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# Preface

The purpose of this technical report is to provide current documentation of the Sensor Intercomparison and Merger for Biological and Interdisciplinary Oceanic Studies (SIMBIOS) Project activities, NASA Research Announcement (NRA) research status, satellite data processing, data product validation, and field calibration. This documentation is necessary to ensure that critical information is related to the scientific community and NASA management. This critical information includes the technical difficulties and challenges of validating and combining ocean color data from an array of independent satellite systems to form consistent and accurate global bio-optical time series products. This technical report is not meant as a substitute for scientific literature. Instead, it will provide a ready and responsive vehicle for the multitude of technical reports issued by an operational project. This particular document focus on the variability in chlorophyll pigment measurements resulting from differences in methodologies and laboratories conducting the pigment analysis.

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# *Prologue*
# An Overview of SIMBIOS Project Chlorophyll Round Robin Activities

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The Sea-viewing Wide Field-of-view Sensor (SeaWiFS) and Sensor Intercomparison and Merger for Biological and Interdisciplinary Oceanic Studies (SIMBIOS) Projects have invested heavily in activities focused on the improvement of *in situ* radiometric data (Hooker and McClain, 2000; Hooker and Maritorena, 2000). The encouraging results achieved with the optical round robins of SeaWiFS Intercalibration Round-Robin Experiments (SIRREXs) have turned attention to the uncertainties in the pigment measurements. More specifically, the SIMBIOS Project wished to evaluate the variance in pigment data, particularly the data submitted to the SeaWiFS Bio-optical Archive and Storage System (SeaBASS) database by the SIMBIOS Team under the NRA-96 contracts. SeaBASS data holdings are typically used for algorithm development and post launch validation of several ocean color missions, and it has become clear that pigment analyses were subject to more uncertainty that originally thought.

During the SIMBIOS Science Team meeting in San Diego (1998) there was considerable discussion on high performance liquid chromatography (HPLC) –derived versus fluorometrically (FL) - derived chlorophyll determinations. Some questions included:

- What combination of HPLC derived pigments are needed to compare to FL derived chlorophyll ?
- How consistent are the chlorophyll determinations by different laboratories (HPLC and FL)?
- What protocols must be used to process HPLC measurements? Several recommendations were made concerning the measurement of pigments, including adopting Joint Global Ocean Flux Study (JGOFS) protocols (UNESCO, 1994).

Optical methods of chlorophyll detection (such as FL) can significantly under- or overestimate chlorophyll *a* concentrations, because of the overlap of the absorption and fluorescence bands of co-occurring chlorophyll *b* and *c*, chlorophyll degradation products, and accessory pigments (Trees et al., 1985; Smith et al. 1987; Hoepffner and Sathyendranath, 1992; Bianchi et al., 1995; Tester et al. 1995; Mantoura et al., 1997; Trees et al. 2000; Van Heukelem and Thomas, 2001).

In spring of 1999, the SIMBIOS Project set up a joint round robin with the Office of Naval Research (ONR) that included eight SIMBIOS funded Principal Investigators (PI's) and one investigator from the Hyperspectral Coastal Ocean Dynamics Experiment project (HyCODE). The round robin was conducted by the University of Maryland, Horn Point Environmental Laboratory (HPL). The SIMBIOS Project set the goals of the round robin and worked on issues with the team and HPL. The round robin goals were: (1) to evaluate the discrepancies between FL and HPLC methods while measuring a chlorophyll *a* (chl *a*) standard and ocean samples; (2) to do an inter-calibration and inter-comparison among current SIMBIOS PIs (FL and HPLC methods); and (3) to document the procedure used, from collecting the field data to the laboratories analyses, with several questionnaires. Discrepancies between HPLC and fluorometrically

derived chl *a* were investigated based on the following procedure: HPL prepared filters (simulating field samples) and unknown solutions (of chl *a* only and chl *a* + divinyl (DV) chl *a*) and distributed these along with chl *a* standards (to normalize calibrations). The results of laboratory-prepared unknowns aided the understanding of HPLC/fluorometer discrepancies when participants analyzed field samples, which they collected from their SIMBIOS funded sites for this study (total = 18 sites). Each participant sent replicate sets of the field samples to the reference laboratory (HPL), where they were analyzed using HPLC and fluorometer procedures currently used at HPL (Van Heukelem and Thomas, 2001). HPLC field results from the reference laboratory averaged 4% ($\pm$ 16%) less than the fluorometer values, whereas HPLC field results from participants (considered collectively) averaged 6% ($\pm$57%) less than the fluorometer values ($\pm$ indicates 95% confidence limits). Some HPLC results were inaccurate because of injection conditions, inability to accurately quantify DV chl *a*, reporting practices and inaccurate assessment of extraction volumes. HPLC methods not affected by these limitations were inaccurate relative to all laboratory-prepared unknowns by no more than 7% and fluorometer methods were inaccurate by no more than 11%. Factors affecting HPLC/fluorometer discrepancies specifically related to field samples included lack of homogeneity among replicate filters, extraction procedures that differed between HPLC and fluorometer filters, and inherent differences between HPLC and fluorometer analyses. The range of discrepancies associated with extraction mode or homogeneity among filters was 5 times that associated with inherent differences between analysis modes.

Furthermore, in the following years, the SIMBIOS Project supported a revision of the "Ocean optics protocols for satellite ocean color sensor validation, revision 2 and revision 3" (Fargion and Mueller, 2000 and 2002) and supports a new strategy of having one laboratory process all the Science Team's pigment data using the latest HPLC technology implemented under the SIMBIOS NRA-99 contracts.

Concurrent with the SIMBIOS round-robin, the SeaWiFS Project field program under Stan Hooker conducted a limited pigment HPLC round robin between the international laboratories the project collaborates with. These laboratories include the with Joint Research Centre (Ispra, Italy); University of Maryland Center of Environment Studies (Horn Point, Maryland); Marine and Coastal Management (Cape Town, South Africa); and Laboratoire de Physique et Chimie Marines (Villefrance-sur-Mer, France). The samples used for this round robin were collected during the Productivité des Systèmes Océanonique Pélagiques (PROSOPE) cruise which took place between 4 September and 4 October 1999 in the Mediterranean Sea (Hooker et al. 2000). The samples were separated into three concentration regimes [eutrophic (ET), mesotrophic (MT) and oligotrophic (OT)] based on the total chlorophyll a concentration ($C_{Ta}$ in milligrams per cubic meter). The seawater was collected from 12L Niskin bottles fired in the lower water column (1 time at 0m, 3 times at 5m, 3 times in the range of 10-20m, and 11 times over 30m) with the objective to collect 12 replicates at each sampling opportunity with 3 replicates going to each of the four laboratories (total of 142 replicates). After receiving the replicates, each laboratory extracted and analyzed them using their own particular analytical method. All analyses were performed and received by the end of February 2000. The average percent difference for all pigments showed sensitivity to the concentration regimes (13.8% ET, 18.3 %MT and 32.1% OT). This round robin did not include standard pigment samples (i.e., a control data set) nor fluorometric determination analyses.

While preliminary SIMBIOS round robin results were summarized at the SIMBIOS Science Team meeting in Greenbelt (2000) and published in the annual project report (chapter 22 in Fargion and McClain, 2001) the overall results of the sources of variability in chlorophyll analysis by fluorometric and high performance liquid chromatography experiment are presented in the following chapters.

# *Chapter 1*
# SIMBIOS Round Robin Experimental Design

## 1.1 INTRODUCTION

Accurate chlorophyll *a* (chl *a*) measurements are important to algorithm development as used with ocean color remote sensing. In support of this, an inter-calibration exercise was recently conducted to identify sources of discrepancy between fluorometrically and high performance liquid chromatography (HPLC) derived chl *a*. Discrepancy affected by such variables as seasonal cycles is unavoidable (Trees et al. 2000), as variations in phytoplankton community structure result in changes in accessory pigment content which in turn can affect fluorometer chl *a* values (Lorenzen and Jeffrey 1980, Trees et al. 1985). Trees et al. (2000) showed discrepancies varied among three diverse geographical areas where coefficient of determination ($r^2$) ranged from 0.73 to 0.94 and slopes from 0.82 to1.07 (log/log regressions of fluorometric chl *a* v. HPLC total chl *a*). Insights into the many sources of such discrepancy require an understanding of uncertainties associated individually with HPLCs and fluorometers.

It is important to know what particular features of analytical methods are most important to accurate results, and as some discrepancy is to be expected, to know what minimum level of discrepancy is unavoidable. Therefore, the focus of this round robin was to assess accuracy of analytical methods of participating laboratories and to identify common features among methods that were important to good results. Sources of discrepancy in field sample results were investigated using samples collected by participants for analysis by them and a reference laboratory. Factors contributing to increases in discrepancies were investigated and included filter replication, bias in extraction procedures and water type effects. Results of this inter-calibration exercise were considered in the context of previous inter-calibration studies (Latasa et al. 1996, Hooker et al. 2000). Investigators participating in these activities were from the SIMBIOS Team (NRA-96) and from the Hyperspectral Coastal Ocean Dynamics Experiment (HyCODE) project.

## 1.2 EXPERIMENTAL DESIGN

Identifying sources of uncertainty in HPLC and fluorometric chl *a* measurements requires conclusions to be drawn with regard to accuracy. However, standard reference materials for chl *a* with which to assess accuracy of analytical instruments are not available and it is not possible to truly know chl *a* concentrations in natural samples. Alternatively, with natural samples, accuracy is estimated by the degree to which laboratories are able to reproduce results of others (Taylor 1987). In this study, Horn Point Laboratory (HPL) served as a reference laboratory and field sample results of each participating laboratory and HPL were compared for the purpose of identifying factors that contributed to variability in results. HPL methods used in this round robin were consistent with the guidelines in the Ocean Optics Protocols for Satellite Ocean Color Sensor Validation (Mueller et al. 2002) that were developed for HPLC and fluorometric chl *a* measurements (Bidigare et al. 2002, Trees et al. 2002). Furthermore, uncertainties in HPLC measurements by HPL had been assessed by Hooker et al. (2000).

A certain level of discrepancy between HPLC and fluorometer results is to be expected, but beyond this baseline, inaccuracies resulting from calibrations, analytical procedures, extractions, sample collection and water type can cause increases in the range of discrepancies seen. To minimize variability from chl *a* calibrations, chl *a* standards were given to each participant to normalize their calibrations with HPL. Laboratory-

prepared standard solutions of undisclosed content (unknown solutions) and laboratory-prepared filters that simulated field samples were distributed. The accuracy of participating laboratories' analytical methods was assessed with unknown solutions and the ability of all laboratories to yield equivalent results with the analysis of filters (when extraction procedures were standardized) was assessed with laboratory-prepared filters. These exercises were a pre-requisite to understanding sources of uncertainty associated with the analysis of field sample filters.

Each laboratory analyzed the field sample filters they had collected and the reported results of all laboratories were considered collectively for comparison with the analysis of filters at HPL (which were replicates to those analyzed by each laboratory). The HPLC/fluorometer discrepancies were assessed in each data set and investigations were conducted at HPL to identify factors which contributed to discrepancy. These factors included extraction procedures (as many varying extraction procedures were used by laboratories), complex pigment content in the sample extracts and filter replication. Differences in results due to variations in extraction procedures were evaluated at HPL by implementing participants' extraction procedures and comparing results with those acquired using standardized extraction procedures. The degree to which pigments known to interfere with fluorometric chl *a* (Lorenzen and Jeffrey 1980, Trees et al. 1985) affected the range of discrepancy was investigated by quantifying these pigments in the HPLC sample extracts and then analyzing all HPLC extracts fluorometrically (after dilution). The relative abundance of interfering pigments, which included chlorophyll *c* products (chl *c*1 + chl *c*2 + chl *c*3), chlorophyll *b* products (chl *b* + DV chl *b*) and chlorophyllide *a* (chlide *a*), was then compared to the discrepancy between the HPLC and fluorometer chl *a* value for that sample extract. DV chl *a* was also quantified in each extract so that its effect on fluorometric chl *a* could also be evaluated. It was also possible to evaluate the effects of filter replication on the range of discrepancy, as filters from most sites had been sampled in triplicate.

As calibration standards are frequently formulated from a solution of chl *a* in which the concentration has been determined spectrophotometrically, an unknown solution of chl *a* was provided to evaluate participants' spectrophotometer accuracy when guidelines specified for use were followed (Clesceri et al. 1998, Bidigare et al. 2002, Trees et al. 2002).

## 1.3 METHODS

Details are presented with regard to calibration standards and laboratory-prepared unknowns, participants level of involvement, field sample collection and handling, analysis methods, instrument details, extraction procedures and quality assurance monitoring at HPL.

*Laboratory-prepared unknowns and calibration standards*

Calibration packages sent to participants contained chl *a* calibration standards and laboratory-prepared filters (including supplies to extract them) for the fluorometer and HPLC, unknown solutions for the spectrophotometer, HPLC and fluorometer, and an HPLC DV chl *a* standard. Unknown solutions and standards were prepared at HPL from primary stock pigment solutions (stored at -15°C) which were always allowed to come to room temperature (20-25°C) before use. The concentrations of the stock solutions were determined spectrophotometrically and then dilutions were made (using 90% acetone formulated volume to volume with HPLC grade acetone and filtered de-ionized water) with calibrated Class A volumetric pipettes and glass syringes and Class A volumetric flasks. Calibration standards for the HPLC and fluorometer (of at least 5 different concentrations each) were uniquely prepared for each calibration package and dispensed into vials shown to limit evaporation of acetone to no more than 0.1 µ l per day. Packages were sent to participants (on dry ice) by overnight delivery.

*Primary pigment solutions*

The stock chl *a* solutions were prepared from chl *a* granules (Fluka 25730) dissolved in 90% acetone and the concentrations were determined in triplicate (extinction coefficient = 87.67 l $g^{-1}cm^{-1}$, Jeffrey and Humphrey 1975). DV chl *a* was isolated from chlorophyll-deficient maize leaves (Bazzaz 1981), transferred into 100% acetone, analyzed (extinction coefficient = 88.15 l $g^{-1}cm^{-1}$, Jeffrey et al. 1997) and diluted with 90% acetone for distribution to participants. Spectrophotometer procedures at HPL were consistent with Ocean Optics Protocols (Bidigare et al. 2002, Trees et al. 2002) and other suggested guidelines (Clesceri et al. 1998) for optimizing spectrophotometric accuracy. These guidelines include using a monochromator type spectrophotometer with bandwidths of 0.5 to 2 nm, correcting for light scattering and using a solution sufficiently concentrated such that the absorbance is between 0.1 and 1.0 (optical density 664 nm) (Clesceri et al. 1998, Trees et al. 2002), or more conservatively 0.2 and 0.8 (Bidigare et al. 2002).

*Unknown solutions*

Unknown solutions were prepared in lots, stored in freezers (-15 or -25°C) and used until gone (lots sent to participants were recorded). The spectrophotometer unknowns shipped to participants were the same as the primary chl *a* stock solutions used by HPL (concentrations were in the range of 4-9 µg $ml^{-1}$) and were accompanied by spectrophotometric procedures for determining the concentration and a 90% acetone reference solution for zeroing the spectrophotometer. Three different lots of an unknown solution containing chl *a* only were formulated for HPLCs and fluorometers. The concentration of each lot varied slightly, but approximated 117 µg $l^{-1}$. Unknown solutions containing approximately equal portions of DV chl *a* and chl *a* were also prepared for the HPLC and fluorometer, but the fluorometer unknown solutions (3 different lots) approximated 100 µg $l^{-1}$ total chl *a* and the HPLC unknown solutions (2 different lots) approximated 400 µg $l^{-1}$ total chl *a*. All unknown solutions were formulated to be within the range of concentrations spanned by the calibration standards. (The concentrations of HPLC and fluorometer unknowns shipped to participants are shown in Appendix B).

*Laboratory-prepared filter unknowns*

Seventy laboratory-prepared filters were prepared by filtering 10 ml of a culture of *Aureococcus anaphagefferens* onto 25 mm GF/F glass fiber filters (Whatman 1825 025) at the beginning of the study. The concentration of chl *a* in these sample extracts were within the range of concentrations spanned by the calibration standards. Filters were folded in half, given a unique number and stored (-75 to -80°C) until needed.

## 1.4 PARTICIPATING LABORATORIES

A calibration package was shipped to each participant. One was also sent to HPL to evaluate the effects of shipping. Nine different laboratories in this report responded to a questionnaire distributed by HPL regarding their analytical methods, but some laboratories did not participate in other activities to the fullest extent. Labs 3 and 7 did not collect field samples for both HPLC and fluorometer, so their field sample results are not included in this report. Labs 4, 5, 6, 8 and 9 analyzed laboratory-prepared unknowns and field samples; Lab 1 did not analyze laboratory-prepared unknowns but did analyze field samples; Labs 2 and 3 only analyzed fluorometer laboratory-prepared unknowns and Lab 7 only analyzed HPLC laboratory-prepared unknowns.

## 1.5 FIELD SAMPLE COLLECTION AND HANDLING

Participants collected replicate filters from their typical field sites (Table 1.1). Samples were sent to HPL on liquid nitrogen or dry ice by overnight delivery and were then stored (-75 to -80°C) until

Table 1.1. Details of field sample collection as provided by participants. Site and collection bottle numbers are shown so results are traceable to both site and bottle. All filters were GF/F. HPLC filter diameters are shown (all fluorometer filters were 25 mm). "Kept" indicates the number of filters retained for analysis by the collecting laboratory and "HPL" indicates the number sent to HPL. Cells are empty if details were not provided. Information for Labs 2, 3, and 7 are not shown.

| | | | | Fluorometer filters | | | HPLC filters | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Lab Code | Site # | CTD or Bucket | Bottle # | Volume Filtered (ml) | # collected Kept | # collected HPL | Filter (mm) | Volume Filtered (ml) | # collected Kept | # collected HPL |
| 1 | 1 | CTD | 4-7[1] | 100 | 3 | 6 | 25 | 200 | 3 | 6 |
| | 2 | CTD | 5-8[1] | 25 | 3 | 6 | 25 | 50 | 3 | 6 |
| | 3 | CTD | 2-3[2] | 50 | 3 | 6 | 25 | 150 | 3 | 6 |
| | 4 | Bucket | -- | 150 | 3 | 6 | 25 | 200 | 3 | 6 |
| | 5 | Bucket | -- | 150 | 3 | 6 | 25 | 200 | 3 | 6 |
| 4 | 1 | Bucket | -- | 200 | 3 | 6 | 25 | 200 | 3 | 6 |
| | 2 | CTD | 9 | 1020 | 1 | 2 | 25 | 1020 | 1 | 1 |
| | 2 | CTD | 10 | 1020 | 1 | 3 | 25 | 1020 | 1 | 3 |
| | 2 | CTD | 11 | 1020 | 1 | 1 | 25 | 1020 | 1 | 2 |
| | 3 | Bucket | -- | 1020 | 3 | 6 | 25 | 1020 | 3 | 6 |
| 5 | 1 | CTD | 17 | 500 | 1 | 1 | 47 | 4000 | 1 | 1 |
| | 1 | CTD | 18 | 500 | 1 | 1 | 47 | 4000 | 1 | 1 |
| | 1 | CTD | 19 | 500 | 1 | 1 | 47 | 4000 | 1 | 1 |
| | 1 | CTD | 20 | 500 | 1 | 1 | 47 | 4000 | 1 | 1 |
| 6 | 1 | CTD | 21 | 550 | 2 | 2 | 25 | 1600 | 2 | 2 |
| | 1 | CTD | 22 | 550 | 0 | 4 | 25 | 1600 | 0 | 4 |
| | 1 | CTD | 21 | 550 | 2 | 2 | 25 | 1600 | 2 | 2 |
| | 1 | CTD | 22 | 550 | 0 | 4 | 25 | 1600 | 0 | 4 |
| | 1 | CTD | 14 | 280 | 3 | 6 | 25 | 550 | 3 | 6 |
| 8 | 1 | CTD | 10 | 100 | 3 | 6 | 25 | 150 | 3 | 6 |
| | 2 | CTD | 10 | 200 | 3 | 6 | 25 | 300 | 3 | 6 |
| | 3 | CTD | 10 | 100 | 3 | 6 | 25 | 200 | 3 | 6 |
| 9[3] | 1a | | | 250 | 3 | 6 | 47 | 1000 | 3 | 6 |
| | 2a | | | 250 | 3 | 6 | 47 | 1000 | 3 | 6 |
| | 3a | | | 250 | 3 | 6 | 47 | 1000 | 3 | 6 |
| | 1b | | | 250 | 3 | 6 | 47 | 1000 | 3 | 6 |
| | 2b | | | 250 | 3 | 6 | 47 | 1000 | 3 | 6 |
| | 3b | | | 250 | 3 | 6 | 47 | 1000 | 3 | 6 |
| | 1c | | | 250 | 3 | 6 | 47 | 1000 | 3 | 6 |
| | 2c | | | 250 | 3 | 6 | 47 | 1000 | 3 | 6 |
| | 3c | | | 250 | 3 | 6 | 47 | 1000 | 3 | 6 |

[1]Bottle contents mixed in 50 1 carboy. [2]Bottle contents mixed in 20 1 carboy. [3]Lab 9 collected filters from these 3 sites on each of 3 successive days (indicated by a, b, and c). Only results from day (b) were used when results from all laboratories were considered collectively.

analyzed. Most participants stored samples in temperatures for fluorometer filters: -30°C (Lab 1) freezers (-80°C) or under liquid nitrogen until and 0°C (Lab 8). For filters to be considered as analyzed. Two laboratories used freezers at other replicates for evaluating precision, they had to be

collected from the same bottle. Thus, for site 2 of Lab 4 (where replicate filters from the same bottle were not retained by the participant) it was not possible to assess their precision in the analysis of filters from this site. If replication among filters from different bottles was as good as that typically seen for replication among filters from the same bottle (for chl *a* and accessory pigments) then it was deemed acceptable to average results of filters from different bottles for measurements other than precision.

*Field sample extraction procedures*

Participants extracted field sample filters with their usual methods. All participants extracted fluorometer filters differently from HPLC filters. Filters sent to HPL were extracted with standardized procedures (HPLC and fluorometer extraction procedures differed only in the volume of solvent added). The standardized extraction procedures were selected for use in this study exclusively and were not necessarily consistent with those suggested by Bidigare et al. (2002) and Trees et al. (2002). However, the standardized extraction procedures were easily implemented and this was a necessary requirement, as all laboratories used these procedures to extract laboratory-prepared filters. When a second set of replicate filters was sent to HPL, they were extracted with participants' methods.

Extraction volumes were estimated in three ways, classified here as "added", "measured" or "assumed". "Added" means that the volume of solvent added to the filter was used as the extraction volume. "Measured" means the extraction volume was observed by reading a meniscus in a graduated tube or in some HPLC analyses, where an internal standard was used. "Assumed" includes the volume of solvent added to the filter plus the average estimate of the volume of water contributed by a sample filter. Summaries of the extraction procedures used in this report are given for fluorometer filters (Table 1.2) and for HPLC filters (Table 1.3).

*HPLC analytical methods*

Features of HPLC analytical methods addressed include instrument configurations and procedures related to injection, separation and detection. Labs 2 and 3 did not participate in HPLC aspects of this study. The HPLC instrument, methods and analyst used by Lab 1 were the same as those used by HPL.

*HPLC injection conditions*

For accurate resolution and quantitation of early eluting pigments (such as chlide *a*), the sample extracts must be adjusted with a polar solvent (water or buffer) prior to injection. All but one laboratory (Lab 9) did this, but procedures varied according to the HPLC injector capabilities (Table 1.4). With the manual injector, sample and polar solvent were combined (with automatic pipettes) by the analyst, who then injected the mixture and started the analysis. With partially automated systems, the analyst mixed sample with polar solvent (with automatic pipettes) and placed the samples in the autosampler compartment where they resided (up to 24 h) until injected. With fully automated systems, vials of polar solvent and separate vials of sample were placed in the autosampler compartment where they resided (up to 24 h). Immediately prior to injection, a portion of the sample to be analyzed next was automatically combined with polar solvent in the injector's sample loop, the mixture injected and the analysis begun.

*HPLC detection*

HPLC detectors used by participants were of three types: photodiode array (PDA), ultraviolet/visible spectrophotometric (UV/Vis) or fluorometric (FLD) (Table 1.5). Lab 4 used two detectors simultaneously to quantify chl *a*. Lab 1 and HPL each used one detector programmed to acquire data from two wavelengths (665 for chl *a* products and 450 for other pigments). Reference wavelengths were sometimes used to suppress noise.

Table 1.2. Methods used by participants in this study to extract fluorometer filters. All laboratories added 90% acetone to filters. "Grad" indicates graduated and "vol" indicates volumetric. Most laboratories clarified by centrifugation; Lab 1 filtered through a GF/F filter and HPL filtered through a PTFE HPLC syringe cartridge filter with a glass fiber pre-filter. Field data from Labs 2, 3, and 7 are not included in this report.

| Lab Code | Solvent added (ml) | Solved added with | Filter disruption | Soak time (h) | Extraction volume |
|---|---|---|---|---|---|
| 1 | ~8 | Squirt bottle | Grinding | None | Measured[1] |
| 4 | 10 | Grad cylinder | Grinding | 24 | Added |
| 5 | 5 | Not known | None | Overnight | Added |
| 6 | 10 | Re-pipette | None | 24 | Added |
| 8 | 7 | Auto-pipette | None | 24 | Added |
| 9 | 4 (grinding) 2 (rinsing) | Vol pipettes | Grinding | 12-24 | Added (6 ml) |
| HPL[2] | 10 | Vol pipette | None[3] | 3-4 | Assumed[4] |

[1]Lab 1 added solvent non-quantitatively with a squirt bottle for grinding and rinsing and each transfer of the homogenate from the grinding tube was clarified by filtration through a GF/F filter and the filtrate received in a conical, graduated tube used for measuring extraction volume. [2] The standardized method. [3] Samples were mixed vigorously for 30 s before and after soaking. [4] 10.145 ml

Table 1.3. Methods used by participants to extract filters for HPLC analysis. All laboratories used acetone (of varying acetone/water ratios) for extractions. Lab 4 clarified the sample extracts with a nylon HPLC syringe cartridge filter and Labs 1, 8 and 9 used those made of PTFE. Labs 5 and 6 clarified with centrifugation. Empty cells indicate information was not provided. Field data from Labs 2, 3, and 7 are not included in this report.

| Lab code | Solvent | | | | | Extraction volume |
|---|---|---|---|---|---|---|
| | Acetone (%) | Vol (ml) | Added with | Filter disruption | Soak time (h) | |
| 1 | 95 | 3 | Vol pipette | Ultrasonic probe | 3-4 | Assumed[1] |
| 4 | 100 | 1.5 | Auto-pipette | Grinding | 2-12 | Assumed[2] |
| 5 | 90 | 5 | | Sonicating bath | Overnight | added |
| 6 | 100 | 1.5 | Re-pipette | Grinding | 0.5 | Measured[3] |
| 8[4] | 100 | 8 | | None | 24 | measured |
| 9 | 100 | 5 | Vol pipette | Sonicating bath | 12 to 18 | added |
| HPL | Varied[5] | 3 or 5 | Vol pipette | None[6] | 3-4 | Varied[7] |

[1] 3.145 ml. [2] 1.6 ml. [3] Canthaxanthin was used as an internal standard. [4] Eight ml of solvent was added to the filter. After soaking, the solution was clarified and transferred quantitatively to a concentrator tube where the solution was reduced to 3 ml with nitrogen gas. [5] The water content in acetone was specific to the filter size so that water from the filter and the solvent added would yield approximately 90% acetone. [6] Samples were mixed vigorously for 30s before and after soaking. [7] Assumed extraction volumes were used at HPL (3.145 ml for 25 mm filters and 5.700 ml for 47 mm filters) except with filters from Lab 5 where an internal standard was used to measure extraction volume.

Table 1.4. The HLPC configurations used by participants. The different modes of injection are fully described in Chapter 3.5.1. (N/A = not applicable). Labs 2 and 3 did not participate in HPLC aspects of this study.

| Lab Code | HPLC manufacturer, model | Mode of injection | Autosampler compartment Temperature ($^{o}$C) |
|---|---|---|---|
| 1, HPL | Hewlett Packard series 1100 | Fully automated | 4 |
| 4 | Hewlett Packard 1050 | Partially automated | Not controlled |
| 5 | Waters | Manual | N/A |
| 6 | Waters | Fully automated | 5 |
| 7 | Hewlett Packard series 1100 | Fully automated | 4-5 |
| 8 | Dionex | Partially automated | Not controlled |
| 9 | Hewlett Packard series 1100 | Fully automated | Not controlled |

Table 1.5. The HPLC detector settings used by participants. Detector type abbreviations are PDA = photo diode array, FLD = fluorescence, UV/VIS = ultraviolet/visible. EX = excitation, EM = emission. Labs 2 and 3 did not participate in HPLC aspects of this study.

| Lab Code | Detector type | Detector wavelenghts and bandwidth (nm) | Reference Wavelength (nm) |
|---|---|---|---|
| 1, HPL | PDA | 450 $\pm$ 10 and 665 $\pm$ 10 | none |
| 4 | PDA, FLD | PDA 440$\pm$2; FLD 421 EX, 666 EM | 550 $\pm$ 5 |
| 5 | UV/Vis | 446 $\pm$ 5 | none |
| 6 | UV/Vis | 440 $\pm$ 4 | none |
| 7 | PDA | 436 $\pm$ 2 | 550 $\pm$ 5 |
| 8 | PDA | Maxplot[1] $\pm$ 10 | none |
| 9 | PDA | 436 $\pm$ 4 | none |

Table 1.6. The HPLC separation conditions used by partecipants. Codes used for HPLC column sources are: A=Agilent Technologies, B=Alltech, C=Waters, D=Phenomenex, E=VYDAC. Column dimension are given for length (l) and internal diameter (i.d.). Codes to mobile phase references are footnoted and were often modified from those as published.Lab2 and 3 did not participate in HPLC aspect of this study.

| Lab Code | HPLC Column | Column Source | Column Dimension L x i.d. (mm) | Reference For mobile Phase[1] | Column Temperature (°C) |
|---|---|---|---|---|---|
| 1, HPL | Eclipse XDB $C_8$ | A | 150 x 4.6 | 1 | 60 |
| 4 | Alltima $C_8$ | B | 250 x 4.6 | 23 | Not controlled |
| 5 | S50DS $C_8$ | C | 250 x 4.6 | 3 | Not controlled |
| 6 | Adborboshpere $C_8$ | D | 100 x 4.6 | 4 | Not controlled |
| 7 | Ultromex %)DS $C_8$ | E | 250 x 3.2 | 5 | 38 |
| | 201TP54 $C_8$ | | 250 x 4.6 | | |
| 8 | Allsphere ODS-2 $C_8$ | B | 250 x 4.6 | 3 | 40 |
| 9 | Sphereclone ODS-2 $C_8$ | D | 250 x 4.6 | 3 | 30 |

[1]1-Van Heukelem and Thomas (2001), 2-Mantoura & Lleweellyn (1983), 3-Wright et al. (1991), 4-Goericke and Repeta (1993) and 5-Pinckney et al. (1996), modified from Mantoura and Llewellyn (1983).

*HPLC separation conditions*

HPLC separation conditions used by participants varied (Table 1.6). Lab 7 used two HPLC columns connected in series. Of the methods shown, only those employing $C_8$ columns chromatographically separated DV chl *a* from chl *a* (Labs 1, 6 and HPL). With the HPLC method used by Labs 5, 8 and 9 (Wright et al. 1991), it is suggested (Bidigare et al. 2002) that amounts of chl *a* and DV chl *a* be quantified using a simultaneous equation based on their spectral differences (Latasa et al. 1996), but participants in this study did not use this approach.

*Fluorometer analytical methods*

Participants used fluorometers from Turner Designs, Inc. and had equipped them appropriately (Turner Designs, Inc., pers. comm.) with optical kits and lamps specified for the type of analysis. Lab 4 used the non-acidification analysis method (Welschmeyer 1994) while all others used the acidification method (Strickland and Parsons 1972). The instrument used by Lab 1 was the same one used by HPL. Models of fluorometers used included the following: 10-AU-005 CE Labs 1, 3, 9 and HPL; 10-AU-005 Labs 4, 5 and 8; 10-005 Labs 2 and 6.

On occasion, a TD-700 instrument, equipped with the filter and optical kit for the non-acidification method was used at HPL and is so specified in results. At HPL, a constant time interval (1.5 min) was used after the acid was added before the second reading was recorded (Trees et al. 2002). It was not known if participants did this.

*Quality assurance at Horn Point Laboratory*

Quality assurance measurements were conducted with regard to preparation and analysis of chl *a* calibration standards and laboratory-prepared unknowns. Daily instrument performance was monitored as was instrument reproducibility over the duration of this study (November 1999 to January 2001).

*Preparation and analysis of calibration standards and unknown solutions*

The primary factors affecting accuracy and precision in the preparation of calibration standards and laboratory-prepared unknowns were considered to be spectrophotometric measurements, dilution procedures and the stability of standards during storage. All unknown solutions prepared for participants were analyzed prior to shipping.

Spectrophotometric absorbance accuracy was validated with NIST traceable neutral density filters (Starna Cells, Inc. RM-N1N35N, RM-1N2N3N) (Latasa et al. 1999). These filters did not bracket 664 nm (the wavelength used for chl *a*), but did measure absorbance accuracy at 635 nm, where expected absorbance deviated from observed absorbance by $\leqslant 0.003$. Considering that all stock solutions had absorbance values between 0.4 and 0.8, it is unlikely that absorbance inaccuracies exceeded 1% at 664 nm. Wavelength ($\lambda$) accuracy was found to be within 1 nm when the observed $\lambda_{max}$ of chl *a* in 90% acetone was compared to the published $\lambda_{max}$ (Jeffrey and Humphrey 1975). Spectrophotometric measurements were conducted in triplicate and average precision was 0.05% relative standard deviation, or %RSD (%RSD = ($s$ * mean$^{-1}$)*100). Dilutions of these stock solutions were performed only with devices that had been calibrated for accuracy and precision with replicate ($n \geqslant 7$) gravimetric measurements of 100% acetone. The mean accuracy of each measuring device differed by $\leqslant 0.9\%$ from the volume specified. The 95% confidence limits, or warning limits (WL), were used to describe the range within which replicate measurements of these devices should lie. The measuring device with the poorest precision exhibited WL of ± 0.4% from its mean accuracy.

The stability of standard solutions stored for long durations (up to 286 days) was monitored. Three solutions containing either chl *a* or DV chl *a* (in 90% acetone) were monitored for changes in

total peak area (by HPLC) on many occasions during their extended storage. There was no significant effect of analysis date on total peak area ($p > 0.1$ and $r^2 = 0.00$). Even with the standard held for 286 days, changes in total peak area were minimal and varied only ± 2% (WL) from the mean. This standard exhibited a significant ($p < 0.001$) increase in the proportion of allomers and epimers relative to the total peak area, but this increase was small (2.2%).

*Daily instrument performance*

HPLC and fluorometer instrument performance was monitored at HPL by analyzing quality control (QC) standards several times every day instruments were used, and by analyzing solid secondary standards (Turner Designs, Inc.) on the fluorometer. The concentrations of QC standards (as measured) were compared to their formulated (or known) concentrations and values of % difference (%D) computed (%D = chl $a_{\text{MEASURED}}$ - chl $a_{\text{KNOWN}}$)* chl $a_{\text{KNOWN}}^{-1}$) * 100). Values of %D for HPLC QC standards fell within ± 4.0% (WL) and within ∀ 6.2% (WL) for fluorometer QC standards. The average precision associated with replicate analyses of QC standards on the same day was described using the term %RSD. Average daily precision was observed on several days ($n \geqslant 19$) and WL determined. The WL for average daily precision were ± 1.5%RSD (HPLC QC standards), ± 1.0%RSD (fluorometer QC standards) and ± 1.5%RSD (fluorometer analyses of solid secondary standards).

*Reproducibility of chl* a *calibration factors and instrument variability*

Instruments were calibrated with each use. Records of chl *a* calibration factors were kept. Laboratory-prepared filters were analyzed at HPL at frequent intervals to describe the variability of HPL methods in the analysis of filters over the duration in which participants' field sample filters were analyzed at HPL (304 days).

Several sets of calibration standards were prepared at HPL for the fluorometer and HPLC. These included sets sent to participants and sets used for the analysis of field samples at HPL. Thirteen different HPLC calibration curves were uniquely prepared and analyzed during the study. The average slope (± WL) was 3.451 ± 1.6%. All *y* intercepts were near 0 and represented injected amounts less than the limit of detection for chl *a* (0.8 ng, S:N ≅ 10). Calibration regression $r^2$ values were > 0.999. Records of all fluorometer calibration factors were maintained, even though they were expected to vary as the fluorescent lamp aged or was changed. Nevertheless, over a 2 month period, variability of fluorometer calibration factors was confined to ± 5% (WL) and response factors did not vary over the range of concentrations spanned by each set of calibration standards (regression $r^2$ values were > 0.999).

# *Chapter 2*
# Results of Method Assessment

## 2.1 INTRODUCTION

Identifying causes for inaccuracies in analytical methods was simplified because participants' chl *a* calibrations had been normalized with HPL. The potential for agreement among laboratories, had calibrations not been normalized, was assessed with results from the spectrophotometer chl *a* unknown solutions. Results of laboratory-prepared filters demonstrated the reproducibility attainable with HPL analytical methods and the ability of each laboratory and HPL to produce similar results in filter analysis when extraction procedures were standardized. Results of the unknown solutions were useful when identifying causes for analytical inaccuracies.

## 2.2 SPECTROPHOTOMETER UNKNOWNS

When laboratories complied with all spectrophotometer guidelines (Clesceri et al. 1998, Bidigare et al. 2002, Trees et al. 2002), results of spectrophotometer unknown solutions of chl *a* varied by no more than -1.0 to 3.2% from the values measured at HPL before distribution and, on average, these 7 laboratories values were within 1.4% of values measured at HPL. These results suggest that if chl *a* calibration standards had been prepared by each laboratory and if all guidelines for accuracy in spectrophotometric measurements of chl *a* were followed, participants' calibration standards could have yielded similar results to those distributed by HPL, assuming that accurate and precise dilution devices were also used.

Two laboratories (whose results are not included above) used spectrophotometers with fixed bandwidths of 4 and 5 nm and these laboratories' measured values were -3.3% and -7.1%, respectively, of the concentrations measured at HPL before distribution. Bandwidths wider than 2 nm are inconsistent with suggested guidelines, as wide bandwidths are known to suppress chl *a* concentration (Clesceri et al. 1998, Marker et al. 1980). (The laboratory using a spectrophotometer with a 5 nm bandwidth was not a SIMBIOS or HyCODE investigator and results from this laboratory do not appear elsewhere in this report.)

Differences in this study were confined to a narrower range than differences in the spectrophotometric study of Latasa et al. (1996). According to Dunne (1999), bandwidth had not been evaluated in Latasa et al. (1996) and this could have contributed variability to results. Additionally, the solution distributed by Latasa et al. (1996) was less concentrated (absorbance = 0.17) than those used in the current study (absorbance ranged from 0.4 to 0.8) and this also could have contributed to greater variability in results.

## 2.3 ANALYTICAL METHODS

The participants' responses to questionnaires revealed that some HPLC-related procedures had the potential to compromise accuracy (Table 2.1). These limiting procedures were inconsistent with guidelines suggested in HPLC Ocean Optics Protocols (Bidigare et al. 2002) and included: 1) HPLC injection conditions whereby the analyst premixed sample extracts with buffer or water up to several hours before analysis, with the effect that non-polar pigments (such as chl *a*) could precipitate out of solution (Mantoura et al. 1997, Wright and Mantoura 1997, Latasa et al. 2001), 2) HPLC methods whereby DV chl *a* was not individually quantified and if present could cause quantitation of total chl *a* to be inaccurate, 3) HPLC reporting practices where not all chl *a* products (chl *a* and DV

Table 2.1. Feature of some laboratories' HPLC methods were incosistent with the Ocean Optic Protocols (Bidigare et al., 2002; Trees et al., 2002) and had the potential to limit accuracy. Laboratories whose HPLC methods were potentially affected by these limitations are indicated by "X". Lab 2 and 3 did not conduct HPLC analyses.

| | | Laboratory code | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Inconsistency | HPL | 1 | 4 | 5 | 6 | 7 | 8 | 9 |
| Injection procedure | | | X | | | | X | |
| Quantitation of DV chl *a* | | | X | X | | X | X | X |
| Total chl *a* reporting practice | | | X | X | | | X | X |
| HPLC extraction volume determination | | | | X | | | | X |

Table 2.2. Analytical methods of HPL and each participant were considered to yield equivalent results if the participant's % difference (%D) for an unknown solution or laboratory-prepared filter was within WL (95% confidence limits) at HPL for that unknown (n = the number of observations used to describe WL). Laboratories not conducting an analysis = N/A and those with results within WL = √. When a result was outside the WL, a value is shown that indicates the %D that a result for an unknown solution was from the formulated value or that a result for a laboratory-prepared filter was from the mean value at HPL for that instrument.

| Unknown | N | WL (±%D) | Laboratory reporting results[1] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Fluorometer** | | | 2 | 3 | 4[2] | 5 | 6 | 7 | 8 | 9 |
| chl *a* only | 6 | ± 5 | 11 | √ | √ | √ | √ | √ | √ | √ |
| chl *a* + DV chl *a* | 6 | ± 12 | √ | √ | √ | √ | √ | √ | √ | √ |
| Laboratory-prepared filters | 14 | ± 8 | √ | √ | √ | √ | √ | √ | √ | N/A[4] |
| **HPLC** | | | | | | | | | | |
| chl *a* only | 6 | ± 4 | N/A | N/A | 22 | √ | N/A[5] | √ | -11 | √ |
| chl *a* + DV chl *a* | 6 | ± 3 | N/A | N/A | 103 | 15 | √ | 4 | -6 | 11 |
| Laboratory-prepared filters | 17 | ± 5 | N/A | N/A | 20 | -7 | √ | √ | -19 | √ |

[1]Lab 1 did not analyze laboratory-prepared unknowns, Labs 2 and 3 did not analyze HPLC laboratory prepared unknowns. [2]HPLC results shown for Lab 4 are from their PDA detector.[3]Lab 6 analyzed all 4 laboratory-prepared filters by HPLC then diluted the extracts for fluorometric analysis. [4]Lab 9 did not receive flourometer laboratory-prepared filters. [5]Lab 6 received incorrect instructions for the analysis of the HPLC chl *a* solution.

chl *a*, their allomers and epimers and chlide *a*) were included in total chl *a*, thereby exacerbating negative discrepancies and 4) HPLC filter extractions where the water retained by the sample filter (47 mm GF/F) was not accounted for in extraction volume, causing chl *a* results to be underestimated (47 mm G/FF filters retain approximately 700 $\bar{\chi}\bar{X}$ $\mu$l of water, Bidigare et al. 2002). Guidelines pertinent to these issues are given in the newest versions of Ocean Optics Protocols (Bidigare et al. 2002, Trees et al. 2002) and aided efforts to identify potentially problematic procedures.

## 2.4 LABORATORY UNKNOWNS

Accuracy of HPL instruments was assessed through quality assurance measurements over the time period (10 mo) in which calibration packages were sent to participants. The calibration standards and unknown solutions in each participant's package were sub-sampled and analyzed at HPL before shipment and the resulting chl *a* calibration factors were used to measure the concentration of unknowns in that package. Percent differences (%D) were determined by comparing the measured concentration of an unknown with its formulated concentration. After all packages were shipped, the WL associated with all measurements of %D at HPL for each unknown were described (see Table 8). Results of laboratory-prepared filters were considered to cumulatively reflect the sum of all variables that could have affected results at HPL, such as filter replication, sample storage, variations in calibration and instrument performance. Homogeneity among replicate laboratory-prepared filters was assessed by analyzing 7 of these filters by HPLC at the beginning of the study. The chl *a* content among these 7 filters varied by ± 5% (WL). The mean chl *a* content and WL were determined for all laboratory-prepared filters analyzed at HPL by HPLC (n =17) and all filters analyzed by fluorometer (n = 10) over the duration of the study. The % difference (%D) that each individual filter result was from the mean chl *a* value at HPL (for each instrument) is shown according to the analysis date (for results from HPL and participants) in Fig. 2.1. There was no visible effect of analysis date on chl *a* content. If a participant's mean value of %D was within WL at HPL for that instrument ($\pm$ 5% for HPLC filters and $\pm$ 8% for fluorometer filters), their analytical methods were considered to yield filter results that were not significantly different from filter results at HPL (under conditions where extraction procedures were standardized and samples were devoid of DV chl *a*). In 2 of the 3 instances where participants' values of %D for laboratory-prepared filters were outside WL at HPL, their values of %D for the chl *a* unknown solution were also outside WL for that unknown at HPL. Individual results of all unknowns (and their respective WL at HPL) are detailed in Appendix B and C and summarized in Table 2.2. The results of unknowns in the package prepared to evaluate effects of shipping were all within WL.

Precision, measured as relative standard deviation (%RSD), of participants' analytical methods was evaluated based on triplicate analyses of the chl *a* unknown solution and 2 laboratory-prepared filters each for HPLC and fluorometer. The mean %RSD for both unknowns on both instruments was 2% (excluding HPLC results of Lab 8). HPLC precision of Lab 8 was not typical of other laboratories, (19%RSD with the unknown solution and 6% with the laboratory-prepared filters). All other laboratories' values of %RSD were $\leq$ 4% with HPLC and fluorometer results.

## 2.5 FACTORS CONTRIBUTING TO INACCURACIES

In contrast to the fluorometer results, which were similar for both HPL and participants, HPLC results of unknowns varied among laboratories. HPLC variability was due in part to problems quantifying DV chl *a*. With unknowns devoid of DV chl *a*, Labs 5, 6, 7 and 9 were generally within or near WL. However, results of Labs 4 and 8 exhibited a high and low bias, respectively, with all laboratory-prepared unknowns. It is possible that

the biased HPLC results of Labs 4 and 8 were related to limitations of their HPLC calibrations. Evidence for this exists in the $r^2$ values of their calibration regressions, which were 0.994 (Lab 4) and 0.996 (Lab 8). In contrast, regressions of all other participants and HPL had $r^2 > 0.999$. The injection conditions used by Labs 4 and 8 are not recommended (Mantoura et al. 1997, Wright and Mantoura 1997, Latasa et al. 2001, Bidigare et al. 2002) and this could have contributed to the inaccuracies seen with laboratory-prepared unknowns (Table 2.2).

Laboratories that did not chromatographically separate DV chl *a* from chl *a* (Labs 4, 5, 7, 8 and 9) reported results that were outside WL for the unknown solution containing both. These laboratories did not recognize that DV chl *a* was present and quantified the concentration of total chl *a* using chl *a* calibration factors. However, the magnitude of HPLC inaccuracies varied and was related to the detector response of DV chl *a* relative to chl *a*. This relation is made evident with results from Lab 4 (Appendix B, Table 4), where a single HPLC injection was performed and data were acquired with two detectors. Amounts reported from each detector differed by a factor of 1.4.

The response of DV chl *a* and chl *a* in an HPLC detector depends on the HPLC solvent used and the particular wavelength and bandwidth selected. To illustrate the degree to which different HPLC detector settings discriminate between DV chl *a* and chl *a*, a DV chl *a* standard was intentionally quantified using chl *a* calibration factors (Fig. 2.2). Results shown are from laboratories whose %D was no greater than ± 4% with the unknown solution containing chl *a* only. Accuracy with 440 nm ± 4 (Lab 6), 436 nm ± 5 (Lab 5) and 436 nm ± 4 (Lab 9) was poor, but accuracy with 436 nm ± 2 (Lab 7) and 665 nm ± 10 (HPL) was within the range seen for solutions of chl *a* only (± 4%D). To achieve accurate total chl *a* measurements with the first 3 detector settings (if DV chl *a* were present), it would be necessary to chromatographically resolve DV chl *a* from chl *a* and use discrete calibration standards for each, as was normally done by Lab 6. However, as seen with HPLC methods of Lab 7 and HPL, which used detector settings of 436 nm ± 2 and 665 nm ± 10, respectively, it is possible to accurately measure total chl *a* when DV chl *a* is present by acquiring data from a single wavelength that does not discriminate between chl *a* and DV chl *a*. This explains why the result attained by Lab 7 for the unknown solution containing DV chl *a* and chl *a* (Table 2.2) was within WL for the solution containing only chl *a*, even though they did not chromatographically separate chl *a* and DV chl *a*.

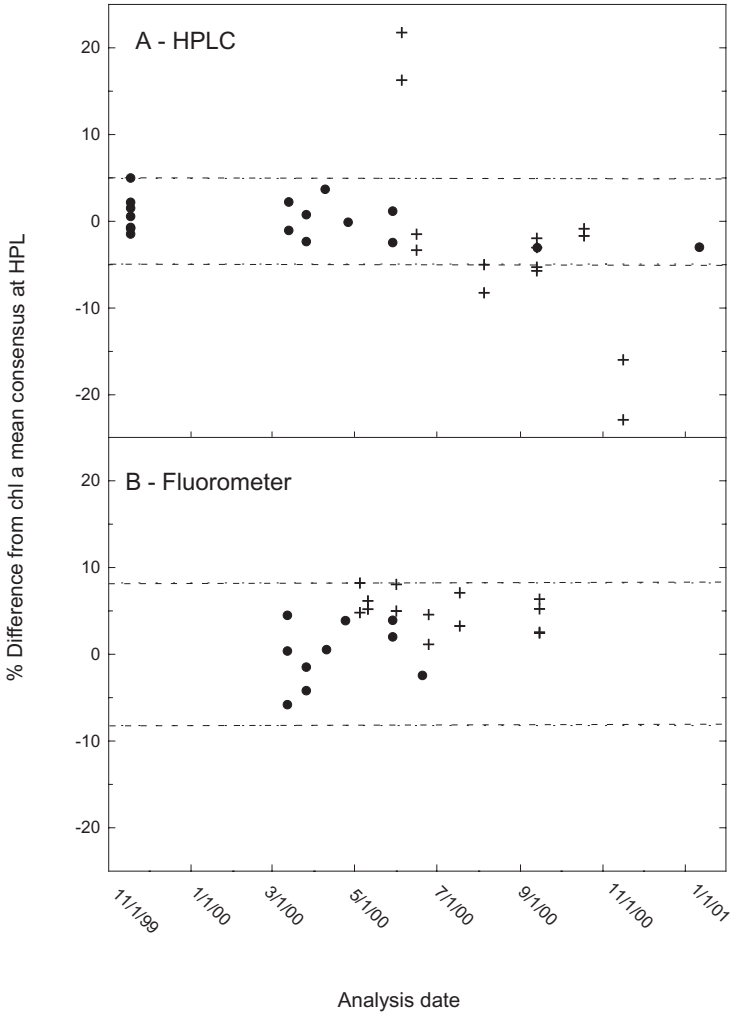


Fig 2.1. The % difference between chl *a* in a laboratory-prepared filter and the mean chl *a* value of all laboratory-prepared filters analyzed at HPL on that instrument: (A) HPLC or (B) fluorometer. Filters analyzed by HPL (±) and by participants (+) are sorted by analysis date (x axis). The 95% confidence limits (dashed lines) for analyses at HPL are ± 5% (A) and ± 8% (B).

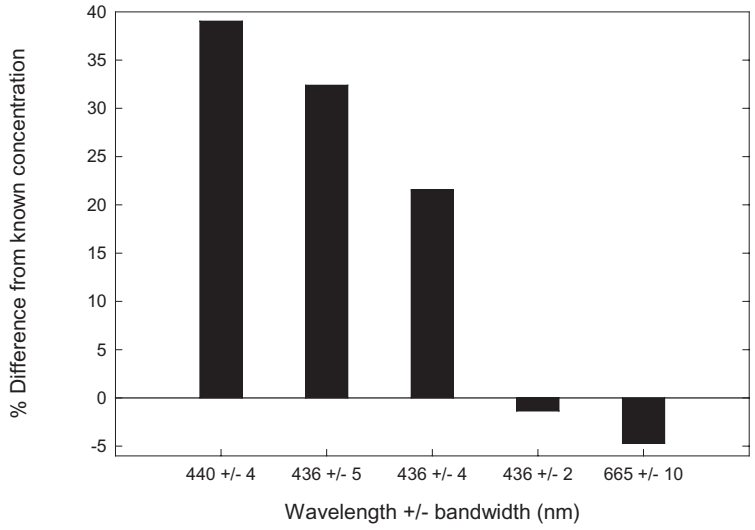


Fig. 2.2. The % difference between the known concentration and the HPLC measured concentration of a DV chl *a* standard analyzed by 5 laboratories whose HPLC methods and detector settings varied. DV chl *a* was quantified with chl *a* calibration factors to show effects of detector settings. HPLC methods were otherwise accurate to within 4% with solutions of chl *a*.

# *Chapter 3*
# Results of Field Samples

## 3.1 INTRODUCTION

Eighteen field sites from diverse geographical locations are represented in this study (Fig. 3.1). So that results from each site remained unique, each site was given an identifier with an initial number indicating the collecting laboratory and a second number differentiating sites collected by that laboratory. Labs 1, 4, 6, 8 and 9 collected filters from 3 to 5 sites and Lab 5 collected from 1 site (see Chapter 1, Table 1.1). Either 2 or 3 sets of filters were collected from each site; each set contained replicate filters for each instrument (HPLC and fluorometer). One filter set was retained by the collecting laboratory and the remaining sets (or set) were sent to HPL. Each collecting laboratory analyzed the filters they had collected with their usual methods. At HPL, a set of filters was extracted with standardized procedures (Tables 1.2 and 1.3) and analyzed with HPL methods. When HPL received 2 sets of filters, the extra set was extracted with the participant's procedures and analyzed with HPL methods. Also at HPL, extracts of all HPLC filters were diluted to sufficient volumes and analyzed fluorometrically.

## 3.2 DISCREPANCY BETWEEN HPLC AND FLUOROMETER CHL *a*

Discrepancy between HPLC and fluorometer chl *a* is described for 3 sets of data: 1) results reported by 6 different laboratories (for the sites where they had collected filters) for which all results were considered collectively (referred to as data from multiple laboratories), 2) results of filters from all 18 sites extracted with standardized procedures and analyzed at HPL and 3) results of HPLC filters from each site that were extracted at HPL and analyzed fluorometrically. For the first 2 data sets, the mean HPLC chl *a* concentration (chl $a_H$) was compared with the mean fluorometric chl *a* concentration (chl $a_F$) from the same site. For the third data set, chl $a_H$ for each HPLC filter extract was directly compared to its chl $a_F$ value (details in Appendices D, E, F). All 3 data sets represented the same 18 field sites. Linear and log/log regressions are shown for these data (Fig. 3.2, details of the regressions are in Table 3.1).

It is important that such regressions accurately predict chl $a_H$ from observed chl $a_F$. Two of the 3 linear regressions (lines 1 and 3, Table 3.1) are incapable of doing so because of the large negative *y* intercepts. The ability of log/log regressions to yield accurate predictions are not intuitively obvious. To illustrate this, the log/log regressions were used to predict chl $a_H$ from the observed chl $a_F$ value at each site. This was done with the data set for multiple labs and the data set for the analysis of filters at HPL. The inaccuracy associated with each chl $a_H$ predicted value, relative to the observed chl $a_H$ for that site, was determined based on the absolute difference in chl *a* $\mu$g $l^{-1}$. When inaccuracies for all sites in each data set were summed, the cumulative inaccuracy associated with the log/log regression of multiple labs was 11 times that associated with the log/log regression of the analysis of filters at HPL. In terms of biomass, the inaccuracies associated with the chl $a_H$ predictions for all 18 sites added up to 109 $\mu$g chl *a* (multiple labs) and 10 $\mu$g chl *a* (the analysis of filters at HPL).

Subsequent HPLC and fluorometer relationships in this report are based on percentage differences using the term % discrepancy (%Dsc = ((chl $a_H$ - chl $a_F$) * chl $a_F^{-1}$)*100). The chl $a_H$ for each site is plotted against the %Dsc at that site for results from multiple labs (Fig. 3.3A), for filter results acquired at HPL (Fig. 3.3B) and for the fluorometric analysis of HPLC extracts at HPL (Fig. 3.3C). Overall, it is evident that the range of %Dsc was smaller with results from HPL, where fewer variables with the potential to affect outcome existed. Results in Fig.

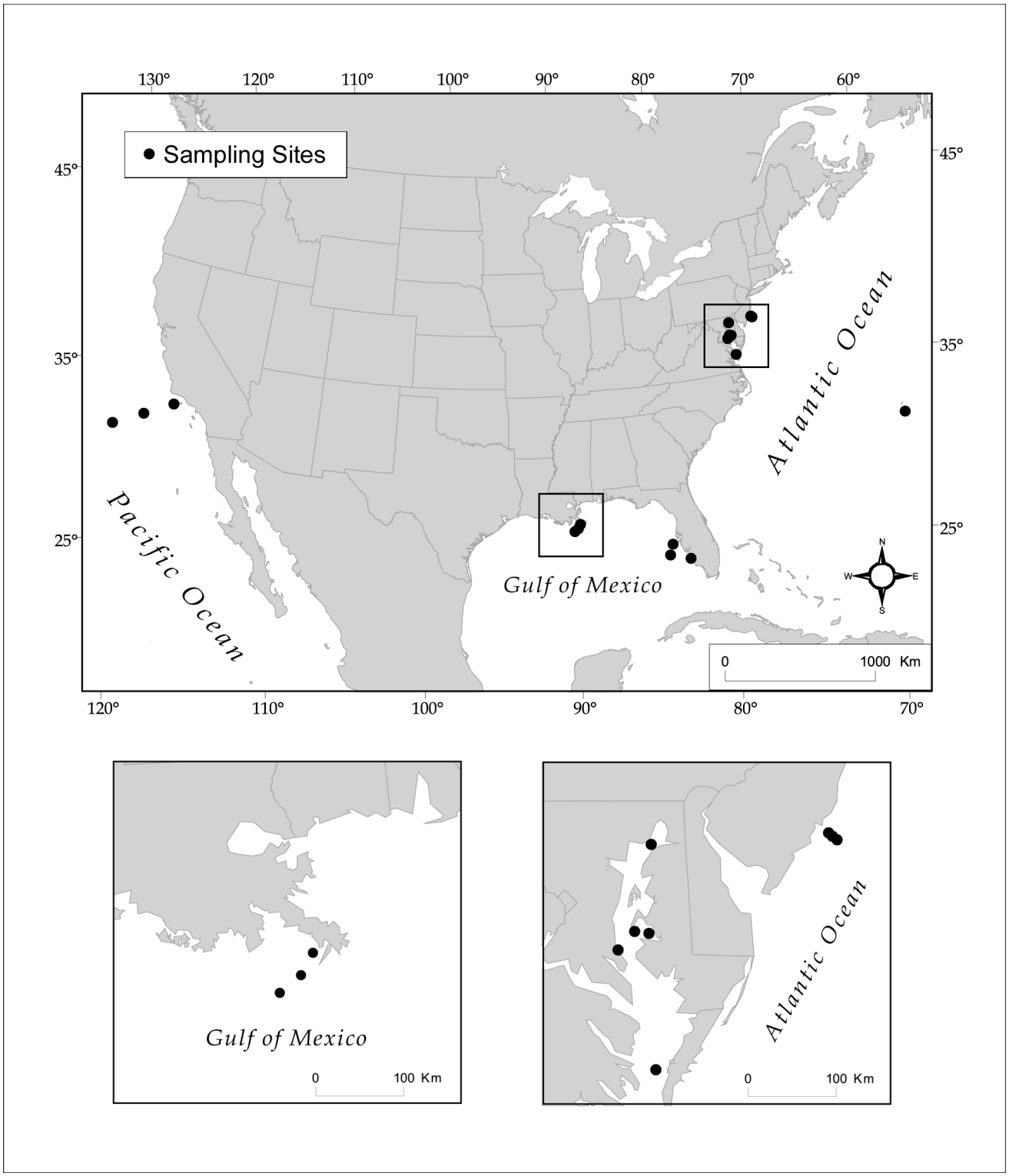


Fig. 3.1. The 18 filter collection sites represented in this study. The number of sites sampled by each of 6 laboratories varied from 1 to 5.

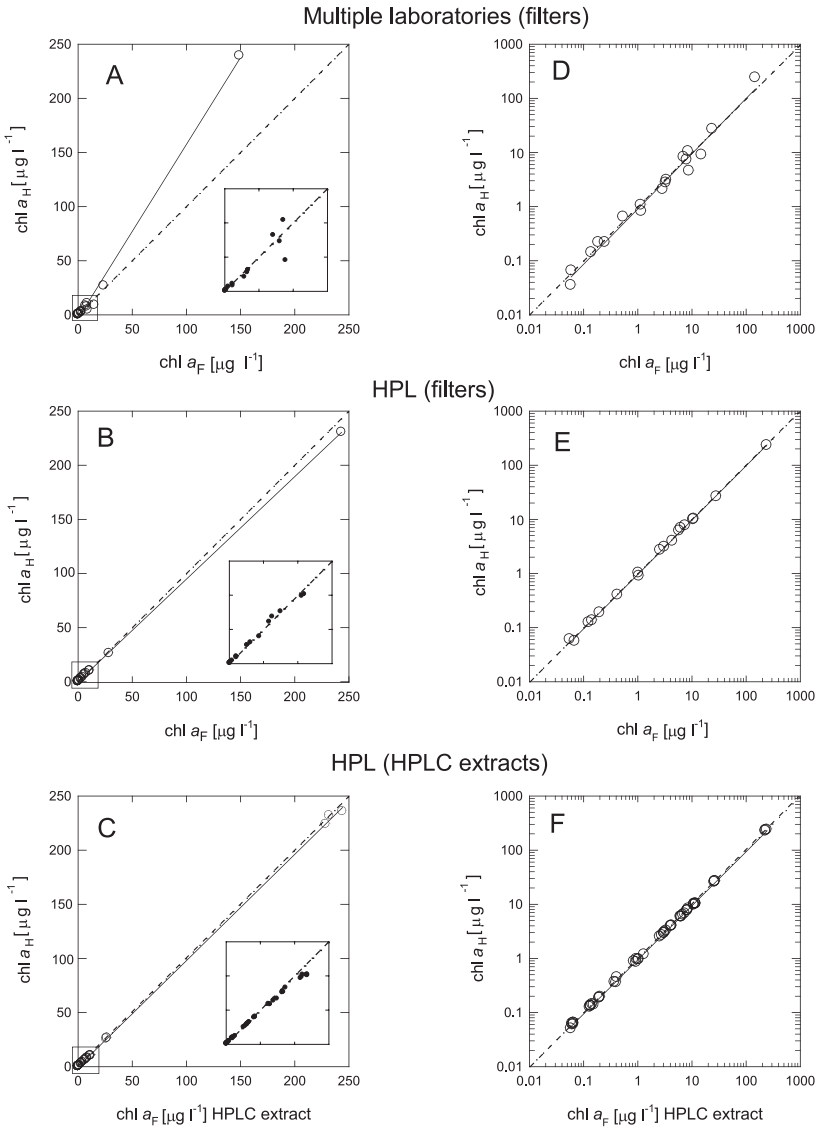


Fig. 3.2. Chl $a_H$ as a function of chl $a_G$ in field samples. Linear regressions (A, B, C) and log/log regressions (D, E, F) are shown. Multiple laboratories (filters) = results reported by 6 laboratories of filters they had collected (chl $a_H$ does not necessarily represent total chl *a*); HPL (filters) = results of filters all analyzed at HPL (chl $a_H$ = total chl *a*); each datum compares mean values of replicate filters. HPL (HPLC extracts) = fluorometric analysis of HPLC filter extracts at HPL (chl $a_H$ = total chl *a*) and each datum represents one filter (3 filters per site). Data in each regression represent filters from the same 18 field sites. Regression equations are in Table 3.1.

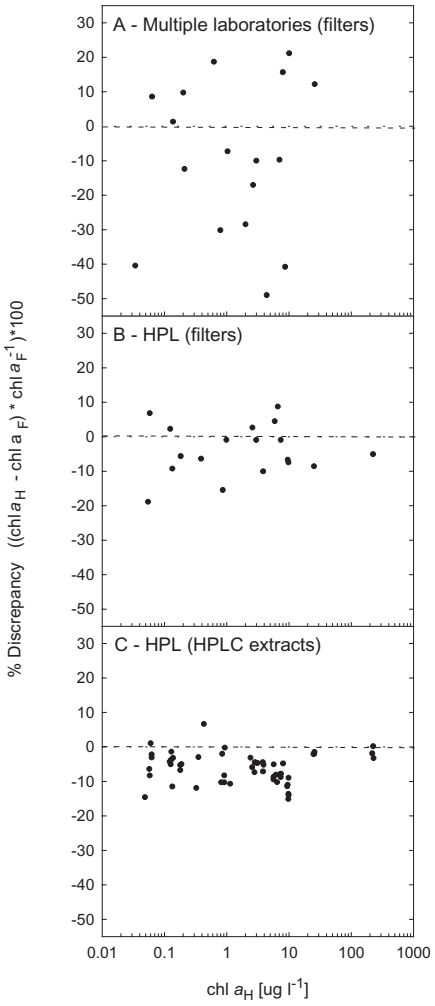


Fig. 3.3. The % discrepancy between results of HPLC and fluorometer filters from the same site as a function of chl $a_H$ at that site for (A) results reported by 6 laboratories of filters they had collected (chl $a_H$ does not necessarily represent total chl *a*), (B) results of filters all analyzed at HPL (chl $a_H$ = total chl *a*), and (C) results of HPLC filter extracts analyzed fluorometrically at HPL (chl $a_H$ = total chl *a*). Data in each panel represent filters from the same 18 sites. In A and B, each datum compares mean values of replicate filters and in C, each datum represents one filter (3 filters per site).

Table 3.1. The linear and log/log regressions of chl $a_F$ v. chl $a_H$. Multiple labs = results of filter analyses reported by 6 different laboratories considered collectively. HPL:filters = the analysis of filters at HPL. HPL:HPLC extracts = the fluorometric analysis of HPLC extracts. Regressions compare mean results of fluorometer filters with mean results of HPLC filters (lines 1, 2, 4 and 5) or compare a fluorometer result with an HPLC result from the same filter (lines 3 and 6). In all cases, the same 18 sites were represented. Each site is represented by 3 individual filter results in lines 3 and 6, therefore, n = 54.

| # | Source of data | Line type | n | Slope | *y* int | $r^2$ |
|---|---|---|---|---|---|---|
| 1 | Multiple labs: filters | Linear | 18 | 1.607 | -3.314 | 0.993 |
| 2 | HPL: filters | Linear | 18 | 0.946 | 0.039 | >0.999 |
| 3 | HPL: HPLC extracts | Linear | 54 | 0.981 | -0.250 | >0.999 |
| 4 | Multiple labs: filters | Log | 18 | 1.021 | -0.047 | 0.983 |
| 5 | HPL: filters | Log | 18 | 1.003 | -0.020 | 0.999 |
| 6 | HPL: HPLC extracts | Log | 54 | 0.999 | -0.028 | >0.999 |

3.3A are from multiple laboratories using different analytical methods and extraction procedures that varied between HPLC filters and fluorometer filters, results in Fig. 3.3B were acquired from filters that were extracted with standardized procedures and analyzed by the same analysts with same instruments and methods, and results in Fig. 3.3C were also performed by the same analysts with the same instruments and methods but were additionally unaffected by inaccuracies associated with extraction volume determinations, poor filter replication or differences between HPLC and fluorometer filters. The systematic reduction in the range of %Dsc associated with these 3 data sets is defined by the mean %Dsc $\pm$ WL. These were -5% $\pm$ 58% (multiple labs), -4% $\pm$ 16% (filter results at HPL) and -6% $\pm$ 9% (fluorometric analysis of HPLC extracts at HPL). (Data are in Appendices D, E, F). On a percentage basis, it would not be surprising for larger differences to occur with dilute samples. But in this study, concentration had little effect on %Dsc, as the slopes of the linear regressions of chl $a_H$ v. %Dsc were not significantly different from 0 ($p > 0.7$ for data in Fig. 3.3A, B and $p = 0.07$ with data in Fig. 3.3C). In all cases, $r^2$ values were low ($r^2 < 0.00$).

## 3.3 FACTORS AFFECTING DISCREPANCY BETWEEN HPLC & FLUOROMETER CHL. *a*

Several factors can affect %Dsc, most of which are related either to sample collection or sample analysis. Factors pertinent to sample collection include lack of homogeneity among replicate filters and differences between HPLC and fluorometer filters. This study addressed homogeneity among filters (as evidenced by poor filter replication) but did not address the important effects of filtration volumes, which often differ between HPLC and fluorometer filters. This topic is addressed by Bidigare and Trees (2002) and Trees et al. (2002). Factors related to sample analyses with the potential to affect %Dsc include such things as instrument imprecision and inaccuracy, effects of extraction procedures and effects of accessory pigments on fluorometric chl *a*. Laboratories' instrument precision and accuracy had been addressed with laboratory-prepared unknowns (Chapter 4). Additional experiments were conducted with field samples at HPL to determine if using extraction procedures that differ between HPLC and fluorometer filters contributed to an increase in the

range of %Dsc and to determine the extent to which pigments known to interfere with fluorometric chl *a* values also contributed to %Dsc. For this, total chl *c*, total chl *b* and DV chl *a* were quantified in each HPLC extract and their abundance (relative to total chl $a_H$) was considered in the context of the magnitude of the %Dsc seen for that sample.

*Sample collection and % discrepancy*

Homogeneity among replicate filters was evaluated for 16 sites, as filters had been collected in triplicate (or duplicate, sites 6-1 and 6-2) from the same collection bottle at each of these sites. The precision (%RSD) associated with the analysis of replicate filters is shown for results reported from HPL (Fig. 3.4A) and participants (Fig. 3.4B). The average HPLC %RSD was 6% (with results from participants and HPL) and the average fluorometer %RSD was 7% (HPL) and 11% (participants). When poorer than average precision co-occurred with results from the participant and HPL for the same site, the cause was considered primarily a result of poor homogeneity among filters. Four sites exhibited poor filter replication by this criteria: site 4-1 with HPLC and fluorometer filters, sites 8-2 and 8-3 with HPLC filters, and 9-1b with fluorometer filters. (Data are in Appendices D, E.)

The conclusion that poor precision at these sites was primarily related to poor filter replication was supported by 3 observations. First, it had been shown, with one exception (HPLC results of Lab 8), that all laboratories were able to achieve %RSD values $\leq$4% when they extracted and analyzed duplicate laboratory-prepared filters. Second, since all laboratories had used the standardized extraction procedures (with laboratory-prepared filters), the standard extraction procedure (used by HPL with field samples) had been proven to produce precise results. Third, when replicate HPLC extracts were analyzed fluorometrically at HPL, the precision associated with the analysis of replicate filter extracts on each instrument differed by no more than 2% and filter replicates exhibiting poor precision with HPLC analyses also did so with fluorometric analyses, indicating that the lack of precision was not due to instrument variability.

So that precision and %Dsc can be considered together for each site, the %Dsc for all 18 sites is shown for results from HPL (Fig. 3.5A) and participants (Fig. 3.5B), with sites that exhibited poor filter replication indicated by "•" (4-1, 8-2, 8-3 and 9-1b). These sites did not necessarily have high values of %Dsc (see site 4-1, 9-1b). The mean %Dsc $\pm$ WL for results from HPL (-4 $\pm$16%) are indicated on this figure so that sites with a large %Dsc are more easily identified. If replicate filters had not been available and %Dsc at each site had instead been calculated by comparing the result of one HPLC filter with one fluorometer filter exhibiting the most disparate result, the mean %Dsc $\pm$ WL (for results from HPL) would have been -5% $\pm$44% instead of that observed from the analysis of replicate filters (-4% $\pm$16%). Thus, not using replicate filters could have increased the range in %Dsc from 32% to 88%.

*Sample analysis and % discrepancy*

The effects of using extraction procedures that differ between HPLC filters and fluorometer filters on the range of %Dsc was determined. Twelve of the 18 sites sampled by participants were represented in these comparisons (Appendix G). These included 3 sites each from Labs 4, 6, 8 and 9. Participants' extraction procedures were duplicated at HPL, then the %Dsc for each site was calculated. When extraction procedures differed between HPLC and fluorometer filters, the mean %Dsc $\pm$WL was -6% $\pm$41%, compared to -1% $\pm$13% for results of filters from these same 12 sites that were extracted with standardized procedures. For quality assurance purposes, the extraction procedures being compared were always performed within the same week.

To evaluate the effects of accessory pigments on inter-instrument variability, all HPLC filter extracts (at HPL) were analyzed fluorometrically (after dilution) and %Dsc of filters from each site were compared to the accessory pigments in filters at that site. For this, total chl *c* and chl *b* and DV chl *a* were quantified by HPLC and their abundance,

relative to HPLC total chl *a*, was calculated (data are in Appendix H). To determine if the difference between chl $a_H$ and chl $a_F$ was significant (p=0.05), a paired t-test was performed with all filters from each site (Table 3.2). Total chl *c* from these 18 sites ranged from 9 to 32%, total chl *b* from 0 to 11% and DV chl *a* from 0 to 42% of total chl *a*. The mean %Dsc for all filters from the same site ranged from -2 to -13% among the 18 sites evaluated, and the magnitude of the %Dsc could not be related to whether the difference between chl $a_H$ and chl $a_F$ was significant (p = 0.05) or to the amounts of accessory pigments present. For example, chl *c* is known to suppress fluorometric chl *a* values and chl *b* is known to have the opposite effect (Lorenzen and Jeffrey 1980, Trees et al. 1985), yet, for the site with the highest chl *c* value (site 1-2) %Dsc was -2% and for the site with the highest chl *b* value (site 8-2) the %Dsc was -5%. For sites 1-1 and 1-4, with the most disparate %Dsc, the accessory pigments were similar. When considered in the context of other variables contributing to an increase in the range of %Dsc (such as filter replication or effects of differing extraction procedures), inter-instrument variability at HPL contributed little to %Dsc. However, as shown by Trees et al. (2000) contribution of fluorometer results to such uncertainties is variable among instruments.

Table 3.2. Inter-instrument variability was assessed by analyzing HPLC extracts fluorometrically (n = the number of HPLC filters per site sampled). Pigments in these extract (total chl c, chl b and DV chl a) were quantified by HPLC and their results are shown as % of total chl a. A paired t-test was performed with HPLC vs. fluorometer chl a results to determine if differences were significant (s = significant differences, p = 0.05).

| | Laboratory collecting filters and the site designation | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Lab 1 | | | | | Lab 4 | | | Lab 5 | Lab 6 | | | Lab 8 | | | Lab 9 | | |
| Site # | 1 | 2 | 3 | 4 | 5 | 1 | 2 | 3 | 1 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| n | 6 | 6 | 5 | 6 | 6 | 6 | 3 | 6 | 4 | 6 | 4 | 6 | 6 | 4 | 6 | 6 | 6 | 6 |
| %Dsc | -2 | -2 | -6 | -13 | -7 | -4 | -7 | -6 | -12 | -4 | -2 | -9 | -13 | -5 | -12 | -7 | -6 | -6 |
| %Chl c | 17 | 32 | 9 | 16 | 14 | 12 | 10 | 15 | 18 | 2 | 19 | 21 | 18 | 11 | 18 | 20 | 23 | 20 |
| %Chl b | 1 | 0 | 0 | 1 | 2 | 3 | 6 | 5 | 4 | 13 | 3 | 6 | 2 | 11 | 2 | 5 | 5 | 6 |
| %DV chl a | 0 | 0 | 0 | 0 | 0 | 0 | 42 | 6 | 0 | 1 | 28 | 0 | 0 | 5 | 0 | 0 | 0 | 0 |
| Significant | s | s | | s | s | s | | s | s | 18 s | | s | s | | | s | s | s |

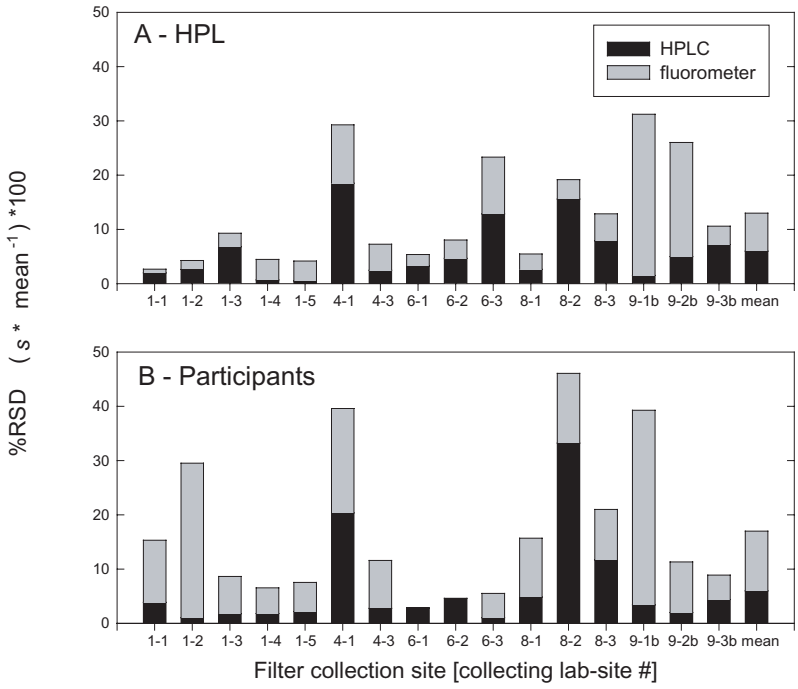


Fig. 3.4. The precision (%RSD) associated with the analysis of replicate filters from each of 16 filter collection sites for (A) analyses conducted at HPL and for (B) results reported by participants. Mean %RSD values represents the average across all sites for each instrument.

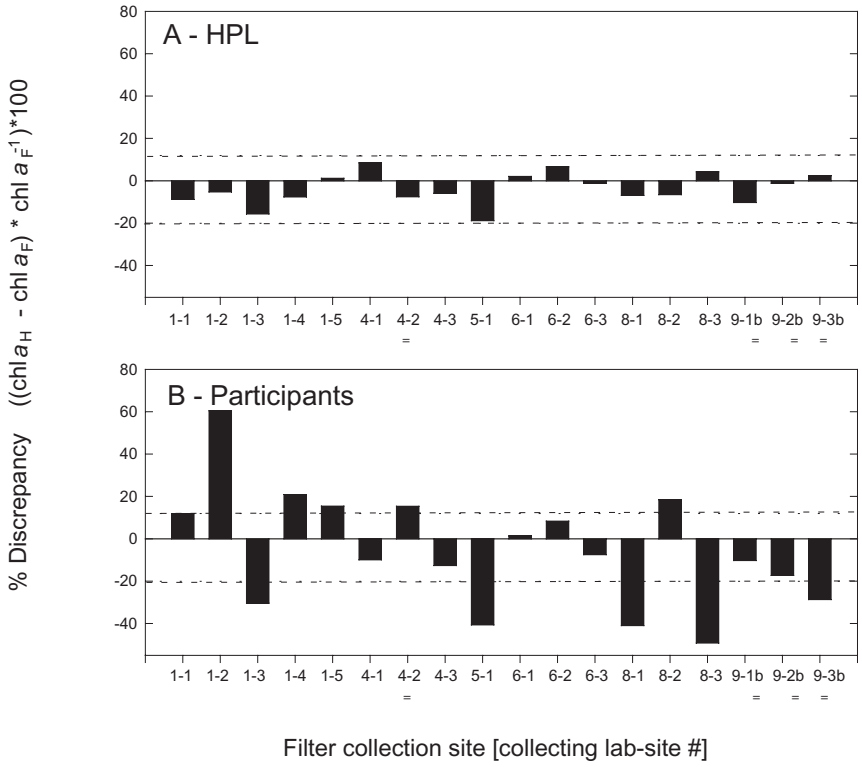


Fig. 3.5. The % discrepancy between chl *a* results of HPLC and fluorometer filters from each of 18 collection sites. Results are from (A) HPL and (B) participants. Dashed lines indicate the 95% confidence limits (± 16%) associated with the mean % discrepancy (-4%) at HPL. Dots (•) indicate sites exhibiting poor filter replication (as seen in Fig. 6).

# *Chapter 4*
# Results Of Inter-Laboratory Variability Analysis

## 4.1 INTRODUCTION

Inter-laboratory variability, as used in this report, is described by the difference between results of field sample filters from the same site analyzed on the same instrument type by two different laboratories and is important to %Dsc, as bias in either instrument (HPLC or fluorometer) has the potential to affect %Dsc. Factors evaluated for their effects on inter-laboratory variability included extraction procedures (which differed between the participant and HPL), complex pigment composition, and differences between fluorometer analysis methods. After identifying factors uniquely affecting results from each site, results of all sites from the same laboratory were considered collectively for the purpose of relating the inter-laboratory variability seen in this study to other inter-calibration exercises.

## 4.2 EFFECTS OF DIFFERING EXTRACTION PROCEDURES ON INTER-LABORATORY VARIABILITY

HPLC extraction procedures of Labs 4, 6 and 9 were implemented at HPL (with the second set of field sample filters provided) and results were compared with those from the same sites for which the standardized extraction procedures had been used. Such comparisons were also made with the fluorometric procedures of Labs 4 and 6. It was possible to identify the effects of differing extraction procedures free from calibration changes, analyst changes and filter changes over time, as comparisons were only made when the participant's procedure had been implemented within 2 days of the standardized procedure. Mean chl *a* values of filters from the same site that had been extracted with the standardized procedures (at HPL) were compared with those (from the same site) extracted with the participant's procedures (at HPL) and the % difference between extraction procedures ($\%D_{EXT}$) was determined ($\%D_{EXT}$ = chl $a_{\text{EXT-PARTICIPANT}}$ - chl $a_{\text{EXT-STANDARD}}$) *chl $a_{\text{EXT-STANDARD}}^{-1}$)*100). The mean $\%D_{EXT}$ ($\pm$ *s*) for each laboratory's extraction procedure was compiled from results of all sites from the same laboratory. Labs 4 and 6 were represented by 3 sites each, and Lab 9 by 3 sites sampled on each of 3 days. (Data are in Appendix I.)

The HPLC extraction procedures of Labs 4 and 6 were unbiased relative to the standardized extraction procedures, as the mean $\%D_{EXT}$ were 1% $\pm$ 3 (Lab 4) and 3% $\pm$ 8 (Lab 6). The fluorometer extraction procedures of Labs 4 and 6 were unbiased relative to the standardized procedure, as the mean $\%D_{EXT}$ were -1% $\pm$ 4 (Lab 4) and -3% $\pm$ 3 (Lab 6). Differences between extraction procedures were minimal, even though procedures varied with regard to whether filters were disrupted or not and the length of soak time (Tables 1.2 and 1.3).

The HPLC extraction procedure of Lab 9, when implemented at HPL produced results that were significantly different ($p \# 0.05$) than the standardized procedure. Procedures of Lab 9 were, on average, 27% $\pm$ 11 lower than the standardized procedure. These differences were attributed primarily to calculations and reporting practices rather than to differences in extraction efficiency, as an incorrect extraction volume had been used, chlide *a* was not included in total chl *a* and acetone was cold when pipetted. It was found at HPL that pipetting 90% acetone when cold significantly increased the volume delivered (by 2%) over that when it was pipetted at room temperature ($p = 0.003$, $n = 7$). It was therefore possible to revise results from extraction procedures of Lab 9 by increasing the volume of solvent delivered (5 ml) by 2%, adding the volume of water retained by a 47

mm GF/F filter (700 $\mu$l, Bidigare et al. 2002) and including chlide *a* in total chl *a*. With these changes, the HPLC extraction procedures of Lab 9 produced results that were, on average, 7% $\pm 7$ lower than results of the standardized procedure.

*Other factors affecting inter-laboratory variability*

Field sample results of each participant and HPL were compared to describe inter-laboratory variability. For this, the participant's mean result and HPL's mean result for the same site (for a particular instrument) were averaged to calculate a mean consensus value. The % difference (%D) from the mean consensus (for each site) was calculated (%D = ((chl *a* $_{\text{PARTICIPANT}}$ - chl *a* $_{\text{MEAN CONSENSUS}}$) * chl *a* $_{\text{MEAN CONSENSUS}}^{-1}$) * 100). Previously, it had been demonstrated (with few exceptions) that laboratories yielded results for filter analyses within $\pm 5$% (HPLC) and $\pm 8$% (fluorometer) when laboratory-prepared filters were extracted with standardized procedures. Field site results where %D from the mean consensus exceeded these ranges were investigated for factors contributing to the larger differences. Values of %D for each field site are shown for HPLC (Fig. 4.1A) and fluorometer results (Fig. 4.1B).

In some instances, when a participant's %D was large and of the same sign with results from the HPLC and fluorometer, the effects on %Dsc were minimal. This can be seen with results from sites 4-2, 8-2 and 9-1b, where the %Dsc (15%, 18%, and -10%, respectively, Fig. 3.5B) does not reflect the magnitude of %D (Fig. 4.1). In contrast, if %D for each instrument was small, but of the opposite sign (as with site 1-3, Fig. 8) the effects on %Dsc were greater (%Dsc = -30%, Fig. 3.5B) than if values of %D for each instrument were of the same sign. (Data are in Appendix J).

In many cases it was possible to identify reasons why values of HPLC %D were larger than $\pm 5$%. As had been previously noted (Table 2.1), Labs 5 and 9 did not account for the water contributed by the HPLC sample filter when determining extraction volumes and did not include chlide *a* in total chl *a*. When corrections were made to their HPLC results (by changing extraction volumes from 5.0 ml to 5.7 ml and by including chlide *a*), all but 1 of the revised values of HPLC %D of Lab 9 were within $\pm$ 5% and the HPLC %D of Lab 5 was reduced from -24% to -18%. While this %D is still high, the revised value from Lab 5 was only 0.016 $\mu$g l$^{-1}$ different from the average total chl $a_H$ value at HPL for this site (0.056 $\mu$g l$^{-1}$ chl *a*). Labs 4 and 8 had used HPLC injection procedures known to contribute uncertainties (Table 2.1) and some of their field sample results were complicated by poor filter replication (Fig. 3.4) and the presence of DV chl *a*, which comprised 40% of total chl *a* at site 4-2 and 5% of total chl *a* at site 8-2. Labs 4 and 8 had been unable to accurately quantify total chl $a_H$ when DV chl *a* was present (Table 2.2). The elevated HPLC %D with site 6-1 may have been influenced by the fact that only one filter was available for analysis at HPL from the field sample collection bottle used for these comparisons.

Many fluorometer field sample %D values exceeded the range seen with laboratory-prepared filters ($\pm 8$%) (Fig. 4.1B). These included 2 site results from Lab 1, all from Labs 4 and 8 and 3 sites from Lab 9. No reasons could be found for the large differences between fluorometer results of Lab 8 and HPL. It is not known why chl $a_F$ reported by Lab 1 for site 1-4 (8.6 $\mu$g l$^{-1}$) was lower than chl $a_F$ from HPL (11 $\mu$g l$^{-1}$). It is possible with site 1-2 that chl $a_F$ reported by the participant (149 $\mu$g l$^{-1}$) resulted from inaccurate dilution of the sample extract prior to analysis (this extract was serially diluted twice with un-calibrated measuring devices). There was no evidence of poor filter replication at this site with total chl $a_H$ (from Lab 1 or HPL) or with chl $a_F$ from HPL, as %RSD was < 3% in all 3 instances. The elevated values of %D of site 9-1b may have been related to poor filter replication, as %RSD was > 30% with results of Lab 9 and HPL. In fact, poor fluorometer filter replication may have occurred at other Lab 9 sites, as poor precision (> 20%RSD) occurred frequently with results at HPL and with results reported by Lab 9 (Appendices D, E). It is not surprising that chl $a_F$ values of Lab 4 differed from those of HPL, as Lab 4 used a non-acidification method (Welschmeyer 1994) for

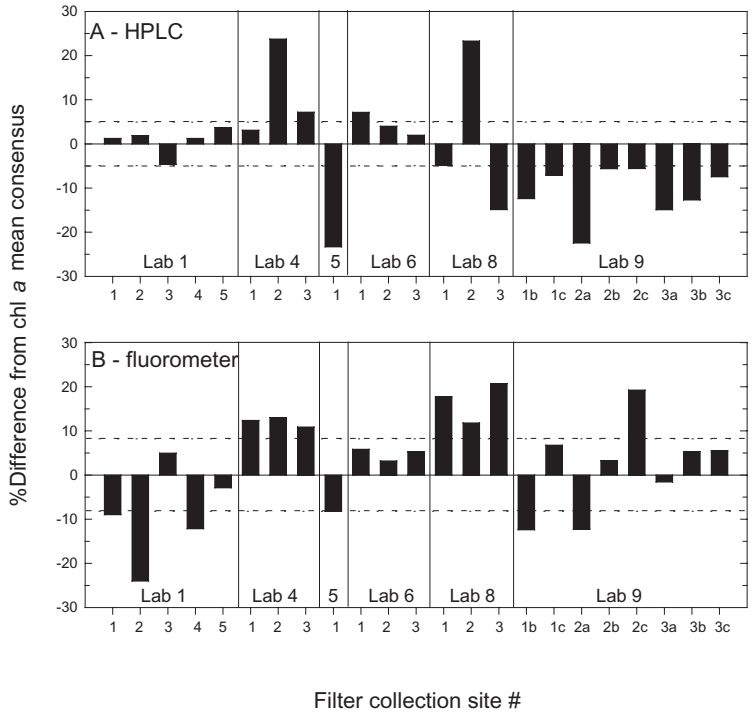


Fig 4.1. The % difference between chl *a* reported by the participant and the mean consensus value for each site for (A) HPLC filters and (B) fluorometer filters representing 18 filter collection sites. (For Lab 9, a, b, and c indicate collection day). Results are discussed in the context of the 95% confidence limits (dashed lines) associated with the analysis of laboratory-prepared filters at HPL (± 5% HPLC, ± 8% fluorometer).

Table 4.1. Inter-laboratory variability was defined by the absolute 5 difference (Abs%D) that each laboratory's results was from the mean consensus for that site. The Abs%D was averaged across all sites sampled by a laboratory to determine a mean Abs%D for each laboratory. The HPLC data subset excludes laboratories whose results were adversely affected by HPLC limitations (see Table 2.1).

| | Mean Abs%D per laboratory | | | | | | |
|---|---|---|---|---|---|---|---|
| Lab Code | 1 | 4 | 5 | 6 | 8 | 9 | Overall mean Abs%D |
| Fluorometer | 10.6 | 12.1 | 8.2 | 4.8 | 16.7 | 7.9 | 10.0 |
| HPLC | 2.5 | 11.3 | 23.3[1] | 4.4 | 14.4 | 11.4 | 11.2 |
| HPLC data subset | 2.5 | ---- | 16.9[1] | 4.4 | ---- | 4.0 | 7.0 |

[1] The high mean Abs%D for HPLC results of Lab 5 is related to the fact that this laboratory had only one site and chl a was very dilute (0.05 $\mu$ gl$^{-1}$).

fluorometer analysis, which is designed to overcome the effects of interfering pigments, and HPL used an acidification method. At HPL, extracts of fluorometer filters (n = 18) and HPLC filters (n = 9) of Lab 4 were analyzed with both fluorometric methods. The results from the non-acidification method were significantly different from the acidification method (p = 0.05, paired t-test) and were, on average, higher by 7%. However, Trees et al. (2000) had previously shown an approximate 6% difference between results of 2 different fluorometers, both of which used an acidification method. Trees et al. (2000) attributed these differences to subtle differences in excitation filters.

*Inter-laboratory variability in the context of other inter-calibration exercises*

Latasa et al. (1996) and Hooker et al. (2000) addressed variability in HPLC chl *a* results among laboratories. Although Latasa et al. (1996), Hooker et al. (2000) and the current study had different objectives, experimental design and data presentation, it is possible to make some comparisons among these studies. In the study by Latasa et al. (1996), HPLC standards containing chl *a* were distributed to 8 laboratories and then participants analyzed unknown solutions containing chl *a*. 90% of results were within ± 20% of the median value. In the study of Hooker et al. (2000), 4 laboratories analyzed replicate filters from 12 field sites, but no attempt was made to control variables, as neither chl *a* calibrations or extraction procedures were standardized. 97% of these results were within ± 20% of mean consensus values. In the current study, laboratory-prepared filters were analyzed by all laboratories (n = 7), chl *a* calibrations were normalized and all laboratories used the same extraction procedures. 86% of these HPLC results and 100% of fluorometer results were within ± 20% of the mean consensus. Results of these 3 studies suggest that diversity exists in the accuracy of HPLC methods among laboratories, as there was a greater consensus of agreement in the study of Hooker et al. (2000) even though results were affected by more variables.

Expressing results as above addresses the range of inaccuracies, but does not address average accuracy among laboratories. It was not possible to compare data of Latasa et al. (1996) on the basis of average accuracy, but comparing average accuracy with laboratory-prepared filters in the current study and average accuracy with field samples in Hooker et al. (2000) was possible. In Hooker et al. (2000), and in the current study, average accuracy was

computed by converting the % difference that each laboratory's result was from a mean consensus value to an absolute number (to preserve the variance) and then averaging results among laboratories. The term, mean absolute % difference (mean Abs%D), describes the average difference between a particular laboratory's results and a mean consensus value. The overall mean Abs%D for chl *a* across all sites and all laboratories in the study of Hooker et al. (2000) was 7.9%. This overall mean Abs%D was reduced to 6.7% when sites high in DV chl *a* were not included. In the current study, the overall mean Abs%D for HPLC results of laboratory-prepared filters among all laboratories was 6.9% (n = 7 laboratories). After excluding 2 laboratories that had been inaccurate when analyzing chl *a* unknown solutions, the HPLC overall mean Abs%D was reduced to 1.9%. The overall mean Abs%D for fluorometer results of laboratory-prepared filters was 1.4% (n = 7 laboratories). These laboratory-prepared filters did not contain DV chl *a*.

In the current study, the mean Abs%D for each laboratory was calculated based on results of the field sites each had analyzed. These results are tabulated for each instrument (Table 4.1). The overall mean Abs%D is shown for HPLC results from all laboratories and also for a subset of laboratories whose HPLC results had not been adversely affected by inconsistencies with HPLC Ocean Optics Protocols (Bidigare et al. 2002). Results reported by Labs 5 and 9 that had been revised to comply with guidelines in Ocean Optics Protocols (see Section 4.2) were also included in this subset. A comparison of the HPLC overall mean Abs%D for all laboratories (11.2%) with that of laboratories in the data subset (7.0%) reveals that HPLC methods unaffected by limitations (Table 2.1) were better able to reproduce results of another laboratory. It is possible that the poor precision seen with some fluorometer field sample results (Fig. 3.4B) and inherent differences between fluorometers, as illustrated in this study with differences between the acidification method and the non-acidification method and as seen previously by Trees et al. (2000), contributed to uncertainties in fluorometer results (Table 4.1).

Field sample results in this study can be considered in the context of results presented in Hooker et al. (2000) but with attention to 3 major differences between the 2 studies. In the current study, the values of Abs%D (from the mean consensus) were based on results of only 2 laboratories (HPL and each participant), different participants analyzed different field samples (which varied in concentration and pigment content) so complexities associated with their analyses also varied, and chl *a* calibrations between each participant and HPL had been normalized. The overall mean Abs%D for the most accurate HPLC results in Hooker et al. (2000) was 6.7%; in this study it was 7.0%.

# *Chapter 5*
# Conclusions

Ocean Optics Protocols for Satellite Ocean Color Sensor Validation (Mueller et al. 2002) for the analysis of chl *a* (Bidigare et al. 2002, Trees et al. 2002) were important to accurate results in this study. Average inter-laboratory variability in chl *a* spectrophotometric analyses, when all laboratories followed suggested protocols, was 1.4%. Some laboratories' HPLC procedures were inconsistent with protocols. When mean chl *a* values between field site results of these laboratories and the reference laboratory were calculated, laboratories differed from the mean, on average, by 15%. When HPLC field site results were limited exclusively to laboratories that used HPLC methods unaffected by these procedural inconsistencies, this average was 7%. The average difference between each laboratory and the mean value with analogous fluorometer field site results was 11%, yet no inconsistencies with suggested fluorometer procedures were found. The HPLC procedures that adversely affected results were related to injection conditions, quantitation of DV chl *a*, chl *a* reporting practices and HPLC filter extraction volumes.

Studies with replicate field samples from 18 sites revealed that discrepancies between HPLC and fluorometer values increased when a greater number of variables with the potential to affect results existed. The mean % discrepancy ($\pm$ 95% confidence limits) was -5% $\pm$ 58% with data affected by the most variables, as it represented results of several laboratories considered collectively. The mean % discrepancy was –4% $\pm$ 16% when all samples were analyzed by one laboratory, extraction procedures were standardized and analytical procedures were consistent with suggested guidelines (Bidigare et al. 2002, Trees et al. 2002). When HPLC extracts were analyzed fluorometrically, thereby removing variables related to sample collection, the mean % discrepancy was –6% $\pm$ 9%. Some variables had a great influence on discrepancy. For example, variation of extraction procedures (between HPLC and fluorometer filters) caused the range of discrepancies to increase threefold over that seen when extraction procedures between filters were standardized.

Poor homogeneity among filters was an important variable affecting discrepancy. Evidence for poor filter replication occurred at 25% of sites for which replicate filters had been collected. The average relative standard deviation (%RSD) associated with filters from sites exhibiting poor filter replication was 18% (HPLC filters) and 24% (fluorometer filters), whereas the average %RSD for replicate filters from other sites was 3.4% (HPLC filters) and 7.0% (fluorometer filters). If filters had not been collected in triplicate and % discrepancy per site had been determined by comparing the result of one HPLC filter with one fluorometer filter, discrepancies could have increased approximately threefold.

Without inter-calibration exercises, laboratories cannot know if the accuracy and precision of their analytical methods are typical of other laboratories. Such exercises are therefore important when identifying what modifications to analytical procedures effect the greatest improvements to accuracy. To assess the complexities of fluorometer and HPLC discrepancies, future inter-calibration exercises should include both field samples and laboratory-prepared samples, as accuracy with fluorometric analyses of laboratory-prepared unknown solutions did not necessarily predict a laboratory's ability to approximate fluorometer field sample results of another laboratory. In contrast, HPLC methods that were accurate with laboratory-prepared unknown solutions were able to closely reproduce field sample results of another laboratory. Additionally, in future studies it may be warranted to further address the effects of different filtration volumes, as has been described by Bidigare et al.

(2002) and Trees et al. (2002) and to address the effects of filter types that differ between HPLC filters and fluorometer filters.

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# Appendix A-Manufacturer's List

Agilent Technologies, Inc.
1601 California Avenue
Palo Alto, CA 94304 USA
Voice: 800-227-9770
Fax: 800-633-8696
Net: http://www.agilent.com/chem

Alltech Associates, Inc.
2051 Waukegan Road
Deerfield, IL 60015 USA
Voice: 800-255-8324
Fax: 847-948-1078
Net: http://www.alltechweb.com

Dionex Corporation
1228 Titan Way
Sunnyvale, CA 94088 USA
Voice: 408-737-0700
Fax: 408-730-9403
Net: http://www.dionex.com

Fluka Chemical Corporation
1001 West St. Paul Avenue
Milwaukee, WI 53233 USA
Voice: 414-273-5013
Fax: 414-273-4979
Net: http://www.sigma-aldrich.com/

Hewlett Packard, see Agilent Technologies, Inc.

Phenomenex, Inc.
2320 West 205$^{th}$ Street
Torrance, CA 90501 USA
Voice: 310-212-0555
Fax: 310-328-7768
Net: http://www.phenomenex.com

Starna Cells, Inc.
P.O. Box 1919
Atascardero, CA 93423 USA
Voice: 800-228-4482
Fax: 805-461-1575
Net: http://www.starna.com

Turner Designs, Inc.
845 W. Maude Avenue
Sunnyvale, CA 94085 USA
Voice: 877-316-8049
Fax: 408-749-0998
Net: http://www.turnerdesigns.com

VYDAC/The Separations Group, Inc.
17434 Mojave Street
Hesperia, CA 92345 USA
Voice: 800-247-0924
Fax: 760-244-1984
Net: http://www.vydac.com

Waters Corporation
34 Maple Street
Milford, MA 01757 USA
Voice: 508-478-2000
Fax: 508-872-1990
Net: http://www.waters.com

Whatman Inc.
9 Bridewell Place
Clifton, NJ 07014 USA
Voice: 973-773-5800
Fax: 973-472-6949
Net: http://www.whatman.com

# Appendix B-Unknown Solutions Analyzed By Participants

Tables 1-4 show the concentrations of unknowns as formulated, as measured by HPL before shipping and as measured by the participant after receiving. The relative standard deviation (%RSD), when given, is the estimate of precision associated with replicate analyses conducted by the participants. The % difference is the difference between the measured and formulated concentrations. Results for Lab code "HPL" are from the shipment prepared to test effects of shipping. Lab 4 reported results from 2 HPLC detectors, PDA and FLD.

Table 1. Unknown solution containing chl *a* only analyzed on the fluorometer

| | | Measured $\bar{X} \pm S$ ($\mu$ g l$^{-1}$) | | | % Difference | |
|---|---|---|---|---|---|---|
| Lab Code | Formulated $\mu$ g l$^{-1}$ | HPL | Participant | %RSD | HPL | Participant |
| 2 | 118.8 | 119.3 ± 0.981 | 132.2 | | 0.45 | 11.3 |
| 3 | 117.5 | 113.8 ± 0 | 117.9 ± 3.274 | 2.78 | -3.12 | 0.32 |
| 4 | 118.8 | 119.3 ± 0.981 | 113.7 ± 2.398 | 2.11 | 0.45 | -4.24 |
| 5 | 117.5 | 114.1 ± 1.566 | 117.2 ± 0.760 | 0.65 | -2.92 | -0.29 |
| 6 | 117.5 | 116.3 ± 0.437 | 117.8 ± 0.62 | 0.53 | -1.06 | 0.29 |
| 8 | 117.5 | 119.5[1] ± 0.245 | 119.2 ± 0.871 | 0.73 | 1.68 | 1.44 |
| 9 | 117.9 | 115.9[1] | 121.8 ± 4.684 | 3.85 | -1.68 | 3.30 |
| HPL | 117.5 | 119.5[1] ± 0.245 | 119.3 ± 0.406 | 0.34 | 1.68 | 1.56 |

[1] Analyzed at HPL on a TD-700 fluorometer with a non-acidification method.

Table 2. Unknown solution containing chl *a* only analyzed on the HPLC

| | | Measured $\bar{X} \pm S$ ($\mu$ g l$^{-1}$) | | | % Difference | |
|---|---|---|---|---|---|---|
| Lab code | Formulated $\mu$ g l$^{-1}$ | HPL | Participant | %RSD | HPL | Participant |
| 4 (PDA) | 118.8 | 115.3 ± 0.569 | 145.5 ± 4.921 | 3.38 | -2.95 | 22.5 |
| 4 (FLD) | 118.8 | 115.3 ± 0.569 | 145.3 ± 5.898 | 4.06 | -2.95 | 22.3 |
| 5 | 117.5 | 115.2 ± 1.996 | 112.5 ± 0.308 | 0.27 | -1.96 | -4.26 |
| 7 | 118.8 | 115.3 ± 0.569 | 118.1 ± 0.856 | 0.72 | -2.95 | -0.59 |
| 8 | 119.8 | 119.4 ± 0.515 | 106.7 ± 19.83 | 18.6 | -0.33 | -10.6 |
| 9 | 119.8 | 121.7 ± 0.591 | 121.8 ± 0.346 | 0.28 | 1.59 | 1.70 |
| HPL | 117.5 | 116.2 ± 0.368 | 116.8 ± 0.956 | 0.82 | -1.11 | -0.56 |

Table 3. Unknown solution containing DV chl *a* and chl *a* analyzed on the fluorometer

| | | Measured $\bar{X} \pm S$ ($\mu$ g l$^{-1}$) | | | % Difference | |
|---|---|---|---|---|---|---|
| Lab code | Formulated $\mu$ g l$^{-1}$ | HPL | Participant | | HPL | Participant |
| 2 | 107.9 | 101.3 ± 0.981 | 116.2 | | -6.16 | 7.65 |
| 3 | 102.9 | 93.04 ± 15.79 | 102.2 ± 1.589 | | -0.64 | -9.56 |
| 4 | 107.9 | 101.3 ± 0.981 | 113.4 ± 0 | | -6.16 | 5.10 |
| 5 | 102.9 | 101.5 ± 0.523 | 104.6 ± 0.745 | | -1.33 | 1.69 |
| 6 | 102.3 | 95.70 ± 0.535 | 93.84 ± 1.322 | | -6.44 | -8.26 |
| 8 | 101.9 | 107.7[1] ± 0.344 | 106.2 ± 0.403 | | 5.74 | 4.24 |
| 9 | 107.9 | 101.6[1] ± 0.212 | 104.8 | | -5.83 | -2.91 |
| HPL | 101.9 | 102.3[1] ± 1.491 | 107.7 ± 0.344 | | 5.74 | 0.45 |

[1] Analyzed at HPL on a TD-700 fluorometer with a non-acidification method.

Table 4. Unknown solution containing DV chl *a* and chl *a* analyzed on the HPLC

| | | Measured $\bar{X}$ ± S ($\mu$ g l$^{-1}$) | | % Difference | |
|---|---|---|---|---|---|
| Lab code | Formulated $\mu$ g l$^{-1}$ | HPL | Participant | HPL | Participant |
| 4 (PDA) | 409.2 | 403.5 ± 0.999 | 829.8 | -1.39 | 103 |
| 4 (FLD) | 409.2 | 403.5 ± 0.999 | 589.0 | -1.39 | 43.9 |
| 5 | 409.2 | 396.2 | 471.5 | -3.18 | 15.2 |
| 6 | 409.2 | 400.6 | 421.0 | -2.10 | 2.88 |
| 7 | 409.2 | 403.5 ± 0.999 | 424.1 | -1.39 | 3.64 |
| 8 | 349.7 | 343.1 | 329.3 | -1.89 | -5.83 |
| 9 | 349.7 | 345.4 ± 0.557 | 386.3 | -1.23 | 10.5 |
| HPL | 409.2 | 408.5 ± 1.218 | 394.5 | -0.17 | -3.47 |

# Appendix C - Laboratory-Prepared Filter Analyzed By Participants And HPL

Participants analyzed at least 2 replicate filters of an algal culture distributed by HPL (n = the number of filters analyzed). Two filters were analyzed at HPL to evaluate the effects of shipping and several others (n = 10, fluorometer and n = 17, HPLC) were analyzed during the study to evaluate the reproducibility of HPL analytical methods . Lab 4 reported results from 2 types of HPLC detectors, PDA and FLD.

Table 1. Laboratory-prepared filters analyzed on the fluorometer

| Lab code | n | Mean ± *s* (ng chl *a* per filter) | % RSD |
|---|---|---|---|
| 2 | 2 | 1186 ± 30.50 | 2.57 |
| 3 | 2 | 1192 ± 7.625 | 0.64 |
| 4 | 2 | 1160 ± 27.35 | 2.36 |
| 5 | 2 | 1201 ± 27.21 | 2.27 |
| 6[1] | 4 | 1175 ± 22.13 | 1.88 |
| 8 | 2 | 1202 ± 24.46 | 2.04 |
| HPL | 2 | 1160 ± 15.29 | 1.32 |
| HPL | 10 | 1128 ± 40.20 | |

[1] Lab 6 extracted all filters for HPLC, therefore they diluted HPLC extracts to analyze on the fluorometer.

Table 2. Laboratory-prepared filters analyzed on the HPLC

| Lab code | n | Mean ± *s* (ng chl *a* per filter) | % RSD |
|---|---|---|---|
| 4 (FLD) | 2 | 1244 ± 43 | 3.46 |
| 4 (PDA) | 2 | 1277 ± 41.57 | 3.29 |
| 5 | 2 | 1002 ± 24.66 | 2.50 |
| 6 | 4 | 1030 ± 19.29 | 1.84 |
| 7 | 2 | 1047 ± 14.01 | 1.34 |
| 8 | 2 | 864 .4 ± 52.45 | 6.02 |
| 9 | 2 | 1059 ± 6.269 | 0.56 |
| HPL | 2 | 1065 ± 27.49 | 2.54 |
| HPL | 17 | 1073 ± 24.98 | |

# Appendix D - Participants' Field Sample Results As Reported By Them

Data from these 18 sites were considered collectively in Figs. 4A, 4D and 5A and when calculating mean % discrepancy (%Dsc) from "multiple laboratories". The code means: 1st number = laboratory code, 2nd number = site number, 3rd number = bottle number. In the case of Lab 9, letter = collection day. Results for Lab 4 are from their PDA detector (440 nm). In some instances, participants provided raw data and calculations were performed at HPL. All reported data were checked for calculation errors and corrections were made if necessary.

| | HPLC | | Fluorometer | | | |
|---|---|---|---|---|---|---|
| Code | $\bar{x} \pm s$ ($\mu$ g $l^{-1}$ chl *a*) | % RSD | $\bar{x} \pm \mu$ s ($\mu$ g $l^{-1}$ chl *a*) | % RSD | %Dsc | |
| 1-1 | 26.74 ± 1.036 | 3.87 | 23.88 ± 2.731 | 11.4 | 12.0 | |
| 1-2 | 239.2 ± 2.505 | 1.05 | 149.0 ± 42.43 | 28.5 | 60.5 | |
| 1-3 | 0.813 ± 0.015 | 1.80 | 1.169 ± 0.080 | 6.84 | -30.4 | |
| 1-4 | 10.37 ± 0.189 | 1.82 | 8.581 ± 0.405 | 4.71 | 20.8 | |
| 1-5 | 8.190 ± 0.178 | 2.18 | 7.093 ± 0.381 | 5.37 | 15.5 | |
| 4-1 | 7.264 ± 1.480 | 20.4 | 8.067 ± 1.550 | 19.2 | -10.0 | |
| 4-2-10[1] | 0.228 | | 0.188 | | 21.3 | |
| 4-2-11[1] | 0.207 | | 0.189 | | 9.52 | |
| 4-3 | 0.217 ± 0.006 | 2.77 | 0.248 ± 0.022 | 8.83 | -12.5 | |
| 5-1-17[2] | 0.039 | | 0.061 | | -36.1 | |
| 5-1-18[2] | 0.034 | | 0.067 | | -49.3 | |
| 5-1-19[2] | 0.036 | | 0.055 | | -34.5 | |
| 5-1-20[2] | 0.031 | | 0.053 | | -41.5 | |
| 6-1-21 | 0.142 ± 0.004 | 2.83 | 0.14 ± 0 | 0 | 1.43 | |
| 6-2-21 | 0.065 ± 0.003 | 4.62 | 0.06 ± 0 | 0 | 8.33 | |
| 6-3 | 1.061 ± 0.011 | 1.04 | 1.15 ± 0.051 | 4.47 | -7.47 | |
| 8-1 | 8.947 ± 0.434 | 4.85 | 15.17 ± 1.642 | 10.8 | -41.0 | |
| 8-2 | 0.641 ± 0.213 | 33.2 | 0.541 ± 0.070 | 12.9 | 18.4 | |
| 8-3 | 4.514 ± 0.529 | 11.7 | 8.890 ± 0.824 | 9.26 | -49.2 | |
| 9-1-b | 3.079 ± 0.105 | 3.41 | 3.431 ± 1.230 | 35.9 | -10.3 | |
| 9-2-b | 2.740 ± 0.054 | 1.99 | 3.312 ± 0.309 | 9.32 | -17.3 | |
| 9-3-b | 2.069 ± 0.092 | 4.40 | 2.902 ± 0.130 | 4.49 | -28.7 | |

[1] Results of these 2 bottles were averaged. [2] Results of these 4 bottles were averaged and Lab 5 was represented by one site.

# Appendix E - Results of field samples analyzed at HPL

Data from these 18 sites were used in Figs. 4B, 4E and 5B and when calculating mean % discrepancy (%Dsc) from "HPL - filters". All filters were extracted with the standardized procedures (unless otherwise noted). The code means: 1st number = laboratory code, 2nd number = site number, 3rd number = bottle number. In the case of Lab 9, letter = collection day.

| | HPLC | | Fluorometer | | |
|---|---|---|---|---|---|
| Code | $\bar{x} \pm s$ ($\mu$ g $l^{-1}$ total chl *a*) | % RSD | $\bar{x} \pm s$ ($\mu$ g $l^{-1}$ chl *a*) | % RSD | %Dsc |
| 1-1 | 26.08 ± 0.517 | 1.98 | 28.60 ± 0.196 | 0.68 | -8.81 |
| 1-2 | 230.4 ± 6.019 | 2.61 | 243.3 ± 3.998 | 1.64 | -5.30 |
| 1-3 | 0.892 ± 0.061 | 6.81 | 1.058 ± 0.026 | 2.47 | -15.7 |
| 1-4 | 10.12 ± 0.069 | 0.68 | 10.96 ± 0.415 | 3.79 | -7.66 |
| 1-5 | 7.603 ± 0.038 | 0.50 | 7.515 ± 0.275 | 3.66 | 1.17 |
| 4-1 | 6.825 ± 1.263 | 18.5 | 6.290 ± 0.676 | 10.8 | 8.51 |
| 4-2-10[1] | 0.130 ± 0 | 0 | 0.138 | | -5.45 |
| 4-2-11[1] | 0.138 | | 0.153 | | -9.51 |
| 4-3 | 0.188 ± 0.005 | 2.46 | 0.199 ± 0.010 | 4.81 | -5.87 |
| 5-1-17[2] | 0.05 | | 0.069 | | -27.7 |
| 5-1-18[2] | 0.059 | | 0.071 | | -16.9 |
| 5-1-19[2] | 0.06 | | 0.068 | | -11.9 |
| 5-1-20[2] | 0.056 | | 0.070 | | -19.9 |
| 6-1-22 | 0.128 ± 0.004 | 3.28 | 0.125 ± 0.003 | 2.07 | 2.07 |
| 6-2-22 | 0.060 ± 0.003 | 4.67 | 0.056 ± 0.002 | 3.37 | 6.57 |
| 6-3 | 1.020 ± 0.131 | 12.9 | 1.032 ± 0.108 | 10.5 | -1.16 |
| 8-1 | 9.866 ± 0.260 | 2.63 | 10.60 ± 0.300 | 2.83 | -6.95 |
| 8-2 | 0.399 ± 0.062 | 15.6 | 0.427 ± 0.015 | 3.58 | -6.60 |
| 8-3 | 6.093 ± 0.482 | 7.90 | 5.844 ± 0.290 | 4.95 | 4.26 |
| 9-1-b | 3.950 ± 0.060 | 1.51 | 4.403 ± 1.308 | 29.7 | -10.3 |
| 9-2-b | 3.066 ± 0.153 | 4.98 | 3.103 ± 0.652 | 21.0 | -1.19 |
| 9-3-b | 2.671 ± 0.193 | 7.21 | 2.608 ± 0.088 | 3.39 | 2.42 |

[1] Results of these 2 bottles were averaged. [2] Results of these 4 bottles were averaged and Lab 5 was represented by one site.

# Appendix F - Fluorometric Analysis Of HPLC Extracts At HPL

Data from these 18 sites were used in Figs. 4C, 4F and 5C and when calculating mean % discrepancy (%Dsc) from the fluorometric analysis of HPLC extracts at HPL. Each site is represented by 3 filters. The code means: 1st number = laboratory code, 2nd number = site number, 3rd number = bottle number. In the case of Lab 9, the letter indicates collection day.

| Code | HPLC $\bar{x} \pm s$ ($\mu$ g $l^{-1}$ total chl *a*) | Fluorometer $\bar{x} \pm s$ ($\mu$ $\mu$ g $l^{-1}$ chl *a*) | %Dsc |
|---|---|---|---|
| 1-1 | 26.24 | 26.84 | -2.24 |
| 1-1 | 25.50 | 26.10 | -2.29 |
| 1-1 | 26.49 | 26.95 | -1.70 |
| 1-2 | 235.6 | 244.2 | -3.50 |
| 1-2 | 231.8 | 231.9 | -0.05 |
| 1-2 | 223.8 | 228.7 | -2.11 |
| 1-3 | 0.960 | 0.955 | 0.47 |
| 1-3 | 0.842 | 0.941 | -10.5 |
| 1-3 | 0.875 | 0.856 | 2.22 |
| 1-4 | 10.19 | 11.88 | -14.2 |
| 1-4 | 10.11 | 11.74 | -13.9 |
| 1-4 | 10.06 | 11.88 | -15.4 |
| 1-5 | 7.613 | 8.365 | -8.99 |
| 1-5 | 7.561 | 8.224 | -8.07 |
| 1-5 | 7.635 | 8.305 | -8.07 |
| 4-1 | 5.870 | 6.200 | -5.32 |
| 4-1 | 6.349 | 6.924 | -8.31 |
| 4-1 | 8.257 | 8.696 | -5.04 |
| 4-2-11 | 0.138 | 0.156 | -11.7 |
| 4-2-10 | 0.130 | 0.137 | -5.32 |
| 4-2-11[1,2] | 0.141 | 0.146 | -3.43 |
| 4-3 | 0.185 | 0.199 | -6.99 |
| 4-3 | 0.185 | 0.196 | -5.52 |
| 4-3 | 0.193 | 0.204 | -5.25 |
| 5-1-17 | 0.050 | 0.059 | -14.8 |
| 5-1-18 | 0.059 | 0.063 | -6.65 |
| 5-1-19 | 0.060 | 0.066 | -8.54 |
| 6-1-22[2] | 0.133 | 0.135 | -1.63 |
| 6-1-22 | 0.131 | 0.137 | -4.03 |
| 6-1-22 | 0.125 | 0.131 | -4.51 |
| 6-2-22 | 0.062 | 0.062 | 0.81 |
| 6-2-22[2] | 0.064 | 0.066 | -2.44 |
| 6-2-22[2] | 0.064 | 0.066 | -3.32 |
| 6-3 | 1.171 | 1.315 | -10.9 |
| 6-3 | 0.947 | 1.059 | -10.5 |
| 6-3 | 0.941 | 1.029 | -8.52 |
| 8-1 | 9.805 | 11.05 | -11.2 |
| 8-1 | 10.15 | 11.18 | -9.21 |
| 8-1 | 9.643 | 10.91 | -11.6 |
| 8-2 | 0.443 | 0.416 | 6.41 |
| 8-2 | 0.355 | 0.404 | -12.2 |
| 8-2[2] | 0.363 | 0.375 | -3.20 |
| 8-3 | 5.806 | 6.375 | -8.93 |
| 8-3 | 5.824 | 6.446 | -9.65 |
| 8-3 | 6.649 | 7.426 | -10.5 |
| 9-1-b | 3.934 | 4.247 | -7.37 |

| Code | HPLC $\bar{x} \pm s$ ($\mu$ g l$^{-1}$ total chl *a*) | Fluorometer $\bar{x} \pm s$ ($\mu$ $\mu$ g l$^{-1}$ chl *a*) | %Dsc |
|---|---|---|---|
| 9-1-b | 3.900 | 4.091 | -4.67 |
| 9-1-b | 4.016 | 4.247 | -5.44 |
| 9-2-b | 3.054 | 3.210 | -4.86 |
| 9-2-b | 2.919 | 3.062 | -4.67 |
| 9-2-b | 3.224 | 3.390 | -4.90 |
| 9-3-b | 2.490 | 2.577 | -3.38 |
| 9-3-b | 2.649 | 2.822 | -6.13 |
| 9-3-b | 2.874 | 3.110 | -7.59 |

[1] Extract was clarified with a PTFE HPLC syringe cartridge filter. [2] HPLC filter was extracted with participant's method.

# Appendix G - % Discrepancy And Variations In Extraction Procedures

Participants' HPLC and fluorometer extraction methods were implemented at HPL to assess effects on % discrepancy (%Dsc) of using extraction procedures that vary between HPLC and fluorometer filters. The code means: 1st number = laboratory code, 2nd number = site number, 3rd number = bottle number. In the case of Lab 9, the letter indicates collection day.

| Code | HPLC $\bar{x} \pm s$ ($\mu$ g $l^{-1}$ chl *a*) | % RSD | Fluorometer $\bar{x} \pm s$ ($\mu$ g $l^{-1}$ chl *a*) | % RSD | %Dsc |
|---|---|---|---|---|---|
| 4-1 | 7.005 ± 0.092 | 1.31 | 6.053 ± 0.493 | 8.15 | 15.7 |
| 4-2-10 | 0.114 | | 0.141 ± 0.001 | 0.92 | -19.5 |
| 4-3 | 0.194 ± 0.008 | 4.24 | 0.195 ± 0.013 | 6.47 | -0.67 |
| 6-1-22 | 0.137 ± 0.005 | 3.59 | 0.123 ± 0.002 | 1.38 | 11.2 |
| 6-2-22 | 0.064 ± 0 | 0 | 0.055 ± 0.0002 | 0.36 | 16.4 |
| 6-3 | 0.949 ± 0.087 | 9.20 | 1.033 ± 0.069 | 6.71 | -8.10 |
| 8-1 | 8.341 ± 0.544 | 6.53 | 9.088 ± 0.119 | 1.30 | -8.22 |
| 8-2 | 0.391 ± 0.078 | 20.0 | 0.346 ± 0.024 | 6.99 | 12.9 |
| 8-3 | 5.259 ± 0.027 | 0.52 | 5.274 ± 0.033 | 0.62 | -0.28 |
| 9-1-b | 3.097 ± 0.045 | 1.46 | 3.866 ± 0.296 | 7.65 | -19.9 |
| 9-2-b | 2.380 ± 0.275 | 11.5 | 3.571 ± 0.335 | 9.38 | -33.4 |
| 9-3-b | 1.736 ± 0.142 | 8.19 | 2.845 ± 0.105 | 3.70 | -39.0 |

# Appendix H – HPLC Analysis Of Accessory Pigments At HPL

All HPLC filter extracts were analyzed for accessory pigment content by HPLC and were also analyzed fluorometrically to determine the effects of accessory pigments on % discrepancy (%Dsc). These results are summarized in Section 5.2.2, Table 10. The code means: 1st number = laboratory code, 2nd number = site number, 3rd number = bottle number. In the case of Lab 9, the letter indicates collection day. Each value represents the analysis of one filter extracted at HPL either with the standard procedure or the participant's procedure. The extraction method of Lab 9 was modified to include the water contributed by the sample filter. Pigment ratio represents the amount of that pigment relative to HPLC total chl *a*. "Trace" indicates that a pigment was detected but the amount was too low to quantify.

Table 1. HPLC filter extracts analyzed by HPLC and fluorometer

| | HPLC | Fluorometer | | Pigment ratio | | | Extraction |
|---|---|---|---|---|---|---|---|
| Code | $\mu$ g $l^{-1}$ total chl *a* | $\mu$ g $l^{-1}$ chl *a* | % Dsc | Chl *c* | Chl *b* | DV chl *a* | mode |
| 1-1 | 26.24 | 26.84 | -2.24 | 0.171 | 0.012 | 0 | standard |
| 1-1 | 25.50 | 26.10 | -2.29 | 0.165 | 0.013 | 0 | standard |
| 1-1 | 26.49 | 26.95 | -1.70 | 0.174 | 0.013 | 0 | standard |
| 1-1 | 27.53 | 28.58 | -3.67 | 0.169 | 0.012 | 0 | participant's |
| 1-1 | 25.57 | 25.73 | -0.62 | 0.174 | 0.012 | 0 | participant's |
| 1-1 | 27.11 | 27.56 | -1.63 | 0.169 | 0.012 | 0 | participant's |
| 1-2 | 235.6 | 244.2 | -3.50 | 0.325 | 0 | 0 | standard |
| 1-2 | 231.8 | 231.9 | -0.1 | 0.314 | 0 | 0 | standard |
| 1-2 | 223.8 | 228.7 | -2.11 | 0.314 | 0 | 0 | standard |
| 1-2 | 236.5 | 242.6 | -2.48 | 0.325 | 0 | 0 | participant's |
| 1-2 | 239.5 | 249.9 | -4.18 | 0.326 | 0 | 0 | participant's |
| 1-2 | 241.5 | 241.7 | -0.1 | 0.333 | 0 | 0 | participant's |
| 1-3 | 0.960 | 0.955 | 0.47 | 0.099 | 0 | 0 | standard |
| 1-3 | 0.842 | 0.941 | -10.5 | 0.107 | 0 | 0 | standard |
| 1-3 | 0.875 | 0.856 | 2.22 | 0.102 | 0 | 0 | standard |
| 1-3 | 0.830 | 1.007 | -17.6 | 0.112 | 0 | 0 | participant's |
| 1-3 | 0.804 | 0.926 | -13.2 | 0.106 | 0 | 0 | participant's |
| 1-4 | 10.19 | 11.88 | -14.2 | 0.165 | 0.008 | 0 | standard |
| 1-4 | 10.11 | 11.74 | -13.9 | 0.164 | 0.007 | 0 | standard |
| 1-4 | 10.06 | 11.88 | -15.4 | 0.162 | 0.008 | 0 | standard |
| 1-4 | 10.23 | 11.86 | -13.7 | 0.167 | 0.015 | 0 | participant's |
| 1-4 | 10.31 | 11.86 | -13.1 | 0.163 | 0.015 | 0 | participant's |
| 1-4 | 10.59 | 11.86 | -10.7 | 0.160 | 0.014 | 0 | participant's |
| 1-5 | 7.613 | 8.365 | -8.99 | 0.141 | 0.012 | 0 | standard |
| 1-5 | 7.561 | 8.224 | -8.07 | 0.142 | 0.012 | 0 | standard |
| 1-5 | 7.635 | 8.305 | -8.07 | 0.144 | 0.012 | 0 | standard |
| 1-5 | 8.043 | 8.659 | -7.11 | 0.138 | 0.021 | 0 | participant's |
| 1-5 | 8.138 | 8.646 | -5.87 | 0.140 | 0.021 | 0 | participant's |
| 1-5 | 8.388 | 8.733 | -3.94 | 0.140 | 0.021 | 0 | participant's |
| 4-1 | 5.906 | 6.230 | -5.20 | 0.107 | 0.030 | 0 | participant's[1] |
| 4-1 | 6.644 | 6.842 | -2.89 | 0.067 | 0.027 | 0 | participant's[1] |
| 4-1 | 6.489 | 6.420 | 1.07 | 0.107 | 0.032 | 0 | participant's[1] |
| 4-1 | 5.870 | 6.200 | -5.32 | 0.147 | 0.038 | 0 | standard |
| 4-1 | 6.349 | 6.924 | -8.31 | 0.157 | 0.037 | 0 | standard |
| 4-1 | 8.257 | 8.696 | -5.04 | 0.150 | 0.032 | 0 | standard |
| 4-2-11 | 0.138 | 0.156 | -11.7 | 0.116 | trace | 0.420 | standard |
| 4-2-10 | 0.130 | 0.137 | -5.32 | 0.108 | trace | 0.454 | standard |
| 4-2-11 | 0.141 | 0.146 | -3.43 | 0.085 | 0.064 | 0.390 | participant's[1] |
| 4-3 | 0.185 | 0.199 | -6.99 | 0.157 | 0.065 | 0.054 | standard |
| 4-3 | 0.185 | 0.196 | -5.52 | 0.162 | 0.060 | 0.049 | standard |
| 4-3 | 0.193 | 0.204 | -5.25 | 0.161 | trace | 0.047 | standard |
| 4-3 | 0.202 | 0.211 | -4.26 | 0.158 | 0.050 | 0.050 | participant's[1] |

| HPLC Code | Fluorometer $\mu$ g l$^{-1}$ total chl *a* | $\mu$ g l$^{-1}$ chl *a* | Pigment ratio % Dsc | Chl *c* | Chl *b* | Extraction DV chl *a* | mode |
|---|---|---|---|---|---|---|---|
| 4-3 | 0.175 | 0.185 | -5.41 | 0.126 | 0.051 | 0.051 | participant's[1] |
| 4-3 | 0.200 | 0.213 | -6.10 | 0.135 | 0.050 | 0.100 | participant's[1] |
| 5-1-17 | 0.050 | 0.059 | -14.8 | 0.174 | 0.044 | 0 | standard |
| 5-1-18 | 0.059 | 0.063 | -6.65 | 0.155 | 0.035 | 0 | standard |
| 5-1-19 | 0.060 | 0.066 | -8.54 | 0.179 | 0.036 | 0 | standard |
| 5-1-20 | 0.056 | 0.067 | -16.0 | 0.196 | 0.039 | 0 | standard |
| 6-1-21 | 0.123 | 0.135 | -8.89 | 0.203 | trace | 0.187 | standard |
| 6-1-21 | 0.136 | 0.145 | -6.21 | 0.196 | 0.037 | 0.216 | participant's |
| 6-1-22 | 0.133 | 0.135 | -1.63 | 0.233 | 0.038 | 0.165 | participant's |
| 6-1-22 | 0.140 | 0.139 | 0.79 | 0.214 | 0.036 | 0.171 | participant's |
| 6-1-22 | 0.131 | 0.137 | -4.03 | 0.206 | 0.023 | 0.183 | standard |
| 6-1-22 | 0.125 | 0.131 | -4.51 | 0.216 | trace | 0.176 | standard |
| 6-2-21 | 0.060 | 0.064 | -6.25 | 0.117 | trace | 0.300 | standard |
| 6-2-22 | 0.062 | 0.062 | 0.81 | 0.129 | trace | 0.290 | standard |
| 6-2-22 | 0.064 | 0.066 | -2.44 | 0.219 | trace | 0.281 | participant's |
| 6-2-22 | 0.064 | 0.066 | -3.32 | 0.219 | 0.031 | 0.281 | participant's |
| 6-3 | 1.171 | 1.315 | -10.9 | 0.207 | 0.060 | 0 | standard |
| 6-3 | 0.947 | 1.059 | -10.5 | 0.215 | 0.067 | 0 | standard |
| 6-3 | 0.941 | 1.029 | -8.52 | 0.203 | 0.066 | 0 | standard |
| 6-3 | 0.858 | 0.949 | -9.61 | 0.212 | 0.069 | 0 | participant's |
| 6-3 | 0.958 | 1.053 | -9.02 | 0.229 | 0.066 | 0 | participant's |
| 6-3 | 1.032 | 1.110 | -7.06 | 0.216 | 0.064 | 0 | participant's |
| 8-1 | 8.850 | 10.35 | -14.5 | 0.128 | 0.017 | 0 | participant's |
| 8-1 | 8.405 | 9.767 | -14.0 | 0.206 | 0.019 | 0 | participant's |
| 8-1 | 7.767 | 9.673 | -19.7 | 0.222 | 0.019 | 0 | participant's |
| 8-1 | 9.805 | 11.05 | -11.2 | 0.180 | 0.015 | 0 | standard |
| 8-1 | 10.15 | 11.18 | -9.21 | 0.183 | 0.014 | 0 | standard |
| 8-1 | 9.643 | 10.91 | -11.6 | 0.183 | 0.014 | 0 | standard |
| 8-2 | 0.443 | 0.416 | 6.41 | 0.095 | 0.088 | 0.043 | standard |
| 8-2 | 0.355 | 0.404 | -12.2 | 0.130 | 0.110 | 0.048 | standard |
| 8-2 | 0.363 | 0.375 | -3.20 | 0.088 | 0.113 | 0 | participant's |
| 8-2 | 0.331 | 0.376 | -12.0 | 0.121 | 0.118 | 0 | participant's |
| 8-3 | 5.806 | 6.375 | -8.93 | 0.187 | 0.022 | 0 | standard |
| 8-3 | 5.824 | 6.446 | -9.65 | 0.194 | 0.021 | 0 | standard |
| 8-3 | 6.649 | 7.426 | -10.5 | 0.167 | 0.019 | 0 | standard |
| 8-3 | 5.234 | 6.187 | -15.4 | 0.199 | 0.028 | 0 | participant's |
| 8-3 | 5.255 | 6.222 | -15.5 | 0.196 | 0.025 | 0 | participant's |
| 8-3 | 5.288 | 6.222 | -15.0 | 0.157 | 0.019 | 0 | participant's |
| 9-1-b | 3.934 | 4.247 | -7.37 | 0.204 | 0.051 | 0 | standard |
| 9-1-b | 3.900 | 4.091 | -4.67 | 0.194 | 0.050 | 0 | standard |
| 9-1-b | 4.016 | 4.247 | -5.44 | 0.197 | 0.049 | 0 | standard |
| 9-1-b | 3.800 | 4.130 | -7.99 | 0.204 | 0.044 | 0 | participant's |
| 9-1-b | 3.689 | 3.896 | -5.31 | 0.207 | 0.044 | 0 | participant's |
| 9-1-b | 3.693 | 4.091 | -9.73 | 0.203 | 0.044 | 0 | participant's |
| 9-2-b | 3.054 | 3.210 | -4.86 | 0.225 | 0.045 | 0 | standard |
| 9-2-b | 2.919 | 3.062 | -4.67 | 0.211 | 0.045 | 0 | standard |
| 9-2-b | 3.224 | 3.390 | -4.90 | 0.227 | 0.044 | 0 | standard |
| 9-2-b | 2.940 | 3.144 | -6.49 | 0.226 | 0.052 | 0 | participant's |
| 9-2-b | 3.054 | 3.129 | -2.38 | 0.231 | 0.053 | 0 | participant's |
| 9-2-b | 2.733 | 3.043 | -10.2 | 0.232 | 0.054 | 0 | participant's |
| 9-3-b | 2.490 | 2.577 | -3.38 | 0.199 | 0.057 | 0 | standard |
| 9-3-b | 2.649 | 2.822 | -6.13 | 0.185 | 0.055 | 0 | standard |
| 9-3-b | 2.874 | 3.110 | -7.59 | 0.202 | 0.055 | 0 | standard |

| HPLC Code | Fluorometer $\mu$ g l$^{-1}$ total chl *a* | $\mu$ g l$^{-1}$ chl *a* | Pigment ratio % Dsc | Chl *c* | Chl *b* | Extraction DV chl *a* | mode |
|---|---|---|---|---|---|---|---|
| 9-3-b | 2.258 | 2.394 | -5.69 | 0.207 | 0.060 | 0 | participant's |
| 9-3-b | 2.130 | 2.336 | -8.85 | 0.216 | 0.062 | 0 | participant's |
| 9-3-b | 1.964 | 2.091 | -6.08 | 0.218 | 0.062 | 0 | participant's |

[1] These filters were extracted with the participant's method, with the exception that an HPLC PTFE syringe cartridge filter was used instead of a nylon HPLC syringe cartridge filter.

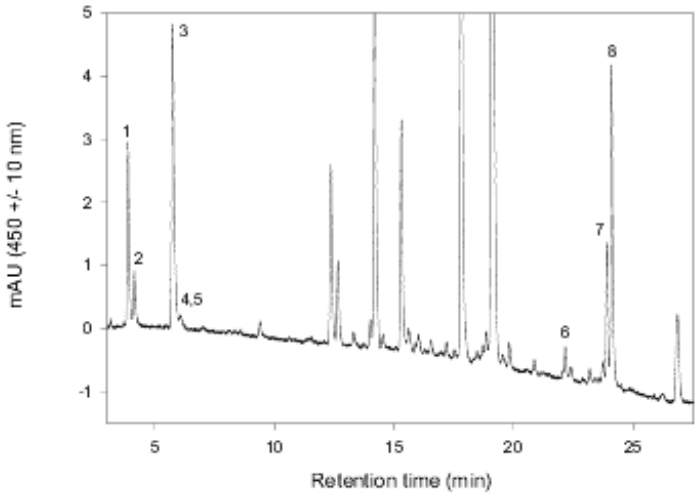


Figure 1. Chromatogram from the HPLC analysis of a field sample filter (site 6-2) showing elution position of pigments quantified and represented in Appendix H, Table 1. This filter was extracted at HPL with the participant's method. Codes to pigment identities are: 1=DV chl c3, 2 = chl c3, 3 = chl c2, 4 = chl c1, 5 = chlide a, 6 = chl b + DV chl b, 7 = DV chl a, 8 = chl a. A simultaneous equation (as in Latasa et al. 1996) was used to determine amounts of chl c1 and chlide a (using two detector settings, 665 nm and 450 nm). Details of quantitation are in Hooker et al. (2000).

# Appendix I - Participant's V. Standardized Extraction Procedures

Replicate field sample filters from sites of Labs 4, 6 and 9 were extracted at HPL with participant's procedures and with standardized procedures and % differences were calculated: ($\%D_{ext}$) = ((chl *a* $_{EXT\text{-}PARTICIPANT}$ - chl *a* $_{EXT\ STANDARD}$)/chl *a* $_{EXT\text{-}STANDARD}$)*100. HPLC extraction procedures (Table 1) and fluorometer extraction procedures (Table 2) were evaluated. The code means: 1st number = laboratory code, 2nd number = site number, 3rd number = bottle number. In the case of Lab 9, letter = collection day. HPLC values shown for participants do not necessarily represent total chl *a*, as participant's usual reporting practices were used. n = the number of replicate filters analyzed. "New" values for Lab 9 reflect revisions to correct for extraction volumes and chlide *a* (see Section 6.1). When a site was represented by more than one bottle, values of all bottles from that site were averaged (as with site 4-2, 6-1 and 6-2).

Table 1. Comparison of HPLC extraction procedures

| | | participant procedures | | standard procedures | |
|---|---|---|---|---|---|
| Code | n | $\mu$ g $l^{-1}$ chl *a* | n | $\mu$ g $l^{-1}$ total chl *a* | % $D_{EXT}$ per bottle |
| 4-1 | 3 | 7.005 ± 0.092 | 3 | 6.825 ± 1.263 | 2.64 |
| 4-2-10 | 1 | 0.114 | 2 | 0.130 ± 0 | -12.5 |
| 4-2-11 | 1 | 0.148 | 1 | 0.138 | 7.46 |
| 4-3 | 3 | 0.194 ± 0.008 | 3 | 0.188 ± 0.005 | 3.14 |
| 6-1-21 | 1 | 0.136 | 1 | 0.123 | 10.6 |
| 6-1-22 | 2 | 0.137 ± 0.005 | 2 | 0.128 ± 0.004 | 6.64 |
| 6-2-21 | 1 | 0.063 | 1 | 0.060 | 5.00 |
| 6-2-22 | 2 | 0.064 ± 0 | 2 | 0.060 ± 0.003 | 6.67 |
| 6-3 | 3 | 0.949 ± 0.087 | 3 | 1.020 ± 0.131 | -6.93 |
| 9-1-a | 3 | 2.283 ± 0.085 | 3 | 3.137 ± 0.193 | -27.2 |
| 9-2-a | 3 | 3.576 ± 0.264 | 3 | 6.955 ± 0.399 | -48.6 |
| 9-3-a | 3 | 2.053 ± 0.168 | 3 | 2.939 ± 0.012 | -30.2 |
| 9-1-b | 3 | 3.097 ± 0.045 | 3 | 3.950 ± 0.060 | -21.6 |
| 9-2-b | 3 | 2.380 ± 0.275 | 3 | 3.066 ± 0.153 | -22.4 |
| 9-3-b | 3 | 1.736 ± 0.142 | 3 | 2.671 ± 0.193 | -35.0 |
| 9-1-c | 3 | 1.326 ± 0.012 | 3 | 1.637 ± 0.029 | -19.0 |
| 9-2-c | 3 | 1.155 ± 0.118 | 3 | 1.309 ± 0.018 | -11.8 |
| 9-3-c | 3 | 1.426 ± 0.068 | 3 | 1.868 ± 0.078 | -23.7 |
| 9-1-a new | 3 | 2.882 ± 0.080 | 3 | 3.137 ± 0.193 | -8.13 |
| 9-2-a new | 3 | 6.082 ± 0.157 | 3 | 6.955 ± 0.399 | -12.6 |
| 9-3-a new | 3 | 2.581 ± 0.155 | 3 | 2.939 ± 0.012 | -12.2 |
| 9-1-b new | 3 | 3.792 ± 0.064 | 3 | 3.950 ± 0.060 | -4.00 |
| 9-2-b new | 3 | 2.960 ± 0.166 | 3 | 3.066 ± 0.153 | -3.46 |
| 9-3-b new | 3 | 2.155 ± 0.150 | 3 | 2.671 ± 0.193 | -19.3 |
| 9-1-c new | 3 | 1.600 ± 0.015 | 3 | 1.637 ± 0.029 | 2.26 |
| 9-2-c new | 3 | 1.368 ± 0.138 | 3 | 1.309 ± 0.018 | 4.51 |
| 9-3-c new | 3 | 1.749 ± 0.020 | 3 | 1.868 ± 0.078 | -6.37 |

Table 2. Comparison of fluorometer extraction procedures

| | | participant procedures | | standard procedures | |
|---|---|---|---|---|---|
| Code | n | $\mu$ g $l^{-1}$ chl *a* | n | $\mu$ g $l^{-1}$ chl *a* | % $D_{EXT}$ per bottle |
| 4-1 | 3 | 6.053 ± 0.493 | 3 | 6.290 ± 0.676 | -3.77 |
| 4-2-9 | 1 | 0.145 | 2 | 0.141 | 3.41 |
| 4-2-10 | 1 | 0.141 ± 0.001 | 1 | 0.138 | 2.84 |
| 4-3 | 3 | 0.195 ± 0.013 | 3 | 0.199 ± 0.010 | -2.26 |
| 6-1-21 | 1 | 0.112 | 1 | 0.125 | -10.2 |
| 6-1-22 | 2 | 0.123 ± 0.002 | 2 | 0.125 ± 0.003 | -2.07 |
| 6-2-21 | 1 | 0.054 | 1 | 0.056 | -3.04 |
| 6-2-22 | 2 | 0.055 ± 0.0002 | 2 | 0.056 ± 0.002 | -2.31 |
| 6-3 | 3 | 1.033 ± 0.069 | 3 | 1.032 ± 0.108 | 0.10 |

# Appendix J - Inter-Laboratory Variability

Inter-laboratory variability was defined for each site as the % difference (%D) from the mean consensus chl *a* value for that site (the mean consensus = the average between the value reported by the participant and HPL for that site). %D = ((chl $a_{\text{REPORTED}}$ - chl $a_{\text{MEAN CONSENSUS}}$) * chl $a_{\text{MEAN CONSENSUS}}^{-1}$) * 100. The code means: 1st number = laboratory code, 2nd number = site number, 3rd number = bottle number. In the case of Lab 9, letter = collection day.

| | HPLC $\mu$ g $l^{-1}$ chl *a* | | | fluorometer $\mu$ $\lambda$ g $l^{-1}$ chl *a* | | |
|---|---|---|---|---|---|---|
| Code | participant | HPL | % D | participant | HPL | % D |
| 1-1 | 26.74 | 26.08 | 1.25 | 23.88 | 28.60 | -8.99 |
| 1-2 | 239.2 | 230.4 | 1.86 | 149.0 | 243.3 | -24.0 |
| 1-3 | 0.813 | 0.892 | -4.64 | 1.169 | 1.058 | 4.96 |
| 1-4 | 10.37 | 10.12 | 1.24 | 8.581 | 10.96 | -12.2 |
| 1-5 | 8.190 | 7.603 | 3.72 | 7.093 | 7.515 | -2.89 |
| 4-1 | 7.264 | 6.825 | 3.11 | 8.067 | 6.290 | 12.4 |
| 4-2-10[1] | 0.228 | 0.130 | 27.4 | 0.188 | 0.138 | 15.3 |
| 4-2-11[1] | 0.207 | 0.138 | 19.7 | 0.189 | 0.153 | 10.5 |
| 4-3 | 0.217 | 0.188 | 7.17 | 0.248 | 0.199 | 10.9 |
| 5-17 | 0.039 | 0.050 | -12.4 | 0.061 | 0.069 | -6.15 |
| 5-18 | 0.034 | 0.060 | -27.7 | 0.067 | 0.071 | -2.90 |
| 5-19 | 0.036 | 0.060 | -25.0 | 0.055 | 0.068 | -10.6 |
| 5-20 | 0.031 | 0.060 | -31.2 | 0.053 | 0.070 | -13.8 |
| 5-17 new[2] | 0.045 | 0.050 | -5.26 | --- | --- | |
| 5-18 new[2] | 0.039 | 0.060 | -21.2 | --- | --- | |
| 5-19 new[2] | 0.041 | 0.060 | -18.8 | --- | --- | |
| 5-20 new[2] | 0.035 | 0.060 | -26.3 | --- | --- | |
| 6-1-21 | 0.142 | 0.123 | 7.17 | 0.140 | 0.125 | 5.82 |
| 6-2-21 | 0.065 | 0.060 | 4.00 | 0.060 | 0.056 | 3.18 |
| 6-3 | 1.061 | 1.020 | 1.96 | 1.150 | 1.032 | 5.28 |
| 8-1 | 8.947 | 9.866 | -4.89 | 15.17 | 10.60 | 17.7 |
| 8-2 | 0.641 | 0.399 | 23.3 | 0.541 | 0.427 | 11.8 |
| 8-3 | 4.514 | 6.093 | -14.9 | 8.890 | 5.844 | 20.7 |
| 9-1-a | 2.337 | 3.137 | -14.6 | 3.433 | ---- | |
| 9-2-a | 4.404 | 6.955 | -22.5 | 5.692 | 7.288 | -12.3 |
| 9-3-a | 2.176 | 2.939 | -14.9 | 2.795 | 2.885 | -1.58 |
| 9-1-b | 3.079 | 3.950 | -12.4 | 3.431 | 4.403 | -12.4 |
| 9-2-b | 2.740 | 3.066 | -5.62 | 3.312 | 3.103 | 3.25 |
| 9-3-b | 2.069 | 2.671 | -12.7 | 2.902 | 2.608 | 5.34 |
| 9-1-c | 1.421 | 1.637 | -7.06 | 1.965 | 1.716 | 6.76 |
| 9-2-c | 1.170 | 1.309 | -5.61 | 2.066 | 1.399 | 19.2 |
| 9-3-c | 1.609 | 1.868 | -7.45 | 1.985 | 1.778 | 5.50 |
| 9-1-a new[3] | 2.866 | 3.137 | -4.51 | --- | --- | |
| 9-2-a new[3] | 5.675 | 6.955 | -10.1 | --- | --- | |
| 9-3-a new[3] | 2.670 | 2.939 | -4.80 | --- | --- | |
| 9-1-b new[3] | 3.845 | 3.950 | -1.35 | --- | --- | |
| 9-2-b new[3] | 3.285 | 3.066 | 3.45 | --- | --- | |
| 9-3-b new[3] | 2.469 | 2.671 | -3.93 | --- | --- | |
| 9-1-c new[3] | 1.706 | 1.637 | 2.06 | --- | --- | |
| 9-2-c new[3] | 1.405 | 1.309 | 3.54 | --- | --- | |
| 9-3-c new[3] | 1.936 | 1.868 | 1.79 | --- | --- | |

[1] Values from these 2 bottles were averaged before calculating the mean consensus and %D. [2] HPLC values were re-calculated with a revised extraction volume. [3] HPLC values were re-calculated with a revised extraction volume and to include chlide *a*.

# Glossary

| | |
|---|---|
| %D | percent Difference between two values for the same instrument<br>$\%D = (\text{chl } a_{\text{MEASURED}} - \text{chl } a_{\text{KNOWN}}) * \text{chl } a_{\text{KNOWN}}^{-1}) * 100$<br><br>$\%D = ((\text{chl } a_{\text{PARTICIPANT}} - \text{chl } a_{\text{MEAN CONSENSUS}}) * \text{chl } a_{\text{MEAN CONSENSUS}}^{-1}) * 100$ |
| $\%D_{EXT}$ | percent Difference between extraction procedures<br>$\%D_{EXT} = (\text{chl } a_{\text{EXT-PARTICIPANT}} - \text{chl } a_{\text{EXT-STANDARD}}) * \text{chl } a_{\text{EXT-STANDARD}}^{-1}) * 100$ |
| %Dsc | percent Discrepancy, $\%Dsc = ((\text{chl } a_H - \text{chl } a_F) * \text{chl } a_F^{-1}) * 100$ |
| %RSD | percent Relative Standard Deviation, $\%RSD = (s * \bar{x}^{-1}) * 100$ |
| Abs%D | Absolute value of the percent Difference |
| CTD | Conductivity, Temperature and Depth |
| EM | Emission wavelength |
| EX | Excitation wavelength |
| FL | Fluoremetric |
| FLD | Fluorometer |
| HPL | Horn Point Laboratory |
| HPLC | High Performance Liquid Chromatography |
| HyCODE | Hyperspectral Coastal Ocean Dynamics Experiment |
| JGOFS | Joint Global Ocean Flux Study |
| MAU | Milli absorbance unit |
| NIST | National Institute of Standards and Technology |
| NRA | NASA Research Announcement |
| ONR | Office of Naval Research |
| PDA | Photo Diode-Array detector |
| PTFE | PolyTetraFluoroEthylene |
| QC | Quality Control |
| SeaBASS | SeaWiFS Bio-optical Archive and Storage System |
| SeaWiFS | Sea-viewing Wide Field-of-view Sensor |
| SIMBIOS | Sensor Intercomparison and Merger for Biological and Interdisciplinary Oceanic Studies |
| SIRREX | SeaWiFS Intercalibration Round-Robin Experiment |

| | |
|---|---|
| S:N | Signal-to-Noise ratio |
| UMCES | University of Maryland Center for Environmental Science |
| UV/Vis | UltraViolet/Visible |
| WL | Warning Limits (95% confidence limts), WL = ± student's *t* value (for n-1) * s |

# Symbols

| | |
|---|---|
| chl *a* | monovinyl chlorophyll *a* |
| chl $a_F$ | total chl *a* determined fluorometrically |
| chl $a_H$ | chl *a* determined by HPLC |
| chl *b* | chlorophyll *b* |
| chl *c* | chlorophyll *c* |
| chl *c*1 | chlorophyll *c*1 |
| chl *c*2 | chlorophyll *c*2 |
| chl *c*3 | chlorophyll *c*3 |
| chlide *a* | chlorophyllide *a* |
| DV chl *a* | divinyl chlorophyll *a* |
| DV chl *b* | divinyl chlorophyll *b* |
| grad | graduated |
| i.d. | internal diameter |
| L | length |
| Lab | laboratory |
| N | normality |
| vol | volumetric |
| v. | versus |
| $\lambda$ (lambda) | wavelength |
| $\lambda$ $(lambda)_{max}$ | wavelength maximum |