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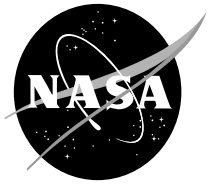

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December 2010

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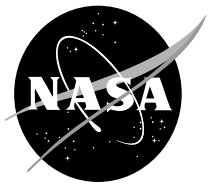

The Fourth SeaWiFS HPLC Analysis Round-Robin Experiment (SeaHARRE-4)

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Abstract

Ten international laboratories specializing in the determination of marine pigment concentrations using high performance liquid chromatography (HPLC) were intercompared using *in situ* samples and a mixed pigment sample. Although prior Sea-viewing Wide Field-of-view Sensor (SeaWiFS) High Performance Liquid Chromatography (HPLC) Round-Robin Experiment (SeaHARRE) activities conducted in open-ocean waters covered a wide dynamic range in productivity, and some of the samples were collected in the coastal zone, none of the activities involved exclusively coastal samples. Consequently, SeaHARRE-4 was organized and executed as a strictly coastal activity and the field samples were collected from primarily eutrophic waters within the coastal zone of Denmark. The more restrictive perspective limited the dynamic range in chlorophyll concentration to approximately one and a half orders of magnitude (previous activities covered more than two orders of magnitude). The method intercomparisons were used for the following objectives: a) estimate the uncertainties in quantitating individual pigments and higher-order variables formed from sums and ratios; b) confirm if the chlorophyll *a* accuracy requirements for ocean color validation activities (approximately 25%, although 15% would allow for algorithm refinement) can be met in coastal waters; c) establish the reduction in uncertainties as a result of applying QA procedures; d) show the importance of establishing a properly defined referencing system in the computation of uncertainties; e) quantify the analytical benefits of performance metrics, and f) demonstrate the utility of a laboratory mix in understanding method performance. In addition, the remote sensing requirements for the *in situ* determination of total chlorophyll *a* were investigated to determine whether or not the average uncertainty for this measurement is being satisfied.

PROLOGUE

The first Sea-viewing Wide Field-of-view Sensor (SeaWiFS) High Performance Liquid Chromatography (HPLC) Round-Robin Experiment (SeaHARRE-1) took place in 1999 (Hooker et al. 2000). It emphasized oligotrophic and mesotrophic regimes (northwest African upwelling and the Mediterranean Sea), involved four laboratories using four different methods (three C_8 and one C_{18}), and was based on 11 duplicates and triplicates (12 triplicates were planned). The dynamic range in the total chlorophyll *a* (TChl *a*) concentration, denoted $\left[\text{TChl } a\right]$, spanned 0.044–2.089 mg m^{-3}.

Field sampling for SeaHARRE-2 took place in 2002 (Hooker et al. 2005) and emphasized mesotrophic and eutrophic regimes (Benguela Current), involved eight laboratories using eight different methods (four C_8 and four C_{18}), although some of the methods were based on the same general method resulting in five rather distinct methods. Sample distribution involved 12 duplicates (12 triplicates were planned) spanning a $\left[\text{TChl } a\right]$ dynamic range of 0.357–26.185 mg m^{-3}.

Field sampling for SeaHARRE-3 took place in 2004 (Hooker et al. 2009) and emphasized the oligotrophic waters of the South Pacific Ocean central gyre. Additional samples were collected in mesotrophic waters and in the higher productivity of the Chilean upwelling. A total of 24 samples were distributed in triplicate covering a $\left[\text{TChl } a\right]$ dynamic range of 0.020–1.366 mg m^{-3}. Seven laboratories participated, but one of them executed two different methods, so a total of eight methods were intercompared.

Field sampling for SeaHARRE-4 took place in 2006 and was the first activity to emphasize coastal sampling, in particular the shallow waters associated with near-shore ecosystems. Unlike prior SeaHARRE field sampling campaigns, which took advantage of already scheduled oceanographic expeditions, the field sampling for SeaHARRE-4 was executed as a separate activity. The samples were collected from the fjords, estuaries, and bays within the coastal zone of Denmark.

Ten international laboratories agreed to participate in SeaHARRE-4 with so-called *validated* HPLC methods:

1. The Australian Commonwealth Scientific and Industrial Research Organisation (CSIRO);
2. The Danish DHI Institute for Water and Environment (DHI);
3. The American University of South Florida (USF) Florida Institute of Oceanography (FIO);
4. The American National Aeronautics and Space Administration (NASA) Goddard Space Flight Center (GSFC);
5. The American Horn Point Laboratory (HPL) University of Maryland Center for Environmental Science (UMCES);
6. The European Joint Research Centre (JRC), which is located in Italy;
7. The French *Laboratoire d'Océanographie de Villefranche* (LOV);
8. The Canadian Dalhousie University (DalU);

9. The American Center for Hydro-Optics and Remote Sensing (CHORS) at the San Diego State University (SDSU); and
10. The American University of South Carolina (USC).

The scientists involved in SeaHARRE-4 are listed in App. A.

The other laboratories that participated in past SeaHARRE intercomparisons are the Canadian Bedford Institute of Oceanography (BIO), the South African Marine and Coastal Management (MCM), and the British Plymouth Marine Laboratory (PML). As shown in Table 1, of all the laboratories involved with SeaHARRE activities, only HPL and LOV have participated in every one, although CSIRO, DHI, JRC, MCM, and SDSU have participated in three each.

Table 1. The laboratories, with their corresponding countries and assigned one-letter codes, that have participated in the four SeaHARRE activities executed to-date.

Laboratory and Country		*Code*	*SeaHARRE*			
BIO	Canada	*B*		2		
CSIRO	Australia	*C*		2	3	4
DHI	Denmark	*D*		2	3	4
FIO	United States	*F*				4
GSFC	United States	*G*				4
HPL	United States	*H*	1	2	3	4
JRC	Italy	*J*	1		3	4
LOV	France	*L*	1	2	3	4
MCM	South Africa	*M*	1	2	3	
DalU	Canada	*N*				4
PML	United Kingdom	*P*		2		
SDSU	United States	*S*		2	3	4
USC	United States	*U*				4

There are advantages in having a recurring set of *core* participants within the overall SeaHARRE activity, most notably because they provide an established capability and knowledge base that can be counted on during data analysis and workshop discussions (there have been almost as many workshops as round robins). The experience of the core analysts has provided invaluable learning opportunities for new analysts and they have helped steer the evolving objectives of each activity. In addition, however, emphasis is placed during the planning stages of each SeaHARRE activity to try and recruit new practitioners because another objective of the SeaHARRE activity is to provide the broadest investigation of community capabilities as possible.

The aforementioned concept of a validated method requires some additional explanation, because there is no external process or independent agency that certifies an HPLC phytoplankton pigment method is validated. The validation process is currently conceived and executed by the individual laboratory based on the sampling requirements and research objectives associated with the method. Consequently, validation occurs largely in isolation and relies heavily on a temporal evaluation of the calibration procedures, although some laboratories use more sophisticated evaluation criteria. As first demonstrated during SeaHARRE-1 (Hooker et al. 2000), intercomparing methods is a more robust mechanism for demonstrating the degree of validation for a particular method, and this is a permanent objective of the SeaHARRE activity.

Method validation procedures are important because they describe the level of measurement uncertainty associated with reported data products, i.e., individual pigment concentrations, sums, ratios, and indices. In the absence of the aforementioned external process or independent agency, however, validation activities of individual laboratories have emerged with varying emphases, often tailored to the specific research conducted by the individual laboratory. The products of validation, therefore, may not always yield the kind of information useful to intercomparing a diverse set of laboratory results over time or between laboratories. Consequently, for a more thorough understanding of measurement uncertainty and its relationship to accuracy in the analysis of field samples, intercalibration exercises are necessary, but the methods of the participating laboratories are best evaluated according to a common set of procedures and products.

The culmination of this philosophy of quantitative assessment was the drafting of a set of *performance metrics* during SeaHARRE-2 (Hooker et al. 2005), which have been continuously reviewed and updated (Hooker et al. 2009), since their inception. The evaluation of the performance metrics—in particular the corresponding accuracy and precision parameters—resulted in some participants replacing the methods they were initially using for a single (more modern) method with superior performance parameters: the Van Heukelem and Thomas (2001) method. In the more recent SeaHARRE activities, since SeaHARRE-1, the maximum diversity in the combination of laboratories and methods was achieved during SeaHARRE-2, and the least during SeaHARRE-3. Consequently, an emphasis was placed on recruiting new laboratories and methods for SeaHARRE-4, particularly methods that would help counterbalance the evolving predominance of C_8 methods over C_{18} methods (Table 2).

The reduction in method diversity over time was not expected. There was a strong feeling in the early planning for SeaHARRE-1 that the approach used in the joint Global Ocean Flux Study (JGOFS 1994)—selecting one method, in this case the Wright et al. (1991) method, and making it the protocol—should not be repeated, even unintentionally, because it stifles creativity. Two principal concerns with having too many practitioners using the same method was a) a bias in the predominant method would go undetected, and b) the state of the art would not continue to evolve. The practical benefit of adopting

Table 2. The methods used by all of the laboratories in the four HPLC round-robin intercomparisons executed to-date. The laboratory codes under the citations indicate the method used by a particular laboratory as a function of the four round robins.

Round Robin	*Gieskes and Kraay (1989)*[1]	*Wright et al. (1991)*[1]	*Pinckney et al. (1996)*[1]	*Vidussi et al. (1996)*[2]	*Barlow et al. (1997)*[2]	*Van Heukelem and Thomas (2001)*[2]
1		*J*		*L*	*M*	*H*
2	*B*	*C D S*		*L*	*M P*	*H*
3		*J S*[3]			*M*	*C D H L S*[3]
4		*N S*[4]	*F U*			*C D G H J L*

[1] A C_{18} column method.
[2] A C_8 column method.
[3] SDSU switched from the Wright et al. (1991) method to the Van Heukelem and Thomas (2001) method, but executed both, so they could be compared.
[4] SDSU switched back to the Wright et al. (1991) method.

a proven method instead of investing an unknown amount of time and resource in trying to improve a method, however, was simply too alluring. The potential pitfall of this approach—which is quantified here in many aspects—is underestimating the difficulty of implementing a new method with all its attendant detail.

The overall results of SeaHARRE-4 are presented in Chap. 1 and the individual methods of the 10 laboratories are presented in Chaps. 2–11, respectively. A summary of the material presented in each chapter is given below.

1. SeaHARRE-4 Methods, Data, and Analysis

The focus of this study was the estimation of uncertainties in quantifying a diverse set of chlorophyll and carotenoid pigment concentrations for a variety of HPLC methods and related procedures used in the analysis of predominantly coastal waters. The SeaHARRE-4 activity was designed to investigate the following objectives: a) estimate the uncertainties in quantitating individual pigments and higher-order variables formed from sums and ratios; b) confirm if the chlorophyll *a* accuracy requirements for ocean color validation activities (approximately 25%, although 15% would allow for algorithm refinement) can be met in coastal waters; c) establish the reduction in uncertainties as a result of applying QA procedures; d) show the importance of establishing a properly defined referencing system in the computation of uncertainties; e) quantify the analytical benefits of performance metrics, and f) demonstrate the utility of a laboratory mix in understanding method performance. All of these objectives were satisfied and the results associated with each are presented.

2. The CSIRO Method

The CSIRO method is a modified version of the Van Heukelem and Thomas (2001) method (VHT) and has the capacity to resolve approximately 35 different pigments with baseline resolutions of divinyl and monovinyl chlorophyll *a*, and zeaxanthin and lutein. Partial separation of divinyl and monovinyl chlorophyll *b*, and chlorophyll c_2 and chlorophyll c_1 can also be achieved. The method used for the samples and standards analyzed for SeaHARRE-4 was the same method used for SeaHARRE-3. Samples were extracted over 15–18 hours in an acetone solution before analysis by HPLC using a C_8 column and binary gradient system with an elevated column temperature. Pigments were identified by retention time and absorption spectrum from a photodiode array (PDA) detector. The method is regularly validated with the use of internal and external standards and individual pigment calibration. The detection limit of most pigments was within the range of 0.001–0.005 $\mathrm{mg\,m^{-3}}$. This method is applicable to the study of pigment composition and concentration in samples from all water types including freshwater, estuarine, upwelling coastal regions, and oligotrophic open ocean, as well as in the microphytobenthos of shallow coastal regions.

3. The DHI Method

The DHI HPLC method is a modified version of the HPL method. The method provides good separation of more than 30 of the most important pigments in freshwater, estuarine, and oceanic environments. Validation steps include four injections of a chlorophyll *a* standard to verify the calibration of the HPLC, use of an internal standard for correcting evaporation errors, and injection of a mixture of pigments to verify correct elution and retention times, and for documenting the precision of the HPLC and response factor stability. During analysis of the SeaHARRE-4 samples, the method provided satisfactory results on the precision of both mixed pigments and natural samples, and on the accuracy of TChl *a* measurements. The accuracy of PPig, however, was much higher when comparing the results to SeaHARRE-3, where DHI first used the HPL method. This increase in uncertainty is ascribed to the complexity of samples derived from transitional areas such as the Danish estuaries, where the SeaHARRE-4 samples were taken.

4. The FIO Method

The FIO method used multiple reversed-phase C_{18} columns connected in series. The configuration uses a single

monomeric and two polymeric columns, and was originally devised to enhance the separation of numerous structurally similar pigments and degradation products. The combination of the coarse and fine molecular properties of the two different column types optimizes pigment separation. The monomeric column provides strong retention and high efficiency, while the polymeric columns provide the selection for similar compounds. The latter are temperature sensitive and require the use of a column heater. A nonlinear binary gradient, adapted from Van Heukelem et al. (1992), was used for pigment separations. Solvent A consisted of 80% methanol:20% ammonium acetate (0.5 M adjusted to pH 7.2) and solvent B was composed of 80% methanol:20% acetone. Absorption spectra and chromatograms were acquired using a photodiode array detector. Pigment peaks were identified by comparing retention times and absorption spectra with standards or culture extracts (depending on the pigment involved).

5. The GSFC Method

GSFC participated in SeaHARRE-4 as an HPLC novice, with no previous experience in actual HPLC pigment analysis. The primary objective of the GSFC participation was to strictly adhere to the established performance metrics for quantitative analysis, as well as the published protocols of the selected method (Van Heukelem and Thomas 2001), to demonstrate whether or not both were sufficiently robust to allow any practitioner to produce results in keeping with the QA subset. Preparation at GSFC prior to sample analysis included servicing the instrument, making solvents, buffers, and standards, and running single- and multipoint regressions of known standards to create a calibration table and update a spectral library used to identify pigments in the 36 SeaHARRE unknown samples. Unexpected pressure issues resulted in recalibration and validation, as well as the SeaHARRE samples being analyzed on the Agilent 1100 HPLC at HPL. Multiple Chl *a* regression curves were run, and residuals calculated, in order to identify the dynamic range of the detector, as well as the overall precision of the instrument. Overall, 35 Chl *a* standards of varying concentrations were injected with average residuals of 1.5% for all concentrations. A second measurement of Chl *a* concentration, using a spectrophotometer and a trichomatic equation was completed at GSFC using the remaining sample extractions from the HPLC analysis. GSFC obtained state-of-the-art results, which established the capability of a novice laboratory to produce high-quality pigment data for use in calibration and validation exercises associated with remote sensing derivations of global chlorophyll concentrations.

6. The HPL Method

The HPL HPLC method employed by Horn Point Laboratory was developed for use with a variety of water types. Many pigments important to freshwater, estuarine, and oceanic systems are baseline resolved and quantitatively reported, including divinyl and monovinyl chlorophyll *a*. The method is built around a C_8 HPLC column, and incorporates a methanol-based reversed-phase solvent system, a simple linear gradient, and an elevated column temperature (60°C) to achieve separation of the pigments to be quantitated. The method can provide quantitative results for up to 25 pigments with qualitative information for additional pigments. Quality assurance measurements are made during sample analysis to confirm that the method performance is within expectations. Investigations into the uncertainties in the method show the 95% confidence limits are estimated as a) 0.5–3.8% for precision of replicate injections within and across sequences, b) 3.2% for chlorophyll *a* calibration reproducibility, and c) 5.1% for chlorophyll *a* method precision, including filter extraction and analysis.

7. The JRC Method

The HPLC method adopted by the JRC is the Van Heukelem and Thomas (2001) method, as modified for SeaHARRE-3 (Van Heukelem and Thomas 2009). This method has been successfully applied to a wide range of pigment concentrations from oligotrophic to eutrophic coastal waters (Van Heukelem and Thomas 2009). Here, it allowed for the separation and the quantification of 22 different pigments and, in particular, the monovinyl and divinyl forms of chlorophyll *a*. The samples are extracted in a 100% acetone solution including an internal standard (vitamin E acetate) and analyzed by HPLC using a C_8 column with a binary solvent gradient. The different pigments are identified using a diode array detector on the basis of the absorption spectra at two different wavelengths (450 and 665 nm). The quality control of the data is assured by injecting a chlorophyll *a* standard at the beginning of each sequence, in order to check the calibration, as well as a mixture of pigments in order to check the retention times, and the system accuracy and precision.

8. The LOV Method

The LOV method, derived from the technique described by Van Heukelem and Thomas (2001), applies a sensitive reversed-phase HPLC technique for the determination of chloropigments and carotenoids within 28 min. The different pigments, extracted in methanol, are detected by diode array detector (DAD), which allows for automatic identification to be carried out on the basis of absorption spectra. Optical densities are monitored at 450 nm (chloropigments and carotenoids), 667 nm (chlorophyll *a* and derived pigments), 770 nm (bacteriochlorophyll *a*) and 222 nm (vitamin E acetate internal standard). The method provides good resolution between most pigments, but uncertainties may arise because of the partial separation of chlorophyll *b* and divinyl chlorophyll *b*, for the resolution of chlorophyll *c* pigments, and for the separation of α- and β-carotenes. It has proven to be efficient over a wide range of trophic conditions, from eutrophic upwelling waters, to the hyperoligotrophic South Pacific Subtropical Gyre. Short-term and

long-term quality control is monitored regularly to ensure state-of-the-art analyses. The injection precision of the method is estimated at 0.3%, and the limits of detection for most pigments are low ($0.019\,\mathrm{ng\,inj^{-1}}$ for chlorophyll *a*, and $0.033\,\mathrm{ng\,inj^{-1}}$ for carotenoids).

9. The DalU Method

The HPLC pigment technique used at Dalhousie University follows the method developed by Wright et al. (1991). It is a reversed-phase HPLC procedure (C_{18} column), based on a tertiary gradient and includes a cooled autosampler, and photodiode array and fluorescence detectors. Samples are disrupted with a sonic probe in 100% methanol including an internal standard (vitamin E acetate). The method is validated with the use of internal and external standards. It does not allow for the separation of divinyl chlorophylls *a* and *b* from their respective monovinyl forms. In addition, chlorophyll c_1 and chlorophyll c_2 are not separated, and neither is lutein from zeaxanthin; however, it offers good separation of a variety of pigments in a relatively short period of time (29 min).

10. The SDSU (CHORS) Method

The CHORS method was developed to provide HPLC phytoplankton pigment analyses for the NASA Sensor Intercomparison and Merger for Biological and Interdisciplinary Oceanic Studies (SIMBIOS) program, following the protocols presented in the *Ocean Optics Protocols for Satellite Ocean Color Sensor Validation* (Bidigare et al. 2002). The method (Wright et al. 1991) was designed to support a wide range of pigment concentrations from waters sampled throughout the world ocean, but it does not chromatographically separate the divinyl chlorophylls *a* and *b* from their corresponding monovinyl forms. The Latasa et al. (1996) dichromatic equations were used to spectrally resolve divinyl chlorophyll *a* from monovinyl chlorophyll *a*. The method uses a reversed-phase C_{18} column, with a tertiary solvent gradient; a temperature-controlled autosampler provides continuous sample injection to maintain a quota of analyzing 4,000 samples per year. System calibration is routinely monitored and recorded each month to ensure repeatability and consistency of data products.

11. The USC Method

The USC method was developed to provide a universal protocol that could be used to analyze freshwater, estuarine, coastal, and ocean habitats, as well as sediment and microbial mat samples. The method employs a unique combination of both monomeric and polymeric C_{18} columns combined with a variable flow binary gradient. This column configuration was originally devised to enhance photopigment separations from sediment samples containing numerous (greater than 150) photopigment and pigment degradation products. The combination of columns provides strong retention and high efficiency (monomeric columns) while selecting for similar compounds with minor differences in molecular structure (polymeric columns). The variable flow binary gradient allows baseline separation of most major pigments including lutein and zeaxanthin, and chlorophyll c_3. Chlorophylls c_1 and c_2 plus divinyl chlorophylls *a* and *b* are not completely separated. For HPLC analysis, filters are placed in disposable polypropylene microfuge tubes and lyophilized to remove all water from the filters. The primary advantages of this method are long column life (greater than 2,000 injections), inexpensive and non-hazardous solvents and reagents, no uncertainty regarding the water retained on the filter, and reliability across a range of sample types.

Chapter 1

SeaHARRE-4 Methods, Data, and Analysis

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Abstract

The focus of this study was the estimation of uncertainties in quantifying a diverse set of chlorophyll and carotenoid pigment concentrations for a variety of HPLC methods and related procedures used in the analysis of predominantly coastal waters. The SeaHARRE-4 activity was designed to investigate the following objectives: a) estimate the uncertainties in quantitating individual pigments and higher-order variables formed from sums and ratios; b) confirm if the chlorophyll *a* accuracy requirements for ocean color validation activities (approximately 25%, although 15% would allow for algorithm refinement) can be met in coastal waters; c) establish the reduction in uncertainties as a result of applying QA procedures; d) show the importance of establishing a properly defined referencing system in the computation of uncertainties; e) quantify the analytical benefits of performance metrics, and f) demonstrate the utility of a laboratory mix in understanding method performance. All of these objectives were satisfied and the results associated with each are presented.

1.1 INTRODUCTION

The results obtained in the first three SeaHARRE activities established a continuing interest in estimating and understanding the sources of uncertainties associated with the principal terms in the equation governing the calculation of the concentration (C) of an individual pigment (P_i) from a field sample. Because prior activities used open ocean samples, SeaHARRE-4 was organized around coastal samples.

1.2 THE DATA SET

The SeaHARRE-4 analyses were derived from field samples and a laboratory mixture of natural pigments. The activity involved 10 laboratories (Table 1) using three different methods (Table 2); a total of six C_8 and four C_{18} methods were executed. In some parts of this document, abbreviations from the Scientific Committee on Oceanic Research (SCOR) Working Group (WG) are used for pigment presentations (Appendix B), but the majority of the analysis results are presented using a more compact lexicon. This lexicon was developed to satisfy the diversity of presentation requirements spanning text, tables, and formulas. The latter is particularly important to summarizing the statistical description of the results.

1.2.1 The Field Samples

The SeaHARRE-4 sampling plan emphasized coastal waters. The samples were collected as part of a dedicated field campaign in the estuaries and fjords of Danish coastal waters. Details concerning individual stations where samples were collected are presented in Table 3. The last three stations (`K`, `L`, and `M`) were collected during a recurring Danish coastal observation activity and were not part of the collection of the other samples. This was done to further broaden the types of waters sampled. The resulting diversity in ecosystems is well described by the range in salinity, which spanned 8.0–28.0 PSU.

The locations of the sampling stations are shown in Fig. 1, but only the last 12 were used for SeaHARRE-4. All samples were collected on 25 mm GF/F filters and stored in liquid nitrogen as soon as filtration was completed. A batch of 12 replicates were collected at each station, with

Table 3. The sampling log for the 13 batches of field samples, which were all collected in triplicate. Samples were collected for 12 laboratories, so each batch contained 36 filters. The individual filters were identified alphabetically from `A` to `M`, but only `B`–`M` were used during SeaHARRE-4 (batch `A` was retained for another purpose). The water samples were collected from Niskin bottles during conductivity, temperature, and depth (CTD) profiles. The sampling depth is shown with the volume of water filtered, V_f, for all filters within a batch.

Station Code and Name	*Date and Time* [GMT]	*Longitude* [°]	*Latitude* [°]	V_f [mL]	*Salinity* [PSU]	*Water Depth* [m]
`A` Hvalpsund	05 Sep 2006 0707	9.1778	56.7046	300.0	25.2	10.0
`B` Virksund	05 Sep 2006 0920	9.2954	56.6084	84.5	18.4	7.0
`C` Skive	05 Sep 2006 1444	9.0660	56.5799	168.4	24.6	5.0
`D` Mariager	06 Sep 2006 0752	9.9736	56.6627	252.8	16.1	28.5
`E` Hadsund	06 Sep 2006 0921	10.1914	56.6969	450.0	20.2	5.5
`F` Randers Fjord	06 Sep 2006 1416	10.2281	56.5232	252.8	8.0	4.0
`G` Kattegat	06 Sep 2006 1455	10.3564	56.6037	500.0	22.2	6.5
`H` Horsens Fjord	07 Sep 2006 0706	9.9003	55.8536	500.0	25.7	4.0
`I` Vejle Fjord 1	07 Sep 2006 0938	9.6837	55.6956	700.0	27.4	10.8
`J` Vejle Fjord 2	07 Sep 2006 1049	9.5648	55.7023	400.0	28.0	2.0
`K` Roskilde Fjord	28 Sep 2006 0941	12.0336	55.8862	700.0	17.4	7.0
`L` Isefjord	28 Sep 2006 0725	11.8262	55.9146	700.0	19.1	9.8
`M` Ven	03 Oct 2006 0855	12.7492	55.8598	1,500.0	15.4	20.2

four stored in so-called *histoprep* filter holders and eight in folded foil packets. Both H and L received foil and histoprep samples, C and G received histopreps only, and the remaining laboratories received foil packets. The histoprep samples were collected to investigate if this storage method might be a source of elevated uncertainties.

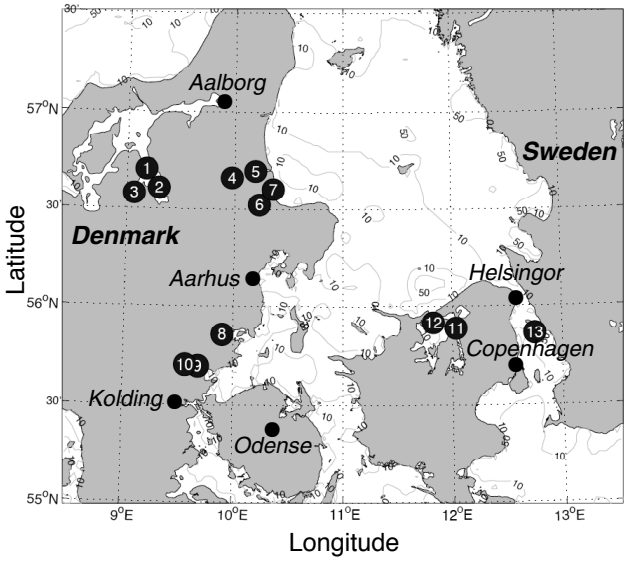

Fig. 1. Station locations for SeaHARRE-4 wherein the 1–13 numeric bullets correspond to stations `A`–`M`, respectively. Depth contours are in meters.

The filters within each batch type were randomly selected for each laboratory and distributed using (liquid nitrogen) dry shippers. There were no anomalies in the shipment of the samples—all filters were received properly frozen by each laboratory.

1.2.2 The Laboratory Mix

A recommendation from the SeaHARRE-2 activity was to find a source for an algal mixture of the primary pigments. After SeaHARRE-2, DHI successfully produced a laboratory mix of pigments and made it commercially available. The mix is made from cultures of different phytoplankton species, and each lot contains approximately 20 different pigments, all of which can be present in oligotrophic oceans: Chl c_3, Chl c_2, Peri, But, Fuco, Neo, Pras, Viola, Hex, Diad, Allo, Diato, Zea, Lut, Chl b, Chl a, $\beta\beta$-Car, and $\beta\varepsilon$-Car. The content varies slightly in different lots, and may also contain Phide a, Phytin a, Chlide a, MgDVP, plus Chl c_1 and other Chl c-type pigments.

The mixed pigments allow an HPLC analyst to check detection of, and resolution between, pigments. They can also be used for quality assurance, for example, monitoring retention time and response factor stability, verifying the correct elution and identity of peaks, and for documenting the precision of the HPLC. The mix is not really a proxy for a natural field sample because they contain less of some pigments and more of some others, so the relative abundance is not in keeping with a natural sample.

The mix analyzed during SeaHARRE-4 is denoted DHI Mix-102. Each laboratory received 10 ampules of the mix and was requested to make at least three analyses of the mix, and to use it to whatever quality assurance (QA) advantage was deemed appropriate. For example, some analysts make an analysis of the mix at the start of the field sample analyses and after every 20 samples (with at least one mix sample analyzed in each *sample set* of field samples, e.g., for each complete analysis of an autosampler compartment). In most cases, more than three analyses were performed.

1.2.3 The Pigments

Each participating laboratory established and validated an HPLC method based on the pigment content of the samples they typically analyze. This is an important point, because some laboratories were exposed to atypical pigment types or concentrations. The variety of methods means some pigments were analyzed by only a few methods, whereas others were analyzed by all methods. The latter constitute a group of pigments that are routinely useful to many aspects of marine studies and, following the nomenclature of Claustre et al. (2004), are referred to here as the *primary pigments* (PPig).

The utility of the pigments for biogeochemical inquiries, along with the number of methods that actually quantitated a particular pigment, were used to separate the pigments into four groups:

- The *primary* pigments are the total chlorophylls and the carotenoids most commonly used in chemotaxonomic or photophysiological studies in the open ocean or in coastal waters (Gieskes et al. 1988, Barlow et al. 1993, Claustre et al. 1994, and Bidigare and Ondrusek 1996);
- The *secondary* pigments are the individual pigments used to create a primary pigment composed of separate contributions (e.g., the total chlorophylls);
- The *tertiary* pigments are those pigments not included in the composition of the primary and secondary pigments for which three or more laboratories provided quantitations; and
- The *ancillary* pigments are those remaining pigments only analyzed by one or two laboratories.

This nomenclature implies a precedence or ranking of the pigments, but this is only true from the perspective of the SeaHARRE activity and marine phytoplankton pigment research, for which certain pigments are routinely used more often than others (e.g., chlorophyll *a*). The primary reason for establishing a unique vocabulary is to provide a categorization scheme for grouping the analytical results.

A listing of the secondary, tertiary, and ancillary pigments are given in Table 4. The ancillary pigments are not used in this study; they are included to summarize the complete capabilities of each method. Only a subset of the tertiary pigments deemed representative of the types of tertiary pigments of interest to the marine phytoplankton community are presented and discussed. Table 4 also provides the methods used for the quantitation of each pigment, the names and abbreviations of each pigment, and the corresponding variable forms, which are used to indicate the concentration of each pigment.

All laboratories quantitated the individual primary pigments, which are used to create the higher-order pigment associations: sums, ratios, and indices (Table 5). The grouping of pigments to form sums permits the formulation of variables useful to different perspectives. For example, the pool of photosynthetic and photoprotective carotenoids (PSC and PPC, respectively) are useful to photophysiological studies (Bidigare et al. 1987) and the total amount of accessory (non-chlorophyll *a*) pigments (TAcc) are useful in remote sensing investigations (Trees et al. 2000). The ratios derived from these pooled variables, e.g., $\left[\mathrm{PSC}\right]/\left[\mathrm{TChl}\,a\right]$, are dimensionless, and have the advantage of automatically *scaling* the comparison of results from different areas and pigment concentrations.

An important pigment sum is the total diagnostic pigments (DP), which was introduced by Claustre (1994) to estimate a pigment-derived analog to the f-ratio (the ratio of new-to-total production) developed by Eppley and Peterson (1979). The use of DP was extended by Vidussi et al. (2001) and Uitz et al. (2006) to derive size-equivalent indices that roughly correspond to the biomass proportion factors of pico-, nano-, and microphytoplankton, which are denoted $\left[\mathrm{pPF}\right]$, $\left[\mathrm{nPF}\right]$, and $\left[\mathrm{mPF}\right]$, respectively. They are composed of sums and are ratios, so they should be useful in reconciling databases from different oceanic regimes.

Together with the individual primary pigments, the pigment sums, ratios, and indices are presented in Table 5. Note that $\left[\mathrm{TChl}\,a\right]$, $\left[\mathrm{TChl}\,b\right]$, and $\left[\mathrm{TChl}\,c\right]$ do not represent individual concentrations—each is a group of pigments roughly characterized by the same absorption spectra (including some degradation products). These sums allow for the comparison of results originating from HPLC methods that differ in the way the pigments within the same family are quantitated (e.g., chlorophyll *c* types) or whose extraction procedures might or might not generate degradation forms (e.g., chlorophyllide *a*). The sums also allow for the comparison of methods that differ in their capability of differentiating monovinyl from divinyl forms.

The symbols used to indicate the concentration of the so-called primary pigments, which were reported by all of the laboratories, are as follows:

C_{T_a} Total chlorophyll *a*,
C_{T_b} Total chlorophyll *b*,
C_{T_c} Total chlorophyll *c*,
C_C Carotenes,
C_A Alloxanthin,
C_B 19′-Butanoyloxyfucoxanthin,
C_{Dd} Diadinoxanthin,
C_{Dt} Diatoxanthin,
C_F Fucoxanthin,
C_H 19′-Hexanoyloxyfucoxanthin,
C_P Peridinin, and
C_Z Zeaxanthin.

These are the same 12 pigments given in the topmost portion of Table 5. The first three are the (total) pigment

Table 4. The secondary (top portion) and tertiary (middle portion) pigments shown with their variable forms, names, and calculation formulas (if applicable). The absence of allomers and epimers for pigments other than Chl *a* is not indicative of a lack of understanding that they might be present—it is simply a reflection that the SeaHARRE participants have agreed to quantitate and include the allomers and epimers in the definition of [Chl *a*]. The methods used to quantitate the various pigments are indicated by their one-letter codes. The variable forms, which are used to indicate the concentration of the pigment, are patterned after the nomenclature established by the SCOR WG 78 (Jeffrey et al. 1997b). Abbreviated pigment forms are shown in parentheses. The presence of [Neo+Vio] and [Zea+Lut] is to maintain continuity with prior SeaHARRE activities, for which these sums were important.

Variable	*Method*	*Secondary Pigment*	*Calculation*
[Chl *a*]	*C D F G H J L N S U*	Chlorophyll *a* (Chl *a*)	Including allomers and epimers
[DVChl *a*]	*C D G H J L* §	Divinyl chlorophyll *a* (DVChl *a*)	
[Chlide *a*]	*C D F G H J L N S U*	Chlorophyllide *a* (Chlide *a*)	
[Chl *b*]	*C D F G H J L N S U*	Chlorophyll *b* (Chl *b*)	
[Chl c_{1+2}]	*C D F G H J L N S U*	Chlorophyll c_{1+2} (Chl c_{1+2})	[Chl c_1] + [Chl c_2]
[Chl c_3]	*C D G H J L N S U*	Chlorophyll c_3 (Chl c_3)	
[$\beta\varepsilon$-Car]	*F S U*	$\beta\varepsilon$-Carotene† ($\beta\varepsilon$-Car)	
[$\beta\beta$-Car]	*F S U*	$\beta\beta$-Carotene‡ ($\beta\beta$-Car)	
Variable	*Method*	*Tertiary Pigment*	*Calculation*
[Chl c_1]	*C G H*	Chlorophyll c_1 (Chl c_1)	
[Chl c_2]	*C D G H S U*	Chlorophyll c_2 (Chl c_2)	
[Lut]	*C D F G H J L N S U*	Lutein (Lut)	
[Neo]	*C D F G H J L N S U*	Neoxanthin (Neo)	
[Neo+Vio]	*C D F G H J L N S U*	Neoxanthin and Violaxanthin (Neo+Viola)	[Neo] + [Viola]
[Phide *a*]	*C D H J L N S*	Phaeophorbide *a* (Phide *a*)	
[Phytin *a*]	*C D G H J L N S*	Phaeophytin *a* (Phytin *a*)	
[Pras]	*C D F G H J L N S U*	Prasinoxanthin (Pras)	
[Viola]	*C D F G H J L N S U*	Violaxanthin (Viola)	
[Zea+Lut]	*C D F G H J L N S U*	Zeaxanthin and Lutein (Zea+Lut)	[Zea] + [Lut]
[Gyro]	*F H U*	Gyroxanthin (Gyro)	
Variable	*Method*	*Ancillary Pigment*	*Calculation*
[DVChl *b*]	*G H*	Divinyl chlorophyll *b* (DVChl *b*)	
[Myxo]	*D*	Myxoxanthophyll (Myxo)	
[MgDVP]	*C F*	Mg 2,4-divinyl phaeoporphyrin a_5 monomethyl ester (MgDVP)	
[BChl *a*]	*L*	Bacterial Chlorophyll *a* (BChl *a*)	
[Pyro]	*C*	Pyro-phaeopigments¶	
[Phytin *b*]	*C*	Phaeophytin *b* (Phytin *b*)	
[Anth]	*D U*	Antheraxanthin (Anthera)	
[Cantha]	*D*	Canthaxanthin (Cantha)	
[Croco]	*F*	Crocoxanthin (Croco)	
[Monado]	*U*	Monadoxanthin (Monado)	

§ Method *S* used the Latasa et al. (1996) simultaneous equations to estimate [DVChl *a*].

† Also referred to as α-Carotene.

‡ Also referred to as β-Carotene.

¶ Pyro-phaeophorbide *a* and pyro-phaeophytin *a*.

Table 5. The (individual) primary pigments, pigment sums, pigment ratios, and pigment indices shown with their variable forms, names, and calculation formulas (if applicable). All methods produced all of these variables. The variable forms, which are used to indicate the concentration of the pigment or pigment association, are patterned after the nomenclature established by the SCOR Working Group 78 (Jeffrey et al. 1997b). Abbreviated forms for the pigments are shown in parentheses.

Variable	*Primary Pigment (PPig)*	*Calculation*
[TChl *a*]	Total chlorophyll *a*† (TChl *a*)	[Chlide *a*] + [DVChl *a*] + [Chl *a*]
[TChl *b*]	Total chlorophyll *b*† (TChl *b*)	[DVChl *b*] + [Chl *b*]
[TChl *c*]	Total chlorophyll *c*† (TChl *c*)	[Chl c_1] + [Chl c_2] + [Chl c_3]
[Caro]	Carotenes† (Caro)	[$\beta\beta$-Car] + [$\beta\varepsilon$-Car]
[Allo]	Alloxanthin (Allo)	
[But]	19′-Butanoyloxyfucoxanthin (But-fuco)	
[Diad]	Diadinoxanthin (Diadino)	
[Diato]	Diatoxanthin (Diato)	
[Fuco]	Fucoxanthin (Fuco)	
[Hex]	19′-Hexanoyloxyfucoxanthin (Hex-fuco)	
[Peri]	Peridinin (Perid)	
[Zea]	Zeaxanthin (Zea)	
Variable	*Pigment Sum*	*Calculation*
[TChl]	Total Chlorophyll (TChl)	[TChl *a*] + [TChl *b*] + [TChl *c*]
[PPC]	Photoprotective Carotenoids (PPC)	[Allo] + [Diad] + [Diato] + [Zea] + [Caro]
[PSC]	Photosynthetic Carotenoids (PSC)	[But] + [Fuco] + [Hex] + [Peri]
[PSP]	Photosynthetic Pigments (PSP)	[PSC] + [TChl]
[TAcc]	Total Accessory Pigments (TAcc)	[PPC] + [PSC] + [TChl *b*] + [TChl *c*]
[TPig]	Total Pigments (TPig)	[TAcc] + [TChl *a*]
[DP]	Total Diagnostic Pigments (DP)	[PSC] + [Allo] + [Zea] + [TChl *b*]
Variable	*Pigment Ratio*	*Calculation*
[TAcc]/[TChl *a*]	The [TAcc] to [TChl *a*] ratio	[TAcc]/[TChl *a*]
[TChl *a*]/[TPig]	The [TChl *a*] to [TPig] ratio	[TChl *a*]/[TPig]
[PPC]/[TPig]	The [PPC] to [TPig] ratio	[PPC]/[TPig]
[PSC]/[TPig]	The [PSC] to [TPig] ratio	[PSC]/[TPig]
[PSP]/[TPig]	The [PSP] to [TPig] ratio	[PSP]/[TPig]
Variable	*Pigment Index*	*Calculation*
[mPF]	Microplankton Proportion Factor‡ (MPF)	$\frac{[\text{Fuco}] + [\text{Peri}]}{[\text{DP}]}$
[nPF]	Nanoplankton Proportion Factor‡ (NPF)	$\frac{[\text{Hex}] + [\text{But}] + [\text{Allo}]}{[\text{DP}]}$
[pPF]	Picoplankton Proportion Factor‡ (PPF)	$\frac{[\text{Zea}] + [\text{TChl } b]}{[\text{DP}]}$

† Considered as individual pigments, although computed or equivalently represented as sums by some methods.
‡ As a group, also considered as *macrovariables*.

associations for the chlorophylls and the other nine are all carotenoids, of which only Caro is a sum.

The secondary and tertiary pigments that are also of interest to this study, in terms of the presentation of some of the statistical analysis of the results and a historical perspective for the SeaHARRE activity, are as follows:

C_a Chlorophyll *a*,
C_{Da} Divinyl chlorophyll *a*,
C_{Ca} Chlorophyllide *a*,
C_L Lutein,
C_N Neoxanthin,
C_{N+V} Neoxanthin plus violaxanthin,
C_{Pba} Phaeophorbide *a*,
C_{Pta} Phaeophytin *a*,
C_{Pr} Prasinoxanthin,
C_V Violaxanthin, and
C_{Z+L} Zeaxanthin plus lutein.

These 11 pigments are a mixture of chlorophylls and carotenoids. They are not the full subset of secondary and tertiary pigment analyzed by three or more laboratories, but are representative of the most important secondary pigments for marine studies (the chlorophyll *a* family of pigments) plus some of the usually minor pigments that many methods quantitate (the tertiary carotenoids).

The symbology presented here is used primarily to represent the final pigment concentrations for each field sample, because this is the way most laboratories report their results, and replicate sampling is not a normal procedure in field campaigns. The symbology does not include the concentrations associated with the individual samples that were used to determine the final sample value.

1.3 THE GOVERNING EQUATIONS

Ignoring the specific details of the basic HPLC processes, because they are presented in detail by Jeffrey et al. (1997a) and Bidigare et al. (2003), the formulation for determining pigment concentration begins with the terms describing the calibration of the HPLC system:

$$\tilde{C}_{P_i} \;=\; \hat{A}_{P_i}\, R_{P_i}, \tag{1}$$

where $\tilde{C}_{P_i}$ is the amount of pigment injected (usually in units of nanograms), $\hat{A}_{P_i}$ is the area of the parent peak and associated isomers for pigment P_i (usually in milliabsorbance units (denoted mAU) or microvolts as a function of time), and R_{P_i} is the response factor. The latter is the calibration coefficient for the HPLC system, and it takes on a separate value for each pigment being quantitated. For the general problem, the response factor is denoted R, but for the specific problem of a particular pigment, it is denoted R_{P_i}. The R values are usually expressed as the amount of pigment divided by the peak area.

The formulation given in (1) is based on a single-point calibration wherein one or more injections of a calibration standard at a known concentration is injected onto the HPLC column. An alternative approach is to create a dilution series of the pigment standard, inject these one at a time, and then fit the response of the HPLC system to a linear function ($y = mx + b$) using least-squares analysis (this is also referred to as a *multipoint* calibration). In this case, pigment concentration is computed as

$$\tilde{C}_{P_i} \;=\; \frac{\hat{A}_{P_i} \;-\; b_i}{m_i}, \tag{2}$$

where m_i is the slope (equating change in peak area with change in amount) and b_i is the y-intercept.

The formulation presented in (2) can be expressed to follow (1) as follows:

$$\tilde{C}_{P_i} \;=\; \hat{A}_{P_i} \left[\frac{1 \;-\; (b_i/\hat{A}_{P_i})}{m_i}\right], \tag{3}$$

where the equivalent R_{P_i} for (1) is given by the terms in brackets. If the linear regression is forced through zero, $b_i = 0$, and (3) becomes

$$\tilde{C}_{P_i} \;=\; \frac{\hat{A}_{P_i}}{m_i}, \tag{4}$$

and $R_{P_i} = 1/m_i$ (note that the inverse slope is change in amount divided by change in peak area, which matches the definition for R). In this context, it is convenient to reconsider the definition of R_{P_i}, which some authors have done (Bidigare et al. 2003), as the inverse of the original definition, that is, $F_{P_i} = 1/R_{P_i}$ and (1) becomes

$$\tilde{C}_{P_i} \;=\; \frac{\hat{A}_{P_i}}{F_{P_i}}. \tag{5}$$

The advantage of this approach is F_{P_i} follows directly from the slope of the linear calibration curve and, for the common case of forcing the slope through zero, $F_{P_i} = m_i$. For the purposes of this study, the majority of the methods used the original definition of R, so it is retained hereafter.

The governing equation for the determination of pigment concentration can be expressed as

$$C_{P_i} \;=\; \frac{V_x}{V_f}\,\frac{\tilde{C}_{P_i}}{V_c}, \tag{6}$$

where V_x is the extraction volume, V_c is the volume of sample extract injected onto the HPLC column (measured in the same units as V_x), and V_f is the volume of water filtered in the field to create the sample (usually through a 0.7 µm pore size glass-fiber filter and measured in liters).

The variables used to compute R_{P_i} in (1), or the terms in brackets in (3), follow from the procedures used to determine the concentration of pigment standards, which most

often are done spectrophotometrically based on principles of the Lambert-Beer Law. The latter states that the fraction of the incident light at a particular wavelength λ that is absorbed by a solution depends on the thickness of the sample, the concentration C of the absorbing compound in the solution, and the chemical nature of the absorbing compound (Segel 1968). This relationship can be expressed as:

$$A_{P_i}(\lambda) \;=\; a_{P_i}(\lambda)\, l_c\, C_{P_i}, \tag{7}$$

where $A_{P_i}(\lambda)$ is absorbance, $a_{P_i}(\lambda)$ is the absorption coefficient (a constant), and l_c is the sample thickness in centimeters (the cuvette pathlength). To compute concentration from a measured absorbance, (7) is rewritten as

$$C_{P_i} \;=\; \frac{A_{P_i}(\lambda)}{a_{P_i}(\lambda)\, l_c}, \tag{8}$$

where the units for C_{P_i} depend on the expression of $a_{P_i}(\lambda)$. For example, if the concentration is expressed in molarity, a becomes the molar absorption coefficient (ε) and if the concentration is expressed as grams per liter, a is the specific absorption coefficient (α). Absorption coefficients vary depending on wavelength and the solvent in which the compound is suspended, and they are always provided with the solvent and wavelength used.

Pigment standards used for HPLC calibration are either a) purchased in solution (with concentrations provided by the manufacturer), b) isolated from natural sources, or c) purchased in solid form. In the latter two cases, pigments are suspended in the solvents specified for use with the selected absorption coefficients, which also determines the wavelength used to measure the absorbance spectrophotometrically. Assuming the specific absorption coefficient is used, computing the concentration of a pigment standard S_i requires the wavelength of maximum absorbance for the pigment, λ_m (specified with α_{S_i}), plus a correction measurement for the absorbance of the pigment at 750 nm:

$$C_{S_i} \;=\; \frac{A_{S_i}(\lambda_m) \;-\; A_{S_i}(750)}{\alpha_{S_i}(\lambda_m)\, l_c}, \tag{9}$$

where A_{S_i} is the absorbance of the internal standard.

The calibration process continues with a volume of the standard, V_c, being injected onto the HPLC column. This assumes that the range of peak areas (for the pigment) over which a linear response can be attained has already been determined. With that knowledge, it will be known whether or not the peak area of the standard (when injected undiluted) is within the linear range. For calibration purposes, the standard is injected at the wavelength and bandwidth used for the quantitation of that pigment. Chromatographic purity also needs to be assessed and can be performed at additional wavelengths—most notably the wavelength specified for use with the absorption coefficient or wavelengths permitting detection of other contaminating pigments. It is rare that a pigment standard is chromatographically pure, meaning no isomers, allomers, epimers or other such degradation products are present. It is desirable that the parent peak (or main peak) represents no less than 90% of the total of all peaks (excluding the injection peak). The sum of the parent peak and the area of the alteration products is denoted as $\Sigma\hat{A}_{S_i}$.

Returning to the HPLC calibration equation, (1) can be converted to concentration by including the V_c term:

$$C_{S_i} \;=\; \frac{\hat{A}_{S_i}\, R_{P_i}}{V_c}, \tag{10}$$

and remembering that the same pigment is involved for S_i and P_i (the former is used to make it clear that a measurement is made on the pigment standard). The response factor is now accessible, but there are two basic procedures used for determining response factors, which are distinguished by how the peak area information is used and are denoted as follows:

- $R^{\Sigma}_{P_i}$ The amount injected onto the column divided by the total peak area (including the sum of the parent peak and degradants).
- $R^{\%}_{P_i}$ The purity-corrected amount injected onto the column divided by the area of the main (or parent) peak alone.

With acceptably pure standards, $R^{\Sigma}_{P_i}$ is sufficiently similar to $R^{\%}_{P_i}$ so that either approach is valid. In fact, as will be shown below, they are computationally equivalent.

To derive $R^{\Sigma}_{P_i}$, the definition from above is applied, which produces the following:

$$R^{\Sigma}_{P_i} \;=\; \frac{V_c C_{S_i}}{\Sigma\hat{A}_{S_i}}, \tag{11}$$

where the numerator on the right side is the amount injected on the column and the denominator is the total peak area. To make use of the spectrophotometric work that began the calibration process, (9) is substituted for C_{S_i} in (11), and the terms rearranged to yield:

$$R^{\Sigma}_{P_i} \;=\; \frac{A_{S_i}(\lambda_m) \;-\; A_{S_i}(750)}{\alpha_{S_i}(\lambda_m)\, l_c}\, \frac{V_c}{\Sigma\hat{A}_{S_i}}, \tag{12}$$

and noting that $\Sigma\hat{A}_{S_i}$ can be used directly in (10) for the peak area term, and then (9) and (10) can be equated to provide $R^{\Sigma}_{P_i}$.

To derive $R^{\%}_{P_i}$, a formulation for purity needs to be established, and the typical definition is the ratio of the main (or parent) peak divided by the sum of the parent peak plus degradants:

$$\hat{A}^{\%}_{S_i} \;=\; \frac{\hat{A}_{S_i}}{\Sigma\hat{A}_{S_i}}, \tag{13}$$

where the numerator is the main peak and the denominator is the sum of peaks. The definition for $R^{\%}$ requires the

purity-corrected amount injected onto the column, which is the product of $C_{S_i} V_c \hat{A}^{\%}_{S_i}$, where the V_c term converts the concentration to amount, and the purity term provides the needed correction factor. Using the definition for $R^{\%}_{P_i}$ produces

$$R^{\%}_{P_i} = \frac{C_{S_i} V_c \hat{A}^{\%}_{S_i}}{\hat{A}_{S_i}}, \tag{14}$$

but once (13) is substituted into (14), and (9) is applied, the relationship becomes:

$$R^{\%}_{P_i} = \frac{A_{S_i}(\lambda_m) - A_{S_i}(750)}{\alpha_{S_i}(\lambda_m)\, l_c} \frac{V_c}{\Sigma \hat{A}_{S_i}}, \tag{15}$$

which is equivalent to (12).

Given the equivalence of (12) and (15), it is logical to wonder why two techniques for calculating response factors are desirable. It is primarily a function of the need to be cognizant of the chromatographic purity of standards, for it is not uncommon for purity to diminish as the standard ages. In this case, a change in $R^{\Sigma}_{P_i}$ may be observed and can be a direct result of the absorption coefficient used for the standard not being completely accurate for the alteration products associated with the aged standard. More importantly, the purity-corrected approach must be used when the standard is diluted to the point where the alteration products are not detectable and only the main peak can be integrated. In this case, the amount injected is $C_{S_i} V_c \hat{A}^{\%}_{S_i}$ and the peak area of the standard is $\hat{A}_{S_i}$. In these instances, it is important to determine chromatographic purity with an undiluted standard that is near the upper limit of the linear range so that alteration products are detectable.

The formulations for response factors can be combined with a typical quantitation equation for pigment concentration (6) to produce a governing equation involving all the terms associated with computing the concentration of a particular pigment in an individual sample:

$$C_{P_i} = \frac{V_x}{V_f} \hat{A}_{P_i} \left[\frac{A_{S_i}(\lambda_m) - A_{S_i}(750)}{\alpha_{S_i}(\lambda_m)\, l_c\, \Sigma \hat{A}_{S_i}} \right], \tag{16}$$

where it is assumed V_c is the same for field samples and laboratory standards, and the terms in brackets represent the response factor.

Additional complexities occur, because an internal standard can be used to improve the determination of V_x. There are two common procedures for using an internal standard, and they are distinguished here by the number of laboratory steps involved: a) the extraction solvent and internal standard are contained together in a mixture (prepared beforehand), which is added to the sample in one step; or b) the extraction solvent and internal standard are added separately in two steps.

In the one-step approach, a volume of solvent and internal standard is mixed together in a batch, and a small portion of the mixed volume, V_m, is added to the sample. In the two-step approach, a volume of the extraction solvent, V_e, is added to the sample followed by a small volume of internal standard, V_s. The filter, now soaking in the solvent–standard mixture, is disrupted (most commonly with a sonic probe), clarified (to remove filter and sample debris), and a volume of the clarified sample extract, V_c, is injected onto the HPLC column.

The internal standard permits a correction for the presence of residual water retained on the filter (plus any variations in extract volume caused by evaporation) by using a) the peak area of the internal standard when it is injected onto the HPLC column ($\hat{A}_c$) prior to its addition to the sample, and b) the peak area of the internal standard in the sample ($\hat{A}_s$). In the one-step approach, $\hat{A}_c$ is determined by injecting the solvent–standard mixture onto the HPLC column, whereas for the two-step approach, the internal standard is injected directly onto the column. For the one-step approach, the internal standard is diluted by the extraction solvent, so $V_{x_1} = V_m \hat{A}_{c_1} / \hat{A}_{s_1}$, where the "1" in the subscripts indicates the one-step methodology. For the two-step approach, $V_{x_2} = V_s \hat{A}_{c_2} / \hat{A}_{s_2}$ (the "2" in the subscripts indicates the two-step methodology).

If an internal standard is not used, an estimate of the volume of water retained on the filter, V_w, is added to the volume of extraction solvent, V_e, so $V_{x'} = V_e + V_w$. For a 25 mm filter, water retention is usually assumed to be 0.2 mL (Bidigare et al. 2003). Whether or not this is a realistic value is directly related to the protocols used in the field. If filters are allowed to sit under vacuum after all water has been exhausted, they can be dried too much.

Considering a one-step internal standard methodology for determining V_x, (16) becomes

$$C_{P_i} = \frac{\hat{A}_{c_1}}{\hat{A}_{s_1}} \frac{V_m}{V_f} \hat{A}_{P_i} \left[\frac{A_{S_i}(\lambda_m) - A_{S_i}(750)}{\alpha_{S_i}(\lambda_m)\, l_c\, \Sigma \hat{A}_{S_i}} \right], \tag{17}$$

and now all terms associated with a rather complicated protocol are accessible (assuming V_c is a constant). This means an analyst can begin to assign uncertainties to each term and ultimately estimate a final uncertainty for the quantitated concentrations of each pigment. This is not to imply that any terms not present in the governing equation should be ignored (e.g., the cancellation of V_c for the assumptions presented here). It is important to scrutinize each step of the protocol to ensure all parts are properly understood.

1.4 LABORATORY METHODS

SeaHARRE is based on a global perspective, so it is likely that some laboratories would receive samples that were atypical of those for which their HPLC method was originally intended. For example, it would not necessarily be true that a method developed for oligotrophic samples would perform optimally with eutrophic samples. The

Table 6. A summary of the extraction specifications for each of the methods. The volume of solvent added is given in milliliters. Each filter was disrupted for the indicated amount of time, allowed to soak for the specified number of hours, and then clarified. For this and subsequent presentations, 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.

Meth. Code	*Volume Added*	*Extraction Solvent*	*Internal Standard*	*Mode and Time of Disruption*	*Soak Time [h]*	*Clarification*
C	4.2	97.5% Acetone	Vitamin E acetate	Sonicating bath 15 min	15–18	Centrifuge, 0.2 µm Teflon syringe filter
D	3.0	95% Acetone	Vitamin E acetate	Sonicating bath ∼10 min	24	0.45 µm Teflon syringe filter
F	0.6	90% Acetone	*trans*-β-apo-8′-carotenal	None	24	0.45 µm Teflon syringe filter
G	1.8	95% Acetone	Vitamin E acetate	Sonic probe ∼15 s	3–4	0.45 µm Teflon syringe filter
H	4.2	95% Acetone	Vitamin E acetate	Sonic probe ∼15 s	4	0.45 µm Teflon syringe filter
J	2.5	100% Acetone	Vitamin E acetate	Sonic probe 90 s	4	0.45 µm Teflon syringe filter
L	3.0	100% Methanol	Vitamin E acetate	Sonic probe 10 s	1†	0.7 µm GF/F filter
N	2.0	100% Methanol	Vitamin E acetate	Sonic probe 20 s	2	Centrifuge 3 min (13,000 rpm)
S	4.0	100% Acetone	*trans*-β-apo-8′-carotenal	Sonic probe <15 s	≥ 24	Centrifuge 4 min (5,100 rpm)‡
U	0.6	90% Acetone	*trans*-β-apo-8′-carotenal	None	24	0.45 µm Teflon syringe filter

† The sum of soaking for 0.5 h, sonicating, and then soaking for another 0.5 h.

‡ Plus a 0.2 µm Teflon membrane filter.

HPLC methods presented here (Chaps. 2–11) are based on diverse objectives, but are most commonly used with samples from a variety of environmental regimes:

C Based on the Van Heukelem and Thomas (2001) method and used predominantly with temperate water samples;

D Based on the Van Heukelem and Thomas (2001) method and used mostly with samples from freshwater, estuaries, and coastal areas;

F Based on the Pinckney et al. (1997) method and used with a wide range of marine samples;

G Based on the Van Heukelem and Thomas (2001) method and used primarily for methodological research with a wide variety of aquatic samples;

H Based on the Van Heukelem and Thomas (2001) method and used with a wide variety of water samples from freshwater lakes, estuarine ecosystems, and the oligotrophic ocean;

J Based on the Van Heukelem and Thomas (2001) method and used predominantly for the analysis of coastal samples;

L Based on the Van Heukelem and Thomas (2001) method and used initially with Case-1 (open ocean) samples, but also successfully with Case-2 (coastal) waters;

N Based on the Wright et al. (1991) method and used with a wide range of oceanic samples;

S Based on the Wright et al. (1991) method and used with a wide range of pigment concentrations from water types throughout the world ocean; and

U Based on the Pinckney et al. (1997) method and used with a wide range of samples from marine ecosystems.

Note there are 10 laboratories, but essentially only three distinctly different methods. It is important to remember, however, that the implementation of a common method always results in differences that will distinguish seemingly identical methods from one another over time.

A summary of the filter extraction procedures is presented in Table 6. All of the methods used acetone as an extraction solvent, except for *L* and *N*, which used methanol. Sonic disruption predominated, although two methods (*F* and *U*) used none. The soak time for the extract ranged from 0.5 h to more than 24 h, and clarification was mostly accomplished by filtration with a few methods including centrifugation in the procedure. The

Table 7. A summary of the HPLC separation procedures used by the SeaHARRE-4 methods. Column particle size (P_s) is in units of micrometers, and column length (L_c) and diameter (D_c) are given in millimeters. Column temperature is denoted as T_c, and wavelength is denoted as λ.

Meth. Code	*Stationary Phase*	*Column*				*Detector and Monitoring Wavelength*	
		P_s	L_c	D_c	T_c	*Manufacturer and Model*	λ [nm]
C	C_8	3.5	150	4.6	55°C	Waters PDA 996	436
D	C_8	3.5	150	4.6	60°C	Shimadzu SPD-M10A VP-DAD	222†, 450, and 665
F	C_{18}	3.0	100§	4.6	40°C	Shimadzu SPD-M10A VP	440
G	C_8	3.5	150	4.6	60°C	Agilent 1100¶	222†, 450, and 665
H	C_8	3.5	150	4.6	60°C	Agilent 1100	222†, 450, and 665
J	C_8	3.5	150	4.6	60°C	Agilent 1100	222†, 450, and 665
L	C_8	3.5	150	3.0	60°C	Agilent 1100	222†, 440, 667, and 770
N	C_{18}	5.0	150	4.6	Room‡	Agilent 1100	436 and 450
S	C_{18}	5.0	250	4.6	Room‡	ThermoQuest UV6000	436 and 450
U	C_{18}	3.0	100§	4.6	40°C	Shimadzu SPD-M10A VP	440

† Used for monitoring vitamin E (the internal standard).
‡ Maintained at 18°C by a specialized air conditioner.
§ The monomeric column is 15 mm.
¶ The *G* method was executed on the same hardware used by *H*.

Table 8. A summary of the HPLC solvent systems used with the SeaHARRE-4 methods: MeCN is acetonitrile, NH_4Ac is ammonium acetate, EtOAc is ethyl acetate, MeOH is methanol, and TbAA is tetrabutyl ammonium acetate. The flow rate is in units of milliliters per minute.

Meth. Code	*Injection Buffer*	*Flow Rate*	*Mobile Phase Solvent*			*Initial Conditions*
			A	B	C	
C	TbAA:MeOH†	1.1	70:30 28 mM TbAA:MeOH	MeOH		95% A:5% B
D	TbAA:MeOH†	1.1	70:30 28 mM TbAA:MeOH	MeOH		95% A:5% B
F	1.0 M NH_4Ac	0.8‡	80:20 MeOH:0.5 M NH_4Ac§	80:20 MeOH:Acetone		100% A
G	TbAA:MeOH†	1.1	70:30 28 mM TbAA:MeOH	MeOH		95% A:5% B
H	TbAA:MeOH†	1.1	70:30 28 mM TbAA:MeOH	MeOH		95% A:5% B
J	TbAA:MeOH†	1.1	70:30 28 mM TbAA:MeOH	MeOH		95% A:5% B
L	aqueous TbAA¶	0.55	70:30 28 mM TbAA:MeOH	MeOH		95% A:5% B
N	0.5 M NH_4Ac	1.0	80:20 MeOH:0.5 M NH_4Ac	90:10 MeCN:Water	EtOAc	100% A
S	Water	1.0	80:20 MeOH:0.5 M NH_4Ac	90:10 MeCN:Water	EtOAc	100% A
U	1.0 M NH_4Ac	0.8‡	80:20 MeOH:0.5 M NH_4Ac§	80:20 MeOH:Acetone		100% A

† 28 mM TbAA:MeOH in a 90:10 (v:v) mixture, and the TbAA has a 6.5 pH.
‡ Variable flow rate of 0.8–1.5 mL min^{-1}.
§ Adjusted to 7.2 pH.
¶ 28 mM aqueous TbAA with 6.5 pH.

internal standard used by *C*, *D*, *G*, *H*, *J*, *L*, and *N* was vitamin E acetate, whereas the *F*, *S*, and *U* methods all used *trans*-β-apo-8′-carotenal.

A summary of the HPLC column separation procedures and solvent systems are given in Tables 7 and 8, respectively. The type of stationary phase divides the methods into two groups, C_8 and C_{18}, with the former predominating. Additional distinctions can be seen with a) column temperature (*N* and S_{18} did not control column temperature while *C*, *D*, *G*, *H*, *J*, and *L* used high temperature control), and b) multiple- versus single-wavelength monitoring systems (*C* only used 436 nm). Note also the diversity in equipment manufacturers. All methods used an injection buffer, and the majority of the methods were two-solvent systems, with S_{18} using a three-solvent system. The flow rates of the methods are very similar except for laboratory *L*, which had a flow rate that is half as much as the others (a lower flow rate is required with the narrower internal diameter column used by *L*).

1.5 DATA ANALYSIS METHODS

This study used a laboratory mix and field samples, both with unknown concentrations. Each laboratory participated as if the analyses were performed as a result of normal operations; that is, a single concentration value was reported by each laboratory for each pigment in each filter. For subsequent data analyses, solitary pigment concentrations per batch were used and were determined as the average concentration of a pigment across a set of triplicates,

also referred to as a "sample." To ensure a consistency in reporting, all values were converted to concentrations of milligrams per cubic meter.

In the analytical approach adopted here for field samples, no one laboratory (or result) is presumed more correct than another—all the methods were considered properly validated by the individual analysts. Furthermore, there is no absolute truth for field samples, so an unbiased approach is needed to intercompare the 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 contributing laboratories or methods:

$$\bar{C}_{P_i}^{L_j}(S_k) \;=\; \frac{1}{N_R}\sum_{l=1}^{N_R} C_{P_i}^{L_j}(S_{k,l}), \tag{18}$$

where P_i identifies the pigment or pigment association (following the symbology established in Sect. 1.2.3); L_j is the laboratory (or method) code; $S_{k,l}$ sets the batch (or station) number and replicate number, using the k index for the former (following Table 3) and l for the latter ($l = 1$, 2, or 3); and N_R is the total number of replicates (3).

In (18), the i index represents an arbitrary ordering of the pigments, and the j index is used for summing over the 10 laboratory (or method) codes. Although any ordering for the pigments and methods is permissible, the former are ordered following their presentation in Table 6; for the latter, $j = 1, 2, \ldots, 10$ corresponds to the C, D, F, G, H, J, L, N, S, and U methods, respectively (which is based on a simple alphabetic ordering of the one-letter codes).

Only one value for each pigment is computed for each station, and this is generically referred to as a "sample," so the number of samples equals the number of stations (or batches). Averages of a sample across the methods reporting a particular pigment in a sample are used to estimate the true value of the pigment for each sample (or station):

$$\bar{C}_{P_i}^{A}(S_k) \;=\; \frac{1}{N_L}\sum_{j=1}^{N_L} \bar{C}_{P_i}^{L_j}(S_k), \tag{19}$$

where the superscript A denotes an average across all (applicable) methods, and N_L is the number of laboratories or methods quantitating a pigment. For the primary pigments, $N_L = 10$, but for the secondary and tertiary pigments N_L is frequently less than 10 (Table 4).

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

$$\psi_{P_i}^{L_j}(S_k) \;=\; 100\,\frac{C_{P_i}^{L_j}(S_k) \;-\; \bar{C}_{P_i}^{A}(S_k)}{\bar{C}_{P_i}^{A}(S_k)}. \tag{20}$$

Note that the formulation in (20) provides a relative percent difference (RPD), because it is signed: 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 laboratory pigment concentration was less than the average). Although $\bar{C}_{P_i}^{A}$ is not considered truth, it is the reference value or proxy for truth by which the performance of the methods with respect to one another are quantified.

When RPD values for methods that do not present any trend relative to the average consensus are summed, however, there is the risk of destroying some or all of the variance in the data. To preserve an appropriate measurement of the variance in the data, absolute UPD values, $|\psi|$, are averaged over the number of samples (N_S) to give the average absolute percent difference (APD) of each laboratory for each pigment across all the samples:

$$|\bar{\psi}|_{P_i}^{L_j} \;=\; \frac{1}{N_S}\sum_{k=1}^{N_S} \left|\psi_{P_i}^{L_j}(S_k)\right|, \tag{21}$$

where S_k is the k^{th} batch (or station) number (Table 3) associated with pigment P_i. For the analysis of field samples, $N_S = 12$, but when the analysis is extended to mixed laboratory samples, N_S is less and depends on the number of times a laboratory analyzed and reported results for the mixed laboratory sample (e.g., the DHI mix).

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 concentrations, but also in terms of a general philosophy: the primary measure of dispersion between the methods are the ψ and $|\bar{\psi}|$ values, so it is important to ensure they are not underestimated.

Another useful parameter is the average of the APD, $|\bar{\psi}|$, values for a particular pigment across the number of laboratories or methods (N_L) reporting the pigment involved:

$$|\bar{\psi}|_{P_i}^{A} \;=\; \frac{1}{N_L}\sum_{j=1}^{N_L} |\bar{\psi}|_{P_i}^{L_j}, \tag{22}$$

where the A code indicates all the laboratories were averaged (and $\bar{\psi}_{P_i}^{A}$ values are formed in a similar fashion from the UPD values). In general, (22) is only computed for the primary pigments, so $N_L = 10$.

To examine the replicate data for each method more closely, the coefficient of variation (CV), ξ, is used, which is expressed as the percent ratio of the standard deviation in the replicate (σ) with respect to the average concentration ($\bar{C}$):

$$\xi_{P_i}^{L_j}(S_k) \;=\; 100\,\frac{\sigma_{P_i}^{L_j}(S_k)}{\bar{C}_{P_i}^{L_j}(S_k)}, \tag{23}$$

where S_k is the k^{th} sample number, and the number of replicates is three for all methods. Individual ξ values are computed for each pigment, for each sample, and for each method; and then all the ξ values for a particular

method are averaged to yield an average precision ($\bar{\xi}$) for the method and pigment:

$$\bar{\xi}_{P_i}^{L_j} \;=\; \frac{1}{N}\sum_{k=1}^{N_S}\xi_{P_i}^{L_j}(S_k). \tag{24}$$

Subsets of the laboratories (or methods) involved are also used for different aspects of the analysis. One of the most important results is the estimation of the uncertainties in the reported data products, which requires an estimate or *proxy* for the truth. From the perspective of pigment concentration, there is no *a priori* understanding of truth with a field sample, so a procedure must be adopted to determine it. For the SeaHARRE activity, the performance metrics established during SeaHARRE-2 (Hooker et al. 2005) and expanded during SeaHARRE-3 (Hooker et al. 2009) are used to determine the quality of the results from the participating laboratories. The methods satisfying the quantitative level of performance (Table 9) are considered to have uncertainties sufficiently low to be considered acceptable representatives of truth and are hereafter referred to as the QA subset. The results from the other methods are considered to be not validated (NV) at the performance level associated with the QA subset. The methods in the NV subset are not used in determining truth.

To compute uncertainties, (19) is modified to include only the contributions from the QA subset:

$$\bar{C}_{P_i}^{A'}(S_k) \;=\; \frac{1}{N_L}\sum_{j=1}^{N_L}\bar{C}_{P_i}^{L_j}(S_k), \tag{25}$$

where the A' code indicates the QA subset, and N_L is set to index over the laboratories within the QA subset. The UPD values (relative uncertainties) are then computed by using (25) in (20):

$$\psi_{P_i}^{L_j}(S_k) \;=\; 100\,\frac{C_{P_i}^{L_j}(S_k) \;-\; \bar{C}_{P_i}^{A'}(S_k)}{\bar{C}_{P_i}^{A'}(S_k)}, \tag{26}$$

where the reference value for truth in each calculation is given by $\bar{C}_{P_i}^{A'}(S_k)$. APD values are determined using (21).

Summary averages for the performance of the QA and NV subsets are derived from (22) by setting N_L to reflect the number of laboratories (or methods) within each subset. Although it is a notable achievement to be included in the QA subset—which is associated with a data quality level in keeping with calibration and validation activities—there are many research inquiries that do not require this level of expertise. A significant advantage of not initially being in the QA subset is all subsequent evaluations are truly independent, so a laboratory (or method) that is evaluated against the QA subset and found to have data products in keeping with the quantitative level of performance has achieved a singularly remarkable result. Indeed, one of the most useful aspects of the SeaHARRE activity is to provide the opportunity of any method to be evaluated against established performance capabilities and criteria.

The formulations presented in (18)–(26) are for the field samples, but they are applicable to the laboratory standards by redefining the indexing limits and setting S_k to match the laboratory samples.

1.6 RESULTS

Before presenting any results, it is useful to clarify the definitions of certain key terms required for arriving at any statistical description of the various methods. Although not all of these terms are used in this study explicitly, they are all defined to provide complete clarification:

- *Accuracy* is the estimation of how close the result of the experiment is to the *true* value.
- *Precision* is the estimation of how exactly the result is determined independently of any *true* value.
- *Repeatability*, also called *within-run* precision, is obtained from a single operator, using the same instrument, and analyzing the same samples from the same batch.
- *Reproducibility*, also called *between-run* precision, is obtained from different operators, using different instruments and analyzing separate samples from the same batch.

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

Two of the most important variables in the results presented here are accuracy and precision, and a simpler definition for these parameters is:

> *Accuracy is telling a story truthfully*, and *precision is how similarly the story is repeated over and over again*.

For the analytical approach adopted here, the average accuracy is represented by the average APD values across the 12 samples, $|\bar{\psi}|$, and the average precision is given as the average CV across the 12 samples, $\bar{\xi}$. These are the principal parameters for determining method performance and the uncertainties in the methods.

The first step in the analysis of the SeaHARRE data is to establish the QA subset using the performance metrics (Table 9). This is initially based on the precision obtained with the field samples. A method with an average precision not satisfying the semiquantitative performance metric—more than 8% plus 2% for field sample variability (or approximately 10%)—is excluded from the QA subset (F and U). In addition, a laboratory with three or more primary pigments with a precision exceeding routine capabilities (13%) is considered for exclusion (N).

The second step is to use a permutation analysis of the results versus different selections of which laboratories constitute the reference set for computing uncertainties. For

Table 9. The current performance metrics (for the four categories established during SeaHARRE-2) for validating the determination of marine pigments using an HPLC method (left to right): concentration (average precision, $\bar{\xi}$, and accuracy, $|\bar{\psi}|$, for TChl *a* and PPig); pigment sums (also for pigment indices); pigment ratios; separation (minimum resolution, $\check{R}_s$, and average retention time precision, $\bar{\xi}_{t_R}$); injection precision, $\bar{\xi}_{\text{inj}}$ (the average of an early- and late-eluting pigment standard, e.g., Perid and Chl *a*); and calibration (average residual, $|\bar{\psi}|_{\text{res}}$, for Chl *a* and the precision of the dilution devices, $\bar{\xi}_{\text{cal}}$). The PPig and TChl *a* performance metrics are based on using the analysis of a laboratory mixture of pigments and replicate field samples with approximately equal weights applied to each (remembering that uncertainties are assumed to combine in quadrature and that the latter presupposes the inclusion of replicate filter collection during field sampling). The corresponding values for method *H* during SeaHARRE-2 are given as an example (selected because SeaHARRE-2 represented an exhaustive inquiry into method performance). The overall performance of *H* is considered "state-of-the-art," because the average score of the weights is 3.6, $(4+4+4+3+4+3+4+3+3+4+4+4+3+4)/14$.

Performance Weight, Category, and Score		*TChl* a $\bar{\xi}$	*TChl* a $\lvert\bar{\psi}\rvert$	*PPig* $\bar{\xi}$	*PPig* $\lvert\bar{\psi}\rvert$	*Sums* $\bar{\xi}$	*Sums* $\lvert\bar{\psi}\rvert$	*Ratios* $\bar{\xi}$	*Ratios* $\lvert\bar{\psi}\rvert$	*Sep.*† $\check{R}_s$	*Sep.*† $\bar{\xi}_{t_R}$	*Inj.*‡ ($\bar{\xi}_{\text{inj}}$) *Perid*	*Inj.*‡ ($\bar{\xi}_{\text{inj}}$) *Chl* a	*Cal.*§ $\lvert\bar{\psi}\rvert_{\text{res}}$	*Cal.*§ $\bar{\xi}_{\text{cal}}$
1. Routine	0.5	8%	25%	13%	40%	8%	20%	5%	15%	0.8	0.18%	10%	6%	5%	2.5%
2. Semiquantitative	1.5	5	15	8	25	5	12	3	9	1.0	0.11	6	4	3	1.5
3. Quantitative	2.5	3	10	5	15	3	8	2	6	1.2	0.07	4	2	2	0.9
4. State-of-the-Art	3.5	≤2	≤5	≤3	≤10	≤2	≤4	≤1	≤3	≥1.5	≤0.04	≤2	≤1	≤1	≤0.5
Method H		*1*	*5*	*2*	*12*	*2*	*5*	*1*	*5*	*1.2*	*0.02*	*<1*	*<1*	*1.1*	*0.4*

† The $\check{R}_s$ parameter is the minimum resolution determined from a critical pair for which one of the pigments is a primary pigment. The retention time precision, $\bar{\xi}_{t_R}$, values are based on sequential replicate injections of pigments identified in a laboratory mix. In the absence of a diverse set of early- through late-eluting pigments, a practical alternative is to compute $\bar{\xi}_{t_R}$ based on three sequential injections of Perid, Fuco, Diad, Chl *a*, and $\beta\beta$-Car.

‡ The $\bar{\xi}_{\text{inj}}$ terms are calculated from the average of replicate injections of an early- and late-eluting pigment in the same run. (Perid is chosen to include the possible effects of peak asymmetry, which is not presented as a separate parameter.)

§ The $|\bar{\psi}|_{\text{res}}$ values presented here are based on calibration points within the range of concentrations typical of the SeaHARRE-2 field samples. To determine this metric for an arbitrary sample set, $|\bar{\psi}|_{\text{res}}$ is computed using those calibration points within the range of concentrations expected in the field samples to be analyzed.

this inquiry, methods with an established QA capability (i.e., satisfy the quantitative analysis performance metrics in Table 9) are intercompared for consistency. The results from the other methods with respect to the QA laboratories are then evaluated individually and as a group. The threshold for retention within the QA subset is the average uncertainty of the primary pigments must be to within 25% (which is the semiquantitative performance metric) or to within 5% of the average for the reference group. From an overall performance perspective, these thresholds are based on an approximately equal evaluation of both laboratory standards and field samples, but because the difference between methods is not always as distinctive with laboratory standards as it is with field samples, greater weight is given to the results achieved with field samples when it comes to establishing the QA subset. This procedure removes *J* and *N* for inclusion in the QA subset.

The objective of the second step is to make sure methods close to a QA capability are carefully considered for inclusion. One of the criteria considered is to investigate beyond the primary pigments and look at the convergence of the candidate method with respect to established QA results with some of the tertiary pigments, like [Neo], [Viola], and Neo+Vio. This is considered important, because method-to-method differences can conceivably produce biases within the referencing system—particularly if the QA subset is dominated by one particular method—and one of the most important objectives of the intercomparison activity is to identify biases and resolve them. As shown in Tables 2 and 7 and discussed in Sect. 1.4, the majority of the methods used during SeaHARRE-4 were based on C_8 columns, and more specifically, were based on a single C_8 method (Van Heukelem and Thomas 2001).

Finally, the remaining laboratories in the QA subset are intercompared. Any method with more than three individual pigments exceeding an uncertainty of 25% is normally removed from the QA subset (if one of the pigments is TChl *a*, the allowed maximum uncertainty is 15%), and a new reference set for all pigments is computed. For SeaHARRE-4, anomalously poor results for But required this threshold to be relaxed to 30%. This procedure leaves only the *C*, *D*, *G*, *H*, and *L*. All ensuing results presented in the follow-on sections are based on these five laboratories as the quality-assured reference set (and are denoted as the QA subset of methods or laboratories), except as noted for specialized discussions.

A few aspects to note about the five laboratories in the QA subset, in terms of the results that will be presented in subsequent sections, are as follows:

- All of them, except *G*, participated in SeaHARRE-2 and SeaHARRE-3, and two of them (*H* and *L*) participated in SeaHARRE-1;

- With the exception of G, all routinely analyze a large number of samples per year and have significant commitments to an established user base;
- All used the same C_8 method (Van Heukelem and Thomas 2001); and
- The participation of G was an experiment to demonstrate what a laboratory that had never done HPLC analysis was capable of achieving if the method protocols and the performance metrics for quantitative analysis were strictly followed.

The latter point is particularly relevant because it shows the power of the performance metrics. The analyst in this case was an established biogeochemist who had never done an HPLC analysis, but who was familiar with the concept of following *best practices* in the laboratory.

The introduction of the QA subset automatically establishes another subset: the laboratories (or methods) that are not part of the QA subset are denoted A^+, which is composed of methods F, J, N, S, and U. In comparison, for the laboratories not in the QA subset:

- Only J and S participated in one or more prior SeaHARRE activities;
- Both C_8 and C_{18} methods were used, with different methods for both column types; and
- Sample analysis varied for a large number of users (S) at the production level of analysis (thousands of samples per year) to exclusive analyses for a very small user base (J).

The importance of these distinctions are considered in more detail during the subsequent presentations of the results.

1.6.1 Average Pigment Concentrations

The analytical process begins with computing the reference or proxy for truth needed for evaluating the analysis of the field samples by the individual methods, i.e., the average pigment concentrations $\bar{C}_{P_i}^{A'}$ in (25) are the reference values and these are computed from the QA subset. Normally, any pigments with significant coelution or specificity problems are not included in producing the overall averages, but for SeaHARRE-4 no such limitations existed for the QA subset.

The average concentrations for the primary pigments quantitated by the QA subset are presented in Table 10. A recurring objective of field sampling for SeaHARRE activities is to have a wide dynamic range in $\left[\text{TChl } a\right]$, typically two orders of magnitude, and the table shows this was partially achieved for SeaHARRE-4. Although the dynamic range in chlorophyll is not as large as desired, the dynamic range in several of the other primary pigments exceeds what has been experienced in prior SeaHARRE activities. The dynamic range in Fuco, for example, is almost two orders of magnitude with elevated concentrations across the entire range.

Table 10 also shows the laboratories not part of the QA subset are typified by higher concentrations on average, which can be on the order of 100% or more (TChl c and Caro, as well as But and Hex), but not in all cases. For example, the results for TChl b, TChl c, and Zea are very similar between the two subsets. Given the objective of average $\left[\text{PPig}\right]$ uncertainties to within 25% and $\left[\text{TChl } a\right]$ uncertainties to within 15%, the preponderance of large differences represent significant performance challenges.

From a generalized perspective, any pigment with an average concentration less than $0.050\,\text{mg}\,\text{m}^{-3}$ can be considered to be at a low concentration. Table 10 shows some of the carotenoids are below this limit with But and Hex being notably so, and Diato being nearly so. The other carotenoids have average concentrations above this limit and usually do not fall below it. The persistent low concentrations of But and Hex provide a significant opportunity for false positives, i.e., a pigment is reported present, when in fact it is not. Alternatively, a seemingly false positive pigment result can occur when a method with exceptional detectability quantifies a result, while other methods cannot detect it; although, the latter is a rare occurrence.

False positives are especially onerous to the computation of uncertainties because the larger false value is differenced with respect to the much smaller reference value, which yields a relatively large number in the numerator of (15). The denominator is the much smaller reference value, so the computed uncertainty is a large number divided by a much smaller number, which frequently yields an uncertainty of many hundreds of percent. So the large overestimation of $\left[\text{TChl } c\right]$, Caro, and But within the A^+ subset is going to lead to very large uncertainties with respect to the relatively smaller A' values.

A false negative occurs when a laboratory reports a pigment is not present when in fact it is. False negatives are not as damaging as false positives, because a false small number is differenced with respect to a larger reference value, which yields a relatively large number in the numerator of (26). The denominator is the larger reference value, so the computed uncertainty is a larger number divided by a slightly larger number, which yields an uncertainty a bit less than 100%.

The influence of false positives and negatives on method uncertainty is usually minimal for pigments that are almost always in an oceanic sample (e.g., Chl a), but they are particularly important for pigments whose presence and abundance changes significantly from sample to sample. The average concentrations of the secondary and tertiary pigments are presented in Table 11. Although there are some low results for these pigments, all of them have average concentrations above $0.050\,\text{mg}\,\text{m}^{-3}$ (except DVChl a). The A^+ subset results for the secondary and tertiary pigments are characterized by a mix of lower and higher concentrations with respect to the A' subset, but the magnitude of the differences are not as great as was seen with the PPig results (Table 10).

Table 10. The average individual PPig concentrations for the QA subset (C, D, G, H, and L) as a function of the batch sample number for the field samples (in units of milligrams per cubic meter). The overall averages for the individual pigments for the QA subset, $\bar{C}_{P_i}^{A'}$, as well as the range in maximum and minimum values, $\hat{C}_{P_i}^{A'}$ and $\check{C}_{P_i}^{A'}$, respectively, are computed across the 12 samples. Corresponding overall averages for the methods not in the QA subset are presented in the last three rows and are denoted by the A^{+} notations.

No.	[TChl *a*]	[TChl *b*]	[TChl *c*]	[Caro]	[But]	[Hex]	[Allo]	[Diad]	[Diato]	[Fuco]	[Peri]	[Zea]
1	42.704	9.971	2.958	2.432	0.137	0.059	0.222	0.857	0.381	10.185	0.097	1.471
2	13.598	0.293	1.424	0.345	0.033	0.021	0.179	0.384	0.039	5.179	0.999	0.146
3	19.244	0.463	2.706	0.458	0.001	0.001	0.288	0.588	0.035	6.145	0.003	0.007
4	2.626	0.530	0.205	0.120	0.007	0.001	0.113	0.075	0.018	0.500	0.021	0.031
5	4.971	0.645	0.187	0.282	0.001	0.001	0.186	0.097	0.007	0.452	0.018	0.199
6	3.944	0.708	0.325	0.137	0.002	0.003	0.162	0.085	0.017	0.702	0.032	0.057
7	5.504	0.433	0.843	0.176	0.020	0.016	0.059	0.266	0.028	1.535	0.867	0.096
8	5.671	0.213	1.022	0.137	0.001	0.020	0.038	0.437	0.041	1.743	1.208	0.044
9	20.836	0.521	1.614	1.008	0.003	0.019	7.438	0.295	0.125	1.049	0.408	0.030
10	3.226	0.314	0.426	0.123	0.002	0.002	0.202	0.134	0.004	0.652	0.380	0.052
11	2.671	0.459	0.305	0.130	0.004	0.006	0.086	0.093	0.001	0.225	0.455	0.204
12	1.896	0.110	0.361	0.061	0.006	0.039	0.060	0.112	0.006	0.611	0.077	0.031
$\hat{C}_{P_i}^{A'}$	42.704	9.971	2.958	2.432	0.137	0.059	7.438	0.857	0.381	10.185	1.208	1.471
$\bar{C}_{P_i}^{A'}$	10.574	1.222	1.031	0.451	0.018	0.015	0.753	0.285	0.059	2.415	0.380	0.197
$\check{C}_{P_i}^{A'}$	1.896	0.110	0.187	0.061	0.001	0.001	0.038	0.075	0.001	0.225	0.003	0.007
$\hat{C}_{P_i}^{A^{+}}$	51.141	11.724	74.519	21.039	2.166	0.434	9.907	1.748	0.699	20.171	3.696	1.935
$\bar{C}_{P_i}^{A^{+}}$	11.275	1.135	4.221	1.889	0.059	0.033	0.870	0.335	0.036	2.990	0.554	0.187
$\check{C}_{P_i}^{A^{+}}$	1.643	0.001	0.064	0.060	0.001	0.001	0.001	0.079	0.001	0.220	0.001	0.001

Table 11. A subset of the average secondary and tertiary pigment concentrations for the field samples analyzed by the QA subset. The presentation follows the scheme established in Table 10.

No.	[Chl *a*]	[DVChl *a*]	[Chlide *a*]	[Lut]	[Neo]	[Neo+Vio]	[Phytin *a*]	[Phide *a*]	[Pras]	[Viola]	[Zea+Lut]
1	40.601	0.001	2.103	2.530	0.473	0.848	7.144	14.833	0.142	0.375	4.001
2	12.867	0.001	0.731	0.015	0.008	0.038	0.632	4.429	0.001	0.030	0.160
3	14.970	0.001	4.274	0.010	0.032	0.103	0.266	1.905	0.007	0.071	0.017
4	2.404	0.001	0.222	0.010	0.057	0.105	0.169	0.316	0.123	0.048	0.040
5	4.822	0.001	0.149	0.162	0.084	0.170	0.190	0.152	0.121	0.087	0.362
6	3.774	0.001	0.170	0.024	0.081	0.148	0.090	0.100	0.181	0.067	0.081
7	4.815	0.001	0.689	0.091	0.061	0.110	0.083	0.283	0.022	0.049	0.187
8	5.095	0.001	0.575	0.022	0.034	0.065	0.043	0.113	0.034	0.031	0.067
9	20.368	0.001	0.474	0.048	0.045	0.130	4.567	0.605	0.032	0.086	0.078
10	2.982	0.001	0.245	0.003	0.041	0.079	0.068	0.091	0.078	0.039	0.055
11	2.587	0.001	0.084	0.023	0.055	0.097	0.029	0.031	0.053	0.043	0.226
12	1.586	0.001	0.310	0.043	0.022	0.041	0.017	0.033	0.023	0.019	0.073
$\hat{C}_{P_i}^{A'}$	40.601	0.001	4.274	2.530	0.473	0.848	7.144	14.833	0.181	0.375	4.001
$\bar{C}_{P_i}^{A'}$	9.739	0.001	0.835	0.248	0.083	0.161	1.108	1.908	0.068	0.079	0.446
$\check{C}_{P_i}^{A'}$	1.586	0.001	0.084	0.003	0.008	0.038	0.017	0.031	0.001	0.019	0.017
$\hat{C}_{P_i}^{A^{+}}$	49.594	0.001	6.824	7.161	1.406	1.928	5.982	21.667	0.392	0.522	8.824
$\bar{C}_{P_i}^{A^{+}}$	10.125	0.001	0.849	0.400	0.089	0.157	0.829	1.253	0.058	0.067	0.588
$\check{C}_{P_i}^{A^{+}}$	1.180	0.001	0.001	0.001	0.001	0.001	0.023	0.001	0.001	0.001	0.001

Notes: 1. The F, N, S, and U methods are not included in the A^{+} [DVChl *a*] results, because these methods did not chromatographically separate DVChl *a* (S used a simultaneous equation for information purposes only).

2. The A' [Phide *a*] entries do not include values from G, and the A^{+} [Phytin *a*] and [Phide *a*] entries do not include values from F and U.

Table 12. The method precision results for the individual PPig $\bar{\xi}$ values (coefficients of variation in percent) across all 12 batches of field samples. The data are presented as a function of the laboratory (or method) with the last column presenting the overall (horizontal) method average across the pigments. The overall (vertical) QA subset averages (C, D, G, H, and L) are given in the A' entries, and the methods that were not validated at the QA level (F, J, N, S, and U) in the A^+ entries. The lowest precision values for each pigment in the QA subset constitute a hypothetical *best method* and are shown in bold typeface, which are summarized for all pigments by the A^- entries in the bottom row. The analyses made by laboratory L on the particulate absorption filters are indicated by the L' results, which are not included in the determination of the QA subset or any of the summary averages; they are presented separately and are shown in slanted typeface. Exceptions to the computation of any of the averages are indicated by a special symbol and explained below the table (most exceptions attempt to deal with the fact that not all methods produce all data products, particularly for the secondary and tertiary pigments).

Meth.	[TChl *a*]	[TChl *b*]	[TChl *c*]	[Caro]	[But]	[Hex]	[Allo]	[Diad]	[Diato]	[Fuco]	[Peri]	[Zea]	*Avg.*
C	4.3	**2.9**	3.6	**2.8**	**1.4**	**1.9**	**4.2**	4.3	8.9	4.6	4.4	**4.2**	**4.0**
D	3.6	8.2	4.8	6.0	0.5†	5.1	4.9	5.6	12.2	5.8	4.7	5.9	6.1
G	4.8	5.9	**3.4**	4.9	0.0†	0.1†	5.7	5.1	**4.1**	4.3	3.7	6.1	4.8
H	5.8	4.9	5.5	5.9	17.9	4.1	5.4	7.3	8.0	5.0	8.3	4.4	6.9
L	**2.8**	5.0	4.3	2.8	2.9	2.0	10.9	**4.0**	7.2	**3.6**	**3.3**	5.1	4.5
L'	*1.8*	*5.7*	*2.4*	*3.1*	*6.2*	*1.9*	*4.5*	*8.9*	*5.9*	*2.6*	*2.7*	*4.1*	*4.2*
F	8.6	12.6	61.9	16.6	35.5	39.8	33.5	12.6	28.0	14.3	34.0	26.4	27.0
J	3.5	6.8	11.1	6.1	6.1	4.3	4.7	5.8	22.7	4.0	3.5	6.4	7.1
N	7.5	6.6	9.4	18.6	1.0	3.8	16.3	16.7	9.2	10.2	6.4	5.7	9.3
S	2.6	3.5	6.1	4.1	3.3	6.5	4.9	5.9	14.0	2.5	8.8	5.9	5.7
U	6.6	19.6	12.2	9.5	0.0†	0.2†	15.2	9.0	37.5	8.9	5.9	26.2	15.1
A'	4.3	5.4	4.3	4.5	7.4	3.3	6.2	5.3	8.1	4.7	4.9	5.1	5.3
A^+	5.8	9.8	20.1	11.0	11.5	13.6	14.9	10.0	22.3	8.0	11.7	14.1	12.7
A^-	2.8	2.9	3.4	2.8	1.4	1.9	4.2	4.0	4.1	3.6	3.3	4.2	3.2

† Artificially low precision, because of a preponderance of false negatives—not included in averages.

1.6.2 Method Precision

Some laboratories use a field sample collected for HPLC analysis for more than one purpose. For example, it is not unusual for an HPLC filter to also be used to measure the particulate absorption of seawater, which has the advantage of providing two measurements from the same water sampling. LOV follows this practice and there has always been some concern as to whether or not the additional handling and different storage of the filter—it is kept in a small plastic histoprep holder, so it can lay flat within a protective shell—causes differences in the determination of pigment concentrations.

Consequently, for SeaHARRE-4, LOV analyzed two sets of filters (H was supposed to do the same, but was unable to do so): a) the first set was collected and stored following normal SeaHARRE practices (i.e., folded into foil packets), and b) the second set was collected as usual, but stored in histopreps. Both sets of filters were stored in liquid nitrogen. The histoprep analyses are designated L' in all of the following presentations of results, and are not included in the determination of the QA subset or any of the summary averages—they are presented separately. Separating the L' results is not an *a priori* determination that they are different—it is simply done to preserve an equal weighting of the methods used to determine the reference values for computing uncertainties and to provide an independent assessment of the LOV histoprep samples.

Method precision for each pigment is estimated by averaging the values computed from the sample triplicates across all 12 samples, which is denoted by $\bar{\xi}_{P_i}$. Table 12 presents the method precision for the primary pigments plus three types of overall precision: a) the average (Avg.) across all primary pigments for each laboratory, b) the average values for the methods in the A' and A^+ subsets, and c) the best precision obtained for each pigment within the A' subset, which is denoted A^-. The latter represent a theoretical *best method* threshold first tested and found to be a satisfactory criteria for a *state-of-the-art method* during prior SeaHARRE activities.

In most cases, the average method precision results for the primary pigments show superior precision is associated with [TChl *a*], and there is a general worsening of precision for the other chlorophylls and carotenoids—this is particularly true for the A^+ subset. The best average precision in the A' subset is for Hex, which was the primary pigment present in the lowest average concentration (Table 10). As shown in SeaHARRE-3 (Hooker et al. 2009), this result is somewhat artificial, because much of the data was at the limit of detection, so as long as a laboratory correctly

Table 13. The $\bar{\xi}$ values (coefficients of variation in percent) across all 12 batches of field samples for a subset of the secondary and tertiary pigments, following the presentation scheme established in Table 12. A blank entry indicates a particular laboratory (or method) did not quantitate the pigment indicated; none of the averages include contributions from blank entries.

Meth.	[Chl *a*]	[DVChl *a*]	[Chlide *a*]	[Lut]	[Neo]	[Neo+Vio]	[Phytin *a*]	[Phide *a*]	[Pras]	[Viola]	[Zea+Lut]
C	4.3	0.0	**2.7**	3.6	**3.8**	**6.1**	**1.2**	7.6	3.6	4.9	**5.5**
D	10.4	0.0	22.5	6.4	6.5	8.0	7.9	12.9	3.0	**4.7**	8.7
G	6.6	0.0	16.2	4.1	4.1	6.7	7.2		**1.7**	5.4	7.4
H	9.8	0.0	46.2	6.0	4.4	10.0	9.6	**7.5**	14.7	9.0	7.4
L	**2.9**	0.0	12.9	**3.1**	4.0	6.7	3.4	8.6	6.8	5.4	6.0
L′	*1.9*	*0.0*	*8.3*	*3.9*	*2.5*	*19.1*	*2.1*	*7.0*	*3.7*	*18.9*	*5.7*
F	9.5		42.4	26.4	16.3	53.2			16.5	50.6	37.4
J	4.8	0.0	26.0	15.3	4.5	6.8	19.1	6.8	5.6	5.1	16.6
N	10.4		18.2	6.1	8.0	20.8	6.3	4.7	7.0	19.2	8.3
S	3.6	8.6‡	18.3	6.3	5.9	10.8	3.8	2.9	9.5	9.0	8.7
U	6.6		25.3	32.6	0.4†	20.9			0.9†	21.3	32.9
A′	6.8	0.0	20.1	4.6	4.5	7.5	5.9	9.2	6.0	5.9	7.0
A^+	7.0	4.3	26.0	17.4	8.7	22.5	9.7	4.8	9.6	21.1	20.8
A^-	2.9	0.0	2.7	3.1	3.8	6.1	1.2	7.5	1.7	4.7	5.5

† Artificially low precision, because of a preponderance of false negatives—not included in averages.

‡ Derived from a simultaneous equation (Latasa et al. 1996) and provided for information purposes only.

identifies the pigment is absent or minimally observed in those samples, the precision will be very good.

The A' average precisions fall within a narrow range of outcomes, 4.0–6.9%, with the L' results very close to the best overall average. The C and G results, which like L' are histoprep samples, have six and two of the lowest precision results, respectively. The C, G, and L' results indicate storage in histopreps and absorption measurements prior to HPLC analysis do not have a detrimental effect on pigment precision. Despite the tight grouping within the A' averages, each individual method exhibits at least one anomalously high precision value, e.g., But for H and Diato for D. These distinctions are important indicators as to where additional work needs to be done by the individual analysts in particular, and the SeaHARRE activity in general.

The individual instances of excellent precision for specific methods and pigments within the QA subset (and also in some of the entries for the other methods), show excellent precision is a recurring outcome across all pigments. The precision of the selected best results in the QA subset (the bold entries in Table 12) ranged from 1.4–4.2%, with an overall average of 3.2% (the overall average for SeaHARRE-3 was 3.4%). The narrow range of precision excellence, and because it occurs across three methods (C, G, and L), suggests filter inhomogeneity is not a significant component of the variance, and that methodological differences account for the majority of the diversity in the precision results. The fact that the C, L, and L' results have an overall precision very close to the A^- average shows the best method approach is achievable and is a useful indicator of the state of the art.

The A^+ average precisions for each primary pigment show a larger range of outcomes. The J and S results are similar to the A' averages, the N results are slightly worse, and the F and U results are significantly poorer. This demonstrates the difficulty of using precision exclusively as a criteria for evaluating method performance. The point to remember here is that precision is the starting point, and it is very difficult for a method to achieve a suitable accuracy if method precision is poor, but that does not mean a good precision will ensure good accuracy—*it is a necessary, but not sufficient, criteria.*

Table 13 presents the precision obtained by the methods for a subset of the secondary and tertiary pigments using the format established for Table 12. These results largely confirm the precision conclusions obtained with the primary pigments, but there are some differences. The best precision of the QA subset occurs over a slightly larger range (1.2–7.5%) and is spread across all five laboratories. The worst results are seen with [Chlide *a*], which was present in reasonably high abundances (Table 10). Once again, areas for additional research can be identified by anomalously poor precision for individual methods. In this regard, the L' results for [Viola], and by extension [Neo+Vio], stand out because of significantly degraded values with respect to the L results. Although L' has degraded precision for [Viola] and [Neo+Vio], the C and G results have the lowest or near-lowest precision for both parameters, which suggests the poorer L' values are not a consequence of histoprep storage. Overall, however, the L' method precision is in keeping with the A' averages.

As anticipated from the PPig results, the A^+ results are usually worse than the A' results—almost by a factor

Table 14. The $\bar{\xi}$ values (coefficients of variation in percent) for DHI Mix-102 as a function of the method for the primary pigments using the presentation scheme from Table 12. The lowest $\bar{\xi}$ values from the QA subset are given in the last row, A^-.

Meth.	[TChl *a*]	[TChl *b*]	[TChl *c*]	[Caro]	[But]	[Hex]	[Allo]	[Diad]	[Diato]	[Fuco]	[Peri]	[Zea]	*Avg.*
C	0.8	0.5	1.6	1.3	0.9	**0.5**	0.9	0.7	0.9	0.9	1.0	0.7	0.9
D	0.9	0.9	0.6	0.9	0.9	1.4	0.9	**0.4**	0.8	1.0	1.4	1.9	1.0
G	0.9	0.7	1.0	1.6	1.3	0.6	1.4	0.6	0.9	1.6	1.1†	0.9	1.1
H	0.9	1.3	1.1	1.1	0.9	0.8	**0.7**	1.1	0.9	1.3	1.1	1.0	1.0
L	**0.1**	**0.2**	**0.4**	**0.2**	**0.2**	0.7	1.2	1.0	**0.8**	**0.7**	**0.2**	**0.3**	**0.5**
F	4.4	4.5	13.3	4.4	3.8	4.7	2.5	4.2	3.9	3.6	5.8	4.5	5.0
J	2.7	0.7	1.2	0.6	0.7	1.3	1.2	0.5	0.6	1.2	3.5	0.6	1.2
N	0.2	0.2	0.2	1.3	0.1	1.2	0.2	0.2	0.1	0.3	0.5	0.0‡	0.4
S	4.0	4.5	9.3	10.8	4.5	4.9	5.1	4.1	5.2	4.5	6.7	4.3	5.7
U	1.3	1.9	7.5	3.4	4.1	3.7	2.8	3.3	1.0	4.6	4.3	2.7	3.4
A'	0.7	0.7	0.9	1.0	0.9	0.8	1.0	0.8	0.9	1.1	0.9	1.0	0.9
A^+	2.5	2.3	6.3	4.1	2.6	3.2	2.3	2.5	2.2	2.9	4.1	3.0	3.2
A^-	0.1	0.2	0.4	0.2	0.2	0.5	0.7	0.4	0.8	0.7	0.2	0.3	0.4

† Revised GSFC Peri precision if the secondary Peri peak is quantitated in addition to the parent peak.
‡ Artificially low precision, because of a persistent nondetection—not included in averages.

of two on average. This is not true for all A^+ methods and all pigments, however. Many of the A^+ results are within the range of the QA subset, with the recurring exception of the F data. If the F data are excluded from an A^+ overall average, the remaining methods have an average precision that is within about 50% of the A' overall average–so approximately half the variance is coming from a single method. Comparable reductions are achieved with many of the individual PPig method precision values if the same exclusion is applied to the A^+ data in Table 12.

Table 14 presents the method precision for the primary pigments quantitated for DHI Mix-102. The results show the A' subset has better average precision than the A^+ subset, although some of the individual methods and pigments of the latter are superior or are similar to the former (J and N). The range in average precision for the A' subset is not very large (0.5–1.1%), and the A^+ results are distinguished primarily by the three methods (F, S, and to a lesser extent U) that are notably different with respect to the A' values. Although the three methods with the poorest precision are C_{18} methods, N is also a C_{18} method and the results obtained were excellent.

The DHI mix does not have the full complexity of a natural sample and the pigment concentrations are significantly elevated with respect to open-ocean surface waters, particularly for many carotenoids. Identifying peaks in the mix is easier than in a natural sample, so false positives or negatives are unlikely. Consequently, the precision obtained with the mix should be closer to the injection precision than the precision of a field sample. Nonetheless, as will be shown later, the precision obtained with the mix is an important QA opportunity, which can provide a lot of information about method performance.

The A^- results (which are a proxy for a theoretical best method) are always to within 0.7% and have an overall average of 0.4%. This means they are within the 2% performance metric for state-of-the-art injection (Table 9), and all the QA methods satisfy this metric for every pigment—as does the N method (except for Zea). The fact that so many methods achieved precision results close to or within this value is a notable indicator that using the best results from the QA subset produces a realistic and achievable metric for what a method should be capable of achieving.

1.6.3 Method Accuracy

Method accuracy for pigment products are determined using $|\bar{\psi}|$, the average APD between the reported laboratory values and the average concentrations constructed from the QA subset, that is, (26) is used in (21). Although the limitations already noted in determining method precision (Sect. 1.6.2) are relevant to understanding accuracy, most do not actually change the computations. There are exceptions, however, and the two most important are the aforementioned false positives and negatives (Sect. 1.6.1), which are both specificity problems.

Note that the primary difference between false negatives and false positives is the uncertainty of a false negative is bounded (it cannot be more than 100%), whereas the uncertainty for a false positive is unbounded (it can be many times more than 100%). In both cases, the actual uncertainty will be somewhat mitigated if the true concentration of the pigment is close to detection limits, because the multiplicative factor relating the observation to the reference value will be a small number. The false positive situation, however, will usually be the most serious in terms of average uncertainties.

Table 15. The $|\bar{\psi}|$ values (average APD in percent) across all 12 batches of field samples for the primary pigments following the presentation scheme established in Table 12. Exceptionally large uncertainties with truncated digits of precision are shown in a red typeface.

Meth.	[TChl *a*]	[TChl *b*]	[TChl *c*]	[Caro]	[But]	[Hex]	[Allo]	[Diad]	[Diato]	[Fuco]	[Peri]	[Zea]	*Avg.*
C	4.5	16.0	31.2	6.8	79.0	78.1	26.7	16.0	56.4	12.5	53.4	21.2	33.5
D	5.4	14.5	30.2	18.5	65.2	45.8	9.2	7.8	30.7	6.0	103.7	17.2	29.5
G	7.8	6.5	9.4	4.4	75.7	61.8	14.4	8.4	66.0	3.3	51.6	21.0	27.5
H	6.8	6.8	16.5	12.3	144.6	57.0	9.1	7.4	64.0	7.9	67.6	15.1	34.6
L	7.4	18.2	14.8	5.8	71.4	44.9	5.8	6.9	32.1	3.3	56.5	20.3	23.9
L′	*7.5*	*22.6*	*14.0*	*4.2*	*92.5*	*45.7*	*12.4*	*11.8*	*31.8*	*5.0*	*56.7*	*20.9*	*27.1*
F	24.6	19.7	55.3	1,318	36,471	122.7	29.8	18.0	86.7	33.2	93.1	56.8	3,194
J	22.7	20.6	13.2	10.1	441.7	66.3	32.1	18.3	32.7	16.7	57.9	30.4	63.6
N	6.9	26.2	12.2	9.0	653.3	897.0	20.0	21.5	94.0	16.0	95.2	271.4	176.9
S	34.1	23.9	32.3	67.3	2,750	3,068	5.1	13.6	30.6	7.5	284.1	55.1	530.9
U	10.7	4.2	1,328	325.2	75.7	64.2	37.1	31.8	65.8	27.0	68.6	32.5	172.6
A′	6.4	12.4	20.4	9.6	87.2	57.5	13.0	9.3	49.8	6.6	66.6	19.0	29.8
A^+	19.8	19.0	288.2	345.9	8,078	843.6	24.8	20.7	62.0	20.1	119.8	89.2	827.6
A^-	4.5	6.5	9.4	4.4	65.2	44.9	5.8	6.9	30.7	3.3	51.6	15.1	20.7

1.6.3.1 Individual Pigments

The performance metrics for QA (quantitative) performance require an average [TChl *a*] and [PPig] uncertainty to within 10% and 15%, respectively (Table 9). Recalling the allowed variance in establishing the QA subset (Sect. 1.6)—no more than three individual primary pigments were permitted to exceed an uncertainty of 25% and the [TChl *a*] uncertainty must be within 15%—the expectation is all QA methods will have PPig uncertainty results satisfying these criteria.

The individual and summary PPig uncertainties are presented in Table 15, which shows the QA subset (A') comes close to, but does not satisfy, the quantitative performance metric for accuracy—the overall uncertainty is 29.8%. Furthermore, only one of the overall individual method uncertainties, L, is less than 25% (the semiquantitative performance threshold); although G is within the 2–3% expected variance from natural variability for this level of performance. The results for [TChl *a*] are more promising. All QA laboratories are well within the 15% threshold, as is the A' average. Furthermore, the overall A' average and all the individual A' methods have TChl *a* uncertainties within the 2–3% sampling variance for the state-of-the-art performance metric.

The two most problematic pigments within the A' subset are But and Hex. If either had an uncertainty more in keeping with expectations, the overall A' average would be closer to 25% (for SeaHARRE-3, But and Hex had QA uncertainties of 14.6 and 6.0%, respectively). The results for Peri are also high, but not as anomalous as for But and Hex (the QA uncertainty for Peri in SeaHARRE-3 was 30.5%). The values for Diato are also high, but Diato has been a problematic pigment in the past, e.g., during SeaHARRE-1 where the average A' uncertainty was even higher. Although there are many But and Hex samples that are detection limited, this is not true for some of the Diato samples and most of the Peri samples. Consequently, the challenges go beyond a single simplistic explanation.

The results from the A^+ methods are significantly different from the A' subset and are characterized by higher or significantly higher uncertainties. The overall average PPig uncertainty (827.6%) does not satisfy any of the performance metrics (Table 9), and neither do any of the individual methods (although the J method is the closest). The routine performance metric for TChl *a* is satisfied as an overall average, and the N and U methods satisfy the quantitative and semiquantitative performance requirements, respectively, while the F and J methods are within the routine analysis threshold.

The problematic pigments within the A^+ subset are the same ones seen with the A' subset with the addition of TChl *c* and Caro. For the latter two, most of the difficulty arises from the F and U results, which are based on the same method. The implementation of the method at the two laboratories appears notably different, because the magnitude of the uncertainties do not covary that closely. The individual pigment results for the A^+ subset are further distinguished by all methods contributing very large uncertainties to one or more of the problematic pigments (only J has large uncertainties for only one pigment).

The best possible outcomes from the QA subset, the A^- entries, have an overall average TChl *a* and PPig uncertainty of 4.5 and 20.7%, respectively. These results are within the requirements for quantitative and semiquantitative performance, respectively. The L' overall PPig average uncertainty also satisfies this metric to within the 2–3% sampling variance. Note that the invocation of adding a margin of 2–3% variance for the performance discussions

is simply a reflection that only field samples are being considered and there is a small amount of variance that comes from the preparation of field replicates—they are not perfect replicates—and this variance is beyond the control of the methods being intercompared.

As noted earlier, the J method stands out within the A^+ subset, because it has the lowest overall average uncertainty in the A^+ subset. If the But results were not so anomalous (i.e., closer to the A' average), the overall PPig average uncertainty for J would be within the range of uncertainties for the A' subset. What this shows is the value of a proper referencing system: it highlights which parts of a method need additional attention and which parts are performing adequately. The latter qualification is simply a reminder that although the J method has been intercompared before, SeaHARRE-4 was the first intercomparison for J after switching to the Van Heukelem and Thomas (2001) method. Consequently, additional intercomparisons are needed to verify the findings emerging here.

Within the context of needing more than one intercomparison to provide confidence in the capabilities of a method, it is worth reviewing the histories of the methods being intercompared here (Tables 1 and 2):

- C Participated in SeaHARRE-2, but switched to the Van Heukelem and Thomas (2001) method for the SeaHARRE-3 activity; only the latter results were part of the QA subset.
- D Participated in SeaHARRE-2 and SeaHARRE-3, and both results were part of the QA subset.
- F Had not participated in a prior SeaHARRE activity.
- G Had not participated in a prior SeaHARRE activity and participated in SeaHARRE-4 as a novice practitioner.
- H Participated in all prior SeaHARRE activities and always produced results as part of the QA subset.
- J Participated in SeaHARRE-1 and SeaHARRE-3, and the former results were part of the QA subset.
- L Produced results that were part of the QA subset for SeaHARRE-1, but not for SeaHARRE-2; switched to the Van Heukelem and Thomas (2001) method for SeaHARRE-3, and results were part of the QA subset.
- N Had not participated in a prior SeaHARRE activity.
- S Participated in SeaHARRE-2 with results that were part of the QA subset; the inability to chromatographically separate Chl a and DVChl a led to the use of the Van Heukelem and Thomas (2001) method for SeaHARRE-3 along with the original method, but neither method produced results as part of the QA subset.
- U Had not participated in a prior SeaHARRE activity.

Out of all of these laboratories, H and L are the only ones to have participated in all prior SeaHARRE activities. Laboratories C, D, J, and S have now participated in three round robins, but C, D, and J are no longer using their original methods; S switched back to their original method for SeaHARRE-4.

The participation history of the various methods establishes how difficult it is to a) confidently evaluate a method using only one intercomparison, b) maintain a QA status over time, and c) further develop or troubleshoot the capabilities of a method—several analysts simply changed to a proven method rather than try to refine an existing method. For the methods considered here, only H has consistently produced QA results over an extensive (many year) time span, although C, D, and L are now emerging with sufficiently long time series of quality control variables. This means comparisons to individual established methods, or the time series of the results from those methods, help establish confidence in many of the findings presented here.

One of the important results from the intercomparison of the PPig analysis is how similar the data within each of the two A' and A^+ subsets are, and how distinctly different the two subsets are with respect to one another. For example, each method in the A^+ subset has at least one pigment with uncertainties that are many hundreds of percent, and three have uncertainties exceeding 1,000%. The A' subset has much lower uncertainties, although D and H have large values for Peri and But, respectively. If the referencing system was flawed, at least one of the methods within one of the two groups would appear out of place with a set of results that would not be in keeping with the group in which it was placed.

If the referencing system involved all of the methods, the aforementioned distinctions would become less notable, because the variance between the populations of results would be shared between the individual contributors. For example, the average uncertainty in PPig for the A' subset would increase to 38.7%, whereas it would decrease to 67.8% for the A^+ subset. Consequently, both sets would not be within the limits for routine analysis, but the A^+ subset would have improved by more than an order of magnitude (the prior value was 827.6%). The effects on TChl a would be less significant, because the A^+ results were not as dramatically different: the uncertainty for the A' subset would increase to 6.8%, and decrease to 17.9% for the A^+ subset. Both results would stay within their respective original quality categories of quantitative and routine analysis. The significant change for the PPig average uncertainties, however, shows the importance of selecting and defending a proper referencing system. If the results are blindly pooled together, the methods with reduced performance metrics are improved at the expense of the higher performing methods and a false understanding of uncertainties is presented.

This concept of whether or not the referencing system is properly established is further evaluated by investigating the uncertainties for the secondary and tertiary pigments,

Table 16. The $|\bar{\psi}|$ values (average APD in percent) across all 12 batches of field samples for a subset of the secondary and tertiary pigments (following the presentation scheme established in Tables 12, 13, and 15).

Meth.	[Chl *a*]	[DVChl *a*]	[Chlide *a*]	[Lut]	[Neo]	[Neo+Vio]	[Phytin *a*]	[Phide *a*]	[Pras]	[Viola]	[Zea+Lut]
C	9.7	0.0	97.5	42.4	29.4	22.1	87.0	82.5	**34.0**	18.8	23.9
D	9.8	0.0	91.2	**25.6**	70.2	30.2	62.1	40.3	45.5	17.0	17.9
G	13.4	0.0	57.8	37.3	30.6	18.4	25.0		38.5	12.3	20.4
H	**8.5**	0.0	**27.3**	35.0	**28.6**	**11.3**	**24.7**	39.3	51.6	**9.6**	**16.1**
L	9.0	0.0	40.6	43.6	37.4	18.1	38.5	**34.8**	77.2	15.3	24.6
L′	*10.6*	*0.0*	*35.6*	*40.9*	*38.7*	*22.8*	*40.6*	*81.5*	*54.1*	*20.3*	*22.3*
F	34.9		38.7	209.6	71.7	48.8			66.7	51.7	48.5
J	25.6	0.0	21.1	179.4	80.5	27.2	28.7	74.3	1,330	6.8	82.1
N	10.5	0.0	107.4	93.0	78.0	45.5	74.1	82.3	553.2	42.0	109.4
S	36.7	78,461†	42.8	154.5	534.9	170.8	23.8	88.8	2,148	53.5	70.2
U	10.8		90.0	141.3	91.8	90.3			78.1	87.1	44.7
A′	10.1	0.0	62.9	36.8	39.3	20.0	47.5	49.2	49.4	14.6	20.6
A^+	23.7	39,231	60.0	155.6	171.4	76.5	42.2	81.8	835.2	48.2	71.0
A^-	8.5	0.0	27.3	25.6	28.6	11.3	24.7	34.8	34.0	9.6	16.1

† Derived from a simultaneous equation (Latasa et al. 1996) and provided for information purposes only.

which are presented in Table 16. Unlike the PPig results, these data are not supplied by all the methods, so some of the results cannot be used at the same level of efficacy. The A' uncertainties are usually significantly lower than the corresponding A^+ values, but not in all cases. For example, note the good *J* results for Chlide *a* and Viola, as well as the good *S* results for Phytin *a*.

Despite some good results within the A^+ subset, the general distinction between the A' and A^+ results is each of the A^+ methods have one or more pigments for which the uncertainty is anomalously poor, both with respect to the A' methods and to the other A^+ methods:

F Lut;

J Lut and Pras;

N Chlide *a* and Pras; and

S DVChl *a*, Neo, Neo+Vio, and Pras; and

U Lut.

Although DVChl *a* is listed above, it is important to note that *S* provided the associated data for information purposes only, and higher uncertainties were expected based on the results from SeaHARRE-2 (Hooker et al. 2005).

The presence of the anomalously high uncertainties in the A^+ subset further reinforces the need for a properly established referencing system. A separate check on the capabilities of the methods and, in particular, the veracity of establishing two subsets of analyses (A' and A^+), is provided by the results from the DHI mix. With respect to field samples, the DHI mix is distinguished by higher concentrations (larger peaks) and less coeluting contaminants. This means the pigments are easier to quantify and quantitate, so the results should almost be at the highest quality level a method can achieve. In practice what this means is any method should quantitate the DHI mix at one level of performance better than expected for field samples.

A review of the governing equations, (1) and (6), reveals why results for the DHI Mix should be better: fewer variables are needed to compute the concentrations of pigments in the DHI Mix relative to a field sample, because the filtration volume (V_f) and extraction volume (V_x) are not applicable to the former. The variables in common are injection volume (V_c), pigment peak area ($\hat{A}_{P_i}$), and the calibration response factor (R_{P_i}). For the QA subset, the expectation is the results will satisfy the state-of-the-art performance metric on average, and for many of the individual pigments. The A^+ subset is expected to be at a lower level of performance (on average and for many individual pigments), but should be within the 15% threshold.

Given an investigative perspective, uncertainties in V_c, for example, are in part described by the imprecision of replicate injections of the DHI Mix (Sect. 1.6.2 and Table 14). Uncertainty sources affecting $\hat{A}_{P_i}$ differ somewhat between field sample and DHI Mix analyses. For example, effects of filter inhomogeneity and the efficiency with which pigments are extracted from cells are unique to field samples and contribute to interlaboratory differences. For methods with a restricted limit of linearity (or nonlinear methods), however, the DHI Mix at full strength can produce erroneous results and dilution with validated dilution devices may be needed so peak areas fall within the limit of linearity for the method.

Method uncertainties from the DHI Mix-102 analyses are presented in Table 17. The QA subset has overall TChl *a* and PPig uncertainties of 4.2% and 4.6%, respectively, which are within the performance specifications for state-of-the-art analyses (Table 9). The QA subset is also distinguished by a small range in the overall PPig averages

Table 17. The $|\bar{\psi}|$ values (average APD in percent) for DHI Mix-102 as a function of the HPLC method for the primary pigments using the presentation scheme established in Tables 12, 13, and 15. The uncertainties are computed using the average concentrations of the pigments determined by the QA subset as the reference in the uncertainty calculations.

Meth.	[TChl *a*]	[TChl *b*]	[TChl *c*]	[Caro]	[But]	[Hex]	[Allo]	[Diad]	[Diato]	[Fuco]	[Peri]	[Zea]	*Average*
C	6.5	5.7	24.4	5.5	2.8	5.7	4.7	0.7	**0.9**	**1.6**	3.8	**0.6**	5.2
D	6.2	**2.0**	8.0	6.0	8.5	6.6	1.5	7.7	1.8	3.1	5.4	17.1	6.1
G	2.7	5.0	5.6	4.7	2.4	**0.9**	5.6	2.5	1.4	2.3	**0.0**	5.4	**3.2**
H	**1.6**	10.2	**4.4**	**0.3**	6.9	3.3	3.3	5.0	3.7	4.2	4.4	9.3	4.7
L	3.9	7.5	6.3	4.5	**1.1**	1.6	**1.0**	**0.5**	4.2	5.0	4.7	3.1	3.6
F	5.2	13.9	39.3	998.7	30.1	26.6	7.2	17.4	29.5	28.2	11.8	19.7	102.3
J	12.7	26.4	4.0	9.9	1.4	8.7	14.6	1.6	24.8	2.4	10.3	0.1	9.7
N	11.6	6.3	20.0	29.5	6.2	20.0	12.8	22.3	0.5	2.6	17.5	99.8	20.8
S	5.6	4.6	4.8	11.8	8.3	9.5	13.3	1.5	6.1	3.4	19.3	7.8	8.0
U	2.3	19.4	606.6	149.4	9.0	4.6	1.9	0.2	9.2	4.1	6.2	6.7	68.3
A'	4.2	6.1	9.7	4.2	4.3	3.6	3.2	3.3	2.4	3.2	3.7	7.1	4.6
A^+	7.5	14.1	134.9	239.9	11.0	13.9	10.0	8.6	14.0	8.1	13.0	26.8	41.8
A^-	1.6	2.0	4.4	0.3	1.1	0.9	1.0	0.5	0.9	1.6	0.0	0.6	1.2

of 2.9%. The low value of the theoretical best method, 1.2%, shows how many high-quality results were achieved for the individual pigments.

All of the overall A' average PPig uncertainties for DHI Mix-102 are within the state-of-the-art performance metric (Table 9). In terms of the individual methods and individual pigments, virtually all of the results are within, or close to, the state-of-the-art performance metric. The *best method* entries are spread across all five methods. The small number of notable exceptions are TChl *c* for *C*, Zea for *D*, and TChl *b* for *H*.

The difference between the A' uncertainties for robust field samples (i.e., those without quantitation problems) and their corresponding uncertainties in the DHI Mix-102 analyses can be used to estimate the contribution of sampling variance to the uncertainty budgets. For example, the difference for TChl *a* is 2.2% and the difference for Fuco is 3.4%. This range of differences establishes a variance estimate of approximately 2–3% in field sample filtering (which is similar to the filtering variance experienced during SeaHARRE-2 and SeaHARRE-3).

The overall A^+ averages for the DHI Mix-102 analyses are 7.5% for TChl *a* and 41.8% for PPig; the former are within expectations, but the latter are significantly not. Closer inspection shows the *J* and *S* methods are performing as expected, however, and with the exception of the Zea results, the *N* method is very nearly doing so. The *F* and *U* methods each have at least one pigment for which the results are sufficiently anomalous as to prevent higher quality results. Again, these kinds of distinctions reinforce why a proper referencing system is so important.

The uncertainties for the analysis of secondary and tertiary pigments within DHI Mix-102 are presented in Table 18. These data further reinforce the results already presented with the field samples and the PPig results for the DHI mix: a) the QA subset almost always has significantly lower overall uncertainties than the A^+ subset (Chlide *a* is the notable exception) and is usually at a state-of-the-art level of performance; b) the distribution of the *best method* results are spread across more than one QA method (although *C* predominates); and c) there are numerous examples of A^+ methods achieving state-of-the-art performance, but this is countered by other instances of elevated uncertainties, some of which are significant (e.g., Lut for *J* and *N*).

1.6.3.2 Pigment Sums

Prior SeaHARRE activities established a functional relationship in pigment uncertainties: *PPig overall uncertainties decreased significantly as individual pigments were summed.* This is a direct reflection of a) how summing cancels the highs and lows associated with the biases in the individual pigments, and b) more abundant pigments are more important to the sums and they are usually quantitated with lower uncertainties (i.e., smaller biases), because they are not detection limited. If an abundant pigment is also a problematic pigment, summing will frequently not ameliorate the uncertainties involved.

In this cascade towards lower uncertainties, a method having problems with a class of pigments (e.g., the carotenoids), is expected to have elevated uncertainties for sums predominated by that class of pigments (e.g., PPC). These elevated uncertainties can easily influence other sums if the pigments involved are particularly abundant. For example, chlorophyll *a* is usually the most abundant pigment in marine ecosystems, so if there is a problem with the quantitation of this pigment, there will be elevated uncertainties in the formulation of TChl or TPig.

Table 18. The $|\bar{\psi}|$ values (average APD in percent) across the DHI Mix-102 analyses for a subset of the secondary and tertiary pigments (following the presentation scheme established in Tables 12, 13, and 15).

Meth.	[Chl *a*]	[DVChl *a*]	[Chlide *a*]	[Lut]	[Neo]	[Neo+Vio]	[Phytin *a*]	[Phide *a*]	[Pras]	[Viola]	[Zea+Lut]
C	4.7	10.7	20.1	**0.7**	**0.1**	**0.1**	**59.5**		1.7	**0.4**	**0.7**
D	4.4	13.8	68.3	5.4	4.5	4.2			0.6	3.7	10.7
G	2.8	1.3		1.1	8.1	6.3	188.3		0.9	3.7	1.8
H	**1.9**	**0.7**	**1.5**	3.5	2.1	2.7	63.2	75.3	**0.1**	3.6	6.1
L	4.4	2.5	10.1	3.7	5.6	4.9	65.6	75.3	1.9	4.0	3.5
F	30.3		30.5	33.2	33.2	22.0			45.3	6.3	9.1
J	14.7	6.9	25.8	117.8	2.0	5.5	63.0		6.6	10.5	64.1
N	54.1		3.6	99.9					1.8	9.7	99.9
S	3.5	10.3†	33.7	4.6					14.3	3.0	6.1
U	42.6			1.8					39.4	0.8	4.0
A′	3.6	5.8	25.0	2.9	4.1	3.6	94.1	75.3	1.0	3.1	4.6
A^+	29.0	8.6	23.4	51.5	15.3	11.3	63.0		21.5	6.0	36.6
A^-	1.9	0.7	1.5	0.7	0.1	0.1	59.5	75.3	0.1	0.4	0.7

† Derived from a simultaneous equation (Latasa et al. 1996) and provided for information purposes only.

Table 19. The $|\bar{\psi}|$ values (average APD in percent) across all 12 batches of field samples as a function of the laboratory method for the pigment sums (following the presentation scheme established in Tables 12, 13, and 15). The PPig $|\bar{\psi}|$ values for field samples (Table 15) are given in the first column for easy comparison. Method averages for the pigment sums are given in the last column.

Meth.	[PPig]	[TChl]	[PPC]	[PSC]	[PSP]	[TCaro]	[TAcc]	[TPig]	[DP]	*Avg.*
C	33.5	4.8	10.1	13.6	4.3	11.7	4.7	4.2	10.3	8.0
D	29.5	**4.5**	4.2	9.2	**3.1**	6.2	6.0	**3.1**	8.6	5.6
G	27.5	6.8	**3.3**	7.0	6.7	**4.8**	**4.0**	5.9	**5.5**	**5.5**
H	34.6	6.8	7.8	9.1	6.8	9.0	7.2	6.8	7.0	7.6
L	**23.9**	7.7	5.7	**4.9**	6.3	5.7	5.5	6.2	6.4	6.0
L′	*27.1*	*8.4*	*6.5*	*4.9*	*6.9*	*5.2*	*5.8*	*6.7*	*6.3*	*6.3*
F	3,194	25.3	336.5	32.0	16.6	112.1	67.2	14.6	16.1	77.5
J	63.6	18.3	15.8	16.3	18.3	17.4	11.6	18.0	11.9	15.9
N	176.9	7.0	14.7	18.6	7.2	12.8	10.0	7.3	14.7	11.5
S	530.9	33.2	30.3	12.4	29.3	18.7	21.3	28.8	15.5	23.7
U	172.6	117.7	107.6	26.8	97.9	52.9	241.0	98.8	17.7	95.0
A′	29.8	6.1	6.2	8.8	5.4	7.5	5.5	5.2	7.6	6.5
A^+	827.6	40.3	101.0	21.2	33.8	42.8	70.2	33.5	15.2	44.7
A^-	20.7	4.5	3.3	4.9	3.1	4.8	4.0	3.1	5.5	4.2

The uncertainties in the pigment sums for the field samples are presented in Table 19. The overall averages from the QA subset show a decrease in average uncertainties from PPig to pigment sums of 29.8% to 6.5%. The range of the uncertainties within the individual sums is frequently rather small, with the exception of some of the *C* results. The uncertainties in the individual pigment sums are usually less than 8%, as are the overall averages for the individual sums or laboratories, which is the performance level for quantitative analysis. The range of the individual averages are rather similar (they differ by a maximum of only 2.5%). The *best method* results are spread across three of the QA methods with *G* predominating. The difference between the overall A^- and A' averages is 1.3%.

The A^+ subset shows a more dramatic decrease in uncertainties from the overall PPig average of 827.6% to 44.7%. The individual method decreases are also large with *F* decreasing the most. Such substantial decreases establish the utility of using sums in databases to minimize uncertainties, particularly if the source data has an unknown quality, but some caution is needed: with the exception of the *N* method, the overall averages are not to within 12%, the threshold for semiquantitative analysis (Table 9). In addition, many of the individual uncertainties and laboratory averages exceed 20%, the threshold for routine analysis. For uncertainties with sums, the latter is an indication of significant biases in the method. For example, the problems with the individual carotenoids for

Table 20. The $|\bar{\psi}|$ values (average APD in percent) across all 12 batches of field samples as a function of the laboratory method for the pigment ratios and indices (following the presentation scheme established in Tables 12, 13, and 15).

Lab. Method	$\frac{[\text{TAcc}]}{[\text{TChl } a]}$	$\frac{[\text{PSC}]}{[\text{TCaro}]}$	$\frac{[\text{PPC}]}{[\text{TCaro}]}$	$\frac{[\text{TChl}]}{[\text{TCaro}]}$	$\frac{[\text{PPC}]}{[\text{TPig}]}$	$\frac{[\text{PSP}]}{[\text{TPig}]}$	$\frac{[\text{TChl } a]}{[\text{TPig}]}$	*Avg. Ratio*	[pPF]	[nPF]	[mPF]	*Avg. Index*
C	4.1	2.4	4.6	15.3	9.8	0.9	1.6	5.5	14.4	10.7	5.4	10.2
D	9.3	3.3	4.9	7.2	4.5	0.4	3.6	4.7	5.9	9.6	2.2	5.9
G	6.6	3.0	5.1	6.6	8.0	0.9	2.6	4.7	**5.7**	8.6	**1.9**	**5.4**
H	**3.6**	**1.3**	2.6	**5.5**	**3.1**	0.3	**1.4**	**2.5**	8.4	9.6	2.9	6.9
L	4.9	1.9	**2.3**	7.2	4.0	**0.3**	1.9	3.2	12.2	**8.1**	4.9	8.4
L'	5.7	3.1	4.2	7.7	5.4	0.5	2.3	4.1	14.7	11.6	5.0	10.4
F	125.7	40.4	105.9	63.8	283.6	24.7	31.6	96.5	22.3	91.2	16.9	43.5
J	12.6	2.8	4.5	6.9	4.8	0.5	5.3	5.3	23.8	29.9	7.4	20.4
N	9.5	7.1	12.1	13.0	13.0	1.3	3.7	8.5	12.9	13.9	6.4	11.1
S	11.4	6.2	10.9	16.1	10.9	1.4	5.0	8.8	13.6	47.4	9.4	23.5
U	220.2	17.0	36.9	49.0	11.5	1.1	46.1	54.5	12.7	31.0	9.3	17.7
A'	5.7	2.4	3.9	8.4	5.9	0.6	2.2	4.1	9.3	9.3	3.5	7.4
A^+	75.9	14.7	34.1	29.7	64.8	5.8	18.3	34.8	17.1	42.7	9.9	23.2
A^-	3.6	1.3	2.3	5.5	3.1	0.3	1.4	2.5	5.7	8.1	1.9	5.3

the F method produce anomalously large uncertainties for PPC and TCaro, and the large uncertainties in TChl c results in a large TChl uncertainty.

1.6.3.3 Pigment Ratios and Indices

The aforementioned functional relationship in uncertainties established by prior SeaHARRE activities extended beyond pigment sums and into higher-order data products: *PPig overall uncertainties decreased as individual pigments were summed, decreased further when pigment ratios were formed, and then increased slightly with the formation of pigment indices.* The latter are a combination of sums and ratios (Table 6), so the small increase is expected to be on the order of the uncertainty in pigment sums (or less).

The uncertainties in pigment ratios and indices for the SeaHARRE-4 field samples are presented in Table 20. The QA subset has overall average uncertainties of 4.1% and 7.4% for the ratios and indices, respectively. Both results are to within their respective anticipated performance thresholds for quantitative analysis, which are to within 6% and 8%, respectively (Table 9). The individual A' method uncertainties for the ratios are almost always within performance limits and span a narrow range. The notable exception are the C results for TChl/TCaro and PPC/TPig, which exceed the 6% threshold. The individual method uncertainties for the indices are quite similar and are within, or close to, expectations, except for the pPF results for C and L.

The uncertainties in the A^+ pigment ratios and indices provide additional clarity about the unique problems with the methods. As a group, the overall average A^+ uncertainties for ratios and indices are much higher than the corresponding QA subset values, particularly for the ratios, and the values are above the performance thresholds even for routine analysis. Although the overall average decreases in the progression from PPig, to sums, to ratios, it does not increase with the ratios. This deviation from the expected functional form of the uncertainties has been an indicator of methodological (Hooker et al. 2009) or sampling problems (Hooker et al. 2005) in the past.

The individual J, N, and S overall averages do not exhibit a deviation from the expected functional form of the uncertainties—they behave as expected. The average uncertainties for the ratios puts J at a quantitative level of performance, while N and S are at the semiquantitative level; for the indices, J and S are close to the routine performance level, while N is at the semiquantitative level. The F method never satisfies a performance threshold—even for the PSP/TPig ratio, which is one of the least sensitive ratios. The U does satisfy some performance levels for some ratios and indices, and has an overall average uncertainty for the indices that is at the routine level of performance.

The more abundant a pigment is naturally, the more important it is to the net uncertainty of a ratio or index, but not in all cases. As noted in prior SeaHARRE activities, problematic pigments can elevate uncertainties in ratios. This is particularly notable if the problematic pigment or pigments appear exclusively in the numerator or the denominator. If the sources of uncertainty appear in both the numerator and denominator, uncertainties can increase, decrease, or be nullified depending on the biases

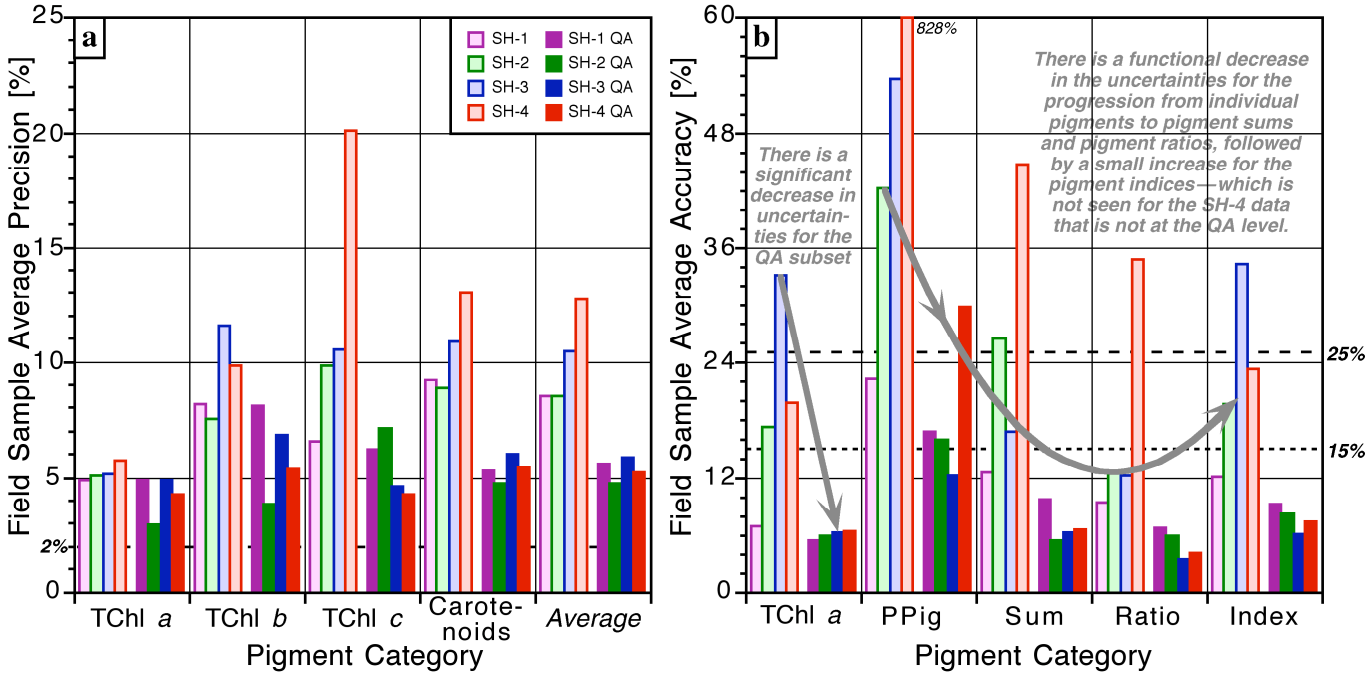

Fig. 2. Overall performance results for the A^+ and A' (QA) subsets (light and dark bars, respectively) for all four SeaHARRE (SH) activities: **a)** method precision, with the approximate 2% variance associated with the preparation of the field samples shown as a dotted line, and **b)** accuracy, with the 15% quantitative and 25% semiquantitative performance metrics for PPig shown as dotted and dashed lines, respectively.

the uncertainties represent (i.e., overestimation versus underestimation) and the magnitudes involved.

1.7 SUMMARY AND DISCUSSION

The diversity of objectives for the SeaHARRE-4 activity, along with the large number of separate analyses involved, make it appealing to summarize and discuss the most important aspects of the results in one place. The discussion presented here is not intended to be a substitute for reading the greater detail presented in the preceding sections, and follows the original presentation of the objectives (Sect. 1.1) and much of the organizational scheme used to present the results (Sect. 1.6).

1.7.1 Precision

A recurring aspect of the results presented here has been to discuss the importance of a proper referencing system for establishing method uncertainties. This has been emphasized, because in round robins involving natural samples, there is no *a priori* understanding of truth. A foundation of the SeaHARRE activity is that truth can be estimated by assuming a properly validated HPLC method is capable of providing a good estimate of truth, and the deviations from truth are mostly due to small sources of random errors, so the pooled estimate of truth from several methods should tend towards a realistic proxy value of truth. The potential pitfall in this logic is if a method that has not been properly validated is included in the methods used to estimate the referencing system. There is no evidence in the results presented that the QA subset was corrupted by an improperly validated method.

Consequently, an important attribute of the subset of methods used as the referencing system for computing uncertainties is that they have rather uniform results across the broadest suite of data products possible. The standard deviation in the uncertainties within the QA subset averaged 1.4% for TChl *a*, 10.5% for PPig, 1.1% for pigment sums, 1.2% for pigment ratios, and 1.9% for pigment indices. The corresponding values for the A^+ subset were 11.0%, 1,551.1%, 38.7%, 40.1%, and 12.2%, respectively. The latter represents approximately a 6- to 150-fold increase in variance with respect to the former, and is one of the distinctive mechanisms for showing the difference between the two subsets. This is not to say all methods within the A^+ subset are associated with the same levels of variance. As has already been shown, there are numerous examples of excellent results from a subset of A^+ methods and for a subset or variety of pigment data products.

The precision of the individual methods is another important indicator of the distinct differences between the A' and A^+ subsets. Figure 2a shows the average precision of the two subsets as a function of the primary pigments grouped as three chlorophylls, the nine carotenoids, and the overall average. To establish a wider context, summary data are shown for all four SeaHARRE activities executed to-date. The precision of the A' subset for SeaHARRE-4

is largely indistinguishable from the first three activities. The SeaHARRE-4 A^+ results are characterized by being more imprecise on average and for all cases shown except TChl *b*.

There are examples for which the two subsets in Fig. 2a have similar precision, for example, the TChl *a* results are not widely different. The majority of the data, however, shows the QA subset has distinctly better precision, and the overall A' average is about 4.7% better on average than the A^+ average. The latter represents almost the entire performance budget for the PPig precision metric for quantitative analysis (5%). Given that precision is the first step in the uncertainty budget, it also represents one-third of the PPig accuracy metric for quantitative analysis, so it is a significant difference.

1.7.2 Accuracy

The most significant difference between the A' and A^+ subsets is in the accuracy (uncertainty) of the derived data products, which is shown in Fig. 2b for all four SeaHARRE activities. A substantial decrease in uncertainties from the A^+ to A' subset is seen in all data products, but very notably for the individual pigments (the largest decrease is for the SeaHARRE-4 PPig results). In fact, the QA methods are within the approximately 2% sampling variance for the 10% and 15% quantitative analysis requirements for TChl *a* and PPig, except for SeaHARRE-4, which are a bit above this threshold. The A^+ subset frequently does not satisfy the TChl *a* and PPig semiquantitative thresholds (15% and 25%, respectively), particularly for the SeaHARRE-3 and SeaHARRE-4 results.

With the exception of the PPig results obtained during SeaHARRE-4, the A' accuracies for all pigment categories within each SeaHARRE activity are rather similar. The closest agreement is seen for TChl *a*, which is important, because it is currently the most significant pigment for ocean color remote sensing. The A^+ results are typified by much greater variability with each pigment category—even for TChl *a*.

The most troubling aspect of the SeaHARRE-4 A^+ results is the absence of the established functional form of the uncertainties—that is, the decrease in uncertainties from PPig to sums to ratios, followed by a small increase with the indices—is seen in all past SeaHARRE activities, but not in SeaHARRE-4. Prior SeaHARRE investigations suggested this represents a corruption of the natural relationships between the pigments (Hooker et al. 2005 and 2009). A likely explanation for the corruption is the presence of significant biases in one or more of the pigment data products, which can occur if a method is put into service without proper validation or if a quality assurance plan is not implemented and rigorously applied over time.

As already noted, the four SeaHARRE activities executed to-date span a wide dynamic range in trophic systems, e.g., the TChl *a* concentration range is approximately 0.020–42.704 $\mathrm{mg\,m^{-3}}$, which represents a little more than four decades in concentration. The sampling for the first three activities involved oceanic regimes emphasizing the mesotrophic Mediterranean Sea, the eutrophic Benguela Current, and the oligotrophic South Pacific gyre, respectively. The SeaHARRE-4 activity emphasized eutrophic Danish coastal waters (fjords and estuaries). Note the international extent of the sampling was conducted.

It is also important to remember that the composition of each SeaHARRE activity changes over time as the overall objectives evolve. The largest number of new analysts participating in a round robin occurred during the SeaHARRE-2 and SeaHARRE-4 exercises, and the greatest diversity in the individual methods occurred during SeaHARRE-2 (Table 2). Some qualifications regarding the results need to be remembered, because the largest variances in the A^+ results frequently come from a minority of methods. For some activities, this was caused by known hardware problems, but in other cases it was simply caused by methods whose performance was compromised by unknown or improperly investigated problems that led to degraded data products.

Additional details regarding the uncertainty in individual primary pigments for the A' and A^+ subsets across all four SeaHARRE activities are presented in Table 21, and within the perspective of a generalized overview, some notable results are as follows:

- The lowest uncertainty for both the A' and A^+ subsets is for TChl *a*, which is largely invariant to water type for the A' data.
- The only carotenoid with an uncertainty to within state-of-the-art PPig performance (to within 10%), is Fuco for the A' subset, and it is also largely invariant to water type.
- The highest uncertainty for both the A' and A^+ subsets is for But, which has a lot of variability within each subset.
- In between the highest and lowest uncertainty for both the A' and A^+ subsets, Diato, Peri, Hex, and TChl *c* emerge as problematic pigments.
- The ranking within the chlorophyll uncertainties is the same for the A' and A^+ subsets; the lowest uncertainty is for TChl *a*, followed by TChl *b*, and TChl *c*.
- The lowest average uncertainties for the A' and A^+ subsets are associated with oligotrophic and mesotrophic waters, respectively. The latter corresponds to SeaHARRE-1, which had the fewest number of participants and the closest agreement between the A' and A^+ classifications.
- The highest average uncertainties for both the A' and A^+ subsets are associated with coastal waters.

Table 21. The $|\bar{\psi}|$ values (average APD in percent) for field samples across all four SeaHARRE activities for the primary pigments following the presentation scheme established in Tables 12, 13, and 15. The lowest individual pigment uncertainties across the four SeaHARRE activities and within the A' subset are shown in bold typeface and summarized in the A^- entries.

Meth.	[TChl *a*]	[TChl *b*]	[TChl *c*]	[Caro]	[But]	[Hex]	[Allo]	[Diad]	[Diato]	[Fuco]	[Peri]	[Zea]	*Avg.*
A' SH-1	7.0	14.0	26.5	17.6	23.6	24.8	38.9	16.0	55.8	8.8	**13.0**	11.4	21.5
A' SH-2	**5.9**	16.5	21.8	16.8	30.6	9.5	20.3	8.7	20.6	**4.7**	15.4	21.4	16.0
A' SH-3	6.3	13.9	**14.9**	13.2	**14.6**	**6.0**	**4.2**	**5.0**	**17.8**	10.6	30.5	**9.6**	**12.2**
A' SH-4	6.4	**12.4**	20.4	**9.6**	87.2	57.5	13.0	9.3	49.8	6.6	66.6	19.0	29.8
A^+ SH-1	7.9	18.0	32.7	20.2	29.5	24.8	38.9	24.9	59.0	11.5	32.3	21.5	26.8
A^+ SH-2	17.2	20.8	25.6	23.5	63.7	40.4	95.4	30.5	49.3	39.1	56.9	44.3	42.2
A^+ SH-3	33.1	36.8	22.4	24.3	112.6	31.1	111.5	22.0	64.3	58.7	112.1	13.7	53.6
A^+ SH-4	19.8	19.0	288.2	345.9	8,078	843.6	24.8	20.7	62.0	20.1	119.8	89.2	827.6
A' Avg.	6.4	14.2	20.9	14.3	39.0	24.5	19.1	9.7	36.0	7.7	31.4	15.3	19.9
A^+ Avg.	19.5	23.7	92.2	103.5	2,071	235.0	67.6	24.5	58.7	32.4	80.3	42.2	237.5
A^-	5.9	12.4	14.9	9.6	14.6	6.0	4.2	5.0	17.8	4.7	13.0	9.6	9.8

- The lowest individual pigment uncertainties within the A' subset are spread across all four SeaHARRE activities, but SeaHARRE-3 predominates with the greatest number of best results.
- The lowest individual pigment uncertainties within the A' subset and across the four SeaHARRE activities establish a *best method* average of 9.8%, which is within the state-of-the-art performance metric for PPig accuracy (Table 9).
- The problematic pigments within the A^- results are almost exactly the same as noted above using the A' and A^+ joint results; the difference is the exclusion of Hex and the inclusion of TChl *b*.
- The difference between the A^- overall average uncertainty (9.8%) and the best A' overall result for an individual SeaHARRE activity (12.2%) is within the 2–3% sampling variability that has been estimated for each round robin.

The importance of some of these findings are discussed in the following sections.

1.7.3 DHI Mix-102

The concept of using the natural relationships between pigments to discern problems with a method is difficult to apply to the DHI Mix-102 results, which are derived from an artificial mixing of pigments from cultured stocks. Nonetheless, the reduction in uncertainties as pigments are summed and then ratioed should occur, and the uncertainties should be considerably lower than in natural samples because of the (mostly) artificially high abundance of the pigments in the mix, plus V_x and V_f do not interfere, i.e., the 15% threshold should always be satisfied and a QA method should have higher-order uncertainties on the order of 5% or less.

A summary of the average uncertainties for the A' and A^+ results for DHI Mix-102 is presented in Table 22. The results for the QA subset satisfy the expected performance thresholds and exhibit a decreasing relationship similar to the functional form presented in Fig. 2b, and includes a small increase in uncertainties associated with the indices. The lowest values are close to the precision of the individual methods, which have an average value of approximately 0.9% for the DHI mix, so the ability to distinguish them further is not really possible.

Table 22. The average uncertainties in percent for DHI Mix-102 as a function of the A' and A^+ subsets.

Subset	*PPig*	*Sums*	*Ratios*	*Indices*
A'	4.6	2.8	2.5	3.1
A^+	41.8	25.0	23.9	8.3

The A^+ subset has an average precision for the DHI Mix of about 3.2%, so there are no resolution issues for the entries in Table 22. With the exception of the results for the indices, the A^+ subset results a) exceed the 15% threshold; and b) do not follow the expected functional form, both in terms of the relationships and the magnitudes. Uncertainties do decrease in the progression from the primary pigments to the pigment ratios, but do not increase for the pigment indices. The magnitude and aberrant functional form of the uncertainties are further evidence of one or more corrupted methods within the A^+ subset.

1.7.4 Ocean Color Requirements

The invariance of [TChl *a*] uncertainties to water type for the A' subset and the fact that it has the lowest overall uncertainty for both the A' and A^+ subsets are important results for the remote sensing problem set. Equally important is the average uncertainty for the A' and A^+ subsets are less than 15 and 25%, respectively. This implies

much of the worldwide contributions of $[\mathrm{TChl}\,a]$ values are, on average, compliant with the SeaWiFS calibration and validation specification, and contributions from QA laboratories can probably be used in algorithm refinement exercises.

The difficulty with the above compliance statement is the range of variance that has been established for methods that are not part of the QA subset can be significant, and most algorithm validation work—particularly in the early stages of validating a new algorithm—proceed more effectively with higher-quality data. The same is true for the very important work of first establishing a new algorithm. Being able to partition databases between A' and A^+ contributions would make establishing and refining an algorithm easier to do.

1.7.5 Performance Metrics

The participation of laboratory G was an experiment to demonstrate what a laboratory that had never done HPLC analysis was capable of achieving if the Van Heukelem and Thomas (2001) protocols and the performance metrics for quantitative analysis were strictly followed. The analyst in this case was an established biogeochemist who had never done an HPLC analysis, but who was familiar with the concept of following *best practices* in the laboratory. The fact that the G results were in the A' subset and were repeatedly some of the best results within that subset demonstrates how adherence to protocols and performance metrics can result in significantly lower uncertainties in the production of data products from field samples, even for novice practitioners. It also shows the performance metrics are achievable and realistically set.

1.7.6 Conclusions and Recommendations

The SeaHARRE-4 activity was designed to investigate the following objectives: a) estimate the uncertainties in quantitating individual pigments and higher-order variables formed from sums and ratios; b) confirm if the chlorophyll *a* accuracy requirements for ocean color validation activities (approximately 25%, although 15% would allow for algorithm refinement) can be met in coastal waters; c) establish the reduction in uncertainties as a result of applying QA procedures; d) show the importance of establishing a properly defined referencing system in the computation of uncertainties; e) quantify the analytical benefits of performance metrics; and f) demonstrate the utility of a laboratory mix in understanding method performance. All of these objectives were satisfied and the most salient aspects involved are as follows:

- Estimates of the uncertainties in quantitating individual pigments and higher-order variables formed from sums and ratios show the latter provide a significant opportunity to reduce uncertainties in larger databases (Fig. 2). In addition, the results showed that the use of histopreps for sample storage does not produce a significant degradation in the uncertainties (or precision) with respect to foil packet storage. In fact, some of the most superior results were obtained for the histoprep samples.
- The chlorophyll *a* accuracy requirements for ocean color validation activities are significantly satisfied in coastal waters, but some additional investigation is needed to reduce uncertainties when detection by absorption spectra is sufficiently degraded that analysts cannot reasonably be expected to make similar choices as to the presence or absence of a pigment (the so-called *two-sentence rule*† is a starting point for this work).
- The reduction in uncertainties as a result of applying QA procedures is demonstrated by the significantly lower uncertainties associated with the QA subset across all four SeaHARRE activities executed to-date (Table 21).
- The importance of establishing a properly defined referencing system in the computation of uncertainties is revealed by the large difference between the A' and A^+ subsets, because if the partition had not been properly defined, the larger variance of the A^+ subset (most notably F and S) would have been spread across all the methods.
- The analytical benefits of the performance metrics are shown by the excellent results achieved with method G (Tables 15–20), which involved a first-time HPLC analyst that was guided primarily by strict adherence to the performance metrics.
- The utility of the laboratory mix was demonstrated by how the differences between the A' and A^+ subsets are still seen in the average uncertainties for DHI Mix-102 (Table 22) even though the mix is significantly easier to analyze than a field sample (no extraction procedures).

The SeaHARRE-4 results were more difficult to integrate, because they contained more unknown pigments than prior SeaHARRE activities. This was probably due to the stations being in eutrophic and estuarine environments. Frequently, the content in the samples were most likely a mixture of freshwater and marine algae, which means they were more complicated to analyze than samples from high salinity areas. By itself this is a challenging analysis scenario, but for an analyst who is not used to these samples, it is doubly challenging, because interpretation is usually more reliable if experience is also a factor. Consequently, a likely reason for the QA subset increase in uncertainties for SeaHARRE-4 was having so many analysts confronting such a complex sample set for the first time.

† The two-sentence rule is as follows (Hooker et al. 2009): If a peak is good and it can be proved to not be the pigment for that retention time, do not report it; otherwise report it. If a peak is bad and it cannot be disproved to be the correct pigment, report it; otherwise do not report it.

Chapter 2

The CSIRO Method

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Abstract

The CSIRO method is a modified version of the Van Heukelem and Thomas (2001) method (VHT) and has the capacity to resolve approximately 35 different pigments with baseline resolutions of divinyl and monovinyl chlorophyll *a*, and zeaxanthin and lutein. Partial separation of divinyl and monovinyl chlorophyll *b*, and chlorophyll c_2 and chlorophyll c_1 can also be achieved. The method used for the samples and standards analyzed for SeaHARRE-4 was the same method used for SeaHARRE-3. Samples were extracted over 15–18 hours in an acetone solution before analysis by HPLC using a C_8 column and binary gradient system with an elevated column temperature. Pigments were identified by retention time and absorption spectrum from a photodiode array (PDA) detector. The method is regularly validated with the use of internal and external standards and individual pigment calibration. The detection limit of most pigments was within the range of 0.001–0.005 mg m^{-3}. This method is applicable to the study of pigment composition and concentration in samples from all water types including freshwater, estuarine, upwelling coastal regions, and oligotrophic open ocean, as well as in the microphytobenthos of shallow coastal regions.

2.1 INTRODUCTION

For SeaHARRE-4, CSIRO was asked to analyze the samples to determine the total particulate absorption coefficient, the pigment composition and concentration by HPLC, and the spectrophotometric total chlorophyll *a* concentration. On 6 December 2006, 12 triplicate samples were received, flat in histopreps (Fisher), together with 10 vials of DHI mixed standards on dry ice. The samples were immediately transferred to liquid nitrogen for storage until the start of HPLC analysis. The standards were stored in a −20°C freezer.

Because only one sample was received for three different analyses, the samples were analyzed in sets of 12 (four groups of triplicate samples). All of the HPLC analyses were completed from 26–30 March 2007, inclusive. The samples were removed from liquid nitrogen and stored on ice in the dark to thaw. Once thawed, an ultraviolet–visible (UV/Vis) spectrophotometer, equipped with an integrating sphere, was used to scan the samples from 200–900 nm. These measurements were used to determine the optical density of the total particulate matter on each filter. The filters were then extracted for pigment analysis. The final extracts were measured at four wavelengths in a UV/Vis spectrophotometer to determine the total chlorophyll *a* concentration before analysis by HPLC to determine pigment concentration for a wide variety of pigments.

2.2 METHODS

Three different methods were used by CSIRO to complete the SeaHARRE-4 work plan: a) spectral absorption, b) spectrophotometric chlorophyll *a*, and c) HPLC pigment analysis. Brief summaries of the first two are presented in the following two sections. The remainder of the material that is presented deals with the HPLC pigment analysis method.

2.2.1 Spectral Absorption

Optical density spectra for total particulate matter were obtained using a GBC 916 UV/Vis dual beam spectrophotometer equipped with an integrating sphere. Quartz glass plates were used to hold the sample and blank filters against the integrating sphere. The optical density of the total particulate matter of each sample was obtained using a blank filter as a reference (from the same batch number as the sample filters) wetted with filtered seawater (0.2 µm) and scanned from 200–900 nm with a spectral resolution of 1.3 nm. The optical density scans were converted to absorption spectra by first normalizing the scans to zero at 750 nm and then correcting for the path length amplification using the coefficients of Mitchell (1990). Particulate spectra were smoothed using a running box-car filter with a width of 10 nm.

2.2.2 Spectrophotometric Chlorophyll *a*

The absorbance of the pigment extract (see details in the next section) was measured using a GBC 916 UV/Vis spectrophotometer with 40 mm path length optical glass microcells. Absorbance was read at wavelengths 750, 664, 647, and 630 nm. The absorbance at 750 nm was subtracted from the absorbance at each of the other three wavelengths and substituted into the equations of Jeffrey and Humphrey (1975).

$$\left[\text{Chl } a\right]_e = \frac{11.85A_{664} - 1.54A_{647} - 0.08A_{630}}{l_c}, \quad (27)$$

$$\left[\text{Chl } b\right]_e = \frac{21.03A_{647} - 5.43A_{664} - 2.66A_{630}}{l_c}, \quad (28)$$

and

$$\left[\text{Chl } c\right]_e = \frac{24.52A_{630} - 1.67A_{664} - 7.60A_{647}}{l_c}, \quad (29)$$

where e indicates the extract, A is the corrected absorbance, and l_c is the path length of the cuvette in centimeters.

The concentration of each chlorophyll pigment in the sample in micrograms per liter was obtained by the following equation:

$$\left[\text{Chl } x\right]_s = \frac{V_e}{V_f}\left[\text{Chl } x\right]_e, \quad (30)$$

where s indicates the sample, V_e is the volume of extract in milliliters, and V_f is the volume of seawater filtered in liters.

2.2.3 HPLC Pigment Analysis

The CSIRO method separated pigments on a C_8 column using a two-solvent gradient system. Pigments were verified by the retention time and absorption spectra (using a PDA detector) of each chromatographic peak and quantified by the detector signal at 436 nm. Analysis time was 31 min per sample with a further 5 min injection delay to ensure no carryover between samples. Separation was achieved for most pigments, the exceptions being $\beta\beta$- and $\beta\varepsilon$-carotene that coelute. In samples where one of the two carotenes is dominant, however, the two pigments can be separated by their absorption spectra.

Additional separation problems routinely occurred between chlorophyll c_2, MgDVP, chlorophyll c_1, and chlorophyllide *a*, for which baseline resolution cannot always be achieved. It is not common for all four pigments to be present in any one particular chromatogram, however, thereby allowing for good resolution between chlorophyll c_2 and chlorophyll c_1, and partial resolution between chlorophyll c_1 and chlorophyllide *a* when both were present. While baseline resolution of divinyl and monovinyl chlorophyll *a* was achieved, only partial separation of divinyl and monovinyl chlorophyll *b* can be achieved.

2.3 EXTRACTION

To extract the pigments, the thawed filters were cut into three or four pieces and covered with 100% acetone (3 mL) in a 10 mL centrifuge tube. Scissor and forcep blades were cleaned between samples. The tube was covered with parafilm and vortexed for about 30 s, followed by sonication for 15 min in an ice-water bath in the dark. The samples were then kept in the dark at 4°C for approximately 15 h. After this time, 200 µL water was added to the acetone such that the extract mixture was 90:10 acetone:water (vol:vol), including the water held in the filter from the sample filtering process, and sonicated once more for 15 min in an ice-water bath in the dark.

The extract was transferred to a Biorad column, (a small column containing a scintered glass disc) sitting in a clean centrifuge tube. The original centrifuge tube was rinsed twice with 0.5 mL 90:10 acetone:water, with the rinses added to the column. The column and centrifuge tube were centrifuged for 5 min at 2,500 rpm and −2°C to separate the filter paper from the extract. At this stage, the final extract volumes were recorded from the centrifuge tube graduations.

The centrifuged extracts were passed through a 0.2 µm Teflon syringe filter (Advantec), which had been rinsed with acetone and air, directly into a 2 mL amber HPLC vial. The remaining extract from each sample remained in a centrifuge tube, covered with parafilm, and was stored at −20°C until the HPLC analysis had been successfully completed.

2.4 HPLC ANALYSIS

The CSIRO hardware was based on a Waters–Alliance HPLC system, comprising a 2695XE separations module with a column heater and a refrigerated autosampler, plus a model 2996 PDA. The column temperature was 55°C and the refrigerated autosampler was set to 4°C.

Immediately prior to injection, the sample extract was mixed with a buffer solution (90:10 28 mM aqueous tetrabutyl ammonium acetate, TbAA, pH 6.5:methanol) within the sample loop. The injector was programmed by the software to draw up alternating microliter volumes of buffer and sample in the following order: 150, 75, 75, 75, and 150 starting with the buffer.

After injection, pigments were separated using a Zorbax Eclipse XDB C_8 stainless steel 150×4.6 mm internal diameter (ID) column with 3.5 µm particle size (Agilent Technologies) and a gradient elution procedure as shown in Table 23. The gradient was held in an isocratic mode from 11–15 min to improve the resolution between violaxanthin and 19′-hexanoyloxyfucoxanthin. The flow rate was 1.1 mL min^{-1}. The separated pigments were detected at 436 nm and identified against standard spectra using Waters `Empower` software. Concentrations of chlorophyll *a*, chlorophyll *b*, and $\beta\beta$-carotene in sample chromatograms

were determined from Sigma-Aldrich (St. Louis, Missouri) standards, while all other pigment concentrations were determined from DHI standards (Hørsholm, Denmark). The analysis time of each sample was 31 min with an additional 5 min injection delay of the next sample to ensure there is no carryover between samples.

Table 23. The gradient elution program for the CSIRO laboratory method as executed during the SeaHARRE-4 activity. Solvent A is 70:30 28 mM TbAA (pH 6.5):methanol, and solvent B is 100% methanol.

Time [min]	A [%]	B [%]
0	95	5
11	45	55
15	45	55
22	5	95
29	5	95
31	95	5

Peak integration and identification was initially performed by the automated features of Waters `Empower` software, which produces an electronic report. Each sample was manually inspected for correct integration markers and identification of pigments. For a few samples, where the pigment concentration was very low, baselines were corrected manually to optimize integration.

2.5 CALIBRATION

As noted earlier, calibration standards of chlorophyll *a*, chlorophyll *b*, and $\beta\beta$-carotene were obtained from Sigma-Aldrich, while all other pigment standards were obtained from DHI. Standards were not available for all pigments reported. CSIRO did not have standards for MgDVP and chlorophyll c_1.

The concentration of all standard stock solutions was determined using a GBC 916 UV/Vis dual-beam spectrophotometer with a 2 nm bandwidth. An absorption spectrum of each pigment was recorded from 350–900 nm. From these stock solutions, a series of 4–6 standard solutions were prepared and analyzed both spectrophotometrically and by HPLC. Calibration curves were obtained with coefficient of determination, r^2, values never less than 0.99 and response factors (RF) for each pigment were determined from these calibration curves.

The concentration of each pigment was calculated using the absorption coefficient from the established literature (Jeffrey et al. 1997b) together with the absorption measured at the corresponding wavelength. Absorption coefficients, wavelengths, and solvents used for each pigment are listed in Table 24. The absorbance at the wavelength used, nominally the wavelength of maximum absorbance, is corrected for any absorption measured at 750 nm.

Table 24. Absorption coefficient (α) values in liters per gram per centimeter used with the CSIRO method for the pigments listed as a function of wavelength (λ). The rightmost column provides the literature reference.

Pigment	*Solvent*	λ	α	*Ref.*
Peri	100% Ethanol	472.0	4.00	†
But	100% Ethanol	446.0	160.00	§
Fuco	100% Ethanol	449.0	160.00	§
Hex	100% Ethanol	447.0	160.00	§
Neo	100% Ethanol	439.0	224.30	§
Pras	100% Ethanol	454.0	160.00	§
Viola	100% Ethanol	443.0	255.00	§
Diad	100% Ethanol	446.0	262.00	§
Allo	100% Ethanol	453.0	262.00	§
Diato	100% Ethanol	449.0	262.00	§
Lut	100% Ethanol	445.0	255.00	§
Zea	100% Ethanol	450.0	254.00	§
Cantha	100% Ethanol	476.0	207.50	§
Gyro. diester	100% Ethanol	445.0	262.00	§
Asta	100% Acetone	482.0	210.00	†
$\beta\varepsilon$-Car	100% Acetone	448.0	270.00	§
$\beta\beta$-Car	100% Acetone	454.0	250.00	†
Chlide *a*	90% Acetone	664.0	127.00	§
Chl *b*	90% Acetone	647.0	51.36	†
DVChl *a*	90% Acetone	664.3	87.67	§
MVChl *a*	90% Acetone	664.0	87.67	†
Chl c_3	90% Acetone	453.0	346.00	§
Chl c_2	90% Acetone	443.8	374.00	§
Phytin *b*	90% Acetone	657.0	31.80	†
Phytin *a*	90% Acetone	667.0	51.20	†
Phide *a*	90% Acetone	667.0	74.20	§

† Jeffrey et al. (1997b) § DHI (Hørsholm, Denmark)

2.6 VALIDATION

At the start of every set of samples analyzed by HPLC, a pigment mixture was qualitatively analyzed to determine if there was any movement in the retention time of approximately 30 pigments. A mixture of known concentrations of astaxanthin, chlorophyll *a*, chlorophyll *b*, and $\beta\beta$-carotene was also analyzed to determine that the HPLC system, including the column, was working appropriately. Between 7 March and 1 May 2007, this pigment mixture was analyzed 20 times yielding a precision (percent CV) of 1.47, 1.84, 1.18, and 1.74 for astaxanthin, chlorophyll *a*, chlorophyll *b*, and $\beta\beta$-carotene respectively. Multipoint calibrations of chlorophyll *a*, chlorophyll *b*, and $\beta\beta$-carotene are done approximately every three months, while calibrations of a selection of other pigments are done approximately once every 12–18 months.

In the case of the SeaHARRE-4 experiment, a mixed pigment standard, supplied by DHI (Denmark) was analyzed three times within a set of 12 samples analyzed, resulting in a total of nine injections. The precision (percent

CV) of the nine injections was <1.0 for all major pigments and many of the minor pigments.

2.7 CARRYOVER

After having analyzed several thousand field samples, carryover between the samples had not been seen. However, the concentration of the pigments in the DHI-supplied mixed standard were so much higher than the field samples that very small peaks of DVChl *a*, MVChl *a*, and $\beta\beta$-carotene were seen in the chromatogram of the injection immediately following the standard. To avoid contamination of the first of the triplicate field samples, the DHI-mixed standard injection was always followed by an acetone blank. Percent carryover from the standard to the blank was <0.25% for both DVChl *a* and MVChl *a*, and <0.5% for $\beta\beta$-carotene of the concentration of the individual pigments in the DHI standard mix. If the blank had not followed the standard, the carryover would have equated to an approximate increase in concentration of 1% for the first of the `B` and `C` samples, to 3% for the first of the `F` samples (Table 3). Because DVChl *a* was not present in any of the SeaHARRE-4 samples, the carryover of this pigment into the next sample to be analyzed would have resulted in large errors for DVChl *a*.

2.8 DATA PRODUCTS

Waters `Empower` software created an electronic file in which each chromatographic peak had its retention time, peak area, and peak height recorded together with initial pigment identification. Once the chromatograms had been manually checked, the peak areas were transferred to an `Excel` spreadsheet in which the pigment concentrations are calculated using the appropriate RF:

$$C'_{P_i} \;=\; \hat{A}_{P_i} R_{P_i}, \tag{31}$$

where C'_{P_i} is the amount of pigment injected (units are nanograms per injection); $\hat{A}_{P_i}$ is the area of the chromatographic peak corresponding to the pigment P_i; and R_{P_i} is the response factor for the pigment P_i. The concentration of the pigment P_i in the sample was then determined using the following equation:

$$C_{P_i} \;=\; \frac{V_x}{V_f}\,\frac{D_f}{V_c}\,C'_{P_i}, \tag{32}$$

where V_x is the final extraction volume (in microliters), V_f is the volume of sample filtered (in milliliters), V_c is the volume of sample extract injected onto the HPLC column (in microliters), and D_f is the dilution factor. The dilution factor was rarely used and was only applied if the color of the extract was dark green and it was likely that the sample chlorophyll *a* concentration would be outside the linear range that was used for the calibration of chlorophyll *a*.

For pigments MgDVP and chlorophyll c_1, the RF value determined from the calibration curve for chlorophyll c_2 was used for quantitation.

2.9 CONCLUSIONS

Since mid-2004, CSIRO changed its HPLC method for routine analysis of pigments from the Wright et al. (1991) method to a slightly modified version of the Van Heukelem and Thomas (2001) method. The main reason for changing the method was to have one method that could analyze complete samples from different regions—tropical and temperate oceanic, coastal, estuarine, and freshwater. The CSIRO method is now able to resolve the DV and MV forms of chlorophyll *a*, and lutein from zeaxanthin. Resolution between chlorophyll c_2 and chlorophyll c_1 still remains problematic. Accurate identification and quantification of all pigments is important in determining phytoplankton community composition from pigment composition. The method has been proven to provide a good balance between accuracy of pigment composition and concentration, and sample throughput.

Chapter 3

The DHI Method

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Abstract

The DHI HPLC method is a modified version of the HPL method. The method provides good separation of more than 30 of the most important pigments in freshwater, estuarine, and oceanic environments. Validation steps include four injections of a chlorophyll *a* standard to verify the calibration of the HPLC, use of an internal standard for correcting evaporation errors, and injection of a mixture of pigments to verify correct elution and retention times, and for documenting the precision of the HPLC and response factor stability. During analysis of the SeaHARRE-4 samples, the method provided satisfactory results on the precision of both mixed pigments and natural samples, and on the accuracy of TChl *a* measurements. The accuracy of PPig, however, was much higher when comparing the results to SeaHARRE-3, where DHI first used the HPL method. This increase in uncertainty is ascribed to the complexity of samples derived from transitional areas such as the Danish estuaries, where the SeaHARRE-4 samples were taken.

3.1 INTRODUCTION

For analyzing the SeaHARRE-4 samples, DHI used a modified version of the Van Heukelem and Thomas (2001) method (VHT). (In SeaHARRE-2, the Wright et al. (1991) method was used, but in SeaHARRE-3, DHI participated with the newly adapted VHT method.) The advantages of the VHT compared to the Wright et al. (1991) method are primarily a much better chlorophyll *c* separation, and that DVChl *a* is separated from MVChl *a*. Unfortunately, when adapted to the DHI HPLC, the VHT method was, however, not providing the expected excellent results for SeaHARRE-3 samples when considering the precision of the results. Subsequently, troubleshooting using DHI-mixed pigments located a fault in the autoinjector. Furthermore, it was found that the HPLC vials used were not tight, which caused solvent evaporation. This emphasizes the usefulness of pigment mixtures and the necessity of QA for detecting method problems that have impact on the results. DHI mixed pigments have become a part of the DHI QA program, because they are well suited for documenting the precision of the HPLC. Furthermore, the mixed pigments are used to check that all pigments are eluted, detected, and separated, and that the retention time and response factors are stable. DHI holds a Danish Accreditation and Metrology Fund (DANAK) accreditation for carrying out accredited measurements of pigment concentration in aquatic environments. DHI performs pigment analyses by HPLC in accordance with ISO 17025†, accredited by DANAK, which is handling the administration of accreditation and metrology in Denmark.

3.2 EXTRACTION

The SeaHARRE-4 filters were stored at 80°C until analysis. The filters were extracted in 3 mL 95% acetone containing approximately 0.025 $\mu g\,mL^{-1}$ vitamin E acetate (Van Heukelem and Thomas 2005) as the internal standard. The samples were sonicated in a sonication bath, precooled with ice to approximately 4°C, for 10 min, placed at 4°C for 24 h, and mixed on a vortex mixer. The filters and cell debris were filtered from the extracts using disposable syringes and 0.2 µm Teflon syringe filters directly into HPLC vials, and the vials were placed in the cooling rack of the HPLC together with a parallel set of vials with the injection buffer (90:10, 28 mM aqueous TbAA, pH 6.5:methanol).

3.3 HPLC ANALYSIS

The DHI HPLC system is a Shimadzu LC-10ADVP HPLC composed of one pump (LC-10ADVP), PDA detector (SPD-M10A VP), SCL-10ADVP System controller

† ISO 17025 is an international standard (published by the International Organization for Standardization) that specifies the general requirements for the competence to carry out tests and calibrations.

with LC Solution software version 1.24 SP1, temperature controlled autosampler (set at 4°C), a column oven (CTO-10ASVP) (set at 60°C), and a degasser. The samples were mixed with buffer by the autoinjector by programming it to withdraw 150 µL buffer, 72 µL sample, 57 µL buffer, 71 µL samples, 150 µL buffer, and inject the entire amount (500 µL in total, sample volume injected was 143 µL) onto the column. The method used for HPLC analysis was the VHT method, where solvent B was methanol, and solvent A was (70:30) methanol:28 mM aqueous TbAA, pH 6.4 (Table 25). The column was an Eclipse XDB C_8, 4.6×150 mm (Agilent Technologies), the flow rate was 1.1 mL min^{-1}, and the temperature of the column oven was set to 60°C.

Table 25. The gradient system used with the DHI method. Solvent A is 70:30 methanol:28 mM aqueous TbAA, pH 6.4, and solvent B is 100% methanol.

Step	*Time*	A [%]	B [%]
Start	0	95	5
2	5	95	5
3	27	5	95
4	34	5	95
5	35	0	100
6	38	0	100
End	39.5	95	5

Mixed pigments (DHI Mix-102) were analyzed with the samples: eight in total were randomly distributed in between the samples, followed by a blank (extraction solvent with vitamin E).

Chlorophyllide *a*, phaeophytin *a*, phaeophorbide *a*, divinyl chlorophyll *a*, and monovinyl chlorophyll *a* were determined at 665 nm; the internal standard at 222 nm; and the rest of the pigments were determined at 450 nm. Peak identities were routinely confirmed by online PDA analysis.

3.4 CALIBRATION

The HPLC was calibrated with pigment standards from DHI (Denmark), prior to analysis of the SeaHARRE-4 samples. Four pigment standards were mixed, and a series of six dilutions were made of each standard mixture: three different concentrations at the range of expected concentrations of the natural samples, and three different concentrations at low concentrations near the limit of quantitation (LOQ) and limit of detection (LOD). The linearity of the series was verified, and the response factors were calculated from the regression forced through zero.

The concentrations of the calibration standards were determined using a Shimadzu UV-2401PC dual-beam, monochromator-type spectrophotometer, which is subjected to a regular set of quality control procedures. The absorption coefficients used are shown in Table 26. Selected batch numbers of the standards are controlled for purity and concentration by an independent laboratory.

Table 26. Absorption coefficient (α) values in liters per gram per centimeter used with the DHI method for the pigments listed as a function of wavelength (λ).

Pigment	*Solvent*	λ	α
Chl c_3	90% Acetone	452.9	346.00
Chl c_2	90% Acetone	443.8	374.00
Chlide *a*	90% Acetone	664.0	127.00
Peri	100% Ethanol	472.0	132.50
Phide *a*	90% Acetone	667.0	74.20
But	100% Ethanol	446.0	160.00
Fuco	100% Ethanol	449.0	160.00
Neo	100% Ethanol	439.0	224.30
Pras	100% Ethanol	454.0	160.00
Viola	100% Ethanol	443.0	255.00
Hex	100% Ethanol	447.0	160.00
Diad	100% Ethanol	446.0	262.00
Allo	100% Ethanol	453.0	262.00
Myxo	100% Acetone	478.0	216.00
Diato	100% Ethanol	449.0	262.00
Zea	100% Ethanol	450.0	254.00
Lut	100% Ethanol	445.0	255.00
Cantha	100% Ethanol	476.0	207.50
Chl *b*	90% Acetone	646.8	51.36
DVChl *a*	90% Acetone	664.0	87.67
MVChl *a*	90% Acetone	664.3	87.67
Phytin *a*	90% Acetone	667.0	51.20
β-Caro	100% Ethanol	453.0	262.00

3.5 VALIDATION

DHI holds a DANAK accreditation for carrying out accredited measurements of pigment concentration in the aquatic environment. DHI performs pigment analyses by HPLC in accordance with ISO 17025 (accredited by DANAK). Validation follows the DHI Standard Operating Procedure (SOP) No. 30/852:01: "Accredited measurements of pigment concentrations in the aquatic environment." Validation steps include four injections of a chlorophyll *a* standard to verify the calibration of the HPLC, use of an internal standard for correcting evaporation errors, and injection of a mixture of pigments (DHI mixed pigments) to verify correct elution, retention times, and for documenting the precision of the HPLC and response factor stability.

3.6 DATA PRODUCTS

The peak areas and pigment identities were transferred to an Excel file, and based on the response factors, the pigment concentrations were calculated:

$$C_{P_i} = \frac{V_x}{V_f} \frac{\hat{A}_c}{\hat{A}_s} \frac{\hat{A}_{P_i}}{V_c} R_{P_i}, \quad (33)$$

where $\hat{A}_{P_i}$ and R_{P_i} are the peak area and the response factor of pigment P_i, respectively, V_x is the extraction volume, V_f is the volume of sample filtered, V_c is the amount of sample injected, $\hat{A}_c$ is the peak area of the internal standard in the extraction solvent, and $\hat{A}_s$ is the peak area of the internal standard in the sample.

The effective LOD and LOQ were calculated as described in the SeaHARRE-2 report (Hooker et al. 2005) for all pigments. Results were submitted in separate spreadsheets with zero values indicating that a pigment was not detected, and results where the values below LOD were replaced by the effective LOD values, while values below LOQ were replaced by the effective LOQ values.

3.7 CONCLUSIONS

The method used at DHI for the SeaHARRE-4 analyses was the VHT method, which in SeaHARRE-2 and SeaHARRE-3 was shown to provide 'state-of-the-art' results. An average precision of 1.0% was attained at DHI when determined by DHI mixed pigments. Because the variability of natural samples where the filtered volume and the extraction procedures are applied, uncertainties—as well as the resulting precision of the SeaHARRE-4 samples—were higher, i.e., PPig ξ (CV) of 6.1%. The overall uncertainty attained in the analysis of field samples were, on average, 5.4% and 29.5% for TChl *a* and PPig, respectively. While the uncertainty of the DHI TChl *a* was comparable to the results obtained in SeaHARRE-3, the uncertainty of PPig was much higher (7.8% in SeaHARRE-3). This increase in uncertainty was seen for all laboratories (Table 21, Chap. 1), and the major problem can be ascribed to the complexity of the samples.

Samples from transitional areas, such as the estuaries (where the samples for the present SeaHARRE-4 activity were taken), contain a complex mixture of freshwater and marine algae of which some undergo degradation due to the changes in the environment, e.g., salinity gradients. Such samples pose a particular challenge in the HPLC analyses because they contain relatively many pigments (freshwater specific pigments as well), and increased amounts of partly degraded pigments. These derivatives may coelute and interfere with the quantification of the nondegraded pigments; therefore, problems with false positives and false negatives may be larger in such samples, because the baseline is sprinkled with small peaks of uncertain origin.

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Chapter 4

The FIO Method

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Abstract

The FIO method used multiple reversed-phase C_{18} columns connected in series. The configuration uses a single monomeric and two polymeric columns, and was originally devised to enhance the separation of numerous structurally similar pigments and degradation products. The combination of the coarse and fine molecular properties of the two different column types optimizes pigment separation. The monomeric column provides strong retention and high efficiency, while the polymeric columns provide the selection for similar compounds. The latter are temperature sensitive and require the use of a column heater. A nonlinear binary gradient, adapted from Van Heukelem et al. (1992), was used for pigment separations. Solvent A consisted of 80% methanol:20% ammonium acetate (0.5 M adjusted to pH 7.2) and solvent B was composed of 80% methanol:20% acetone. Absorption spectra and chromatograms were acquired using a photodiode array detector. Pigment peaks were identified by comparing retention times and absorption spectra with standards or culture extracts (depending on the pigment involved).

Lead Author's Note

The SeaHARRE activity was conceived as an opportunity for the HPLC community to investigate problems with quantifying pigments extracted from aquatic phytoplankton and algae. In particular, the activity was established to estimate the uncertainties in the derivation of standard data products associated with chlorophylls *a, b,* and *c,* plus a diversity of carotenoids. The main perspective was the uncertainty in field samples collected primarily—but not exclusively—from the marine environment. Such samples do not have an *a priori* understanding of truth, so proxy values must be determined from a rigorous application of performance metrics to the candidate analyses.

The basic approach is to rely on community volunteers to provide the field samples needed to address the objectives established for each activity, and a larger group of volunteers are relied on for the subsequent HPLC analyses using validated methods. The sample sets are collected as part of either dedicated activities or in conjunction with an already scheduled field campaign. In either case, considerable extra effort and attentiveness is needed to collect all the samples in the requisite number of replicates. At each step of the process, one or more volunteers step up for the good of the community and the SeaHARRE activity to ensure the highest quality samples are obtained and distributed.

Much of the dialogue for evolving the activity is established during workshops that are open to the SeaHARRE participants and the wider community. The workshops are usually held after the analysis of field samples have been completed, so there have been almost as many workshops as field campaigns. The volunteer HPLC analysts are recruited from a core group with established capabilities plus a subset of new practitioners. The latter are a combination of entirely new analysts and recent participants who have expressed an interest in remaining a part of the overall process. The union of these diverse sets of analysts presumably provides a valid *snap shot* of community capabilities and diversity during each SeaHARRE activity.

All participants agree to an open documentation process, where all laboratories and methods are presented without anonymity. The forums for presenting the results includes the workshops, scientific conferences, technical reports, and peer-reviewed publications. The most detailed information for community-wide consumption is in the technical reports, so at some level they are the most precious. SeaHARRE activities need active participation and full disclosure from all participants, in order that other community members can glean useful information. Repeated efforts were made to obtain a description of the FIO method, but no documentation was provided before the completion of this report. The FIO chapter abstract and this note were provided by the lead author of the report. The abstract is based on details concerning the method that are available in Pinckney et al. (1996 and 2001); additional information is available in Chap. 11.

Chapter 5

The GSFC Method

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Abstract

GSFC participated in SeaHARRE-4 as an HPLC novice, with no previous experience in actual HPLC pigment analysis. The primary objective of the GSFC participation was to strictly adhere to the established performance metrics for quantitative analysis, as well as the published protocols of the selected method (Van Heukelem and Thomas 2001), to demonstrate whether or not both were sufficiently robust to allow any practitioner to produce results in keeping with the QA subset. Preparation at GSFC prior to sample analysis included servicing the instrument, making solvents, buffers, and standards, and running single- and multipoint regressions of known standards to create a calibration table and update a spectral library used to identify pigments in the 36 SeaHARRE unknown samples. Unexpected pressure issues resulted in recalibration and validation, as well as the SeaHARRE samples being analyzed on the Agilent 1100 HPLC at HPL. Multiple Chl *a* regression curves were run, and residuals calculated, in order to identify the dynamic range of the detector, as well as the overall precision of the instrument. Overall, 35 Chl *a* standards of varying concentrations were injected with average residuals of 1.5% for all concentrations. A second measurement of Chl *a* concentration, using a spectrophotometer and a trichomatic equation was completed at GSFC using the remaining sample extractions from the HPLC analysis. GSFC obtained state-of-the-art results, which established the capability of a novice laboratory to produce high-quality pigment data for use in calibration and validation exercises associated with remote sensing derivations of global chlorophyll concentrations.

5.1 INTRODUCTION

The SeaHARRE-4 samples were shipped from Denmark by DHI to GSFC on 4 December 4 2006, and arrived at GSFC on 6 December 2006. All samples were placed in a −80°C freezer until analysis began, which was completed in September 2007. Analysis for the GSFC samples were to be performed on an Agilent 1100 Series HPLC using the Van Heukelem and Thomas (2001) method. The GSFC HPLC instrument had not been used for approximately 2 y, however, so an extensive preliminary work plan was formulated (Sect. 5.3) to calibrate and validate this instrument. Even with this effort, several unexpected pressure issues of known and unknown causes resulted in recalibration and validation runs, as well as the GSFC samples being ultimately analyzed on an Agilent 1100 HPLC at HPL.

An elevated pressure during the initial start-up procedure (i.e., purging of the instrument, with the pump valve open at a flow rate of 15 mL min^{-1}), exposed the first pressure problem. This pressure issue was easily corrected with a change of the pump filter frit. An increasing pressure at initial conditions (i.e., 95% solvent A and 5% solvent B, at a flow rate of 1.1 mL min^{-1}) was the second pressure issue. From the initial start-up of the instrument on 22 June through 17 July, initial pressure remained approximately 150 bar. From 19–26 July, initial pressure slightly increased (156–196 bar), and then decreased with the changing of the pump frit on 26 July (146 and 149 bar on 6 and 7 August, respectively). The pressure issue with the initial conditions returned on 10 August and eventually reached maximum pressure at 200 bar on 13 August.

An assessment of the instrument by Agilent, based on the description of the problem via teleconferences, resulted in a diagnosis of contamination throughout the system. The solution recommended by Agilent was to replace the column, most of the tubing, and several major pump parts, and then to purge the system for a day with a mixture of organic solvents. These steps were to be followed by a thorough inspection of the system by an authorized Agilent technician.

Because of time constraints, and with no guarantee that the suggested solution to the pressure issue would result in the GSFC system being operable, the decision was made to take all necessary supplies, standards, and samples (via dry shipper), to HPL for a week to rerun calibration and validation injections, and perform the analysis of the sam-

ples on an Agilent 1100 HPLC that was known to be functioning properly.

5.2 EXTRACTION

Extraction of the 36 SeaHARRE-4 samples was performed at HPL, and followed the HPL extraction method. Samples were extracted over three days (17–19 September) in three random batches of 12. Each filter was placed in a solution of −20°C chilled 100 µL water and 2.5 mL of acetone for approximately 1 h. Next, the chilled samples were sonicated to completely macerate the filter, and then placed back in the −20°C freezer for 3.5–4.0 h. After this time period, sample slurries were individually filtered through a 0.45 µm pore size polytetrafluoroethylene (PTFE)syringe cartridge filter into a 7 mL glass amber scintillation vial. Filtered sample extracts were then vortex mixed, and approximately 500 µL of extract were transferred into an HPLC vial for overnight HPLC analysis. The remaining extracts were stored in the −20°C freezer, and returned to GSFC for spectrophotometric chlorophyll *a* analysis (Sect. 5.6).

5.3 HPLC ANALYSIS

Before beginning the SeaHARRE-4 sample analysis on the GSFC HPLC, several steps were required because of the instrument being idle for a long period of time. All solvent bottles were cleaned and dried, and all glass solvent frits were replaced with new ones. A new Eclipse XDB C_8 4.6 × 150 mm 3.5 µm column was placed in the instrument, and all solvent lines, system tubing, and the column were purged with a 70:30 methanol and water solution. All pipettes, syringes, and dispensers were calibrated. Finally, the following solutions were prepared for QA and QC purposes during sample analysis: a) a vitamin E solution for use as an internal and working standard; b) the injection buffer composed of 90:10 of 28 mM TbAA and methanol; c) solvent A, a solution of 70:30 methanol and 28 mM TbAA; and d) a Chl *a* standard.

The original HPL method gradient and instrument parameters were transferred directly to the GSFC HPLC. The HPL injector program, however, needed to be modified to accommodate the injection of 525 µL of sample and buffer to the GSFC hardware, which used a 100 µL syringe head and a multidraw loop. An 18-step injector program was designed, providing maximum draws of 100 µL or less, with a draw speed of 130 µL min^{-1} and an eject speed of 250 µL min^{-1}, for a total injector program time of approximately 10 min.

Once the injector program was in place and tested with acetone blanks, a series of samples from DHI Mix-102 and -103 were analyzed to determine instrument retention times for all pigments. From this analysis, 2 min were added to the overall run time to accommodate capturing carotene, the last pigment to elute off the column. With the retention times established, eight pure culture samples (*Tahitian isochrysis*, *Emiliania huxleyi*, *Pycnococcus*, *Pelagococcus*, *Prorocentrum*, *Duneliella*, cryptophyte, and mutant corn) were extracted and analyzed. These samples were designed to evaluate the GSFC instrument performance by checking elution of early- (Chl *c*) and late-eluting (carotene) peaks, the chromatography of less common peaks (DVChl *a* and DVChl *b*) and coeluting peaks (Chlide *a* and Chl c_1), and the resolution of critical pigment pairs (Zea and Lut, as well as Viola and Hex).

Multiple Chl *a* regression curves were run, and residuals calculated, in order to identify the dynamic range of the detector, as well as the overall precision of the instrument. The Chl *a* standard was produced by the dissolution of crystallized Fluka (25730) Chl *a* in 90% acetone. Subsequent HPLC analysis determined the purity of the standard to be 98.2%. Five-point regressions, which signified concentrations ranging from 0.017–1.264 mg L^{-1} (2.533–186.194 ng inj^{-1}), were performed.

Overall, 35 Chl *a* standards of varying concentrations were injected with average residuals of 1.5% for all concentrations, and an average response factor of 0.2988 (with an average standard deviation of 0.005). This information, combined with triplicate single-point runs of individual DHI pigment standards, formulated the calibration table, which was used to update the HPL spectral library designed to identify unknown pigments in the SeaHARRE-4 samples.

At this point in the instrument calibration and validation process, the unresolved pressure issues at initial conditions rendered the completion of the SeaHARRE-4 samples on the GSFC HPLC impossible. The remaining analyses, except for the spectrophotometer work (Sect. 5.6), was performed at HPL by the GSFC participant using GSFC supplies and standards (except where noted) along with the nearly identical Agilent 1100 HPLC at that facility.

5.4 CALIBRATION

Upon arrival at HPL and the inspection of the dry shipped vials of GSFC standards, it was found that two standards—Fluka Chl *a* and Fuco—were damaged in shipment, and were therefore, unusable. The HPL Fluka Chl *a* and an HPL Fuco standard based on culture extractions were used for the GSFC calibrations. Four calibration curves for Chl *a* were run, two regressions on the first day (17 September) and two regressions on the last day (19 September), with Chl *a* concentrations ranging from 0.014–1.002 mg L^{-1} (1.9–146.1 ng inj^{-1}). The average residuals were always less than 1%, and ranged from 0.81% (0.014 mg L^{-1}) to 0.13% (1.002 mg L^{-1}). The average response factor was 0.288 ($\xi = 0.540$).

Single point individual DHI standards, and the HPL fucoxanthin standard were also interspersed throughout the sample runs, in duplicate, to have sufficient data to create a calibration table and update the spectral library,

or later chromatogram interpretation and data analysis calculations of SeaHARRE-4 sample pigment concentrations. The absorption coefficients (α) used with the GSFC method are presented in Table 27.

Table 27. The α values used by the GSFC method for a variety of pigments as a function of λ. The units for α are liters per gram per centimeter and the units for λ are nanometers.

Pigment	*Solvent*	λ	α
Chl c_3	90% Acetone	452.9	346.00
Chl c_2	90% Acetone	443.8	374.00
Peri	100% Ethanol	472.0	132.50
But	100% Ethanol	446.0	160.00
Fuco	100% Ethanol	449.0	160.00
Neo	100% Ethanol	439.0	224.30
Pras	100% Ethanol	454.0	160.00
viola	100% Ethanol	443.0	255.00
Hex	100% Ethanol	447.0	160.00
Diad	100% Ethanol	446.0	262.00
Allo	100% Ethanol	453.0	262.00
Diato	100% Ethanol	449.0	262.00
Zea	100% Ethanol	450.0	254.00
Lut	100% Ethanol	455.0	255.00
Chl b	90% Acetone	646.8	51.36
DVChl a	90% Acetone	664.3	87.67
Chl a	90% Acetone	664.0	87.67
Phaeo a	90% Acetone	667.0	51.20
$\beta\beta$-Car	100% Acetone	454.0	250.00

5.5 VALIDATION

For both the validation of samples, and to verify instrument precision, during the three days of SeaHARRE-4 sample runs, both Chl a standards ($0.547\,\mathrm{mg\,L^{-1}}$ or $79.7\,\mathrm{ng\,inj^{-1}}$) and vitamin E injections were interspersed throughout each batch every four to six samples. Vitamin E standards had an average area of 3,171.7 mAU ($\xi = 0.2\%$), and Chl a had an average area of 277.8 mAU ($\xi = 0.4\%$) for all three runs.

5.6 DATA PRODUCTS

With limited past experience in pigment chromatogram interpretation, a set of reprocessing guidelines were devised prior to data analysis to provide the most consistency. First, a set of integration parameters was established based on the most number of primary peaks initially identified and labeled via retention times. Second, library spectral peak matches were attempted for these time-identified peaks, with large and well-formed peaks investigated first; small and well-formed peaks second; and small and poorly-formed peaks were looked at last. The chromatogram was then examined for pigment peaks not labeled via integration parameters and retention times. Little resizing of peak areas was performed, and peaks not quantified, but identified by the software, were removed. Finally, reports for each sample were consolidated and printed.

A spreadsheet was then created in `Excel`, and individual pigment concentrations (in units of micrograms per liter) were quantified via the following equation:

$$C_{P_i} = \frac{V_x}{V_f}\,\frac{\hat{A}_{P_i}}{V_c}\,R_{P_i}, \tag{34}$$

where $\hat{A}_{P_i}$ is the area of pigment P_i; R_{P_i} is the response factor; V_x is the extraction volume in liters; V_f is the filtration volume in liters; and V_c is the injection volume in milliliters. Furthermore, because of the fact that the method used for pigment analysis did not separate Chl c_1 and Chlide a, the quantitation of these two pigments was determined using a set of simultaneous equations (Hooker et al. 2000). For each pigment triplicate, the average, standard deviation, and CV were calculated.

An additional calculation of Chl a was accomplished using a spectrophotometer and the trichromatic equation of Humphrey and Jeffrey (1997). After HPLC pigment analysis at HPL, all remaining SeaHARRE-4 pigment extracts were carefully wrapped and then placed in a dry shipper and brought back to GSFC. Upon arrival, all extracts were read on a PerkinElmer Lambda 35 spectrophotometer, and absorption at 630, 647, 664, and 750 nm were recorded.

Absorption at 750 nm was used as a baseline and was subtracted from the other three wavelengths. These adjusted absorption measurements were used to calculate $\left[\mathrm{SChl}a\right]$ for each of the 36 SeaHARRE-4 pigment samples using the following equation:

$$\left[\mathrm{SChl}a\right] = 11.85A(664) - 1.54A(647) - 0.08A(630). \tag{35}$$

5.7 CONCLUSIONS

Initially, the 36 SeaHARRE-4 samples were to be analyzed at GSFC, using the Van Heukelem and Thomas (2001) method, on an Agilent 1100 HPLC. Unresolved pressure issues, however, created an obstacle to this plan. With the calibration and validation of the GSFC instrument complete, the pressure issues became insurmountable; recalibration, validation, and analysis of the SeaHARRE-4 samples were, therefore, performed at HPL using GSFC supplies and standards. A second measurement of Chl a concentration, using a spectrophotometer and a trichomatic equation, was completed at GSFC using the remaining sample extractions from the HPLC analysis. Finally, state-of-the-art results by the GSFC HPLC novice participant, highlighted the importance of establishing guideline performance metrics for biogeochemical methods.

Chapter 6

The HPL Method

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Abstract

The HPL HPLC method employed by Horn Point Laboratory was developed for use with a variety of water types. Many pigments important to freshwater, estuarine, and oceanic systems are baseline resolved and quantitatively reported, including divinyl and monovinyl chlorophyll *a*. The method is built around a C_8 HPLC column, and incorporates a methanol-based reversed-phase solvent system, a simple linear gradient, and an elevated column temperature (60°C) to achieve separation of the pigments to be quantitated. The method can provide quantitative results for up to 25 pigments with qualitative information for additional pigments. Quality assurance measurements are made during sample analysis to confirm that the method performance is within expectations. Investigations into the uncertainties in the method show the 95% confidence limits are estimated as a) 0.5–3.8% for precision of replicate injections within and across sequences, b) 3.2% for chlorophyll *a* calibration reproducibility, and c) 5.1% for chlorophyll *a* method precision, including filter extraction and analysis.

6.1 Introduction

The HPL HPLC method (Van Heukelem and Thomas 2001) and modifications to it have been extensively described in previous SeaHARRE reports (Hooker et al. 2005 and 2009). The HPL method uses a C_8 column and a reversed-phase, methanol-based, gradient solvent system. A linear gradient is used because segmented gradients offered no advantages—and increased baseline instability—which can interfere with the quantitation of pigments, particularly those in low concentrations. Elevated column temperature is used to facilitate separation selectivity. High column temperature, however, shortens the useful column lifetime and special handling should be considered (Wolcott et al. 2000). The HPL procedures are occasionally modified as new types of samples and activities lead to new understandings, as have been the case with the SeaHARRE activities.

6.2 Methodology

HPLC methodology includes descriptions of sample extraction procedures, quality assurance (which includes calculation equations), and HPLC analysis, which is further subdivided into procedures for injection, pigment separation, detection, calibration, and pigment identification. Data products and data reporting practices specific to the SeaHARRE-4 activity are also described.

The SeaHARRE-4 samples were received frozen in liquid nitrogen dry shippers the week of 21 February 2007. The filters were immediately placed in a freezer at −80°C until processed in March 2007. DHI pigment standards, including the Mix-102 standard, were received well frozen on dry ice the week of 21 February and held at HPL in a −25°C freezer until analyzed.

6.2.1 Extraction

At HPL, the 'default' volumes used during sample extraction are 2.5 mL 100% acetone and 100 µL water. These volumes are sometimes adjusted depending on the individual sample set. Several different extraction volumes have been previously tested and validated. During SeaHARRE-4, the volumes were adjusted to 4.0 mL 100% acetone and 160 µL water, after noting that the filters appeared visibly darker than normal (and therefore more concentrated). Increasing the extraction volume also ensured that sufficient sample extract would be available for spectrophotometric analysis.

The procedures for extractions were as follows:

1. A frozen filter sample was placed in a thick-walled 12 mL glass centrifuge tube.
2. The extraction solvent, 100% HPLC-grade acetone (J.T. Baker 9002-03, Phillipsburg, New Jersey) containing DL-α-tocopherol acetate (vitamin E acetate,

Sigma-Aldrich 95250, St. Louis, Missouri) in a concentration of approximately 0.02 mg mL^{-1} was kept in a 1 L brown glass bottle. The bottle was removed from a −15°C freezer and brought to room temperature. A 4.0 mL aliquot of room-temperature extraction solvent was added to a centrifuge tube with a glass volumetric pipette that had been calibrated gravimetrically with 100% acetone.

3. An automatic pipette was used to deliver 0.16 mL of deionized water to the tube.
4. To prevent evaporation, the top of the centrifuge tube was covered with Parafilm immediately after the acetone and water were added.
5. The tube was placed in an ice bath shielded from the light as soon as the top was covered with Parafilm.

After all filters in a set were processed following steps 1–5:

6. Samples in the ice bath were placed in a −25°C freezer for at least 1 h.
7. Samples were removed from the freezer. Each sample filter was individually disrupted for approximately 20 s with a Branson 450 Sonifier (Danbury, Connecticut) equipped with a 0.125 in microtip sonic probe, using control settings that resulted in approximately a 30–40 W output.
8. The filter extracts were re-covered with Parafilm and stored in a −25°C freezer for 3–4 h.
9. Samples were removed from the freezer. The filter slurry of each sample was then transferred to a clean 5 mL disposable syringe with a Luer-Lok tip (Becton-Dickinson 309603). The slurry was clarified by pushing it through a PTFE Titan HPLC syringe cartridge filter attached to each Luer-Lok syringe. The pore size of all filters used for clarification was 0.45 µm, and the diameter was 17 mm (Sun SRI 44504-NP, Rockwood, Tennessee). The clarified extract was collected in clear, 7 mL scintillation vials with cork-backed, foil-lined screw caps (Fisher Scientific 03-337-26, Pittsburgh, Pennsylvania).

After all the samples extracted in a day were clarified (steps 6–9), the final sample preparation procedures were as follows:

10. The sample extract, contained in a 7 mL scintillation vial, was vortexed gently for approximately 1 s. Using an autopipette, approximately 0.5 mL was transferred to an amber HPLC vial (National Scientific C4011-6W, Rockwood, Tennessee) and capped with a PTFE and silicone snap cap (National Scientific C4011-54B). A clean, unused tip was used with each extract.
11. The vials were placed in the HPLC temperature-controlled autosampler (TCAS) compartment, set at 4°C, and analyzed within approximately 24 h.
12. The remaining unused sample extracts were recapped and stored at −15°C until they were used for spectrophotometric analysis.

6.2.2 HPLC Analysis

The filter extracts and standards were analyzed using a fully automated Agilent 1100 HPLC equipped with a quaternary pump, programmable autoinjector, TCAS, Peltier temperature-controlled column oven compartment, PDA detector, and `ChemStation` software revision A.10.02 (all from Agilent Technologies, Inc., Santa Clara, California).

6.2.2.1 Injection

The HPL HPLC has been modified with a metering device and sample loop capable of drawing up to 900 µL. The injector is programmed to draw successive aliquots of sample extract and buffer into the sample loop. A mix function was tested during SeaHARRE-3, but because it offered no apparent advantages, HPL returned to the injector program used in SeaHARRE-2 (Hooker et al. 2005).

Several steps have been identified at HPL that contribute to excellent injector precision, and are therefore always followed:

a. Place sample and buffer vials in the TCAS at least 1 h prior to starting analyses;
b. Disregard the first injection of a sequence;
c. Maintain a sufficient and constant column re-equilibration volume between injections;
d. Use vial and cap combinations that have been tested and proven to prevent evaporation (Hooker et al. 2009);
e. Use injection draw speeds that prevent a vacuum from forming when sample is withdrawn from the vial; and
f. Limit the vial residence time in the TCAS to approximately 24 h or less.

The injection buffer solution is 28 mM TbAA (pH 6.5) and methanol, mixed 90:10 (vol:vol), respectively. This solution is filtered in batches through an Acrodisc 25 mm syringe filter with a 1 µm glass fiber membrane (Pall 4523T, East Hills, New York). Snap caps on the buffer and sample vials are formulated of layered PTFE and silicone, but caps used with buffer vials are preslit (National Scientific C4011-59) to facilitate accurate draw volumes of the more viscous buffer mixture. Snap caps on sample vials are not preslit, to prevent evaporation of the sample.

Rather than use a large reservoir from which to draw buffer, buffer is put in the TCAS in the same type of HPLC vials as samples. Each HPLC vial contains enough buffer for four separate injections. Using these smaller buffer reservoirs helps control potential contamination of the buffer by sample extract, as well as minimize the impacts of this type of carryover if it were to occur. The

needle is also dipped in a vial of 100% acetone (with caps with Teflon septa, National Scientific C4011-52R) after each time the needle draws from a sample vial, in order to help control carryover. Occasionally, peak splitting with chlorophylls c_1, c_2, and c_3 is encountered, and MgDVP is similarly affected if present.

6.2.2.2 Separation

The separation conditions used in SeaHARRE-4 were the same as those used in SeaHARRE-2 and -3. Solvent A is formulated (vol:vol) from HPLC-grade methanol (Fisher Scientific A452-4, Fair Lawn, New Jersey) and 28 mM TbAA (pH 6.5) in a ratio of 70:30, respectively. The aqueous TbAA solution is made by diluting 0.4 M tetrabutyl ammonium hydroxide (J.T. Baker V365-7) with high-purity deionized water (HPLC-grade equivalent) and adjusting the pH to 6.5 with acetic acid.

Solvent A is thoroughly mixed and then filtered through a 0.2 µm pore size, 47 mm diameter nylon membrane filter (Millipore GNWP04700, Billerica, Massachusetts). The number of batches of solvent A needed to complete the work associated with one week of analyses are combined in a large glass carboy, so all solvent A to be used in a week is homogeneous. This procedure promotes day-to-day retention time stability. This container is kept on a stir plate at a very low setting to prevent layering from occurring in the solvent container over the course of the week. Any solvent A not fully consumed within the week is either discarded or refiltered and combined with the next batch of solvent A, to avoid potential precipitates forming and clogging the column.

Table 28. The HPL pump gradient used during SeaHARRE-4. Solvent A is 70:30 methanol:28 mM TbAA (pH 6.5), solvent B is methanol, and solvent C is acetone. (After SeaHARRE-3, Solvent C was added as a rinse to help alleviate carryover.)

Time	A [%]	B [%]	C [%]	*Flow*
0	95	5	0	1.1
22.00	5	95	0	1.1
24.50	5	95	0	1.1
24.75	5	65	30	1.3
25.75	5	65	30	1.3
25.85	5	65	30	1.1
26.10	95	5	0	1.1
29.10	95	5	0	1.1

The pump gradient used in SeaHARRE-4 is presented in Table 28. The total run time, including the injector program, from the beginning of one injection to the next, is 44.9 min. A total of 15.2 mL of the solvents at the initial condition flows through the column in between the time of the completion of one sample injection and the injection of the next sample. Column temperature is set at 60.0°C ±0.8°C. The HPLC column is a Zorbax Eclipse XDB-C8, 3.5 µm particle size, 4.6×150 mm^2 (Agilent 963967-906, Santa Clara, California). The column is used without a guard column or prefilter, because those have been tested at HPL and offered no advantages.

6.2.2.3 Detection

Chromatograms are plotted using the 450 and 665 nm wavelengths (each with a 20 nm bandwidth), as well as at 222 nm with a 10 nm bandwidth. Tungsten and deuterium lamps are both used and absorbance spectra are collected between 350–750 nm, with a range step and slit width of 2 nm. The flow cell capacity is 13 µL and the pathlength is 1 cm.

The wavelength at 222 nm is used exclusively for quantitation of the internal standard. This wavelength is not associated with the maximum response for vitamin E, but it allows good detection and minimizes baseline drift. The baseline drift is caused by solvent effects of the gradient and is approximately -3 mAU min^{-1}. The signal height of vitamin E at the concentration used is approximately 600 mAU, and sufficiently high so that baseline drift does not interfere with accurate peak area determinations. The vitamin E solution is formulated at a concentration such that the resulting peak height corresponds to about two-thirds of the height threshold where the response becomes nonlinear.

6.2.2.4 Calibration

The retention times of pigments to be quantified, and pigments with a potential to interfere with the quantitation process, were originally documented using algal monocultures and standards (Van Heukelem and Thomas 2001). This information is summarized in the SeaHARRE-3 report, Table 31, as well as additional details on chromatographic problems that can cause large uncertainties for some pigments in natural samples (Hooker et al. 2009).

The HPLC was calibrated with both purchased standards and those isolated at HPL. Concentrations of pigment standards were determined with the absorption coefficients given in Table 29. For standards that were purchased from DHI, the spectrophotometrically determined concentrations provided by DHI were used to compute the response factors observed from their standards at HPL.

The spectrophotometer used for determining the concentrations of standards isolated at HPL, or purchased from Sigma-Aldrich or Carotenature and dissolved at HPL, was a Shimadzu 2401-PC (Columbia, Maryland), with a bandwidth of either 1 or 2 nm, a medium sampling rate, 1 nm sampling interval, and turbidity correction at 750 nm. Absorbance of stock standards is typically between 0.2–0.8, a range recommended by Marker et al. (1980) for best spectrophotometric accuracy.

Table 29. The HPL α values (in units of liters per gram per centimeter), used for spectrophotometrically measuring pigment concentrations. Also shown are the solvents and maximum wavelengths (λ_m) specified for use with α values. Absorption coefficients and reference wavelengths used at HPL match those used at DHI. The units for λ_m are nanometers. Standards isolated at HPL, or purchased from DHI, Sigma-Aldrich or Carotenature, are denoted H, D, S, and C respectively, in the source column.

Pigment	*Solvent*	λ_m	α	*Source*
Chl c_3	90% Acetone	453	346.00	H D
Chl c_2	90% Acetone	444	374.00	H
Chl c_1	90% Acetone	443	318.00	H
Chlide *a*	90% Acetone	664	127.00	D
Phide *a*	90% Acetone	667	74.20	D
Peri	100% Ethanol	475	132.50	H D
But	100% Ethanol	447	160.00	D
Fuco	100% Ethanol	449	160.00	H
Neo	100% Ethanol	439	227.00	H
Pras	100% Ethanol	438	160.00	H
Viola	100% Ethanol	443	255.00	D
Hex	100% Ethanol	447	160.00	D
Diad	100% Ethanol	446	262.00	D
Allo	100% Ethanol	453	262.00	H
Diato	100% Ethanol	449	262.00	H
Zea	100% Ethanol	450	254.00	H
Lut	100% Ethanol	445	255.00	D
DVChl *b*	100% Acetone	645	52.50	H
Chl *b*	90% Acetone	647	51.36	H D
DVChl *a*	90% Acetone	664	87.67	H
Chl *a*	90% Acetone	664	87.67	S
Phytin *a*	100% Acetone	667	51.20	H
$\beta\beta$-Car	100% Acetone	454	250.00	H C

Single-point response factors are generally used at HPL, because previously it has been demonstrated that the samples being analyzed were within the linear range and, in addition, the linear regressions associated with the multipoint calibration curves exhibited y-intercept values very near zero. The values of the response factors being used are based on averages obtained from using the same method for many years. The validity of the average response factors is checked frequently, and the uncertainties for calibration checks are expected to be within 5%. This uncertainty represents the cumulative effects of spectrophotometric determinations of stock standard concentrations, dilution, and the subsequent HPLC analyses. Uncertainties greater than 5% are investigated and, if necessary, response factors in the calibration table are changed.

Discrete calibration standards were not available for all of the pigments quantitated during the SeaHARRE-4 activity, including:

- Chlide *a*;
- A chlorophyll c_3-like pigment which elutes immediately after chlorophyll c_3 and was summed with chlorophyll c_3;
- Four pigments with Phide *a* spectra that elute after the retention time of the Phide *a* standard purchased from DHI (quantitated with the response factor from DHI Phide *a* standard);
- Chl *a* and DVChl *a* allomers and epimers (quantitated as part of total Chl *a*); and
- A Phytin *a* epimer (quantitated with Phytin *a* response factor).

Because HPL had no discrete standards for these pigments, retention times were updated as they were observed in the DHI retention time mix or in natural samples (as identified with in-line visible absorbance spectra).

During the analysis of SeaHARRE-4 samples, retention times were documented on a daily basis using DHI Mix-102. This mix was injected near the beginning of a sequence and then approximately once every 24 h thereafter. Pigment identification in natural samples was based primarily on retention time; absorbance spectral match with known standards was also used when possible (e.g., where the signal was adequate to produce usable absorbance spectra for peaks in sample extracts). In other words, if a pigment appeared at a retention time for a particular standard, but the absorbance spectrum was clearly inconsistent with what should be apparent for that standard, the pigment was reported as not present (i.e., a limiting value was assigned).

Pigments with an SNR of 4 or less (at the wavelength used for their quantitation) were considered "not present" (i.e., a limiting value was assigned).

If a symmetrical, well-shaped peak appeared at the retention time of a standard, with an SNR $\geq$ 4, but the SNR was too low to confirm or reject pigment identity on the basis of absorbance spectra, the pigment was still quantitated and reported. Small peaks that were clearly contaminated with an interference peak, as evidenced by peak shape distortion, were reported with a limiting value (this most frequently occurred with Diato).

6.3 Quality Assurance

The quality assurance discussion addresses replicate injections and carryover.

6.3.1 Replicate Injections

The average precision of replicate injections of the internal standard (ISTD) was 0.41% for dates during and immediately following the analysis of SeaHARRE-4 field samples.

As part of standard procedures, duplicate sample extract injections are performed daily. Aliquots of the extract of the first sample to be analyzed in a sequence of injections are added to two HPLC vials, then both vials are

placed in the TCAS at the same time, along with all other sample extracts prepared on that day (referred to here as the "daily sample set"). The first vial (of the two vials that contain the same sample extract) is the first injection of the daily sample set (but not the first injection of the sequence), and the second vial is injected after all other samples in the daily sample set have been injected.

The timing of the duplicate injections may differ by as much as 20–30 h. For SeaHARRE-4 samples, only one sample extract was injected two times in the manner described, therefore, data are included for other duplicate extract injections that were made on the same sequence as the SeaHARRE-4 field samples. The CV (in percent) was determined for TChl *a*, as well as the primary pigments (which were averaged and reported as an overall average PPig CV). For these replicate injections, which represent samples extracted on 5, 6, 7, 8, and 9 March 2007, the CV of TChl *a* was 0.31, 0.01, 0.28, 0.56, and 0.04%, respectively; the corresponding CV for PPig was 2.15, 3.88, 6.02, 1.64, and 1.88%, for the respective dates. The overall average CV for TChl *a* was 0.24% and for PPig it was 3.11%.

Four separate vials of the DHI Mix-102 were used for quality assessment. In all cases, when making HPLC injections, the contents of a DHI Mix-102 vial was dispersed into one or more HPLC vials (500 µL per vial); only one injection was performed from each HPLC vial. Both intravial injections (i.e., multiple injections from the contents of one vial of DHI Mix) and intervial injections (one injection each from contents of multiple DHI Mix vials) were made. Three intravial injections were performed on 4 March 2007, for which the CV was 0.2% (TChl *a*) and 0.2% (PPig); and six intervial injections were performed from 7–8 March 2007, for which the CV was 0.7% (TChl *a*) and 1.0% (PPig).

6.3.2 Carryover

In the HPL SeaHARRE-3 chapter, it was noted that HPL experienced substantial carryover immediately following SeaHARRE-3, which was alleviated by adding an acetone rinse to the solvent gradient (Table 28). During SeaHARRE-4, carryover for pigments was monitored by inspecting chromatograms for extraneous pigments of any internal standard or single standard injection. Carryover of ISTD was monitored by inspecting chromatograms that followed an ISTD injection, if the proceeding injection would not normally contain ISTD (such as a Chl *a* quality control, QC, or retention time mix). No carryover was observed during SeaHARRE-4.

6.4 Data Reporting

Pigments denoted as "not found" or ones with an SNR that is less than four were reported with a limiting value ($0.0001\,\mathrm{mg\,m^{-3}}$). Concentrations were rounded to four digits to the right of the decimal.

6.4.1 Governing Equation

Previously, HPL used multiple equations during the pigment calculation process. (See the SeaHARRE-2 and SeaHARRE-3 reports, Hooker et al. 2005 and 2009, respectively, for the equations and descriptions thereof.) Some of these equations are reiterated here. The amount of pigment injected ($\tilde{C}_{P_i}$) was calculated as

$$\tilde{C}_{P_i} \;=\; \hat{A}_{P_i}\, R_{P_i}, \tag{36}$$

where $\hat{A}_{P_i}$ is the area of the parent peak (and associated isomers) and R_{P_i} is the response factor (calibration factor).

To calculate the concentration of a pigment in a natural sample (C_{P_i}),

$$C_{P_i} \;=\; \frac{V_x}{V_f}\,\frac{\tilde{C}_{P_i}}{V_c}, \tag{37}$$

was used, where V_x is the extraction volume, V_c is the volume of sample extract injected onto the HPLC column, and V_f is the filtration volume.

Using a 'one-step' addition approach, an internal standard was used to correct V_x for residual water retained on the filter paper (plus any variations in volume caused by evaporation):

$$V_{x_1} \;=\; \frac{\hat{A}_{c_1}}{\hat{A}_{s_1}}\, V_m, \tag{38}$$

where $\hat{A}_{c_1}$ is the peak area of the internal standard (within the internal standard batch mixture) when it is injected onto the HPLC column prior to its addition to the sample (determined using a one-step internal standard methodology); $\hat{A}_{s_1}$ is the peak area of the internal standard in the sample; and V_m is the volume of extraction solvent (with internal standard) added to each sample filter.

While all of these basic equations are still in use, these equations have been merged with other basic calculation equations into one governing equation:

$$C_{P_i} = \frac{\hat{A}_{c_1}}{\hat{A}_{s_1}}\,\frac{V_m}{V_f}\,\hat{A}_{P_i}\left[\frac{A_{S_i}(\lambda_m) - A_{S_i}(750)}{\alpha_{S_i}(\lambda_m) l_c \Sigma \hat{A}_{s_i}}\right], \tag{39}$$

where α_{S_i} is the absorption coefficient for the pigment standard S_i associated with the pigment of interest P_i, λ_m is the reference wavelength associated with the absorption coefficient, l_c is the pathlength of the spectrophotometer cuvette (usually 1 cm), and A_{S_i} is the spectrophotometric absorbance value of the pigment standard (Sect. 1.3).

It can be noted that some of the variables from the previous equations do not appear in the governing equation. It is not that the variables are no longer of importance, but rather that the variables *drop out* or *cancel* (from the numerator and denominator) once multiple formulas with some of the same variables are combined. Quality control measurements are still made to ensure the accuracy and precision of such variables.

6.4.2 Data Products

The data products reported with the HPL method include: a) individual pigments, b) pigment sums (these are individually quantified pigments summed together), and c) pigments resulting from one or more unresolved pigments that are quantified collectively using a response factor for the most frequently abundant pigment at that retention time (e.g., Caro and Chl c_2).

Pigment standards frequently contain small isomers that are usually less than 10% of the total peak area for that standard. It is rather likely that isomers are also present in natural samples. Most often, however, these isomers are undetectable, because their peak areas are small relative to the parent peak, or they coelute with the main peak of a more dominant pigment than the parent pigment. Only isomers for MVChl *a*, DVChl *a*, Phytin *a*, and Peri are routinely quantitated for the HPL method, although the potential of isomers for coelution is described in Table 11 of Hooker et al. (2005). Isomers for Chl *b* have also been observed. In the SeaHARRE-4 samples, a relatively large peak was observed eluting near the main Peri peak. The retention time of this peak, however, did not match what was expected for the Peri isomer, therefore, it was not quantitated as part of Peri. Some other SeaHARRE-4 participants did identify this peak as the Peri isomer, so in these instances the Peri concentration reported by HPL would be lower than the average.

In SeaHARRE-4, the total area of all MVChl *a* allomers and epimers, plus the main Chl *a* peak, were summed; the nanograms per injection was determined by multiplying the resulting total peak area times the Chl *a* response factor. No DVChl *a* was observed in the SeaHARRE-4 samples, but DVChl *a* was in DHI Mix-102.

6.5 Discussion

Previous SeaHARRE HPL method chapters have discussed problems of pigment quantitation encountered by HPL (Hooker 2005 and 2009). Here, the discussion is limited to the problems that most probably contributed to degradation of accuracy with SeaHARRE-4 samples. During SeaHARRE-4, using a "can't say no" de-facto acceptance criteria, HPL did not reject small peaks (SNR $\leq$ 4) that had no, or inconclusive, spectra. These acceptances likely explain some of HPL's false-positive results relative to other A' laboratories, if they either rejected or did not integrate these small peaks. Similarly, there were some small Peri peaks that only yielded a spectral match if manual referencing were used. Differences among the laboratories' quantitative analysis procedures and differences in available software features, or the use of such, can lead to differences in quantitation, particularly of small peaks.

In SeaHARRE-4, all A' laboratories, including HPL, experienced undesirable average inaccuracy ($>$ 25% APD) for But, Hex, Diato, and Peri, with an overall A' average APD for these pigments of 65%. This behavior was largely a result of laboratories differing regarding whether a pigment was reported as present or not at the 12 sampling locations. The number of sites for which A' laboratories reached a consensus of agreement for each of these four pigments ranged between one and six, depending on pigment. In contrast, for the other eight primary pigments, A' laboratories agreed unanimously in all but three instances (one site for Allo and two for Zea). For each of these other eight primary pigments, the A' average accuracy was always 20% or less; the overall average among A' labs was 12%. These data emphasize the need for implementing measures that reduce the instances of false-negative and false-positive results (see Chap. 1 for definitions).

Pigments that are only weakly present in a sample extract (e.g., the SNRs are low) are detected by some laboratories, but may not be detected by others, and they are subject to differences in reporting practices. The "two-sentence rule" was intended to minimize such inconsistencies (Hooker et al. 2009), but it is evident that reporting practices in SeaHARRE-4 were still inconsistent when SNRs were low. For example, in SeaHARRE-4, 48 reporting opportunities existed for the pigments But, Hex, Diato, and Peri. Laboratories *G* and *H* reported these pigments, collectively, as present in 23% and 75% of instances, respectively, despite the fact that both laboratories conducted analyses with the same analysis method (Van Heukelem and Thomas 2001) on the same HPLC. Considering the specific example of But (for which the HPL average APD was 144.6%), HPL had five false-positive results. In each false-positive instance, the SNR was 11 or less. Because an SNR of this magnitude does not yield adequate absorption spectra, these peaks that were at the But retention time were reported by HPL (to be in compliance with the two-sentence rule) because they could not be proved to be other than But. This problem is not unique to HPL; any laboratory that is less aggressive in rejecting small peaks in a SeaHARRE activity is at risk of a higher frequency of false-positive results.

A "true" false-positive result would occur if a laboratory reported a pigment as present when the identity of the pigment was not supported by the absorption spectra and/or retention time. As SNR decreases, there is deterioration in the absorption spectra and there is a point at which interpretation of spectra becomes too ambiguous to make a determination. This so-called point-of-ambiguity varies with pigment, method, and spectral features of the chromatography software. To elaborate using the specific example of Peri in SeaHARRE-4—for which average A' APD was 65%—the first six sites exhibited much higher than normal APDs (A' average APD = 118%), but the last six sites did not (average A' APD = 12%). It is notable that the Peri peak height at the last six sites was sufficiently high to yield adequate spectra (at HPL SNR was $>$200), but the peak height was relatively low at the first six sites (SNRs ranged from 13–70 at HPL). An SNR of 70

is typically sufficient to yield unambiguous Peri spectra at HPL, but in the HPL Peri result for site 1 (for which HPL had a "false-positive" result and an APD of 398%), an unambiguous positive-identification spectra was only obtainable by disabling the "automatic referencing" feature of the software.

Most HPLC reprocessing software packages have the built-in capability to override automatic referencing during spectral analysis with a manual referencing function. This function allows analysts to select their own pair of reference points in the chromatogram before checking spectra for the peak of interest (typically points immediately before and after the peak of interest are chosen).

The utility of manual referencing when checking low-level spectra is undeniable, but should be used with prudence. It is improbable that even the same user can recreate the exact referencing conditions more than once, and even a minor change in referencing can lead to a difference in spectral interpretation. If an analyst uses a combination of automatic referencing and the two-sentence rule to interpret chromatograms, it is likely that the analyst may reach the conclusion of "can't say the pigment is not there" with many low SNR peaks. The use of manual referencing might provide some clarity on some of these spectral interpretations. It is likely, therefore, that the analyst will be able to come to a greater number of definitive "yes/no" assessments as opposed to a "can't say no" de-facto acceptance of a peak identity. This difference could cause an analyst's results to diverge with those of other analysts who use only automatic referencing, or who interpret their manual referencing results differently. The difference can even lead to variability in interpreting the spectrum of the same peak by the same analyst on multiple occasions, given that it is unlikely that the analyst will be able to select the same reference points (which must be selected each time the peak is inspected) multiple times. Manual referencing is a valuable tool, but has the potential to introduce more false positives and negatives to one's results.

6.6 Conclusions

The HPLC method used at HPL has been in use since 1999 and is capable of providing quantitative results for up to 25 different phytoplankton pigments. HPL documented the elution position and absorbance spectra of 58 pigments in a spectral library that can be used during the analysis of samples to assist pigment identification. HPLC pigment analysis is conducted at HPL within the framework of a detailed quality assurance plan. Limitations of the method are regularly reassessed as more diverse water types are analyzed. Such limitations cannot be known until an analysis method is used with all possible combinations of samples—a process that is not possible during initial method validation. As such, unforeseen coelution problems were encountered, which required HPL to disregard some pigments that were originally intended for quantitation.

Following SeaHARRE-4, several changes in the reporting practices of HPL were made and are summarized herein. Chromatographic peaks representing Chl c_2, Chl c_1, and MgDVP are now quantified as one entity, referred to as Chl c_{12}, because MgDVP, also a Chl c pigment, is frequently found in oligotrophic samples and interferes with accurate quantitation of both Chl c_2 and Chl c_1. Pigments are now categorized as either primary, secondary, tertiary, or ancillary—terms defined and used within the SeaHARRE activities.

Digits of precision have been reduced from four places (e.g., 0.0001) to three (e.g., 0.001). This action is consistent with recommendations of other SeaHARRE pigment analysts. Pigments are rarely quantifiable at concentrations less than $0.0005\,\mu g\,L^{-1}$, and, in fact, these low concentrations are often associated with uncertainties of similar magnitude. As an example, the average standard deviation of pigments in mesotrophic and oligotrophic samples is on the order of $0.0005\,\mu g\,L^{-1}$, as determined from replicate injections of sample extracts—a quality control analysis performed daily with all samples.

A new null value of $0.0009999\,\mu g\,L^{-1}$ is now used to represent pigments that are "not detected," as well as pigments that are present, but with concentrations less than $0.0005\,\mu g\,L^{-1}$. To put this in perspective, for primary pigments, the average limits of detection (SNR = 3) and quantitation (SNR = 10) for filtration and extraction volumes typical of oligotrophic samples are 0.0004 and $0.0013\,\mu g\,L^{-1}$, respectively. A value of 0.0009999, while it would give the appearance of a concentration of 0.001, is easily discernible from a value that would represent a concentration less than 0.0014, but greater than $0.0005\,\mu g\,L^{-1}$ (the range of values that would round to $0.001\,\mu g\,L^{-1}$). The null value is inserted after results have been rounded to three digits and higher-order data products have been computed.

Chapter 7

The JRC Method

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Abstract

The HPLC method adopted by the JRC is the Van Heukelem and Thomas (2001) method, as modified for SeaHARRE-3 (Van Heukelem and Thomas 2009). This method has been successfully applied to a wide range of pigment concentrations from oligotrophic to eutrophic coastal waters (Van Heukelem and Thomas 2009). Here, it allowed for the separation and the quantification of 22 different pigments and, in particular, the monovinyl and divinyl forms of chlorophyll *a*. The samples are extracted in a 100% acetone solution including an internal standard (vitamin E acetate) and analyzed by HPLC using a C_8 column with a binary solvent gradient. The different pigments are identified using a diode array detector on the basis of the absorption spectra at two different wavelengths (450 and 665 nm). The quality control of the data is assured by injecting a chlorophyll *a* standard at the beginning of each sequence, in order to check the calibration, as well as a mixture of pigments in order to check the retention times, and the system accuracy and precision.

7.1 INTRODUCTION

The HPLC method adopted by the JRC in 2007 and first tested during the present SeaHARRE-4 exercise is the Van Heukelem and Thomas (2001) method (VHT), as modified for SeaHARRE-3 (Van Heukelem and Thomas 2009). This method allows for the separation and the quantification of a larger number of taxonomically relevant pigments than the previously used Wright et al. (1991) method. In particular, the VHT method permits the chromatographic separation of the monovinyl and divinyl forms of chlorophyll *a*. The VHT method has been successfully applied to a wide range of pigment concentrations ranging from the oligotrophic central South Pacific Ocean sampling conducted for SeaHARRE-3 (Hooker et al. 2009) to the eutrophic coastal South African waters for SeaHARRE-2 (Hooker et al. 2005).

The main changes introduced with respect to Wright et al. (1991) are related to the extraction procedure, the column characteristics, and the mobile phase. The extraction time is shortened (4 h instead 24 h) and the samples are extracted in less solvent together with a different internal standard (vitamin E acetate). The filter used for the extract clarification has a lower surface area and allows for a more efficient recovery of the sample elute.

The column phase changed from C_{18} to C_8 with a length decreasing from 250–150 mm. The particle size decreased from 5 µm to 3.5 µm, thus inducing a better peak resolution. The change of mobile phase results in a very regular baseline not requiring a blank subtraction before the peak quantification.

The adoption of the VHT method resulted in an increase of method stability and repeatability, and shortened the analysis time.

7.2 EXTRACTION

The SeaHARRE-4 samples were received on 24 January 2007 and were stored at −80°C until they were analyzed (15–19 October 2007). The routine extraction procedure used for 47 mm GF/F filters was adapted, in terms of extraction volume used, to the 25 mm GF/F delivered for the SeaHARRE-4 exercise. All pipettes used in the extraction were gravimetrically calibrated using the same solvent used in the extraction procedure, as well as a precision balance (A200S, Sartorius Analytic).

The sample extraction procedure consisted of the following steps:

1. To start, 2.5 mL of a 250 mg mL^{-1} concentration of vitamin E acetate (Fluka) dissolved in 100% acetone (HPLC grade, Merck) were put, using a calibrated pipette (Dispensette 0–10 mL Brandt), into a 10 mL polypropylene plastic tube (Falcon) of 14 mL. Then, 100 µL of bidistilled water, produced using a Milli-Q system manufactured by Millipore (Billerica, Massachusetts),

were added using a calibrated pipette (Eppendorf). Accounting for the water retained on the 25 mm filter (Wright et al. 1991), the samples were extracted in a 86% acetone solution. The plastic tubes were placed for 30 min at −20°C.

2. The samples were cut into small pieces within the tubes. Each tube, after the samples were added, was capped and made airtight with parafilm, and soaked at −20°C for 1 h. The content of the tubes was disrupted by sonication using a Sonicator SonoPlus model GM2070 manufactured by Bandelin electronic (Berlin, Germany). The sonication lasted 90 s with the tube immersed in ice (approximately 0°C) in order to avoid the sample warming. The power was set at 50% (approximately 35 W), the pulsed cycle was set to 5 (at approximately 0.5 pulse s^{-1}), the time was 1 min, and the probe immersion was 10–15 mm. The sonicator probe was cleaned with wipes and acetone after each sample. After sonication, the tubes were capped and closed with parafilm. The samples were then soaked at −20°C for 4 h.
3. The samples were clarified by transferring the contents of the tubes into a plastic syringe (Plastibrand), connected to a Millex-FH filter, 0.45 µL (Millipore). The elute was collected in 4 mL amber vials. The 4 mL amber vials were mixed and an amount of approximately 1.5 mL was transferred by glass pipette into HPLC 2 mL amber vials. Mixing was accomplished using a Vortex Reax 2000 manufactured by Heidolph Brinkmann, LLC (Elk Grove Village, Illinois).

Finally, the sample extracts contained in the HPLC vials were transferred to the prechilled HPLC autosampler for HPLC analysis.

7.3 HPLC ANALYSIS

The HPLC system used for the analysis is an HPLC 1100 series Agilent Technologies running `ChemStation` software (B.01.03). The HPLC system is assembled with a microdegasser, a quaternary pump, a temperature-controlled autosampler compartment (set at 4°C), a programmable autoinjector (900 µL syringe and 900 µL injection loop), a temperature-controlled column oven compartment (set at 60°C), and a DAD.

The samples were maintained at 4°C in the autosampler in 2 mL amber vials with rubber caps, waiting for analysis. Each sample was mixed in the injector loop, alternated with the buffer, following the sequence in Table 30; the final amount injected onto the column was 375 µL. The column was a Zorbax Eclipse XDB-C8, which has a 3.5 µm particle size, 150 mm length, and a diameter of 4.6 mm, and is manufactured by Agilent Technologies (Santa Clara, California). No precolumn was used.

Table 30. The JRC HPLC injector program. The buffer solution is 90:10 (vol.) of 28 mM TbAA and methanol, respectively. The Metering Device Speed (MDS) is in units of microliters per minute, and the needles were washed in 90% acetone.

Step	*Injector Action*	*MDS*
1	Draw 150 µL from buffer	500
2	Draw 75 µL from sample	130
3	Needle rinse (Acetone 90%)	
4	Draw 75 µL from buffer	130
5	Draw 75 µL from sample	130
6	Needle rinse (Acetone 90%)	
7	Draw 150 µL from buffer	500
8	Inject loop contents onto column	

The gradient adopted is the three solvents gradient adopted by Van Heukelem and Thomas in SeaHARRE-3. Solvent A was a solution 70:30 volume of methanol (HPLC grade, Merck) and 28 mM TbAA (pH 6.5). The 28 mM solution was obtained by diluting 0.4 M TbAA hydroxide tritant (HPLC reagent, J.T. Baker) with bidistilled water (Milli-Q system, Millipore). The pH was adjusted to 6.5 by adding acetic acid.

Solvent A was filtered (mild vacuum) through a 0.2 filter of nitrocellulose (GNWP Millipore) and stocked in 5 L amber bottles waiting for the analysis: Solvent A was not stocked longer than two weeks in order to avoid algal growth. Solvent B was methanol (HPLC grade, Merck), and Solvent C is acetone (HPLC grade, Merck). The HPLC gradient is described in Table 31.

Table 31. The gradient system used with the JRC method. Solvent A is 70:30 methanol:28 mM TbAA (pH 6.5), solvent B is methanol, and solvent C is acetone; the flow is in units of milliliters per minute.

Time	A [%]	B [%]	C [%]	*Flow*
0	95	5	0	
22.00	5	95	0	1.1
24.50	5	95	0	1.1
24.75	5	65	30	1.3
25.75	5	65	30	1.3
25.85	5	65	30	1.3
26.10	95	5	0	1.1
29.10	95	5	0	1.1

The DAD measured absorbance at the 450, 665, and 222 nm wavelengths, with a bandwidth of 5 nm. Note that the 222 nm wavelength was used only for quantifying the internal standard.

7.4 CALIBRATION

When the new column was installed (June 2007), the system was calibrated over a 22 pigment standards set (DHI, Denmark; Fluka for chlorophylls *a* and *b*) including

all the pigments quantified. For each pigment, a multipoint calibration curve was determined based on nine dilutions. The dilutions were made from the standard using precalibrated syringes (Hamilton) while trying to minimize evaporation of the solvent. The syringes used for preparing the dilution were gravimetrically calibrated using the same solvent used for the dilution, as well as a precision balance (A200S, Sartorius Analytic). The calibration curve points were distributed between the expected LOD and the standard concentration. After injection into the HPLC system, the concentration of the standard and of its dilutions were checked using a dual-beam monochrometer spectrophotometer (Lambda 12, PerkinElmer). The content of each vial was transferred into a quartz cuvette, and two scan spectra were collected over the range 350–700 nm (2 nm bandwidth). The determination of the pigment concentration of each dilution was made considering the specific absorption coefficient indicated by DHI for the standard (Table 32) and the absorption measured at the corresponding wavelength.

Table 32. Absorption coefficient (α) values from DHI certificates as used with the JRC method for the pigments listed as a function of wavelength (λ).

Pigment	*Solvent*	λ	α
Chl c_3	90% Acetone	453.0	374.00
Chl c_1 + c_2	90% Acetone	443.0	262.00
Chlide *a*	90% Acetone	664.0	127.00
Phide *a*	90% Acetone	667.0	74.20
Peri	Ethanol	472.0	132.50
But	Ethanol	446.0	160.00
Fuco	Ethanol	449.0	160.00
Neo	Ethanol	439.0	224.30
Pras	Ethanol	454.0	160.00
Viola	Ethanol	443.0	255.00
Hex	Ethanol	447.0	160.00
Diad	Ethanol	446.0	262.00
Allo	Ethanol	453.0	262.00
Diato	Ethanol	449.0	262.00
Zea	Ethanol	450.0	254.00
Lut	Ethanol	445.0	255.00
Chl *b*	90% Acetone	646.0	51.36
DVChl *a*	90% Acetone	664.3	87.76
MVChl *a*	90% Acetone	664.3	87.76
Phytin *a*	90% Acetone	667.0	51.20
α-Caro	100% Acetone	448.0	270.00

The HPLC results and the standard concentration were corrected for pigment purity on the basis of the spectrophotometric data. The response factor for each pigment was determined from a linear fit performed on this multipoint calibration curve.

After the SeaHARRE-4 exercise, the HPLC instrument was recalibrated using the monovinyl chlorophylls *a* and *b* standards from DHI. The SeaHARRE-4 samples were reanalyzed after this second calibration. On average, the concentration of monovinyl chlorophyll *a* in the requantified samples was 14% lower than the value obtained with the Fluka standard, whereas the monovinyl chlorophyll *b* was 13% higher than the value obtained with the Fluka standard.

7.5 QUALITY CONTROL

Some quality control procedures were standardized for each sequence of analysis.

1. At the beginning of each sequence, a solution of $1\,\mathrm{mg\,L^{-1}}$ of chlorophyll *a* (Fluka) was analyzed;
2. An injection of a mix standard was performed every 10 analyses to check the retention time and the analysis reproducibility;
3. After the first injection of the mix standard, an internal standard was injected for checking the carryover;
4. The first sample injected at the beginning of the sequence was injected at the end to check for any degradation or variation in quantification.

All the data relative to the quality control, together with the data relative to the noise control for each sequence, were statistically recorded.

7.6 LOD AND LOQ

The LOD and LOQ were experimentally determined by creating a dilution series for each analyzed pigment with known concentrations corresponding to 3 and 10 times the SNR. Subsequent to this system verification, during the routine analysis, the LOD was set as four times the SNR relative to the sequence. Because the SNR is expected to change with the instrument condition, the SNR is calculated separately for each sequence and is determined by the average of the SNR sampled at different time intervals after the first injection of the internal standard. Regarding the SeaHARRE-4 sequences, the effective LOD was varied from 0.0003–0.0028 $\mathrm{mg\,m^{-3}}$ for the pigment quantified at 450 nm, and from 0.0004–0.004 $\mathrm{mg\,m^{-3}}$ for the pigment quantified at 665 nm.

7.7 DATA PRODUCTS

The chromatographic information (peak area, peak height, retention time, and compound amount) relative to every sample was exported from the `ChemStation` Software (Agilent) and transferred into an `Excel` spreadsheet. Pigment concentrations were calculated as:

$$C_{P_i} = \frac{V_x}{V_f}\,\frac{\hat{A}_c}{\hat{A}_s}\,\frac{\hat{A}_{P_i}}{V_c}\,R_{P_i}, \qquad (40)$$

where $\hat{A}_{P_i}$ and R_{P_i} are the peak area and the response factor of pigment P_i, respectively; V_x is the extraction volume; V_f is the volume of filtered sample; V_c is the amount of sample injected onto the column; A_c is the peak area of the internal standard in the extraction solvent; and A_s is the peak area of the internal standard in the sample.

7.8 CONCLUSIONS

Since the SeaHARRE-3 activity, the JRC changed the HPLC method that was being used and adopted the Van Heukelem and Thomas (2009) method. This allowed the separation and the quantification of a larger number of taxonomically relevant pigments than the previously used Wright et al. (1991) method. In particular, the new method permitted the separation of the monovinyl and divinyl forms of chlorophyll *a*, plus lutein and zeaxanthin. During SeaHARRE-4, the effective LOD varied from 0.0003–0.0028 mg m^{-3} for pigments quantified at 450 nm, and from 0.0004–0.004 mg m^{-3} for pigments quantified at 665 nm.

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Chapter 8

The LOV Method

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Abstract

The LOV method, derived from the technique described by Van Heukelem and Thomas (2001), applies a sensitive reversed-phase HPLC technique for the determination of chloropigments and carotenoids within 28 min. The different pigments, extracted in methanol, are detected by diode array detector (DAD), which allows for automatic identification to be carried out on the basis of absorption spectra. Optical densities are monitored at 450 nm (chloropigments and carotenoids), 667 nm (chlorophyll *a* and derived pigments), 770 nm (bacteriochlorophyll *a*) and 222 nm (vitamin E acetate internal standard). The method provides good resolution between most pigments, but uncertainties may arise because of the partial separation of chlorophyll *b* and divinyl chlorophyll *b*, for the resolution of chlorophyll *c* pigments, and for the separation of α- and β-carotenes. It has proven to be efficient over a wide range of trophic conditions, from eutrophic upwelling waters, to the hyperoligotrophic South Pacific Subtropical Gyre. Short-term and long-term quality control is monitored regularly to ensure state-of-the-art analyses. The injection precision of the method is estimated at 0.3%, and the limits of detection for most pigments are low (0.019 ng inj^{-1} for chlorophyll *a*, and 0.033 ng inj^{-1} for carotenoids).

8.1 INTRODUCTION

The present method applied at the LOV is derived from the method described by Van Heukelem and Thomas (2001), with modifications aimed at increasing the sensitivity. It is a reversed-phase HPLC technique (C_8 column), based on a binary gradient and allows for the determination of most chloropigments (including degradation products) and carotenoids, which are extracted in 100% methanol and analyzed within 28 min. Detection is carried out at four wavelengths:

- 450 nm for carotenoids and chlorophylls *b* and *c*;
- 667 nm for chlorophyll *a* and derived products;
- 770 nm for bacteriochlorophyll *a*, and
- 222 nm for the internal standard (vitamin E acetate).

A very good resolution is achieved for most pigments ($R_s < 1.5$), although only partial resolution ($R_s < 1$) can be obtained for chlorophyll *b* and divinyl chlorophyll *b* and chlorophyll c_2, chlorophyll c_1, and MgDVP. Coeluting pairs include chlorophyllide *a* and chlorophyll c_1 (but they are quantified at different wavelengths), as well as $\beta\varepsilon$-carotene and $\beta\beta$-carotene. Pigment identification is based both on absorption spectra and retention time.

8.2 EXTRACTION

The SeaHARRE-4 *in situ* samples (stored and transported in liquid nitrogen) were stored at −80°C until analysis. The filters were extracted and analyzed between the 25 June and 2 July 2007.

The extraction process involved the following steps:

1. The 25 mm GF/F filter was placed into a 10 mL disposable Falcon tube.
2. A 3 mL volume of 100% methanol, including an internal standard (vitamin E acetate, Sigma-Aldrich), was added to each tube using an Eppendorf pipette, while making sure that the filter was completely covered. The tube was closed with an airtight cap.
3. The samples were placed in a −20°C freezer for a minimum of 30 min.
4. The filters were then disrupted using an ultrasonic probe (Bandelin Sonopuls HD2200† for 10 s maximum. The probe was rinsed with methanol and then wiped between each sample. In order to preserve the pigments from heating due to ultrasounds, the tubes were placed in an ice-filled beaker during sonication.

† Bandelin Electronic (Berlin, Germany).

5. The tubes were returned to the freezer for another 30 min minimum.
6. The samples were clarified by vacuum filtration through GF/F (0.7 µm particle retention size, 25 mm diameter) filters using a Millipore‡ filtration unit. A glass tube (cleaned with methanol and wiped between each sample) was used to press the sample slurry. The filtrate was collected in 10 mL Falcon tubes, and closed with airtight caps.
7. Clarified extracts were stored at −20°C until HPLC analysis (within 24 h).

8.3 HPLC ANALYSIS

Pigment analyses were carried out on a complete Agilent Technologies 1100 series HPLC system, which is made up of the following components:

- Degasser;
- Binary pump;
- Automated sampler, including Peltier temperature control (set at 4°C) and a programmable autoinjector with sample preparation prior to injection;
- programmable column oven compartment;
- DAD; and
- `ChemStation for LC` (A.09.03).

The sample extracts and standards were transferred into 2 mL glass vials using disposable glass Pasteur pipettes. The vials were then placed in the autosampler at 4°C for less than 24 h. Sample preparation (mixture between buffer (28 mM aqueous TbAA) and sample on a 1:1 basis) was carried out in the 500 µL loop just before injection, with alternating volumes of buffer and sample in order to favor mixing in the loop. The total injection volume was 250 µL. Although this injection mixture is optimized for methanolic extracts, standard solutions in ethanol or acetone, which are used for calibration, do not present the peak fronting problems that were encountered during SeaHARRE-2.

In order to increase sensitivity, the modifications to the Van Heukelem and Thomas (2001) method included injection onto a narrow diameter, "solvent saving," Zorbax Eclipse XDB-C8 column (3×150 mm, 3.5 µm), and a 0.55 µL min^{-1} flow rate. The fact that the pigments were in methanol also allowed for a larger injection volume. The column temperature was maintained at 60°C.

Separation was based on a linear gradient between a 70:30 methanol-to-TbAA 28 mM mixture and a 100% methanol solution (solvent A and B respectively), ranging from 10–95% B in 22 min, followed by an isocratic hold at 95% B for 5 min more. At the end of the run, the mobile phase returned to initial conditions (10% B) for a column equilibration time of 5 min (Table 33).

‡ Millipore (Billerica, Massachusetts).

Table 33. The gradient used for the LOV method. 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	90	10
2	22	5	95
3	27	5	95
4	28	90	10
End	33	90	10

Detection was carried out at three different wavelengths (10 nm bandwidths): 450 nm for all carotenoids, chlorophylls *c* and *b*; 667 nm for divinyl chlorophyll *a*, chlorophyll *a*, and associated allomers and epimers, as well as chlorophyllide *a* and phaeopigments; and 770 nm for bacteriochlorophyll *a*. The internal standard was detected at 222 nm where there is no interference from phytoplankton pigments. For all signals, a reference at 850 nm was applied to compensate for fluctuations caused by baseline absorbance.

The offline version of the `ChemStation` software was used for verification and eventual correction of the peak integrations in each chromatogram. Automatic spectral identification was also applied and then checked; only then would the individual pigment concentrations be calculated using a `Visual Basic` program under `Excel`.

8.4 CALIBRATION

A calibration was performed in July 2007, shortly after the analysis of the SeaHARRE-4 samples. The concentrations for nine pigment standards (Peri, But, Fuco, Hex, Allo, Zea, Pras, chlorophyll *a*, and bacteriochlorophyll *a*) provided by DHI International Agency for ^{14}C determination and Sigma-Aldrich, were determined by spectrophotometry using a PerkinElmer Lambda 19 dual-beam spectrophotometer (2 nm slit, 400–800 nm spectral range, with a correction at 700 nm). The multipoint calibration curves were composed of 4–10 points, and the corresponding response factors at 440, 667, and 770 nm were determined by HPLC analysis of each standard solution.

The response factors for divinyl chlorophyll *a* and divinyl chlorophyll *b* were computed from the following:

- Knowing the specific absorption coefficients of chlorophyll *a* (or chlorophyll *b*);
- Accounting for the absorption of chlorophyll *a* and divinyl chlorophyll *a* (or chlorophyll *b* and divinyl chlorophyll *b*) at 667 nm when the spectra are both normalized at their red maxima; and
- Considering that both pigments have the same molar absorption coefficient at this red maximum.

The same process was used for determining the Chlide *a* absorption coefficient relative to chlorophyll *a*. For the remaining pigments, their specific absorption coefficients

Table 34. The α values (in liters per gram per centimeter), associated with their respective wavelengths (λ, in nanometers), used in the LOV method for a variety of pigments that are listed in the same order as their retention times (t_R).

Pigment	*Solvent*	λ	α	t_R [min]	*Reference*
Chl c_3	90% Acetone	630.6	42.60	4.33	As for Chl c_2
Chl c_2	90% Acetone	630.6	42.60	5.83	DHI
Mg DVP	90% Acetone	630.6	42.60	5.86	As for Chl c_2
Chl c_1	90% Acetone	630.6	42.60	5.90	DHI
Chlide *a*	90% Acetone	664.0	127.00	5.90	Jeffrey et al. 1997
Phide *a*	90% Acetone	667.0	74.20	7.30	Jeffrey et al. 1997
Peri	100% Ethanol	472.0	132.50	8.97	DHI
But	100% Ethanol	446.0	160.00	11.91	DHI
Fuco	100% Ethanol	449.0	160.00	12.16	DHI
Neo	100% Ethanol	437.0	224.30	12.86	DHI
Pras	100% Ethanol	454.0	160.00	13.10	DHI
Viola	100% Ethanol	441.0	255.00	13.49	DHI
Hex	100% Ethanol	447.0	160.00	13.75	DHI
Diad	100% Ethanol	446.0	262.00	14.80	DHI
Allo	100% Ethanol	453.0	262.00	16.30	DHI
Diato	100% Ethanol	452.0	262.00	16.80	DHI
Zea	100% Ethanol	450.0	254.00	17.34	DHI
Lut	Diethyl Ether	445.0	248.00	17.53	Jeffrey et al. 1997
BChl *a*	100% Acetone	770.0	54.67	21.60	Oelze 1985
DVChl *b*	90% Acetone	646.8	51.47	21.61	Derived from Chl *b*
Chl *b*	90% Acetone	646.8	51.36	21.72	DHI
DVChl *a*	90% Acetone	664.3	87.87	23.49	Derived from Chl *a*
Chl *a*	90% Acetone	664.3	87.67	23.68	DHI
Phytin *a*	90% Acetone	667.0	51.20	25.22	Jeffrey et al. 1997
$\beta\varepsilon$-Car	100% Ethanol	453.0	262.00	26.32	As for $\beta\beta$-Car.
$\beta\beta$-Car	100% Ethanol	453.0	262.00	26.38	DHI

were either derived from previous calibrations or from the literature (Jeffrey et al. 1997a). The absorption coefficients for the LOV standard pigments are listed in Table 34.

Because $\beta\varepsilon$-Car and $\beta\beta$-Car coelute, the peak was first identified spectrally. The spectral shape, therefore, pointed to either one dominant pigment or the other and it was quantified as such.

8.5 VALIDATION

Short-term quality control (during a sequence run) was monitored using the methanol-plus-internal standard solution, which was injected twice at the beginning of the sequence and once after 10 sample injections. This was done to verify retention time reproducibility, peak area precision (should be less than 1%), and instrument stability during the analytical sequence. The first two injections of the sequence, however, were discarded because they generally tended to lack reproducibility. For troubleshooting purposes, the pressure signal was also monitored during the analyses.

The identification of individual pigments was manually checked by retention time comparison and observation of the absorption spectra using the `ChemStation` spectral library. This pigment library comprises the retention times and spectral information of different pigments obtained from the analysis of standard solutions or identified phytoplankton cultures.

Long-term quality control is carried out using a mixed pigment standard supplied by DHI. This standard is regularly injected, at least in triplicate, to monitor the quality of the column and of the instrument performance. This is represented by a number of parameters, including a) the initial backpressure, b) the noise level at 450 and 667 nm, c) the injection precision of chlorophyll *a* and fucoxanthin, d) the accuracy of these pigments, e) their plate numbers, peak widths, and retention times, and f) the resolution for two critical pairs (DVChl *a*–Chl *a* and But–Fuco). Signs of deterioration of the column can, therefore, be rapidly detected. Generally, a column is changed every 2,000 samples, although they have shown to last even longer.

The calibration of the volumetric measuring devices (pipettes, syringes, etc.) is carried out annually. These data are used to provide the performance metrics that were determined during the previous SeaHARRE-2 exercise (Table 42 in Hooker et al. 2005). In this way, the

objective is to evaluate and maintain the state-of-the-art level of analysis at the LOV.

If a technical problem should prevent the analysis of already extracted samples within the 24 h limit, the extracts are stored under nitrogen gas and placed in a −80°C freezer until routine analysis is re-established.

8.6 DATA PRODUCTS

The `ChemStation for LC` program produces a spreadsheet 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 P_i, as in the following equation:

$$C_{P_i} = \frac{\hat{A}'_{P_i}}{V_f} R_{P_i}, \tag{41}$$

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

$$\hat{A}'_{P_i} = \frac{\hat{A}_{c_1}}{\hat{A}_{s_1}} \hat{A}_{P_i}, \tag{42}$$

where $\hat{A}_{P_i}$ is the uncorrected peak area (mAU), $\hat{A}_{c_1}$ is the reference area (in mAU) of the internal standard (established as the average of internal injections over a single day) and $\hat{A}_{s_1}$ is the area of the internal standard in the sample.

8.7 CONCLUSIONS

During this exercise, the LOV method has proven to be well adapted to the high chlorophyll, particle-rich, coastal samples. Generally, a very good resolution was obtained for most pigments, although uncertainties may arise for the resolution of chlorophyll *c* pigments or $\beta\varepsilon$-Car and $\beta\beta$-Car, which tend to coelute. Problems did occur because of the presence of many unidentified small peaks that sometimes influenced integration, for example, neoxanthin. This also pointed to a lack of consistency that existed in reporting or not reporting the very small peaks. The limits of detection are estimated to be $0.019\,\mathrm{ng\,inj^{-1}}$ for chlorophyll *a* and $0.033\,\mathrm{ng\,inj^{-1}}$ for carotenoids. Effective LOD is not mentioned this time because of the high variability in filtration volumes for these samples.

Chapter 9

The DalU Method

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Abstract

The HPLC pigment technique used at Dalhousie University follows the method developed by Wright et al. (1991). It is a reversed-phase HPLC procedure (C_{18} column), based on a tertiary gradient and includes a cooled autosampler, and photodiode array and fluorescence detectors. Samples are disrupted with a sonic probe in 100% methanol including an internal standard (vitamin E acetate). The method is validated with the use of internal and external standards. It does not allow for the separation of divinyl chlorophylls *a* and *b* from their respective monovinyl forms. In addition, chlorophyll c_1 and chlorophyll c_2 are not separated, and neither is lutein from zeaxanthin; however, it offers good separation of a variety of pigments in a relatively short period of time (29 min).

9.1 INTRODUCTION

The Dalhousie University protocol follows the method detailed in Wright et al. (1991). It is a reversed-phase HPLC procedure using a C_{18} column), which is based on a tertiary gradient. Chlorophyll c_1 and chlorophyll c_2 are not separated, and neither is lutein from zeaxanthin. Chlorophyllide *a* coelutes with chlorophyll c_2, but the identification and quantification can be done on different detectors; therefore, the interference between the two pigments is avoided.

It does not allow the separation of divinyl chlorophylls *a* and *b* from monovinyl chlorophylls *a* and *b*, however, this does not interfere with the routine analysis conducted at Dalhousie University because most phytoplankton samples analyzed come from temperate coastal waters where the producer of divinyl chlorophylls, i.e., *Prochlorococcus,* is not abundant. *Prochlorococcus* has not been found in the well-studied Nova Scotian inlet, Bedford Basin (Li and Dickie 2001).

9.2 EXTRACTION

The SeaHARRE-4 samples were received on 16 January 2007, stored immediately in liquid nitrogen, and analysis was started on 30 March 2007. All procedures were carried out under dim light and samples were kept on ice. Only 12 samples per batch were extracted at a time, which minimized the risk for pigment degradation while samples sat in the autosampler. Filters were taken out of liquid nitrogen, cut in to three pieces and put in a 4 mL amber glass vial; 2 mL of extraction solvent was added to the vials with a gravimetrically calibrated automatic pipette. This solvent consisted of 100% methanol with approximately 0.125 mg L^{-1} vitamin E acetate internal standard. After tightly capping the vials, the samples were stored for 2 h in a −20°C freezer. After this time period, samples were transferred into a 10 mL BD syringe, the tip of which was closed with Parafilm before sonicating them on pulse mode for 20 s with a sonicator probe. Samples were vortexed for 5 s and transferred into a 2.5 mL Eppendorf centrifuge tube and centrifuged at 13,000 rpm for 3 min. The supernatant was transferred into a 2 mL autosampler vial and stored at −20° until all samples were ready to be analyzed.

9.3 HPLC ANALYSIS

The Agilent 1100 series HPLC system consisted of a cooled autosampler (4°C), quaternary pump, and photodiode array and fluorescence detectors. Immediately prior to injection, 100 µL of sample was mixed three times with 40 µL of buffer solution within the 500 µL sample loop. The buffer solution consisted of 0.5 M ammonium acetate (pH 7.2). A total volume of 140 µL was injected in the HPLC system. Pigments were separated with a Zorbax Eclipse XDB-C18 150 mm×4.6 mm column with a 5 µm particle size (Agilent Technologies). The column temperature was not controlled by a thermostat, and pigments were separated at ambient temperature. The flow rate was 1 mL min^{-1} and the gradient elution program is given in Table 35.

Table 35. The gradient elution program for the DalU method (Wright et al. 1991). The time is in minutes, and the percentages of solvents A, B, and C are given in the last three columns where solvent A is 80:20 100% methanol:0.5 M ammonium acetate (pH 7.2); solvent B is 90:10 100% acetonitrile:Milli-Q water; and solvent C is 100% ethyl acetate.

Time [min]	A [%]	B [%]	C [%]
0	100	0	0
4	0	100	0
18	0	20	80
21	0	100	0
24	100	0	0
29	100	0	0

Peak integration was initially performed by Agilent `ChemStation` software, and all peaks were manually identified by retention time and spectra signature comparison with known pigment standards. Absorption peaks of chlorophyll *a* and phaeopigments were detected at 436 nm, and carotenoids were detected at 450 nm.

9.4 CALIBRATION

All standards, except for chlorophyll *a*, were provided by DHI. Concentration of the chlorophyll *a* standard (Sigma-Aldrich) stock solution was determined using a Cary 3 UV/Vis dualbeam spectrophotometer with a 2 nm bandwidth. The absorption spectrum was recorded between 300–800 nm. The chlorophyll *a* concentration was calculated using a specific absorption coefficient of 79.95 L $g^{-1}cm^{-1}$ at 665.2 nm for a solution of 100% methanol (Porra et al. 1989). Any absorption measured at 750 nm was subtracted from the absorption measured at 665.2 nm. Concentrations provided by DHI for all other stock standard pigment solutions were used to calculate concentrations for the 4–6 points dilution series. Dilutions were prepared with gravimetrically calibrated automatic pipettes and Hamilton glass syringes.

More dilutions at low concentrations for chlorophyll *a* and fucoxanthin permitted calculation of the relative limits of detection (LOD) and quantification (LOQ) for all pigments (Hooker et al. 2005). Signal-to-noise ratios (SNRs) of 3 and 10 were used for the LOD and LOQ, respectively. The detection limit for most pigments ranged from 0.5–1.1 $\mu g\,L^{-1}$ per injection, and 1.3–1.9 $\mu g\,L^{-1}$ for the LOQ. All calibration curves were linear regressions of area versus concentrations forced through zero. Their correlation coefficients (r^2) were always better than 0.99 and generally, absolute percent residuals were no more than 2% as recommended by Hooker et al. (2005).

9.5 VALIDATION

At the beginning of an HPLC sequence run, four injections preceded the sample set. The first injection was of 100% methanol. This first chromatogram was discarded and served as the instrument "warm-up." Next, a chlorophyll *a* standard solution was used to verify the stability in the response factor of this pigment. Then, a mixed standard solution (that was provided by DHI) was injected to ensure good retention time and pigment separation. Finally, the vitamin E acetate internal standard was used in the quantification equation. After the 12 samples were injected, the internal standard, mixed pigment standard, and chlorophyll *a* standard were reinjected to ensure that no degradation or retention time shift occurred during the sequence.

9.6 DATA PRODUCTS

The concentration (C_{P_i} in micrograms per liter) of each pigment in the sample was determined using the following equation:

$$C_{P_i} = \frac{V_x}{V_f}\,\frac{\hat{A}_c}{\hat{A}_s}\,\frac{\hat{A}_{P_i}}{F_{P_i}}, \tag{43}$$

where the inverse response factor F_{P_i} is the slope obtained by plotting the peak area with the concentrations of a specific pigment standard injected; $\hat{A}_{P_i}$ is the peak area; V_x is the extraction volume (in milliliters); V_f is the volume of sample filtered (in milliliters); $\hat{A}_c$ is the peak area of the internal standard when it is injected onto the HPLC column; and $\hat{A}_s$ is the peak area of the internal standard in the sample.

Standard deviation and unbiased percent differences were calculated on all replicates and were used as quality control measures. High deviants were revisited to ensure that no errors in pigment identification, integration, or quantification were present. Concentration values less than the LOQ were not reported and were replaced by the respective LOQ values for the pigment.

In the SeaHARRE-4 samples, partial coelution was frequent between 19′-hexanoloxyfucoxanthin, prasinoxanthin, and violaxanthin, especially when peak areas were large. In the samples routinely processed at Dalhousie University, prasinoxanthin is generally absent, or is in very low concentrations; thus, coelution with this group of pigments is not a problem.

9.7 CONCLUSIONS

This SeaHARRE exercise demonstrated that the reversed-phase HPLC method used by Dalhousie University is a useful method for routine analysis of pigments as long as the separation of divinyl chlorophyll *a* and *b* from monovinyl chlorophylls *a* and *b*, and lutein from zeaxanthin is not critical to the research objectives. Indeed, it provides good resolution of most marine pigments, as well as consistent and repeatable pigment compositions and concentrations.

Chapter 10

The SDSU (CHORS) Method

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Abstract

The CHORS method was developed to provide HPLC phytoplankton pigment analyses for the NASA Sensor Intercomparison and Merger for Biological and Interdisciplinary Oceanic Studies (SIMBIOS) program, following the protocols presented in the *Ocean Optics Protocols for Satellite Ocean Color Sensor Validation* (Bidigare et al. 2002). The method (Wright et al. 1991) was designed to support a wide range of pigment concentrations from waters sampled throughout the world ocean, but it does not chromatographically separate the divinyl chlorophylls *a* and *b* from their corresponding monovinyl forms. The Latasa et al. (1996) dichromatic equations were used to spectrally resolve divinyl chlorophyll *a* from monovinyl chlorophyll *a*. The method uses a reversed-phase C_{18} column, with a tertiary solvent gradient; a temperature-controlled autosampler provides continuous sample injection to maintain a quota of analyzing 4,000 samples per year. System calibration is routinely monitored and recorded each month to ensure repeatability and consistency of data products.

10.1 INTRODUCTION

The current focus of HPLC analysis at CHORS is to characterize phytoplankton pigments in the water column collected as part of SIMBIOS field campaigns. The method follows the HPLC protocols established in the *Ocean Optics Protocols for Satellite Ocean Color Sensor Validation* (Bidigare et al. 2002). The chromatographic analyses do not separate monovinyl and divinyl chlorophyll *a*; instead, these pigments are quantified using dichromatic equations at 436 and 450 nm.

Because of the large number of samples processed, approximately 4,000 or more per year, the CHORS HPLC laboratory procedures have been streamlined to accommodate the variety and quantity of water samples collected by the SIMBIOS research community. By using larger HPLC solvent reservoirs, multiple sample tube racks, and running the system 24 h a day, the CHORS laboratory can accommodate 500 samples a month, with the system running continuously over a 3–4 week period.

10.2 EXTRACTION

The SeaHARRE-4 samples were stored in liquid nitrogen pending extraction and analysis upon receipt at CHORS. The filters were extracted in 4.0 mL of 100% acetone using a Brinkmann Bottletop Dispenser (with a coefficient of variation of 0.321%). An internal pigment standard, *trans*-β-apo-8′-carotenal, was added to the acetone extract prior to pipetting the 4.0 mL, to correct for any extraction volume changes during sample processing.

After 24 h of extraction in a freezer (maintained at $-20°$C), the samples were sonicated for 10 s using an ultrasonic microprobe tip at a 60% duty cycle. The samples were then extracted for an additional 24 h (stored in a freezer at $-20°$C). Glass-fiber particles, generated during filter sonication, were removed from the extract by centrifuging the samples at 5,100 rpm for 4 min and then subsequently filtering the extract using 0.2 µm PTFE in-line filters.

10.3 HPLC ANALYSIS

The equipment used with the CHORS method is as follows:

- ThermoQuest HPLC system with membrane degasser and P4000 quaternary pump;
- AS3000 temperature-controlled autosampler;
- UV6000 PDA detector (scanning 436 and 450 nm);
- FL3000 fluorescence detector; and
- System controller with `ChromQuest` (v3.0) software.

The HPLC gradient program was the same one specified in the Ocean Optics Protocols for pigment analysis (Bidigare et al. 2002). Pigments were separated on a reversed-phase, Waters Spherisorb ODS-2 (5 µm) C_{18} column (250 mm long with a 4.6 mm internal diameter), using a three-solvent gradient system and a 1.0 mL min^{-1} flow rate (Table 36).

A ThermoQuest P4000 pump and an AS3000 Autosampler (maintained at 4°C) were used for processing all samples. Each sample extract was mixed (605 mL:195 mL of extract to water), and 100 mL of this mixture was injected onto the HPLC column.

Table 36. The tertiary gradient elution system used with the CHORS HPLC method. Solvent A is 80:20 methanol:0.5 M ammonium acetate; solvent B is 90:10 acetonitrile:water; and solvent C is ethyl acetate.

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	20.0	0	31	69
6	22.0	0	100	0
End	25.0	100	0	0

The separation of the various pigments requires approximately 25 min with the pigment peaks being detected by an absorption detector: a ThermoQuest UV6000 scanning DAD (190–800 nm at 1 nm resolution). Pigments were monitored at 436 and 450 nm, with peak retention time or spectrophotometric recognition used to assign peak identification. In addition, a ThermoQuest FL3000 scanning fluorescence detector was used to detect the various chlorophyll degradation products, which occur at lower concentrations.

Although the absorption peaks for monovinyl and divinyl chlorophyll *a* coelute, each compound absorbs differently at 436 and 450 nm, so it was possible to correct for the divinyl chlorophyll *a* contribution by monitoring changes in the 450/436 ratio as a function of changes in the divinyl percentage (Latasa et al. 1996).

Accuracy for each pigment compound was based on the availability of pigment standards and the selection of pigment-specific absorption coefficients. The standard fluorometric method of Hølm-Hansen et al. (1965) was used to calculate chlorophyll and phaeopigment concentrations on an aliquot (100 µL) of the pigment extract. These concentrations were also corrected for extraction volume changes using the internal standard. The Ocean Optics Protocols (Trees et al. 2003) for fluorometric chlorophyll *a* was also followed.

10.4 CALIBRATION

The HPLC pigment calibration standards were purchased from Sigma-Aldrich and DHI. System calibrations were performed to determine individual standard response factors for each compound. Each pigment response factor was determined using a multipoint calibration. The concentration of the standards were provided by DHI, or verified spectrophotometrically (for chlorophyll *a* and chlorophyll *b*) using published absorption coefficients (Table 37). The coefficient of determination for the chlorophyll *a* calibration curve was $r^2 = 0.999$. The coefficient of variation for the internal standard average was about 3.8%.

Table 37. The α values used with the CHORS method for a variety of pigments as a function of λ. The units for α are liters per gram per centimeter, and the units for λ are nanometers.

Pigment	*Solvent*	λ	α
Chlide *a*	90% Acetone	664.0	127.00†
Chl c_3	90% Acetone	452.9	346.00†
Chl c_2	90% Acetone	630.9	40.40†
Peri	100% Ethanol	472.0	132.50†
But	100% Ethanol	446.0	160.00†
Fuco	100% Ethanol	449.0	160.00†
Neo	100% Ethanol	439.0	224.30†
Hex	100% Ethanol	447.0	160.00†
Pras	100% Ethanol	454.0	160.00†
Viola	100% Ethanol	443.0	255.00†
Diad	100% Ethanol	446.0	262.00†
Allo	100% Ethanol	453.0	262.00†
Diato	100% Ethanol	449.0	262.00†
Lut	100% Ethanol	445.0	255.00†
Zea	100% Ethanol	450.0	254.00†
Chl *b*	90% Acetone	646.6	51.36‡
MVChl *a*	90% Acetone	663.5	87.67‡
DVChl *a*	90% Acetone	664.3	87.67†
Phytin *a*	90% Acetone	667.0	51.20†
$\beta\varepsilon$-Car	100% Acetone	448.0	270.00†
$\beta\beta$-Car	100% Ethanol	453.0	262.00†

† DHI Water and Environment
‡ Jeffrey et al. (1997a)

10.5 VALIDATION

The integration of pigment peaks was performed using `ChromQuest` (v3.0) software, and manually checked to ensure each peak was properly integrated. Retention times and spectral signatures of the standards were used to verify peak identification. Peak areas were quantified using software-aided integration. Response factors generated during the system calibration were used for final concentration calculations.

10.6 DATA PRODUCTS

Each sample data file included the integrated peak area, which was used with the pigment response factors to compute pigment concentration:

$$C_{P_i} = \frac{V_m}{V_f}\frac{V_c}{V_s}\frac{\hat{A}_{c_1}}{\hat{A}_{s_1}}\hat{A}_{P_i}/F_{P_i}, \tag{44}$$

where V_m is the volume of extract (solvent plus standard) added (in milliliters); V_f is the volume of sea water filtered

(in liters); V_c is the volume of sample extract injected onto the column; V_s is the volume of standard injected to determine the inverse response factor, F_{P_i}; $\hat{A}_{c_1}$ is the average peak area of 25 internal standard injections; $\hat{A}_{s_1}$ is the peak area of the internal standard in the sample; and $\hat{A}_{P_i}$ is the peak area of pigment P_i. For the CHORS method, $V_c = V_s$, so (1001) can be simplified to:

$$C_{P_i} = \frac{V_m}{V_f} \frac{\hat{A}_{c_1}}{\hat{A}_{s_1}} \frac{\hat{A}_{P_i}}{F_{P_i}}. \quad (45)$$

The following pigments are reported:

- Chlorophyllide *a*,
- Chlorophyll c_3,
- Chlorophyll c_2,
- Chlorophyll $c_{1+}c_2$,
- Peridinin,
- 19′-Butanoyloxyfucoxanthin,
- Fucoxanthin,
- 19′-Hexanoyloxyfucoxanthin,
- Prasinoxanthin,
- Violaxanthin,
- Diadinoxanthin,
- Alloxanthin,
- Diatoxanthin,
- Lutein,
- Zeaxanthin,
- Chlorophyll *b*,
- Chlorophyll *a*,
- $\beta\varepsilon$-Carotene,
- $\beta\beta$-Carotene,
- Monovinyl chlorophyll *a*,
- Divinyl chlorophyll *a*,
- Total chlorophyll *a*,
- Fluorometric chlorophyll, and
- Fluorometric phaeopigments.

Chlorophyll *a* is computed from the absorbance at 436 nm, assuming no divinyl chlorophyll *a* is present with a single response factor. Total chlorophyll *a* is computed as the sum of monovinyl chlorophyll *a*, divinyl chlorophyll *a*, chlorophyllide *a*, chlorophyll *a* allomer, and chlorophyll *a* epimer. Divinyl chlorophyll *a* is not chromatographically separated; it is computed using the Latasa et al. (1996) simultaneous equations.

10.7 CONCLUSIONS

The CHORS method provides consistent and repeatable HPLC and fluorometric pigment concentrations in support of the SIMBIOS project, and accommodates the high number of samples, and timely turnaround of final data products. Overall, bulk pigment properties of the community structure can be determined for a variety of oceanographic water types.

Chapter 11

The USC Method

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Abstract

The USC method was developed to provide a universal protocol that could be used to analyze freshwater, estuarine, coastal, and ocean habitats, as well as sediment and microbial mat samples. The method employs a unique combination of both monomeric and polymeric C_{18} columns combined with a variable flow binary gradient. This column configuration was originally devised to enhance photopigment separations from sediment samples containing numerous (greater than 150) photopigment and pigment degradation products. The combination of columns provides strong retention and high efficiency (monomeric columns) while selecting for similar compounds with minor differences in molecular structure (polymeric columns). The variable flow binary gradient allows baseline separation of most major pigments including lutein and zeaxanthin, and chlorophyll c_3. Chlorophylls c_1 and c_2 plus divinyl chlorophylls a and b are not completely separated. For HPLC analysis, filters are placed in disposable polypropylene microfuge tubes and lyophilized to remove all water from the filters. The primary advantages of this method are long column life (greater than 2,000 injections), inexpensive and non-hazardous solvents and reagents, no uncertainty regarding the water retained on the filter, and reliability across a range of sample types.

11.1 INTRODUCTION

The HPLC method used at USC for photopigment separations is derived from the Van Heukelem et al. (1992 and 1994) and Pinckney et al. (1996) protocols. Two different reversed-phase C_{18} columns are connected in series. A single monomeric guard column is followed by a monomeric reversed-phase C_{18} column and a polymeric reversed-phase C_{18} column. This column configuration was originally devised to enhance photopigment separations from sediment samples containing numerous (greater than 150) photopigment and pigment degradation products. Monomeric columns provide strong retention and high efficiency, while polymeric columns select for similar compounds with minor differences in molecular structure (Van Heukelem et al. 1992 and Jeffrey et al. 1997b).

In addition to providing for an increase in the number of theoretical plates, the combination of both monomeric and polymeric columns optimizes photopigment separations based on two different molecular properties (coarse and fine structure). This method allows for the baseline separation of most major pigments including lutein and zeaxanthin, as well as chlorophyll c_3. Chlorophylls c_1 and c_2, however, are not completely separated. Divinyl chlorophylls a and b are not completely resolved, but occur as "shoulders" on the monovinyl chlorophylls a and b and can be visually identified in chromatograms.

11.2 EXTRACTION

The SeaHARRE-4 samples were immediately stored in a $-80°C$ freezer upon receipt. For HPLC analysis, filters were placed in disposable polypropylene microfuge tubes (2 mL) and lyophilized ($-50°C$, 0.57 mbar, 12 h; Labconco FreeZone 2.5) to remove all water from the filters. After lyophilization, filters were cut into six equal sections and placed in microfuge tubes. Samples were extracted in 90% acetone (600 µL), and stored at $-20°C$ for 18–20 h. Each sample also received 50 µL of the synthetic carotenoid *trans*-β-apo-8′-carotenal (Sigma-Aldrich, 10810) in 90% acetone as an internal standard using a gastight syringe (Hamilton) and click dispenser (Hamilton, PB600-1). After extraction, the extract was clarified using a 0.45 µm PTFE filter (Gelman Acrodisc). A known volume of the extract (400 µL) was then dispensed into amber glass autosampler vials (2.0 mL) and sealed with PTFE-silicone caps.

11.3 HPLC ANALYSIS

The instrumentation was manufactured by Shimadzu and was part of their Validation and Productivity (VP) series. It consisted of a binary gradient pump (dual LC10-AT and controller SCL-10A), temperature-controlled autosampler (SIL10-A) with a 500 µL injection loop, column

oven (CTO-10AS), and PDA (SPD-M10A with a 200–800 nm range). For the PDA, spectra (380–700 nm) were obtained at 2 s intervals for the duration of each run and photopigment peaks were quantified at 440±4 nm.

Two different reversed-phase C_{18} columns were connected in series. A single monomeric guard column (Rainin Microsorb, 0.46 × 1.5 cm, 3 µm packing) was followed by a monomeric reversed-phase C_{18} column (Varian Microsorb-MV 100-3, 0.46 × 10 cm, 3 µm packing) and a polymeric reversed-phase C_{18} column (Vydac 201TP54, 0.46 × 25 cm, 5 µm packing). The column oven maintained a constant 40°C for the duration of the gradient.

A nonlinear binary gradient, which was adapted from Van Heukelem et al. (1992), was used for pigment separations (Table 38). Solvent A consisted of 80% methanol and 20% ammonium acetate (0.5 M adjusted to pH 7.2), and solvent B was composed of 80% methanol and 20% acetone (Table 38). Solvents were degassed with an in-line degasser (Shimadzu DGU 14A). All solvents were HPLC-grade and chemicals were analytical grade.

Table 38. The gradient used with the USC method. The time is in minutes, the flow rate is in milliliters per minute, and the percentages of solvents A and B are given in the last two columns.

Step	*Time*	*Flow*	A [%]	B [%]
Start	0	0.80	100	0
2	0.5	0.80	50	50
3	35	1.25	0	100
4	36	1.50	0	100
5	37	0.80	0	100
6	38	0.80	100	0
End	50	0.80	100	0

Just prior to the HPLC run, an ion-pairing (IP) solution (1 M ammonium acetate) was added to the vial in a ratio of four parts extract to one part ammonium acetate. Prior work has shown there is negligible pigment degradation within 12 h of adding the IP solution if the sample is placed in a refrigerated autosampler (4.0°C). The IP solution, however, should not be added to the sample if the time until sample analysis is greater than 18 h.

11.4 CALIBRATION

Peaks were identified based on retention time and spectral matches with pigment spectra obtained from DHI standards (Table 39). Peak areas were quantified using Shimadzu `SP1` v7.2.1 software. The PDA was calibrated using a multipoint calibration procedure for a range of injection volumes (25–300 µL) of pigment standards. Regressions were performed using known pigment concentration (y) versus integrated peak area (x), and were of the form $y = mx + b$, where m is the slope and b is the y-intercept.

Table 39. The α values used by the USC method for a variety of pigments as a function of λ. The units for α are liters per gram per centimeter and the units for λ are nanometers. All solvents are at a 100% purity unless indicated otherwise. Not all of the pigments listed were identified and reported for SeaHARRE-4.

Pigment	*Solvent*	λ	α
$\beta\beta$-Car	Acetone	454	250.00
$\beta\varepsilon$-Car	Acetone	448	270.00
$\beta\psi$-Car	Pet Ether	459	318.00
$\varepsilon\varepsilon$-Car	Pet Ether	440	290.00
$\psi\psi$-Car	Acetone	474	344.60
Allo	Acetone	454	250.00
Anth	Ethanol	446	235.00
Asta	Hexane	468	210.00
BChl *a*	Ace./Meth.	771	59.40
But	Acetone	445	147.00
Cantha	Pet Ether	466	220.00
Chl *a*	90% Acetone	664	87.67
Chl *b*	90% Acetone	647	51.36
Chl c_{12}	90% Acetone	631	42.60
Chl c_3	90% Acetone	453	346.00
Chlide *a*	90% Acetone	664	127.00
Chlide *b*	90% Acetone	645	74.07
Croco	Ethanol	443	250.00
Diadchr	Acetone	428	250.00
Diad	Methanol	445	225.00
Diato	Acetone	452	210.00
Dino	Acetone	442	210.00
DVChl *a*	90% Acetone	664	87.67
DVChl *b*	90% Acetone	647	51.36
Echin	Pet Ether	458	215.80
Fuco	Acetone	443	166.00
Gyro	Ethanol	445	262.00
Hex	Acetone	445	142.00
Lut	Ethanol	445	255.00
MgDVP	Methanol	623	58.90
Monado	Diethyl Ether	446	250.00
Myxo	Acetone	478	216.00
Neo	Ethanol	438	227.00
P-457	Acetone	457	164.00
Peri	Acetone	466	134.00
Phide *a*	90% Acetone	667	74.20
Phide *b*	90% Acetone	657	46.37
Phytin *a*	90% Acetone	667	51.20
Phytin *b*	90% Acetone	657	31.80
Pras	Diethyl Ether	446	250.00
Pyrophytin	Diethyl Ether	667	60.29
Siphx	Acetone	445	250.00
Siphn	Ethanol	462	192.00
Vauch	Acetone	444	250.00
Viola	Acetone	442	240.00
Zea	Acetone	452	234.00

All regressions had a coefficient of determination of $r^2 > 0.98$. The slope of the fitted line was used as the response factor for all pigment concentration calculations. The concentrations of pigments for which standards were unavailable were estimated using the ratio method outlined in Jeffrey et al. (1997b).

11.5 VALIDATION

Carotenal blanks (*trans*-β-apo-8′-carotenal in 90% acetone) were run after every 10 samples to verify peak time reproducibility, peak area precision, and instrument performance during the sequence run. Peaks were identified based on retention time and comparison of absorbance spectra with a spectral library derived from pure pigment standards (DHI). Long-term quality control was achieved by analyzing pure standards for chlorophyll *a* and the DHI mix at monthly intervals. Instrument performance was measured and compared with previous measures to determine changes in performance metrics. Volumetric measuring devices were checked weekly.

11.6 DATA PRODUCTS

Pigment concentrations were calculated for each identifiable peak using the following equation:

$$C_{P_i} = \frac{R_I}{V_c} \frac{V_m}{V_f} \frac{\hat{A}_c}{\hat{A}_s} \hat{A}_{P_i} R_{P_i}, \tag{46}$$

where C_{P_i} is the pigment concentration in micrograms per liter; $\hat{A}_{P_i}$ is the pigment peak area; R_{P_i} is the response factor; V_c is the injection volume in microliters; V_m is the total extract volume (volume of added acetone plus volume of internal standard in milliliters); R_I is the ratio of the volume of ion-pairing (IP) solution plus V_m divided by V_m; V_f is the volume of seawater filtered (in liters); $\hat{A}_c$ is the average peak area for carotenal standards; and $\hat{A}_s$ is the peak area of carotenal in the sample.

11.7 CONCLUSIONS

This method has been employed by USC for approximately 15 y to analyze a broad spectrum of sample types from marine and freshwater habitats. The execution of the method is straightforward and involves minimum manipulation of the samples and extracts, is relatively inexpensive, and does not generate hazardous waste products. The primary weakness of the method is the inability to completely separate chlorophylls c_1 and c_2, and divinyl chlorophylls *a* and *b*.

Acknowledgments

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Appendices

A. The SeaHARRE-4 Science Team
B. The SCOR WG 78 Pigment Abbreviations
C. Commercial HPLC Manufacturers and Pigment Suppliers

Appendix A

The SeaHARRE-4 Science Team

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*,
DVChl *a*	Divinyl chlorophyll *a*,
DVChl *a*′	Divinyl chlorophyll *a* epimer,
DVChl *b*	Divinyl chlorophyll *b*,
DVChl *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,
MgDVP	Mg 2,4-divinyl phaeoporphyrin a_5 monomethyl ester,
Monado	Monadoxanthin,
Myxo	Myxoxanthophyll,
Neo	Neoxanthin,
Perid	Peridinin,
Pras	Prasinoxanthin,
Viola	Violaxanthin,
Zea	Zeaxanthin,
$\beta\beta$-Car	$\beta\beta$-Carotene (β-Carotene), and
$\beta\varepsilon$-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 Road
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
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Voice: 49–800–569–9000
Fax: 49–721–560–6149
Net: http://www.carl-roth.de

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Fluka Chemical Corporation[3]
1001 West St. Paul Avenue
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Voice: 414–273–3850
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.
5100 Franklin Drive
Pleasanton, CA 94588-3355
Voice: 925–218–2800
Fax: 925–218–2900
Net: http://www.hitachi-hta.com

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

[1] Formerly the Hewlett-Packard Analytical Division.

[2] Formerly the VKI Water Quality Institute.

[3] Part of Sigma-Aldrich.

Shimadzu Scientific Instruments
7102 Riverwood Drive
Columbia, MD 21046–1245
Phone Number: 800–477–1227
Fax Number: 410–381–1222

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

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

Glossary

APD	Absolute Percent Difference
BIO	Bedford Institute of Oceanography (Canada)
CHORS	Center for Hydro-Optics and Remote Sensing
CSIRO	Commonwealth Scientific and Industrial Research Organisation (Australia)
CTD	Conductivity, Temperature, and Depth
CV	Coefficient of Variation
DAD	Diode Array Detector
DANACK	Danish Accreditation and Metrology Fund
DalU	Dalhousie University (Canada)
DHI	Not an acronym, but the abbreviation for the DHI Water and Environment Institute (Denmark).
DP	(Total) Diagnostic Pigments
EtOAc	Ethyl Acetate (alternate abbreviation)
FIO	Florida Institute of Oceanography
GF/F	Not an acronym, but a type of glass fiber filter.
GNWP	Not an acronym, but a filter code.
GSFC	Goddard Space Flight Center
HPL	Horn Point Laboratory
HPLC	High Performance Liquid Chromatography
JGOFS	Joint Global Ocean Flux Study
JRC	Joint Research Centre (Italy)
ID	Internal Diameter
IP	Ion-Pairing
ISO	International Organization for Standardization
ISTD	Internal Standard
LOD	Limit of Detection
LOQ	Limit of Quantitation
LOV	*Laboratoire d'Océanographie de Villefranche* (Oceanographic Laboratory of Villefranche, France)
MCM	Marine and Coastal Management (South Africa)
MeCN	Acetonitrile (alternate abbreviation)
MDS	Metering Device Speed
MODIS	Moderate Resolution Imaging Spectroradiometer
MPF	Microplankton Proportion Factor
NASA	National Aeronautics and Space Administration
NPF	Nanoplankton Proportion Factor
NV	Not Validated
PDA	Photodiode Array
PML	Plymouth Marine Laboratory (United Kingdom)
PPC	Photoprotective Carotenoids
PPF	Picoplankton Proportion Factor
PPig	Primary Pigments
PSC	Photosynthetic Carotenoids
PSP	Photosynthetic Pigments
PTFE	Polytetrafluoroethylene
QA	Quality Assurance
QC	Quality Control
RPD	Relative Percent Difference
RF	Response Factor
SCOR	Scientific Committee on Oceanographic Research
SDSU	San Diego State University
SeaHARRE	SeaWiFS HPLC Analysis Round-Robin Experiment
SeaHARRE-1	The first SeaHARRE
SeaHARRE-2	The second SeaHARRE
SeaHARRE-3	The third SeaHARRE
SeaHARRE-4	The fourth SeaHARRE
SeaWiFS	Sea-viewing Wide Field-of-view Sensor
SIMBIOS	Sensor Intercomparison and Merger for Biological and Interdisciplinary Oceanic Studies
SNR	Signal-to-Noise Ratio
SOP	Standard Operating Procedure
TAcc	Total Accessory Pigments
TbAA	Tetrabutyl Ammonium Acetate
TCaro	Total Carotenoids
TCAS	Temperature-Controlled Autosampler
TChl	Total Chlorophyll
TPig	Total Pigments
UMCES	University of Maryland Center for Environmental Science
UPD	Unbiased Percent Difference
USC	University of South Carolina
USF	University of South Florida
UV	Ultraviolet
VHT	Van Heukelem and Thomas (2001) method
Vis	Visible
VP	Validation and Productivity
WG	Working Group

[4] Formerly Sigma Chemical.

[5] Formerly Thermo Separation Products.

Symbols

a The specific absorption coefficient.
A This is used to denote the average of all the methods.
$A(\lambda)$ Absorbance.
A' The methods within the QA subset.
A^{+} The methods not within the QA subset, which are also referred to as the NV methods (not validated at the QA level of performance).
A^{-} The set of best results (e.g., lowest uncertainties or precisions) from a group of methods.
$\hat{A}_c$ The peak area of the internal standard when it is injected onto the HPLC column.
$\hat{A}_{c_1}$ The $\hat{A}_c$ determined using a one-step internal standard methodology.
$\hat{A}_{c_2}$ The $\hat{A}_c$ determined using a two-step internal standard methodology.
A_{P_i} The absorbance of the pigment.
$\hat{A}_{P_i}$ The area of the parent peak and associated isomers for pigment P_i.
$\hat{A}'_{P_i}$ The corrected peak area (in units of milli-absorbance units).
$a_{P_i}(\lambda)$ Absorption coefficient of a pigment (a constant).
$\hat{A}_s$ The peak area of the internal standard in the sample.
$\hat{A}_{s_1}$ The $\hat{A}_s$ determined using a one-step internal standard methodology.
$\hat{A}_{s_2}$ The $\hat{A}_s$ determined using a two-step internal standard methodology.
A_{S_i} The absorbance of the internal standard measured on the spectrophotometer.
$\hat{A}_{S_i}$ The peak area of the internal standard measured on the HPLC.

b_i The y-intercept of a linear equation.

C The CSIRO method, or the concentration of a pigment (depending on usage).
$\bar{C}$ The average concentration of a particular pigment.
C_a The concentration of chlorophyll *a*.
C_A The concentration of alloxanthin.
C_{P_i} The concentration of a particular pigment.
$\tilde{C}_{P_i}$ The amount of pigment injected for pigment P_i, usually in units of nanograms.
$\bar{C}^{A}_{P_i}$ The average concentration of a particular pigment across all methods.
$\hat{C}^{A'}_{P_i}$ The maximum concentration of pigment P_i across all 24 samples.
$\bar{C}^{A'}_{P_i}$ The overall averages for the individual pigments for the QA subset.
$\bar{C}^{A^{+}}_{P_i}$ The overall averages for the individual pigments for the methods not in the QA subset.
$\hat{C}^{A^{+}}_{P_i}$ The average concentration of a particular pigment across all methods for the not in the QA subset.
$\check{C}^{A^{+}}_{P_i}$ The minimum concentration of pigment P_i across all 24 samples not in the QA subset.
$\check{C}^{A'}_{P_i}$ The minimum concentration of pigment P_i across all 24 samples.
C_B The concentration of 19′-butanoyloxyfucoxanthin.
C_C The concentration of the carotenes.
C_{Ca} The concentration of chlorophyllide *a*.
C_{Da} The concentration of divinyl chlorophyll *a*.
C_{Dd} The concentration of diadinoxanthin.
C_{Dt} The concentration of diatoxanthin.
C_F The concentration of fucoxanthin.
C_H The concentration of 19′-hexanoyloxyfucoxanthin.
C_L The concentration of lutein.
C_N The concentration of neoxanthin.
C_{N+V} The concentration of neoxanthin plus violaxanthin.
C_P The concentration of peridinin.
C_{Pba} The concentration of phaeophorbide *a*.
C_{P_i} The concentration of a particular pigment.
C'_{P_i} The amount pigment injected.
$\tilde{C}_{P_i}$ The amount of pigment injected for pigment P_i, usually in units of nanograms.
$\bar{C}_{P_i}$ The average concentration for pigment P_i.
C_{Pr} The concentration of prasinoxanthin.
C_{Pta} The concentration of phaeophytin *a*.
C_{S_i} The concentration of pigment standard for pigment P_i.
C_{T_a} The concentration of total chlorophyll *a*.
C_{T_b} The concentration of total chlorophyll *b*.
C_{T_c} The concentration of total chlorophyll *c*.
C_V The concentration of violaxanthin.
C_Z The concentration of zeaxanthin.
C_{Z+L} The concentration of zeaxanthin plus lutein.

D The DHI method.
D_c The column diameter.
D_f A dilution factor.

e Extract.

F The FIO method.
F_{P_i} The inverse response factor for pigment P_i, i.e., $1/R_{P_i}$.

G The GSFC method.

H The HPL method.

i An array index.

j An array index.
J The JRC method.

k An index indicating the station number.

l An index indicating the replicate number.
L The LOV method.
L' The histoprep analyses.
l_c The pathlength of the cuvette.
L_c The column length.
L_j The laboratory or method code.

m_i The slope of a linear equation (equating change in peak area with change in amount).

N The DalU method.
N_L The number of laboratories quantitating a pigment.
N_R The total number of replicates: 3 for M and 2 for all other methods.
N_S The number of samples.

P_i A particular pigment (referenced using index i).
P_s The column particle size.

r^2 The coefficient of determination.
R The response factor (from a generalized perspective).
$R^{\%}$ The purity corrected response factor.
R_I The ratio of the volume of IP solution plus V_m divided by V_m.

R_{P_i} The response factor for pigment P_i, usually expressed as the amount of pigment divided by the peak area.
$R^{\Sigma}_{P_i}$ The amount injected onto the column divided by the total peak area (including the sum of the parent peak and degradants).
$R^{\%}_{P_i}$ The purity-corrected amount injected onto the column divided by the area of the main (or parent) peak alone.
R_s The resolution (or separation) between peaks.
$\check{R}_s$ The minimum resolution determined from a critical pair for which one of the pigments is a primary pigment.

s The sample.
S The (SDSU) CHORS method.
S_i The pigment standard for pigment P_i.
S_k The k^{th} station or sample number.
$S_{k,l}$ The station or sample number set by k, and the replicate number set by l.

T_c The column temperature.
[TChl a] The concentration of total chlorophyll a.
t_R The retention time.

U The USC method.

V_c The volume of sample extract injected onto the HPLC column.
V_e The volume of the extraction solvent.
V_f The volume of water filtered in the field to create the sample.
V_m The volume of extraction solvent (containing internal standard) added to a filter.
V_s The internal standard.
V_x The extraction volume.
$V_{x'}$ The extraction volume in milliliters.
V_{x1} The extraction volume computed using a one-step internal standard methodology.
V_{x2} The extraction volume computed using a two-step internal standard methodology.
[Vit E] The concentration of vitamin E (in grams per milliliter) in the extraction solvent plus internal standard.

α Specific absorption coefficient a.
α_{S_i} The absorption coefficient for the pigment of interest.

ε The molar absorption coefficient at the specified wavelength for the pigment λ_{P_i}.

λ The spectral wavelength.
λ_m The maximum wavelength.

ξ The coefficient of variation.
$\bar{\xi}$ The average precision.
$\bar{\xi}_{\text{cal}}$ The average CV for gravimetric calibration of dilution devices.
$\bar{\xi}_{\text{inj}}$ The injector precision.
$\bar{\xi}_{P_i}$ The average precision for pigment P_i.
$\bar{\xi}_{t_R}$ The CV of retention time.

σ The standard deviation.
$\Sigma\hat{A}_{S_i}$ The sum of the parent peak and the area of the alteration products.

$\bar{\psi}^{A}_{P_i}$ The average of the RPD values for a particular pigment across the number of laboratories or methods (N_L) reporting the pigment involved.
$|\psi|$ The absolute UPD.
$|\bar{\psi}|^{A}_{P_i}$ The average of the APD, $|\bar{\psi}|$, values for a particular pigment across the number of laboratories or methods (N_L) reporting the pigment involved.
$|\bar{\psi}|$ The average APD.
$|\bar{\psi}|_{\text{res}}$ The average of the absolute residuals.

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14. ABSTRACT

Ten international laboratories specializing in the determination of marine pigment concentrations using high performance liquid chromatography (HPLC) were intercompared using in situ samples and a mixed pigment sample. The method intercomparisons were used for the following objectives: a) estimate the uncertainties in quantitating individual pigments and higher-order variables formed from sums and ratios; b) confirm if the chlorophyll a accuracy requirements for ocean color validation activities (approximately 25%, although 15% would allow for algorithm refinement) can be met in coastal waters; c) establish the reduction in uncertainties as a result of applying quality assurance procedures; d) show the importance of establishing a properly defined referencing system in the computation of uncertainties; e) quantify the analytical benefits of performance metrics, and f) demonstrate the utility of a laboratory mix in understanding method performance. In addition, the remote sensing requirements for the in situ determination of total chlorophyll a were investigated to determine whether or not the average uncertainty for this measurement is being satisfied.

15. SUBJECT TERMS

SeaWiFS, High Performance Liquid Chromatography, HPLC, analysis, round-robin, pigment, chlorophyll, method

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