Designation: D3731 – 87 (Reapproved 2012)

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

This standard is issued under the fixed designation D3731; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

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 concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety, health, and environmental practices and determine the applicability of regulatory limitations prior to use.* Specific precautionary statements are given in Section 7.

1.4 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

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

[1] These practices are under the jurisdiction of ASTM Committee D19 on Water and are the direct responsibility of Subcommittee D19.24 on Water Microbiology.

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

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 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.

[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.