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High Performance Liquid Chromatography (HPLC)

2010 Workshop

Hosted by

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Abstract

Twelve 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 derived from laboratory cultures as part of the fifth SeaWiFS HPLC Analysis Round-Robin Experiment (SeaHARRE-5). The sampling for the SeaHARRE-5 activity was organized and executed as a strictly coastal activity and the field samples were collected from primarily eutrophic waters within the coastal zones of the United States (New England) and Australia (Tasmania). 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.

Introduction

The first Sea-viewing Wide Field-of-view Sensor (SeaWiFS) High Performance Liquid Chromatography (HPLC) Round-Robin Experiment (SeaHARRE-1) took place in 2000 (Hooker et al. 2000) and emphasized the so-called Case-1† waters of the open ocean. Because SeaHARRE-4 had shown an elevation in uncertainties associated with coastal samples (Hooker et al. 2010) with respect to the first three activities, which were all conducted in the open ocean, the SeaHARRE-5 activity again emphasized the optically more complex waters of the coastal zone, the so-called Case-2 waters.

Samples were collected in two different coastal environments, which were anticipated to have very different HPLC baselines. The first set of 12 samples were taken from the New England (United States) coastal waters around Portsmouth (New Hampshire), and the second set of 12 samples were obtained in the near vicinity of the coastal waters around Hobart (Tasmania). The latter were expected to have simpler baselines than the former, because the environmental conditions of the Tasmanian watersheds were expected to be less influenced by anthropogenic sources (as established by prior analyses by the Australia Commonwealth Scientific and Industrial Research Organization, CSIRO). The difference in baseline noise was a desirable feature of the sampling, because the SeaHARRE-4 samples had complicated baselines and the hypothesis was the noisy baselines were elevating pigment uncertainties. The two different types of baselines in the SeaHARRE-5 samples would prove or disprove this theory.

There was a strong opinion in the early planning for SeaHARRE-1 that the approach used in the Joint Global Ocean Flux Study (JGOFS)—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. Consequently, the SeaHARRE activity emphasizes international participation to bring together the widest diversity of HPLC analysts and methods to provide the broadest investigation of community capabilities as possible.

Although diversity is needed, there are advantages in having a recurring set of *core* participants within the overall SeaHARRE activity. Most notably, the core group provides an established capability and knowledge base that can be counted on during data analysis, as well as workshop discussions (there have been almost as many workshops as round robins). The experience of the core analysts has helped steer the evolving objectives of each activity and provided invaluable learning opportunities for new analysts.

Method diversity has been a recurring challenge in terms of trying to balance the presence of core participants and new practitioners. The practical benefit of adopting a proven method instead of investing an unknown amount of time and resources in trying to improve a method, however, is simply too alluring. As one method emerged with outstanding performance capabilities—the Van Heukelem and Thomas (2001) method (VHT)—more and more SeaHARRE participants switched to this method, which resulted in the core participants all using the same method.

† By definition, the optical properties of Case-1 waters are solely determined by the phytoplankton and its derivative products (Morel and Prieur 1977), whereas Case-2 optical properties are also determined by other material, e.g., from terrestrial or bottom origin.

This convergence to a single method is potentially worrisome, because the core participants have been a part of the quality-assured (QA) subset of laboratories used to provide the reference values in the computation of uncertainties.

To counter the trend towards a predominant method, SeaHARRE emphasizes international participation and a diversity of specialized analyses to understand how uncertainties are influenced by the full complexity of the methods being used. The latter has included a) new hardware introduced to an established method; b) new analysts, both experienced or novice, executing an established method; c) unequivocally damaged (defrosted) samples being analyzed by a QA laboratory; d) reanalyses of replicate samples to better understand analysis anomalies, and e) the use of two simultaneous methods by one laboratory. For SeaHARRE-5, the majority of the HPLC analysts were new to the activity (Table 1) and a variety of sample storage and extraction efficiency experiments were conducted.

The insights attained from the approximately biannual SeaHARRE collaborations have brought the HPLC pigment analysis community closer to uniformity. Each laboratory processes and analyzes samples using their standard laboratory practices and HPLC methods. The computation of uncertainties provides a better understanding of the performance level of the method, which directly affects data quality. The ultimate goal of SeaHARRE is a universal adoption of best practices and optimum method performance in the pigment community. Such a convergence can result in higher data quality and overall decreased uncertainties to comply with the validation requirements of ocean color algorithms.

The SeaHARRE-5 activity—hosted by Lesley Clementson, a four-time participant in SeaHARRE—took place from 12–16 April 2010 at the CSIRO facility in Hobart, Tasmania. The attendees consisted of both veteran and new participants. The primary objectives of the workshop were to introduce new participants to the concepts associated with achieving and maintaining QA in marine pigment analysis and quantitation (the performance metrics, accuracy, and precision); discuss method similarities and differences that influence the aforementioned QA parameters; and address outstanding concerns and issues associated with marine pigment quantitation during the working groups.

The major questions and concerns that were posed during the workshop included the following:

- How should pigments quantitated below the limit of quantitation (LOQ) be reported?
- How should small peaks be identified and quantitated?
- What are the methodological factors causing anomalously high uncertainties in certain primary pigments (e.g., diatoxanthin, 19′-hexanoyloxyfucoxanthin and 19′-butanoyloxyfucoxanthin)?
- Is the high uncertainty for prasinoxanthin caused by the interference of another carotenoid, e.g., 4-keto-19′-hexanoyloxyfucoxanthin?
- Would improvements in pigment quantitation or reporting practices (or both) decrease uncertainties?
- Should a new null value be used, so it can be unequivocally identified as a substitution value (e.g., a value with more digits of precision)?
- Would developing a common protocol for summing multiple peaks reduce uncertainties for certain pigments (e.g., when to sum the phaeophorbides and the peridinin main peak and associated epimer)?

The agenda for the workshop was constructed to facilitate the resolution of these questions and is presented in Fig. 1. Much of the discussion for the questions occurred during the break-out sessions for working groups or in the plenary sessions associated with synthesizing the material covered by the working groups.

Working Group One

Working group one was concerned primarily with recommendations for improving the reporting practices for pigment products. It has been suggested that improving and homogenizing the reporting practices of pigments may avoid some uncertainty. Common discrepancies occur, particularly when pigment concentration is at, or near, the instrument LOQ, i.e., when peaks are small or are not confidently identified. The participants were divided into smaller subgroups to discuss three topics of interest: the first subgroup discussed method differences in reporting; the second subgroup discussed the determination of a new null value; and the third subgroup discussed problem pigments.

The first subgroup concluded that the so-called *two-sentence rule*, developed during the SeaHARRE-3 activity (Hooker et al. 2009), is difficult for non-native English speakers and needs additional clarification:

> If a peak is *good* and it can be proved to be the incorrect pigment for that retention time (e.g., the absorption spectrum does not match), do not report it; otherwise report it.
>
> If a peak is *bad* and it cannot be proved to be the incorrect pigment, report it; otherwise do not report it.

The point of the two-sentence rule is to have the burden of proof switch as the quality of the data changes, but in each case to have the simpler task emphasized, so analysts will more likely be doing the same thing while doing less work. When the data is good, the burden is to prove a peak is not going to be correctly identified, and given the good data available, this task will be rather simple. When the data is poor, the burden is to disprove the assumption that the peak is correctly identified, but because the data

Time	13 April (Tue)	14 April (Wed)	15 April (Thu)	16 April (Fri)
0830	Welcome (L. Clementson)	Welcome (L. Clementson)	Welcome (L. Clementson)	Welcome (L. Clementson)
0840	Workshop Agenda (S. Hooker)	Workshop Agenda (S. Hooker)	Workshop Agenda (S. Hooker)	Workshop Agenda (S. Hooker)
0850	SeaHARRE Overview, the Governing Equation, and the NASA Perspective (S. Hooker)	Calculating Uncertainties (S. Hooker)	CHEMTAX (Simon Wright, Australian Antarctic Divison, Kingston)	Estimation of Performance Metrics (S. Hooker, L. Clementson, C. Thomas, A. Neeley, and L. Shlüter)
0900				
0910		Overview of SeaHARRE-5 Analysis Results (S. Hooker)		
0920				
0930	The CSIRO Method (L. Clementson)		Questions and Answers	
0940			New Pigments (Shirley Jeffrey, CSIRO, Hobart)	
0950	The USM Method (S. Lohrenz)	SeaHARRE-5 Results Discussion (S. Hooker)		
1000				
1010	The Scripps Method (W. Kozlowski)	SeaHARRE Technical Reports (S. Hooker)		
1020			Questions and Answers	
1030	*Break*	*Break*	*Break*	*Break*
1100	The Bodø Method (E. Egeland)	Reduced Performance from Reporting Issues (S. Hooker)	IMOS (Simon Allen, CSIRO, Hobart)	Future Plans for the SeaHARRE Activity (S. Hooker)
1110				
1120	The HPL Method (C. Thomas)			
1130		Recommendations for Improving Reporting Practices of Pigment Products (S. Hooker, L. Clementson, C. Thomas, A. Neeley, and L. Shlüter)	Ocean Color Validation (Thomas Schroeder, CSIRO, Canberra)	
1140				
1150	The FURG Method (V. Garcia)			
1200			Algal Cultures and Pigments (Susan Blackburn, CSIRO, Hobart)	
1210	The IO Method (V. Brotas)			
1220				
1230	*Lunch*	*Lunch*	*Lunch*	*Lunch*
1300				
1330				
1400	The Dalhousie Method (C. Normandeau)	Estimation of Uncertainties for the Terms Within the HPLC Governing Equation (S. Hooker, L. Clementson, C. Thomas, A. Neeley, and L. Shlüter)	Tour of CSIRO (L. Clementson)	Local Field Trip (L. Clementson)
1410				
1420	The GSFC/CVO Method (A. Neleey)			
1430				
1440				
1450	Quantitation Problems with Prasinoxanthin (M. Maddox)			
1500				
1530	*Break*	*Break*	*Break*	
1600	The DHI Method (L. Shlüter)	Performance as a Function of Concentration and SNR (S. Hooker)	HPLC Issues Identified by SeaHARRE Activities (S. Hooker)	
1610				
1620				
1630	Methods Discussion (C. Thomas)	Reducing Uncertainties in the Quantitation of Small Peaks (S. Hooker, L. Clementson, C. Thomas, A. Neeley, and L. Shlüter)		
1640			Round Table Discussion with Invited Speakers	
1650				
1700				
1710				
1720				
1730	Adjourn	Adjourn	Adjourn	

Fig. 1. The agenda for the SeaHARRE-5 workshop showing informal meeting times (blue), plenary sessions (green), break-out sessions for working group discussions (yellow), invited presentations (orange), and alternative scheduling (purple). Individual method presentations were the primary focus of the first day, followed by working group meetings in break-out sessions, invited presentations, final discussions and future plans, and a local field trip.

Table 1. The participants of the workshop organized according to the organizations they represented (acronyms are defined in the glossary). Core participants (HPLC analysts who have participated in three or more SeaHARRE activities) are shown in bold face typeface and new participants (HPLC analysts for which SeaHARRE-5 was their first intercomparison) are shown in slanted typeface (Fig. 2).

Organization	*Country*	*Participant*	*E-mail Address*
CSIRO	Australia	**Lesley Clementson**§	lesley.clementson@csiro.au
	Australia	Tasha Waller	tasha.waller@csiro.au
	Australia	Shirley Jeffrey†	shirley.jeffrey@csiro.au
	Australia	Simon Allen†	simon.allen@csiro.au
	Australia	Thomas Schroeder†	thomas.schroeder@csiro.au
	Australia	Susan Blackburn†	susan.blackburn@csiro.au
AAD	Australia	Simon Wright†	simon.wright@aad.gov.au
FURG	Brazil	*Virginia Garcia*	docvmtg@furg.br
	Brazil	*Raphael Mendes*	rmendes@fc.ul.pt
Dalhousie University	Canada	Claire Normandeau	c.normandeau@dal.ca
DHI	Denmark	**Louise Schlter**	lsc@dhigroup.com
	Denmark	**Marete Allerup**	mea@dhigroup.com
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	France	**Joséphine Ras**‡	jras@obs-vlfr.fr
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Bodø University	Norway	*Einar Egeland*	einar.skarstad.egeland@hibo.no
University of Lisbon	Portugal	*Vanda Brotas*	vbrotas@fc.ul.pt
	Portugal	*Paolo Cartaxana*	pcartaxana@fc.ul.pt
GSFC/CVO	USA	Stanford Hooker§	stanford.b.hooker@nasa.gov
	USA	*Aimee Neeley*	aimee.neeley@nasa.gov
HPL	USA	**Crystal Thomas**	cthomas@hpl.umces.edu
	USA	Meg Maddox	mmaddox@umces.edu
SIO	USA	*Wendy Kozlowski*	wkozlowski@ucsd.edu
USM	USA	*Steve Lohrenz*	steven.lohrenz@usm.edu
	USA	*Sumit Chakraborty*	sumit.chakraborty@usm.edu

§ A meeting organizer.
† An invited speaker.
‡ Did not attend the workshop, but participated in the data analysis.

are poor, there will be little chance this will be possible, so the usual outcome will be the straightforward solution of simply reporting it.

The four cases of the two-sentence rule can also be summarized in terms of absorption spectra matching (the most common means for accepting or rejecting peak identities) as follows:

1. If a peak is *good* and it matches the spectrum, report it;
2. If a peak is *good* and it does not match the spectrum, do not report it;
3. If a peak is *bad* and it cannot be proved to not match the spectrum, report it; and
4. If a peak is *bad* and it can be proved to not match the spectrum, do not report it.

The language of the two-sentence rule is purposely vague, in terms of the definitions of a "good" versus "bad" peak. The idea was to use it as a motivator for analysts to start establishing what these definitions should be. Although aspects of the definitions were discussed, these terms are expected to be more completely defined in a future working group.

The second subgroup determined that all quantitated values will have three digits of precision and the null value will have six digits, which will flag the pigment as not quantitated. The *new* null value will be 0.000999.

The third subgroup was assigned the task of addressing the topic of problematic pigments, or those pigments that consistently produce high uncertainties. The subgroup suggested that one recurring problem that may contribute to high uncertainties was associated with the rules for reporting coeluting pigments and summing pigments. For example, there are discrepancies among analysts on whether to sum all identified phaeophorbide peaks (usually three or more) or quantitate them separately. Moreover, there is a lack of agreement on whether to include the peri-

Fig. 2. The participants for the HPLC workshop who were responsible for producing the analytical results: (top to bottom and left to right: Virginia Garcia, Merete Allerup, Louise Schlüter, Steve Lohrenz; Paolo Cartaxana, Raphael Mendes, Sumit Chakraborty, Stanford Hooker; Wendy Kozlowski, Claire Normandeau, Crystal Thomas, Einar Egeland, Tasha Waller; Aimee Neeley, Lesley Clementson, Vanda Brotas, and Meg Maddox.

dinin epimer that follows the main (parent) peridinin peak in the final quantitation, or quantitate them separately.

The third subgroup also suggested that the problem of uncertainties might be associated with coeluting peaks, which are method dependent. The question was posed as to whether or not there should be a threshold that determines when to report these pigments as a sum and when to report them as individual pigments. It was also suggested that a list of the limitations of each method should be compiled and then determine what can be done to optimize these methods through modifications, although it is understood that some of these issues are hardware dependent.

One concern of the participants was the reporting practices used to provide data products. For example, if the presence of a pigment is uncertain, particularly when it is at a low concentration where spectra matching can become dubious, how should it be reported? At low concentrations, the spectral matches are noisy, so the ambiguity in reporting practices increases significantly. One suggestion was to devise a similarity index, particularly for those pigments that possess similar spectra. To accomplish this task, a separate spectral library would be constructed using diluted pigment standards to simulate low concentrations observed in some field samples.

Working Group Two

Working group two was concerned primarily with defining a *good* and *bad* peak. Following the first discussion about the reporting practices associated with the clarifications to the two-sentence rule, it was obvious that objective definitions of 'good' and 'bad' peaks should be determined. These questions prompted a second working group that was divided into the following three subgroups: the first subgroup was to define a good peak; the second subgroup was to define a bad peak; and the third subgroup was to assess software applications that are available for defining these parameters.

The first subgroup proposed the following criteria for a good peak: on the basis of the peak itself, a good peak should have a symmetrical shape, a good signal-to-noise ratio (SNR), and the same spectrum all over the peak. In comparison with the reference standard, a good peak should have the same peak width, retention time, spectral wavelength(s), and spectral shape.

The second subgroup defined a bad peak as having a retention time outside the normal range; an abnormal elution order; an asymmetric or distorted peak—asymmetry includes peak doubling, peak tailing, unexpected shouldering, and peak broadness in relation to height; poor resolution (e.g., coelution); low SNR; and baseline distortion or anomaly.

The bad peak spectral characteristics were defined as low similarity to candidate library spectra; low SNR of the spectrum (the spectral form is not clear because of excessive noise); multiple spectrum matches with library spectra; an incomplete absorption spectrum (i.e., a partial match); and inconsistencies of the spectrum in different regions of the peak.

The second subgroup also developed a method that may be used to decide when a peak is bad:

- If the peak is big and does not exhibit any of the bad peak characteristics defined above, move to assessing the spectral characteristics.
- For small peaks (as defined by SNR) the peak shape characteristics are often bad, so they should be evaluated based on SNR and spectra.

Future considerations for defining bad peaks that must be discussed further include establishing how many characteristics define a peak as bad; determining whether good peaks are only defined by peak characteristics other than spectra, and whether bad peaks are defined by both shape and spectral characteristics; and investigating if there is a gradient between the good and bad peak. For the latter, this involves discerning whether or not there is a breaking point between the two, is it always in the same place, or does the pigment determine it?

The third subgroup assessed software features that may help with defining good and bad peaks and decided that SNR, spectra match (a numerical value), and tailing factor are features of most software packages that may help to characterize good and bad peaks. Thresholds may be set using these parameters to determine good and bad peaks. Next, the software manufacturers should be contacted to elucidate other possible features of the software to meet these parameters. The automation of these values into the reporting system would be ideal.

Working Group Three

Working group three was concerned primarily with applying definitions of good and bad peaks to chromatograms. After the second working group discussion concluded, the participants were given the opportunity to review their chromatograms from the SeaHARRE-5 activity and choose three examples of what they considered good and bad peaks. Each participating laboratory presented these chosen peaks to the rest of the group to test the applicability of the previously determined definitions of good and bad to the identification and quantification of marine pigments.

Working Group Four

Working group three was concerned primarily with the estimation of performance metrics. The participants discussed the current version of the performance metrics and whether it should be revised, i.e., should parameters be removed or added for further refinement. All agreed that software-dependent parameters, such as peak symmetry, should not be used because of differences among software manufacturers on how these values are calculated. Moreover, it was acknowledged that the performance metrics are based (in part) on the analysis of standards, which are more representative of analyzing samples from cultures rather than the field. The current weighting is more towards the latter than the former, because the latter represents a more complex sample set and the majority of the SeaHARRE samples are field samples.

It was suggested that the performance metrics should be more explicitly weighted based on the analysis type. For example, if 50% of the phytoplankton samples from a particular laboratory are cultured and 50% are sourced from the field, then the performance metrics for each analytical environment would be equally weighted (multiplied by 0.5) and summed to produce a more representative overall performance; or, the two different performance capabilities could be reported separately (in which case, there would be no weighting).

It was suggested that the exchange of field samples with other laboratories would act as a periodic quality check of precision and accuracy. In this same context, DHI could collect field samples and sell them for this type of comparison. If this idea were adopted, the samples would need to cover all water types (oligotrophic, mesotrophic, eutrophic, and coastal waters). Another suggestion was to use 13 mm filters instead of 25 mm filters to decrease filtration time and extraction volume. It was also suggested that zeaxanthin and lutein individual standards could be mixed together and then analyzed to assess resolution.

Future SeaHARRE Activities

One of the objectives of the SeaHARRE activities is to address problem sets frequently encountered by the pigment community. Previous activities have examined the effects of damaged samples, storage methods, and extraction efficiency on data quality; however, many other scenarios exist and should be addressed. The participants were asked to list topics or problem sets they would like to address, and the resulting suggestions for the future include the analysis of the following:

- Samples from regions of high chlorophyll *a* (i.e., bloom conditions with chlorophyll *a* concentrations approaching or exceeding $50\,\mathrm{mg\,m^{-3}}$);
- Samples from harmful algal blooms;
- Samples from algal cultures;
- Samples from freshwater ecosystems;

- Samples from marshland communities at the land–sea boundary;
- Samples from coastal waters;
- Samples from both the Arctic and Antarctic regions;
- Samples from anywhere, but with the inclusion of microscopy;
- Samples from a large-scale Arabian Sea bloom of the dinoflagellate *Noctiluca miliaris*;
- Samples consisting of a mixture of phytoplankton cultures that reflect a field sample scenario, which should include such problematic pigments as 4-keto-19′-hexanoyloxyfucoxanthin and prasinoxanthin;
- Samples from the Chesapeake Bay; and
- Samples selected to emphasize the comparison of extraction methods.

Glossary

AAD Australian Antarctic Division
CSIRO Commonwealth Scientific and Industrial Research Organization
CVO Calibration and Validation Office (NASA)
FURG Federal University of Rio Grande (Brazil)
HPLC High Performance Liquid Chromatography
JGOFS Joint Global Ocean Flux Study
LOQ Limit of Quantitation
NIO National Institute of Oceanography
PIs Principal Investigators
QA Quality Assurance
QC Quality Control
SeaBASS Bio-optical Algorithm Storage System
SeaHARRE SeaWiFS HPLC Analysis Round-Robin Experiment
SeaWiFS Sea-viewing Wide Field-of-view Sensor
SIO Scripps Institution of Oceanography
SNR Signal-to-Noise Ratio
USM University of Southern Maine
VHT Van Heukelem and Thomas (2001) method

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