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Standard Operating Procedures for Fluorometric Determination of Chlorophyll α in waters and sediments of Fresh/Estuarine/Coastal Areas.

(References: EPA 445.0, SM10200H.3)

Document #: NASLDoc-035

Revision 2023-1
Replaces Revision 2022-1
Effective May 1, 2023
I attest that I have reviewed this standard operating procedure and agree to comply with all procedures outlined within this document.

Employee (Print)	Employee (Signature)	Date
Employee (Print)	Employee (Signature)	Date
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Revised by: _____ Date: _____

Reviewed by: _____ Date: _____

Laboratory Supervisor: _____ Date: _____

Revisions affecting 2023-1

No changes were made.

1. SCOPE and APPLICATION

1.1 This is an acetone extraction method to determine chlorophyll α in water and sediments.
1.2 Method Detection Limits (MDL) of 0.68 µg/L total chlα, 0.46 µg/L phaeophytin, 0.69 µg/L active chlα, and 0.84 µg/L non-acid chlα, 0.13 mg/m^2 total sediment chlα, 1.04 mg/m^2 phaeophytin in sediment, 0.69 mg/m^2 active sediment chlα were determined using the Student's t value X the standard deviation of at least seven replicates.
1.3 The quantitation limit for chlα is dependent upon sample volume. The reporting limit is equal to the MDL.
1.4 This procedure should be used by analysts experienced in the theory and application of chlorophyll analysis. A three-month training period with an analyst experienced in the analysis using the fluorometer is required.
1.5 This method can be used for all programs that require fluorometric analysis of chlorophyll α.
1.6 This procedure references EPA Method 445.0 and SM10200H.3, 22nd edition.

2. SUMMARY

2.1 Chlorophyll α is extracted from phytoplankton cells using a 90% solution of acetone. The cells are physically disrupted by mechanical grinding or sonication. The samples are refrigerated in the dark from 2 to 24 hours (overnight is preferable). After the appropriate time, the samples are then centrifuged to separate the sample material from the extract. The extract must be at room temperature before analysis. The extract is analyzed on a fluorometer. To determine phaeophytin and active chlα, the extract is then acidified using 5% HCl, and reread. The concentrations are then calculated.

3. DEFINITIONS

3.1 Acceptance Criteria – Specified limits placed on characteristics of an item, process, or service defined in a requirement document. (ASQC)
3.2 Accuracy – The degree of agreement between an observed value and an accepted reference value. Accuracy includes a combination of random error (precision) and systematic error (bias) components which are due to sampling and analytical operations; a data quality indicator. (QAMS)
3.3 Aliquot – A discrete, measured, representative portion of a sample taken for analysis. (EPA QAD Glossary)
3.4 Analytical Range – The analytical range is dependent on the volume of water filtered and the volume of acetone used in the extraction.
3.5 Batch – Environmental samples, which are prepared and /or analyzed together with the same process and personnel, using the same lot(s) of reagents. An **analytical batch** is composed of prepared environmental

samples (extracts, digestates, or concentrates) and/or those samples not requiring preparation, which are analyzed together as a group using the same calibration curve or factor. An analytical batch can include samples originating from various environmental matrices and can exceed 20 samples. (NELAC/EPA)

3.6 Blank- A sample that has not been exposed to the analyzed sample stream in order to monitor contamination during sampling, transport, storage, or analysis. The blank is subjected to the usual analytical and measurement process to establish a zero baseline or background value and is sometimes used to adjust or correct routine analytical results. (ASQC)

3.7 Calibrate- To determine, by measurement or comparison with a standard, the correct value of each scale reading on a meter or other device, or the correct value for each setting of a control knob. The levels of the applied calibration standard should bracket the range of planned or expected sample measurements. (NELAC)

3.8 Calibration – The set of operations that establish, under specified conditions, the relationship between values indicated by a measuring device. The levels of the applied calibration standard should bracket the range of planned or expected sample measurements. (NELAC)

3.9 Calibration Curve – The graphical relationship between known values, such as concentrations, or a series of calibration standards and their analytical response. (NELAC)

3.10 Calibration Method – A defined technical procedure for performing a calibration. (NELAC)

3.11 Calibration Standard – A substance or reference material used to calibrate an instrument. (QAMS)

- 3.11.1 Initial Calibration Standard (STD) – A series of standard solutions used to initially establish instrument calibration responses and develop calibration curves for individual target analytes.
- 3.11.2 Initial Calibration Verification (ICV) – An individual standard, analyzed initially, prior to any sample analysis, which verifies the acceptability of the calibration curve or previously established calibration curve.
- 3.11.3 Continuing Calibration Verification (CCV) – An individual standard that is analyzed after every 10-15 field sample analysis.

3.12 Certified Reference Material (CRM) – A reference material one or more of whose property values are certified by a technically valid procedure, accompanied by or traceable to a certificate or other documentation which is issued by a certifying body. (ISO 17025)

3.13 Corrective Action – Action is taken to eliminate the causes of an existing nonconformity, defect, or other undesirable situation in order to prevent a recurrence. (ISO 8402)

3.14 Deficiency – An unauthorized deviation from acceptable procedures or practices. (ASQC)

3.15 Demonstration of Capability – A procedure to establish the ability of the analyst to generate acceptable accuracy. (NELAC)

3.16 Detection Limit – The lowest concentration or amount of the target analyte that can be determined to be different from zero by a single measurement at a stated degree of confidence.

3.17 Duplicate Analysis – The analyses of measurements of the variable of interest were performed identically on two subsamples (aliquots) of the same sample. The results from duplicate analyses are used to evaluate analytical or measurement precision but not the precision of sampling, preservation, or storage internal to the laboratory. (EPA-QAD)

3.18 Epiphytic growth – Fouling organisms that adhere to solid surfaces in natural waters.

3.19 External Standard (ES) – A pure analyte (Anacystis nidulans algae, or equivalent) that is measured in an experiment separate from the experiment used to measure the analyte(s) in the sample. The signal observed for a known quantity of the pure external standard is used to calibrate the instrument response for the corresponding analyte(s). The instrument response is used to calculate the concentrations of the analyte(s) in the unknown sample.

3.20 Field Duplicates (FD1 and FD2) – Two separate samples collected at the same time and place under identical circumstances and treated exactly the same throughout field and laboratory procedures. Analyses of FD1 and FD2 provide a measure of the precision associated with sample collection, preservation, and storage, as well as with laboratory procedures.

3.21 Field Reagent Blank (FRB) – An aliquot of reagent water or other blank matrix that is placed in a sample container in the laboratory and treated as a sample in all respects, including shipment to the sampling site, exposure to the sampling site conditions, storage, preservation, and all analytical procedures. The purpose of the FRB is to determine if method analytes or other interferences are present in the field environment.

3.22 Fluorescence – Fluorescence is a physical property of certain atoms and molecules. A fluorescent molecule has the ability to absorb light at one wavelength and almost instantly emit light at a new and usually longer wavelength. (Turner Designs TD700 manual)

3.23 Holding time – The maximum time that samples may be held prior to analysis and still be considered valid. (40 CFR Part 136) The time elapsed from the time of sampling to the time of extraction or analysis, as appropriate.

3.24 Instrument Detection Limit (IDL) – The minimum quantity of analyte of the concentration equivalent which gives an analyte signal equal to three times the standard deviation of the background signal at the selected wavelength, mass, retention time absorbance line, etc.

3.25 Laboratory Duplicates (LD1 and LD2) – Two aliquots of the same sample taken in the laboratory and analyzed separately with identical procedures. Analyses of LD1 and LD2 indicate precision associated with laboratory procedures, but not with sample collection, preservation, or storage procedures.

3.26 Laboratory Reagent Blank (LRB) and Reagent Blank (RB) – The Reagent Blank is a matrix blank (i.e., 90% acetone) that is treated exactly like a sample including exposure to all glassware, equipment, solvents, and reagents that are used with other samples. The RB is used to determine if method analytes or other interferences are present in the laboratory environment, the reagents, or the instrument. The LRB consists of a blank filter pad extracted in 90% acetone at the end of the loading process. It is analyzed at the end of each run. LRB data are used to assess contamination from the laboratory environment.

3.27 Laboratory Control Sample (LCS) – A sample matrix, free from the analytes of interest, spiked with verified known amounts of analytes from a source independent of the calibration standard or a material containing known and verified amounts of analytes. The LCS is generally used to establish intra-laboratory or analyst-specific precision and bias or to assess the performance of all or a portion of the measurement system. (NELAC)

3.28 Limit of Detection (LOD) – The lowest concentration level that can be determined by a single analysis and with a defined level of confidence to be statistically different from a blank (ACS), also known as MDL.

3.29 Limit of Quantitation (LOQ) – The minimum levels, concentrations, or quantities of a target variable (target analyte) that can be reported with a specified degree of confidence. The LOQ is set at 3 to 10 times the LOD, depending on the degree of confidence desired. Also known as Quantitation Limit.

3.30 Linear Dynamic Range (LDR) – The absolute quantity over which the instrument response to an analyte is linear. This specification is also referred to as the Linear Calibration Range (LCR).

3.31 May – Denotes permitted action, but not required action. (NELAC)

3.32 Method Detection Limit (MDL) – The minimum concentration of an analyte that can be identified, measured, and reported with 98% confidence that the analyte concentration is greater than zero.

3.33 Must – Denotes a requirement that must be met. (Random House College Dictionary)

3.34 Precision – The degree to which a set of observations or measurements of the same property, obtained under similar conditions, conform to themselves; a data quality indicator. Precision is usually expressed as standard deviation, variance, or range, in either absolute or relative terms. (NELAC)

3.35 Preservation – Refrigeration, freezing, and/or reagents added at the time of sample collection (or later) to maintain the chemical and or biological integrity of the sample.

3.36 Quality Control Sample (QCS) – A sample of analytes of known and certified concentrations. The QCS is obtained from a source external to the laboratory and is different from the source of calibration standards. It is used to check laboratory performance with externally prepared test materials. Also known as CRM.

3.37 Quenching – A phenomenon that occurs when the fluorescence measurements decrease even though the analyte concentration is increasing. (Turner Designs TD700 manual)

3.38 Raw fluorescence – Refers to the "relative" fluorescence of a substance being read, rather than the actual concentration. (Turner Designs TD700 manual)

3.39 Run – One sample analysis from start to finish, including printout.

3.40 Run Cycle – Typically a day of operation – the entire analytical sequence of runs from the first run to the last run and including the transfer of run cycle data to the disc.

3.41 Safety Data Sheets (SDS) – Written information provided by vendors concerning a chemical's toxicity, health hazards, physical properties, fire, and reactivity data including storage, spill, and handling precautions.

3.42 Sample Volume – Volume of water filtered.

3.43 Sensitivity – The capability of a test method or instrument to discriminate between measurement responses representing different levels (concentrations) of a variable of interest.

3.44 Shall – Denotes a requirement that is mandatory whenever the criterion for conformance with the specification requires that there be no deviation. (ANSI)

3.45 Should – Denotes a guideline or recommendation whenever noncompliance with the specification is permissible. (ANSI)

3.46 Standard Reference Material (SRM) – Material that has been certified for specific analytes by a variety of analytical techniques and/or by numerous laboratories using similar analytical techniques. These may consist of pure chemicals, buffers, or compositional standards. The materials are used as an indication of the accuracy of a specific analytical technique. Also known as CRM.

4. INTERFERENCES

4.1 Light and heat cause the chlorophyll molecule to break down. Therefore, the samples should be kept cold in the dark and care should be taken during cell disruption so as not to overheat the sample. When ready to analyze, the extract must be at room temperature, and the analysis performed under reduced lighting. Chlorophyll *b* also fluoresces within an overlapping range of wavelengths, possibly leading to an overestimation of chlorophyllα.

5. SAFETY

5.1 Safety precautions must be taken when handling reagents, samples, and equipment in the laboratory. Protective clothing including lab coats, safety glasses, and enclosed shoes should be worn. In certain situations, it will be necessary to also use gloves and/or a face shield. If solutions come in contact with eyes, flush with water continuously for 15 minutes. If solutions come in contact with skin, wash thoroughly with soap and water. Contact Solomons Rescue Squad (911) if emergency treatment is needed and also inform the CBL Associate Director of Administration and Facilities of the incident. Contact the CBL Associate Director of Administration and Facilities if additional treatment is required.

5.2 The toxicity or carcinogenicity of each reagent used in this procedure may not have been fully established. Each chemical should be regarded as a potential health hazard and exposure should be as low as reasonably achievable. Cautions are included for known hazardous materials and procedures.

5.3 Do not wear jewelry when troubleshooting electrical components. Even low voltage points are dangerous and can injure if allowed to short circuit.

5.4 The following hazard classifications are listed for the chemicals used in this procedure. Detailed information is provided on Safety Data Sheets (SDS).

Table 1:

Chemical	Health Hazard	Fire Hazard	Instability Hazard	Specific Hazard	
Hydrochloric Acid	3	0	2	ACID, COR	
Acetone	1	3	0		

On a scale of 0 to 4 the substance is rated on four hazard categories: health, flammability, reactivity, and contact. (0 is non-hazardous and 4 is extremely hazardous)

HAZARD RATING
Health Hazard - Blue: 4 – deadly, 3 – extreme danger, 2 – hazardous, 1 – slightly hazardous, 0 – normal material

Fire Hazard - Red: Flash Points: 4 – below 73° F, 3 – below 100° F, 2 – below 200° F,
1 – above 200° F, 0 – will not burn

Instability Hazard - Yellow: 4 – may detonate, 3 – Shock and heat may detonate, 2 – violent chemical change, 1 – unstable is heated, 0 - stable

Specific Hazard - White: Acid = ACID, Alkali = ALK, Corrosive = COR, Oxidizer = OXY

6. EQUIPMENT AND SUPPLIES

6.1 A fluorometer equipped with the proper light source and filters for chlorophyll analysis. This laboratory uses a Turner Designs Trilogy fluorometer equipped with either the non-acid or the acid optical module.
6.2 Freezer, capable of maintaining -20° ± 5° C and refrigerator, capable of maintaining 4° ± 2° C.
6.3 A centrifuge.
6.4 Appropriate apparatus for grinding, such as a Teflon pestle, either by hand or power, and/or a sonicator.

7. REAGENTS AND STANDARDS

7.1 Purity of Water – Unless otherwise indicated, references to water shall be understood to mean reagent water conforming to ASTM Specification D 1193, Type I. Freshly prepared water should be used for making the standards intended for calibration. The detection limits of this method will be limited by the purity of the water and reagents used to make the standards.

7.2 Purity of Reagents – Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society, where such specifications are available. Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without compromising the accuracy of the determination.

7.3 Acetone ($H_2C=O=CH_2$), 90% v/v

Acetone, reagent grade	900 ml
Reagent water	100 ml

Using a graduated cylinder, add 100 ml reagent water to 900 ml acetone.

7.4 Hydrochloric Acid, 5 % (v/v) –

Hydrochloric acid (HCl), concentrated,	5.0 ml
Reagent water, q.s.	100 ml

In a 100 ml volumetric flask, add 5.0 ml of concentrated hydrochloric acid to ~60 ml of reagent water. Dilute to 100 ml with reagent water.

7.5 Blanks – A reagent blank of 90% acetone is used.

7.6 Standards – Standards used are one of the following:

7.6.1 Turner Designs Fluorometer standards, PN 10-850. These include a set of 2 ampoules, 1 high and 1 low concentration, accompanied by a certification from Turner Designs.

7.6.2 Chlorophyll α from Anacystis nidulans algae, PN C6144-1MG, ordered from Sigma-Aldrich. If chlorophyll α from algae is not available, chlorophyll α from spinach, PN C5753-1MG, may be substituted.

7.7 Quality Control Sample (QCS) – For this procedure, the QCS can be any certified sample that is obtained from an external source. If a certified sample is not available, then use the standard material. A red filter secondary standard (PN8000-952) from Turner Designs is used. User instructions for the secondary standard can be found at http://docs.turnerdesigns.com/t2/doc/instructions/998-0025.pdf.

8 SAMPLE COLLECTION, PRESERVATION, AND STORAGE

8.1 Water collected for chlα should be filtered through a Whatman GF/F glass fiber filter (nominal pore size 0.7 μm), or equivalent.

8.2 Sediments collected for chlα should be sampled to a known depth and area, or known wet weight.

8.3 Solid matrices used to determine epiphytic deposition, should not be soluble in acetone and should be of a size to fit within a 50 ml centrifuge tube and fit below the 40 ml mark. Mylar sheets are commonly used.

8.4 Water collected for chlα should be filtered as soon as possible. If immediate filtration is not possible, the water samples should be kept on ice in the dark and filtered within 24 hours.

8.5 The sample is kept frozen at -20° C or lower. Filter pads may be stored in folded aluminum foil pouches. Sediments and solid matrix samples may be stored in 50 ml polypropylene centrifuge tubes. Do NOT use polystyrene tubes.

8.6 Frozen chlα samples should be analyzed within 28 days of collection.

9 QUALITY CONTROL

9.1 The laboratory is required to operate a formal quality control (QC) program. The minimum requirements of this program consist of an initial demonstration of laboratory capability and the continued analysis of laboratory instrument blanks and calibration standard material, analyzed as samples, as a continuing check on performance. The laboratory is required to maintain performance records that define the quality of data generated.

9.2 Initial Demonstration of Capability

9.2.1 The initial demonstration of capability (iDOC) – is used to characterize instrument performance (MDLs) and laboratory performance (analysis of QC samples) prior to the analyses conducted by this procedure.

9.2.2 Quality Control Sample (QCS/SRM) – When using this procedure, a quality control sample is required to be analyzed at the beginning, after every 20 samples, and end of the run, to verify data quality and acceptable instrument performance. (See section 7.7) If the determined concentrations are not within ± 10% of the certified values, the performance of the determinative step of the method is unacceptable. The source of the problem must be identified and corrected before either proceeding with the initial determination of MDLs or continuing with analyses.

9.2.3 Linear Dynamic Range (LDR) - The linear dynamic range for chlα should be established by using a blank and a minimum of two and a maximum of five appropriate standards for the calibration curve.

9.2.4 Method Detection Limits (MDLs) – MDLs should be established for chlα using a low-level ambient water sample. To determine the MDL values, analyze a minimum of seven replicate aliquots of water. Perform all calculations defined in the procedure (Section 11) and report the concentration values in the appropriate units. Calculate the MDL as follows:

$$MDL = S t_{(n-1,1-\alpha=0.99)}$$

Where,

t(n-1,1-α=0.99) = the Student's t-value appropriate for a single-tailed 99th percentile

t statistic and a standard deviation estimate with n-1 degrees of freedom.

n = number of replicates

S = Standard Deviation of the replicate analyses.

9.2.5 MDLs should be determined yearly.

9.3 Assessing Laboratory Performance

9.3.1 Laboratory Reagent Blank (LRB) and Reagent Blank (RB) – The laboratory must analyze at least one RB at the beginning, after every 20 samples, and at the end with each batch of samples. The RB consists of 90% acetone. The LRB is a blank filter loaded and extracted with 90% Acetone at the end of the loading process. It is analyzed at the end of each run. LRB data are used to assess contamination from the laboratory environment.

9.3.2 Quality Control Sample (QCS)/ Standard Reference Material (SRM) – when using this procedure, a quality control sample is required to be analyzed at the beginning of the run, after every 20 samples, and end of the run, to verify data quality and acceptable instrument performance. (See Section 7.7) If the determined concentrations are not within ± 3σ of the certified values, the performance of the determinative step of the method is unacceptable. The source of the problem must be identified and corrected before either proceeding with the initial determination of MDLs or continuing with the analyses. The results of these samples shall be used to determine batch acceptance.

9.3.3 The QCS will be obtained from a source external to the laboratory and different from the source of calibration standards.

9.3.4 Continuing Calibration Verification (CCV) – The CCV is the red filter secondary standard which is also the quality control sample. If the beginning CCV reading is off by more than ±10.0%, the instrument should be recalibrated. Failure to meet the criteria constitutes correcting the problem and reanalyzing the samples. If not enough sample exists, the data must be qualified if reported.

9.4 Data Assessment and Acceptance Criteria for Quality Control Measures

9.4.1 The Acceptance Criteria for chlα is 0.9990. If the correlation coefficient is less than acceptable, the standards must be made again.

9.5 Corrective Actions for Out of Control Data

9.5.1 If the samples do not fall within the range of the standards, they must be diluted.

10 CALIBRATION AND STANDARDIZATION

10.1 Calibration – Quarterly calibrations with standards of known concentration are performed. Checks with a red filter secondary standard are made daily. If the red filter standard has drifted more than 10.0 % from the original calibration, the instrument should be recalibrated.

10.2 The fluorometric standards received from Turner Designs are certified for concentration, and are ready for use on the fluorometer.

10.3 Make a stock solution of chlorophyll α using Anacystis nidulans algae, dissolving 1 mg in 100 mls of 90% acetone. If chlorophyll α from A. nidulans algae is not available, chlorophyll α from spinach is acceptable. The concentrations of the stock and STD1 and STD2 must be determined by spectrophotometer. Use the Jeffrey and Humphrey trichromatic equation:

11.85(X664) - 1.54(X647) - 0.08(X630)

(subtracting the 750 nm absorbance from each wavelength) to determine the concentration. Once the concentrations are known, make a series of standards by serial dilution. Dilution instructions are listed on the calibration bench sheets.

STD1 = 20 ml STOCK IN 100mls			F1 = 20 ml STD1 IN 100mL
STD2 = 10 ml STOCK IN 100mls			F2= 10ml STD1 IN 100ml
			F3 = 10ml STD2 IN 100 ml
			F4 = 10ml F1 IN 100 ml
			F5 = 10ml F3 IN 100 ml

Use a rolling average of the last three calibrations to determine the acid ratio (rb/ra) and, <u>if from the same lot number and batch of standard</u>, the f_s, which is the standard concentration divided by the fluorescence reading.

10.4 To calibrate the Trilogy fluorometer, refer to the operating manual found online at https://www.turnerdesigns.com/trilogy-laboratory-fluorometer. Follow the link to the user manual under Technical Documentation.

11 PROCEDURE

11.1 Sample Preparation – water column

11.1.1 Filter a known volume of water through a Whatman GF/F filter pad (nominal pore size 0.7 μm) or equivalent. Only a faint color is needed on the pad. Do not rinse the pad.

11.1.2 Fold pad in half, sample inside, wrap in aluminum foil, label, and store at minus 20°C or lower for analysis within 28 days.

11.1.3 Before analysis, briefly thaw pads and then place them in a 15 ml glass or polypropylene centrifuge tube. DO NOT use polystyrene. Using a repipettor, add 10 ml of 90% acetone, including the drip.

11.1.4 Record all information in the lab bench sheet.

11.1.5 Acceptable methods of cell disruption are either mechanical grinding or sonication. This lab uses mechanical grinding by hand. Using a Teflon pestle, grind the filter against the side of the tube until the filter is well ground. When hand grinding, 10-15 seconds is all that is necessary. Power grinding requires vigilance because excess heat will degrade the chlorophyll. Shake the sample after cell disruption. Allow the sample to extract for a minimum of 2 hours and not to exceed 24 hours in the dark under refrigeration of 4° ± 2° C. Overnight is recommended.

11.1.6 Remove from the refrigerator, shake tubes, and then centrifuge at between 500 and 675g for 20 minutes. The present centrifuge in this laboratory is set to 1700 rpm. To calculate rpm, use this formula: $RCF = 1.12r(rpm/1000)^2$,
Where: RCF = relative centrifugal force
r = radius of the rotor in millimeters (usually found on the manufacturer's website)
rpm = speed of rotation
If a refrigerated centrifuge is used, a longer spin time may be used, but not necessary. Allow the samples to come to room temperature. Pipette liquid into a 2 ml capped vial for the Trilogy fluorometer. Avoid pipetting the pad fines within the sample tube. Refer to User Manual (link found in Section 10.4) for how to operate the instrument. Take an initial reading. If sample concentration or raw fluorescence is greater than the linear range of the standard curve, dilute with 90% acetone to fall within the range.

fluorometer dilutions		
2.0 mls total volume		
dilution	mls acetone	mls sample
x2	1.00	1.00
x4	1.50	0.50
x7	1.50	0.25
x10	1.80	0.20
x20	1.90	0.10

11.1.7 If phaeopigments are to be measured, add 1 drop of 5% Hydrochloric Acid, shake and read on the fluorometer again. If using the Welschmeyer (non-acid) technique, **do not** acidify the sample.

11.2 Chlorophyll α in Sediments and Epiphytes
Along with filtered water samples, sediments and epiphyte Mylar strips are also analyzed for chlorophyll α and phaeophytin. The method is modified in the volume of extract used, and the samples are not ground.

11.2.1 Procedure - Sediments:

11.2.1.1A known volume of sediment, usually the top 1 cm section of a core (diameter of the coring device and depth of core must be provided to determine area), or a known wet weight is placed in a polypropylene 50 ml centrifuge tube and frozen. The sample may be kept frozen for up to 28 days before analysis.

11.2.1.2 Forty milliliters of 90% acetone are delivered into the centrifuge tube. The tube is shaken and placed in a dark

box and put in the refrigerator to extract for 2-24 hours. Overnight is recommended.

11.2.1.3 After extraction, weigh the tubes to balance the centrifuge. Centrifuge for 20 minutes at 500-675g. Note: If using a refrigerated centrifuge, spin time may be longer.

11.2.1.4 Allow the samples to come to room temperature before analyzing.

11.2.1.5 Pipette liquid into a 2 ml capped vial for analysis on the fluorometer, being careful not to aspirate any particles. Dilute the sample as needed. Take initial reading. Add 1 drop of 5% HCl to the vial and read again to determine phaeopigments and corrected chlα.

11.2.2 Epiphyte Mylar strips – Mylar strips are deployed in the water column for a set length of time to allow epiphytic algae to attach and grow.

11.2.2.1 Cut Mylar strips short enough to stay below the 40-ml mark on a plastic 50-ml centrifuge tube. Freeze until ready to analyze, up to 28 days.

11.2.2.2 Add 40 ml of 90% acetone, shake well, and place in a dark box in the refrigerator for 2-24 hours. Overnight is recommended.

11.2.2.3 Follow the procedure for sediments after extraction. Dilute the sample as needed.

11.3 Pollution Prevention and Waste Management

11.3.1 This method generates hazardous waste.

11.3.2 Acetone waste is stored in 4-liter jugs in the cabinet under the hood and transferred to the hazardous waste area of the Storage Facility on campus.

11.3.3 Do not pour acetone down the sink.

11.3.4 Decant the waste acetone into the waste jugs, and then allow the remaining ground filter pad or sediment to dry in the hood.

11.3.5 The dried waste may then be put in the trash.

11.4 How to operate the Turner Designs Trilogy

11.4.1 Connect the Trilogy to the computer by the serial port cable(s).

11.4.2 Turn on the instrument. The instrument must be turned on before the software program is opened. The instrument has a touch screen. Select the appropriate module, CHL-A for the acid method, and CHL-NA for the non-acid method. The Trilogy must be recalibrated each time an optical module is changed.

11.4.3 Open the SIS for Trilogy software on the computer. Click START. An EXCEL spreadsheet will open. Minimize the SIS file.

11.4.4 Allow the Trilogy to warm up for about 10 minutes.

11.4.5 Confirm that the instrument and the computer are talking to each other.

11.4.6 Before analyzing samples, analyze an acetone blank and the red filter standard. Press Read Raw Fluorescence for the acid method. Record the RFU values.
For the non-acid method, first, press the CALIBRATE button to select a standard curve; press EXISTING for a saved curve or NEW to proceed with a new calibration. Up to 5 standards may be used. Once selected press View Curve, and then select it to record in the spreadsheet. This puts the instrument into concentration mode. Press Measure Fluorescence. Follow instructions in Section 11.4.9.

11.4.7 Pipette the samples into the 2 ml screw-cap vials, and cap. Wipe the vial with lens paper to ensure it is dry and has no smudges. Enter the sample name by pressing the sample ID button on the upper left side of the screen.

11.4.8 A red filter secondary standard is used as a check (QCS) on the calibration. Take out the vial adaptor to insert the red filter standard. Read the red filter secondary standard after the initial blank, after every 20 samples, and again at the end after the final blank.

11.4.9 Read either Raw Fluorescence or Direct Concentration. To read direct concentration, press the MEASURE FLUORESCENCE button. Enter the volume filtered and press OK. (For standards and blanks enter 1.) Enter the extract volume. (For standards and blanks enter 1.) The instrument will then confirm the choices and begin the analysis. This is the direct concentration mode, so only dilutions need to be factored into the extract volume.

11.4.10 After all samples are run, SAVE the file to the desktop in the Trilogy chla folder. Do not close the SIS software until the EXCEL spreadsheet has been saved.

11.4.11 It is now safe to close the SIS software. It will also close the spreadsheet.

11.4.12 Turn off the instrument.

11.5 Calculations for the Turner Designs Trilogy

11.5.1 Filtered water samples:

11.5.1.1 Total chla:

(R_B * F_S * 10 * dil.)/vol. filtered (ml) = conc. µg/L

11.5.1.2 Active chla:

((R_B - R_A) * F_S* (r/(r-1)) * 10 * dil.)/vol. filtered (ml) = µg/L

11.5.1.3 Phaeophytin:

(((R_A * r) - R_B) * F_S * (r/(r-1)) * 10 * dil.)/vol. filtered (ml) = µg/L

Where: R_B = Reading before adding acid
R_A = Reading after adding acid
10 = extract volume in ml
r = acid ratio: R_B/R_A of calibrating std
F_S = calibrating std conc. / reading of std

11.5.2 Sediments:

11.5.2.1 Total chlα:

$$\frac{R_B \times F_s \times 40 \times dil.}{area.of.core \times 1000} = mg/m^2$$

11.5.2.2 Active or corrected chlα:

$$\frac{(R_B - R_A) \times F_s \times (\frac{r}{r-1}) \times 40 \times dil.}{area.of.core \times 1000} = mg/m^2$$

11.5.2.3 Phaeopigments:

$$\frac{((R_A \times r) - R_B) \times F_s \times (\frac{r}{r-1}) \times 40 \times dil.}{area.of.core \times 1000} = mg/m^2$$

11.5.2.4 To determine mg/g total sample:

$$\frac{\dfrac{R_B \times F_S \times 40 \times dil. \times \dfrac{total.wt(g)}{extract.wt(g)}}{extract.wt(g)}}{1000} = mg / g$$

11.5.3 Epiphyte Mylar strips:

11.5.3.1 Total chlα:

$$\frac{R_B \times F_S \times 40 \times dil.}{1000} = \mu g / strip$$

By dividing by the area of the strip, the units then become μg/$m^{2.}$

Where: R_B = reading before adding acid

R_A = reading after adding acid

40 = extract volume in milliliters

r = acid ratio: R_B/R_A

F_S = calibrating std/reading of std

12 References:

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12.3 EPA Method 445.0: In Vitro Determination of Chlorophyll a and Phaeophytin a in Marine and Freshwater Algae by Fluorescence.

12.4 Welschmeyer, N.A. 1994. Fluorometric analysis of chlorophyll *a* in the presence of chlorophyll *b* and phaeopigments. Limnol. Oceanogr., 39: 1985-1992.

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