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Ocean Optics Protocols For Satellite Ocean Color Sensor Validation, Revision 2

Giulietta S. Fargion
SAIC General Sciences Corporation, Beltsville, Maryland

James L. Mueller
Center for Hydro-Optics and Remote Sensing
San Diego, State University, San Diego, California

National Aeronautical and
Space administration

Goddard Space Flight Space Center
Greenbelt, Maryland 20771

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Chapter 14

Fluorometric Chlorophyll *a*: Sampling, Laboratory Methods, and Data Analysis Protocols

Charles C. Trees[1], Robert R. Bidigare[2], David M. Karl[2] and Laurie Van Heukelem[3]

[1]*Center for Hydro-Optics & Remote Sensing, San Diego State University, California*
[2] *Department of Oceanography, University of Hawaii, Hawaii*
[3]*Horn Point Laboratory, University of Maryland Center for Environmental Science, Horn Point, Maryland*

14.1 INTRODUCTION

In addition to HPLC analyses, it is recommended that the standard fluorometric methodology used for measuring chlorophylls and pheopigments also be applied to (i) the same extracted pigment samples used for HPLC analysis, and (ii) additional independent samples. Analysis of fluorometric chlorophyll *a* concentration is a far simpler procedure than HPLC analysis, especially at sea. On a given research cruise, therefore, it is economically feasible to acquire and process many more fluorometric than HPLC samples and to statistically relate fluorometric and HPLC chlorophyll a concentrations using linear regression analysis. This additional analysis will also enable a direct link to the historical bio-optical algorithms and database development during the CZCS validation experiments.

Protocols for fluorometric determination of the concentrations of chlorophyll and pheopigments were developed initially by Yentsch and Menzel (1963) and Holm-Hansen et al. (1965), and are described in detail by Strickland and Parsons (1972). Holm-Hansen et al. (1965) and Strickland and Parsons (1972) used first principles of fluorescence spectroscopy to derive these fluorometric equations. The equation proposed by Yentsch and Menzel (1963) is only indirectly linked to first principles, through debatable assumptions, and its use is not recommended. Although these measurements have been shown to contain errors as compared to HPLC determinations (Trees et al. 1985; Smith et al. 1987; Hoepffner and Sathyendranath 1992; Bianchi et al. 1995; Tester et al. 1995), the CZCS phytoplankton pigment concentration algorithms were based on them entirely. The SeaWiFS protocols for this analysis will be those given in Strickland and Parsons (1972) as updated by this protocol.

Pigment databases generally show a log-normal distribution, which is consistent with that proposed by Campbell (1995) for bio-optical properties. Therefore, it is appropriate to perform log-linear regressions on HPLC determined total chlorophyll *a* (chlorophyllide *a*, chlorophyll *a* epimer, chlorophyll *a* allomer, monovinyl chlorophyll *a* and divinyl chlorophyll *a*) and fluorometrically determined chlorophyll, using model I regressions. Standard Model I regressions were selected because HPLC determined total chlorophyll *a* concentrations are to be predicted from fluorometrically determined chlorophyll [Model I regressions are appropriate for both predictions and determining functional relationships, whereas Model II regressions should not be used to predict values of y given x (page 543, Sokal and Rohlf, 1995)]. Examples of regression models predicting log HPLC total chlorophyll *a* (following Chapter 20 HPLC protocols) from log fluorometric chlorophyll *a* for three cruises in different geographic areas are shown in Figures. 14.1, 14.2, and 14.3. There are statistically significant differences, although for the Gulf of California (GoCal November 1996, Figure 14.3) there seems to be a reasonably good agreement. One to one ratios have been found for other geographic areas, although it can have a seasonal cycle within each area. Therefore, the offset between HPLC total chlorophyll *a* and fluorometric chlorophyll must be determined for a selected number of samples for each cruise, so that a scaling factor can be applied to other fluorometric samples, if necessary.

Absorption of light in seawater, or any other medium, is a volumetric process, even though the volume absorption coefficient may vary with the density of the medium. For ocean color and optical analyses, therefore, the concentration of chlorophyll a shall be expressed in units of mass per unit volume of seawater, either in $\mu g\ L^{-1}$, or $mg\ m^{-3}$.

This differs from the JGOFS protocols, which specify that concentrations in seawater of chlorophyll a and pheopigments should be expressed in $\mu g\ kg^{-1}$.

14.2 SAMPLING ACQUISITION AND STORAGE

Water samples should be taken using Niskin bottles or equivalents at the site of, and simultaneously with, the surface in-water upwelled radiance and reflectance measurements, and at depth increments sufficient to resolve variability within at least the top optical depth.

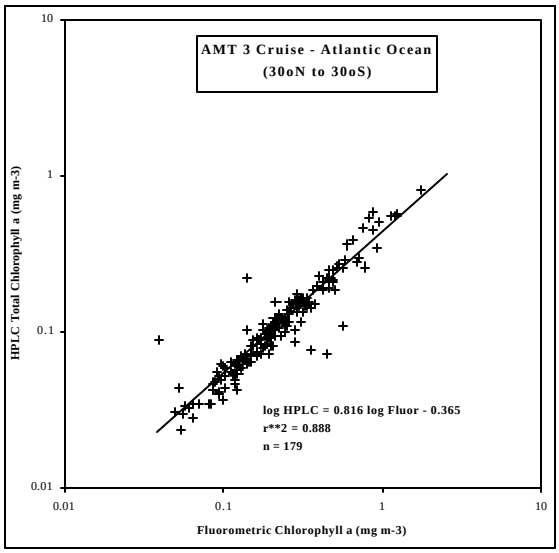


Figure 14.1 Comparisons between fluorometrically determined chlorophyll and HPLC determined total chlorophyll *a* (chlorophyllide *a*, chlorophyll *a* epimer, chlorophyll *a* allomer, monovinyl chlorophyll *a*, and divinyl chlorophyll *a*) from samples collected during Atlantic Meridional Transect 3 cruise (30°N to 30°S, October 1996).

The K(z), profiles over this layer will be used to compute optically weighted, near-surface pigment concentration for bio-optical algorithm development (Gordon and Clark 1980). When possible, samples should be acquired at several depths distributed throughout the upper 200 m of the water column [or in turbid water, up to seven diffuse attenuation depths, $\ln((E(0)/E(z))=7$], to provide a basis for relating fluorescence signals to pigment mass concentration.

Samples should be filtered as soon as possible after collection. If processing must be delayed (>1 hr), hold samples on ice or at 4°C and protected from exposure to light. For periods longer than several hours, the samples should be stored in liquid nitrogen. Use opaque sample bottles, because even brief exposure to light during sampling and/or storage might alter pigment values.

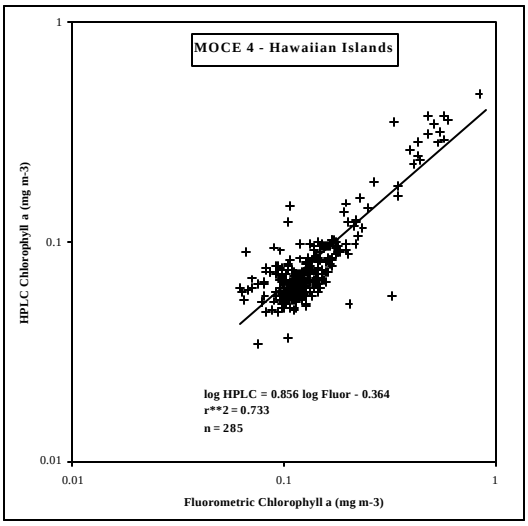


Figure 14.2 Same as Figure 14.1 for data collected during the Marine Optical Characterization Experiment (MOCE) 4 cruise.

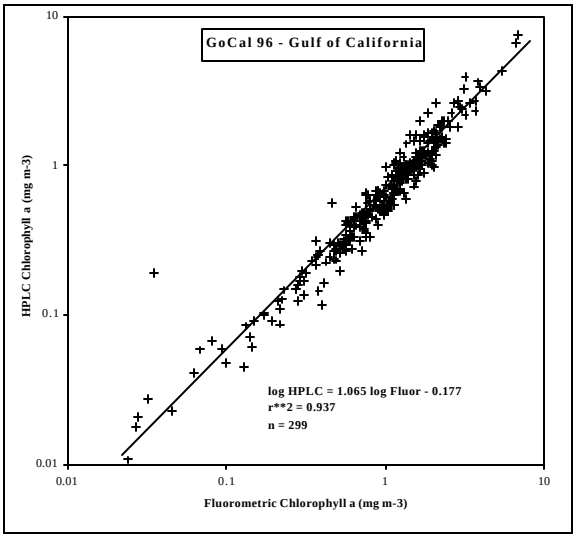


Figure 14.3 Same as Figure 14.1 for data collected during the Gulf of California cruise (Gulf of California, November 1996).

Filtration

Whatman GF/F glass fiber filters, approximately 0.7 µm pore size, are preferred for removing phytoplankton from water. The glass fibers assist in breaking the cells during grinding

and no precipitate forms after acidification. Inert membrane filters, such as polyester filters, may be used when size fraction filtration is required. In addition, it is also recommended to filter a replicate sample through a GF/F to determine the total concentration (summing the various size fractionated concentrations may not produce an accurate estimate of the total, because of the potential for cell disruption during filtration). Twenty-five mm diameter GF/F glass fiber filters should be used with a vacuum or positive pressure with a pressure differential equivalent to 180-200 mm of mercury. Large filtration volumes are not required, because of the increased sensitivity of the fluorescence measurement.

There has been an ongoing discussion on filter types and retention efficiencies for natural samples. Phinney & Yentsch (1985) showed the inadequacy of GF/F filters for retaining chlorophyll *a* in oligotrophic waters, as did Dickson and Wheeler (1993) for samples from the North Pacific. In response to Dickson and Wheeler (1993), Chavez et al. (1995) compared samples collected in the Pacific Ocean using GF/F and 0.2 μm membrane filters with small filtered volumes (100-540 mL). Their results for small volumes showed a very close agreement between the two filter types with GF/F filters having only a slightly positive 5% bias.

Filtration volume can directly affect the retention efficiency for GF/F filters. Particles can be retained by filters through a variety of ways, such as filter sieving, filter adsorption, electrostatic and van der Waals attractions (Brock, 1983). It is known that Whatman GF/F filters can retain particles much smaller than their rated pore size. With Nuclepore filters, when water flows through the pores, streamlines are formed that can align small particles longitudinally (cell diameter becomes important with these filters). Generally, at small volumes (100-300 mL) filter adsorption, and electrostatic and van der Waals attractions are important, whereas at larger volumes (> 2,000 mL) sieving dominates. This has been tested in oligotrophic waters off Hawaii in which small (< 500 mL) and large volumes (> 2-4 liters) retained similar amounts of chlorophyll *a*, whereas intermediate volumes had lower concentrations. As a general rule, it is recommended that the following volumes be filtered for these water types: 0.5-1.0 liter for oligotrophic, 0.2-0.5 liter for mesotrophic, and 0.1 liter and less for eutrophic water.

It is recommended that seawater samples not be pre-filtered to remove large zooplankton and particles as this might result in the exclusion of pigment-containing colonial and chain-forming phytoplankton, e.g., diatoms and *Trichodesmium* sp. Large zooplankton should be removed following filtration using forceps.

Sample Handling, and Storage

Samples should be filtered as quickly as possible after collection and stored immediately in liquid nitrogen. Liquid nitrogen is the best method for storing samples with minimum degradation for short, as well as, longer storage times (e.g. 1 year). Placing samples in liquid nitrogen also assists in pigment extraction by weakening the cell wall and membrane during this rapid temperature change. Ultra-cold freezers (-90^{o}C) can be used for storage, although they have not been tested for longer than 60 days (Jeffrey et al. 1997). Conventional deep freezers should not be used for storing samples more than 20 hours before transferring them to an ultra-cold freezer, or liquid nitrogen. Again, storage of samples in liquid nitrogen immediately after filtration is the preferred method. The addition of $MgCO_3$ at the end of the filtration process to stabilize chlorophyll has not been used for many years as a routine oceanographic method, because of the uncertainty in pigment absorption by $MgCO_3$.

If samples are to be stored for any length of time prior to fluorometric analysis, they should be folded in half with the filtered halves facing in. This eliminates problems of rubbing particles off the filter during placement in sample containers and storage. The easiest and least expensive sample container is aluminum foil. Cut small pieces of heavy-duty aluminum foil into approximately 4 cm squares. Fold this in half and using a fine-point permanent marker write a short sample identifier (e.g. first letter of the cruise and a sequential numbering scheme) on the foil. Writing on the folded foil, prior to placement of the filter, alleviates problems of puncturing the foil with the marking pen, as well as improving the legibility of the sample identifier. Take the folded filter and place it in the aluminum foil. Fold the three open

sides to form an envelope that is only slightly larger than the folded filter (~3cm x 1.5cm).

This protocol will minimize the size requirement of the storage container. Cryogenic tubes, or HistoPrep tissue capsules, can be used, although they require a larger storage space, and unless reused, are more expensive than aluminum foil. If fluorometric analysis is to be done soon after collection, it is still recommended to place the samples in liquid nitrogen to assist in pigment extraction and then place them immediately in chilled 90% acetone.

Recordkeeping

Information regarding sample identification should be logged in a laboratory notebook with the analyst's initials. For each filter sample record the sample identifier (as written on the sample container), station number for the cruise, water volume filtered (V_{FILT}) in mL, and depth of the water sample, together with the date, time, latitude, and longitude of the bottle cast during which the sample was acquired.

14.2 LABORATORY METHODS FOR FLUOROMETRIC DETERMINATION OF CHL. *a* AND PHEOPIGMENT CONCENTRATIONS

Chlorophyll and pheopigments can be determined using either a Turner Designs (or Sequoia) fluorometers equipped with the standard light sources and Corning excitation and emission filters, following the manufacture's recommendation for measuring extracted chlorophyll. The fluorometric instrument should be warmed-up for at least 30 to 45 minutes prior to making measurements. Because of the acidification requirement for the standard fluorometric method (Holm-Hansen et al., 1965), differences in excitation and emission wavelength bands between fluorometers can produce uncertainties (Trees et al., 1985). The sensitivity of an instrument to differentiate between chlorophyll and pheopigment , which is a function of the excitation wavelength, is measured during calibration of the fluorometer and is called the tau factor (τ). Saijo and Nishizawa (1969) have shown that τ can vary from 1 to 11.5, depending upon the excitation wavelength (410-440 nm). A comparison between Turner Designs analog (Model 10-005R) and Turner Designs digital (Model 10-AU-005, Black) fluorometers showed statistically significant differences for 42 oceanic samples (slope = 1.06), even though both were calibrated with exactly the same standards (Figure 14.4). Obviously, there were some differences in the excitation bands for the two fluorometers.

Fluorometer Calibrations

Bench fluorometers used to measure concentrations of extracted chlorophyll and pheopigments should be calibrated using authentic chlorophyll *a* standards as prescribed in the HPLC Protocols. Chlorophyll *a* can be purchased from Sigma Chemical Co. (St. Louis, MO 63178, USA). The concentration of the standard, in the appropriate solvent, must be determined using a monochromator-based spectrophotometer prior to calibration of the fluorometer. The recommended extinction coefficient for chlorophyll *a* in several solvents can be found in Appendix E of Jeffrey et al. (1997). Absorbance is measured in a 1 cm cuvette at the peak wavelength λ_{max} and 750 nm (to correct for light scattering). The bandwidth of the spectrophotometer should be between 0.5 and 2 µm with the standard concentration beign such that the absorbance value ranges between 0.08 to 1.0 optical density. Concentration of the standard is calculated as follows:

$$C_{STD} = \frac{1\,0^6[A(\lambda_{max}) - A(750)]}{b\ x\ E_{1cm}}, \qquad (14.1)$$

where C_{STD} is the concentration (µg L^{-1}) of the chlorophyll *a* standard, $A(\lambda_{max})$ and $A(750)$ are absorbances at λ_{max} and 750 nm, *b* is the pathlength of cuvette (cm), and E_{1cm} is the specific absorption coefficient (L g^{-1} cm^{-1}) of chlorophyll *a* in 90% acetone. For 90% acetone E_{1cm}=87.67 L g^{-1} cm^{-1} , and for 100% acetone E_{1cm}=88.15 L g^{-1} cm^{-1}, when applied to the absorption measured at the peak wavelength λ_{max} (Jeffrey et al. 1997, Appendix E). The peak wavelength λ_{max} must be determined by inspection of the measured spectrum, because its shift results from interactions

between the particular solvent and pigment compounds measured. Standards stored under nitrogen in the dark at -20°C are stable for about one month. If the fluorometer has been shipped for a cruise, or if it has been unused for several weeks, it is strongly recommended that it be recalibrated with an authentic chlorophyll *a* standard. The use of solid standards, like those provided by Turner Designs and others, can only provide a check for instrumental drift. They cannot be used as primary pigment standards. The stock chlorophyll *a* standard, with its concentration measured on a spectrophotometer as described above, should be diluted using calibrated gas-tight syringes, and Class A volumetric pipettes and flasks. The minimum number of dilutions of the stock standard for calibrating a fluorometer depends on whether it is a digital model (Turner Designs 10-AU-005), or it is an analog model with a mechanical mode (e.g. Turner Designs 10-005) for changing sensitivity. A minimum of 5 dilutions is required for calibrating a digital fluorometer. Fluorometers with a variety of door settings, such as the Turner Designs Model 10-005, must be calibrated for each door setting using at least three standard concentrations per door. The diluted standard pigment concentrations used in calibrating the fluorometer must bracket the range of concentrations found in the samples being analyzed.

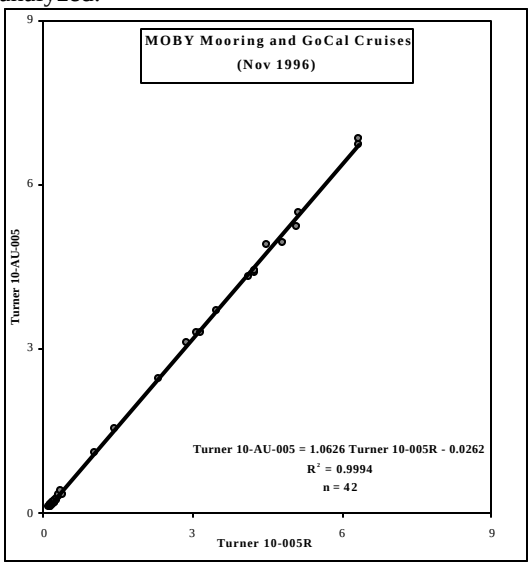


Figure 14.4 Comparison of fluorometrically determined chlorophyll a using the VisLab Turner Fluorometer (10-005R) and the Moss Landing Marine Laboratory Turner Fluorometer (10-AU-005). Samples were analyzed from a MOBY Nov 96 cruise and a Gulf of California cruise (Mueller, Nov 96).

Each diluted chlorophyll *a* standard is placed in the fluorometer and the signal (F_b) is recorded, after waiting a short period of time (60 seconds) for it to stabilize. The standard is removed and diluted HCL acid (2 drops of 5%, or 1 drop of 10%) is added and mixed within the test tube. The tube is then placed back into the fluorometer, and after stabilization, the acidified fluorescence signal (F_a) is recorded. Following acidification of the chlorophyll *a* standard, the fluorescence signal stabilizes relatively quickly. This is not the case for natural samples that contain a mixture of pigment compounds, however, and stabilization time may vary from sample to sample. Stabilization time has to be the same for both pigment standards and for natural samples. To minimize this source of uncertainty, and to standardize this measurement technique, it is recommended that both acidified natural sample and acidified pigment standards be allowed to react with the acid for one minute prior to recording the acidified fluorescence signal (F_a). Two drops of 5% v/v hydrochloric acid is added to each of the pigment standards and natural samples. Once the acid is added, the sample in the test tube should be mixed by inverting the tube several times, using parafilm as a stopper. All fluorometric measurements for both pigment standards and natural samples should be carried out at room temperature. A 90% acetone blank (Blk_b) and a acidified acetone blank (Blk_a) should also be measured. Generally, the acidified blank (Blk_a) has been found to be equal to the non-acidified blank (Blk_b). The fluorometer's sensitivity to pheopigments, τ, is calculated as

$$\boldsymbol{t} = \frac{F_b - Blk_b}{F_a - Blk_a}, \qquad (14.2)$$

and is averaged over all concentrations of the chlorophyll *a* standard. For the mechanical door model fluorometers, data from the higher gain door settings will often become noisy and computed τ values will begin to decrease. These data should be excluded from the average. The fluorometer's response factor, F_R (μg L^{-1} per fluorescence signal) is determined as the slope of the simple linear regression equation

$$C_{STD} = F_R \left(F_b - Blk_b \right), \qquad (14.3)$$

calculated for the sample of diluted concentrations of the pigment standard, and forcing a zero intercept. With a digital fluorometer, the regression analysis is applied to the data from the entire 5, or more, concentrations and a single F_R factor is

determined for the instrument. With a mechanical fluorometer, the regression is applied to the data from the 3, or more, concentrations of the standard, and a separate F_R factor is determined, for each door setting. As a means of monitoring an instrument's performance, F_R factors from successive calibrations should be charted as functions of time. These quality control graphs should be retained with the data analysis logbooks to document the quality of each data set for which that fluorometer is used.

Solvent Preparation.

It is recommended that 90% acetone (v/v) be used to extract pigments for the fluorometric analysis. Richard and Thompson (1952) were the first to propose 90% acetone as a solvent to extract pigments from marine phytoplankton. Their results indicated improved extraction efficiencies, as well as minimizing the activity of the naturally-occurring chlorophyllase enzyme, which degrades the pigment. With a graduated cylinder, make up 90% acetone by first pouring in the distilled water, followed by 100% acetone. Using volumetric pipettes, or auto-pipettes, accurately measure 8 to 10 mls of 90% acetone and place it in a centrifuge tube. Record this volume as V_{EXT}. A number of such tubes containing acetone are then stored in a freezer and individually removed as filter samples are collected. Pre-chilling the solvent in this way reduces the possibility of temperature induced pigment degradation.

Extraction

Filters are removed from liquid nitrogen and placed in the chilled centrifuge tubes for extraction in V_{EXT} mL of 90% acetone. Samples are disrupted by sonication and allowed to extract at 0^oC for 24 h. Alternatively, the cells can be mechanically disrupted using a glass/Teflon tissue grinder and allowed to extract at 0^oC for 24 h. If after disrupting the cells, it is necessary to rinse the tissue grinder, or mortar and pestle, then a known volume of 90% acetone, measured using a Class A volumetric pipette, should be used. The ease at which the pigments are removed from the cells varies considerably with different phytoplankton. In all cases, freezing the sample filters in liquid nitrogen improves extraction efficiency. Prior to analysis, pigment extracts are swirled into a vortex to remove particles from the sides of the tube, and then centrifuged to minimize cellular debris.

Measurement

Following the same measurement procedure described above, under *Fluorometer Calibration*, each extracted sample is placed in the fluorometer and its non-acidified and acidified responses, Fb and Fa, are measured and recorded. The concentration of chlorophyll [Chl] ($\mu g\ L^{-1}$) in the sample is calculated as

$$[Chl] = (F_b - F_a - Blk_b + Blk_a)\frac{t}{t-1}F_R\frac{V_{EXT}}{V_{FILT}}, \quad (14.4)$$

and pheopigments concentration [Pheo] ($\mu g\ L^{-1}$) as

$$[Pheo] = \{(F_a - Blk_a)t - (F_b - Blk_b)\}\frac{t}{t-1}F_R\frac{V_{EXT}}{V_{FILT}}, \quad (14.5)$$

where volumes extracted V_{EXT} and filtered V_{FILT} are in mL. Pheopigment concentrations determined using the standard fluorometric method of Holm-Hansen et al. (1965) have not been reported in published articles for many years. This is based on the fact that (i) there is always a residual amount of pheopigments in all natural samples (Smith and Baker, 1978; 25% of the summed chlorophyll plus pheopigment), (ii) pheopigment concentrations are overestimated in the presence of chlorophyll *b* (Lorenzen and Jeffrey, 1980; Vernet and Lorenzen, 1987), and (iii) HPLC measured pheopigments, generally contribute very little to the chlorophyll *a* pigment pool (e.g., Hallegraeff, 1981; Everitt et al., 1990; and Bricaud et al., 1995). Trees et al. (2000) assembled an extensive HPLC pigment database (5,617 samples) extending over a decade of sampling and analysis, and includes a variety of environments ranging from freshwater to marine, oligotrophic to eutrophic, and tropical to polar, and found that the average pheopigment to chlorophyll *a* ratio was only 0.037. This global scale result emphasizes the problems associated with estimating pheopigments using the standard fluorometric method.

14.3 *In Situ* CHLOROPHYLL a FLUORESCENCE PROFILES

An *in situ* fluorometer should be employed to measure a continuous profile of chlorophyll fluorescence. The fluorometer should be mounted

on the same underwater package as the water sampler, ideally together with a CTD, transmissometer and other IOP sensors. In some cases it may be desirable to also include a radiometer on this package, if shading effects associated with the package and/or ship are not significant.

In situ fluorometers produce nearly continuous profiles of artificially stimulated fluorescence. Fluorometer data (in volts) should be corrected by subtracting an offset, determined by shading the instrument on deck. These unscaled fluorescence responses are adequate to provide guidance in K-profile analysis and interpretation.

To produce vertical continuous profiles of pigment concentration, HPLC-derived pigment concentrations from water samples taken at discrete depths may be interpolated, with the aid of *in situ* fluorescence profiles. These *fluorescence interpolated* profiles should then be used with $K_d(z,\lambda)$ profiles to compute optically weighted pigment concentration over the top attenuation length (Gordon and Clark 1980).

The A/D channel used to acquire and record signal voltages from the *in situ* fluorometer must be calibrated, and its temperature-dependent response to known voltage inputs characterized. The range dependent A/D bias coefficients should be determined at approximately 5^0 C intervals over the range from 0-25^0 C to characterize the temperature sensitivity of the data acquisition system.

Zero fluorescence offsets should be measured on deck before and after each cast; the optical windows should be shaded to avoid contamination of the zero offset value by ambient light. Before each cast, the fluorometer windows should be cleaned following the manufacturer's instructions.

14.4 PROTOCOL STATUS AND FUTURE DIRECTIONS FOR RESEARCH

In order to minimize interferences caused by the overlapping excitation and emission wavebands of chlorophylls *a*, *b*, *c* and pheopigments, Turner Designs (Sunnyvale, CA) recently introduced the multi-spectral fluorometer TD-700. This instrument was recently beta-tested using samples collected at the US JGOFS Hawaii Ocean Time-series Station ALOHA (22.75^0N, 158^0W). An annual set of monthly pigment samples (0-200 m) was analyzed by HPLC using the protocols described in Chapter 20. Extracts were subsequently diluted and analyzed with the TD-700. Model I regression analyses, which summarize these results are given below (pigment concentrations are expressed as ng L-1).

HPLC Chl *a* = 0.80[TD-700 Chl *a*] + 25.98 (r^2 = 0.916),

HPLC Chl *b* = 0.80[TD-700 Chl *b*] + 11.74 (r^2 = 0.843),

HPLC Chl *c* = 1.21[TD-700 Chl *c*] + 3.40 (r^2 = 0.867).

It is interesting noteworthy that the TD-700 did not detect pheopigments in any of the samples analyzed.

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