Bermuda Biological Station for Research, Inc.
Bermuda Atlantic Time-series Study

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

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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 chlorophyll *b* and/or chlorophyll *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. Another source of interference is divinyl-chlorophyll *a*, which is measured 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 different environments.

2.0 Definition

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

3.0 Principle of Analysis

Some algal pigments, particularly chlorophyll *a*, fluoresce in red wavelengths after extraction in acetone when excited by blue wavelengths of light. The fluorometer excites the extracted sample with a broadband blue light and the resulting red fluorescence is detected by a photomultiplier. The fluorescence of the sample is corrected for phaeopigments by acidifying the sample, which converts all of the chlorophyll *a* to phaeopigments. The fluorescence values before and after acidification can be used to calculate the chlorophyll *a* and phaeopigment concentrations by applying a known conversion ratio.

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 (GE F4T4-BL), 5-60 blue filter and 2-64 red filter.

5.0 Reagents

5.1 *100% acetone*

5.2 *90% acetone*

5.3 *1.2 M HCl* (100 ml HCl in 900 ml Milli-Q water)

6.0 Sample Collection and Storage

Water samples are collected from the OTE bottles into 4 liter polypropylene bottles equipped with a 1/4" outlet and Tygon® tubing at the base. Filtration is "in line"; each bottle is connected via the tubing to a polycarbonate filter holder (Gelman) holding a 47mm GF/F filter, and a vacuum system. Samples are filtered promptly using a vacuum of less than 100 mm Hg. Filters are folded in half twice, wrapped in aluminum foil, labeled, and stored in liquid nitrogen until analysis on shore. Alternatively, if analysis is to be carried out onboard ship, filters can be placed immediately into 100 % acetone for pigment extraction.

In oligotrophic waters 4 liters are filtered for this measurement along with HPLC analysis. For fluorometric analysis alone, a smaller volume (0.5 -1.0 l) is sufficient. In coastal regions, a volume of 0.1-0.5 l may be adequate and use of 25 mm GF/F filters 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, approximately 0.8 ml of water is retained, adjusting the final extraction solution to approximately 90 %

acetone and the final extraction volume to approximately 5.8 ml. The samples are covered with Parafilm, sonicated (0°C, subdued light) and allowed to extract overnight in the dark at -20°C. Following extraction, the samples are vortexed, the filters are pressed to the bottom of the tube with a stainless steel spatula and the samples centrifuged for 10 minutes to remove cellular debris. For fluorometric analysis (not HPLC), decantation can replace centrifuging.

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 one hour prior to use.

7.3 The fluorometer is zeroed with 90% acetone at the start and each time the door setting is changed.

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 90. 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:

$$Chla = \left(\frac{(A_{max} - A_{750nm})}{E \cdot l}\right)\left(\frac{1000mg}{1g}\right)$$

Where:

A_{max} = absorption maximum (664 nm)

$A_{750\,nm}$ = absorbance at 750 nm to correct for light scattering

E = extinction coefficient for chl *a* in 90% acetone at 664 nm (87.67 l g^{-1} 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 of 1.2 M HCl.

7.5.4 Linear calibration factor (K_x) is 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:

$$Chla = \left(\frac{T}{(T-1)}\right)(Rb - Ra)\ (Fd)\left(\frac{vol_{ex}}{vol_{filt}}\right)$$

$$Phaeo = \left(\frac{T}{(T-1)}\right)(T \cdot Ra - Rb)\ (Fd)\left(\frac{vol_{ex}}{vol_{filt}}\right)$$

Where:

T	=	acidification coefficient (Rb/Ra) for pure Chl *a* (usually 2.2).
Rb	=	reading before acidification
Ra	=	reading after acidification
Fd	=	door factor from calibration calculations
vol_{ex}	=	extraction volume (5.8 ml)
vol_{filt}	=	sample volume (4 l)

8.1 The units of μg kg^{-1} can be obtained by dividing the calculated chlorophyll and phaeopigment concentration by the density of the seawater.

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.

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