GUAM ENVIRONMENTAL PROTECTION AGENCY
EMAS ANALYTICAL PROGRAM

STANDARD OPERATING PROCEDURE

In Vitro Determination of Chlorophyll *a* in Marine and Freshwater Algae by Fluorescence

Prepared by: _____ 4-13-2018
Edelisa S. Yanit, Chemist II — Date

Peer Reviewed by: _____ 4-13-2018
Rodolfo B. Paulino, Chemist II — Date

Reviewed by: _____ 4/13/2018
Jesse T. Cruz, Administrator
EMAS Division — Date

Approved by: _____ 04/19/18
Walter Leon Guerrero, Administrator
Guam Environmental Protection Agency — Date

Periodic Review:

Signature	Title	Date

Revision Page

Date	Rev. No.	Summary of Changes	Sections

CONTENTS

1.0 SCOPE AND APPLICATION

1.1 This method provides a procedure for low level determination of chlorophyll *a* (chl *a*) in marine and freshwater phytoplankton by fluorescence detection.

1.2 The GEPA Laboratory shall primarily analyze for chlorophyll *a* using the Non-acidification technique (referred to as the "modified fluorometric" technique in Method 445.0) provided with the use of the Trilogy Laboratory Fluorometer (Turner Designs, Sunnyvale, CA). This instrument is equipped with narrow bandpass excitation and emission filters that nearly eliminates the spectral interference caused by the presence of pheophytin *a* and chlorophyll *b*.

1.3 This method uses 90% acetone as the extraction solvent because of its efficiency for most types of algae.

1.4 Instrumental detection limit (IDL) in µg chl *a*/L shall be determined by this laboratory.

1.5 The estimated detection limit (EDL) in µg chl *a*/L shall be determined in 10 mL of final extraction solution.

1.6 The upper limit of the linear dynamic range (LDR) for the instrumentation used in this method evaluation shall also be determined prior to sample analyses.

2.0 METHOD SUMMARY

2.1 Chlorophyll-containing phytoplankton in a measured volume of sample water are concentrated by filtering at low vacuum through a glass fiber filter. The pigments are extracted from the phytoplankton in 90% acetone with the aid of a mechanical tissue grinder and allowed to steep for a minimum of 2 h, but not to exceed 24 h, to ensure thorough extraction of the chlorophyll *a*. The filter slurry is centrifuged at 675 *g* for 15 min (or at 1000 *g* for 5 min) to clarify the solution. An aliquot of the supernatant is transferred to a glass cuvette and fluorescence is measured. Sensitivity calibration factors, which have been previously determined on solutions of pure chlorophyll *a* of known concentration, are used to calculate the concentration of chlorophyll *a* in the sample extract. The concentration in the natural water sample is reported in µg/L.

3.0 INTERFERENCES

3.1 Any substance extracted from the filter or acquired from laboratory contamination that fluoresces in the red region of the spectrum may interfere in the accurate measurement of chlorophyll *a*.

3.2 The relative amounts of chlorophyll *a*, *b* and *c* vary with the taxonomic composition of the phytoplankton. Chlorophylls *b* and *c* may significantly interfere with chlorophyll *a* measurements depending on the amount present. Due to the spectral overlap of chlorophyll *b* with pheophytin *a* and chlorophyll *a*, underestimation of chlorophyll *a* occurs.

3.3 Quenching effects are observed in highly concentrated solutions or in the presence of high concentrations of other chlorophylls or carotenoids. Minimum sensitivity settings on the fluorometer should be avoided; samples should be diluted instead.

3.4 Fluorescence is temperature dependent with higher sensitivity occurring at lower temperatures. Samples, standards, LRBs and QCSs must be at the same temperature to prevent errors and/or low precision. Analyses of samples at ambient temperature is recommended in this method. Ambient temperature should not fluctuate more than ± 3°C between calibrations or recalibration of the fluorometer will be necessary.

3.5 Samples must be clarified by centrifugation prior to analysis.

3.6 All photosynthetic pigments are light and temperature sensitive. Work must be performed in subdued light and all standards, QC materials and filter samples must be stored in the dark at -20°C or -70°C to prevent degradation.

3.7 Method interferences may be caused by contaminants in the reagent water, reagents, glassware, and other sample processing apparatus that bias analyte response.

4.0 DEFINITIONS

4.1 Estimated Detection Limit (EDL) -- The minimum concentration of an analyte that yields a fluorescence 3X the fluorescence of blank filters which have been extracted according to this method.

4.2 Linear Dynamic Range (LDR) -- The absolute quantity or concentration range over which the instrument response to an analyte is linear.

4.3 Linear Range -- is the concentration range in which the fluorometer output is directly proportional to the concentration of the fluorophore. The linear range begins with the smallest detectable concentration, and spans to an upper limit (concentration) that is dependent upon: the properties of the fluorescent material; the filter used; and the path length.

4.4 Quenching signal – A non-linear relationship is seen at very high concentration where the fluorescence signal does not increase at a constant rate in comparison to the

change in concentration. At even higher concentration, the fluorescence signal will decrease even though the sample concentrations are continuing to increase. This effect is known as "signal quenching".

4.5 Instrument Detection Limit (IDL) -- The minimum quantity of analyte or 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. For this method the background is a solution of 90% acetone.

4.6 Stock Standard Solution (SSS) -- A concentrated solution containing the analyte prepared in the laboratory using assayed reference materials or purchased from a reputable commercial source.

4.7 Primary Dilution Standard Solution (PDS) -- A solution of the analytes prepared in the laboratory from stock standard solutions and diluted as needed to prepare calibration solutions and other needed analyte solutions.

4.8 Calibration Standard (CAL) -- A solution prepared from the primary dilution standard solution or stock standard solutions containing the analytes. The CAL solutions are used to calibrate the instrument response with respect to analyte concentration.

4.9 Response Factor (RF) -- The ratio of the response of the instrument to a known amount of analyte.

4.10 Laboratory Reagent Blank (LRB) -- An aliquot of reagent water or other blank matrices that are treated exactly as a sample including exposure to all glassware, equipment, solvents, reagents, internal standards, and surrogates that are used with other samples. The LRB is used to determine if method analytes or other interferences are present in the laboratory environment, reagents, or apparatus.

4.11 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 give a measure of the precision associated with sample collection, preservation and storage, as well as with laboratory procedures.

4.12 Quality Control Sample (QCS) -- A solution of method analytes of known concentrations which is used to fortify an aliquot of LRB or sample matrix. Ideally, 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.

4.13 Safety Data Sheet (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

5.0 HEALTH AND SAFETY

5.1 The toxicity or carcinogenicity of the chemicals used in this method have not been fully established. Each chemical should be regarded as a potential health hazard and handled with caution and respect.

5.2 Protective clothing including lab coats, safety glasses and gloves must always be worn. Contact lenses must not be worn.

5.3 If solutions come into contact with your skin, wash thoroughly with soap and water. Contact your Supervisor or Health and Safety Coordinator to determine if additional treatment is required.

5.4 The grinding of filters during the extraction step of this method should be conducted in a fume hood due to the volatilization of acetone by the tissue grinder.

5.5 For details on how to properly handle acetone and other toxic or hazardous chemicals, consult SDS. A file of SDS should be made available to all personnel involved in the chemical analysis.

6.0 EQUIPMENT AND SUPPLIES

6.1 Trilogy Laboratory Fluorometer -- A Turner Designs Model equipped with the following:

6.1.1 Blue Mercury Vapor Lamp - has narrow lines of emission which optically excite chlorophyll *a* at 436nm and minimally excite other interfering compounds

6.1.2 Filters - Narrow band interference filters are selected to only allow the specific excitation (436nm) and emission (680nm) wavelengths of chlorophyll *a* to pass.

6.1.3 Chlorophyll *a* Non-Acidification Module – is not sensitive to high chlorophyll *b* concentrations and is well suited for freshwater and open ocean environment.

6.14 12 mm x 75 mm Round Glass test Tubes

6.2 Centrifuge, capable of 675 *g*.

(Note: GEPA Lab uses a Becton Dickinson Clay Adams Compact II Centrifuge capable of 1163 *g*)

6.3 Tissue grinder, Teflon pestle (50 mm X 20 mm) with grooves in the tip with 1/4" stainless steel rod long enough to chuck onto a suitable drive motor and 30-mL capacity glass grinding tube.
Manual disposable tissue grinders are also available for purchase.

6.4 Whatman GF/F Filters, glass fiber, 47-mm or 25-mm, nominal pore size of 0.7 µm unless otherwise justified by data quality objectives

6.5 Petri dishes, plastic, 50 X 9-mm, or some other solid container for transporting and storing sampled filters.

6.6 Aluminum foil.

6.7 Laboratory tissues.

6.8 Tweezers or flat-tipped forceps.

6.9 Vacuum pump or source capable of maintaining a vacuum up to 6 inches Hg.

6.10 Room thermometer.

6.11 Lab ware -- All reusable lab ware (glass, polyethylene, Teflon, etc.) that comes in contact with chlorophyll solutions should be clean and acid free. An acceptable cleaning procedure is soaking for 4 h in laboratory grade detergent and water, rinsing with tap water, distilled deionized water and acetone.

6.11.1 Assorted Class A calibrated pipets.

6.11.2 Graduated cylinders, 500-mL and 1-L.

6.11.3 Volumetric flasks, Class A calibrated, 25-mL, 50mL, 100-mL and 1-L capacity.

6.11.4 Glass rods.

6.11.5 Pasteur type pipets or medicine droppers.

6.11.6 Disposable glass cuvettes for the Trilogy fluorometer.

6.11.7 Filtration apparatus consisting of 1 or 2-L filtration flask, 47-mm fritted glass disk base and a glass filter tower.

6.11.8 Centrifuge tubes, polypropylene or glass, 15-mL capacity with non-pigmented screw-caps.

6.11.9 Polyethylene squirt bottles.

7.0 REAGENTS AND STANDARDS

7.1 Acetone, HPLC grade, (CASRN 67-64-1).

7.2 Chlorophyll *a* free of chlorophyll *b* -- May be obtained from a commercial supplier such as Sigma Chemical (St. Louis, MO). Turner Designs (Sunnyvale, CA) supplies ready-made standards.

7.3 Reagent Water -- ASTM Type I water (ASTM D1193) water is required. Suitable water may be obtained by passing distilled water through a mixed bed of anion and cation exchange resins.

Note: GEPA Lab produces its own reagent water by passing the tap water through the water softener before distillation, then through the Barnstead NANOpure ultrapure water system that produces water with an electrical resistivity of ≥18 MΩ-cm at 25°C.

7.4 Aqueous Acetone Solution -- 90% acetone /10% reagent water. Carefully measure 100 mL of reagent water into the 1-L graduated cylinder. Transfer to a 1-L flask or storage bottle. Measure 900 mL of acetone into the graduated cylinder and transfer to the flask or bottle containing the water. Mix, label and store.

7.5 Chlorophyll Stock Standard Solution (SSS) -Chlorophyll *a* from a commercial supplier will be shipped in an amber glass ampoule which has been flame sealed. This dry standard should be stored at -20 or -70°C in the dark and the SSS prepared just prior to use. Tap the ampoule until all the dried chlorophyll is in the bottom of the ampoule. In subdued light, carefully break the tip off the ampoule. Transfer the entire contents of the ampoule into a 50-mL volumetric flask. Dilute to volume with 90% acetone, label the flask and wrap with aluminum foil to protect from light. The concentration of the solution must be determined spectrophotometrically using a multi-wavelength spectrophotometer. When stored in a light and airtight container at freezer temperatures, the SSS is stable for at least six months. The concentration of all dilutions of the SSS must be determined spectrophotometrically each time they are made.

7.6 Laboratory Reagent Blank (LRB) -- A blank filter which is extracted and analyzed just as a sample filter. The LRB should be the last filter extracted of a sample set. It is used to assess possible contamination of the reagents or apparatus.

7.7 Chlorophyll *a* Primary Dilution Standard Solution (PDS) -- Add 1 mL of the SSS (Sect. 7.5) to a clean 100-mL flask and dilute to volume with the aqueous acetone solution (Sect. 7.4). If exactly 1 mg of pure chlorophyll *a* was used to prepare the SSS, the concentration of the PDS is 200 µg/L. Prepare fresh just prior to use. Determine actual concentration by spectrophotometer and using the Jeffrey and Humphrey's Trichromatic Equation from EPA Method 446.0 (See Section 12.1).

7.8 Quality Control Sample (QCS) -- Since there are no commercially available QCSs, dilutions of a stock standard of a different lot number from that used to prepare calibration solutions may be used.

8.0 SAMPLE COLLECTION, PRESERVATION AND STORAGE

8.1 **Water Sample Collection** -- Water may be obtained by a pump or grab sampler. Data quality objectives will determine the depth at which samples are taken. Healthy phytoplankton, however, are generally obtained from the photic zone (depth at which the illumination level is 1% of surface illumination).

8.1.1 Enough water should be collected to concentrate phytoplankton on at least three filters so that precision can be assessed. Filtration volume size will depend on the particulate load of the water.

8.1.2 Four liters may be required for open ocean water where phytoplankton density is usually low, whereas 1 L or less is generally sufficient for lake, bay or estuary water.

8.2 **Filtration**

8.2.1 All apparatus should be clean and acid-free. Filtering should be performed in subdued light as soon as possible after sampling since algal populations, thus chlorophyll *a* concentration, can change in relatively short periods of time. Field filtration is highly recommended.

8.2.2 Assemble the filtration apparatus and attach the vacuum source with vacuum gauge and regulator. Vacuum filtration should not exceed 6 inches Hg (20 kPa). Higher filtration pressures and excessively long filtration times (> 10 min) may damage cells and result in loss of chlorophyll.

8.2.3 Prior to drawing a subsample from the water sample container, thoroughly but gently agitate the container to suspend the particulates (stir or invert several times).

8.2.4 Pour the subsample into a graduated cylinder and accurately measure the volume.

8.2.5 Pour the subsample into the filter tower of the filtration apparatus and apply a vacuum (not to exceed 6 inches Hg or 20 kPa). A sufficient volume has been filtered when a visible green or brown color is apparent on the filter.

8.2.6 Do not suck the filter dry with the vacuum; instead slowly release the vacuum as the final volume approaches the level of the filter and completely release the vacuum as the last bit of water is pulled through the filter.

8.2.7 Remove the filter from the fritted base with tweezers, fold once with the particulate matter inside, lightly blot the filter with a tissue to remove excess moisture and place it in the petri dish or other suitable container.

8.2.8 If the filter will not be immediately extracted, then wrap the container with aluminum foil to protect the phytoplankton from light and store the filter at -20 or -70°C. Short term storage (2 to 4 h) on ice is acceptable, but samples should be stored at -20 or -70°C as soon as possible.

8.3 **Preservation** -- Sampled filters should be stored frozen (-20°C or -70°C) in the dark until extraction.

8.4 **Holding Time** -- Filters can be stored frozen at -20°C or -70°C for as long as 3½ weeks without significant loss of chlorophyll *a*.

9.0 QUALITY CONTROL PROCEDURES

9.1 GEPA Lab operates a formal quality control (QC) program. The QC program consists of an initial demonstration of laboratory capability, and the continued analysis of laboratory reagent blanks and quality control samples as a continuing check on performance. The laboratory is required to maintain performance records that define the quality of the data that are generated.

9.2 Initial Demonstration of Performance (Mandatory)

9.2.1 The initial demonstration of performance is used to characterize instrument performance (instrumental detection limits and linear dynamic range) and laboratory performance (analyses of QCSs) prior to sample analyses.

9.2.2 **Linear Dynamic Range (LDR)** -- The LDR should be determined by analyzing a minimum of 5 calibration standards ranging in concentration from 0.2 µg/L to 200 µg chl *a*/L across all sensitivity settings of the fluorometer.

9.2.2.1 If using an analog fluorometer or a digital fluorometer requiring manual changes in sensitivity settings, normalize responses by dividing the response by the sensitivity setting multiplier.

9.2.2.3 Perform the linear regression of normalized response vs. concentration and obtain the constants m and b, where m is the slope and b is the y-intercept.

9.2.2.4 Incrementally analyze standards of higher concentration until the measured fluorescence response, R, of a standard no longer yields a calculated concentration, C_c, that is ± 10% of the known concentration, C, where $C_c = (R - b)/m$. That concentration defines the upper limit of the LDR for your instrument.

9.2.2.5 Should samples be encountered that have a concentration which is 90% of the upper limit of the LDR, these samples must be diluted and reanalyzed.

9.2.3 **Instrumental Detection Limit (IDL)** -- Zero the fluorometer with a solution of 90% acetone on the maximum sensitivity setting. Pure chlorophyll a in 90% acetone should be serially diluted until it is no longer detected by the fluorometer on a maximum sensitivity setting.

9.2.4 **Estimated Detection Limit (EDL)** -- Several blank filters should be extracted according to the procedure in Sect. 11, using clean glassware and apparatus, and the fluorescence measured. A solution of pure chlorophyll *a* in 90% acetone should be serially diluted until it yields a response which is 3X the average response of the blank filters.

9.2.5 **Quality Control Sample (QCS)** -- When beginning to use this method, on a quarterly basis or as required to meet data quality needs, verify the calibration standards and acceptable instrument performance with the analysis of a QCS (Sect. 7.8). If the determined value is not within the confidence limits established by project data quality objectives, then the determinative step of this method is unacceptable. The source of the problem must be identified and corrected before continuing analyses. The QCS is obtained from a source external to the laboratory and different from the source of calibration standards.

9.2.6 **Extraction Proficiency** -- Personnel performing this method for the first time should demonstrate proficiency in the extraction of sampled filters (Sect. 11.1).

Twenty to thirty natural samples should be obtained using the procedure outlined in Sect. 8.1 of this method. Sets of 10 or more samples should be extracted and analyzed according to Sections 11.1 and 11.2. The percent relative standard deviation (%RSD) of chlorophyll *a* values (non-acidified method) should not exceed 15% for samples that are approximately 10X the IDL.

Alternatively, ten or more blank filters are spiked with different concentrations of chlorophyll *a* that are approximately 10X the IDL, are extracted and analyzed according to Sections 11.1 and 11.2. The percent relative standard deviation (%RSD) of chlorophyll *a* values should not exceed 15%.

9.3 Assessing Laboratory Performance (Mandatory)

9.3.1 Laboratory Reagent Blank (LRB) -- The laboratory must analyze at least one blank filter with each sample batch. The LRB should be the last filter extracted. LRB data are used to assess contamination from the laboratory environment. LRB values that exceed the IDL indicate contamination from the laboratory environment. When LRB values constitute 10% or more of the analyte level determined for a sample, fresh samples or field duplicates must be analyzed after the contamination has been corrected and acceptable LRB values have been obtained.

10.0 CALIBRATION AND STANDARDIZATION

10.1 Calibration -- When calibrated the Trilogy fluorometer converts the fluorescence signal to concentration units based on the standards used for calibration.

10.1.1 Calibrate or check calibration using a secondary solid standard before running a new batch of samples.

10.1.2 Calibration should be performed once every three months; or:
--- when accuracy becomes unacceptable after calibration verification by a secondary solid standard;
--- after changing Optical Application Module or when there has been an adjustment made to the instrument, such as replacement of lamp, filters or photomultiplier;
--- if the ambient temperature changes by $\pm$10°C.
--- if you make measurements on a new analyte

10.2 Trilogy Fluorometer Calibration Procedures

10.2.1 Direct Calibration Procedure – The following procedure applies to Trilogy Fluorometer Multi Point calibration, Chlorophyll Non-Acidification Modules:

a. Prepare 0.2, 2, 5, 20 and 200 µg chl *a*/L calibration standards from the PDS (Sect. 7.7).

b. Turn on the Trilogy fluorometer and allow the instrument to warm up for at least 15 min.

c. Select the module "**Chl-NA**" and confirm by touching "**OK**".

d. On the home screen, touch "**Calibrate**" to begin a calibration sequence.

e. Select "**Run New Calibration**".

f. Select the unit of measurement "**µg/l**"

g. Insert the calibration "blank" and touch "**OK**"

h. Enter the concentration for the first standard. Be sure to use standards in order of increasing concentration.

i. Follow the screen prompt indicating that the standard should be inserted, touch "**OK**".

j. After the calibration is complete, either select "**Proceed with Current Calibration**" or select "**Enter More Standards**", in which case, return to "g" above.

k. Name and save the calibration for future use (OPTIONAL).

l. Subsequent readings in the Direct Concentration mode should reflect the actual concentration of the analyte.

m. Confirm successful completion of the calibration by measuring the same Standard. The displayed concentration should equal the value used in step "g" above.

n. Optionally, the solid Secondary Standard could now be adjusted to give the same reading for future calibrations. (See Section 10.2.2.5)

10.2.2 Using the Secondary Standard

10.2.2.1 The Solid Secondary Standards provides a very stable fluorescent signal. It has an adjustment screw so that you can tune the Solid Standard to provide a signal to match a specific sample.

10.2.2.2 It can be used in place of a primary liquid standard once a correlation between a primary and the solid standard has been established.

10.2.2.3 It can be used to check the fluorometer stability, and/or check for loss in sensitivity resulting from instrument/optical module problems.

10.2.2.4 It should be noted that each Solid Standard/Fluorometer relationship is unique. This means that a given Solid Standard cannot be used to calibrate multiple fluorometers or modules for identical readings of a given solution.

10.2.2.5 The following procedure describes how to use the Solid Secondary Standard (P/No. 8000-952) with the Trilogy fluorometer fluorescence module:

a. Establish a correlation between a known chlorophyll *a* concentration and the fluorometer reading by measuring a chlorophyll *a* solution of known concentration (say 20 *u*g chl *a*/L from Section 10.2.1 a) and note the fluorometer reading.
b. Insert the Solid Secondary Standard in the Optical Module. **Wait for 10 seconds and measure**. Turn the secondary standard adjustment screw to produce the same reading on the fluorometer as in step **a** above (turning the secondary standard adjustment screw clockwise reduces the displayed concentration).
c. Next perform a chlorophyll *a* extraction using the Trilogy fluorometer to determine the actual chlorophyll *a* concentration.
d. The solid secondary standard can now be used to recalibrate and/or check the stability of the Trilogy fluorometer.

11.0 PROCEDURES

11.1 Extraction of Filter Samples

11.1.1 If sampled filters have been frozen, remove them from the freezer but keep them in the dark. Workspace lighting should be the minimum that is necessary to read instructions and operate instrumentation.

11.1.2 Set up the tissue grinder and have on hand tissues and squirt bottles containing water and acetone.

11.1.3 Remove a filter from its container and place it in the glass grinding tube. The filter may be torn into smaller pieces to facilitate extraction. Push it to the bottom of the tube with a glass rod.

11.1.4 With a volumetric pipet, add 4 mL of the aqueous acetone solution (90% acetone) (Sect. 7.4) to the grinding tube.

11.1.5 Grind the filter until it has been converted to a slurry.

(NOTE: Although grinding is required, care must be taken not to overheat the sample. Good judgement and common sense will help you in deciding when the sample has been sufficiently macerated.)

11.1.6 Pour the slurry into a 15-mL screw-cap centrifuge tube and, using a 6-mL volumetric pipet, rinse the pestle and the grinding tube with 90% acetone. Add the rinse to the centrifuge tube containing the filter slurry. Cap the tube and shake it vigorously.

11.1.7 Place it in the dark before proceeding to the next filter extraction.

11.1.8 Before placing another filter in the grinding tube, use the acetone and water squirt bottles to thoroughly rinse the pestle, grinding tube and glass rod. The last rinse should be with acetone. Use a clean tissue to remove any filter residue that adheres to the pestle or to the steel rod of the pestle.

11.1.9 Shake each tube vigorously before placing them steep in the dark at 4°C. Samples should be allowed to steep for a minimum of 2 h but not to exceed 24 h. The tubes should be shaken at least once during the steeping period.

11.1.10 After steeping is complete, shake the tubes vigorously and centrifuge samples for 5 minutes at 1000 g.

11.1.11 Samples should be allowed to come to ambient temperature before analysis. This can be done by letting them stand at room temperature for 30 min.

11.1.12 Recalibrate the fluorometer if the room temperature fluctuated ± 3°C from the last calibration date.

11.2 Sample Analysis

11.2.1 After the Trilogy fluorometer has warmed up for at least 15 min, use the 90% acetone solution to zero the instrument on the sensitivity setting that will be used for sample analysis.

11.2.2 Pour or pipet the supernatant of the extracted sample into a test tube (or sample cuvette).

11.2.3 Choose a sensitivity setting that yields a midscale reading when possible and avoid the minimum sensitivity setting.

11.2.4 If the concentration of chlorophyll *a* in the sample is 90% of the upper limit of the LDR, then dilute the sample with the 90% acetone solution and reanalyze.

11.2.5 Record the fluorescence measurement and sensitivity setting used for the sample.

12.0 DATA ANALYSIS, CALCULATIONS AND REPORTING

12.1 Jeffrey and Humphrey's Trichromatic Equation – Subtract the absorbance value at 750 nm from the absorbance values at 664,647 and 630 nm. Calculate the concentration (mg/L) of chlorophyll *a* in the Chlorophyll *a* Primary Dilution Standard (PDS) (Section 7.7) by inserting the 750 nm-corrected absorbance values into the following equations:

$$C_a = 11.85\ (Abs\ 664) - 1.54\ (Abs\ 647) - 0.08\ (Abs\ 630)$$

Where: C_a = concentration (mg/L) of chlorophyll *a* in the PDS or in the extract solution analyzed

12.2 Calculate the chlorophyll *a* concentration in the total volume of sample from the total scraped area as:

$$\text{Chlorophyll } a\ (\mu g/cm^2) = \frac{\text{Trilogy reading } (\mu g/L) \times \text{total volume of sample (L)}}{\text{Total scraped area } (cm^2)}$$

12.3 LRB and QCS data should be reported with each sample data set.

12.4 Review the results for all QC samples for compliance with the criteria specified in Section 9. If results are not acceptable, take appropriate corrective action.

12.5 All results should be reported using no more than three significant figures; however, no values of less significance than the EDL may be reported. Results less than the EDL should be reported as <EDL values.

12.6 Before releasing the results, the laboratory conducts data verification and validation. This is done through peer review of the data and validation by another analyst. The QA Manager makes the final audit and validation prior to the release of the results.

13.0 DOCUMENTATION

13.1 When samples are received, the laboratory personnel verify that the chain of custody is properly filled out. Laboratory personnel may then receive and sign the chain of custody.

13.2 Each standard and reagent prepared for the analysis is recorded in the Standard Preparation Logbook and Reagent Preparation Logbook respectively.

13.3 A QC Summary Report that contains the QC sample results and evaluations must be prepared for every analytical run.

13.4 Sample results are entered in the Laboratory Information Management System (LIMS) to facilitate storage and retrieval of data. An analytical results report is generated from the LIMS or in a spreadsheet as required by customer.

14.0 REFERENCES

14.1 EPA Method 445.0, *In Vitro* Determination of Chlorophyll *a* and Pheophytin *a* in Marine and Freshwater Algae by Fluorescence, Revision 1.2, September 1997

14.2 EPA Method 446.0, *In Vitro* Determination of Chlorophylls *a*, *b*, $c_1 + c_2$ and Pheopigments in Marine and Freshwater Algae by Visible Spectrophotometry, Revision 1.2, September 1997

14.2 Turner Designs Trilogy Fluorometer User's Manual, Version 1.2, September 15, 2010