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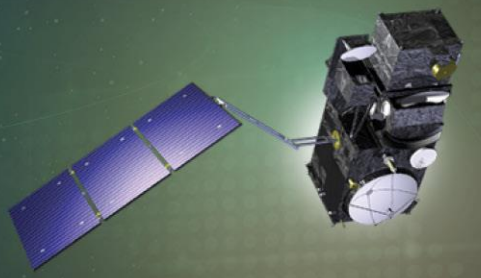

7th Sentinel-3 Validation Team Meeting 2022

18-20 October 2022 | ESA-ESRIN | Frascati (Rm), Italy

Overview of two HPLC inter-comparison exercises on chlorophyll a and Marine Pigments organized to support the validation of data products from Copernicus Sentinel-3

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Ocean Color data product validation and satellite bio-optical algorithm development require availability of high quality *in-situ* measurements of **chlorophyll *a*** and **accessory pigments**.

Liquid Chromatography is considered the techniques that could provide the most accurate analysis in terms of chlorophyll *a* (JGOFS, 1994).

Uncertainty of 25% associated with **chlorophyll *a*** concentration measurements is considered acceptable for validation of satellite data and **15 % is requested for algorithm refinement** (Hooker&McLain, 2000)

HIP Validation Objective: evaluate uncertainties in support to remote sensing cal/val activities

The **JRC Marine Optical Laboratory** has regularly organized (since 2010) inter-comparison exercises on **H**PLC **P**hytoplankton **P**igments (HIP) analysis to support the validation of satellite data products.
"[…] evaluate the uncertainties of *in situ* Chlorophyll *a* in support of the MERIS CAL/VAL activities"
"[…] to support the validation of **marine phytoplankton data products** from Copernicus Sentinel-3 missions"

HIPs OBJECTIVES:

- Quantify the uncertainties of the single laboratory and evaluate the level of agreement (or discrepancy) with respect of other participants;
- Become a reference exercise for establishing whether or not the accuracy requirements for validating the satellite data product is achieved;
- Networking and assure continuity in the inter-comparison activities

HIPs Overview

The phytoplankton pigments considered for the present exercise are the chlorophylls, pheopigments and carotenoids most commonly used in marine chemotaxonomic and photophysiological studies.

The laboratories are compared both on **certified standards** and **natural samples** representative of wide range of water kind. Specific and focussed secondary exercises are proposed at each HIP.

A common Standard Operative Procedure is adopted for collecting the samples to minimize the variability intra-series due different operators and filtering apparatus.

HIP-5 has been launched in 2019 with the general objective to support the validation of marine phytoplankton data products from Copernicus Sentinel-3 missions. The **HIP-6** has been launched in 2020 and **HIP-7** (ongoing) at the end of 2021.

Laboratories involved in the HIPs inter-comparison during the years

- **DHI**, Danish Hydraulic Institute, **Denmark**
- **LOV**, Laboratoire d'Océanographie de Villefranche, IMEV-CNRF, **France**
- **NIVA**, Norwegian Institute for Water Research, **Norway**
- **HZG**, Helmholtz-Zentrum Geesthacht – Centre for Materials and Coastal Research, **Germany**
- **ENEA**, Agenzia nazionale per le nuove tecnologie, l'energia e lo sviluppo economico sostenibile, **Italy**
- **CIMA,** Centre for Marine and Environmental Research - University of Algarve, **Portugal**
- **ULISBOA**, Univeristy of Lisbon, **Portugal**
- **AWI**, Alfred Wegener Institute, **Germany**
- **FURG**, Federal University of Rio Grande, **Brazil**
- **BIO**, Bedford Institute of Oceanography, **Canada**
- **CEAB**, Centro de Estudios Avanzados de Blanes, **Spain**
- **JRC**, Joint Research Centre, **European Commission**

	Reference Method	Extraction solvent	Sample injected	Wavelength	Stationary phase and Temp.	Mobile phase
A	**Van Heukelem et *al*. (2001)**	Acetone and vit. E, 2.5 mL + 150 µl of double distilled water	135 µL	450 nm Carotenoids, Chlorophylls *c* and *b;* 665 nm Chlorophyll *a* and derived	C8, 3.5 µm, 4.6x150 mm, 60 °C	**A:** 70:30 Methanol: 0.028M TBAA **B:** Methanol **C:** Acetone Flow 1,1 mL/min
B	**Van Heukelem et *al*. (2001)**	Acetone and vit. E, 3 mL	135 µL	450 nm Carotenoids, Chlorophylls	C8, 3.5 µm, 4.6x150 mm, 60 °C	**A:** 70:30 Methanol: 0.028M TBAA **B:** Methanol **C:** Acetone Flow 1,1 mL/min
C	**Ras et al. [Van Heukelem mod.]**	Methanol and vit. E, 3 mL	125 µL	450 nm Carotenoids, Chlorophylls *c* and *b;* 676 nm Chlorophyll *a* and derived; 770 nm, Bacteriochlorophyll *a*	C8, 3.5 µm, 3x150 mm, 60 °C	**A:** 70:30 Methanol: 0.4M TBAA **B:** Methanol Flow 0.8 mL/min
D	**Zapata et *al*. (2000)**	Methanol	100 µL	430, 440, 450 nm	C8, 3.5 µm, 4.6x150 mm	**A:** 50:25:25 Methanol: Aqueous Pyridine: Acetonitrile **B:** 20:60:20 Methanol: Acetonitrile: Acetone Flow 1 mL/min
E	**Barlow et *al*. (1997)**	Acetone 90%, 2 mL	50 µL	440 nm	C8, 3 µm, 4.6x100 mm, 30 °C	**A:** 70:30 Methanol: 1 M Ammonium Acetate **B:** Methanol Flow 1 mL/min
F	**Zapata et *al*. (2000)**	Methanol (buffered Ammonium Acetate 2%), 3 mL	100 µL	430, 440, 450 nm	C8, 3.5 µm, 4.6x150 mm	**A:** 50:25:25 Methanol: Aqueous Pyridine: Acetonitrile **B:** 20:60:20 Methanol: Acetonitrile: Acetone Flow 1 mL/min
G	**Wright (1991)**	Acetone, 2 mL	100 µL	436, 664 nm (MV and DV Chl. a); 436 (Chlide a); 667 nm (Pheopigments); 450 nm (Chlorophylls and carotenoids);	C18, 5 µm, 4.6x250 mm, 22 °C	**A:** 70:30 Methanol: 0.5M Ammonium Acetate **B:** Methanol Flow 1 mL/min
H	**Gieskes and Kraay (1989)**	Acetone 95%, 1.5 mL	75 µL	430 nm	C18, 3 µm, 4.6x70 mm, room temp.	**A:** 80:20 Methanol: 0.5 M Ammonium Acetate **B:** 70:30 Methanol: Ethyl Acetate Flow 1 mL/min
I	**Zapata et al. (2000)**	Acetone 95%, 5 mL	10 µL	434 nm rest of the pigmnets, 668 nm Pheophytin a, Pheophorbide a	Guard column: C8 3.5 µm, 2.1x10 mm Column: C8 3.5 µm , 4.6x150 mm	**A:** 50:25:25 Methanol: Acetonitrile: 0.25M pyridine solution **B:** 80:20 Acetonitrile: Acetone Flow 1 mL/min
L	**Jeffrey et al. (1997) mod.**	Acetone 90%, 3 mL	50 µL	410 nm (Pheophorbide, Pheophytin a); 436 nm (Phaeopigments, Chlorophyllide a); 450 nm (Chlorophyll and Carotenoids, Chlorophyll b, Chlorophyll c2 and c3)	C18, 3.5 µm, 4.6x150 mm	**A:** 80:20 Methanol: 0.5M Ammonium Acetate **B:** 90:10 Acetonitrile: Water **C:** Ethyl Acetate Flow 1 mL/min
M	**Jeffrey et al. (1997)**	Acetone 90%, 3 mL	100 µL	410 nm (Pheophorbide, Pheophytin a); 436 nm (Phaeopigments, Chlorophyllide a); 450 nm (Chlorophyll and Carotenoids, Chlorophyll b, Chlorophyll c2 and c3)	C18, 5 µm, 4.0×250 mm	**A:** 80:20 Methanol: 0.5M Ammonium Acetate **B:** 90:10 Acetonitrile: Water **C:** Ethyl Acetate Flow 1 mL/min
	Van Heukelem et *al*. (2001)	Acetone 90%, 2 mL	25 µL	410 nm (Pheophorbide, Pheophytin a); 436 nm (Phaeopigments, Chlorophyllide a); 450 nm (Chlorophyll and Carotenoids, Chlorophyll b, Chlorophyll c2 and c3)	C8, 3.5 µm, 4.6×150 mm	**A:** 80:20 Methanol: 0.5 M Ammonium Acetate **B:** 70:30 Methanol: Ethyl Acetate Flow 1.1 mL/min
O	**Kray et al. (1992) mod. For UPLC**	Acetone 100%, 4 mL	7.5 µL	440 nm	UPLC HSS C18 SB column (dimensions: 100 x 2.1 mm, particle size: 1.8 µm	**A**70 : 30 ethyl acetate : Acetonitrile **B:** 51 : 36 : 13 methanol : acetonitrile : MilliQ water 0.3 M ammonium acetate Flow 0.7 mL/min

Reference Chemo-metric and Statistic

- EURACHEM "[…] international traceability of chemical measurements and the promotion of good quality practices."
  -EURACHEM The Fitness for Purpose of Analytical Methods
  -EURACHEM CITAC Guide CG 4: Quantifying Uncertainties in Analytical Measurements

- ISO International Organization for Standardization
  - ISO 9000 Quality Management System
  - ISO/IEC 17025:2017 general requirements for the competence of testing and calibration laboratories
  - ISO /IEC GUIDE 99:2007 VIM (International Vocabulary of Metrology)

- IUPAC Intern. Union for Pure and Applied Chemistry
  - Protocol for the design, conduct and interpretation of method –performance studies, Pure &Appl. Chem., 1995.
  - Harmonized Guidelines for Single-Laboratory validation of Methods of Analysis, Pure &Appl. Chem., 2002.

Reference Chemo-metric and Statistic

Performance metrics (SeaHARRE-2, Hooker et al. 2005)

Performance Weight, Category, and Score		*TChl a* $\bar{\xi}$	*TChl a* $\lvert\bar{\psi}\rvert$	*PPig* $\bar{\xi}$	*PPig* $\lvert\bar{\psi}\rvert$	*Separation*† $\check{R}_s$	*Separation*† $\bar{\xi}_{t_R}$	*Injection*‡ ($\bar{\xi}_{\mathrm{inj}}$) *Perid*	*Injection*‡ ($\bar{\xi}_{\mathrm{inj}}$) *Chl a*	*Calibration*§ $\lvert\bar{\psi}\rvert_{\mathrm{res}}$	*Calibration*§ $\bar{\xi}_{\mathrm{cal}}$
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	≤2	≤5	≤3	≤10	≥1.5	≤0.04	≤2	≤1	≤1	≤0.5
Method H		*1*	*5*	*2*	*12*	*1.2*	*0.02*	*<1*	*<1*	*1.1*	*0.4*

Comparison

Single lab

$$\lvert\psi\rvert_{Pi}^{L_j}(S_k) = 100\left|\frac{\widetilde{C}_{Pi}^{L_j}(S_k) - \overline{C}_{\mathrm{Pi}}^{\mathrm{A}}(S_k)}{\overline{C}_{\mathrm{Pi}}^{\mathrm{A}}(S_k)}\right|$$

$$\xi_{Pi}^{L_j}(S_k) = 100\frac{\sigma_{Pi}^{L_j}(S_k)}{\overline{C}_{Pi}^{L_j}(S_k)}$$

HIP-5 Samples and Standards

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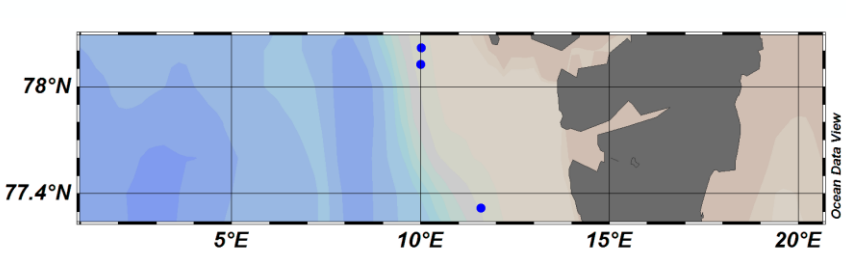
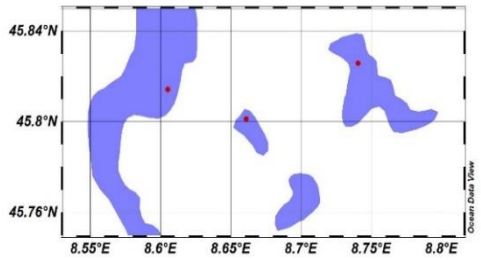
Main Exercise

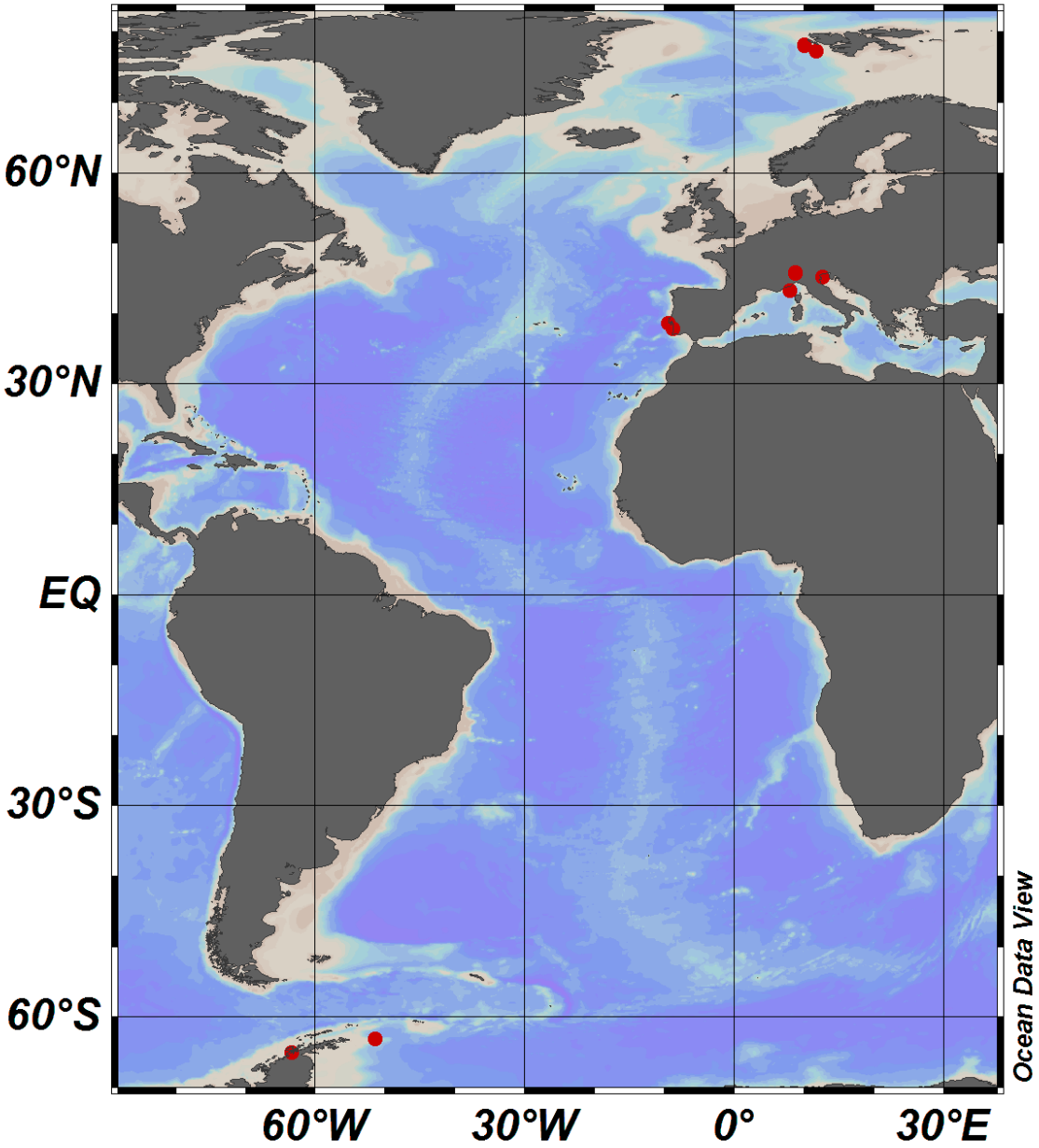
-13 serie of natural samples triplicates (39 samples to each lab): 5 batches collected from participants;
- 1 mix standard (DHI, Denmark) at c.a. 0.3 mg/L conc. of TChl *a*
-3 mix standards (DHI) at c.a. 3.5 mg/L conc. of TChl *a*

Complementary Exercises

-3 series of Artic replicates; 4 Labs
-3 series of Lakes replicates; 3 Labs; PP + target pigments analyzed.







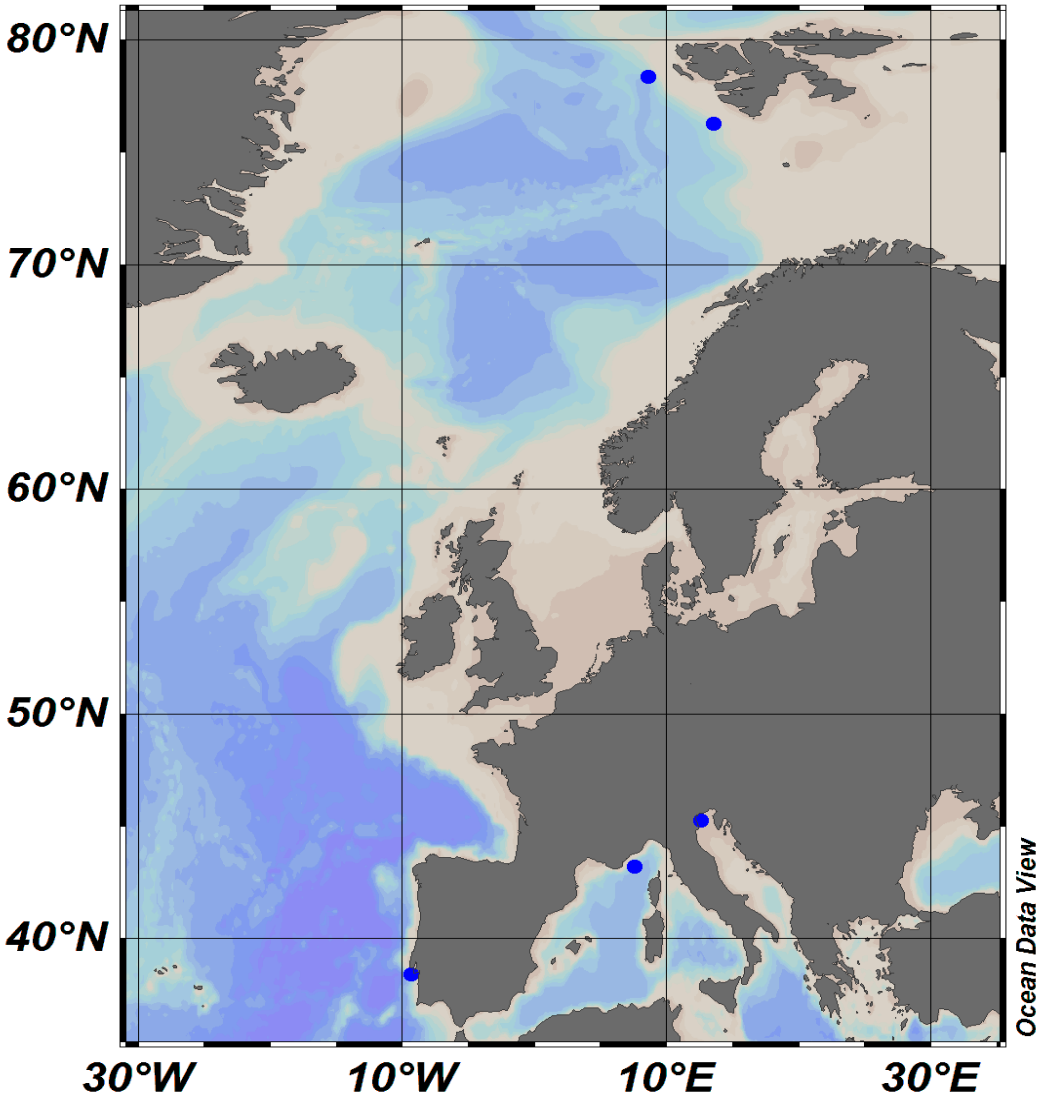
HIP-6 Samples and Standards

Main Exercise

-12 series of natural samples triplicates (36 sample to each lab): 2 batches collected from participants;
- 1 mix standard (JRC) at c.a. 0.3 mg/L conc. of TChl *a*
-3 mix standards (DHI, Denmark) at c.a. 3.5 mg/L conc. of TChl *a*

Complementary Exercises

- 1 series of estuarine samples triplicates



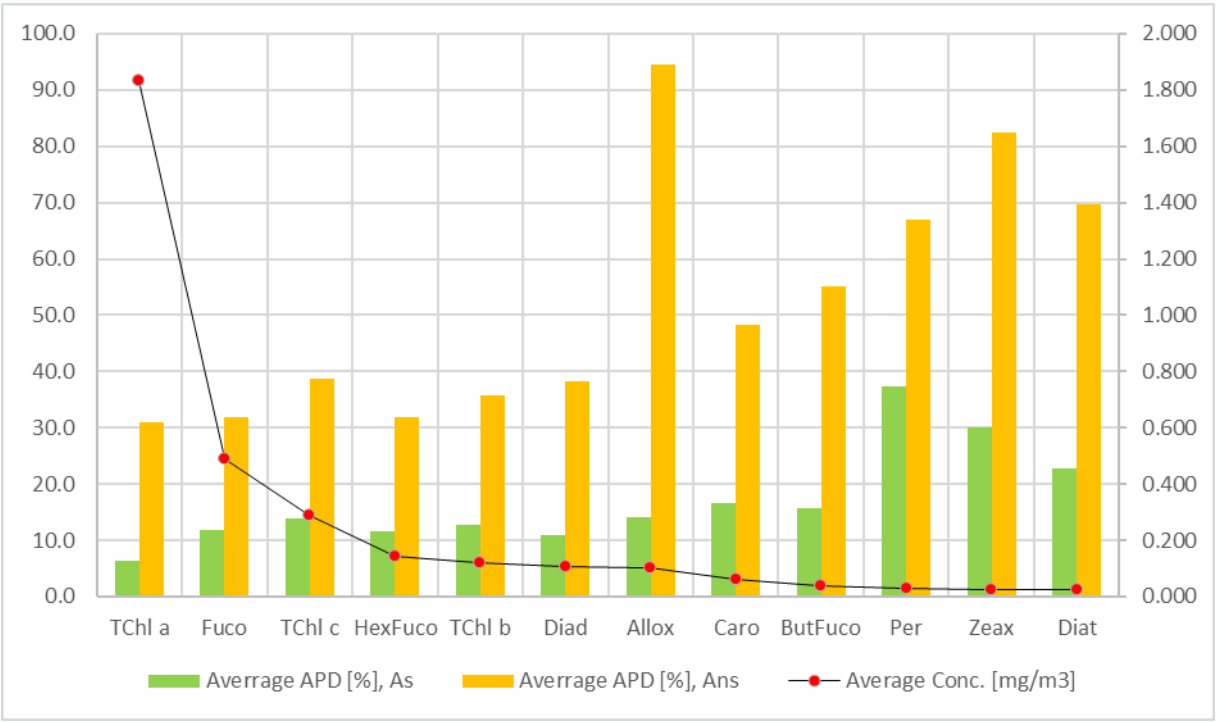
HIP-5 Results

In **orange** the laboratories out of the quality reference subset i.e., 3 Pigments > 25% PD respect the mean value); in ***light green*** resubmission

APD (std. HIP-5)	Tot Chlorophyll a	Tot Chlorophyll b	Tot Chlorophyl c	Carotenes	but-fucoxanthin	19-hex-fucoxanthin	Alloxanthin	Diadinoxanthin	Diatoxanthin	Fucoxanthin	Peridinin	Zeaxanthin
A	3.1	14.0	0.4	7.0	12.1	0.0	7.7	4.1	10.2	0.4	5.8	46.7
B	10.6	1.2	7.1	3.0	7.1	2.1	1.3	8.0	3.8	2.6	17.9	2.8
C	11.7	1.9	5.4	11.6	9.8	2.5	2.4	6.3	3.7	2.7	5.3	36.4
C'	*1.1*	*1.8*	*2.5*	*13.3*	*9.7*	*2.8*	*2.7*	*6.9*	*4.1*	*4.0*	*5.1*	*35.7*
D	0.8	15.1	8.3	27.3	3.6	2.0	12.2	8.4	2.4	5.2	1.3	0.0
E	31.5	52.5	6.7	42.1	39.8	35.3	46.1	37.6	38.3	34.0	33.5	44.1
F	7.4	2.5	14.7	12.1	24.3	2.3	1.5	11.0	29.2	9.2	12.3	19.6
F'	*1.0*	*2.6*	*14.2*	*11.8*	*24.3*	*2.3*	*1.5*	*11.1*	*29.5*	*9.0*	*12.3*	*13.6*
G	12.3	4.2	10.9	6.4	8.2	4.9	17.3	25.1	29.5	9.7	6.9	27.0
H	56.7	23.7	29.7	73.7	3.9	23.4	34.0	>100	58.1	25.6	27.3	44.4

APD (sample HIP-5)												
A	2.8	18.1	8.8	15.9	11.5	13.6	25.0	14.1	35.8	14.0	23.3	42.6
B	4.9	9.0	16.9	14.0	16.7	15.6	16.0	15.2	25.6	16.1	36.9	24.8
C	9.4	15.8	11.6	19.5	18.3	10.4	11.8	9.1	11.4	8.9	35.9	28.7
C'	*10.2*	*15.6*	*12.8*	*19.2*	*20.1*	*12.4*	*10.6*	*12.2*	*11.7*	*9.6*	*35.9*	*32.9*
D	8.3	7.8	18.3	17.4	15.9	6.9	3.2	5.4	18.2	8.2	53.3	24.2
E	59.1	58.2	38.2	49.6	66.1	55.8	60.4	56.7	76.6	58.9	59.8	71.2
F	9.8	13.8	14.3	11.4	27.7	13.1	>100	22.4	47.3	9.6	64.2	80.2
F'	*8.1*	*11.2*	*11.3*	*9.2*	*22.9*	*10.6*	*16.7*	*17.2*	*31.2*	*7.9*	*61.3*	*34.5*
G	42.3	32.1	81.9	58.4	33.9	30.5	49.9	58.6	60.7	44.3	51.8	83.0
H	12.2	39.2	20.1	73.4	92.5	27.9	27.0	15.4	94.2	15.0	92.3	95.8

TChl *a* :- 6.3% quality subset (As);
- 30.9% (Ans)



HIP-6 Results

In **orange** the laboratories out of the quality reference subset i.e., 3 Pigments > 25% PD respect the mean value)

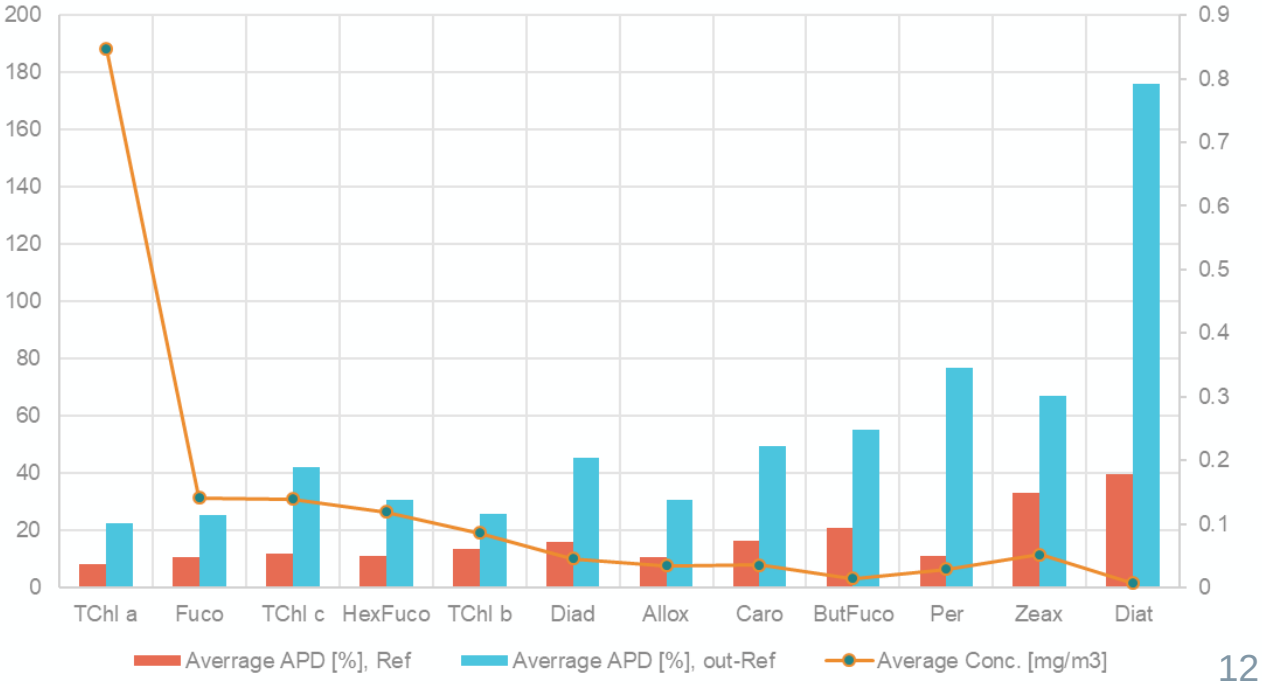
RPD (std. HIP-6)

	Tot Chlorophyll a	Tot Chlorophyll b	Tot Chlorophyl c	Carotenes	but-fucoxanthin	19-hex-fucoxanthin	Alloxanthin	Diadinoxanthin	Diatoxanthin	Fucoxanthin	Peridinin	Zeaxanthin
A	-8.2	-0.4	-12.7	3.7	-0.5	-0.3	8.1	7.1	6.9	-4.6	8.7	38.9
C	9.6	11.4	3.9	-0.2	11.1	-3.8	2.6	-5.3	6.9	-6.9	8.0	-2.8
E	-4.1	-6.0	9.2	-1.4	10.8	5.4	2.5	22.3	-0.4	32.6	43.6	-6.2
F	-6.6	3.6	1.4	-23.4	-10.3	-18.6	-1.5	-8.7	-1.6	-41.4	-99.4	10.0
G	-8.6	5.0	5.8	41.6	24.2	0.4	-16.9	-11.8	-23.5	13.0	11.6	-15.6
O	2.0	-29.3	-29.7	-19.7	-52.2	19.4	-11.2	0.0	-8.6	18.8	27.1	-16.0
I	15.9	15.6	22.1	-0.6	16.9	-2.3	16.4	-3.6	20.2	-11.4	0.4	-8.2
H	>100	>100	>100	>100	76.9	>100	>100	>100	>100	94.7	>100	>100

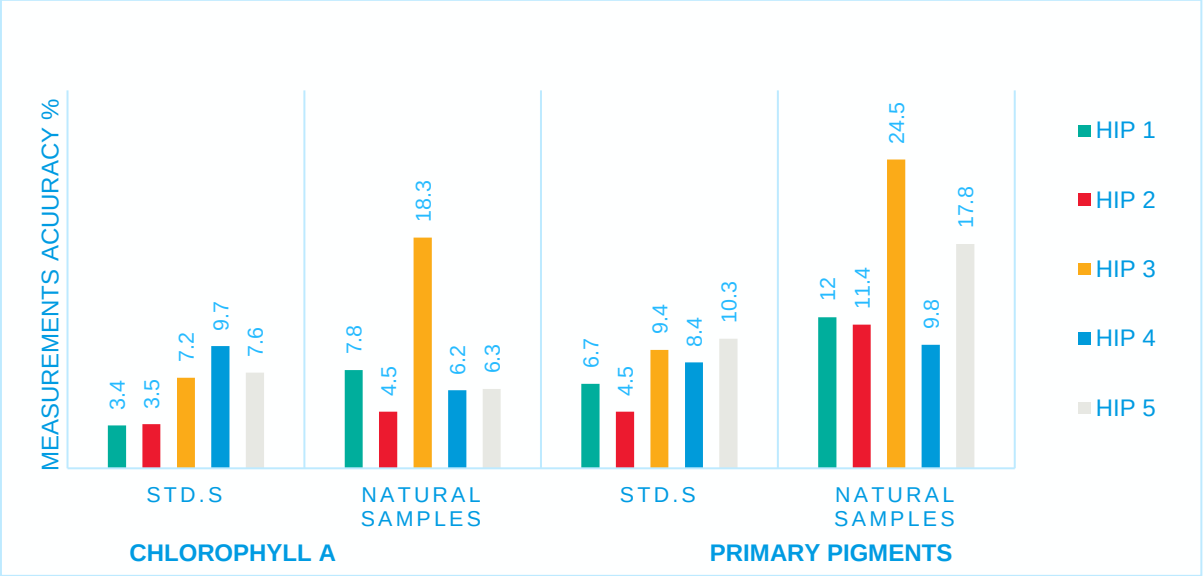
APD (sample HIP-6)

A	4.3	10.4	12.3	23.6	21.7	20.2	22.2	18.4	43.5	19.8	13.6	38.9
B	9.8	7.8	5.4	20.1	25.7	11.0	14.4	4.0	71.7	4.6	7.9	26.8
C	5.4	14.9	8.9	9.3	14.1	6.4	14.1	3.9	18.5	4.8	7.1	18.5
E	8.2	7.9	22.5	42.6	73.2	21.5	12.4	18.0	41.5	22.1	>100	34.3
F	11.7	23.6	16.3	31.0	47.4	18.9	100.0	11.9	>100	30.1	86.7	59.1
G	23.4	7.0	68.3	47.0	23.0	11.8	26.8	25.7	37.3	15.3	12.9	75.5
O	60.4	59.0	45.4	52.3	39.1	18.7	36.5	46.1	61.6	29.3	36.2	79.1
I	13.8	11.4	21.0	13.5	23.0	16.5	12.7	16.4	25.1	14.0	16.7	48.5
H	8.7	55.7	58.3	74.0	93.4	57.4	52.1	52.0	100.0	30.6	91.7	86.5

TChl *a* :- 8.3% quality subset (As);
- 22.4% (Ans)



Conclusion



Measurement uncertainties evolution through the HIPs

In the last 10 years the HIP exercises

- helped laboratories in improving their procedures, which led to more confidence in their methods;
- introduced some common standard evaluation of laboratory performance and offered the opportunity to validate newly implemented methods;
- The first four inter-comparisons focused on European waters (TChl *a* from 0.4 to 2.4 mg/m^3) confirmed that TChl *a* uncertainties requirement for satellite data validation and algorithm refinement (15% of uncertainty) is achievable (Canuti et al., 2016);
- The HIP-5 and the HIP-6 (preliminary) results confirmed the trend extending the comparison to Arctic and Antarctic waters (Canuti et al., in preparation)

Final recommendation to laboratories involved in phytoplankton pigments analysis in support to data satellite validation is **to take part in similar comparison exercises on regular basis.**

How to take part in the next comparison?

Participants requirements
-Performance metrics as described in SeaHARRE-2: qualify at least routine;
-Quantify the so-called Primary Pigment (TChl *a*, TChl *b*, TChl *c*, caro, zeax, but-fuco, diadino, diato, allo, fuco, hex-fuco, perid);
-Analyze the 36 natural samples distributed at least once;
-Analyze the mix std. normal conc. at least 3 times;
-Analyze the mix std. at low conc. at least 3 times

Each laboratory should **submit his method description prior to participation,** the performance metric evaluation and indicate a reference contact person.

The 4th HPLC Intercomparison on Phytoplankton Pigments Workshop: March 2023, Hybrid, Joint Research Center, Ispra (Italy)

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Thank you!