

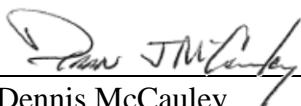
**STANDARD OPERATING PROCEDURE FOR THE DETERMINATION OF  
CHLOROPHYLL *a* AND PHEOPHYTIN *a* BY THE FLUOROMETRIC METHOD FOR  
THE NEON PROGRAM, ABBREVIATED**

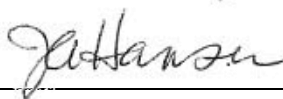
**CHM 2041A**

**Method Reference: EPA Method 445.0**

**April 7, 2025**

**Great Lakes Environmental Center, Inc.  
(GLEC)**

	4/7/2025
Dennis McCauley GLEC President	Date

	4/7/2025
Jennifer Hansen GLEC Quality Assurance Officer	Date

*Changes made in this version:*

- Added to the equipment list: Eppendorf pipettes - adjustable (100 to 1000 microliter [μL]) with new disposable solvent-safe plastic tips.
- Changed in the standard solutions sections from purchasing the QC standard from Turner Designs to preparing it at GLEC: “Prepare in acetone from purchased solid chlorophyll a, from a different lot than was used to prepare standards. Store in flame-sealed ampules wrapped in aluminum foil at  $\leq -10^{\circ}\text{C}$  until use.”
- Edited the instructions for zeroing the instrument to read as follows: Zero the Sequoia Turner instrument or record readings and subtract the blank concentration from the sample concentrations for the Turner instrument. Fill a clean cuvette with the 90% aqueous acetone (solvent blank solution) and place it into the sample well. On the Sequoia Turner, set the sensitivity setting to 200 and turn the ‘zero’ knob until instrument is zeroed.

*Changes made in version October 22, 2024:*

- Changed the management approval from Robin Silva-Wilkinson to Dennis McCauley

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## **I. SCOPE AND APPLICATION**

- 1.1 This Standard Operating Procedure (SOP) describes the procedure for the fluorometric determination of chlorophyll *a* and pheophytin *a* in water samples for the National Ecological Observatory Network (NEON) Program. The analyst may choose from two different fluorometers for analysis: The Sequoia Turner or the Turner Designs Trilogy with acidification module.
- 1.2 Phytoplankton pigments are extracted off a filter, from the field filtration of a water sample, using acetone and maceration. Chlorophyll *a* and pheophytin *a* in the extract are measured by fluorescence before and after acidification. The concentrations of chlorophyll *a* and pheophytin *a* are calculated from the fluorometer results.

## **II. EQUIPMENT AND SUPPLIES**

- 2.1 Aluminum foil.
- 2.2 Ampules – 15 milliliter [mL] capacity, glass.
- 2.3 Beaker – 50 mL capacity, glass.
- 2.4 Centrifuge – refrigerated, Beckman J2-21, or equivalent, with a rotor capable of holding 15 mL centrifuge tubes.
- 2.5 Centrifuge tubes - 15 mL glass, graduated, screw cap.
- 2.6 Cuvettes.
- 2.7 Eppendorf pipettes - adjustable (100 to 1000 microliter [ $\mu$ L]) with new disposable solvent-safe plastic tips.
- 2.8 Fluorometer
  - 2.8.1 Sequoia Turner Model 450 modified for chlorophyll *a* analysis. The emission filter is a Turner SC665 and the excitation filter is a Turner NB440; or
  - 2.8.2 Turner Designs Trilogy, with acidification module.
- 2.9 Forceps.
- 2.10 Lamp - green filtered.

- 2.11 Pasteur pipettes – disposable, glass.
- 2.12 Pipettes – glass, graduated, 10 mL capacity.
- 2.13 Syringes - 10 mL, solvent resistant, gas-tight.
- 2.14 Tissue grinder - 7 mL glass, loose (or B) pestles, and tight (or A) pestles.
- 2.15 Volumetric flasks – 25, 100, and 1000 mL.

### **III. REAGENTS AND STANDARDS**

#### **3.1 Reagents**

- 3.1.1 90% Aqueous acetone.
- 3.1.2 6 N Hydrochloric acid (HCl).
- 3.1.3 0.6 N HCl.

#### **3.2 Standard Solutions**

- 3.2.1 Stock solution (1 mg/L) – Prepare in acetone from purchased solid chlorophyll *a*. Store in flame-sealed ampules wrapped in aluminum foil at  $\leq -10^{\circ}\text{C}$  until use.
- 3.2.2 Working stock solution (50  $\mu\text{g/L}$ ) – Prepare from the stock solution in 90% aqueous acetone in a volumetric flask.
- 3.2.3 2, 5, 25, 60, and 200  $\mu\text{g/L}$  calibration standards - Prepare from the stock solution and working stock solution, as appropriate, in 90% aqueous acetone in volumetric flasks.
- 3.2.4 Chlorophyll *a* QC standard - Prepare in acetone from purchased solid chlorophyll *a*, from a different lot than was used to prepare standards. Store in flame-sealed ampules wrapped in aluminum foil at  $\leq -10^{\circ}\text{C}$  until use .

### **IV. SAMPLE RECEIPT, STORAGE AND HOLDING TIME**

#### **4.1 Sample Receipt**

- 4.1.1 Upon receipt, ensure all samples (foil wrapped filters) are in good condition (e.g., dry-ice still present in cooler, foil packaging not

damaged, sample identification present and legible). If samples are received in compromised condition, Reply All, to the shipment notification email to notify the Battelle Technical Representative within two business days.

- 4.1.2 Complete the client receipt form, emailed to GLEC, to document the condition of samples. Upload the completed form to the NEON Data Portal.
- 4.2 Sample storage – Store the filters containing the concentrated samples between -20°C and -70 °C in a freezer with an auto-notification system.
- 4.3 Sample holding time – Extract samples within 14 days of collection, counting the day of collection as Day 1. If this holding time is exceeded, proceed with extraction and analysis, flag data, and contact the Battelle Technical Representative within 48 hours of the incident.

## V. QUALITY CONTROL

- 5.1 Laboratory reagent blank (LRB) - Analyze an LRB sample at the beginning and end of each analytical batch to determine if any interferences are present in the laboratory environment or the equipment. Prepare the LRB by filtering 100 mL of DI water using the same procedure as is used for field samples. The raw fluorescence unit reading for LRBs must be < 0.5; if it is not, reanalyze the LRB. If the result is again ≥ 0.5 raw fluorescence units, determine the source of the problem. If the corrective action fails, report data with a quality flag.
- 5.2 Laboratory fortified blank unfiltered (LFBUF, equivalent to the NEON QC Standard) – For each analytical batch, use the instrument reading for a calibration standard in the expected mid-range of sample concentrations as an unknown to back-calculate the measured concentration. Calculate the percent recovery (R) using Equation 1, where D is the result for the LFBUF sample and C is the fortified concentration:

$$R = \frac{D}{C} \times 100 \qquad \text{Equation 1}$$

The calculated result must be within 5% of the known value. If the result falls outside of this criterion, re-prepare and reanalyze the calibration standards and recalculate the LFBUF concentration. If the corrective action fails, analyze samples and report data with a quality flag.

- 5.3 Quality Control (QC) Standard - Analyze a purchased or prepared QC Standard after the calibration standards, prior to analyzing field samples, as a second source check of the calibration standards. Concentration varies by lot. Chlorophyll

recovery must be within 10% of the true value to be considered acceptable. If the result is outside this criterion, determine the cause of the discrepancy and report data with a quality flag.

## **VI. CALIBRATION**

- 6.1 On both the Sequoia Turner and Turner Designs Trilogy (Trilogy) instruments, use the chlorophyll *a* standard solutions to determine the fluorometer calibration factor  $F_s$ . Use the same procedures for these measurements as for the sample extracts. Take readings before and after acidification so that  $r_s$  can be determined.
- 6.2 Allow the fluorometer to warm up for fifteen minutes.
- 6.3 Zero the Sequoia Turner instrument or record readings and subtract the blank concentration from the sample concentrations for the Turner instrument. Fill a clean cuvette with the 90% aqueous acetone (solvent blank solution) and place it into the sample well. On the Sequoia Turner, set the sensitivity setting to 200 and turn the 'zero' knob until instrument is zeroed.
- 6.4 Remove the cuvette and empty it, discarding the solvent blank solution.
- 6.5 Fill the cuvette with the lowest concentration standard, place it in the sample well, and record the fluorescence reading as  $R_b$ .
- 6.6 Remove the cuvette from the instrument. Acidify the standard in the cuvette using one drop from a Pasteur pipet (0.025 mL) of 0.6 N HCl. Gently mix without aeration, and reinsert the cuvette into the instrument. After 90 seconds, take a fluorescence reading and record it as  $R_a$ . Empty the cuvette, discarding the acidified standard.
- 6.7 Repeat steps in Sections 6.5 and 6.6 with each of the next four standards, progressing from low to high concentration.
- 6.8 Enter the calibration information into the Google bench sheet/calculation sheet.

## **VII. PROCEDURE**

- 7.1 Extraction
  - 7.1.1 Extract samples in a fume hood in a dark room dimly illuminated by a green lamp to avoid chlorophyll degradation.
  - 7.1.2 Using forceps, place the filter containing the concentrated sample in a tissue grinder and cover it with 2 mL of 90% aqueous acetone solution.

Macerate with the loose fitting (B) pestle then the tight fitting (A) pestle for about 3 minutes or, if a GF/F filter was used, until it has been converted to a slurry.

- 7.1.3 Transfer the extract to a screw-capped graduated centrifuge tube. Rinse the grinder with a few mLs of 90% aqueous acetone and add the rinsate to the extract. Bring all sample extracts up to the same volume with 90% aqueous acetone, and record this volume.
- 7.1.4 Wrap the test tubes with aluminum foil to prevent light exposure.
- 7.1.5 Let the samples steep for at least two hours, and not more than 24 hours, in a refrigerator ( $\leq 6^{\circ}\text{C}$ ) in the dark. Shake samples at least once during the steeping period.
- 7.1.6 Clarify the samples by centrifuging the tubes for 10 minutes at 2800 rpm.
- 7.2 Fluorometric chlorophyll *a* analysis with acidification for pheophytin *a*, following calibration.
  - 7.2.1 Allow sample extracts to come to ambient temperature.
  - 7.2.2 Set the sensitivity level to the mid-point.
  - 7.2.3 If the instrument was not calibrated on the same day as sample analysis, insert a solvent blank and zero the instrument. Check the zero reading periodically and adjust as needed.
  - 7.2.4 Fill a clean cuvette with an aliquot of LRB extract. Place the cuvette in the sample well. Record the fluorometric units reading as Fluorescence Before Acid (Rb).
  - 7.2.5 Remove the cuvette from the instrument. Acidify the LRB extract by adding one drop from a Pasteur pipet (0.01 mL) of 0.6 N HCl. After 90 seconds, take a fluorescence reading and record it as Fluorescence After Acid (Ra).
  - 7.2.6 Repeat steps in Sections 7.2.4 and 7.2.5 with the QC standard, LFBUF, each field sample extract, ending with a second aliquot of LRB extract.

## VIII. DATA ANALYSIS AND CALCULATIONS

- 8.1 For each calibration standard, calculate the fluorometer response factor ( $F_s$ ) and the acidification response factor using Equations 2 and 3. This is performed automatically in the Google sheet created in Section 6.8.

$$F_s = \frac{\text{Concentration of chlorophyll } a \text{ std } (\mu\text{g/L})}{R_b} \quad \text{Equation 2}$$

Where:  $F_s$  is the fluorometer response factor

$R_b$  is the fluorometer reading of the standard before acidification.

$$r_s = R_b/R_a \quad \text{Equation 3}$$

Where:  $r$  is the acidification response factor

$R_b$  is the fluorometer reading of the standard before acidification

$R_a$  is the fluorometer reading of the standard after acidification.

- 8.2 Calculate the concentrations of chlorophyll *a* and pheophytin *a* in the samples. The Google sheet created in Section 6.8 is set up to perform automated calculations.

- 8.2.1 The calculation for the corrected chlorophyll *a* concentration in the sample extract ( $Ca$ ) uses Equation 4.

$$Ca (\mu\text{g/L}) = F_{ave} * (r_s/(r_s-1)) * (R_b - R_a) \quad \text{Equation 4}$$

Where:  $F_{ave}$  is the average of the  $F_s$  values of the 5 standards;

$r_s$  is the average of the acidification response factors for the 5 standards;

$R_b$  is the fluorometer reading of the sample extract before acidification; and

$R_a$  is the fluorometer reading of the sample extract after acidification.

- 8.2.2 The calculation to convert  $Ca$  to the concentration of chlorophyll *a* in the original water sample uses Equation 5.

$$\text{Chlorophyll } a (\mu\text{g/L}) = Ca (\mu\text{g/L}) * D * V/W \quad \text{Equation 5}$$

Where:  $Ca$  is the chlorophyll *a* determined in Section 8.2.1;

$D$  is the dilution factor for the sample extract (for example, if the dilution is 1:10, then  $D = 10$ ). An extract is only diluted if the result for the undiluted extract is above the range of the fluorometer;

$V$  is the volume of the sample extract (mL);

$W$  is the volume of the water sample filtered (mL).

- 8.2.3 The calculation to convert the pheophytin *a* concentration in the sample extract (*Pa*) uses Equation 6.

$$Pa (\mu\text{g/L}) = F_{\text{ave}} * (r_s / (r_s - 1)) * (r_s * Ra - Rb) \quad \textbf{Equation 6}$$

Where:  $F_{\text{ave}}$  is the average of the  $F_s$  values of the 5 standards;  
 $r_s$  is the average of the acidification response factors for the 5 standards;  
 $Rb$  is the fluorometer reading of the sample extract before acidification; and  
 $Ra$  is the fluorometer reading of the sample extract after acidification.

- 8.2.4 The calculation to convert *Pa* to the concentration of pheophytin *a* in the original water sample uses Equation 7.

$$\text{Pheophytin } a (\text{mg/L}) = Pa (\text{mg/L}) * D * V/W \quad \textbf{Equation 7}$$

Where: *Pa* is the pheophytin *a* determined in Section 8.2.3;  
 $D$  is the dilution factor for the sample extract (for example, if the dilution is 1:10, then  $D = 10$ ). An extract is only diluted if the result for the undiluted extract is above the range of the fluorometer;  
 $V$  is the volume of the sample extract (mL);  
 $W$  is the volume of the water sample filtered (mL).

### 8.3 Generate reports

- 8.3.1 Download the Google sheet to an Excel workbook.
- 8.3.2 Upload the data from the workbook into the GLEC laboratory information management system (LIMS).
- 8.3.3 Use NEON-specific queries to populate the sample result and QC result tables with new data. These tables follow the field and data criteria specifications required by NEON for data ingest upload.
- 8.3.4 Export the two tables created in Section 8.3.3 to Excel, and format the Excel sheets for data ingest upload.
- 8.3.5 Upload the final lab ingest files created in Section 8.3.4 to the NEON Data Portal at <https://NEON/NEONscience.org/myaccount>.

## **IX. QUALITY ASSURANCE**

- 9.1 Data reports are reviewed by a qualified GLEC staff member before submission to the client. This QA Review is an independent review; it is performed by someone not associated with the data generation. This review evaluates the computations performed, and the accuracy and traceability of the data. It is the responsibility of the person who generated the report to satisfactorily address any of the QC reviewer's comments and concerns and to generate the final report.
- 9.2 Hard copies of original documents are maintained in secure files at GLEC. Electronic data or other information are filed and stored by the project name on GLEC's computer server, which is backed-up daily.

## **X. WASTE MANAGEMENT/POLLUTION PREVENTION**

- 10.1 Let small amounts (e.g., < 10 mL) of acetone evaporate in the fume hood. Larger amounts should be poured into a designated waste container and stored in the chemical storage shed for disposal. Dispose of hazardous material by appointment with RecycleSmart.
- 10.2 This method should be conducted with active pollution prevention as an objective, by: modifying processes to reduce or eliminate waste at the source, promoting the use of non-toxic or less-toxic substances, implementing conservation techniques, and re-using materials when possible, rather than disposing of them.

## **XI. REFERENCES**

- 11.1 EPA Method 445.0. *In vitro* Determination of Chlorophyll a and Pheophytin a in Marine and Freshwater Algae by Fluorescence, Revision 1.2. September 1997. National Exposure Research Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, Cincinnati, Ohio.
- 11.2 Turner Designs Trilogy Manual.