

AN INTERCOMPARISON OF *IN VITRO* CHLOROPHYLL-A DETERMINATIONS - PRELIMINARY RESULTS

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ABSTRACT

An investigation of the capability of the MERIS validation teams to determine chlorophyll-a, using the latest measuring protocols and advanced high performance liquid chromatography (HPLC) and spectrophotometric methods, has been performed. Two intercomparisons, the NIVACal 1 and 2, were made in 2002 as parts of the activities of the MERIS and AATSR validation team (MAVT). For a correct validation of the MERIS L2 data products it is important to make accurate determinations of chlorophyll-a from the collected water samples. The algal pigment indices Chl1 and Chl2 were determined by HPLC, and Chl2 was also determined by a spectrophotometric method. In the intercomparisons samples from algal cultures and natural samples collected off the coast of Norway have been used.

Eleven validation teams, representing twenty laboratories, participated in the intercomparisons. Of the twenty laboratories ten used HPLC methods, eight used spectrophotometric methods and three reported results from fluorometric methods, even if fluorometry is not a method recommended by the MERIS protocols. The successful preparation and distribution of samples during the two periods in 2002 were based on the use of dry ice and courier transport, making the samples arrive at the participants within 2-3 days.

The results show that a coefficient of variation for the determined values of Chl2, calculated as the standard deviation divided by the median value and expressed in per cent, varied from 15 to 25 % for the HPLC (Chl2.hplc) and from 5 to 25 % for the spectrophotometric (Chl2.sp) method. For the overall variation the spectrophotometric results were more consistent between the laboratories. For the Chl1.hplc results the range of variation was larger with values from 5 to 35%.

The determination of chlorophyll-a extracts showed variations of less than 10%, excluding some "outliers". When the same laboratories analysed both extracts and ordinary samples, the results indicated that also extraction procedures could influence the variation.

For both the HPLC and spectrophotometric results the systematic errors are dominating, and a few laboratories showed random errors and "outliers" and should check their laboratory procedures and methods. These laboratories have to show if and how this will affect the use of their chlorophyll-a data in the validation of the MERIS data products.

1 INTRODUCTION

Two intercomparisons of the chlorophyll-a methods used for the MERIS validation have been performed as parts of the activities in the MERIS and AATSR validation team (MAVT). The objective was to compare the capability of the different MAVT team laboratories to perform pigment analyses based on the validation protocols [1,2]. Before MERIS chlorophyll-a products can be quantitatively used, e.g. for environmental monitoring, the data products have to be of high quality, and a validation based on *in situ* measurements is necessary. The different teams involved should use the same methods and demonstrate that their results meet a specific standard.

The NIVACal 1 and 2 intercomparisons include the two *in vitro* phytoplankton pigment indices Chl1 and Chl2 for CASE I and CASE II waters and also the phytoplankton pigment absorption $a_{pig}(442)$ that emerges from the CASE II Neural Network processing. The algal pigment indices Chl1 and Chl2 are defined in the MERIS validation protocol [1]. Chl2 includes only chlorophyll-a, whereas Chl1 in

addition includes divinyl-chlorophyll-a, the chlorophyll-a phaeopigments (phaeophorbide-a, phaeophytin-a, chlorophyllide-a) and the epi- and allomer of chlorophyll-a.

Eleven MAVT validation teams participated, representing twenty different laboratories (Table 1). Out of the twenty laboratories ten used a HPLC method, eight a spectrophotometric method and three reported results of fluorometric methods. Four of the laboratories using an HPLC method also reported the Chl1 pigment index for CASE I water.

Table 1. The participants in the NIVACal 1 and NIVACal 2 chlorophyll-a intercomparisons in 2002

Institute or laboratory	Principal investigator	AO-project	HPLC methods	Spectrophotometric method	Fluorometric method
SOC (Southampton Oceanographic Centre)	Weeks	290	Chl1, Chl2		
CEAB (Centre d'Etudes Avancés de Blanes)	Weeks	290		Chl2	
LPCM (Laboratoire d'Océanographie de Villefranche sur mer)	Antoine	322	Chl1, Chl2		
SU (Stockholm University)	Kratzer	371		Chl2	
IOW (Baltic Sea Research Institute Warnemünde)	Siegel	468	Chl2		
NIVA (Norwegian Institute for Water Research)	Sørensen	609	Chl2	Chl2	
UIB (University of Bergen)	Sørensen	609		Chl2	
IMR (Institute of Marine Research, Norway)	Sørensen	609			Chlorophyll-a
GKSS (GKSS Research Centre)	Doerffer	647	Chl1, Chl2		
MUMM (Management Unit for the North Sea Mathematical Models)	Roose	698	Chl2		
PML (Plymouth Marine Laboratories)	Aiken	9001	Chl2		
JRC (JRC IES/IMW)	Zibordi	9020	Chl1, Chl2		
IVM (Institute for Environmental Studies, Netherlands)	Peters	9096	Chl2		
AquaSense (Netherlands)	Peters	9096		Chl2	
Steins lab (Denmark)	Fell	9106		Chl2	
ILAT (Institute für Lebensmittel, Arzneimittel und Tierseuchen)	Fell	9106		Chl2	
NTNU (Norwegian University of Science and Technology)	Fell	9106	Chl2	Chl2	
IOLR (I) (Israel Oceanographic and Limnological Research Institute)	Fell	9106			Chlorophyll-a
IOLR (II)	Fell	9106			Chlorophyll-a

This paper describes the preliminary results and conclusions from the HPLC and spectrophotometric intercomparisons in the NIVACal 1 and 2. The results from the NIVACal 2 are not complete (January 2003), since data from the participants are still pending.

2 PREPARATION OF SAMPLES AND METHODS

2.1 Preparation of the intercomparison samples

Both natural samples (NIVACal 2) and samples from algal cultures (NIVACal 1) were prepared at the laboratory of NIVA. The samples were filtered onto 47 mm GF/F Whatman filters. The filters were then transferred to vials and immediately frozen in liquid nitrogen, before storing at -80°C until transportation to the participants. During this sub-sampling for filtration the samples were kept in the dark in a 50 l container under continuous stirring. For each set of 30-100 replicates the filtration was completed within 0.5 to 1.5 hour. Every 10th sample was used to control the variation due to filtration, handling and storing. The samples were transported to the European participants in an isopore box of minimum 50 mm wall thickness and with about 5 kg dry ice, which was sufficient to keep the samples deep-frozen for 3-4 days.

2.2 Algal cultures used in NIVACal 1

A total of five sample sets (10 samples, A-J) was prepared from algal cultures during the NIVACal 1 in May/June 2002. Eight samples were prepared from cultures with different cell concentrations of the diatom *Skeletonema costatum*. Two samples of slightly different pigment concentration (sample pair IJ) were prepared from a mixture of *S. costatum*, the chlorophyte *Dunaliella tertiolecta* and the cyanobacteria *Chroococcus* sp. All species were taken from the NIVA culture collection. Different concentrations were obtained by dilution with natural seawater of a salinity of 34. The concentration range of the samples was from 0.5 mg/m^3 to 7 mg/m^3 .

The results from the variations due to filtration, handling and storing are shown for all the 10 concentrations in Fig. 1. For this control a spectrophotometric method was used based on extraction in 90 % acetone. Fig. 1 shows the mean and standard deviation of the chlorophyll-a concentrations in the samples.

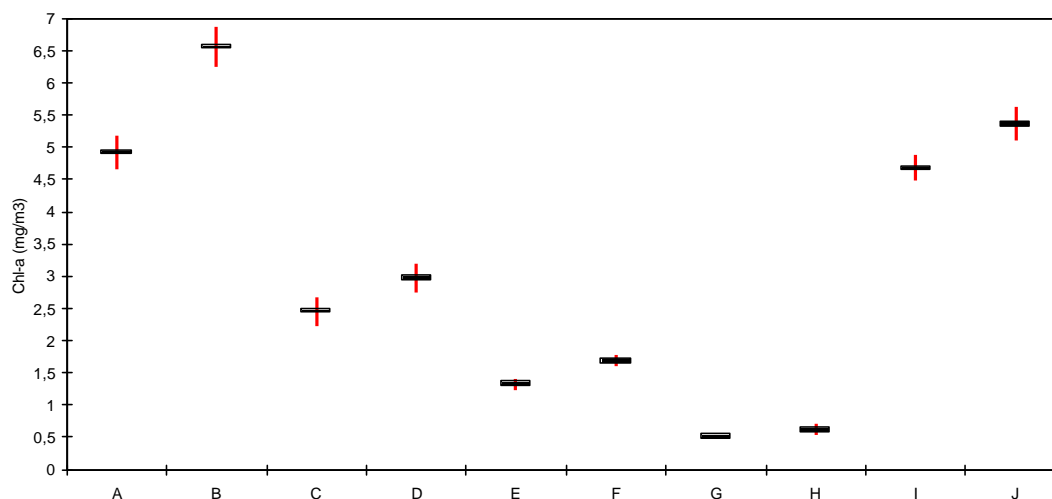


Fig. 1. The variation of chlorophyll-a in algal culture samples in the NIVACal 1. The horizontal bars represent the mean values and the vertical bars 4 times the standard deviation ($\pm 2 s$).

2.3 Natural samples used in NIVACal 2

Natural water samples were collected in September 2002 from four Norwegian fjords situated in southern Norway. The samples were from the outer Oslofjord, the Singlefjord in the Hvaler archipelago, the inner part of the Sandefjord and the central part of the Frierfjord. The preparation and the test of variation were performed as for the algal cultures. The sample pairs (AB, CD, EG and GH) had concentrations in the range from 1 to 12 mg/m³ chlorophyll-a.

2.4 Algal extracts used in NIVACal 2

Samples of extracted pigments for the NIVACal 2 were prepared from algal cultures at GKSS (Geesthacht, Germany) in November 2002 and sent out together with the natural samples from NIVA. Since the participating laboratories used both 90 % and 100 % acetone for extraction, two sample sets were prepared; one in 90 % and one in 100 % acetone. Two samples of a fresh culture of the diatom *Thalassiosira weissflogii* were therefore filtrated on Whatman GF/F filters. The filters were shock-frozen in liquid nitrogen and afterwards extracted in glass vials with 30 ml of cold 100 % acetone (Extract 1) or 90 % acetone (Extract 2). The extraction lasted one hour at -30°C. The extracts were then filtered through 0.22 μ m-filters (Spartan, 13A), then bubbled with nitrogen. The two extracts were respectively divided into autosampler vials, closed and sealed under nitrogen. All samples were stored at -80 °C until transport in dry ice.

Five aliquots of both sets were regularly measured by HPLC at GKSS to monitor the variation in the samples and the degradation of the pigments. The results are illustrated in Fig. 2 for the 90 % acetone extract (Extract 2). During the period from November 15th to January 8th no significant change in the mean concentration could be detected and no degradation of the pigment was observed.

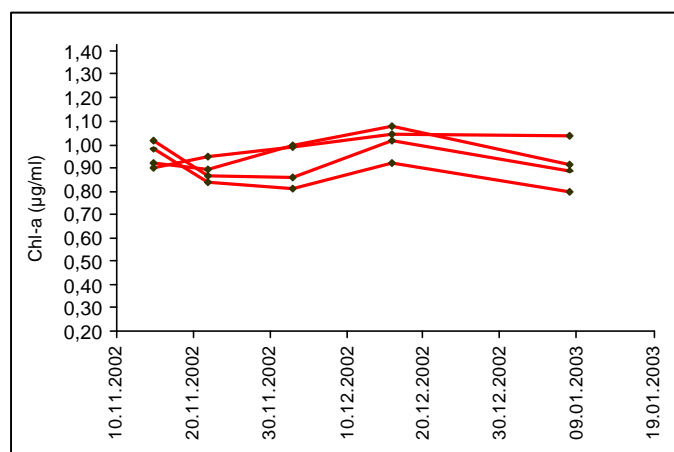


Fig. 2. Concentrations of chlorophyll-a ($\mu\text{g/ml}$) in 90 % acetone extract measured by HPLC in different intervals.

2.5 Statistical methods

The results from the laboratories were treated according to the method of Youden [3]. The participants analyse samples in pairs, and the result for each pair is presented as a point in a Youden plot. Each laboratory's location in the diagram gives information regarding the kind and magnitude of error. The use of pair of samples of the same or slightly different concentrations and the way of presenting the results according to [3] allow us to determine whether the results have a random or a systematic error. In case of only random errors, the points will be evenly distributed between the quadrants, whereas a grouping along the 45° line expressing the deviation from the "true" values indicates mainly systematic errors.

If one or both values of a sample pair deviated more than 50% from the median value, the pair was excluded from the calculation of the median ("outliers"). Of the remaining values a new median (\bar{x}) and a standard deviation (s) were calculated. Pairs of results in which one or both values were outside $\bar{x} \pm 3s$ were excluded before calculation of the final median value (= "true value"), standard deviation and other statistical parameters.

The variation between the participants is presented as a coefficient of variation (CV), defined as the standard deviation divided by the median ("true") value, and expressed in per cent. "Outliers" are not included in the calculation of the CV.

3 RESULTS

3.1 HPLC results

3.1.1 Chl2.hplc from NIVACal 1 with algal cultures

Five pairs of samples in different concentrations were analysed by 10 different laboratories in the concentration range from ca. 0.5 mg/m^3 to 7 mg/m^3 . The results are presented in Youden plots [3] as shown in Fig. 3 for the sample pairs AB and IJ. Each pair of results is represented by a point in the diagrams and "outliers" are marked grey. The circle showing a deviation of 20 % from the median value is included in the plots. In sample pair AB laboratory 8 and 14 are "outliers" and in sample pair IJ also laboratory 8 becomes an "outlier". Laboratory 20 is systematically low in both sample pair.

When the "outliers" are excluded, the CV between the participants is between 15% and 23% for all concentrations (A-J). The number of "outliers" was 1 for the sample pair IJ, 2 for AB, 3 for CD and GH, and 4 for EF.

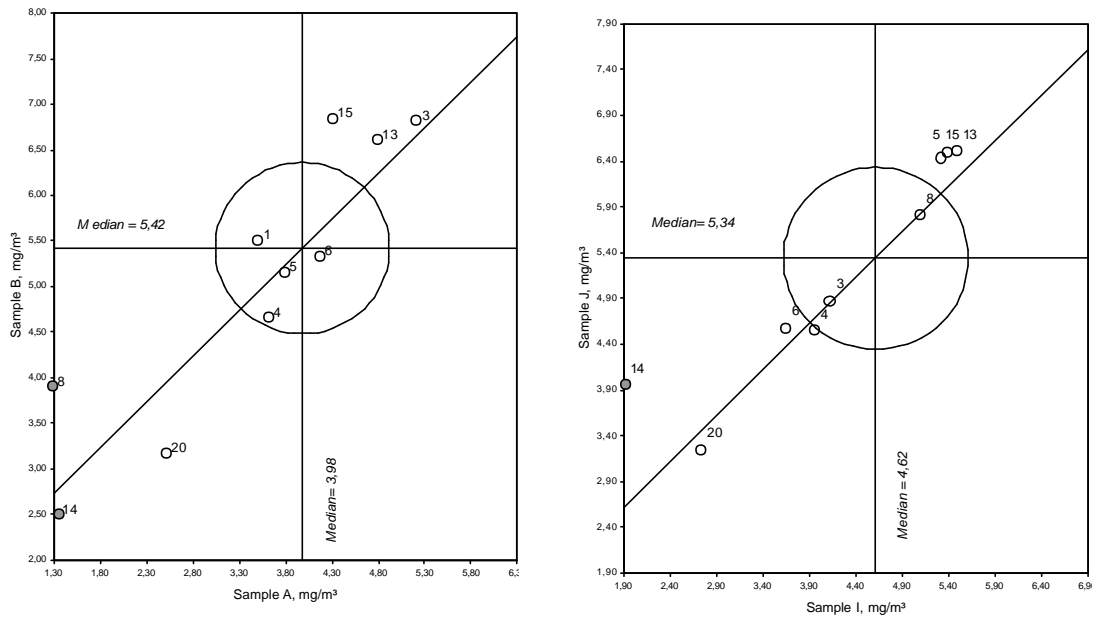


Fig. 3. Youden plots of two pairs of results from the NIVACal 1 for HPLC analysis of chlorophyll-a (Chl2.hplc). The left graph presents the sample pairs AB, the right the graph sample pairs IJ. “Outliers” are marked grey.

3.1.2 Chl2.hplc from NIVACal 2 with natural samples

The participants measured four pairs of natural water samples with chlorophyll-a concentrations ranging from 1 to 12 mg/m³. The results for the two sample pairs with a low concentration of chlorophyll-a (AB and CD) are shown in Fig. 4. When “outliers” are excluded from the calculation, the CV between the participants is between 17 and 25% for all the four sample pairs (8 concentrations). The number of “outliers” was 1 for the pairs EF and CD, and 2 for the pair GH, otherwise none. A typical random error is shown in the sample pair AB for laboratory 8.

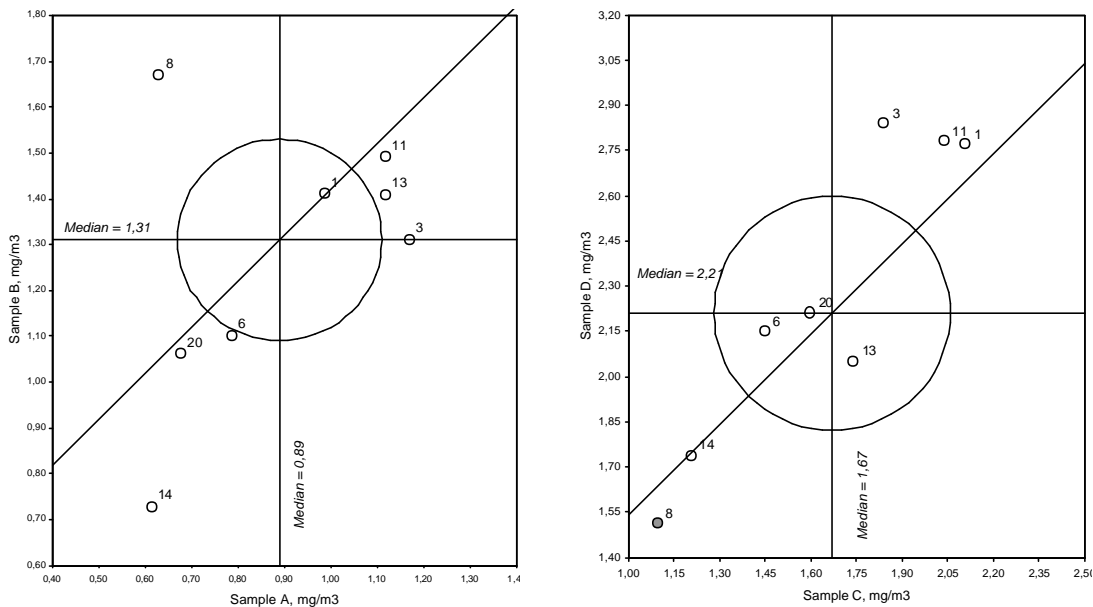


Fig. 4. Youden plots of results from sample pair AB (left) and CD (right) from the NIVACal 2 for HPLC analysis of chlorophyll-a (Chl2.hplc), based on natural samples. “Outliers” are marked grey.

3.1.3 Chl1.hplc from NIVACal 1 and 2

Four of the laboratories that analysed Chl2 (only chlorophyll-a) by HPLC also measured Chl1 (which includes more chlorophyll-a pigments). In the NIVACal 1 with algal cultures the CV between laboratories is between 5 and 35% when “outliers” are excluded. There is one “outlier” (the same laboratory) in the pairs AB, GH and IJ. This laboratory reported consistently lower values than the other laboratories.

Results are still pending from the NIVACal 2 so only very few and preliminary results are available. In the NIVACal 2 the CV between the laboratories is between 6 and 22% when excluding the “outliers”. There was one “outlier” in the pair EF and two in the pair GH.

3.1.4 HPLC determination of chlorophyll-a extract

Six laboratories have reported HPLC results for the 90 % acetone extracts depicted in Fig. 5. The overall CV between the laboratories is 17 %. Apart from one of the results (laboratory 13), the CV between the laboratories is acceptable (10 %).

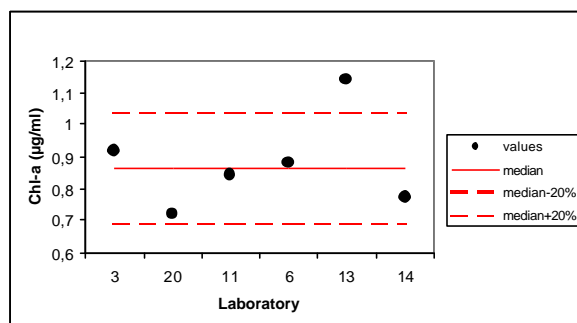


Fig. 5. Results of the HPLC analyses of the chlorophyll-a extracts in 90 % acetone during the NIVACal 2

3.2 Spectrophotometric results

3.2.1 Chl2.sp from NIVACal 1 with algal cultures

Eight laboratories reported spectrophotometric values for Chl2 with the algal cultures in NIVACal 1. The results for sample pair AB and GH are shown in Fig. 6. The CV between reported values was between 9 and 24 % when the “outliers” were excluded for all the 10 concentrations in these tests. There was one “outlier” in samples of the pair EF and two in GH. Laboratory 9, 12 and 10 had random error in their analyses, and in sample pair GH laboratory 2 and 10 are “outliers”.

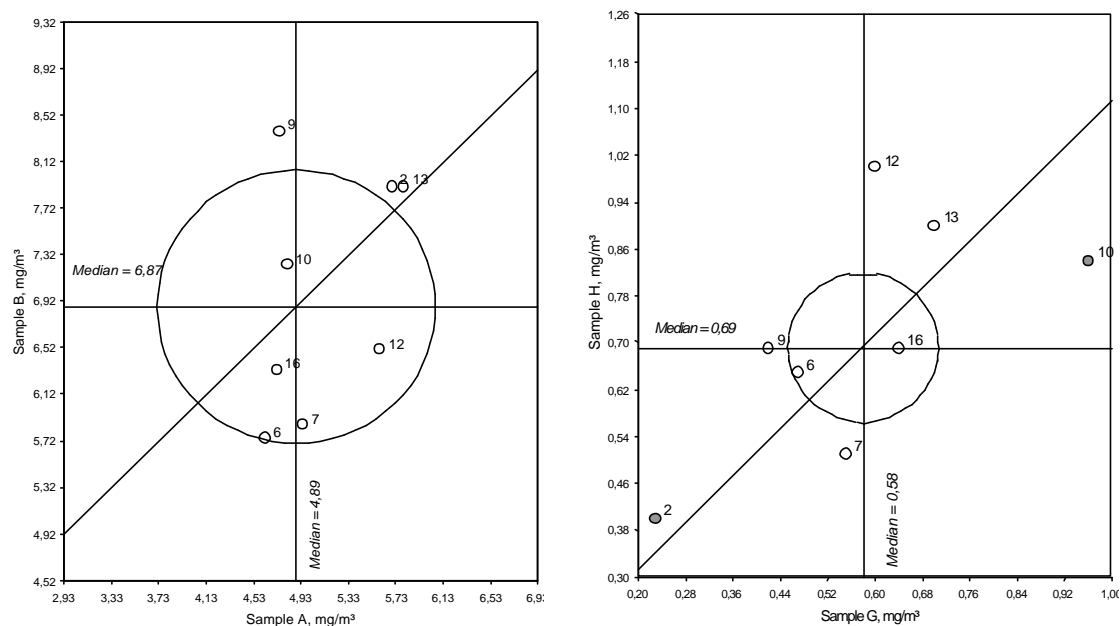


Fig. 6. Youden plots for results of the pairs AB (left) and GH (right) from the NIVACal 1 obtained by spectrophotometric analysis of chlorophyll-a (Chl2.sp).

3.2.2 Chl2.sp from NIVACal 2 with natural samples

The results from this round are similar to those from the NIVACal 1 with algal cultures. The CV for the results between the laboratories varies from 5 to 18%, and there are only two “outliers”; one in the pair EF and one in the pair GH.

3.2.3 Spectrophotometric determination of chlorophyll-a in 90 % acetone

Four laboratories have reported absorbance values of the 90 % acetone extracts of chlorophyll-a, and the results obtained by using a specific extinction coefficient of $87,67 \text{ l} \cdot \text{g}^{-1} \cdot \text{cm}^{-1}$ [2] are presented in Fig. 7. The CV for the few results reported was 4 % between the laboratories.

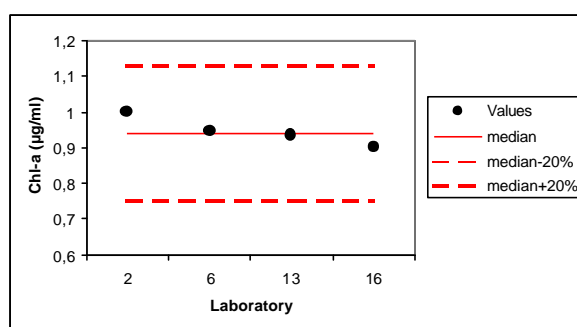


Fig. 7. Results of the spectrophotometric analyses of chlorophyll-a extracts in 90 % acetone during the NIVACal 2.

4 DISCUSSION AND PRELIMINARY CONCLUSION

The average variation (CV) in the samples caused by filtration, handling and storing during the test periods is less than 7 % for all samples. The transport in dry ice to laboratories in Europe was successful. On an average the samples arrived at the laboratories within 2-3 days. There was only a

problem with shipment of dry ice to Australia and Canada, which had to be excluded for the intercomparisons.

For both the HPLC and spectrophotometric results the systematic errors (high or low) are dominating. A few labs for the HPLC in the NIVACal 1 and 2 show unacceptable deviations ($> \sim 30\%$) from the median value, which could be caused by improper extraction procedures or problems with calibration of the HPLC systems. A few laboratories have shown random errors and “outliers” and should check their laboratory procedures and methods. In the NIVACal 1 the number of “outliers” seems to increase with decreasing concentration in the samples, but in the NIVACal 2 the “outliers” are found also in the two highest concentrations. These laboratories have to show if and how this will affect the use of their chlorophyll-a data in the validation of the MERIS data products.

On an average the variation for each laboratory expressed by a CV varies between 15 and 25 % for chlorophyll-a measured by HPLC (Chl2.hplc), and between 5 and 35 % for Chl1.hplc (the sum of chlorophyll-a and other chlorophyll-a pigments). For the spectrophotometric determination of chlorophyll-a (Chl2.sp) the variation ranges from 5 to 25 %, and in general the spectrophotometric results show a lower variation and are in better agreement than the HPLC results. Five out of eight laboratories are within a 20 % deviation from the median for samples with concentrations above 1 mg/m^3 .

There are problems with the determination of low chlorophyll-a concentration for 5-6 laboratories, but this does not necessarily mean that their validation results are affected since the laboratories normally tune their systems and filtration volumes to the concentration range in their investigation area.

The results from the few laboratories that have analysed the chlorophyll-a extract are in good agreement between the laboratories and show a CV of 4 % for the spectrophotometric method and 10 % for the HPLC method, when excluding one laboratory. Since the error in analysing the extract is lower than the total error for the samples, this indicates that also factors as e.g. the extraction procedures contribute significantly to the total error. This will be analysed further when all the results from NIVACal 2 are available.

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