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An intercomparison of HPLC phytoplankton pigment methods using in situ samples: application to remote sensing and database activities

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Abstract

Whether for biogeochemical studies or ocean color validation activities, high-performance liquid chromatography (HPLC) is an established reference technique for the analysis of chlorophyll *a* and associated phytoplankton pigments. The results of an intercomparison exercise of HPLC pigment determination, performed for the first time on natural samples and involving four laboratories (each using a different HPLC procedure), are used to address three main objectives: (a) estimate (and explain) the level of agreement or discrepancy in the methods used, (b) establish whether or not the accuracy requirements for ocean color validation activities can be met, and (c) establish how higher order associations in individual pigments (i.e., sums and ratios) influence the uncertainty budget while also determining how this information can be used to minimize the variance within larger pigment databases. The round-robin test samples (11 different samples received in duplicate by each laboratory) covered a range of total chlorophyll *a* concentration, [TChl *a*], representative of open ocean conditions from 0.045 mg m^{-3}, typical of the highly oligotrophic surface waters of the Ionian Sea, to 2.2 mg m^{-3}, characteristic of the upwelling regime off Morocco. Despite the diversity in trophic conditions and HPLC methods, the agreement between laboratories, defined here as the absolute percent difference (APD), was approximately 7.0% for [TChl *a*], which is well within the 25% accuracy objective for remote sensing validation purposes. For other pigments (mainly chemotaxinomic carotenoids), the agreement between methods was 21.5% on average (ranging from 11.5% for fucoxanthin to 32.5% for peridinin), and inversely depended on pigment concentration (with large disagreements for pigments close to the detection limits). It is shown that better agreement between methods can be achieved if some simple procedures are employed: (a) disregarding results less than the effective limit of quantitation (LOQ, an alternative to the method detection limit, MDL), (b) standardizing the manner in which the concentration of pigment standards are determined, and (c) accurately accounting for divinyl chlorophyll *a* when computing [TChl *a*] for those methods which do not chromatographically separate it from monovinyl chlorophyll *a*. The use of these quality-assurance procedures improved the agreement between methods, with average APD values dropping from 7.0% to 5.5% for [TChl *a*] and

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from 21.5% to 13.9% for the principal carotenoids. Additionally, it is shown that subsequent grouping of individual pigment concentrations into sums and ratios significantly reduced the variance and, thus, improved the agreement between laboratories. This grouping, therefore, provides a simple mechanism for decreasing the variance within databases composed of merged data from different origins. Among the recommendations for improving database consistency in the future, it is suggested that submissions to a database should include the relevant information related to the limit of detection for the HPLC method.

Keywords: Pigments; HPLC; Phytoplankton; Database; Methods

1. Introduction

Because phytoplankton concentration is an important variable in the study of marine biogeochemical cycles, the accurate quantification of its biomass is a fundamental requirement. Phytoplankton biomass is typically approximated by quantifying chlorophyll *a* concentration, [Chl *a*], for which many methods ranging from the single cell to thesynoptic (remote sensing) scale have been developed (Yentsch and Menzel, 1963; Parsons and Strickland, 1963; Olson et al., 1983; O'Reilly et al. 1998).

The taxonomic composition of phytoplankton influences many biogeochemical processes, so it is essential to simultaneously determine phytoplankton biomass and composition over the continuum of phytoplankton size (approximately 0.5–100 μm). The determination of chlorophyll and carotenoid pigment concentrations by high-performance liquid chromatography (HPLC) is a method which fulfills most of these requirements. Indeed, many carotenoids and chlorophylls are taxonomic markers of phytoplankton taxa, which means community composition can be evaluated at the same time that [Chl *a*] is accurately quantified.

Since the initial methodological paper by Mantoura and Llewellyn (1983), the possibility of determining community composition and biomass has resulted in the HPLC method rapidly becoming the technique of choice in biogeochemical and primary production studies. The use of HPLC methods in marine studies has also been promoted, because the international Joint Global Ocean Flux Study (JGOFS) program recommended HPLC in the determination of [Chl *a*] (JGOFS, 1994) and, more precisely, to use the protocol of Wright et al. (1991). Since the start of the JGOFS decade in the 1980s, HPLC techniques have evolved considerably (Jeffrey et al., 1999), and some JGOFS contributors decided not to follow the original JGOFS recommendation in order to take full benefit of the ongoing methodological evolutions. In particular, the C8 method of Goericke and Repeta (1993) was an important improvement, because it allowed the separation of divinyl chlorophyll *a* from its monovinyl form. Subsequent adaptations of this method were proposed (e.g., Vidussi et al., 1996; Barlow et al., 1997) and used for a variety of JGOFS cruises. More recently, new methods have also been proposed that rely on C8 phase and elevated column temperature to achieve the desired separation selectivity (Van Heukelem and Thomas, 2001) or on mobile phase modified with pyridine to resolve chlorophyll *c* pigments (Zapata et al., 2000).

Because the analysis of marine pigment concentration by the HPLC method was a new and rapidly changing research field, it was necessary to carefully check the performance consistency between the evolving methods and, if necessary, propose corrective recommendations. Such a review was also required, because HPLC was becoming the reference method for calibration and validation activities of [Chl *a*] remote sensing measurements, for which accuracy was an essential requirement. For example, 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} (Hooker and Esaias, 1993). This value is based on inverting the optical measurements to derive pigment concentrations using a bio-optical algorithm, so the in situ pigment observations will always be one of two axes to derive or validate the pigment relationships (Hooker and McClain, 2000). Given this, it seems appropriate to reserve approximately half of the uncertainty budget for the in situ pigment measurements. The sources of uncertainty

are assumed to combine independently (i.e., in quadrature), so an upper accuracy range of 25% is acceptable, although 15% would allow for significant improvements in algorithm refinement.

The SCOR UNESCO Working Group 78 for determining the photosynthetic pigments in seawater was established in 1985 and culminated with the publication of a monograph with many methodological recommendations (Jeffrey et al., 1997). Similarly, JGOFS sponsored an intercomparison exercise involving the distribution and the analysis of pigment standards among several laboratories, which also resulted in some analytical recommendations (Latasa et al., 1996). More recently, the National Aeronautics and Space Administration (NASA) established and has incrementally refined a set of protocols for measurements in support of oceanic optical measurements, including the use of the HPLC method for phytoplankton pigment analysis (Bidigare et al., 2002). Checking the reliability of different HPLC methods on natural samples has never been performed. Such an evaluation is essential, because the purpose of methodological revisions and testing is to improve the accuracy in pigment determinations on natural samples.

As part of the Productivité des Systémes Océaniques Pélagiques (PROSOPE) JGOFS-France cruise, which took place from 4 September to 4 October 1999, four laboratories, using four different methods, participated in an intercomparison exercise based solely on natural samples. The samples used for this exercise were collected over a large gradient of trophic conditions, from the high-productivity regime off the northwestern coast of Africa to the highly oligotrophic conditions of the Ionian Sea (Fig. 1). The range in oceanic ecosystems ensured a diversity of pigment compositions that were explored as part of this exercise.

Using such a geographically diverse data set, a priori representative of many oceanic conditions, the main objectives of the present study were (a) to estimate the uncertainties in the HPLC method used and explain the level of agreement (or discrepancy) achieved; (b) to establish whether or not the uncer-

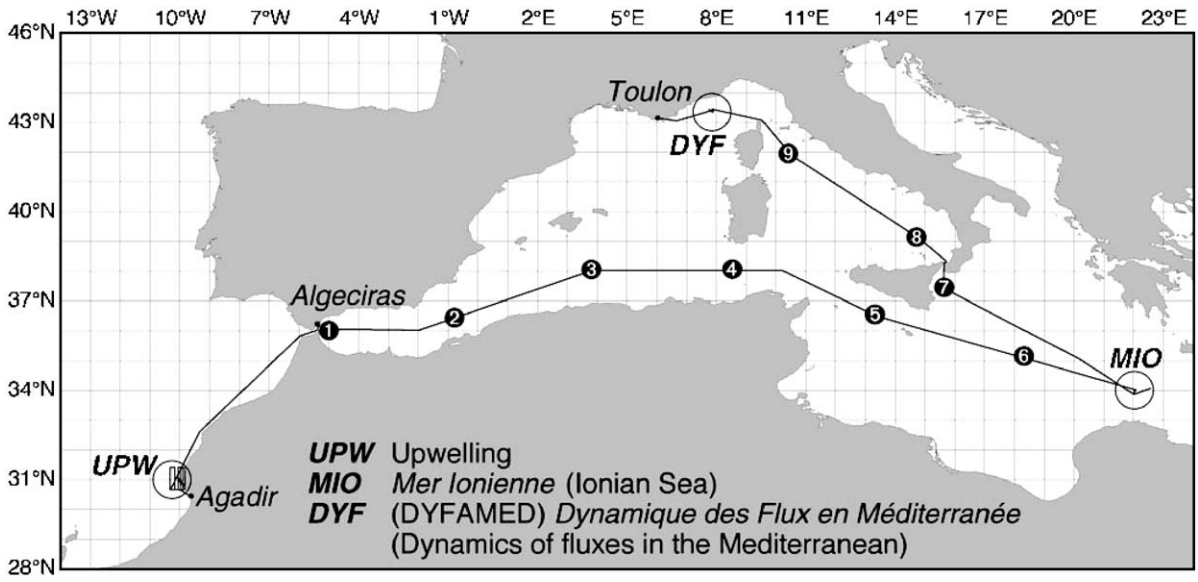

Fig. 1. The PROSOPE cruise track showing the three long stations (open circles), which lasted a few days each, and the nine short along-track stations (numbered bullets), which lasted 1 day each. Data collected at the latter are identified by "S" codes (e.g., sample S4 was collected at the fourth along-track station), and data from the former are identified by three unique letters plus a number: "U" for UPW, "M" for MIO, "D" for DYF, and the number is a sequential index to keep track of the number of days on station. The total data set used in this study encompasses water samples from the upwelling zone (U1 and U3), the short along-track stations (S4, S8, and S9), the Ionian Sea (M2 and M4), and the DYFAMED site (D1, D3, D4, and D5).

tainty objectives for ocean color validation activities can be met with the HPLC technique; and (c) to quantify how higher order associations of the individual pigments (sums and ratios) influence the uncertainty budget while also determining how the results can be applied to a larger database to keep uncertainties at a minimum.

2. Methods

Four laboratories, which had contributed to various aspects of SeaWiFS calibration and validation activities, participated in the round-robin: (a) the American Horn Point Laboratory (HPL) of the University of Maryland Center for Environmental Science; (b) the European Joint Research Centre (JRC) Inland and Marine Waters unit of the Institute for Environmental Sustainability, which was formerly the Marine Environment unit of the Space Applications Institute; (c) the French Laboratoire d'Océanographie de Villefranche (LOV), which was formerly the Laboratoire de Physique et Chimie Marines; and (d) the South African Marine and Coastal Management (MCM) Ocean Environment Unit. Each laboratory is identified according to a one-letter code: H for HPL, J for JRC, L for LOV, and M for MCM.

2.1. Sampling and sample distribution

Glass fiber filters (25 mm GF/F) were used to collect seawater samples, which varied in volume from 1.0 to 2.8 l depending on the sampling region (Hooker et al., 2000). Eleven geographical locations were sampled, and at each location, 12 replicates were taken, so triplicates could be distributed to each laboratory. One set of 12 replicates is referred to here as a batch and corresponds to all the samples collected at a particular station. Only 10 replicates were collected for the 6th batch and the 12th batch could not be distributed to all the laboratories, so a total of 130 individual filter samples were distributed and analyzed for this study.

2.2. Laboratory methods

None of the laboratories used exactly the same HPLC procedures as another. Details of each method are presented in Hooker et al. (2000), so only method-related procedures and performance evaluation criteria are emphasized here.

2.2.1. Sample handling and extraction

Filters were shipped to participants in liquid nitrogen dry shippers. Filters were stored and extracted according to procedures summarized (Table 1). Laboratory H estimated extraction volume as the volume of solvent added plus the average volume of water (145 μl) contributed by a 25-mm GF/F, as previously observed at H. Laboratories J, L, and M each used an internal standard to determine extraction volumes. The water content in the sample extracts for all laboratories was limited to approximately 10%.

All laboratories used automated HPLC injectors, which mixed sample extract with buffer immediately prior to injection. In addition, all laboratories used temperature-controlled autosampler compartments (set at 2 or 4 °C), where samples resided up to

Table 1
A summary of the extraction specifications for each of the four laboratories (or methods)

Laboratory code	Storage temperature (°C)	Extraction solvent	Internal standard	Disruption mode and extraction time		Clarification	Sample and buffer mix
H	− 80	95% acetone	none	ultrasonic probe	4 h	0.45μm Teflon syringe filter	sample loop
J	− 80	100% acetone	*trans*-β-apo-8′ -carotenal	tissue grinder	24 h	0.45μm Teflon syringe filter	sample loop
L	− 20	100% methanol	*trans*-β-apo-8′ -carotenal	ultrasonic probe	1 h	GF/C 1.3 μm filter	separate vial
M	− 80	100% acetone	canthaxanthin	ultrasonic probe	0.5 h	centrifugation (10 min)	separate vial

The volume of solvent added is given in milliliters. Each filter was disrupted, allowed to soak, and then clarified.

Table 2
A summary of accuracy and precision of pipettes and HPLC injectors

Parameter	H	J	L	M
Pipette setpoint volume (ml)	3.0	1.5	3.0	2.0
Pipette observed volume (ml)	3.009	1.530	2.987	2.005
Percent of setpoint volume delivered (%)	100.3	102.0	99.57	100.3
Pipette precision (95% confidence limits) (%)	0.11	2.06	0.38	1.11
HPLC average injector precision (%)	0.7	1.5	1.5	1.8
HPLC injection volume (μl)	150	97.5	133	100

Average precision of injector programs (set to deliver the specified volume of sample) was assessed with replicate injections of chlorophyll *a* (H and M) or internal standard (J and L).
The accuracy and precision of the pipettes used for adding extraction solvent was assessed by weighing (in replicate, $N=10$ or 7) the solvent delivered, and correcting for specific gravity to determine volume delivered.

approximately 24 h before injection. Details pertaining to extraction volumes, pipette calibrations, and injector precision are given in Table 2.

2.2.2. Separation conditions

Each laboratory selected an HPLC method based on the pigment content of the samples they typically analyzed. The methods for laboratories H, J, L, and M were based on Van Heukelem and Thomas (2001), Wright et al. (1991), Vidussi et al. (1996), and Barlow et al. (1997), respectively. Method details are given in Tables 3 and 4.

Most of the principal pigments (Table 5) were well resolved, i.e., the resolution, Rs (Snyder and Kirkland, 1979), between adjacent pigments was adequate for quantitation by peak area (Rs>1). Exceptions included Chlide *a* and Chl c_1 (H and J); Chl c_1, Chl c_2, and Chlide *a* (L and M); Chl *b* and DVChl *b* (J and L); Chl *a* and DVChl *a* (J); ββ-car and βε-car (all methods). Chl *b* and DVChl *b* were partially resolved (Rs < 1) by H and M.

Table 4
A summary of the solvent systems used by each laboratory

Laboratory code	Solvent A	Solvent B	Solvent C
H	70:30 methanol/ 28 mM aqueous TBAA[a]	100% methanol	100% ethyl acetate
J	80:20 methanol/ 0.5 M ammonium acetate	90:10 acetonitrile/ water	
L	70:30 methanol/ 0.5 M ammonium acetate	100% methanol	
M	70:30 methanol/ 1.0 M ammonium acetate	100% methanol	

[a] Tetrabutyl ammonium acetate.

2.2.3. Detection and quantitation

All laboratories used photodiode array detectors set to acquire data at two wavelengths. The detectors used were either a Hewlett-Packard (HP) series 1100 (H, J, and L) or a Thermo Separations UV6000 (M). Simultaneous equations, described by Latasa et al. (1996), were used by H to quantify the co-eluting pigments chlorophyllide *a* and chlorophyll c_1 (Hooker et al., 2000) and by J (exclusively for this study) to determine the relative proportions of [DVChl *a*] and [Chl *a*].

The criteria used to approximate a method detection limit (MDL), referred to here as a limit of quantitation (LOQ), was based on the amount of injected pigment (in nanograms) corresponding to a

Table 3
A summary of the separation specifications for each of the four laboratories (or methods)

Laboratory	Column phase	Particle size (μm)	Internal diameter (mm)	Column length (mm)	Flow rate (ml min^{-1})	Column temperature (°C)
H	C8	3.5	4.6	150	1.1	60
J	C18	5.0	4.6	250	1.0	not controlled
L	C8	3.0	3.0	100	0.5	not controlled
M	C8	3.0	4.6	100	1.0	25

Table 5
The chlorophyll and carotenoid pigments of importance to the present study shown with their symbols, names, and calculation formulae (if applicable)

Symbol	Methods				Pigment	Calculation
[Chl *a*]	H		L	M	chlorophyll *a*[a] (Chl *a*)	
[Chl *b*]	H			M	chlorophyll *b* (Chl *b*)	
[Chl c_1]	H				chlorophyll c_1 (Chl c_1)	
[Chl c_2]	H				chlorophyll c_2[b] (Chl c_2)	
[Chl c_3]	H	J	L	M	chlorophyll c_3 (Chl c_3)	
[Chlide *a*]	H	J		M	chlorophyllide *a* (Chlide *a*)	
[DVChl *a*]	H		L	M	divinyl chlorophyll *a* (DVChl *a*)	
[DVChl *b*]	H			M	divinyl chlorophyll *b* (DVChl *b*)	
[TChl *a*]	H	J	L	M	■ total chlorophyll *a* (TChl *a*)	[Chlide *a*]+[DVChl *a*]+[Chl *a*]
[TChl *b*]	H	J	L	M	■ total chlorophyll *b* (TChl *b*)	[DVChl *b*]+[Chl *b*]
[TChl *c*]	H	J	L	M	■ total chlorophyll *c* (TChl *c*)	[Chl c_1]+[Chl c_2]+[Chl c_3]
[Allo]	H	J	L	M	alloxanthin (Allo)	
[But]	H	J	L	M	■ 19′-butanoyloxyfucoxanthin (But-fuco)	
[Caro]	H	J	L	M	■ carotenes (ββ-car and βε-car)	[ββ-car]+[βε-car]
[Diad]	H	J	L	M	■ diadinoxanthin (Diadino)	
[Diato]	H	J	L		diatoxanthin (Diato)	
[Fuco]	H	J	L	M	■ fucoxanthin (Fuco)	
[Hex]	H	J	L	M	■ 19′-hexanoyloxyfucoxanthin (Hex-fuco)	
[Lut]	H		L		lutein (Lut)	
[Neo]	H		L		neoxanthin (Neo)	
[Peri]	H	J	L	M	■ peridinin (Perid)	
[Pras]	H		L		prasinoxanthin (Pras)	
[Viola]	H		L	M	violaxanthin (Viola)	
[Zea]	H	J	L	M	■ zeaxanthin (Zea)	

The methods (laboratories) that reported the various pigments are identified by their single character codes (H for HPL, J for JRC, L for LOV, and M for MCM). The pigments which comprise the so-called individual pigments in this study are identified by the square bullet (■). Pigments shown without bullets were reported by at least one laboratory, but are not statistically compared, except some are used in specialized analyses or implicitly considered through summed or derived variables. The pigment symbols, which are used to indicate the concentration of the pigment (in milligrams per cubic meter), are patterned after the nomenclature established by the Scientific Committee on Oceanographic Research (SCOR) Working Group 78 (Jeffrey et al., 1997). Abbreviated forms for the pigments are given in parentheses.

[a] Monovinyl chlorophyll *a* (MVChl *a*) plus allomers and epimers.

[b] Plus Mg-2,4-divinyl phaeoporphyrin a_5 monomethyl ester (Mg DVP).

signal-to-noise ratio (SNR) of 10 (at the wavelength used for quantitation). Each laboratory measured the LOQ for Chl *a* and Fuco. Short-term instrument noise (Snyder and Kirkland, 1979) occurring after the elution of carotenes, where wander and drift were minimal, was used in SNR computations. The amount of pigment (in nanograms per liter of seawater) that resulted in an injected amount equivalent to a particular method LOQ is referred to here as the effective LOQ and was determined for each filtration volume used. Method LOQ and an example of effective LOQ are given in Table 6.

Pigment standards used by J and L (and some used by M) were purchased from the DHI Water and Environment Institute (Hørsholm, Denmark). Each laboratory spectrophotometrically analyzed their DHI standards and used the observed concentrations (instead of those provided by DHI) for computing HPLC response factors (RFs). Chlorophyll *a* (H and M), chlorophyll *b* (H), and ββ-carotene (H) were purchased in solid form from Sigma (St. Louis, MO) or Fluka Chemie (Buchs, Switzerland). Other standards used by H were isolated from natural sources (Van Heukelem and Thomas, 2001).

The extinction coefficients used by the laboratories are summarized in Table 7. Some pigments, for which laboratories had no discrete standards, were quantified based on RFs derived from other standards (with similar spectra) with adjustments for differences in molecular weight. Pigments so quantified were Chlide *a* (all methods), Chl c_3 (H, J, and L), and DVChl *a* and DVChl *b* (L).

Table 6
HPLC PDA detector settings are based on center method LOQ, and the effective LOQ (for a filtration volume of 2.8 l) for the four laboratories

Product		H	J	L	M
Chlorophyll *a* Pigments	$\lambda c \pm \Delta\lambda$ (nm)	665 ± 10	436 ± 4	667 ± 15[a]	440 ± 7
	$\lambda c \pm \Delta\lambda$ (nm)		450 ± 4		
	method LOQ (ng)	0.5	0.5[b]	0.3	1.2
Other Pigments	$\lambda c \pm \Delta\lambda$ (nm)	450 ± 10	436 ± 4	440 ± 15[a]	440 ± 7
	$\lambda c \pm \Delta\lambda$ (nm)		450 ± 4		
	method LOQ (ng)	0.6	0.4[b]	0.3	0.5
Chlorophyll *a*	effective LOQ (ng l^{-1})	3.6	2.7	2.4	8.6
Fucoxanthin	effective LOQ (ng l^{-1})	4.3	2.2	2.4	3.6

The former are based on center wavelength (λc) and bandwidths ($\Delta\lambda$), used to quantify pigments and method LOQ (defined as the nanograms of injected pigment that correspond to an SNR of 10).

[a] A reference wavelength was also used, 750 ± 5 nm.

[b] Measured at 436 ± 4 nm.

All laboratories used gas-tight glass syringes for diluting stock pigment solutions, validated HPLC response factors were linear over the range of sample concentrations, and verified quantitation was unaffected by carry-over between injections. The R^2 values for chlorophyll *a* calibration curves observed by all laboratories were typically near 0.999.

2.3. Analytical variables

Together with the individual pigments (Table 5), the pigment groups and higher order variables used in this study are presented in Table 8. [TChl *a*], [TChl *b*], and [TChl *c*] do not represent individual pigment concentrations—each represents a group of pigments roughly characterized by the same absorption spectra (including some degradation products). These sums allow the comparison of results originating from various HPLC methods that differ in the way the pigments within the same family are distinguished (e.g., chlorophyll *c* types) or whose extraction procedures might or might not generate degradation forms (e.g., chlorophyllide *a*). Perhaps most importantly, the sums permit the comparison of methods that differ in

Table 7
Details of the pigment standards used by the four laboratories and their sources (DHI or "Other")

Pigment name	Pigment source					Extinction coefficient	
	DHI			Other		DHI	Other
Chl *a*	J	L		H	M	87.67, 90% acetone	88.15 in 100% acetone (M)
DVChl *a*				H	M		100% acetone: 88.15 (H[a]); 88.35 (M[b])
Chl *b*	J	L	M	H		51.36, 90% acetone	52.5 in 100% acetone (H)
DVChl *b*				H	M		100% acetone: 52.5 (H[a]); 51.47 (M[b])
Chl c_1				H			39.2 in 100% acetone (H)
Chl c_2				H			37.2 in 100% acetone (H)
Chl c_1+c_2	J		M			42.6, 90% acetone	
But-fuco	J	L	M	H		160 ethanol	134.6 ethanol (M)
Diadino	J		M	H	L	262 ethanol	233.7 ethanol (M), 223 in 100% acetone (L)
Fuco	J	L	M	H		160 ethanol	152 ethanol (M)
Hex-fuco	J	L	M	H		160 ethanol	130 ethanol (M)
Perid	J	L	M	H		132.5 ethanol	
Zea	J	L	M	H		254 ethanol	234 in 100% acetone (H)
ββ-car	J		M	H		262 ethanol	180 ethanol (L[c])

The extinction coefficients (in units of liters per gram per centimeter) were the same as those provided by DHI unless indicated in the "Other" column.

[a] Isolated by HPL.

[b] Standards used were from HPL and the University of Hawaii.

[c] Based on a previous calibration.

Table 8
The higher order pigments shown with their symbols, names, and calculation formulae

Symbol	Pigment sum	Calculation
[PPC]	photoprotective carotenoids (PPC)	[Allo]+[Diad]+[Diato]+[Zea]+[Caro]
[PSC]	photosynthetic carotenoids (PSC)	[But]+[Fuco]+[Hex]+[Peri]
[PSP]	photosynthetic pigments (PSP)	[PSC]+[TChl *a*]+[TChl *b*]+[TChl *c*]
[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*]
Symbol	**Pigment ratio**	**Calculation**
[TAcc]/[TChl *a*]	total accessory pigments to total chlorophyll *a*	[TAcc]/[TChl *a*]
[PPC]/[TPig]	photoprotective carotenoids to total pigments	[PPC]/[TPig]
[PSP]/[TPig]	photosynthetic pigments to total pigments	[PSP]/[TPig]
[mPF]	microplankton proportion factor[a]	([Fuco]+[Peri])/[DP]
[nPF]	nanoplankton proportion factor[a]	([Hex]+[But]+[Allo])/[DP]
[pPF]	picoplankton proportion factor[a]	([Zea]+[TChl *b*])/[DP]

Abbreviated forms for the pigments are given in parentheses.
[a] As a group, also considered as indices or macrovariables.

their capability of differentiating monovinyl from divinyl forms.

The carotenoids (Table 5) were chosen based on the fact that they are the most common pigments used in chemotaxonomic or photophysiological studies in open ocean or coastal waters (Gieskes et al., 1988; Bidigare and Ondrusek, 1996; Barlow et al., 1993; Claustre et al., 1994). Some carotenoids are implicitly considered in the analysis of summed and ratioed variables, e.g., Allo and Diato.

Subsequent grouping of pigments (including chlorophyll 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 (nonchlorophyll *a*) pigments (TAcc) are useful in remote sensing investigations (Trees et al., 2000). The ratios that can be derived from these pooled variables, e.g., [PSC]/[TChl *a*], are dimensionless and have the advantage of automatically scaling the comparison of results from different areas and pigment concentrations.

The [DP] pigment criteria was introduced by Claustre (1994) to estimate a pigment-derived analog to the *f*-ratio (the ratio of new production to total production) developed by Eppley and Peterson (1979). The use of [DP] was extended by Vidussi et al. (2001) to derive size-equivalent pigment indices which roughly correspond to the biomass proportions of pico-, nano-, and micro-phytoplankton, [pPF], [nPF], and [mPF], respectively. The latter so-called macrovariables are composed of pigment sums and are ratios, so they should be particularly useful in reconciling inquiries applied to databases from different oceanic regimes.

2.4. Data reporting and statistics

Each laboratory participated as if the analyses were performed as a result of normal operations—that is, a single concentration value was reported for each pigment, for each laboratory, and for each batch of replicates. The solitary concentrations were the averages of the individual replicated filters (two or three) analyzed for each batch. To ensure consistency in reporting, all values were converted to concentrations of milligrams per cubic meter, and any no-detection (null) result was replaced with a value of 0.0005 mg m^{-3}.

One of the primary objectives of this study is to determine whether or not the HPLC methods under consideration meet the remote sensing accuracy objectives. Accuracy is the degree of agreement of a measured value with the true or expected value (Taylor, 1987), so a representation for the true concentration of each batch of samples is needed. No one laboratory (or result) is assumed to be more correct

than another—there is no absolute truth, because standards were not part of the sample set—so an unbiased approach is needed to compare the differences between the methods. The first step in developing an unbiased analysis is to calculate the average concentration for each pigment, $\bar{P}_j$, for each batch (or station) reported by the four contributing laboratories:

$$[\bar{P}_j(S_k)] = \frac{1}{4}\sum_{i=1}^{4}[P_j^i(S_k)], \quad (1)$$

where the i superscript identifies the laboratory (or method), which is used for summing over the four possible laboratory (or method) codes; the j subscript identifies the pigment or pigment association (Tables 5 and 8); and S_k sets the batch (or station).

The relative percent difference (RPD) for each pigment of the individual laboratories with respect to the average values are then calculated for each batch as

$$\psi_j^i(S_k) = 100\frac{[P_j^i(S_k)] - [\bar{P}_j(S_k)]}{[\bar{P}_j(S_k)]}. \quad (2)$$

Although $[\bar{P}_j(S_k)]$ is not considered truth, it is the surrogate for truth and is the reference value by which the performance of the methods with respect to one another are quantified. A positive RPD value indicates the pigment concentration for a particular laboratory is greater than the average for that pigment (a negative value indicates the opposite). Consequently, the RPD statistic retains the information on the sign of the dispersion of a particular result (with respect to the average) and provides insight into methodological biases (i.e., identification of methods that are systematically high or low relative to the average consensus).

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, the absolute percent difference (APD), which is simply the absolute value of the RPD, is used when averaging over the number of batches (N):

$$|\psi_j^i| = \frac{1}{N}\sum_{k=1}^{N}|\bar{\psi}_j^i(S_k)|. \quad (3)$$

The average APD is an estimate of the accuracy of each laboratory for each pigment across the number of batches selected for analysis. The lower the APD, the greater the accuracy or, equivalently, the lower the uncertainties. When the entire round-robin data set is considered, $N = 11$ (the total number of batches or stations).

In addition to the size and recurrence of any methodological bias, the performance of each method can be measured by precision. Precision is the degree of agreement within multiple measurements of a quantity (Taylor, 1987), and is frequently computed as the deviation of a set of estimates from their average value. The precision is estimated here using the standard deviation in the replicate analysis, divided by the average concentration for each replicate (as determined by the individual methods). This relative standard deviation (RSD) is expressed as a percent and is also known as the coefficient of variation (the lower the RSD, the better the precision).

3. Results

The results are organized according to the individual pigments (Table 5) and the higher order pigment sums and ratios (Table 8). A particular emphasis is placed on the identification of systematic biases and the relationship of accuracy (RPD and APD) and precision (RSD) with the individual pigment concentrations.

3.1. Individual pigments

The round-robin data cover a range of [TChl *a*] extending over almost two orders of magnitude from 0.045 mg m^{-3}, typical of the Ionian Sea oligotrophic surface waters, to 2.2 mg m^{-3}, characteristic of the upwelling regime off Morocco (Fig. 1). The analysis of the RPD values for [TChl *a*] estimation (Fig. 2a) shows some generalized systematic biases with respect to the average: L is systematically below the average (9.6%), J (except for the M4 batch in the Ionian Sea) is systematically above the average (9.5%), and M and H are balanced around the average (zero). For the four laboratories, the RPD values do not present any obvious trend as a function of [TChl *a*]. If the RPD analysis is extended to the

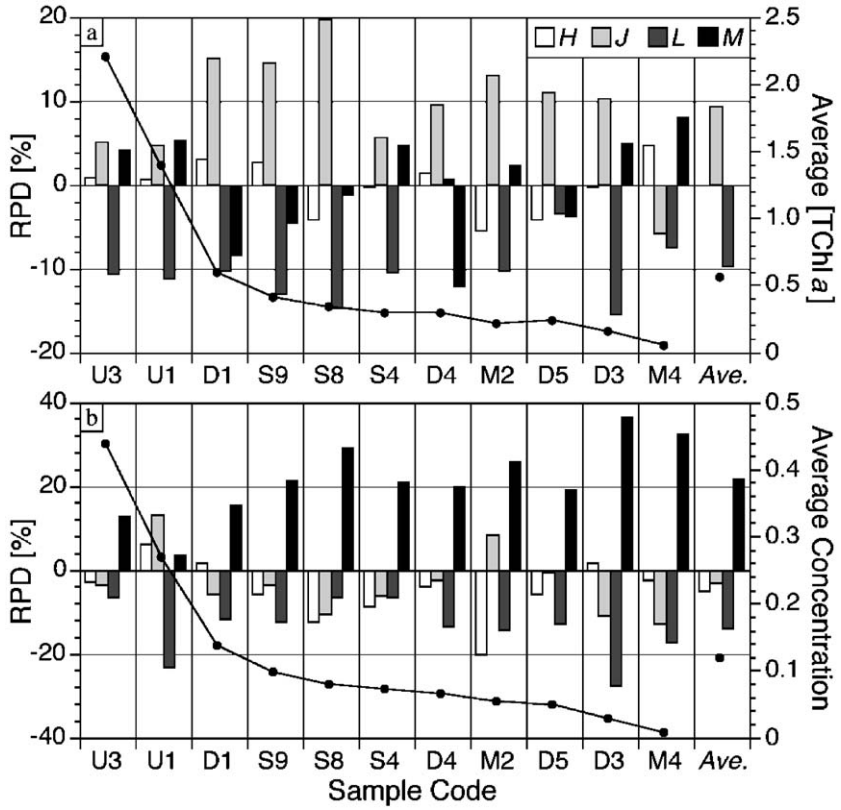

Fig. 2. The relative percent difference (RPD) values for the four methods and 11 samples for (a) [TChl *a*], and (b) the average individual pigment concentrations. The concentrations for the former and latter are given as the overplotted symbols and lines, with the corresponding axis scales to the right in units of milligrams per cubic meter. Averages across all samples are given in the rightmost column in each panel.

average of all the individual pigments (identified by the ■ symbol in Table 5), M is systematically above (typically 21%) and L systematically below (about 15%) the consensus average (Fig. 2b). In comparison, H and J are almost always below the consensus average (the average RPD is less than 5% for both laboratories).

Individual pigment concentrations had an important effect on method differences (Fig. 3). For many pigments, there is an inverse relationship between the APD value and the concentration value—there is approximately a 22% decrease in APD for every decade increase in pigment concentration. The APD values for [TChl *a*] show almost no sensitivity over the range of concentrations (and, thus, of trophic regimes) observed in this study—the APD range is confined to 5.3–9.9% with an average value of 7.0%.

Fig. 4 presents precision (RSD) as a function of the pigment concentration for the analysis of carotenoids and TChl *a*. Pigments occurring at low concentrations tend to exhibit poor precision (higher RSD values) more frequently than pigments at high concentrations. With a precision poorer than ± 12.5% (RSD), the 25% remote sensing uncertainty objective would be met infrequently if it was based on the analysis of solitary, not replicate, filters. The average RSD for pigments less than 0.01 mg m^{-3} was 12%, but it was 6.6% among pigments of higher concentrations. It is important to note the average precision for [TChl *a*] was 5.4% (RSD), which suggests the precision for [TChl *a*] was completely adequate for the remote sensing accuracy objectives.

The APD distributions for individual pigments are considered as a function of [TChl *a*] in Fig. 5a. The

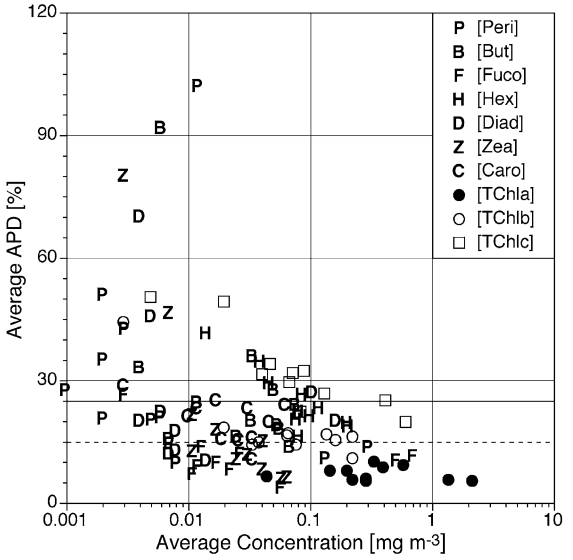

Fig. 3. The average absolute percent difference (APD) values (across all methods) for the individual pigments as a function of the average concentration of the pigment. The chlorophyll pigments are shown as symbols (with [TChl *a*] as the only solid symbol, so it can be easily distinguished), and the other pigments by letters. From a generalized perspective, it is useful to adopt the aforementioned SeaWiFS accuracy objectives criteria for all the pigments considered in this study, even though they are only strictly applicable to [TChl *a*]; thus, the dashed and solid horizontal lines give the 15% and 25% accuracy objectives, respectively.

majority of the data are within the 25% remote sensing objective, particularly for the moderate and high [TChl *a*] concentration regimes. As seen with precision, however, there is a trend for poor accuracy (increasing APD) as a function of decreasing [TChl *a*]. Some carotenoids do not follow this trend, do not covary with [TChl *a*], and rather consistently exceed the 25% objective. For example, the APDs of [Zea] and [But] are high even in the high [TChl *a*] regime, as are the APDs of [Peri] in the moderate [TChl *a*] regime. The high APDs of these carotenoids likely occurred because the algal classes they represent were of minor importance in the trophic regime in which they occurred.

3.2. Pigment sums and ratios

The average APD values for the summed variables as a function of [TChl *a*] (Fig. 5b) show a substantial improvement in accuracy with respect to the individual pigments (Fig. 5a): the majority of the data are below the 15% accuracy objective, and almost all of the data are below the 25% objective. The reason for the improvement in accuracy is that the summing process reduces the statistical weight of the low-concentration pigments (which have the greatest uncertainty). The [PSC] results are the only data with a clear trend as a function of concentration. The trend is primarily due to [Hex], which provides the most important statistical weight in the determination of [PSC] for the samples considered here.

Average APD values decrease further when pigment ratios are considered (Fig. 5c). This is particularly notable for the [PSP]/[TPig] results, which are confined to an average APD range of 0.7–3.0% with an overall average value of 1.6%. The [TAcc]/[TChl *a*] and [PPC]/[TPig] ratios are also confined to narrow APD ranges of 11.3–18.8% and 7.9–16.3%, respectively, with overall average values of 12.9% and 14.1%, respectively. The biomass proportion factors, however, extend over a larger APD range

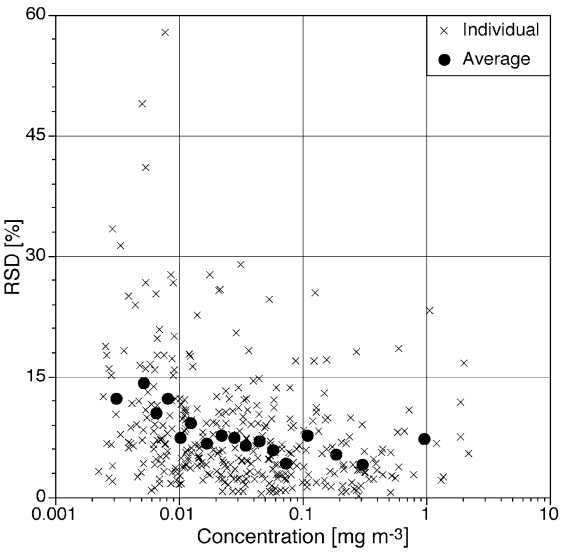

Fig. 4. The relative standard deviation (RSD), expressed in percent, associated with all the carotenoids (the individual and ancillary carotenoids in Table 5) plus the total chlorophyll *a* values reported by each laboratory, but not discriminated for each (×). The average RSD values (●) are based on ranking the data as a function of concentration, and then binning 21 consecutive data points to form the average.

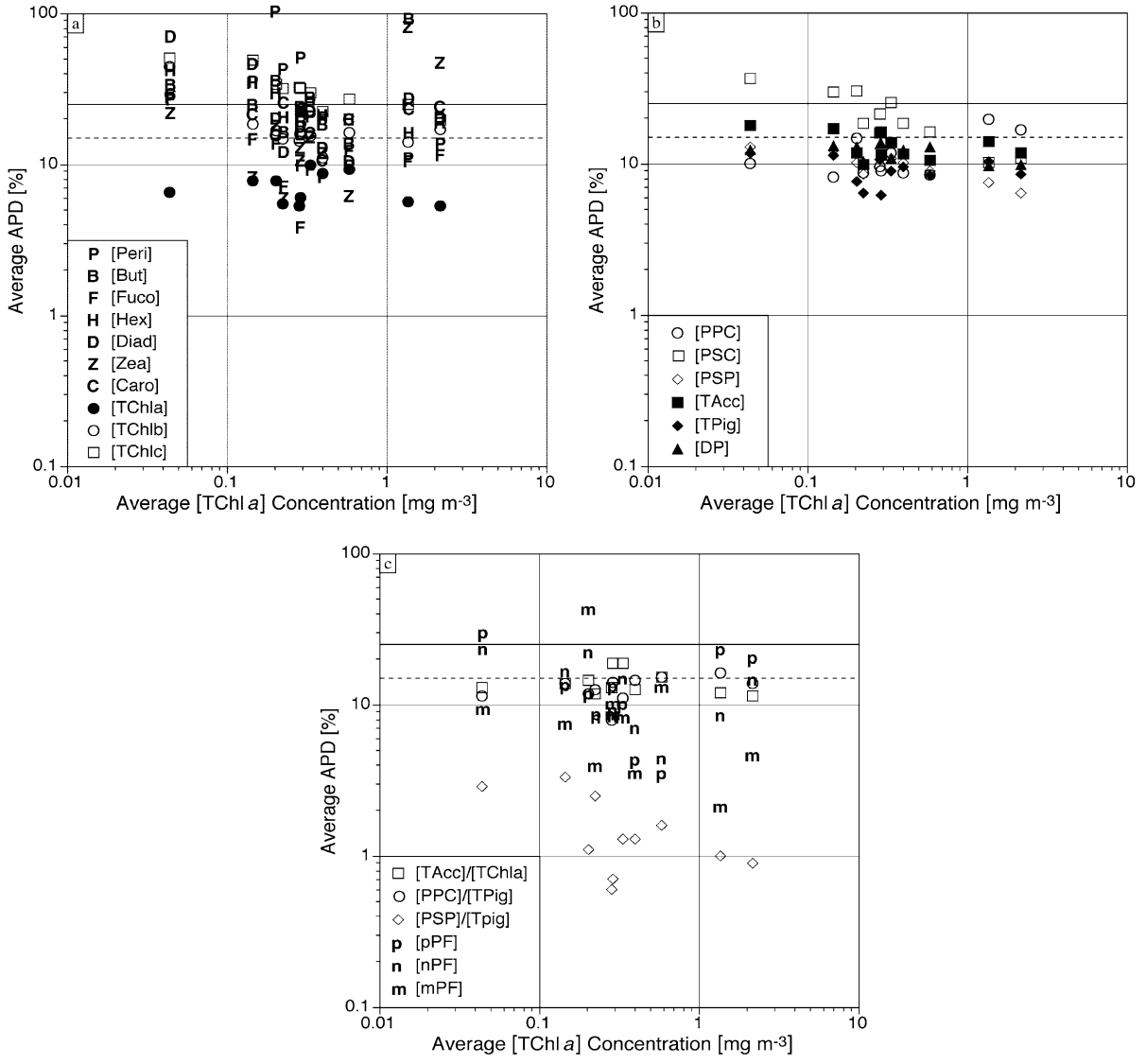

Fig. 5. The average absolute percent difference (APD) values (across all methods) as a function of the average [TChl *a*] value for (a) individual pigments, (b) pigment sums, and (c) pigment ratios. The dashed and solid horizontal lines give the 15% and 25% accuracy objectives, respectively.

and are the primary variables to exceed the accuracy thresholds.

4. Discussion

Three principal topics are considered for discussion: (a) identifying the factors influencing the agreement between methods; (b) determining whether the level of agreement between the methods is in accordance with the requirements for remote sensing validation activities; and (c) applying the knowledge gained from this intercomparison exercise to the more general problem of minimizing the variance associated with pigment data from different origins when they are merged into a larger database.

4.1. Methodological factors

Factors investigated for their effects on accuracy (APD values) are limited here to (a) the inclusion of pigment concentrations in the database less than or equal to the effective LOQ, (b) variations in the procedures for determining the concentrations of pigment standards, and (c) not individually quantifying [DVChl *a*] when determining [TChl *a*]. In addition, the effects of method precision are also addressed.

4.1.1. Limit of quantitation

Estimating the MDL for a particular method is useful, because it describes the quality of the results at low concentrations. For example, values below the MDL are not significantly different from zero; whereas values above—but near—the MDL are frequently associated with high uncertainty. Ocean color remote sensing protocols suggest measuring an MDL (Bidigare et al., 2002), but none of the laboratories in this study did so. Consequently, it was not initially known how much of the reported data was acquired at or below the MDL.

A variety of procedures can be used to estimate the MDL (Cleseri et al., 1998). For this study, the effective LOQ was used as a proxy for the MDL. To ensure uniform reporting, batches not satisfying the effective LOQ were removed (i.e., if one laboratory reported a value less than the effective LOQ for the pigment under consideration, the batch was not retained). This analytical approach is hereafter referred to as the batch LOQ. The method LOQ of Fuco was used as a proxy for all carotenoids, which included Peri, But-fuco, Fuco, Hex-fuco, Diad, Zea, and Caro. Because [TChl *a*] is a summation of pigments, the components of the sum that were less than the method LOQ for [Chl *a*] were replaced with the null value (0.0005 mg m^{-3}) before summing. There was no method LOQ determined for the Chl *b* and Chl *c* parent pigments and their derivatives, so they were excluded from the evaluation.

The original and recomputed APD values are presented in Table 9 and show there was a small reduction in the [TChl *a*] APD values. The greatest reduction in APD, from 32.2% to 13.8%, was for [Peri], which was often present in low concentrations and thus most affected by a batch LOQ test. The average APD across all pigments and laboratories was reduced from 21.5% to 16.2%, with the reduction for each laboratory ranging from 4.4% to 6.2%.

4.1.2. Determination of standard concentrations

The concentration of HPLC calibration standards are determined spectrophotometrically. In addition, many laboratories remeasure the concentration of standards purchased from DHI, and in some instances, use extinction coefficients different from DHI. Variance in spectrophotometric measurements plus the choice of extinction coefficients can therefore contribute to HPLC uncertainties. The importance of these two sources of uncertainty are explored in Table 10. When all the laboratories used concentrations provided by DHI, the overall average APD for individual pigments was 13.1%. When the laborato-

Table 9
The original and recomputed APD values for eight pigments

Pigment	*N*	H	J	L	M	Average
[TChl *a*]	11 (11)	2.5 (2.5)	10.5 (10.5)	9.7 (9.5)	5.4 (5.3)	7.0 (7.0)
[But]	11 (9)	20.3 (13.2)	15.5 (6.4)	33.4 (27.0)	48.7 (41.9)	29.5 (22.1)
[Caro]	11 (10)	6.8 (6.0)	34.7 (33.9)	11.5 (9.6)	28.0 (28.1)	20.2 (19.4)
[Diad]	11 (9)	15.9 (11.9)	22.8 (20.0)	12.0 (9.8)	48.7 (39.8)	24.9 (20.4)
[Fuco]	11 (10)	9.3 (8.0)	9.1 (9.0)	10.4 (9.4)	17.2 (13.7)	11.5 (10.0)
[Hex]	11 (11)	23.4 (23.4)	6.2 (6.2)	22.9 (22.9)	46.7 (46.6)	24.8 (24.8)
[Peri]	11 (4)	19.9 (5.8)	38.8 (21.0)	41.8 (18.6)	28.4 (9.9)	32.2 (13.8)
[Zea]	11 (9)	15.9 (8.2)	22.9 (14.0)	17.0 (9.1)	30.1 (17.6)	21.5 (12.2)
Average	11 (9)	14.3 (9.9)	20.1 (15.1)	19.8 (14.5)	31.6 (25.4)	21.5 (16.2)

The recomputed APD values are shown in parentheses and are based on only using the batches for which all reported values were greater than the effective LOQ values for each method. The number of batches involved is given by *N*; the original 11 is followed by the number (in parentheses) passing the batch LOQ quality assurance threshold. The overall averages are based on all methods (horizontal) or all pigments (vertical).

Table 10
The average APD across all methods, except for [Chl *a*] for which J is omitted, caused by variations in the procedures by which concentrations of standards from DHI were determined

Pigment	N_M	Concentration	*A*	*B*	*C*
[Chl *a*]	2	0.473	5.1	5.4	5.4
[But]	3	0.046	16.5	15.4	22.1†
[Caro]	2	0.029	18.7	19.4	19.4
[Diad]	2	0.036	14.5	15.4	20.4†
[Fuco]	3	0.138	9.2	9.0	10.0†
[Hex]	3	0.096	16.1	16.1	24.8†
[Peri]	3	0.113	13.8	13.8	13.8
[Zea]	3	0.034	11.2	12.2	12.2
Overall average APD across all pigments			13.1	13.3	16.0

The batches used included only those for which the results were greater than the effective LOQ. N_M is the number of laboratories (methods) using the indicated standard from DHI, and the concentration is the average of the batches from the data as reported in milligrams per cubic meter. Standard concentrations were determined by DHI (*A*), from spectrophotometric measurements by laboratories using the same extinction coefficients as DHI (*B*), and the same as in *B* except extinction coefficients used by MCM for some pigments (indicated by the † symbol) differed from those of DHI (*C*).

ries used their own spectrophotometric measurements—but the same extinction coefficients as DHI—the overall average APD was 13.3%. Remeasuring the concentrations of standards purchased from DHI, therefore, may not improve accuracy. For the data as originally reported, however, the laboratories used their observed concentrations, and M also used different extinction coefficients for four pigments, and the resulting overall average APD was 16.0%.

4.1.3. Divinyl forms

Although the average determination of [TChl *a*] is within the remote sensing and algorithm refinement accuracy objectives, the J method does not separate the monovinyl from the divinyl form of chlorophyll *a*. The importance of this is well established (Latasa et al., 1996) and is explored in Fig. 6. This figure shows the [TChl *a*] RPD values for J (solid circles) versus the corresponding average H, L, and M [DVChl *a*]/[TChl *a*] values. The least-squares linear regression of the data indicates about a 4.0% increase in JRC uncertainty for a 10% increase in [DVChl *a*]/[TChl *a*].

The [TChl *a*] values for J were subsequently recomputed using the simultaneous equations of Latasa et al. (1996). For these calculations, values of Chlide *a* less than or equal to the effective LOQ were replaced with the null value (0.0005 mg m^{-3}). These revised results are shown in Fig. 6 (open circles) and reflect an overall threefold improvement with respect to the data as originally reported. Furthermore, the average RPD for the J [TChl *a*] values (across all batches) was reduced from 9.4% to 4.2%, the average APD for J was reduced from 10.5% to 4.2%, and the average [TChl *a*] APD across all laboratories was reduced from 7.0% to 5.5%. Unexpectedly, the simultaneous equation only improved [TChl *a*] RPD values if allomers and epimers were excluded.

4.1.4. Method precision

The average precision of each laboratory was 9.9% (H), 9.7% (J), 6.6% (L), and 4.3% (M). These averages were based upon the RSD observed for individual pigments in replicate filters, but for which results less than or equal to each laboratory's effective LOQ had been discarded. The laboratory averages were, therefore, not affected by the inclusion of results less than an MDL, so differences between

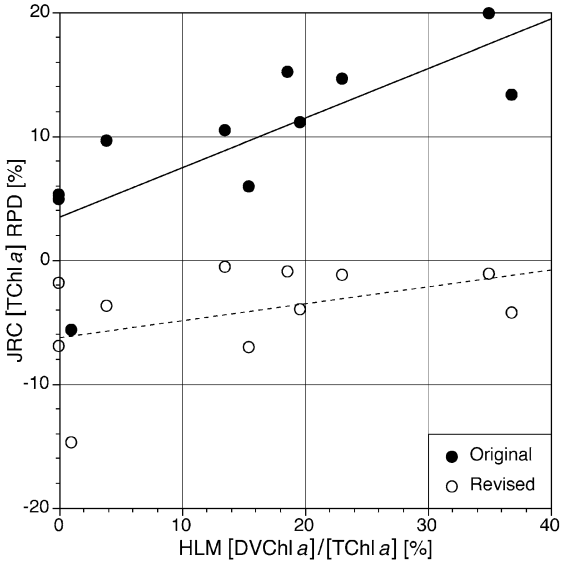

Fig. 6. The relative percent difference (RPD) values for the JRC determination of [TChl *a*] using the original analysis (solid circles) and the revised analysis (open circles) based on the Latasa et al. (1996) simultaneous equations and plotted as a function of the average [DVChl *a*]/[TChl *a*] values derived from the H, L, and M methods. A least-squares linear regression of the original and revised data are given by the solid and dashed lines, respectively.

laboratories represent the effects of HPLC instrument precision, extraction volume determinations, and filter inhomogeneity.

It is notable that the H method had the poorest sample precision (9.9%), despite having the best HPLC injection precision (0.7%), and was probably a consequence of method H not using an internal standard to assess extraction volumes. Conversely, the excellent sample precision of method M (4.3%)—the method with the poorest injection precision (1.8%)—suggests extraction volume estimates were very precise and that filter inhomogeneity contributed little to the variance in replicate filter analyses across all methods.

4.1.5. Cumulative effects

The cumulative effect of the quality-assurance procedures discussed above (application of the batch LOQ, unification of extinction coefficients, and quantification of DVChl *a* by all laboratories) was a 7.6% reduction in the average uncertainty relative to the results for the same set of pigments in the original data set. The APD values of the individual pigments are shown as a function of concentration in Fig. 7 (for quality-assured data) and in Fig. 3 (for data as originally reported). The most striking difference is that almost all the data in Fig. 3 are within the 25% uncertainty objective, and the majority of the data in Fig. 7 are within the 15% uncertainty objective.

Application of the quality-assurance procedures also reduced systematic differences between laboratories. Previously, the RPD values of methods J and L for [TChl *a*] (Fig. 2a) exhibited a high and low bias, respectively, which was nearly systematic across all stations (H and M RPD values nearly coincided with the average). With the application of the quality-assurance procedures, all four laboratories had a similar dispersion with respect to the average [TChl *a*]: 4.7% (H), −4.3% (J), −5.1% (L), and 4.7% (M). In addition, the average carotenoid RPD of M had been systematically high, but was improved from 24.7% to 15.7% primarily because M values were recomputed using extinction coefficients in common with other laboratories. The average APD of laboratory M for all individual pigments showed an even greater improvement and was reduced from 35.4% to 19.1% by the application of the quality-assurance procedures—an improvement of 16.3%.

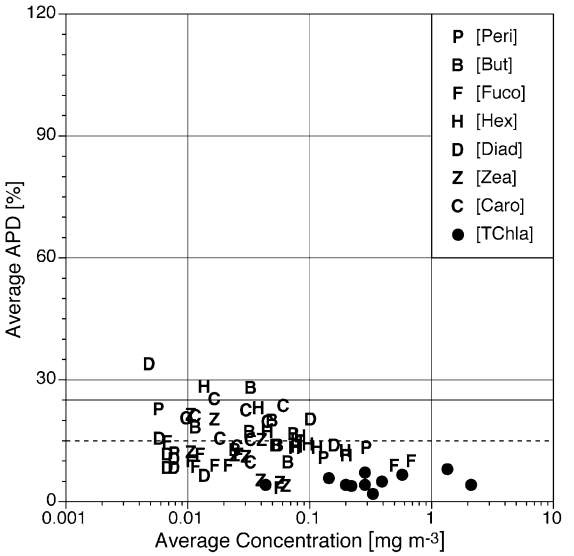

Fig. 7. The average absolute percent difference (APD) values (across all methods) for the individual pigments (excluding chlorophyll *c* and chlorophyll *b*) after recomputing the results from each method using procedures that reduced uncertainties: only batches for which individual concentrations were above the effective LOQ were retained. In addition, where standards from DHI were used for quantitation, the concentrations provided by DHI were used for determining response factors. The dashed and solid horizontal lines give the 15% and 25% accuracy objectives, respectively.

4.1.6. Recommendations for methodological improvements

The results presented above show it is possible to merge data from different HPLC methods and achieve a 25% uncertainty objective, but consistency among laboratories in ancillary procedures is important. Also implicit in the results is the importance of such things as pigment resolution, injector precision, and pipette calibrations.

Variability can be reduced if all laboratories use the same extinction coefficients for determining the concentration of pigment standards. This seemingly simple choice is, in fact, rather complex. For example, when standards are purchased from commercial vendors, the choices of extinction coefficients and solvents in which the standards are suspended are beyond the analyst's control. There is also ambiguity in the HPLC pigment protocols established for remote sensing objectives (Bidigare et al., 2002) in that many

of the recommended carotenoid extinction coefficients are based on dissolution in acetone, not ethanol, and such standards may not be commercially available in acetone.

Applying an MDL threshold (here the effective LOQ) improved the quality of merged data. However, the approach used here only describes uncertainties associated with detection, it does not adequately describe the variance associated with sample collection, storage, extraction, etc. The MDL procedure recounted by Bidigare et al. (2002), which has not been adopted by analysts, may not adequately define the variance at low concentrations (Zorn et al., 1997). Efforts to redefine the measurement and use of pigment MDL values are warranted.

4.2. Application to remote sensing activities

To achieve the SeaWiFS objective of agreement to within 35% between the remote and in situ chlorophyll *a* determinations, an upper limit of 25% uncertainty was considered acceptable, although 15% was desirable for algorithm refinement. The overall average [TChl *a*] uncertainty of 7% resulting from this study is well within both limits, with the average uncertainties for each laboratory ranging from 5.3% to 9.9% and could be decreased further if all laboratories separated the monovinyl and divinyl forms of chlorophyll *a*.

It is important to remember the round-robin data set is limited, however, and covers a reduced range of concentration (0.04–2.1 mg m^{-3}) with respect to the one associated with the 35% objective (0.05–50 mg m^{-3}). This exercise may not be fully representative of the end points within the concentration range, particularly very oligotrophic areas, where the uncertainties increase as the methodologies approach the sensitivity thresholds. A mitigating factor, however, is the uncertainty in [TChl *a*] proved to be largely insensitive to the concentration level for the concentrations and filtration volumes used.

One fundamental use of in situ [TChl *a*] estimates is to validate the empirical algorithms linking marine apparent optical properties (e.g., reflectances at different wavelengths combined in ratios) with [TChl *a*]. The low uncertainty in [TChl *a*] achieved in this study (7%) underestimates the combined variance associated with the determination of [TChl *a*] within the large data sets used for the development of global (predominantly open ocean) algorithms, e.g., the so-called OC4v4 algorithm (O'Reilly et al., 2000). The latter usually contain both HPLC and fluorometric determinations of [TChl *a*] from a large number of participants using a diversity of optical equipment to sample a wide range of oceanic regimes. When the large source of contributions (and thus, variance) for a global data set is restricted to an individual inquiry, the uncertainty associated with the in-water optical measurements can be as low as 3% (Hooker and Maritorena, 2000).

Using the individualized uncertainty estimates for the optical and pigment variables constituting bio-optical algorithms (3% and 7%, respectively), the largest part of the uncertainty is intrinsic to the variability inherent in empirical algorithms. Part of the variance is natural and originates from the variability between the physical signal (the reflectance) and the concentration of total chlorophyll *a*, but part of it is artificial and originates from differences in the methodologies used. The former is due to the spatial and temporal aspects of the sampling, as well as the changing contribution of the different optically significant substances (phytoplankton, particulate detritus, colored dissolved organic matter, and mineral particles in coastal waters).

4.3. Application to database analyses

The continuous evolution of HPLC methods during the data acquisition phase of the JGOFS program, coupled with the ongoing requirement to complete the data synthesis activities, establishes the problem of merging data acquired by different laboratories into metadata. One purpose of the data synthesis effort is to extract generic oceanic properties; conversely, another purpose is to highlight specific regional (spatio-temporal) features, in keeping with what was recently initiated by Trees et al. (2000).

Given this level of advanced analysis, it is legitimate to question if the desired syntheses and resulting parameterizations will be biased, because they deal with data acquired by different methods. It is therefore timely to investigate the issue of database consistency and the potential for bias. In particular, it is relevant to evaluate the benefit of building and using higher order variables (e.g., pigment sums, ratios, and indices or

macrovariables) as alternatives to the use of individual pigment concentrations. This intercomparison exercise provides the opportunity to investigate this important issue using an admittedly restricted, but unique, data set.

4.3.1. The round-robin data as a subset of the PROSOPE data set

For the complete PROSOPE data set (Ras et al., 2000), 50 samples were from the high [TChl *a*] regime, 333 from the moderate regime, and 152 from the low regime. Note that this distribution approximately parallels the round-robin data set in the sense that the majority of the data are from the moderate regime and the high [TChl *a*] regime has the smallest number of samples. There is a disparity in the amount of sampling within the round-robin low [TChl *a*] regime, but this might not be so important, because this regime is expected to have a smaller range in pigment properties than the other two. Consequently, the round-robin data set can probably be considered as a representative subset of the whole PROSOPE data set.

The round-robin subset can subsequently be used as a reference, or baseline, to investigate whether the distributions of uncertainties among pigment categories (individual, sum, ratio, and macro) are similar in the subset versus the entire PROSOPE data set; in other words, to determine whether or not the entire data set has a functional form similar to the round-robin subset. A functional form is defined here as a recurring relationship between the pigment uncertainties and the pigment category, which can occur across or within pigment categories. Although the amplitude of the relationship might differ as a function of the pigment category, the basic shape of the relationship is expected to be the same. For this inquiry, the overall average concentration of each pigment or pigment group derived from the four laboratories across all batches ($N=11$) is used as the reference value in (2), and the average APD is the average across all the PROSOPE samples ($N=535$).

A presentation of these alternative analyses and how they compare to one another is given in Fig. 8. The pigment categories follow what was presented in Section 3 (including individual pigments, sums, and ratios), except the pigment ratios have been further subdivided; the macrovariables, [mPF], [nPF], and [pPF], have been combined into one category and presented separately from the pigment ratios. The results from the round-robin subset follow directly from what was presented in Fig. 5 and are denoted the baseline, because they are the starting point for evaluating the analytical approaches. Almost all of the pigment categories (on average) in Fig. 8 are within the 25% accuracy objective and, with the exception of the individual pigments, are within the 15% objective. The lowest uncertainty is achieved with the pigment ratios (9.3%), which have an uncertainty that is more than a factor of two smaller than the individual pigments (22.0%).

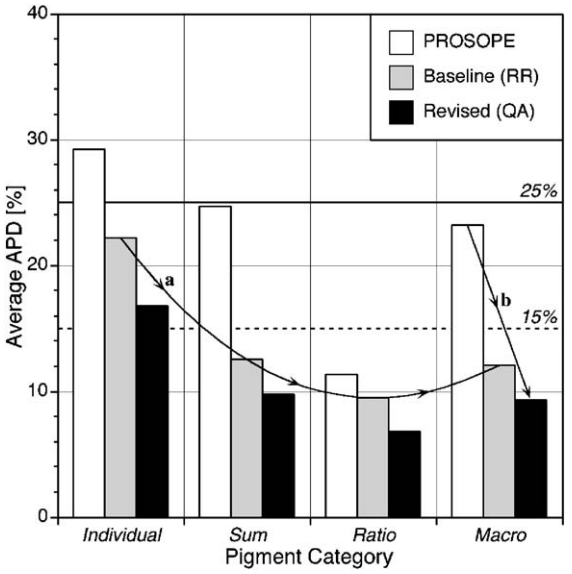

Fig. 8. The average absolute percent difference (APD) values for the round-robin (RR) data set using baseline analysis (gray bars), quality assurance (QA) procedures with the baseline analysis (black bars), and applying the round-robin results as a reference (or model) for the entire PROSOPE database (white bars). The quality-assured data were subjected to the batch LOQ threshold tests and, for J, use [TChl *a*] values based on a simultaneous equation (Latasa et al., 1996). The bold dashed and solid horizontal lines give the 15% and 25% accuracy objectives, respectively. The functional forms for the behavior of the uncertainties are distinguished by the curve and line with arrows, respectively: (a) across the pigment categories, and (b) within each pigment category. Both functional forms are shown as single examples, but are individually applicable across all the pigment categories or within a category for the three data sets.

There are two functional forms in the uncertainties in Fig. 8: (a) within a pigment category, the uncertainty decreases going from the entire PROSOPE data

(high natural variability), to the baseline round-robin data, and then to the quality-assured data (low methodological variability); and (b) between pigment categories, the uncertainty decreases going from the individual pigments to the sums and then to the ratios, but then slightly increases for the macrovariables. The consistency of these two relationships across the various analytical schemes used here is an important indicator of the robustness of the results and the conclusions that can be derived from them. Furthermore, they show that the round-robin subset is a good reference for the entire data set and all pigment categories.

The apparent degradation in accuracy for the macrovariables is primarily the result of the statistical weight of the pigments in the biomass proportions associated with the accessory pigments (see above). For example, in the eutrophic regime, where large phytoplankton predominate, it is expected that the APD for [mPF] would be lower, because Fuco and [Peri] have a low APD (Fig. 5a); conversely, higher APD values would be expected for [mPF] in a low [TChl *a*] regime. The present data set, however, is not extensive enough to go deeper in the interpretation of the observed patterns. Note, however, that the accuracy for the macrovariables is similar to the pigment sums in terms of the range of APD values (Fig. 5b).

4.3.2. Developing and applying quality-assurance criteria

The lowest uncertainties for almost all the pigment categories are achieved with the quality-assured data (black bars in Fig. 8), i.e., the data were screened for an effective LOQ threshold on a batch-by-batch basis (Section 4.1.1), and the same extinction coefficients were applied (Section 4.1.2). All higher order variables have uncertainties well below 15%, and the lowest value, 6.7%, is for the pigment ratios. The reduction in uncertainties of the quality-assured data with respect to the (original) baseline analysis is 5.4% (individual), 2.8% (sum), 2.7% (ratio), and 2.8% (macro). In terms of a percent reduction with respect to the original baseline, the quality-assured data have uncertainties that are on average about 29.8% lower.

An alternative to the Fig. 8 quality-assured data can be performed by changing how the effective LOQ threshold is applied. In the batch LOQ analysis, if any one laboratory failed the LOQ threshold, the affected pigment was not retained for subsequent analysis. This logic is only applicable to a round-robin data set and could not be duplicated by individual analysts independently contributing data to a database, because they would necessarily be analyzing different samples. An independent analysis can be constructed by identifying those pigments for which the reported concentrations were less than the effective LOQ for the individual methods. The concentration of the pigments so identified can then be replaced with an acceptable LOQ derived from Fig. 7 (approximately 0.006 mg m^{-3}). This replacement is not an assertion that this value should be a reference value, but is made strictly to demonstrate the consequence of an LOQ approach. In general, it emphasizes the need to define rules for reporting pigment data, and in particular, it establishes the capability of exporting not only the numerical values but also a numerical representation of data quality.

With this alternative approach (which could be implemented within a larger database drawn from multiple sources), the average APD values again reproduce the results shown for the quality-assured results in Fig. 8 (black bars), but the individual pigment uncertainties are further decreased by 5.9%. The effect on the pigment sums, ratios, and macrovariables, however, are insignificant (1% or less). The latter is to be expected because the higher order variables are less influenced by the low-concentration (individual) pigments. Despite any limitations, the primary point is uncertainties in individual pigments can be reduced if the small absolute differences in low concentrations are properly managed; many schemes are possible, but concentrations below a reasonable level of quantitation need to be replaced with an appropriate alternative.

In addition to quality-assurance procedures based on detection limits, some established and robust relationships can also be used as quality-assurance procedures. For example, Trees et al. (2000) demonstrated a log-linear relationship is found between [TAcc] and [TChl *a*]. This robust and generic relationship can be used as reference criteria to identify and screen, in a merged data set, those data that are potentially biased. Figure 9 shows this relationship for the round-robin data set, and the dependence is very nearly log-linear. A least-squares linear fit to the log-transformed data yields a slope of $m = 0.96$, a *y*-

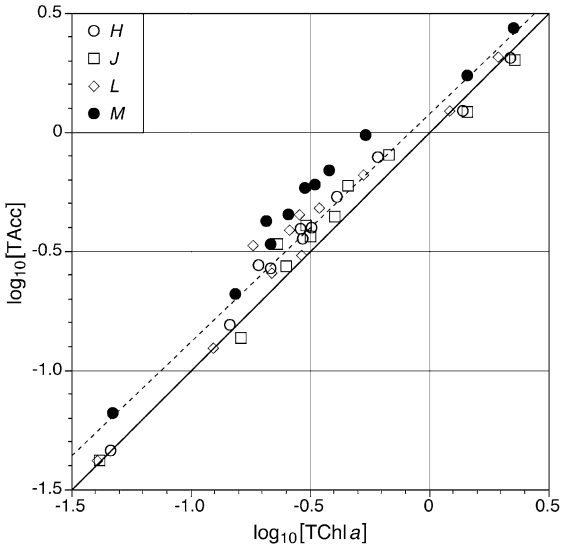

Fig. 9. The distribution of [TChl *a*] versus [TAcc] for the four methods and 11 different batches. A least-squares linear fit to the data is given by the dashed line. The solid diagonal line is the 1:1 correlation.

intercept of $b = 0.072$, and a coefficient of determination of $R^2 = 0.948$. Interestingly, the slight shift of the M data with respect to the other methods is another way to identify quickly the carotenoid overestimation by this laboratory (relative to the other laboratories), as discussed before. This observation shows that the evaluation of such a global relationship for any data set is an efficient and reliable way to identify data deserving additional analysis.

5. Conclusions

This study was designed to intercompare, within a round-robin context, the HPLC determination of pigments useful to biogeochemical studies, as well as the uncertainty requirements for validating the SeaWiFS remote sensing chlorophyll *a* product. This activity was expected to estimate the uncertainties in the HPLC method used and give reasons for the level of agreement (or discrepancy) achieved. The analysis was also designed to quantify how methodological uncertainties propagate within a database originating from different sources and demonstrate how this information can be exploited.

Some HPLC procedures reduced the variance for individual pigments. These procedures included disregarding results less than the effective LOQ (a method detection limit alternative), standardizing the manner in which the concentration of pigment standards are determined, and accurately accounting for [DVChl *a*] when computing [TChl *a*] in cases where [DVChl *a*] is present in significant proportions. When data reported by laboratories were modified according to these procedures, the average uncertainty across all laboratories (for all individual pigments) was reduced by about 6.6% (from 22.2% to 15.6%). Notably, the uncertainty in determining [TChl *a*] (0.045–2.200 mg m^{-3}) was only 7.0%, which was reduced to 5.5% when all laboratories individually quantified [DVChl *a*]. These findings suggest that, on average, it is possible for HPLC techniques to meet uncertainty objectives for ocean color validation activities and that HPLC quality-assurance practices are important to the reduction of uncertainty levels.

Another mechanism for reducing the uncertainties is to form the individual pigments into higher order variables. In every case, the average APD decreased as pigments were grouped from individual concentrations into sums and then ratios, with the latter showing the most robust statistical properties. The macrovariables always performed as well as the sums. This strongly suggests that as larger databases are constructed from incremental contributions (i.e., from different geographic locations and analysts), the most reliable use of the data will likely be with the higher order variables. Furthermore, the low uncertainties (and standard deviations) with the pigment ratios indicate they can also be used for quality-assurance purposes (at least in open ocean data).

Finally, the reproduction of the principal aspects of the baseline analysis with the alternative approaches (using the round-robin data set as a model for the entire PROSOPE data set) suggests the round-robin data represent an acceptable subsampling of the conditions encountered during the PROSOPE cruise. This confirms the results achieved with a statistically valid subset can be applied successfully to a superset and justifies a recommendation for a group of laboratories to incrementally analyze a set of replicates from a variety of field campaigns over time. A continuing series of round-robins based on diverse geographical samples would provide a better statistical description

of how uncertainties propagate through increasingly extensive databases, particularly as a function of trophic levels and methodologies.

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