Method 446.0

In Vitro Determination of Chlorophylls *a*, *b*, $c_1 + c_2$ and Pheopigments in Marine And Freshwater Algae by Visible Spectrophotometry

Adapted by

Elizabeth J. Arar

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National Exposure Research Laboratory
Office of Research and Development
U.S. Environmental Protection Agency
Cincinnati, Ohio 45268

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1.0 Scope and Application

1.1 This method provides a procedure for determination of chlorophylls *a* (chl *a*), *b* (chl *b*), $c_1 + c_2$ (chl $c_1 + c_2$) and pheopigments of chlorophyll *a* (pheo *a*) found in marine and freshwater phytoplankton. Chlorophyllide *a* is determined as chl *a*. Visible wavelength spectrophotometry is used to measure the pigments in sub-parts per million (ppm) concentrations. The trichromatic equations of Jeffrey and Humphrey[1] are used to calculate the concentrations of chl *a*, chl *b*, and chl c_1+c_2. Modified monochromatic equations of Lorenzen[2] are used to calculate pheopigment-corrected chl *a* and pheo *a*.

1.2 This method differs from previous descriptions of the spectrophotometric technique in several important aspects. Quality assurance/quality control measures are described in Sect. 9.0. Detailed sample collection and extraction procedures are described in Sect. 8.0, and most importantly, interference data, heretofore only presented in research journals, is included so the analyst may know the potential limitations of the method. Multilaboratory data is included in Section 13.

Analyte	Chemical Abstracts Service Registry Number (CASRN)
Chlorophyll *a*	479-61-8
Chlorophyll *b*	519-62-0
Chlorophyll c_1	18901-56-9
Chlorophyll c_2	27736-03-4

1.3 Instrumental detection limits (IDLs) of 0.08 mg chl *a*/L, 0.093 mg chl *b*/L and 0.085 mg pheo *a*/L in pure solutions of 90% acetone were determined by this laboratory using a 1-cm glass cell. Lower detection limits can be obtained using 2, 5 or 10-cm cells. An IDL for chlorophylls c_1+c_2 was not determined due to commercial unavailability of the pure pigments. Estimated detection limit (EDL) determinations were made by analyzing seven replicate filtered phytoplankton samples containing the pigments of interest. Single-laboratory EDLs (S-EDL) were as follows: chl *a* - 0.037 mg/L, chl *b* - 0.07 mg/L, chl $c_1 + c_2$ - 0.087 mg/L, pheopigment-corrected chl *a* - 0.053 mg/L, and pheo *a* - 0.076 mg/L. The trichromatic equations lead to inaccuracy in the measurement of chlorophylls *b* and c_1+c_2 at chl *a* concentrations greater than ~5X the concentration of the accessory pigment or in the presence of pheo *a*. The upper limit of the linear dynamic range (LDR) for the instrumentation used in this method evaluation was approximately 2.0 absorbance units (AU) which corresponded to pigment concentrations of 27 mg chl *a*/L, 30 mg chl *b*/L and approximately 45 mg pheo *a*/L. No LDR for chl $c_1 + c_2$ was determined. It is highly unlikely that samples containing chl c_1+c_2 at concentrations approaching the upper limit of the LDR will be encountered in nature.

1.4 Chl c_1+c_2 is not commercially available, therefore, the minimum indicator of laboratory performance for this pigment is precision of chl $c_1 + c_2$ determinations in natural samples known to contain the pigments.

1.5 This method uses 90% acetone as the extraction solvent because of its efficiency for extracting chl *a* from most types of algae. (**NOTE:** There is evidence that certain chlorophylls and carotenoids are more thoroughly extracted with methanol[3-5] or dimethyl sulfoxide.[6] Using high performance liquid chromatography (HPLC), Mantoura and Llewellyn[7] found that methanol led to the formation of chl *a* derivative products, whereas 90% acetone did not. Bowles, et al.[5] found that for chl *a* 90% acetone was an effective solvent when the steeping period was optimized for the predominant species present.)

1.6 One of the limitations of absorbance spectrophotometry is low sensitivity. It may be preferable

to use a fluorometric[8-10] or HPLC[11-15] method if high volumes of water (>4 L) must be filtered to obtain detectable quantities of chl *a.* The user should be aware of the inaccuracies of fluorometric methods when chl *b* is also present in the sample.

1.7 This method is for use by analysts experienced in handling photosynthetic pigments and in the operation of visible wavelength spectrophotometers 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 filtration 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 exceeding 24 h, to ensure thorough extraction of the pigments. 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 cell and absorbance is measured at four wavelengths (750, 664, 647 and 630 nm) to determine turbidity, chlorophylls *a*, *b*, and $c_1 + c_2$, respectively. If pheopigment-corrected chl *a* is desired, the sample's absorbance is measured at 750 and 664 nm before acidification and at 750 and 665 nm after acidification with 0.1 N HCl. Absorbance values are entered into a set of equations that utilize the extinction coefficients of the pure pigments in 90% acetone to simultaneously calculate the concentrations of the pigments in a mixed pigment solution. No calibration of the instrument with standard solutions is required. Concentrations are reported in mg/L (ppm).

3.0 Definitions

3.1 Field Replicates -- Separate samples collected at the same time and place under identical circumstances and treated exactly the same throughout field and laboratory procedures. Analyses of field replicates give a measure of the precision associated with sample collection, preservation and storage, as well as with laboratory procedures.

3.2 Instrument Detection Limit (IDL) -- The minimum quantity of analyte or the concentration equivalent that gives an analyte signal equal to three times the standard deviation of a background signal at the selected wavelength, mass, retention time, absorbance line, etc. In this method the instrument is zeroed on a background of 90% acetone resulting in no signal at the measured wavelengths. The IDL is determined instead by serially diluting a solution of known pigment concentration until the signal at the selected wavelength is between .005 and .008 AU.

3.3 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. For this method the LRB is a blank filter that has been extracted as a sample.

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

3.5 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.

3.6 Estimated Detection Limit (EDL) -- The EDL is determined in a manner similar to an EPA MDL. It is not called an MDL in this method because there are known spectral interferences inherent to this method that make 99% confidence that the chlorophyll concentration is greater than zero impossible.

3.7 Quality Control Sample (QCS) -- A solution of method analytes of known concentrations that 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. The USEPA no longer provides QCSs for this method.

4.0 Interferences

4.1 Any compound extracted from the filter or acquired from laboratory contamination that absorbs light between 630 and 665 nm may interfere in the accurate measurement of the method analytes. An absorbance measurement is made at 750 nm to assess turbidity in the

sample. This value is subtracted from the sample's absorbance at 665, 664, 647 and 630 nm. A 750 nm absorbance value that is $\geq$ .005 AU indicates a poorly clarified solution. This is usually remedied by further centrifugation or filtration of the sample prior to analysis.

4.2 The relative amounts of chlorophyll *a*, *b* and c_1 + c_2 vary with the taxonomic composition of the phytoplankton. Due to the spectral overlap of the chlorophylls and pheo *a*, over- or underestimation of the pigments is inevitable in solutions containing all of these pigments.

Chl *a* is overestimated by the trichromatic equation of Jeffrey and Humphrey when pheo *a* is present (Figure 1). Lorenzen's modified monochromatic equation only slightly overestimates chl *a* in the presence of chl *b* (Figure 2). The degree of error in the measurement of any pigment is directly related to the concentration of the interfering pigment. Knowledge of the taxonomic composition of the sample, proper storage and good sample handling technique (to prevent degradation of chl *a*) can aid in determining whether to report trichromatic or pheopigment-corrected chl *a*. If no such knowledge exists, it is advisable to obtain values for all of the pigments and to compare the chl *a* results in light of the apparent concentrations of the other pigments. Obviously, if the chl *a* values vary widely, sound judgement must be used in deciding which pigments, chl *b* and chl c_1 + c_2, or pheo *a*, are in greatest abundance relative to each other and to chl *a*. The method of standard additions, explained in most analytical chemistry textbooks, is recommended when greater accuracy is required.

Accuracy of chl *b* measurements is highly dependent upon the concentration of chl *a* and pheo *a*.[16] In pure solutions of chl *a* and *b*, underestimation of chl *b* is observed with increasing concentrations of chl *a* (Figure 3). Using the method of standard additions, the same phenomenon was confirmed to occur in natural samples. The underestimation of chl *b* is due in part to the spectral component of chl *a* that is subtracted from chl *b* as chl c_1 + c_2 in the trichromatic equation. Chl *a* concentrations that range from 4 to 10 times the concentration of chl *b* lead to 13% to 38% underestimation of chl *b*. The highest chl *b*:chl *a* ratio likely to occur in nature is 1:1.

Pheo *a*:chl *a* ratios rarely exceed 1:1. Pheo *a* is overestimated in the presence of certain carotenoids[16] and when chl *b* is converted to pheo *b* in the acidification step required to determine pheopigment-corrected chl *a* and pheo *a*. The rate of conversion of chl *b* to pheo *b*, however, is slower than that of chl *a* to pheo *a*. It is important, therefore, to allow the minimum time required for conversion of chl *a* to pheo *a* before measuring absorbance at 665 nm. Ninety seconds is recommended by this method.

When a phytoplankton sample's composition is known (i.e., green algae, diatoms, dinoflagellates) Jeffrey and Humphrey's dichromatic equations for chl *a*, *b*, and c_1 + c_2 are more accurate than the trichromatic equations used here.[1]

4.3 Precision and recovery for any of the pigments is related to efficient maceration of the filtered sample and to the steeping period of the macerated filter in the extraction solvent (Table 1). Precision improves with increasing steeping periods. A drawback to prolonged steeping periods, however, is the extraction of interfering pigments. For example, if the primary pigment of interest is chl *a*, extended steeping periods may extract more of the other pigments but not necessarily more chl *a*. Statistical analysis revealed steeping period to be a significant factor in the recovery of chl *b* and pheo *a* from a mixed assemblage containing these pigments in detectable quantities, but not a significant factor in the recovery of chl *a*. Chl *b* and pheo *a* are mutual interferents so that an actual increase in the recovery of chl *b* leads to a slight apparent increase in pheo *a*.

4.4 Sample extracts must be clarified by centrifugation prior to analysis.

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

5.0 Safety

5.1 Each chemical used in this method 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 also should 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 Spectrophotometer -- Visible, multiwavelength, with a bandpass (resolution) not to exceed 2 nm.

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 round-bottomed, 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 (20 KPa).

6.10 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.

6.10.1 Assorted Class A calibrated pipets.

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

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

6.10.4 Glass rods.

6.10.5 Disposable Pasteur type pipets or medicine droppers.

6.10.6 Glass cells for the spectrophotometer, 1, 2, 5 or 10 cms in length. If using multiple cells, they must be matched.

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

6.10.8 Centrifuge tubes, polypropylene or glass, 15-mL capacity with nonpigmented screw-caps.

6.10.9 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 Chl *a* free of chl *b* and chl *b* substantially free of chl *a* may be obtained from a commercial supplier such as Sigma Chemical (St. Louis, MO).

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% ASTM Type I water. Carefully measure 100 mL of the 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) -- Chl *a* (MW = 893.5) and chl *b* (MW = 907.5) from a commercial supplier is shipped in amber glass ampules that have been flame sealed. The dry standards must be stored at -20°C in the dark. Tap the ampule until all the dried pigment is in the bottom of the ampule. In subdued light, carefully break the tip off the ampule. Transfer the entire contents of the ampule into a 25-mL volumetric

flask. Dilute to volume with 90% acetone, label the flask and wrap with aluminum foil to protect from light. Pheo *a* may be prepared by the mild acidification of chl *a* (to .003 N HCl) followed by a 1:1 molar neutralization with a base such as dilute sodium hydroxide solution. When stored in a light- and air-tight container at -20°C, the SSS is stable for at least six months. All dilutions of the SSS must be determined spectrophotometrically using the equations in Sect. 12.

7.8 Laboratory Reagent Blank (LRB) -- A blank filter that 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 Quality Control Sample (QCS) -- Since there are no commercially available QCSs, dilutions of a stock standard 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 and frequency[21] 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. 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 populations, thus chlorophyll *a* concentration, can change in a relatively short period 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 or excessively long filtration times (>10 min) may damage cells and result in loss of chlorophyll. Care must be taken not to overload the filters. Do not increase the vacuum during filtration.

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). Typically, 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, wrap the container with aluminum foil to protect the phytoplankton from light and store the filter at -20°C or -70°C. Short term storage (2 to 4 h) on ice is acceptable, but samples should be stored at -20°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°C for as long as 3½ weeks without significant loss of chl *a*.[22]

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 replicates and QC samples as a continuing check on performance. The laboratory is required to maintain performance records that define the quality of the data generated.

9.2 Initial Demonstration of Performance (Mandatory)

9.2.1 The initial demonstration of performance is used to characterize instrument performance (IDLs and LDRs) and laboratory performance (MDLs and analyses of QCSs) prior to sample analyses.

9.2.2 Standard Reference Material (SRM) 930e (National Institute of Standards and Technology,

Gaithersburg, MD) or other suitable spectrophotometric filter standards that test wavelength accuracy must be analyzed yearly and the results compared to the instrument manufacturer's specifications. If wavelength accuracy is not within manufacturer's specifications, identify and repair the problem.

9.2.3 Linear Dynamic Range (LDR) -- The LDR should be determined by analyzing a minimum of 5 standard solutions ranging in concentration from 1 to 15 mg/L. Perform the linear regression of absorbance response (at pigment's wavelength maximum) 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 absorbance 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 and absorbance response defines the upper limit of the LDR for your instrument. Absorbance responses for samples should be well below the upper limit of the LDR, ideally between .1 and 1.0 AU.

9.2.4 Instrumental Detection Limit (IDL) -- Zero the spectrophotometer with a solution of 90% acetone. Pure pigment in 90% acetone should be serially diluted until it yields a response at the selected wavelength between .005 and .008 AU.

9.2.5 Estimated Detection Limit (EDL) -- At least seven natural phytoplankton samples known to contain the pigments of interest should be collected, extracted and analyzed according to the procedures in Sects. 8 and 11, using clean glassware and apparatus. The concentration of the pigment of interest should be between 2 and 5 times the IDL. Dilution or spiking of the sample extract solution to the appropriate concentration may be necessary. Inaccuracies occur in the measurement of chlorophylls *b* and $c_1 + c_2$ when the chl *a* concentration is greater than ~5X the concentration of the accessory pigment. Perform all calculations to obtain concentration values in mg/L in the extract solution. Calculate the EDL as follows[23]:

$$EDL = (3) X (S)$$

S = Standard deviation of the replicate analyses.

9.2.6 Quality Control Sample (QCS) -- When beginning to use this method, on a quarterly basis or as required to meet data quality needs, verify instrument performance with the analysis of a QCS (Sect. 7.9). 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.

9.2.7 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 trichromatic chl *a* should not exceed 15% for samples that are at least 10X the IDL.

9.2.8 Corrected Chl a -- Multilaboratory testing of this method revealed that many analysts do not adequately mix the acidified sample when determining the corrected chl *a*. The problem manifests itself by highly erratic pheo *a* results, high %RSDs for correctetd 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 2.0 ppm chl *a* solution in 90% acetone. The new analyst should analyze 5-10 separate aliquots, using carefully rinsed 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 acceptable results are obtained.

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 in a sample, fresh samples or field replicates must be analyzed after the contamination has been corrected and acceptable LRB values have been obtained.

10.0 Calibration and Standardization

10.1 Daily calibration of the spectrophotometer is not required when using the equations discussed in this method. It is extremely important, therefore, to perform regular checks on instrument performance. By analyzing a standard reference material such as SRM 930e (National Institute of Standards and Technology, Gaithersburg, MD) at least quarterly, wavelength accuracy can be compared to instrument manufacturer's specifications. Filter kits that allow stray light, bandpass and linearity to be evaluated are also commercially available. Although highly recommended, such kits are not required for this method if the LDR is determined for the pigment of interest and QCSs are routinely analyzed.

10.2 Allow the instrument to warm up for at least 30 min. Use a 90% acetone solution to zero the instrument at all of the selected wavelengths. 750 nm, 664 nm, 647 nm and 630 nm are used for the determination of chl *a*, chl *b* and chl $c_1 + c_2$. 750 nm, 665 nm and 664 nm are used for the determination of pheopigment-corrected chl *a* and pheo *a*. The instrument is now ready to analyze samples.

11.0 Procedure

11.1 Extraction of Filter Samples

11.1.1 For convenience, a 10-mL final extraction volume is described in the following procedure. A larger extraction volume may be necessary if using a low-volume 10-cm cell. On the other hand, a smaller extraction volume can be used to obtain a concentration factor. The filter residue retains 2-3 mL of solution after centrifugation and a 1-cm cell requires approximately 3 mL of solution so that a recommended minimum extraction volume is 6 mL.

11.1.2 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 laboratory 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. After the filter has been converted to a slurry, grind the filter for approximately 1 min at 500 rpm. (**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 the aqueous 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. To reduce the volume of reagent grade solvents used for rinsing between extractions, thoroughly rinse the grinding tube and glass rod with tap water prior to a final rinse with ASTM Type I water and acetone. 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 last filter extracted should be a blank. The entire extraction with transferring and rinsing takes approximately 5 min. Approximately 500 mL of acetone and water waste are generated per 20 samples from the rinsing of glassware and apparatus.

11.1.3 Shake each tube vigorously again before placing them to 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. Tubes should be shaken at least once, preferably two to three times, during the steeping period to allow the extraction solution to have maximum contact with the filter slurry.

11.1.4 After steeping is complete, centrifuge samples for 15 min at 675 g or for 5 min at 1000 g.

11.2 Sample Analysis

11.2.1 The instrument must be zeroed on a 90% acetone solution as described in Sect. 10.2. In subdued lighting, pour or pipet the supernatant of the extracted sample into the glass spectrophotometer cell. If the absorbance at 750 nm exceeds .005 AU, the sample must be recentrifuged or filtered through a glass fiber filter (syringe filter is recommended). The volume of sample required in the instrument's cell must be known if the pheopigment-corrected chl *a* and pheo *a* will be determined so that acidification to the correct acid concentration can be performed. For example, a cell that

holds 3 mL of extraction solution requires .09 mL of the .1 N HCl solution to obtain an acid concentration of .003 N. Measure the sample's absorbance at the selected wavelengths for chl *a*, chl *b* and chl $c_1 + c_2$. Dilute and reanalyze the sample if the signal at the selected wavelength is $\geq$90% of the signal previously determined as the upper limit of the LDR. If pheopigment-corrected chl *a* and pheo *a* will be determined, acidify the sample in the cell to .003 N HCl using the .1 N HCl solution. Use a disposable 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 and measure the sample's absorbance at 750 and 665 nm.

NOTE: Proper mixing of the acidified sample is critical for accurate and precise results.

12.0 Data Analysis and Calculations

12.1 Jeffrey and Humphrey's Trichromatic Equations -- Subtract the absorbance value at 750 nm from the absorbance values at 664, 647 and 630 nm. Calculate the concentrations (mg/L) of chl *a*, *b*, and $c_1 + c_2$ in the extract solution by inserting the 750 nm-*corrected* absorbance values into the following equations:

$C_{E,a}$ = 11.85 (Abs 664) - 1.54 (Abs 647) - .08 (Abs 630)

$C_{E,b}$ = 21.03 (Abs 647) - 5.43 (Abs 664) - 2.66 (Abs 630)

$C_{E,c}$ = 24.52 (Abs 630) - 7.60 (Abs 647) - 1.67 (Abs 664)

where:

$C_{E,a}$ = concentration (mg/L) of chlorophyll *a* in the extraction solution analyzed,

$C_{E,b}$ = concentration (mg/L) of chlorophyll *b* in the extract solution.

$C_{E,c}$ = concentration (mg/L) of chlorophyll $c_1 + c_2$ in the extract solution analyzed.

12.2 Lorenzen's Pheopigment-corrected Chl *a* and Pheo *a* -- Subtract the absorbance values at 750 nm from the absorbance values at 664 and 665 nm. Calculate the concentrations (mg/L) in the extract solution, C_E, by inserting the 750 nm *corrected* absorbance values into the following equations:

$C_{E,a}$ = 26.7(Abs 664_b - Abs 665_a)

$P_{E,a}$ = 26.7 [1.7 X (Abs 665_a) - (Abs 664_b)]

where,

$C_{E,a}$ = concentration (mg/L) of chlorophyll *a* in the extract solution measurted,

$P_{E,a}$ = concentration (mg/L) of pheophytin *a* in the extraction measured.

Abs 664_b = sample absorbance at 664 nm (minus absorbance at 750 nm) measured before acidification, and

Abs 665_a = sample absorbance at 665 nm (minus absorbance at 750 nm) measured after acidification.

12.3 Calculate the conentration of pigment in the whole water sample using the following generalized equation:

$$C_s = \frac{C_E \text{ (a,b, or c) X extract volume (L) X DF}}{\text{sample volume (L) X cell length (cm)}}$$

where:

C_s = concentration (mg/L) of pigment in the whole water sample.

$C_{E(a,b,or\ c)}$ = concentration (mg/l) of pigment in extract measured in the cuvette.

.

extract volume = volume (L) of extract (before any dilutions), typically 0.0104).

DF = any dilution factors.

sample volume = volume (L) of whole water sample that was filtered, and

cell length = optical path length (cm) of cuvette used (typically 1 cm).

For example, calculate the conentration of chlorophyll *a* in the whole water sample as:

$$C_{s,b} = \frac{C_{E,a}\ X\ extract\ volume\ (L)\ DF}{sample\ volume\ (L)\ X\ cell\ length\ (cm)}$$

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

13.0 Method Performance

13.1 Single Laboratory Performance

13.1.1 Replicate analyses were performed on low level dilutions of the pure pigments in 90% acetone. The results, contained in Table 2, give an indication of the variability not attributable to sampling and extraction or pigment interferences.

13.1.2 The IDLs and S-EDLs for the method analytes are reported in Table 3.

13.1.3 Precision (%RSD) for replicate analyses of two distinct mixed assemblages are contained in Table 4.

13.1.4 Three QCS ampules were obtained from the USEPA, analyzed and compared to the reference values in Table 5. (**NOTE:** The USEPA no longer provides pigment QCSs.)

13.2 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 24 volunteer participants in the spectrophotometric methods component that returned data. The primary goals of the study were to determine detection limits and to assess precision and bias (as percent recovery) for select unialgal species, and natural seawater.

13.2.1 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.[24] method that does not assume 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 6. It is referred to as the 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 pooled EDL (p-EDL) for each method. Results (in ppm) are given in Table 6. The standard fluorometric and HPLC methods gave the lowest p-EDLs while the spectrophotometric (monochromatic equations) gave the highest p-EDLs.

13.2.2 To address precision and bias in chlorophyll *a* determination for different algal species three pure uniagal cultures (amphidinium, dunnnaliella 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 respectiave methods. The “true” concentration was assigned by taking the average of the HPLC results for the highest concentration algae sample since chlorophyll *a* is separatead from other interfereing pigments prior to determination. Pooled precision data (%RSD) are presented in Tables 7-9 and accuracy data (as percent recovery) are presented in Table 10. No significant differences in precision were observed across conentrations 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 10) and in this case the median is a better measurement 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 chlorophylide *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 tahe 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 11. Only one filtration volume (100 mL) was provided in duplicate to partaicpant 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 USEPA 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 U.S. 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

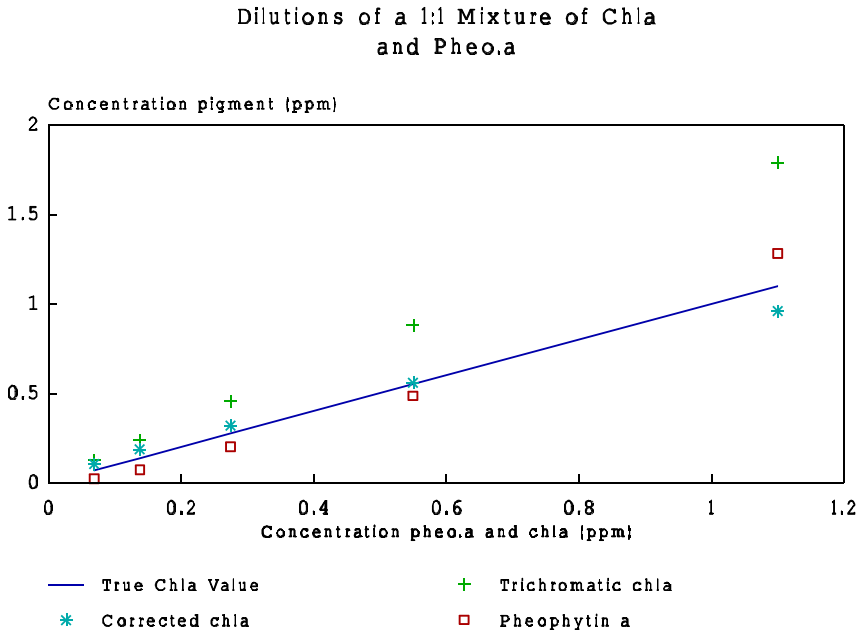

FIGURE 1 - The effect of pheo *a* on calculated pigment concentrations.

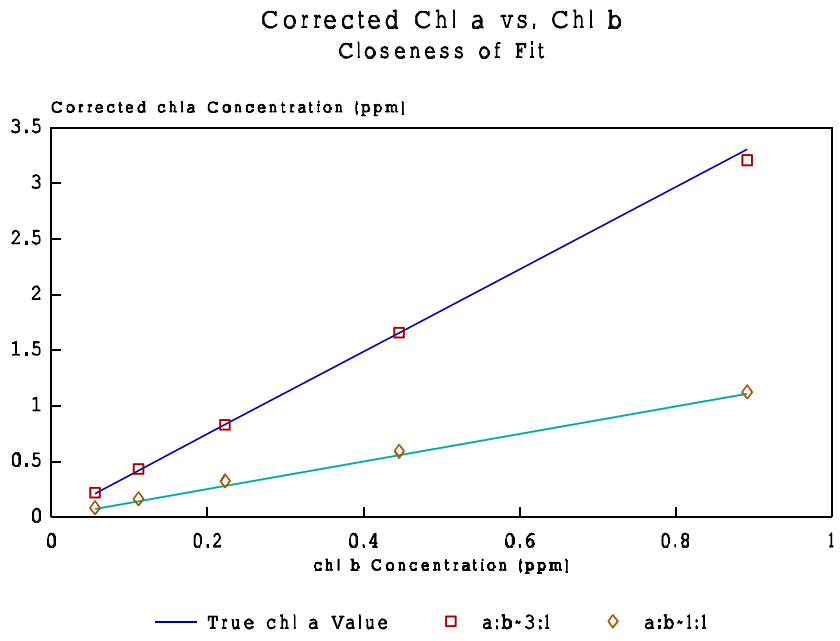

FIGURE 2 - The effect of Chl *b* on pheopigment - corrected Chl *a*.

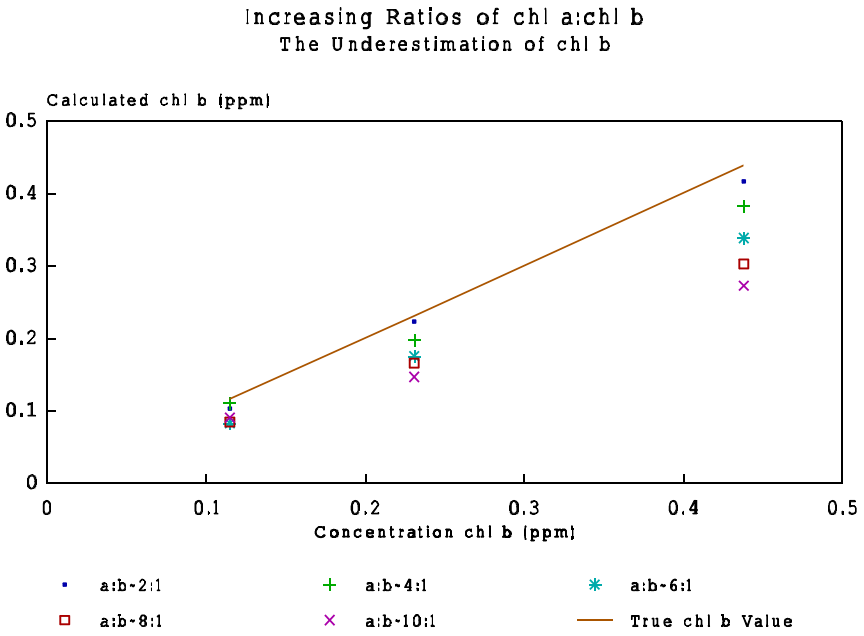

FIGURE 3 - The underestimation of Chl *b* with increasing concentrations of Chl *a*.

TABLE 1. COMPARISON OF PRECISION AND RECOVERY OF PIGMENTS FOR 4 h AND 24 h STEEPING PERIODS

	chl *a*		chl *b*		chl c_1+c_2		pheo *a*		corr *a*	
	4h	24h	4h	24h	4h	24h	4h	24h	4h	24h
N	6	6	6	6	6	6	6	6	6	6
SD	1.22	0.88	0.42	0.21	0.44	0.37	1.08	1.23	1.46	1.04
Mean	26.14	25.73	0.49	1.72	5.87	5.26	1.38	2.88	24.47	23.29
%RSD	24.67	3.40	6.35	12.00	7.43	7.04	78.35	42.62	5.97	4.47

N - Number of samples
SD - Standard deviation
Mean - Concentration in natural water, mg/L
%RSD - Percent relative standard deviation

TABLE 2. REPLICATE ANALYSES OF PURE PIGMENTS AT LOW CONCENTRATIONS

Trichromatic Equations			Modified Monochromatic Equations		
	chl *a*	chl *b*		chl *a*	chl *b*
N	7	7	N	7	6
SD	.000612	.009792	SD	.010091	.011990
Mean	.102 mg/L	.109 mg/L	Mean	.103 mg/L	.171 mg/L
%RSD	.60	8.9	%RSD	9.8	7.0

TABLE 3. INSTRUMENTAL AND METHOD DETECTION LIMITS

INSTRUMENTAL DETECTION LIMITS[1]
(Concentrations in mg/L)

Trichromatic Equations		Modified Monochromatic Equation	
chl *a*	.080	pheo *a*	.085
chl *b*	.093		

S-ESTIMATED DETECTION LIMITS[1]
(Concentrations in mg/L)

Trichromatic Equations		Modified Monochromatic Equation	
chl *a*	.037[2]	chl *a*	.053[2]
chl *b*	.070[2]	pheo *a*	.076[2]
chl $c_1 + c_2$	.087[3]		

[1] Determinations made using a 1-cm path length cell.
[2] Mixed assemblage samples from San Francisco Bay.
[3] Predominantly diatoms from Raritan Bay.

TABLE 4. ANALYSES OF NATURAL SAMPLES

SAN FRANCISCO BAY

	Trichromatic Equations			Modified Monochromatic Equations	
	chl *a*	chl *b*	chl c_1+c_2	pheo *a*	corr *a*
N	7	7	7	7	7
SD	0.0118	0.0062	0.0096	0.0244	0.0168
Mean	0.2097	0.04271	0.03561	0.0806	0.1582
%RSD	5.62	14.50	26.82	30.21	0.64

RARITAN BAY

	Trichromatic Equations			Modified Monochromatic Equations	
	chl *a*	chl *b*	chl c_1+c_2	pheo *a*	corr *a*
N	7	7	7	7	7
SD	0.0732	0.0223	0.0277	0.0697	0.0521
Mean	1.4484	0.0914	0.2867	0.1720	1.3045
%RSD	5.06	24.43	9.65	40.53	3.99

Mean concentrations (mg/L) reported in final extraction volume of 10 mL. Samples were macerated and allowed to steep for approximately 24 h.

N - Number of samples
SD - Standard deviation
Mean - Concentration in natural water
%RSD - Percent relative standard deviation

TABLE 5. ANALYSES OF USEPA QC SAMPLES

Ampule 1 (3 separate ampules, chl *a* only)

	Trichromatic Equations				Modified Monochromatic Equations		
	Mean	Reference	%RSD		Mean	Reference	%RSD
chl *a*	2.54 mg/L	2.59	.61	chl a	2.56 mg/L	2.70	.8
				pheo *a*	ND		

ND - None detected

Ampule 2 (3 separate ampules, all method pigments)

	Trichromatic Equations				Modified Monochromatic Equations		
	Mean	Reference	%RSD		Mean	Reference	%RSD
chl a	4.87 mg/L	4.86	.1	chl *a*	3.70 mg/L	3.76	2.3
chl b	1.12 mg/L	1.02	1.3	pheo a	1.79 mg/L	1.70	4.4
chl $c_1 + c_2$	.29 mg/L	.37	4.9				

TABLE 6. 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	9	0.082
HPLC	4	0.081
SP-M	15	0.229
SP-T	15	0.104

(1) See Section 13.2.1 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 7. POOLED PRECISION FOR AMPHIDINIUM CARTERAE SAMPLES

Method[1]	mLs of culture filtered	N[2]	Mean (mg chla/L)	Std. Dev.	%RSD
SP-M	5	17	0.068	0.026	37.8
	10	19	0.139	0.037	26.6
	50	19	0.679	0.150	22.1
	100	19	1.366	0.205	15
SP-T	5	16	0.059	0.021	35.1
	10	18	0.130	0.027	20.8
	50	18	0.720	0.102	14.2
	100	18	1.408	0.175	12.4

(1) SP-M = Pheophytin *a* - corrected chlorophyll *a* method using monochromatic equations.

SP-T = Trichromatic equations method.

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

TABLE 8. POOLED PRECISION FOR DUNALIELLA TERTIOLECTI SAMPLES

Method[1]	mLs of culture filtered	N[2]	Mean (mg chl*a*/L)	Std. Dev.	%RSD
SP-M	5	19	0.166	0.043	26.0
	10	19	0.344	0.083	24.0
	50	19	1.709	0.213	12.5
	100	19	3.268	0.631	19.3
SP-T	5	18	0.161	0.030	18.4
	10	18	0.339	0.058	17.1
	50	18	1.809	0.190	10.5
	100	18	3.500	0.524	15.0

(1) SP-M = Pheophytin *a* corrected chlorophyll *a* method using monochromatic equations.

SP-T = Trichromatic equationss method.

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

TABLE 9. POOLED PRECISION FOR PHAEODACTYLUM TRICORNUTUM SAMPLES

Method[1]	mLs of culture filtered	N[2]	Mean (mg chl*a*/L)	Std. Dev.	%RSD
SP-M	5	19	0.223	0.054	24.1
	10	19	0.456	0.091	19.9
	50	19	2.042	0.454	22.2
	100	19	4.083	0.694	17.0
SP-T	5	18	0.224	0.031	14.0
	10	18	0.465	0.077	16.5
	50	18	2.223	0.217	9.7
	100	18	4.422	0.317	7.2

(1) SP-M = Pheophytin *a* corrected chorophyll *a* method using monochromatic equations.

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

TABLE 10. 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
		SP-M	240	247	247	243

Table 10 cont'd

Species	Statistic	Method	Percent Recovery			
			Conc. Level 1	Conc. Level 2	Conc. Level 3	Conc. Level 4
		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 11. 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.