SOP-C-112

Determination of Chlorophyll-a and Pheophytin-a

Revision 15

Approval:

_____	4.12.23
Laboratory Manager	Date
_____	04-12-2023
Concurrence	Date

Effective date: 4-14-23

Renewal date: _____ Initials: _____

i. Identification of the method

a. SM 10150A&B (approved 2022)
b. A modification of the method is the storage of frozen filters for 24 days rather than 28 days. This is a requirement of client QAPPs.

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, "Laboratory Quality Control."

iv. Scope and application, including parameters to be analyzed

a. Chlorophyll-a and pheophytin-a in water

v. Summary of the method

a. Spectrophotometric utilizing a filtration apparatus for concentration of the biomass from a water sample, a grinding apparatus for maceration of the biomass, and a spectrophotometer 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

b. Turbidity is corrected for by taking the readings at 750nm before and after acidification. A value of > 0.005 absorbance at 750nm indicates poorly clarified sample or the presence of excess water. Re-clarify if necessary.
c. This optical method can significantly under- or overestimate chlorophyll-*a* concentrations, in part because the absorption and fluorescence bands of co-occurring accessory pigments and chlorophyll degradation products overlap. At a spectral band width of 20 nm, the chlorophyll-*a* concentration may be underestimated by as much as 40%.

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

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. Spectrophotometer- an instrument with a narrow band (pass) width (0.5 to 2.0 nm) used to identify the relatively narrow chlorophyll-a absorption peak. (At a spectral bandwidth of 20 nm the chlorophyll-a concentration may be underestimated by as much as 40%.)

i. Cuvette (with lid)- A sample vessel with a 1 cm 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 $\mu g/L$): Add 100 mL of saturated $MgCO_3$ to a 1000 mL volumetric flask. Open glass vial containing dry chlorophyll-a from algae (or spinach) and empty contents into the flask. (Normal purchased amounts are about 1 mg per vial.) Rinse the vial with acetone until the acetone runs clear (at least two 1-ml portions) and add to the volumetric flask. Immediately bring to volume with acetone, and wrap the flask with foil 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. Refer to the Calibration and Standardization section below. When not in use, store the stock solution and any dilutions in the dark, flammable storage cabinet. Other concentrations and volumes may be used as long as these are documented and confirmed.

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: $C_1V_1 = C_2V_2$ 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. Pheophytin-a LOQ/LOQcv Standard (3.0 mg/M^3) – Dilute a portion of the stock solution with aqueous acetone solution to the require concentration using the formula:

$C_1V_1 = C_2V_2$ 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).

Ensure that the concentration has been checked by the spectrometric method. Adjust concentration through addition of extra stock solution or aqueous acetone until final value is within 10% of desired value. Then acidify this standard with 0.1 N HCl to convert the chlorophyll-a to Pheophytin-a. Record the standard in the Standards Log and analyze.

vi. Aliquots of both the Chlorophyll and Pheophytin standards are analyzed twice with each run to serve as the LCSD/LOQ duplicates.

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 >0.0-≤6.0° 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. At least one laboratory or field duplicate for every analytical batch of ten samples will be analyzed each day that samples are submitted, as QAM-Q-101, “Laboratory Quality Control,” 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.

d. The analyst should refer to QAM-I-103, "Operation and Calibration of the UV-Vis Spectrophotometer", or the Beckman DU-600/60 Series 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 on UV-Vis on each of the seven aliquots described in the Standards section above. Using the formula in the calculations section, calculate the concentration of chlorophyll-a in each portion. 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 >0.0-≤6.0° C since sample collection.
c. Preferred sample volume is 1 liter.
d. Samples are stored in opaque containers at >0.0-≤6.0° C until they are processed. Samples are filtered within 48 hours of collection.
e. Concentration of biomass
    i. Use glassware and cuvettes that are clean and acid-free.
    ii. Allow samples to come to room temperature for accurate volume measurement.
    iii. Shake samples well before filtering.
    iv. 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. Turn the setscrew on the pump exhaust to adjust.

v. Add 2 mL of $MgCO_3$ solution to sample just before the filtering process is completed. $MgCO_3$ solution acts as a pH buffer to keep chlorophyll from degrading.

vi. If entire sample is filtered, stop and measure the volume of sample filtered. Then rinse the sample container with about 20 mL organic-free DI (which is also passed through the same sample filter to make sure all cells are collected). Add 2 mL of MgCO3 solution just before the filtering process is completed.

vii. Measure and document the volume of sample filtered, and the date and time of filtration in the Chl-a/Pheo-a Prep Log. Do not include any rinse water or $MgCO_3$ in the volume.

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

ix. Samples on filters taken from water having pH of 6 or higher may be placed in airtight bags and stored frozen at least 24 hours and not longer than 28 days.

x. Samples from acidic water must be processed promptly to prevent chlorophyll-a degradation. If the pH measured in the field for chlorophyll-a or pheophytin-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 >0.0-≤6.0° 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. Spectrophotometric determination of chlorophyll-a in presence of pheophytin-a:

i. Zero the instrument by placing filtered aqueous acetone in the sample cell of the spectrophotometer and zeroing the instrument absorbance reading at the 750 nm wavelength, prior to sample and standard readings. Zeroing is performed at a minimum at the beginning of every day on which Chl-a/Pheo-a samples are analyzed.

ii. The 750 nm reading allows for compensation of turbidity and is subtracted from the respective 664 or 665 reading before calculating the chlorophyll-a concentration.

iii. Place 3.0 mL of sample into sample cell using a syringe through a 0.45 µm syringe filter.

iv. Take readings at 750 and 664 nm and document in the log. This documentation may consist of readouts from the spectrophotometer taped into the log. The initial reading

at 750 nm is less than or equal to 0.005. If not, the sample contains suspended matter. Re-filter and take another reading. The absorbance 664 before acidification should be between 0.1 and 1.0. For very dilute extracts or to obtain lower detection levels, use cuvettes with a longer path. If a larger cell is used, add a proportionately larger volume of acid. Correct absorbance obtained with larger cuvettes to 1 cm before making calculations.

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 readings at 665 and 750 nm 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:

$$\text{Chlorophyll-a, mg/M}^3 = 26.7[(664b-750b)-(665a-750a)]V_1 /(V_2)L$$

$$\text{Pheophytin-a, mg/M}^3 = 26.7[1.7(665_a-750b)-(664_b-750a)]V_1 /(V_2)L$$

Where:

V_1 = volume of extract (L), usually 10 mL
V_2 = volume of sample (m^3)
L = light path length or width of cuvette (1 cm)
664_b = optical density of 90% acetone extract before acidification at 664 nm
665_a = optical density of 90% acetone extract after acidification at 665 nm
750_b = optical density of filtered sample before acidification at 750 nm
750_a = optical density of filtered sample after acidification at 750 nm

i. A spreadsheet (Excel Chl-a/Pheo-a) or log may be used to calculate the Chl-a/Pheo-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 10150A&B (approved 2022).

b. National Environmental Laboratory Accreditation Conference TNI standard, The NELAC Institute, 2016.

xxiii. Any tables, diagrams, flowcharts and validation data

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

b. Example Chl-a/Pheo-a Preparation Log

Attachment 1 Example Chl-a/Pheo-a Spreadsheet

Chlorophyll/Pheophytin A Worksheet
Start date/time:
Analyst:
Revised jrh 3/26/21

Acceptance limits **LCS** **LCSd** **LOQ**
Chlorophyll **2.4-3.6** **20%** **2.1-3.9**
Pheophytin **1.93-2.89** **20%** **1.69-3.13**

If you use less than 1 Liter, adjust LOD accordingly

Aq. Acetone 022321e, A-7, P-24, 0.1 N HCl 010621b, Acetone 3308

SAMPLE #	VOLUME IN LITERS	750 BEFORE ACID	664 BEFORE ACID	665 AFTER ACID	750 AFTER ACID	SAMPLE #	CHL A MG/M3	PHEO A MG/M3	664b/665a	Maceration Date	Maceration Time	Comments
Instrument Blk	1.000					**Instrument Blk**	0.00	0.00				na
cLCS	1.000					**cLCS**	0.00	0.00	#DIV/0!	na	na	na
cLCSd	1.000					**cLCSd**	0.00	0.00	#DIV/0!	na	na	na
cLOQ	1.000					**cLOQ**	0.00	0.00	#DIV/0!	na	na	na
						0	#DIV/0!	#DIV/0!	#DIV/0!			na
						0	#DIV/0!	#DIV/0!	#DIV/0!			na
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Attachment 2 Example Chl-a/Pheo-a Preparation Log

CHLOROPHYLL/PHEOPHYTIN PREP LOG

DATE	[illegible]	SAMPLE ID	SAMPLE VOLUME (L)	Analysis Method	Graduated Cylinder ID (TC-To Contain) Class A	GF/F filter manufacturer	GF/F lot number	Sat. MgCO3 ID	Pipetter ID	Batch Start time	Batch End time	Storage Freezer ID	Grinding Date	[illegible]	Aq. Acetone ID	GC TD ID	Grind Start Time	Grind End Time	Extraction Date	Extraction Time	[illegible]	Nylon filter manufacturer	Nylon filter lot number	comments	QA review	date
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