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Chesapeake Biological Laboratory
Nutrient Analytical Services
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**Standard Operating Procedures for
Fluorometric Determination of Chlorophyll α in waters and sediments of
Fresh/Estuarine/Coastal Areas.**

(References: SM10200H.3, EPA 445.0)

Document #: NASLDoc-035

**Revision 2018-1
Replaces Revision 2017-4
Effective May 1, 2018**

**I attest that I have reviewed this standard operating procedure and agree to comply with
all procedures outlined within this document.**

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Laboratory Supervisor: _____	Date: _____
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Revisions 2018

Section 1.2 Changed the MDLs for 2018.

9.2.4 Replaced “Student’s t value for the 99% confidence level with $n-1$ degrees of freedom ($t = 3.14$ for 7 replicates)” with “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.”

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 $\mu\text{g/L}$ total chl α , 0.46 $\mu\text{g/L}$ phaeophytin, 0.69 $\mu\text{g/L}$ active chl α , and 0.84 $\mu\text{g/L}$ non-acid chl α , 1.37 mg/m^2 total sediment chl α , 2.42 mg/m^2 phaeophytin in sediment, and 0.71 mg/m^2 active sediment chl α were determined using the Student's t value (3.14, n=7) X the standard deviation of seven replicates. If more replicates are used, refer to the Student t test table for the appropriate value.
- 1.3 The quantitation limit for chl α 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, 19th edition.

2. SUMMARY

- 2.1 Chlorophyll α is extracted from phytoplankton cells using a 90% solution of acetone. The samples are refrigerated in the dark from 2 to 24 hours (over night is preferable). After the appropriate time, the samples are allowed to come to room temperature, and then centrifuged to separate the sample material from the extract. The extract is analyzed on a fluorometer. To determine phaeophytin and active chl α , 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 which 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 acceptability of the calibration curve or previously established calibration curve.
 - 3.11.3 Continuing Calibration Verification (CCV) – An individual standard which 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 taken to eliminate the causes of an existing nonconformity, defect or other undesirable situation in order to prevent 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 performed identically on two sub samples (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 which 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) – A matrix blank (i.e., 90% acetone) that is treated exactly as a sample including exposure to all glassware, equipment, solvents, and reagents that are used with other samples. The LRB is used to determine if method analytes or other interferences are present in the laboratory environment, the reagents, or the instrument.
- 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 Material Safety Data Sheets (MSDS) – Written information provided by vendors concerning a chemical's toxicity, health hazards, physical properties, fire, and reactivity data including storage, spill, and handling precautions.
- 3.32 May – Denotes permitted action, but not required action. (NELAC)
- 3.33 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.
- 3.34 Must – Denotes a requirement that must be met. (Random House College Dictionary)
- 3.35 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)
- 3.36 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.
- 3.37 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 different from the source of calibration standards. It is used to check laboratory performance with externally prepared test materials. Also known as CRM.

- 3.38 Quenching – A phenomenon which occurs when the fluorescence measurements decrease even though the analyte concentration is increasing. (Turner Designs TD700 manual)
- 3.39 Raw fluorescence – Refers to the “relative” fluorescence of a substance being read, rather than the actual concentration. (Turner Designs TD700 manual)
- 3.40 Run – One sample analysis from start to finish, including printout.
- 3.41 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.
- 3.42 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 which 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 when grinding the samples 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 chlorophyll α .

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 Material Safety Data Sheets (MSDS).

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 $-20^{\circ} \pm 5^{\circ}$ C.
- 6.3 Lab ware – All reusable lab ware (glass, Teflon, plastic, etc) should be sufficiently clean for the task objectives.
- 6.4 A centrifuge.

6.5 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 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 ($\text{H}_2\text{C}=\text{O}=\text{CH}_2$), 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 certification from Turner Designs.

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

7.7 Quality Control Sample (QCS) – For this procedure, the QCS can be any certified sample which 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.

8 SAMPLE COLLECTION, PRESERVATION, AND STORAGE

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

- 8.2 Sediments collected for chl α 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 chl α 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 chl α samples should be analyzed within 4 weeks.

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 (DOC) – 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 and end of the run, to verify data quality and acceptable instrument performance. If the determined concentrations are not within $\pm 3\sigma$ of the certified values, 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 chl α should be established by using a blank and a minimum of two and maximum of five appropriate standards for the calibration curve.
 - 9.2.4 Method Detection Limits (MDLs) – MDLs should be established for chl α 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:

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

Where,

$t_{(n-1, 1-\alpha=0.99)}$ = 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) – The laboratory must analyze at least one LRB at the beginning and the end with each batch of samples. The LRB consists of 90% acetone. 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 and end of the run, to verify data quality and acceptable instrument performance. If the determined concentrations are not within $\pm 3\sigma$ of the certified values, 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 $\pm 10.0\%$ or more, adjust the set screw of the red filter standard reading to that acquired at calibration, by using the Allen wrench found in the storage box. Re-read after each adjustment until the readout equals the reading at calibration. 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 chl α 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 5.0 % from the original calibration, the instrument is reset to that setting.
- 10.2 If using *Anacystis nidulans* algae, the concentration must be determined by spectrophotometer.
- 10.3 The fluorometric standards received from Turner Designs are certified for concentration, and are ready for use on the fluorometer.
- 10.4 To calibrate the Trilogy fluorometer, refer to the operating manual found online at <http://www.turnerdesigns.com/t2/doc/manuals/998-7210.pdf>.

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 μ m). 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 4 weeks.
 - 11.1.3 Before analysis, briefly thaw pads, and then place in a 15 ml glass or polypropylene centrifuge tube. DO NOT use polystyrene. Add 10 ml of 90% acetone.
 - 11.1.4 Record all information in the lab bench sheet.
 - 11.1.5 Using a Teflon pestle, grind the filter against the side of the tube until the filter is well ground. If hand grinding, 10-15 seconds is all that is necessary. Power grinding requires vigilance, because excess heat will degrade the chlorophyll. Allow the sample to extract for 2 - 24 hours in the dark under refrigeration. Overnight is recommended.
 - 11.1.6 Remove from the refrigerator and shake tubes, and then centrifuge at between 500 and 675g for 20 minutes. 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. Avoid pipetting the pad fines within the sample tube. Take an initial reading. If sample concentration or raw fluorescence is greater than the range of the standard curve, dilute with 90% acetone to fall within range.

- 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:

11.2.1.1 A 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 4 weeks 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. Spin 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 cuvette 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 cuvette and read again to determine phaeopigments and corrected chl α .

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 4 weeks.

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 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 desktop computer by the serial port cable(s).
 - 11.4.2 Turn on the instrument. 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 desktop. Click START. An EXCEL spreadsheet will open. Minimize the SIS file.
 - 11.4.4 The Trilogy does not need a long warm up period.
 - 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, new or existing. Up to 5 standards may be used. Once selected, press View Curve, and then select it to record in the spreadsheet.
 - 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:

$$(R_B * F_S * 10 * \text{dil.}) / \text{vol. filtered (ml)} = \text{conc. } \mu\text{g/L}$$

11.5.1.2 Active chla:

$$((R_B - R_A) * F_S * (r/(r-1)) * 10 * \text{dil.}) / \text{vol. filtered (ml)} = \mu\text{g/L}$$

11.5.1.3 Phaeophytin:

$$(((R_A * r) - R_B) * F_S * (r/(r-1)) * 10 * \text{dil.}) / \text{vol. filtered (ml)} = \mu\text{g/L}$$

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 chl α :

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

11.5.2.2 Active or corrected chl α :

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

11.5.2.3 Phaeopigments:

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

11.5.2.4 To determine mg/g total sample:

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

11.5.3 Epiphyte Mylar strips:

11.5.3.1 Total chl α :

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

By dividing by the area of the strip, the units then become $\mu\text{g}/\text{m}^2$.

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:

12.1 Strickland, J.D.H. and T.R. Parsons. 1972. A practical handbook of seawater analysis. Bulletin 167 (2nd Ed.). Fisheries Research Board of Canada, Ottawa, Canada.

12.2 Parsons, T.R., Y. Maita and C.M. Lalli. 1984. Determination of chlorophylls and total carotenoids: Spectrophotometric method. pp. 101 - 112 in Parsons, T.R., Y. Maita and C.M. Lalli. A manual of chemical and biological methods for seawater analysis. Pergamon Press, Oxford.

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.

12.5 APHA, Standard Methods for the Examination of Water and Wastewater, Method #10200H, 19th Edition.

FLUOROMETER ACID CHLA BENCH SHEET p.						LOAD DATE:	
CRUISE:						ANALYSIS DATE:	
						ANALYST:	
SAMPLE	DATE	TUBE #	RB	DILUTION	RA	MLS FILTERED	COMMENTS
1							BEGINNING BLK
2							SOLSTD
3							
4							
5							
6							
7							
8							
9							
10							
11							
12							
13							
14							
15							
16							
17							
18							
19							
20							BLK
21							SOLSTD
22							
23							
24							
25							
26							
27							
28							
29							
30							
31							
32							
33							
34							
35							
36							
37							
38							ENDING BLK
39							SOLSTD
40							

FLUOROMETER CHLA BENCH SHEET NON ACID p.					LOAD DATE:
CRUISE:					ANALYSIS DATE:
					ANALYST:
SAMPLE	DATE	TUBE #	DILUTION	MLS FILTERED	COMMENTS
1					BEGINNING BLK
2					SOLSTD
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					
13					
14					
15					
16					
17					
18					
19					
20					BLK
21					SOLSTD
22					
23					
24					
25					
26					
27					
28					
29					
30					
31					
32					
33					
34					
35					
36					
37					
38					ENDING BLK
39					SOLSTD
40					