SeaWiFS† HPLC Analysis Round-Robin Experiment (SeaHARRE) Overview and NASA Perspective

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†*SeaWiFS is the Sea-viewing Wide Field-of-view Sensor (a NASA ocean color satellite launched 1 August 1997).*

Welcome

Code	*Organization*	*Principal Investigator*	*Country*	*E-mail Address*
C	CSIRO	L. Clementson	Australia	lesley.clementson@csiro.au
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		M. Allerup	Denmark	mea@dhigroup.com
E	Bodø University	E. Egeland	Norway	einar.skarstad.egeland@hibo.no
G	GSFC/CVO	S. Hooker	USA	stanford.b.hooker@nasa.gov
		A. Neeley	USA	aimee.neeley@nasa.gov
H	HPL	C. Thomas	USA	cthomas@hpl.umces.edu
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I	NIO	S.G.P. Matondkar	India	sgpm@nio.org
		S. Parab	India	psushma@nio.org
K	Scripps	W. Kozlowski	USA	wkozlowski@ucsd.edu
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N	Dalhousie Univ.	C. Normandeau	Canada	c.normandeau@dal.ca
O	IO/U. Lisbon	V. Brotas	Portugal	vbrotas@fc.ul.pt
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	FURG	V. Garcia	Brazil	docvmtg@furg.br
		R. Mendes	Brazil	rmendes@fc.ul.pt
T	USM	S. Lohrenz	USA	steven.lohrenz@usm.edu
		S. Chakraborty	USA	sumit.chakraborty@usm.edu

Workshop Agenda

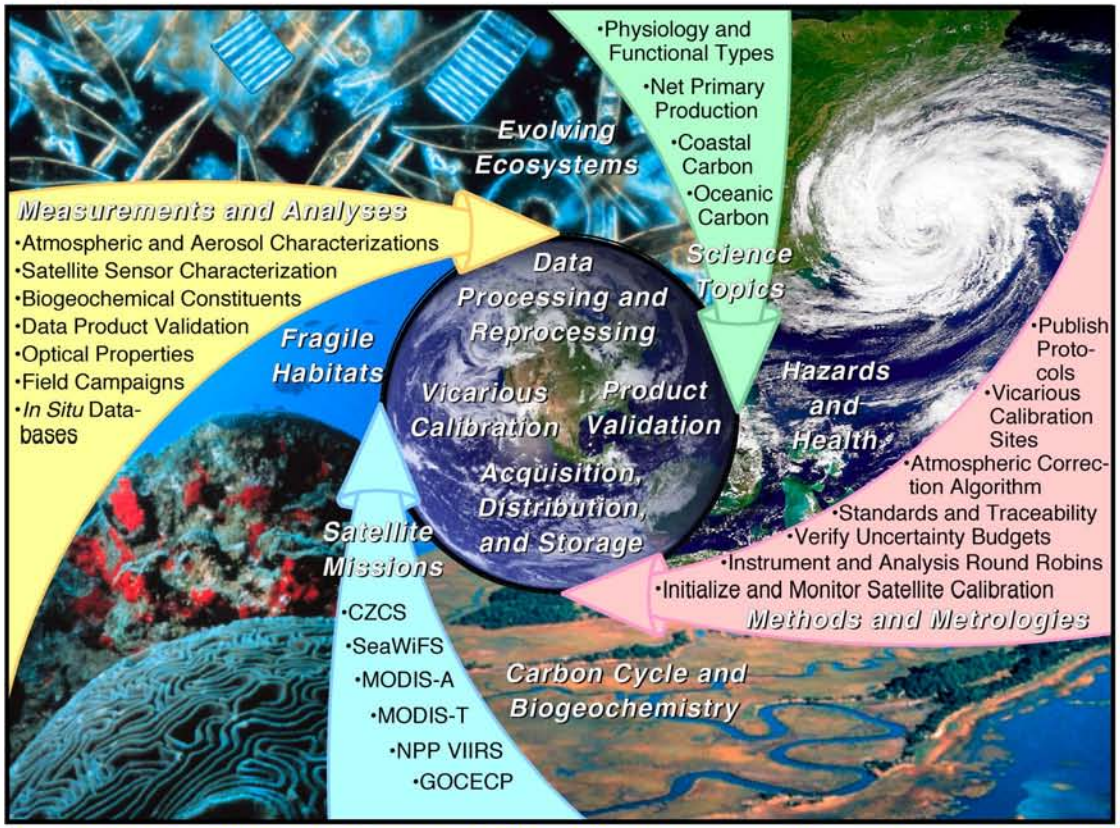
The HPLC Workshop is a combination of plenary and break-out sessions (working groups). The former centers around presentations, which include invited speakers, and discussions by all the participants; whereas the latter are concerned with reaching a consensus on specific topics of interest to the assembled scientists. Separate time for alternative scheduling is also included and involves a tour of the CSIRO facilities and a local field trip. Days begin at 0830 and end at 1730.

Key:

- Informal Meeting Time
- Invited Presentations (Plenary)
- Plenary
- Break-out Session (Working Groups)
- Alternative Scheduling

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)	Recommendations for Improving Reporting Practices of Pigment Products (S. Hooker, L. Clementson, C. Thomas, A. Neeley, and L. Shlüter)		
1130			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)	Round Table Discussion with Invited Speakers	
1640				
1650				
1700				
1710				
1720				
1730	Adjourn	Adjourn	Adjourn	

A Strategic Plan to Ensure Ocean Color Advanced Science has Calibration and Validation Support



NASA/SP–2007–214152

NASA Strategic Planning Document: A Comprehensive Plan for the Long-Term Calibration and Validation of Oceanic Biogeochemical Satellite Data

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National Aeronautics and Space Administration

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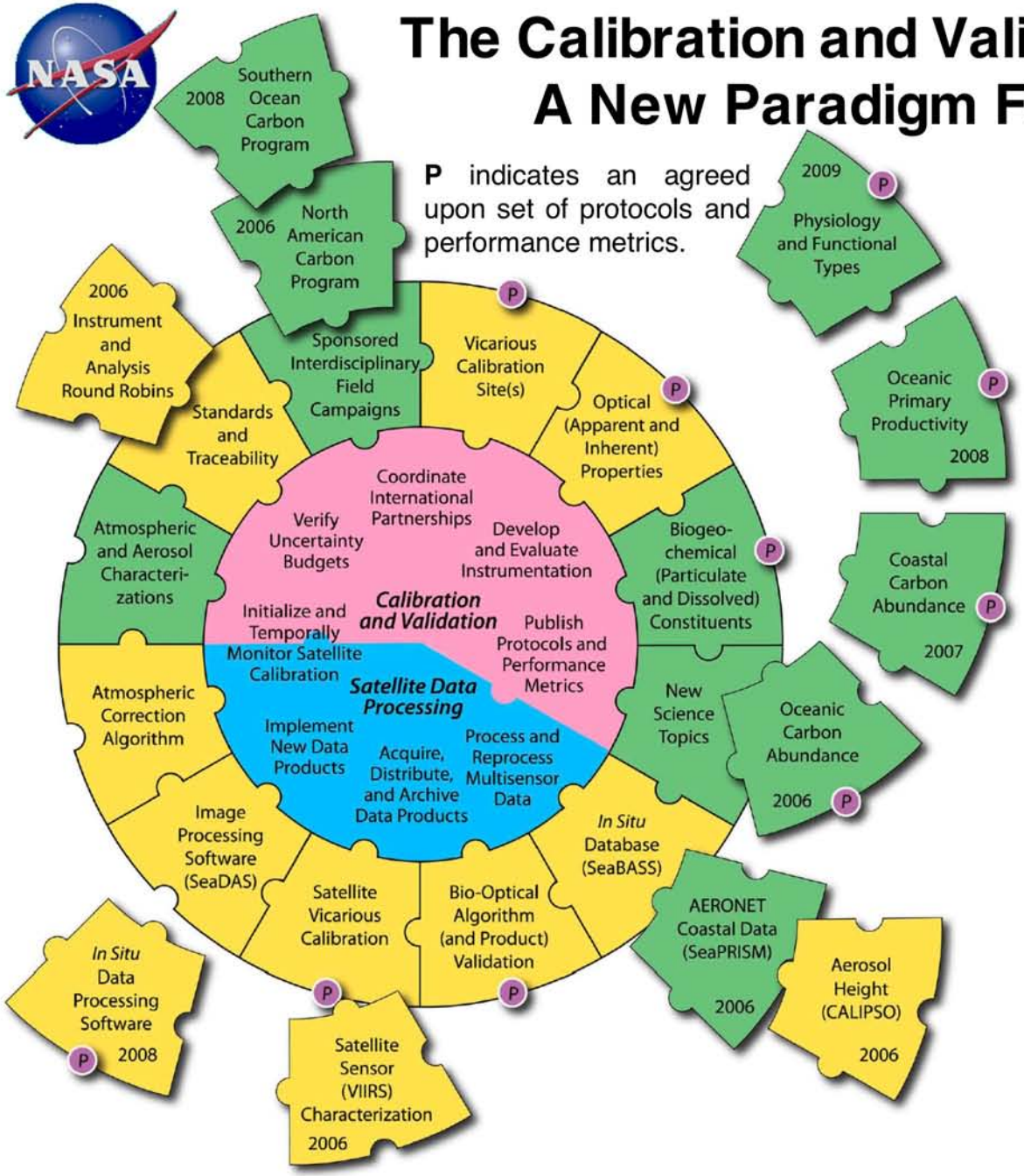
Available from the following URL: http://oceancolor.gsfc.nasa.gov/DOCS

The NASA Headquarters Ocean Biology and Biogeochemistry Program Manager has established an Advanced Science Plan (left) and a Calibration and Validation Office at the Goddard Space Flight Center. The latter is responsible for establishing a long-term capability for calibrating and validating oceanic biogeochemical satellite data, and has published a detailed plan for implementing the tasks involved (right).

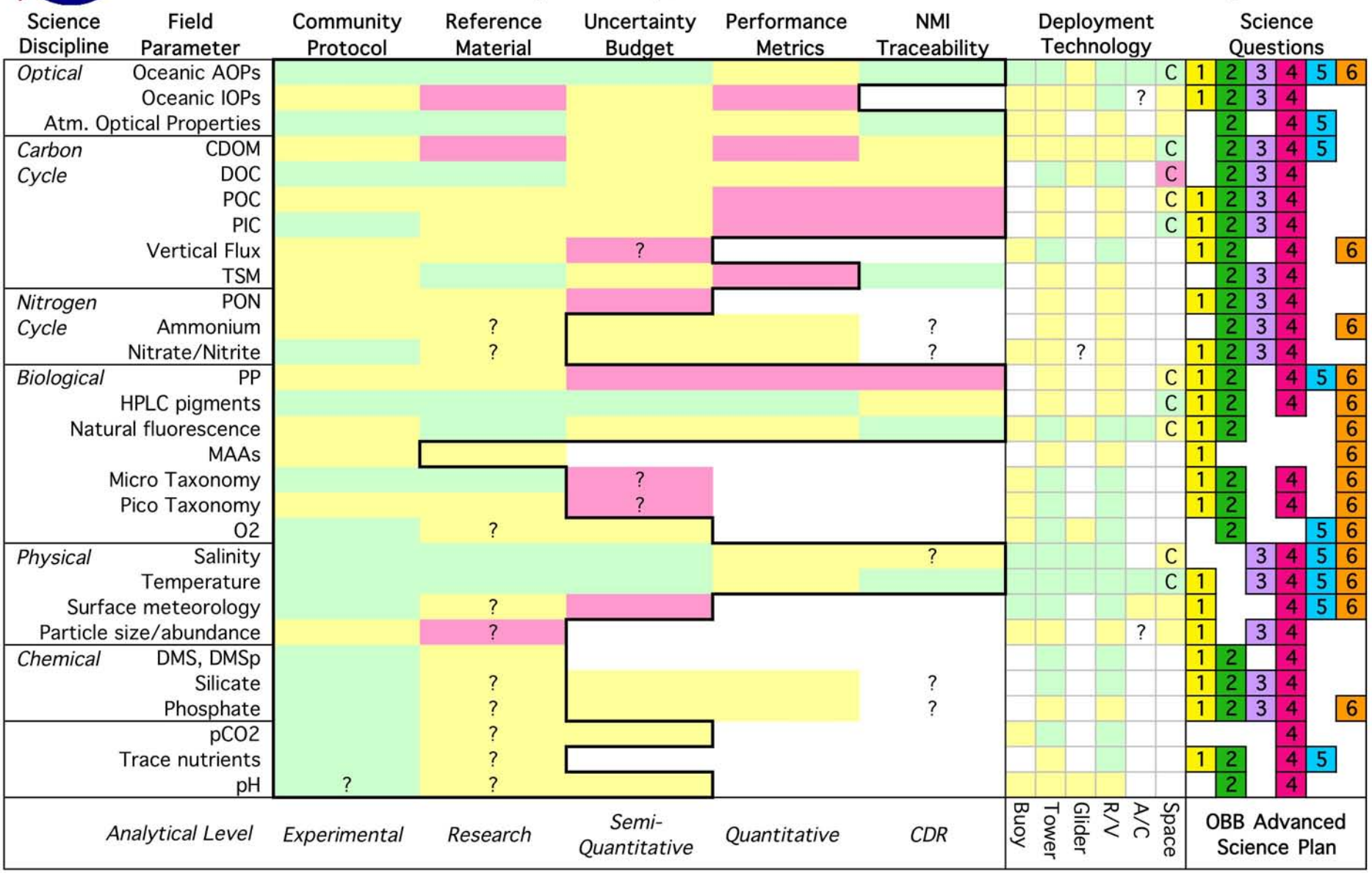
The Calibration and Validation Office (CVO): A New Paradigm For Ocean Color



P indicates an agreed upon set of protocols and performance metrics.

All elements of the CVO are interdependent, that is, they connect like puzzle pieces, and when properly joined, a comprehensive capability for the activity emerges. No one component is predetermined to be more important than a-nother. Consequently, a hori-zontal organizational scheme is anticipated and the entire enterprise is split into two components of equal stature: calibration and validation plus satellite data processing.

All Analyses, from Experimental to a Climate-Quality Data Record (CDR), are Assessed for Quality

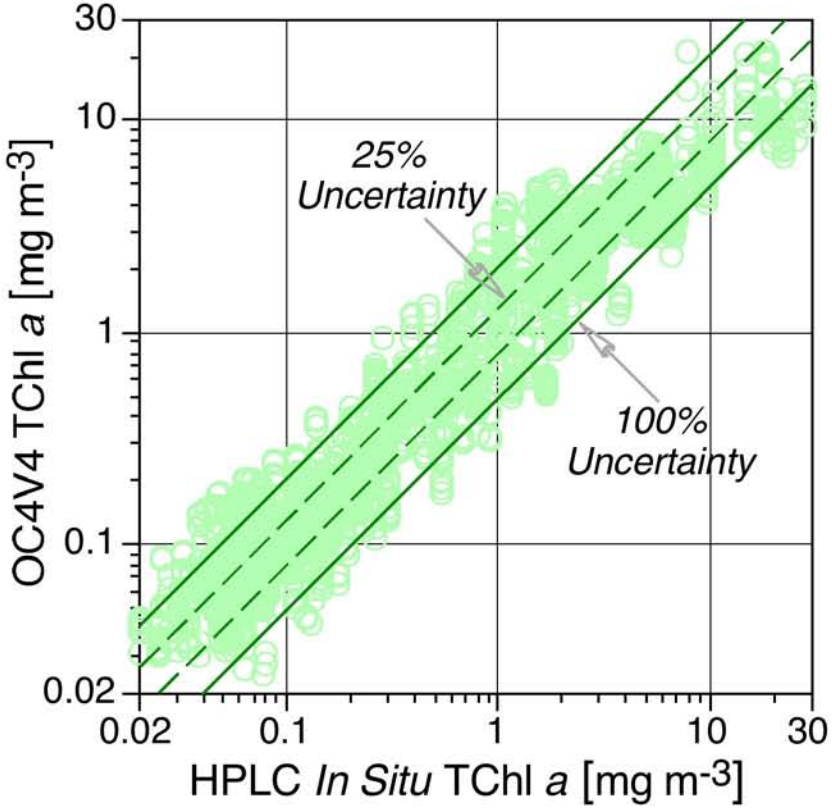


The Motivation for the SeaHARRE Activity

The primary motivation for the SeaHARRE activity was to determine whether or not the ground truth requirement for ocean color remote sensing was being satisfied.

The Sea-viewing Wide Field-of-view Sensor (SeaWiFS) Project requires agreement between the in situ and remotely-sensed observations of chlorophyll a concentration to within 35% over the range of 0.05–50.0 mg m^{-3}.

This 35% value is based on inverting the optical measurements to derive pigment concentrations using a bio-optical algorithm, so the *in situ* pigment observations are one of two axes to derive or validate the pigment relationships. Given this, it seems appropriate to reserve approximately half of the uncertainty budget for the field data. The uncertainties are assumed to combine independently (i.e., in quadrature), so *an upper accuracy of 25% is acceptable, although 15% would (presumably) permit significant improvements in algorithm refinement.*



SeaHARRE Participants Summary

SeaHARRE emphasizes a) quality-assured (QA) laboratories for computing uncertainties, b) international participation (blue text), plus c) the use of new hardware (orange), new analysts (green), and novice practitioners (yellow). There have also been specialized investigations of damaged samples (D), reanalyses to better understand analysis anomalies (R), and the use of two simultaneous methods (2).

Code	*Organization (and Country)*	*PI*	*SH-1*	*SH-2*	*SH-3*	*SH-4*	*SH-5*
B	Bedford Institue of Oceanography (Canada)	V. Stuart					
C	Common. Scientific and Indust. Res. Org. (Australia)	L. Clementson			Q	Q	R
D	DHI Water Environment Health (Denmark)	L. Schlüter		Q	Q	Q	Q
E	Bodø University College (Norway)	E. Egeland					
F	USF/Florida Institute of Oceanography (USA)	D. Millie					
G	NASA Goddard Space Flight Center (USA)	M. Russ, A. Neeley				Q	Q
H	University of Maryland Horn Point Laboratory (USA)	LVH, C. Thomas	Q	Q,D	Q	Q	Q
I	National Institute of Oceanography (India)	S.G.P. Matondkar					
J	Joint Research Centre (Italy)	J-F. Berthon	Q		R		
K	Scripps Institue of Oceanography (USA)	W. Kozlowski					
L	Laboratoire d'Océanographie de Villefranche (France)	H. Claustre, J. Ras	Q		Q	Q	Q
M	Marine and Coastal Management (South Africa)	R. Barlow	Q	Q	R		
N	Dalhousie University (Canada)	C. Normandeau					
O	IO/University of Lisbon (Portugal/Brazil)	V. Brotas					
P	Plymouth Marine Laboratory (United Kingdom)	J. Aiken					
S	San Diego State University/CHORS (USA)	J. Perl		Q	2		
T	University of Southern Mississippi (USA)	S. Lohrenz					2
U	University of South Carolina (USA)	J. Pinckney					

SeaHARRE Method Diversity as a Function of Time

A diversity of methods have been used by SeaHARRE participants, but the majority of them have been based on C_8 columns. Consequently, there has been a recurring emphasis to add new practitioners who are using C_{18} methods, so method diversity can be maintained over time.

Column	*Method Citation*	*SH-1*	*SH-2*	*SH-3*	*SH-4*	*SH-5*	*Total*
C_{18}	Gieskes and Kraay (1989)		B				1
C_{18}	Wright et al. (1991)	J	C,D,S	J,S_{18}	N,S	I,N,T_{18}	11
C_{18}	Pinckney et al. (1996)				F,U		2
C_{18}, C_8	Egeland et al. (1995)					E	1
C_8	Vidussi et al. (1996)	L	L				2
C_8	Barlow et al. (1997)	M	M,P	M			4
C_8	Zapata et al. (2000)					K,O	2
C_8	Van Heukelem and Thomas (2001)	H	H	C,D,H,L,S_8	C,D,G,H,J,L	C,D,G,H,L,T_8	19

Laboratory codes for new participants are shown in red.

Despite an explicit effort to maximize method diversity and a strong initial desire to not have the SeaHARRE community produce a unified method, there has been a significant movement by the analysts to adopt the *Van Heukelem and Thomas (2001)* method (VHT) above all others. *For the last three SeaHARRE activities, all the QA laboratories used VHT*. Consequently, a majority of the SeaHARRE data comes from the use of a C_8 method. This evolution was the reason for recruiting as many practitioners using a C_{18} method as possible for SeaHARRE-5.

Pigment Products

The intercomparisons are performed on four different types of products: individual pigments, pigment sums, pigment ratios, and pigment indices. The individual pigments are further divided into primary, secondary, and tertiary pigments. The primary pigments—*which all participants must quantitate to be eligible for inclusion in the referencing system to compute uncertainties*—are as follows:

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)	

Secondary pigments are used to produce primary pigments, and tertiary pigments are any other pigments for which at least three laboratories quantitated results.

The Higher-Order Pigment Products are Computed from the Primary Pigments

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)	$\frac{[\text{Zea}] + [\text{TChl } b]}{[\text{DP}]}$

Performance Metrics

The culmination of the SeaHARRE inquiries into using QA procedures to minimize uncertainties is a proposed set of performance metrics applicable to any HPLC method. The four different categories are arbitrary, and are used simply to provide a range of capabilities. Each category is assigned a weight and score, so the ultimate performance is based on summing the weights for each parameter, dividing by the number of parameters, and comparing the result to the category scores.

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

Based on the robustness of the original performance metrics and the continuing close agreement of the QA subset for most data products across all SeaHARRE activities, performance metrics for higher-order data products have been proposed.

Performance Weight, Category, and Score		*Sums*†		*Ratios*	
		$\bar{\xi}$	$\lvert\bar{\psi}\rvert$	$\bar{\xi}$	$\lvert\bar{\psi}\rvert$
1. Routine	0.5	8%	20%	5%	15%
2. Semiquantitative	1.5	5	12	3	9
3. Quantitative	2.5	3	8	2	6
4. State-of-the-Art	3.5	$\leq$2	$\leq$4	$\leq$1	$\leq$3

† Also for pigment indices.

Strict Adherence to Performance Metrics Produces Excellent Results Under Challenging Circumstances

The importance of performance metrics has been well demonstrated in several SeaHARRE work plans: ED, an experienced analyst analyzed an unequivocally damaged (defrosted) set of pigments for comparison with quality-assured (QA) methods and methods not validated (NV) at the QA level; NP, an established biogeochemist, but novice practitioner (who had never done HPLC), was tasked with implementing a method on an Agilent 1100 HPLC; and EN, an experienced HPLC analyst was tasked with implementing a new method using brand-new Agilent 1200 series HPLC hardware.

Category	*SeaHARRE-2*			*SeaHARRE-4*			*SeaHARRE-5*		
	ED	*QA*	*NV*	*NP*	*QA*	*NV*	*EN*	*QA*	*NV*
TChl *a*	*18.4*	**6.9**	*17.8*	7.8	**6.4**	*19.8*	5.9	**5.4**	*17.1*
PPig	22.9	**19.2**	*37.4*	**27.5**	29.8	*827.6*	**12.5**	14.8	*43.1*
Sum	14.5	**6.2**	*26.7*	**5.5**	6.5	*44.8*	**4.8**	7.5	*15.8*
Ratio	10.8	**6.2**	*11.4*	4.7	**4.2**	*34.8*	**4.0**	4.2	*13.4*
Index	7.6	**9.8**	*19.7*	**5.4**	7.4	*23.2*	**5.0**	5.9	*20.5*

QA = Quality-assured method and analyst
NV = Not validated at QA performance
ED = Experienced (QA) analyst analyzing unequivocally defrosted samples
NP = Novice practitioner strictly adhering to QA performance metrics
EN = Experienced analyst using new method and QA metrics on brand-new hardware

Formulating The Governing Equation for a Field Sample

The formulation for determining the concentration of a pigment, P_i, begins with the terms describing the calibrated response of an HPLC system:

The Amount of Pigment Injected (usually in ng)

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

Peak Area times Response Factor

Volumetric terms must now be added to provide the concentration, *C*, of a natural sample (acquired on a glass fiber filter in the field):

$$C_{P_i} = \frac{V_x}{V_f} \frac{\tilde{C}_{P_i}}{V_c}$$

V_x is the extraction volume,
V_f is the filtration volume, and
V_c is the volume injected onto the column.

The first two equations can be combined to provide the governing equation for determining the concentration of a field sample:

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

Including Details Associated With Calibrating the HPLC

The Lambert-Beer Law states that the fraction of the incident light at a particular wavelength 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:

Absorbance $$A_{P_i}(\lambda) = a_{P_i}(\lambda)\, l_c\, C_{P_i}$$

a is the absorption coefficient, and
l_c is the thickness of the sample.

The terms in the equation can be rearranged to solve for the concentration of the solution:

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

The concentration of standard, S_i, used to calibrate for pigment P_i is determined on a spectrophotometer using the above equation and a correction in the near-infrared. To derive grams per liter, *a* is replaced with the specific absorption coefficient, α, where λ_m sets the wavelength of maximum absorption:

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

Establishing the Response Factor

To derive the response factor, *R*, the concentration of the standard on the HPLC is used based on the governing equation presented at the beginning:

$$C_{S_i} = \frac{\hat{A}_{S_i} R_{P_i}}{V_c}$$

This concentration is set equal to the spectrophotometric concentration from the prior equation, the terms rearranged to solve for *R*, and the peak area of the standard expressed as a sum to reflect the computation of the total peak area. There are at least two different procedures for the latter, but they both yield the same formulation for *R*:

$$R_{P_i} = \frac{A_{S_i}(\lambda_m) - A_{S_i}(750)}{\alpha_{S_i}(\lambda_m)\, l_c} \frac{V_c}{\Sigma \hat{A}_{S_i}}$$

Complete Formulation for the Governing Equation

The full governing equation is derived by substituting the expression for *R* into the earlier formulation of the governing equation and assuming V_c is always the same:

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

For methods using an internal standard to improve the computation of the extraction volume, V_x, additional terms are involved in the formulation, which depend on how the internal standard is used. For the Van Heukelem and Thomas (2001) method, which uses a so-called one-step procedure, the governing equation becomes:

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

The green, blue, and red colors, respectively, denoted low, medium, and high anticipated uncertainties in the individual terms.

Workshop Objectives

Although the SeaHARRE activity has produced an increasingly sophisticated set of criteria to evaluate HPLC methods, the primary emphasis is still to understand the sources of uncertainty and to agree on procedures to reduce them. This objective is only possible if the participants are willing to share their problems, discuss potential solutions in an open forum, and then quantitatively assess their ideas in follow-on activities. In that spirit, the following objectives seem timely and appropriate:

- Agree on an objective set of criteria for quantitating peaks with coelution problems or signal-to-noise problems—*both of which are frequent features of small peaks, but not exclusive to small peaks*—so the uncertainty budget is not dominated by false positives and false negatives;
- Agree on reporting practices and the numerical resolution of the results;
- Agree on which pigments should be reported and whether or not the pigments should be classified (e.g., primary, secondary, and tertiary) with differing reporting or performance requirements for each classification;
- Agree on performance metrics and what criteria should be applied to the agreed upon classification scheme; and
- Agree on whether algal cultures should be part of SeaHARRE samples.

Working Groups, Breakout Sessions, and Future Planning

Although the SeaHARRE activity has produced an increasingly sophisticated set of criteria to evaluate HPLC methods, the primary emphasis is still to understand the sources of uncertainty and to agree on procedures to reduce them. This objective is only possible if the participants are willing to share their problems, discuss potential solutions in an open forum, and then quantitatively assess their ideas in follow-on activities. In that spirit, the following objectives seem timely and appropriate:

- Recommending improvements in reporting practices for pigment products (recognizing that almost every laboratory blundered in reporting their results),
- Estimating uncertainties for the terms within the HPLC governing equation (understanding the methods have different governing equations),
- Reducing uncertainties in the quantitation of small peaks (remembering the two-sentence rule is just a starting point for the discussion),
- Estimating performance metrics (assuming each analyst is using a validated method and has established accuracy requirements), and
- Planning a future SeaHARRE activity (recalling prior accomplishments and noting that both coastal analyses, which will usually involve the most complicated water types, have proved challenging).