QUASIMEME **BEQUALM**

Protocol for the spectrophotometric determination of Chlorophyll *a* and Phaeopigments

based on the ICES Techniques in Marine Environmental Sciences (TIMES) publication No. 31:

"Standard procedure for the determination of chlorophyll *a* by spectroscopic methods"

by Alain Aminot[1] and Francisco Rey[2]

[1]IFREMER, Centre de Brest, BP 70, F-29280 Plouzané, France
[2]Institute of Marine Research, P.O. Box 1870 Nordnes, N-5817 Bergen, Norway

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adapted for use in the QUASIMEME/BEQUALM programme by

Marcus Reckermann and Franciscus Colijn
Research and Technology Centre Westcoast of Kiel University, Hafentörn, D-25761 Büsum, Germany

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Preliminary remark

This manual is a condensed version of the original ICES TIMES document No. 31

"Standard procedure for the determination of chlorophyll a by spectroscopic methods"

by Aminot and Rey (2000). It was adapted from the original version for the Monitoring QA programme of QUASIMEME/BEQUALM. Although it is undisputed that fluorometric and HPLC methods provide better sensitivity in low-chlorophyll waters, only the spectrophotometric methods of Jeffrey and Humphrey (1975) and Lorenzen (1967) will be applied, for the sake of robustness and simplicity.

The sub-headings of omitted sections (which are not relevant have been for the present purpose) kept. To provide the participants of the QUASIMEME/BEQUALM Chlorophyll-a Laboratory Performance Study with an overview over the contents of the original article.

The original version can be viewed and downloaded from the ICES web site:

http://www.ices.dk/ocean/procedures/chlorophyll/

Please note that Sections 1, 2, 4, 5 and 6 contain general information and a discussion of the methods, while ***section 3*** *contains the actual analytical procedures.*

1 INTRODUCTION

Chlorophyll *a* is the principal pigment in plants. In converting light energy to chemical energy, it allows photosynthesis, i.e., light-induced carbon fixation (primary production) to take place. As a biomass indicator of aquatic micro-algae that support food webs in the sea, it is probably the most frequently measured biochemical parameter in oceanography (Jeffrey and Mantoura, 1997).

Whatever the method used for the determination of algal pigments, the measure itself relies on their spectroscopic characteristics: light absorption or fluorescence. In discrete samples, photosynthetic pigments can be measured either by the traditional non-separative spectroscopic methods or after chromatographic separation, using HPLC. Detection by either remote sensing or *in situ* probes is also used, but these relative data must be calibrated against discrete 'chemical' measurements on samples at a frequency appropriate to local conditions.

As chlorophyll *a* determination is complex but non-specific, traceability and quality controls are difficult to establish. Quality Assurance (QA) relies strongly on rigorous application, at each step, of recommended protocols checked by specialists, and good appraisal of the limits of validity of the method. Specific publications from international bodies have reviewed the procedures and/or proposed guidelines and recommendations (SCOR-UNESCO, 1966; BMB, 1979). However, an updated UNESCO publication, entitled 'Phytoplankton pigments in oceanography: guidelines to modern methods', presents a very detailed review on phytoplankton pigments issued by the SCOR Working Group 78 on 'Determination of photosynthetic pigments in seawater' (Jeffrey *et al*., 1997). As this book contains the latest methodological developments and recommendations, it provides the benchmark for pigment studies.

2 CONTEXT AND DIFFICULTIES OF CHLOROPHYLL *a* DETERMINATION

Measurement of chlorophyll *a* requires its extraction from planktonic cells, which involves filtering the water as the first step, then extracting the filter with an appropriate solvent. Once the extracts are obtained, chlorophyll *a* can be measured using single or multi-wavelength spectrophotometric or fluorometric procedures. These procedures overcome some of the interferences from other pigments extracted together with chlorophyll *a* and having similar spectroscopic properties. Since pigments are light-sensitive molecules, protection from light should be a constant concern throughout all analytical steps.

2.1 Interfering Chlorophylls and Degradation Products

Three types of chlorophylls have been identified: *a*, *b*, and *c* (there are six known types of chlorophyll *c*).

The basic structure of chlorophylls is a tetrapyrrole macrocycle chelating a magnesium ion. Differing radicals characterize the three types of chlorophylls. Chlorophylls *a* and *b* have a side phytol chain, unlike chlorophylls *c*. When the chlorophyll (*a*, *b*, or *c*) molecule loses its magnesium ion, the resulting product is a pheophytin (*a*, *b*, or *c)* respectively). Dephytylation produces chlorophyllides. Pheophorbides are both dephytylated and magnesium-free. Pheophytins and pheophorbides constitute the pheopigments.

2.2 Occurrence and Significance of the Chlorophyll Group Pigments

Degradation of chlorophylls can occur either naturally in the medium or within the analytical chain. Table A2.1 shows the natural occurrence of the main pigments.

Table A2.1 *Natural occurrence of the main pigments of the chlorophyll group.*

Pigment	**Occurrence**
Chlorophyll *a*	All photosynthetic algae (except prochlorophytes) and higher plants
Chlorophyll *b*	Higher plants, green algae, symbiotic prochlorophytes
Chlorophylls *c*	Chromophyte algae, brown seaweeds
Chlorophyllide *a*	Senescent tissue, damaged centric diatoms, zooplankton faecal pellets
Chlorophyllide *b*	Senescent tissue, zooplankton faecal pellets
Pheophytin *a*	Photosynthetic reaction centres of higher plants, plant and algal detritus
Pheophytin *b*	Terrestrial plant detritus, protozoan faecal pellets
Pheophorbide *a*	Marine detritus, zooplankton and protozoan faecal pellets
Pheophorbide *b*	Terrestrial plant detritus, protozoan faecal pellets

From Table A2.1, it is clear that several pigments and degradation products may be found simultaneously in a sample. For research studies, analysis of the detailed pigment composition may significantly improve knowledge of phytoplankton composition and physiology. Although this is not necessary for routine use of chlorophyll *a* as a biomass indicator, the following should be kept in mind:

- as the only pigment present in all microalgae, chlorophyll *a* is currently the right biomass indicator (the chlorophyll *a* derivative found in prochlorophytes is measured as chlorophyll *a*);

- in marine samples, pheopigments result from grazing and are, consequently, inactive pigments, thus determination of pheopigments *a* together with chlorophyll *a* may be useful;

- in turbid estuaries, higher plant detritus may contribute pigment concentrations (pheophytin *a* being known as an active plant pigment, misinterpretation may occur); this contribution, however, is assumed to be minor in comparison with river phytoplankton inputs;

- chlorophyllides, not spectroscopically distinct from chlorophyll *a*, lead to an overestimation of chlorophyll *a*.

2.3 Spectroscopic Characteristics and Resulting Measurement Methods

2.3.1 Spectrophotometry

Chlorophylls exhibit two major light absorption bands, one on the blue side of the visible spectrum (< 460 nm) and one in the red (630–670 nm). As carotenoids, co-extracted with chlorophylls, have also strong absorption maxima in the blue, spectrophotometric

measurements are limited to the red absorption bands. Because of overlapping of the main absorption bands and of secondary maxima in the range 630–670 nm, several spectrophotometric procedures have been developed to determine the three chlorophylls in the same extract. They are based on measurement of absorbances at three wavelengths, then computation of chlorophylls using three equations (called 'trichromatic equations').

Unfortunately, degradation products have spectroscopic characteristics close to those of their parent chlorophyll. The spectra of chlorophyllides in the red wavelengths are so close to those of their parent chlorophylls that there is no way of differentiating the forms spectrophotometrically. Pheopigments *a* and *b* (no data available for *c*) also show spectra similar to those of the corresponding chlorophylls, but with a slight red shift and a decrease of the molar extinction coefficients to about 0.6 times those of chlorophylls.

Although methods, that take into account pheopigments in the extracts, have been developed for chlorophyll *a*, none of the spectrophotometric methods is quite accurate in the presence of degradation products.

2.3.2 Fluorometry

Section omitted (no relevance for QUASIMEME/BEQUALM intercalibrations)

2.4 Critical View of the Preparation of Pigment Extracts

2.4.1 Sampling and sub-sampling

Section omitted (no relevance for QUASIMEME/BEQUALM intercalibrations)

2.4.2 Filtration

Section omitted (no relevance for QUASIMEME/BEQUALM intercalibrations)

2.4.3 Storage of the filters

Storage temperature and time are critical points, with chilling being of obvious importance. Storage at room temperature, even after freeze-drying, is not recommended because it results in extensive degradation of pigments.

With the availability of deep freezers, storage at –18 °C to –20 °C is a very convenient, widespread practice that has been recommended in several handbooks. An increasing proportion of marine scientists now use a lower storage temperature for phytoplankton samples.

A systematic study by the SCOR Working Group 78 checked storage conditions for up to 11 months at various temperatures (+22 °C, –20 °C, –90 °C, and –196 °C). Pigment composition was assessed using HPLC. The main findings are summarized as follows:

- the lower the temperature, the longer the storage time can be; usual deep-freezing conditions (about –20 °C) are above the eutectic point of the cell fluid, allowing biochemical reaction to proceed in the concentrated residual medium;

- degradation products of chlorophyll *a* were always dominated by chlorophyllide *a* and chlorophyll *a*-allomers, but after 11 months they accounted for only a quarter of the chlorophyll decline; the resulting decrease in total pigment suggested production of colourless degradation end-products;

- pheopigments *a* were never produced during storage;

- a 'bound' pool of pigments (up to 20 %), not extracted by methanol in fresh filters, was freed by extended freezing.

The above findings suggest that the effects of short-term storage of filters are of no consequence if chlorophyll *a* is to be determined by spectroscopic methods. Indeed, since early degradation products have spectral characteristics close to those of chlorophyll *a*, the original value of chlorophyll *a* in the sample will not be severely affected. This condition can be met for short storage times. However, if further degradation occurs, predominantly colourless products may be formed and erroneous data will be obtained.

The SCOR Working Group 78 recommended that storage of pigments for periods up to one year was done by freezing at the temperature of liquid nitrogen (–196 °C). Storage at –18 °C to –20 °C was acceptable only up to one week. However, when considering chlorophyll *a* alone, experiments with a natural phytoplankton community yielded ~100 % recovery over at least 1 month. This agrees with the findings of several authors that chlorophyll *a* decreased by less than 5–10 % in a deep freezer for up to eight weeks (see Mantoura *et al*., 1997b).

Finally, for storage not exceeding several weeks, it may be recommended that filters for chlorophyll *a* and pheopigments *a* be stored at –20 °C. Use of ultracold freezers allows extended storage times.

2.4.4 Extraction of the filters

Extraction of pigments from planktonic diatoms and naked flagellates is easy, but some algae are difficult to extract (e.g., armoured dinoflagellates, heavily silicified benthic diatoms, cyanobacteria, thick-walled green algae). Extraction of pigments from filters has given rise to numerous tests and procedures published in the literature. Various solvents have been used, with or without mechanical action (sonication or grinding), at various temperatures, and for various lengths of time. Not all of these extraction parameters are independent. It must be noted that the extracting solvent is necessarily the solvent in which spectroscopic measurements will be made.

The SCOR Working Group 78 considered six criteria for the extraction technique applied to phytoplankton:

1) extractability: extraction of all pigments should be complete, irrespective of the algae;

2) fidelity: the pigments should not be altered by the process (stability up to one day);

3) compatibility: the solvent must be compatible with the materials (especially for HPLC);

4) precision: replication should be satisfactory;

5) simplicity: the technique should be rapid, with few handling steps;

6) safety: solvent should have low toxicity and flammability; few transfers.

In the context of the determination of chlorophyll *a* and pheopigments as bio-indicators for natural communities of phytoplankton, a comparative assessment of the entire set of protocols, using HPLC as a reference method, was developed by the SCOR Group (Mantoura *et al.*, 1997a). Results obtained with protocols using grinding of filters in 90 % acetone (Holm-Hansen *et al.*, 1965; Lorenzen, 1967) matched those of HPLC for chlorophyll *a* and pheopigments *a*. This solvent was therefore validated for these pigments as biomarkers in routine field work. Moreover, in this solvent absorption peaks are narrower and extinction coefficients are larger and precisely determined. In addition, 90 % acetone has little toxicity.

Note that, if acetone is used, it is strongly recommended to grind the filters instead of sonicating or soaking overnight. In a glass homogeniser with a motor-driven teflon pestle, complete disruption of the filter is obtained in about 1 minute Extraction time may be prolonged to 30–60 minutes after transfer into the centrifuge tubes kept tightly closed and protected from heat and light (Lorenzen, 1967).

2.5 Spectroscopic Measurements

2.5.1 Choice of the method

Spectrophotometry and fluorometry can be equally used for chlorophyll *a* and pheopigments *a* determination. However, spectrophotometry is less sensitive and therefore requires the filtering of a much larger volume of sample in oligotrophic areas. Modern high performance spectrophotometers measure low absorbances with a 10^{-4} resolution, which allows chlorophyll *a* determination down to ~0.1 mg m^{-3} in samples of about 1 litre, with a 5 cm light-path cuvette. Fluorometry must be used when the available volume of water is insufficient for reliable absorbance measurement. It should be noted that, unlike spectrophotometers, fluorometers have to be calibrated with chlorophyll standards.

There are two types of spectrophotometric methods suitable for routine use: trichromatic and monochromatic. *Trichromatic methods* have been developed in order to determine the three types of chlorophyll (*a*, *b*, and *c*) in the absence of degradation products. Absorbances must be measured at the three maximum wavelengths of the three chlorophylls, plus a blank wavelength, then a set of three equations is used to calculate the concentrations. *Monochromatic methods* have been developed to correct chlorophyll *a* for pheopigment *a*. Absorbances are measured at the red maximum (plus a blank wavelength) before and after acidification. It is assumed that acidification degrades all chlorophyll-like pigments into pheopigments by eliminating the magnesium ion from the tetrapyrrole complex. The drop in absorbance allows both chlorophyll *a* and pheopigment *a* to be calculated.

The classic fluorometric method for routine work is similar, in its principle, to the spectrophotometric monochromatic method. Fluorescence of the extract is measured at a unique wavelength before and after acidification, then concentrations of chlorophyll *a* and pheopigments *a* can be calculated.

As stated above reminder that none of the spectroscopic methods corrects for chlorophyllide *a*. However, from method comparisons (see Section 2.4) and field data available in the literature, such interference does not appear to be a serious problem. Overestimating chlorophyll *a* by trichromatic equations, due to the presence of pheopigments, is certainly the major problem encountered in coastal and estuarine waters. Pheopigment-correcting methods can therefore be recommended in these areas. Using fluorometry, the presence of chlorophyll *b* may result, if a standard lamp is used, in significant overestimation of pheopigments *a*, thus underestimating chlorophyll *a* (this is due to the wavelength shift of chlorophyll *b*, under acidification, into a band of strong energy of the lamp). As a quality control rule, in order to validate data obtained with pheopigment-correcting methods, it is suggested to perform occasional controls using the trichromatic spectrophotometric equations (or even multi-wavelength spectrofluorimetry).

2.5.2 Spectrophotometry

Instrumental characteristics

Since the determination of chlorophyll relies on absolute absorbance values (no calibration), it requires high performance spectrophotometers. The bandwidth should not exceed 2 nm. Significant underestimation of chlorophyll *a* occurs with large bandwidths (Brown *et al*., 1980). As long-path cuvettes are generally required to increase sensitivity, operators must make sure that the entire light beam passes through the extract. Low volume (thick-wall) cuvettes may produce erroneous data in spectrophotometers having very converging beams.

Trichromatic method

Four sets of trichromatic equations have been published, following improvements in the values of maximum absorption wavelengths and extinction coefficients. Coefficients are determined for pigments dissolved in 90 % acetone. Equations of Richards and Thompson (1952) are obsolete and should not be used. Those of Parsons and Strickland (1963) and SCOR-UNESCO (1966) can be used for chlorophyll *a* only. The equations of Jeffrey and Humphrey (1975) are the only ones recommended for the three chlorophylls.

Trichromatic measurements allow the presence of chlorophyll *b* to be detected, hence, its possible interference in the measurement of chlorophyll *a* using monochromatic methods. When chlorophyll *b* is low, then:

[trichromatic chlorophyll *a*] $\cong$ [monochromatic chlorophyll *a*] + 0.6 $\times$ [pheopigments].

Monochromatic (pheopigment-correcting) method

Monochromatic methods are recommended for chlorophyll *a* in coastal and estuarine waters. The correction equations for pheopigments have been published by Lorenzen (1967). They are suitable for pigments dissolved in 90 % acetone. As mentioned in the UNESCO Monograph (Jeffrey et al., 1997; annex F), the specific extinction coefficient used by Lorenzen (91.1 l g^{-1} cm^{-1}) is about 4 % higher than the presently accepted coefficient of Jeffrey and Humphrey (1975), i.e. 87.7 l g^{-1} cm^{-1}. In order to keep consistency between the spectrophotometric and the fluorometric methods described below, the corresponding factor of the Lorenzen's equation was set at 11.4 instead of 11.0 in the

original method. A derived set of equations was subsequently established by Holm-Hansen and Riemann (1978) for methanol.

Specific points of the spectrophotometric procedure

The blank should be determined with care. It is intended to correct for background absorption and/or turbidity produced by the filters and any particulate matter. With soluble membrane filters, the contribution of the filter to the blank must be accurately determined. With glass fibre filters, centrifugation is critical for avoiding the transfer of fibres into the optical cuvette. The blank (750 nm) should be checked for stability over the time required for measuring the sample. Decreasing blanks denote the presence of particles.

In terms of acidification methods, it has been shown (Moed and Hallegraeff, 1978) that the acid concentration should be well controlled (pH 2.6-2.8 in the extract). Optimum conditions are obtained with $[H^+]$ = 0.003 mol/L (Holm-Hansen and Riemann, 1978) to 0.006 mol/L (Nusch, 1980) in the extract, and a reaction time of 2-3 min. A very important point in acidification methods is to ensure that any acid residue has been thoroughly rinsed out of the optical cuvette before the next sample extract is introduced into it (otherwise, chlorophyll would be degraded and underestimated; any suspect excess of pheopigment may indicate such an eventuality).

2.5.3 Fluorometric measurement

Section omitted (not required for QUASIMEME/BEQUALM Laboratory Performance Study)

3 PROCEDURE

General precautionary note

- ***As the pigments are both photo- and heat-sensitive, care to protect them from direct sunlight and from warming must be taken at each step of the procedure.***

3.1 Sampling

Section omitted (not relevant to this for QUASIMEME/BEQUALM Laboratory Performance Study)

3.2 Filtration

Section omitted (not relevant to this for QUASIMEME/BEQUALM Laboratory Performance Study)

3.3 Storage of the Filters

- Keep storage time as short as possible. Filters frozen at –20 °C can be kept for up to a 3–4 week period without significant decrease in chlorophyll *a*. For longer periods, colder temperatures (–70 °C) should be used.

Precautionary note

- *Every laboratory should check the freezing conditions by randomly analysing duplicate samples against filters extracted and analysed immediately without storage.*

3.4 Pigment Extraction

- Extract pigment by grinding the filters in a few millilitre of 90 % acetone in a glass homogenizer with a motor-driven Teflon pestle, for 1 minute, in an ice bath and under subdued light.
- After grinding, carefully transfer the extract to a stoppered and graduated centrifuge tube, rinse the glass homogenizer and the pestle with 90 % acetone and add rinsing to the centrifuge tube.
- Adjust the extract volume in the centrifuge tube to exactly 10 ml 90 % acetone (i.e. 10 ml + dead volume of filter) and stopper the tube.

Precautionary notes

- *Soaking of the filters overnight is not recommended unless the extraction efficiency of this procedure is thoroughly checked against grinding for the actual working conditions.*

- *If the extracts are not measured immediately after grinding, e.g., if the measurements are done in batches, then they can be kept in a tightly stoppered tube in cold and dark for up to one hour.*

3.5 Centrifugation

- Immediately before measurement, mix the sample thoroughly and centrifuge the extract for 10 minutes at 500×*g*, where *g* is the gravitational acceleration. Assuming *g* to be 9.81 m s^{-2}, then the centrifugation velocity (rpm) for a particular centrifuge can be estimated by $668.8/R^{0.5}$ where R is the radius, the distance (in metre units) between the axis of the centrifuge head and the mid-point of the centrifuge tube.

Precautionary notes

- *When working with glass fibre filters, centrifugation is critical as fibres must not be transferred into the optical cuvette. The blank (at 750 nm) should be checked for stability over the time required for measuring the sample. Decreasing blanks denote the presence of particles.*

- *After centrifugation, some glass fibres often remain on the tube wall, above the solvent surface. These may fall into the extract during transfer to the optical cell. In such case, tubes should be centrifuged twice: after a first brief centrifugation the tubes should be gently swirled to collect remaining fibres and then centrifuged again.*

3.6 Spectroscopic Measurement

Spectrophotometric methods are usually preferred when an ample water sample volume is available (as a rule, about one litre of water is needed for a single measurement). The trichromatic method is recommended for seawater samples containing chlorophyll *a*, *b*, and *c* as the major pigments and where chlorophyll degradation products are absent. The monochromatic method is recommended for seawater samples containing significant amounts of degradation products. When only small sample volumes are available, fluorometry is generally the only satisfactory method, unless a high performance (very sensitive) spectrophotometer is used. Fluorometry is also recommended for seawater samples containing significant amounts of degradation products.

Note: Fluorometric methods will not be used for the present QUASIMEME / BEQUALM Ring test.

3.6.1 Spectrophotometry: Trichromatic method

- Use a spectrophotometer of 2 nm maximum bandwidth and stoppered cuvettes with path length up to 5 cm (such a path length is required in most instances for satisfactory measurements).

- Transfer the sample extracts from the centrifuge tubes to the cuvette by careful pipeting.

- Measure the absorbance of the sample extract at 750, 664, 647, and 630 nm against a 90 % acetone blank.

- Calculate the concentration of chlorophyll *a*, *b* and *c*, according to the equations of Jeffrey and Humphrey (1975):

Chlorophyll *a* = $(11.85*(E_{664} - E_{750}) - 1.54*(E_{647} - E_{750}) - 0.08(E_{630} - E_{750}))*V_e / L*V_f$

Chlorophyll *b* = $(-5.43*(E_{664} - E_{750}) + 21.03*(E_{647} - E_{750}) - 2.66(E_{630} - E_{750}))*V_e / L*V_f$

Chlorophyll *c* = $(-1.67*(E_{664} - E_{750}) - 7.60*(E_{647} - E_{750}) + 24.52(E_{630} - E_{750}))*V_e / L*V_f$

Where:
L = Cuvette light-path in centimetre.
V_e = Extraction volume in millilitre.
V_f = Filtered volume in litre.
Concentrations are in unit mg m^{-3}.

3.6.2 Spectrophotometry: Monochromatic method with acidification

- Use a spectrophotometer of 2 nm maximum bandwidth and stoppered cuvettes with path length up to 5 cm (such a path length is required in most instances for satisfactory measurements).

- Transfer the sample extracts are from the centrifuge tubes to the cuvette by careful pipeting.

- Measure the absorbance of the sample extract at 750 nm (E_{750o}) and 665 nm (E_{665o}) against a 90 % acetone blank.

- Add 0.2 ml 1 % v/v hydrochloric acid in the cuvette and mix.

- Wait 2-5 min (but not more).

- Measure again the absorbance at 750 nm (E_{750a}) and 665 nm(E_{665a}) against a 90 % acetone blank.

- Calculate the concentration of chlorophyll *a* and pheopigments *a* according to the equations of Lorenzen (1967):

Chlorophyll *a* = $11.4*K*((E_{665o} - E_{750o}) - (E_{665a} - E_{750a}))*V_e / L*V_f$

Pheopigments *a* = $11.4*K*((R*(E_{665a} - E_{750a})) - (E_{665o} - E_{750o}))*V_e / L*V_f$

Where:
L = Cuvette light-path in centimetre.
V_e = Extraction volume in millilitre.
V_f = Filtered volume in litre.
R = Maximum absorbance ratio of E_{665o}/E_{665a} in the absence of pheopigments = 1.7.
K = R/(R – 1) = 2.43.
Concentrations are in unit mg m^{-3}.

Precautionary notes

- *Do not pour the sample from the tube into the cuvette as this can transfer glass fibres.*
- *Closed optical cuvettes reduces cooling due to evaporation and, hence, absorbance variability due to the Schlieren effect.*
- *Check that optical cuvettes are filled sufficiently to use the entire light beam.*
- *A very important point in the acidification method is to ensure that all acid residue are thoroughly rinsed from the optical cuvette before the next sample is transferred. Otherwise chlorophyll* a *would be degraded and underestimated. Excess of pheopigment values would indicate that this has occurred.*
- *It is recommended to check the delay for completeness of reaction after acidification (stable absorbance).*

3.6.3 Fluorometry

Section omitted (not for QUASIMEME/BEQUALM LPs)

4 SUMMARY OF OPERATIONAL CONDITIONS

OPERATION	MATERIAL	CONDITIONS	
Sampling	Opaque sampling bottle		Q/B
Sub-sampling	Opaque bottle	optional: pre-filtration 100–150 µm	Q/B
Temporary storage of water		protected from heat and light; max. one hour	Q/B
Filtration	0.7 µm glass fibre filter (e.g., Whatman GF/F)	> 0.7 bar residual pressure; subdued light; remove large zooplankton from filter with forceps	Q/B
Storage of filter	Deep freezer	–20 °C; maximum two months	Q/B
Extraction of the filter	Solvent: 90 % acetone; homogenizer with teflon pestle	chill; subdued light; grinding: ~1 minute; until analysis (within 1 hour), keep the chill extract in darkness	P
Centrifugation or filtration of the extract	Graduated, stoppered tubes		P
Measurement	- spectrophotometer (bandwidth ≤ 2 nm) - fluorometer - both	Lorenzen (1967); Holm-Hansen *et al.* (1965); calibration with pure chlorophyll *a* test (spectrophotometry): Jeffrey and Humphrey (1975)	P

Q/B Done by QUASIMEME/BEQUALM

P To be completed by participants

5 QUALITY CONTROL

Since a stable reference material is not available, replicated samples may be used to collect information on the repeatability of the procedure. A control chart can be constructed using these data, by plotting the differences between two double samples, with zero as the expected mean. Such a control chart provides information on measurement uncertainty and also on the validity of the sampling procedure.

When the trichromatic method is used, the presence of pheopigments should be checked from time to time, or on selected samples, in order to validate the data.

Tests for the presence of chlorophyll *b* may also be useful for validation of the data obtained by the fluorometric method, if the combination of lamp and filters is not optimized.

During the spring bloom in open areas, or in algal cultures in the exponential growth phase, pheopigments should be at very low levels. Excessive concentrations of pheopigments relative to chlorophyll could indicate potential procedural errors (note that storage by freezing does not generate pheopigments). In the acidification methods, for instance, errors can occur from insufficient or excessive acidification or from poor rinsing of residual acid from optical cuvette after each sample.

Participation in intercomparison exercises is strongly recommended. For this purpose the trichromatic method is recommended as the reference method, unless significant pheopigment concentrations in the samples are suspected. It is also highly recommended that each laboratory performs occasional checking of its adapted procedures against the trichromatic method (if another method is chosen) or, even better, against HPLC pigment analysis (Jeffrey *et al*., 1997), if available.

It is recommended that every laboratory develops its own quality assurance (QA) routines for the particular form by which the present proposed procedure is employed for measuring chlorophyll *a.* These QA routines should include all steps of the procedure, from sampling to the final result. Laboratories that use procedures deviating from the present protocol should indicate the nature of these differences and provide comparability of their method against the procedure described here.

6 ALTERNATIVE PROCEDURES

Extraction by soaking in 96 % ethanol instead of 90 % acetone has been recommended by HELCOM (Baltic Monitoring Programme, 1998). Unfortunately few papers on this procedure have been published in the international literature, and ethanol extraction was not tested by the SCOR Working Group 78. However, because 90 % acetone may poorly extract chlorophyll *a* in some algal species growing frequently in the Baltic Sea, 90-96 % ethanol may be used as an alternative solvent, with reference to the work of Nusch (1980). The above protocol for monochromatic spectrophotometric measurement with acidification can be applied directly to ethanol extraction. The only change is the numerical factor used in the Lorenzen equation which should be 12.0 (for consistency with the HELCOM method). It must be noted that there is no actual consensus on the specific extinction coefficient (SEC) of chlorophyll *a* in ethanol and that, according to values of the SEC found in literature, the above factor varies between 11.5 and 12.2

Direct fluorometric determination of chlorophyll *a*, after 90 % acetone extraction but without acidification, has been developed by Welschmeyer (1994); interference from chlorophyll *b* and pheopigments is eliminated but the latter are not measured. The method uses a classic filter fluorometer equipped with a specific combination of lamp and interference filters. The SCOR Working Group 78 did not test this method, but it is

mentioned as an interesting development for routine assays after the recommended acetone extraction.

7 ACKNOWLEDGEMENT

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