Horn Point Analytical Services Laboratory
Version 1.4 (Battelle Service Contract);
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CHLOROPHYLL *a*

Fluorometric methods are available for analysis of chlorophyll *a* (hereafter referred to as chl *a*). Chlorophyll, in a measured volume of water, is concentrated by filtering through a glass fiber filter, and the pigments are extracted in acetone. Fluorescence is proportional to chlorophyll concentration.

Methodology

Arar, E. J. and G. B. Collins. 1997. Method 445.0, Revision 1.2: In vitro determination of chlorophyll *a* and pheophytin *a* in marine and freshwater algae by fluorescence. National Exposure Research Laboratory, USEPA, Cincinnati, OH, 22 p.

Instrumentation

Fluorometer: Turner Designs Model 10-AU digital is calibrated against a spectrophotometer using pure chlorophyll *a* from spinach (Sigma Chemical Company, 10865) or pure chlorophyll *a* (Turner Designs). We acknowledge that the Turner Designs Model 10-AU is no longer being manufactured; however, Turner Designs will continue to support this instrument for the foreseeable future. There are 3 Turner Designs 10-AU fluorometers at Horn Point Laboratory (HPL) which may be used by HPL Analytical Services in case of instrument problems.

Sample Preparation and Storage

Note: The following procedure is specific for the NEON samples (Battelle service contract). These standard operating procedures are kept on file in the HPL Analytical Services laboratory.

1. Algae samples are collected by NEON, filtered and frozen within 24 hours of collection. Filters used for algae (25 mm GF/F) will be frozen at -20 ºC, folded in aluminum foil packets, and shipped on dry ice to HPL Analytical Services. Frozen filters will be shipped overnight to the Contractor (HPL Analytical Services) within 7 days of sampling. Most samples will be shipped between the months of March and November. Shipments will include a hard-copy manifest and will be preceded by an email notification of shipment containing an electronic copy of the manifest and receipt form.

2. Within 4 hours of sample delivery, HPL Analytical Services will ensure all samples are in good condition (e.g., dry-ice still present in cooler, samples not damaged, sample identification present and legible, etc.). If samples are received in compromised condition, HPL Analytical Services will notify the Battelle Technical Representative within two business days and enter remarks accordingly in the electronic receipt form. Upon completion of sample custody, the receipt form (that accompanies the electronic shipping manifest) will to be uploaded to the NEON Data Portal within 48 hours of receiving shipment.

Sample Storage

While in HPL Analytical Services' custody, samples will be stored according to the conditions in Table 1.

Table 1. Storage Conditions, Hold Times, and Analytical Methods: Algal Chlorophyll a and Pheophytin *a*

Grouping	Analyte	Analysis Approach	Method or Standard Operating Procedure (SOP)	Required Method Detection Limit	Hold Time	Storage Conditions
Periphyton, Seston	Chlorophyll *a* (GF/F filter)*	Acetone extraction/ fluorometric detection	EPA 445.0 or similar	0.5 ug/L	14 days from date of sample collection	-20 °C**
	Pheophytin *a* (GF/F filter)*					

*Chlorophyll *a* and Pheophytin *a* will be analyzed from the same filter sample.
**Freezer should be equipped with a back-up generator or an auto-notification system to protect against failure.

To avoid pigment degradation, **samples should be analyzed within 7 days from receipt so that the holding time of 14 days from collection is not exceeded**. If this holding time is exceeded, HPL Analytical Services should proceed with analysis, flag data appropriately, and contact the Battelle Technical Representative within 48 hours of the incident.

During pigment extraction, samples are kept in a refrigerator (4°C) in the HPL Analytical Services laboratory for 20-28 hours (typically overnight).

General Analytical Procedure

During extraction, light exposure is minimized by turning overhead lighting off. The HPL Analytical Services Laboratory room where sample analysis is performed does not have windows. To further minimize light, samples will be kept shaded and artificial lighting reduced whenever samples are outside of freezers or refrigerators.

1. When ready for analysis, prepare an extraction summary sheet to include internal ID number, NEON sample ID, volume filtered, extraction volume, and comments regarding sample condition.

2. Pre-label centrifuge tubes (15mL) to correspond with the internal ID numbering system.

3. Prepare a cooler with an ice bath and rack to hold centrifuge tubes, and a second cooler with only ice, to keep the samples cold while working through the extraction process.

4. Each sample filter is placed into its corresponding pre-labeled centrifuge tube using clean forceps. Add 7mL of 90% acetone to the tube. Cap and seal top with parafilm. Place tube in rack in ice bath and repeat step #4 for the remaining samples.

5. A 'Blank' sample is prepared by placing a new, clean 25mm GF/F filter into a centrifuge tube and following the same extraction procedure as noted in step 4 above.

6. Vortex each sample for 20 secs and place back in the ice bath until all samples have been vortexed. Transfer rack of samples into a dry cooler, cover with a dark towel, and place in 4°C refrigerator to soak overnight, at a minimum of 20 hours or up to 28 hours.

7. After the 20-28 hour soaking period, turn the fluorometer on and allow it to warm up for 15 minutes. A chl *a* QC standard of known concentration is prepared at room temperature from the chl *a* stock solution kept under -20°C conditions.

8. Tubes are transferred in their cooler from refrigerator to benchtop, and vortexed for 20 seconds. Samples are then centrifuged for 10 minutes, at 3000rpm.

9. After centrifugation, samples are placed in a dry cooler prior to fluorometric reading on a Turner Design 10-AU.

10. Before analysis begins with the samples, record three consecutive readings of both the 'High' and 'Low' settings of the Turner Design Solid State Standard.

11. Transfer 5mL of the chl *a* QC standard into a glass test tube. Insert test tube into the fluorometer and record this reading as the pre-acidification (Fo) reading. Acidify sample with 150µL of 0.1N HCl. Wait 90 seconds and record post-acidification (Fa) reading. Repeat this entire step for an additional chl *a* QC reading.

12. Transfer 5mL of the 'Blank' supernatant into a glass test tube and cover with parafilm. Record two pre-acidification (Fo) readings.

13. For each sample, transfer 5mL of the supernatant to a glass test tube. Record a pre-acidification (Fo) reading, then acidify sample with 150µL of 0.1N HCl. Wait 90 seconds and record a post-acidification (Fa) reading.

14. After 20 samples have been analyzed, record a reading for the Solid State Standard, both "High" and "Low" settings.

15. Next, record the Fo and Fa readings of a chl *a* QC standard and the Fo reading of the 'Blank'. [The daily blank pre-acidification readings were not used to adjust any functions on the Turner Design 10-AU fluorometer, instead the averaged daily blank readings were used during the calculation of concentrations for sample chl a and pheo a, as well as chl a concentrations for the chl a qa checks. See Calculations section below for the use of an averaged daily blank value.]

16. Repeat steps 11-13 for the next 20 samples, followed by steps 14-15 for the Solid State Standard and chl *a* QC check readings after every 20 samples.

17. If a sample concentration exceeds the highest concentration of the chlorophyll standards used for calibration, quantitatively dilute the sample with 90% acetone and reread on the fluorometer. Record dilution factor along with Fo and Fa readings.

Instrument Quality Control

To document the instrument is performing satisfactorily during sample analysis, read a chl *a* QA standard of known concentration, as well as the secondary solid state standard at both the high and low readings. Do this at the start of analysis and after every 20 samples on the fluorometer. Chl *a* QA readings should correspond to concentrations within +/- 5% of the known chl *a* stock concentration. If they are outside of this parameter, do one or more of the following, in this order, until readings correspond to chl *a* concentrations within the set tolerance: prepare a new chl *a* QA check, re-determine the chl *a* stock concentration, and lastly, prepare a new chl *a* stock solution. Bimonthly prepare a standard curve using chl *a* stock solution (detailed in section below).

Calibration of the Fluorometers

Determine the concentration of the chlorophyll *a* stock solution on the spectrophotometer. The concentration of the stock solution is calculated from a published extinction coefficient and the absorbance reading at 664 nm, corrected for turbidity with an absorbance reading taken at 750 nm. [The extinction coefficient is consistent with what investigators use who are involved with the Joint Global Ocean Flux Study (JGOFS) and Sea-Viewing-Wide-Field-of-View Sensor (SeaWIFS). Chlorophyll *a* stock solution is prepared to read in the range of 0.1 to 0.9, in accordance with Standard Methods of the Examination of Water and Wastewater (1992). The chromatographic purity of the chl *a* standard is monitored by HPLC. Use up to six standards, that span the range of expected sample concentration, and one blank to determine the standard curve. Calculate a linear regression and residuals of the standard curve.

Calculations:

The formulas used to calculate chl *a* and phaeophytin *a* concentrations are from Standard Methods for the Examination of Water and Wastewater (1998) Section 20200H.3 and EPA Method 445.0.

$$\text{Chl } a \text{ :µg/l seawater} = ((R_b - R_a) - \text{y intercept}) * \text{slope}^{-1}) * V_e * V_f^{-1}$$

or

$$\text{Chl } a \text{ :µg/l seawater} = F_s * (((r / (r-1)) * (R_b - R_a) * (V_e * V_f^{-1}))$$

$$\text{Phaeophytin a :µg/l} = F_s * (((r/(r-1) * (rR_a - R_b) * (V_e * V_f^{-1}))$$

$$F_s = C_a * R_b^{-1}$$

Where:
F_s = calibration factor for sensitivity setting S,
R_a = blank corrected fluorescence of sample extract standard after acidification,
$r = R_b/R_a$, as determined with pure chlorophyll *a* standard,
R_b = blank corrected fluorescence of sample extract before acidification,
V_e = extraction volume (in ml),
V_f = volume of water filtered onto filter (in ml), and
C_a = concentration of pure chlorophyll *a* standard.