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Standard Practices for Measurement of Chlorophyll Content of Algae in Surface Waters[1]

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1. Scope

1.1 These practices include the extraction and the measurement of chlorophyll *a*, *b*, and *c*, and pheophytin *a* in freshwater and marine plankton and periphyton. Three practices are provided as follows:

1.1.1 Spectrophotometric, trichromatic practice for measuring chlorophyll *a*, *b*, and *c*.

1.1.2 Spectrophotometric, monochromatic practice for measuring chlorophyll *a* corrected for pheophytin *a*; and for measuring pheophytin *a*.

1.1.3 Fluorometric practice for measuring chlorophyll *a* corrected for pheophytin *a*; and for measuring pheophytin *a*.

1.2 The values stated in SI units are to be regarded as the standard. The values given in parentheses are provided for information purposes only.

1.3 *This standard does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.* Specific precautionary statements are given in Section 7.

2. Terminology

2.1 *Definitions:*

2.1.1 *plankton*—nonmotile or weakly swimming organisms, usually microscopic, that drift or are carried along by currents in surface waters, commonly consisting of bacteria, algae, protozoa, rotifers, and microcrustacea.

2.1.2 *periphyton*—microorganisms growing on submerged objects, commonly consisting of bacteria, algae, protozoa, and rotifers.

3. Summary of Practices

3.1 The chlorophyll and related compounds are extracted from the algae with 90 % aqueous acetone. The concentration of the pigments is determined by measuring the light absorption or fluorescence of the extract.

4. Significance and Use

4.1 Data on the chlorophyll content of the algae have the following applications:

4.1.1 To provide estimates of algal biomass and productivity.

4.1.2 To provide general information on the taxonomic composition (major groups) of the algae, based on the relative amounts of chlorophyll *a*, *b*, and *c*, and the physiological condition of algal communities, which is related to the relative abundance of pheopigments.

4.1.3 To determine long-term trends in water quality.

4.1.4 To determine the trophic status of surface waters.

4.1.5 To detect adverse effects of pollutants on plankton and periphyton in receiving waters.

4.1.6 To determine maximum growth rates and yields in algal growth potential tests.

5. Interferences and Special Considerations

5.1 *Pigment Extraction*—The chlorophylls are only poorly extracted, if at all, from some forms of algae, such as the coccoid green algae, unless the cells are disrupted, whereas other algae, such as the diatoms, give up their pigments very readily when merely steeped in acetone. Since natural communities of algae usually consist of a wide variety of taxa that differ in their resistance to extraction, it is necessary to disrupt the cells routinely to ensure maximum recovery of the chlorophylls. Failure to do so may result in a systematic underestimation of 10 % or more in the chlorophyll content of the samples. **(1, 2, 3)**[2]

5.2 *Grinders*—The cells of many common coccoid green algae resist disruption by most methods, but usually yield their pigments after maceration with a tissue grinder. The routine use of grinders, therefore, is recommended. Glass-to-glass grinders are more rigorous in disrupting cells in plankton concentrated by centrifugation, and in periphyton scrapings, than are TFE-fluorocarbon-to-glass grinders, and their use for this purpose is preferred. However, TFE fluorocarbon-to-glass grinders perform well with glass-fiber filters. Other cell disruption methods, such as sonication, may be used if, for each

[1] These practices are under the jurisdiction of ASTM Committee E47 on Biological Effects and Environmental Fate and are the direct responsibility of Subcommittee E47.01 on Aquatic Assessment and Toxicology.

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[2] The boldface numbers in parentheses refer to the list of references at the end of this standard.

type of sample, it is demonstrated that the chlorophyll recovery is comparable to that obtained with tissue grinders **(4)**.

5.3 *Filters*—Glass-fiber filters usually provide a higher recovery of chlorophyll than is obtained with membrane filters when extraction-resistant algae are present in the samples, and should be employed routinely **(4)**.

5.4 *Chlorophyll-Related Pigments*—Naturally occurring, structurally related chlorophyll precursors and degradation products, such as the chlorophyllides, pheophytins, and pheophorbides, commonly occur in pigment extracts and may absorb light in the same region of the spectrum as the chlorophylls. These compounds may interfere with the analysis by indicating falsely high chlorophyll concentrations.

5.4.1 This practice includes a correction for pheophytin *a* only. Pheophytin *a* is similar in structure to chlorophyll *a*, but lacks the magnesium atom (Mg) in the porphyrin ring. The magnesium can be removed from chlorophyll in the laboratory by acidifying the extract. When a solution of pure chlorophyll *a* is converted to pheophytin *a* by acidification, the absorption peak is reduced to approximately 60 % of its original value and shifts from 664 to 665 nm, resulting in a before:after acidification absorption peak ratio (OD664/OD665) of 1.70. This phenomenon is utilized in correcting the apparent concentration of chlorophyll *a* for the presence of pheophytin *a*. Unwanted degradation of chlorophyll to pheophytin in the phytoplankton on filters, or in periphyton samples, or in the acetone extract, by the occurrence of acidic conditions can be prevented by the addition of a magnesium carbonate suspension to the plankton sample before filtering or to the periphyton samples before grinding, and by adding a small amount of a sodium bicarbonate solution to the aqueous acetone when it is prepared. Addition of magnesium carbonate may also aid in clarifying the samples following steeping **(5)**.

5.5 *Turbidity*—The optical density of the extract is measured at 750 nm to correct for turbidity.

5.6 *Spectrophotometer Resolution*—The absorption peak of acetone solutions of chlorophyll extracts is relatively narrow, and a spectrophotometer with a resolution of 2 nm or better is required to obtain accurate results. If instruments of lower resolution are employed, the concentration of chlorophyll *a* may be significantly underestimated depending on the band width. At a spectral band width of 20 nm, the error in the estimate of the chlorophyll *a* concentration may be as large as 40 %.

5.7 *Fluorometer Filters*—In the fluorometric practice, interferences from light emitted by chlorophyll *b* and chlorophyll *c* are greatly reduced by the use of a sharp cut-off red filter[3] that blocks all light with a wavelength of less than 650 nm **(6)**.

5.8 *Light Sensitivity of Extracts*—Chlorophyll solutions degrade rapidly in strong light. Work with these solutions, therefore, should be carried out in subdued light, and all vessels, tubes, and so forth, containing the pigment extracts should be covered with aluminum foil or other opaque substance.

6. Apparatus

6.1 *Filters*, Glass-fiber filters, providing quantitative retention of particles equal to or greater than 0.45 µm in diameter.

6.2 *Filtering Apparatus* suitable for use with glass-fiber filters.

6.3 *Tissue Homogenizer*—Tissue grinder consisting of a motor-driven pestle and enclosing glass tube (glass to glass or TFE-fluorocarbon-to-glass grinder).[4]

6.4 *Spectrophotometer* suitable for use over the range from 600 to 750 nm, with a resolution of 2 nm or better, and equipped with sample cells having a light path of 1, 5, and 10 cm, with a capacity of 10 mL or less.

6.5 *Fluorometer (Optional)*:

6.5.1 *Spectrophotofluorometer* that provides an excitation wavelength of 430 nm and detection of emission over the range from 600 to 700 nm, or:

6.5.2 *Filter Fluorometer* equipped with a blue light source and blue excitation filter[5] and a sharp cut off filter[3]

6.6 *Centrifuge* that can provide a centrifugal force of 1000 g; head with swing-out buckets preferred.

6.7 *Centrifuge Tubes*, screw-cap or stoppered, conical, graduated, 15-mL. Avoid cap liners soluble in acetone and neoprene rubber stoppers.

7. Reagents and Materials

7.1 *Aqueous Acetone, 90 %*—Add 1 volume of distilled water to 9 volumes of reagent grade acetone. Add 5 drops of 1 *N* sodium bicarbonate solution per litre. (**Caution—**the volume:volume relationship between the acetone and water must be strictly followed to prevent shifts in the absorption peaks.)

7.2 *Hydrochloric Acid (1 N)*—Add one volume of concentrated hydrochloric acid (HCl, sp gr 1.19) to eleven volumes of distilled water.

7.3 *Magnesium Carbonate Suspension*—Add 1 g of finely powdered magnesium carbonate to 100 mL of distilled water in a stoppered Erlenmeyer flask. Shake immediately before use.

7.4 *Sodium Bicarbonate Solution (1 N)*—Prepare by dissolving 8.4 g of sodium bicarbonate in 100 mL of distilled water.

8. Sampling

8.1 *Plankton*:

8.1.1 *Collection*—Collect samples with a water bottle, diaphragm pump, or other suitable device. To protect the chlorophyll from degradation prior to extraction and analysis, immediately add 1 mL of magnesium carbonate suspension per L of sample, and protect from the direct sunlight.

8.1.2 *Concentration*—Immediately concentrate the plankton by filtering or by centrifuging for 20 min at 1000 g. To avoid cell damage and loss of contents during filtration, do not exceed a vacuum of ½ atm (50 kPa). After centrifuging, check the samples for buoyant cells that may resist sedimentation.

[3] Corning CS-2-64 filter or its equivalent, has been found suitable for this purpose. Available from Corning Glass Works, 388 Beartown Rd., Painted Post, NY 14870.

[4] Kontes type C, glass-to-glass grinder or its equivalent, has been found suitable for this purpose. Available from Kontes Manufacturing Co., Spruce St., Vineland, NJ 08360.

[5] Corning CS-5-60 filter has been found satisfactory. Equivalent filters may be used.

8.1.3 *Holding Time*—Samples that cannot be concentrated immediately after collection may be held at 0 to 4°C in the dark for 24 h before the plankton are concentrated. The centrifugate or residue on the filter may be stored in the dark at −20°C for 30 days before extracting the pigment.

8.2 *Periphyton*:

8.2.1 *Collection*—Take samples from natural or artificial substrates and immediately ice, freeze, or place in cold (iced) aqueous acetone in the dark.

8.2.2 *Holding Time*—Iced samples may be held 24 h before they are further processed. Frozen samples may be held at −20°C for 30 days.

9. Pigment Extraction

9.1 Algal cells in plankton concentrates (from centrifugation or filtration) and periphyton scrapings are disrupted by grinding in a tissue homogenizer for 3 min at approximately 500 r/min in 4 to 5 mL of 90 % aqueous acetone. Use a glass-to-glass tissue grinder for macerating plankton concentrate obtained by centrifugation and for macerating periphyton scrapings. A TFE-fluorocarbon-to-glass or glass-to-glass grinder may be used to macerate plankton concentrated on glass-fiber filters.

9.2 Wash the homogenate into a vial or into a 15-mL centrifuge tube, rinse the pestle and grinding tube with a small amount of aqueous acetone, bring the volume of the extract to 10 mL by adding 90 % aqueous acetone, cap or stopper the tube, and mix and place the material in the dark at 4°C to steep.

9.3 Steep not less than 15 min or more than 24 h. Mix the homogenate by inverting the tube several times, and clarify the extract by centrifuging 20 min at 1000 g, or by filtering. If the clarified extract is not analyzed immediately, store in the dark at −20°C in a tightly stoppered tube.

9.4 After clarification, decant the extract directly into a cuvette or a screw-cap or stoppered tube. If the analysis can not be carried out immediately, the extract can be stored for 1 year without appreciable chlorophyll degradation if held in the dark at −20°C.

10. Extract Analysis

10.1 The two practices of extract analysis commonly employed are visible spectrophotometry and fluorometry **(6, 7)**. Each practice has its advantages and disadvantages. The trichromatic, spectrophotometric practice, has the advantage of providing a simple procedure for the simultaneous estimation of chlorophyll *a*, *b*, and *c*, which, at the current state of technology, cannot be obtained as easily by the fluorometric practice, but does not correct for chlorophyll degradation products. The monochromatic, spectrophotometric practice corrects the chlorophyll *a* for pheophytin, *a*, but does not measure chlorophyll *b* and *c*. The fluorometric practice, is one to three orders of magnitude more sensitive than the spectrophotometric practices, when using the instrumentation commonly employed for chlorophyll analyses, but the practices for simultaneously measuring chlorophyll *b* and *c* are much more complex.

10.2 *Spectrophotometric, Trichromatic Practice*:

10.2.1 The chlorophyll concentration is a function of the absolute optical density (OD) of the extract at the specified wavelengths, rather than the relative OD as is commonly the case in colorimetric analyses. For quantitative chlorophyll determinations, it is essential, therefore, to check the instrument or chart OD readings, or both, at several wavelengths in the range from 600 to 750 nm across the full span of the absorbance (OD = 0.0–1.0), using a set of filters[6] of known optical density.

10.2.2 Transfer the extract to a sample cell and measure the optical density at 750, 664, 647, and 630 nm. If possible, choose a cell-path length or dilution to provide an OD664 greater than 0.2 and less than 1.0.

10.2.3 Subtract the OD750 from each of the other ODs. Then divide by the cell-path length in centimetres.

10.2.4 Calculate the concentration of chlorophyll *a*, *b*, and *c* in the extract by inserting the 1-cm OD664, OD647, and OD630 into the following Jeffrey and Humphrey equations **(6)**:

$$\text{Chl } a, \text{ mg/L} = 11.85(\text{OD664}) - 1.54(\text{OD647}) - 0.08(\text{OD630}) \quad (1)$$

$$\text{Chl } b, \text{ mg/L} = 21.03(\text{OD647}) - 5.43(\text{OD664}) - 2.66(\text{OD630}) \quad (2)$$

$$\text{Chl } c, \text{ mg/L} = 24.52(\text{OD630}) - 1.67(\text{OD664}) - 7.60(\text{OD647}) \quad (3)$$

10.2.5 Express the concentration of pigments in a plankton sample as milligrams per cubic metre (mg/m^3) and calculate as follows:

$$\text{Chl } a, \text{ mg/m}^3 = \frac{\text{Ca} \times E}{G} \quad (4)$$

where:

Ca = concentration of chlorophyll *a* in the extract, mg/L,
E = extract volumes, L, and
G = grab sample volume, m^3.

10.2.6 Express the concentration of pigments in a periphyton sample as milligrams per square metre (mg/m^2) and calculate as follows:

$$\text{Chl } a, \text{ mg/m}^2 = \frac{\text{Ca} \times E}{A} \quad (5)$$

where:

Ca = concentration of chlorophyll *a* in the extract, mg/L, and
A = substrate area sampled, m^2.

10.3 *Spectrophotometric, Monochromatic Practice*:

10.3.1 Measure the optical density of the chlorophyll extract at 750 and 664 nm.

10.3.2 Acidify the extract by adding 2 drops of 1 *N* HCl (amount added to a 1-cm cell; if a larger cell is used, add a proportionately larger volume of acid), stir well, and measure the OD at 750 and 665 nm not sooner than 1 min or later than 2 min after acidification.

[6] Filters, Standard Reference Material 930, Glass Filters for Spectrophotometry, available from the National Bureau of Standards, Office of Product Standards, Administration Building A603, Gaithersburg, MD 20899, or equivalent have been found suitable for this purpose.

10.3.3 Calculate the concentration of chlorophyll *a* (Ca) corrected for the presence of pheophytin *a*, and the concentration of pheopigments expressed as pheophytin *a* (Pa) in the extract by inserting the 1-cm ODs in the following equations **(5)**:

$$\text{Ca, mg/L} = 26.7(\text{OD664}b - \text{OD665}a) \quad (6)$$

$$\text{Pa, mg/L} = 26.7[1.7(\text{OD665}a) - \text{OD664b}] \quad (7)$$

where:

OD*664b* = OD664 − OD750 measured before acidification, and

OD*665a* = OD665 − OD750 measured after acidification.

10.3.4 Calculate the concentration of these pigments in plankton and periphyton samples as described in 10.2.5 and 10.2.6, respectively.

10.4 *Fluorometric Practice*:

10.4.1 The fluorometric practice is 10 to 1000 times more sensitive than the spectrophotometric practices and requires proportionately smaller amounts of sample. The method has important disadvantages, however, which include the inability to easily determine chlorophyll *b* and *c* concentrations, and the need to calibrate the instrument with "reference" chlorophyll solutions containing a known concentration of chlorophyll *a* determined by previous spectrophotometric analysis **(7)**.

10.4.2 To calibrate the instrument, carefully dilute the "reference" extract to provide solutions that give midscale readings in each sensitivity range of the fluorometer. Use the readings to determine a calibration factor, *F* (*s*), for each sensitivity level (*s*) as follows:

$$F(s) = \frac{\text{Ca}}{R} \quad (8)$$

where:

Ca = concentration of chlorophyll *a, µg/L, and*

R = fluorometer reading.

10.4.3 To correct for the presence of pheopigments, expressed as pheophytin *a*, determine *a* before:after acidification fluorescence ratio, *r*, as described in 10.3.2 using an extract that is free of pheophytin *a*, where the before:after acidification ratio is 1.70, based on the OD664*b*/OD665*a*, as determined with a spectrophotometer. Use the fluorometric before:after acidification ratio (*r*) and the calculation factor, *F* (*s*), in the following equations:

$$\text{Ca, µg/L} = F(s)\frac{r}{r-1}(Rb - Ra) \quad (9)$$

$$\text{Pa, µg/L} = F(s)\frac{r}{r-1}(rRa - Rb) \quad (10)$$

where:

Ca and Pa = concentration of chlorophyll *a* and pheophytin *a*, respectively, in the extract,

Rb = fluorometer reading before acidification; and

Ra = fluorometer reading after acidification.

11. Precision and Bias

11.1 It is not practicable to specify the precision of the procedures in Practices D 3731 for measuring chlorophyll content of algae since there are no interlaboratory data sets available at this time.

12. Keywords

12.1 algae surface water; chlorophyll; pheophytin

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