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Chlorophyll *a*: Determination by spectroscopic methods

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Chlorophyll *a*: Determination by spectroscopic methods

Alain Aminot and Francisco Rey

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Abstract

Chlorophyll *a* is the principal pigment in plants. As a biomass indicator of aquatic microalgae which support food webs in the sea, it is probably the most frequently measured biochemical parameter in oceanography.

This document describes a procedure for the routine determination of chlorophyll *a* in sea water for use by ICES Member Countries. It has been developed from a review of current methodology for measuring chlorophyll *a* undertaken by the Working Group on Phytoplankton Ecology (WGPE) and the Marine Chemistry Working Group (MCWG). This work was led by A. Aminot (MCWG) and F. Rey (WGPE) and both groups based their discussions around a recently published work on this topic (Jeffrey, Mantoura, and Wright, 1997). The present document represents the consensus between the two groups.

This document draws attention to critical points of chlorophyll *a* determination and reviews recommendations concerning the use of this pigment as a biomass marker. In addition, it proposes a standard procedure for chlorophyll *a* determination. Although many points in the procedure can apply to any other method, the document is devoted to the determination of chlorophyll *a* in discrete samples, after extraction and spectroscopic measurement of the pigments.

It is not presently possible or desirable to recommend a single method for measuring chlorophyll *a* in seawater samples. Instead, a procedure incorporating three spectroscopic analytical methods is proposed. Apart from these alternatives, all other steps in the procedure are similar.

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Key words: chlorophyll, phaeopigment, spectrophotometry, fluorometry

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 microalgae 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 high performance liquid chromatography (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; Edler, 1979). However, an updated UNESCO publication, entitled "Phytoplankton pigments in oceanography: guidelines to modern methods", presents a very detailed review of 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.

The present paper draws attention to critical points of chlorophyll *a* determination and reviews recommendations concerning the use of this pigment as a biomass marker. It draws heavily on the UNESCO monograph (Jeffrey *et al.*, 1997) and several other sources referred to below for adequate QA/quality control (QC) practices. Finally, it proposes a procedure for the routine determination of chlorophyll *a* based on 90 % acetone extraction. More complex methods can be used, but they are out of the scope of this document.

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 phaeophytin (*a*, *b*, or *c*, respectively). Dephytylation produces chlorophyllides. Phaeophorbides are both dephytylated and magnesium-free. Phaeophytins and phaeophorbides constitute the phaeopigments.

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 1 shows the natural occurrence of the main pigments.

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

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

From Table 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 correct biomass indicator (the chlorophyll *a* derivative found in prochlorophytes is measured as chlorophyll *a*);
- in marine samples, phaeopigments result from grazing and are, consequently, inactive pigments, thus determination of phaeopigments *a* together with chlorophyll *a* may be useful;
- in turbid estuaries, higher plant detritus may contribute pigment concentrations (as phaeophytin *a* is 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, also have strong absorption maxima in the blue, spectrophotometric measurements are limited to the red absorption bands. Owing to 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 the 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. Phaeopigments *a* and *b* (no data are 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 phaeopigments 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

Fluorescence assays are one or two orders of magnitude more sensitive than spectrophotometric methods. This makes fluorometry more attractive for oligotrophic areas.

Excitation maxima for the pigments of the chlorophyll group are very close to their wavelengths of the blue absorption maximum, while emission maxima occur close to the strong red absorption maxima. As is the case for spectrophotometry, fluorometric methods for the measurement of chlorophyll *a*, with corrections for phaeopigments, have been developed. However, also in this case, chlorophyllide cannot be differentiated from chlorophyll. Multi-wavelength methods exist for the determination of the three chlorophylls and their phaeopigments, but these methods require high performance fluorometers and complicated calibration procedures and, consequently, they are not suitable for routine work.

3 CRITICAL VIEW OF THE PREPARATION OF PIGMENT EXTRACTS

3.1 Sampling and Sub-sampling

Water samples can be collected using any non-toxic sampling bottle (preferably opaque) or a pump (up to 50 m). Sub-samples should be collected preferably in opaque bottles, protected from heat and light, and filtered without delay. As zooplankton contains chlorophyll pigments, its presence may induce greater variability in the results. Pre-filtering through a nylon net of 100 µm to 150 µm mesh size eliminates most of the problems, but cannot be applied if large or colonial phytoplankton dominate. Subsequent removal of large zooplankton from the filter using forceps is suggested.

The sample volume will depend on the spectroscopic method chosen for the measurement. For fluorometry, about 0.25-litre samples are suitable for most purposes. For spectrophotometric measurements, at least one-litre samples are generally needed, but modern spectrophotometers with a resolution ten times better than that of the old generation (0.0001 AU) enable analysis of volumes comparable to those used with fluorometry.

3.2 Filtration

3.2.1 Filter material and pore size

Glass-fibre filters are widely used owing to their large filtration capacity and flow rate. In addition, they are inert towards extracting solvents. Membrane filters, particularly those composed of cellulose esters, have often been preferred for fluorometric determination since they dissolve in the extracting solvent. However, both types of filters may alter the analytical blank (see Section 4.2.4, below). The pore size should be small enough to prevent the loss of picoplankton (0.2–2 μm), especially in the open ocean. Comparison of filter retention yields concluded that use of the “depth” Whatman GF/F glass-fibre filter (0.7 μm nominal pore size) resulted in concentrations of chlorophyll *a* that were indistinguishable from those obtained using Millipore 0.45 μm membranes. GF/F filters even recovered >94 % of picoplanktonic chlorophyll *a*. Therefore, GF/F filters (or an equivalent) can be recommended for spectroscopic determination of chlorophyll *a*.

3.2.2 Magnesium carbonate

Magnesium carbonate has often been added on the filters, with the idea of preventing acid degradation of chlorophyll. Experiments show that it has no positive effect but, on the contrary, adsorbs the degradation pigments. Its use is now discouraged.

3.2.3 Vacuum pressure

To avoid damaging cells during filtration, vacuum pressure should be limited. While Jeffrey *et al.* (1997) mention keeping residual pressure under the filter not lower than 0.5 bar, most users recommend that it be kept higher than 0.7 bar.

3.3 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 $-18\text{ }^{\circ}\text{C}$ to $-20\text{ }^{\circ}\text{C}$ 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 SCOR Working Group 78 checked storage conditions for up to eleven months at various temperatures ($+22\text{ }^{\circ}\text{C}$, $-20\text{ }^{\circ}\text{C}$, $-90\text{ }^{\circ}\text{C}$, and $-196\text{ }^{\circ}\text{C}$). 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 $-20\text{ }^{\circ}\text{C}$) are above the eutectic point of the cell fluid, allowing biochemical reactions to proceed in the concentrated residual medium;
- degradation products of chlorophyll *a* were always dominated by chlorophyllide *a* and chlorophyll *a*-allomers, but after eleven months they accounted for only a quarter of the chlorophyll decline; the resulting decrease in total pigment suggested production of colourless degradation end-products;
- phaeopigments *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.

SCOR Working Group 78 recommended that the storage of pigments for periods up to one year be 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 one 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 can be recommended that filters for chlorophyll *a* and phaeopigments *a* be stored at −20 °C. Use of ultracold freezers allows extended storage times.

3.4 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.

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

- 1) extractability: the 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: the solvent should have low toxicity and flammability; few transfers.

SCOR Working Group 78 performed extensive trials (using HPLC for identifying the pigments) with four microalgae extracted using seven solvents (in particular, acetone, methanol, dimethylformamide, and dimethylsulfoxide) and various treatments (soaking, grinding, sonication). They showed that complex interactions between solvent and treatment precluded a simple step-by-step optimization of the process.

For a detailed HPLC analysis of the pigments, it appeared that sonication in dimethylformamide (DMF) should be regarded as a reference extraction method, since it gave the best pigment

recovery of all protocols, and fulfilled criteria 1) – 5), above. Sonication in methanol appeared to be a satisfactory alternative. Therefore, the SCOR Working Group recommended methanol for routine HPLC measurements of field samples. Acetone was significantly less effective on the algae tested.

In the context of the determination of chlorophyll *a* and phaeopigments 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 Working 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 phaeopigments *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 homogenizer 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).

4 SPECTROSCOPIC MEASUREMENTS

4.1 Choice of the Method

Spectrophotometry and fluorometry can be equally used for chlorophyll *a* and phaeopigments *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 $\sim 0.1 \text{ mg m}^{-3}$ 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 phaeopigment *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 phaeopigments by eliminating the magnesium ion from the tetrapyrrole complex. The drop in absorbance allows both chlorophyll *a* and phaeopigment *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 phaeopigments *a* can be calculated.

As stated above, none of the spectroscopic methods corrects for chlorophyllide *a*. However, from method comparisons (see Section 3.4, above) 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 phaeopigments, is certainly the major problem encountered in coastal and estuarine waters. Phaeopigment-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 phaeopigments *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 phaeopigment-correcting methods, it is suggested to perform occasional controls using the trichromatic spectrophotometric equations (or even multi-wavelength spectrofluorometry).

4.2 Spectrophotometry

4.2.1 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.

4.2.2 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. The 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, resulting in its possible interference in the measurement of chlorophyll *a* using monochromatic methods. When chlorophyll *b* is low, then:

$$[\text{trichromatic chlorophyll } a] \cong [\text{monochromatic chlorophyll } a] + 0.6 \times [\text{phaeopigments}].$$

4.2.3 Monochromatic (phaeopigment-correcting) method

Monochromatic methods are recommended for chlorophyll *a* in coastal and estuarine waters. The correction equations for phaeopigments 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 ($91.1 \text{ l g}^{-1} \text{ cm}^{-1}$) is about 4 % higher than the presently accepted coefficient of Jeffrey and Humphrey (1975), i.e., $87.7 \text{ l g}^{-1} \text{ cm}^{-1}$. In order to maintain consistency between the spectrophotometric and the fluorometric methods described below (all based on 90 % acetone extraction), 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.

4.2.4 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 $[H^+] = 0.003 \text{ mol l}^{-1}$ (Holm-Hansen and Riemann, 1978) to 0.006 mol l^{-1} (Nusch, 1980) in the extract, and a reaction time of 2–3 minutes. 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 phaeopigment may indicate such an eventuality).

4.3 Fluorometric Measurement

4.3.1 Fluorometer characteristics

Characteristics equivalent to those of the popular Turner Sequoia model 111, model 112, or Turner Designs model 10 filter fluorometers are suitable with red sensitive photomultiplier R446, light source F4T5BL (preferably to the standard F4T5B or F4T5D), excitation filter 430–450 nm (e.g., Corning CS-5–60), and emission filter 650–680 nm (e.g., Corning CS-2-64).

It is important to note that any new setting or change to the optical design of the fluorometer will involve re-calibrating the instrument.

Equations for chlorophyll *a* and phaeopigments have been proposed by Holm-Hansen *et al.* (1965).

4.3.2 Specific points of the fluorometric procedure

Blank problems are similar to those encountered in spectrophotometry. The filter fluorescence blank must be evaluated when solvent-soluble membranes are used.

Calibration of the fluorometer is easily done with a solution of pure chlorophyll *a*. The true concentration should be determined spectrophotometrically. Calibration with algal extracts can also be done provided that their chlorophyll *a* concentration is known. However, as the solution should be free of phaeopigments, chlorophyll *b* and preferably chlorophyll *c*, this calibration is less reliable and not recommended for routine use.

The acidification conditions and precautions defined for spectrophotometry are also valid for fluorometry.

5 PROCEDURE

General precautionary note

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

5.1 Sampling

- 1) Collect seawater samples using a non-toxic water sampler or a pump.
- 2) Withdraw sub-samples, preferably into opaque plastic bottles of known volume. The appropriate sample volume will depend on the spectroscopic method chosen (see Section 3.1, above).
- 3) Until filtration, protect sub-samples from warmth and light.

Precautionary notes

- *Pre-rinse the sample bottle with the sample before sub-sampling.*
- *As filtration of the entire sub-sample is recommended, the volume of the sample bottles should be checked before use. Mark each sample bottle to the desired volume. Another common procedure is to randomly select a number of bottles from the batch routinely used for collecting the samples and carefully measure their whole volume. The volume of sea water used for the measurements is then expressed as the measured average volume of the sampling bottles with the corresponding standard deviation.*
- *If pre-filtration through nylon net is used (see Section 3.1, above), it is preferable to conduct it on-line during sub-sampling.*

5.2 Filtration

- 1) Carry out filtration within one hour of collection of the sample.
- 2) Place a glass-fibre filter (Whatman GF/F type, 25 mm or 47 mm in diameter) on the filter holder, using forceps.
- 3) Gently mix the seawater sample.
- 4) Filter the sample at a residual pressure of 0.7 bar (maximum vacuum of 0.3 bar).
- 5) Gently suck the last part of the water sample through the filter.
- 6) Remove carefully any visible zooplankton from the filter with forceps.
- 7) Take off the filter from the holder. The filter is then folded once with the algae inside, blotted with absorbent paper to remove most of the water, and placed in a properly labelled clean container.

Precautionary notes

- *If the samples are not filtered immediately after collection, they can be kept for a few hours in cold and dark storage, in a refrigerator or an ice bath. The time between sampling and filtering must be as short as possible and no longer than 24 hours.*
- *Pre-filtration of the samples for removal of large zooplankters is not recommended without knowing the phytoplankton species composition of the samples, since large phytoplankton cells or chain-forming species could also be removed (see Section 3.1, above).*
- *Filtration should be carried out under subdued light.*
- *Magnesium carbonate should not be used as a filter aid.*
- *All handling of the filters should be done using forceps.*
- *The filtration time should be kept as short as possible. Clogging of the filters should be avoided.*
- *In turbid coastal regions, filtration of one sample may take a long time. In such cases, it is preferable to use smaller volumes on different filters and then to extract the filters together.*

5.3 Storage of the Filters

- 1) If the extraction is not to be carried out right after filtration, the filters should be immediately frozen (at least $-20\text{ }^{\circ}\text{C}$).
- 2) Keep the storage time as short as possible. Filters frozen at $-20\text{ }^{\circ}\text{C}$ can be kept for up to a 3–4 week period without a significant decrease in chlorophyll *a*. For longer periods, colder temperatures ($-70\text{ }^{\circ}\text{C}$) should be used.

Precautionary note

- *Every laboratory should check the freezing conditions by randomly running, from time to time, within their normal runs, a few duplicate samples against unstored filters extracted and analysed immediately after filtration.*

5.4 Pigment Extraction

- 1) Carry out extraction by grinding the filters in a few millilitres of 90 % acetone in a glass homogenizer with a motor-driven Teflon pestle, for 1 minute, in an ice bath and under subdued light.
- 2) After grinding, carefully transfer the extract to a stoppered and graduated centrifuge tube, rinse properly the glass homogenizer and the pestle with 90 % acetone and add the rinsing volumes to the centrifuge tube.
- 3) Make up 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, for instance, if the measurements are done in batches, they can be kept tightly stoppered in cold and dark storage for up to one hour.*
- *Working with solvents such as acetone poses a health risk. Therefore, all work should be carried out in well-ventilated conditions, preferably under a hood.*

5.5 Centrifugation

Immediately before measurement, mix thoroughly and centrifuge the extracts for 10 minutes at $500 \times g$, 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 stay on the tube wall, above the solvent surface. These may fall into the extract during transfer to the optical cell. In such cases, tubes are centrifuged twice: after a first brief centrifugation, the tubes are gently swirled to collect remaining fibres and then centrifuged again.*

5.6 Spectroscopic Measurement

Within their respective application fields, all three methods mentioned below are equally satisfactory.

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 chlorophylls *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.

5.6.1 Spectrophotometry: Trichromatic method

- 1) Use a spectrophotometer of 2 nm maximum bandwidth and stoppered cuvettes with a path length of up to 5 cm (such a path length is required in most instances for satisfactory measurements).
- 2) Transfer the sample extracts from the centrifuge tubes to the cuvette by careful pipetting.
- 3) Measure the absorbance of the sample extract at 750 nm, 664 nm, 647 nm, and 630 nm against a 90 % acetone blank.

- 4) Calculate the concentrations of chlorophylls *a*, *b*, and *c*, according to the equations of Jeffrey and Humphrey (1975):

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

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

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

where

L = Cuvette light-path in centimetres

V_e = Extraction volume in millilitres

V_f = Filtered volume in litres

Concentrations are in the unit mg m⁻³.

5.6.2 Spectrophotometry: Monochromatic method with acidification

- 1) Use a spectrophotometer of 2 nm maximum bandwidth and stoppered cuvettes with a path length of up to 5 cm (such a path length is required in most instances for satisfactory measurements).
- 2) Transfer the sample extracts from the centrifuge tubes to the cuvette by careful pipetting.
- 3) Measure the absorbance of the sample extract at 750 nm (E_{750o}) and 665 nm (E_{665o}) against a 90 % acetone blank.
- 4) Add 0.2 ml 1 % v/v hydrochloric acid in the cuvette and mix.
- 5) Wait 2–5 minutes (but not more).
- 6) Measure again the absorbance at 750 nm (E_{750a}) and 665 nm (E_{665a}) against a 90 % acetone blank.
- 7) Calculate the concentrations of chlorophyll *a* and phaeopigments *a* according to the equations of Lorenzen (1967):

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

$$\text{Phaeopigments } a = 11.4 K ((R (E_{665a} - E_{750a})) - (E_{665o} - E_{750o})) V_e/L V_f$$

where

L = Cuvette light-path in centimetres

V_e = Extraction volume in millilitres

V_f = Filtered volume in litres

R = Maximum absorbance ratio of E_{665o}/E_{665a} in the absence of phaeopigments = 1.7

K = R/(R - 1) = 2.43

Concentrations are in the unit mg m⁻³.

Precautionary notes

- *Pouring from the tube into the cuvette is not advisable since it can transfer glass fibres.*

- The use of closed optical cuvettes reduces cooling due to evaporation and, hence, absorbance variability due to the Schlieren effect.
- Check that optical cuvettes are filled high enough so that the entire light beam passes below the extract surface.
- A very important point in the acidification method is to ensure that any acid residue has been thoroughly rinsed from the optical cuvette before the next sample is transferred. If not, chlorophyll *a* would be degraded and underestimated. A suspect excess of phaeopigments should alert to such an eventuality.
- It is recommended to check the delay for completeness of reaction after acidification (stable absorbance).

5.6.3 Fluorometry

- 1) Use a fluorometer equipped as described in Section 4.3.1, above. Spectrofluorometers can also be used instead of filter fluorometers. The excitation wavelength should be 430 nm (10 nm bandwidth) and the emission wavelength 680 nm (10 nm bandwidth).
- 2) Calibrate the fluorometer using a commercial solution of pure chlorophyll *a* (Sigma UK, Aldrich UK, Fluke Chemie Switzerland, or other manufacturer). The concentration of that solution (in 90 % acetone) is determined spectrophotometrically using an extinction coefficient of 87.67 l g⁻¹ cm⁻¹ (Jeffrey and Humphrey, 1975) at 664 nm against a 90 % acetone blank. The calibration should be carried out with different chlorophyll *a* concentrations covering the entire linear range for the relationship between chlorophyll concentration and instrument output. Also, the maximum acid ratio must be determined by measuring the fluorescence of the standard before and after acidification (see Section 5.6.2, above). Ready-to-use primary chlorophyll *a* standards are now available from Denmark (DKI, Copenhagen) and the USA (Turner Designs Inc.).
- 3) Transfer the sample extracts from the centrifuge tubes to the fluorometer cuvette by careful pipetting.
- 4) Measure the fluorescence of the sample extract against a 90 % acetone blank.
- 5) Add 0.2 ml 1 % v/v hydrochloric acid in the cuvette and mix.
- 6) Wait 2–5 minutes (but not more).
- 7) Measure again the fluorescence of the sample extract against a 90 % acetone blank.
- 8) Calculate the concentrations of chlorophyll *a* and phaeopigments *a* according to the equations of Holm-Hansen *et al.* (1965):

$$\text{Chlorophyll } a = K (F_m / (F_m - 1)) \times V_e \times (F_o - F_a) / V_f$$

$$\text{Phaeopigments } a = K (F_m / (F_m - 1)) \times V_e \times ((F_m \times F_a) - F_o) / V_f$$

where

- K = calibration coefficient = µg Chl *a* per ml 90 % acetone per instrument fluorescence unit
- F_m = maximum acid ratio (F_o/F_a) of pure chlorophyll *a* standard
- F_o = sample fluorescence before acidification

- F_a = sample fluorescence after acidification
- V_e = extraction volume in millilitres
- V_f = filtered volume in litres

Concentrations are in the unit mg m^{-3} .

Precautionary notes

- *In fluorometry, if a standard lamp is used, the presence of chlorophyll b may result in significant overestimation of phaeopigments. Hence, an underestimation of chlorophyll a may result. In order to validate data obtained with phaeopigment-correcting methods, occasional control of the presence of chlorophyll b using the trichromatic spectrophotometric equations should be performed.*
- *Any new setting or change of the optical design of the fluorometer implies re-calibrating the instrument. The same applies when moving the instrument.*
- *In fluorometry, it is very important to keep the same temperature for both the calibration and the measurement of the samples. The use of a water bath covered from direct light is recommended for this purpose. The extracts should also be measured at a fixed time after being introduced in the fluorometer. If not, the heat in the instrument will cause the sample temperature to increase, thereby decreasing the fluorescence.*
- *The cuvettes should always be placed in the same position in the fluorometer to avoid scratching the cuvette wall, which could affect the readings.*
- *When calibrating the fluorometer, care must be taken in checking the chlorophyll solution for the presence of degradation products. This can be easily done by scanning the solution with a scanning spectrophotometer or by checking the absorbance of the solution at 665 nm both before and after adding hydrochloric acid. The ratio between the two measurements should be about 1.7.*
- *A secondary standard should be measured at regular intervals during each measurement run in order to keep track of the fluorometer stability. Any statistically significant change in the secondary standard will require a re-calibration of the fluorometer. Turner Designs Inc. (USA) can provide a solid secondary standard that simplifies this task.*
- *A very important point in the acidification method is to ensure that any acid residue has been thoroughly removed from the cuvette before the next sample is transferred; if not, chlorophyll a would be degraded and underestimated. A suspect excess of phaeopigments should alert to such an eventuality.*
- *It is recommended to check the delay for completeness of reaction after acidification (stable absorbance).*

6 SUMMARY OF OPERATIONAL CONDITIONS

OPERATION	MATERIAL	CONDITIONS
Sampling	Opaque sampling bottle	
Sub-sampling	Opaque bottle	optional: pre-filtration 100–150 µm
Temporary storage of water		protected from heat and light; maximum one hour
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
Storage of filter	Deep freezer	–20 °C; maximum two months
Extraction of the filter	Solvent: 90 % acetone; homogenizer with Teflon pestle	chill; subdued light; grinding: ~1 minute; until analysis (within 1 hour), keep the chilled extract in darkness
Centrifugation or filtration of the extract	Graduated, stoppered tubes	
Measurement	- spectrophotometer (bandwidth ≤ 2 nm) - fluorometer - both	Lorenzen (1967) Holm-Hansen <i>et al.</i> (1965); calibration with pure chlorophyll <i>a</i> test (spectrophotometry): Jeffrey and Humphrey (1975)

7 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 phaeopigments 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, phaeopigments should be at very low levels. Excessive concentrations of phaeopigments relative to chlorophyll could indicate potential procedural errors (note that storage by freezing does not generate phaeopigments). In the acidification methods, for instance, errors can occur from insufficient or excessive acidification or from poor rinsing of residual acid from the 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 phaeopigment concentrations in the samples are suspected. It is also highly recommended that each laboratory perform 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 develop 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 documentation of the comparability of their method against the procedure described here.

8 ALTERNATIVE PROCEDURES

Extraction by soaking in 96 % ethanol instead of 90 % acetone has been recommended by HELCOM (1998). Unfortunately few papers on this procedure have been published in the international literature, and ethanol extraction was not tested by SCOR Working Group 78. However, because 90 % acetone may poorly extract chlorophyll *a* in some algal species frequently growing in the Baltic Sea, 90–96 % ethanol may be used as an alternative solvent, with reference to the work of Nusch (1980) and Jespersen and Christoffersen (1987). The protocol for monochromatic spectrophotometric measurement with acidification (see Section 5.6.2) can be applied directly to ethanol extraction. The only change with regard to 90 % acetone extraction 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 the 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 phaeopigments 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. 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.

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