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14. ABSTRACT

Several laboratories, including but not limited to SIMBIOS and HyCODE investigators, participated in an inter-calibration for HPLC and fluorometer conducted by Horn Point Laboratory of the University of Maryland Center for Environmental Science. The goal of this exercise was to investigate causes of discrepancy between HPLC and fluorometer chlorophyll *a* values. This exercise was conducted in two phases: analysis of laboratory-prepared standards and samples (presented in Part I) and analysis of field-collected natural samples (presented in Part II). It was found that when methods and instrumentation were not limiting, good reproducibility among laboratories was attained. HPLC values were typically 7-10% lower than fluorometer values. When all analyses were performed at one laboratory with one extraction procedure, results agreed within 19%. When laboratories, extraction procedures and analytical methods varied, results agreed within 60%. Factors that affected discrepancy included: varying extraction procedures between HPLC and fluorometer, inaccurately determining extraction volume, inconsistent HPLC chlorophyll a reporting practices and inaccurate analytical methods.

15. SUBJECT TERMS

HPLC, fluorometer, chlorophyll a, calibration, round-robin

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Results of Simbios Project Round Robin: Part I

Results of Laboratory-Prepared Standards and Samples

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Table of Contents

1.0 INTRODUCTION

Accuracy in phytoplankton pigment measurements is important to algorithm development as used with ocean color remote sensing. In support of this effort, an inter-calibration exercise was recently conducted by Hooker et al. (2000), assessing uncertainties in the HPLC analysis of pigments in field samples in SeaHARRE-1. In this study, we extend the efforts of Hooker et al. (2000) with new study participants by investigating factors that cause discrepancy between HPLC and fluorometer chl *a* values in the analysis of standards and field samples.

Several studies describe the relationships between fluorometric and HPLC chl *a* values for field samples, as discussed in Trees et al. (2000a). These authors show data from three large sets ($n \geq 179$) from three geographical areas and where protocols suggested for use (Fargion and Mueller 2000) were used. With these data, correlations (r^2) varied from 0.73 to 0.94, chl *a* concentrations were < 10 μg/l and linear regressions relating log HPLC chl *a* to log fluorometric chl *a* gave slopes which ranged from 0.82 to 1.07. In a study of Chesapeake Bay sample ($n = 560$) chl *a* concentration varied from 0.1 to 160 μg/l, correlation (r^2) between fluorometric and HPLC derived chl *a* was 0.94 and slope of HPLC versus fluorometric chl *a* was 1.02 (data courtesy of Lawrence W. Harding, Jr.). Trees et al. (2000a) caution that variability is affected by seasonal cycles, presumably as a result of changes in phytoplankton community structure, and, as has been shown, variations in accessory pigment content can affect fluorometer chl *a* values (Lorenzen and Jeffrey 1980; Trees et al.1985).

Ascertaining sources of discrepancy between fluorometrically and HPLC derived chl *a* values is difficult, as many factors affect outcome, including such things as water type, filtration effects (Bidigare and Trees 2000), methodological procedures and instrument limitations. Some work has been done to describe uncertainties in HPLC pigment measurements. Latasa et al. (1996) found that 90% of HPLC chl *a* values (after calibrations among HPLCs had been normalized) were within ± 20% of the median value, when standards were distributed to eight laboratories. In SeaHARRE-1 (Hooker et al., 2000), triplicate field sample filters from each of 12 field sites were distributed to 4 laboratories and it was found that 97% of all HPLC chl *a* values were within ± 20% of the mean consensus value. In a separate study, Trees et al. (2000b) demonstrated that with HPLC samples where the number of measurable accessory pigments was less than four, low signal to noise ratios (SNRs) existed and resulted in diminished quantitative accuracy.

Protocols exist for use with such analyses (Bidigare and Trees 2000; Trees et al. 2000a) and are designed to foster consistency in chl *a* analysis and minimize discrepancies. However, the more complicated an analysis, the more difficult it is to exactly reproduce. Thus, our investigation into sources of discrepancy between two analytical instruments, HPLC and fluorometer, was complex and required considerable effort by the participating laboratories, for which we are very appreciative. One of the benefits of inter-laboratory comparisons is that they allow analysts to see if the uncertainties associated with their methods are limited to what is

generally attainable in the community at large and they provide insights into such things as instrument limitations, which may not be identifiable otherwise.

2.0 EXPERIMENTAL DESIGN

Our primary objective was to identify sources of discrepancy between HPLC and fluorometrically derived chl *a* values among specific SIMBIOS project investigators whose names we had been given. Other participants were either funded by the HyCODE project or, in some cases, provided their own money for the cost of participation.

Before proceeding, we feel it necessary to qualify our use of the term, accuracy, as we realize a true measure of accuracy cannot be known. Accuracy is often assessed with standard reference materials, SRMs (standards whose concentrations are known to be true and correct). Unfortunately, these are not currently available for chl *a*. However, it is necessary to estimate a method's ability to be accurate. An alternate approach to SRMs is based on the premise that for a method to be accurate, it must be able to reproduce results attained by others in a manner which is unbiased and precise (Taylor 1987). Thus, we compared a laboratory's results with others and with our own. For this, we conducted quality assurance measurements to validate our capabilities, which we include in this report.

We conducted this inter-calibration with the idea that to elucidate causes of discrepancies between two analytical instruments (fluorometer and HPLC), sources of uncertainty arising from either one individually must first be known and that this is a prerequisite to understanding sources of discrepancy in the analysis of field samples. Therefore, we divided the project into two components. First we evaluated uncertainties that were more likely to result from instrument configurations and analytical procedures (results which we discuss in this document). Second we evaluated uncertainties that were more likely to arise from field samples themselves and the extractions thereof (results which we discuss in a separate report). These included the effects of water type, the effects of extraction procedures which differ between sample filters destined for HPLC versus fluorometric analysis and the attainable precision in the analysis of replicate samples.

When conducting analytical measurements, imprecision and bias can result from such factors as: 1) limitations in instrument configurations, 2) inaccuracies in instrument calibrations, 3) instrument drift which goes uncorrected and 4) inappropriate comparisons when results of fluorometric and HPLC chl *a* values are compared. For example, all pigments quantified by HPLC that respond as chl *a* in a fluorescence detector should be summed (Trees et al., 2000a). We addressed these four topics by first distributing questionnaires to participants to gather pertinent information about instrumentation, calibration practices, how instrument performance was monitored and how chl *a* (by HPLC) was reported. Next we distributed standards, unknowns and filtered samples, which simulated field samples. Our purpose for distributing calibration standards was to normalize each laboratory's calibration with ours and to provide consistency in calibrations among all laboratories and instruments. The resulting calibration

factors were to be used for all measurements conducted during the round robin, as this simplified our ability to identify common features among instrument configurations and procedures which were important to results. The results from the unknowns provided information regarding the accuracy of the chl *a* calibrations, the accuracy with which DV chl *a* was reported by HPLC and the effects of differential discrimination toward chl *a* and DV chl *a*. The results of the filtered samples provided information regarding results attainable when extraction procedures were standardized among laboratories and between filters destined for HPLC or fluorometric analysis.

As we imposed a degree of artificiality on laboratories by distributing chl *a* calibration standards, we examined the likelihood that participants' results would have been similar if calibration standards had not been distributed. It is typical that chl *a* standards are prepared using a spectrophotometer to determine the concentration of a stock solution, followed by subsequent dilutions. Thus, we provided a chl *a* unknown for spectrophotometric analysis and we asked participants to formulate their own HPLC and fluorometer calibration standards (using their usual procedures) and to then measure their concentrations using calibration factors derived from the HPLC and fluorometer calibration standards distributed by us. We then determined the discrepancies between results from the HPL-prepared and participant-prepared calibration standards.

3.0 METHODS

We shipped calibration kits (containing standards, unknowns and sample surrogate filters) on different dates over the course of seven months to accommodate the various schedules of participants. In this section we present details of how calibration kits were prepared, definitions used in data analysis and a summary of how data were analyzed. We give an overview of methods used at HPL but the specific details about instruments and associated procedures used by HPL and participants are in the results sections pertinent to the instrument being discussed. We use the SCOR Working Group 78 abbreviations for pigment names (Appendix 1).

3.1 Participating laboratories and their schedules

Initially, questionnaires were sent to all laboratories to obtain information about their instruments, cruise schedules and portions of the round robin in which they would participate. In all, 13 laboratories participated in various aspects of this round robin, as shown in Table 1. Laboratories were given a unique code for the purpose of anonymity.

Participants' cruise schedules varied and, as we intended for them to calibrate their instruments immediately prior to analyzing field samples collected for use in the round robin, we shipped calibration kits convenient to their schedules. In some cases, field cruise schedules changed after shipping and calibration kits were stored by participants in freezers or liquid nitrogen for varying lengths of time prior to analysis. With Laboratory 8, we shipped two different sets of calibration standards (when cruise schedules changed) and in the instance of

Laboratory D, when fluorometric analyses were performed first, followed by HPLC analyses in a subsequent month, fluorometer and HPLC kits were sent on different dates. The time elapsed between the date shipped and the last date of analysis by the participant are illustrated in Fig. 1.

Table 1. Phases of the inter-calibration exercises in which laboratories participated

	Laboratory code												
Phase of the Round Robin	1	2	3	4	5	6	7	8	9	A	B	C	D
Questionnaires	x	x	x	x	x	x	x	x	x	x	x	x	x
Spectrophotometer comparison		x	x	x	x	x	x	x			x	x	x
Fluorometer unknowns		x	x	x	x	x		x		x		x	x
Fluorometer calibration inter-comparison		x	x	x	x	x		x					x
Fluorometer sample surrogate filters		x	x	x	x	x		x		x			
Fluorometer analysis of field samples	x		x	x	x	x		x					x
HPLC unknowns				x	x	x	x	x		x	x		x
HPLC calibration inter-comparison				x		x		x			x		x
HPLC sample surrogate filters				x	x	x	x	x		x			x
HPLC analysis of field samples	x			x	x	x	x	x					x

3.2 Overview of instrumental methods used at Horn Point Laboratory

The HPLC method used at HPL is fully described in Hooker et al. (2000) and Van Heukelem and Thomas (2001). Divinyl chl *a* and chl *a* are chromatograpically resolved and both are quantified from the response at 665 nm (±10 nm). We use the same response factor for both chlorophylls at this wavelength because we have seen that their responses are sufficiently similar to warrant this approach (within ~2%). We routinely use the total area of both peaks (including allomers and epimers), then determine the relative proportions of chl *a* and DV chl *a* from their respective peak heights.

The fluorometer method used is based on that of Strickland and Parsons (1972) and detailed in Trees et al. (2000a). In most cases we used a fluorometer with a "before" and "after" acid reading. When adding acid, we consistently added 150 μl of 0.1 N HCl to approximately 5 ml of standard or sample and recorded the "after" acid value after precisely 1.5 min. At one point during the round robin, our primary fluorometer (10-AU-005-CE) failed and it was necessary to use a TD-700 instrument with the non-acidification method (Welschmeyer 1994) until a replacement for our primary fluorometer arrived. We have noted as such in results when this was done.

3.3 Calibration kits

A complete calibration kit contained items for use with a spectrophotometer, HPLC and fluorometer and included chl *a* calibration standards and sample surrogate filters (including supplies to extract them) for the fluorometer and HPLC, unknowns for all instruments and DV chl *a* and accessory pigment standards for the HPLC. Unknowns and standards were prepared from primary stock pigment solutions (stored in a freezer at -15 °C in PTFE bottles or glass vials) which were always allowed to come to room temperature (20-25 °C) before use. Dilutions were made (using 90% acetone formulated volume to volume with HPLC grade acetone and 18.3 megaohm/cm filtered de-ionized water) with calibrated Class A glass volumetric pipettes and glass syringes and Class A glass volumetric flasks. Unknowns were prepared in lots, stored in freezers (-15 or -25 °C) and used until gone (lots sent to participants were recorded). Calibration standards were uniquely prepared for each calibration kit within 5 days of shipping. They were dispensed into amber glass vials (4 ml capacity, Supelco 27115-U, or 22 ml capacity, Supelco 27073-U) with PTFE-lined screw caps (as were unknowns) and shipped to participants overnight on dry ice.

3.3.1 Primary chl a stock solutions and spectrophotometer unknowns

Our primary chl *a* stock solutions were also used as spectrophotometer unknowns for participants. We used published guidelines for attaining maximum spectrophotometric accuracy when determining concentrations of all pigment standards. These guidelines included using a monochromator type spectrophotometer (Latasa et al. 1996, Bidigare and Trees 2000, Trees et al. 2000a) with bandwidth not exceeding 2 nm (Brown et al. 1980, Clesceri et al. 1998, Trees et al. 2000a), correcting for light scattering (Clesceri et al. 1998, Bidigare and Trees 2000, Trees et al. 2000a) and using a solution sufficiently concentrated such that the absolute absorbance is between 0.1 and 1.0 (Clesceri et al. 1998) or more conservatively, between 0.2 and 0.8 (Marker et al. 1980).

Three different lots of primary chl *a* stock solutions were used and each was formulated from chl *a* (Fluka 25730) dissolved in 90% acetone such that absorbance values were between 0.4 and 0.8 (OD 664 nm). The concentration was determined in triplicate (extinction coefficient = 87.67 $g^{-1}cm^{-1}$, Jeffrey and Humphrey 1975) within 3 days of using them to prepare the unknowns and calibration standards. A 90% acetone reference solution (also shipped to participants) was used for zeroing the spectrophotometer.

3.3.2 Divinyl chl a standards for the HPLC

Divinyl chl *a* was isolated from chlorophyll-deficient maize leaves (Bazzaz 1981) grown in our laboratory and transferred to 100% acetone. The concentration was determined spectrophotometrically (extinction coefficient = 88.15 $g^{-1}cm^{-1}$, Jeffrey et al. 1997). The standard was then diluted with 90% acetone for distribution to participants (one lot only was prepared).

3.3.3 *HPLC and fluorometer unknowns*

Three unknowns were distributed for fluorometric analysis. Unknowns #1 and #2 contained chl *a* only and Unknown #3 contained equal portions of chl *a* and DV chl *a*. It was necessary to formulate three different lots of Unknowns #1 and #2 and four lots of Unknowns #3. The concentrations of unknowns varied slightly among lots; the concentrations of Unknowns #1 and #2 were near 117 and 7 μg/l, respectively, and the concentration of Unknown #3 was near 100 μg/l total chl *a* (DV chl *a* + chl *a*).

There were two HPLC unknowns distributed. HPLC Unknown #1 was the same as fluorometer Unknown #1 and HPLC Unknown #3 contained approximately equal portions of chl *a* and DV chl *a* (there was no HPLC unknown #2). HPLC Unknown #3 was formulated twice and the concentrations of both formulations were near 400 μg/l total chl *a*. All unknowns were formulated to be within the range of concentrations spanned by the calibration standards. The precise concentrations of all lots of unknowns are given in pertinent discussions and Appendices.

3.3.4 *Chlorophyll a calibration standards*

We attempted to provide standards that would bracket the range of concentrations typically used by participants. This meant that in some cases more than five calibration standards were provided for each instrument such that when analyzed, at least five calibration levels per instrument would be useable by the participant.

3.3.5 *Accessory pigment calibration standards for the HPLC*

Two different accessory pigment calibration mixtures were distributed for HPLC analysis, referred to as "Mix 82" and "the retention time mix". Mix 82 (one lot only) was used by participants (and HPL) to determine response factors for quantifying accessory pigments in the sample surrogate filters, which included chl *c*2, but-fuco, fuco, diadino and β, β-car. The retention time mix was used qualitatively for assessing elution position and separation between pigments. Both mixtures were formulated from individual primary stock pigment solutions which were either purchased in solid form or isolated from natural sources and suspended in the solvent specific to the extinction coefficient to be used for determining its concentration spectrophotometrically (listed in Hooker et al. 2000). Guidelines specified in Section 3.3.1 were used. Details of these two mixtures are given in Appendix 2.

3.3.6 *Sample surrogate filters*

Sample surrogate filters were prepared by filtering 10 ml of a culture of *Aureococcus anaphagefferens* onto 25 mm GF/F glass fiber filters. Sufficient material was collected on the filter such that the concentration of chl *a* in the sample extract was within the range of concentrations spanned by either the fluorometer or HPLC calibration curves. Filters were folded in half, given a unique number (1-70), individually placed in HD PolyChron™ envelopes (Light Impressions, 5631), and then placed in a resealable bag and placed in a freezer (-75 to

-80°C) until needed.

As these filters were to be extracted by participants (and HPL) with specified procedures, we provided the instructions for extraction (Appendix 3) and the required supplies. These included four empty, 7 ml capacity amber glass vials (Supelco, 27072-U) with PTFE-lined screw caps for HPLC extractions and 22 ml capacity vials (Supelco 27073-U) with PTFE-lined screw caps for fluorometer extractions. Calibrated Class A glass volumetric pipettes were provided for adding extraction solvent to filters, 5 and 10 ml capacity, respectively, for HPLC and fluorometer extractions. HPLC syringe cartridge filters with glass fiber pre-filters (SRI, Inc., 44525) attached to 20 ml capacity syringes were provided for clarifying the filter extracts.

3.4 Data analysis

Our data analysis methods and associated terms are based on those discussed in Taylor (1987) and Clesceri et al. (1998) and we refer the reader to the glossary for definitions of terms.

For the analysis of unknowns, sample surrogate filters and quality assurance measurements at HPL were assessed for accuracy, bias and imprecision. When estimating variability in a set of measurements, we calculated the 95% and 99.73% confidence limits. We estimated accuracy by the degree to which a participant's measured result for a particular unknown differed from the concentration of that unknown as formulated. Accuracy in the analysis of sample surrogate filters was estimated by the degree to which a laboratory's result differed from the mean consensus value. These estimates of accuracy were expressed as values of %D (the percent difference) as modified from Clesceri et al. (1998), where:

%D = ((measured value - formulated value)/formulated value) * 100.

As values of %D include sign (either positive or negative), this term was also used to describe bias relative to a formulated value or a mean value. In instances where we estimated bias in a set of measurements, we use the term mean %D which is calculated from the average of all values of %D. When estimating the average accuracy attained in a series of measurements, the term mean |%D| was used, which is calculated from individual values of %D after converting them to their absolute value. When estimating the imprecision associated with replicate measurements, we use %RSD (percent relative standard deviation) which is calculated as ($s/\bar{x}$) * 100. The expressions *warning limits* (WL) and *control limits* (CL) are used to describe the distance from a mean value within which 95% and 99.73%, respectively, of data should lie. These limits, which are only used when $n \geq 7$, are calculated as:

± student's *t* value (for 95 or 99.73% confidence) * *s* (Taylor 1987).

4.0 Quality Assurance at Horn Point Laboratory

To assess uncertainties in analyses at HPL, we conducted many quality assurance

measurements pertinent to the unknowns, calibration standards, sample surrogate filters and shipping. We summarize these results here and include additional information in appendices. These additional details are summarized in Section 4.4.

4.1 Unknowns and calibration standards

For each calibration kit sent, we analyzed the calibration standards in that shipment, computed the calibration factors and then used them to measure the concentration of the unknowns, also in that shipment. From this we calculated values of %D for each unknown in each in of the 8 fluorometer and 8 HPLC calibration kits sent out (see results in Appendices 4 and 5). Limits within which uncertainties should be contained for these measurements at HPL were computed (see WL and CL as defined in Section 3.4). The limits determined for each unknown and the number of lots of each unknown formulated are shown in Table 2. We also show the mean |%D| (the average difference from the formulated value) and the mean %D (indicating average bias) for each.

Table 2. Data from the analysis of unknowns prior to shipping to participants

Instrument	Unknown	No. of lots	95% limits (WL)	99.73% limits (CL)	mean |%D|	mean %D
Fluorometer	#1	3	± 4.2%	± 8.0%	1.6%	-0.8%
Fluorometer	#2	3	± 6.3%	± 12.6%	2.1%	-0.3%
Fluorometer	#3	4	± 9.6%	± 18.4%	4.2%	-2.8%
HPLC	#1	3	± 3.8%	± 7.3%	1.4%	-0.6%
HPLC	#3	2	± 2.9%	± 5.5%	2.1%	-2.1%

The fluorometers and HPLC were calibrated every time they were used, even though there was no indication that this was necessary. The linear regressions for all calibration curves and their correlation coefficients are shown in Appendix 6. (Note: we show linear regressions for fluorometer standards to illustrate linearity, however, calibration factors used with analyses were determined according to Trees et al., 2000a). These data include that from calibration standards shipped to participants and calibration standards which were used at HPL for the analysis of field samples. Additionally, to estimate the likely accuracy with which these linear regressions could measure samples of varying concentration, we used each to measure the concentration of the calibration standards from which they were derived and we calculated the %D for each calibration level. These results are shown in Appendix 7.

4.2 Sample surrogate filters

We assessed homogeneity of the sample surrogate filters prior to the round robin by randomly selecting seven for analysis. These were extracted on the same day (procedures given in Appendix 3) and evaluated for pigment content by HPLC. We calculated the %RSD for each

pigment from all 7 filters and from this we calculated the WL and CL to describe the limits within which the variability in pigment content among the remaining filters analyzed at HPL should be contained. Results are shown in Table 3.

Table 3. Variability among sample surrogate filters (n=7) as analyzed by HPLC at HPL prior to distribution to participants

	chl *a*	chl *c*2	but-fuco	fuco	diadino	β, β-car
WL	±5.4%	±7.3%	±6.6%	±5.1%	±6.3%	±16%
CL	±11%	±15%	±13%	±10%	±13%	±33%

The sample surrogate filters were distributed to participants over a period of 184 days. To determine the effects of long term storage on changes in pigment content in these filters, 9 different filters were analyzed by HPLC on 6 different days spanning the duration of time in which filters were shipped to participants. Regressions of pigment concentration versus analysis date indicated that slopes were not significantly different from zero ($0.1 < p < 0.5$). Furthermore, the pigment amounts in these filters were all within CL and 93% of all pigment values were within WL previously established for filter homogeneity (Table 3, above.)

4.3 Shipping

To assess the effects of shipping on the integrity of unknowns and standards, we prepared a calibration kit for ourselves, shipped it to ourselves, stored it in a freezer (-15 °C) for 14 days and then analyzed the contents as if we were a participant. We report these results as "self" along side participants' results. When calculating mean |%D| for participants' results, we exclude values reported as "self".

4.4 Bias and precision

Other factors affecting bias and precision in our measurements were evaluated at HPL and are described in Appendices 8 and 9. These evaluations were conducted previous to and/or concurrent with this round robin.

5.0 Fluorometer Results

We report details about participants' fluorometers, the procedures they used (regarding calibrations and monitoring instrument performance) and the results of the analysis of unknowns and sample surrogate filters. These findings provide information concerning the ability of the chl *a* calibration factors (derived from standards distributed by us) to accurately predict the concentration of unknowns, the response of the various fluorometers to solutions containing DV chl *a* and agreement attainable among laboratories in the analysis of filters when the same extraction procedures are used and the filtered material is lacking in the pigments chlide *a* and DV chl *a*, which can cause high fluorometer discrepancy relative to HPLC chl *a* values.

5.1 Survey of participants' fluorometers and methods

Most participants used instruments from Turner Designs, Inc. (Sunnyvale, CA, USA) and had equipped them with optical kits and lamps appropriate to the type of analysis, acidification or non-acidification (developed by Welschmeyer 1994). Instruments from Turner Designs equipped for the non-acidification method have excitation and emission filters of 436 ± 10 nm and 680 ±10 nm, respectively. With the instrument used by Laboratory A (from Barnstead/Thermolyne) the specifications of filters used with the non-acidification method were different: the excitation filter was 440 ± 5 nm and the emission filter, > 665 nm. The types of fluorometers and methods used by participants are shown in Table 4. The primary fluorometer used at HPL was a 10-AU-005 CE. On occasion, we used a TD-700 from Turner Designs with the non-acidification method and we have specified as such in pertinent Appendices.

Table 4. Fluorometer configurations used by participants

Lab code	Instrument model	Acidification	Non-acidification
1, 3, D, HPL	10-AU-005 CE	X	
2, 6	10-005	X	
5, 8, 9	10-AU-005	X	
A	Barnstead/Thermolyne, Turner Model 450-003		X
4, C	10-AU-005		X
HPL	TD-700		X

5.2 Results of fluorometer unknowns

Unknowns #1 and #2, containing chl *a* only, were used to assess the accuracy with which the chl *a* calibrations (derived from standards distributed by us) measured the concentrations of solutions near the upper and lower limits of those spanned by the chl *a* calibration standards. Fluorometer Unknown #3 contained approximately equal portions of chl *a* and DV chl *a*, the concentration of which (chl *a* + divinyl chl *a*) was near the upper range of concentrations spanned by the calibration standards.

All laboratories analyzed Unknowns #1 and #2 three or more times except Laboratory D (n=2) and Laboratory 2 (n=1, for Unknown #1; n=2 for Unknown #2). We show all values of %D for Unknowns #1 and #2 in Figs. 2 and 3, respectively. For Unknown #1, all laboratories' values (except Laboratory 2) were within ± 6% of the formulated concentrations. This was also true for Unknown #2, except for Laboratory 2 (who showed a high bias for both unknowns) and Laboratories A and D who exhibited one value each < -6% (%D). The average that all laboratories differed from the formulated values (the mean |%D|) were 2.4 and 4.8%, for Unknown #1 and #2, respectively. This indicates that uncertainties in participants' results were only slightly in excess of those seen at HPL, where mean |%D| for each unknown was 1.6 and

2.4% (see Section 4.1, Table 2). As Unknowns #1 and #2 were of disparate concentrations, it is not surprising that differences, on a percentage basis, were slightly higher for Unknown #2. All participants' data are shown in Appendix 10.

All laboratories analyzed Unknown #3 at least twice, except Laboratories 2 and D (n=1) and most values of %D were within ± 9% of the formulated concentrations, as seen in Fig. 4. The exceptions include Laboratories A, C and one value for Laboratory #3. Relative to results from Unknowns #1 and #2, there was greater variability with Unknown #3 in participants' data and in data from HPL. Again, the mean |%D| from all laboratories' results for Unknown #3 (6.8%, excluding Laboratory A) was only slightly higher than that seen with analyses for this unknown at HPL (4.2%). (See Section 5.4 for reasons why results from Laboratory A were excluded). All participants' data are shown in Appendix 10.

To estimate average precision attained by laboratories, we determined the %RSD for each set (n=27) of replicate observations for each laboratory for each unknown. The average for all sets was 2% and only 6 sets of replicate observations exceeded this average. In only one instance was the %RSD unusually high (17%RSD with Laboratory 3, Unknown #3). (All observations for precision when analyzing these unknowns are shown in Appendix 10).

5.3 Analysis of sample surrogate filters by fluorometry

Seven participants analyzed at least two sample surrogate filters by fluorometry, as well as HPL, who analyzed seven filters over the duration of time in which filters were shipped to participants. A mean consensus was determined from the mean values reported from laboratories, including HPL. Results from Laboratory A were excluded from the mean consensus because we felt their fluorometer configuration (Section 5.1, Table 4) may have been exhibiting a high, positive bias when pigments other than chl *a* were present (see results for Unknown #3) and that this was not typical of values attained on the other fluorometers used (all from Turner Designs, Inc.). Results from Laboratory 6 were also excluded from the mean consensus because they had inadvertently extracted all four filters shipped to them using the HPLC extraction procedure. They analyzed these HPLC extracts on the fluorometer as well by expanding the extract volumes.

Participants' mean results differed from the mean consensus from -4.5% to 2.0%. The mean |%D| that all laboratories were from the mean consensus was 1.5% (excluding results from Laboratory A, who differed from the mean consensus by 18.4%). The average precision in replicate filter analysis was 2.1% and ranged from 0.6% to 3.5%. Data are illustrated in Fig. 5 and shown in Appendix 11.

5.4 Fluorometer discussion

Uncertainties in participants' results differed only slightly from those seen at HPL. Results from Unknowns #1 and #2 demonstrated that participants' fluorometer calibrations (from

standards provided) accurately reproduced values attained at HPL, in fact 96% of values fell within the CL and 87% fell within the WL for uncertainties in their measurements at HPL. Furthermore, consistency in fluorometer calibrations among laboratories was also very good, as indicated with the results from the sample surrogate filters, where no one laboratory (except A) differed from the mean consensus by more than 4.5%. Additionally, sample surrogate filter results show the level of agreement attained among laboratories when the same extraction procedure was used and filters are homogeneous. These results were attained even though some laboratories held their calibration kits for extended periods of time before analyzing them (refer to Fig. 1, Section 3.1).

The increased variability in results for Unknown #3 (by participants and HPL) led us to investigate whether there was differential discrimination toward DV chl *a* in results from laboratories using the non-acidification method (Laboratories 4, C and A). We illustrate this apparent bias in Fig. 6 where values of %D for Unknown #3 are plotted alongside values of %D for Unknown #1 (containing chl *a* only). Here it is evident that the differences between Unknowns #1 and #3 (in a positive direction) are greatest with Laboratories 4, C and A. (Note: results from Laboratories 4 and C were still within the WL and CL, respectively, for uncertainties in the analysis of Unknown #3 at HPL.)

To clarify this, we evaluated the effects of DV chl *a* on fluorometric chl *a* values by analyzing 10 standard solutions where DV chl *a* comprised from 0 to 90% of the combined chl *a* value (chl *a* + divinyl chl *a*). These solutions were analyzed with the acidification method (on our 10-AU-005-CE and TD-700 fluorometers) and with the non-acidification (on our TD-700 fluorometer). We saw that, with the acidification method on our 10-AU-005-CE, there was no discrepancy in the accuracy of the "total chl *a*" measured relative to the proportion of DV chl *a* present ($p > 0.3$). With the TD-700 there was discrepancy ($p < 0.001$), but the magnitude of the discrepancy was minimal (approximately 5% when DV chl *a* was 50% of the "total chl *a*" solution). There was no clear difference between results from the non-acidification and the acidification methods used with the TD-700 instrument.

The discrepancy seen with Unknown #3 (38%) by Laboratory A may have resulted from the excitation filter used in this fluorometer, as its specifications (440 ± 5 nm) are nearer the excitation maximum for DV chl *a* than chl *a*, which likely enhanced detection of DV chl *a*. (For comparison, the excitation filter used by Turner Designs, Inc. for the non-acidification method is 436 ± 10 nm). (Note: Laboratory A is unaffected from potential interference by DV chl *a* as they do not sample from areas where the DV chl *a* containing *Prochlorophytes* are present.)

6.0 HPLC Results

We report details about participants' HPLCs, their procedures with regard to HPLC analyses (including injection, separations and detector settings) and procedures associated with calibrations and monitoring instrument performance. The results of the analysis of unknowns provides information concerning the ability of the chl *a* calibration factors (derived from

standards distributed by us) to make accurate measurements. Unknown #3 was used to assess the accuracy with which total chl *a* was determined when DV chl *a* was present. Results of the analysis of sample surrogate filters assessed the agreement attainable among laboratories in the analysis of filters when the same extraction procedures are used and the filtered material is lacking in the pigments chlide *a* and DV chl *a*.

6.1 Survey of HPLC methods

For an HPLC method to be accurate and precise, several criteria should be met. For example, retention times must be reproducible for correct pigment identification, and for accurate quantitation, resolution between pigments must be adequate, signal to noise ratios (SNRs) sufficiently high, peak areas reproducible and calibrations valid. These factors are affected by many things, including instrument configurations and associated procedures as they relate to injection, separation and detection. We addressed factors affecting these variables in our survey of methods.

6.1.1 HPLC injection conditions

In this section we discuss features of the HPLC injection configurations, the way a sample is prepared for injection and the precision associated with injection conditions. Precision is affected by such things as the instrument's capabilities, sample stability, the solvent in which the standard or sample is suspended, and the accuracy of diluting devices used when preparing samples for injection.

Adjusting the sample extract with water or an aqueous buffer prior to injection improves peak shapes of the pigments which elute early in the chromatogram and allows them to be quantified, if resolution between them is also adequate. Typically, pigments which are affected include chl *c* compounds and chlide *a*. All but one laboratory in this study (Laboratory D) modified the sample extract with water (or buffer) prior to injection. How samples were modified with buffer varied among participants and was partially dependant on the configuration of the HPLC injector, which was either manual, partially automated or fully automated.

With manual injections, the sample and buffer are combined by the analyst, who then injects the mixture and then starts the analysis. With partially automated systems, the injector can draw and inject a specified volume of sample from a vial, but it cannot mix sample with buffer prior to injection. We refer to this mode of injection as "manual-mix/auto-inject", as the analyst must manually mix the sample with buffer in an HPLC vial and then place this vial in the automated injector sample compartment (the autosampler compartment) where it resides until the sample is injected and the analysis activated. With fully automated systems, the injector can combine buffer with the sample extract. In these instances, vials containing sample and separate vials containing buffer are placed in the autosampler compartment and the injector automatically combines sample with buffer immediately prior to injection. The mixture is then injected and the analysis started.

We show details of the HPLCs used by participants, including the manufacturer, mode of injection and whether the autosampler compartment was controlled for temperature (Table 5). Laboratory 6 used an HPLC equipped with both a manual injector and a fully automated injector which were used for different purposes.

Table 5. HPLC configurations and injection mode used by participants and HPL (N/A = not applicable)

Lab code	HPLC manufacturer model	Mode of injection	Autosampler compartment temperature (°C)
1	Hewlett Packard series 1100	fully automated	4°
4	Helwett Packard 1050	manual-mix/auto-inject	not controlled
5	Waters	manual	N/A
6	Waters	fully automated (for samples)	5°
6	Waters	manual (for chl *a*)	N/A
7	Hewlett Packard series 1100	fully automated	4-5°
8	Dionex	manual-mix/auto-inject	not controlled
A	Beckman Gold	manual	N/A
B	Hewlett Packard series 1100	fully automated	4°
D	Hewlett Packard series 1100	fully automated	not controlled
HPL	Hewlett Packard series 1100	fully automated	4°

When manual and manual-mix/auto-inject modes were used, the required volumes of sample and buffer were measured with automatic pipettes (except for Laboratory A who used glass syringes). With manual injections in Laboratories 5 and A, analysts allowed the sample and buffer to equilibrate for a specified time after mixing and before injecting (5-10 min, as suggested in Bidigare and Trees, 2000). When manual-mix/auto-inject modes were used, samples pre-mixed with buffer typically resided in the autosampler compartments for periods of time not exceeding approximately 24 hrs before being injected. (Vials exclusively containing sample extract resided in the autosampler compartments of the fully automated injectors for the same duration).

Ancillary procedures associated with injections included sample load (the volume of sample extract actually injected), the type and pH of the injection buffer and the ratio of sample extract to buffer used. These features are summarized in Table 6.

Table 6. Features of participants' injection procedures. Sample load refers to the volume (s) of sample extract or standard injected onto the column during analyses for the round robin; TBAA refers to tetrabutylammonium acetate; AmAce refers to ammonium acetate; N/A means not applicable

Lab code	Injector sample load (μl)	Buffer type	Buffer pH	Ratio (sample: buffer or water)
1	150	28 mM TBAA	6.5	1:1
4	60, 100, 200	0.5 M AmAce	7.6	2:1
5	357	water	N/A	2.5:1
6[1] (samples)	210	0.5 M AmAce	6.0	3:2
6[1] (chl *a*)	100	none added	N/A	N/A
7	75 (possibly others)	0.5 M AmAce	not given	2.3:1
8	400	0.5 M AmAce	7.2	2:1
A	100	28 mM TBAA	6.5	1:1
B	100, 200, 300	0.5 M AmAce	not given	2.3:1
D	100	none added	N/A	N/A
HPL	150	28 mM TBAA	6.5	1:1

[1] This laboratory used an HPLC equipped with both a manual injector and a fully automated injector. The manual injector was used for injecting standards, determining calibration factors and evaluating instrument linearity, as this mode of injection was more accurate (personal communication). The fully automated mode of injection was used with field sample analyses, as more samples could be analyzed per day. This injection mode required that internal standard be added during sample extraction. Internal standard calibration factors were validated at frequent intervals during the automated analysis of samples.

6.1.2 HPLC detection

HPLC detectors used in this inter-calibration were of three different types: photodiode array (PDA), ultraviolet/visible spectrophotometer (UV/Vis) or fluorometer (FLD). Laboratory 4 used two detectors in line. Laboratory 1 and HPL used one detector but monitored more than one wavelength (665 ± 10 nm for quantification of all chl *a* products and 450 ± 10 nm for all other pigments). With some PDAs, a reference wavelength was used to suppress baseline noise. The detector settings used by each laboratory are shown (Table 7).

Table 7. HPLC detectors and settings used during inter-calibration exercises

Lab code	Type	Detector wavelengths (nm)	Reference wavelength (nm)
1	PDA	450 ± 10 and 665 ± 10	None
4	PDA, FLD	PDA 440 ± 2, 405 ± 2; FLD 421 excitation, 666 emission	550 ± 5
5	UV/Vis	436 ± 5	none
6	UV/Vis	440 ± 4	None
7	PDA	436 ± 2	550 ± 2
8	PDA	Maxplot[1] ± 10	None
A	PDA	450 ± 10	None
B	PDA	436 ± 2	550 ± 2
D	PDA	436 ± 4	None
HPL	PDA	450 ± 10 and 665 ± 10	None

[1] PDA detectors can be set to monitor absorbance spectra for each peak in the chromatogram and thus the wavelength of maximal absorbance for each peak can be known. The MaxPlot™ feature used by Laboratory 8 identifies the wavelength of maximal absorbance for each peak and then plots the chromatogram such that the area and height of each peak is derived from its wavelength of maximal absorbance. Hence, wavelengths used varied among peaks and among injections. This feature was selected for use by this Laboratory to help improve detectability and to provide absorbance spectra for confirmation of peak identity.

6.1.3 HPLC separation conditions

Separation between pigments is affected by such parameters as column choice, mobile phase composition, gradient shape and column temperature. A summary of separation conditions used by participants is shown (Table 8). In the case of Laboratory 7, two columns were connected in series and with Laboratory B, three. Of the methods described here, only those employing C_8 columns separated DV chl *a* from chl *a* (Laboratories 1, 6, A and HPL). (Personal communication per Laboratory 7: after these results were given to us, they modified their method and are reporting DV chl *a* as well as chl *a*. This newer modified method was used with field samples only.)

Table 8. Summary of HPLC separation conditions employed by participants in the inter-calibration exercises. The reference for mobile phases are given as a footnote to this Table and were often modified from that as published

Lab code	Column (source)	Column dimensions (L x i.d. mm)	Reference for mobile phase[1]	Column temperature (°C)
1	Eclipse XDB C_8 (Agilent Technologies)	150 x 4.6	4	60
4	Alltima C_{18} (Alltech)	250 x 4.6	1	not controlled
5	S50DS2 C_{18} (Waters)	250 x 4.6	2	not controlled
6	Adsorboshpere C_8 (Alltech)	100 x 4.6	3	not controlled
7	Ultromex 50DS C_{18} (Phenomenex) 201TP54 C18 (Vydac)	250 x 3.2 250 x 4.6	5	38
8	Allsphere ODS-2 C_{18} (Alltech)	250 x 4.6	2	40
A	Eclipse XDB C_8 (Agilent Technologies)	150 x 4.6	4	60
B	201TP54 C_{18} (Vydac) 201TP54 C_{18} (Vydac) Hypersil ODS C_{18} (Hewlett Packard)	250 x 4.6 250 x 4.6 200 x 4.6	1	38
D	Sphereclone ODS-2, C_{18} (Phenomenex)	250 x 4.6	2	30
HPL	Eclipse XDB C_8 (Agilent Technologies)	150 x 4.6	4	60

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

6.2 Results of chl *a* calibration standards

Participants analyzed the chl *a* calibration levels provided by HPL, provided us the raw data and in some cases, the calculated linear regressions, which we proofed for calculation errors and then corrected if necessary. All calculations performed at HPL were according to participants' usual procedures. Linear regressions yielded r^2 values >0.9999 for Laboratories 5, 6, 7 and D; for Laboratory B, $r^2 = 0.9997$; and Laboratories A, 8 and 4, r^2 values were 0.998, 0.996 and 0.994, respectively.

We determined how accurately each participant's calibration curve could measure all concentrations spanned by the calibration standards. We did this by using the linear regressions to measure the concentration of the calibration standards from which they were derived. The measured concentrations were compared to the formulated concentrations and values of %D were determined for each calibration level. The %D values from each calibration level were then averaged over all levels to determine the mean |%D| for each calibration curve. These data indicate that for all laboratories but 4, 8 and A, calibration curves were likely to be very accurate over the entire range of concentrations spanned, as the mean |%D| was generally ≤ 3%. For

Laboratories 4, 8 and A the mean |%D| was 24%, 24% and 16%, respectively. These three calibration curves exhibited inaccuracies (≤|6%|) when the injected amounts of chl *a* were ≥ 45, 203 and 34 ng (Laboratories 4, 8 and A, respectively) but when the amounts injected were lower than this, the values of |%D| were much higher (see Appendix 12).

6.3 Results of Unknown #1 (containing chl *a* only)

The purpose of Unknown #1 was to estimate the accuracy of each participant's HPLC chl *a* calibration factors (derived from standards distributed by us) and to assess the precision attained when it was analyzed in triplicate (Laboratories B and D analyzed it in duplicate). The %D values for Unknown #1 (from the concentrations measured by participants relative to the concentration as formulated at HPL) varied from -5% to 2% for Laboratories 5, 7, B, D and self and from -22 to 45% for Laboratories 4, 8 and A. With these latter three laboratories, the amount of chl *a* injected when analyzing Unknown #1 was less than that required for these laboratories' calibration factors to be accurate (see Section 6.2) and it is possible that this influenced the results seen here. Individual data points and mean values are illustrated in Fig. 7 and detailed in Appendix 13. The average that all laboratories' mean values differed from the formulated concentrations (mean |%D|) was 9.8%; when Laboratories A, 8 and 4 were excluded, it was 2.2%. This latter value shows that uncertainties in the analysis of Unknown #1 by these participants were very similar to that seen at HPL (mean |%D| = 1.6%) prior to shipping. This indicates that these laboratories very accurately reproduced results attained at HPL.

The precision seen with replicate injections (%RSD) ranged from 0.3 to 4% except for Laboratories 8 and A where %RSD values were 19 and 14%, respectively. As seen with results from Laboratory 8 (Fig. 7) one of the three injections provided very different results from the other two. The two with similar results (3.4%RSD) the solution had resided in the autosampler compartment for typical durations, and the third, variant injection had not. In fact, this sample had been mixed with buffer immediately prior to injection, which is not typical procedure for this laboratory (personal communication).

We do not show results for Unknown #1 from Laboratory 6, even though they analyzed it in triplicate. We had advised them to analyze it under the same injection conditions as samples. However, in Laboratory 6, samples are always co-injected with an internal standard and quantitation of pigments in samples relies on calibration factors which use the internal standard response from the same injection as the sample. It was therefore not possible to accurately quantify chl *a* in Unknown #1, as it did not contain an internal standard. Instead, because we knew the formulated concentration of this unknown, we used its results (as injected in the fully automated mode) to create calibration factors which could be used to quantify chl *a* in the other solutions also injected in this mode and which did not contain internal standard. This included the sample surrogate filters and Unknown #3.

6.4 HPLC analysis of sample surrogate filters (chl *a*)

These filters were analyzed for chl *a* (discussed here) and accessory pigments (Section 6.5). With one exception, each participant analyzed two replicate filters; Laboratory 6 analyzed 4 filters. Nine filters were analyzed at HPL over the duration of time in which filters were sent to participants. The mean chl *a* concentration was determined for each laboratory, from which the mean consensus value was determined. The mean value for HPL was included in the mean consensus, but that from "self" was not. In the case of Laboratory 4, who reported values from two different HPLC detectors, we used the mean of the mean from each detector to incorporate into the mean consensus.

We determined the %D that each laboratory's individual result was from the mean consensus. These data are shown in Fig. 8 and detailed in Appendix 14. Most values differed by no more than ± 6% from the mean consensus with the exception of Laboratories 4 and 8, whose values exhibited bias (+20% and -17%, respectively) of the same sign as that seen with Unknown #1. We had speculated that the inability of these laboratories' calibration factors to accurately measure concentrations when the injected amounts were below a certain concentration (Section 6.2) had affected their results for Unknown #1, but this pattern was not seen with results from Laboratory 4, as the amount injected in the analysis of these filters was sufficiently high that the calibration curve predictions should have been accurate (Appendix 12). The precision seen with replicate filter analysis ranged from 0.6 to 6% with an average for all laboratories of 3%.

6.5 HPLC analysis of sample surrogate filters (accessory pigments)

Participants identified and quantified accessory pigments in the sample surrogate filters using calibration factors derived from the analysis of the standard we had provided (Mix 82). Hence, these analyses provided observations of a laboratory's ability to provide accurate results with calibration factors independent of those used for all other measurements, the chl *a* calibration curves.

In most cases, each laboratory identified the same pigments as being present in each filter. However, in some cases, pigments which were not present were identified (Laboratories 4, 8 and D) and some pigments which were present in very low levels (chlide *a*, diato and β,β-car) were not identified by all laboratories. We had not indicated to participants that these filters were replicates, hence, with Laboratory 4, who was experiencing poorer than usual retention time reproducibility during these analyses (personal communication), some pigments in one of the filters (filter # 64) were mis-identified. A summary of all pigments reported by laboratories is shown in Table 9. The accessory pigments documented as present in this *Aureococcus* culture are chl *c*3, chl *c*2, but-fuco, fuco, diadino, diato and β, β-car.

The quantitative results of accessory pigments in the sample surrogate filters are detailed in Appendix 15. We calculated mean consensus values for those pigments consistently reported by laboratories (chl *c*2, but-fuco, fuco and diadino). (Quantitative results for chl *c*3 and

diato are not included as they were not in Mix 82.) We excluded Laboratory 8 results from the mean consensus values, as their results exhibited a consistently large negative bias and were not representative of results reported by other laboratories. With Laboratory 4, who had mis-identified pigments in one filter replicate, we correctly identified these pigments and quantified the results using their calibration factors before including their results in the mean consensus, as not doing so would have caused the mean consensus values to be unduly biased and would not have represented a correct assessment of variability in quantitative analysis among laboratories. In Fig. 9, we show the %D that mean pigment values were from the mean consensus. Excluding results from Laboratory 8, values of |%D| were less than 10% from the mean consensus for but-fuco, fuco and diadino. In fact, the mean |%D| for these pigments from these laboratories was 3.7%. There was more variability with chl *c*2, which may partially be explained by the biased results introduced to the mean consensus by Laboratory D, who experienced exceptionally poor precision with this pigment. The high positive bias (relative to the mean consensus) seen for chl *a* by Laboratory 4 is not seen with the accessory pigments. However, with results from Laboratory 8, there is a consistent negative bias with all pigments reported, including chl *a*.

Table 9. Accessory pigments identified by participants in the HPLC sample surrogate filters

Lab code	chlide *a*	chl *c*3	chl *c*2	but-fuco	fuco	diadino	diato	β, β-car	other
HPL	x	x	x	x	x	x	x	x	
4 (# 25)			x	x	x	x			lut
4 (# 64)			x		x				hex-fuco, lut
6		x	x	x	x	x		x	
7		x	x	x	x	x			
8				x	x	x		x	hex-fuco, phytin *a*
A			x	x	x	x	x	x	
D	x		x	x	x	x	x	x	divinyl chl *a*

The precision associated with all sets of replicate accessory pigment analyses (n=27) was 2.7% (excluding one value of %RSD of 50% for chl *c*2 from Laboratory D). For 90% of all replicate filters, the values of %RSD for accessory pigment analysis were <5.5%. (Laboratory D does not modify sample extract with buffer, thus the peak shape and resolution of early eluting pigments are compromised. This likely affected their precision in the analysis of chl *c*2.)

6.6 Results of unknowns containing divinyl chl *a*

Participants analyzed a solution containing 100% DV chl *a* and one containing approximately 50% DV chl *a*, Unknown #3 (different formulations were either 43 or 50% DV chl *a* and the remaining percentage was from chl *a*). We emphasize results from laboratories not separating and quantifying DV chl *a* (all but Laboratories 6 and self). However, in this example,

we calculated results for 'self' as if we did not separate DV chl *a* and chl *a* to demonstrate the minimal differential detector discrimination seen with the detector settings we use. Therefore, only Laboratory 6 separately quantified chl *a* and DV chl *a* in Unknown #3; their results for DV chl *a* + chl *a* were within -2.1% of the value as formulated for this unknown (DV chl *a* + chl *a*).

For laboratories not distinguishing DV chl *a* from chl *a* and using chl *a* calibration factors only, the results for chl *a* will be biased to the degree that DV chl *a* is present and to the degree that the detector settings discriminate differentially toward these two pigments. This bias can either be positive or negative. We show in Fig. 10, the degree that laboratories methods caused bias in results for chl *a* when DV chl *a* was present at ~50% (Unknown #3) and 100% (details for results of Unknown #3 are in Appendix 16). These results show the likely over or under estimations that would result from these laboratories' findings if DV chl *a* were present in natural samples at realistic levels (50%), as DV chl *a* has been reported as present at levels from 10 to 60% in natural samples (Bidigare and Trees, 2000). The magnitude of the bias among laboratories results can be related to their detector settings (see Table 7, Section 6.1.2).

One would expect the error to be greater when the proportion of DV chl *a* in the solution is higher, and the relationship between the %error and the proportion of DV chl *a* in the solution should be linear if there is differential discrimination toward the two chlorophylls and if these differences are greater than other uncertainties associated with the analyses. Inspection of Fig. 10 shows that for all laboratories but 4 and 7, the error is greater with the 100% solution relative to the 50% solution. That laboratories 4 and 7 were exceptions, led us to evaluate all laboratories methods for the ability to provide this linear relationship. We therefore computed the linear regression of the response factors (RF = amount injected/peak area) versus the proportion of DV chl *a* in solution: 0%, 50% (or 43% depending on formulation) and 100%. We found that for all laboratories but 4 and 7, relationships were linear. However, for Laboratory 7, the slope was not significantly different from zero ($p > 0.5$), the r^2 was 0.15 and the difference between any two RF values < 7%. Thus, we conclude that there was no discernable differential discrimination by this laboratory's detector settings. However, with results from Laboratory 4, we suspect an error of large magnitude occurred from an unidentifiable source. In Fig. 11 we show the RF values for each solution for each of the two HPLC detectors used (PDA and FLD) by Laboratory 4. Inspection of this figure reveals that the relationship between the three solutions was not linear. Additionally, the fact that these two detectors responded differently to DV chl *a* enabled us to show that the ratio of the response of the two detectors (PDA/FLD) versus the proportion of DV chl *a* in solution was linear. From this we concluded that the correct solutions had been injected and that they had been correctly formulated. These results are also shown in Fig. 11. (For a review of the detector settings used by Laboratory 4, see Table 7).

6.7 HPLC discussion

Laboratories reproduced results attained at HPL with standards containing only chl *a* and reproduced results of other laboratories when analyzing the laboratory-prepared filtered samples on average in both instances to within 2%. Additionally, with these laboratories, r^2

values > 0.999 were seen with chl *a* calibration curves. These results exclude data from Laboratories A, 4 and 8, whose accuracy and precision were often not representative of what was generally attainable by others. While it is not possible to identify the specific causes for these laboratories' difficulties, we can speculate based on objective observations. These limitations were often related to instrument capabilities. With Laboratory A, the detector and data integration system were outdated causing poor detectability and poor repeatability when integrating peak areas (personal communication for the latter). With Laboratories 4 and 8, the mode of injection used may have adversely affected results, as Wright and Mantoura (1997) caution against using the mode of injection implemented by these laboratories (which we refer to as the manual mix/auto-inject configuration). Wright and Mantoura (1997) state that when sample is pre-mixed with buffer, hydrophobic pigments precipitate out of solution with duration in the TCAS compartment, and, as shown by Mantoura et al. (1997), losses of up to 10% can occur with a chl *a* solution (30% buffer) within 4 hr. Laboratories 4 and 8 both used this sample:buffer ratio and their samples resided in autosampler compartments (not controlled for temperature) for up to 24 hr.

However, excellent results were attained with other modes of injection used, fully automated and fully manual. Wright and Mantoura (1997) describe fully automated injection systems as very desirable. Some fully automated injection systems cannot mix sample and buffer in the sample loop and must mix portions of each in a separate, empty vial, from which an aliquot is then withdrawn and injected. Others can mix buffer and sample in the sample loop. We have used both injection schemes at HPL over the years and have found, with our instruments, that the precision is much better with those that "mix in the loop". This injection scheme was exclusively used by participants with fully automated injectors and may have contributed to the excellent results generally attained by them.

We also saw accurate and precise results from Laboratory 5 with manual injections. The HPLC protocols described in Bidigare and Trees (2000) address procedures to enhance performance when using manual injectors. Both laboratories using manual injectors in this study (Laboratories 5 and A) followed these guidelines. Some results for Laboratory A were less accurate and precise (as previously discussed), but results for Laboratory 5 exhibited uncertainties similar to those from laboratories using fully automated injection systems.

HPLC chl *a* values can be over or under estimated when DV chl *a* is present in samples and not accounted for. Six of the eight laboratories in this study did not chromatographically separate DV chl *a* from chl *a* and could be subject to these errors. In these situations, Bidigare and Trees (2000) suggest that the relative proportions of each chlorophyll be quantified using spectral deconvolution by monitoring two different wavelengths (436 and 450 nm). However, none of these six laboratories used this approach. The potential for error with these six laboratories results is dependent on the abundance of DV chl *a* in a sample and the HPLC detector and wavelengths used (Section 6.1.2, Table 7). When analyzing an unknown containing ~50% DV chl *a*, the greatest error was seen with the PDA detector in Laboratory 4, where chl *a* was overestimated by 100%. On the other hand, detector settings used by Laboratory 7 and B

caused no discernable uncertainties above what would otherwise be seen. Settings used by Laboratories 5 and D caused 10 to 15% error and that used by Laboratory 8, -6% error. (Note: with results reported for field samples by Laboratory 4 (who used two detectors), the discrepancy would depend on which detector's result was reported and this varied according to the analyst's discretion; personal communication).

7.0 SPECTROPHOTOMETER RESULTS

The spectrophotometer unknown distributed by us was used to assess how closely another laboratory's measured value compared with that attained at HPL prior to shipping when guidelines for improved spectrophotometric accuracy in the analysis of chl *a* were followed (as detailed in Section 3.3.1). We provided instructions (shown in Appendix 17) and gathered information about participants' spectrophotometers to ascertain when measurements were performed according to these guidelines.

7.1 Survey of spectrophotometers

Procedures for improved spectrophotometric accuracy suggest that spectrophotometers with monochromator type optics (Latasa et al. 1996) and bandwidths not greater than 2 nm (Brown et al. 1980, Clesceri et al. 1998, Trees et al. 2000a) be used. Our survey results showed that all participants spectrophotometers were in accordance with these guidelines except for Laboratories 3, 9 and B who had fixed bandwidths in excess of 2 nm. Spectrophotometer instrument configurations used by participants are shown in Table 10.

Table 10. Spectrophotometers used by participants

Lab code	Manufacturer and model	Dual beam or single beam	Adjustable bandwidth	Fixed bandwidth (nm)
1	Hitachi U-3110	Dual	Yes	
2	Perkin Elmer Lambda 40	Dual	Yes	
3	Camspec M330	Single	No	4 nm
4	Shimadzu UV-1601	Dual	No	2 nm
5	Perkin Elmer Lambda 18	Dual	Yes	
6	Varian Cary 1E	Dual	Yes	
7	Shimadzu UV-2501PC	Dual	Yes	
8	Perkin Elmer Lambda 18	Dual	Yes	
9	Bausch Lomb SP21	Single	No	10 nm
B	Pharmacia LKB Ultraspec Plus	Single	No	5 nm
C	Varian Cary 50	Single	No	2 nm
D	Shimadzu UV 2501PC	Dual	Yes	
HPL	Hitachi U-3110	Dual	Yes	

7.2 Spectrophotometer analysis of unknowns

Participants analyzed the chl *a* solution on their spectrophotometers to determine its concentration and they recorded absorbance values at wavelengths of 660, 662, 664, 666 and 668 nm to roughly assess the wavelength accuracy of their instruments. All laboratories' spectrophotometers exhibited λ_{max} at 664 nm for the chl *a* solutions except Laboratory 3, where it was 662 nm; and the absorbance at 664 nm (the λ_{max} for chl *a* in 90% acetone) was 3% lower than at 662 nm.

The mean chl *a* concentration measured by each participant was compared to the concentration determined at HPL for the same solution prior to shipment. From this we calculated %D for each laboratory (Fig. 12). For laboratories meeting all guidelines for improved accuracy, %D values ranged from -1 to 3%, with a mean |%D| of 1.4%. We excluded laboratories 3, B and "9" from the mean |%D| as their spectrophotometers utilized bandwidths greater than 2 nm and as shown in Fig. 12, the %D values for these laboratories ranged from -13 to -3%. Laboratory 9 was not able to participate in this phase of the study, but we felt it was important to demonstrate the capabilities of the type of spectrophotometer they use. Hence, the value shown for them as "9" was actually acquired at HPL using the same type of spectrophotometer as theirs, which has a fixed bandwidth of 10 nm. The precision (%RSD) associated with each laboratory's triplicate spectrophotometric measurements ranged from 0.0 to 2.3% with an average of 0.5% (for all laboratories). All data for spectrophotometer unknowns shown in Appendix 18.

7.3 Spectrophotometer discussion

Latasa et al. (1996) and Turner Designs (personal communication) conducted spectrophotometric inter-calibrations with chl *a* standards. In the study conducted by Latasa et al. (1996) among 8 participants, several pigments were evaluated and 90% of results were within ±6% of the mean consensus. Values for chl *a*, when analyzed by diode array type spectrophotometers were ~6% lower than results attained from instruments with monochromator type optics. Turner Designs, when evaluating chl *a*, reported that all participants (n=4) were within ±4% of the mean consensus, and the lowest value was attained from an instrument with diode array optics. The results of the current round robin are not directly comparable, as we did not use mean consensus values, yet, all laboratories (with 2 nm bandwidths) were within 3% of values attained at our laboratory.

Dunne (1999) investigated the response of chl *a* in spectrophotometers with diode array and monochromator type optics and found only slightly suppressed values for chl *a* on a diode array type spectrophotometer. In Dunne (1999), as suggested by Rebel (1997), the absorbance depression by this diode array type instrument (Hewlett Packard 8452A) may be a function of its wider bandwidth (where this author states that the actual slit width was 2 nm, but effective resolution was 4 nm). Dunne (1999) also suggests the low absorbance (0.18) and differences in bandwidths among spectrophotometers may have contributed to variation in data presented by Latasa et al. (1996). When wide bandwidths are used with pigments having sharp absorbance

spectra (as with chl *a*) the concentration measured spectrophotometrically will be suppressed, and as Clesceri et al. (1998) notes, this can be up to 40% for chl *a* when a 20 nm bandwidth is used.

The results of the current study corroborate the importance of bandwidth, therefore we suggest all guidelines described in Section 3.3.1 be followed. Furthermore, Latasa et al. (1999) suggests that absorbance accuracy be validated with NIST traceable neutral density filters and Trees et al. (2000a) suggests that the λ_{max} of the chl *a* solution be experimentally determined and used for measuring its concentration. This latter approach would have corrected for minor inaccuracies in wavelength calibration (such as with Laboratory 3).

8.0 PARTICIPANTS' CALIBRATION PROCEDURES

The results thus far are based on chl *a* calibration standards distributed by HPL and where calibrations are normalized with HPL and are consistent among laboratories. In this section we discuss how chl *a* standards from the participants' usual procedures compared with standards distributed by HPL. Thus, we collected details regarding the preparation (and purchase) of their standards and, as it is also important to ascertain that calibrations remain valid during the analysis of samples, we asked participants to share information regarding instrument monitoring. Finally, we asked participants to analyze a chl *a* standard from their own laboratory (which we refer to as the quality control (QC) standard) and to use the calibration standards distributed by us to measure its concentration. The difference between the formulated and measured concentrations were determined (%D). This was done for both fluorometers and HPLCs, both of which are discussed in the following sections.

8.1 Survey of calibration procedures

Participants shared details of their calibrations with regard to the source of the chl *a* standards, the extinction coefficient used for determining its concentration and whether single point of multi-point calibrations were performed. The sources for standards varied as some participants purchased standards already in solution with the concentrations specified (from Turner Designs, Inc. or DHI), while others purchased chl *a* as a solid and dissolved it in the same solvent as that used with extractions, which was acetone in all cases except with Laboratory 9, where it was methanol. The extinction coefficients used by all participants with all HPLC calibrations was the same (87.67 $g^{-1}cm^{-1}$), but that used with fluorometer calibrations varied. In all cases multi-point calibrations were used for the HPLCs, but in some cases, single point or two point calibrations were performed with the fluorometers. We summarize these findings for those which varied among participants in Table 11.

Laboratories prepared calibration standards in a variety of ways. For those laboratories who purchased fluorometer standards from Turner Designs, Inc., it was not necessary prepare the standards further. However, Laboratory 3 validated the concentration provided by Turner Designs, Inc. by analyzing the standard of highest concentration spectrophotometrically. (Turner Designs, Inc. makes two concentrations available.) With all other HPLC and fluorometer calibration standards (even those purchased in solution from DHI for HPLC analysis), dilutions

were made of the stock solution such that multi-level calibrations were performed. When making dilutions, most laboratories used automatic pipettes and in some instances, volumetric glassware was used in conjunction with them. When Laboratories 5 and D prepared their HPLC chl *a* standards for this round robin, they made a dilute solution and then analyzed this on the spectrophotometer to determine its concentration. In these cases, the absorbance values observed were 0.021 and 0.049, respectively. (We show no data for the HPLC standard prepared by Laboratory 5, as the HPLC failed during its analysis; personal communication).

Table 11. Summary of chl *a* calibration procedures used by participants. (Cells which are blank indicate either that that laboratory does not do this type of analysis or did not participate in this phase of the round robin.)

	HPLC	Fluorometer		
Lab code	Source	Source	Extinction coefficient	Single, two or multi-level calibrations
1	Fluka or Sigma	Fluka or Sigma	87.67	multi
2		Sigma	87.67	multi
3		Turner Designs, Inc.	Lorenzen (1967)[1]	two
4	Sigma	Sigma	87.67	multi
5	Sigma	Sigma	87.67	multi
6	Sigma	Sigma	87.67	multi
7	Sigma			
8	DHI	Turner Designs, Inc.	Lorenzen (1967)	two
9	NA	Sigma	74.5 (methanol)	multi
A	Sigma	Sigma	87.67	multi
B	DHI			
C		Turner Designs, Inc.	Lorenzen (1967)	single
D	Sigma	Sigma	87.67	multi or two
HPL	Fluka or Sigma	Fluka or Sigma	87.67	multi

[1] Uses a before and after acidification reading, as described in Clesceri et al. (1998)

With chl *a* analysis by HPLC, there are allomers and epimers associated with the main chl *a* peak. These are degradation products which always co-occur and they have similar excitation and emission spectra as chl *a* and they respond as chl *a* in a fluorescence detector. The amount of these degradation products varies (relative to the total peak area chl *a*) but is typically not more than 10%, the percentage of which is related to such factors as the integrity of the chl *a* standard, the method used and the concentration of the samples analyzed. This has a potential affect on HPLC fluorometer relationships and Trees et al. (2000a) suggest that these degradation

products be included in the chl *a* response. Therefore, we asked participants what they included when tabulating the peak areas for chl *a* (Table 12).

Table 12. Peaks included when tabulating chl *a* peak areas for HPLC analysis

	Laboratory code									
	1	4	5	6	7	8	A	B	D	HPL
main chl *a* peak only		x	x			x[2]	x			
main chl *a* peak + allomers + epimers	x	x[1]		x	x	x[3]		x	x	x

[1] Sometimes
[2] For standards
[3] For samples

8.2 Monitoring instrument performance

As it cannot be assumed that calibrations will remain valid during analysis, we asked participants how they monitored stability during sample analyses. For fluorometric analyses, many participants analyzed secondary standards at intervals bracketing the analysis of samples. The secondary standards used were either the solid standards recently introduced by Turner Designs, Inc. or coproporphyrin. Other laboratories stated that they re-calibrated their instruments (at intervals which differed) rather than monitoring daily calibration. In the case of Laboratory 2, the fluorometer was re-calibrated every time it was used for field sample analyses. Data are summarized Table 13. (Note: to facilitate a laboratory's ability to monitor instrument drift, we purchased secondary standards from Turner Designs, Inc. for those participants who did not have them.)

Table 13. Procedures used by participants to monitor the performance of their fluorometer calibrations

	Secondary standards		
	Turner Designs, Inc.	Coproporphyrin	Recalibration
Lab code	1, 3, 8, C, D, HPL	6	2, 4, 5, 9, A

Table 14 describes how participants monitor their HPLC calibrations. In most cases, participants prepared a quality control (QC) standard which was analyzed during the analysis of samples and its concentration, as measured by the current chl *a* calibration factors, was used to validate that these remained accurate.

Table 14. Ways in which participants monitored the performance of their HPLC calibrations. We have added a few of our comments in parentheses

Lab code	Participant's method of calibration monitoring
1	A QC check is injected every 10th injection.
4	Injection of known quantity of chl *a* - concentration determined spectrophotometrically
5	With each days analyses. (Procedures for preparing the QC standard is specified in Laboratory 5's HPLC procedures manual)
6	Within run variability is monitored through external standard injections performed at regular intervals with each day's analysis. Chl *a* calibrations are performed prior to each project. Calibrations have not changed over ~6 years.
7	Typically re-inject standards at different time intervals
8	Fresh standards are diluted for at least three points over the calibrated range. These standards are then run as samples. Analysis is continued if the standards are within 20% of their known value. At minimum, one standard and one blank are run at the beginning of a run, at the end of a run and every 20 samples. Calibration is repeated when QC is either consistently out of range, shows a consistent trend on either side of the mean, or the method has been out of use for more than one month.
D	Redo calibration curve for chl *a* every 100 or so samples or when solvents/columns are changed/added.
A	QC standard is injected every ~20 samples. Calibration is performed every month.
HPL	During this intercalibration exercise, a set of calibration standards was analyzed every time the HPLC was used. A QC check or calibration standard was injected every 10th injection.

8.3 Results of the analysis of participants' chl *a* calibration standards

Participants formulated (or purchased and diluted) their own QC standards and analyzed them using the calibration standards distributed by us. Values of %D were calculated to express the measured concentration relative to the formulated concentration. The HPLC QC standards differed from the formulated concentrations from -4.3 to 6.2%, except for results from Laboratory 8, who had injected their standard two times and the mean difference was 18.6% (the first injection was within 4.6% and the second injection within 33% of the formulated concentration). The average difference (mean |%D|) for all laboratories was 6%, and 3% (when Laboratory 8 was excluded).

Laboratories 3, 8 and D used fluorometer QC standards as purchased from Turner Designs, Inc.; others prepared their own. Laboratory 8 analyzed QC standards on two separate occasions, as they had received two sets of fluorometer calibration standards from us due to changes in their field sampling schedule. The standards used by participants as QC standards varied in concentration from 10 to 197 μg/l. Values of %D ranged from -5 to 10%, relative to the formulated concentrations. The average difference (mean |%D|) for values reported was 4%. Values of %D for HPLC and fluorometer are illustrated in Fig. 13 and detailed in Appendix 19.

8.4 Discussion

Calibration accuracy is affected by the accuracy and precision of the spectrophotometric determinations when assessing concentrations of the stock solutions and the accuracy and precision of the dilution devices used for preparing standards for the HPLC and fluorometer. Likewise, it is as important that there is proof that calibrations have remained valid during the analysis of samples. Therefore, we discuss results in the context of these issues.

The results for the participants' chl *a* QC standards indicate that, had they calibrated their instruments with their own standards and not those provided by us, their results for the laboratory-prepared unknowns discussed in this report would likely have been different by no more than 10% and on average, the differences would likely have been <4% (with both of these observations, Laboratory 8's HPLC result is omitted). Interestingly, the results from Laboratory 4 for their HPLC QC standard was more accurate than their HPLC results for the Unknown #1. We speculate that this is due to the fact that the amount injected when analyzing the QC standard was sufficiently high that the calibration curve made accurate predictions (see discussion in Section 6.2) and this was not true when analyzing the Unknown #1.

Some spectrophotometric procedures pertinent to calibrations and used by participants were inconsistent with guidelines for improved spectrophotometric accuracy (see Section 3.3.1). These inconsistencies include the use of absorbance values < 0.1 by Laboratories 5 and D, and the use of extinction coefficients other than those suggested for use in the Ocean Optics Protocols (see Turner Designs, Inc. and Laboratory 9, Table 11). It is not necessarily true that these inconsistencies will contribute to greater uncertainties, and the data in this study are too few to validate that they had an effect. However, these inconsistencies in procedures are easily modified and doing so may have a positive benefit on minimizing discrepancies in future studies.

Reproducibility in calibration factors over time is important to consistency of results and is affected by many variables, including instrument imprecision, spectrophotometer accuracy, stability of the standards during storage and accurate dilutions. With regard to sample stability during storage, we have found standards to be stable for extended periods of time (184 days, See Appendix 8 and 9). However, analysts should document that storage vessels used prevent evaporation and, before use, the concentration of the standards should be validated either spectrophotometrically (if sufficiently concentrated) or by cross referencing with freshly made standards. Bidigare and Trees, 2000 suggest that concentrations of all standards be validated spectrophotometrically. This poses a dilemma for analysts using standards purchased from DHI or Turner Designs, Inc., as these standard solutions are not necessarily sufficiently concentrated to yield absorbance values >0.1 and therefore procedures may be inconsistent with spectrophotometric guidelines for maximal accuracy (See section 3.3.1). Furthermore, if spectrophotometric readings are highly accurate, and dilution devices inaccurate and imprecise, then the calibration standards used for the HPLC and fluorometer will be in error. We therefore recommend using only devices which are calibrated for accuracy and capable of excellent precision. We tested several dilution devices (a gas-tight glass syringe and three different automatic pipettes) for the precision attainable when used in accordance with typical laboratory

procedures (e.g., changing pipette tips between each use with automatic pipettes and pre-wetting the tip). We then calculated the WL and CL associated with the replicate measurements (n = 7 each) to describe expected variability about the mean volume (500 μl) when measuring 100% acetone. We found these dilution devices to vary tremendously and we summarize these findings in Table 15.

Table 15. Precision attainable with various dilution devices when measuring 500 μl of 100% acetone

Measuring device	WL	CL
glass syringe	±0.4%	±0.7%
Automatic pipette 1	±4.3%	±8.7%
Automatic pipette 2	±1.6%	±3.2%
Automatic pipette 3	±1.4%	±2.7%

We do not imply that in all cases where automatic pipettes are used, the results will be inaccurate or imprecise, and this was not the case in this study. However, data from our precision testing suggest that the probability is greater that measurements will be more precise when using glass syringes. At minimum, laboratories should determine the accuracy and precision (with acetone) of any dilution device used for quantitative measurements and use only those with excellent performance (as seen here with the glass syringe).

All calibrations should be validated during sample analysis to ascertain that the instrument was operating within limits which were generally attainable for that instrument and method, yet it was not clear that all participants did this (except Laboratories 6 and 8, see Table 14). These limits can be estimated through multiple observations of "daily calibrations" or QC standards and can easily be done with fluorometer performance by using the solid secondary standard (Turner Designs, Inc.). Such data are used to construct WL and CL within which daily readings for these standards should lie when the instrument is in statistical control (as was done in Appendix 9). However, it is important for analysts to know the instrument performance generally attained by others if their limits are to have useful meaning in the context of others results. For example, Laboratory 8 constructed such limits, and yet their limits allowed uncertainties in their analyses which were generally greater than others. This illustrates a need for frequent inter-calibrations among laboratories. (Note: the analyst from Laboratory 8, while experienced with HPLC analyses and analytical methods, had little experience in pigment analyses and had not previously participated in HPLC pigment inter-calibration exercises.)

9.0 CONCLUSIONS

We estimated a laboratory's ability to be accurate by how closely their fluorometer and HPLC results reproduced those attained by others when standard solutions containing chl *a*, chl *a* plus DV chl *a*, and laboratory-prepared filtered samples were analyzed. Our premise was that to understand discrepancies between fluorometric and HPLC results, factors affecting the accuracy

of each instrument individually must be first be determined. Having done this, we can now summarize results as they relate to fluorometer/HPLC discrepancies. We include all participants (and HPL results) in these discussions and we identify the average uncertainties which reflect what can be attained among laboratories when instrumentation and procedures are not limiting. Thus, when calculating these averages, we excluded results from laboratories when they were not representative of others, but only when we could identify a probable cause for the additional uncertainty. All conclusions pertaining to measurements were based on chl *a* calibrations which had been normalized with HPL.

Participants analyzed a solution (Unknown #1) containing only chl *a* to identify the accuracy with which the chl *a* calibration factors could measure its formulated concentration. Nine of the 10 laboratories fluorometer results exhibited inaccuracies < 4.3% and 5 of the 8 laboratories' HPLCs did so. The average uncertainty for both fluorometers and HPLCs was approximately 2%. Higher uncertainties were seen with one fluorometer result (11%, Laboratory 2) and three HPLC results (-11% to 26%, Laboratories A, 4 and 8). These HPLC results were omitted from the aforementioned average (for reasons to be discussed later), however, we did include the fluorometer result from Laboratory 2, as we could not identify a probable cause for its result.

Once the accuracy of the chl *a* calibration curves were established, we could identify uncertainties associated with other variables. Thus, we determined how comparable results were among laboratories (and between HPLCs and fluorometers) when homogeneous laboratory-prepared filters were extracted and analyzed. Inaccuracies were estimated by determining the difference that an individual laboratory's result was from the mean consensus for a particular instrument, HPLC or fluorometer. Seven of the 8 laboratories reporting fluorometric results were within 4.5% of the corresponding mean consensus and 6 of the 8 laboratories reporting HPLC results were so. The average uncertainties among laboratories for both instruments was <2%. We excluded three laboratories when calculating these average uncertainties, as they differed more than 4.5% from the mean consensus values. This included one fluorometric result (Laboratory A) and two HPLC results (Laboratory 4 and 8) and in all three cases, we had identified probable reasons for their higher uncertainties which approximated ± 20%.

It is surprising that the average uncertainties in the analysis of laboratory-prepared filtered samples was no greater than that seen with the chl *a* standard solution. Undoubtedly, two factors contributed to these excellent results: 1) that filters were devoid of pigments which, by HPLC, should be summed and reported as total chl *a* (such as chlide *a* and DV chl *a*) and 2) that standardized extraction procedures were used by all laboratories. However, these results also demonstrate consistency in the preparation of the chl *a* calibration standards at HPL and that shipping did not affect them measurably, otherwise uncertainties in results among laboratories would have been greater. It is inappropriate to say that calibrations among laboratories were normalized, as participants received standards from HPL which were formulated on separate occasions. Thus we conclude that the procedures used to prepare the standards at HPL yielded very reproducible results.

That uncertainties in results from the chl *a* solution and the laboratory-prepared filters were low allow us to confidently describe the level of discrepancy between fluorometric and HPLC chl *a* values, as caused by their inherent differences. As one would expect, there was no bias in results between fluorometers and HPLCs for the chl *a* standard solution. However, the HPLC mean consensus value for chl *a* in the filtered samples was 89% of the mean consensus value for the fluorometers. This illustrates a limitation of fluorometric analyses, that other fluorescing pigments (in this case chl *c* compounds) can interfere with the chl *a* result (Lorenzen and Jeffrey 1980; Trees et al. 1985)

It is well known that DV chl *a* can cause inaccuracy in chl *a* values reported by HPLC if it is not individually quantified and summed in a total chl *a* value (as discussed in Bidigare and Trees, 2000). When standard solutions containing equal portions of DV chl *a* and chl *a* were analyzed, we found that with both HPLCs and fluorometers inaccuracies were more frequent and of greater magnitude than those seen with the solution containing only chl *a*. When DV chl *a* was present, 40% of the fluorometer and HPLC values exhibited inaccuracies <4.3%, yet when a solution contained only chl *a*, 90% of fluorometers and 60% of HPLCs exhibited these low levels of uncertainty. When considering those laboratories whose results had been accurate with the chl *a* solution (and whose results here we feel reliably reflect effects of DV chl *a*), fluorometer inaccuracies varied from -10% to 38% and HPLC inaccuracies varied from -3.5% to 15%.

Two laboratories individually quantified each chlorophyll type and their "total chl *a* values" were accurate to within 3.5%. However, it was not necessarily true that HPLC "total chl *a*" was inaccurate when DV chl *a* was not individually quantified, as two laboratories not separating DV chl *a* reported results for "total chl *a*" as accurate as those who individually quantified it. These results demonstrate that the magnitude of the inaccuracies seen when DV chl *a* was present (and not individually quantified) was highly related to the detector settings used, as wavelengths (and associated bandwidths) differ in the degree to which they differentially discriminate toward these two chlorophyll pigments. At detector settings where their response is very similar, total chl *a* was accurately reported and at detector settings which differentiate substantially, the result for "total chl *a*" was biased in either a positive or negative sign. We feel that the fluorometer exhibiting the highest uncertainty (38%, Laboratory A) was also affected by differential discrimination, as its poor results are likely related to the wavelength specifications for the excitation filter. (This was the only fluorometer not from Turner Designs, Inc.)

Several factors with regard to HPLC instrumentation and methods used affected chl *a* results. The three laboratories (A, 4 and 8) whose HPLC results often exhibited uncertainties higher than others may have been limited by their instrumentation. The older HPLC used by Laboratory A exhibited lower SNR than others for similar amounts injected and it was difficult to attain repeatable results with their peak integration system (personal communication). Poor SNRs may also have affected results from Laboratory 8. However, with Laboratories 4 and 8, the injection procedures used by them can result in pigment losses during sample analysis (Mantoura et al. 1997) and for this reason, it is recommended by Wright and Mantoura (1997) that these injection procedures not be used. These issues are not addressed in Bidigare and Trees (2000). Additionally, when DV chl *a* and chl *a* were not separated, total chl *a* results were inaccurate to

the degree that the detector settings differentially discriminated between the two chlorophylls. Other differences in HPLC procedures and configurations, such as column choice and gradient conditions, had no apparent effect on results for total chl *a*.

Thus far, all conclusions have been based upon calibration standards distributed by HPL. Participants also analyzed their own calibration standards. It was found that, on average, the participants' calibration standards would have provided results within 2.8% of those predicted by our calibration standards and individual observations varied by no more than 8%. This average value includes fluorometric and HPLC results, but excludes results from Laboratories 4 and 8 (for reasons previously described). These results suggest that participants calibrations would have been, in general, very similar to ours. However, we caution that these data were not collected "blind" as had results for unknowns. These results should not be used to make assumptions about previous calibrations in these laboratories.

As all calibrations are ultimately based on a spectrophotometer reading, participants tested the accuracy with which their spectrophotometers reproduced results attained at HPL by analyzing a spectrophotometer unknown distributed by us. These results indicate that when guidelines for improved spectrophotometric accuracy were followed (See Section 3.3.1), the average inaccuracy was 1.4% and individual discrepancies were not greater than 3.2%. However, when spectrophotometers were equipped with bandwidths greater than 2 nm (and therefore were inconsistent with guidelines), accuracy was compromised and values as low as -7% and -13% were reported when bandwidths of 5 and 10 nm, respectively, were used. These results have grave implications for Laboratory 9 who uses a spectrophotometer with a 10 nm bandwidth.

The results of this inter-calibration exercise reveal that, when calibrations are consistent among laboratories, instrumentation is not limiting and methods are properly optimized, excellent reproducibility among laboratories can be attained. This study shows that it is important to validate the accuracy of fluorometers and HPLCs individually before accurate assumptions about fluorometer/HPLC discrepancies can be made. In cases where laboratories exhibited elevated uncertainties, this was often related to limitations in instrumentation, as with Laboratory A's HPLC and Laboratory 9's spectrophotometer. The higher uncertainties with HPLC results from Laboratory 4 and 8 are likely related to the mode of injection used. However, these analysts could improve results if they modified their injection procedures. These results show limitations in the analysis of laboratory-prepared samples and provides a basis for understanding discrepancies seen with the analysis of field samples.

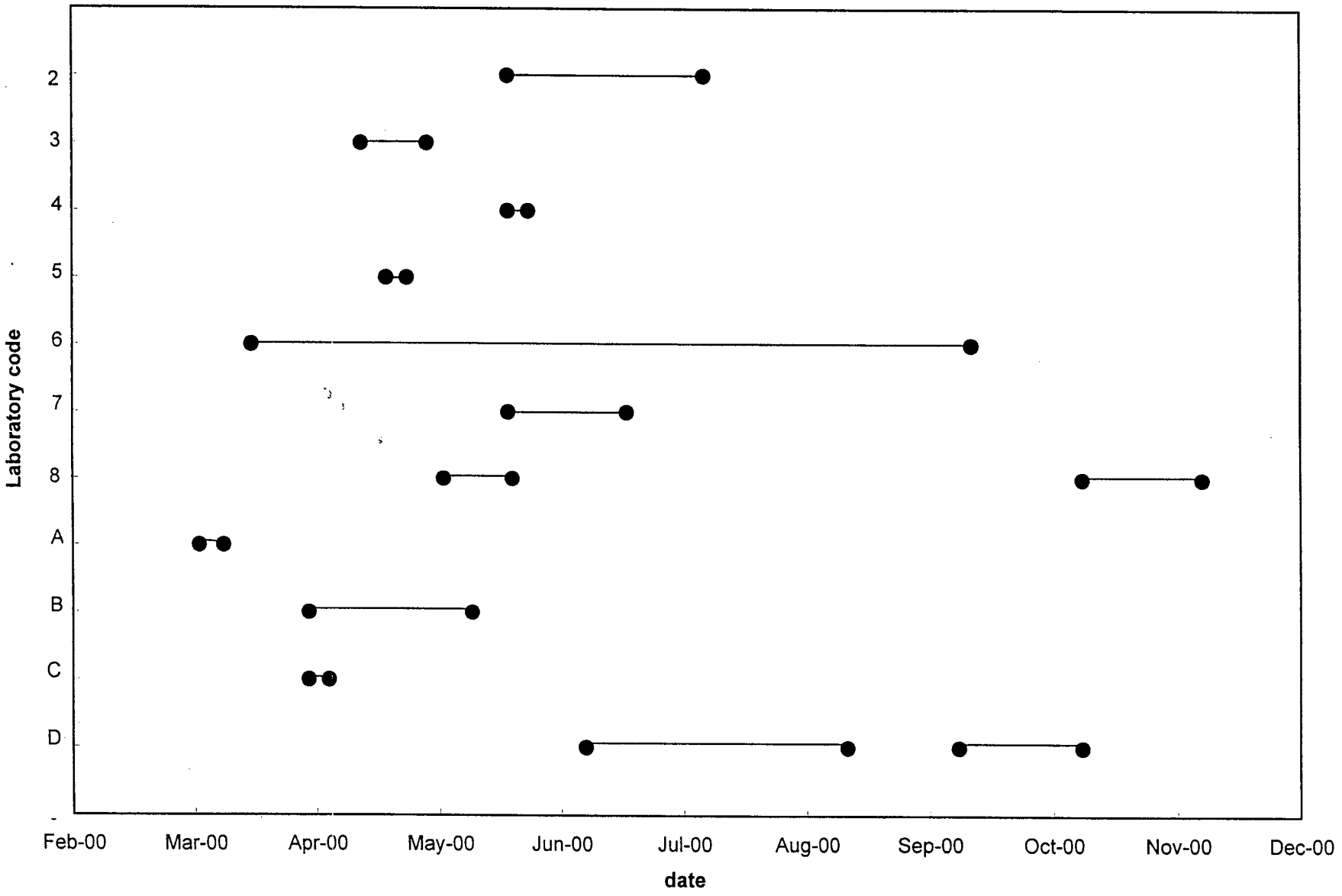


Fig. 1. Timetable depicting when participants received their calibration packets (first dot) and when they completed their analyses (last dot). In some instances, participants received more than one kit (Lab 8, D).

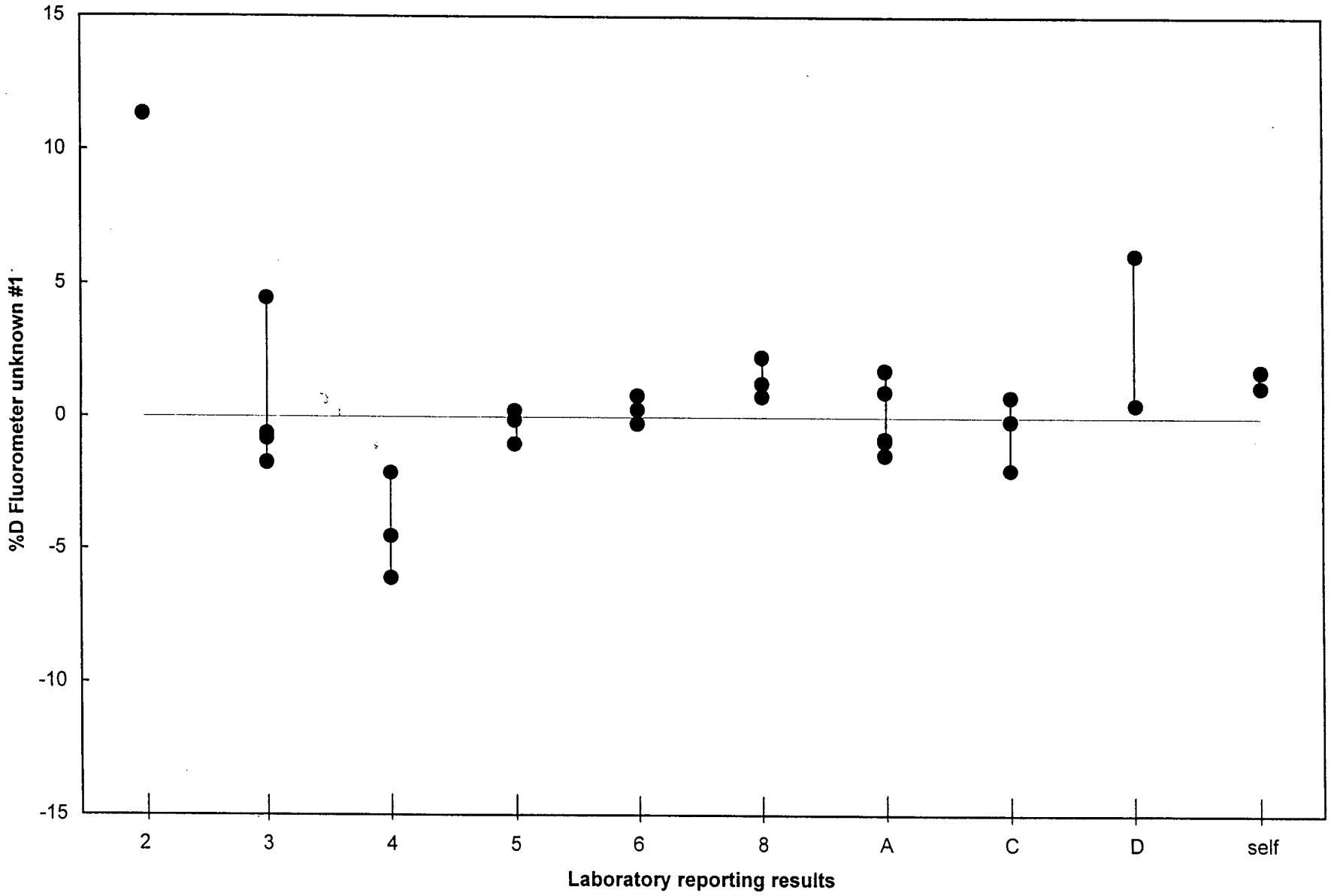


Fig. 2. Participants' results of analysis of fluorometer Unknown #1. %D is relative to the formulated value.

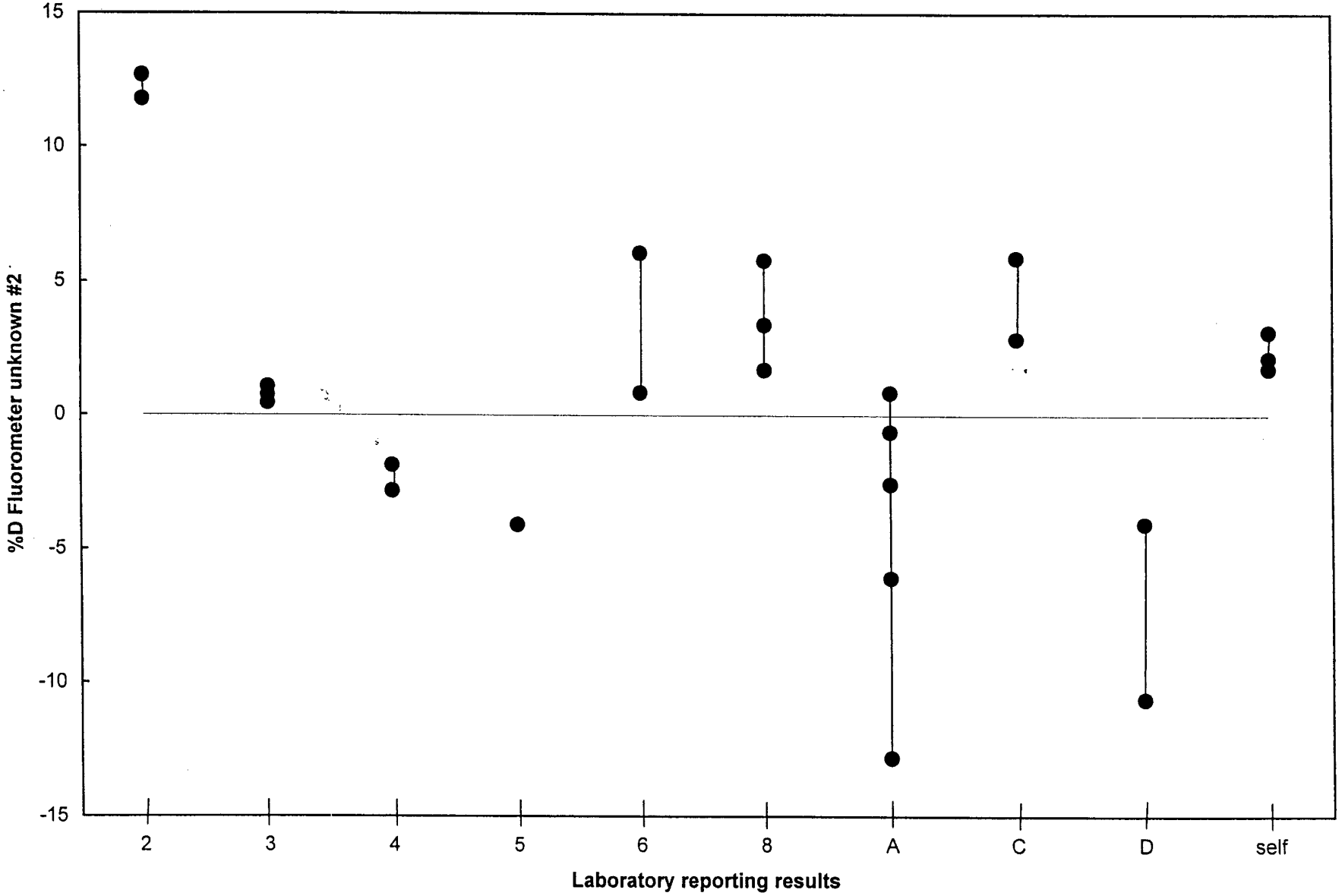


Fig. 3. Participants' results of analysis of flurometer Unknown #2. %D is relative to the formulated value.

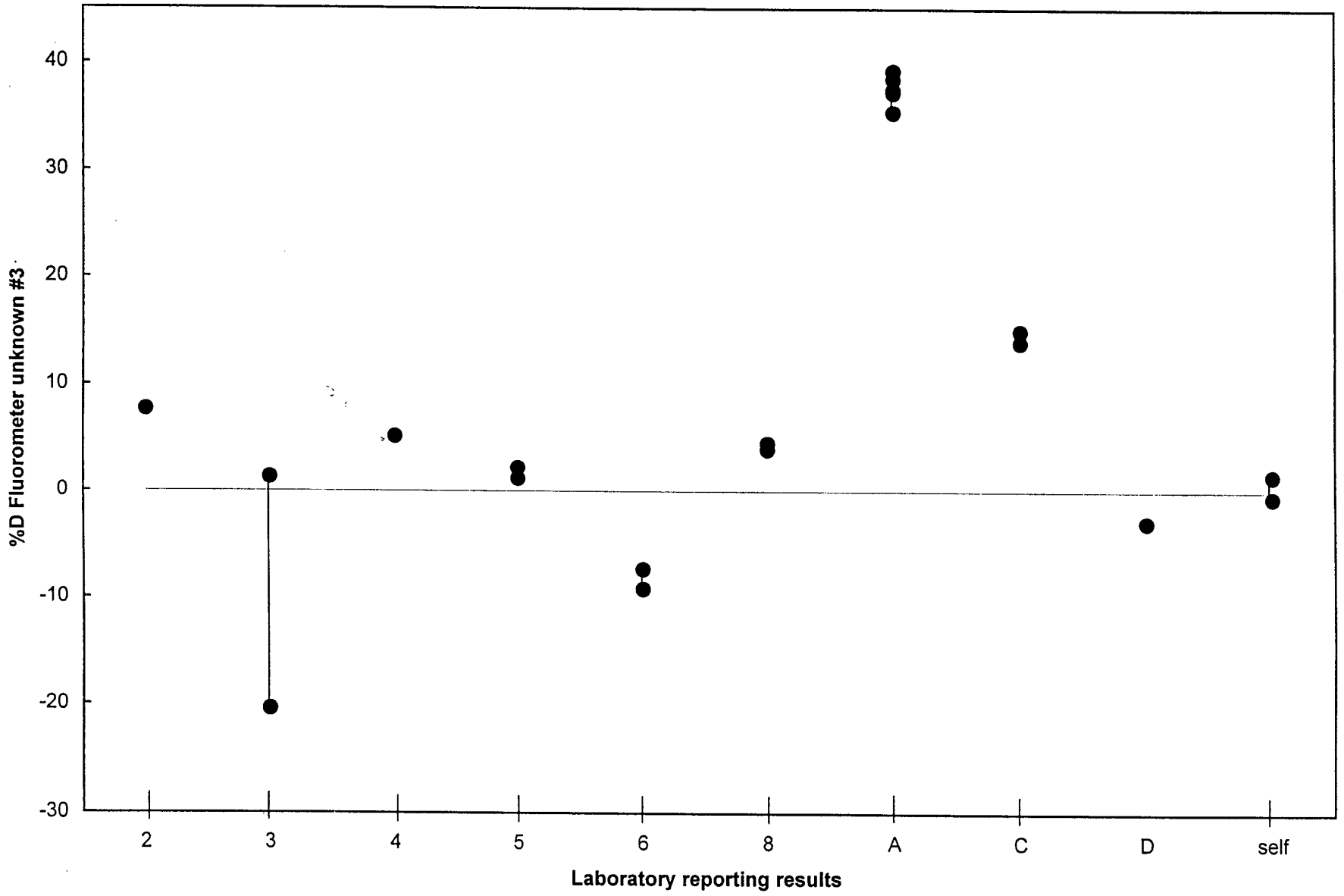


Fig. 4. Participants' results of analysis of fluorometer Unknown #3. %D is relative to the formulated value.

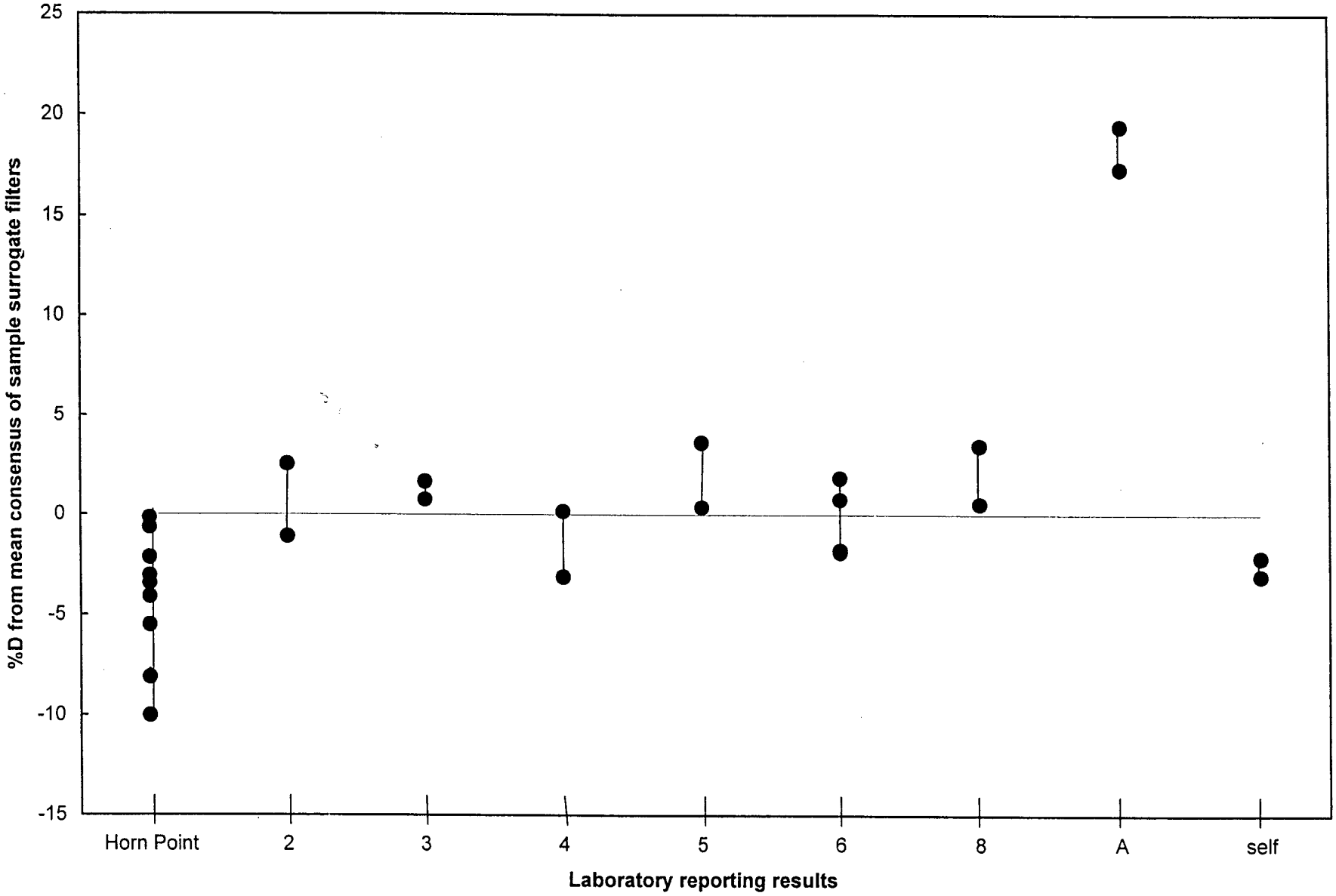


Fig. 5. Results of analysis of sample surrogate filters by fluorometry. Values are expressed as %D from the mean consensus. Mean consensus was calculated without values from Laboratory 6, A and self.

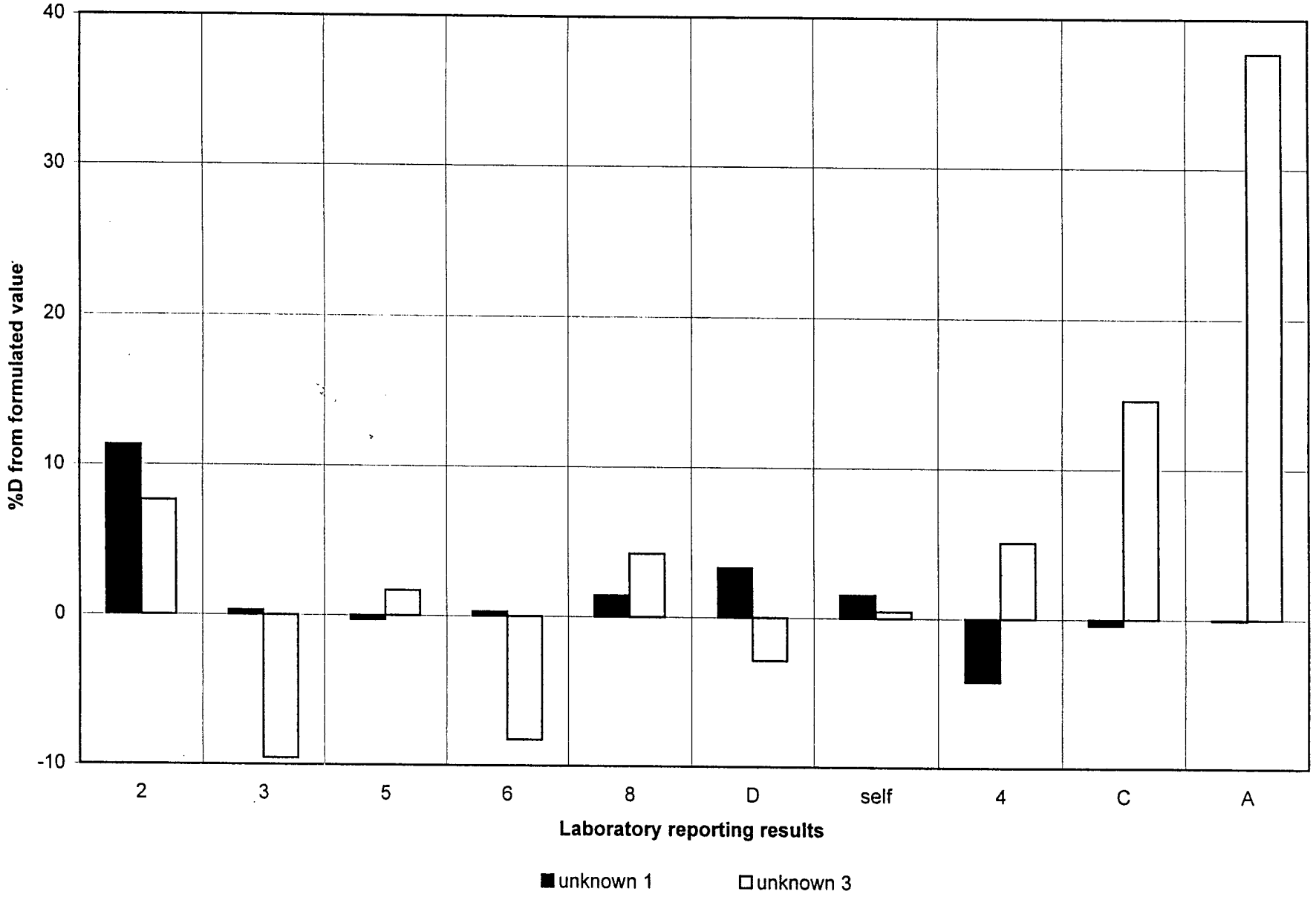


Fig. 6. Participants' results of analysis of fluorometer Unknowns #1 and #3.

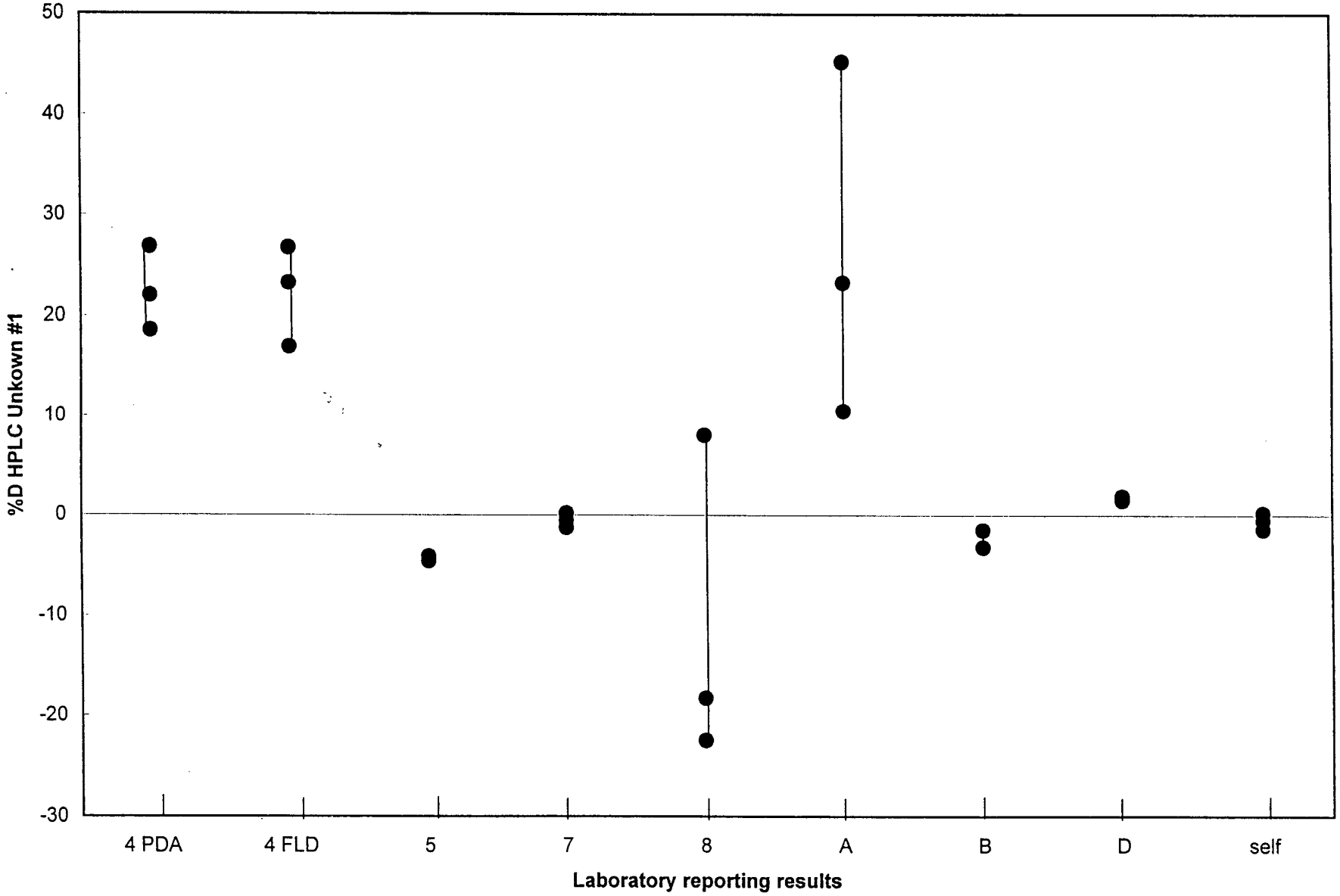


Fig. 7. Participants' results of analysis of HPLC Unknown #1. Laboratory 4 used 2 detectors (described in Table 7). %D is relative to the formulated value.

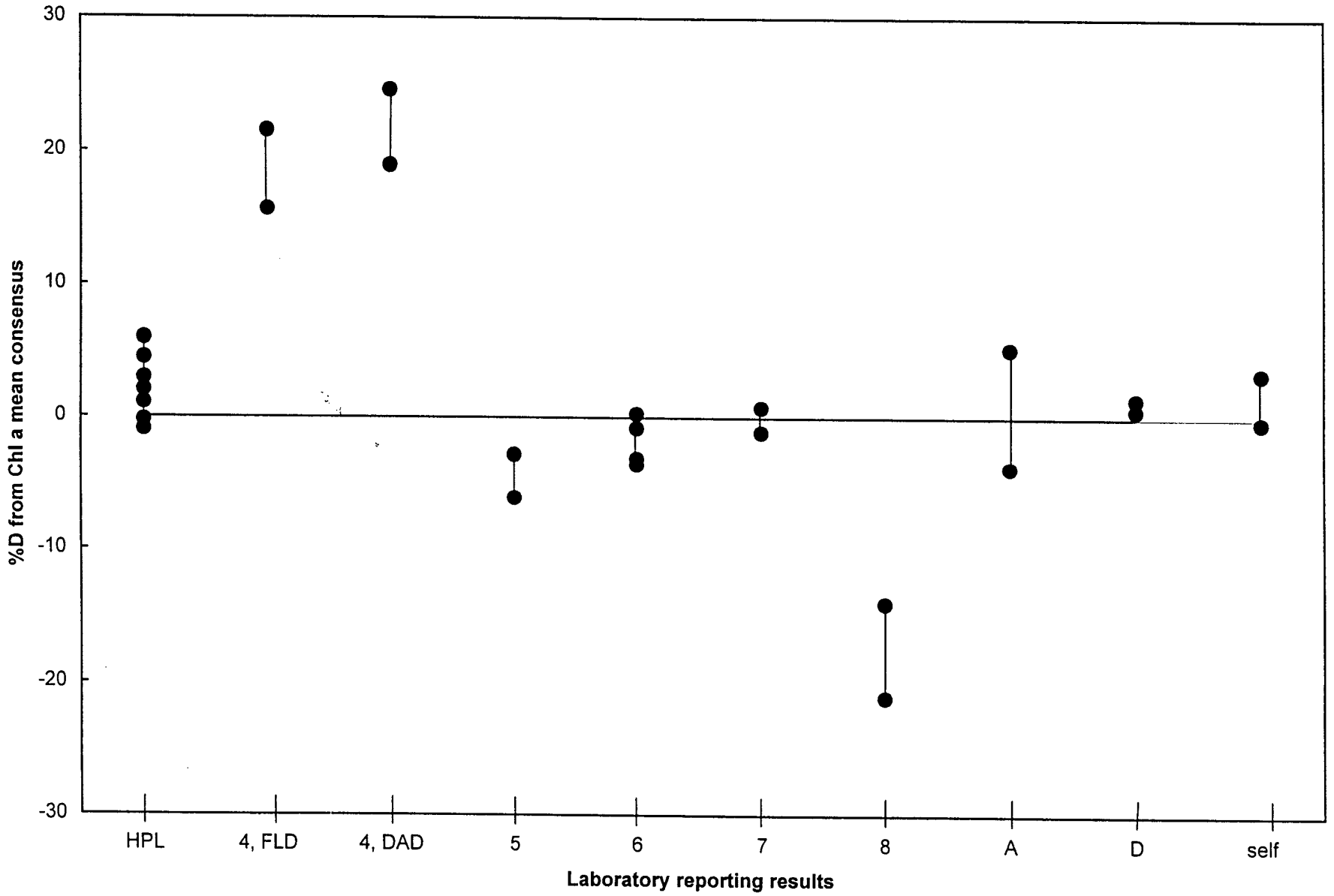


Fig. 8. Participants' results of chl a analysis of sample surrogate filters by HPLC. Values are expressed as %D from the mean consensus. The mean consensus was calculated without values from 'self'.

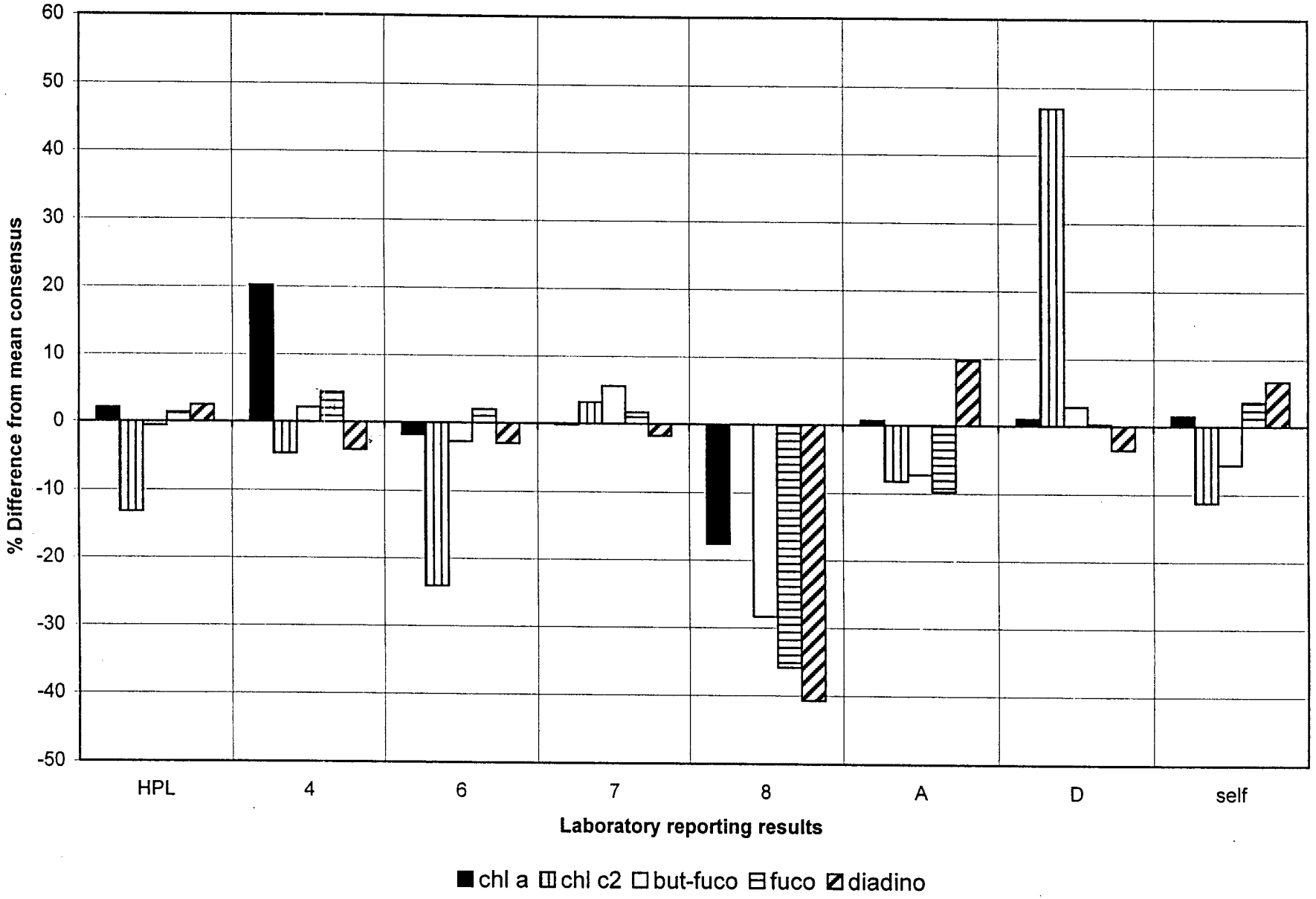


Fig. 9. Participants' accessory pigment analysis of sample surrogate filters by HPLC. The accessory pigment mean consensuses were calculated without values from Laboratory 8 or self. Chl a values are shown for comparison.

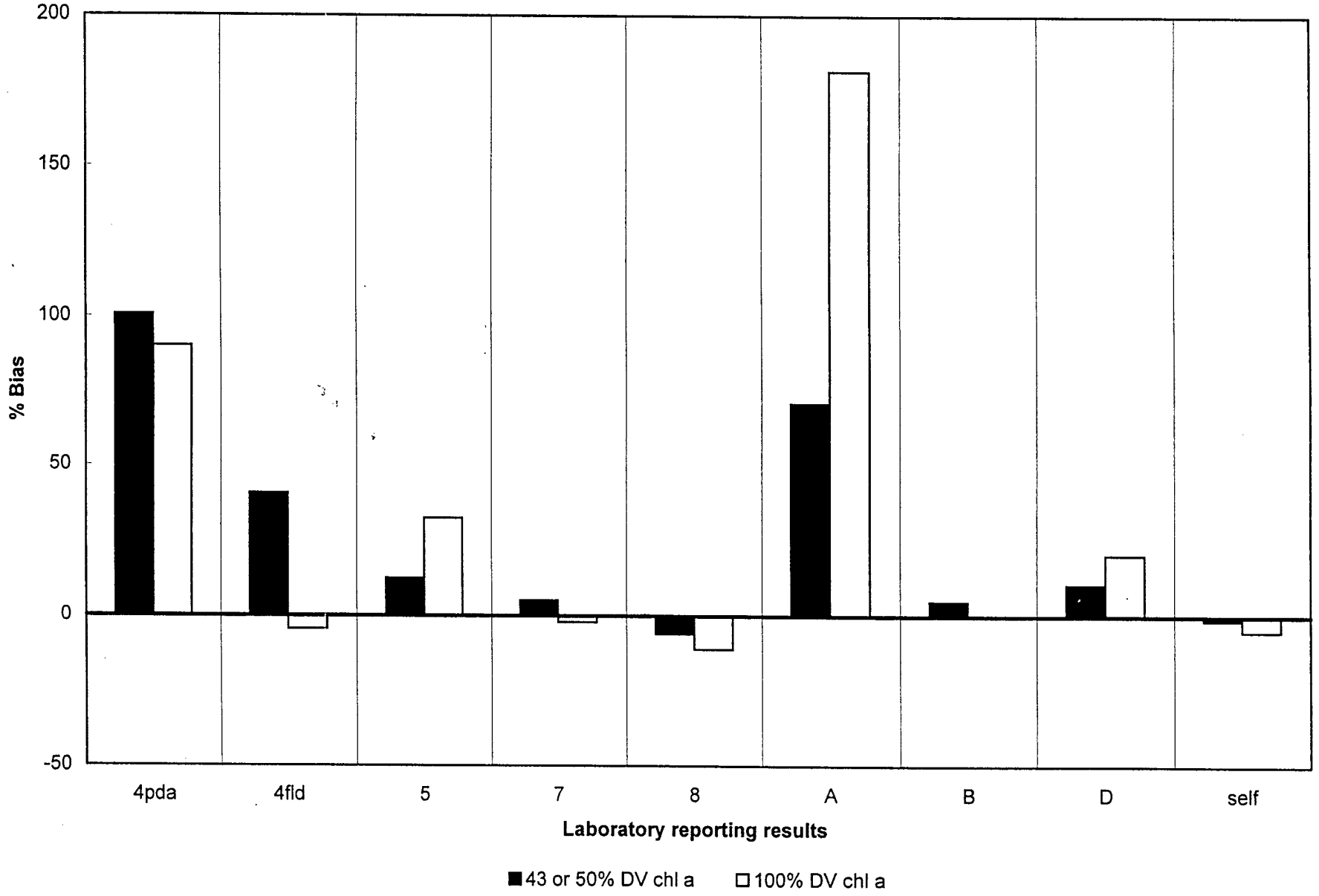


Fig. 10. Participants' results of analysis of HPLC unknowns containing DV chl *a*.

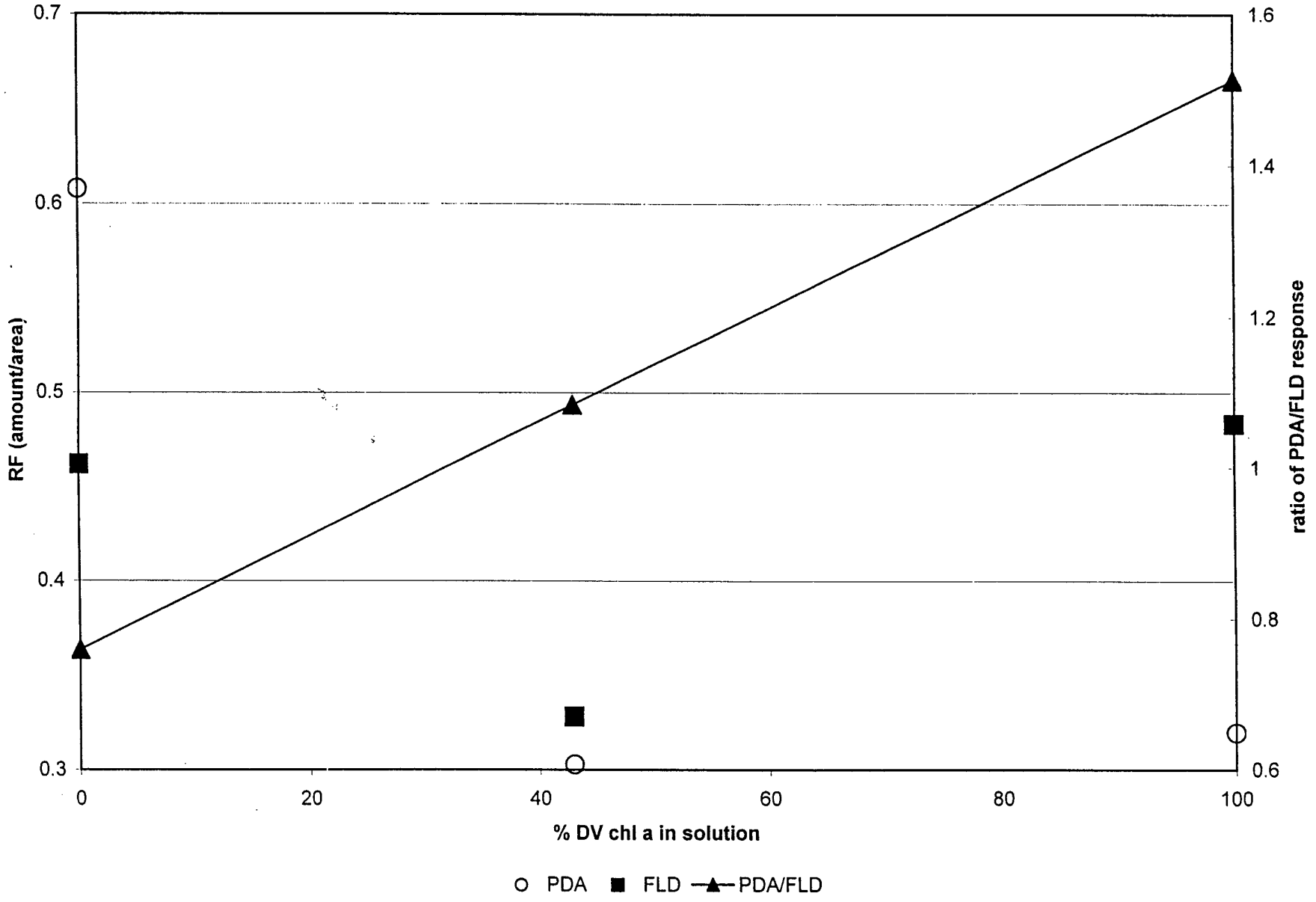


Fig. 11. Further analysis of Laboratory 4 data. See Section 6.6 for explanation.

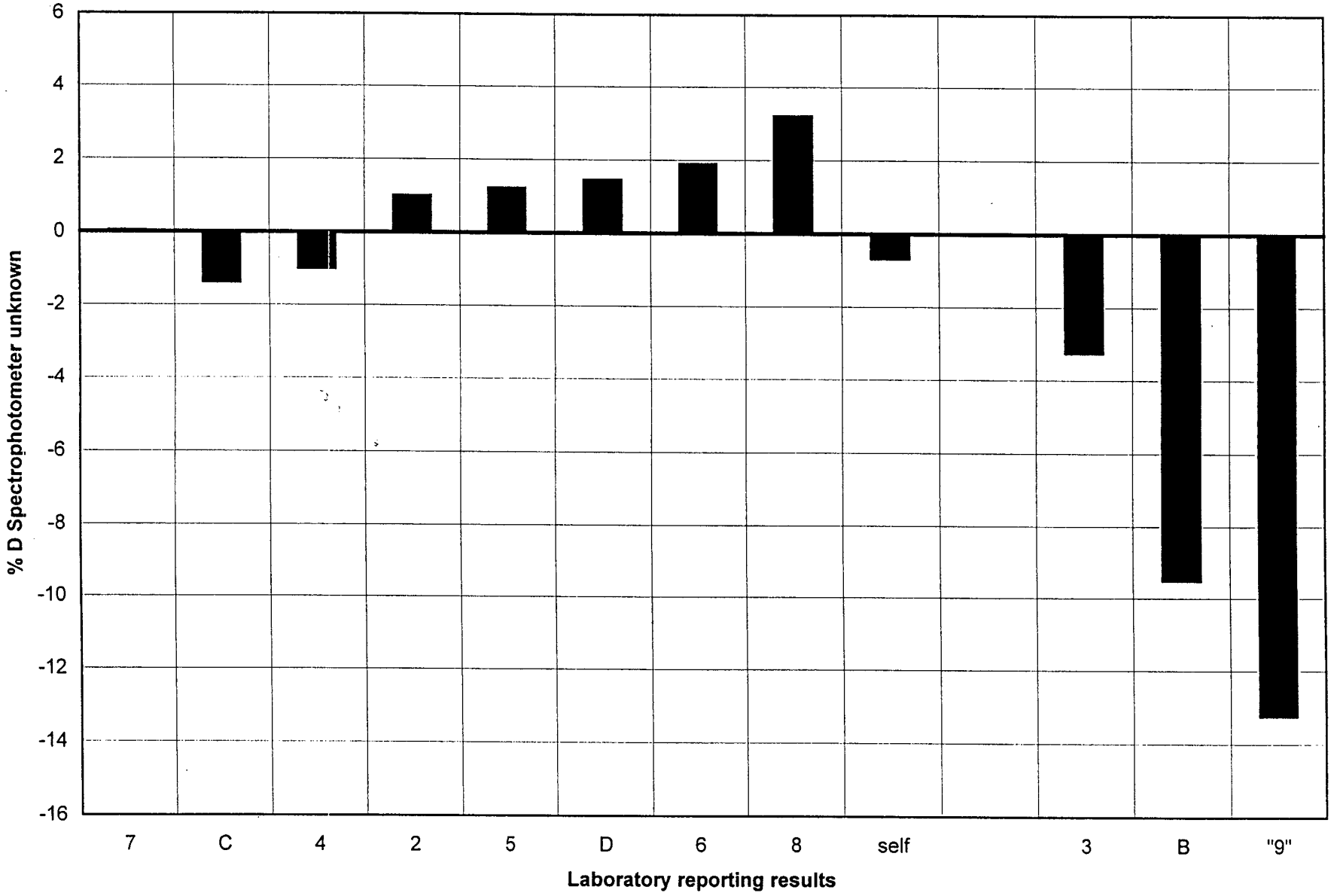


Fig. 12. Participants' results of analysis of spectrophotometer unknown. Results reported as Laboratory "9" were actually analyzed at HPL using the same type spectrophotometer that Laboratory 9 uses.

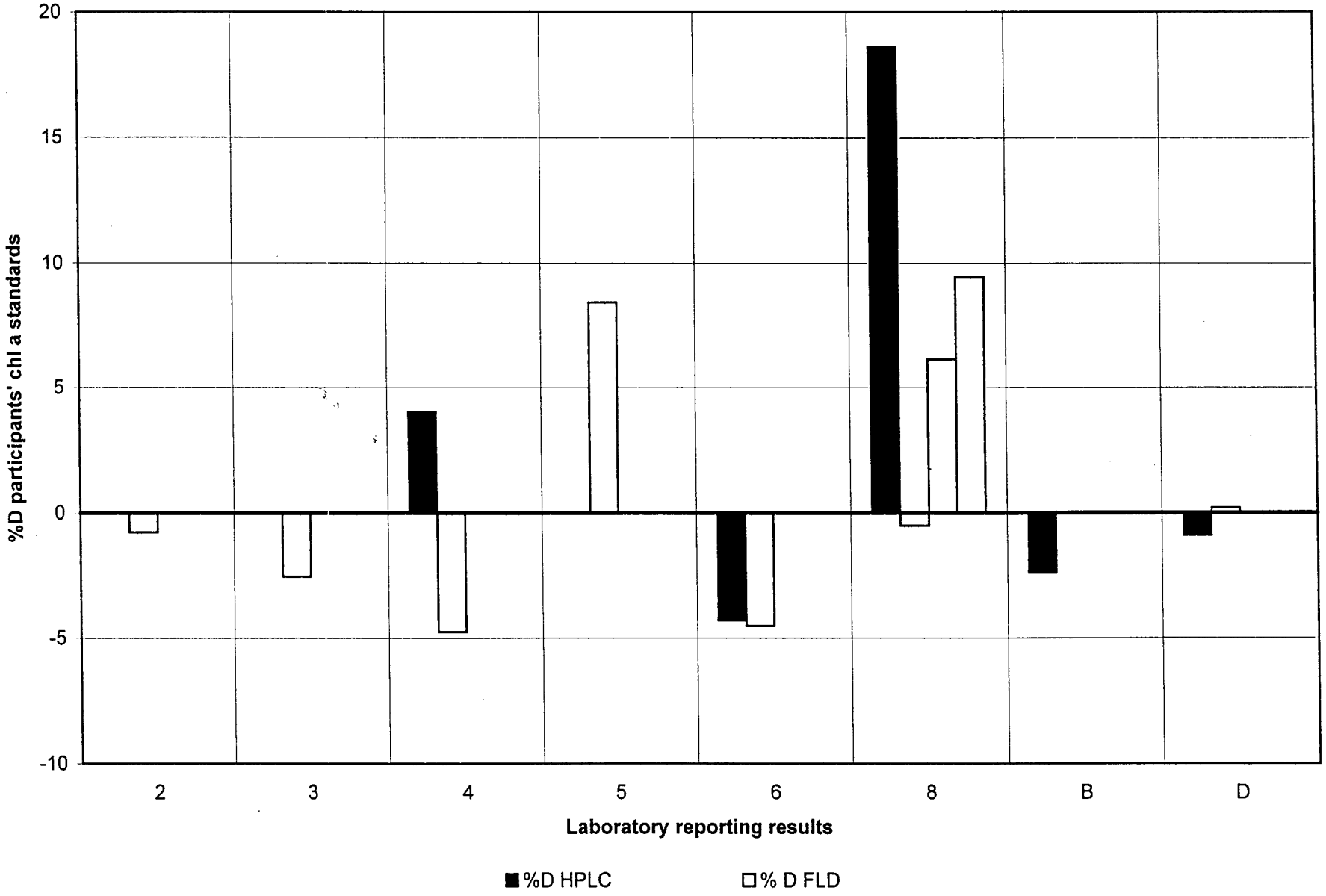


Fig. 13. Results of analysis of participants' chl a standards they formulated, showing differences between calibration standards distributed by HPL and those formulated by participants.

Appendix 1

Pigment abbreviations used in text

Pigment abbreviations are the same abbreviations adopted by Scientific Committee on Oceanic Research (SCOR) Working Group 78.

Pigment	Abbreviation
Chlorophylls	
Chlorophyll *a*	Chl *a*
Chlorophyll *b*	Chl *b*
Chlorophyll *c*1	Chl *c*1
Chlorophyll *c*2	Chl *c*2
Chlorophyllide *a*	Chlide *a*
Divinyl chlorophyll *a*	DV chl *a*
Divinyl chlorophyll *b*	DV chl *b*
Phaeophytin *a*	Phytin *a*
Carotenoids	
Alloxanthin	Allo
19'-butanoyloxyfucoxanthin	But-fuco
Canthaxanthin	Cantha
β, β-carotene	β, β-car
Diadinoxanthin	Diadino
Diatoxanthin	Diato
Fucoxanthin	Fuco
19'-hexanoyloxyfucoxanthin	Hex-fuco
Lutein	Lut
Neoxanthin	Neo
Peridinin	Perid
Prasinoxanthin	Pras
Violaxanthin	Viola
Zeaxanthin	Zea

Appendix 2

Accessory pigment calibration standards for the HPLC (see section 3.3.5)

Two Accessory pigment calibration standards were distributed, each of which contained multiple pigments. "Mix 82" was distributed to be used with the quantitative analysis of the accessory pigments in the sample surrogate filters. The "retention time mix" (Appendix 2.2) was distributed for use in determining elution positions of 20 different pigments.

Appendix 2.1. "Mix 82" formulation

The concentration of each pigment in this mix is shown. While lutein was present, it was not needed for quantitative purposes.

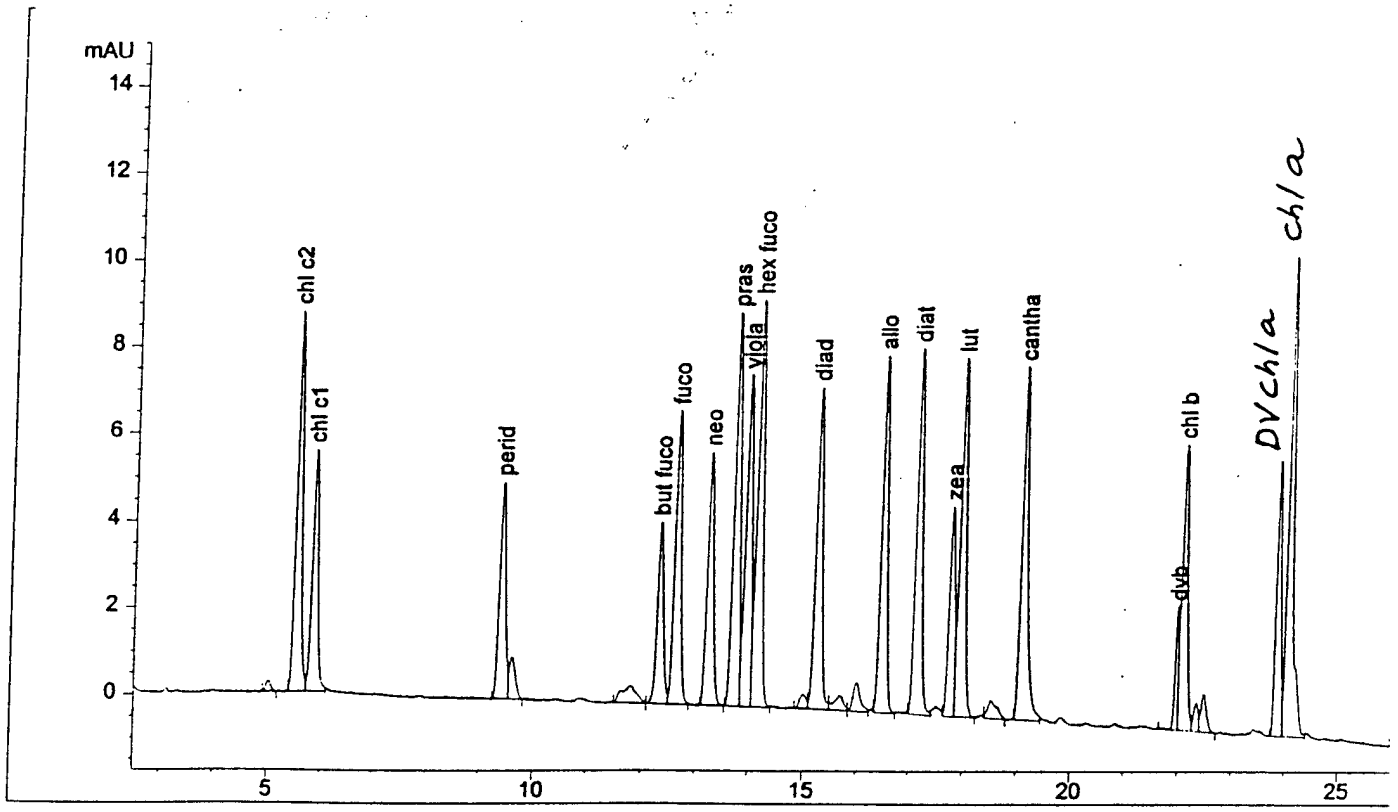
pigment name	concentration (ng/μl)
chlorophyll c_2	0.03327
19'- butanoyloxyfucoxanthin	0.05195
fucoxanthin	0.04650
19'- hexanoyloxyfucoxanthin	0.05372
diadinoxanthin	0.04366
lutein	0.06776
chlorophyll *a*	not quantitative in this mix
beta carotene	0.02928

Appendix 2 (cont.)

Accessory pigment calibration standards for the HPLC (see section 3.3.5)

The "retention time mix" contained 20 pigments, as indicated by numbers above the peaks in this chromatogram generated at HPL. The numbers given correspond with the pigment names listed below (abbreviations given in Appendix 1).

Appendix 2.2 Chromatogram of "retention time mix"



1 - chl *c*2	6 - neo	11 - allo	16 - DV chl *b*
2 - chl *c*1	7 - pras	12 - diato	17 - chl *b*
3 - perid	8 - viola	13 - zea	18 - DV chl *a*
4 - but-fuco	9 - hex-fuco	14 - lut	19 - chl *a*
5 - fuco	10- diadino	15 - cantha	20 - β, β-car

Appendix 3

Extraction procedures provided to and used by participants and HPL for the sample surrogate filters (see section 3.3.6). These procedures were also used by HPL as the "standard extraction procedure" used with participants' field samples. The extraction volumes used in calculations were considered to be the volume of acetone added plus the water contributed by the glass fiber filters (145μl for 25 mm GF/F; 500μl for 47 mm GF/F).

Appendix 3.1 SIMBIOS/HyCODE chlorophyll *a* round robin

HPLC Extraction Procedure for Surrogate Sample Filters

1. Keep track of filter numbers.
2. With forceps, place filter in 7 ml amber glass vial (provided). Note filter number on vial.
3. Add 5 ml 90% acetone (using 5 ml, Class A volumetric pipette provided to you).
4. Make sure filter is submerged and cap tightly.
5. Vortex vigorously for 15 seconds. Make certain filter is still submerged.
6. Again, make certain filter is submerged.
7. Place tubes in freezer (~ -10 to -20°C) for three hours (note time placed in freezer).
8. Remove from freezer (note time). Check cap tightness. Vortex each tube vigorously for 15 s.
9. Attach filter cartridge to end of syringe.
10. Decant filter and extract into syringe and force extract through filter with plunger.
11. Collect filtrate in empty 7 ml amber glass vial (provided). Cap tightly. Vortex well.
12. Remove aliquot for HPLC analysis.

Note: avoid bright light

Supplies:
- 90% acetone (vol:vol)
- Amber glass vials (4 provided)
- Forceps
- Glass, 5 ml volumetric Class A pipette (provided)
- Freezer (-10 to -20°C)
- Filtration cartridges and syringes (2 provided)
- Vortex genie

Appendix 3 (cont.)

Appendix 3.2 SIMBIOS/HyCODE chlorophyll *a* round robin

Turner fluorometer Extraction Procedure for Surrogate Sample Filters

1. Keep track of filter numbers.
2. Using forceps, place folded filter in 22 ml amber vial (provided). Note filter number on vial.
3. Add 10 ml 90% acetone (using 10 ml, Class A volumetric pipette provided to you).
4. Make certain filter is submerged and cap tightly.
5. Vortex vigorously for 15 seconds. Make certain filter is still submerged.
6. Place tubes in freezer (-10 to -20°C) for three hours (note time placed in freezer).
7. Remove from freezer (note time). Check cap tightness. Vortex each vial vigorously for 15 s.
8. Attach filter cartridge to end of syringe.
9. Decant filter and extract into syringe and force extract through filter with plunger.
11. Collect filtrate in empty 22 ml amber glass vial (provided). Cap tightly. Vortex well.
12. Read on calibrated Turner fluorometer

Note: avoid bright light

Supplies:
- 90% acetone (vol:vol)
- Amber glass vials (4 provided)
- Forceps
- Glass, 10 ml volumetric Class A pipette (provided)
- Freezer (-10 to -20°C)
- Vortex genie
- Filtration cartridges and syringes (2 provided)

Appendix 4

HPLC Unknowns Analyzed at HPL

All HPLC unknowns were analyzed by HPLC at HPL before shipping to participants. The measured concentration was compared to the formulated concentration and %D was computed (see Section 3.4 for terms). Details of these analyses are provided in the following tables, where n = the number of replicate analyses of that unknown at HPL for that shipment date, the measured value represents the mean of those observations and the %RSD is from the replicate analyses for that unknown in a particular shipment.

HPLC Unknown #1

Lab receiving shipment	Date shipped	Formulated Concentration µg/l	Measured Concentration ($\bar{x}$)µg/l	% RSD	n	% D
A	3/15/00	117.5	116.2 ± 1.9233	1.66%	2	-1.11%
6	3/29/00	117.5	116.6 ± 0.6465	0.56%	2	-0.77%
B	4/12/00	117.5	119.5		1	1.70%
5	5/3/00	117.5	115.2 ± 1.9964	1.73%	2	-1.96%
self	5/18/00	117.5	116.2 ± 0.3675	0.32%	3	-1.11%
4, 7	5/31/00	118.8	115.3 ± 0.5689	0.49%	3	-2.95%
D	9/18/00	119.8	121.7 ± 0.5913	0.49%	3	1.59%
8	10/18/00	119.8	119.4 ± 0.5149	0.43%	3	-0.33%

HPLC Unknown #3

A	3/15/00	409.2	397.6 ± 1.8036	0.45%	2	-2.83%
6	3/29/00	409.2	400.6		1	-2.10%
B	4/12/00	409.2	392.9		1	-3.98%
5	5/3/00	409.2	396.2		1	-3.18%
self	5/18/00	409.2	408.5 ± 1.2183	0.30%	2	-0.17%
4, 7	5/31/00	409.2	403.5 ± 0.9989	0.25%	2	-1.39%
D	9/18/00	349.7	345.4 ± 0.5566	0.16%	2	-1.23%
8	10/18/00	349.7	343.1		1	-1.89%

Appendix 5

Fluorometer Unknowns Analyzed at HPL

All fluorometer unknowns were analyzed fluorometrically at HPL before shipping to participants. The measured concentration was compared to the formulated concentration and %D was computed (see section 3.4 for terms). Details of these analyses are provided in the following tables, where n = the number of replicate analyses of that unknown at HPL for that shipment date, the measured value represents the mean of those observations and the %RSD is from the replicate analyses for that unknown in a particular shipment.

Fluorometer Unknown #1

Lab receiving shipment	Date shipped	Formulated Concentration μg/l	Measured Concentration ($\bar{x}$)μg/l	% RSD	% D	n	instrument used
A	3/15/00	117.5	118.7 ± 0.440	0.37%	1.01%	3	10-au-005
6	3/29/00	117.5	116.3 ± 0.437	0.38%	-1.06%	3	10-au-005
C	4/12/00	117.5	116.5 ± 1.030	0.88%	-0.88%	2	10-au-005
3	4/26/00	117.5	113.8 ± 0.000	0.00%	-3.12%	2	10-au-005
5	5/3/00	117.5	114.1 ± 1.566	1.37%	-2.92%	2	10-au-005
8, self	5/18/00	117.5	119.5 ± 0.245	0.21%	1.68%	3	TD-700
2, 4	5/31/00	118.8	119.3 ± 0.9808	0.82%	0.45%	2	10-au-005
D	6/21/00	117.9	115.9		-1.68%	1	TD-700

Fluorometer Unknown #2

A	3/15/00	7.050	7.136 ± 0.1589	2.23%	1.22%	3	10-au-005
6	3/29/00	7.050	6.784 ± 0.1903	2.81%	-3.78%	3	10-au-005
C	4/12/00	7.050	6.995 ± 0.0721	1.03%	-0.78%	2	10-au-005
3	4/26/00	7.050	7.249 ± 0.1273	1.76%	2.82%	2	10-au-005
5	5/3/00	7.050	7.132 ± 0.0834	1.17%	1.17%	2	10-au-005
8, self	5/18/00	7.050	6.794 ± 0.1517	2.23%	-3.64%	2	TD-700
2, 4	5/31/00	6.905	6.985 ± 0.1075	1.54%	1.15%	2	10-au-005
D	6/21/00	6.875	5.941 ± 0.0219	0.37%	-13.59%	2	TD-700

Appendix 5 (cont.)

Fluorometer Unknowns Analyzed at HPL

Fluorometer Unknown #3

Lab receiving shipment	Date shipped	Formulated Concentration μg/l	Measured Concentration ($\bar{x}$)μg/l	% RSD	% D	n	instrument used
A	3/15/00	102.3	97.97 ± 1.6172	1.65%	-4.22%	2	10-au-005
6	3/29/00	102.3	95.70 ± 0.5346	0.56%	-6.44%	2	10-au-005
C	4/12/00	102.9	99.36 ± 6.6906	6.73%	-3.43%	2	10-au-005
3	4/26/00	102.9	102.2 ± 1.5889	1.55%	-0.64%	2	10-au-005
5	5/3/00	102.9	101.5 ± 0.5226	0.52%	-1.33%	2	10-au-005
8, self	5/18/00	101.9	107.7 ± 0.3439	0.32%	5.74%	2	TD-700
2, 4	5/31/00	107.9	101.3 ± 0.9808	0.97%	-6.16%	2	10-au-005
D	6/21/00	107.9	101.6 ± 0.2121	0.21%	-5.83%	2	TD-700

Appendix 6

Chlorophyll *a* calibration standards analyzed at HPL

Linear regression data is given for all chl *a* calibration standards prepared and analyzed at HPL. Those standards sent to participants are indicated by "date sent" and the Laboratory receiving that set of standards is indicated under the column heading "used for". If a date sent does not appear, that set of calibration standards was used for the analysis of field samples (at HPL) for the Laboratory appearing in the column "used for". Date made indicates the date the calibration standards were prepared.

Data for HPLC chl *a* calibration standards

Date made	Date analyzed	Date sent	Used for	Slope	*y* intercept	r^2
3/13/00	3/13/00	3/15/00	A	3.442	-0.4802	0.9999
3/27/00	3/27/00	3/29/00	6	3.424	-0.9687	0.9999
4/10/00	4/10/00	4/12/00	B	3.440	-2.227	0.9999
4/27/00	4/27/00	5/3/00	5	3.532	-1.141	0.9999
5/15/00	5/15/00	5/18/00	self	3.374	0.5708	0.9999
5/25/00	5/26/00	5/31/00	4, 7	3.424	-0.3746	0.9999
7/6/00	7/7/00		1, 5	3.472	0.8650	0.9993
7/24/00	7/25/00		D	3.461	0.7248	0.9998
7/27/00	7/27/00		D	3.454	-0.3434	0.9996
8/1/00	8/3/00		4	3.498	-0.4571	0.9999
9/14/00	9/14/00	9/18/00	D	3.428	-1.005	0.9999
9/14/00	9/20/00		6	3.471	-1.597	0.9999
10/12/00	10/13/00	10/18/00	7, 8	3.441	-0.4241	0.9999
1/12/01	1/12/01		8	3.316	0.2267	0.9999

Appendix 7

Quality Assurance assessment of chl *a* calibration standards at HPL

All sets of calibration standards prepared for participants were analyzed at HPL (before shipping) on the instrument for which they were prepared. The resulting calibration curves were then used to measure the concentration of each of the individual calibration standards (or calibration levels) from which it was derived. Values of %D were determined for each calibration level to determine the accuracy with which the calibration curve was likely to measure chl *a* of varying concentrations. See section 3.4 for definition of mean |%D|. "Lab" refers to the laboratory receiving that set of calibration standards, "ng" refers to the ng of chl *a* injected for that calibration level (for HPLC) and μg/l refers to the concentration of that calibration level for fluorometer standards.

Values of %D for HPLC chl *a* calibration standards

	Level 6		Level 5		Level 4		Level 3		Level 2		Level 1		
Lab	ng	%D	ng	%D	ng	%D	ng	%D	ng	%D	ng	%D	Mean |%D|
A			4	-2%	8	-2%	51	1%	84	-1%	160	0.3%	1%
6			4	1%	8	-2%	48	0.3%	92	-0.4%	169	-0.4%	1%
B			4	4%	8	0.3%	48	-0.3%	91	-0.3%	167	0.1%	1%
5			3	4%	6	1%	35	-0.3%	66	3%	121	0.2%	2%
self			3	-9%	6	-6%	35	1%	67	1%	123	-0.4%	3%
4, 7			3	-2%	6	-2%	35	<0.1%	67	0.5%	123	-0.1%	1%
D	4	2%	11	0.3%	27	0.5%	52	-0.1%	99	-0.4%	182	0.1%	1%
7, 8			4	<0.1%	7	-1%	27	0.3%	76	<0.1%	143	<0.1%	0.3%

Appendix 7 (cont.)

Quality Assurance assessment of chl *a* calibration standards at HPL

Values of %D for fluorometer chl *a* calibration standards

	Level 7		Level 6		Level 5		Level 4		Level 3		Level 2		Level 1		Mean
Lab	µg/l	%D	µg/l	%D	µg/l	%D	µg/l	%D	µg/l	%D	µg/l	%D	µg/l	%D	|%D|
A			0.7	60%	3	3%	14	1%	35	1%	71	-3%	117	0.5%	11%
6	0.4	-6%	2	-8%	8	-1%	20	<0.1%	40	0.2%	67	0.3%	216	-0.1%	2%
C	0.4	17%	2	22%	8	3%	20	2%	40	1%	67	-3%	213	0.3%	7%
3	0.4	18%	2	-4%	8	0%	20	3%	41	-1%	68	-0.4%	218	0.1%	4%
5	0.5	10%	2	-16%	10	-3%	24	0.5%	48	9%	77	-3%	231	0.2%	6%
8, self			2	-10%	10	-5%	25	-0.2%	49	1%	74	1%	197	-0.1%	3%
2, 4			2	5%	10	1%	25	2%	49	-1%	74	-0.3%	197	0.1%	2%
D			2	-15%	10	2%	25	0.3%	49	1%	74	1%	196	0.2%	2%
8			3	-12%	15	-2%	36	-1%	73	2%	109	<0.1%	219	-0.2%	3%

Appendix 8

Quality Assurance at Horn Point Laboratory (to evaluate bias)

Sources of uncertainty likely to cause bias

Factors likely to perpetuate bias in our data were spectrophotometer absorbance and wavelength inaccuracy, effects of long term storage on standards and unknowns and inaccuracy of diluting devices used when preparing standards and unknowns.

Spectrophotometric accuracy

We validated absorbance and wavelength accuracies in spectrophotometric measurements. NIST traceable neutral density filters (Starna Cells, Inc. RM-N1N35N, RM-1N2N3N) (Latasa et al. 1999) were used to evaluate absorbance accuracy between 0.303 and 1.032 at wavelengths of 440, 465, 546.1, 590 and 635 nm. The observed absorbance values varied by $\leq$ 0.0042 from values given for these filters. As the optical density (664 nm) of our standards ranged from 0.4 to 0.8, our absorbance inaccuracies were not likely to be >1%. Wavelength accuracy was assessed by scanning for λ_{max} of a chl *a* solution in 90% acetone and was found to be within 1 nm of the published λ_{max} (664.3 nm, Jeffrey and Humphrey 1975).

Effects of long term storage on standards

All standards evaluated for changes during long-term storage were held in freezers (-15°C or -25°C), in PTFE bottles or amber glass vials, shielded from the light and opened periodically for use. These standards were evaluated either by spectrophotometer or HPLC. When determining whether the effects of long term storage were statistically significant, we calculated linear regressions of the parameter being evaluated versus analysis date and determined if the slope was significantly different from zero.

Three primary chl *a* stock solutions were evaluated spectrophotometrically for concentration at intervals during their storage (total storage time = 60 to 123 days). These standards ranged in approximate concentration from 5 to 8 μg/ml and with two of them, there was a significant increase in concentration over time ($0.02<p<0.04$). The standard with the greatest daily change in concentration was 0.0023 μg/ml per day, as predicated from the linear regression. As we determined the concentration of the primary stock chl *a* solutions within 3 days of using them to prepare calibration standards and unknowns for participants, we anticipated that this level of change was likely to not affect the concentration of the standards by more than +0.15%.

Three solutions containing either chl *a* or DV chl *a* (in 90% acetone) were held for durations $\geq$63 and $\leq$286 days and were evaluated for changes in total peak area by HPLC. When calculating regressions of peak area versus analysis date, the slopes were not significantly different from zero ($p>0.4$). With the standard held for the longest duration (286 days) there was

a significant ($p<0.001$) increase in the proportion of allomers and epimers relative to the total peak area, but this change was small (2.2%).

Mix 82 was analyzed by HPLC 20 times over the time interval in which it was distributed to participants (184 days). The pigments in this mixture used for quantitative analysis of the pigments in the sample surrogate filters were monitored for change in concentration. These pigments included chl *c*2, but-fuco, fuco, diadino and β,β-car. The linear regressions indicated an increase in concentration for chl *c*2 and diadino which were statistically significant. However, with both pigments, the magnitude of the increase in concentration was <5%.

Accuracy of diluting devices

Glass volumetric pipettes (Class A, 5 or 10 ml capacities, total n = 22) and two glass syringes (500 and 100 μl, Hamilton 81230, 81030) were used for diluting unknowns and calibration standards from the primary pigment solutions. These diluting devices were calibrated gravimetrically with water before use. The glass pipettes were found to deviate by no more than 0.7% from the volume specified and the glass syringes by no more than 0.1%. The glass syringes were also calibrated with 100% acetone (where the weight was corrected for the specific gravity of acetone to determine the volume delivered) and they were found to deliver within 0.7% (500 μl syringe) and 0.9% (100 μl syringe) of the volume specified.

Appendix 9

Quality Assurance at Horn Point Laboratory (to evaluate precision)

Sources of uncertainty likely to affect precision

We discuss three primary sources of imprecision in our analyses. These include the precision associated with the diluting devices used for preparing unknowns and calibration standards and the repeatability and reproducibility associated with instrumental analyses. We also assessed the precision with which each of the 8 sets of HPLC calibration standards sent to participants measured the concentration of the same test standard. (Unfortunately we were not able to do this with the fluorometers). We define the warning limits and control limits within which variability contributed by these factors are likely to be contained.

Imprecision of dilution devices

Glass syringes and several volumetric pipettes (the same as in Appendix 8) were assessed for precision. The WL and CL for precision in replicate gravimetric measurements of water with the glass pipettes were ±0.13% and ±0.22%, respectively and with the 500 μl glass syringes, with acetone, these limits were ±0.37% and ±0.74% and with the 100 μl syringe they were ±0.87 and ±1.76%, respectively.

Instrument variability

Variability in instrumentation is referred to in terms of repeatability and reproducibility. Repeatability measures variability occurring over a short duration (typically one day) and may vary from reproducibility, that which occurs over longer durations (typically multiple days). Typically, the same calibration factors are used throughout a day and the frequency of re-calibrations are based on an understanding of the instruments reproducibility. We assessed repeatability and reproducibility of the fluorometers and HPLC, exclusive from re-calibrations.

With fluorometers, we define repeatability as that which occurs on the same day (typically within a 10 hr period) and with the HPLC, as that which occurs during the same sequence, where a sequence refers to a series of analyses conducted in an automated fashion, which (in our case) may collectively require up to 32 hr for completion. We define fluorometer reproducibility as that which occurs over days or months, but when no lamp changes or changes in sensitivity settings have been made (factors which would necessarily require that re-calibration be performed). We define HPLC reproducibility as that which occurs over multiple sequences, and may be affected by changes in columns (which vary in lot number or serial number only).

HPLC instrument variability. Repeatability was assessed by injecting sample extracts or standards multiple times on the same sequence. These analyses were distributed among 26 different sequences where vials had resided in the temperature controlled auto-sampler compartment (TCAS) for random durations of time which were typical of our routine practices

Appendix 9 (cont.)

Quality Assurance at Horn Point Laboratory (to evaluate precision)

(from 1 hr to approximately 24 hr). For each sequence we calculated the %RSD associated with the total chl *a* peak area (including allomers and epimers) for the replicate injections ($2 \leq n \leq 7$) of each different sample or standard (n=42). The limits for expected repeatability (about the mean total peak area) were ±1.47% (WL) and ±2.33% (CL).

HPLC reproducibility was assessed by analyzing the same standard on 8 different sequences and determining the WL and CL from the relative standard deviation (%RSD) associated with chl *a* total peak area among all sequences. These sequences were conducted over a period of 213 days and during which time 6 different HPLC columns were used. Furthermore, it had been previously demonstrated that this standard exhibited no significant changes in concentration during storage over the duration in which these sequences were conducted. The limits for expected reproducibility about the mean total peak area were ±2.53% (WL) and ±5.07% (CL).

Fluorometer instrument variability. The repeatability of the Turner Designs model 10-AU-005-CE fluorometers were assessed primarily in two ways: with the red solid standard that Turner Designs sells and with chl *a* quality control (QC) standards prepared at HPL. The solid standard was analyzed three times at each of its two settings (low and high) at the beginning of each day's analysis and at regular intervals during the day (approximately every 10th sample reading). The chl *a* QC standards were analyzed at least two times on each day. The %RSD was calculated for each days replicate analyses of the solid standards and chl *a* QC standards and from this, the limits for both the low and high readings of the solid standard were found to be approximately ±0.7 (WL) and ±1.1% (CL) (n=20 days). For chl *a* QC standards ranging in concentration from 22 to 117 μg/l, the WL and CL were ±1.0 and ±1.7%, respectively (n=19 days). (The performance of each 10-AU-005-CE fluorometer used were similar, so we report the results from one only).

It is typical for fluorescence output to diminish as the lamp ages and this constitutes one of the reasons for periodic instrument re-calibration. We quantified the magnitude of this drift on one of our 10-AU-005-CE fluorometers by regressing the fluorescence reading of the solid standard (both the low and high settings) against analysis date over a time period of 166 days. These regressions indicated a significant ($0.001<p<0.002$) average decrease in fluorescence output (0.019% per day). From this average rate of change in fluorescent output per day, this instrument would have exhibited solid standard fluorescence readings outside the CL (±1.1%) for same-day repeatability at approximately 60 days and outside the WL (±0.7%) at 36 days. These data indicate that for this instrument, under the conditions of use at HPL, calibrations should be performed at intervals of less than 36 days to remain within the WL for repeatability. We state these results here to emphasize the utility of using solid standards for monitoring instrument variability, however, we conducted fluorometer re-calibrations (with no changes to sensitivity settings) every time an instrument was used.

Appendix 9 (cont.)

Quality Assurance at Horn Point Laboratory (to evaluate precision) (cont.)

Variation in calibration factors

We evaluated uncertainties in results predicted from the various HPLC calibration curves by analyzing the same test standard on different sequences, during which a unique set of chl *a* calibration standards was also analyzed (those sent to participants) and whose resulting calibration curve was used to quantify chl *a* in the test standard. In each case, the measured value for the test standard was compared to the formulated value and %D was calculated. From these observations (n=8) we determined that each calibration curve would likely measure the concentration of this standard within ±2.37% (WL) and ±4.69% (CL) of its formulated concentration.

Appendix 10

Participants' Fluorometer Unknown Results

Participants' results of fluorometer unknown analyses. The measured concentration was compared to the formulated concentration (as determined at HPL) and %D was computed. Details of these analyses are provided in the following tables, where n = the number of replicate analyses of that unknown performed by the participating laboratory, the measured value represents the mean of those observations and the %RSD is from the replicate analyses for that unknown.

Participants' Fluorometer Unknown #1

Lab code	Formulated μg/l	Measured ($\bar{x}$) μg/l	% RSD	% D	n
A	117.5	117.4 ± 1.599	1.36%	-0.07%	5
6	117.5	117.8 ± 0.62	0.53%	0.29%	3
5	117.5	117.2 ± 0.760	0.65%	-0.29%	3
3	117.5	117.9 ± 3.274	2.78%	0.32%	4
C	117.5	117.0 ± 1.640	1.40%	-0.44%	3
8	117.5	119.2 ± 0.871	0.73%	1.44%	3
D	117.9	121.8 ± 4.684	3.85%	3.30%	2
4	118.8	113.7 ± 2.398	2.11%	-4.24%	3
2	118.8	132.2		11.32%	1
self	117.5	119.3 ± 0.406	0.34%	1.56%	3

Appendix 10 (cont.)

Participants' Fluorometer Unknown Results

Participants' Fluorometer Unknown #2

Lab code	Formulated μg/l	Measured ($\bar{x}$) μg/l	%RSD	% D	n
A	7.050	6.750 ± 0.384	5.69%	-4.25%	5
6	7.050	7.357 ± 0.214	2.90%	4.35%	3
5	7.050	6.763 ± 0.000	0.00%	-4.07%	3
3	7.050	7.098 ± 0.021	0.29%	0.68%	4
C	7.050	7.324 ± 0.124	1.69%	3.89%	3
8	7.050	7.307 ± 0.146	1.99%	3.64%	3
D	6.875	6.371 ± 0.321	5.04%	-7.33%	2
4	6.905	6.732 ± 0.038	0.57%	-2.51%	3
2	6.905	7.751 ± 0.043	0.56%	12.24%	2
self	7.050	7.216 ± 0.050	0.70%	2.35%	3

Participants' Fluorometer Unknown #3

Lab code	Formulated μg/l	Measured μg/l	%RSD	%D	n
A	102.3	$\bar{x}$ = 141.6 ± 1.484	1.05%	37.67%	5
6	102.3	$\bar{x}$ = 93.84 ± 1.322	1.41%	-8.26%	2
5	102.9	$\bar{x}$ = 104.6 ± 0.745	0.71%	1.69%	2
3	102.9	$\bar{x}$ = 93.04 ± 15.790	16.97%	-9.56%	2
C	102.9	$\bar{x}$ = 117.9 ± 0.759	0.64%	14.57%	2
8	101.9	$\bar{x}$ = 106.2 ± 0.403	0.38%	4.24%	2
D	107.9	104.8		-2.91%	1
4	107.9	$\bar{x}$ = 113.4 ± 0.00	0.00%	5.10%	2
2	107.9	116.2		7.65%	1
self	101.9	$\bar{x}$ = 102.3 ± 1.491	1.46%	0.45%	2

Appendix 11

Participants' Fluorometer Surrogate Filter Results

Participants analyzed at least 2 replicate filters of an algal culture with a simple pigment signature. HPL represents all filters that were analyzed at HPL during the course of time over which samples were shipped to participants. 'Self' is also HPL, after we sent samples to ourselves to mimic being a participant. Mean consensus = 1177.7 ug/l chl *a* and includes all but self and Laboratory A.

Lab code	n	mean ug/l chl a	% RSD	$\bar{x}$ %D from mean consensus
HPL	10	1124.5 ± 39.0	3.47%	-4.5%
2	2	1186.3 ± 30.5	2.57%	0.7%
3	2	1192.1 ± 7.6	0.64%	1.2%
4	2	1160.3 ± 27.3	2.36%	-1.5%
5	2	1201.4 ± 27.2	2.27%	2.0%
6	4	1174.7 ± 22.1	1.88%	-0.3%
8	2	1201.5 ± 24.5	2.04%	2.0%
A	2	1394.8 ± 17.5	1.26%	18%
self	2	1146.9 ± 7.6	0.66%	-2.6%

Appendix 12

Participants' HPLC calibration curve results

Participants' results of analysis of HPLC calibration standards. Level refers to calibration level, ng refers to ng chl *a* per injection for the specified calibration level and %D refers to % difference from formulated concentrations. Laboratory 4 used two detectors and therefore two calibration curves were determined (PDA = photodiode array detector, FLD = fluorescence detector). (See Section 3.4 for additional definitions.)

	Level 6		Level 5		Level 4		Level 3		Level 2		Level 1		
Lab code	ng	%D	ng	%D	ng	%D	ng	%D	ng	%D	ng	%D	Mean |%D|
A			3	-49%	5	18%	34	-5%	56	6%	107	-1%	16%
6			3	8%	5	2%	32	-0.5%	61	-0.7%	112	0.3%	2%
B			3	12%	5	2%	32	-0.7%	60	-0.9%	111	0.3%	3%
5			7	2%	14	1%	82	-2%	157	1%	288	-0.2%	1%
4 (PDA)			2	53%	4	39%	23	-19%	45	3%	82	0.5%	23%
4 (FLD)			2	65%	4	41%	23	-18%	45	1%	82	1%	25%
D	3	9%	7	1%	18	-0.1%	35	-0.1%	66	-1%	121	0.3%	2%
7			2	7%	3	5%	18	-0.7%	34	-1%	62	0.4%	3%
8			10	36%	18	63%	71	17%	203	4%	380	-2%	24%

Appendix 13

Participants' HPLC Unknown #1 Results

Lab code	Approximate ng chl *a* injected	Measured μg/l	%RSD	% D	n
A	12	$\bar{x}$=148.5 ± 20.63	13.89%	26.38%	3
B	24, 35	$\bar{x}$=114.8 ± 1.414	1.23%	-2.30%	2
5	40	$\bar{x}$=112.5 ± 0.308	0.27%	-4.26%	3
4 (PDA)	24	$\bar{x}$=145.5 ± 4.921	3.38%	22.5%	3
4 (FLD)	24	$\bar{x}$=145.3 ± 5.898	4.06%	22.3%	3
7	9	$\bar{x}$=118.1 ± 0.856	0.72%	-0.59%	3
D	12	$\bar{x}$=121.8 ± 0.346	0.28%	1.70%	2
8	48	$\bar{x}$=106.7 ± 19.83	18.58%	-10.6%	3
self	17	$\bar{x}$=116.8 ± 0.956	0.82%	-0.56%	3

Appendix 14

Sample Surrogate Filters Analyzed by HPLC for chl *a*

Results of sample surrogate filters analyzed by HPLC for chl *a* content. Percent difference is calculated as the difference from the mean consensus. All filters for HPL were analyzed over the duration of time in which samples were shipped to participants. All laboratories but self were included in the mean consensus calculation. Mean consensus value = 1048 ng chl a per filter.

Lab code	Approximate ng chl *a* injected	Mean ± *s* ng chl *a* per filter	n	% RSD	% D
4 (FLD)	50	1244 ± 43	2	3.5%	16.0%
4 (PDA)	50	1277 ± 42	2	3.3%	19.1%
5	70	1002 ± 25	2	2.5%	-4.5%
6	42	1030 ± 19	4	1.9%	-1.8%
7	15	1047 ± 14	2	1.3%	-0.16%
8	67	864 ± 52	2	6.1%	-18%
A	21	1056 ± 67	2	6.3%	0.72%
D	21	1059 ± 6	2	0.6%	1.0%
Self		1065 ± 27	2	2.6%	1.5%
HPL	31	1070 ± 25	9	2.3%	2.1%

Appendix 15

Sample Surrogate Filters Analyzed by HPLC for Accessory Pigment Content

Quantitative HPLC analysis of accessory pigments in surrogate sample filters by participants. Mean ± s for each pigment is shown. Concentrations are expressed as ng pigment per filter.

Laboratory performing analysis	n	ng chl *c*2	ng but-fuco	ng fuco	ng diadino	ng β, β-car
HPL	9	318 ± 7	95 ± 3	761 ± 17	101 ± 3	20 ± 1
4	2	349 ± 26	98 ± 1	784 ± 19	95 ± 1	not reported
6	4	278 ± 6	93 ± 2	766 ± 13	96 ± 4	30 ± 2
7	2	377 ± 4	101 ± 3	764 ± 14	97 ± 5	not reported
8	2	not reported	69 ± 8	481 ± 21	59 ± 0.9	85 ± 35
A	2	336 ± 5	89 ± 0.4	678 ± 0.4	108 ± 4	40 ± 2
D	2	537 ± 266	99 ± 0.6	753 ± 6	95 ± 0.55	22 ± 2
Self	2	325 ± 1	91 ± 2	778 ± 8	105 ± 1	20 ± 1

Appendix 16

Participants' HPLC Unknown #3 Results

Results of participants' analysis of HPLC Unknown #3, containing chl *a* and DV chl *a*. All concentrations are expressed as total concentration (chl *a* + DV chl *a*).

Lab code	Formulated μg/l	Measured μg/l	% D
A	409.2	685.1	67.5%
6	409.2	421.0	2.88%
B	409.2	418.8	2.35%
5	409.2	471.5	15.2%
4 (FLD)	409.2	589.0	43.9%
4 (PDA)	409.2	829.8	103%
7	409.2	424.1	3.64%
D	349.7	386.3	10.5%
8	349.7	329.3	-5.83%
Self	409.2	394.5	-3.47%

Appendix 17

Spectrophotometer results

Instructions for Determining Concentration of Primary Chl *a* Standard on **Spectrophotometer**

Your Name: Date of Analysis:

Model of Spectrophotometer: Type: please check which applies:

diode array: _____

monochromator: _____

dual beam: _____

single beam: _____

1. Turn on spectrophotometer and lamp and allow to warm up for ~ 1/2 hour.

2. Set wavelength to 664. Can bandwidth be adjusted? If yes, set to 2 nm (**very important**).

Yes: _____

No: Bandwidth is fixed at: nm

3. Remove primary chl *a* standard from freezer and allow to come to room temperature.

4. Use clean, dry, quartz or glass cuvettes with a 1 cm pathlength. Cuvettes with a 3 ml capacity are preferred to limited volume cuvettes.

5. If using a dual beam spec, dedicate one cuvette to the reference and one to the sample. Regardless of type of spec, always place cuvettes in same orientation with regard to light source.

6. Place 90% acetone (provided) in reference and sample cuvettes (sample only for single beam specs). Inspect sides for smears or spilled acetone.

7. Place in spec and, with wavelength set to 664 nm, set absorbance to zero. Record all absorbance readings in table # 1 on next page.

8. Without removing cuvette(s), change wavelength to 750 and record absorbance.

9. Change wavelength back to 664. Remove sample cuvette and discard contents. Shake out excess acetone.

10. Place ~ 1 ml of primary chl *a* standard in cuvette. Discard.

Appendix 17 (cont.)

11. Re-fill cuvette with primary chl *a* standard, inspect sides for spillage and place in sample holder. Record absorbance at 664. Change to 750 nm and record absorbance. Discard contents.

12. Repeat step # 11.

13. Repeat step # 11 again, but after recording absorbance values, leave chl *a* in cuvette in spec and check wavelength accuracy according to instructions on next page.

Table 1. Raw data from spectrophotometer

Sample	Absorbance 664 nm	Absorbance 750 nm
90% acetone		
primary chl a standard (observation 1)		
primary chl a standard (observation 2)		
primary chl a standard (observation 3)		

Checking wavelength accuracy of your spectrophotometer

The published absorbance maximum for chl *a* in 90% acetone is 664 nm. If your spectrophotometer can do wavelength scanning, scan from 660 nm to 670 nm and identify the wavelength yielding maximum absorbance. If it does not do wavelength scans, read and record absorbance at 660, 662, 664, 666 and 668 nm. Use a 1 or 2 nm bandwidth if your spec allows.

Absorbance				
660	662	664	666	668

Wavelength of maximum absorbance:

Comments:

Appendix 18

Spectrophotometer Unknown Results

Results of the spectrophotometer unknown chl *a* standard analyzed at HPL prior to shipping to participants. The unknown was analyzed in triplicate each time it was shipped.

Laboratory receiving shipment	Date shipped	Mean concentration µg/ml	Standard deviation	% RSD
2, 4, 7	5/31/00	6.9053	0.0033	0.05%
8, Self	5/18/00	6.8479	0.0022	0.03%
6	3/29/00	6.7461	0.0076	0.11%
B	4/12/00	6.8073	0.0064	0.09%
C	4/12/00	6.8073	0.0064	0.09%
3	4/26/00	6.8073	0.0064	0.09%
5	5/3/00	6.8073	0.0064	0.09%
D	9/18/00	7.2853	0.0023	0.03%

Results of the spectrophotometer unknown chl *a* standard analyzed by participants. The mean concentration (n=3 analyses, for Lab 5 n=2 analyses), the standard deviation, % relative standard deviation and %D relative to the HPL concentration are shown.

Laboratory reporting results	Mean concentration (µg/ml)	Standard deviation	%RSD	%D
7	6.909	0.0263	0.38%	0.05%
C	6.713	0.1515	2.26%	1.27%
4	6.836	0.0461	0.67%	-1.00%
2	6.975	0.0082	0.12%	1.01%
5	6.891	0.0042	0.06%	1.23%
D	7.391	0.0000	0.00%	1.46%
6	6.875	0.0350	0.51%	1.90%
8	7.067	0.0654	0.92%	3.21%
3	6.585	0.0066	0.10%	-3.26%
B	6.160	0.0114	0.19%	-7.08%
Self	6.802	0.0020	0.03%	-0.68%

Appendix 19

Participants' chl *a* QC Standard

The % difference that the mean values for chl *a* standards from participants' laboratories were from their formulated concentrations when quantified using chl *a* calibration standards distributed by HPL. Three different standards were analyzed by fluorometer by Laboratory 8.

Lab code	Fluorometer %D	HPLC %D
2	-0.8%	not done
3	-2.5%	not done
4	-4.8%	6.2% (PDA)
4		1.8% (FLD)
5	8.4%	not done
6	-4.5%	-4.3%
8	-0.5%, 6.1%, 9.5%	18.2%
B	not done	-2.4%
D	0.19	-0.9%

Appendix 20

Manufacturer's List

Agilent Technologies, Inc.
(formerly Hewlett Packard)
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

Amersham Pharmacia Biotech, Inc.
800 Centennial Avenue
P.O. Box 1327
Piscataway, NJ 08855 USA
Voice: 732-457-8000
Fax: 732-457-0557
Net: http://www..apbiotech.com

Barnstead/Thermolyne
2555 Kerper Boulevard
Dubuque, Iowa 52001 USA
Voice: 800-446-6060
Fax: 319-589-0516
Net: http://www.barnsteadthermolyne.com

Beckman Coulter, Inc.
4300 North Harbor Boulevard
P.O. Box 3100
Fullterton, CA 92834 USA
Voice: 800-742-2345
Fax: 800-643-4366
Net: http://www.beckmancoulter.com

Brinkmann Instruments, Inc. (merged with Eppendorf Scientific)
One Cantiague Road
P.O. Box 1019
Westbury, NY 11590 USA

Voice: 800-645-3050
Fax: 516-334-7506
Net: http://www.brinkmann.com

Camspec
11, High Street
Sawston
Cambridge
CB2 4BG UK
Voice: 44 (0) 1223 836971
Fax: 44 (0) 1223 836414
Net: http://www.camspec.co.uk

DHI Water and Environmental Institute
(formerly VKI Water Quality Institute)
Agern Allé11,
DK-2970 Hørsholm
Denmark
Voice: 45-4516-9200
Fax: 45-4516-9292
Net: http://www.c14.dhi.dk

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

Hamiliton Company
P.O. Box 10030
Reno, NV 89502 USA
Voice: 800-648-5950
Fax: 775-856-7259

Net: http://www.hamiltoncomp.com

Hitachi Instruments, Inc.
3100 North First Street

San Jose, CA 95134 USA
Voice: 800-548-9001
Fax: 408-432-8258
Net: http://www.hii-hitachi.com

Light Impressions
439 Monroe Avenue, P. O. Box 940
Rochester, NY 14604-0940 USA
Voice: 800-828-6216
Fax: 800-828-5539
Net: http://www.lightimpressionsdirect.com/

Nalge Nunc International
2000 North Aurora Road
Naperville, IL 60563 USA
Voice: 630-983-5700
Fax: 630-416-2519
Net: http://www.nalgenunc.com

Perkin Elmer
761 Main Avenue
Norwalk, CT 06859
Voice: 203-762-4000
Fax: 203-762-4228
Net: http://www.perkin-elmer.com

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

Rainin Instrument Company, Inc.
Rainin Road, Box 4026
Woburn, MA, 01888 USA
Voice: 781-935-3050
Fax: 781-938-1152.
Net: http://www.rainin.com

Scientific Resources, Inc.
1 Industrial Way
Bldg. E, Unit G
Eatontown, NJ 07724 USA
Voice: 800-637-7948
Fax: 770-476-4571

Net: http://www.sciresources.com/sri/

Shimadzu Scientific Instruments
7102 Riverwood Drive
Columbia, MD 21046 USA
Voice: 800-477-1227
Fax: 410-381-1222
Net: http://www.shimadzu.com

Sigma-Aldrich Company
3050 Spruce Street
St. Louis, MI 63103 USA
Voice: 314-771-5765
Fax: 314-771-5757
Net: http://www.sigma-aldrich.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

Supelco
Supelco Park
Bellefonte, PA 16823 USA
Voice: 800-247-6628
Fax: 800-447-3044
Net: http://www.sigma-aldrich.com

Thermo Spectronic
(formerly Bausch and Lomb)
820 Linden Avenue
Rochester , NY 14625 USA
Voice: 716-248-4000
Fax: 716-248-4200
Net: http://www.thermo.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

Varian Chromatography Systems
2700 Mitchell Drive
Walnut Creek, CA 94598 USA
Voice: 800-367-4752
Fax: 510-945-2360
Net: http://www.varianinc.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

Glossary

Accuracy - "the degree of agreement of a measured value with the true or expected value of the quantity of concern" (Taylor 1987)

Bias - caused by systematic errors in a method, which can be either positive or negative in sign.

Formulated value - the concentration of a pigment in a solution which is prepared by diluting primary stock pigment solution (whose concentration was determined spectrophotometrically); calculated from the concentration of the stock pigment solution

Mean consensus value - the average of all mean values reported by laboratories for a particular measurement

Measured value - the concentration of a pigment in a standard, unknown or sample extract that is predicted from the calibration factors used for its analysis

Precision - "the degree of mutual agreement characteristic of independent measurements where repeated applications of a specified measurement process under investigation are applied" (Taylor 1987)

Repeatability - used when describing variables affecting results from a single laboratory that are short term in nature, as with the precision attained with analyses conducted on the same day

Reproducibility - used when describing factors affecting results from a single laboratory that are long term in nature (as over multiple days and calibrations) and when comparing results among laboratories

s - sample standard deviation

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Results of Simbios Project Round Robin: Part II

Results of Field Samples

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1.0 INTRODUCTION

A field sample inter-calibration exercise was constructed to compliment a concurrent pigment standard inter-calibration. Investigators collected water samples from collection sites they typically frequent and split those samples between themselves and HPL. Although the ability of investigators to accurately measure the concentration of several pigment standards was assessed (Round Robin Report Part 1), it was necessary to also assess discrepancy between HPLC and fluorometer results using natural samples, as natural samples may behave somewhat differently than standards. Laboratories who had participated in Part 1 of the study but were not affiliated with either the SIMBIOS or HyCODE programs did not participate in this phase of the study. One SIMBIOS investigator who had participated in Part 1 was unable to participate in field studies.

There are many variables involved in natural sample collection and analysis, and each step may cause an increase in uncertainty in the analysis of replicate filters. Because of these many steps and the inherent differences in HPLCs and fluorometers, some discrepancy should be expected. We therefore identified at HPL a level of expected discrepancy which we feel is the best attainable given present technology, as conditions used for this assessment were highly standardized and subject to strict quality assurance guidelines. We used this baseline discrepancy for comparisons when investigating the effects of factors known to cause discrepancies and when investigating the effects that participants' procedures had on results which they reported.

2.0 EXPERIMENTAL DESIGN

The objectives of this phase of the inter-calibration exercise were to, 1) collectively investigate factors universally affecting HPLC/ fluorometer discrepancies in the analysis of field samples and 2) identify specific factors that affected an individual laboratory's ability to maintain discrepancies within expected limits. To do this, replicate field samples were collected from participants' usual collection sites; these samples were then analyzed by the participating laboratories and by HPL.

To evaluate causes of discrepancy in field sample analysis, it was first necessary to identify limits within which expected discrepancies were likely to fall. We assessed these limits under conditions likely to minimize discrepancies. For this, we used standardized procedures at HPL with analytical methods that were subjected to strict quality assurance practices. Next we established the typical levels of discrepancies seen when participants implemented their usual procedures. By collectively comparing the results of these two treatments, we identified the magnitude of the additional uncertainties seen when multiple laboratories provide results, relative to the uncertainties seen when analytical procedures are standardized and all results are from one laboratory. Furthermore, as interfering pigments can affect fluorometric results, we analyzed all HPLC extracts for the presence of these pigments, followed by fluorometric analysis of the same extracts, to determine the degree to which they affected discrepancies. When identifying factors contributing specifically to a laboratory's discrepancies, we reviewed their methods, referred to their results in the analysis of laboratory-

prepared samples, and implemented their extraction procedures at HPL when necessary to determine if their extraction procedures induced bias.

3.0 METHODS

Participants collected replicate filters of natural samples from their typical field sites for fluorometric and HPLC analysis. The number of sets of replicate filters collected at each site varied and was dependent on the volume of water required per filter, such that detection would be adequate when they were analyzed. After samples were collected, participants retained one set of replicate filters from each site and sent others to HPL for analysis. Sometimes only one set of replicate filters (for HPLC and fluorometer) were sent to HPL from each site, in which case they were extracted and analyzed at HPL with standardized procedures (Appendix 1). When two sets of replicate filters were sent to HPL, the second set was extracted at HPL with procedures used by participants (Section 4.1.1, Table 4; Section 4.1.2, Table 5) and analyzed with HPL analytical methods. Filters retained by participants were extracted and analyzed with their usual methods, except that chl *a* calibration factors generated from chl *a* standards distributed by HPL were used. All HPLC extracts prepared at HPL were diluted so they could also be analyzed by fluorometer. To ensure accuracy and precision in these dilutions, we used only a 500 µl gas-tight glass syringe and either 5 or 10 ml Class A glass volumetric pipettes which had been tested for accuracy and precision (Round Robin Report Part 1, Appendix 8). A description of all analytical methods can also be found in Round Robin Report Part 1. Here we present the participating laboratories, details with regard to sample collection, storage, date of analysis and data analysis methods.

3.1 Participating laboratories and sample collection

Participants were given instructions for sample collection and a numbering system which uniquely identified each filter with regard to collection site, bottle collection number, whether the filter was collected for HPLC or fluorometric analysis, the filter replicate number and the participants' identity. This documentation was necessary so we could ascertain that appropriate comparisons were made between filters. Only filters considered homogeneous were used as replicates and only filters collected from the same bucket or the same bottle were deemed homogeneous, unless several buckets or bottles were combined and well mixed. Participants reported collection date and time, the latitude and longitude of the collection site, the mode of sampling (bucket or CTD bottle), the filter type and diameter and the volumes filtered. This information is summarized along with the number of filters retained by the participant and the number sent to HPL (Table 1). All laboratories used 25 mm glass fiber filters (GF/F) for fluorometric analyses except Laboratory 3 who used HA membrane filters. All laboratories used GF/F filters for HPLC samples of either 25 or 47 mm diameter.

Table 1. Details of field sampling. The column headings indicate: collection date; collection time and, where known, the time zone (EST = Eastern standard time, GMT = Greenwich mean time, PST = Pacific standard time); the site location in latitude and longitude; the mode of sample collection, CTD cast or bucket (bottle number is shown such that water homogeneity is traceable). Also shown are details of filters collected for fluorometric and HPLC analysis. In all cases, fluorometer filters were 25 mm GF/F unless otherwise noted and all HPLC filters were GF/F (filter diameters in mm are shown). "Kept" indicates the number of filters retained for analysis by the collecting laboratory and "sent to HPL" indicates the number which were sent to HPL. Where cells are empty, information is not known.

						Fluorometer			HPLC			
							# of filters collected				# of filters collected	
Lab Code	Site #	Date (M-D-Y) Time	Site Location (Lat/Long)	CTD or bucket	Bottle #	Volume filtered (ml)	kept	sent to HPL	Filter (mm)	Volume filtered (ml)	kept	sent to HPL
1	1	04-29-00	37°30.04 76°05.14	CTD	4-7[1]	100	3	6	25	200	3	6
	2	05-01-00	38°30.00 78°28.83	CTD	5-8[1]	25	3	6	25	50	3	6
	3	05-02-00	39°21.98 76°08.02	CTD	2-3[2]	50	3	6	25	150	3	6
	4	2-27-01	38°39.12' 76°18.36'	bucket	--	150	3	6	25	200	3	6
	5	2-27-01	38°38.10' 76°09.55'	bucket	--	150	3	6	25	200	3	6
	6	2-27-01	38°40.92' 75°58.20'	bucket	--	50	3	6	25	75	3	6
3	1	06-04-00 10:30 EST	43°47.82 N 66°38.43 W	bucket	--	100	3 HA 3GF/F	3 HA 3GF/F	--	--	--	--
	2	06-04-00 13:30 EST	43°42.295 N 67°48.78 W	bucket	--	100	3 HA 3GF/F	3 HA 3GF/F	--	--	--	--
	3	06-04-00 15:30 EST	43°40.5 N 68°34.956 W	bucket	--	100	3 HA 3GF/F	3 HA 3GF/F	--	--	--	--
4	1	04-04-00 14:00 GMT	26°24.830 N 82°02.968 W	bucket	--	200	3	6	47	200	3	6
	2	04-06-00 12:05 GMT	26°46.460 N 83°23.678 W	CTD	9	1020	1	2	47	1020	1	1
	2	04-06-00 12:05 GMT	26°46.460 N 83°23.678 W	CTD	10	1020	1	3	47	1020	1	3
	2	04-06-00 12:05 GMT	26°46.460 N 83°23.678 W	CTD	11	1020	1	1	47	1020	1	2
	3	04-06-00 23:15 GMT	27°23.349 N 83°07.976 W	bucket	--	1020	3	6	47	1020	3	6
5	1	05-12-00 12:45	31°47.568 64°44.745	CTD	17	500	1	1	47	4000	1	1
	1	05-12-00 12:45	31°47.568 64°44.745	CTD	18	500	1	1	47	4000	1	1
	1	05-12-00 12:45	31°47.568 64°44.745	CTD	19	500	1	1	47	4000	1	1
	1	05-12-00 12:45	31°47.568 64°44.745	CTD	20	500	1	1	47	4000	1	1

Table 1. (cont.) Details of field sampling

						Fluorometer			HPLC			
							# of filters collected				# of filters collected	
Lab Code	Site #	Date (M-D-Y) Time	Site Location (Lat/Long)	CTD or bucket	Bottle #	Volume filtered (ml)	kept	sent to HPL	Filter (mm)	Volume filtered (ml)	kept	sent to HPL
6	1	04-16-00 19:30 PST	31°54.7 N 124 10.2 W	CTD	21	550	2	2	25	1600	2	2
	1	04-16-00 19:30 PST	31°54.7 N 124 10.2 W	CTD	22	550	0	4	25	1600	0	4
	2	04-17-00 14:54 PST	32 54.6 N 122 7.8 W	CTD	21	550	2	2	25	1600	2	2
	2	04-17-00 14:54 PST	32 54.6 N 122 7.8 W	CTD	22	550	0	4	25	1600	0	4
	3	04-18-00 10:31 PST	33 52.6 N 120 08.1 W	CTD	14	280	3	6	25	550	3	6
8	1	10-25-00 17:00 GMT	29.05383 -89.4702	CTD	10	100	3	6	25	150	3	6
	2	10-26-00 18:32 GMT	28.68 -89.8992	CTD	10	200	3	6	25	300	3	6
	3	10-29-00 15:45 GMT	28.8425 -89.6318	CTD	10	100	3	6	25	200	3	6
7, 9	1	10-10-00 9:00	10:30:011 64:40:089	CTD	1	500	0	4	47	1500	0	4
	1	10-10-00 9:00	10:30:011 64:40:089	CTD	2	500	4	0	47	1500	4	0
	2	10-10-00	10:47:372 64:18:133	CTD	2	500	0	4	47	1500	0	4
	2	10-10-00	10:47:372 64:18:133	CTD	3	500	4	0	47	1500	4	0
D	1 day 1	07-10-00 12:00 EST	39:27.75 74:15.65			500	3	6	47	1000	3	6
	1 day 2	07-11-00 10:25 EST	39:27.66 74:15.78			250	3	6	47	1000	3	6
	1 day 3	07-12-00 19:55 EST	39:21.98 74:12.30			250	3	6	47	1000	3	6
	2 day 1	07-10-00 12:45 EST	39:25.82 74:13.2			250	3	6	47	1000	3	6
	2 day 2	07-11-00 12:25 EST	39:25.83 74:13.22			250	3	6	47	1000	3	6
	2 day 3	07-12-00 19:00 EST	39:24.72 74:13.69			250	3	6	47	1000	3	6
	3 day 1	07-10-00 13:25 EST	39:24.06 74:10.54			250	3	6	47	1000	3	6
	3 day 2	07-11-00 14:30 EST	39:24.21 74:10.42			250	3	6	47	1000	3	6
	3 day 3	07-12-00 19:55 EST	39:27.75 74:15.65			250	3	6	47	1000	3	6

[1] Bottle contents mixed in 50 L carboy.

[2] Bottle contents mixed in 20 L carboy.

Samples were shipped on liquid nitrogen or dry ice to HPL by overnight delivery where they were stored until analyzed (-75 to -80°C). Samples were stored under varying conditions by participants until analyzed (Table 2). The dates samples were collected and the dates they were analyzed are summarized in Table 3.

Table 2. Manner in which filters were stored prior to analysis. Information is given verbatim (except that which is in parentheses). We were not provided this information by Laboratory 5.

Lab code	Fluorometer filters	HPLC filters
1	-30°C	-80°C
3	Filters are stored at -5°C during the one day cruises and then at ~ -17°C until analyzed.	(HPLC not done)
4	liquid nitrogen	liquid nitrogen
6	Stored on liquid nitrogen until extraction (round robin samples). Ordinarily, chl samples submitted to SIMBIOS are extracted immediately after filtration.	If at sea we store samples in liquid nitrogen until ready to analyze. Most all samples are stored in liquid nitrogen until extraction.
7	(Fluorometry not done)	-80°C
8	0°C.	Liquid nitrogen. Extracts are stored in a freezer at -5°C.
9	Filters are stored at -10°C and analyzed within 10 days of collection.	(HPLC not done)
D	liquid nitrogen/-80°C	After filtration stored in liquid nitrogen on ship until reaching shore then at -80°C.
HPL	-80°C	-80°C

Table 3. Dates when samples were collected and the dates they were analyzed by participants and HPL.

		Dates for analysis of field samples	
Lab code	Field sample collection dates	Participant	HPL
1	5/1/00, 2/27/01	7/7/00, 6/13/01	7-7-00, 4/11/01, 6/4-5/01, 6/13/01
3	6/4/00	6/4-5/00	7/7/00
4	4/5/00	6/5-6/00	8/3/00
5	5/12/00	5/15/00, 6/1/00	7/7/00
6	4/16-17/00	9/14-15/00, 9/18/00	9/20-22/00
7	10/10/00	mid 6/01	6/4/01
8	10/25/00	11/16/00	1/12/01, 6/7/01
9	10/10/00	--	6/7/01
D	7/10-12/00	8/9-10/00, 10/18/00	7/24-25/00, 9/6/00

3.2 Data analysis

Our overall approach was to compare participants' results to those attained at HPL. In this process, we calculated differences in very specific ways, used linear regressions where possible to quantify relationships, determined 95% confidence limits and estimates of precision. Values of %D were used with two different meanings, % difference or % discrepancy. The term % difference was used to make comparisons between an individual laboratory's results with those attained at HPL from the same sites with the same instrument type (HPLC or fluorometer) or in some cases to determine the difference between results (both acquired at HPL) but where results from the participant's extraction procedures were compared to the standard procedures. Secondly, %D was used to identify % discrepancy between HPLC and fluorometric values from the same site but within the same laboratory. In each case, our intended use of the term %D is given and were based on the following formulas:

% difference (%D) = ((participant's HPLC value-HPL's HPLC value)/HPL's HPLC value)*100

% difference (%D) = ((participant's FLD value-HPL's FLD value)/HPL's FLD value)*100

% discrepancy (%D) = ((HPLC value - FLD value)/FLD value) *100

In most instances, replicate filters were analyzed, so comparisons were most often between mean values. This allowed us to estimate the precision associated with replicate analyses, using the percent relative standard deviation (%RSD), which is calculated as:

$\%RSD = (s/\bar{x}) * 100$, where s = the sample standard deviation and $\bar{x}$ = the mean.

However, in other cases where replicate filters were not provided or when each HPLC extract was analyzed on the fluorometer at HPL, individual values were compared. The HPLC value used for comparisons varied and was either HPLC total chl *a*, HPLC chl *a* or HPLC chl *a* "as reported" by participants. In all discussions, we specify what the HPLC value reflects in discussions.

After determining % discrepancy values for sites from each laboratory, we calculated the mean % discrepancy for the treatment under consideration. This describes the average bias between HPLC and fluorometer values for a specific treatment and is calculated from individual values of % discrepancy. The *warning limits* (within which 95% of all values of % discrepancy for a treatment should lie) are plotted around the mean % discrepancy and are calculated according to Taylor (1987):

warning limits (WL) = ± student's t value (for n-1) * s.

When evaluating specific factors affecting results, we often used subsets of data as to include all would have biased results due to disproportionate representation by different laboratories. In these cases, when selecting subsets of data for use, we specify which data is included in that particular evaluation and all data is shown in Appendices. We refer the reader to the glossary and data analysis

section (Section 3.4) in Round Robin Report Part 1 for additional clarification and definitions.

4.0 RESULTS OF SURVEY OF METHODS

As one of our objectives was to identify specific factors likely to influence a participant's HPLC/ fluorometer discrepancy, we needed a detailed understanding of the manner in which participants extracted field samples and reported HPLC chl *a* values. Participants responded to our questionnaires by describing their procedures. We report these findings here.

4.1 Extraction procedures

Extraction procedures affect results in many ways. The efficiency with which pigments are extracted from cells affects the ultimate concentration in the sample extract and is affected by such steps as the mode of disruption and the solvent used for extraction. The concentration of pigments can also be affected by filter storage conditions prior to the analysis. Furthermore, as the volume used for extractions is used in calculating results, it is important that it is accurately determined, yet, for a variety of reasons, extraction volume is difficult to accurately assess. Several factors need to be considered when determining extraction volumes. The exact volume is that which results from the amount added to the filter plus the amount of residual water contributed by the sample filter minus solvent losses. There were three ways in which participants estimated extraction volumes. We classify these as *added*, *measured* and *assumed*. We use the term *added* when an analyst used the volume of extraction solvent added to the filter as the extraction volume. We consider *measured* to be when the analyst read the meniscus of the solvent in a measuring device after the homogenate had been clarified, and, in HPLC analysis, where an internal standard was used. We use *assumed* to mean that which includes the volume of solvent added to the filter plus the average estimate of the volume of residual water contributed by the sample filter. The manner in which the sample homogenate is clarified also has the potential to affect outcome. We surveyed how filters were stored, the type of solvent added, the measuring devices used for adding solvent, the mode of disruption, whether the filter was allowed to soak in extraction solvent, how the filter homogenate was clarified and how the extraction volume was determined.

4.1.1 Fluorometer filters

All laboratories added 90% acetone to filters for extraction except Laboratory 9, who added methanol. With all laboratories but 1, 4 and D, the volume used for extractions was added in one step (measuring devices varied) and no physical disruption of the filter was conducted. Laboratories 1, 4 and D disrupted the filters by grinding. With Laboratory 4, the entire volume used for extraction was measured ahead of time in a 10 ml graduated cylinder. Laboratory D used glass volumetric pipettes for adding solvent (4 ml for grinding and 2 ml for rinsing) as specified in the extraction method they followed. With both laboratories, the filter was initially disrupted in a portion of solvent, the slurry transferred to a centrifuge tube and subsequent aliquot(s) added to the grinding tube and pestle for

rinsing. The rinsings were then combined with the first homogenate in a conical tube for clarification by centrifugation. Laboratory 1 added solvent 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. The only other laboratory to use filtration for clarification of the homogenate was HPL, who used a PTFE HPLC syringe cartridge filter equipped with a glass fiber pre-filter. The fluorometer extraction information provided by participants is summarized in Table 4.

Table 4. Summary of extraction procedures used with filters for fluorometric analysis.

Lab code	Vol solvent added (ml)	Measuring device for adding solvent	Mode of disruption	Soak time and T (°C) where given	Means for estimating extraction volume
1	~ 8	squirt bottle	grinding	none	measured
3	10	not provided	none	24 hr	added
4	10	graduated cylinder	grinding	24 hr	added
5	5	not known	none	overnight	added
6	10	re-pipette	none	24 hr	added
8	7	auto-pipette	none	24 hr	added
9	~ 10	not provided	none	24 hr / 5°C	measured
D	4 grinding, 2 rinsing	volumetric pipettes	grinding	12-24 hr	added (6 ml)
HPL[1]	10	volumetric pipette	none	3-4 hr / -15°C	assumed (10.145 ml[2])

[1] The standardized method.

[2] The average volume of water estimated to be contributed by a 25 mm GF/F filter is 145 µl.

4.1.2 HPLC filters

All laboratories used acetone for extractions, but the acetone/water ratio varied with laboratory, as did the measuring devices used for adding solvent to the filters. All but two laboratories (8 and HPL) used some mode of disruption and all laboratories allowed filters to soak in extraction solvent for times which varied. Two laboratories disrupted filters with grinding, but their procedures differed. Laboratory 4 ground the filter in 500 µl of solvent and then used two additional aliquots (of 500 µl each) to quantitatively transfer residual homogenate from the grinding apparatus to a centrifuge tube. Laboratory 6 added solvent to the filter, ground the filter, assumed that the filter slurry was homogeneous and qualitatively transferred it in one step (with no rinsing) to a centrifuge tube using a disposable pipette. All laboratories but 4, 5 and 6 clarified the filter extract by filtration through PTFE HPLC syringe cartridge filters. Laboratories 5 and 6 clarified the sample extracts by centrifugation. Laboratory 4 also centrifuged the extract, and further clarified 500 µl of it with a nylon HPLC syringe cartridge filter. After this, 250µl of injection buffer was pushed through the same cartridge filter and both filtrates were collected directly in the HPLC vial. All three methods for determining extraction

volumes (as defined in Section 4.1.1) were used: added, measured and assumed. While the standard method used at HPL (Appendix 1) ordinarily assumed extraction volume, we used an internal standard and measured the extraction volume with HPLC samples from Laboratory 5. We did this because the samples were very dilute, the filters were large (47 mm) and we added a small volume of solvent (3 ml) so our usual method of calculating extraction volume would likely have incorporated too much error. The HPLC extraction information provided by participants is summarized in Table 5.

Table 5. Extraction procedures used with filters for HPLC analysis.

Lab code	Solvent			Mode of disruption	Soak time/ T (°C)	Means for estimating extraction volume (ml)
	Type	Vol added	Measuring device			
1	95%	3 ml	volumetric pipette	ultrasonic probe	3-4 hr / -15°C	assumed (3.145)
4	100%	1.5 ml	500 µl auto-pipette	grinding	2-12 hr / ~ -20°C	assumed (1.6)
5	90%	5 ml	not known	sonicating bath	overnight in freezer	added
6	100%	1.5 ml	re-pipette (1.5 ml)	grinding	0.5 hr / -20°C	measured
7	100%	not given	not given	ultrasonic probe	overnight in freezer	not given
8	100%	8 ml[1]	not given	none	24 hr / -4°C	measured (3)
D	100%	5 ml[2]	volumetric pipette	sonicating bath	12 to 18 hr / -20°C	added
HPL	90-100%[3]	3, 5 ml	volumetric pipettes	none	3-4 hr / -15°C	assumed[4], measured[5]

[1] Eight ml of solvent was added to the filter, which was then allowed to soak. After this, the solution was filtered through an HPLC syringe cartridge filter. The extraction tube was rinsed with an additional 2 ml of 100% acetone which was also passed through the syringe cartridge filter. The 10 ml of extraction solvent was reduced under a stream of N_2 gas to 3 ml (as indicated by the meniscus and gradation on the concentrator tube).

[2] Solvent was cold when measured.

[3] The type of solvent added was adjusted according to the filter size and volume of solvent added such that the final concentration of acetone was ~90%.

[4] It had previously been determined in this laboratory that, on average, a 25 mm GF/F added 145µl and a 47 mm GF/F added 500µl of water to the volume of solvent added for extraction.

[5] An internal standard was used to measure extraction volume with HPLC samples from Laboratory 5.

4.2 HPLC chl *a* reporting practices

Several pigments respond in a fluorometer as chl *a* (DV chl *a*, chl *a* and DV chl *a* allomers and epimers, and chlide *a*) and thus with HPLC analyses it is important that they be accurately quantified and included in a total chl *a* value (Trees et al. 2000), especially when comparisons are made between HPLC and fluorometric results. We asked participants whether they quantified these additional pigments. We failed to ask whether these pigments were summed with chl *a* and reported as total chl *a*, however. It is possible that in instances where these pigments were quantified, that total chl *a* was not routinely reported as such, as the need for this approach may not be deemed important unless the analyst knows that HPLC/ fluorometer comparisons are to be made. Participants' responses are

summarized in Table 6.

Table 6. Reporting ractices used by participants for pigments analyzed by HPLC but which respond as chl *a* when analyzed by fluorometry. Responses are stated verbatim.

Lab code	What peaks are included in the chl *a* response?	Do you quantify and report chlide *a*?
1	Chl *a*, allomers and epimers. DV chl *a* is individually quantified and included in total chl *a*.	Yes. Prior to 2000, reported but not included in total chl *a*.
4	Chl *a* only. Sometimes I add epimers and allomers	Yes
5	Chl *a* main peak only.	Not usually present. If present, it is quantified using extinction coefficient 68.7.
6	Chl *a*, allomers and epimers. DV chl *a* is individually quantified and included in total chl *a*.	Yes. Included in total chl *a*.
7	Typically include all allomers and epimers with chl *a* and report as total chl *a*.	Yes. Typically quantify as chl *a*
8	Chl *a* main peak only for calculating calibration curve. Allomers and epimers are used with samples.	No
D	Use allomers and epimers and chl *a* main peak.	Yes. Use same response factor as chl *a*.
HPL	Chl *a*, allomers and epimers. DV chl *a* is individually quantified and included in total chl *a*.	Yes. Included in total chl *a*.

4.3 Discussion

The fact that participants' extraction procedures differed between filters collected for fluorometer analysis and HPLC analysis is likely to impose discrepancy on results between the two analytical methods. Smaller extraction volumes are commonly used with HPLC filters to create a more concentrated sample extract to overcome the limitations of HPLC detectors, which are typically less sensitive than fluorometers. However, it can be more difficult to accurately assess extraction volumes when they are low, as imprecision and inaccuracies with measuring devices and inaccuracies when accounting for the water contributed by the sample filter have a greater effect percentage-wise on the extraction volume used in calculations. Also, when the extraction solvent is delivered in more than one step (as was done with Laboratory 4, where three 500 µl measurements were performed to add extraction solvent), errors with the measuring device are of special concern as their effects are compounded. Furthermore, if large filters are used with low extraction volumes and the water contributed by the filter is not accounted for, large errors will be incorporated. When larger extraction volumes are used with small filters (as with fluorometric extractions) the effects of water retained by the sample filter and errors in measuring devices have less effect on results.

Two specific practices used by laboratories concerned us. Laboratories 5 and D did not

account for the contribution of water retained in the glass fiber filters they used (47 mm) to the HPLC extraction volume. These filters can add as much as 640 µl to 700 µl to the extraction volume (observations in our laboratory; and suggestions by Bidigare (1991), respectively). Thus, that both laboratories added 5 ml of extraction solvent means that their HPLC results would automatically be biased by ~ -14%. Secondly, the practice of measuring extraction solvent cold (as was done with HPLC extractions by Laboratory D) is not advised as pipettes are typically calibrated at 20 or 25°C and would therefore be less accurate at other temperatures.

There should be consistency in assessments of chl *a* products. Procedures suggested for use (Bidigare and Trees 2000, Trees et al. 2000) recommend that all chl *a* allomers and epimers, DV chl *a* and chlide *a* be quantified and reported in HPLC total chl *a*. In our survey of methods, we found inconsistency with regard to inclusion of chl *a* allomers and epimers, as Laboratories 4, 5 and 8 did not consistently include them, Laboratories 4, 5, 7, 8 and D did not report DV chl *a* and Laboratory 8 did not report chlide *a*. Laboratories 1, 6 and HPL currently include all products and report a total chl *a* value. While it is clear that the other laboratories do not include DV chl *a* in their reports, it is not known whether they report total chl *a*, where chlide *a* is included (however, it is known that Laboratory 8 does not include chlide *a* as they do not report it).

5.0 Results of Field Sample Analyses Considered Collectively

Initially we inspected results of field sample analyses collectively to estimate average discrepancies seen with results from participants and with results from HPL. We evaluated the effects of chl *a* concentration on % discrepancy using data acquired at HPL. Furthermore, as accessory pigment content can universally affect fluorometer results, we investigated the effects of several accessory pigments on discrepancy by analyzing all HPLC extracts by fluorometry as well. This approach eliminated variability arising from differences in filters and extractions. Finally, we determined the effect that variations in extraction procedures had on discrepancy, relative to that attained when extraction procedures were standardized.

5.1 Estimates of discrepancy: standard method vs. multiple methods

We evaluated the results of replicate filters from the same site, 1) when all filters were extracted and analyzed with standard procedures at HPL, and 2) when participants analyzed filters using their usual methods. We refer to these two treatments using the abbreviated terms "standard" and "multiple" methods, respectively. When comparing results, we eliminated data from sites that were not represented by both treatments. Thus, data from 18 different sites were compared representing six different laboratories (1, 4, 5, 6, 8 and D). However, the number of results per laboratory was disproportionally distributed. Five sites were represented for Laboratory 1 and three for Laboratories 4, 6, 8 and D. Laboratory 5 collected from one site only, but data from this site was represented four times. With Laboratory D we used results from three sites, but day 2 only, as to include all days would have biased results unnecessarily. Samples were analyzed in either duplicate or triplicate by HPLC and

fluorometer from all but seven sites (standard method) or all but five sites (multiple methods). (Data are shown in Appendix 2 for standard method and Appendix 3 for multiple methods.)

Linear regressions were determined on $\log_{10}$ transformed chl *a* values (µg/l). Total chl *a* was used to represent the HPLC values for data from the standard method and HPLC values used with data from the multiple methods were according to the participants' typical reporting practices (see Table 6). With the standard method, the linear regression was log HPLC = 1.013 log fluorometric chl *a* - 0.031, $r^2 = 0.999$, n=21 (Fig. 1). For data derived from multiple methods, the linear regression was log HPLC = 1.048 log fluorometer chl *a* - 070, $r^2 = 0.986$, n = 21 (Fig. 2).

We also determined the % discrepancy between the HPLC and fluorometer values for each of the 21 observations under each treatment. From these values, we calculated the mean % discrepancy and the warning limits within which 95% of discrepancies should be contained. For the results where extractions and analyses were standardized, the HPLC total chl *a* values were, on average, -6.4% of the fluorometer values, and warning limits were ± 19% (Fig. 3). For results reported by participants, representing multiple methods and laboratories, the HPLC values were, on average, -10% of the fluorometer values, and warning limits were ± 60% (Fig. 4).

5.2 Pigment effects

We determined whether the chl *a* concentration had an effect on % discrepancy by using regression analysis with the 21 observations (representing18 sites) discussed in the previous section. These sites ranged in chl *a* concentration from approximately 0.05 to 240µg/l chl *a*. Using the trophic classification scheme presented in Hooker et al. (2000), 11 sites were eutrophic (> 1.0 µg/l chl *a*), five were mesotrophic (0.1 < µg/l chl *a* < 1.0) and two were oligotrophic (< 0.1 µg/l chl *a*). We found that the slope was not significantly different from zero ($0.2 < p < 0.1$) and r^2 was 0.091 when the linear regression of % discrepancy versus $\log_{10}$ fluorometric chl *a* value (from the standard method at HPL) was computed. Even so, we did see increased % discrepancy with very dilute samples, as seen with inspection of Fig. 3, where one oligotrophic result is outside the lower warning limit.

Some accessory pigments are known to affect fluorometric chl *a* values (Trees, et al. 1985). We evaluated the effects of the relative abundance of some of these pigments on % discrepancy. These pigments included chl *c*, chl *b*, DV chl *a* and chlide *a*. When investigating each pigment, we excluded samples high in the others to reduce variability so that a trend, if any, would be more apparent. Additionally, we indirectly evaluated the effect of chl *a* degradation products by inspecting the relationships between % discrepancy and the fluorometer "before acid" to "after acid" ratio (Fo/Fa ratio). Data for all these evaluations were derived from samples analyzed by HPLC with either the standard or participant's method (n = 142, representing 19 sites) that were also analyzed by fluorometer at HPL (where the HPLC extracts were diluted to provide sufficient volume for fluorometric analysis). Regression analysis was used to quantify the effects of chlide *a*, but not with other pigments, as these data were not normally distributed (even with transformation). Before

proceeding with our analysis of individual pigments, we determined the linear regression for the results of all 142 sample extracts. We found that log HPLC = 1.000 log fluorometer chl *a* -0.031, $r^2 = 0.998$ (Fig. 5). We found that the mean % discrepancy was -6.5% and the warning limits were ± 16% (Fig. 6). Thus, when exploring the effects of interfering pigments, we compared results to the mean % difference and to the warning limits on either side of it, which encompassed -23% to 10%.

5.2.1 Chlorophyll c

We evaluated the effects of chl *c* concentration on HPLC/ fluorometer discrepancy, as chl *c* accessory pigments are known to cause the chl *a* values attained by fluorometry to be overestimated (Trees et al. 1985). There are several naturally occurring chl *c* compounds, so we determined a total chl *c* value by summing all chl *c* products present, as measured by HPLC (this included combinations of chl *c*1, chl *c*2, and chl *c*3). We then determined the ratio of total chl *c* to total chl *a* for each sample and compared these ratios with % discrepancy, after excluding samples containing either DV chl *a*, high amounts of chlide *a* (>10%) or high amounts of chl *b* (>10%). However, visual inspection of these data (Fig. 7) shows no obvious trend. Chl *c* ranged from 3% to 33% of the HPLC total chl *a*.

5.2.2 Chlorophyll b

We evaluated the effects of chl *b* concentration on HPLC/ fluorometer discrepancy, as chl *b* is known to cause the chl *a* values attained by fluorometry to be underestimated (Trees et al. 1985). Thus, in cases where chl *b* is abundant, the % discrepancies, which are typically negative, may be less so or even positive. We determined the ratio of chl *b* to the HPLC total chl *a* for each sample and compared these ratios with % discrepancy after excluding samples containing either DV chl *a*, high amounts of chlide *a* (>10%) or high amounts of chl *c* (>20%). Visual inspection of these data (Fig. 8) shows that with increasing ratios of chl *b*, the sign of the % discrepancies changes in a positive direction and many values are above the mean % difference. However, this apparent trend could not be confirmed with regression analysis, as it was not possible to achieve a normal distribution. Chl *b* was present in 17 of the 19 sites represented in this illustration in concentrations ranging from 0.3% to 27% of the total chl *a*.

5.2.3 Divinyl chlorophyll a

We evaluated the effects of DV chl *a* concentration on HPLC/ fluorometer discrepancy. Five of the 19 sites represented in this comparison contained DV chl *a* in amounts which ranged from 2 to 45% of monovinyl chl *a* and DV chl *a* combined. We determined the ratio of DV chl *a* to monovinyl + DV chl *a* for each sample and compared these ratios with % discrepancy, after excluding samples high in either chlide *a* (>10%), chl *b* (>10%) or chl *c* (>20%). Visual inspection of these data (Fig. 9) indicated that most discrepancies seen with samples containing DV chl *a* fell above the mean % discrepancy (-6.5%).

5.2.4 Chlorophyllide a

We evaluated the effects of chlide *a* concentration on HPLC/ fluorometer discrepancy. Chlide *a* was present in sample extracts from 17 of the 19 sites tested in amounts ranging from 0.2 to 43% of HPLC total chl *a*. As chlide *a* responds as chl *a* in a fluorometer, we estimated the effect that it would have on % discrepancy if it were not accounted for and total chl *a* not reported. To do this, we determined the % discrepancy (with chlide *a* not included in the HPLC component) versus the proportion of chlide *a* in the sample (chlide *a* / total chl *a*). We conducted regression analysis (with data from samples containing chlide *a*) after transforming values to attain normal distributions (see Fig. 10). Transformation of the chlide *a* ratio required $\log_{10}$ conversions and the % discrepancy values were scaled and then squared. From the resulting linear regression ($r^2 = 0.540$) we determined that at 2%, chlide *a* would cause % discrepancies to drop below the mean % discrepancy (-6.5%) and at 19% it would cause the negative % discrepancy to be at the lower warning limit seen with these samples (-23%) (see Fig. 6 for illustration of warning limits). Only one site consistently had chlide *a* concentrations in excess of 19%, but 60% of all sites had chlide *a* concentrations in excess of 2%.

5.2.5 Chlorophyll a *degradation products*

We indirectly evaluated whether the abundance of phaeopigments affected the HPLC/ fluorometer discrepancy by using the Fo/Fa ratio from the fluorometric analysis of sample extracts as an indicator of relative abundance of phaeopigments. We found that for samples from all sites but one, Fo/Fa ratios varied between 1.6 and 2.0 ($\bar{x} = 1.80 \pm 0.087$, 5%RSD). The variation seen in these sample extracts was only slightly greater than that observed as the average for chl *a* calibration standards ($\bar{x} = 1.99 \pm 0.080$, 4%RSD). Thus, variability in the abundance of phaeopigments was likely confined to a narrow range. For the one site which differed, the average Fo/Fa ratio was 1.2, indicating that the abundance of phaeopigments was greater at this site. This was corroborated by inspecting the HPLC chromatograms from this site where an abundance of phaeophorbide peaks were seen. However, discrepancy between HPLC and fluorometric values for this site still were within warning limits.

5.3 Effects of variations in extraction procedures

We considered variations in extraction procedures as having a strong potential to affect discrepancy. We therefore evaluated the degree to which using a variety of extraction procedures collectively contributed to uncertainty. To do this we implemented participants' extraction procedures at HPL (Appendix 4) and compared results to replicate sets of filters also analyzed at HPL using standardized extraction procedures. There were 12 sites from which we could make these comparisons (two sites are represented twice, so n = 14) and four laboratories' samples and extraction procedures were represented. With each treatment, Laboratory 6 was represented 5 times and Laboratories 4, 8 and D three times. With the standardized extraction method, log HPLC = 0.990 log fluorometric chl *a* - 0.007, $r^2 = 0.999$ (Fig. 11) and with results seen when participants' extraction

methods were used at HPL, log HPLC = 0.948 log fluorometric chl *a* - 0.023, $r^2 = 0.991$ (Fig. 12). When these data were reinspected for % discrepancy between HPLC total chl *a* and fluorometric chl *a*, the mean % discrepancy seen with the standardized extractions was -1.2% with warning limits of ± 14% (Fig. 13) and with the participants' extraction methods, the mean % discrepancy was -1.5% with warning limits of ± 39% (Fig. 14).

5.4 Discussion

We found that % discrepancies were lowest when all analyses were performed at HPL and when extraction procedures used with HPLC filters and fluorometer filters were standardized. With these conditions, the warning limits (describing the 95% confidence limits) varied from ±14% to ± 19% (depending on the number of samples included in the analysis). When various extraction methods were used, but analyses still conducted at HPL, the warning limits for % discrepancies were ± 39% and when multiple laboratories using many different extraction procedures reported results, the warning limits for % discrepancies were ± 60%. These results suggest that for laboratories to reduce uncertainties, using standardized extraction procedures with HPLC filters and fluorometer filters would be of benefit.

We were unable to quantify the degree to which chl *c*, chl *b* and DV chl *a* contributed to % discrepancies through fluorometric interferences, even though these pigments were widely distributed. However, chlide *a* was present in many samples and we demonstrated that it could contribute to large discrepancies if not included in total chl *a*. Our observations support the suggestion by Trees et al. (2000) that total chl *a* values are reported for HPLC analyses.

6.0 CONCLUSIONS

• HPLC values in this study were typically 7 to 10% lower than fluorometer values. Negative discrepancies are consistent with historical relationships.

• Under the most controlled conditions (all analyses performed at one laboratory and with extraction procedures standardized) the 95% confidence limits for % discrepancy were ± 19%. When participants reported data from the same sites, the 95% confidence limits for % discrepancy were ± 60%.

• The $\log_{10}$ linear regressions of HPLC vs. fluorometer chl *a* (µg/l) for the two above data sets exhibited very similar values, where slopes were 1.0 and $r^2 = 0.99$. Log_{10} linear regressions, while commonly used to present these relationships, are not descriptive of the magnitude of the % discrepancies seen.

• The % discrepancies in this study may be smaller than would otherwise be seen, as all calibrations were consistent between fluorometers and HPLCs. The % differences between results from the participant and Horn Point Laboratory, for each instrument, are also undoubtedly reduced, as calibrations among laboratories were also consistent.

• We identified several factors affecting % discrepancies. These included the use of extraction procedures that differ between HPLC and fluorometric filters, inaccurate assessments of extraction volumes, inconsistency among laboratories with regard to HPLC total chl *a* reporting pracitces (DV chl *a* and chlide *a* were not commonly quantified) and inaccuracies resulting from analytical methods.

• Low % discrepancies do not necessarily indicate that results are accurate, if both instruments exhibit inaccuracies of the same sign.

• Participants more accurately reproduced HPLC results at Horn Point Laboratory than fluorometer results.

• The average precision by participants in the analysis of replicate HPLC filters was 6% (median = 3%) and for fluorometer filters the average precision was 11% (with a median of 9%).

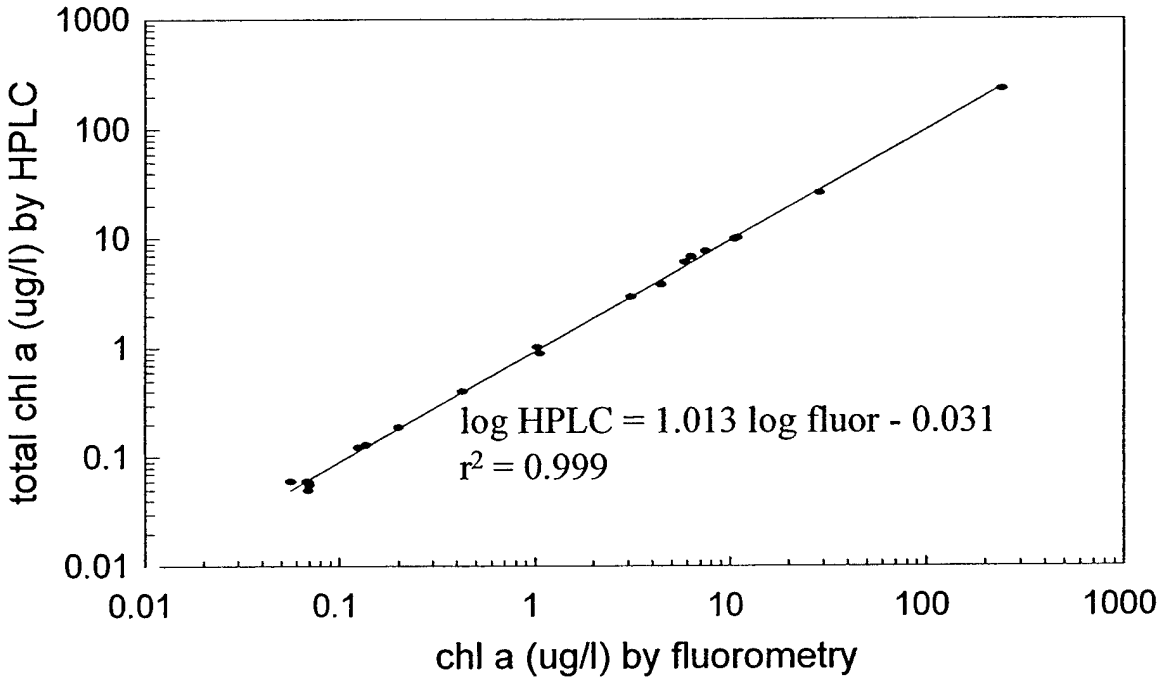

Fig. 1. Results at HPL of filters collected for HPLC and fluorometric analysis and extracted with standardized procedures. All but 7 points are the mean of replicate analyses.

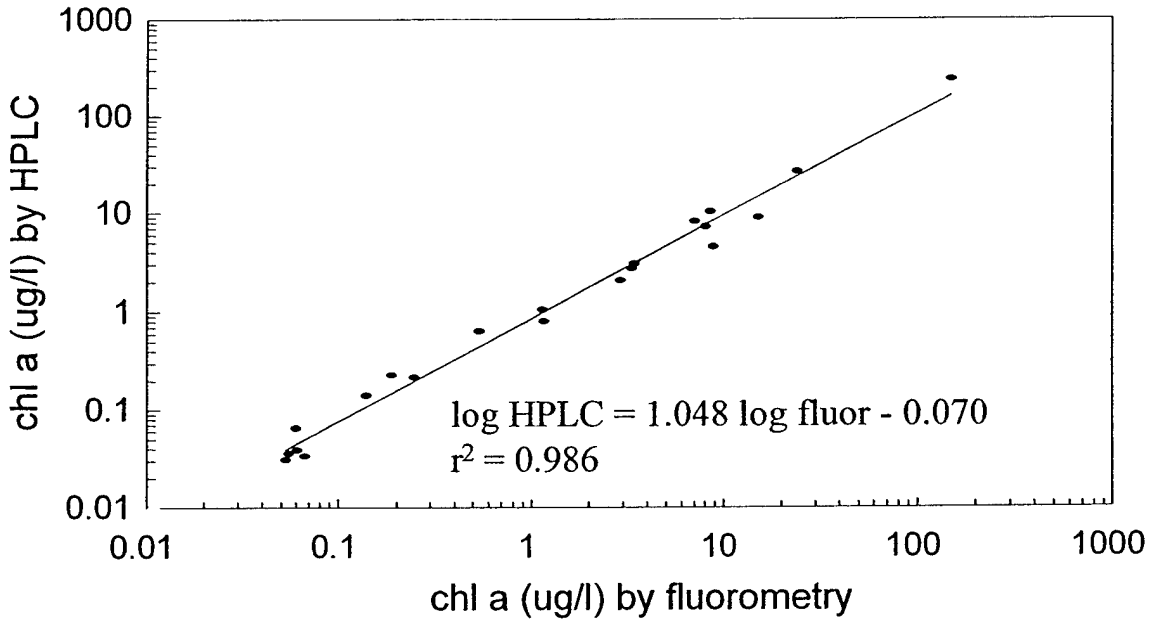

Fig. 2. Participants' results as reported by them (for the same sites shown in Fig. 1) for HPLC and fluorometer. All but 5 points are the mean of replicate analyses.

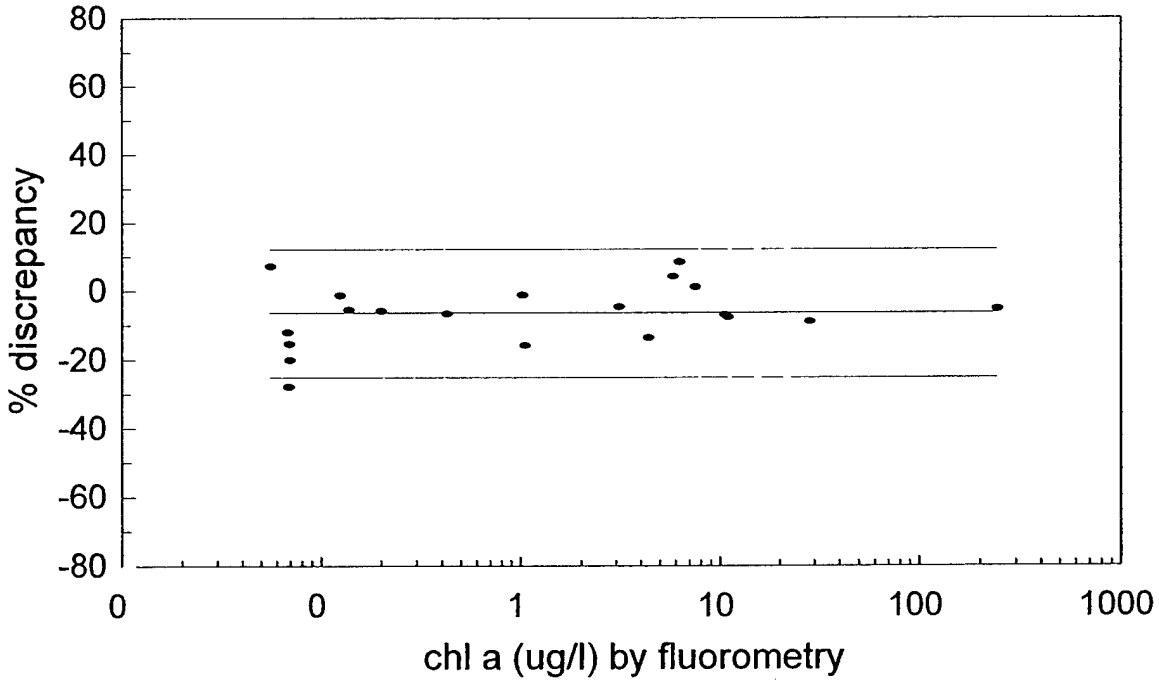

Fig. 3. Control chart for results at HPL of filters collected for HPLC and fluorometric analysis and extracted using standardized procedures. All but 7 points are the mean of replicate analyses. Outside lines depict warning limits and middle line depicts mean % discrepancy.

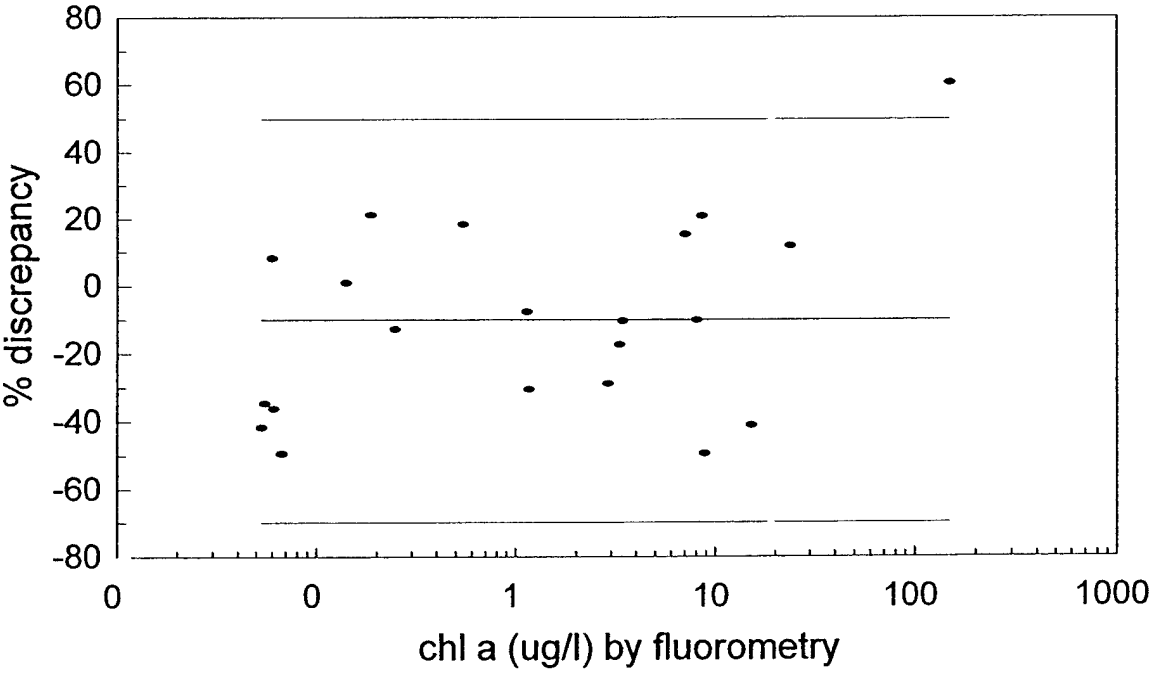

Fig. 4. Control chart for data as reported by participants (for same sites as in Fig. 3) for HPLC and fluorometer. All but 5 points are the mean of replicate analyses. Outside lines depict warning limits and middle line depicts mean % discrepancy.

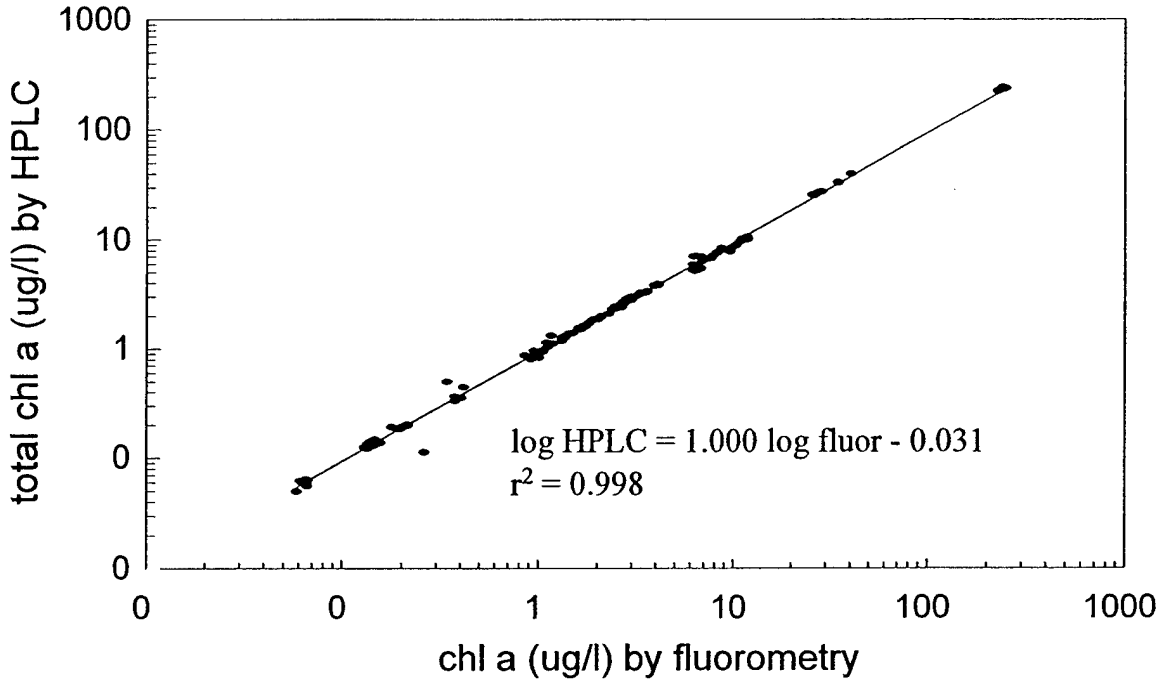

Fig. 5. HPLC filter extracts diluted and read on the fluorometer at HPL.

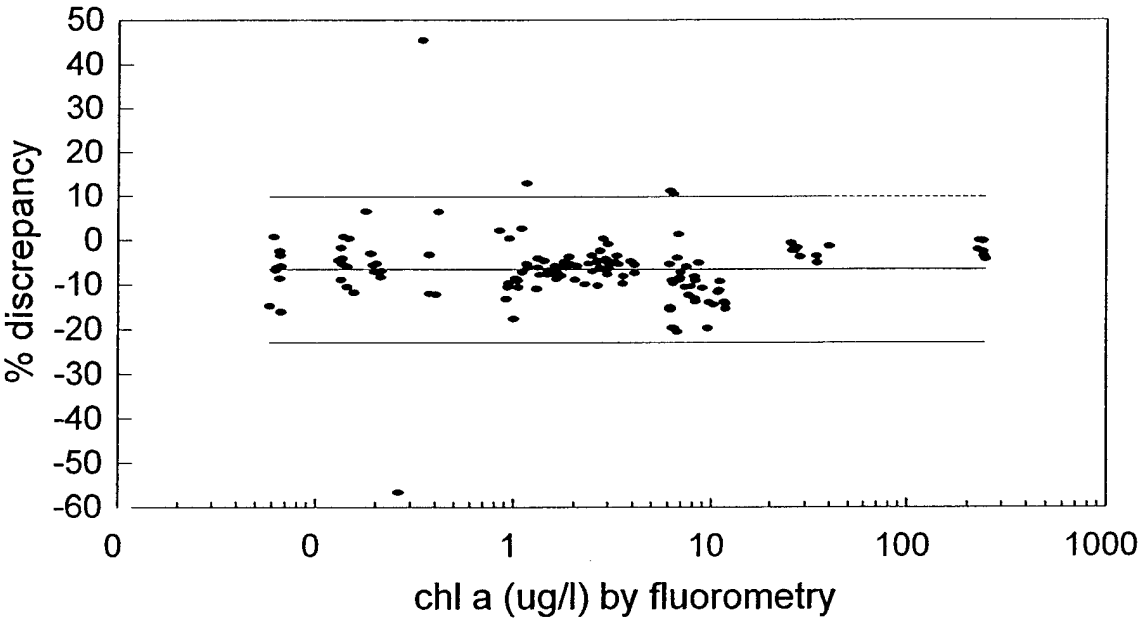

Fig. 6. Control chart for HPLC filter extracts diluted and analyzed on the fluorometer at HPL. Outside lines depict warning limits. Middle line depicts mean % discrepancy.

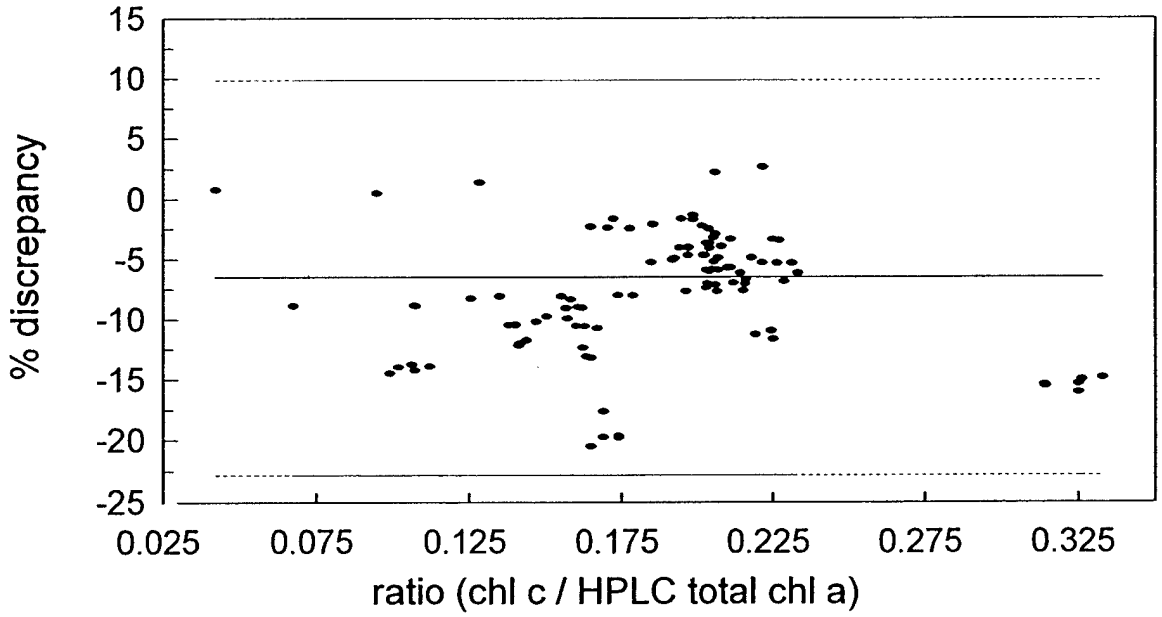

Fig. 7. Control chart relating % discrepancy to the ratio (chl *c*/HPLC total chl *a*) in a sample. Outside lines depict warning limits. Middle line depicts mean % discrepancy.

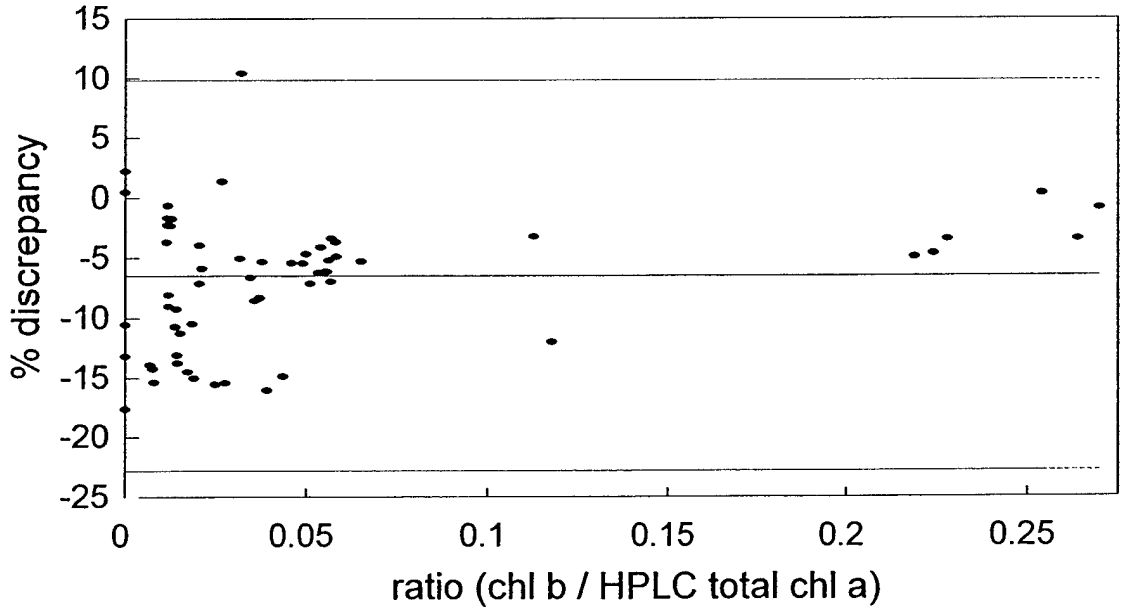

Fig. 8. Control chart relating % discrepancy to the ratio (chl *b*/HPLC total chl *a*) in a sample. Outside lines depict warning limits. Middle line depicts mean % discrepancy.

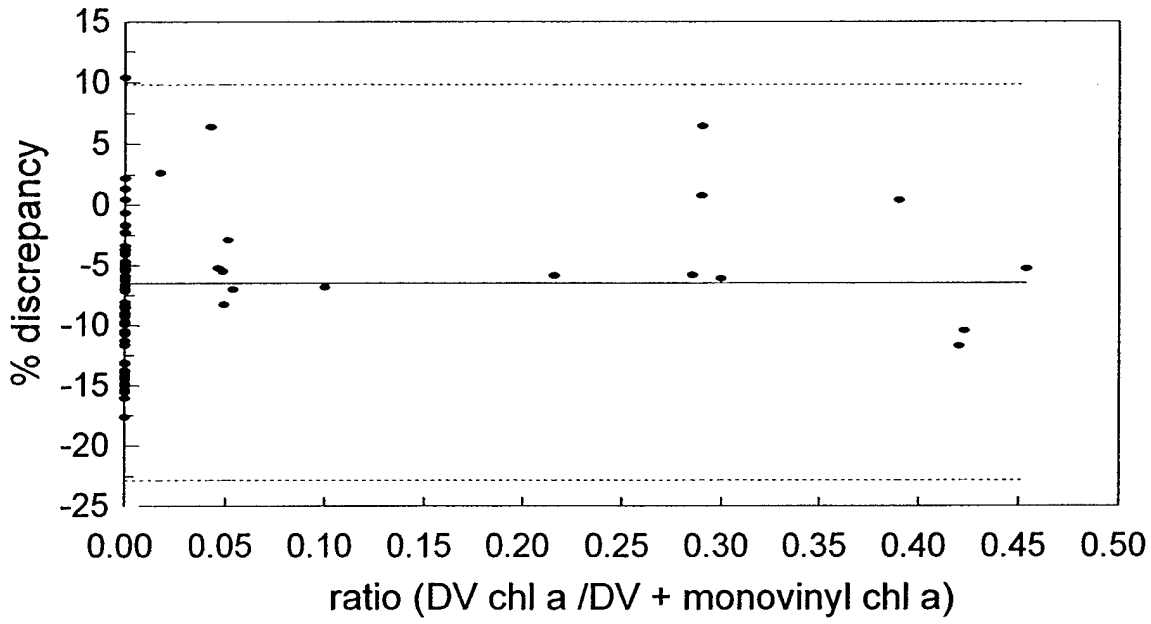

Fig. 9. Control chart relating % discrepancy to the ratio (DV chl *a* /DV + monovinyl chl *a*) in a sample. Outside lines depict warning limits. Middle line depicts mean % discrepancy.

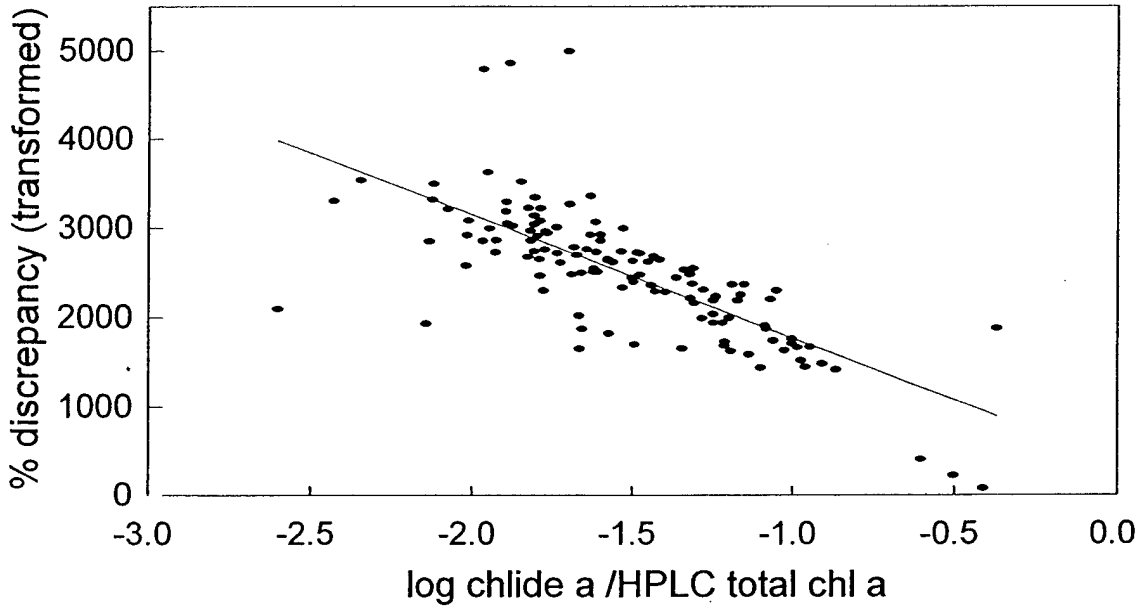

Fig. 10. % discrepancy occurring (with increasing ratios of chlide *a*) when chlide *a* is not included in total chl *a* . Data are transformed to yield normal distributions. $r^2 = 0.540$.

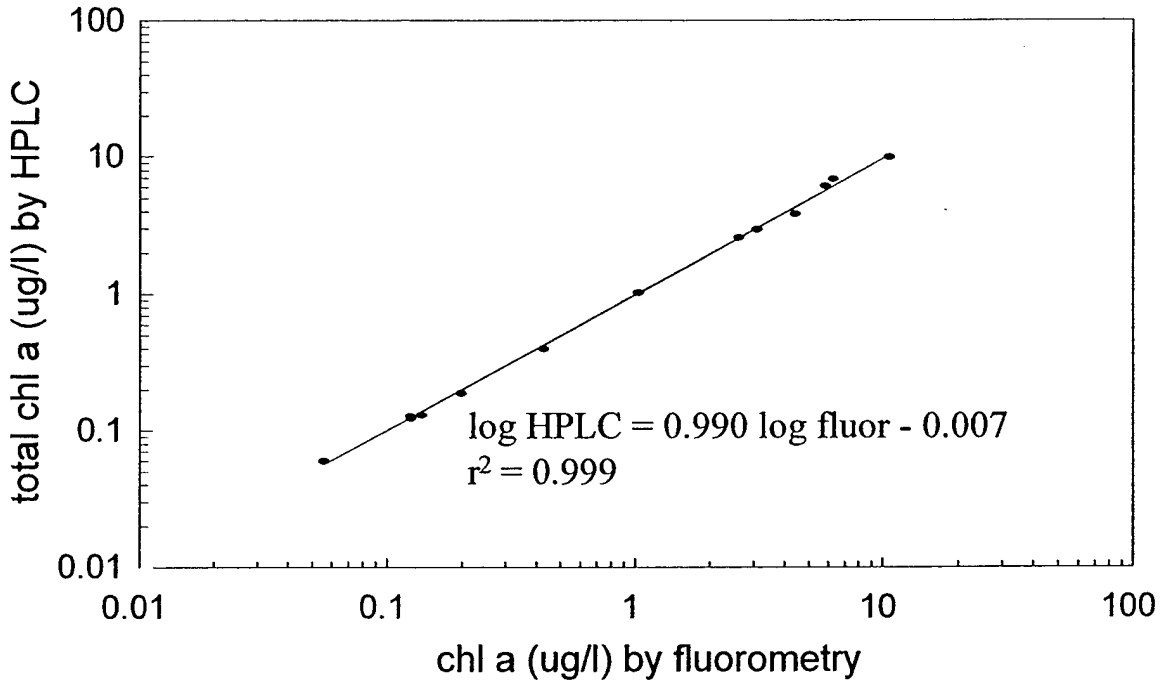

Fig. 11. Results at HPL of filters collected for HPLC and fluorometric analysis and extracted with standardized procedures. All but 3 points are the mean of replicate analyses.

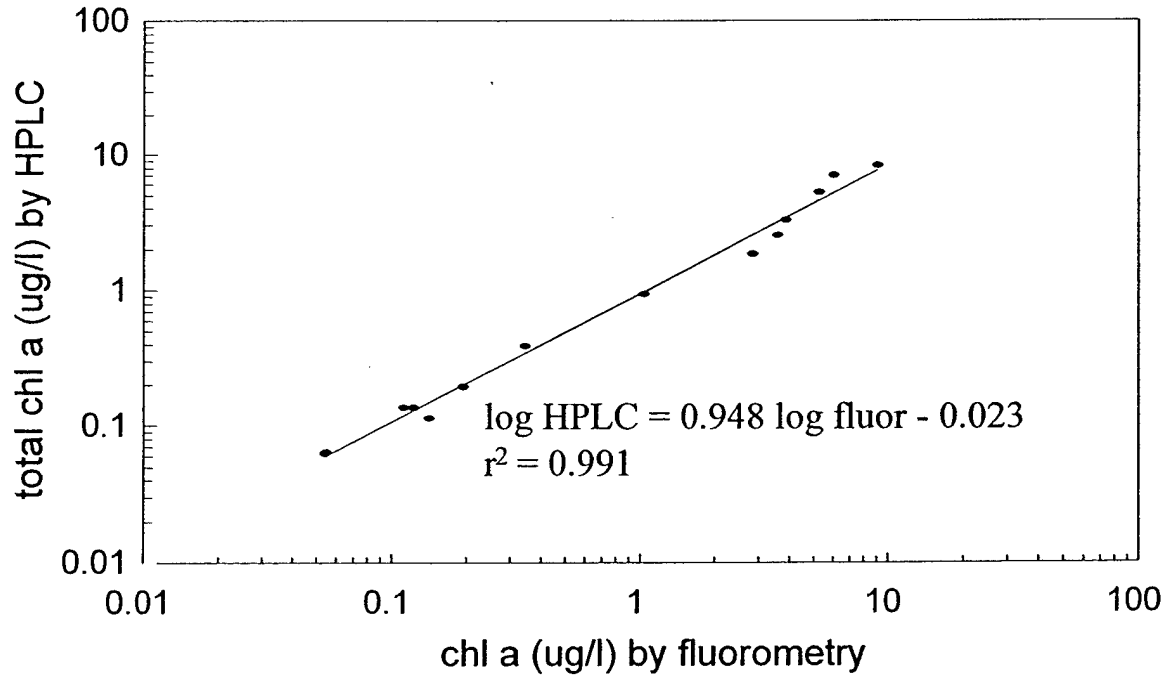

Fig. 12. Results of participants' extraction procedures at HPL (for the same sites as in Fig. 11) for HPLC and fluorometer. All but 3 points are the mean of replicate analyses.

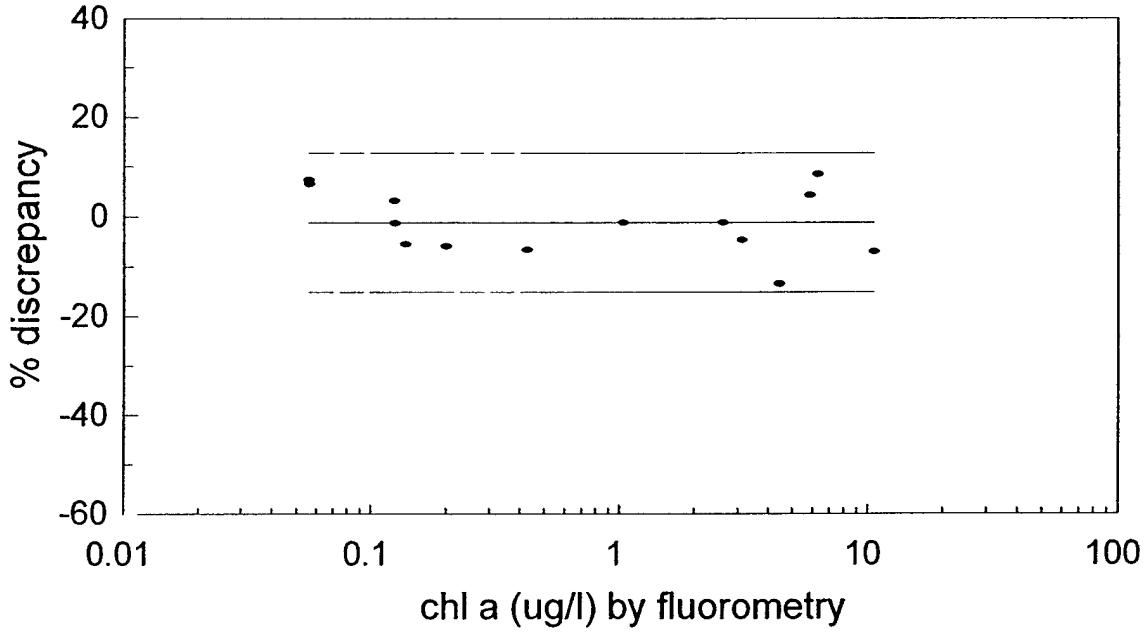

Fig. 13. Control chart for results at HPL of filters collected for HPLC and fluorometric analysis and extracts with standardized procedures. All but 3 points are the mean of replicate analyses. Outside lines depict warning limits. Middle line depicts mean % discrepancy.

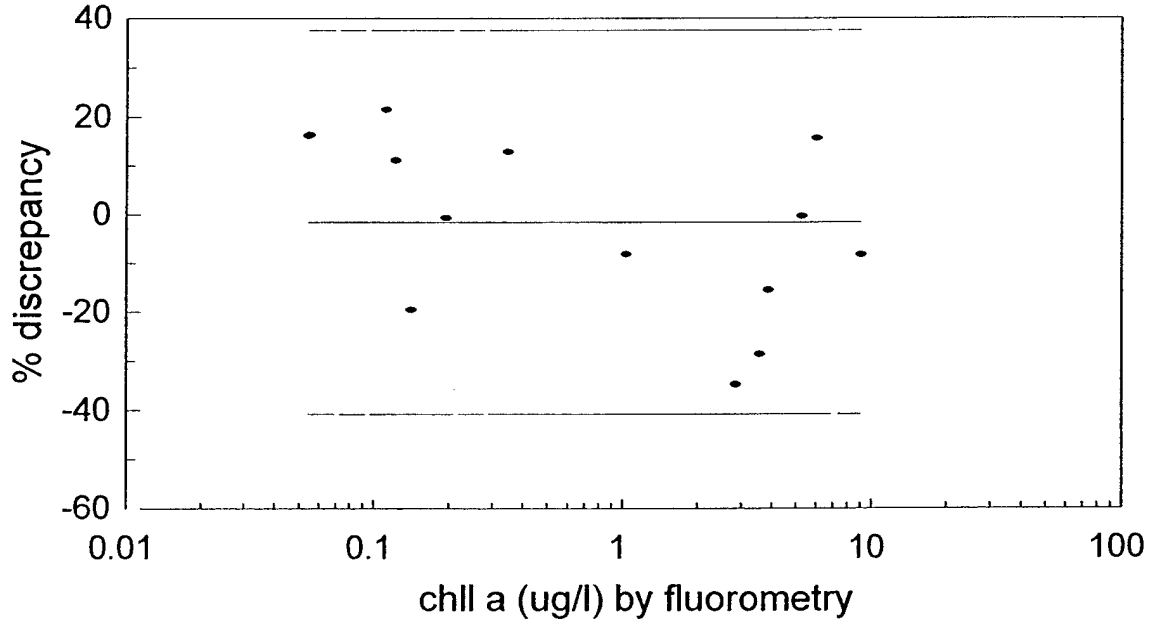

Fig. 14. Control chart for participants' extraction procedures at HPL (for same sites shown in Fig. 13) for HPLC and fluorometer. All but 3 points are the mean of replicate analyses. Outside lines depict warning limits. Middle line depicts mean % discrepancy.

Appendix 1

Extraction procedures used as the "standard extraction procedure" for participants' field samples at HPL. The extraction volumes used in calculations were considered to be the volume of acetone added plus the water contributed by the glass fiber filters (145 μl for 25 mm GF/F; 500 μl for 47 mm GF/F). Although Bidigare (1991) suggests that a 47 mm GF/F filters contributes 700 μl to the total volume, we had experimentally determined that the water contribution was 500 μl and used that value for calculations of HPL values.

Appendix 1.1 SIMBIOS/HyCODE chlorophyll *a* round robin

HPLC Standardized Extraction Procedure

1. Keep track of filter numbers.

2. With forceps, place filter in 7 ml amber glass vial. Note filter number on vial.

3. Add 5 ml 90% acetone using 5 ml, Class A volumetric pipette.

4. Make sure filter is submerged and cap tightly.

5. Vortex vigorously for 15 seconds. Make certain filter is still submerged.

6. Again, make certain filter is submerged.

7. Place tubes in freezer (~ -10 to -20°C) for three hours (note time placed in freezer).

8. Remove from freezer (note time). Check cap tightness. Vortex each tube vigorously for 15 s.

9. Attach filter cartridge to end of syringe.

10. Decant filter and extract into syringe and force extract through filter with plunger.

11. Collect filtrate in empty 7 ml amber glass vial. Cap tightly. Vortex well.

12. Remove aliquot for HPLC analysis.

Note: avoid bright light

Supplies:
- 90% acetone (vol:vol)
- Amber glass vials
- Forceps
- Glass, 5 ml volumetric Class A pipette
- Freezer (-10 to -20°C)
- Filtration cartridges and syringes
- Vortex genie

Appendix 1 (cont.)

Appendix 1.2 SIMBIOS/HyCODE chlorophyll *a* round robin

Turner fluorometer Standard Extraction Procedure

1. Keep track of filter numbers.

2. Using forceps, place folded filter in 22 ml amber vial. Note filter number on vial.

3. Add 10 ml 90% acetone using 10 ml, Class A volumetric pipette.

4. Make certain filter is submerged and cap tightly.

5. Vortex vigorously for 15 seconds. Make certain filter is still submerged.

6. Place tubes in freezer (-10 to -20°C) for three hours (note time placed in freezer).

7. Remove from freezer (note time). Check cap tightness. Vortex each vial vigorously for 15 s.

8. Attach filter cartridge to end of syringe.

9. Decant filter and extract into syringe and force extract through filter with plunger.

11. Collect filtrate in empty 22 ml amber glass vial. Cap tightly. Vortex well.

12. Read on calibrated Turner fluorometer

Note: avoid bright light

Supplies:
- 90% acetone (vol:vol)
- Amber glass vials
- Forceps
- Glass, 10 ml volumetric Class A pipette
- Freezer (-10 to -20°C)
- Vortex genie
- Filtration cartridges and syringes

Appendix 2

Results of Standard Extraction at HPL

The code means: 1st number = laboratory code, 2nd number = site number, 3rd number = either bottle number, or in the case of Laboratory D, day.

	HPLC		Fluorometer		
Code	$\bar{x} \pm s$ (μg/l total chl *a*)	% RSD	$\bar{x} \pm s$ (μg/l chl *a*)	% RSD	% discrepancy
1-1	26.08 ± 0.5167	1.98%	28.60 ± 0.1955	0.68%	-8.81%
1-2	230.4 ± 6.0187	2.61%	243.3 ± 3.9984	1.64%	-5.30%
1-3	0.8922 ± 0.0608	6.81%	1.058 ± 0.0261	2.47%	-15.7%
1-4	10.12 ± 0.0688	0.68%	10.96 ± 0.4149	3.79%	-7.66%
1-5	7.603 ± 0.0382	0.50%	7.515 ± 0.2753	3.66%	1.17%
1-6	35.50 ± 3.788	10.7%	42.24 ± 5.478	13.0%	-16.0%
4-1	6.825 ± 1.263	18.5%	6.290 ± 0.6764	10.8%	8.51%
4-2-9	didn't have		0.1406		
4-2-10	0.1300 ± 0	0%	0.1375		-5.45%
4-2-11	0.1380		0.1525		-9.51%
4-3	0.1877 ± 0.0046	2.46%	0.1994 ± 0.0096	4.84%	-5.87%
5-1-17	0.0500		0.0692		-27.7%
5-1-18	0.0590		0.0710		-16.9%
5-1-19	0.0600		0.0681		-11.9%
5-1-20	0.0560		0.0699		-19.9%
6-1-22	0.1280 ± 0.0042	3.31%	0.1254 ± 0.0026	2.11%	2.07%
6-1-21	0.1230		0.1246		-1.28%
6-2-22	0.0600 ± 0.0028	4.71%	0.0563 ± 0.0019	3.39%	6.57%
6-2-21	0.0600		0.0559		7.33%
6-3	1.020 ± 0.1311	12.9%	1.032 ± 0.1079	10.5%	-1.16%
7-1-1	0.1199 ± 0.0085	7.07%	0.1259 ± 0.0050	1.51%	-4.77%
7-1-2	didn't have		didn't have		
7-2-2	0.4875 ± 0.0671	13.8%	0.5328 ± 0.0240	4.51%	-8.50%
7-2-3	didn't have		didn't have		

Appendix 2 (cont.)

Results of Standard Extraction at HPL

8-1	9.866 ± 0.2595	2.63%	10.60 ± 0.2995	2.82%	-6.95%
8-2	0.3990 ± 0.0622	15.6%	0.4272 ± 0.0153	3.58%	-6.60%
8-3	6.093 ± 0.4816	7.90%	5.844 ± 0.2894	4.95%	4.26%
D-1-1	3.027 ± 0.1861	6.15%	didn't have		
D-1-2	3.811 ± 0.0576	1.51%	4.403 ± 1.308	29.7%	-13.4%
D-1-3	1.580 ± 0.0284	1.79%	1.716 ± 0.0396	2.31%	-7.91%
D-2-1	6.711 ± 0.3854	5.74%	7.288 ± 0.2630	3.61%	-7.91%
D-2-2	2.958 ± 0.1473	4.98%	3.103 ± 0.6523	21.0%	-4.66%
D-2-3	1.263 ± 0.0171	1.35%	1.399 ± 0.0405	2.89%	-9.71%
D-3-1	2.836 ± 0.0114	0.40%	2.885 ± 0.0581	2.01%	-1.69%
D-3-2	2.577 ± 0.1859	7.21%	2.608 ± 0.0883	3.39%	-1.18%
D-3-3	1.802 ± 0.0750	4.16%	1.778 ± 0.0782	4.40%	1.35%

Appendix 3

Participants' Data as Reported by Them

The code means: 1st number = laboratory code, 2nd number = site number, 3rd number = either bottle number, or in the case of Laboratory D, day. Laboratory 4 used two detectors at the same time, and the analyst decided whether to report results from the FLD or the DAD. We report results for both detectors, but when analyzing data collectively, we used the DAD results.

	HPLC		Fluorometer		
Code	$\bar{x} \pm s$ (μg/l chl *a*)	% RSD	$\bar{x} \pm s$ (μg/l chl *a*)	% RSD	% discrepancy
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	0.8131 ± 0.0146	1.80%	1.169 ± 0.0799	6.83%	-30.4%
1-4	10.37 ± 0.1888	1.82%	8.581 ± 0.4045	4.71%	20.8%
1-5	8.190 ± 0.1784	2.18%	7.093 ± 0.3808	5.37%	15.5%
1-6			39.36 ± 4.214	10.7%	
3-1	not done		1.035 ± 0.0421-HA 1.003 ± 0.0372-GFF	4.07% 3.71%	
3-4	not done		0.6847 ± 0.0505-HA 0.6797 ± 0.0246-GFF	7.38% 3.62%	
3-6	not done		2.738 ± 0.2570-HA 3.164 ± 0.3311-GFF	9.38% 10.5%	
4-1	5.715-FLD 7.264 ± 1.480-DAD	 20.4%	8.067 ± 1.550	19.2%	-29.2%[1] -10.0%
4-2-9	0.135-FLD 0.182-DAD		0.178		-24.2% 2.3%
4-2-10	0.181-FLD 0.228-DAD		0.188		-3.72% 21.3%
4-2-11	0.147-FLD 0.207-DAD		0.189		-22.2% 9.5%
4-3	0.201 ± 0.004-FLD 0.217 ± 0.006-DAD	2.01% 2.93%	0.248 ± 0.0219	8.84%	-19.0% -12.5%
5-1-17	0.039		0.061		-36.1%
5-1-18	0.034		0.067		-49.3%
5-1-19	0.036		0.055		-34.5%
5-1-20	0.031		0.053		-41.5%

Appendix 3 (cont.)

Participants' Data as Reported by Them

5-1-17 new[2]	0.045		0.061		-26.2%
5-1-18 new[2]	0.039		0.067		-41.8%
5-1-19 new[2]	0.041		0.055		-25.5%
5-1-20 new[2]	0.035		0.053		-34.0%
6-1-22	didn't have		didn't have		
6-1-21	0.142 ± 0.004	2.50%	0.14 ± 0	0%	1.43%
6-2-22	didn't have		didn't have		
6-2-21	0.065 ± 0.003	4.35%	0.06 ± 0	0%	8.33%
6-3	1.061 ± 0.011	1.03%	1.15 ± 0.0513	4.48%	-7.47%
7-1-1	didn't have		not done		
7-1-2	0.1401 ± 0.0266	19.0%	not done		
7-2-2	didn't have		not done		
7-2-3	0.5021 ± 0.0456	9.08%	not done		
8-1	8.947 ± 0.434	4.85%	15.17 ± 1.642	10.8%	-41.0%
8-2	0.641 ± 0.213	33.2%	0.5414 ± 0.0696	12.9%	18.4%
8-3	4.514 ± 0.5289	11.7%	8.890 ± 0.8235	9.26%	-49.2%
D-1-1	2.337 ± 0.0901	3.85%	3.433 ± 0.0823	2.40%	-31.9%
D-1-2	3.079 ± 0.1051	3.41%	3.431 ± 1.230	35.9%	-10.3%
D-1-3	1.421 ± 0.0006	0.04%	1.965 ± 0.1177	5.99%	-27.7%
D-2-1	4.404 ± 0.0637	1.45%	5.692 ± 0.0706	1.24%	-22.6%
D-2-2	2.740 ± 0.0544	1.99%	3.312 ± 0.3087	9.32%	-17.3%
D-2-3	1.170 ± 0.0072	0.62%	2.066 ± 0.6653	32.2%	-43.4%
D-3-1	2.176 ± 0.088	4.05%	2.795 ± 0.9538	34.1%	-22.2%
D-3-2	2.069 ± 0.091	4.41%	2.902 ± 0.1303	4.49%	-28.7%
D-3-3	1.609 ± 0.087	5.40%	1.985 ± 0.0992	5.00%	-18.9%
D-1-1 new[2]	2.851		3.433 ± 0.0823	2.40%	-17.0%
D-1-2 new[2]	3.830		3.431 ± 1.230	35.9%	11.6%
D-1-3 new[2]	1.691		1.965 ± 0.1177	5.99%	-13.9%

Appendix 3 (cont.)

Participants' Data as Reported by Them

D-2-1 new[2]	5.660	5.692 ± 0.0706	1.24%	-0.56%
D-2-2 new[2]	3.270	3.312 ± 0.3087	9.32%	-1.30%
D-2-3 new[2]	1.391	2.066 ± 0.6653	32.2%	-32.7%
D-3-1 new[2]	2.655	2.795 ± 0.9538	34.1%	-5.00%
D-3-2 new[2]	2.454	2.902 ± 0.1303	4.49%	-15.4%
D-3-3 new[2]	1.921	1.985 ± 0.0992	5.00%	-3.20%

[1] This value was not used in collective comparisons because there was only one value, there was poor filter replication at this site and we did not feel this value was truly representative of the fluorometer's capabilities.

[2] Data as reported for HPLC only was recalculated. For Laboratory 5, numbers were recalculated to incorporate a change in extraction volume from 5 to 5.7 ml. For Laboratory D, numbers were recalculated to incorporate a change in extraction volume from 5 to 5.7 ml and include chlide *a* in total chl *a*.

Appendix 4

Results of Participants' Extraction Methods Implemented at HPL

The code means: 1st number = laboratory code, 2nd number = site number, 3rd number = either bottle number, or in the case of Laboratory D, day.

	HPLC		Fluorometer		
Code	$\bar{x} \pm s$ (μg/l chl *a*)	% RSD	$\bar{x} \pm s$ (μg/l chl *a*)	% RSD	% discrepancy
1-1	26.74 ± 1.036	3.87%	didn't have		
1-2	239.2 ± 2.505	1.05%	didn't have		
1-3	0.8131 ± 0.0146	1.80%	didn't have		
1-4	10.37 ± 0.1888	1.82%	didn't have		
1-5	8.190 ± 0.1784	2.18%	didn't have		
3-1	not done		0.8882 ± 0.0649-HA 0.9450 ± 0.0383-GF/F	7.31% 4.06%	
3-2	not done		0.4808 ± 0.0132-HA 0.5840 ± 0.0283-GF/F	2.74% 4.84%	
3-3	not done		1.783 ± 0.0561-HA 1.788 ± 0.0871-GF/F	3.15% 4.87%	
4-1	7.005 ± 0.0919	1.31%	6.053 ± 0.4931	8.15%	-15.7%
4-2-9	0.1912		0.1454		31.5%
4-2-10	0.1138		0.1414 ± 0.0013	0.90%	-19.5%
4-2-11	0.1483		didn't have		
4-3	0.1936 ± 0.0082	4.26%	0.1949 ± 0.0126	6.47%	-0.67%
6-1-22	0.1365 ± 0.0049	3.63%	0.1228 ± 0.0017	1.38%	11.2%
6-1-21	0.1360		0.1119		21.5%
6-2-22	0.0640 ± 0	0%	0.0550 ± 0.0002	0.39%	16.4%
6-2-21	0.0630		0.0542		16.2%
6-3	0.9493 ± 0.0873	9.20%	1.033 ± 0.0693	6.71%	-8.10%
7-1-1	didn't have		0.1260 ± 0.0050	3.99%	
7-1-2	didn't have		didn't have		
7-2-2	didn't have		0.5835 ± 0.0040	0.69%	
8-1	8.341 ± 0.5444	6.53%	9.088 ± 0.1185	1.30%	-8.22%

Appendix 4 (cont.)

Results of Participants' Extraction Methods Implemented at HPL

8-2	0.391 ± 0.0782	20.0%	0.3463 ± 0.0242	6.99%	12.9%
8-3	5.259 ± 0.0273	0.52%	5.274 ± 0.0329	0.62%	-0.29%
D-1-1	2.485 ± 0.0687	2.76%			
D-1-2	3.269 ± 0.0552	1.69%	3.866 ± 0.2957	7.65%	-15.4%
D-1-3	1.379 ± 0.0128	0.93%	1.807 ± 0.0622	3.44%	-23.7%
D-2-1	5.243 ± 0.1356	2.59%	9.165 ± 1.432	15.63%	-42.8%
D-2-2	2.552 ± 0.1430	5.60%	3.571 ± 0.3350	9.38%	-28.5%
D-2-3	1.180 ± 0.1191	10.1%	1.528 ± 0.0735	4.81%	-22.8%
D-3-1	2.225 ± 0.1334	6.00%	3.216 ± 0.1104	3.43%	-30.8%
D-3-2	1.857 ± 0.1293	6.96%	2.845 ± 0.1054	3.70%	-34.7%
D-3-3	1.508 ± 0.0176	1.17%	1.968 ± 0.0636	3.23%	-23.4%

Appendix 5

% Difference Between Participants' and HPL Values for Each Instrument

Participants' data was 'as reported' and HPL data was derived using the standardized extraction technique. Participants' mean value was compared to HPL's mean value for that instrument (see Section 3.2 for a complete explanation). The code means: 1st number = laboratory code, 2nd number = site number, 3rd number = either bottle number, or in the case of Laboratory D, day.

Code	% difference HPLC	% difference fluorometer
1-1	2.53%	-16.50%
1-2	3.80%	-38.75%
1-3	-8.87%	10.44%
1-4	2.52%	-21.67%
1-5	7.72%	-5.61%
4-1	6.43%	28.25%
4-2-9		26.60%
4-2-10	75.38% - DAD 39.23% - FLD	36.73%
4-2-11	50.00% - DAD 6.52% - FLD	23.93%
4-3	15.45% - DAD 7.09% - FLD	24.37%
5-1-17	-22.00%	-11.85%
5-1-18	-42.37%	-5.63%
5-1-19	-40.00%	-19.24%
5-1-20	-44.64%	-24.18%
5-1-17 new[1]	-10.0%	
5-1-18 new[1]	-33.9%	
5-1-19 new[1]	-31.7%	
5-1-20 new[1]	-37.5%	
6-1-21	15.04%	12.36%
6-2-21	8.33%	7.33%
6-3	4.02%	11.17%

Appendix 5 (cont.)

% Difference Between Participants' and HPL Values for Each Instrument

8-1	-9.32%	43.04%
8-2	60.73%	26.73%
8-3	-25.91%	52.13%
D-1-1	-22.79%	
D-1-2	-19.22%	-22.08%
D-1-3	-10.04%	14.52%
D-2-1	-34.37%	-21.90%
D-2-2	-7.40%	6.73%
D-2-3	-7.36%	47.70%
D-3-1	-23.26%	-3.10%
D-3-2	-19.71%	11.27%
D-3-3	-10.74%	11.65%
D-1-1 new[1]	-5.81%	
D-1-2 new[1]	0.51%	
D-1-3 new[1]	7.10%	
D-2-1 new[1]	-15.7%	
D-2-2 new[1]	10.6%	
D-2-3 new[1]	10.1%	
D-3-1 new[1]	-6.38%	
D-3-2 new[1]	-4.77%	
D-3-3 new[1]	6.60%	

[1] Data as reported for HPLC only was recalculated. For Laboratory 5, numbers were recalculated to incorporate a change in extraction volume from 5 to 5.7 ml. For Laboratory D, numbers were recalculated to incorporate a change in extraction volume from 5 to 5.7 ml and include chlide *a* in total chl *a*.

Appendix 6

% Difference Between Results from Participants' Extraction Methods at HPL and the Standardized Extraction Method at HPL

For some laboratories with elevated % discrepancies, it was necessary to implement the participant's extraction method at HPL in an effort to determine the cause of the high % discrepancies. The participant's method was implemented at HPL and these values were compared to results when the standardized extraction technique was implemented, for the same instrument. The code means: 1st number = laboratory code, 2nd number = site number, 3rd number = bottle number.

Code	% difference HPLC	% difference fluorometer
4-1		-3.77%
4-2-9		3.41%
4-2-10		2.84%
4-3		-2.26%
4-1 [1]		16.9%
4-2-9 [1]		10.7%
4-2-10 [1]		16.3%
4-3 [1]		13.9%
8-1	-15.5%	-14.3%
8-2	-2.01%	-18.9%
8-3	-13.7%	-9.74%

[1] Laboratory 4's second set of data compares fluorometer values when the same extract (those from when the participant's procedures were implemented at HPL) was read using an acidification analysis method (on a 10-AU) and using a non-acidification analysis method (on a TD-700). Non-acidification values were compared to the acidification values. In all other fluorometric analyses, an acidification method was used.

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