Standard Operating Procedure for In Vitro Determination of Chlorophyll *a* in Freshwater Phytoplankton by Fluorescence

LG405

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1.0 SCOPE AND APPLICATION

1.1 This method provides a procedure for the fluorometric determination of chlorophyll *a* in freshwater phytoplankton, employing narrow band optical filters and a high intensity blue-lamp as part of the non-acidification method for extractive chlorophyll.

1.2 This method is based on that originally published by Welschmeyer (1994), and is applicable to waters from the Great Lakes.

2.0 SUMMARY OF METHOD

2.1 Chlorophyll-containing phytoplankton in a measured volume of sample water are concentrated onto a glass fiber filter by low-vacuum filtration.

2.2 After sonication, pigments are extracted in 90% buffered acetone, for 16 to 24 hours at -20 ^{o}C. The extracted slurry is removed by filtration, and fluorescence of the extract read. The concentration in the natural water sample is reported in μg/L.

3.0 SAMPLE HANDLING AND PRESERVATION

3.1 Samples should be stored frozen (-20 ^{o}C) in the dark until analyzed. Filters can be stored frozen for as long as 3½ weeks without significant loss of chlorophyll *a* (Weber *et al.*, 1986). If samples are being returned from the field to the laboratory they should remain frozen and wrapped in aluminum foil during transport.

3.2 Analysis should be carried out under subdued (green) light to prevent photo-decomposition of chlorophyll *a*.

4.0 INTERFERENCES

4.1 Spectral interferences resulting from fluorescence of the accessory pigment chlorophyll *b*, and the chlorophyll *a* degradation product pheophytin *a*, can result in overestimation of chlorophyll *a* concentrations. However, the highly selective optical filters used in this method minimize these interferences.

4.2 Previous work with this method has shown maximum interferences from chlorophyll *b* and pheophytin *a* to be +6% and +10%, respectively (Welschmeyer, 1994; Arar, 1994).

5.0 EQUIPMENT REQUIRED

5.1 Turner Designs Digital Fluorometer equipped with:

10-113 Excitation Filter (436-nm), and 10-115 Emission Filter (680-nm), high intensity F4T.5 blue lamp
Plastic Filter Funnel, Gelman (300-mL with magnetic base)
Vacuum System (1-4 psi)
GF/F Filters, Whatman (47-mm)
16 x 100 mm Screw Cap Culture Tubes
13 x 100 mm Culture Tubes, Disposable
250-mL Filter Flask with Sidearm

Nalgene Tubing
1 200-mL Volumetric Flask
5 100-mL Volumetric Flasks
1 50-mL Volumetric Flask
Aluminum Foil
Parafilm
Spectrophotometer
Disposable Glass Pipettes
20.0 µg/L Chlorophyll *a* Calibration Standard (available from Turner Designs)
Solid secondary Chlorophyll *a* Standards (available from Turner Designs)
High Purity Grade Acetone (1 L)
Magnesium Carbonate
Filter Forceps
Vacuum Source

6.0 REAGENTS

6.1 **Saturated Magnesium Carbonate Solution:** Add 10 grams magnesium carbonate to 1000 mL of de-ionized water. The solution is allowed to settle for a minimum of 24 hours. **Only the clear "powder free" solution is used during subsequent steps.**

6.2 **90 % (v/v) Buffered Acetone:** Add 100 mL of the Magnesium Carbonate solution (6.1) to 900 mL of acetone in a 1-L volumetric flask.

7.0 PREPARATION OF CHLOROPHYLL *a* STANDARDS

7.1 Primary standards of chlorophyll *a* in 90% acetone will be ordered from Turner Designs (845 W. Maude Avenue Sunnyvale CA 94086, 408 749-0994). If stored at -20 ^{o}C in the dark, standards are good for one month from their receipt from Turner Designs.

7.1.1 Standards come in foil wrapped ampules, which are broken and poured directly into the cuvette and read on the 10-AU. Standards typically come as a set of 1 low and 1 high concentration. Approximate concentrations are 15 – 20 µg/L for the low standard and 140 – 160 µg/L for the high standard. Actual concentration varies by lot, and is listed on a certificate of analysis to three significant figures.

7.2 Solid secondary standards, also available from Turner Designs, are used for daily calibration check standards. According to the company this standard is stable indefinitely.

7.2.1 Secondary standards come sealed in a holder that fits directly into the 10-AU 13-mm test tube adaptor. This two-sided solid standard contains two levels of ready-to-use fluorometric standard concentrations: a high value of approximately 30.0 µg/L and a low value of approximately 4.0 µg/L.

8.0 CALIBRATION

8.1 The following instructions apply to the Turner Designs 10-AU digital fluorometer. Calibration with a certified primary standard should be performed once each survey prior to the analysis of any samples from that survey. The standard concentration range selected for calibration does impact the calibration slope, and for consistency among all historical data, **the low standard concentration (15 – 20 µg/L) should be used**. This calibrant is also the better match for most field sample readings. BEFORE the calibration procedure is initiated the instrument should be returned to its default settings (see Section 8.1.1) and readings of the solid standard recorded. These values for the low and high solid secondary standard under the default calibration settings are the best way to assess long-term instrument drift. In general, the 10-AU is known to have a <0.5% electronic drift per month and the calibration will be good for all analyses within a given survey. Have a blank of 90% buffered acetone and the calibration standard ready. These are to be maintained at room temperature (22 – 25 ^{o}C), a water bath is recommended.

8.1.1 Before initiating a new calibration, reset the instrument to default calibration settings

8.1.2 Press <ENT> to reach Main Menu screen.

8.1.3 Press <2> to reach the Calibration Menu screen.

8.1.4 Reset unit to default settings by pressing <6> on the Calibration Menu.

8.1.5 On the next screen press <9> five times to confirm reset to calibration defaults.

8.1.6 Once the calibration default is set, <Esc> to the home screen and get readings for the LOW and HIGH solid secondary standards, record the values on a calibration log sheet.

8.2 Start Calibration with Primary Standard

8.2.1 Press <ENT> to reach Main Menu screen, and then press <2> to reach the Calibration Menu screen.

8.2.2 Set the concentration range control to AUTO. From screen 2.0, press <4> to bring up screen 2.4, then <3> to bring up screen 2.43 (set conc. range control), and press <ENT> to toggle.

8.2.3 Press <Esc> to screen 2.0, press <2> to access screen 2.2 (standard solution concentration). Enter the **actual** concentration of the primary liquid calibration standard (e.g., 15.0 µg/L).

8.3 Run Blank

8.3.1 Press <Esc> to return to screen 2.0. Then press <1> to access screen 2.1 and confirm that option #2 on screen 2.1 reads YES. Press <Esc> to return to HOME screen.

8.3.2. While on the HOME screen, fill a clean 13-mm culture tube with the blank of 90% acetone. Put the blank in the sample chamber and replace the light cap.

(Because temperature affects fluorescence, do not allow the blank to remain in the instrument any longer than necessary for a stable reading.)

8.3.3 Then access screen 2.11 by pressing <ENT> , <2>, <1>, and <1>. After the Blank % reading is stable ("TC" on screen 2.11 cycles from 1 to 8 seconds) and assuming the Blank % is less than 200%, press <0>. When "FINISHED" appears, press <ESC> all the way to HOME.

8.3.4 Remove the blank. Set cuvette aside.

8.4 Run Calibration Standard Solution

8.4.1. While on the HOME screen, fill a clean 13-mm culture tube with the LOW primary chlorophyll *a* standard, place in the sample chamber and replace the light cap.

(Because temperature affects fluorescence, do not allow the blank to remain in the instrument any longer than necessary for a stable reading.)

8.4.2 Then access screen 2.3 by pressing <ENT>, <2>, and <3>.

8.4.3 FS is the maximum concentration or relative fluorescence you will be able to read for a particular range, and it is not necessary or likely that FS match the value of the calibration standard. Adjust the Span % using the UP and DOWN arrows until the FS readings for each range are satisfactory. (Typical span setting range from 45 – 60 and the typical FS table readings used are approximately 2.5, 25, and 250). Pressing the UP arrow increases Span % (sensitivity), but decreases FS. Pressing the DOWN arrow decreases Span %, but increases FS. Wait until readings are stable ("TC" on screen cycles from 1 to 8 sec.), then press <*>. When "FINISHED" appears, press <ESC> all the way to HOME screen.

8.4.4 Get a reading of the primary liquid standard on the home screen by pressing <*>, to confirm that the calibration was properly set and record the calibrant and reading on the calibration log sheet.

8.5 CALIBRATION OF SECONDARY STANDARD FOR BATCH COMPARISONS

8.5.1 This procedure should be done for each solid standard following calibration with liquid standard. These standards are stable for at least two years, and can subsequently be used as daily check standards for multiple analytical batches during the survey once the 10-AU has been calibrated as above.

8.5.2 Place the solid secondary standard in the instrument, so that the LOW value (approximately 5.0 µg/L) is to be read. Replace the light cap.

8.5.3 Wait 15 seconds for the reading to stabilize. Record the reading on the solid standard calibration record sheet.

8.5.4 Reorient the solid standard so that the HIGH value (approximately 30.0 µg/L) is to be read. Replace the light cap.

8.5.5 Wait 15 seconds for the reading to stabilize and record the reading of the solid standard on the calibration record sheet.

9.0 SAMPLE ANALYSIS PROCEDURE

9.1 Sample Preparation

9.1.1 Add 10 mL of 90% buffered acetone (6.2) to the tube containing the filter. Recap tube tightly and invert tube 3 times, making sure that the filter is totally submerged in buffered acetone solution.

9.1.2 Place each tube in an ultrasonic bath that had been previously filled with water and ice and maintaining near zero ^{o}C temperatures, and sonicate for 20 minutes. Water depth in the bath should cover level of liquid in the tubes. Keep samples in the dark during this procedure.

9.1.3 After 20 minutes, cover sample tubes with foil and return to freezer (-20 ^{o}C) to steep for between 16 to 24 hours.

9.2 Instrument Preparation

9.2.1 Turn on the fluorometer and allow it to warm up for at least 60 minutes prior to analysis to reduce any variability in internal temperature.

9.2.2 Remove samples from freezer and allow them to come to room temperature (approximately 20-30 minutes) before being analyzed.

9.2.3 Samples should be kept covered and maintained at room temperature during analysis.

9.3 Daily Check Standard

9.3.1 Once the 10-AU is warmed up, place the solid secondary standard in the instrument, so that the LOW value is to be read. Replace the light cap.

9.3.2 Wait 15 seconds for the reading to stabilize and log the reading on the chlorophyll *a* data sheet.

9.3.3 Reorient the solid standard so that the HIGH value is to be read. Replace the light cap.

9.3.4 Wait 15 seconds for the reading to stabilize and log the reading on the chlorophyll *a* data sheet.

9.3.5 Daily check of the secondary solid standard values should be < 10% of their previously determined post calibration values. If they are not, the instrument may have to be recalibrated. Speak to the Biology Team Leader before proceeding with sample analysis.

9.4 Sample Analysis

9.4.1 Invert the sample test tube completely 6 times to thoroughly mix the extracted sample.

9.4.2 Using a side-arm filter flask or bell jar unit attached to a vacuum pump, filter the entire contents of the sample through a GF/F (47-mm) filter, **directly into the 13 x 100 mm disposable culture tube** used for analysis. Rinse filter holder and funnel with acetone prior to filter of the next sample to prevent any carryover between samples. This step is critical and should be practiced to ensure proper rinsing and drying in between subsequent samples.

NOTE: *Do not let the vacuum pressure exceed 1-2 psi or the sample volume will be affected.*

9.4.3 Wipe the outside of the cuvette dry with a lab wipe, and place in the instrument. Replace the light cap.

9.4.4 Wait about 15 seconds for the reading to stabilize, and log the reading.

Remember, because of temperature effects, for greatest accuracy, read all samples after they have been in the fluorometer for approximately the same length of time.

9.4.5 If the display reads OVER, it means the sample is too concentrated to be read at the current calibration, and is probably over the linear range of the instrument. In this case, dilute the sample by 75% (1 part sample to 3 parts buffered acetone), and reread it.

9.4.6 Record the fluorescence measurement, and the dilution factor, if any, on the Analytical Chlorophyll *a* Calculation sheet. Also record the date and time that the analysis is completed for that batch of samples.

NOTE: *It is helpful to electronically copy the sample IDs from the sample lists provided by GLNPO prior to the cruise onto the calculation data sheets to prevent extra writing and possible transcription errors.*

9.4.7 Readings obtained are the actual concentration of extracted chlorophyll *a* in the cuvette. To arrive at the environmental chlorophyll *a* for each sample, corrections for the amount of water filtered and extraction volume of acetone used must be applied, as described below.

10.0 CALCULATIONS

10.1 The concentration of chlorophyll *a* in the lake water sample is calculated by multiplying the results obtained above by 10 mL (the extraction volume) and dividing this by the volume (in mL) of the lake water sample (typically 250 ml for lakes Michigan, Huron, Ontario and Superior and 150 ml for lake Erie) that was filtered on the boat. If the sample was diluted, multiply the reading by the necessary dilution factor.

e.g. CHLa (ug/L) = [Chlor Analysis Reading x (Extract Volume / Volume Lakewater filtered)] x Dilution Factor

10.2 All data, including readings of the secondary calibration standards, should be entered in the electronic (Excel 5.0) version of the chlorophyll *a* data sheet. This spreadsheet will automatically perform all calculations. A printout of this spreadsheet will then serve as the permanent data record.

10.3.1 The relative percent difference (RPD) for laboratory and field duplicates is calculated according to the equation below:

$$RPD = \frac{\left| high\ value - low\ value \right|}{average\ value} \times 100$$

11.0 QUALITY CONTROL

11.1 The following audits are to be performed:

QC Type	Minimum Frequency	Acceptance Criteria
Calculation Check	Daily	± 10%
Laboratory Duplicate	Once per basin	Relative Percent Difference (RPD) 25%*
Field Duplicate	Once per basin	Relative Percent Difference (RPD) 25%*
Field Blank	Once per basin	0.00 µg/L ± 0.11 µg/L

* These limits are interim limits that will be used until there is enough data to calculate performance limits for this procedure.

12.0 WASTE DISPOSAL

12.1 Follow all laboratory waste disposal guidelines regarding the disposal of acetone solutions.

13.0 REFERENCES

13.1 Arar, Elizabeth J. and G.B. Collins. 1992. In Vitro Determination of Chlorophyll *a* and Pheophytin *a* in Marine and Freshwater Phytoplankton by Fluorescence In: Methods for the Determination of Chemical Substances in Marine and Estuarine Environmental Samples. Environmental Monitoring and Support Laboratory, Office of Research and Development, U.S. EPA Cincinnati, OH EPA/600/R-92/121.

13.2 Arar, E.J. 1994. Evaluation Of A New Fluorometric Technique That Uses Highly Selective Interference Filters For Measuring Chlorophyll *a* In The Presence Of Chlorophyll *b* And Pheopigments. Environmental Monitoring and Support Laboratory, Office of Research and Development, U.S. EPA Cincinnati, OH.

13.3 Turner Designs Model 10-AU-005 Field Fluorometer User's Manual/November 1992 (P/N 10-AU-075).

13.4 Weber, C.I., L.A. Fay, G.B. Collins, D.E. Rathke and J. Tobin. 1986. A Review of Methods for the Analysis of Chlorophyll in Periphyton and Plankton of Marine and Freshwater Systems, Ohio Sea Grant Program, Ohio State University Grant; No. NA84AA-D-00079, 54 pp.

13.5 Welschmeyer, N. 1994. Fluorometric analysis of Chlorophyll *a* in the presence of Chlorophyll *b* and pheopigments. Limnol. Oceanogr. 39:1985-1992.

ANALYTICAL CHLOROPHYLL *a* CALCULATION SHEET

Analytical Chlorophyll *a* Calculation Sheet

Survey:		*Batch Number:*	
Lake:		*Date/Time Analyzed:*	
		Analyst:	

Solid Secondary (low):	
Solid Secondary (high):	

Sample ID	*Dilution Factor (1,2,3,4...)*	*Volume Filtered (mL)*	*Extract Volume (mL)*	*Chlorophyll a* Analysis Reading	Chlorophyll *a* (µg/L) Calculated Result

10-AU FLUOROMETER SOLID STANDARD CALIBRATION RECORD

10-AU Fluorometer Solid Standard Calibration Record

Instrument Serial Number: _____

Date Standard Received: _____

Date	Analyst	"L" reading	"H" Reading