Method 445.0

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

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1.0 Scope and Application

1.1 This method provides a procedure for low level determination of chlorophyll *a* (chl *a*) and its magnesium-free derivative, pheophytin *a* (pheo *a*), in marine and freshwater phytoplankton using fluorescence detection.(1,2) Phaeophorbides present in the sample are determined collectively as pheophytin *a*. For users primarily interested in chl *a* there is currently available a set of very narrow bandpass excitation and emission filters (Turner Designs, Sunnyvale, CA) that nearly eliminates the spectral interference caused by the presence of pheo *a* and chlorophyll *b*. The difference between the modified method and the conventional fluorometric method is that the equations used for the determination of chlor *a* without pheo *a* correction (uncorrected chlor *a*), are used instead of the equations for "corrected chlor *a*". This EPA laboratory has evaluated the modified filters and found the technique to be an acceptable alternative to the conventional fluorometric method using pheo *a* correction.(3)

Analyte	Chemical Abstracts Service Registry Number (CASRN)
Chlorophyll *a*	479-61-8

1.2 Instrumental detection limits (IDL) of 0.05 µg chl *a*/L and 0.06 µg pheo *a*/L in a solution of 90% acetone were determined by this laboratory. Method detection limits (MDL) using mixed assemblages of algae provide little information because the fluorescence of other pigments interferes in the fluorescence of chlorophyll *a* and pheophytin *a*.(4) A single lab estimated detection limit for chlorophyll *a* was determined to be 0.11 µg/L in 10 mL of final extraction solution. The upper limit of the linear dynamic range for the instrumentation used in this method evaluation was 250 µg chl *a*/L.

1.3 This method was multilaboratory validated in 1996.(5) Results from that study may be found in Section 13. Additional QC procedures also have been added as a result of that study.

1.4 This method uses 90% acetone as the extraction solvent because of its efficiency for most types of algae. There is evidence that certain chlorophylls and carotenoids are more thoroughly extracted with methanol(5-8) or dimethyl sulfoxide.(9) Bowles, et al.(8) found that for chlorophyll *a*, however, 90% acetone was an effective extractant when the extraction period was optimized for the dominant species present in the sample.

1.5 Depending on the type of algae under investigation, this method can have uncorrectable interferences (Sect. 4.0). In cases where taxomonic classification is unavailable, a spectrophotometric or high performance liquid chromatographic (HPLC) method may provide more accurate data for chlorophyll *a* and pheophytin *a*.

1.6 This method is for use by analysts experienced in the handling of photosynthetic pigments and in the operation of fluorescence detectors or by analysts under the close supervision of such qualified persons.

2.0 Summary of Method

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 before and after acidification to 0.003 N HCl with 0.1 N HCl. 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* and pheophytin *a* in the sample extract. The concentration in the natural water sample is reported in µg/L.

3.0 Definitions

3.1 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.

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

3.3 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.

3.4 Stock Standard Solution (SSS) -- A concentrated solution containing one or more method analytes prepared in the laboratory using assayed reference materials or purchased from a reputable commercial source.

3.5 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.

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

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

3.8 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.

3.9 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.

3.10 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.

3.11 Material Safety Data Sheet (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.

4.0 Interferences

4.1 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 both chlorophyll *a* and pheophytin *a*.

4.2 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 accompanied by overestimation of pheophytin *a* when chlorophyll *b* is present in the sample. The degree of interference depends upon the ratio of *a*:*b*. This laboratory found that at a ratio of 5:1, using the acidification procedure to correct for pheophytin *a*, chlorophyll *a* was underestimated by approximately 5%. Loftis and Carpenter(10) reported an underestimation of 16% when the *a*:*b* ratio was 2.5:1. A ratio of 1:1 is the highest ratio likely to occur in nature. They also reported overestimation of chlorophyll *a* in the presence of chlorophyll *c* of as much as 10% when the *a*:*c* ratio was 1:1 (the theoretical maximum likely to occur in nature). The presence of chlorophyll *c* also causes the underestimation of pheophytin *a*. The effect of chlorophyll *c* is not as severe as the effect of chlorophyll *b* on the measurement of chlorophyll *a* and pheophytin *a*. Knowledge of the taxonomy of the algae under consideration will aid in determining if the spectrophotometric method using trichromatic equations to determine chlorophyll *a*, *b*, and *c* or an HPLC method would be more appropriate.(11-16) In the presence of chlorophyll *b* or pheopigments, the modified fluorometric method described here is also appropriate.(5)

4.3 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.

4.4 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.

4.5 Samples must be clarified by centrifugation prior to analysis.

4.6 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.

5.0 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. Each laboratory is responsible for maintaining a current awareness file of Occupational Safety and Health Administration (OSHA) regulations regarding the safe handling of the chemicals specified in this method.(17-20) A file of MSDS should also be made available to all personnel involved in the chemical analysis.

5.2 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.

6.0 Apparatus and Equipment

6.1 Fluorometer -- Equipped with a high intensity F4T.5 blue lamp, red-sensitive photomultiplier, and filters for excitation (CS-5-60) and emission (CS-2-64). A Turner Designs Model 10 Series fluorometer was used in the evaluation of this method. The modified method requires excitation filter (436FS10) and emission filter (680FS10).

6.2 Centrifuge, capable of 675 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.

6.4 Filters, glass fiber, 47-mm or 25-mm, nominal pore size of 0.7 µm unless otherwise justified by data quality objectives. Whatman GF/F filters were used in this work.

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 in. Hg.

6.10 Room thermometer.

6.11 Labware -- All reusable labware (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, 50-mL, 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 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 nonpigmented screw-caps.

6.11.9 Polyethylene squirt bottles.

7.0 Reagents and Standards

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

7.2 Hydrochloric acid (HCl), concentrated (sp. gr. 1.19), (CASRN 7647-01-0).

7.3 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.

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

7.5 ***0.1 N HCl Solution*** -- Add 8.5 mL of concentrated HCl to approximately 500 mL water and dilute to 1 L.

7.6 ***Aqueous Acetone Solution*** -- 90% acetone /10% water. Carefully measure 100 mL of 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.

7.7 ***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 multiwavelength spectrophotometer.(10) 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.

7.8 ***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.

7.9 ***Chlorophyll*** *a* ***Primary Dilution Standard Solution*** (PDS) -- Add 1 mL of the SSS (Sect. 7.8) to a clean 100-mL flask and dilute to volume with the aqueous acetone solution (Sect. 7.7). 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.

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

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

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

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

8.3 ***Holding Time*** -- Filters can be stored frozen at -20 or -70°C for as long as 3½ weeks without significant loss of chlorophyll *a*.(21)

9.0 Quality Control

9.1 Each Laboratory using this method 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 reagent blanks, field duplicates 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 thus 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, linear dynamic range and MDLs) 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. 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. 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. 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. 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. 7.10). 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.

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 Sect. 11.2. The percent relative standard deviation (%RSD) of uncorrected values of chlorophyll *a* should not exceed 15% for samples that are approximately 10X the IDL. RSD for pheophytin *a* might typically range from 10 to 50%.

9.2.7 Corrected Chl *a* -- Multilaboratory testing of this method revealed that many analysts do not adequately mix the acidified sample when determining corrected chl *a*. The problem manifests itself by highly erratic pheo-*a* results, high %RSDs for corrected chl *a* and poor agreement between corrected and uncorrected chl *a*. To determine if a new analyst is performing the acidification step properly, perform the following QC procedure:

Prepare 100 mL of a 50 ppb chl *a* solution in 90% acetone. The new analyst should analyze 5-10 separate aliquots, using separate cuvettes, according to instructions in Section 11.2. Process the results according to Section 12 and calculate separate means and %RSDs for corrected and uncorrected chl *a*. If the means differ by more than 10%, then the stock chl *a* has probably degraded and fresh stock should be prepared. The %RSD for corrected chl *a* should not exceed 5%. If the %RSD exceeds 5%, repeat the procedure until the %RSD 5%.

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*** -- Calibration should be performed bimonthly or when there has been an adjustment made to the instrument, such as replacement of lamp, filters or photomultiplier. Prepare 0.2, 2, 5, 20 and 200 µg chl *a*/L calibration standards from the PDS (Sect. 7.11). Allow the instrument to warm up for at least 15 min. Measure the fluorescence of each standard at sensitivity settings that provide midscale readings. Obtain response factors for chlorophyll *a* for each sensitivity setting as follows:

$$F_s = C_a/R_s$$

where:

F_s = response factor for sensitivity setting, S.

R_s = fluorometer reading for sensitivity setting, S.

C_a = concentration of chlorophyll *a*.

NOTE: If you are using special narrow bandpass filters for chl *a* determination, **DO NOT** acidify. Use the "uncorrected" chl *a* calculation described in Section 12.1.

If pheophytin *a* determinations will be made, it will be necessary to obtain before-to-after acidification response ratios of the chlorophyll *a* calibration standards as follows: (1) measure the fluorescence of the standard, (2) remove the cuvette from the fluorometer, (3) acidify the solution to .003 N HCl(6) with the 0.1 N HCl solution, (4) use a pasteur type pipet to thoroughly mix the sample by aspirating and dispensing the sample into the cuvette, keeping the pipet tip below the surface of the liquid to avoid aerating the sample, (5) wait 90 sec and measure the fluorescence of the standard solution again. Addition of the acid may be made using a medicine dropper. It will be necessary to know how many drops are equal to 1 mL of acid. For a cuvette that holds 5 mL of extraction solution, it will be necessary to add 0.15 mL of 0.1 N HCl to reach a final acid concentration of 0.003N in the 5 mL. Calculate the ratio, r, as follows:

$$r = R_b/R_a$$

where:

R_b = fluorescence of pure chlorophyll *a* standard solution before acidification.

R_a = fluorescence of pure chlorophyll *a* standard solution after acidification.

11.0 Procedure

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. Set up the tissue grinder and have on hand tissues and squirt bottles containing water and acetone. Workspace lighting should be the minimum that is necessary to read instructions and operate instrumentation. 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. With a volumetric pipet, add 4 mL of the aqueous acetone solution (Sect. 7.6) to the grinding tube. 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.) 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. Place it in the dark before proceeding to the next filter extraction. 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. Proceed to the next filter and repeat the steps above. The entire extraction with transferring and rinsing steps takes 5 min. Approximately 500 mL of acetone and water waste are generated per 20 samples from the rinsing of glassware and apparatus.

11.1.2 Shake each tube vigorously before placing them to steep in the dark at 4^{o}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.

11.1.3 After steeping is complete, shake the tubes vigorously and centrifuge samples for 15 min at 675 g or for 5 min at 1000 g. Samples should be allowed to come to ambient temperature before analysis. This can be done by placing the tubes in a constant temperature water bath or by letting them stand at room temperature for 30 min. Recalibrate the fluorometer if the room temperature fluctuated ± 3°C from the last calibration date.

11.2 SAMPLE ANALYSIS

11.2.1 After the 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 sample cuvette. The volume of sample required in your instrument's cuvette should be known so that the correct amount of acid can be added in the pheophytin *a* determinative step. For a cuvette that holds 5 mL of extraction solution, 0.15 mL of the 0.1 N HCl solution should be used. Choose a sensitivity setting that yields a midscale reading when possible and avoid the minimum sensitivity setting. 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. Record the fluorescence measurement and sensitivity setting used for the sample. Remove the cuvette from the fluorometer and acidify the extract to a final concentration of 0.003 N HCl using the 0.1 N HCl solution. Use a pasteur type pipet to thoroughly mix the sample by aspirating and dispensing the sample into the cuvette, keeping the pipet tip below the surface of the liquid to avoid aerating the sample. Wait 90 sec before measuring fluorescence again. NOTE: Proper mixing is critical for precise and accurate results. Twenty-five to thirty-five samples can be extracted and analyzed in one 8 hr day.

NOTE: If you are using special narrow bandpass filters for chl *a* determination, **DO NOT** acidify samples. Use the “uncorrected” chl *a* calculations described in Section 12.1.

12.0 Data Analysis and Calculations

12.1 For “uncorrected chlorophyll *a*,” calculate the chlorophyll *a* concentration in the extract as:

$$C_{E,u} = R_b \times F_s$$

where $C_{E,u}$ = uncorrected chlorophyll *a* concentration (µg/L) in the extract solution analyzed,

R_b = fluoresence response of sample extract before acidification, and

F_s = fluoresence respnse factor for sensitivity setting S.

Calculate the “uncorrected” concentration of chlorophyll *a* in the whole water sample as follows:

$$C_{S,u} = \frac{C_{E,u} \ x \ extract \ volume \ (L) \ X \ DF}{sample \ volume \ (L)}$$

where $C_{S,u}$ **=** uncorrected chlorophyll *a* concentration (µg/L) in the whole water sample,

extract volume = volume (L) of extraction prepared before any dilutions,

DF = dilution factor,

sample volume = volume (L) of whole water sample.

12.2 For “corrected chlorophyll *a*”, calculate the chlorophyll *a* concentration in the extract as :

$C_{E,c} = F_s \ (r/r\text{-}1) \ (R_b - R_a)$

where:

$C_{E,c}$ = corrected chlorophyll *a* concentration (µg/L) in the extract solution analyzed,

F_s = response factor for the sensitivity setting S,

r = the before-to-after acidification ratio of a pure chlorophyll *a* solution (Sect. 10.1),

R_b = fluorescence of sample extract before acidification, and

R_a = fluorescence of sample extract after acidification.

Calculate the “corrected” concentration of chlorophyll *a* in the whole water sample as follows:

$$C_{s,c} = \frac{C_{E,u} \ x \ extract \ volume \ (L) \ X \ DF}{sample \ volume \ (L)}$$

where $C_{S,c}$ = corrected chlorophyll *a* concetration (µg/L) in the whole water sample,

extract volume = volume (L) of extract prepared before dilution,

12.3 Calculate the pheophytin *a* concentration as follows:

$P_E = F_s \ (r/r\text{-}1) \ (rR_a - R_b)$

$$P_s = \frac{P_E \ X \ extract \ volume \ (L) \ X \ DF}{sample \ volume \ (L)}$$

where P_E = pheophytin *a* concentration (µg/L) in the sample extract; and

P_s = pheophytin *a* concentration (µg/L) in the whole water sample.

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

13.0 Method Performance

13.1 The single lab EDL for the instrument used in the evaluation of this method was 0.05 µg/L for chlorophyll *a* and 0.06 µg/L pheophytin *a*.

13.2 The precision (%RSD) for chlorophyll *a* in mostly blue-green and green phytoplankton natural samples which were steeped for 2 h vs 24 h is reported in Table 1. Although the means were the same, precision was better for samples which were allowed to steep for 24 h prior to analysis. Since pheophytin *a* was found in the samples, the chlorophyll *a* values are "corrected" (Sect. 12.2). Table 2 contains precision data for pheophytin *a*. A statistical analysis of the pheophytin *a* data indicated a significant difference in the mean values at the 0.05 significance level. The cause of the lower pheophytin *a* values in samples extracted for 24 h is not known.

13.3 Three QCS ampoules obtained from the USEPA were analyzed and compared to the reported confidence limits in Table 3. **NOTE:** The USEPA no longer provides these QCSs.

13.4 **Multilaboratory Testing** - A multilaboratory validation and comparison study of EPA Methods 445.0, 446.0 and 447.0 for chlorophyll *a* was conducted in 1996 by Research Triangle Institute, Research Triangle Park, N.C. (EPA Contract No. 68-C5-0011). There were 21 volunteer participants in the fluorometric methods component that returned data; 10 that used the modified fluorometric method and 11 that used the conventional method. The primary goals of the study were to determine estimated detection limits and to assess precision (%RSD) and bias (as percent recovery) for select unialgal species, and natural seawater.

13.4.1 The term, pooled estimated detection limit (p-EDL), is used in this method to distinguish it from the EPA defined method detection limit (MDL). An EPA MDL determination is not possible nor practical for a natural water or pure species sample due to known spectral interferences and to the fact that it is impossible to prepare solutions of known concentrations that incorporate all sources of error (sample collection, filtration, processing). The statistical approach used to determine the p-EDL was an adaptation of the Clayton, et.al.(22) method that does not assume constant error variances across concentration and controls for Type II error. The statistical approach used involved calculating an estimated DL for each lab that had the desired Type I and Type II error rates (0.01 and 0.05, respectively). The median DLs over labs was then determined and is reported in Table 4. It is referred to as pooled-EDL (p-EDL).

Solutions of pure chlorophyll *a* in 90% acetone were prepared at three concentrations (0.11, 0.2 and 1.6 ppm) and shipped with blank glass fiber filters to participating laboratories. Analysts were instructed to spike the filters in duplicate with a given volume of solution and to process the spiked filters according to the method. The results from these data were used to determine a p-EDL for each method. Results (in ppm) are given in Table 4. The standard fluorometric and HPLC methods gave the lowest p-EDLs while the spectrophotometric (monochromatic equations) gave the highest p-EDLs. Due to the large dilutions required to analyze these solutions, the fluorometric p-EDLs are unrealistically high compared to what is achievable by a single lab. Typical single lab EDLs can easily be 1000 fold lower than the p-EDL reported in Table 4.

13.4.2 To address precision and bias in chlorophyll *a* determination for different algal species, three pure unialgal cultures (Amphidinium, Dunaliella and Phaeodactylum) were cultured and grown in the laboratory. Four different “concentrations” of each species were prepared by filtering varying volumes of the algae. The filters were frozen and shipped to participant labs. Analysts were instructed to extract and analyze the filters according to the respective methods. The “true” concentration was assigned by taking the average of the HPLC results for the highest concentration algae sample since chlorophyll *a* is separated from other interfering pigments prior to determination. Pooled precision (as determined by %RSD) data are presented in Tables 5-7 and accuracy data (as percent recovery) are presented in Table 8. No significant differences in precision were observed across concentrations for any of the species. It should be noted that there was considerable lab-to-lab variation (as exhibited by the min and max recoveries in Table 8) and in this case the median is a better measure of central tendency than the mean.

In summary, the mean and median concentrations determined for Amphidinium carterae (class dinophyceae) are similar for all methods. No method consistently exhibited high or low values relative to the other methods. The only concentration trend observed was that the spectrophotometric method-trichromatic equations (SP-T) showed a slight percent increase in recovery with increasing algae filtration volume.

For Dunaliella tertiolecti (class chlorophyceae) and Phaeodactylum tricornutum (class bacillariophyceae) there was generally good agreement between the fluorometric and the spectrophotometric methods, however, the HPLC method yielded lower recoveries with increasing algae filtration volume for both species. No definitive explanation can be offered at this time for this phenomenon. A possible explanation for the Phaeodactylum is that it contained significant amounts of chlorophyllide *a* which is determined as chlorophyll *a* in the fluorometric and spectrophotometric methods. The conventional fluorometric method (FL-STD) showed a slight decrease in chlorophyll *a* recovery with increasing Dunaliella filtration volume. The spectrophotometric-trichromatic equations (SP-T) showed a slight increase in chlorophyll *a* recovery with increasing Dunaliella filtration volume. The fluorometric and the spectrophotometric methods both showed a slight decrease in chlorophyll *a* recovery with increasing Phaeodactylum filtration volume.

Results for the natural seawater sample are presented in Table 9. Only one filtration volume (100 mL) was provided in duplicate to participant labs.

14.0 Pollution Prevention

14.1 Pollution prevention encompasses any technique that reduces or eliminates the quantity or toxicity of waste at the point of generation. Numerous opportunities for pollution prevention exist in laboratory operation. The EPA has established a preferred hierarchy of environmental management techniques that places pollution prevention as the management option of first choice. Whenever feasible, laboratory personnel should use pollution prevention techniques to address their waste generation (e.g., Sect. 11.1.1). When wastes cannot be feasibly reduced at the source, the Agency recommends recycling as the next best option.

14.2 For information about pollution prevention that may be applicable to laboratories and research institutions, consult *Less is Better: Laboratory Chemical Management for Waste Reduction*, available from the American Chemical Society's Department of Government Relations and Science Policy, 1155 16th Street N.W., Washington D.C. 20036, (202)872-4477.

15.0 Waste Management

15.1 The Environmental Protection Agency requires that laboratory waste management practices be conducted consistent with all applicable rules and regulations. The Agency urges laboratories to protect the air, water, and land by minimizing and controlling all releases from hoods and bench operations, complying with the letter and spirit of any sewer discharge permits and regulations, and by complying with all solid and hazardous waste regulations, particularly the hazardous waste identification rules and land disposal restrictions. For further information on waste management consult *The Waste Management Manual for Laboratory Personnel*, available from the American Chemical Society at the address listed in the Sect. 14.2.

16.0 References

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17.0 TABLES, DIAGRAMS, FLOWCHARTS, AND VALIDATION DATA

TABLE 1. COMPARISON OF PRECISION OF TWO EXTRACTION PERIODS

CORRECTED CHLOROPHYLL *a*

	Sample A(1)		Sample B(2)	
	2 h(3)	24 h(3)	2 h(3)	24 h(3)
Mean Concentration (µg/L)	49.6	52.9	78.6	78.8
Standard Deviation (µg/L)	4.89	2.64	6.21	2.77
Relative Standard Deviation (%)	9.9	5.0	7.9	3.5

[1] Values reported are the mean measured concentrations (n=6) of chlorophyll *a* in the natural water based on a 100-mL filtration volume.

[2] Values reported are the mean measured concentrations (n=9) of the extraction solution. Sample filtration volume was 300 mL.

[3] The length of time that the filters steeped after they were macerated.

TABLE 2. COMPARISON OF PRECISION OF TWO EXTRACTIONS PERIODS FOR Pheophytin *a*

Pheophytin ***a***

	Sample A(1)		Sample B(2)	
	2 h(3)	24 h(3)	2 h(3)	24 h(3)
Mean Concentration (µg/L)	9.22	8.19	13.1	10.61
Standard Deviation (µg/L)	2.36	3.55	3.86	2.29
Relative Standard Deviation (%)	25.6	43.2	29.5	21.6

[1] Values reported are the mean measured concentrations (n=6) of pheophytin *a* in the natural water based on a 100-mL filtration volume.

[2] Values reported are the mean measured concentrations (n=9) of pheophytin *a* the extraction solution. Sample filtration volume was 300 mL.

[3] The length of time that the filters steeped after they were macerated.

TABLE 3. ANALYSES OF USEPA QC SAMPLES

ANALYTE	REFERENCE VALUE	CONFIDENCE LIMITS
Chlorophyll *a*	2.1 µg/L	0.5 to 3.7 µg/L
Pheophytin *a*	0.3 µg/L	-0.2 to 0.8 µg/L

ANALYTE	MEAN MEASURED VALUE	% Relative Standard[1] Deviation
Chlorophyll *a*	2.8 µg/L	1.5
Pheophytin *a*	0.3 µg/L	33

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[1] N = 3

TABLE 4. POOLED ESTIMATED DETECTION LIMITS FOR CHLOROPHYLL *A* METHODS(1)

Method(2)	N(3)	p-EDL(4) (mg/L)
FL -Mod(5)	8	0.096
FL - Std(5)	9	0.082
HPLC	4	0.081
SP-M	15	0.229
SP-T	15	0.104

(1) See Section 13.4.1 for a description of the statistical approach used to determine p-EDLs.

(2) FL-Mod = fluorometric method using special interference filters.

FL-Std = conventional fluorometric method with pheophytin *a* correction.

HPLC = EPA method 447.0

SP-M = EPA method 446.0, monochromatic equation.

SP-T = EPA method 446.0, trichromatic equations.

(3) N = number of labs whose data was used.

(4) The p-EDL was determined with p = 0.01 and q (type II error rate) = 0.05.

(5) Due to the large dilutions required to analyze the solutions by fluorometry, the fluormetric p-EDLs are unrealistically high.

TABLE 5. POOLED PRECISION FOR DUNALIELLA TERTIOLECTI SAMPLES

Method(1)	mLs of culture filtered	N(2)	Mean (mg chl*a*/L)	Std. Dev.	%RSD
Fl-Mod	5	7	0.163	0.037	22.8
	10	7	0.298	0.080	26.7
	50	7	1.684	0.385	22.9
	100	7	3.311	0.656	19.8
Fl-Std	5	8	0.185	0.056	30.4
	10	8	0.341	0.083	24.4
	50	8	1.560	0.311	19.9
	100	8	3.171	0.662	20.9

(1) Fl-Mod = fluorometric method using special interference filters.

Fl-Std = conventional fluorometric method with pheophytin *a* correction.

(2) N = number of volunteer labs whose data was used.

TABLE 6. POOLED PRECISION FOR AMPHIDINIUM CARTERAE SAMPLES

Method(1)	mLs of culture filtered	N(2)	Mean (mg chl*a*/L)	Std. Dev.	%RSD
Fl-Mod	5	7	0.066	0.010	14.6
	10	7	0.142	0.045	31.5
	50	7	0.757	0.208	27.5
	100	7	1.381	0.347	25.1
Fl-Std	5	8	0.076	0.018	23.2
	10	8	0.165	0.040	24.3
	50	8	0.796	0.140	17.5
	100	8	1.508	0.324	21.5

(1) Fl-Mod = fluorometric method using special interference filters.

Fl-Std = conventional fluorometric method with pheophytin *a* correction.

(2) N = number of volunteer labs whose data was used.

TABLE 7. POOLED PRECISION FOR PHAEODACTYLUM TRICORNUTUM SAMPLES

Method[1]	mLs of culture filtered	N[2]	Mean (mg chl*a*/L)	Std. Dev.	%RSD
Fl-Mod	5	7	0.221	0.040	18.0
	10	7	0.462	0.094	20.3
	50	7	2.108	0.491	23.3
	100	7	3.568	1.186	33.2
Fl-Std	5	8	0.214	0.053	24.8
	10	8	0.493	0.091	18.4
	50	8	2.251	0.635	28.2
	100	8	4.173	0.929	22.3

(1) Fl-Mod = fluorometric method using special interference filters.

Fl-Std = conventional fluorometric method with pheophytin *a* correction.

(2) N = number of volunteer labs whose data was used.

NOTE: The phaeodactylum extract contained significant amounts of chlorophyll *c* and chlorophyllide *a* which interferes in chlorophyll *a* measurement in the fluorometric method, therefore, the concentration of chlorophyll *a* is overestimated compared to the HPLC method which separates the three pigments. The FL-Mod interference filters minimize this interference more so than the conventional filters.

TABLE 8. MINIMUM, MEDIAN, AND MAXIMUM PERCENT RECOVERIES BY GENERA, METHOD, AND CONCENTRATION LEVEL

Species	Statistic	Method	Percent Recovery			
			Conc. Level 1	Conc. Level 2	Conc. Level 3	Conc. Level 4
Amphidinium	Minimum	FL-MOD	70	73	75	76
		FL-STD	66	91	91	90
		HPLC	82	85	87	88
		SP-M	36	48	68	64
		SP-T	21	63	71	70
	Median	FL-MOD	105	112	105	104
		FL-STD	109	107	111	109
		HPLC	102	106	112	105
		SP-M	99	101	101	101
		SP-T	95	96	106	107
	Maximum	FL-MOD	121	126	143	146
		FL-STD	156	154	148	148
		HPLC	284	210	131	116
		SP-M	141	133	126	125
		SP-T	115	116	119	117
Dunaliella	Minimum	FL-MOD	162	159	157	156
		FL-STD	179	171	165	164
		HPLC	165	109	64	41
		SP-M	120	188	167	164
		SP-T	167	169	166	165
	Median	FL-MOD	206	246	227	223
		FL-STD	250	228	224	210
		HPLC	252	177	89	80

Table 8 cont'd

Species	Statistic	Method	Percent Recovery			
			Conc. Level 1	Conc. Level 2	Conc. Level 3	Conc. Level 4
		SP-M	240	247	247	243
		SP-T	225	244	256	256
Dunaliella	Maximum	FL-MOD	295	277	287	288
		FL-STD	439	385	276	261
		HPLC	392	273	172	154
		SP-M	342	316	296	293
		SP-T	291	283	283	283
Phaeodactylum	Minimum	FL-MOD	216	183	157	154
		FL-STD	189	220	223	219
		HPLC	150	119	84	75
		SP-M	161	138	156	160
		SP-T	203	195	216	244
	Median	FL-MOD	292	285	250	245
		FL-STD	296	263	254	254
		HPLC	225	203	114	90
		SP-M	287	274	254	253
		SP-T	286	281	277	274
	Maximum	FL-MOD	357	337	320	318
		FL-STD	371	415	415	334
		HPLC	394	289	182	139
		SP-M	446	344	330	328
		SP-T	357	316	318	299

TABLE 9. CHLOROPHYLL *A* CONCENTRATIONS IN MG/L DETERMINED IN FILTERED SEAWATER SAMPLES

Method	Con.[1]	No. Obs.	No. Labs	Mean	Std. Dev.	RSD(%)	Minimum	Median	Maxium
FL-MOD	100	14	7	1.418	0.425	30.0	0.675	1.455	2.060
FL-STD	100	15	8	1.576	0.237	15.0	1.151	1.541	1.977
HPLC	100	10	5	1.384	0.213	15.4	1.080	1.410	1.680
SP-M	100	38	19	1.499	0.219	14.6	0.945	1.533	1.922
SP-T	100	36	18	1.636	0.160	9.8	1.250	1.650	1.948
All Methods	100	113	57	1.533	0.251	16.4	0.657	1.579	2.060

(1) Con = mLs of seawater filtered.