# SECTION D.3
# CHLOROPHYLL AND PHEOPHYTIN

1.1 Scope and Application

1.1.1 This spectrophotometric method is used in the determination of chlorophyll *a*, *b*, and *c*, and pheophytin *a*. This method can be used to estimate phytoplankton biomass.

1.2 Summary of Method

1.2.1 Algal cells are filtered on a glass fiber and ground in aqueous acetone to extract the pigments. The extract is analyzed using a spectrophotometer to measure the absorbances at the specified wavelengths.

1.3 Apparatus and Materials

1.3.1 Laboratory coat: worn at all times and with an apron when handling acids.

1.3.2 Protective eye wear: worn at all times.

1.3.3 PVC gloves: worn at all times.

1.3.4 Glass fiber filters: Whatman GF/F or equivalent, 0.7µm nominal pore size.

1.3.5 Centrifuge. (A centrifuge capable of cooling to 4°C is recommended.)

1.3.6 Scanning spectrophotometer - Visible, multi-wavelength, with a bandpass not to exceed 2 nm. Glass cells for the spectrophotometer can be 1, 2, 5 or 10 cm. in length. If using multiple cells, they must be matched.

1.3.7 Tissue Grinder - Teflon pestle with attached stainless steel rod.

1.3.8 Filtration apparatus with fritted glass disk base.

1.3.9 Solvent resistant glass fiber syringe filter (optional).

1.4 Reagents

1.4.1 Aqueous acetone (90%): add 1 part water to 9 parts of reagent grade acetone (v/v), made within 24-48 hours of time of use.
*Note: Incorrect preparation of this solution may cause erroneous 750 nm readings.*

1.4.2 Hydrochloric acid (1N), HCl.

1.4.3 Magnesium carbonate suspension, (10 mg/L $MgCO_3$): add 1 g finely powdered magnesium carbonate to 100 mL reagent grade water.

1.4.5 Reagent Grade Water: ASTM Type II. See section 1.9.

1.5 Sample Processing

1.5.1 Samples are filtered in the field according to procedures in section 3.4, (p.IV-13) which are modified as follows:

Immediately after collecting the sample, filter a known volume of sample water (measured with a graduated cylinder) onto a glass fiber filter to concentrate the algae. Use sufficient sample (100-1500 mL) to produce a green color on the filter pad. To avoid cell damage and loss of contents during filtration, do not exceed a vacuum of 10 psi, or a filtration duration greater than 5 minutes. If sampling non-saline water (< 0.5 ppt salinity), add 1mL of saturated $MgCO_3$ solution during the last few seconds of filtering. Do not suck the filter dry with the vacuum; instead slowly release the vacuum as the final volume approaches the level of the filter and completely release the vacuum as the last bit of water is pulled through the filter.

1.5.2 Remove the filter from the fritted base with tweezers, fold once with the particulate matter inside, lightly blot the filter with a tissue to remove excess moisture and place it in a petri dish or other suitable container. Wrap the container in aluminum foil to protect the phytoplankton from light and store the filter at -20°C. Processed filters may be stored for 2-4 hours on ice before storing at -20 °C.

Samples that cannot be filtered immediately after collection may be held at 0 to 4°C in the dark for 4 hours before the plankton are concentrated, however, any delay is strongly discouraged. The residue on the filter is to be stored in the dark at -20 ± 2°C for up to 28 days before extracting and analyzing the pigments. Some studies indicate degradation after a few week so the less time in storage the better.

1.6 Grinding Procedure

1.6.1 Remove frozen samples from the freezer but keep them in the dark. Keep workspace lighting to a minimum. Place filter into a glass centrifuge tube and add 2-3 ml of 90% acetone using a volumetric pipet.

1.6.2 Insert pestle into centrifuge tube and turn on grinder.

1.6.3 Grind filter for approximately 1 to 2 minutes being sure there are no discernible pieces remaining. If the tube gets warm from the friction of grinding, place the tube in a beaker of ice while grinding.

1.6.4 Pull pestle from vessel, rinse with 90% acetone if necessary while adding an exact volume with a volumetric pipet. Record the total volume of acetone added for grinding and extraction.

1.6.5 Cap the centrifuge tube and shake vigorously before steeping overnight at 4°C in the dark.

1.6.6 Centrifuge the extract prior to spectrophotometric analysis. If the centrifuge has a temperature control, cool the unit to 4 ± 2°C; centrifuge samples for approximately 15 minutes at 675 g. Keep centrifuged samples cool and protected from light. The centrifuged extract can be stored at -20 ± 2°C for up to 28 days after sample collection. If this time is exceeded, report and flag the data.

1.7 Instrument Optimization

1.7.1 Allow the instrument to warm up for at least 30 min. prior to use. Daily calibration of the spectrophotometer with known standards is not required.

1.7.2 Absorbance responses, i.e., optical densities, for samples should be between 0.1 and 1.0 absorbance units to ensure a linear response. Alternatively, the instrument's actual linear range may be demonstrated using a series of diluted samples. A higher absorbance response can be obtained by using a longer path length cell, a smaller extract volume, or a larger sample size.

1.7.3 Check the accuracy of the wavelength readings using a standard reference material such as holium oxide filter (NIST SRM 930e) at least quarterly.

1.8 Procedure

1.8.1 Use a 90% acetone solution to zero the instrument at each of the wavelengths 750 nm, 665 nm, 664 nm, 647 nm and 630 nm. (See section 12.7 for checks on instrument performance.) If using a dual beam instrument leave one of the cuvettes in the reference cell.

1.8.2 Carefully pour or dispense the supernatant of the extracted sample into the cuvette. If the initial absorbance reading at 750 nm exceeds 0.007, recentrifuge or filter the extract through a solvent resistant glass fiber syringe filter to remove turbidity interference.

If recentrifugation and filtering does not remove the 750 nm turbidity interference, continue to measure the absorbances at the rest of the wavelengths and write in the comment section that the sample had been recentrifuged and/or filtered.

1.8.3 Measure absorbances at the following wavelengths: 750 nm, 664 nm, 647 nm, 630 nm. If necessary, re-zero the spectrophotometer with 90% acetone before reading at each wavelength.

1.8.4 After the 630 nm reading is taken, add the volume of 1N HCL that results in a final normality of 0.02 N in the cuvette. Mix well.
*Example: 5 mL cuvette*:

mL 1N HCL = (0.02N)(cuvette volume(mL)).
mL 1N HCL = (0.02N)(5mL) = 0.1mL

0.1 mL(~20 drops/mL) = ~ 2 drops

1.8.5 90 seconds after acidification and mixing, measure sample absorbances at 750 nm and 665 nm. If necessary, re-zero the spectrophotometer with 90% acetone before reading at each wavelength.

1.9 Quality Control

1.9.1 Samples should have an optical density (OD) ratio of OD $664_b$ (before acidification) to OD $665_a$ (after acidification) ratio between 1.0 and 1.7. Ratios outside of this range may be caused by interfering pigments or in low level samples, from variability near detection levels. Ratios near 1.7 are considered to have no pheophytin and to be in excellent physiological condition.[1]

1.9.2 It is especially important to maintain the spectrophotometers in peak operating condition. This should be confirmed by the following guidelines:

1.9.2.1 Analyze a reference standard or SRM for chlorophyll analysis.

1.9.2.2 Periodic evaluation of the slopes of calibration curves from spectrophotometer analyses for other parameters for which there are reliable standards (e.g. orthophosphate, nitrite, etc.). If significant slope deviation or consistent unidirectional slope change over time is noted, an alternate spectrophotometer should be used until the problem is corrected by an authorized repair person.

1.9.2.3 The holmium oxide absorption spectrum needs to be measured quarterly or when problems are suspected. Details are not provided here since this and subsequent evaluation should be performed only by or under direct supervision of experienced personnel.

1.9.3 Method detection limits (MDL): Method detection limits should be established using the guidelines in Chapter II, Section D.

1.9.4 Method blank: see Chapter II, Section C.

1.9.5 Laboratory duplicate: see Chapter II, Section C.

1.10 Calculation and Reporting

1.10.1 The Chesapeake Bay Program staff use Lorenzen's pheopigment-corrected equations to calculate chlorophyll *a* and pheophytin *a.* The $750_b$ nm and $750_a$ OD values are subtracted from the readings before (OD 664 nm) and after acidification (OD 665). Lorenzen's equations are:

$$\text{Chlorophyll } a, \mu g/L = \frac{26.7\,(664_b - 665_a) \times V_1}{V_2 \times L}$$

$$\text{Pheophytin } a, \mu g/L = \frac{26.7\,[1.7\,(665_a) - 664_b) \times V_1}{V_2 \times L}$$

where:

$V_1$ = volume of extract, mL
$V_2$ = volume of sample, L
L = light path length or width of cuvette, cm, and
$664_b$, $665_a$ = optical densities of extracts before and after acidification, respectively.

1.10.2 Laboratories report all optical densities, volumes and light path length so that chlorophyll *a, b*, and *c* may be calculated by the trichromatic method.

1.11 References

[1]American Public Health Association, 1995. Standard Methods for the Examination of Water and Wastewater, 19th Edition.

[2]EPA, 1997. Methods for the Determination of Chemical Substances in Marine and Estuarine Environmental Matrices - 2nd Edition, Method 446.0. EPA/600/R-97/072.

[3]Parsons, T., Y. Maita and C. Lalli. 1984. A Manual of Chemical and Biological Methods for Seawater Analysis. Pergamon Press, pp. 101-112.