SOP-C-136

Determination of Chlorophyll-a by Fluoroscopy

Revision 0

Approval:

_____ 10-23-18

Laboratory Manager Date

_____ 10-23-2018

Concurrence Date

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11-3-22

i. Identification of the method

a. SM 10200H (approved 2011); EPA 445.0 (approved 1997)
b. A modification of the method is the storage of frozen filters for 28 days rather than 21 days. This is a requirement of client QAPPs. If projects require a 21 day freezing period maximum, that period will be used.

ii. Applicable matrix or matrices

a. water samples, or filters of samples

iii. Limits of detection and quantitation

a. Applicable range without dilution is LOD to about 200 mg/M^3 without diluting or using less sample volume. LOD is determined annually per QAM-Q-101.

iv. Scope and application, including parameters to be analyzed

a. Chlorophyll-a and pheophytin-a in water

v. Summary of the method

a. Fluorometric utilizing a filtration apparatus for concentration of the biomass from a water sample, a grinding apparatus for maceration of the biomass, and a Fluorometer to measure the concentration of chlorophyll-a/pheophytin-a.

vi. Definitions

a. Chlorophyll-a (Chl-a) - A compound contained in all green plants, which constitutes approximately 1-2 % of the dry weight of planktonic algae.
b. Pheophytin-a (Pheo-a) - A degradation byproduct of Chl-a which is indicative of previously existing Chl-a, but lacks the chelated magnesium ion.
c. Glass fiber filter- A fiberglass filter which aids in macerating the algal cells to release cell constituents including the Chl-a/Pheo-a
d. Maceration- A mechanical process that breaks up the planktonic algal cells to release Chl-a/Pheo-a as well as other constituents.
e. Steep- A Chl-a/Pheo-a extraction process achieved by soaking ground biomass in an acetone solution.
f. Refer to QAM-Q-101 for standard QC definitions

vii. Interferences

a. Chlorophyll-b and other pheopigments, degradation byproducts, and humic substances

viii. Safety

a. Acetone is toxic and highly flammable. Exposure to acetone can be hazardous. Refer to MSDS literature in lab about specifics prior to analysis.

b. The analyst should wear protective eyewear, gloves, and a laboratory coat or apron while performing the analysis operations.

c. Grinding of filters should be performed in a vent hood.

ix. Equipment and supplies

a. Filtration apparatus- A filtering system to concentrate the biomass from the water sample onto a filter and to separate the glass fiber filter from the filtrate.
   i. Vacuum pump- A vacuum pump capable of sustaining at least 20 inches of vacuum
   ii. Filter assembly- A side-arm flask with solvent resistant filter holder capable of holding a 47 mm diameter filter.
   iii. Separation filter- A nylon (0.45µm porosity, 47 mm diameter) filter (Whatman or equivalent) to separate the glass fibers from the concentrated filtrate.
   iv. Concentration filter- a glass fiber filter (Whatman GF/B or equivalent) used to concentrate biomass

b. Grinding apparatus- an apparatus to macerate the biomass which releases the Chl-a/Pheo-a and other constituents from the algal cells.
   i. Drill assembly- a mounted drill with a 1/40 horsepower rating which is capable of 500 rpm and able to hold a pestle (Glas-col® or equivalent).
   ii. Grinding tube- A round-bottom glass grinding tube (15mm x 15cm).
   iii. Grinding pestle- A round bottom pestle that has grooves or grains cut in the Teflon (TFE) or glass tip and which matches the grinding tube.

c. Fluorometer with a 340-500nm excitation filter, a >665nm emission filter and a daylight white lamp.

i. Cuvette (with lid)- A sample vessel with a 13 mm pathlength made of a material that does not interfere with the wavelength used for pigment concentration analysis.

d. Aluminum foil
e. Borosilicate vials with Teflon caps (40 – 60 mL)
f. Opaque or dark sample bottles
g. Glass rods
h. Graduated cylinders, Class A, 10 mL and 1000 mL
i. Timer
j. Calibrated micropipetter capable of accurate delivery of 0.1 mL.

x. Reagents and standards

a. Reagents

i. Saturated Magnesium carbonate solution- Disperse 1.0 g finely powdered magnesium carbonate ($MgCO_3$) in DI water and dilute to 100 mL.

ii. Aqueous acetone solution- Mix 90 parts acetone (reagent grade-boiling point 56°C) with 10 parts saturated magnesium carbonate solution (by volume) above. Store in flammable storage cabinet.

iii. Hydrochloric acid, HCl, 1.0 N- Add 8.3 mL concentrated HCl to 50 mL of DI water and dilute to 100 mL.

iv. Hydrochloric acid, HCl, 0.1 N- Add 5.0 mL of 1.0N HCl and dilute to 50 mL in a volumetric flask.

b. Standards

i. Dehydrated chlorophyll-a standards are available and are prepared as per manufacturers' instructions. Spinach and algae sources should be available. The Laboratory Manager may determine which is most efficient. At present, the only known standard source for pheophytin-a is chlorophyll-a standard that has been acidified by the laboratory.

ii. Standards (LCS and LCSD at the RL/LOQ) are analyzed at the beginning of the first run each day

that Chl-a/Pheo-a samples are analyzed and with at least every preparation batch of 20 samples thereafter, depending on QAPP requirements. Concentrations vary from the manufacturer, but are usually prepared by weighing the entire contents of a sealed ampule (around 1 mg) and dissolving in 1 L of acetone/$MgCO_3$ solution. Pre-mixed standards may also be commercially available. Other stock concentrations may be used.

iii. Stock Chlorophyll-A Solution (about 1000 mg/M^3 or µg/L): Add 100 mL of saturated $MgCO_3$ and 800 ml of acetone solution to a 1-Liter volumetric flask. Other stock concentrations and volumes may be used as long as these are documented and confirmed. Cover the bottom bulb with aluminum foil to reduce light exposure. Open glass vial containing dry chlorophyll-a from algae (or spinach) and empty contents into volumetric. Normal purchased amounts are about 1 mg per vial. Rinse the vial with at least two 1-ml portions of acetone and add to the volumetric flask. Immediately bring to volume with acetone, and cover rest of volumetric to exclude, as much as possible, light from the stock solution. Invert flask to mix and to dissolve dry algae into solution. After total dissolution, filter enough solution through a nylon filter to perform seven (7) measurements for chlorophyll-a by SOP-C-112, “Determination of Chlorophyll-a and Pheophytin-a.” When not in use, store the stock solution and any dilutions in the dark, flammable storage cabinet. Standards may also be purchased already prepared.

iv. Chlorophyll-a LCS/LCSD as the LOQ/LOQcv Standard (3.0 mg/M^3) – Dilute a portion of the stock solution with aqueous acetone solution to the required concentration using the formula:

$$C_1V_1 = C_2V_2 \text{ where:}$$

C_1 = concentration of stock solution
V_1 = volume of stock solution to use

C_2 = concentration of standard (3.0)
V_2 = volume of standard being made (e.g. 250-ml)
Record the standard in the Standards Log and analyze.

v. Aliquots of the Chlorophyll standards are analyzed twice with each run to serve as the LCS/LCSD and LOQ.

xi. Sample collection, preservation, shipment and storage

a. Sample collection and shipment: refer to field procedures
b. Filter within 48 hours, 28 days frozen for filters from basic waters; analyze immediately for acidic waters (pH<6). Some project QAPPs may allow for a 21 or 30-day filter storage in the freezer. The start time/holding time for analysis is met when the sample is macerated.
c. Refrigerate sample to >0-≤6° C, freeze filter

xii. Quality control

a. Do not remove more than one preparation batch of filters at a time from the freezer for maceration.
b. Duplicate samples (or field splits) may be collected by field staff and submitted for analysis with regular samples. If sufficient volume is submitted, analyze at least one laboratory or field duplicate for every analytical batch of ten samples each day that samples are submitted, as QAM-Q-101 requires. The LCS/LCSD may be used in lieu of actual sample duplicates or splits, if allowed by the project.
c. All aspects of this procedure, including LCS/LCSD or sample duplicate/field split relative percent deviation, method blanks, and LCS/LCSD/CCV recovery comply with QAM-Q-101, "Laboratory Quality Control".
d. The analyst should refer to QAM-I-119, "Operation and Calibration of the Fluorometer", or the Turner Designs 10-AU Instrument Operations Manual, if problems occur during operations.
e. Record all preparation of all reagents in the Reagents Log and standards in the Standards Log.
f. Record all preparations, maceration and filtration in the Chla/Pheo Preparation Log (C-112-2, attachment 2), including supply and reagent lot numbers.

xiii. Calibration and standardization

a. For standard preparation, perform spectrometric determination of chlorophyll-a by SOP-C-112, "Determination of Chlorophyll-a and Pheophytin-a." The average of the seven values will be the concentration of the stock solution. Write the value of the stock solution on the volumetric flask and in the Standards Log. Also record the true value, as determined, in the log, i.e. 3 mg/M^3 (μg/L) as analyzed is actually 0.6 of the 500 mg/M^3 (μg/L) stock (when taking into account the concentration step for samples).

xiv. Procedure

a. Verify that sample containers received are opaque or wrapped in foil. Light energy (hλ) will alter Chl-a/Pheo-a concentration with even a brief period of exposure.

b. Verify that samples have been kept on ice or at >0-≤6° C since sample collection.

c. Preferred sample volume is 1 liter.

d. Samples are stored in opaque containers at >0-≤6° C until they are processed. Samples are filtered within 48 hours of collection.

e. Concentration of biomass

   i. Allow samples to come to room temperature for accurate volume measurement.

   ii. Use glassware and cuvettes that are clean and acid-free.

   iii. Shake samples well before filtering. Filter the sample using a glass fiber filter to concentrate the biomass. Do not vacuum the filter to complete dryness and do not use more than 500 mm Hg (20 in.) vacuum pressure. The setscrew on the pump exhaust may be turned to adjust. If entire sample is filtered, rinse sample storage container with about 20 mL organic-free DI (which is also passed through the same sample filter to make sure all cells are collected).

   iv. Add approximately 2 mL of $MgCO_3$ solution to sample just before filtering process is completed.

$MgCO_3$ solution acts as a pH buffer to keep chlorophyll from degrading.

v. Document the volume of actual sample that passed through the filter, date and time of filtration in the log. Do not add volume of rinse water or $MgCO_3$ to this.

vi. Fold the filter once and wrap in aluminum foil. Label the foil with the sample ID and volume filtered.

vii. Samples on filters taken from water having pH of 6 or higher may be placed in airtight bags and stored frozen for up to 28 days.

viii. Samples from acidic water must be processed promptly to prevent chlorophyll-a degradation. If the pH measured in the field for chlorophyll-a samples is less than 6, a second measurement is made in the field. If the second field measurement is also less than 6, laboratory personnel are alerted when the sample is submitted so they can measure the pH in the laboratory. If the laboratory confirms that the pH is less than 6, the laboratory manager is notified for determination of whether the sample can be run immediately.

f. Maceration of sample

i. Place the filter containing the sample into the grinding tube.

ii. Add 2-3 mL of a 10-mL portion of aqueous acetone solution to the tube. Initially, break up the filter with a glass rod or metal spatula.

iii. Using the pestle that is mounted in the motor assembly, grind the sample for no longer than 1 minute at 500 rpm. Overgrinding the sample may raise the slurry temperature and result in solvent loss.

iv. Pour the ground slurry in a vessel that may be capped to prevent release of volatile solvent.

v. Rinse the grinding tube with the remaining aqueous acetone solution and pour into the vessel with the

rest of the sample. Rinse apparatus with acetone and discard rinsate between samples.

vi. Tightly cap the vessel, shake, and wrap with foil. Place in cooler at >0-≤6° C.

vii. Steep samples at least 2 hours.

g. Extraction of biomass pigments

i. Assemble the separation filtration apparatus using a nylon filter.

ii. Filter the steeped sample and place filtrate in an airtight vessel.

iii. Keep sample in the dark to prevent degradation of chlorophyll-a.

iv. Rinse apparatus with acetone between samples.

h. Fluorometric determination of chlorophyll-a in presence of pheophytin-a:

i. Turn on instrument and allow to warm up for at least 15 minutes.

ii. Zero the instrument by placing filtered aqueous acetone in the sample cell of the fluorometer and zeroing the instrument reading. Zeroing is performed at a minimum at the beginning of every day on which Chl-a/Pheo-a samples are analyzed.

iii. Place about 5.0 mL of sample into sample cell.

iv. Take the initial fluorescence reading and record.

v. Add 0.1 mL of 0.1 N HCl with the micropipetter to the sample cell, cover and gently invert three times, covering with a Teflon lid. Allow a 90-second reaction time before proceeding.

vi. Total sample concentration of acid should not exceed 0.003 N HCl.

vii. Take a final fluorescence reading and document in the log. This documentation may consist of readouts from the spectrophotometer taped into the log or direct entry into a spreadsheet log.

xv. Data analysis and calculations;

a. Calculation of Chl-a/Pheo-a:

$$\text{Chlorophyll-a, mg/M3} = F_s(R_b - R_a)(V_e/V_s)$$

Where:

F_s = The calibration factor for sensitivity setting S, which is equal to the concentration of chlorophyll-a determined spectrophotometrically divided by the fluorometer reading

Rb = The fluorescence of extract before acidification

Ra = The fluorescence of extract after acidification

Ve = The volume of the extract, and

Vs = The volume of the sample.

b. A spreadsheet or log may be used to calculate the Chl-a concentration using the formulas above (Attachment 1). If the calculation is done manually, all formula entries and manipulation are entered into the analyst's Personal or electronic log. If a manual spreadsheet is used, a printout of the variables and results are taped into the Personal Log.

xvi. Method performance

a. Method performance, data assessment and acceptance criteria: refer to QAM-Q-101, "Laboratory Quality Control"

xvii. Pollution prevention

a. Acetone is not evaporated in the hood as a means of disposal. Incidental evaporation is minimized.

xviii. Data assessment and acceptance criteria for quality control measures

a. Method performance, data assessment and acceptance criteria: refer to QAM-Q-101, "Laboratory Quality Control"

xix. Corrective actions for out-of-control data

a. Corrective action: refer to QAM-Q-105, “Corrective Actions.”

xx. Contingencies for handling out-of-control or unacceptable data

a. Corrective action: refer to QAM-Q-105, “Corrective Actions.”

xxi. Waste management

a. All waste is placed into the proper waste receptacle and disposed of in accordance with QAM-W-101, “Disposal of Laboratory Waste”. Acetone waste is considered hazardous and flammable, nonchlorinated solvent waste.

xxii. References

a. Standard Methods for the Examination of Water and Wastewater, latest online edition, Method 10200H (approved 2011).

b. Method 445.0: *In Vitro* Determination of Chlorophyll *a* and Pheophytin *a* in Marine and Freshwater Algae by Fluorescence, USEPA, September 1997.

c. National Environmental Laboratory Accreditation Conference TNI standard, The NELAC Institute, 2009.

xxiii. Any tables, diagrams, flowcharts and validation data

a. Example Excel Chl-a/Pheo-a spreadsheet

Attachment 1 Example Chl-a Spreadsheet

Fluorometer Chlorophyll Worksheet

Start date/time:		
End date/time:		
Analyst:		

LCS/LCSD acceptance limits

Chlorophyll	**2.4-3.6**

F_s =

SAMPLE #	VOLUME IN LITERS	BEFORE ACID	AFTER ACID	SAMPLE #	Before-After	before/after	**Maceration Date**	**Maceration Time**
Instrument Blk	1.000			**Instrument Blk**			na	na

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