

Chapter 14. Measurement of Chlorophyll *a* and Phaeopigments by Fluorometric Analysis

1.0 Scope and field of application

Chlorophyll *a* measurements have historically provided a useful estimate of algal biomass and its spatial and temporal variability. The fluorometric method is extensively used for the quantitative analysis of chlorophyll *a* and phaeopigments. However, errors can be introduced into the results when chlorophylls *b* and/or chlorophylls *c* are present.

Chlorophyll *b* is the main source of error in this method. While generally not abundant in surface waters, chlorophyll *b* can be as high as 0.5 times the chlorophyll *a* concentration in the deep chlorophyll maximum, causing slight underestimations of the chlorophyll *a* concentration, and drastic overestimations of the phaeopigment concentrations. Divinyl-chlorophyll *a* also interferes and is taken as chlorophyll *a* by this method. The procedure described here is appropriate for all levels of chlorophyll *a* concentration in the marine environment. Filtration volumes should be modified for the different environments.

Scientists who employ this or other methods to measure pigments should make themselves aware of the current and historical issues that surround these techniques and make appropriate decisions about specific methodologies for their application based on the scientific requirements and constraints of their individual programs.

2.0 Definition

The concentrations of chlorophyll *a* and phaeopigments in seawater are given as $\mu\text{g kg}^{-1}$.

3.0 Principle of Analysis

Algal pigments, particularly chlorophyll *a*, fluoresce in the red wavelengths after extraction in acetone when they are excited by blue wavelengths of light. The fluorometer excites the extracted sample with a broadband blue light and the resulting fluorescence in the red is detected by a photomultiplier. The significant fluorescence by phaeopigments is corrected for by acidifying the sample which converts all of the chlorophyll *a* to phaeopigments. By applying a measured conversion for the relative strength of chlorophyll and phaeopigment fluorescence, the two values can be used to calculate both the chlorophyll *a* and phaeopigment concentrations.

4.0 Apparatus

4.1 Filtration system and Whatman GF/F filters

4.2 Liquid nitrogen and freezer for storage and extraction

4.3 Glass centrifuge tubes for extraction, 15 ml

4.4 Turner fluorometer, fitted with a red sensitive photomultiplier, a blue lamp, 5-60 blue filter and 2-64 red filter.

5.0 Reagents

5.1 100% acetone

5.2 90% acetone

5.3 1.2M HCl (100 ml HCl in 900 ml de-ionized water)

6.0 Sample Collection and Storage

Water samples are collected from niskins into clean polyethylene bottles with Tygon[®] tubing. Samples are immediately filtered through 47 mm GF/F filters using polycarbonate in-line filters (Gelman) and a vacuum of less than 100 mm Hg. Filters are folded in half twice and wrapped in aluminum foil, labeled, and stored in liquid nitrogen (to avoid formation of degradation products) until shore analysis. Alternatively, filters can be placed immediately in acetone for pigment extraction if analysis is to be carried out onboard ship.

In oligotrophic waters, for this measurement coupled with HPLC determined pigments, 4 liters are filtered. For fluorometric analysis alone, a smaller volume (0.5 -1.0 l) may be sufficient. In coastal regions, a volume of 0.1-0.5 l may be adequate. In this case, use of 25 mm GF/F filters may be appropriate.

7.0 Procedure

7.1 After removal from liquid nitrogen or freezer), the pigments are extracted by placing the filters in 5.0 ml 100% acetone. For 47 mm GF/F filters, 0.8 ml of water is retained adjusting the final extraction solution to 86% acetone and the final extraction volume to 5.8 ml. The samples are covered with Parafilm to reduce evaporation, sonicated (0°C, subdued light) and allowed to extract for 4 hours in the dark at -20°C. Following extraction, samples are vortexed, filters are pressed to the bottom of the tube with a stainless steel spatula and spun down in a centrifuge for 5 minutes to remove cellular debris. For fluorometric analysis (not HPLC), decantation can replace centrifuging.

- 7.1.1 The addition of 5.0 ml acetone for pigment extraction is necessary to completely submerge 47 mm GF/F filters in 15 ml centrifuge tubes. This volume may be altered depending on the size of the filter and volume of the extraction tube.
- 7.2 The fluorometer is allowed to warm up and stabilize for 30 minutes prior to use.
- 7.3 The fluorometer is zeroed with 90% acetone.
- 7.4 1.0 ml of pigment extract is mixed with 4.0 ml 90% acetone in a cuvette and read on the appropriate door to give a reading between 30 and 100. The sample is then acidified with 2 drops of 1.2 M HCl. Further dilutions may be necessary for higher chlorophyll *a* concentrations.

7.5 *Standardization*

- 7.5.1 For laboratory use, the fluorometer is calibrated every 6 months with a commercially available chlorophyll *a* standard (*Anacystis nidulans*, Sigma Chemical Company). If the fluorometer is taken to sea, it is recommended that the fluorometer be calibrated before and after each cruise.
- 7.5.2 The standard is dissolved in 90% acetone for at least 2 hours and its concentration (mg l^{-1}) is calculated spectrophotometrically as follows:

$$\text{chl}a = \frac{(A_{\text{max}} - A_{750\text{nm}})}{E \times l} \times \frac{1000\text{mg}}{1\text{ gram}}$$

where:

- A_{max} = absorption maximum (664 nm)
- $A_{750\text{ nm}}$ = absorbance at 750 nm to correct for light scattering
- E = extinction coefficient for chl *a* in 90% acetone at 664 nm ($87.67\text{ L g}^{-1}\text{ cm}^{-1}$)
- l = cuvette path length (cm)

- 7.5.3 From the standard, a minimum of five dilutions are prepared for each door. Fluorometer readings are taken before and after acidification with 2 drops 1.2 M HCl.
- 7.5.4 Linear calibration factor (K_x) are calculated for each door (x) as the slope of the unacidified fluorometric reading vs. chlorophyll *a* concentration calculated spectrophotometrically.

7.5.5 The acidification coefficient (F_m) is calculated by averaging the ratio of the unacidified and acidified readings (F_o/F_a) of pure chlorophyll *a*.

7.5.6 Samples are read using a door setting that produces a dial reading between 30 and 100. The fluorometer is zeroed with 90% acetone each time the door setting is changed.

8.0 Calculation and expression of results

The concentrations of chlorophyll *a* and phaeopigments in the sample are calculated using the following equations:

$$\text{Chl } (\mu\text{g/l}) = \left(\frac{F_m}{F_m - 1} \right) \times (F_o - F_a) \times K_x \times \left(\frac{\text{vol}_{\text{ex}}}{\text{vol}_{\text{filt}}} \right)$$

$$\text{Phaeo (chl equiv. weights)} = \left(\frac{F_m}{F_m - 1} \right) \times [(F_m \bullet F_a) - F_o] K_x - \text{vol}_{\text{ex}}$$

where:

F_m = acidification coefficient (F_o/F_a) for pure Chl *a* (usually 2.2).

F_o = reading before acidification

F_a = reading after acidification

K_x = door factor from calibration calculations

vol_{ex} = extraction volume

vol_{filt} = sample volume

9.0 References

Herbland, A., A. Le Bouteiller, and P. Raimbault. (1985). Size structure of phytoplankton biomass in the equatorial Atlantic Ocean. *Deep-Sea Res.*, **32**: 819-836.

Holm-Hansen, O., and B. Riemann. (1978). Chlorophyll *a* determination: improvements in methodology. *Oikos*, **30**: 438-447.

ERRATUM

In the equation for phaeopigments in section 8.0 of Chapter 14 (page 122) the last term:

$$- vol_{ex}$$

should be substituted by the following:

$$\left(\frac{vol_{ex}}{vol_{flt}} \right)$$

The correct version of the phaeopigments equation is then:

$$\text{Phaeo (chl equiv. weights)} = \left(\frac{F_m}{F_m - 1} \right) \times [(F_m \cdot F_a) - F_o] K_x \times \left(\frac{vol_{ex}}{vol_{flt}} \right)$$