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Volume 14, The First SeaWiFS HPLC Analysis Round-Robin Experiment (SeaHARRE-1)

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Preface

O ne of the primary objectives of the Sea-viewing Wide Field-of-view Sensor (SeaWiFS) Project, as stated in Volume 1 of the prelaunch *SeaWiFS Technical Report Series* (Hooker et al. 1992) is to "achieve radiometric accuracy of 5% absolute and 1% relative, water-leaving radiances to within 5% absolute, and chlorophyll *a* concentration to within 35% over the range of 0.05–50.0 mg m^{-3}." Much of the SeaWiFS Project's efforts have been devoted to meeting the rather stringent radiometric accuracy goals. The activities have included detailed prelaunch sensor calibration and characterization experiments, *in situ* measurement protocol definition and development, calibration round-robins, advanced instrument technology development, and advanced atmospheric correction algorithm development. The radiometric goals, rather than the chlorophyll *a* concentration objective, were set as the highest priority because these are challenging requirements and the ocean bio-optics community had never demonstrated an ability to make measurements at this level of accuracy. In addition, accurate *in situ* radiometry is required for the chlorophyll *a* algorithm.

More recently, the SeaWiFS Project has turned its attention to the uncertainties in the pigment measurements used for algorithm development and postlaunch validation because steady progress has been made in the radiometry arena and it has become clear that the pigment analyses are subject to more uncertainty than originally thought. The first SeaWiFS HPLC† Analysis Round-Robin Experiment (SeaHARRE-1) is the initial effort by the SeaWiFS Project at understanding the uncertainties in these measurements. The participation was international and SeaHARRE-1 lays the foundation for other round-robins, including the first Sensor Intercomparison and Merger for Marine Biological and Interdisciplinary Oceanic Studies (SIMBIOS) Project pigment round-robin, co-sponsored by the Office of Naval Research, which is being led by one of the SeaHARRE participants, Laurie Van Heukelem.

I would like to thank all of the SeaHARRE participants for volunteering their time and resources to this important activity. Their level of cooperation represents an excellent example of the community's enthusiasm and commitment to assisting the SeaWiFS Project in achieving our mission goals.

Greenbelt, Maryland   — C. R. McClain
26 June 2000

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† High Performance Liquid Chromatography

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Abstract

Four laboratories, which had contributed to various aspects of SeaWiFS calibration and validation activities, participated in the first SeaWiFS HPLC Analysis Round-Robin Experiment (SeaHARRE-1): Horn Point Laboratory (USA), the Joint Research Centre (Italy), the *Laboratoire de Physique et Chimie Marines* (France), and the Marine and Coastal Management group (South Africa). The analyses of the data are presented in Chapter 1 and the individual methods of the four groups are presented in Chapters 2–5. The average (or overall) conclusions of the round-robin are derived from 12 *in situ* stations occupied during a cruise in the Mediterranean Sea, although, only 11 stations are used in the analyses. The data set is composed of 12 replicates taken during each sampling opportunity with 3 replicates going to each of the 4 laboratories. The average (or overall) results from the intercomparison of 15 pigments or pigment associations are as follows (in some cases, data subsets that exclude pigments which were not analyzed by all the laboratories, or that had unusually large variances, are used to exclude a variety of problematic pigments): a) the accuracy of the four methods in determining the concentration of total chlorophyll a is 7.9% (one method did not separate mono- and divinyl chlorophyll a, and if the samples containing significant divinyl chlorophyll a concentrations are ignored, the four methods have an accuracy of 6.7%); b) the accuracy in determining the full set of pigments is 19.1%; c) there is a reduction in accuracy of approximately -12.2% for every decade (factor of 10) decrease in concentration (based on a data subset); d) the precision of the four methods using a subset data is 8.6% (6.2% for an edited subset); e) the repeatability of the four methods using the subset data is 9.2% (7.2% for an edited subset); and f) the reproducibility of the four methods using the subset data is 21.3% (15.0% for an edited subset).

PROLOGUE

The SeaWiFS Project is tasked with executing a program to acquire the global SeaWiFS data set, validate and monitor its accuracy and quality, process the radiometric data into geophysical units using a set of atmospheric and bio-optical algorithms, and distribute the final products to the scientific community through the Goddard Space Flight Center (GSFC) Distributed Active Archive Center (DAAC). One of the primary calibration and validation objectives of the Project is to achieve radiometric accuracy to within 5% absolute and 1% relative, water-leaving radiances to within 5% absolute, and chlorophyll a concentration to within 35% over the range of 0.05–50.0 mg m^{-3} (Hooker and Esaias 1993). The two principal *in situ* data sets for these requirements are measurements of the in-water light field and estimates of near-surface pigment concentrations (Hooker and McClain 2000).

The Project has engaged in a concerted effort to determine and then reduce the uncertainties in the optical measurements, and has relied on field campaigns and SeaWiFS Intercalibration Round-Robin Experiments (SIRREXs) to meet the radiometric objectives (Hooker and Maritorena 2000). The successful results achieved with the optical round-robins suggested a similar approach might be useful for quantifying and reducing the variance in pigment data. The Project has relied on the Joint Global Ocean Flux Study (JGOFS) protocols (JGOFS 1991) for water sampling methods (Hooker et al. 1992) and the HPLC technique for pigment assessment, so the process began with identifying laboratories following the JGOFS principles for HPLC analysis (JGOFS 1994).

The starting point for the evaluation of the results is the SeaWiFS Project pigment objective of agreement to within 35%. This value is based on inverting the optical measurements to derive pigment concentrations using a bio-optical algorithm, so the *in situ* pigment observations will always be one of two axes to derive or validate the pigment relationships. Given this, it seems appropriate to reserve approximately half of the uncertainty budget for the *in situ* pigment measurements. Because the sources of uncertainty combine independently (i.e., in quadrature), an upper accuracy range of 20–25% is probably acceptable, although 15% would allow for significant improvement in algorithm refinement.

Four laboratories that had contributed to various aspects of SeaWiFS calibration and validation activities participated in the HPLC round-robin:

1. The American University of Maryland Center for Environmental Science (UMCES) Horn Point Laboratory (HPL),

2. The European Joint Research Centre (JRC) Marine Environment Unit of the Space Applications Institute,

3. The French *Laboratoire de Physique et Chimie Marines* (LPCM) which is part of the *Observatoire Océanologique de Villefranche-sur-Mer*, and

4. The South African Marine and Coastal Management (MCM) group.

Each laboratory was assigned a one-letter identification code according to H for HPL, J for JRC, L for LPCM, and M for MCM.

The focus of this intercomparison study is the estimation of *in situ* pigment concentrations using HPLC techniques. In the results reported here, different methodologies from four research groups were applied to a set of replicate water samples to evaluate the degree to which procedural differences between laboratories influence the estimation of pigment concentration, and whether any general improvements can be made.

The overall results of SeaHARRE-1 are presented in Chapter 1 and the individual methods of the four groups are presented in Chapters 2–5, respectively. The science team is presented in Appendix A. A summary of the material presented in each chapter is given below.

1.   SeaHARRE-1

The focus of this study was the round-robin intercomparison of a variety of chromatographically determined pigments from four HPLC methods (and laboratories). The primary difference between the methods was one of the laboratories did not separate divinyl chlorophyll a from monovinyl chlorophyll a. A total of 15 pigments or pigment associations were intercompared. The data were separated into three concentration (or trophic) regimes based on the total chlorophyll a concentration (C_{Ta} in milligrams per cubic meter) in the original water samples: eutrophic (ET), $C_{Ta} > 1$; mesotrophic (MT), $0.1 \leq C_{Ta} \leq 1$; and oligotrophic (OT), $C_{Ta} < 0.1$.

The average percent difference for all pigments showed some sensitivity to the concentration regimes, but a general increase in percent differences with decreasing trophic level was obscured by higher percent differences from pigments with very low concentrations, particularly in the ET regime. When the very low concentration pigments of the ET regime were removed, there was an average increase in percent differences with decreasing trophic level: 13.8% (ET), 18.3% (MT), and 32.1% (OT). In terms of total chlorophyll a concentration, the average percent differences for the four methods were all within 13% for the three regimes, and the average percent difference for all four methods was 7.9%. Although these results are well within the SeaWiFS Project requirements for pigment determination, they could be decreased farther if all of the laboratories separated divinyl chlorophyll a from monovinyl chlorophyll a; a comparison of total chlorophyll a determinations for samples with insignificant divinyl chlorophyll a levels was at the 6.7% level.

2.   The HPL Method

The HPL method was developed for use with a variety of water types ranging from freshwater lakes, to estuarine and oligotrophic oceanic samples. As such, pigments important to all these systems are baseline resolved and quantitatively reported, including divinyl and monovinyl chlorophyll a. The method can accommodate samples ranging in concentration by up to 400 fold without changes to system set up or calibrations. The method employs the use of a C_8 HPLC column in combination with a methanol-based, reversed phase binary gradient with a simple linear gradient and elevated column temperature ($60°C$). The method is relatively fast, well suited to automated analyses, and can provide quantitative information for up to 20 pigments with qualitative information for additional pigments. The average method variability is approximately 2% (estimated from data gathered from approximately 300 standard injections and 14 chlorophyll a calibration curves from 4 different columns).

3.   The JRC Method

The HPLC method used by the JRC follows the JGOFS core measurements protocols (JGOFS 1994) and is a modified version of the method presented by Wright et al. (1991). It does not permit the separation of divinyl chlorophyll a and divinyl chlorophyll b from chlorophyll a and chlorophyll b, respectively. The method was designed for coastal Adriatic Sea waters only, and because prochlorophytes are not found in coastal areas, this has not been a relevant disadvantage. Filter disruption is accomplished mechanically using a motorized grinder. The pigments are extracted within a 100% acetone solution including an internal standard (*trans-β*-apo-8′-carotenal). The HPLC system used includes a reversed phase C_{18} column (with a C_{18} guard column), an autosampler (with thermostat), a diode array detector (DAD), a fluorescence detector, and a three-solvent gradient. The JRC method provides measurements of the main pigment concentrations with a detection limit of approximately $0.001\,\mu g\,L^{-1}$, a repeatability (based on the analysis of several samples from the same water volume) of about $7(\pm 6)\%$ for chlorophyll a concentration (Zibordi et al. 2000), and an analysis time of about 35 min.

4.   The LPCM Method

The LPCM method applies a sensitive reversed phase HPLC technique for the determination of chloropigments and carotenoids within approximately 24 min. The different pigments are detected by a DAD which allows for automatic identification to be carried out on the basis of absorption spectra. Optical densities are monitored at 440 nm (chloropigments and carotenoids) and at 667 nm (chloropigments only). The method provides a good resolution between divinyl chlorophyll a and chlorophyll a, but uncertainties may arise for the partial separation of chlorophyll b and divinyl chlorophyll b, and for the resolution of chlorophyll c pigments. Detection limits for most pigments are low (approximately $0.001\,mg\,m^{-3}$). The use of an internal standard has shown to improve the accuracy of the analysis.

5.   The MCM Method

The MCM method uses a reversed phase HPLC technique using a binary solvent system following a linear gradient on a C_8 chromatography column. Baseline separation of mono- and divinyl chlorophyll a and of lutein

and zeaxanthin, partial separation of mono- and divinyl chlorophyll b, and resolution of other key chemotaxonomic chlorophylls and carotenoids are achieved in an analysis time of approximately 30 min. The method provides good resolution of mono- and divinyl chlorophylls a and b, as well as lutein and zeaxanthin, and satisfactorily separates other key pigments within approximately 30 min. The use of a canthaxanthin internal standard improves the accuracy of pigment determinations. Providing a pragmatic balance between good analyte† resolution and acceptable sample throughput, the method is suitable for the analysis of a wide range of oceanographic water samples.

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† The substance being measured.

Chapter 1

SeaHARRE-1

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ABSTRACT

The focus of this study was the estimation of a variety of chromatographically determined pigments from four HPLC methods (and laboratories). The primary difference between the methods was one of the laboratories did not separate divinyl chlorophyll a from monovinyl chlorophyll a. A total of 15 pigments or pigment associations were intercompared. The data were separated into three concentration (or trophic) regimes based on the total chlorophyll a concentration (C_{Ta} in mg m^{-3}) in the original water samples: ET, $C_{Ta} > 1$; MT, $0.1 \leq C_{Ta} \leq 1$; and OT, $C_{Ta} < 0.1$. The average percent difference for all pigments showed some sensitivity to the concentration regimes, but a general increase in percent differences with decreasing trophic level was obscured by higher percent differences from pigments with very low concentrations, particularly in the ET regime. When the very low concentration pigments of the ET regime were removed, there was an average increase in percent differences with decreasing trophic level: 13.8% (ET), 18.3% (MT), and 32.1% (OT). In terms of total chlorophyll a concentration, the average percent differences for the four methods were all within 13% for the three regimes, and the average percent difference for all four methods was 7.9%. Although these results are well within the SeaWiFS Project requirements for pigment determination, they could be decreased farther if all of the laboratories separated divinyl chlorophyll a; a comparison of total chlorophyll a determinations for samples with insignificant divinyl chlorophyll a levels was at the 6.7% level.

1.1 INTRODUCTION

The filtered seawater used for this exercise was collected during the *Productivité des Systèmes Océaniques Pélagiques* (PROSOPE) cruise† which took place between 4 September and 4 October 1999. The cruise started in the high productivity upwelling off the northwestern coast of Africa (Agadir, Morocco), continued through the lower productivity of the western Mediterranean and Ionian Seas (as far east as southwest of Crete), and ended at an offshore site in the Ligurian Sea (near La Seyne, France), as shown in Fig. 1.

The seawater was collected from 12 L Niskin bottles during conductivity, temperature, and depth (CTD) measurements (except on one occasion for which the seawater was collected from a pumping system deployed to different depths). Because the objective was to collect 12 replicates

at each sampling opportunity with 3 replicates going to each of the four laboratories, one set of 12 replicates is referred to here as a *batch*. Each laboratory analyzed the replicates and reported the pigment concentrations as per their normal practices.

The filtered volume varied between 1.0–2.8 L per filter, depending on the concentration of particles seen on the filters during the filtration process. The water budget for each bottle did not permit replicate sampling under many circumstances, so the water from at least four bottles was frequently mixed to yield the required amount of water (the number of bottles depended on the filtering requirements). Seawater was filtered onto 25 mm glass fiber GF/F (0.7 µm) filters, and immediately stored in liquid nitrogen once filtration was completed. Three replicates (hereafter referred to as triplicates) were collected for each laboratory, so 12 replicates were collected for each of the 12 batches (except only 10 were collected for batch 6), giving a total of 142 replicates.

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† Detailed information about the PROSOPE cruise is available from `http//www.obs-vlfr.fr`.
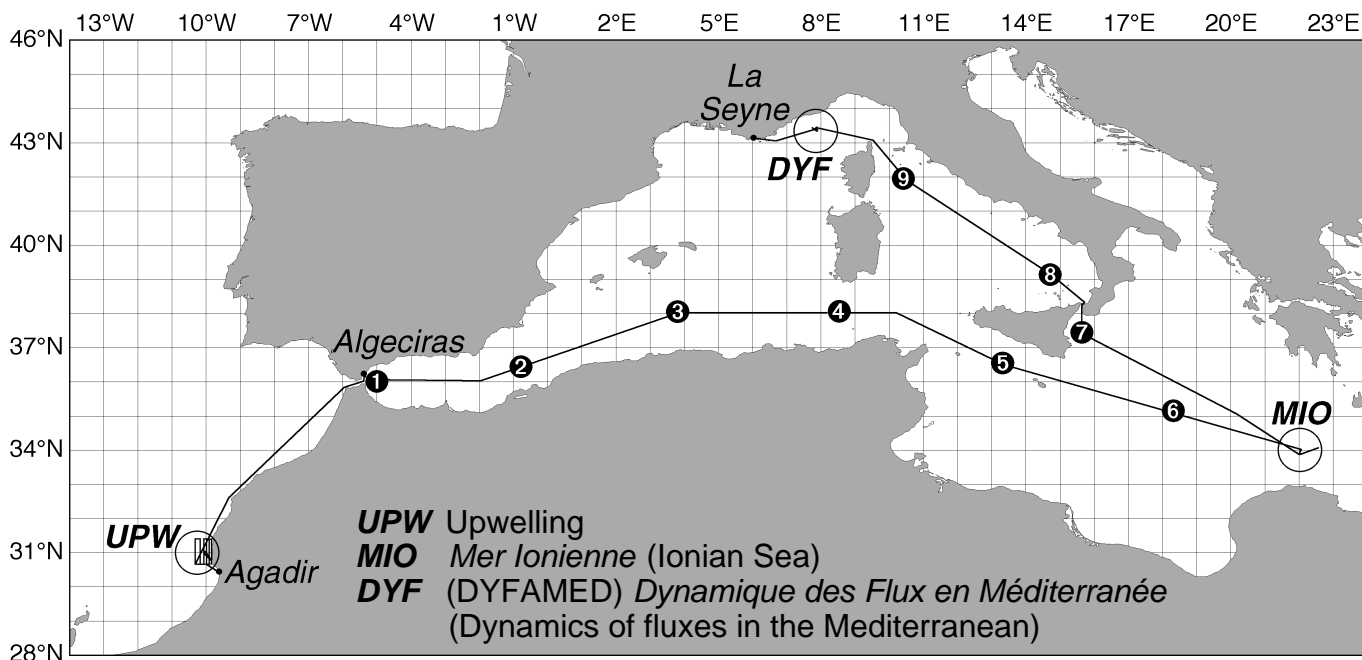
Fig. 1. The PROSOPE cruise track showing the three long (circled) stations, which lasted a few days each, and the nine short along-track (SAT) stations (numbered bullets), which lasted one day each. All of the seawater was collected in the Mediterranean Sea except a little was collected in the upwelling off the northwest African coast.

Table 1. The characteristics of the replicate batches and the sequential day of the year (SDY) they were collected. The original identification (ID) labels are followed by the station names and the final sample codes (which are a combination of a one-letter station code and a one-digit identifier). The CTD depths are in meters, the volume of water filtered is given in liters, and the number of replicates is given in the last column.

Batch	SDY (Date)	ID	Station	Code	CTD	Depth(s)	Volume	No.
1	252 (9 Sep.)	UPW001	UPW1	U1	2	0, 5, 30, 40	2.0	12
2	254 (11 Sep.)	UPW002	UPW3	U3	P1†	5, 10	1.0	12
3	260 (17 Sep.)	CTD021	SAT4	S4	21	45, 75	2.8	12
4	264 (21 Sep.)	MIO002	MIO2	M2	39	100	2.8	12
5	266 (23 Sep.)	MIO004	MIO4	M4	55	5	2.8	12
6	270 (27 Sep.)	INT006	SAT8	S8	67	70, 83, 90	2.8	10
7	271 (28 Sep.)	INT007	SAT9	S9	69	75	2.8	12
8	272 (29 Sep.)	INT008	DYF1	D1	72	47–50	2.8	12
9	274 (1 Oct.)	CTD091	DYF3	D3	91	15	2.8	12
10	275 (2 Oct.)	DYF004	DYF4	D4	99	60	2.8	12
11	276 (3 Oct.)	DYF025	DYF5	D5	103	25	2.8	12

† The U3 sample was taken from a diaphragm pump deployed at different depths.

After receiving the replicates, each laboratory extracted and analyzed them using their own particular analytical method. The results from all four laboratories were supplied by the end of February 2000 to LPCM where the data were processed. A single analyst reviewed the data, applied any quality control (QC) procedures to validate the replicates in each batch, and made the final determination of the individual pigment concentrations, C, for each *sample*. Each sample, then, is a unique determination of the pigment concentrations at a particular geolocation (and point in the water column) for the cruise, and is derived from the number of valid replicates within each batch. In most cases, the individual concentrations were the average of the available replicates (usually three), but in some cases, replicates were removed from the averaging process because of QC constraints.

1.2 THE DATA SET

Table 1 summarizes the particulars of each batch of replicates giving the date when the replicates were collected, the original sample identifier, the station designa-

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tor, and the resulting sample code. In the station designator, a three-letter sequence and a number are used to identify the station: SAT for the short along-track stations, UPW for the (eutrophic) African upwelling, MIO for the (oligotrophic) eastern Mediterranean, and DYF for the (mesotrophic) coastal station. Also presented is the CTD cast number, the depths of the bottles used, and the volume of water filtered.

Because of a faulty (liquid nitrogen) shipping container, a so-called *dry shipper*, 3 of the 12 replicates for each batch were defrosted (except CTD106) for about half a day and were considered as potentially *bad*. These replicates were redistributed, so each lab could have at least two *good* replicates, i.e., properly stored during transportation. The MCM replicates had already been shipped before this problem occurred, so the MCM laboratory was the only one to receive and analyze three *good* replicates from each batch. Because several different kinds of problems can occur during storage and shipment—particularly during international campaigns which expose the filters to unknowns during customs clearance—the *bad* replicates were included in batches, so the variance associated with mistreated filters could be quantified.

When the replicates were shipped to the participating laboratories, the CTD106 batch was inadvertently not distributed to the MCM laboratory. Given the remote location involved (South Africa), the problem already encountered with a faulty dry shipper, and the fact that most groups were participating without additional funding, the decision was made to accept the consequences of the undistributed CTD106 batch and not work out yet another distribution of the samples. For the purposes of this analysis, the CTD106 batch was deleted from the primary analysis (the CTD106 batch does not appear in Table 1), yielding a total of 11 batches of replicates to provide 11 samples of pigment concentrations.

Although each laboratory determined pigment concentrations using HPLC methods, none of the laboratories used exactly the same procedures as another. Table 2 summarizes the main conditions for extraction for each laboratory. Individual differences in the methods were also seen in the analytical equipment and the solvent systems used by each laboratory, which are summarized in Tables 3 and 4, respectively†.

In some parts of this document, the United Nations Educational, Scientific, and Cultural Organization (UNESCO) Scientific Committee on Oceanographic Research (SCOR) Working Group (WG) 78 abbreviations are used for pigment presentations (Appendix B), but the majority of the analysis results are presented using a more compact

symbology. The symbols used to indicate the concentration of the major pigments or pigment associations, which were reported by all of the laboratories, are as follows:

C_P Peridinin,

C_B 19′-butanoyloxyfucoxanthin,

C_F Fucoxanthin,

C_H 19′-hexanoyloxyfucoxanthin,

C_D Diadinoxanthin,

C_Z Zeaxanthin,

C_{Ta} Total chlorophyll a (chlorophyll a plus divinyl chlorophyll a),

C_{Ca} Chlorophyllide a

$C_{\Sigma a}$ Sum chlorophyll a (the sum of total chlorophyll a and chlorophyllide a),

C_{Tb} Total chlorophyll b (chlorophyll b plus divinyl chlorophyll b), and

C_{12} Chlorophyll c_{1+2}.

Note that this nomenclature is used to represent the final pigment concentrations for each sample—they do not represent the concentrations associated with the individual replicates that were used to determine the final sample value.

Some of the laboratory methods permitted the separation of monovinyl chlorophyll a from divinyl chlorophyll a (H, L, and M) and monovinyl chlorophyll b from divinyl chlorophyll b (H and M). The primary reason for these differences in capability was the normal operational requirements of the laboratories—a laboratory supporting field work in an area not requiring the distinction (e.g., in Case-2 conditions) did not have such a capability. The laboratories capable of making the divinyl separation produced the following additional pigment concentrations:

C_a Chlorophyll a,

C_{Da} Divinyl chlorophyll a,

C_b Chlorophyll b, and

C_{Db} Divinyl chlorophyll b.

It is important to remember the total chlorophyll a and b concentrations can be overestimated if the divinyl separation is not made (Latasa et al. 1996), and a wavelength is not used where both chlorophylls give equal response.

The choice of pigments was determined by their usefulness to calibration and validation activities, particularly those involved with the accurate determination of $C_{\Sigma a}$, and their recurring use as taxonomic indicators (or *biomarkers*) for phytoplankton populations. The general taxonomic significance of the most abundant pigments detected in this exercise are as follows:

C_B Pelagophytes, chrysophytes, and prymnesiophytes;

C_H Prymnesiophytes;

C_P Dinoflagellates;

C_F Diatoms;

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† Identification of commercial equipment to adequately specify or document the experimental problem does not imply recommendation or endorsement, nor does it imply that the equipment identified is necessarily the best available for the purpose.

Table 2. A summary of the extraction specifications for each of the four laboratories (or methods). The volume of solvent added is given in milliliters. Each filter was disrupted for the indicated number of seconds, allowed to soak for the specified number of hours, and then clarified.

Lab. Code	Volume Added	Extraction Solvent	Internal Standard	Mode and Time of Disruption		Soak Time [h]	Clarification
H	3.0	95% Acetone	None	Ultra-sonification	$\sim 60\,\mathrm{s}$	4.0	0.45 μm Teflon™ Syringe Filter
J	1.5	100% Acetone	trans-β-apo-8′-carotenal	Grinder	30 s	24.0	0.45 μm Teflon Syringe Filter
L	3.0	100% Methanol	trans-β-apo-8′-carotenal	Ultra-sonification	$\leq 10\,\mathrm{s}$	1.0†	GF/C 1.3 μm Filter
M	2.0	100% Acetone	Canthaxanthin	Ultra-sonification	30 s	0.5†	Centrifugation (10 min)

† Minimum time.

Table 3. The HPLC column characteristics, mode of detection, and injection conditions for each laboratory. The indicated sample volume was mixed with buffer (as specified) prior to injection. The Hewlett-Packard (HP) and Thermo Separations Products (TSP) DAD specifications are given in the last column.

Lab. Code	Buffer	Injection Volume	Column Length	Column Diameter	Particle Size	Phase	DAD Detector
H	28 mM TBAA‡	150 μL	150 mm	4.6 mm	3.5 μm	Reversed C$_8$	HP 1100 at 450 nm [1]
J	1.0 M Ammonium Acetate	97.5	250	4.6	5.0	Reversed C$_{18}$	HP 1100 at 436 nm [2]
L	1.0 M Ammonium Acetate	133	100	3.0	3.0	Reversed C$_8$	HP 1100 at 440 nm [1] [3]
M	1.0 M Ammonium Acetate	100	100	4.6	3.0	Reversed C$_8$	TSP UV 6000 at 440 nm [1]

‡ Tetrabutyl ammonium acetate.   [1] And DAD at 665 nm.   [2] And DAD at 405 nm.   [3] And fluorometer.

Table 4. A summary of the solvent systems used by each laboratory (a blank entry means the indicated solvent is not used). The flow rate is given in milliliters per minute.

Lab. Code	Flow Rate	Solvent A	Solvent B	Solvent C
H	1.1	70:30 Methanol:28 mM aqueous TBAA	100% Methanol	
J	1.0	80:20 Methanol:0.5 M Ammonium Acetate	90:10 Acetonitrile:Water	100% Ethyl Acetate
L	0.5	70:30 Methanol:0.5 M Ammonium Acetate	100% Methanol	
M	1.0	70:30 Methanol:1.0 M Ammonium Acetate	100% Methanol	

C_Z Cyanobacteria and prochlorophytes;

C_b Green flagellates;

C_{Da} Prochlorophytes; and

C_{Db} Prochlorophytes.

The remaining pigments were selected based on maximizing the number of pigments that could be compared.

1.3 DATA ANALYSIS METHODS

This study used field data for the intercomparison exercise, and the assumption made here is that each laboratory participated as if the analyses were performed as a result of normal operations—that is, a single concentration value was reported for each laboratory for each batch of replicates (which constituted a sample), even though multiple (2 or 3) replicates were analyzed by each laboratory within each batch. The solitary (or sample) concentrations were usually the averages of the individual replicates analyzed for each batch.

In the analytical approach adopted here, no one laboratory (or result) is assumed to be more correct than another—there is no absolute truth, because standards were not part of the sample set, so an unbiased approach is needed to compare the various methods. The first step in developing an unbiased analysis is to calculate the average concentration, $\bar{C}$, for each pigment from each sample as a function of the four contributing laboratories:

$$\bar{C}_{P_i} = \frac{1}{4}\sum_{j=1}^{4} C_{P_i}^{L_j}, \qquad (1)$$

where the subscript P_i identifies the pigment or pigment association (following the symbology established in Sect.

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1.2), and L_j is the laboratory (or method) code. In (1), the i index represents an arbitrary ordering of the pigments, and the j index under the summation symbol is used for summing over the four possible laboratory (or method) codes. Although any ordering for the pigments and laboratories is permissible, the former are ordered following their presentation in Sect. 1.2; for the latter, $j = 1, 2, 3$, and 4 corresponds to the H, J, L, and M laboratories, respectively (which is based on a simple alphabetic ordering of their names).

The unbiased percent difference (UPD), ψ, for each pigment of the individual laboratories with respect to the average values are then calculated for each sample as

$$\psi_{P_i}^{L_j} = 100 \, \frac{C_{P_i}^{L_j} - \bar{C}_{P_i}}{\bar{C}_{P_i}}. \tag{2}$$

Note that a positive ψ value indicates the pigment concentration for a particular laboratory was greater than the average for that pigment (a negative value indicates the pigment concentration for a particular laboratory was less than the average for that pigment). Although $\bar{C}_{P_i}$ is not considered truth, it is the reference value by which the performance of the methods with respect to one another are quantified.

The absolute UPD values, $|\psi|$, are averaged over the number of samples (N) to give the average percent difference of each laboratory for each pigment across all the samples:

$$\bar{\psi}_{P_i}^{L_j} = \frac{1}{N} \sum_{k=1}^{N} |\psi_{P_i}^{L_j}(S_k)|, \tag{3}$$

where S_k is the kth sample code (Table 1) associated with pigment P_i. When considering the entire data set, $N = 11$ (the total number of samples). Absolute values are used in the overall averages, so positive and negative ψ values do not cancel out and artificially lower the average difference. The latter is particularly important for pigments with low concentration values, but also in terms of a general philosophy: the primary measure of dispersion between the methods are the ψ values, so it is important to ensure they are not underestimated.

Changes in trophic regimes are considered, in a broad sense, to be a function of pigment concentration, i.e., lower productivity and pigment concentrations are found in oligotrophic conditions, and higher productivity and pigment concentrations are found in eutrophic conditions. For the analysis presented here, three concentration regimes are considered based on the concentration of total chlorophyll a (C_{Ta} in milligrams per cubic meter) at the point of sampling (i.e., not in terms of a vertically integrated description of the water column or a productivity description of the sampling station): ET, $C_{Ta} > 1$; MT, $0.1 \leq C_{Ta} \leq 1$; and OT, $C_{Ta} < 0.1$.

This partitioning is somewhat arbitrary, but the primary reason for doing so is to allow the results to be discussed within a simple and easily understood framework

associated with a key bio-optical parameter. This is important for several reasons:

a) Investigators working in other regions can make use of those results in keeping with their concentration regimes (i.e., the OT analysis results should be applicable to any group working in low concentration waters);

b) Pigment concentration is a fundamental parameter for describing many aspects of oceanic biology, so it provides a common reference for the wider application of the results; and

c) The low pigment concentration data frequently need to be discussed separately, because the signal-to-noise ratio (SNR) for these data are usually low and the percent differences can be anomalously large (which causes problems when average results are considered).

If the UPD values are computed for concentration regimes, (3) is used with N representing the number of samples in the concentration regime. In these instances, the ET, MT, and OT codes are used as regime labels to indicate the eutrophic, mesotrophic, and oligotrophic concentration regimes, respectively.

Another useful parameter is the average of the $\bar{\psi}$ values for a particular pigment across the four laboratories (or methods):

$$\bar{\psi}_{P_i}^{A} = \frac{1}{4} \sum_{j=1}^{4} \bar{\psi}_{P_i}^{L_j}, \tag{4}$$

where the A code indicates all the laboratories were averaged. This parameter can also be calculated for each concentration regime by using the $\bar{\psi}_{P_i}^{L_j}$ values appropriate to the regime in question.

1.4 RESULTS

The comparison of the pigments and pigment associations begins with the C_{Ta} estimates, and is followed by the remaining pigments discussed in Sect. 1.2. The reason for this is the chlorophyll a results are the most important to the satellite calibration and validation activities which motivated the HPLC round-robin. Because the determination of chlorophyll a concentration involves the separation of monovinyl and divinyl components, there is a separate inquiry into the importance of performing this separation, and how that affects the comparison results. To include an understanding of the importance of concentration on the intercomparisons, the results are categorized following the eutrophic, mesotrophic, and oligotrophic concentration regimes discussed earlier.

1.4.1 Total Chlorophyll a

The UPD values for total chlorophyll a, ψ_{Ta}, for the HPL, JRC, LPCM, and MCM laboratories (or methods)

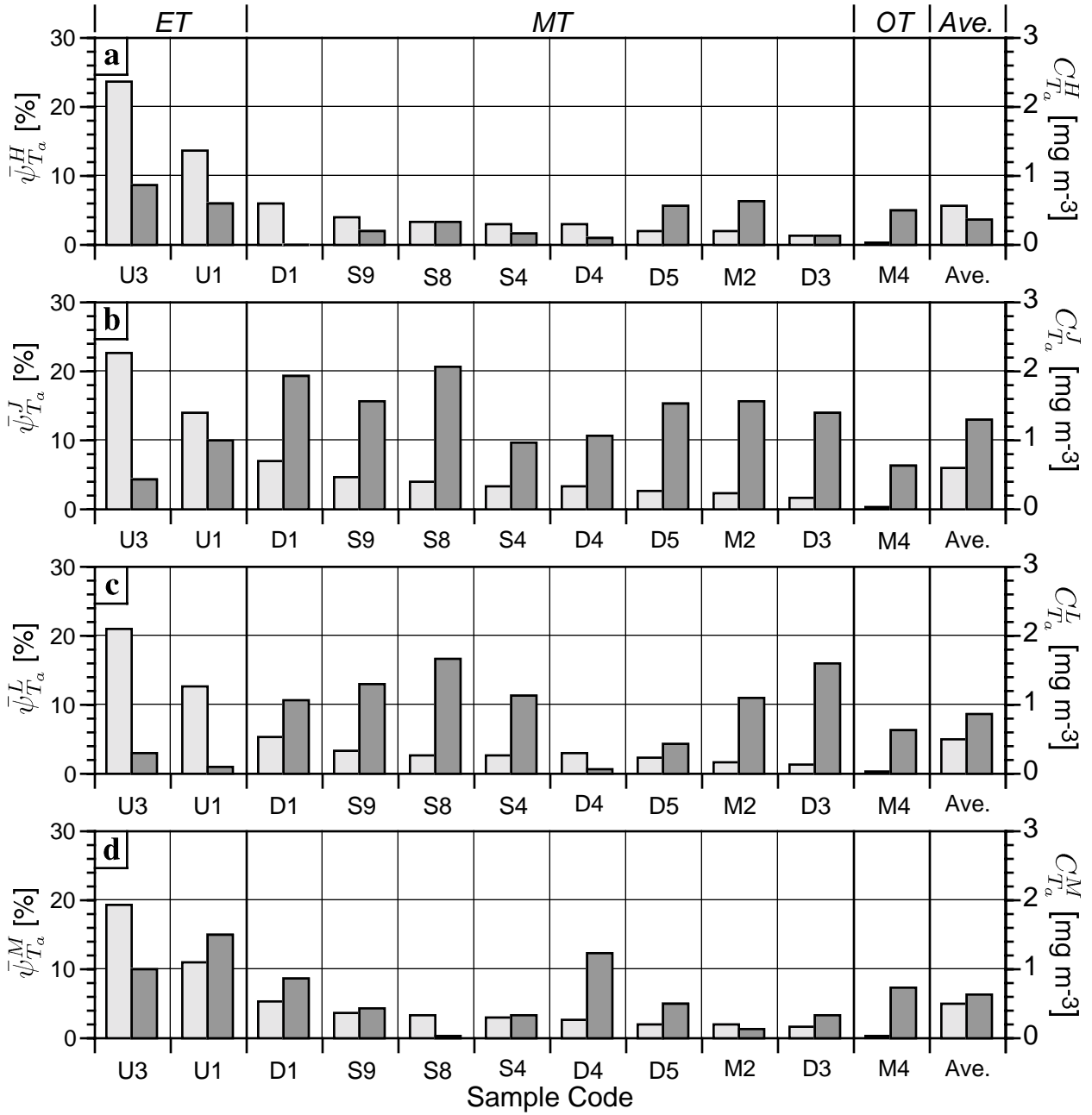**Fig. 2.** The ψ_{T_a} values (dark right columns) shown with C_{T_a} estimates (light left columns) for the four laboratories: a) HPL, b) JRC, c) LPCM, and d) MCM. Average UPD and concentration values for each laboratory are given in the right-most columns. The bold sample codes indicate negative ψ_{T_a} values.

as a function of the C_{T_a} estimates for each sample code are presented in Figs. 2a–2d, respectively. The data are presented from highest (left) to lowest (right) concentration, as determined by the overall concentration average, and are also grouped according to the larger-scale concentration regimes shown along the top-most panel. Some general aspects in the differences between the four methods are easily discerned:

1. All of the differences are within the 20–25% pigment intercomparison objective, and most of the

differences are within the 15% algorithm refinement objective;

2. The J MT results exhibit the largest ψ_{T_a} values, and the J method almost always overestimates C_{T_a} (the only exception is the OT sample);

3. The L method usually underestimates C_{T_a}; and

4. The MT regime has the largest ψ_{T_a} values.

The last result is a little unexpected, because from the perspective of the SNR of the data, the worst results would

be expected for the OT regime. The persistent above and below estimation for the JRC and LPCM methods suggests deterministic differences with these methods. A summary of the Fig. 2 data as a function of the concentration regimes and the overall average is presented in Table 5.

Table 5. The $\bar{\psi}_{T_a}$ values (in percent) for the three trophic regimes. The last column gives the average percent data for all the data—no partitioning into trophic regimes, and the last row gives the average for the four laboratories.

Method	ET	MT	OT	All
H	7.5	2.7	5.1	3.8
J	7.1	15.2	6.3	12.9
L	2.1	10.5	6.3	8.6
M	12.5	4.9	7.4	6.5
A	7.3	8.3	6.3	7.9

The most important results of the C_{T_a} analysis are as follows:

a) If the ET, MT, and OT concentration regimes are considered, the lowest $\bar{\psi}_{T_a}$ values are 2.1 (LPCM), 2.7 (HPL), and 5.1% (HPL), and considering all the data as one group, the HPL method has the lowest $\bar{\psi}_{T_a}$ value (3.8%);

b) The $\bar{\psi}_{T_a}$ values for the H, J, L, and M methods across all the data are within 13% (3.8, 12.9, 8.6, and 6.5%, respectively);

c) The average $\bar{\psi}_{T_a}$ for all the data and methods ($\bar{\psi}_{T_a}^A$) is 7.9%;

d) The average results are not very sensitive to the concentration regimes (the $\bar{\psi}_{T_a}^A$ values for the ET, MT, and OT regimes across all four methods are 7.3, 8.3, and 6.3%, respectively); and

e) For all of the possible summaries—either in terms of laboratory averages, concentration regime averages, or global averages (across all methods and all regimes)—the average agreement is to within less than the 20–25% pigment intercomparison objective, as well as to within less than the 15% algorithm refinement objective.

The latter is the most important, because it shows all four laboratories intercompare at a level in keeping with ocean color calibration and validation requirements.

A cautionary aspect for the the C_{T_a} analysis needs to be remembered. Figure 2 shows the three concentration regimes were not equally sampled (2 ET samples, 8 MT samples, and only 1 OT sample). The uneven sampling distribution places a burden on interpreting the importance of some of the results. As an example, the Table 5 results show the OT data have the smallest average differences and the least variability, as measured by the range in differences between the maximum and minimum values,

but the poor sampling distribution makes the range in differences suspect. The sampling distribution represents an important deficiency in the execution of the round-robin, but a thorough and complete sampling scheme was beyond the scope of the voluntary (mostly unfunded) participation of the four laboratories.

1.4.2 Mono- and Divinyl Chlorophyll a

Figure 3 presents the data in Fig. 2 in a different format: the absolute value of the UPD values for each laboratory are presented together, so the relationships between the methods can be more easily discerned. The recurring larger differences associated with the JRC method are very evident. This result deserves additional consideration, because the JRC method does not separate the monovinyl and divinyl chlorophyll a components.

The samples for which the $\psi_{T_a}^J$ results exceeded the ψ_{T_a} values for the other laboratories are D1, S9, S8, D5, and M2. Using the results from the three laboratories that separate divinyl chlorophyll a, the samples where C_{D_a} was more than 10% of C_{T_a} were D1, S9, S8, S4, D5, M2, and D3. In other words, all of the anomalously high $\psi_{T_a}^J$ values correspond to samples with significant divinyl chlorophyll a concentrations (significant here is in terms of the level of differences being investigated). If the C_{T_a} results are compared for when C_{D_a} was not a significant part of C_{T_a}, $\bar{\psi}_{T_a}^A = 6.7\%$—the four methods intercompare at less than the 7% level.

1.4.3 Complete Pigment Intercomparisons

Figure 4 presents the $\bar{\psi}$ values for all the pigments or pigment associations as a function of the average concentration. In this case, no partitioning of the data according to concentration regimes is included in the analysis—all of the data are taken as independent samples. In general, there is a strong inverse correlation between concentration levels and percent differences: the pigments with high concentration levels have low percent differences, i.e., the SNR appears to determine a significant part of the agreement. The C_P, C_B, C_H, C_D, and C_Z values are all below $0.1\,\mathrm{mg\,m^{-3}}$, and they all have elevated $\bar{\psi}$ values. There are exceptions to this relationship, but they usually involve pigments that were not analyzed by all the laboratories, e.g., C_{D_a} and C_{C_a}.

In terms of method comparisons, the smallest $\bar{\psi}$ values are usually associated with the HPL results, whereas the largest are most frequently associated with the MCM results; the JRC and LPCM results are usually comparable. This is seen in the individual pigments and in the average pigment ($\bar{C}$) results. On average, and in terms of the majority of the individual results, the percent differences are within the 20–25% pigment intercomparison objective. Most importantly, almost all of the chlorophyll a results are within the 15% algorithm refinement objective.
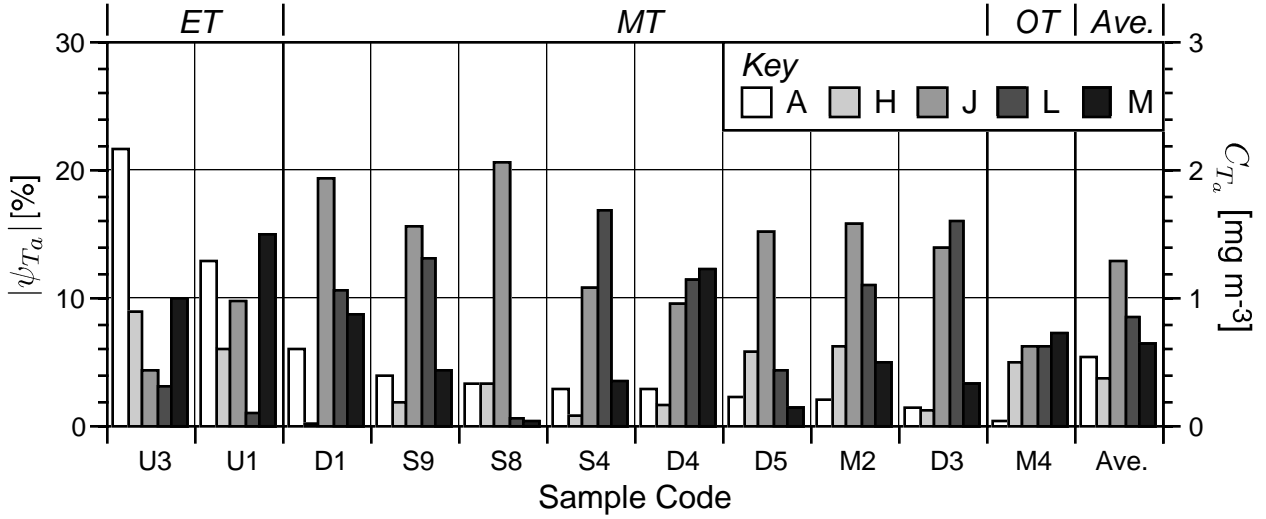
Fig. 3. The absolute value of the UPD values for total chlorophyll a, $|\psi_{Ta}|$, for the four methods (gray bars) as a function of the sampling stations and, thus, the concentration regimes (given along the top). The second y-axis on the right, gives the value of C_{Ta}^A for the average of each sampling station (white bars), which is used to order the data from highest (left) to lowest (right) C_{Ta}^A concentration.

Table 6. A summary of the concentrations (in milligrams per cubic meter) and $\bar{\psi}$ values (in percent) for the three trophic regimes (the latter are calculated from the individual $|\psi|$ values). Averages across all four laboratories are identified by the A code, whereas the individual laboratory results are indicated by their one-letter superscript codes. The eutrophic, mesotrophic, and oligotrophic partitioning of the data is represented by the subsequent ET, MT, and OT regime labels; results for the entire data set are given by the "All" regime label. Missing values correspond to pigments not quantified (or not submitted) by the laboratory involved and are not included in any of the analysis procedures.

| Regime	Code	C_P	C_B	C_F	C_H	C_D	C_Z	C_a	C_{Da}	C_{Ta}	C_{Ca}	$C_{\Sigma a}$	C_b	C_{D_b}	C_{T_b}	C_{12}
ET	$\bar{C}^A$	0.223	0.020	0.621	0.147	0.141	0.005	1.693	0.001	1.729	0.152	1.831	0.051	0.001	0.052	0.422
	$\bar{\psi}^H$	2.7	36.1	6.2	26.3	11.9	46.7	10.1	0.0	7.5	66.7	4.0	7.4	0.0	9.9	5.7
	$\bar{\psi}^J$	21.6	51.5	15.1	0.3	25.1	60.6			7.1	68.2	1.0			20.2	14.5
	$\bar{\psi}^L$	22.3	60.0	8.5	9.4	4.8	66.7	2.1	0.0	2.1		7.9			15.5	30.8
	$\bar{\psi}^M$	6.4	57.0	12.8	30.2	41.7	73.9	10.4	0.0	12.5	134.9	3.2	7.4	0.0	6.6	46.3
	$\bar{\psi}^A$	13.3	51.1	10.6	16.5	20.9	62.0	7.5	0.0	7.3	89.9	4.0	7.4	0.0	13.1	24.3
MT	$\bar{C}^A$	0.005	0.047	0.021	0.095	0.008	0.037	0.236	0.063	0.315	0.001	0.315	0.059	0.054	0.121	0.038
	$\bar{\psi}^H$	28.2	14.2	7.9	20.6	13.2	7.4	4.5	4.0	2.7	13.5	2.7	7.4	13.5	14.1	12.1
	$\bar{\psi}^J$	45.8	6.8	7.2	6.7	18.3	15.7			15.2	6.1	15.1			20.5	24.4
	$\bar{\psi}^L$	42.3	25.7	10.6	25.2	11.6	7.1	6.4	10.2	10.5		10.6			21.4	22.6
	$\bar{\psi}^M$	35.7	42.6	13.4	45.5	35.6	14.9	5.5	11.3	4.9	9.5	5.0	7.4	13.5	7.0	39.4
	$\bar{\psi}^A$	38.0	22.3	9.8	24.5	19.7	11.2	5.4	8.5	8.3	9.7	8.3	7.4	13.5	15.8	24.6
OT	$\bar{C}^A$	0.001	0.004	0.003	0.014	0.004	0.011	0.045	0.001	0.044		0.044	0.003	0.001	0.003	0.004
	$\bar{\psi}^H$	20.0	29.4	27.3	35.7	52.9	14.3	3.0	0.0	5.1		5.1	60.0	0.0	23.1	25.0
	$\bar{\psi}^J$	20.0	5.9	27.3	14.3	52.9	4.8			6.3		6.3			53.8	75.0
	$\bar{\psi}^L$	20.0	29.4	9.1	28.6	29.4	33.3	8.2	0.0	6.3		6.3			7.7	25.0
	$\bar{\psi}^M$	60.0	64.7	45.5	78.6	135.3	42.9	5.2	0.0	7.4		7.4	60.0	0.0	69.2	75.0
	$\bar{\psi}^A$	30.0	32.4	27.3	39.3	67.6	23.8	5.5	0.0	6.3		6.3	60.0	0.0	38.5	50.0
All	$\bar{C}^A$	0.044	0.038	0.128	0.097	0.031	0.029	0.484	0.046	0.548	0.062	0.566	0.053	0.039	0.098	0.105
	$\bar{\psi}^A$	32.8	28.5	11.5	24.4	24.3	21.6	4.4	6.2	7.9	23.4	7.4	12.2	9.8	17.3	26.9
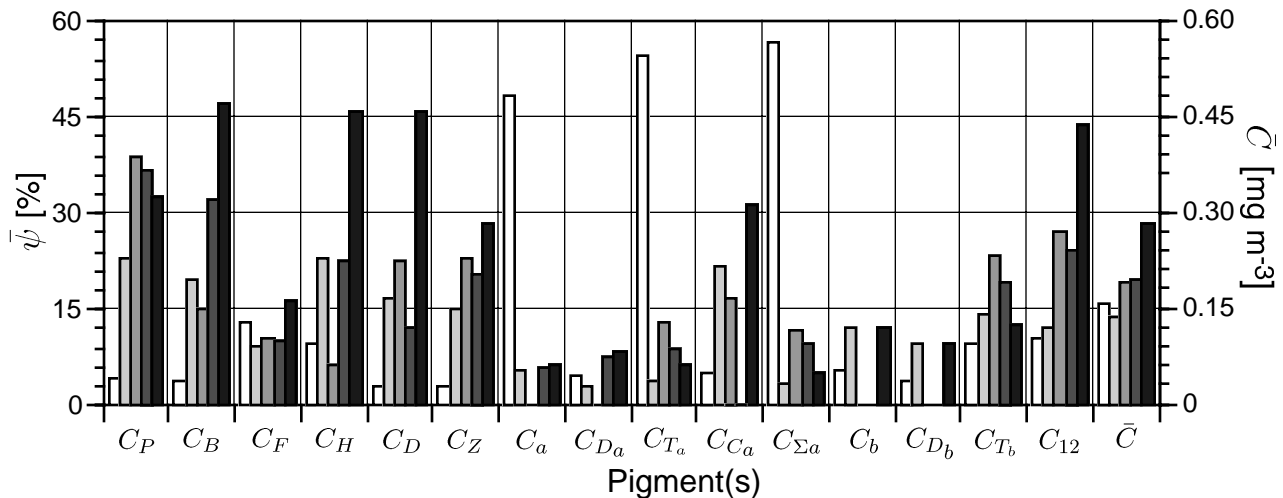
Fig. 4. The UPD values, $\bar{\psi}$, of the four methods as a function of the average pigment concentrations for all the pigments or pigment associations. The coding scheme for the bars is the same as that in Fig. 3 (white is the average of all the methods, and the gray bars, from left to right, correspond to the HPL, JRC, LPCM, and MCM methods). The average concentration of all pigments, $\bar{C}$, for each method is given in the right-most column.

A partitioning of the Fig. 4 data, in terms of concentration regimes, is presented in Table 6 and plotted in Fig. 5. Table 6 gives the percent differences with the average concentrations from the four methods, so the importance of the SNR can be discerned. There is a significant dependence on concentration, and this is well demonstrated by the average concentration of all pigments as a function of the concentration regime, which decreases from 0.473 (ET), to 0.094 (MT), to 0.013 mg m^{-3} (OT); although, individual pigments do not always show this orderly decrease. The change in $\bar{\psi}$ values for the three regimes is 22.3 (ET), 18.3 (MT), and 32.1% (OT).

The larger $\bar{\psi}$ value for the ET regime with respect to the MT regime is a consequence of some of the ET pigments having very low concentrations (some with concentrations even lower than the corresponding MT pigments). The lower concentrations produce lower SNRs which results in higher percent differences. For example, the C_B and C_Z concentrations are lower in the ET regime than in the MT regime, and the corresponding percent differences are higher.

If the low concentration ET pigments are removed from the samples to be averaged, the percent differences increase with decreasing trophic level. If absolute differences are used, following (3), the trend is clearly seen; if relative (or signed) averages are used, the trend is not always apparent, as shown in Table 7. Although relative averages confuse some aspects of the differences between methods and regimes (because of sign cancellation), they do show which methods are high and which are low (with respect to the average). From this perspective, the H, J, and L methods are very similar (usually negative) and the M method is distinctly different (usually positive). Note, this

is also seen in the absolute averages wherein the M method always has the highest $\bar{\psi}_A$ values for each concentration regime.

Table 7. The average $\psi_A^{L_j}$ values as a function of concentration regimes for absolute and relative (signed) differences. Pigments not analyzed by all laboratories are not included and the averages associated with the ψ_B and ψ_Z values for the ET regime are excluded.

Method	Regime	Absolute	Relative
H	ET	9.2	−5.5
	MT	12.3	−8.1
	OT	23.8	−9.3
J	ET	13.3	−5.8
	MT	17.6	2.8
	OT	26.7	−15.9
L	ET	12.7	−4.2
	MT	18.8	−13.0
	OT	19.5	−11.0
M	ET	19.9	15.5
	MT	24.4	18.3
	OT	58.6	36.2

The exclusion of pigments for part of the Table 7 analysis is somewhat artificial, but it demonstrates the sensitivity of the results to low pigment concentrations. If overall averages are formed from the four methods using the absolute averages, the progression in average differences for the ET, MT, and OT regimes is 13.8, 18.3, and 32.1%, respectively.
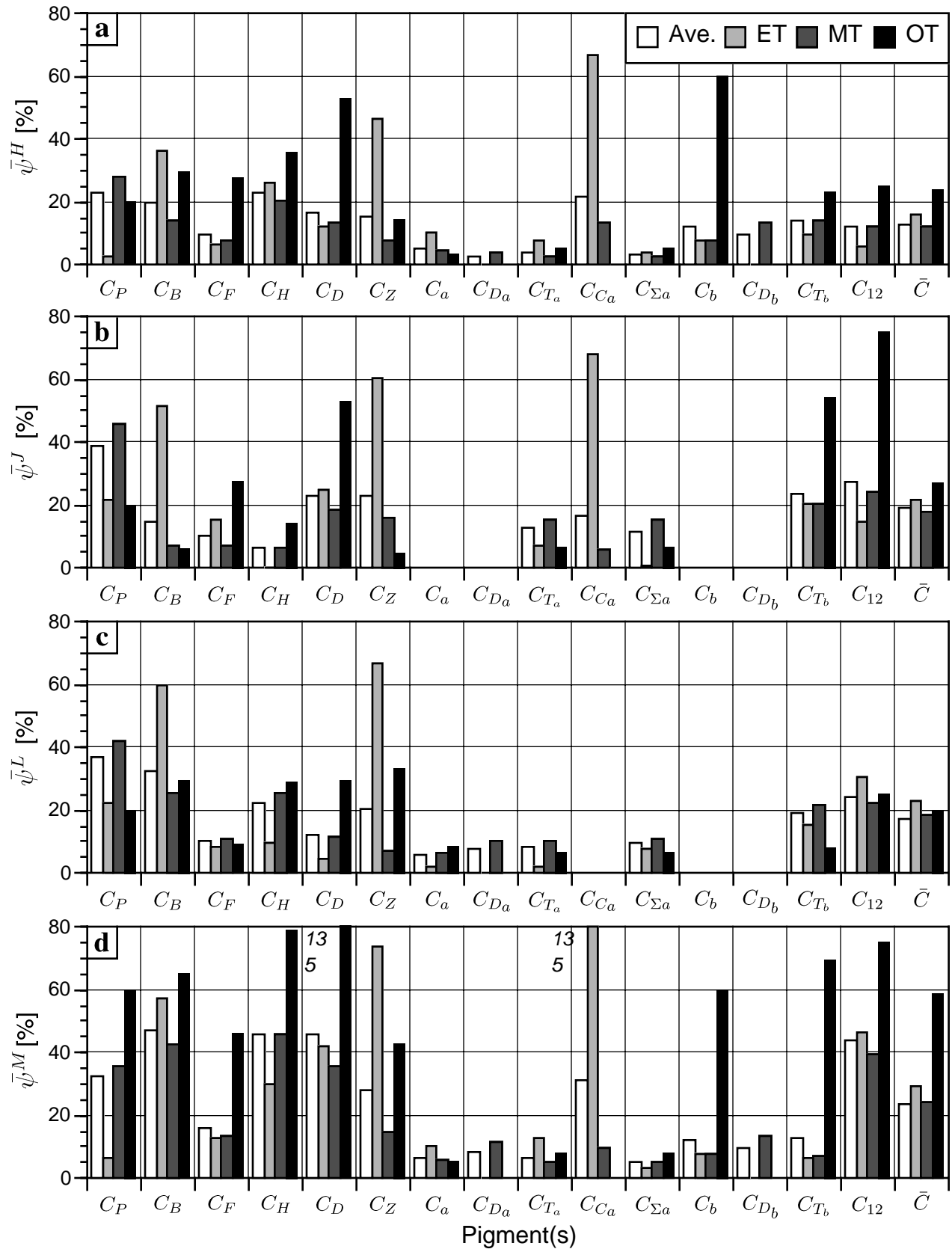
Fig. 5. The $\bar{\psi}$ values as a function of the concentration regimes for the four laboratories: **a)** HPL, **b)** JRC, **c)** LPCM, and **d)** MCM.

One result that is independent of the population sizes is the ranking of the methods from smallest to largest percent differences: H, L, J, and M for ET; H, J, L, and M for MT; and L, H, J, and M for OT. In general, therefore, the HPL method produced the smallest percent differences, the JRC and LPCM methods produced comparable results which were a little larger, and the MCM method produced the largest percent differences.

1.4.4 Pigment Concentration Levels

The correlation between the differences in the methods and the concentration of the pigments is an important point that deserves additional consideration. Figure 6 is a plot of the pigment data used for the averages presented in Table 6, that is, the average percent differences across all methods for each pigment, $\bar{\psi}^A_{P_i}$, are plotted as a function of $\bar{C}_{P_i}$ for each concentration regime. Also included in the plot is the data for the averages across all the pigments, but the plot does not include divinyl chlorophyll a, divinyl chlorophyll b, and chlorophyllide a data, because these pigments were not identified by all of the laboratories.
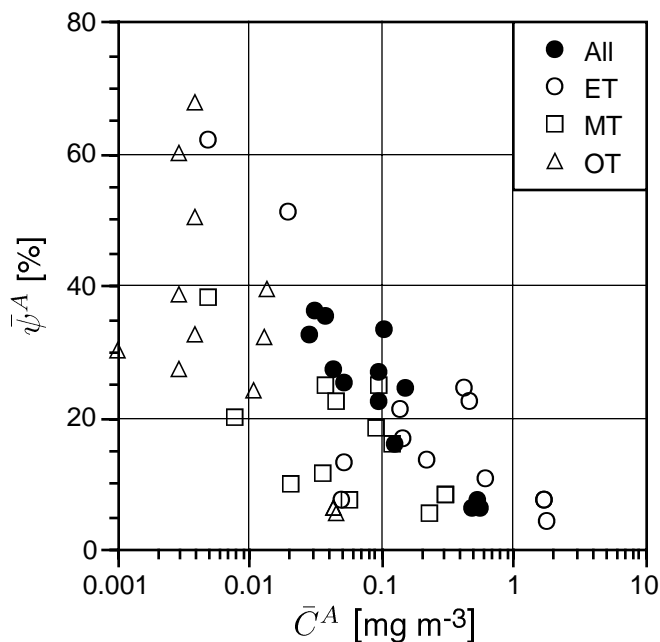

Fig. 6. The $\bar{\psi}^A_{P_i}$ values as a function of average pigment concentration for the three concentration regimes and (average) pigment concentration, $\bar{C}^A_{P_i}$, except $\bar{\psi}_{D_a}$, $\bar{\psi}_{D_b}$, and $\bar{\psi}_{C_a}$. The average for all the data in each concentration regime, as well as the overall average, is given by the solid circles.

There are several aspects of the Fig. 6 data which are important to the understanding of the results in terms of pigment concentration:

a) The distribution of the data reflects the concentration regimes (the OT data are clustered in the low end, the MT data in the middle, and the ET data in the high end);

b) There is an inverse relationship between $\bar{\psi}^A$ and $\bar{C}^A$ (high values of $\bar{\psi}^A$ are associated with low values of $\bar{C}^A$ and low values of $\bar{\psi}^A$ are associated with high values of $\bar{C}^A$); and

c) The range (taken here as a measure of the variance) in the $\bar{\psi}^A$ values is relatively constant, approximately 40%, for low concentrations and about 20% or less for high concentrations.

A least-squares fit to the average data (the solid circles in Fig. 6) indicates the percent error changes by approximately 21.8% for each decade of change (i.e., each factor of 10) in pigment concentration. This estimate is biased somewhat by the poor sampling at high concentrations (there are only a few data points above $1.0\,\mathrm{mg\,m^{-3}}$), but it is a quantitative measure of the effects of concentration level on the intercomparison results.

Figure 7 presents the analytical relationship of Fig. 6 separated into the four methods (as also presented in Table 6). Although the general behavior of decreasing percent differences with increasing pigment concentration is evident in all four methods, there is also the aforementioned general progression of increasing percent differences as a function of the concentration regimes (the OT data are clustered in the low end, the MT data in the middle, and the ET data in the high end).

The Fig. 7 data show the MCM method (Fig. 7d) is distinctly different from the other three methods (Figs. 7a–7c). In addition to the shift to higher differences (many $\bar{\psi}^M$ values are above the arbitrary reference line), the MCM method $\bar{\psi}^A$ values cover a larger range of percent differences, both in terms of the individual pigments (open symbols) and the averages (solid circles), than the other methods. There is also a subtle difference in the HPL results with respect to the JRC and LPCM data: the average values for the former are confined to a narrower range of percent differences (approximately 5–25%), whereas the latter are spread out over a larger range (approximately 5–40%).

The data in Figs. 6 and 7 were not separated according to pigment types, so the source of the higher percent differences is not immediately apparent (although, it can be discerned from the data in Table 6). Figure 8 presents the average results for each pigment as a function of the overall averages and within the three concentration regimes (which do not appear explicitly because of the coding scheme used to indicate the pigments). Although many of the pigments have a distribution of $\bar{\psi}^A$ values that decreases with increasing $\bar{C}^A$ (e.g., chlorophyll c_{1+2}), there are exceptions at different parts within the parameter matrix (e.g., fucoxanthin). The overall distribution, however, comes from the combined set of pigments and not from each pigment behaving in the same fashion.

The most notable exception to the overall relationship of decreasing percent differences with increasing concentration levels is total chlorophyll a—the percent differences for
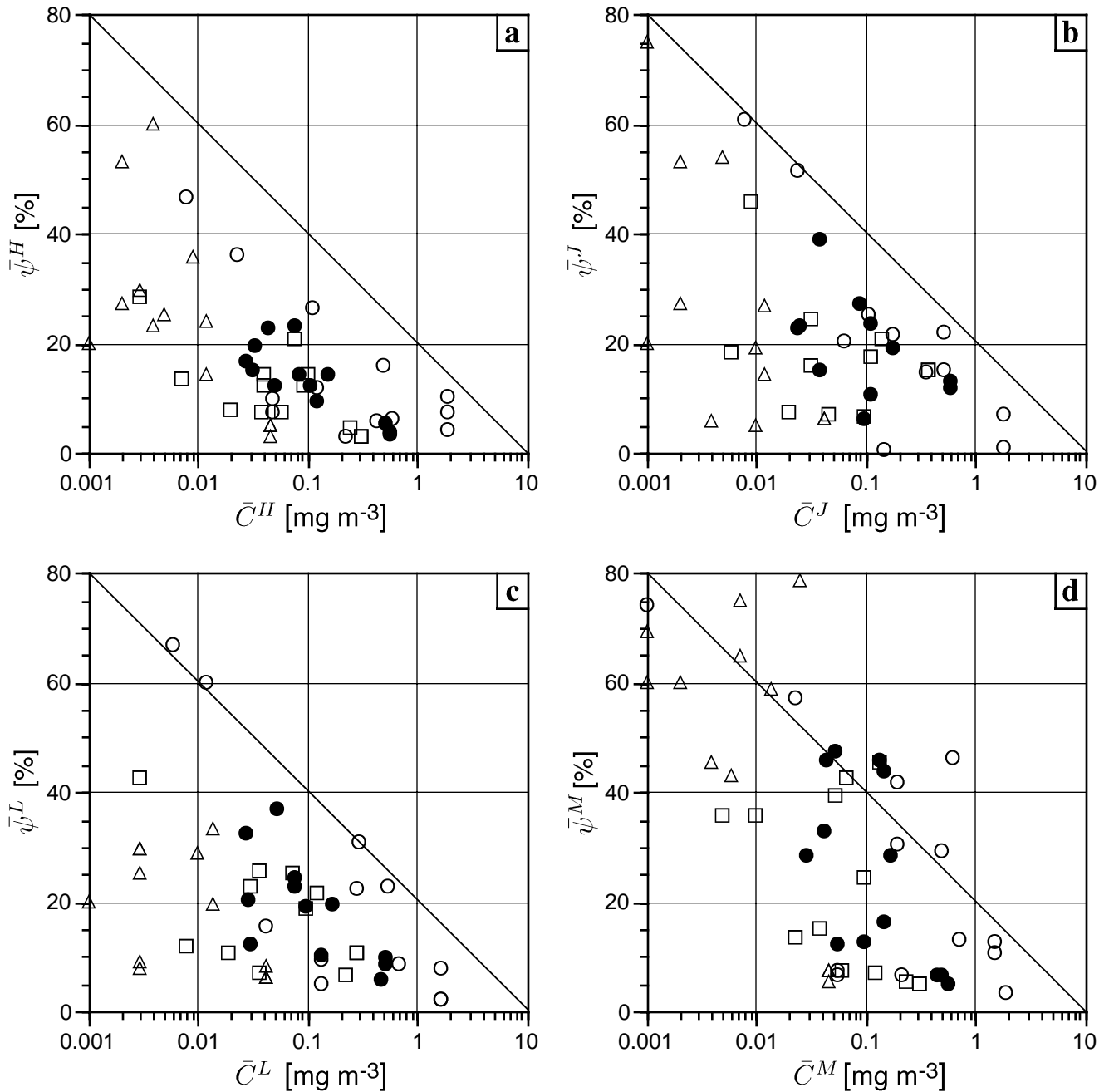
Fig. 7. The data from Fig. 6 separated according to the four individual laboratories (or methods): **a)** HPL, **b)** JRC, **c)** LPCM, and **d)** MCM. The symbol coding is the same as in Fig. 6 (open circle for ET, open square for MT, and open triangle for OT). The averages for all the data in each concentration regime, as well as the overall average, are given by the solid circles (which are not distinguished according to pigment type so the general behavior can be easily discerned). The diagonal line represents a 20% change in $\bar{\psi}$ for a factor of 10 change in $\bar{C}^A$, which is similar to the overall behavior of the Fig. 6 data, and is provided here as an arbitrary reference to make it easier to distinguish differences between the methods.

total chlorophyll a are small and largely insensitive to the concentration level (and, thus, the concentration regime). The insensitivity of the determination of total chlorophyll a as a function of concentration level is a fortunate result, because this is a crucial pigment association for ocean color calibration and validation activities (Sect. 1.4.1).

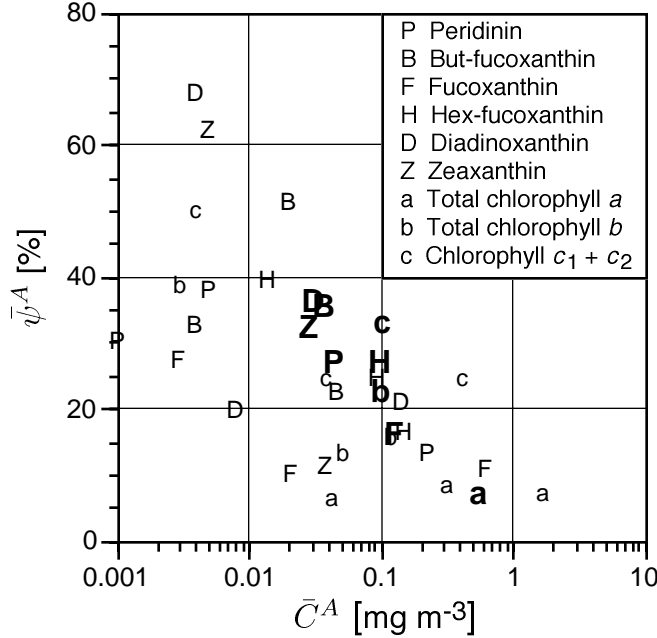**Fig. 8.** The $\bar{\psi}_{P_i}^A$ values for nine pigments as a function of $\bar{C}_{P_i}^A$. The data are separated into concentration regimes and overall averages. The average for all the data are given by the (larger) bold face letters and the three concentration regimes by the (smaller) plain face letters (which are not individually identified).

1.4.5 Repeatability

The results presented so far have included some of the effects associated with the mishandled (so-called *bad*) replicates, because some of them passed the initial QC procedures and were included in the final determination of the pigment concentration for some samples. The issue considered next is whether or not these replicates negatively influenced the results. The analysis for this inquiry is based on separating the data into two groups:

 i) The first group is formed by excluding all of the *bad* replicates, and

 ii) The second group is formed by including all of the replicates.

The former is referred to as the duplicate data set (because, in most cases, only two samples were then available for HPLC analysis), and the latter as the triplicate data set. The MCM replicates were all *good*, so the duplicate set for MCM was formed by removing the corresponding *good* replicate from each of the batches with *bad* replicates.

Figure 9 presents a comparison of the average pigment concentrations for the duplicate and triplicate data sets, $\bar{C}^D$ and $\bar{C}^T$, respectively. The pigments considered are the same as in Fig. 8, except chlorophyll a and divinyl chlorophyll a are included in the Fig. 9 data. All of the methods show very good agreement between the duplicate and triplicate data, and there are minimal effects from the *bad* replicates—all of the data are grouped very close to the 1:1 line. The excellent agreement of the other methods with respect to the M results suggests the *bad* replicates have a minimal influence on the final results.
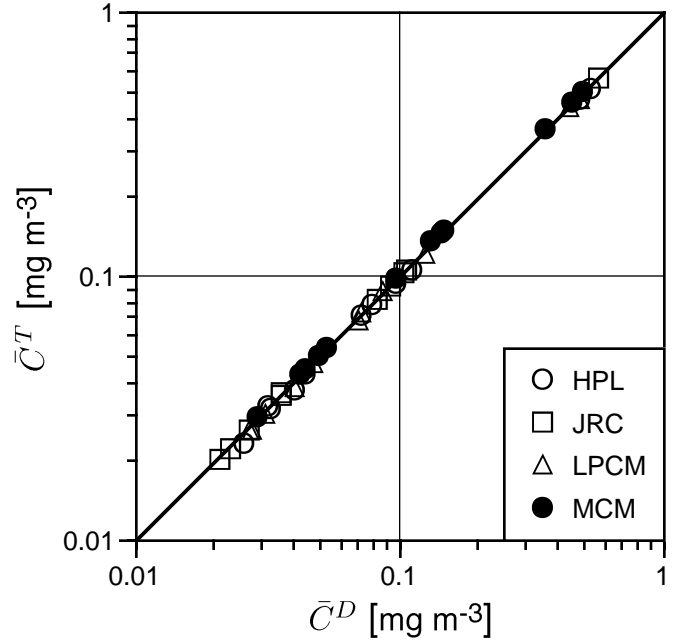
Fig. 9. The comparison of $\bar{C}^T$ (one *bad* replicate) and $\bar{C}^D$ (no *bad* replicates) for the H, J, L, and M methods.

To examine the replicate data more closely, a new parameter, ξ, is considered: ξ is the percent ratio of the standard deviation, σ, in the replicate data set with respect to the average. This variable is also known as the coefficient of variation and is presented in Table 8 for the triplicate data set. The average of ξ for all the samples for a particular pigment is given by $\bar{\xi}_{P_i}$.

The repeatability of the methods is well quantified by ξ, and the Table 8 data shows the overall repeatability in the methods across all pigments, $\bar{\xi}_A^{L_j}$, falls within a narrow range of 7.6–11.3%. If the very high variance associated with the determination of C_{C_a} and C_P is excluded (both appear in very low concentrations), the range is reduced to 3.7–10% (the LPCM average value is not improved by editing the data, because the $\bar{\xi}_P^L$ value is the same as the overall LPCM average and the LPCM method did not detect C_{C_a}). In all cases, the best repeatability is associated with the MCM method (which had no bad replicates), and in almost all cases, the $\bar{\xi}_{P_i}^M$ values are less than 5%. From the

Fig. 10. A comparison of ξ histogram values for the duplicate and triplicate data sets (derived from the Fig. 9 data) from **a)** the H, J, and L methods, and **b)** ξ values from the M method. The largest amplitude bars are always shown in the background, and the smallest amplitude bars in the foreground.
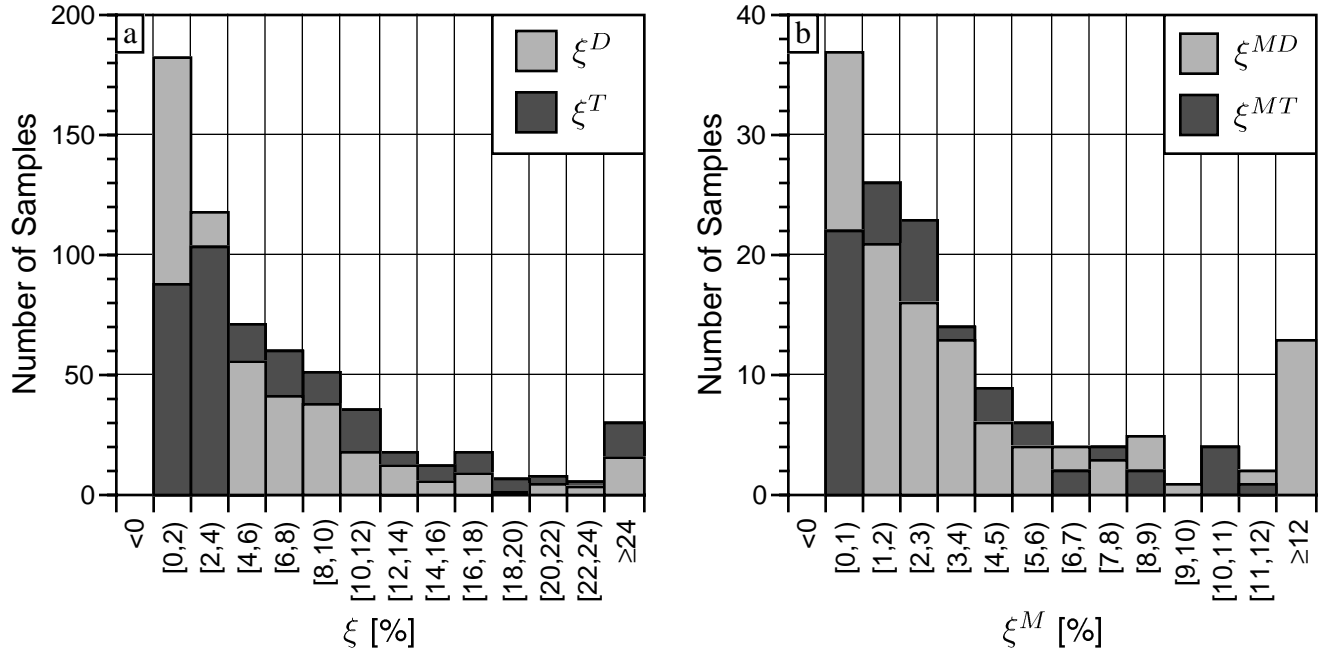
perspective of ocean color calibration and validation activities, the $\bar{\xi}_{Ta}$ values range from 4.3–6.8% with an overall average of 5.4%.

Table 8. The $\bar{\xi}_{P_i}^{L_j}$ values (in percent) for the four laboratories. The last column gives the average across the four methods, and the next to last row, the averages across the pigments. The last row excludes the $\bar{\xi}_{Ca}$ and $\bar{\xi}_P$ data.

$\bar{\xi}$	H	J	L	M	A
$\bar{\xi}_P$	30.1	18.0	7.6	32.5	21.1
$\bar{\xi}_B$	10.7	10.3	6.2	2.0	7.2
$\bar{\xi}_F$	13.5	11.4	7.7	4.0	8.9
$\bar{\xi}_H$	8.1	6.9	4.6	2.8	5.5
$\bar{\xi}_D$	12.8	12.0	11.5	4.7	10.3
$\bar{\xi}_Z$	6.7	9.4	5.5	3.2	6.2
$\bar{\xi}_a$	5.2		5.3	4.8	5.1
$\bar{\xi}_{Da}$	5.2		7.4	1.9	4.9
$\bar{\xi}_{Ta}$	5.1	6.8	5.5	4.3	5.4
$\bar{\xi}_{Ca}$	21.9	8.6		21.4	17.3
$\bar{\xi}_{Tb}$	5.2	7.5	15.3	4.5	8.4
$\bar{\xi}_{12}$	11.7	15.8	6.6	4.8	9.8
$\bar{\xi}_A$	11.3	10.7	7.6	7.6	9.2
$\bar{\xi}_{A'}$	8.4	10.0	7.6	3.7	7.2

A subset of the data is used in Table 8 and for most of the analyses considered hereafter. This was needed because some analytical relationships were artificially improved, because of inequalities in the production of the data sets (i.e., not all pigments were analyzed by each laboratory). The subset was constructed from the full set by a) excluding pigments that were not analyzed by all laboratories; b) removing pigments that could not be calculated completely correctly because of missing pigments; c) including the most important pigments for ocean color calibration and validation (chlorophyll a, divinyl chlorophyll a, and total chlorophyll a); and then adding chlorophyllide a, so the effects of very low concentrations on the products and conclusions can be clearly addressed. A close scrutiny of Table 6 shows the subset well represents the needed diversity in pigments.

The MCM replicate distribution included no *bad* replicates. For discussion purposes, the Table 8 data show the MCM results can be considered as a *control* of sorts. A comparison of ξ values from the H, J, and L methods versus the M method for the duplicate and triplicate data sets is presented in Figs. 10a and 10b, respectively. In terms of the H, J, and L results, the histogram for the duplicate data is different than the histogram for the triplicate data: the 0–2% bin is reduced, the peak in the histogram is shifted to the 2–4% bin, and the remaining bins are slightly elevated. This shift from the lowest bin to higher bins represents a general decrease in overall agreement, which is the expected effect of the *bad* samples. The histograms for the duplicate and triplicate M data sets (Fig. 10b), however, show a similar result: the peak in the duplicate data set is shifted away from the lowest bin, and the larger bin intervals are slightly more elevated.

To quantify the importance of the duplicate and triplicate analyses in terms of the initial UPD results, the differ-

ences between the two data sets is quantified by calculating the percent difference between them:

$$\delta_{P_i}^{L_j} \ = \ 100 \, \frac{C_{P_i}^{L_j D} \ - \ C_{P_i}^{L_j T}}{C_{P_i}^{L_j T}}, \tag{5}$$

where the normalization is formed using the triplicate data set, because this was the starting point for much of the results presented earlier.

The averages of the δ values for each pigment and method over all the samples (calculated from the absolute δ values as was done with the ψ parameter), $\bar{\delta}_{P_i}$, are presented in Table 9. The pigments are (basically) ordered from highest (total chlorophyll a) to lowest (chlorophyllide a) concentration, and there is no evidence of any strong dependence on concentration level (except the poor results with chlorophyllide a and peridinin which are attributed to low concentration levels). The most significant results from the $\bar{\delta}$ data are as follows:

1. The M method has the smallest differences;

2. The fucoxanthin averages are anomalously high;

3. The differences in the H, J, and L results are all similar to one another (particularly when the overall pigment averages are considered with the chlorophyllide a and peridinin results removed); and

4. The H, J, and L results are on average about 3% higher than the M results.

The excellent agreement between the H, J, and L methods, and the very small size of $\bar{\delta}_{A'}$ for the M method, suggest the *bad* replicates contribute approximately 1–3% extra uncertainty to the HPLC intercomparisons (assuming the repeatability results associated with the MCM method are representative of the other methods).

Table 9. The $\bar{\delta}_{P_i}$ values (in percent) for the four laboratories. The last column gives the average across the four methods, and the next to last row, the averages across the pigments. The last row is an average across the pigments with the $\bar{\delta}_{Ca}$ and $\bar{\delta}_P$ data excluded and is denoted by the A' code.

$\bar{\delta}$	H	J	L	M	A
$\bar{\delta}_P$	15.9	4.9	5.3	7.7	8.4
$\bar{\delta}_B$	5.0	5.9	1.6	0.6	3.3
$\bar{\delta}_F$	6.3	8.1	6.0	0.1	5.1
$\bar{\delta}_H$	4.2	2.3	2.6	0.9	2.5
$\bar{\delta}_D$	4.6	3.5	8.2	0.9	4.3
$\bar{\delta}_Z$	3.5	3.0	3.7	0.1	2.6
$\bar{\delta}_a$	3.0		2.7	1.7	2.5
$\bar{\delta}_{Da}$	2.6		4.3	0.8	2.6
$\bar{\delta}_{Ta}$	3.0	2.8	2.8	1.6	2.5
$\bar{\delta}_{Ca}$	2.6	4.6		9.8	5.7
$\bar{\delta}_{Tb}$	2.2	3.1	6.1	2.1	3.4
$\bar{\delta}_{12}$	6.9	4.3	2.8	1.9	3.9
$\bar{\delta}_A$	5.0	4.2	4.2	2.4	3.9
$\bar{\delta}_{A'}$	4.1	4.1	4.1	1.1	3.3

1.5 CONCLUSIONS

Before discussing the results, it is useful to clarify the definitions of certain key terms necessary for arriving at any conclusions about the various methods:

1. *Accuracy* is the estimation of how close the result of the experiment comes to the *true* value.

2. *Precision* is the estimation of how exactly the result is determined independently of any *true* value.

3. *Repeatability*, also called *within-run* precision, is obtained from a single operator, using the same instrument, and analyzing the same samples.

4. *Reproducibility*, also called *between-run* precision, is obtained from different operators, using different instruments and analyzing separate samples.

Note that alternative definitions and quantifications are possible, and the ones advocated above are simply the ones deemed suitable for this study.

The determination of the accuracy of the various methods involved is central to the objectives of any round-robin. Accuracy is affected by systematic uncertainties, and in this study, it is quantified by the $\bar{\psi}_{P_i}^{L_j}$ values in Table 6 and the corresponding averages across all pigments in Table 7. The data from Tables 5 and 6 show the determination of C_{T_a}, the most important product for ocean color calibration and validation exercises, exhibits a narrow range of accuracy for all four methods, 2.1–15.2%, with an average accuracy across all methods and all concentration regimes of 7.9%. Both the average and the range are significant, because they are within the 20–25% pigment intercomparison objective, and almost the entire range is within the 15% algorithm refinement objective. There is a mitigating factor in the $\bar{\psi}_{T_a}$ summaries, however: the JRC method did not include a capability for separating mono- and divinyl chlorophyll a. If the $\bar{\psi}_{T_a}$ results are compared for when C_{D_a} was not a significant part of C_{T_a}, $\bar{\psi}_{T_a}^A = 6.7\%$—the four methods intercompare at less than the 7% level.

For averages across all concentration regimes (Table 7), the accuracy range is larger, 12.3–58.6%, but this is primarily due to very low concentrations in the oligotrophic regime (almost all the $\bar{C}_{P_i}$ values are less than $0.1 \, \mathrm{mg \, m^{-3}}$) plus two very low pigment concentrations in the eutrophic regime ($\bar{C}_B$ and $\bar{C}_Z$ are both less than $0.1 \, \mathrm{mg \, m^{-3}}$). If the latter two are ignored, the accuracy range is 9.2–24.4%, with an average across all four methods of 13.8–18.3%. The reduction in accuracy with decreasing concentration is an important result from the vicarious calibration point of view, because most of the world ocean is classified as oligotrophic. The one encouraging result is the accuracy in determining C_{T_a} does not appear to be very sensitive to concentration levels: the average accuracy for C_{T_a} across all three concentration regimes ranges from 6.3–8.3%. Unfortunately, the sampling distribution of the regimes is not equal; the majority of the samples come from the mesotrophic regime.

The overall reduction in accuracy as a function of decreasing pigment concentration can be quantified by the slope of a least-squares, linear fit of the $\bar{\xi}^{L_j}$ values to the $\bar{C}^{L_j}$ values for each concentration regime. If this is done, the decreases in accuracy (as measured by the slope of the fitted line) for the HPL, JRC, LPCM, and MCM methods are as follows: -10.6, -11.1, -7.3, and -20.1% for every decade (factor of 10) decrease in concentration, respectively. Although the former three fall within a narrow range, the MCM method does not.

The distribution of accuracy as a function of concentration level (Figs. 6–8) also leads directly to a consideration of the precision of the results. Precision is affected by random uncertainties, and in this study, it is quantified by the dispersion (standard deviation) of the $\bar{\xi}^{L_j}_{P_i}$ values, $\sigma^{L_j}_{P_i}$, which are presented in Table 10. The Table 10 data show the precision for all four laboratories falls within a narrow range, approximately 1.7–8.5% (4.1–7.1% for an overall average), if the high values associated with C_P, C_{Ca}, C_{T_b}, and C_{12} are excluded. The former two have already been identified as a source of higher variance, but the latter two are associated with the performance of specific methods: LPCM for C_{T_b}, and JRC for C_{12}. The average precision excluding the C_P and C_{Ca} data (the $\sigma_{A'}$ results) fall within a 4.1–7.8% range, with an overall average of 6.2%.

Table 10. The standard deviation in $\xi^{L_i}_{P_i}$ values (in percent) for the four laboratories. The last column gives the average across the four methods, and the next to last row, the averages across the pigments. The last row excludes the σ_{Ca} and σ_P data.

σ	H	J	L	M	A
σ_P	32.5	9.5	5.0	52.5	24.9
σ_B	5.5	5.3	5.6	1.7	4.6
σ_F	8.2	4.5	8.3	3.3	6.1
σ_H	7.4	3.8	4.2	2.8	4.5
σ_D	8.5	8.1	7.9	3.9	7.1
σ_Z	4.2	5.2	7.4	3.9	5.2
σ_a	4.5		4.6	6.4	5.2
σ_{Da}	4.1		6.7	1.6	4.1
σ_{Ta}	4.5	2.4	4.6	6.5	4.5
σ_{Ca}	12.1	9.2		29.6	17.0
σ_{T_b}	3.4	3.6	25.0	7.1	9.8
σ_{12}	9.3	25.3	3.8	3.6	10.5
σ_A	8.7	7.7	7.5	10.3	8.6
$\sigma_{A'}$	6.0	7.3	7.8	4.1	6.2

Repeatability reflects the homogeneity of the replicates, and in this study is represented by the $\bar{\xi}^{L_j}_{P_i}$ values (Table 8) for each method. The overall repeatability in the methods across all pigments, $\bar{\xi}^{L_j}_A$, falls within a narrow range of 7.6–11.3%, and an even narrower range of 3.7–10% if the higher variance associated with the determination of C_{Ca} and C_P are ignored. In all cases, the best repeatability

is associated with the MCM method, and in almost all cases, the $\bar{\xi}^M_{P_i}$ values are less than 5%. In regards to the needs of ocean color calibration and validation activities, the $\bar{\xi}_{T_a}$ values range from 4.3–6.8% with an overall average of 5.4%.

Another aspect that was covered in the repeatability analysis was the importance of the defrosted (presumably *bad*) replicates on the results. Although the mishandling in this case was not fatal, it nonetheless quantifies some of the effects of shipping on the analysis of pigment samples. The comparison of duplicates (no *bad* replicate) and triplicates (one *bad* replicate) showed this probably contributed a 1–3% uncertainty to the repeatability. The latter assumes the approximately 1% repeatability results associated with the MCM method, which included no *bad* replicates, are applicable to the other methods (Table 9).

Reproducibility reflects the homogeneity of the intercomparisons which is quantified using the dispersion (standard deviation) in the $\bar{\psi}^{L_j}_{P_i}$ values, denoted by $\varsigma^{L_j}_{P_i}$, which are presented in Table 11. The Table 11 data show the best reproducibility occurs for the chlorophyll a pigments, which exhibit a narrow range of values, 3.6–9.6%, and averages, 5.5–7.6%. All the other pigments have a much poorer range of values which raises the average over all pigments to 21.3% (for a range of 16.0–27.7%). Once again the C_P and C_{Ca} have the largest variances, and if these are removed, the overall average improves to 15.0% with a range of 11.1–20.5%. Although the differences between the methods is small compared to the ς values or the averages, the MCM method always has a ς value that is larger than the averages. In comparison, the HPL method almost always has a ς value that is smaller than the averages.

Table 11. The standard deviation in $\psi^{L_i}_{P_i}$ values (in percent) for the four laboratories. The last column gives the average across the four methods, and the next to last row, the averages across the pigments. The last row excludes the ς_{Ca} and ς_P data.

ς	H	J	L	M	A
ς_P	26.6	63.3	43.2	37.7	42.7
ς_B	26.9	31.7	18.8	41.7	29.8
ς_F	10.9	10.4	11.8	12.1	11.3
ς_H	6.3	7.5	11.8	16.8	10.6
ς_D	13.0	12.7	17.2	34.5	19.4
ς_Z	14.6	31.0	26.4	38.2	27.6
ς_a	3.9		5.3	7.3	5.5
ς_{Da}	3.6		9.7	9.6	7.6
ς_{Ta}	4.8	7.6	6.0	7.3	6.4
ς_{Ca}	63.1	36.7		89.3	63.0
ς_{T_b}	13.9	15.1	21.7	22.2	18.2
ς_{12}	13.1	23.5	4.4	15.1	14.0
ς_A	16.7	24.0	16.0	27.7	21.3
$\varsigma_{A'}$	11.1	17.4	13.3	20.5	15.0

A possible reason why the MCM method gave results that were different from the other methods may be due to the choice of detectors. The HPL, JRC, and LPCM methods all used the Hewlett-Packard HP 1100 DAD detector. The MCM method uses the Thermo Separations UV 6000 DAD detector that incorporates a 5 cm flow cell with a 10 μL capacity. The UV 6000 detector, therefore, has approximately a five times greater sensitivity than the other detectors fitted with conventional 1 cm cells, and provides an improved SNR for the detection of low pigment concentrations.

The insensitivity of the determination of total chlorophyll a as a function of the concentration level is a direct consequence of the ubiquitous nature of the chlorophyll a pigment within any oceanic regime, and the range of variation in individual pigments is much larger than that of chlorophyll a. The latter is due to changes in community (pigment) composition as a function of the trophic regime (Claustre 1994). For example, the eutrophic regime is associated with high fucoxanthin and low zeaxanthin (if any) concentrations, and reciprocally for the oligotrophic regime. The insensitivity in total chlorophyll a accuracy is due to the fact that a) the lowest concentrations are still relatively high (as compared to the lowest concentration of other pigments), and b) the range of variation is weaker for this pigment than for the others. This is an important point which means that for any future round-robins based solely on *in situ* samples, a better accuracy can be expected for total chlorophyll a than for any of the other pigments.

Because accuracy is expected to decrease with decreasing trophic level (for most pigments but less evident for total chlorophyll a as discussed above), there is a need for improved methods (better sensitivity for better accuracy) for low concentration regimes. Conversely, in eutrophic regimes, there is also a problem of accuracy that is linked to the presence of phaeopigments, and this was not addressed in the present round-robin. Indeed, in open ocean Case-1 waters, eutrophic regimes are the only ones which include the presence of phaeopigments and sometimes a contribution of chlorophyllide a (a priori from diatom senescence, but also as a result of sample preservation and extraction problems). Some laboratories found chlorophyllide a and some others not; the same was true for phaeopigments.

In general, phaeopigment concentration might reach 10% of total chlorophyll a in eutrophic surface waters, so the importance between phaeopigments and chlorophyllide a needs to be resolved in a future round-robin. In between these trophic domains (i.e., the so-called mesotrophic regime), there are enough pigments and (virtually) no degradation products, so there is no accuracy problem and a future intercomparison exercise need not emphasize mesotrophic sampling. Similarly, from the point of view of optical databases (C_a versus reflectance ratio), there is a lack of data for very low and very high chlorophyll concentrations (O'Reilly et al. 1998). PROSOPE tried to (partially) fill this gap, but additional work on the accuracy of HPLC methods in these low and high concentration domains is still justifiable within the framework of ocean color calibration and validation issues.

Although every effort was made to make SeaHARRE-1 as complete as possible, there were deficiencies in the work plan that should be addressed in a future round-robin. The most obvious recommended improvements are as follows:

1. Greater care in the handling of the samples (the manufacturer of the defective dewar has changed the design of their dry shippers);

2. A more concerted effort to sample oligotrophic and eutrophic regimes (from a remote sensing perspective this is also the concentration levels where the most new data is needed);

3. The inclusion of standard pigment samples, so a control data set is available for analysis (this would permit a more conclusive analysis of the sensitivity issue highlighted by the MCM results); and

4. A systematic sampling for fluorometric determination of total chlorophyll a.

The use of standard pigment samples is particularly important, because several sources of uncertainty are best quantified if the concentration of the sample is already known. Although this was recognized as a needed element of SeaHARRE-1, the voluntary aspect of the work did not permit a level of effort beyond what is presented here.

The addition of fluorometric techniques is suggested for two reasons: a) to address the generally observed discrepancy between HPLC and fluorometric methods, and b) to provide the data for reconciling (merging) fluorometric and HPLC historical databases. Sampling for fluorometric determinations should be restricted to surface waters, because chlorophyll b is virtually absent from surface waters while it is a dominant pigment at the level of the deep chlorophyll maximum. From a historical perspective there was a misinterpretation of chlorophyll b as phaeopigment using the acidification with the fluorometric technique. Because optical databases are restricted to surface waters, there is no need to complicate the exercise (the problem of chlorophyll b interference was not addressed in SeaHARRE-1).

Chapter 2

The HPL Method

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ABSTRACT

The HPL method was developed for use with a variety of water types ranging from freshwater lakes, to estuarine and oligotrophic oceanic samples. As such, pigments important to all these systems are baseline resolved and quantitatively reported, including divinyl and monovinyl chlorophyll a. The method can accommodate samples ranging in concentration by up to 400 fold without changes to system set up or calibrations. The method employs the use of a C_8 HPLC column in combination with a methanol-based, reversed phase binary gradient with a simple linear gradient and elevated column temperature ($60°C$). The method is relatively fast, well suited to automated analyses, and can provide quantitative information for up to 20 pigments with qualitative information for additional pigments. The average method variability is approximately 2% (estimated from data gathered from approximately 300 standard injections and 14 chlorophyll a calibration curves from 4 different columns).

2.1 INTRODUCTION

The method used by HPL was developed for use with a variety of water types with widely varying chlorophyll a concentrations. As such, many taxonomically important pigments common to freshwater, estuarine, and oceanic systems are well resolved. The excellent linearity of the chlorophyll a response and the lack of carry-over between injections allows sequential analysis of samples varying significantly in concentration. Features of this method include:

1. Baseline resolution between key taxonomically important carotenoids, as well as divinyl and monovinyl chlorophyll a, and partial resolution between divinyl and monovinyl chlorophyll b;

2. An analysis time for one sample of approximately 30 min; and

3. Adequate sensitivity for the accurate analysis of dilute oceanic water samples (approximately 0.5 ng of chlorophyll a yields an SNR of 10).

This method uses a C_8 column and a reversed-phase, methanol-based, binary gradient, solvent system. The detector signal at 665 nm is used to quantify chlorophyll a, divinyl chlorophyll a, and chlorophyllide a. All other pigments are quantified from the signal at 450 nm. Limitations of this method include chromatographic co-elution of chlorophyllide a with chlorophyll c_1 and α-carotene with β-carotene. Chlorophyllide a and chlorophyll c_1 are, therefore, quantified using a dichromatic equation based on their

spectral differences similar to that described in Latasa et al. (1996) for divinyl chlorophyll a and chlorophyll a. Other pigments which pose a potential for co-elution are listed in Table 13 and are further detailed in Van Heukelem and Thomas (2000).

2.2 EXTRACTION

The replicates were received on 9 December 1999 and stored in a freezer at $-80°C$ until analyzed on 11–12 January 2000. Each filter (25 mm GF/F) was cut into slivers and placed in a heavy-walled, 15 mL glass tube. Precisely 3 mL of 95% HPLC-grade acetone (v:v with deionized water) was added to the tube using a Class A, 3 mL volumetric pipette. Each tube was then covered with Parafilm™, placed in an ice bath, and kept in the dark until a set of filters (20 or 30) had been processed in the same manner. Each filter was then individually disrupted using an ultrasonic probe (0.25 in. tapered micro tip, approximately 40 W output, Branson model 450) for approximately 1 min. Each tube was submerged in a beaker of ice during disruption to prevent heat accumulation.

After each filter had been disrupted and the tube was again tightly wrapped in Parafilm, the entire set was placed in a freezer ($-15°C$) for 3.5–4 h. Each sample slurry was then well mixed on a vortex mixer and the extract clarified using a Teflon HPLC syringe cartridge filter (0.45 μm pore size, 25 mm diameter, with a glass fiber prefilter from Scientific Resources, Inc.). The extract was collected in

a 7 mL glass vial. After mixing well, an aliquot was removed and placed in an HPLC vial. The remaining extract was stored in the freezer (-15°C) until the HPLC analysis was complete. The HPLC vials were placed directly into the temperature-controlled (5°C) autosampler compartment of the HPLC until analyzed (within 24 h). The total extraction volume was assumed to be 3.145 mL as previous studies at HPL have shown that, on average, the water retained in a 25 mm GF/F filter contributes 145 μL to the total extraction volume.

2.3 HPLC ANALYSIS

The replicates were analyzed using a fully automated HP 1100 HPLC with quaternary pump, programmable autoinjector, temperature-controlled autosampler and column compartments, photodiode array detector, and computer data station. Samples and calibration standards were placed in the autosampler compartment tray (maintained at a temperature of 5°C) and analyzed over a time period not exceeding 24 h. The sample extract (or standard) was mixed with buffer (28 mM aqueous tetrabutyl ammonium acetate, pH 6.5) by the autoinjector immediately prior to injection using an injector program specifically created for use with this method and designed to provide symmetrically shaped peaks with good peak area and retention time reproducibility. The volume of sample injected was 150 μL.

After sample injection, separation was achieved using an Eclipse XDB C$_8$ HPLC column (4.6$\times$150 mm) manufactured by Agilent Technologies† with gradient elution using a linear gradient from 5–95% solvent B in 22 min, where solvent B was methanol and solvent A was (70:30) methanol, 28 mM aqueous tetrabutyl ammonium acetate, pH 6.5 (Table 4). An isocratic hold on 95% B was necessary from 22–29 min for elution of the last pigment (β-carotene) at approximately 27 min. After a return to initial conditions (5% solvent B) by 31 min, the column was equilibrated for 5 min prior to the start of the next analysis (Table 12). The flow rate for the gradient was 1.1 mL min^{-1} and the column temperature was 60°C.

Table 12. The gradient used with the HPL column organized by the steps involved in the complete analysis of a sample and the percentages of solvent A and solvent B.

Step	Time	A [%]	B [%]
Start	0	95	5
2	22	5	95
3	29	5	95
4	31	95	5
End	36	95	5

† Summary information regarding commercial manufacturers and suppliers described in this report is presented in Appendix C.

Data from the first injection in an automated sequence of analyses were always disregarded, because retention time and peak area reproducibility were less precise than in the subsequent analyses. Pigments were detected at 450 and 665 nm (both with 20 nm bandwidths). The signal at 665 nm was used for quantifying divinyl chlorophyll a, and chlorophyll a and its products; the signal at 450 nm was used for quantifying all other pigments. Peaks were integrated using the automated functions of the computer data station. Subsequently, all peaks in all chromatograms were visually inspected to verify the automated integrator had drawn peak baselines correctly and in a fashion consistent with the peak integrations of calibration standards. A paper print-out and an electronic computer data file were retained for each analysis.

2.4 CALIBRATION

External calibration standards were either purchased or isolated from naturally occurring sources (Van Heukelem and Thomas 2000) as listed in Table 13. All pigments listed with a bullet (•) symbol in the first column in Table 13 were reported (the mode of quantitation and potential for interference from co-eluting pigments are also listed). The concentration was determined using a dual beam, monochromator-type spectrophotometer (Hitachi U-3110). A spectral bandwidth of 2 nm was used, the sample was corrected for turbidity at 750 nm, and the standards were sufficiently concentrated such that the absolute absorbance of each pigment fell between 0.1–1.0 (in absolute units) for greatest spectrophotometer accuracy (Greenberg et al. 1992). Absorbance accuracy of the spectrophotometer was checked with neutral density filters traceable to the National Institute of Standards and Technology (NIST) as described in Latasa et al. (1999).

After the pigment concentrations were determined, each pigment was injected individually to determine chromatographic purity, peak purity, and the response factor (RF). All 20 pigments listed with extinction coefficients, E (Table 14), were precisely combined into one mixture for use as a quality control standard with subsequent sample analyses. The RFs for each pigment in this much-diluted sample were compared to the RFs observed from the highly concentrated stock pigment standards to ascertain instrument linearity. Single-point calibration was deemed suitable, and was used for all pigments except chlorophyll a, because the linear dynamic range was observed to range from approximately 0.5 to, in some cases, in excess of 700 ng per injection. For chlorophyll a, five-point calibration curves were used instead of single point RFs, even though the linear dynamic range was observed to be of the same magnitude as other pigments.

In all cases, when formulating standard dilutions and mixtures, volumetric class A glassware was used. Gastight, calibrated glass syringes were used when delivering volumes less than 2 mL. Additionally, the concentrations

Table 13. The pigments identified by the HPL method, the sources used to identify them, and the in-line visible absorbance spectra from the HPLC DAD (350–700 nm). For the latter, parentheses indicate a spectral shoulder. Peak numbers marked with the bullet symbol (•) are reported—the others are not reported. The t_R values refer to pigment retention times (in minutes) on the chromatograms. Pigments generally occurring in low amounts (some of which are unknown to HPL) are also listed, so the potential for co-elution with the pigments that are reported can be seen. The R_s values indicate resolution between adjacent pigments (as indicated by their pigment number and are shown only when $R_s < 1.5$); NR means not resolved. The mode of quantitation indicates whether the pigment was i) quantified based on HPLC RFs, ii) derived from a discrete pigment standard whose concentration had been determined spectrophotometrically with extinction coefficients (those listed with E), or iii) an alternative mode of quantitation. The SCOR WG 78 abbreviations are listed in Appendix B. The pigment extinction coefficients used are given in Table 14.

No.	Pigment	Source	Absorbance	t_R	R_s		Quantitation
1•	Chl c_3	C, J, L, N, S, T	456, 588, (625)	3.88			Chl c_2 RF
2	MV chl c_3	J	448, 585, (626)	4.14			
3•	Chl c_2	A–E, H, J, L–O, R–T	446, 584, 634	5.70	NR	3/4	E
4	Mg DVP	G, P	440, 576, 632	5.81	NR	4/a	
a	Unknown	K		5.92	NR	a/5	
5•	Chl c_1	B, D, E, R	442, 580, 634, 668	6.05	NR	5/6	Simultaneous equation
6•	Chlide a	F, H, N, O	(390), 434, 620, 668	6.06	NR	5/6	Simultaneous equation, part of total chl a
7•	Perid	A, B, M	476	9.32			E
8	Perid isomer	A, B, M	478	9.58			
b	Unknown		456, 476	11.37			
9•	But-fuco	VKI†, C, N, S, T	448, 464	12.31			E
c	Unknown	G	458	12.68	NR	c/10	
10•	Fuco	C, D, E, L, N, R–T	454	12.63	NR	c/10	E
11•	Neo	F, G, P, U	414, 438, 466	13.29	NR	11/12	E
12	4k-hex-fuco	J	448, 470	13.31	NR	11/12	
d	Unknown		446, 468	13.73	NR	d/13	
13•	Pras	VKI†, G, P	462	13.74	NR	d/13	E
14•	Viola	VKI†, F, G, P, Q, U	418, 442, 470	13.99	1.3	14/15	E
15•	Hex-fuco	VKI†, C, J, L	(430), 452, 480	14.16	1.3	14/15	E
16	Asta	Shrimp carapace, G	480	14.53			
e	Unknown	P	466	14.78			
17	Diadchr	M	(410), 428, 456	15.02	NR	17/f	
f	Unknown	J	448, 470	15.09	NR	f/18	
18	Unknown	K	452, 474, 506	15.13	NR	18/19	
19•	Diadino	A–E, J, L–N, R–T	(428), 446, 476	15.23	1.4	19/20	E
20	Dino	A, M	416, 440, 470	15.49			
21	Anth	F	(425), 446, 474	15.99			
22•	Allo	H, O	(430), 452, 480	16.53			E
23•	Diato	C, D, E, M, R, T	(430), 454, 480	17.12	NR	23/24	E
24	Monado	H, O	(422), 444, 472	17.22	NR	23/24	
25•	Zea	C, F, G, I, K, P, Q, V	(430), 452, 478	17.79			E
26•	Lut	F, G, T, V	424, 446, 474	17.98			E
g	Unknown	Q	422, 444, 472	18.24	NR	g/h	
h	Unknown	G	(408), 428, 454	18.32	NR	g/h	
i	Unknown	L, N, S, T	(424), 448, 472	18.84			

Table 13. (cont.) The pigments identified by the HPL method, the sources used to identify them, and the in-line visible absorbance spectra from the HPLC DAD (350–700 nm).

No.	Pigment	Source	Absorbance	t_R	R_s		Quantitation
27•	Cantha	W	480	19.07			E
j	Unknown	Q	422, 444, 472	19.23			
28	Gyroxanthin diester-like	C	(426), 444, 472	19.94			
29	Gyroxanthin diester-like	C	(426), 444, 472	21.00			
30•	DV chl b	U	478, 608, 654	21.92	0.8	30/31	E, based on peak height
31•	Chl b	Fluka†, F, G, P	468, 602, 652	22.03	0.8	30/31	E, based on peak height
32	DV chl b'	U	480, 608, 658	22.29			
33	Croco	H, O	(428), 446, 476	22.42	NR	33/34	
34	Chl b'	Fluka†, F, G, P	470, 602, 652	22.50	NR	33/34	
35	Chl a allom	Fluka†, A–T	(390), 432, 620, 666	23.30			E, part of total chl a
36	Chl a allom	Fluka†, A–T	(390), 432, 620, 666	23.43	NR	36/k	E, part of total chl a
k	Unknown	R	(464–474)	23.52	NR	k/l	
l	Unknown	R	454	23.52	NR	l/37	
37	Phytyl-chl c	J, R	460, 588, 636	23.53	NR	l/37	
38•	DV chl a	U	(390), 442, 622, 666	23.76			E, part of total chl a
m	Unknown	L	458, 588, 638	23.91	NR	m/39	
39•	Chl a	Fluka†, A–T	(390), 432, 620, 666	23.96	NR	m/39	E, part of total chl a
40	DV chl a'	U	(386), 440, 622, 666	24.13			E, part of total chl a
41	Chl a'	Fluka†, A–T	(388), 432, 618, 666	24.33			E, part of total chl a
n	Unknown	P	(422), 442, 470	25.58			
42	$\beta\varepsilon$-Car	Sigma†, G, H, J, L, O, P, S	(422), 446, 474	26.65	NR	42/43	Part of total carotenes
43•	$\beta\beta$-Car	Fluka†, A–G, I–N, P–U	(430), 452, 476	26.71	NR	42/43	E, part of total carotenes

A *Prorocentrum minimum*
D *Thalassiosira pseudonana*
G *Pycnococcus provasolii*
J *Emiliania huxleyi*
M *Amphidinium carterae*
P *Micromonas pusilla*
S *Pelagomonas calceolata*
V Marigold petals
B *Gyrodinium uncatenum*
E *Isochrysis sp.* (Tahiti strain)
H *Pyrenomonas salina*
K *Synechococcus cf. elongatus*
N *Pelagococcus subviridis*
Q *Nannochloropsis sp.* 1
T *Aureococcus anophagefferens*
W Gift from Perdue, Inc.
C *Gyrodinium galatheanum*
F *Dunaliella tertiolecta*
I *Synechococcus sp.*
L *Chrysochromulina polylepsis*
O *Guillardia theta*
R *Isochrysis galbana*
U Mutant corn
† Details given in Appendix C.

of the stock standards were rechecked on the spectrophotometer prior to the formulation of new calibration standards.

The signal at 665 nm (±10 nm) was selected, because divinyl chlorophyll a and chlorophyll a respond similarly (and strongly) at this wavelength, therefore, the same calibration constants were used with each pigment. For reporting total chlorophyll a (the sum of divinyl chlorophyll a and monovinyl chlorophyll a) the peak areas of all products (including all allomers and epimers) were summed and used in the calculations. When reporting the individual contribution of each chlorophyll a type (when both were present), the following formulas (based on the amount per injection, $\tilde{C}$) were used:

$$\tilde{C}_{Da} = \frac{\hat{A}_{Da}}{\hat{A}_a + \hat{A}_{Da}}\tilde{C}_{Ta} \tag{6}$$

and

$$\tilde{C}_a = \frac{\hat{A}_a}{\hat{A}_a + \hat{A}_{Da}}\tilde{C}_{Ta}, \tag{7}$$

where $\hat{A}$ indicates the peak area and the subscript identifies the pigment or pigment association (as per the symbology established in Sect. 1.2).

It should be noted that to the extent that divinyl chlorophyll a epimers are present in a sample, the value for

chlorophyll a will be overestimated and the value for divinyl chlorophyll a will be underestimated, because divinyl chlorophyll a epimer co-elutes with the chlorophyll a main peak. This error, however, does not affect the total chlorophyll a value.

Quantitation was based on peak area for all peaks except chlorophyll b and divinyl chlorophyll b, which were based on peak height because $R_s < 1.0$. Chlorophyll c_2 and Mg DVP co-eluted and were reported as one based on the chlorophyll c_2 RF. In addition, α- and β-carotene also co-eluted and were reported as $carotenes$ based on the β-carotene RF. Other pigments with a potential for co-elution and their potential effect on accuracy in quantitation are given in Table 13.

Table 14. E values used with the HPL method for the pigments in Table 13 as a function of the maximum wavelength (λ_m). The units for E are liters per gram per centimeter and the units for λ_m are nanometers.

No.	Pigment	Solvent	λ_m	E	Ref.
3	Chl c_2	100% Acetone	629.6	37.20	[1]
5	Chl c_1	100% Acetone	629.1	39.20	[1]
7	Perid	100% Ethanol	472.0	132.50	[2]
9	But-fuco	100% Ethanol	447.0	160.00	[3]
10	Fuco	100% Ethanol	449.0	160.00	[4]
11	Neo	100% Ethanol	438.0	227.00	[1]
13	Pras	100% Ethanol	454.0	160.00	[4]
14	Viola	100% Ethanol	443.0	255.00	[4]
15	Hex-fuco	100% Ethanol	447.0	160.00	[3]
19	Diadino	100% Ethanol	446.0	262.00	[4]
22	Allo	100% Ethanol	453.0	262.00	[4]
23	Diato	100% Ethanol	449.0	262.00	[4]
25	Zea	100% Acetone	452.0	234.00	[5]
26	Lut	100% Ethanol	445.0	255.00	[4]
27	Cantha	100% Acetone	474.0	222.90	[6]
30	DV chl b	100% Acetone	644.6	52.50	[1]
31	Chl b	100% Acetone	644.6	52.45	[7]
38	DV chl a	100% Acetone	663.0	88.15	[1]
39	Chl a	100% Acetone	664.0	87.67	[1]
43	$\beta\beta$-Car	100% Ethanol	453.0	262.00	[5]

[1] Jeffrey (1972)
[2] Jeffrey and Haxo (1968)
[3] Vesk and Jeffrey (1987)
[4] Bidigare (1991)
[5] Davies (1976)
[6] Latasa et al. (1996)
[7] Watanabe et al. (1984)

2.5 VALIDATION

During method development, validation of retention times for various pigments was accomplished by analyzing several reference algal cultures recommended by the Scientific Committee on Oceanic Research (Jeffrey and Le Roi 1997) and comparing pigment HPLC in-line photodiode array detector spectra (350–700 nm) with published values (observed spectra and algal sources given in Table 13). Subsequently, during sample analyses, pigment identities were monitored daily by comparing retention times of pigments in the samples with retention times of the 20 pigments in the standard quality control mixture that had been formulated. Chromatograms of the natural samples were overlaid (on screen) with the chromatogram of this standard injection to verify peak identities.

The accuracy of the calibration factors used were monitored by injecting additional, independently-formulated quality control samples (each containing chlorophyll a) at a frequency of every fifth injection (in most cases, the usual frequency is every tenth injection). The RFs observed from these quality control injections were determined not to exceed the average variability associated with this method (a 2% relative standard deviation was observed for all RFs for all pigments). This estimate of method precision takes into account the natural variability associated with such factors as

1. The spectrophotometrically determined concentration of the pigment standards;

2. The stability of the standard over time in the freezer;

3. HPLC precision;

4. The stability of the standards in the temperature-controlled autosampler compartment while waiting for injection; and

5. Uncertainties associated with the dilution of standards.

For a 2% average variability, 95% of all observed RFs should fall within approximately ±4% of the expected values.

2.6 DATA PRODUCTS

All pigments with a bullet ($\bullet$) symbol in Table 14 are reported, those listed with an extinction coefficient are reported quantitatively, and those with a bullet symbol, but no extinction coefficient, are estimated only according to details given in Table 13. The following formulas are used to calculate micrograms of pigment per liter of seawater:

$$\tilde{C}_{P_i} = \hat{A}_{P_i} R_{P_i}, \tag{8}$$

where $\tilde{C}_{P_i}$ is the amount of pigment injected (in units of nanograms per injection), and $\hat{A}_{P_i}$ is the area of the parent peak and associated isomers for pigment P_i.

$$C_{P_i} = \frac{\tilde{C}_{P_i} V_e}{V_i V_f}, \tag{9}$$

where V_e is the extraction volume (in microliters), V_i is the injection volume (in microliters), and V_f is the filtration volume (in milliliters).

2.7 CONCLUSIONS

This method has been developed for use with a wide variety of water sample types and, as such, has the ability to separate pigments important to both estuarine and oceanic samples including divinyl and monovinyl chlorophyll a. Pigments often abundant in estuarine and freshwater systems (such as neo, prasino, viola, allo, and lut) are well resolved from each other and from pigments commonly found in oceanic systems (but-fuco, hex-fuco, fuco, and zea). The method is also designed to accommodate samples ranging in concentration by up to approximately 400 fold, as the linear dynamic range extends nearly to detector saturation and high sample mass does not deleteriously affect peak shape, peak area, or retention time reproducibility. Furthermore, it has been documented that there is no carryover from a highly concentrated injection to a subsequent injection of a dilute sample.

Canthaxanthin is not used as an internal standard, because it occurs naturally in some samples that have been received for analysis (e.g., Assateague Bay and the Baltic Sea). The precision of this method has been well established as has its potential limitations with regard to pigment co-elutions. The method is relatively fast, well suited to automated analyses, and can provide quantitative information for up to 20 pigments with qualitative information for additional pigments.

Chapter 3

The JRC Method

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ABSTRACT

The HPLC method used by the JRC follows the JGOFS core measurements protocols (JGOFS 1994) and is a modified version of the method presented by Wright et al. (1991). It does not permit the separation of divinyl chlorophyll a and divinyl chlorophyll b from chlorophyll a and chlorophyll b, respectively. The method was designed for coastal Adriatic Sea waters only, and because prochlorophytes are not found in coastal areas, this has not been a relevant disadvantage. Filter disruption is accomplished mechanically using a motorized grinder. The pigments are extracted within a 100% acetone solution including an internal standard (*trans-β*-apo-8′-carotenal). The HPLC system used includes a reversed phase C_{18} column (with a C_{18} guard column), an autosampler (with thermostat), a DAD, a fluorescence detector, and a three-solvent gradient. The JRC method provides measurements of the main pigment concentrations with a detection limit of approximately $0.001\,\mu g\,L^{-1}$, a repeatability (based on the analysis of several samples from the same water volume) of about $7(\pm 6)\%$ for chlorophyll a concentration (Zibordi et al. 2000), and an analysis time of about 35 min.

3.1 INTRODUCTION

The HPLC method used at JRC follows the JGOFS core measurements protocols (JGOFS 1994) and is a modified version of the method presented by Wright et al. (1991). This method does not allow the separation of divinyl chlorophylls a and b from chlorophylls a and b, respectively. In addition, chlorophyll c_1 and chlorophyll c_2 are not separated. The HPLC system used includes an autosampler and a DAD.

The samples, which were received on 15 November 1999, were stored in a freezer at $-80°C$ until analysis. The pigments were extracted within an acetone solution including an internal standard.

3.2 EXTRACTION

The pigments were extracted with a solution of 100% acetone including an internal standard. The internal standard, *trans-β*-apo-8′-carotenal, is used to correct possible errors due to evaporation of the solution and for water retained by the filter after sampling. The concentration of the internal standard in 100% acetone is chosen in such a way that the peak areas of the pigments and standard are comparable, in general approximately $0.2\,\mu g\,L^{-1}$.

The extraction process began with the placement of the filter in 1.5 mL of acetone including the internal standard. The filter was then ground for 30 s, in dim light and $0°C$, using a motorized grinder with a Teflon pestle. After grinding, the disrupted filter was allowed to extract for 24 h in the dark at $-20°C$. Between the grinding of the filters, the Teflon pestle was rinsed with 100% acetone. The extract was poured into a syringe and then forced through a $0.45\,\mu m$ filter (Millex FH), mounted on the syringe, and transferred in an amber vial.

3.3 HPLC ANALYSIS

The autosampler was programmed to mix, before the injection, $75\,\mu L$ of the filtrated extract with $22.5\,\mu L$ of ammonium acetate buffer (1 M). This solution was then injected through a $100\,\mu L$ loop into the HPLC system by a temperature-controlled autosampler (set at $4°C$).

The JRC HPLC system is composed of the following components:

- A reversed phase column (250×4.6 mm), $5\,\mu m$ particle size, ODS-2 C_{18} (Spherisorb) coupled with a (15×4.6 mm) ODS-2 C_{18}, $5\,\mu m$ particle size, (Hichrom) guard column (the column temperature is not monitored);

- An autosampler and thermostat (HP 1100);
- A DAD (HP 1100);
- A pumping system and degasser (HP 1050);
- A fluorescence detector (HP 1046A); and
- The data acquisition and analysis software (HP Rev. A.06.03).

The solvents in the mobile phase, which had a flow rate of $1\,\mathrm{mL\,min^{-1}}$, were as follows:

- A 80:20 methanol:0.5 M ammonium acetate (with a pH of 7.2);
- B 90:10 acetonitrile:water; and
- C 100% ethyl acetate.

(This information is summarized in Table 4). The JRC gradient, which used three solvents, is given in Table 15.

Table 15. The gradient used with the JRC column. The time is in minutes, and the percentages of solvents A, B, and C are given in the last three columns.

Step	Time	A [%]	B [%]	C [%]
Start	0.0	100	0	0
2	2.0	0	100	0
3	2.6	0	90	10
4	13.6	0	65	35
5	18.0	0	31	69
6	23.0	0	31	69
7	25.0	0	100	0
8	26.0	100	0	0
End	34.0	100	0	0

The DAD collects the spectra between 350–750 nm. Chlorophyll pigments and carotenoids were detected at 436 nm and phaeopigments at 405 nm. The peak integration was performed automatically according to the following conditions:

a) Area rejection, 0.09 mAU†;

b) Peak width, 0.09 min; and

c) Threshold, 0.09 mAU.

When peaks were not significantly separated (this rarely happened for the present experiment), a manual integration was performed.

3.4 CALIBRATION

The calibration of the HPLC system was performed in December 1999 (before the pigment analyses for the SeaHARRE-1 activity were performed). The internal standard, $trans$-β-apo-8′-carotenal, was purchased from Fluka Chemie AG (Buchs, Switzerland). The external standard pigments, together with their extinction coefficients, were

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† Milli-absorbance unit.

all provided by DHI Water and Environment Institute (Høsholm, Denmark) except for chlorophyll c_{1+2} and ($trans$-β-apo-8′-carotenal).

The purity of each standard pigment was checked by HPLC analysis and computed as:

$$P_{\%} \;=\; \frac{\hat{A}_s}{\hat{A}_s \;+\; \sum \hat{A}_{P_d}}, \tag{10}$$

where $P_{\%}$ is the chromatographic purity (in percent) of the primary pigment standard, $\hat{A}_s$ is the HPLC peak area of the pigment standard (in units of mAU s), and $\hat{A}_{P_d}$ is the HPLC peak area of the degraded pigment (mAU s).

Concentrations were determined using a dual beam monochromator-type spectrophotometer Lambda 12 Perkin Elmer spectrometer, with a 2 nm bandwidth. For each pigment standard, the absorbance at the specific wavelength is corrected for the absorbance at 750 nm, and a calibration linear fit was performed using four measurement points. The E values for the JRC standard pigments are given in Table 16.

Table 16. E values used with the JRC method for a variety of pigments as a function of λ. The units for E are liters per gram per centimeter and the units for λ are nanometers.

Pigment	Solvent	λ	E
Chlorophyll a	90% Acetone	664.0	87.67
Chlorophyll b	90% Acetone	646.8	51.36
Chlor. c_{1+2}	90% Acetone	631.0	42.60†
Peridinin	100% Ethanol	472.0	132.50
But-fucoxanthin	100% Ethanol	447.0	160.00
Fucoxanthin	100% Ethanol	449.0	160.00
Hex-fucoxanthin	100% Ethanol	447.0	160.00
Diadinoxanthin	100% Ethanol	446.0	262.00
Alloxanthin	100% Ethanol	453.0	262.00
Zeaxanthin	100% Ethanol	450.0	254.00
β-carotene	100% Ethanol	453.0	262.00
β-apo-8′-caro.	100% Ethanol	452.0	254.00†

† Mantoura and Repeta (1997).

The areas of the standard pigments isomers (allomers and epimers) were included in the total peak area.

3.5 VALIDATION

A variety of procedures, executed at different time intervals, are used to validate the performance of the JRC HPLC system:

1. The performance of the analytical system is checked by Hewlett-Packard once a year.

2. The standards are only used within one year from the day of production (using information provided by DHI).

3. The variability of the measurements is surveyed by a regular (every four months) performance of successive injections (three) of the same sample.

4. The RF of the internal standard is verified every three months.

5. The correctness of pigment identification is regularly checked by injecting a mixture of different standards and comparing their retention time with the one observed for a sample (this is done approximately every 30 samples).

3.6 DATA PRODUCTS

For every injection, a data file was created wherein the pigment peaks (along with their respective retention times), area, height, and width were listed. The peak areas were transferred into a spreadsheet data file and converted to pigment concentrations (Zibordi et al. 2000).

3.7 CONCLUSIONS

The JRC method provides measurements of the main pigment concentrations with a detection limit of approximately $0.001\,\mu g\,L^{-1}$, a repeatability (based on the analysis of several samples from the same water volume) of about $7(\pm6)\%$ for chlorophyll a concentration (Zibordi et al. 2000), and an analysis time of about $35\,min$. The method does not permit the separation of divinyl chlorophylls a and b from chlorophylls a and b, respectively. Up to now, the JRC method has been applied to the coastal Adriatic Sea waters only (Zibordi et al. 2000) and, as prochlorophytes are not found in coastal areas, this has not been considered a relevant disadvantage.

Chapter 4

The LPCM Method

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Abstract

The LPCM method applies a sensitive reversed phase HPLC technique for the determination of chloropigments and carotenoids within approximately 24 min. The different pigments are detected by a DAD which allows for automatic identification to be carried out on the basis of absorption spectra. Optical densities are monitored at 440 nm (chloropigments and carotenoids) and at 667 nm (chloropigments only). The method provides a good resolution between divinyl chlorophyll *a* and chlorophyll *a*, but uncertainties may arise for the partial separation of chlorophyll *b* and divinyl chlorophyll *b*, and for the resolution of chlorophyll *c* pigments. Detection limits for most pigments are low (approximately $0.001 \, \text{mg m}^{-3}$). The use of an internal standard has shown to improve the accuracy of the analysis.

4.1 INTRODUCTION

The LPCM method applies a sensitive reversed phase HPLC technique (with a C_8 column) for the determination of most chloropigments (including degradation products) and carotenoids (extracted in methanol) within approximately 24 min. This type of analysis, which separates divinyl chlorophyll *a* from chlorophyll *a* has been initially developed for application to open ocean Case-1 waters, especially in oligotrophic areas (low detection limits of approximately $0.001 \, \text{mg m}^{-3}$). This method, however, also performs well in Case-2 waters.

The SeaHARRE-1 samples were stored in liquid nitrogen, then in a freezer before extraction and analysis, which took place between 20–24 December 1999.

4.2 EXTRACTION

The extraction process involved the following steps:

1. The 25 mm GF/F filter was placed into a 10 mL Falcon™ tube.

2. 3 mL of methanol, including an internal standard (*trans*-β-apo-8′-carotenal), was added to each tube using an Ependorf™ pipette, while making sure that the filter was completely covered.

3. The samples were placed in a freezer at $-20°$C for a minimum of 30 min.

4. The filters were then macerated using an ultrasonic probe (Ultrasons-Annemasse), for not more than 10 s. The probe was rinsed with methanol then wiped between each sample.

5. The tubes were placed back into the freezer for another minimum of 30 min.

6. The sample was then clarified by filtering it through a 25 mm GF/C (1.3 μm porosity) filter. A glass tube (rinsed with methanol then wiped between each sample) was used to press the sample slurry. The filtrate was collected in a 10 mL Falcon™ tube.

7. The filtrate was stored in a freezer at $-20°$C until HPLC analysis.

4.3 HPLC ANALYSIS

The LPCM HPLC system is composed of the following:

- An HP *Chemstation for LC* software (A.06.03);

- A Thermoquest Autosampler (AS 3000), including a temperature control (set at 4°C), an autoinjector for mixing the sample with the ammonium acetate (1 M) buffer, a 1 mL preparation syringe, and a 1 mL sample syringe;

- An HP degasser (HP 1100);

- An HP binary pump (HP 1100); and

- Two detectors.

The detectors are HP DADs (HP 1100) with measurements at 440 nm (for carotenoids and chlorophylls) and at 667 nm for phaeopigments, and a Thermoquest fluorometer (AS 3000).

The analytical method, based on a gradient between a methanol–ammonium acetate mixture (70:30) and a 100% methanol solution (solvent A and solvent B, respectively), is similar to that described by Vidussi et al. (2000). Modifications to this method to separate certain peaks and increase sensitivity included a) a flow rate of $0.5\,\mathrm{mL\,min^{-1}}$, and b) a reversed phase chromatographic column (C_8) with a 3 mm internal diameter (Hypersil MOS 3μm). The gradient used is presented in Table 17.

Table 17. The gradient used with the LPCM column. The time is in minutes, and the percentages of solvents A and B are given in the last two columns.

Step	Time	A [%]	B [%]
Start	0	80	20
2	4	50	50
3	18	0	100
End	22	0	100

For analysis, the sample extract was transferred into a glass HPLC vial using a Pasteur pipette, which was then disposed of after use.

4.4 CALIBRATION

A calibration was performed in December 1999 shortly after the analysis of the SeaHARRE-1 samples. This provided LPCM Perkin Elmer Spectrophotometer response factors for peridinin, 19′-butanoyloxyfucoxanthin, fucoxanthin, 19′-hexanoyloxyfucoxanthin, alloxanthin, zeaxanthin, chlorophyll a and chlorophyll b (standards provided by the International Agency for ^{14}C Determination, Denmark). These RFs were then derived to compute the LPCM HPLC RFs at 440 nm.

The RFs for divinyl chlorophyll a and divinyl chlorophyll b were computed

a) Knowing the specific extinction coefficients of chlorophyll a (or chlorophyll b);

b) Accounting for the absorption of chlorophyll a and divinyl chlorophyll a (or chlorophyll b and divinyl chlorophyll b) at 440 nm when the spectra of both pigments are normalized at their red maxima; and

c) Considering that both pigments have the same molar absorption coefficient at this red maximum.

For the remaining pigments, their specific extinction coefficients were either derived from previous calibrations or from the literature (Jeffrey et al. 1997).

The extinction coefficients for the LPCM standard pigments are listed in Table 18 in the same order as their retention times. They were identified spectrally and then quantified in relation to the peak area (the concentrations are given in milligrams per cubic meter). Because chlorophyll c_1 and chlorophyll c_2 co-elute, they were first identified spectrally before being quantified then summed. Similarly, because chlorophyll b and divinyl chlorophyll b literally co-elute, they were first identified spectrally, then

quantified with their respective extinction coefficients, and finally summed.

Table 18. E values used with the LPCM method for a variety of pigments as a function of λ. The units for E are liters per gram per centimeter and the units for λ are nanometers.

Pigment	Solvent	λ	E
Mg DVP			Chl. c ¶
Chlorophyll c_3	90% Acetone	630.6	42.60 †
Chl. c_{1+2}	90% Acetone	630.6	42.60 †
Peridinin	100% Ethanol	472.0	132.50 ‡
But-fucoxanthin	100% Ethanol	446.0	160.00 ‡
Fucoxanthin	100% Ethanol	449.0	160.00 ‡
Prasinoxanthin	Diethyl Ether	446.0	250.00 †
Hex-fucoxanthin	100% Ethanol	447.0	160.00 ‡
Violaxanthin	100% Acetone	442.0	240.00 †
Neoxanthin	100% Ethanol	438.0	227.00 †
Diadinoxanthin	100% Acetone	447.5	223.00 †
Alloxanthin	100% Ethanol	453.0	262.00 ‡
Diatoxanthin	100% Acetone	452.0	210.00 †
Zeaxanthin	100% Ethanol	450.0	254.00 ‡
Lutein	Diethyl Ether	445.0	248.00 †
Chlorophyll b	90% Acetone	646.8	51.36 ‡
Divinyl Chl. b		440.0	28.03
Divinyl Chl. a		440.0	74.20
Sum Chl. a allomers	90% Acetone	664.3	87.67 ‡
Chlorophyll a	90% Acetone	664.3	87.67 ‡
Sum Chl. a epimers	90% Acetone	664.3	87.67 ‡
α-Carotene		440.0	180.00 §
β-Carotene		440.0	180.00 §
Phaeophorbide a	90% Acetone	667.0	74.20 †

¶ Same as chlorophyll c.
† Jeffrey et al. (1997).
‡ DHI Water and Environment Institute.
§ Previous calibration.

4.5 VALIDATION

Two solutions of methanol, including an internal standard, were injected at the beginning, at the end, and every 10 injections. This was done to check the retention times, and the stability and precision of the analysis. The internal standard was $trans$-β-apo-8′-carotenal. The pigment identification was manually verified by retention time comparison and observation of the absorption spectra.

4.6 DATA PRODUCTS

The *Chemstation for LC* program produces an Excel file for each sample comprising the pigment identification, retention times, peak areas, peak heights, peak widths,

and other chromatographic information. This file is used in a Visual Basic™ program to extract the peak areas and names, and then to calculate the internal-standard-corrected concentrations, C (in milligrams per cubic meter) of each pigment as in the following equation:

$$C_{P_i} = \frac{\hat{A}'_{P_i} R_{P_i}}{V_f},$$ (11)

where $\hat{A}'_{P_i}$ is the corrected peak area (mAU s) and R_{P_i} is the pigment response factor $[\text{mg (mAU s)}^{-1}]$, and V_f is in units of cubic meters. The $\hat{A}'_{P_i}$ term is computed as

$$\hat{A}'_{P_i} = \frac{\hat{A}_{P_i} \hat{A}_s}{\hat{A}_m},$$ (12)

where $\hat{A}_p$ is the uncorrected peak area (mAU s), $\hat{A}_s$ is the reference area (mAU s) of the internal standard (established as the average of internal standard injections over a single day), and $\hat{A}_m$ is the measured area (mAU s) of the internal standard.

4.7 CONCLUSIONS

The LPCM method provides very good resolution between divinyl chlorophyll a and chlorophyll a pigments. The detection limits for most pigments are low, approximately $0.001\,\text{mg m}^{-3}$. Uncertainties may arise for the separation of chlorophyll b and divinyl chlorophyll b, and for the resolution of chlorophyll c pigments. The use of an internal standard has shown to improve the accuracy of the analysis. The HPLC method applied by the LPCM has, therefore, provided satisfactory results for the analysis of chlorophyll a and accessory pigments in seawater samples from areas of different concentration levels.

Chapter 5

The MCM Method

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Abstract

The MCM method uses a reversed phase HPLC technique using a binary solvent system following a linear gradient on a C_8 chromatography column. Baseline separation of mono- and divinyl chlorophyll a and of lutein and zeaxanthin, partial separation of mono- and divinyl chlorophyll b, and resolution of other key chemotaxonomic chlorophylls and carotenoids are achieved in an analysis time of approximately 30 min. The method provides good resolution of mono- and divinyl chlorophylls a and b, as well as lutein and zeaxanthin, and satisfactorily separates other key pigments within approximately 30 min. The use of a canthaxanthin internal standard improves the accuracy of pigment determinations. Providing a pragmatic balance between good analyte resolution and acceptable sample throughput, the method is suitable for the analysis of a wide range of oceanographic water samples.

5.1 INTRODUCTION

Pigments are analyzed using a reversed phase HPLC method using a binary solvent system following a linear gradient on a C_8 chromatography column (Barlow et al. 1997). Baseline separation of mono- and divinyl chlorophyll a and of lutein and zeaxanthin, partial separation of mono- and divinyl chlorophyll b, and resolution of other key chemotaxonomic chlorophylls and carotenoids are achieved in an analysis time of approximately 30 min. Chlorophylls c_1 and c_2 and α- and β-carotene are not well separated by this technique. Prior to analysis, samples are stored either in liquid nitrogen or in a $-85°$C freezer.

5.2 EXTRACTION

An appropriate amount of canthaxanthin is dissolved in 0.5 or 1.0 L of 100% acetone and the absorbance (approximately 0.1) is determined at the blue maximum to estimate the concentration of the canthaxanthin internal standard in the acetone. The solution is stored at $5°$C in the dark. Then, 2 mL of the acetone–canthaxanthin are added to the frozen filter samples (25 mm), which contain 0.1–0.2 mL of water after filtration, in graduated centrifuge tubes. Pigments are extracted with the aid of ultrasonication (30 s), soaked for 30 min in the dark, and clarified by centrifugation in a refrigerated centrifuge. Final extract volumes are read from the centrifuge tube graduations, whose graduations have been checked against pipetted volumes of solution.

5.3 HPLC ANALYSIS

Extracts in dark glass vials are loaded into a Thermo Separations AS3000 autosampler and cooled to a temperature of $2°$C. The autosampler incorporates a column compartment containing a $3\,\mu$m Hypersil MOS2 C_8 column and an autoinjector, and both are heated to $25°$C. Prior to injection, the autosampler is programmed to vortex mix $300\,\mu$L of extract with $300\,\mu$L of 1 M ammonium acetate buffer, and $100\,\mu$L of the extract-buffer is injected onto the chromatography column.

The individual pigments are separated at a flow rate of $1\,$mL$\,$min^{-1} by a linear gradient (Table 19) using a Varian ProStar tertiary pump. Before the next injection, the column is reconditioned to its original condition over an additional 7 min. Solvent A consists of 70:30 (v:v) methanol:1 M ammonium acetate and solvent B is 100% methanol (Table 4).

Table 19. The gradient used with the MCM column. The time is in minutes, and the percentages of solvents A and B are given in the last two columns.

Step	Time	A [%]	B [%]
Start	0	75	25
2	1	50	50
3	20	30	70
4	25	0	100
End	32	0	100

The pigments are detected by absorbance at 440 nm using a Thermo Separations UV6000 DAD and any chlorophyll transformation products are simultaneously monitored at 665 nm. Spectral data are collected on every sample between 400–700 nm. Peak areas are initially automatically integrated using instrument default conditions, and then every chromatogram is checked with appropriate peak markers and the baselines are manually moved and placed to optimize the integration.

5.4 CALIBRATION

The canthaxanthin is purchased from Roth Chemicals (Karlsruhe, Germany). The chlorophyll a standard is obtained from Sigma Chemical Co. (St. Louis, Missouri), and a stock solution prepared at approximately 1 mg in 100 mL 100% acetone. A 10% working standard is then prepared in 100% acetone from the stock solution. Divinyl chlorophyll a and divinyl chlorophyll b is obtained from HPL (Cambridge, Maryland) or from the University of Hawaii at Manoa (Honolulu, Hawaii).

The following standards (2.5 mL volume) were obtained from DHI Water and Environment Institute:

a) Chlorophyll b,

b) Chlorophyll c_2,

c) Chlorophyll c_3,

d) Peridinin,

e) Fucoxanthin,

f) 19′-Hexanoyloxyfucoxanthin,

g) 19′-Butanoyloxyfucoxanthin,

h) Violaxanthin,

i) Diadinoxanthin,

j) Diatoxanthin,

k) Alloxanthin,

l) Zeaxanthin,

m) Lutein, and

n) β-Carotene.

Chlorophyll standards are shipped in 90% acetone and carotenoid standards are shipped in 100% ethanol.

Dual or triple single-point calibrations are run with all of the standards, except for chlorophyll a and canthaxanthin for which multipoint calibrations are conducted. The concentrations of all external standards and the canthaxanthin internal standard are determined and checked from absorbance measurements, scanning between 400–750 nm, using a monochromatic, double beam Hitachi U-2000 spectrophotometer with a 2 nm bandwidth. Red and blue wavelength maxima are used for the chlorophylls and carotenoids, respectively, along with the extinction coefficients estimated from data given in Table 20 (Jeffrey et al. 1997).

Table 20. E values used with the MCM method for a variety of pigments as a function of λ. The units for E are liters per gram per centimeter and the units for λ are nanometers.

Pigment	Solvent	λ	E
Canthaxanthin	100% Acetone	470	220.00
Chlorophyll a	100% Acetone	663	88.15
Divinyl chl. a	100% Acetone	662	88.35
Chlorophyll b	90% Acetone	645	51.36
Divinyl chl. b	100% Acetone	650	51.47
Chlorophyll c_2	90% Acetone	630	40.40
Chlorophyll c_3	90% Acetone	453	346.00
Peridinin	100% Ethanol	475	132.50
Fucoxanthin	100% Ethanol	450	152.00
But-fucoxanthin	100% Ethanol	446	134.60
Hex-fucoxanthin	100% Ethanol	446	130.00
Violaxanthin	100% Ethanol	441	255.00
Diadinoxanthin	100% Ethanol	447	233.70
Diatoxanthin	100% Ethanol	450	248.10
Alloxanthin	100% Ethanol	451	262.00
Zeaxanthin	100% Ethanol	452	254.00
Lutein	100% Ethanol	446	255.00
β-Carotene	100% Ethanol	452	262.00

The method separates chlorophyll a from its allomers and epimers and, therefore, these transformation products are not included in the peak areas for chlorophyll a calibration or quantification. Divinyl chlorophyll a is chromatographically separated from chlorophyll a and is independently calibrated and quantified.

5.5 VALIDATION

Pigments are identified by retention time comparison with external and internal standards and from diode array spectra. RFs are calculated relative to the internal standard and a quality control check is run by injecting the chlorophyll a working standard on a daily basis. Variability of the chlorophyll a RF ranges from 2–7% of the original calibration. The concentration of the canthaxanthin internal standard is checked from time to time by absorbance measurement, as described above, to ensure stability under working and storage conditions, and the RF is checked from a chromatographic run. The variability of the canthaxanthin RF ranges from 1–6% of the original calibration.

5.6 DATA PRODUCTS

The files of the chromatographic results report the pigment identification, retention times, peak areas, and peak heights. RFs are computed from the peak areas and pigment standard concentrations, and relative RFs, R'_{P_i}, are calculated by relating individual pigment RFs to the canthaxanthin RF. The weight of the canthaxanthin internal

standard added in each $2\,\text{mL}$ extraction is known from the concentration of canthaxanthin in the acetone extract solution and is of the order of $450\text{--}500\,\text{ng}\,\text{mL}^{-1}$. The pigment concentration (C_{P_i}) of the sample (in nanograms per liter) is then calculated as:

$$C_{P_i} \;=\; \frac{\hat{A}_{P_i}\, R'_{P_i}\, W_s}{\hat{A}_s\, V_f},\tag{13}$$

where W_s is the weight of the (canthaxanthin) standard, and $\hat{A}_s$ is the area of the (canthaxanthin) standard.

5.7 CONCLUSIONS

The reversed phase HPLC method using a C_8 column provides good resolution of mono- and divinyl chlorophylls a and b, as well as lutein and zeaxanthin, and satisfactorily separates other key pigments within approximately a 30 min time interval. The use of a canthaxanthin internal standard improves the accuracy of pigment determinations. Providing a pragmatic balance between good analyte resolution and acceptable sample throughput, the method is suitable for the analysis of a wide range of oceanographic samples.

Editors' Note

There is an emerging replacement of the letters "ae" (derived from the Latin "æ" dipthong) with the letter "e" in some publications, many of which are scholarly treatments of pigment analyses. Such a simplification has relevance to this document, because it allows a choice for spelling "phaeopigment" as "pheopigment," "phaeophytin" as "pheophytin," etc. For this report, the editors consulted a number of experts and interested parties in the publishing field, and a variety of dictionaries and style manuals.

Although leading journals in the field are frequently the best source of acceptable spelling, the general consensus was that there is no good reason for the evolving change in spelling regardless of where it is appearing—even if it is restricted to adjectives and nouns and not adopted for botanical nomenclature (*Phaeophyceae, Phaeothamnion,* and many others)—so, the dipthong was retained. One practical argument supporting the decision to retain the dipthong is the body of existing printed English literature (American or British) cannot be altered, so everyone is going to have to know both spellings even if they only use one. Another argument is it maintains the linkage back to what the original terminology was meant to convey, i.e., phaeophytin means "brown plant material."

Appendices

A. The SeaHARRE-1 Science Team
B. The SCOR WG 78 Pigment Abbreviations
C. Commercial HPLC Manufacturers and Pigment Suppliers
D. The HPL Simultaneous Equation for Quantitation of Chlorophyllide a and Chlorophyll c_1

Appendix A

The Science Team for SeaHARRE-1

The science team is presented alphabetically.

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Appendix B

The SCOR WG 78 Pigment Abbreviations

The chlorophyll pigments used in this report and their SCOR WG 78 abbreviations are presented alphabetically:

Chl a Chlorophyll a,

chl a' Chlorophyll a epimer,

Chl b Chlorophyll b,

chl b' Chlorophyll b epimer,

Chl c_1 Chlorophyll c_1,

Chl c_2 Chlorophyll c_2,

Chl c_3 Chlorophyll c_3,

Chlide a Chlorophyllide a

DV chl a Divinyl chlorophyll a,

DV chl a' Divinyl chlorophyll a epimer,

DV chl b Divinyl chlorophyll b,

DV chl b' Divinyl chlorophyll b epimer,

Phide   Phaeophorbide a, and

Phytin a Phaeophytin a.

The carotenoid pigments and their SCOR WG 78 abbreviations are presented alphabetically (with their trivial names in parentheses):

Allo   Alloxanthin,

Anth   Antheraxanthin,

Asta   Astaxanthin,

But-fuco   19′-Butanoyloxyfucoxanthin,

Cantha   Canthaxanthin,

Croco   Crocoxanthin,

Diadchr   Diadinochrome (Diadinochrome I and II),

Diadino   Diadinoxanthin,

Diato   Diatoxanthin,

Dino   Dinoxanthin,

Fuco   Fucoxanthin,

Hex-fuco   19′-Hexanoyloxyfucoxanthin,

Lut   Lutein,

Mg DVP   Mg 2,4-divinyl phaeoporphyrin a_5 monomethyl ester,

Monado   Monadoxanthin,

Neo   Neoxanthin,

Perid   Peridinin,

Pras   Prasinoxanthin,

Viola   Violaxanthin,

Zea   Zeaxanthin,

$\beta\beta$-Car $\beta\beta$-Carotene (β-Carotene),

$\beta\varepsilon$-Car $\beta\varepsilon$-Carotene (α-Carotene),

Appendix C

Commercial HPLC Manufacturers and Pigment Suppliers

The commercial HPLC manufacturers and pigment suppliers discussed in this report are presented alphabetically.

Agilent Technologies, Inc.[1]
2850 Centreville Rd.
Wilmington, DE 19808
Voice: 800–227–9770
Fax: 800–519–6047
Net: `http://www.agilent.com/chem`

Branson Ultrasonics Corporation
41 Eagle Road
Danbury, CT 06810
Voice: 203–796–0400
Fax: 203–796–0320
Net: `http://www.bransoncleaning.com`

Carl Roth GmbH and Company
Schoemperlenstraße
1-5 D-76185 Karlsruhe
GERMANY
Voice: 49–800–569–9000
Fax: 49–721–560–6149
Net: `http://www.carl-roth.de`

DHI Water and Environment Institute[2]
Agern Allé 11,
DK–2970 Høsholm
DENMARK
Voice: 45–45–16–92–00
Fax: 45–45–16–92–92
Net: `dhi@dhi.dk`

Fluka Chemical Corporation[3]
1001 West St. Paul Avenue
Milwaukee, WI 53233
Voice: 414–273–5013
Fax: 414–273–4979
Net: `flukausa@sial.com`

Hewlett-Packard Company
3000 Hanover Street
Palo Alto, CA 94304–1185
Voice: 650–587–1501
Fax: 650–857–5518
Net: `http://www.hp.com`

Hitachi Instruments, Inc.
3100 North First St.
San Jose, CA 95134
Voice: 800–548–9001
Fax: 408–492–8258
Net: `http://www.hii-hitachi.com`

Scientific Resources, Inc.
P.O. Box 957297
Duluth, GA 30095–7297
Voice: 800–637–7948
Fax: 770–476–4571

Sigma-Aldrich Company[4]
3050 Spruce Street
St. Louis, MI 63103
Voice: 314–771–5765
Fax: 314–771–5757
Net: `sigma@sial.com`

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[1]   Formerly the Hewlett-Packard Analytical Division.

[2]   Formerly the VKI Water Quality Institute.

[3]   Part of Sigma-Aldrich.

[4]   Formerly Sigma Chemical.

ThermoQuest[5]
355 River Oaks Parkway
San Jose, CA 95134–1991
Voice: 408–526–1100
Fax: 408–965–6810
Net: http://www.thermoquest.com

Appendix D

The HPL Simultaneous Equation for Quantitation of Chlorophyllide a and Chlorophyll c₁

Simultaneous equations for the quantitation of chlorophyllide a and chlorophyll c_1 are needed, because they co-elute when the HPL method is used for pigment determination. The formulation begins with defining the peak area for pigment P_i as $\hat{A}_{P_i}(\lambda)$. The calculation for the amount (in nanograms) per injection of chlorophyllide a and chlorophyll c_1 ($\tilde{C}_{C_a}$ and $\tilde{C}_{c_1}$, respectively) are as follows:

$$\tilde{C}_{C_a} \;=\; \frac{1}{R_{c_1}^{C_a}}\left[\frac{\hat{A}_{C_a}(665)}{R_{c_1}(450)} \;-\; \frac{\hat{A}_{C_a}(450)}{R_{c_1}(665)}\right] \qquad (D1)$$

and

$$\tilde{C}_{c_1} \;=\; \frac{1}{R_{c_1}^{C_a}}\left[\frac{\hat{A}_{c_1}(450)}{R_{C_a}(665)} \;-\; \frac{\hat{A}_{c_1}(665)}{R_{C_a}(450)}\right], \qquad (D2)$$

where

$$R_{c_1}^{C_a} \;=\; R_{C_a}^{-1}(665)R_{c_1}^{-1}(450) \;-\; R_{C_a}^{-1}(450)R_{c_1}^{-1}(665). \qquad (D3)$$

Solving for $R_{C_a}(665)$ begins with

$$R_{C_a}(665) \;=\; \bar{R}_a\left[\frac{M_{C_a}}{M_a}\right], \qquad (D4)$$

where M_{C_a} and M_a are the molecular weights of chlorophyllide a and chlorophyll a, respectively, and $\bar{R}_a$ is the mean RF for chlorophyll a. Substituting the value 0.6883 for the M_{C_a}/M_a ratio:

$$\begin{aligned} R_{C_a}(665) &= 0.2946\,(0.6883) \\ &= 0.2028 \end{aligned} \qquad (D5)$$

and, therefore, $R_{C_a}^{-1}(665) = 4.9316$.
Solving for $R_{C_a}(450)$ begins with the formulations associated with the peak areas:

$$\begin{aligned} R_{C_a}(450) &= R_{C_a}(665)\left[\frac{\hat{A}_{C_a}(450)}{\hat{A}_{C_a}(665)}\right] \\ &= 0.2028\,[2.0214] \\ &= 0.4140 \end{aligned} \qquad (D6)$$

and, therefore, $R_{C_a}^{-1}(450) = 2.4156$.
Solving for $R_{c_1}(450)$ begins with

$$R_{c_1}(450) \;=\; 0.0979 \qquad (D7)$$

and, therefore, $R_{c_1}^{-1}(450) = 10.2145$.

Solving for $R_{c_1}(665)$ also begins with the formulations associated with the peak areas:

$$\begin{aligned} R_{c_1}(665) &= R_{c_1}(450)\left[\frac{\hat{A}_{c_1}(450)}{\hat{A}_{c_1}(665)}\right] \\ &= 0.0979\,[86.3729] \\ &= 8.4559 \end{aligned} \qquad (D8)$$

and, therefore, $R_{c_1}^{-1}(665) = 0.1183$.
Substitutions can now be made to solve for $R_{c_1}^{C_a}$ (D3) using the values computed above:

$$\begin{aligned} R_{c_1}^{C_a} &= (4.9316)(10.2145) - (2.4156)(0.1183) \\ &= 50.0881, \end{aligned} \qquad (D9)$$

where the numeric entries are the inverse values calculated for (D5), (D7), (D6), and (D8), respectively.

GLOSSARY

CNRS	*Centre National de la Recherche Scientifique* (the French National Institute of Scientific Research)
CTD	Conductivity, Temperature, and Depth
DAAC	Distributed Active Archive Center
DAD	Diode Array Detector
DYF	DYFAMED
DYFAMED	*Dynamique des Flux en Méditerranée* (Dynamics of fluxes in the Mediterranean)
ET	Eutrophic
GSFC	Goddard Space Flight Center
HP	Hewlett-Packard
HPL	Horn Point Laboratory
HPLC	High Performance Liquid Chromatography
ID	Identification
INSU	*Institut National des Sciences de l'Univers* (the French National Institute of the Science of the Universe)
JGOFS	Joint Global Ocean Flux Study
JRC	Joint Research Centre (of the European Commission)
LPCM	*Laboratoire de Physique et Chimie Marines*
MCM	Marine and Coastal Management (of South Africa)
MIO	*Mer Ionienne* (Ionian Sea)
MT	Mesotrophic
NASA	National Aeronautics and Space Administration
NIST	National Institute of Standards and Technology
NR	Not Resolved
OT	Oligotrophic
PROSOPE	*Productivité des Systèmes Océaniques Pélagiques* (Productivity of Pelagic Oceanic Systems)

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[5] Formerly Thermo Separation Products.

QC Quality Control

RF Response Factor

SAI Space Applications Institute

SAT Short Along-Track (station)

SCOR Scientific Committee on Oceanographic Research

SDY Sequential Day of the Year

SeaHARRE SeaWiFS HPLC Analysis Round-Robin Experiment

SeaHARRE-1 The First SeaWiFS HPLC Analysis Round-Robin Experiment

SeaWiFS Sea-viewing Wide Field-of-view Sensor

SIRREX SeaWiFS Intercalibration Round-Robin Experiment

SNR Signal-to-Noise Ratio

TBAA Tetrabutyl Ammonium Acetate

TSP Thermo Separation Products

UMCES University of Maryland Center for Environmental Science

UNESCO United Nations Educational, Scientific, and Cultural Organization

UPD Unbiased Percent Difference

UPW Upwelling

UV Ultraviolet

WG Working Group

Symbols

A Used to denote an average across all four methods or all pigments.

A' Used to denote a subset average (i.e., a small number of pigments are excluded).

$\hat{A}$ The peak area.

$\hat{A}_m$ The measured area of the internal standard.

$\hat{A}_p$ The uncorrected peak area.

$\hat{A}_{P_d}$ The HPLC peak area of the degraded pigment P_d.

$\hat{A}_{P_i}$ The area of the parent peak and associated isomers for pigment P_i.

$\hat{A}'_{P_i}$ The corrected peak area.

$\hat{A}_s$ The HPLC peak area of the pigment standard.

C The concentration.

$\bar{C}$ The average concentration.

$\tilde{C}$ The amount per injection.

C_{12} The concentration of chlorophyll $c_1 + c_2$ pigment.

C_a The concentration of chlorophyll a pigment.

C_b The concentration of chlorophyll b pigment.

C_B The concentration of 19′-butanoyloxyfucoxanthin pigment.

$\tilde{C}_{c_1}$ The amount of chlorophyll c_1 injected.

C_{C_a} The concentration of chlorophyllide a pigment.

$\tilde{C}_{C_a}$ The amount of chlorophyllide a injected.

$\bar{C}^D$ The average pigment concentrations for the duplicate data sets.

C_D The concentration of diadinoxanthin pigment.

C_{D_a} The concentration of divinyl chlorophyll a pigment.

C_{D_b} The concentration of divinyl chlorophyll b pigment.

C_F The concentration of fucoxanthin pigment.

C_H The concentration of 19′-hexanoyloxyfucoxanthin pigment.

C_P The concentration of peridinin pigment.

C_{P_i} The concentration of pigment P_i.

$\tilde{C}_{P_i}$ The amount of pigment P_i injected.

$\bar{C}_{P_i}$ The average concentration for each pigment, P_i.

$\bar{C}^T$ The average pigment concentrations for the triplicate data sets.

C_{T_a} The concentration of total chlorophyll a (chlorophyll a plus divinyl chlorophyll a) pigment.

C_{T_b} The concentration of total chlorophyll b (chlorophyll b plus divinyl chlorophyll b) pigment.

C_Z The concentration of zeaxanthin pigment.

$C_{\Sigma a}$ The concentration of sum chlorophyll a (the sum of total chlorophyll a and chlorophyllide a) pigment.

D Used to denote the so-called duplicate samples in the analysis.

E Extinction coefficient.

H The HPL method (or laboratory).

i An index for summing, usually over the pigments involved.

j An index for summing, usually over the four methods (H, J, L, and M).

J The JRC method (or laboratory).

k An index for summing, usually over the number of samples.

L The LPCM method (or laboratory).

L_j A particular laboratory (or method).

M The MCM method (or laboratory).

M_{C_a} The molecular weight of chlorophyllide a.

M_a The molecular weight of chlorophyll a.

N The number of samples.

$P_\%$ The chromatographic purity of the primary pigment standard.

P_d The degraded pigment.

P_i The pigment (or pigment association).

R The RF (for a pigment).

R' The relative RF (for a pigment).

$\bar{R}_a$ The mean RF for chlorophyll a.

R_{P_i} The RF for pigment P_i.

R_{c_1} The RF for chlorophyll c_1.

R_{C_a} The RF for chlorophyllide a.

$R_{c_1}^{C_a}$ A difference term equal to $R_{C_a}^{-1}(665)R_{c_1}^{-1}(450) - R_{C_a}^{-1}(450)R_{c_1}^{-1}(665)$.

R'_{P_i} The relative RF for pigment P_i.

R_s The resolution between adjacent pigments.

S_k The kth sample code (associated with a particular pigment).

T Used to denote the so-called triplicate samples in the analysis.

t_R Pigment retention time.

V_e The extraction volume.

V_f The filtration volume.

V_i The injection volume.

W_s The weight of the standard.

$\delta_{P_i}^{L_j}$ The percent difference between the duplicate and triplicate concentrations for a particular laboratory and pigment.

$\bar{\delta}_{P_i}^{L_j}$ The averages of the δ values for each pigment and method over all the samples (calculated from the absolute δ values).

λ Wavelength.

λ_m Maximum wavelength.

ξ The percent ratio of the standard deviation, σ, in the replicate data set with respect to the average value.

$\bar{\xi}_{P_i}$ The average of ξ for all the samples for a particular pigment.

$\xi_{P_i}^{L_j}$ The ratio of the standard deviation to the average for a particular laboratory and pigment (also called the coefficient of variation).

$\bar{\xi}_A^{L_j}$ The average of ξ across all pigments for a particular laboratory.

$\bar{\xi}_{P_i}^{L_j}$ The average of ξ for a particular laboratory and pigment.

σ The standard deviation of a sample set.

$\varsigma_{P_i}^{L_j}$ The standard deviation) in the $\bar{\psi}_{P_i}^{L_j}$ values.

ψ The UPD of a particular method with respect to the average of all methods.

$\bar{\psi}$ The average of the absolute UPD values over the number of samples (usually given for each method and each pigment).

$\psi_{12}^{L_j}$ The UPD of chlorophyll c_{1+c_2} pigment for a particular laboratory.

$\psi_a^{L_j}$ The UPD of chlorophyll a for a particular laboratory.

$\psi_b^{L_j}$ The UPD of chlorophyll b for a particular laboratory.

$\psi_B^{L_j}$ The UPD of 19'-butanoyloxyfucoxanthin for a particular laboratory.

$\psi_{Ca}^{L_j}$ The UPD of chlorophyllide a for a particular laboratory.

$\psi_D^{L_j}$ The UPD of diadinoxanthin for a particular laboratory.

$\psi_{Da}^{L_j}$ The UPD of divinyl chlorophyll a for a particular laboratory.

$\psi_{Db}^{L_j}$ The UPD of divinyl chlorophyll b for a particular laboratory.

$\psi_F^{L_j}$ The UPD of fucoxanthin for a particular laboratory.

$\psi_H^{L_j}$ The UPD of 19'-hexanoyloxyfucoxanthin for a particular laboratory.

$\psi_P^{L_j}$ The UPD of peridinin for a particular laboratory.

$\psi_{P_i}^{L_j}$ The UPD for a particular pigment and a particular laboratory.

$\psi_{Ta}^{L_j}$ The UPD of total chlorophyll a (chlorophyll a plus divinyl chlorophyll a) pigment for a particular laboratory.

$\psi_{T_b}^{L_j}$ The UPD of total chlorophyll b (chlorophyll b plus divinyl chlorophyll b) for a particular laboratory.

$\psi_Z^{L_j}$ The UPD of zeaxanthin for a particular laboratory.

$\psi_{\Sigma a}^{L_j}$ The UPD of sum chlorophyll a (the sum of total chlorophyll a and chlorophyllide a) for a particular laboratory.

$\psi_{P_i}^{L_j}$ The UPD for each pigment of the individual laboratories with respect to the average values for each pigment.

$\bar{\psi}_{P_i}^{A}$ The average of the $\bar{\psi}$ values for a pigment P_i across the four laboratories (or methods).

$\bar{\psi}_{P_i}^{L_j}$ The average UPD of each laboratory for each pigment across all the samples.

$|\psi|$ The absolute value of ψ.

$\bar{\psi}_{P_i}^{L_j}$ The average percent difference of each laboratory for each pigment across all the samples computed from the absolute percent differences.

ψ_{Ta} The UPD value for total chlorophyll a.

$\bar{\psi}_{Ta}$ The average UPD value for total chlorophyll a.

$|\psi_{Ta}|$ The absolute value of the UPD value for total chlorophyll a.

References

Barlow, R.G., D.G. Cummings, and S.W. Gibb, 1997: Improved resolution of mono- and divinyl chlorophylls a and b and zeaxanthin and lutein in phytoplankton extracts using reverse phase C-8 HPLC. *Mar. Ecol. Prog. Ser.,* **161,** 303–307.

Bidigare, R.R., 1991: "Analysis of algal chlorophylls and carotenoids." In: Marine Particles: Analysis and Characterization. *Geophysical Monograph 63,* D.C. Hurd and D.W. Spencer, Eds., American Geophysical Union, Washington, DC, 119–123.

Claustre, H., 1994: Phytoplankton pigment signatures of the trophic status in various oceanic regimes. *Limnol. Oceanogr.,* **39,** 1,207–1,211.

Davies, B.H., 1976: Carotenoids. In: *Chemistry and Biochemistry of Plant Pigments, Volume 2, 2nd Edition.* T.W. Goodwin, Ed., Academic Press, London, 38–165.

Greenberg, A.E., L.S. Clesceri, and A.D. Eaton (Eds.), 1992: *Standard Methods for the Examination of Water and Wastewater, 18th Edition.* American Public Health Association, Washington, DC, 10–19.

Hooker, S.B., W.E. Esaias, G.C. Feldman, W.W. Gregg, and C.R. McClain, 1992: An Overview of SeaWiFS and Ocean Color. *NASA Tech. Memo. 104566, Vol. 1,* S.B. Hooker and E.R. Firestone, Eds., NASA Goddard Space Flight Center, Greenbelt, Maryland, 24 pp., plus color plates.

——, and W.E. Esaias, 1993: An overview of the SeaWiFS project. *Eos, Trans. AGU,* **74,** 241–246.

——, and S. Maritorena, 2000: An evaluation of oceanographic radiometers and deployment methodologies. *J. Atmos. Oceanic Tech.,* **17,** 811–830.

——, and C.R. McClain, 2000: The calibration and validation of SeaWiFS data. *Prog. Oceanogr.,* **45,** 427–465.

Jeffrey, S.W., 1972: Preparation and some properties of crystalline chlorophyll c_1 and chlorophyll c_2 from marine algae. *Biochim. Biophys. Acta.,* **279,** 15–33.

——, and F.T. Haxo, 1968: Photosynthetic pigments of symbiotic dinoflagellates (zooxanthallae) from corals and clams. *Biol. Bull.,* **135,** 149–165.

——, and J-M. LeRoi, 1997: Simple procedures for growing SCOR reference microalgal cultures. In: *Phytoplankton Pigments in Oceanography: Guidelines to Modern Methods.* S.W. Jeffrey, R.F.C. Mantoura, and S.W. Wright, Eds., UNESCO Publishing, Paris, 181–205.

——, and R.F.C. Mantoura, 1997: Appendix A: Pigment abbreviations used by SCOR WG 78. In: *Phytoplankton Pigments in Oceanography: Guidelines to Modern Methods.* S.W. Jeffrey, R.F.C. Mantoura, and S.W. Wright, Eds., UNESCO Publishing, Paris, 447–559.

——, ——, and S.W. Wright, 1997: *Phytoplankton Pigments in Oceanography: Guidelines to Modern Methods.* UNESCO Publishing, Paris, 661 pp.

Joint Global Ocean Flux Study, 1991: JGOFS Core Measurements Protocols. *JGOFS Report No. 6,* Scientific Committee on Oceanic Research, 40 pp.

——, 1994: JGOFS Protocols for the Joint Global Ocean Flux Study Core Measurements. *JGOFS Report No. 29,* Scientific Committee on Oceanic Research, 170 pp.

Latasa, M., R.R. Bidigare, M.E. Ondrusek, M.C. Kennicutt II, 1996: HPLC analysis of algal pigments: A comparison exercise among laboratories and recommendations for improved analytical performance. *Mar. Chem.,* **51,** 315–324.

——, ——, ——, and ——, 1999: On the measurement of pigment concentrations by monochromator and diode-array spectrophotometers. *Mar. Chem.,* **66,** 253–254.

Mantoura, R.F.C., and D.J. Repeta, 1997: Calibration method for HPLC. In: *Phytoplankton Pigments in Oceanography: Guidelines to Modern Methods.* S.W. Jeffrey, R.F.C. Mantoura, and S.W. Wright, Eds., UNESCO Publishing, Paris, 407–428.

O'Reilly, J.R., S. Maritorena, B.G. Mitchell, D.A. Siegel, K.L. Carder, S.A. Garver, M. Kahru, and C. McClain, 1998: Ocean color chlorophyll algorithms for SeaWiFS. *J. Geophys. Res.,* **103,** 24,937–24,953.

Van Heukelem, L., and C.S. Thomas, 2000: Computer-assisted HPLC method development with applications to the isolation and analysis of marine phytoplankton pigments. *J. Chrom. A.,* (in press).

Vesk, M., and S.W. Jeffrey, 1987: Ultrastructure and pigments of two strains of the picoplanktonic alga *Pelagococcus subviridis* (Chrysophyceae). *J. Phycol.,* **23,** 322–336.

Vidussi, G., H. Claustre, J. Bustillos-Guzmàn, C. Cailliau, and J-C. Marty, 2000: Rapid HPLC method for determination of phytoplankton chemotaxonomic pigments: separation of chlorophyll a from divinyl-chlorophyll a and zeaxanthin from lutein. *J. Plankton Res.,* **18,** 2,377–2,382.

Watanabe, T., A. Hongu, K. Honda, N. Masataka, M. Konno, and S. Saitoh, 1984: Preparation of chlorophylls and pheophytins by isocratic liquid chromatography. *Anal. Chem.,* **56,** 251–256.

Wright, S.W., S.W. Jeffrey, F.C. Mantoura, C.A. Llewellyn, T. Bjørnland, D. Repeta, and N. Welschmeyer, 1991: Improved HPLC method for the analysis of chlorophylls and carotenoids from marine phytoplankton. *Mar. Ecol. Prog. Ser.,* **77,** 183–196.

Zibordi, G., J-F. Berthon, J-P. Doyle, S. Grossi, D. van der Linde, C. Targa, L. Alberotanza, P. Cova, and S. Vianello, 2000: Coastal Atmosphere and Sea Time Series (CoASTS): A long-term field project for satellite color data validation in the North Adriatic Sea. *J. Geophys. Res.,* (submitted).

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Firestone, E.R., and S.B. Hooker, 2000: SeaWiFS Postlaunch Technical Report Series Cumulative Index: Volumes 1–5. *NASA Tech. Memo. 2000–206892, Vol. 6,* S.B. Hooker and E.R. Firestone, Eds., NASA Goddard Space Flight Center, Greenbelt, Maryland, 14 pp.

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Johnson, B.C., H.W. Yoon, S.S. Bruce, P-S. Shaw, A. Thompson, S.B. Hooker, R.E. Eplee, Jr., R.A. Barnes, S. Maritorena, and J.L. Mueller, 1999: The Fifth SeaWiFS Intercalibration Round-Robin Experiment (SIRREX-5), July 1996. *NASA Tech. Memo. 1999–206892, Vol. 7,* S.B. Hooker and E.R. Firestone, Eds., NASA Goddard Space Flight Center, 75 pp.

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Hooker, S.B., and G. Lazin, 2000: The SeaBOARR-99 Field Campaign. *NASA Tech. Memo. 2000–206892, Vol. 8*, S.B. Hooker and E.R. Firestone, Eds., NASA Goddard Space Flight Center, 46 pp.

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Vol. 11

O'Reilly, J.E., and 24 Coauthors, 2000: SeaWiFS Postlaunch Calibration and Validation Analyses, Part 3. *NASA Tech. Memo. 2000–206892, Vol. 11,* S.B. Hooker and E.R. Firestone, Eds., NASA Goddard Space Flight Center, 49 pp.

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Vol. 13

Hooker, S.B., G. Zibordi, J-F. Berthon, S.W. Bailey, and C.M. Pietras, 2000: The SeaWiFS Photometer Revision for Incident Surface Measurement (SeaPRISM) Field Commissioning. *NASA Tech. Memo. 2000–206892, Vol. 13,* S.B. Hooker and E.R. Firestone, Eds., NASA Goddard Space Flight Center, Greenbelt, Maryland, 24 pp.

Vol. 14

Hooker, S.B., H. Claustre, J. Ras, L. Van Heukelem, J-F. Berthon, C. Targa, D. van der Linde, R. Barlow, and H. Sessions, 2000: The First SeaWiFS HPLC Analysis Round-Robin Experiment (SeaHARRE-1). *NASA Tech. Memo. 2000–206892, Vol. 14,* S.B. Hooker and E.R. Firestone, Eds., NASA Goddard Space Flight Center, Greenbelt, Maryland, 42 pp.