Standard Operating Procedure

Laboratory Procedures for Chlorophyll *a* and Phycocyanin Analysis in Water

Commonwealth of Kentucky
Kentucky Energy and Environment Cabinet
Department for Environmental Protection
Division of Water

Effective Date: April 18, 2017
Revision Date: March 1, 2020
Version Number: 2.0

Document ID: DOWSOP03048

Action By:	Signature	Date
Jacob Eldridge, Prepared SOP Author Water Quality Branch		02/25/20
Lara Panayotoff, Reviewed Section Supervisor Water Quality Branch		2/12/20
Melanie Arnold, Approved Manager Water Quality Branch	Melanie Arnold	2-19-20
David Cravens, Approved Quality Assurance Coordinator Water Quality Branch		2-19-20
Mary Rockey, Approved Quality Assurance Officer Division of Water	Mary Rockey	2-20-2020
Paul Miller, Approved Director Division of Water		2-21-20
Larry Taylor, Approved Quality Assurance Manager Department for Environmental Protection		2/24/20

Document Revision History

Date of Revision	Page(s) Revised	Revision Explanation	Revision Author
4/21/2016	Entire Document	Replaces in part "Kentucky Ambient/Watershed Water Quality Monitoring SOP, August 2005"	Lara Panayotoff
2/1/2020	Entire Document	-Added steps in protocol for the analysis of phycocyanin -Removed the requirement to calibrate fluorometer after changing optical module -Added DI rinse of graduated cylinder and filter funnel into sample when filtering -Removed language about storing filters in foil packs -Changed LRB to include filtering process and use 10 mL DI water with LRB filter; clarified that LRB should always be done at the end of batch in addition to every 20 samples -Added step of shaking sample before placing in centrifuge -Changed extract volume to reflect volume displaced by filter -Clarified timing and methods of cleaning equipment -Removed instructions to dry filter apparatus and filter with lab tissue -Changed CCV check to include before analysis starts, at least after every 12 samples, and the end of a batch -Changed data recording method to prefer electronic lab log and data entry in K-WADE -Instruction to record calibration information in electronic lab log	Jacob Eldridge
2/1/2020	Appendix G	Added Chlorophyll Quick Guide to SOP appendix	Jacob Eldridge

Suggested Citation: Kentucky Division of Water (DOW). 2020. Standard Operating Procedures: Laboratory Procedures for Chlorophyll *a* and Phycocyanin Analysis in Water, Version 2.0. Kentucky Department for Environmental Protection, Division of Water, Frankfort, Kentucky.

TABLE OF CONTENTS

1.0 SCOPE & APPLICABILITY 4
2.0 SUMMARY OF METHOD 4
3.0 DEFINITIONS & ACRONYMS 4
4.0 HEATH & SAFETY STATEMENT 5
5.0 CAUTIONS & INTERFERENCES 6
6.0 PERSONNEL QUALIFICATIONS 7
7.0 EQUIPMENT & SUPPLIES 8
8.0 STEP-BY-STEP PROCEDURE 9
8.1 Instrument Initial Calibration, Continuing Calibration Verification, and Determination of Estimated Detection Limit 9
8.2 Other Equipment Maintenance and Operation 10
8.3 Sample Collection 10
8.4 Receive Field Sample into Laboratory 10
8.5 Sample Filtration 11
8.6 Sample Grinding and Extraction, Steeping, and Fluorometric Analysis 12
8.6.1 Preparation of Aqueous Acetone Solution (chlorophyll a)- 90% acetone /10% water 12
8.6.2 Preparation of 50nM Phosphate Buffer (phycocyanin) 13
8.6.3 Grinding and Extraction 13
8.6.4 Steeping 14
8.6.5 Fluorometric Analysis 14
8.7 Sample Dilutions 16
9.0 TROUBLESHOOTING 16
10.0 DATA & RECORDS MANAGEMENT 16
11.0 QUALITY CONTROL & QUALITY ASSURANCE 17
12.0 REFERENCES 19
13.0 APPENDICES 20
Appendix A: Chain-of-Custody REQUIREMENTS 20
Appendix B: Chlorophyll *a* log “CHLa log” 21
Appendix C: Chlorophyll *a* log “Zero Log” 22
Appendix D: Chlorophyll *a* log “CCV Log” 23
Appendix E: Chlorophyll *a* log “PD Log” 23
Appendix F: Chlorophyll *a* log “Calibration” 24
Appendix G: Phycocyanin log “PC log” 25
Appendix H: Phycocyanin log “Zero Log” 26
Appendix I: Phycocyanin log “CCV Log” 27
Appendix J: Phycocyanin log “PD Log” 27
Appendix K: Phycocyanin log “Calibration” 28
Appendix L: Chlorophyll Quick Guide 29

LIST OF TABLES

TABLE 1. LABORATORY EQUIPMENT AND SUPPLIES 8
TABLE 2. QAQC REQUIREMENTS FOR CHLOROPHYLL A ANALYSIS 17

1.0 SCOPE & APPLICABILITY

This document outlines procedures for the analysis of water column chlorophyll *a* and phycocyanin samples that are collected by the Kentucky Division of Water. Chlorophyll *a* concentration is used as a surrogate for sestonic algal biomass and a factor in calculating lake trophic status index (TSI). Phycocyanin concentration is used as a cyanobacteria monitoring tool, utilizing phycocyanin to chlorophyll *a* ratio as a cyanobacteria bloom condition indicator. Although samples collected from surfaces (i.e., periphyton) can be analyzed with similar methods, this SOP is intended for use with water column samples. A Turner Trilogy fluorometer is used for sample analysis. The use of other instruments may require different procedures.

2.0 SUMMARY OF METHOD

This method is based on those described in EPA 445 r1.2 (EPA 1997) and SM 10200H (APHA 1999). A measured volume of sample water is filtered within 24 hours of collection with a glass fiber filter to concentrate phytoplankton. The filter is then either extracted immediately or placed in a freezer for storage up to 24 days. Filters for chlorophyll *a* analysis are extracted in 90% acetone and ground mechanically using a tissue grinder. Filters for phycocyanin analysis are extracted in 50nM phosphate buffer and ground mechanically using a tissue grinder. The resulting slurry is then centrifuged to clarify. The clarified extract is transferred to a cuvette and analyzed using a fluorometer with narrow bandpass filters (Turner Designs Trilogy digital fluorometer with Chl NA optical kit or Orange optical kit for phycocyanin). There is not an additional acidification step for chlorophyll *a* analysis (i.e. the “modifed fluorometry method” described in EPA 445 rev 1.2, otherwise known as the Welshmeyer method). The results are a measurement of the concentration of chlorophyll *a* with minimal interference from phaeophytin *a* and other chlorophylls.

3.0 DEFINITIONS & ACRONYMS

CCV: Continuing Calibration Verification
EDL: Estimated Detection Limit
FB: Field Blank Sample
FD: Field Duplicate Sample
FS: Field Sample
DI: De-ionized Water
DQO: Data Quality Objective
DEP: Kentucky Department for Environmental Protection
DOW: Kentucky Division of Water

LRB: Laboratory Reagent Blank
LSS: Laboratory Split Sample
PPE: Personal Protective Equipment
PD: Performance Demonstration
RFU: Relative Fluorescence Units
QA: Quality Assurance
QC: Quality Control
SOP: Standard Operating Procedure

4.0 HEALTH & SAFETY STATEMENT

All lab staff should review "Worksite Hazard Assessment Guidance Document." In addition, each employee will be individually trained by his/her supervisor, or designee, to perform assigned job tasks safely, prior to his/her performing the task.

Lab staff working in and around potentially contaminated surface waters should receive immunization shot for Hepatitis A in accordance with DEP Policy SSE-708. In addition, staff should receive immunization for Hepatitis B and tetanus, to aid in the prevention of contracting those pathogens. All field staff should also be trained in CPR, First Aid, and Blood Borne Pathogens in accordance with DEP Policy SSE-711.

The use of personal protective equipment (PPE) should be used when sampling including:

- Gloves, lab coat, and eye protection should be worn during grinding of filters and anytime handling acetone.
- Use latex or another heavy glove during the grinding/analysis portion of the SOP or anytime when handling acetone. <u>Nitrile gloves do not provide sufficient protection against acetone exposure</u>.
- A heavy glove should be worn when grinding samples to protect the hand in case of grinding tube failure. Eye protection also should be worn.

In addition,

- The grinding of filters during the extraction step must be conducted in a fume hood due to the volatilization of acetone by the tissue grinder. Ensure that the hood sash is set to the inspection mark.
- Store all containers with acetone in the flammables cabinet when not in use.
- Disposal of glass consumables should be done with care, using a sharps container where appropriate.

- If using dry ice to store filters in the field, handle with gloves and keep dry ice in paper wrapping. Use a cooler with a lid that is not airtight to allow gases to escape. Keep cooler in well ventilated area at all times, such as in the vehicle rooftop carrier or on boat. Do not keep inside vehicle or indoors where people are present for more than short periods of time.

5.0 CAUTIONS & INTERFERENCES

General Cautions:

- Samples must be processed within designated time to ensure sample quality. Sample water should be immediately stored on wet ice and filtered as soon as possible but no later than 24 hours after collection. Sampled filters may be kept on wet ice up to 4 hours after filtering if grinding immediately. Otherwise, sampled filters should be immediately placed in a dark freezer (-20°C) for up to 24 days before extraction.

- Thorough mixing of the sample is necessary in several steps during processing for a sample to be representative. Mixing should occur before pouring sample water into the graduated cylinder, before and during steeping, and before placing in the centrifuge.

- All photosynthetic pigments are light and temperature sensitive. Work must be performed in subdued light and all standards, quality control (QC) materials and filter samples must be stored in the dark at -20°C or lower to prevent degradation.

- Gloves must be worn when handling samples, filters, and glassware; all glassware must be clean and acid-free to avoid degradation of pigments by contact with acidic substances. Samples from acidic waters should be filtered and analyzed as soon as possible for this reason.

Sample Filtering:

- Ensure an appropriate amount of water is filtered. Surface water should have a minimum volume of 50 mL filtered, but generally 100 mL is preferred. If 100 mL is filtered and there is no visible color change (green or brown), continue to add measured sample water until color change is visible and record the total volume filtered. More turbid water will likely need less water filtered whereas very clean water may need more water filtered. Never add more water than can pass through the filter in 10 minutes and do not exceed vacuum pressures greater than 6 in. Hg (20 kPa) to avoid damaging cells.

- Regularly dispose of the filtered water in the filter flask to ensure water is not pulled into the vacuum pump. If water is pulled into the pump, immediately unplug the pump and contact the appropriate lab personnel to perform maintenance on the pump.

- Ensure oil level for the vacuum pump is within the specified range. Add more oil if necessary.

- Never directly touch the concentrated particulates on the sampled filter. If the concentrated particulates are touched (e.g. tweezer touches when folding), restart the filtering process for that sample.

Sample Analysis:

- Ensure the correct optical module is inserted and most current valid stored calibration is selected for the fluorometer.

- Any substance extracted from the filter or acquired from laboratory contamination that fluoresces in the red region of the spectrum may interfere with the accurate measurement of chlorophyll *a*. Any substance extracted from the filter or acquired from laboratory contamination that fluoresces in the orange region of the spectrum may interfere with the accurate measurement of phycocyanin.

- Quenching effects are observed in highly concentrated solutions. The linear range per manufacturer specifications is 0-300µg/L. Samples reading greater than 250 µg/L should be diluted and re-run. The need for dilution can be minimized by avoiding filtering too much volume of highly concentrated samples.

- Fluorescence is temperature dependent with higher sensitivity occurring at lower temperatures. Samples, standards, and laboratory reagent blanks (LRB) must be at the same temperature to prevent errors and/or low precision. Analyses of samples at ambient temperature is recommended in this method. Ambient temperature should not fluctuate more than ± 3°C between calibrations or recalibration of the fluorometer will be necessary.

6.0 PERSONNEL QUALIFICATIONS

Personnel performing this method will be trained in this SOP by experienced staff. Training will consist of verbal and in-person instructions including the demonstration of safe work practices. Personnel should have fully read and understood this SOP and the user manual for the Turner Trilogy fluorometer and the IEC HN-SII centrifuge.

Personnel performing this method for the first time will demonstrate proficiency in the extraction of sampled filters prior to analyzing field samples for projects. A 1L water sample

will be obtained from a natural waterbody that is expected to have a chlorophyll concentration at least 10X the instrument detection limit. Ten replicate filters will be extracted and analyzed following the procedures in this SOP. The percent relative standard deviation of chlorophyll *a* concentrations should not exceed 15%. Percent relative standard deviation is calculated as the standard deviation divided by the mean, multiplied by 100. Record performance demonstration data on the "PD LOG" tab of the digital Chlorophyll *a* (Appendix E) or Phycocyanin Log (Appendix J).

7.0 EQUIPMENT & SUPPLIES

Table 1 is a list of supplies required for Chlorophyll *a* and phycocyanin laboratory analysis.

Table 1. Laboratory Equipment and Supplies

Sample Containers
250mL opaque (brown) bottles, or larger as needed
Laboratory Equipment
Turner Trilogy fluorometer with Chl NA lamp installed (Orange lamp installed for phycocyanin)
Turner Adjustable Secondary Standard, Turner 8000-952
6 position centrifuge, capable of 675 g
Vacuum pump with gauge capable of maintaining a vacuum up to 6 in Hg
Filtration apparatus, glass or acetone-resistant plastic, with 47-mm disk base
Tissue grinder assembly
Teflon serrated pestles with matching 50 ml borosilicate mortars (Thomas Size C or similar)
Drive motor on laboratory stand
Graduated cylinders: 100 ml, 500 ml,1000 ml
Volumetric pipets: 1mL, 10 mL
Filter forceps
Scissors
Test tube racks to fit centrifuge tubes and cuvettes
Polyethylene squirt bottles
Low wattage lamp
Fume hood
Refrigerator
Freezer
Laboratory Supplies
Chlorophyll calibration standard, Turner 10-850
Phycocyanin calibration standard, Sigma Aldridge P217210MG
15mL graduated centrifuge tubes with screw caps
Plastic transfer pipets
12mm x 75mm borosilicate cuvettes
Alconox® detergent
Acetone, HPLC grade
50nM Phosphate Buffer
Powderless latex/nitrile gloves
Glass microfiber filters, 47mm diameter, pore size 0.7 µm (Whatman GF/F or similar)
De-ionized water

Laboratory wipes (e.g., Kim wipes®)
Aluminum foil
Gallon zip lock bags
Sharps box
Additional supplies and Equipment for off-site filtration and storage
Vacuum hand pump and tubing
Gallon storage bags
Field freezer or dry ice and cooler

8.0 STEP-BY-STEP PROCEDURE

8.1 Instrument Initial Calibration, Continuing Calibration Verification, and Determination of Estimated Detection Limit

The fluorometer is calibrated shortly before the beginning of the sampling season (Initial Calibration). Additionally, the fluorometer must be calibrated any time the continuing calibration verification (CCV) fails to meet acceptance criteria. Calibration standards for chlorophyll *a* are obtained from the manufacturer (Turner) that have been quantified spectrophotometrically and are used to perform a two-point calibration. The standards are shipped overnight and must be immediately placed in the -20°C freezer upon receipt. Per the manufacturer, the standards are usable for one year if unopened and 1 month once opened, provided they are properly stored in the dark at -20°C. The phycocyanin standards are mixed and diluted to create a standard curve using Sigma Aldrich P2172 C-Phycocyanin from *Spirulina* in 50nM phosphate buffer (Kasinak, et al. 2014). Once the chlorophyll *a* or phycocyanin calibration has been performed (see User Manual, Turner Designs 2016), measure the fluorescence (as µg/L chlorophyll *a* or µg/L phycocyanin) of the solid secondary standard following instructions in the instrument manual. The initial calibration results are recorded in the "Calibration" tab of the Chlorophyll *a* (Appendix F) or Phycocyanin Log (Appendix K).

The solid secondary standard is then used for CCV, which serves to detect possible drift in the instrument (readings should be +/-10% of established value). The CCV check is recommended to be completed prior to initiating sample extractions to allow time for the instrument to be recalibrated if necessary. Checking the calibration with the secondary standard is then done each day before sample analysis, at least after every 12 samples, and after the last sample is analyzed. The calibration details are recorded in the "Calibration" tab of the Chlorophyll *a* (Appendix F) or Phycocyanin Log (Appendix K).

The Estimated Detection Limit (EDL) is determined annually. First, calibration is performed. Then, at least four blank filters are processed in a similar way as routine samples, and their measured relative fluorescence values are averaged. A chlorophyll *a* or phycocyanin solution of known concentration (usually the low-end calibration standard) is serially diluted (using 1, 5, and 10 mL volumetric pipets) until it yields a response (RFU) that is approximately 3x the

average response of the blank filters. This concentration is the EDL. The details of EDL determination and final EDL value are recorded on the "Calibration" tab of the Chlorophyll *a* (Appendix F) or Phycocyanin Log (Appendix K).

8.2 Other Equipment Maintenance and Operation

The vacuum pump requires periodic maintenance. See pump owner's manual for maintenance instructions and also how to reset maximum pressure if the setting is lost.

8.3 Sample Collection

- Water samples are collected per project study plan requirements. Follow the SOP for surface water sampling appropriate for the type of waterbody sampled.
- Use opaque sample containers or wrap containers with foil to block light from reaching the sample.
- Store water samples on wet ice immediately after collection.
- Record sample details on a Chain of Custody (COC) form that contains all required elements listed in Appendix A.
- Samples should be filtered within 24 hours of collection. Samples from eutrophic waters with high levels of algae or samples from acidic waters should be filtered as soon as possible. If samples cannot be returned to the laboratory for filtering within 24 hours (i.e., during overnight sampling trips) then samples should be filtered off-site (e.g., in the hotel at the end of the work day or before starting the next day). The filters should then be placed in a portable freezer or on dry ice for storage until filters can be transferred to the laboratory freezer. Wet ice storage of sampled filters is also acceptable for up to 4 hours.
- If water samples are filtered off-site, include details (e.g. time, volume) on the COC. This information will be transcribed to the sample log when samples are received in the laboratory.

8.4 Receive Field Sample into Laboratory

1. Inspect sample container for any damage.
2. Review sample COC for required collection information (Appendix A).
3. Assign a laboratory ID to the sample. The laboratory ID will be maintained throughout the analysis of the sample in the laboratory and will serve as a unique identifier to the sample. All samples, including any QC samples (i.e. laboratory duplicates, laboratory reagent blanks, etc.), will receive a laboratory ID. Chlorophyll *a* IDs will be formatted as "C", the year (YY) and a sequential number. For example the first laboratory ID for calendar year 2020 will be: C20-001. Phycocyanin IDs will be formatted as "P", the year

(YY) and a sequential number. For example the first laboratory ID for calendar year 2020 will be: P20-001. (See later sections for ID conventions for QC samples.)

4. Log Sample in to Chlorophyll *a* (Appendix B) or Phycocyanin Log (Appendix G), using the digital copy if possible. If using a paper log, keep the log continuous and mark out unused rows if skipping rows to start a new sheet. Transcribe the paper log or filtering information from the COC (if samples filtered remotely) to the digital Chlorophyll *a* or Phycocyanin Log as soon as possible.

8.5 Sample Filtration

1. Assemble the filtration apparatus and use clean forceps to place a filter onto the base. Attach the vacuum source with vacuum gauge and regulator.
2. If processing multiple samples, it is recommended to prepare the number of centrifuge tubes needed for the day by wrapping them in aluminum foil and labeling the caps with sample IDs using a grease pencil. Organize centrifuge tubes in a test tube rack in the order they will be processed. Note: acetone used in the grinding process will remove ink from the tubes so Sharpies® should not be used.
3. Adjust workspace lighting to the minimum level that is necessary to read instructions and operate instrumentation. Keep samples in the refrigerator/cooler until ready to filter.
4. Prior to drawing a subsample from the container, thoroughly but gently agitate the container to suspend the particulates (invert several times).
5. Quickly pour a portion of sample into a graduated cylinder and accurately measure the volume. Volumes for Kentucky's waters that typically produce a mid-range chlorophyll *a* value are 50-100 mL (generally 100 mL). Larger volumes may be required in oligotrophic waters. Do not filter less than 50 mL for surface water samples.
6. Pour the subsample into the filter tower of the filtration apparatus and apply a vacuum. Rinse the graduated cylinder with De-ionized (DI) water into the filter funnel using a squirt bottle. Vacuum filtration should not exceed 6 in. Hg (20 kPa). Higher filtration pressures and excessively long filtration times (> 10 min) may damage cells and result in loss of chlorophyll or phycocyanin. A sufficient volume has been filtered when a faint but visible green or brown color is apparent on the filter.
7. When the final volume approaches the level of the filter, slowly release the vacuum and gently rinse the inside walls of the filter funnel with DI water. Then, completely release the vacuum as the last water is pulled through the filter. Do not suck the filter dry with the vacuum. Regularly dispose of the filtered water in the filter flask to ensure water is not sucked into the vacuum pump.
8. Remove the filter from the base with tweezers, fold in half three times (into a pie shape). Never touch the filter with tweezers or lab tissue inside the circle where particulates were concentrated. Place the folded filter in a 15 mL centrifuge tube wrapped in aluminum foil. If not pre-labeled, use a grease pencil to mark centrifuge tube and cap with the laboratory ID. Transfer to -20°C freezer or onto dry ice, using

plastic storage bags to keep sets of samples together. Label the bags with the site ID, filtered date, and expiration date if frozen (24 days from filtering date).

9. At least 1 in 20 samples per project must be duplicated by filtering a second aliquot from the same sample. This is the Laboratory Split Sample (LSS). Assign a sequential sample ID but assign the sample type as "LSS" in the Chlorophyll *a* or Phycocyanin Log. Plan ahead to ensure that at least one water sample per batch will have sufficient volume to allow for two filtered samples (e.g. use larger field sample container if necessary). If all samples have an adequate volume for the LSS, select sample at random.
10. After all samples in a batch have been filtered (**and** after every 20th sample in a batch if batch is more than 20), prepare a LRB filter. There should always be an LRB at the end of every batch. To make an LRB, repeat steps 5-8 but substitute 10 mL DI water for the sample water. Assign this sample an ID in the form CYY-LRB-MMDDYY for chlorophyll-a samples(e.g., C17-LRB-040117) or PYY-LRB-MMDDYY for phycocyanin samples(e.g., P17-LRB-040117), where the date is the date that the LRB is prepared. If there is more than one LRB in a day then add a sequential number to the end of the ID.
11. **Record the filtration time, volume filtered, analyst (personnel who filtered), and whether filters were frozen within four hours on the Chlorophyll *a* (Appendix B) and Phycocyanin Log (Appendix G).**
12. If filtering numerous samples, the filters in centrifuge tubes can be placed on wet ice until the batch is complete (up to 4 hrs), but filters should be stored at -20°C as soon as possible if not extracting immediately.
13. Clean filter apparatus after every batch by washing filtering equipment and graduated cylinders in laboratory grade detergent and water, rinsing well with tap water, and finally triple-rinsing with deionized water. Let dry. If chlorophyll field sampling bottles are re-used (only within the same project), triple rinse with tap water and then triple rinse with DI water and allow to thoroughly air dry.
14. Preservation -- filters should be stored frozen (-20°C) in the dark until extraction.
15. Holding Time -- Filters can be stored frozen at -20°C for as long as 3½ weeks (24 days) without significant loss of chlorophyll *a*.

8.6 Sample Grinding and Extraction, Steeping, and Fluorometric Analysis

Note: It is recommended to check the calibration of the fluorometer using the solid secondary standard prior to initiating sample extractions so that issues can be addressed or the instrument can be recalibrated before proceeding. The reading should be +/- 10% of the original value.

<u>8.6.1 Preparation of Aqueous Acetone Solution (chlorophyll *a*)- 90% acetone /10% water</u>

1. Measure 100 mL of DI water with a 100 mL graduated cylinder.
2. Transfer to a 1-L flask or storage bottle.
3. Measure 900 mL of acetone into a 1000 mL graduated cylinder

4. Transfer acetone to the flask or bottle containing the water.
5. Mix the solution, then label and store.

8.6.2 Preparation of 50nM Phosphate Buffer (phycocyanin)

1. Measure 40 mL of concentrated phosphate buffer (Ricca 5807-16) with a 100 mL graduated cylinder.
2. Transfer to a 1-L flask or storage bottle.
3. Measure 960 mL of distilled water into a 1000 mL graduated cylinder
4. Transfer water to the flask or bottle containing the phosphate buffer.
5. Mix the solution, then label and store in a refrigerator.

8.6.3 Grinding and Extraction

1. If filters have been frozen, remove them from the freezer when ready to process (no more than a few at a time) but keep in the dark and/or wrapped in foil.
2. Set up the tissue grinder assembly in the hood. Have laboratory wipes, a DI squirt bottle, and 90% acetone squirt bottle (or phosphate buffer bottle for phycocyanin) on hand.
3. Adjust workspace lighting to the minimum that is necessary to read instructions and operate instrumentation.
4. Use clean forceps to remove filter from its container, cut into small pieces with scissors and place in the glass grinding tube. Push into the bottom of the tube with a clean probe or glass rod.
5. Using a squirt bottle, add approximately 6 mL of 90% acetone solution (or phosphate buffer for phycocyanin) to the grinding tube.
6. Seat the grinding tube onto the pestle, submerging the pestle in the acetone. Turn on the motor and gently move the tube up and down to repeatedly bring the pestle end to the bottom and force the filter fragments up the side walls to be ground. (NOTE: Light to moderate pressure should be sufficient and there should not be noticeable heat build-up. The grinding should take 30 seconds or less. If more time or pressure seems necessary then inspect pestle for signs of wear – see Cautions section.)
7. Pour the slurry into a labeled 15-mL screw-cap centrifuge tube. Using a fine-tipped squirt bottle of 90% acetone (or phosphate buffer for phycocyanin), rinse the pestle into the grinding tube and rinse the grinding tube walls, transferring the rinsate into the centrifuge tube. Repeat as necessary, taking care to use as little acetone (or phosphate buffer for phycocyanin) as possible as not to exceed the 15 mL capacity of the centrifuge tube.
8. Using the squirt bottle of acetone (or phosphate buffer for phycocyanin), add solution until the slurry exactly reaches the 15 mL graduation on the centrifuge tube.
9. **Record the final extract volume in the Chlorophyll *a*** or Phycocyanin Log **(usually 14.9 mL).** Note: A dry 47 mm GFF filter has a displacement volume of 0.10 mL (APHA 1999), so a tube filled to 15 mL only has 14.9 mL of extract.

10. Cap the tube tightly and shake it vigorously. Place foil-covered centrifuge tubes in test tube rack and place rack in refrigerator (4°C) to steep.
11. Thoroughly clean the pestle, grinding tube, and probe/rod by triple-rinsing with deionized water and rinsing a final time with acetone. Carefully inspect the pestle and rod for any residue that may be adhered and remove with a clean lab tissue. Clean the forceps and scissors by rinsing with acetone and wiping with a clean lab tissue.

8.6.4 Steeping

1. Samples should be allowed to steep at 4°C for a minimum of 2 hours but not to exceed 24 hours for chlorophyll *a.* Samples should be allowed to steep at 4°C for a minimum of 2 hours but not to exceed 8 hours for phycocyanin.
2. The tubes should be shaken at least once during the steeping period.
3. After steeping, proceed to Fluorometric Analysis.

8.6.5 Fluorometric Analysis

1. Thirty minutes to one hour prior to analyzing in the fluorometer, remove rack of steeped samples from the refrigerator and set in a dark place to allow samples to warm to room temperature.
2. Adjust workspace lighting to the minimum that is necessary to read instructions and operate instrumentation.
3. After samples have come to room temperature, shake tubes vigorously and then load tubes into the centrifuge.
4. Spin tubes for 5 minutes @ 1000 g (2250 rpm with the IEC HN-SII centrifuge). Note: always balance the centrifuge by filling all 6 holders, installing "dummy" centrifuge tubes if necessary that are filled to the same volume as the samples).
5. Turn the fluorometer on and use the touch screen to register that the Chl-NA optical kit is in place for chlorophyll, or the Orange optical module is in place for phycocyanin.
6. Select "Calibrate" and choose the last valid stored calibration for the appropriate module.
7. Pipet 2-3 mL of 90% acetone (phosphate buffer for phycocyanin) into a cuvette, wipe with a Kim wipe® and place in the round receiver. Select "measure fluorescence" and enter 1 mL for sample volume and extract volume. Verify that the measured value is 0.0. **Record the measured value on the "Zero Log" tab of the Chlorophyll *a* (Appendix C) or Phycocyanin Log (Appendix H).**
   NOTE: If the fluorometer does not read 0.0, clean or replace the cuvette and read again. Prepare fresh acetone solution if necessary. If the fluorometer still does not read 0.0, flag the samples in the batch and record the blank value in the flag comments. If the issue cannot be resolved, re-calibrate the fluorometer before analyzing additional samples (Turner Designs 2016).
8. Remove the round cuvette adapter from the optical kit and insert the solid secondary standard, keeping the tab in front. Select "measure fluorescence" and enter 1 mL for

sample volume and extract volume. **Record the measured value for the starting CCV and time on the "CCV Log" tab of the Chlorophyll *a* (Appendix D) or Phycocyanin Log (Appendix I).** Compare this to the value determined during the last calibration recorded in the calibration and maintenance log. The value should be +/- 10% of this value. On the digital version, the "QC Pass" will display a green "Pass" in the column if within 10%. If not, then locate source of problem and rectify before proceeding. Replace the round cuvette adapter when finished.

9. Remove a centrifuge tube from the centrifuge and place in the test tube rack, being careful not to disturb the pellet of filter residue.
10. Using a plastic transfer pipet, remove 2-3mL of sample from the centrifuge tube without disturbing the pellet and transfer to a clean cuvette.
11. Wipe the cuvette with a laboratory wipe and place in the receiver.
12. Select "measure fluorescence". Enter sample volume and extract volume when prompted. The chlorophyll *a* or phycocyanin concentration in µg/L will be displayed. **Record this value in the Chlorophyll *a* (Appendix B) or Phycocyanin Log (Appendix G).** If the digital Chlorophyll *a* or Phycocyanin Log is updated correctly, then the entered value will be highlighted yellow if it is under the EDL.
    Note: If "OVER" is displayed then sample is too concentrated and must be diluted. Estimate the amount of dilution necessary and follow instructions in next section (Sample Dilutions). **If no dilution then record dilution factor as 1.**
13. Remove the cuvette from the fluorometer and pour sample into labeled acetone or phosphate buffer waste container located in the fume hood.
14. Repeat steps 9 through 13 as needed to complete all regular samples, the LSS (if applicable), and the LRB. If batch is more than 12 samples then do next step (CCV) after a minimum of every 12th sample before proceeding.
15. When the batch is complete and at least after every 12 samples, measure the CCV (in addition to the starting CCV). Place the solid secondary standard in the receiver and enter volume and extract volume of 1. **Record the measured CCV values on the "CCV Log" tab of the Chlorophyll *a* (Appendix D) or Phycocyanin Log (Appendix I).** The value should be +/- 10% of the original value. If it is not then locate source of problem and rectify and re-run samples if possible. If samples cannot be re-analyzed and the issue cannot be resolved then samples must be flagged.
16. **For any flagged samples, record the flag type and details about the flag on Chlorophyll *a* (Appendix B) or Phycocyanin Log (Appendix G).** Use the "Flags" tab to determine the appropriate letter of flag to include on the Chlorophyll *a* or Phycocyanin Log.
17. When finished, turn off the fluorometer and discard all empty cuvettes and pipettes in the sharps box. Pour remaining sample left in centrifuge tubes into the acetone or phosphate waste and discard tubes.
18. At the end of a batch of samples (or when waste acetone container is full) arrange with Environmental Services Branch staff for waste acetone to be taken to the central acetone waste storage.

8.7 Sample Dilutions

Label a clean centrifuge tube with the sample ID and the dilution factor. Dilution factors are written as final volume / volume of sample (e.g. 5 mL of sample diluted to a total of 10 mL with 5 mL acetone has dilution factor of 2). Record the dilution factor on the sample log. Transfer the desired amount of sample using a 1, 5, or 10 mL pipet, from either the cuvette or the remainder of sample in the original centrifuge. Using a clean 1 or 10 mL pipet, transfer the desired amount of 90% acetone or 50nM phosphate buffer into the centrifuge tube. Mix solution thoroughly with a clean plastic transfer pipet before transferring to cuvette for measurement. Typical dilutions follow these general guidelines: for "x2 dilution" use 5 mL sample and 5 mL 90% acetone or 50nM phosphate buffer, for "x3 dilution" use 3 mL sample and 6 mL 90% acetone of 50nM phosphate buffer, and for "x4 dilution" use 3 mL sample and 9 mL 90% acetone or 50nM phosphate buffer.

9.0 TROUBLESHOOTING

- If sample water does not all pass through filter or if filtering takes longer than 10 minutes, discard the sample water and filter and restart filtering process with less sample volume.
- If pestle becomes stuck during grinding, immediately unplug the motor. Then, pull the grinding tube off of the pestle and add more acetone if necessary.
- If pestle starts to detach during grinding, immediately unplug the motor and contact appropriate lab personnel for maintenance.

10.0 DATA & RECORDS MANAGEMENT

All sample information is recorded in the Chlorophyll *a* (Appendix B) or Phycocyanin Log (Appendix G), using the current year's shared digital copy in the "Chlorophyll *a*" folder if possible (\\eas.ds.ky.gov\dfs\EEC-DEP-DOW\DW\DOWWQB\Chlorophyll a\Lab Log). If a paper copy is used, information should be transferred as soon as possible to the shared digital copy. Final chlorophyll and phycocyanin results are entered in K-WADE as soon as possible after analysis. The calibration log is located in the "Calibration" tab of the Chlorophyll *a* (Appendix F) or Phycocyanin Log (Appendix K). A scanned copy of the Chlorophyll A Certificate of Analysis for the primary calibration standard should be filed in the "Chlorophyll a" folder.

All records, including hardcopy and electronic files, that are collected by DOW staff or that are collected for the explicit use by DOW must be kept according to DEP record retention policy (KDLA 2006).

11.0 QUALITY CONTROL & QUALITY ASSURANCE

- The linear dynamic range and instrument method detection limit of the Trilogy fluorometer have been determined by the manufacturer to be 0-300 µg/L and 0.025 µg/L for chlorophyll *a*.
- The EDL is determined annually and is recorded in the "Calibration" tab of the Chlorophyll a (Appendix F) or Phycocyanin Log (Appendix K).
- Personnel will demonstrate proficiency in sample extraction when performing this method for the first time.
- The fluorometer will be calibrated with a chlorophyll *a* and phycocyanin standards at the beginning of the season or if the CCV fails to meet acceptance criteria.
- CCV will be accomplished using the Turner solid secondary standard, measured at the start and end of each sample analysis batch (the sample extracts analyzed in a day), and additionally at least after every 12 samples in a batch.
- A LRB will be analyzed by filtering a blank filter with 10 mL DI water, extracting, processing, and analyzing the filter at the end of each sample processing batch (the set of filters processed in a day) and additionally after every 20 samples in a batch.
- A LSS (a duplicate filter processed from the same sample) will be performed at a rate of at least 1 in 20 samples filtered for a project.
- Field duplicate (FD) samples are generally recommended at a rate of 1 in 20 samples (5%) to assess repeatability of field collections. Field duplicates should be specified in program management plans or quality assurance project plans along with their acceptance criteria.
- Field blank (FB) samples are recommended at a rate determined by the project study plan, especially if chlorophyll bottles are reused.
- Quality Assurance/Quality Control (QA/QC) requirements, acceptance criteria, and corrective actions are summarized in Table 2.

Table 2. QAQC requirements for chlorophyll *a* and phycocyanin analysis

QC Requirement	Frequency	Acceptance Criteria	Corrective Action
Extraction Proficiency Demonstration	Each analyst, prior to analyzing samples for the first time (one time only, not annually)	<15% Relative Standard Deviation (std dev / mean x 100) (10 split samples)	Retrain and repeat until pass
EDL determination	Annually	EDL must meet data quality objectives (DQO) for monitoring programs	n/a

QC Requirement	Frequency	Acceptance Criteria	Corrective Action
Initial Calibration	Annually or after CCV fail	Successful calibration following fluorometer user manual instructions	If calibration is not successful then locate source of problem and rectify before proceeding with analyses.
Continuing Calibration Verification (CCV)	At beginning and end of each sample batch; plus at least every 12 samples for large batches.	CCV Recovery = 100 +/- 10	If outside acceptance criteria, locate source of problem and rectify. Recalibrate and re-analyze all samples if possible; if not possible then flag samples as biased high or biased low accordingly.
Laboratory Reagent Blank (LRB)	At the end of every batch; plus every 20 samples for large batches.	Concentration < EDL	If concentration > EDL then investigate sources of contamination in the laboratory; if LRB values are >=10% of chlorophyll or phycocyanin values in samples then samples must be reanalyzed after acceptable LRB is achieved or results must be flagged.
Laboratory Split Sample (LSS)	At least one per 20 samples per project.	RPD <=20% (if sample concentrations are >EDL)	If outside limits then investigate sources of variability; retraining and proficiency testing should be considered if LSS is repeatedly out of bounds for an analyst.

12.0 REFERENCES

APHA. 1999. 10200H, Chlorophyll. In *Standard Methods for the Analysis of Water and Wastewater 17th Ed*.

Kasinak, Jo-Marie, Brittany Holt, Michael F. Chislock, and Alan E. Wilson. 2014. "Benchtop fluorometry of phycocyanin as a rapid approach for estimating cyanobacterial biovolume." *Journal of Plankton Research,* 248-257.

Kentucky Department for Libraries and Archives (KDLA). 2006. *State Agency Records Retention Schedule: Department for Environmental Protection.* State Archives and Records Commission, Public Records Division, Frankfort, KY.

Turner Designs. 2016. *Trilogy Laboratory Fluorometer User's manual*. Version 1.2, P/N 998-7210. Sunnyvale, CA. http://www.turnerdesigns.com/t2/doc/manuals/998-7210.pdf.

US Environmental Protection Agency (EPA). 1997. *Method 445.0 rev. 1.2: In vitro determination of chlorophyll a and pheophytin a in marine and freshwater phytoplankton by fluorescence.* Cincinnati, OH: Environmental Monitoring Systems Laboratory, Office of Research and Development, US Environmental Protection Agency.

13.0 APPENDICES

Appendix A: Chain-of-Custody REQUIREMENTS

Sample COC may be formatted as appropriate to best meet project management needs. At minimum the COC must contain the following information:

- A header that identifies the sample as a chlorophyll or phycocyanin sample and lists the project or program name.
- K-WADE Station ID (where applicable) or GPS coordinates if not an established station
- Sample Location Description
- Sample Primary Collector
- Collection Date and Time
- Sample Type (Field Sample (FS), Field Duplicate (FD), Field Blank (FD), or other type)
- Collection Method (Vertical Discrete Composite, Grab-Direct to Sample Container, Grab-Using Sampler, or other method)
- Collection Depth or Depth Zone (e.g. Euphotic Zone)
- Container Type and Capacity
- Storage (will always be wet ice)
- Check box to identify if sample filtered prior to delivery to lab (off-site)
- Filtering details (where applicable)
    - Date/Time Filtered
    - Filtered By Person
    - Volume Filtered
    - Filter Storage (portable freezer, dry ice, wet ice)
- Blocks for date/time sample relinquished and relinquished by, date/time sample received and received by.
- Lab Sample ID (for lab use when samples logged in to Chlorophyll *a* or Phycocyanin sample log)

Appendix B: Chlorophyll *a* log "CHLa log"

ChlA Lab Sample ID	Sample						Filtration					Macerate/Extract				Analysis					QC		HOLDING TIME		KWADE		NOTES
	Site Sample ID	Project	Date	Time	Sample Type	Final Sample Vol	Date	Time	Analyst	Vol filtered mL	Filter Froz w/in 4 hr?	Date	Time	Analyst	Extract Vol mL	Date	Time	Analyst	Dil Factor	Chl a µg/L	Pass? Y/N	Flags	Holding Time Days	T Flag Needed	Date	By	

Appendix C: Chlorophyll *a* log "Zero Log"

Blank Analysis					QC	
Date	Time	Analyst	Dil Factor	Chl a µg/L	Pass? Y/N	Flags

Appendix D: Chlorophyll *a* log "CCV Log"

CCV Analysis						QC		
Date	Time	Dec Time	Analyst	Dil Factor	Chl a µg/L	Pass?	Flags	Residual

Appendix E: Chlorophyll *a* log "PD Log"

Collection				Filtration					Macerate				Analysis					Rel SD
Sample ID	Date	Time	Sample Type	Date	Time	Analyst	Vol filtered	Filter Froz w/in	Date	Time	Analyst	Extract Vol mL	Date	Time	Analyst	Dil Factor	Chl a µg/L	

Appendix F: Chlorophyll *a* log "Calibration"

Fluorometer Calibration Log: Turner Triology, Division of Water Frankfort Lab

Calibration Date:	
Performed By:	
Standard Lot #:	
Prepared Date:	
Received Date:	
Low Standard Concentration (µg/L):	
High Standard Concentration (µg/L):	

Calibration		
	RFU	Reading After
blank		
low		
high		

File Name:	
Solid Secondary Standard fluorescence (µg/L):	
CCV acceptance range (µg/L):	

Filter Blanks	
Filter	RFU
1	
2	
3	
4	
5	
average	
3x average	

Low Standard Dilutions		
Dilution Factor	Calculated Concentration	RFU

Estimated Detection Limit (µg/L):	

Appendix G: Phycocyanin log "PC log"

		Sample					Filtration					Macerate/Extract				Analysis					QC		HOLDING TIME		KWADE		
ChlA Lab Sample ID	Site Sample ID	Project	Date	Time	Sample Type	Final Sample Vol	Date	Time	Analyst	Vol filtered mL	Filter Froz w/in 4 hr?	Date	Time	Analyst	Extract Vol mL	Date	Time	Analyst	Dil Factor	PC µg/L	Pass? Y/N	Flags	Holding Time Days	T Flag Needed	Date	By	NOTES
P20-001																											

Appendix H: Phycocyanin log "Zero Log"

Blank Analysis					QC	
Date	Time	Analyst	Dil Factor	PC µg/L	Pass? Y/N	Flags

Appendix I: Phycocyanin log "CCV Log"

CCV Analysis						QC		
Date	Time	Dec Time	Analyst	Dil Factor	PC µg/L	Pass?	Flags	Residual

Appendix J: Phycocyanin log "PD Log"

Collection				Filtration					Macerate				Analysis					Rel SD
Sample ID	Date	Time	Sample Type	Date	Time	Analyst	Vol filtered	Filter Froz	Date	Time	Analyst	Extract Vol mL	Date	Time	Analyst	Dil Factor	PC µg/L	

Appendix K: Phycocyanin log "Calibration"

Fluorometer Calibration Log: Turner Triology, Division of Water Frankfort Lab

Calibration Date:	
Performed By:	
Standard Lot #:	
Prepared Date:	
Received Date:	
Low Standard Concentration (µg/L):	
(Optional) 2nd Standard Concentration (ug/L):	
(Optional) 3rd Standard Concentration (ug/L):	
(Optional) 4th Standard Concentration (ug/L):	
High Standard Concentration (µg/L):	

Calibration		
	RFU	Reading After
blank		
low		
(Optional) 2		
(Optional) 3		
(Optional) 4		
high		

File Name:	
Solid Secondary Standard fluorescence (µg/L):	
CCV acceptance range (µg/L):	

Filter Blanks	
Filter	RFU
1	
2	
3	
4	
5	
average	
3x average	

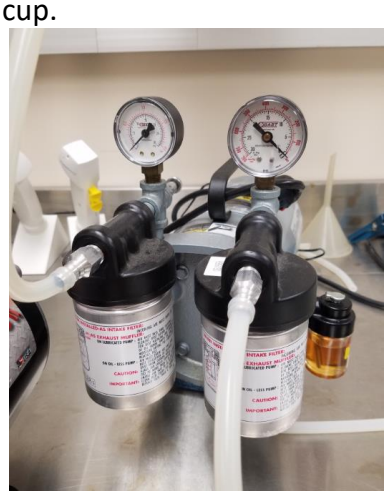
Low Standard Dilutions		
Dilution Factor	Calculated Concentration	RFU

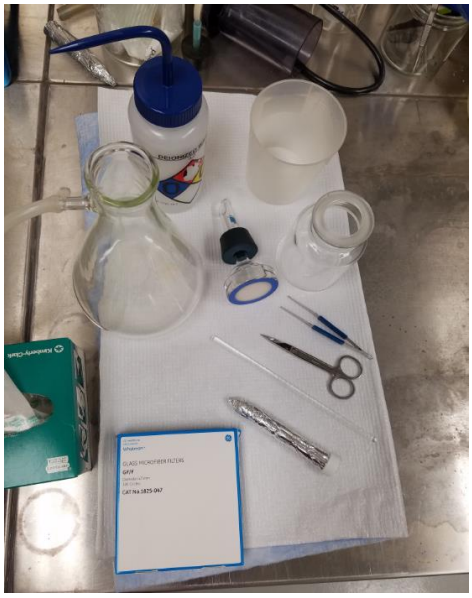
Estimated Detection Limit (µg/L):	

Appendix L: Chlorophyll Quick Guide

Filtering

Set up: Filter flask, filter base, filter tower, filters, scissors, filter forceps, glass rod, Kimwipes, labeled vials wrapped in foil, 100mL graduated cylinder, vacuum pump, DI wash bottle, rinse cup.





1. Get sample from cooler or refrigerator.
2. Load filter on base.
3. Gently agitate sample and mix well.
4. Measure 100 mL of sample and pour into tower. Use more or less volume if necessary.
5. Rinse graduated cylinder with DI water squirt bottle into funnel.
6. Turn on vacuum, check pressure while sample filters, rinse walls of filter funnel with DI when water is almost gone, then stop vacuum just before filter is completely dry.
7. Remove tower upper section, fold filter 3 times, place in centrifuge tube and cap.
8. Take tube to freezer, record time on log.
9. Rinse forceps, probe and scissors with DI and air dry.
10. Rinse filter base, filter tower, and graduated cylinder with DI and air dry.
11. Repeat for remaining samples.
12. Prepare a Lab Rinsate Blank (LRB) at the end of every batch and after every 20 samples in a batch. Substitute 10 mL DI water for sample water for the LRB.
13. Prepare one randomly selected Lab Split Sample (LSS) for every 20 samples per project (or as specified in Study Plan).

Grinding

Set up: Motor and grinding pestle, scissors, sturdy forceps, glass rod, 90% acetone, DI water, rinse cup, grinding tube, racks for grinding tube and sample tubes, Kimwipes acetone waste bottle.

1. Take sample out of freezer a few minutes before processing
2. Snip filter into grinding tube
3. Add ~3 good squirts of acetone (enough to submerge pestle, ~6 mL)
4. Place tube on pestle and start motor, grind to slurry (<30 sec)
5. Rinse pestle into tube 1-2 times and inspect pestle for filter fragments.
6. Rinse tube walls with 1-2 good squirts.
7. Transfer most of slurry to sample tube.
8. Rinse remaining material into sample tube by rinsing ~5 more times.
9. Top off to 15 mL with acetone. Cap and shake vigorously.
10. Put sample tube into rack in refrigerator and enter time in log.
11. Triple rinse with DI, then rinse with acetone the grinding tube, forceps, and scissors. Dry scissors with lab wipe.
12. Rinse pestle into rinse cup and inspect.
13. Repeat for remaining samples.
14. Allow to steep for 2-24hr. Shake samples at least once during the steeping time.

Analysis

Set up: Cuvettes (culture tubes), solid secondary standard, 90% acetone, dummy centrifuge tubes, tube rack, cuvette rack, centrifuge, disposable transfer pipets, Kimwipes, waste acetone container.

1. Take samples out of fridge ~30-45 min before analysis and place in dark.
2. Load samples into centrifuge, balance with dummy tubes if less than 6.
3. Set timer to 5 min, flip switch up (On W/ Timer), check RPMs (2500).
4. Turn on fluorometer, choose chl-NA.
5. Select Calibrate, use existing calibration. Select the file shown on Cal Log.
6. Check zero with 90% acetone – record on log.
7. Remove round adapter, check CCV (tab forward), record on log. Replace adapter.
8. Take one sample from centrifuge, pipet 2-3 ml to cuvette, wipe cuvette.
9. Select “measure fluorescence”, enter filtered volume and extract volume from log.
10. Record chlorophyll µg/L in log.
11. Dump cuvette and sample tube contents into acetone waste container.
12. Check CCV at least every 12 samples and at the end of the batch (in addition to CCV check at beginning of batch).