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

LG405

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TABLE OF CONTENTS

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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 as part of the non-acidification method for extracted 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 °C. The extracted slurry is removed by filtration, and fluorescence of the extract is read. The concentration in the natural water sample is reported in µg/L.

3.0 SAMPLE HANDLING AND PRESERVATION

3.1 After water samples are filtered, samples should be stored frozen (-20 °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 in their capped tubes 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* concentration. 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 Trilogy Fluorometer equipped with CHL-A NA module

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

12 x 75 mm Culture Tubes, Disposable

250-mL Filter Flask with Sidearm

Nalgene Tubing

Aluminum Foil

Parafilm

Disposable Glass Pipettes

Chlorophyll a Calibration Liquid Standard Sets

Solid secondary Standard

High Purity Grade Acetone

Magnesium Carbonate

Filter Forceps (not pointed, blunt edge)

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 decanted from the original container 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 amber pump bottle.

7.0 STANDARDS

7.0.1 Primary standards of chlorophyll *a* in 90% acetone can be ordered from Turner Designs (**Toll Free:** (877) 316.8049 or sales@turnerdesigns.com. They are shipped overnight on dry ice). If stored at -20 °C in the dark, standards are good for one month from their receipt from Turner Designs.

7.0.2 Standards come as two foil wrapped ampules, which are brought to room temperature, and then broken and poured directly into the cuvette and read on the Trilogy. Standards typically come as a set of one low and one 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 a lot and is listed on a certificate of analysis to three significant figures.

7.1 A solid secondary standard for the Trilogy, also available from Turner Designs (Part 8000-952 STD-SOLID ADJ, RED), is used for daily calibration checks. According to the company this standard is stable indefinitely.

8.0 CONVERSION OF RAW SIGNAL TO CHLOROPHYLL *a*

8.1 The Turner Designs Trilogy digital fluorometer output is in raw fluorescence units (RFU). This output is converted to chlorophyll concentration by applying a linear calibration function determined by measuring the raw fluorescence signal of an acetone blank and of a certified standard. The instrument is calibrated once per survey, prior to analyzing the first sample. The stability of the calibration is checked daily during the survey by measuring the fluorescence of a stable solid standard. The nominal

value of the solid standard is determined by measuring it immediately after the initial calibration of the Trilogy. 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.

9.0 SAMPLE ANALYSIS PROCEDURE

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. Set up these spreadsheets prior to adding acetone to vials so that the lists best reflect the order of the samples in the racks*.

9.1 Sample Preparation

9.1.1 Add 10 mL of 90% buffered acetone (6.2) to the tube containing the filter using a 10 ml aliquot repipetter pump. Take care to pump out air bubbles prior to adding to samples. Also be sure to slowly raise and lower the pump to ensure a full aliquot. 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 a culture tube rack, ordered by station and depth code. A typical sample batch includes forty to fifty samples. Place tube rack in an ultrasonic bath that has been previously filled with water and ice to maintain a temperature near zero °C, and sonicate for 20 minutes. Water depth in the bath should cover the level of liquid in the tubes but be below the level of the caps. Keep samples in the dark during this procedure.

9.1.3 After 20 minutes, cover sample tubes with foil and return to freezer (-20 °C) to extract for between 16 to 24 hours. Label the rack with the run number and the date and time samples will be ready for analysis.

9.2 Instrument Preparation

9.2.1 Remove samples from freezer and allow them to come to room temperature (approximately 50-60 minutes) before being analyzed. Loosen foil cover but keep in darkness in drawer.

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

9.2.3 The Trilogy does not require warming up. First lift the lid and check to see that the CHL-A NA module is inserted completely. Close the lid and turn on the Trilogy with the rocker switch on the back of the unit. On the touch screen select CHL-NA. Lift the lid and remove the cuvette holder and place the solid secondary standard in the instrument. Close the lid. Press MEASURE FLUORESCENCE RAW to get a digital reading. Reading should be around 340 RFU. Note values of the solid standard in other modules would be very different.

9.2.4 Log the solid standard reading on the chlorophyll *a* data sheet in the margin where the run begins with the date and time. At the end of the run measure the solid standard again and record on the sheet with the date and time.

9.2.5 Daily check of the secondary solid standard value should be < 10% of their previously determined post calibration values. If it is not, corrective actions such as assurance that the selected module is correct and that the standard cuvette is in the correct orientation (tab in

back) should be made. Speak to the Biology Team Leader before proceeding with further sample analysis.

9.3 Sample Analysis

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

9.3.2 Using a side-arm filter flask or bell jar unit attached to the biolab hood vacuum system or a portable vacuum pump, filter a portion (5 ml) of the sample through a GF/F (47-mm) filter, **directly into the 12 x 75 mm disposable culture tube** used for analysis. Note that this tube cannot hold the complete 10 ml volume. Rinse filter holder and funnel with acetone prior to filter of the next sample to prevent any carryover between samples.

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

9.3.3 Ensure that the black plastic cuvette holder is in the module. Wipe the outside of the cuvette dry with a lab wipe, and place in the instrument. Close the lid.

9.3.4 Press MEASURE FLUORESCENCE RAW, and log the reading including all decimal places that are displayed.

9.3.5 Readings obtained are raw fluorescence units (RFU) in the cuvette, which are then converted to chlorophyll units (µg/L) for the concentrated extract using the equations below. To arrive at the environmental chlorophyll *a* for each sample, conversions for the amount of water filtered and extraction volume of acetone are applied, also described below.

10.0 CALCULATIONS

10.1 The slope of the calibration is determined by the equation below

Slope of Calibration = (Standard Reading (RFU) - Blank Reading (RFU)) / (Value of Standard (µg/L))

10.2 The raw fluorescence value (RFU) of the extract is converted to chlorophyll *a* according to the equation below

Extract Value (µg/L) = ((Extract Reading (RFU) - Blank Reading (RFU)) / (Slope of Calibration)

10.3 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 (µg/L) = (Extract Value) x (Extract Volume / Lake Water Volume)

10.4 All data, including readings of the initial certified standard and the daily secondary solid calibration standard, should be entered in the electronic (Excel) spreadsheet. This spreadsheet will automatically perform all calculations described above.

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

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

11.0 QUALITY CONTROL

11.1 The following audits are to be performed:

QC Type	Minimum Frequency	Acceptance Criteria
Calibration 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 are enough data to calculate performance limits for this procedure.

The samples for these QC samples are preselected to align with QC samples for other water quality parameters. Preprinted labels are provided at the beginning of the surveys.

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 Trilogy Fluorometer User Manual.

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 Initials:	

Solid Secondary Standard (RFU):

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

TRILOGY FLUOROMETER SOLID STANDARD CALIBRATION RECORD

Trilogy Fluorometer Solid Standard Calibration Record

Instrument Serial Number: _____

Date Standard Received: _____

Date	Analyst	Reading (RFU)