

Standard Operating Procedure for:  
Total Chlorophyll, Chlorophyll-a, and Pheophytin

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## 1 Identification of the method

- 1.1 Measurement of Total Chlorophyll, Chlorophyll-a, and Pheophytin via Fluorometry (EPA Method 445.0).

## 2 Applicable matrix or matrices

- 2.1 This method is suitable for the analysis of [environmental samples](#).

## 3 Detection limit

- 3.1 Total Chlorophyll [Method Detection Limit](#): 0.3 µg/L  
3.2 Chlorophyll-a [Method Detection Limit](#): 0.7 µg/L  
3.3 Pheophytin [Method Detection Limit](#): 0.8 µg/L  
3.4 The above [Method Detection Limits](#) were calculated by analyzing 7 replicates of a low concentration environmental sample as described in Section 15. The [Method Detection Limit](#) was calculated as 3\*Standard Deviation of the determined concentrations of the 7 replicates.

## 4 Scope of the method

- 4.1 This standard operating procedure is intended to provide MU Limnology [operators](#), [technicians](#), and [analysts](#) with guidance on the analysis of total chlorophyll, chlorophyll-a, and pheophytin with the Cary Eclipse Fluorometer. This document is not intended to replace individual training in this method by an experienced MU Limnology [technician](#).

## 5 Summary of the method

- 5.1 A known volume of a collected water sample is filtered in order to concentrate sample particulates. Filters are kept frozen with silica desiccant until extraction. Chlorophyll from filters is extracted into known quantities of 95% ethanol using a heated water bath. Samples are analyzed using fluorometry once unacidified and once acidified. Using these readings in conjunction with the standard curve and the acid shift, concentrations of total chlorophyll, chlorophyll-a and pheophytin are determined for each sample.

5.2

- Operating Range: 0.3 µg/L–50 µg/L
- Sample Volume: 8 ml
- Run Time: 1.5–3 hours
- Samples per run: 40–80

## 6 Interferences

- 6.1 Imprecise filtration of samples will lead to poor replication between duplicates.

- 6.2 Exposure of samples to excess light after the addition of ethanol will cause degradation of sample and lead to an underestimation of total chlorophyll and chlorophyll-a and an overestimation of pheophytin.
- 6.3 Overheating of the extraction water bath may cause the ethanol to boil and cause loss of sample integrity.

## 7 Health and Safety

- 7.1 These analyses involve handling freshwater samples that may contain live microorganisms and therefore pose some threat of infection. Laboratory personnel who are routinely exposed to such water samples are encouraged to protect themselves from water borne illnesses by wearing clean disposable gloves and washing hands frequently.
- 7.2 Wear protective gloves, lab coats, and other appropriate [PPE](#) when handling all chemical substances used in this method. All [operators](#) and [technicians](#) performing this method should review the [MSDS](#) for additional information and safety concerns regarding the chemical substances used throughout these procedures.
- 7.3 The following chemicals used in this method are considered especially hazardous and should be handled with extra care:
- 40% Hydrochloric Acid

## 8 Personnel qualifications

- 8.1 Samples will be analyzed by MU Limnology staff who have been trained to the [operators](#) or [technician](#) level in this method and who are familiar with all of the MU Limnology sampling handling and labeling procedures and appropriate [SOPs](#).

## 9 Equipment and supplies

- 9.1 Ethanol, 190 Proof (95%), Decon Labs #2801
- 9.2 Sigma Aldrich Fine Chemicals Biosciences Chlorophyll A, 501786411
- 9.3 Hydrochloric Acid, 35–38%, Fisher Chemical, A144S-500
- 9.4 Silica Gel Desiccant, Fisher Chemical, S162-212
- 9.5 Stock Solution of Pure Chl-a
- 9.6 Precision Scientific Co. Water Bath, Cat. No. 66648
- 9.7 Cary Eclipse Fluorescence Spectrophotometer, Agilent Technologies, Product No. G9900A
- 9.8 Cary 60 UV Vis Spectrophotometer
- 9.9 10 mm Rectangular Quartz Fluorometer Cell, Starna Cells. Inc. Catalog No: 3-Q-10
- 9.10 Strathmore Pure Cotton Wove Paper
- 9.11 Kimax 8 ml Borosilicate Glass Test Tubes
- 9.12 Whatman GF/F Glass Microfiber Filters, 0.7  $\mu\text{m}$  pore size, 47mm, CAT No. 1825-047

- 9.13 47 mm Magnetic Filter Flask, PALL
- 9.14 Internal Vacuum System or Portable Vacuum Pump
- 9.15 250 ml Graduated Cylinder
- 9.16 1 ml pipette

## 10 Reagents and standards

- 10.1 Chlorophyll-a Stock
  - Prepare a stock solution by dissolving 1 mg of Sigma Aldrich Fine Chemicals Biosciences Chlorophyll A in approximately 250 ml of 95% ethanol. This solution should be stored below -18 °C and can be kept for 3 months before being reprepared.
- 10.2 [Calibration Standards](#)
  - Prepare standards as shown below in Table 2 directly into their light proof storage containers. These containers should be made of glass with a tight fitting lid, and should be covered in an opaque material (e.g. duct tape). Concentration of the Chlorophyll-a stock may vary and the below volumes may be adjusted by [operator](#) to achieve the desired concentrations.

Table 2: Nominal Concentration and Preparation of [Calibration Standards](#)

Standard	A	B	C	D	E	F	G	H	Acid Shift
ml 95% Ethanol	50	50	50	50	50	50	50	50	200
ml Chlorophyll-a Stock	0.2	0.5	0.8	1.1	1.4	1.5	1.8	2.1	0.5

- Once standards have been prepared, determine actual concentrations of standards by spectrophotometry. Turn on the Cary 60 spectrophotometer at least 30 minutes before beginning. Thoroughly rinse 1 cm quartz cuvette with 95% ethanol and wipe the sides carefully with a Kimwipe. Set the GENESYS 2 to read at 665 nm. Take readings of each standard, rinsing the cuvette with 95 % ethanol in between.

- Standard concentrations can be calculated as follows:

$$\text{Chlorophyll-a (ug/L)} = 380.8 * \text{ABS665}$$

- Record absorbance and calculated concentrations in the chlorophyll log sheet located on the Desktop of the Cary Eclipse's computer. Label standard containers with concentrations and date of preparation and store standards and acid shift in the freezer. Standards should be remade every two months or when they are depleted, whichever comes first.

#### 10.3 Environmental [Reference](#)

- Filter environmental [reference](#) water through .7 µm GF/F filters. Approximately 4 to 8 filters should be processed. Filters should be extracted per procedure described in Section 13.1. Supernatant from extracted filters should be poured into a light proof container and kept in the freezer with the standards for up to 3 months.

#### 10.4 "40%" Hydrochloric Acid

- Hydrochloric acid (35–38%) can be used as is from purchase. Acid should be added to a [repipette](#) and stored in an acid cabinet.

### 11 Determination of Acid Shift

- 11.1 Prior to each field season the acid shift should be calculated. Remove the acid shift prepared in section 10.2 from the freezer 30 minutes prior to analysis. Analyze a full standard curve as described in Section 13,3 prior to analyzing the acid shift and then analyze the acid shift as if it were a sample as described in Section 13.4.

- 11.2 Calculate the acid shift as

$$AS = \frac{(a * UF^2) + b(UF)}{((a * AF^2) + b(AF))}$$

Where:

a= exponential coefficient of the calibration equation

b= linear coefficient of the calibration equation

UF= Unacidified fluorescence of the acid shift

AF= Acidified fluorescence of the acid shift

- 11.3 The calculated acid shift should be between 1.6 and 1.9. If it is not, it should be recalculated. This acid shift will be used for all runs until it is recalculated.

## 12 Quality Control

### 12.1 Environmental [Reference](#)

The fluorescence of the [reference](#) sample will be measured with every run. [Operators](#) should note when a new environmental [reference](#) has been prepared in the chlorophyll log sheet located on the desktop of the Cary Eclipse computer.

### 12.2 Pre-Disposal Run Check

Since re-analysis of samples is not possible, it is very important that [operators](#) check their measured values at the end of each run before disposing of samples. [Operators](#) will check to make sure that sample duplicates or triplicate readings are within 10% of each other and that the acidified samples produce notably lower measurements than their unacidified readings. If either of these is not the case, the [operator](#) will rerun these samples immediately.

## 13 Analysis

### 13.1 Sample Extraction

- Working in the dark, carefully remove filters from their filter houses and place in a glass test tube. Fill the test tube with 8 ml of 95 % ethanol using a [repipette](#). Once this has been done for all tubes, place them in a water bath at 72 °C for 20 minutes. Samples should sit in the dark after extraction for at least 24 hours as extraction continues while the samples cool down, and not more than 48 hours to avoid the evaporation of ethanol from the tubes.

### 13.2 Set Up

- 30 minutes prior to starting, turn on the Cary Fluorometer and remove the standards from the freezer. Place standards and samples in a dark drawer below the Cary for the duration of the run.
- On the desktop, open the folder labeled “Chlorophyll” and open the file labeled “Chlorophyll Template.”
- Open the tab labeled “Sample List” and enter your standards in column B. Enter samples in order from lowest to highest and type only the calculated concentration as determined in Section 6.
- Add sample information in columns D-H.
- Open the tab labeled “IMPORT” and delete all rows which don’t contain sample names.
- Save the file as an Excel workbook according to the [file name convention](#).
- Save the file as “Text (MS-DOS) file” with the same name and close it.

- On the desktop, open the icon labeled “Chlorophyll Method.”
- Click “Setup” and open the tab labeled “Samples.”
- Click “Import Samples” and select the Text (MS-DOS) file that you just created.
- Parameters for this method are show below in Table 3.

Table 3: Parameters and Settings Included in the Cary Chlorophyll Method

Parameter	Excitation Wavelength (nm)	Emission Wavelength (nm)	Excitation Slit (nm)	Emission Slit (nm)	Read Time (s)	PMT Detector Voltage
Setting	430	663	5	2.5	1	725

### 13.3 Calibration and Standardization

- Standards must be room temperature prior to analysis.
- Cary must be on 30 minutes prior to analysis.
- Handling it only by the top edges, rinse three cuvette times with 95 % ethanol and carefully wipe clean with a Kimwipe.
- Rinse the cuvette with a small portion of the lowest standard.
- Using a transfer pipette, fill the cuvette  $\frac{3}{4}$  full with the lowest standard.
- Place the cuvette in the Cary and select "OK" in the run window.
- Rinse cuvette three times with 95 % ethanol and carefully wipe clean with a Kimwipe.
- Repeat for all standards and the [reference](#) sample working from lowest to highest.

### 13.4 Sample analysis

- During sample analysis, tubes should remain in the lightproof container they are stored in to prevent exposure.
- Remove the first tube and invert three times.
- Rinse the cuvette with a small amount of sample.
- Fill the cuvette  $\frac{3}{4}$  full with the sample.
- Place the cuvette in the Cary and select "OK" in the run window.
- If fluorescence exceeds 600, pour the sample back into the tube and return it to the box. Refer to step 13.6.
- If fluorescence does not exceed 600, use the [repipette](#) to add 0.1 ml of HCl to the sample tube, invert several times, and return to the box.
- Rinse cuvette three times with 95 % ethanol and carefully wipe clean with a Kimwipe.
- Repeat for all samples.

13.5 Once unacidified readings of all samples have been taken, return to the first sample and reanalyze the now acidified samples.

13.6 Once all regular samples have been measured, select "reread" on the run window and select the samples which exceeded a fluorescence of 600 on their initial readings.

- Dilute samples into clean tubes using 95 % ethanol and a calibrated pipette.
- A 2x dilution (3 ml of sample and 3 ml of 95 % ethanol) should be sufficient for most samples.
- Repeat analysis for the diluted samples.
- If further dilution is required, repeat dilution and analysis.

## 14 Data acquisition, calculations, and reporting

14.1 Upon completion of the run, results should be saved as a "Rich Text Format" or "RTF" file.

- 14.2 Reopen your Excel workbook and open to the tab labeled “export.”
- 14.3 From Excel, open the “RTF” file and consult the “Text Import Wizard.”
- 14.4 Select “Fixed Widths” is selected and hit “Next.”
- 14.5 Drag the black bars in the window provided so that one column fully contains your sample names and the other contains your fluorescence readings.
- 14.6 Copy and paste your dilutions from below the run over the initial readings for those samples.
- 14.7 Copy and paste, the sample names and fluorescence readings from the RTF into column B and column H of the “export” tab so that they match the sample names shown in column A.
- 14.8 Enter the calibration information from the graph in the box provided to the left.
- 14.9 Open the “Calculations Tab.”
- 14.10 A [calibration curve](#) should be automatically generated by the Excel file. The curve should have an  $r^2 > 0.99$ . If it does not, contact a supervisor. Enter the calibration information in the box provided to the left.
- 14.11 Enter any dilutions that you performed in Column K.
- 14.12 As noted above, the Excel sheet will automatically calculate the [calibration curve](#) and acid shift. Once the [operator](#) has entered all information, it will also automatically calculate the total chlorophyll, chlorophyll-a, and pheophytin values. The details of these calculations are provided here.
- 14.13 Defined Terms
  - a= exponential coefficient of the calibration equation
  - b= linear coefficient of the calibration equation
  - UF= Unacidified fluorescence of sample
  - AF= Acidified fluorescence sample
  - AS= Acid Shift
  - TC= Total Chlorophyll ( $\mu\text{g/L}$ )
  - ATC= Acidified total chlorophyll
  - Chl-a= Chlorophyll-a ( $\mu\text{g/L}$ )
  - Pheo= Pheophytin ( $\mu\text{g/L}$ )
  - F= Filter Volume
  - D= Dilution Factor

Calibration Curve: A regression of the form  $y = a * x^2 + b * x$  is calculated from the readings of the calibration standards where y is the known concentration of chlorophyll ( $\mu\text{g/L}$ ) and x is the measured fluorescence value. This curve is forced through zero.

$$TC = (a * UF^2) + (b * UF)$$

$$ATC = (a * AF^2) + (b * AF)$$

$$Chl - a = (TC - ATC) * \left(\frac{AS}{(AS-1)}\right) * \left(\frac{250}{F}\right) * D$$

$$Pheo = \left((ATC * AS) * \left(\frac{250}{F}\right) * D\right) - Chla$$

## 15 Computer hardware and software

- 15.1 Windows 7 Enterprise
- 15.2 Cary Eclipse Advance Reads Application, Software Version 1.2 (146)

## 16 Method performance

- 16.1 Total Chlorophyll Method Detection Limit: 0.3  $\mu\text{g/L}$
- 16.2 Chlorophyll-a Method Detection Limit: 0.7  $\mu\text{g/L}$
- 16.3 Pheophytin Method Detection Limit: 0.8  $\mu\text{g/L}$
- 16.4 Precision < 10 %
- 16.5 Calibration  $r^2 > 0.99$

## 17 Pollution prevention

- 17.1 All reagents and standards will be prepared in appropriate volumes so as to reduce waste.
- 17.2 All sample and reagents will be handled according to MU EHS policies in order to ensure proper disposal.

## 18 Data assessment and acceptable criteria for quality control measures and corrective actions for out-of-control or unacceptable data

- 18.1 Excel Cell References and Data Entry  
Poor cell references or improperly entered data will lead to erroneous results. If a problem is noticed with a run, the data entry and the cell references should first be checked by a supervisor and pointed out to an operator if found.
- 18.2  $r^2$  Values  
The mandatory  $r^2$  for valid results is a minimum of 0.99. If the  $r^2$  falls below 0.99, up to two standards with the highest deviation from the standard curve may be eliminated. If this issue continues, analysis by this method should be halted and the problem investigated.

18.3 Comparison of Chl-a and Total Chlorophyll Values

Samples should not have chl-a concentration in great excess of the total chlorophyll value or negative pheophytin values. If more than 10 % of the samples in a run have these issues in excess of 10 %, the acid shift should be remade or the [operator](#) retrained. Samples where this occurs to a small degree may be corrected so that chl-a= Total Chlorophyll and Pheo=0.

18.4 Environmental [Reference](#)

The fluorescence of the environmental [reference](#) should not vary by more than 10 % between runs. If this occurs, it should be re-prepared. If this issue persists, runs should be halted until the issue is fixed.

18.5 High Pheophytin Values

Be wary of samples with pheophytin concentrations in excess of chl-a concentrations. While this is not impossible, it is rare enough that a run with more than 15 % of samples where this occurs should be evaluated carefully. A supervisor should check with the [operator](#) and re-train them on the extraction process as excess light exposure at this stage can cause this issue. Samples where pheophytin calculations are calculated to be in excess of the total chlorophyll value should be considered invalid results. Common causes of this problem are:

1. Excessive light exposure prior to analysis.
2. Failure on the part of the [operator](#) to acidify the sample.

If an [operator](#) has this problem occur twice, they should be re-trained.

## 19 Waste management

19.1 All waste generated is considered hazardous.

19.2 All analyzed standards and reagents should be treated as waste upon completion of the run.

19.3 Waste should be kept in an approved container with proper labeling.

19.4 Waste will not be held for longer than 6 months and [MU Environmental Health and Safety \(EHS\)](#) will be notified an appropriate time before this point so that waste can be collected and disposed of.

## 20 References

20.1 Kentucky Division of Water (DOW). 2020. Standard Operating Procedures: Laboratory Procedures for Chlorophyll a and Phycocyanin Analysis in Water, Version 2.0. Kentucky Department for Environmental Protection, Division of Water, Frankfort, Kentucky.

- 20.2 US Environmental Protection Agency (EPA). 1997. Method 445.0 rev. 1.2: In vitro determination of chlorophyll a and pheophytin a in marine and freshwater phytoplankton by fluorescence. Cincinnati, OH: Environmental Monitoring Systems Laboratory, Office of Research and Development, US Environmental Protection Agency.
- 20.3 Sartory, D.P.; Grobbelaar, J.U. Extraction of Chlorophyll a from Freshwater Phytoplankton for Spectrophotometric Analysis. *Hydrobiologia*, 1984 114, 177-187.  
<http://dx.doi.org/10.1007/BF00031869>

## 21 Tables, diagrams, flow charts, and validation data

- 21.1 Chlorophyll "CHEAT SHEET". The following information is provided to [operators](#) next to the instrument as a quick reference guide:

### Chlorophyll Cheat Sheet

#### SETTING UP THE RUN

1. **30 MINUTES** before beginning, remove standards from the freezer and turn on the Cary Eclipse.
2. On the desktop, open the folder labeled "Chlorophyll" and open the file called "Chlorophyll Template".
3. For a more detailed guide, open the tab labeled "instructions".
4. Open the tab labeled "Sample List" and enter your standards in column B and sample information in columns D-H.
5. Open the tab labeled "IMPORT" and delete all rows which don't contain sample names.
6. Save the file as an Excel workbook with the name "CHL-INITIALS-DATE PROJECT".
7. Save the file as a Text (MS-DOS) file with the same name and close it.
8. On the desktop, click on the icon labeled "chlorophyll method".
9. Click "Setup" and open the tab labeled "Samples".
10. Click "Import Samples" and select the Text (MS-DOS) file that you just created.

#### ZEROING/ACID SHIFT/STANDARD CURVE

1. Rinse the cuvette 3X with 95 % ethanol and carefully clean the exterior with a Kimwipe.
2. Fill the cuvette  $\frac{3}{4}$  of the way with 95 % ethanol and place it in the cuvette holder with the "Q" facing towards you.
3. Hit "Zero" and then "OK" when prompted to. Empty the cuvette.
4. Hit "Start" and you will be prompted to take the unacidified reading of the acid shift.

5. Rinse the cuvette with a small amount of the acid shift and then fill it  $\frac{3}{4}$  of the way.
6. Place the place the cuvette into the cuvette holder with the "Q" facing towards you.
7. Hit "OK" and you will be prompted to take the acidified reading of the acid shift.
8. Remove the cuvette and use the [repipette](#) to add 0.1 ml of 40 % HCl to the cuvette.
9. Place the place the cuvette into the cuvette holder with the "Q" facing towards you and wait 30 seconds.
10. Hit "OK", remove the cuvette and rinse it 3x with 95 % ethanol.
11. Calculate the acid shift as:  $\text{Shift} = (\text{Unacidified Reading})/(\text{Acidified Reading})$
12. The acid shift should be between 1.6 and 2.0. If it is not within these limits, stop the run and restart at step 2.
13. You will now be prompted to run your first standard.
14. Between standards, rinse the cuvette 1X with 95 % ethanol, 1X with a small amount of the next standard, and carefully clean the exterior with a Kimwipe.

#### READING SAMPLES

1. Between samples, rinse the cuvette 1X with 95 % ethanol, 1X with a small amount of the next sample, and carefully clean the exterior with a Kimwipe.
2. Remove the sample from the box and invert it 3X.
3. Carefully pour the sample to fill the cuvette  $\frac{3}{4}$  of the way.
4. Place the cuvette into the cuvette holder with the Q facing towards you and hit "OK".
5. If the reading is above 600, pour the sample back into the tube.
6. If the reading is below 600, use the [repipette](#) to add 0.1 ml of 40 % HCl to the tube and invert 3x.
7. Once you have finished all unacidified readings, take your acidified readings in the same manner ignoring steps 5 and 6.

#### DILUTIONS

1. Once you have finished your run, dilute any samples with readings above 600 by pipetting 3 ml of the sample and 3 ml of 95 % ethanol into a clean tube and invert to mix.
2. Click the "reread" button and use the window to select the samples you need to re-read making sure to include both the unacidified and acidified readings.
3. Taking readings of your dilutions following the same steps 1–7 in the "reading samples" section above.

**DO NOT CLOSE THE PROGRAM WITHOUT SAVING YOUR FILE AS DESCRIBED BELOW!**

### FINISHING YOUR RUN

1. When all samples have been read, save the file as a “Rich Text Format” with the same name as your Excel workbook.
2. Re-open your Excel workbook and move to the tab labeled “export”.
3. Use Excel to open the “RTF” file that you just created and it will open a “Text Import Wizard”.
4. Make sure that “Fixed Widths” is selected and hit “Next”.
5. Drag the black bars in the window provided so that one column fully contains your sample names and the other contains your fluorescence readings.
6. Copy and paste your dilutions from below the run over the initial readings for those samples.
7. Copy and paste the sample names and fluorescence readings from the RTF into column B and column H of the “export” tab so that they match the sample names shown in column A.
8. (Note that you will need to scroll down to enter your acidified readings if you had less than 80 samples).
9. Open the “Calculations Tab”.
10. Enter the calibration information from the graph in the box provided to the left.
11. Enter any dilutions that you performed in Column K.
12. Save the file and use a flash drive to transfer it to the main lab computer.