mistakes is to be used. Indelible ink must be used for all handwritten information.

If there is any indication that the fluorometer is not reading samples within tolerance values - $\pm 10\%$ of known chlorophyll *a* concentrations from quality control checks or calibration verification standards - the problem is identified and corrected.

5.1 Daily QA/QC

Temperature Performance Checks – Temperature for incoming samples are taken from either an infrared thermometer, or a thermometer using Temperature Control bottles included with sample containers, and are recorded on SCDES form 1814. An infrared thermometer must be checked daily against a calibrated thermometer in a sample bottle of water at refrigerator temperatures and readings recorded on the “IR Calibration Log – Sample Receiving” log sheet (SCDES form 1814). Thermometers in both the refrigerator and freezer are checked twice daily, no earlier than 4 hours apart and recorded on the “Temperature Record Chart”). In other words, a check is performed in the AM hours and a check in the PM hours that are over 4 hours apart from one another. All thermometers are calibrated with a NIST traceable reference thermometer annually, as described in the SCDES Environmental Quality Field Quality Plan Protocol section 14.3 (SCDES, BEHSPROC-104). IR thermometers must be calibrated every 6 months.

Laboratory Reagent Blank (LRB) – An aliquot of DI water or other blank matrices that are treated exactly as a sample including exposure to all glassware, equipment, solvents, reagents, internal standards, and surrogates that are used with other samples. The LRB is used to assess contamination from the laboratory environment and determine if method analytes or other interferences are present in the laboratory environment, reagents, or equipment. A blank filter is extracted using DI water and analyzed just as a sample filter. The LRB should be the last filter in each sample set. The laboratory must analyze at least one blank filter with each sample batch, in which no more than 30 minutes has elapsed between filtering samples. LRB values that exceed 10% or more of the chlorophyll *a* concentration determined for a corresponding sample batch may indicate contamination or interference. Sample analysis must be suspended until the problem is identified and acceptable LRB values have been obtained.

Calibration Verification Standard (CVS) – Solid secondary standards are used for calibration and verification of instrument performance. Solid secondary chlorophyll *a* standards (certified and supplied by Turner Designs, Inc.) are used as CVSs on a daily basis. This solid secondary standard will be analyzed before and after analysis of each batch of samples. Currently, there is one solid secondary standard being used in the chlorophyll *a* lab with the Triology. The solid secondary sample provides a very stable fluorescent signal. It has an adjustment screw so you can tune the solid standard to match a specific sample. Follow the steps below on how to use the secondary standard:

1. Calibrate your instrument with a known standard
2. Take the adjustable secondary standard from its storage case. Using the 0.005" Allen wrench provided, loosen the locking screw on the back of the secondary standard by turning it counterclockwise one turn.
3. Place the adjustable secondary standard in the fluorometer sample compartment with the handle towards the rear of the instrument.
4. Wait approximately 10 seconds while the instrument averages the reading, then read the value
5. You can use the 3/32" Allen Wrench provided to adjust the attenuation screw through the hole at the top of the secondary standard to increase or decrease the value displayed on the screen. Turning the screw counterclockwise will increase the reading.
6. Repeat steps 4 and 5 until the secondary standard reads the concentration value of interest. Record the secondary standard reading for future reference.
7. At this point, remove the solid standard and turn the locking setscrew clockwise until it just makes contact with the attenuation screw.
8. Do not overtighten or remove either the hex screw on the Solid Secondary, overtightening may damage the standard.

An average is calculated using the first 20 readings after the yearly Initial Demonstration of Performance, and Relative Percent Difference (%RPD) is determined by the equation: $\%RPD = (\text{Absolute value of (Average - Reading)} / \text{Average})$. Daily CVS reading should be between $\pm 15\%$ of the RPD.

5.2 Bimonthly (two times a month) QA/QC

Calibration Verification Standard (CVS) – Solid secondary standards (referenced in the section above, 5.1) and chlorophyll *a* standards in 90% acetone are used for

calibration and verification of instrument performance before and after fluorometer calibration. Two liquid standards per order are certified and supplied by Turner Designs, Inc. The High standard has a chlorophyll *a* concentration of approximately 180-200 µg/L and the Medium standard has a concentration of approximately 18-22 µg/L. A Low standard is to be made in the laboratory and used as a calibration verification standard. This Low standard should be approximately 1.5-2.5 µg/L and will be made by making a 1/10 dilution of the Medium standard (2 mL of the Medium, diluted to 20 mL with 90% acetone). Chlorophyll-*a* standards in 90% acetone from Turner Designs, Inc. are stable until the ampoule is broken and opened. After use, standards must be disposed of.

Quality Control Sample (QCS) – With this method, on a bimonthly basis or as required to meet data quality needs, verify instrument performance with the analysis of a QCS. Several dilutions of the Stock Standard Solution are used to check instrument performance and are obtained from a source external to the laboratory and different from the source of calibration standards. An acceptable source of QCS is pure chlorophyll-*a* free from chlorophyll *b* obtained from Sigma Chemical (St. Louis, MO). The chlorophyll-*a* from Sigma Chemical is in dry form and must be reconstituted in 90% acetone with its concentration being determined spectrophotometrically. This solution serves as the Stock Standard Solution (SSS) and to obtain QCSs. Dilutions from the SSS will be prepared following the directions in section 3.1 above.

If the determined value is not within $\pm 10\%$ of the known concentration, then the source of the problem must be identified and corrected before continuing analyses.

5.3 Monthly QA/QC

Checks of the eye washes, safety showers, and fire extinguishers are to be performed on a monthly basis. Inspection sheets for the eye washes and safety showers are posted on the wall nearby; inspection tags are connected to the fire extinguishers.

5.4 Yearly QA/QC

An initial demonstration of performance is conducted once annually to characterize instrument performance and laboratory performance prior to sample analyses. PDS and dilution standards prepared above in section 3.1 are used to determine instrument performance. The following quality control investigations are conducted for the initial demonstration of instrument performance:

Linear Dynamic Range (LDR) -- The absolute quantity or concentration range over which the instrument response to an analyte is linear. The LDR should be determined by analyzing a minimum of 5 calibration standards (prepared from section 3.1) ranging in concentration from 0.2 µg/L to 200 µg/L chlorophyll-*a* across all sensitivity settings of the fluorometer (Low ≈ 0-3.0 µg/L, Med ≈ 3.0-30 µg/L, High ≈ 30-300 µg/L). Incrementally analyze standards of higher concentration until the measured fluorescence response, R , of a standard no longer yields a calculated concentration, C_c , that is $\pm 10\%$ of the known concentration, C , where $C_c = (R - b)/m$. That concentration defines the upper limit of the LDR for the instrument. Should samples be encountered that have a concentration which is 90% of the upper limit of the LDR, these samples must be diluted and reanalyzed. EPA Method 445.0 and other research have suggested a LDR upper limit of approximately 250 µg/L for fluorometry of chlorophyll-*a*; however, the exact range must be established for each individual lab for each year.

Instrumental Detection Limit (IDL) -- The minimum quantity of analyte or the concentration equivalent that gives an analyte signal equal to three times the standard deviation of the background signal at the selected wavelength, mass, retention time, absorbance line, etc. For this method the background is a solution of 90% acetone. Zero the fluorometer with a solution of 90% acetone on the maximum sensitivity setting. Pure chlorophyll-*a* in 90% acetone should be serially diluted until it is no longer detected by the fluorometer on a maximum sensitivity setting.

Estimated Detection Limit (EDL) -- The minimum concentration of an analyte that yields a fluorescence 3X the fluorescence of blank filters that have been extracted according to this method. Several blank filters should be extracted according to the methods under Chlorophyll Filtration Procedures using clean glassware and apparatus, and the fluorescence measured. A solution of pure chlorophyll-*a* in 90% acetone should be serially diluted until it yields a response which is 3X the average response of the blank filters.

Extraction Proficiency – Initial Demonstration of Capability (IDOC) -- Personnel performing this method for the first time should demonstrate acceptable proficiency in the extraction of sampled filters by analyzing 10 low to mid-range standards and report results to supervisor for calculation. A set of 5 blank filters should also be extracted and analyzed with acceptable proficiency. The percent relative standard deviation of uncorrected values of chlorophyll-*a* should not exceed 15% for samples

that are approximately 10X the IDL.

For Initial Demonstration of Performance, analyze each solution, including an acetone blank, in the fluorometer and take the RAW fluorometer reading. Record the RAW readings and concentrations determined by spectrophotometer in the “Chlorophyll Lab Calibration Logbook” (SCDES form D-1814). Change back to direct concentration once all readings have been performed and calibrate the fluorometer with the proper calibration standards.

6.0 Calibration

6.1 Fluorometer

The fluorometer calibration is performed bimonthly per the procedure described in the “Calibration Procedures” of the Triology Laboratory Fluorometer User’s Manual. Reference chlorophyll-*a* in 90% acetone used for calibrations and calibration verifications, are obtained and certified from Turner Designs (Sunnyvale, CA). A record of fluorometer calibrations is maintained in the “Chlorophyll Lab Calibration Logbook” (SCDES form D-1817). When calibrated, the Triology uses stored calibration values to automatically convert fluorescence responses to concentration estimates; this saves you from having to manually calculate concentration estimates using RFU. At the end of each analysis series, you are prompted to save calibrations. You can save a total of 18 calibrations into memory to be recalled when the module is being used. Once you turn the triology off, you will need to reselect the calibration you desire for the appropriate module. Remember, the Triology will not recognize the module once it has been inserted, you will need to select what module you’ve inserted and what calibration file to use for that module.

1. Under subdued lighting, allow the standards and the blank to come to room temperature.
2. Take readings of calibration standards, solid secondary standard, quality control standards, and instrument checks prior to calibration and record in the “Chlorophyll Lab Calibration Logbook” (SCDES form D-1817).
3. Blanking the fluorometer – On the home screen, touch “calibrate” to begin a calibration sequence. Select “Run New Calibration.” Select the unit of measurement (ug/L). Open the lid and insert the blank sample. Close the lid and touch “OK”. After the blank has been measured, open the lid and remove the blank.

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4. Enter the concentration for the first standard and touch "OK" (Note: if doing multi-point calibrations, be sure to use standards in order of increasing concentration).
5. Insert the standard, close the lid, and touch "OK"
6. After the standard has been measured, either select "Proceed with Current Calibration" and continue to step #7 or "Enter More Standards" and return to step #4.
7. Name and save the calibration for future use.
8. Subsequent readings in the direct concentration mode should reflect the actual concentration of the fluorophore in relation to the standards used for calibration.
9. Confirm successful completion of the calibration by measuring the same standard used to calibrate the Triology. The displayed concentration should equal the value in step #4.

6.2 Spectrophotometer

The spectrophotometer is used to determine the concentrations of the SSS and primary dilution standards by the following steps:

1. Turn on the spectrophotometer and wait while any prepared standards to be analyzed are allowed to come to ambient temperature either by setting them out in the dark at room temperature or placing them in a water bath equivalent to room temperature.
2. When ready to use the spectrophotometer, click the "Live Display" icon on the software home screen. In the live display mode, the instrument performs continuous absorbance measurements automatically.
3. Carefully fill one glass test-tube with 90% acetone and the other with the chlorophyll-*a* standard to be analyzed.
4. Carefully wipe the sides of the tubes with lab wipes each time before placing them in the spectrophotometer to get a reading.
5. Set the spectrophotometer to 750 nm. Insert the blank solution (90% acetone) into the cell holder and close the sample compartment lid. Press the blue "Blank" button at the bottom of the screen and allow for the spectrophotometer to read the absorbance of the blank.
6. Remove the blanking cell from the unit and replace it with the cell containing the sample solution. The reading will be displayed in about

- ten seconds. Record the value under the 750 nm reading on the "Spectrophotometer Readings in ABS worksheet."
7. Determine and record additional absorbance readings of blank and sample at wavelengths 664 nm, 647 nm, and 630 nm by repeating steps 5 and 6. The spectrophotometer must be zeroed with 90% acetone blank at each wavelength setting before the sample is read.
 8. Perform calculations per the trichromatic method of chlorophyll-*a* determination as determined by the equation: Chlorophyll-*a* (mg/L)= 11.85(ABS664-ABS750) – 1.54(ABS647-ABS750) – 0.08(ABS630-ABS750). Multiply by 1000 to get µg/L.
 9. When the concentration of the Primary Dilution Standard (PDS) is determined, fluorometer calibration can proceed as needed using the PDS or other Calibration Standards carefully diluted from the PDS.
 10. The photometric (absorbance) performance of the spectrophotometer should be calibrated every 2 months during the sampling season using the Spectronic Standards Kit. This kit is used to test accuracy in the visible region.

7.0 References

- United States Environmental Protection Agency. September 1997. In Vitro Determination of Chlorophyll *a* and Pheophytin *a* in Marine and Freshwater Algae by Fluorescence, Method 445.0, Revision 1.2, National Exposure Research Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, Cincinnati, Ohio 45268.
- South Carolina Department of Environmental Services. 2025. Environmental Affairs Standard Operating Procedures. Bureau of Regional and Laboratory Services.
- Turner Designs, May 31, 2019. Triology Laboratory Fluorometer User's Manual, San Jose, CA.
- Turner Designs. Undated. A Procedure for Measuring Extracted Chlorophyll *a* Free From the Errors Associated With Chlorophyll *b* and Pheopigments. Turner Designs, 845 W. Maude Avenue, Sunnyvale, CA 94086

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ThermoFisher Scientific, February 2019. Genesys Family Spectrophotometers: User Guide, Revision A. ThermoSpectronic, 2000. Spectronic® Genesys 8™ UV/Visible Spectrophotometer Operator's Manual, Issue 2a, Rochester, NY.

8.0 Revision History

Date	Revision	Change	Section
June 2018	3.0	Corrected to define a sample batch as no more than 30 minutes elapsing between sample filtrations	4.1, 5.1
June 2018	3.0	Changed "Extraction proficiency" from filtration of 5 water samples to the EPA approved method of 10 or more samples.	5.4
March 2020	4.0	Updated fluorometer type to the Turner Designs Triology Fluorometer	1.0
March 2020	4.0	Updated apparatus and equipment for the Triology Fluorometer	2.0
March 2020	4.0	Updated size of borosilicate glass cuvettes to 12mm x 75mm.	2.0
March 2020	4.0	Updated steps for measuring fluorescence on the Triology	4.3- 2-4
March 2020	4.0	Added Solid Secondary Standard steps for the Triology	5.1
March 2020	4.0	Updated Fluorometer Calibration for the Triology	6.1
May 2021	5.0	Updated to include mortar and pestle for tissue/filter grinding	2.0
May 2021	5.0	Updated to include mortar and pestle in the methods for extraction	4.2
May 2024	6.0	Updated Spectrophotometer type to Genesys 50 UV-Vis Spectrophotometer	2.0
May 2024	6.0	Added Spectrophotometer standards to the Apparatus and Equipment list	2.0
May 2024	6.0	Added information on tracking Acetone lot numbers, expiration dates, and open dates	3.0
May 2024	6.0	Updated Acetone manufacturer name	3.0

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Date	Revision	Change	Section
May 2024	6.0	Updated PDS hold time to six to nine months based on EPA Method 445.0	3.1
May 2024	6.0	Added statement about checking PDS and QCS samples monthly via spectrophotometer to ensure concentrations are within range	3.1
May 2024	6.0	Added lab duplicate description	5.0
May 2024	6.0	Updated SOP referenced for thermometer calibrations	5.1
May 2024	6.0	Added that IR thermometers must be calibrated every 6 months	5.1
May 2024	6.0	Removed sentence saying the spectrophotometer step is unnecessary to calibrate the fluorometer if chlorophyll standards with certificates are available	6.2
May 2024	6.0	Updated Spectrophotometer directions to reflect new Spectrophotometer Genesys 50	6.2
May 2024	6.0	Removed old SOP from citations; added updated SOP reference	7.0
May 2024	6.0	Updated Fluorometer user manual citation	7.0
May 2024	6.0	Updated Spectrophotometer user manual citation	7.0
January 2026	7.0	Changed "SCDHEC" to "SCDES"	Where applicable
January 2026	7.0	Changed SCDHEC SOP reference to SCDES SOP reference BRLSPROC400	4.1
January 2026	7.0	Updated Signatures	Title page