Intergovernmental Oceanographic Commission
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Report of the *In situ* component of the 'Plymouth Chlorophyll Meeting and Workshops (Extended Antares Network)'

Sponsored by GOOS, GEO, PML and POGO

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It is essential that the satellite coverage provided by the proposed network should be complemented by high quality and mutually-consistent *in situ* measurements of chlorophyll-a (chla), and if possible by a set of ancillary bio-optical measurements. Ideally these data could be used to establish regional remote-sensing algorithms for estimating chlorophyll-a from ocean-colour data. It is recognised that this may not be possible from the very beginning, considering that the recommended method for satellite validation is by comparison with chlorophyll-a estimated by High-Performance Liquid Chromatography (HPLC), and that due to its cost not all laboratories in the network will have access to this instrument. Nevertheless, it is important to assure that good quality *in situ* measurements, the best possible according to the infrastructure available, are made and that these should be useful for examining the performance of the satellite algorithms on a regional basis, as well as to provide data for periods when satellite observations are not possible (for example, due to cloud cover).

The objective of the 'in situ component' of the workshop was then, to asses differences in the estimates of chla concentration within the methods in use in the laboratories taking part in the network, with the final aim of establishing the best possible practice rendering good quality and comparable results among the participants.

The exercise was divided into two parts, one taking part during the workshop at PML, and the second an inter-calibration performed after the meeting at each one of the laboratories involved. The main objective of the first exercise at PML was to test some of the protocols for estimating chlorophyll-a in the laboratory, based on discussions about the theoretical and practical considerations that underlie each method. This part provided the rare opportunity that people in charge of chla determination in more than 15 countries could perform the measurements at the same time, and could exchange details not always provided in manuals or publications. At the same time provided a frame of reference for the following inter-calibration exercise, since it showed that different people using the same method would achieve similar results, leaving the interpretation of differences in the laboratory performances only to variations in the efficiency of the methods used and not to the skill of the personnel.

This *'in situ'* section will be divided into four parts: 1- a general introduction to the subject of chla measurements; 2- summary of the results obtained from the exercise at PML; 3- the results from the inter-calibration; and 4- the main conclusions drawn from the whole exercise.

1. Introduction: 'Possible variations in the common *in vitro* fluorometric method to measure chlorophyll-a concentration in seawater'

High-Performance-Liquid-Chromatography is undoubtedly recognized nowadays as the best technique to separate, identify, and quantify phytoplankton pigments in seawater (Jeffrey et al. 1997). It has been, therefore, chosen by NASA as the 'reference' method to develop and validate satellite-retrieved chlorophyll-a concentration. Some drawbacks of this technique are its cost (equipment, maintenance, pigment-standards, and quality of solvents needed), sophistication (it requires a dedicated, well-trained operator), time consumption (it takes about 30 minutes or more to run a single sample). All these characteristics make the HPLC method a less-than ideal method for estimating chlorophyll-a in an operational context. That is why the standard *in vitro* fluorometric method of Holm-Hansen et al. (1965), which is economic, simple and sensitive, continues to be much used in oceanographic studies, including some dedicated to validation of satellite-estimated chlorophyll-a. Thus, in many papers, the *in situ* chlorophyll-a measurements reported are based on the 'Holm-Hansen et al. (1965) method', leading readers to believe that all measurements, from any laboratory in the world, using this same method would be mutually consistent. Nevertheless, it is common practice that every laboratory has its own slightly different protocol to carry out the *in vitro* fluorometric chla measurements. Some of these variations may involve the volume of seawater filtered, the storage of samples, the extraction procedure, the settings on the instrument, and so on. Slight differences in these steps could potentially make a significant difference in the chlorophyll-a concentrations retrieved.

The method of Holm-Hansen et al. (1965) uses an acidification step to determine the amount of 'phaeophytin', or to state it more broadly 'phaeopigments', in the sample, which allows retrieval of a value of chlorophyll-a corrected for 'phaeopigments'. It has been proven convincingly that the acidification can introduce a significant bias in the estimation of 'phaeopigments' and some differences in chla concentrations, according to the concentrations of chlorophyll-b and chlorophyll-c present in the sample (Gibbs, 1979; Trees et al., 1985). A modification to account for the presence of chlorophyll-b was proposed by Welschmeyer (1994). This modified fluorometric technique is also now being used by some laboratories. Therefore, some differences in chla concentrations may be expected between measurements performed using these two techniques.

The *in vivo* fluorometric method of Holm-Hansen et al. (1965), or the modification of Welschmeyer (1994), uses 90% acetone as solvent for the extraction of chlorophyll-a from the phytoplankton sample. Nevertheless, it has been shown in previous comparison exercises for the HPLC method (Wright et al., 1997) that 100% methanol is a better solvent for extraction, especially in the case of green algae and cyanobacteria. That is why 100% methanol has been adopted as standard solvent for extraction in several HPLC methods to determine phytoplankton pigments. Some laboratories are already using 100% methanol for the extraction of samples to measure chla concentration by the common 'fluorometric' technique. In a previous comparison, extraction by 100% methanol yielded higher chlorophyll-a concentrations than those obtained from 90% acetone extraction, with differences at times greater than 35% (19% higher on an average for 22 samples) for natural samples from a cruise covering a wide variety of phytoplankton populations and concentrations (Lutz et al., unpublished results). Again, significant differences in the chlorophyll-a concentrations may arise from the solvent chosen.

There are also differences in the methods used to extract pigments from the phytoplankton cells. Ultrasonication, mechanical grinding and soaking for 24 hours at -20ºC are used in different laboratories to aid extraction. Differences in the extraction efficiency of the methods are another potential source of difference in the estimated chlorophyll concentration.

It is also well-known that the *in vitro* fluorometric determination of chlorophyll-a concentration is dependent on the individual calibration of each fluorometer. The method to calibrate a fluorometer for the determination of chla concentration has been described in detail in several publications (e.g., Trees et al. 2003) and is not going to be repeated here. The basic point to consider is that fluorometric measurements have to be calibrated with pure chlorophyll-a standard based on the spectrophotometrically measured concentration. This is because the fluorescence emission (elicited by the sample) measured by the photomultiplier in a fluorometer (at 90°) will vary according to the efficiency of the lamp used, the type of excitation and emission filters used, and the geometrical configuration of a particular instrument. Hence, there is no single exact fluorescence efficiency factor (fluorescence emitted per unit chlorophyll-a concentration). The factor should be determined by measuring the concentration of a pure chlorophyll-a standard, dissolved in a particular solvent, in a spectrophotometer (for which a known and fixed extinction coefficient exists for absorption per unit chlorophyll-a concentration) and then measuring the fluorescence emitted by the same solution. To establish the linearity of the response, a calibration curve should be performed. For this, a number of dilutions from a solution of known chlorophyll-a concentration are made, and the fluorescence emission measured in each of them. The range of concentrations for the dilutions should be chosen so that they cover the scale of the given fluorometer, and render a linear response. After a calibration curve is made and a chlorophyll-fluoresence relationship has been established for a particular fluorometer, care should be taken that the fluorescence of the samples to be measured falls within the calibration interval. If the fluorescence of a sample is higher than the upper limit of the calibration curve, then the sample should be diluted to avoid measurements out of the calibration range. This is important to guarantee that the assumed linear response is maintained. Furthermore, the lamp loses its efficiency with use (plus some other changes in the instrument, for example through degradation of the photomultiplier, slight shifting of the optical components in transport, etc. may occur with time) and will produce changes in the calculated calibration factor. Therefore, the calibration procedure should be repeated periodically, ideally every time that a set of samples is going to be measured (e.g., for a particular cruise), or when the instrument has been transported, or at least twice a year. Failure in the calibration of an instrument may also render differences in the concentration of chlorophyll-a measured by two fluorometers even if exactly the same method is being used.

A thorough description of different methods to measure chlorophyll-a, and other phytoplankton pigments, has been presented in the book 'Phytoplankton pigments in oceanography: guidelines to modern methods' (Jeffrey et al., 1997), which constitutes an essential reference for anybody working in phytoplankton pigments. The theoretical aspects on pigments there discussed are relevant to any type of chla measurement. Nevertheless, most of the revision and comments on the procedures to measure pigments, and the comparison exercises shown in it, are focused on the HPLC technique. Therefore, there are still some comparisons between variations in the basic *in vitro* fluorometric, and spectrofluorometric, techniques to determine chlorophyll-a concentration which are missing. This exercise aimed at evaluating differences in chlorophyll-a concentration obtained by applying the same basic *in vitro* fluorometric technique but with some of the most common 'variations' applied in the protocol by different laboratories.

2. Chlorophyll-a measurements during the workshop at the Plymouth Marine Laboratory

Samples collected from a site close to PML were filtered in a number of replicates, and were then extracted in 90% acetone and 100% methanol using a combination of ultrasonication, mechanical grinding and 24h soaking at -20 °C to aid the extraction. Fluorometric readings were

made using the special fluorometer kit described by Welschmeyer (1994) and the normal kit used in the Holm-Hansen et al. (1965) method. The average results showed very small differences between the different extraction methods as well as for the solvent used for the extraction, although the coefficient of variation increased when mechanical extraction was performed. A student t-test indicated that the Welschmeyer method provided significant increase in the chlorophyll-a concentration compared with the Holm-Hansen et al. (1965) method when using 90% acetone ($p < 0.01$). However, the difference between the methods when using 100% methanol was relatively small, usually insignificant ($p > 0.05$).

An HPLC analysis on the extracts of the samples used for this exercise showed that the pigment composition was dominated by chlorophyll-a, peridinin and chlorophyll-c2, with small amounts of chlorophyll b, chlorophyll-c3, and 19'hexanoyloxyfucoxanthin. This indicated the dominance of dinoflagellates in the phytoplankton community. Dinoflagellates are known to release their pigments easily during the extraction procedure.

The limited effect of the different treatments on the chlorophyll-a concentration estimation was probably due to the dinoflagellate-dominated community. However, for samples dominated by difficult-to-extract cells, such as cyanobacteria or small coccoid chlorophytes, there may be larger differences between the extraction procedures (ultrasonification vs. soaking and/or methanol vs acetone).

3.   Chlorophyll-a inter-calibration at the different laboratories

The exercise was divided into two parts:

1)   In the first, all laboratories were asked to run the same "standard" method; i.e., an inter-comparison to see the repeatability among different laboratories.

2)   In the second, each laboratory had to run the samples using their own "routine" method, which would give the opportunity to see differences in the efficiency of the different methods.

Each laboratory received two sets of 5 different types of samples (A, B, C, D, E), each one in triplicates, i.e, each laboratory had to analyse 15 samples for part one of the inter-calibration and 15 samples for part two of the study. A summary of the samples sent to the participating laboratories and how they were to be treated are given in Table 1.

Table 1. _Summary of IOC GOOS Chl Pilot Study inter-comparison samples_

SAMPLE	SAMPLE A	SAMPLE B	SAMPLE C	SITE D	SITE E
Fluorometry Method 1 **(home lab protocol)**	3 replicates	3 replicates	3 replicates	3 replicates	3 replicates
Fluorometry Method 2 **("Standard"protocol)**	3 replicates	3 replicates	3 replicates	3 replicates	3 replicates

Type of samples used

Samples A: culture of *Synechococcus* sp.; known to be difficult to extract.
Samples B: culture of *Nannochloris* sp.; containing chlorophyll-b, and known to be difficult to extract.
Samples C: mixed culture of *Phaeodactylum tricornutum, Amphidinium carterae* and *Dicrateria inornata*; expected to be relatively easy to extract.

Site D: field samples; from a site where the phytoplankton community is supposed to be relatively easy to extract.
Site E: field samples; from a site where the phytoplankton community supposed to be relatively easy to extract.

Cultures, acquired from Marine Biological Association of the UK, were chosen to represent different degrees of difficulty for the extraction of chla. Natural samples were collected at sites close to Plymouth. All samples were filtered on GF/F filters and quick-frozen in liquid nitrogen. They were then shipped in dry-ice containers (~ 20 kg) to the different laboratories.

Details of the Fluorometric "Standard" Protocol

1. The filter will be dissolved in 8 ml of 90% Acetone
2. It will be kept for 24 hours in the freezer
3. It will be analysed in the fluorometer using the acidification method of Holm-Hansen et al. (1965).
4. Chlorophyll concentration will be reported both as the corrected value (using the acidification equation), and also as the uncorrected value (not considering acidification).

Table 2. *Laboratories participating in the inter-calibration, person in charge of the analysis, information about the status of the results received, the type of instrument used and whether they were able to perform the 'standard' protocol or not* .

Country	Institution	Person in charge análisis	Status results received	Instrument/standard method (Y or N)
United Arab Emirates	MERC	Anbiah Rajan	Samples spoiled	Not able to perform measurements, due to degradation of samples.
Argentina-EFPU	EFPU	Klaudia Hernandez, Walter Helbling	Received - OK	Fluorometer - Normal kit - **N**
Argentina-INIDEP	INIDEP	Daniel Cucchi-Colleoni, Valeria Segura	Received -OK	Spectrofluorometer (similar Welschmeyer) - **N**
Brazil-FURG	FURG	Virginia Garcia	Received -OK	Fluorometer - Welschmeyer kit - **N**
Brazil-USP	USP	Mayza Pompeu, Salvador Gaeta	Received -OK	Fluorometer - Normal & Welschmeyer kit - **N**
Canada-BIO	BIO	Marie-Helene Forget	Received -OK	Fluorometer - Normal kit - **Y**
Canada-NFL	Fisheries and Oceans	Gary Maillet	Received -OK	Fluorometer - Normal & Welschmeyer kit - **Y**
Chile	PROFC-UDEC	Gadiel Alarcón, Osvaldo Ulloa	Received -OK	Fluorometer - Normal kit - **Y**
India-SAC	SAC	Mini Raman	Received -OK	Fluorometer - Normal kit- **Y**
India – CIFT	CIFT	Muhamed Ashraf, Meenakumari Bharathiamma	Received -OK	Fluorometer - Normal kit - **N**
Mexico	UABC	Adriana Gonzalez-Silvera	Received -OK	Fluorometer - Normal kit- **Y**
Namibia	NatMIRC	Deon.C.Louw	Received -OK	Fluorometer - Welschmeyer kit - **N**
Peru	IMARPE	Georgina Flores Gonzáles, Ruth Calienes	Received -OK	Fluorometer - Normal kit- **Y**
South Africa	MCM	Ray Barlow	Received -OK	Fluorometer - Welschmeyer kit - **N**
Sri-Lanka	NARA	J K Rajapaksha	Received -OK	Spectrophotometer - **N**
Tanzania	IMS Zanzibar	Margareth Kyewalyanga	Results spoiled	Spectrophotometer not working properly
Thailand	Chulalongkorn Universtiy	Nirucha Mongkonsangsuree, Suriyan Saramul	Received -OK	Fluorometer - Normal kit- **Y**
UK	PML	Isobel Cook, Carole Llewellyn	Received -OK	HPLC - **N**
Venezuela	EDIMAR	Juan Carlos Capelo, Ramon Varela	Received -OK	Fluorometer - Normal kit- **Y**
Vietnam	Inst. Oceanog. Viet Nam	Vo Duy Son, Le Lan Huong	Received -OK	Spectrophotometer - **N**

Twenty two laboratories took part in this inter-calibration. One of the laboratories did not report the results yet, and two others could not provide results because the samples arrived spoiled, or the results were meaningless due to malfunctioning of the instrument used. We lack some information to confirm results from one laboratory. That means that we have available confirmed results from 18 laboratories. Of these, two laboratories ran the samples by spectrophotometry, and one by HPLC, leaving 15 laboratories providing fluorometric measurements of chlorophyll. Four laboratories out of these 15 had only the Welschmeyer set up, and one used only 100%-methanol. Two other laboratories introduced some modifications to the "standard" protocol. Hence, only 8 laboratories provided comparable "standard" method results.

Due to the variety of procedures being followed, and the fact that the same laboratory used two or more different methods, it was decided to consider each set of results as a different **'case'**. A list of the main features of the different treatments employed in each of the cases is shown in Table 3.

Table 3. Main features of the treatments followed in each of the study cases.

Case	Solvent	Extraction procedure	Measurement
1	90%-acetone	soak-24 hs	Welschmeyer
2	90%-acetone	soak-24 hs	Welschmeyer
3	90%-acetone	soak-24 hs	H-H-ac
4	90%-acetone	soak-24 hs	H-H-no-ac
5	90%-acetone	soak-24 hs	H-H-ac
6	90%-acetone	soak-24 hs	H-H-no-ac
7	90%-acetone	soak-24 hs	Welschmeyer
8	90%-acetone	soak 24 hs/two extr.	H-H-ac
9	90%-acetone	soak 24 hs/two extr.	H-H-no-ac
10	90%-acetone	soak 24 hs/two extr.	Welschmeyer
11	90%-acetone	soak-24 hs	Spectrofl-ac
12	90%-acetone	soak-24 hs	Spectrofl-no-ac
13	90%-acetone	soak-24 hs	H-H-ac
14	90%-acetone	soak-24 hs	H-H-no-ac
15	90%-acetone	soak-24 hs	H-H-ac
16	90%-acetone	soak-24 hs	H-H-no-ac
17	90%-acetone	soak-24 hs	H-H-ac
18	90%-acetone	soak-24 hs	H-H-no-ac
19	90%-acetone	soak-24 hs	H-H-ac
20	90%-acetone	soak-24 hs	H-H-no-ac
21	90%-acetone	soak-24 hs	Welschmeyer
22	90%-acetone	soak-24 hs	H-H-ac
23	90%-acetone	soak-24 hs	H-H-no-ac
24	90%-acetone	probe-sonic	Welschmeyer
25	90%-acetone	probe-sonic	Welschmeyer
26	100%methanol	probe-sonic	Spectrofl
27	90%acetone	glass-rod-grind	Spectrophotometer
28	90%acetone	glass-rod-grind	H-H-ac
29	90%acetone	glass-rod-grind	H-H-no-ac

30	90%acetone	soak-24hs/bath-sonic	H-H-no-ac
31	90%acetone	soak-24hs/bath-sonic	H-H-no-ac
32	90%acetone	probe-sonic	H-H-ac
33	90%acetone	probe-sonic	H-H-no-ac
34	90%acetone	grind/soak-24 hs/centr	H-H-ac
35	90%acetone	grind/soak-24 hs/centr	H-H-no-ac
36	DMSO:acetone-90% (4 : 6)	soak-24 hs	Welschmeyer
37	100%methanol	bath-sonic	H-H-ac
38	100%methanol	bath-sonic	H-H-no-ac
39	90%acetone	probe-sonic	HPLC
40	90%acetone	soak-24hs/bath-sonic/glass-rod-grind/centr	H-H-ac
41	90%acetone	soak-24hs/bath-sonic/glass-rod-grind/centr	H-H-no-ac
42	90%acetone	probe-sonic/soak-24 hs/centr	Welschmeyer
43	90%acetone	soak-24hs/vortex	H-H-ac
44	90%acetone	soak-24hs/vortex	H-H-no-ac
45	100%methanol	probe-sonic/soak-20 hs/centr	H-H-ac
46	100%methanol	probe-sonic/soak-20 hs/centr	H-H-no-ac
47	90%acetone	soak-24 hs/shake/centr	Spectrophotometer

Comparison of all cases together

The analysis of the results was done in different steps.

First the mean, standard deviation and coefficient of variation (CV) of the replicates for each of the samples, for each of the cases were computed for only fluorometric treatments. This implies that the two cases of spectrophotometric (27, 47) and the one of HPLC (39) determinations of chla were omitted from the first part of the analyisis, and were used in the end for a general comparison. A classification of the cases according to the CV in the replicates was done, separating them into four classes: >30%; ≤30 >25 %; ≤25 >20 %; ≤20 % (see Table 4). This was a first attempt to evaluate the precision of the cases; as a reference value we should consider that a previous HPLC inter-calibration exercise (Sorensen et al., 2003) concluded that a 20% variation in values of chla concentration among laboratories was acceptable.

<u>**Table 4.**</u> *Number, and percentage, of cases falling into the four classes of coefficient of variation in replicates for each sample type. Note that in some samples the total n does not amount 44, this is because some cases missed the analysis of a certain sample type.*

Sample type	>30 %	≤30 >25 %	≤25 >20 %	≤20 %	N total	
A	2	1	6	35	44	*n*
	5	2	14	80		*%*
B	3	0	9	32	44	*n*
	7	0	20	73		*%*
C	1	1	3	39	44	*n*
	2	2	7	89		*%*
D	0	2	2	34	38	*n*
	0	5	5	89		*%*
E	3	0	0	40	43	*n*
	7	0	0	93		*%*

This table shows that the overall precision in the different cases was good, with more than 70% of the cases falling in the ≤20% variation for the replicates for all sample types. It also shows that the performance was somewhat better (>88%) for sample types C, D and E which were assumed to be the 'easiest' to extract. In any case, this first result does not tell us how the actual values (not shown so far) of the chla concentrations vary among cases, or how accurate they were.

Since it was realized that sample types 'D' and 'E' were the easiest to extract (ie., all methods should be expected to perform well), and leaving aside sample type 'C' composed of a mixed culture with high chla concentrations, a first screening was done checking the performance of each of the cases. Those cases, where the CV in the triplicates for samples 'D' or 'E' were higher than 30% were removed. The arbitrary choice of a 30% threshold was chosen as a conservative limit. This left out cases 13, 15 and 16. Then, a median for each of the cases was calculated (for samples 'D' or 'E'), and from there a mean-median. Those cases where the median lay above or below the 'mean-median±2 standard deviations' were removed. This left out cases 17, 20 and 33.

This screening was performed to select the 'best' cases and to use only these for the estimation of the mean-median for each of the sample types, towards which all of the cases were going to be compared. This was done in the next step and the results are shown in Table 5.

<u>**Table 5.**</u> *Mean-median (chla mg m^{-3}) and corresponding standard deviation and coefficient of variation for all the sample types, calculated using the 'selected' cases (see text).*

Sample type	Mean-Median	Stdv	CV
A	145.7	43.4	30
B	390.2	235.9	60
C	665.7	168.3	25
D	4.6	1.0	21
E	2.7	0.8	28

These results show that the highest variation from the mean-median was found for sample type B, which is one of the most difficult cases to extract and also more susceptible to errors in

the determination of chla by the "standard" acidification method, due to the presence of chlorophyll-b, as it was mentioned in the introduction.

Then, the percentage differences between the mean-median and each one of the medians for all of the cases were computed for each sample type, and cases were classified again into ranks of CV. These results are shown in Table 6.

Table 6. Number, and percentage, of cases falling into the four ranks of percentage-difference between each case-median and the mean-median for each sample type. Note that in some samples the total n does not amount 44, this is because some cases missed the analysis of a certain sample type.

Sample type	>30 %	≤30 >25 %	≤25 >20 %	≤20 %	N total	
A	14	8	7	15	44	*n*
	32	*18*	*16*	*34*		*%*
B	32	0	3	9	44	*n*
	73	*0*	*7*	*20*		*%*
C	12	3	4	25	44	*n*
	27	*7*	*9*	*57*		*%*
D	8	3	3	24	38	*n*
	21	*8*	*8*	*63*		*%*
E	14	3	5	21	43	*n*
	33	*7*	*12*	*49*		*%*

This shows that considering all the cases, there were noticeable disparities in chla concentrations provided by all the treatments (cases), demonstrating that the efficiency of the different methods used causes significant differences in the final chla retrieved. Only for the apparently 'easiest' type 'D' did most of the treatments provide close answers, having 63% of the cases within 20% of the mean-median. Although, the number of subtle differences between treatments makes it difficult to evaluate in detail the performance of each one, we provide here as an example, the graph (Figure 1) of mean and standard deviations for all the cases for sample types 'B' (the most difficult) and 'D' (the easiest).

__Figure 1.__ Mean values of chla, and standard deviations, estimated by each of the 44 cases considered, for sample types 'B' and 'D'.
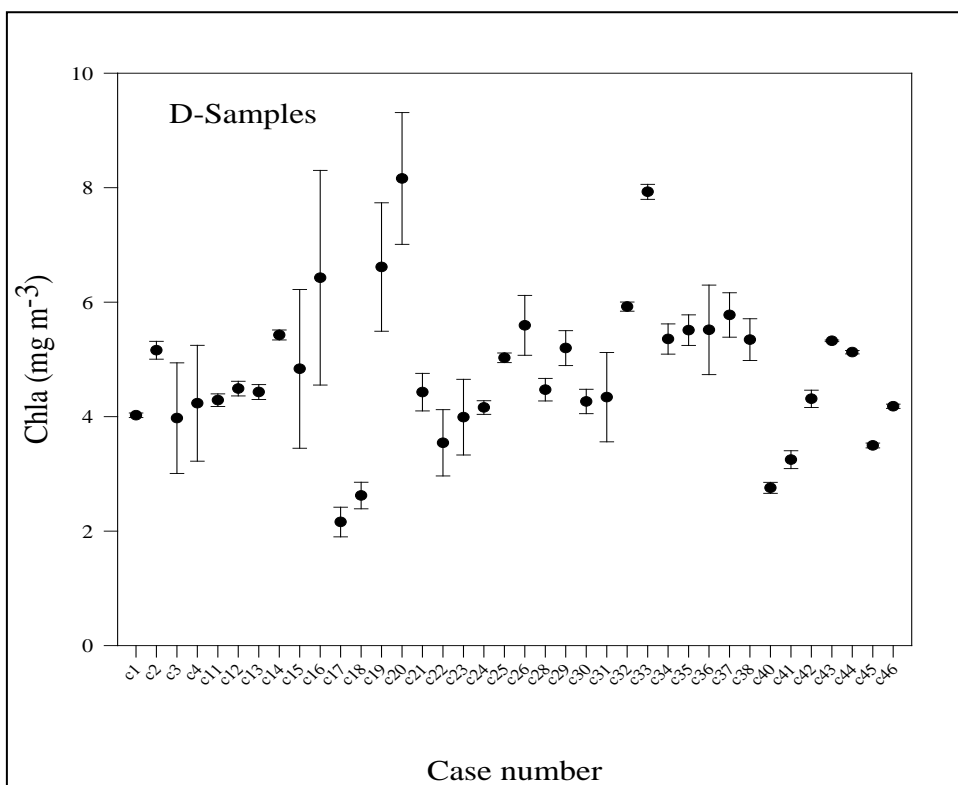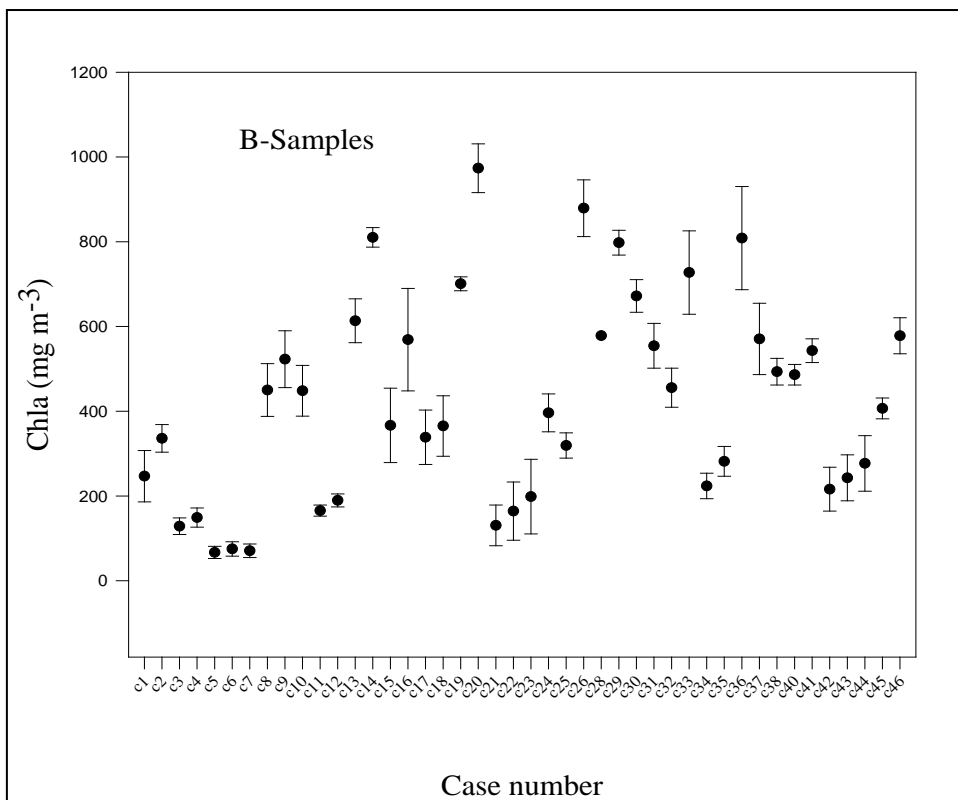
Comparison of separate type of treatments

The next step in the analysis involved separating from all the cases a reduced number of identifiable methods to compare their performances. The five treatments selected were:

1) The "standard" Holm-Hansen et al. method (as previously defined). n=8.
2) The "standard" Holm-Hansen et al. method (as previously defined), but without taking into account the acidification in the calculations (ie., assuming no pheopigments are present). n=8.
3) The Welschmeyer procedure using a "standard" approach, that is 90%-acetone and 24 hs soaking. n=5.
4) The Welschmeyer procedure using 90%-acetone, but adding a probe-sonication disruption of the sample. n=3.
5) The methanol treatment include those where 100%-methanol and sonication was used for the extraction, but it includes one method using a spectrofluorometer (similar to the Welschmeyer approach) and two using the normal-configuration fluorometers (no-acidification). n=3.

Results of this comparison are shown in Table 7.

***Table 7**. Mean-median and coefficient of variation for each of the 5 selected treatments (see text for a more detailed explanation), for each of the sample types. Results for all the cases together are shown for comparison.*

	All-cases	HH-ac	HH-no-ac	Welschmeyer 'standard'	Welschmeyer 'sonication'	Methanol 'sonication'	
A	145.7	142.5	153.0	135.1	132.7	176.0	**mean** **median**
	29	*46*	*55*	*21*	*10*	*34*	*CV*
B	383.3	341.4	452.0	208.8	309.2	642.1	**mean** **median**
	62	*71*	*77*	*54*	*36*	*33*	*CV*
C	661.2	592.9	609.9	573.0	672.2	782.5	**mean** **median**
	25	*45*	*47*	*15*	*10*	*20*	*CV*
D	4.6	4.5	5.2	4.5	4.5	5.1	**mean** **median**
	21	*35*	*37*	*10*	*11*	*15*	*CV*
E	2.7	2.3	2.9	2.7	2.5	3.0	**mean** **median**
	28	*39*	*39*	*17*	*11*	*31*	*CV*

To facilitate comparison, the median of all the cases, including the selected treatments as well as the separate cases of the two spectrophotometric and the one HPLC measurements are shown for the different samples in Figure 2.

***Figure 2**. Median for the values of chla for all-cases, the 'selected' treatments (see text), and separately for the cases of spectrophotometric and HPLC analysis.Panel a shows sample types A,B,C; and panel b shows sample types D and E.*
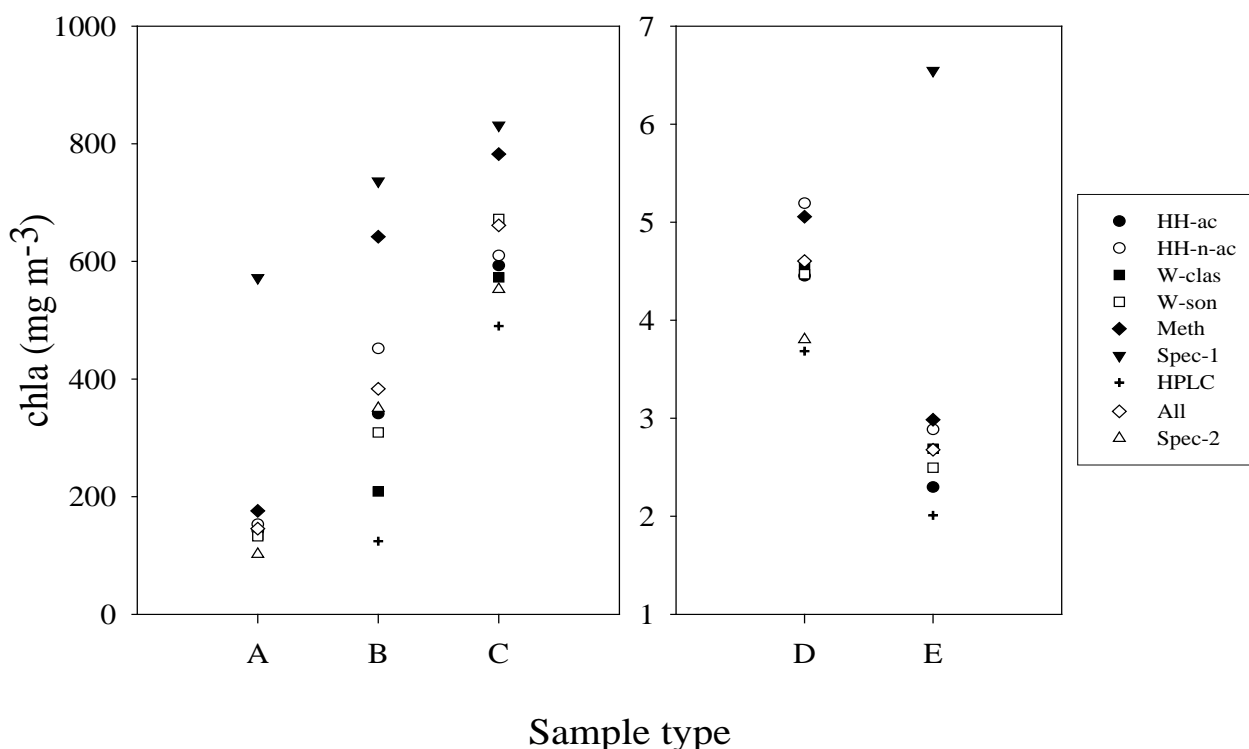
Sample type

The main results from Table 7 and Figure 2 are that:

➢ No large differences were observed between the "standard" method whether acidification was used or not (HH-ac, and HH-no-ac); except for sample type B, where the presence of chlorophyll-b caused 24% underestimation of chla; a 20% difference was also evidenced in samples E.

➢ There seemed to be no large differences between the "standard" and 'sonication' extraction for the Welschmeyer approach; except again for sample type B, where sonication significantly improved the extraction of chla >30%.

➢ There were no large differences in the results from the "standard" Holm-Hansen approachs (HH-ac or HH-no-ac) and Welschmeyer approachs (W-standard, or W-sonication); except for sample type B where the Welschmeyer method rendered 46% lower chla concentrations especially if the HH-no-ac is considered. This is an unexpected result, since the Welschmeyer method should not be affected by the chlb present in these samples.

➢ Overall, the 'Methanol-sonication' procedure rendered the highest chla concentrations. This proves, as it has been reported for HPLC (Jeffrey et al., 1997), the higher efficiency of 100% methanol for the extraction of chla in phytoplankton. This is especially so for 'difficult' to extract species such as green algae and cyanobacteria, represented here by samples B (chlorophyte) where the chla retrieved by 'methanol' was >60% of the total median, and samples A (cyanobacteria) where the chla retrieved by 'methanol' was

>20% of the total median, while for the 'easy' to extract samples results were closer to the total median.

➤ The CVs were high (between 35 and 77%) for the "standard" method whether using acidification or not. Although care was taken to choose cases where the "standard" protocol was performed according to instructions, small differences in the handling and extraction of the samples, as well as differences in the calibration of the fluorometers may have caused these deviations in the final chla concentrations.

➤ The 'methanol-sonication' treatment also showed CVs slightly above 30% for some of the samples, but we should take into account that this group included the use of different types of instrument (fluorometers with the normal-kit, and a spectrofluorometer), besides small differences in the extraction procedure.

➤ The two sets of spectrophotometric measurements gave significantly different results. Case 'spec-1' had the highest results in some instances > 100% than any of the other methods, whereas case 'spec-2' gave results within the range of the other methods, except for samples type A and D, where they were lower. These discrepancies may be due to the lower sensitivity of spectrophotometry compared to fluorometry.

➤ Except for sample type A, HPLC results were lower than for any of the other selected methods and the overall median for chla concentrations. This may be due in part to the fact that the method is able to separate various derivatives of chla, which are measured as part of 'chla' in the rest of the methods. It may also be due to differences in the extraction procedures for the particular HPLC method used. Variations in chla retrieved by different HPLC methods have been reported (Jeffrey et al., 1997; Sorensen et al. 2003; Claustre et al., 2004).

4. Concluding remarks

Variations in chla concentrations retrieved by the different treatments carried out by different laboratories were large, even when the supposedly "standard" Holm-Hansen et al. (1965) method was performed (CVs above 35%; Table 7). This proves the relevance of going through an inter-calibration exercise before comparing results from different laboratories in the network, or even more so, using them as a validation for satellite chla.

To get better quality and comparable results we should look at the sources of variations and decide which would be the best practice. According to the results obtained from the present exercise, including detailed information on every step of the procedures and calculations exchanged between the participants, as well as what is known from literature reviews on pigment determination (e.g., Jeffrey et al., 1997; NASA-report V) a few recommendations can be made:

1- A careful calibration of the fluorometer should be performed, ideally every time that a set of samples is going to be measured (e.g., for one cruise), or when the instrument has been transported, or at least twice a year. This should help to diminish the variations observed even when the same method is used.

2- Probe-sonication would be the best way to help the disruption of the cells to extract their pigments. Although, some of the 'easy' samples showed no difference whether sonication was used or not, in the 'difficult' cases the extraction was improved. It should be borne in

mind that in many oligotrophic waters most of the phytoplankton species present belong to the 'difficult' type.

3- Whenever a sample-disruption step is used (by sonication, mechanical grinding, glass rod, shaking in a vortex, etc.), samples should be centrifuged before reading in the fluorometer; or at least left to sediment during several hours in the freezer. Several laboratories reported using some sort of 'disruption' and then reading the samples immediately; this may also affect the results.

4- 100%-methanol seems to be a better solvent to retrieve chla concentrations by fluorometry, as it had been proved for HPLC, especially so for the difficult types. If this new solvent was to be adopted, care should be taking to perform the calibration of the fluorometer with it. Field samples may become unstable in methanol if left for too long before reading in the fluorometer. It is therefore important to read directly after 24 hour extraction. In this case it is advisable to dissolve and keep the chla standard in 100% acetone (since methylation may alter the pigments kept for long time in methanol); and from a concentrated work-solution dilutions in 100% methanol (taking care that final acetone is < 3% of the final solution) should be prepared to run the calibration curve in the fluorometer.

5- The Welschmeyer approach has been proved in several studies to return more realistic chla concentrations in the presence of chlorophyll b. It provides a more selective and also easier (avoids the acidification step) way of measuring chla.

6- It has long been proved that the use of the acidification to correct the chla from the presence of pheopigments, produces artificially high concentrations of pheopigments in the presence of chlb, together with underestimation of chla; as well as artificial negative values of pheopigments and overestimation of chla in the presence of high concentrations of chlorophyll-c. As a matter of fact, pheopigment concentrations obtained from this inter-calibration exercise were not reported since variations among treatments were extreme (from larger than the chla values to negative ones). For this exercise there were not large differences in chla whether the acidification was applied or not in the case of 'easy' samples, while acidification produced an underestimation of chla in the presence of chlb. It has been reported through HPLC studies (Trees et al., 2000), that concentrations of pheopigments are usually extremely low in seawater, but the presence of chlb cannot be disregarded. There may, nevertheless be, some instances, in high chla waters exposed to a strong grazing pressure or decaying blooms, where pheopigments may be present in significant amounts. Therefore, when applying the "standard" fluorometric method with acidification the chla concentrations should be interpreted with caution according to the conditions of waters studied. It is not recommended to use the values of pheopigments, since they are not reliable. The best solution would be to use the Welschmeyer approach, being more selective and rendering chla concentrations not affected by chlb or pheopigments.

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