Determination of Chlorophyll in Seawater

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I Summary

Chlorophyll *a* is the primary pigment responsible for photosynthesis and is found in all photosynthetic algae and higher plants. Chlorophyll *a* has been used as an indicator of phytoplankton biomass for a number of decades, and is a core measurement for eutrophication monitoring in many international assessments.

Traditionally, chlorophyll *a* was determined using either spectroscopic or fluorometric techniques. High performance liquid chromatography (HPLC) methods for chlorophyll *a* determination were first developed in the 1980s. Remote sensing devices, such as satellites or *in situ* devices, are also becoming increasingly used. In addition to different detection methods, different extraction solvents and extraction methods are also used. There is no single standard analysis technique for chlorophyll *a*, and the choice of method depends on purpose, cost and preference.

Intercomparison exercises to compare the performance of different chlorophyll *a* methods have revealed a high degree of variability. It is therefore important to have robust quality control procedures in place and to maintain a metadata record.

This updated guideline replaces ICES Techniques in Environmental Marine Sciences Vol. 30 (Aminot and Rey, 2001) and draws attention to critical points and issues associated with the different analytical techniques used within the ICES region.

1 Introduction

Phytoplankton are small, single-celled organisms that form the base of the marine foodweb. These organisms contain compounds known as pigments, which are used in photosynthesis. Roy *et al*. (2011) listed 74 pigments across a number of microalgal classes. These pigments have a variety of functions, and some are unique to different microalgal classes. Chlorophyll is the principal pigment used in photosynthesis. It exists in many different forms (*a*, *b*, *c*, *d*, *e* and *f*), but chlorophyll *a* is the primary pigment responsible for photosynthesis, and is found in all photosynthetic algae and higher plants (Jeffrey *et al.,* 1997). The other chlorophylls are accessory pigments in that they transfer any energy they absorb to the primary chlorophyll *a* instead of directly participating in the photosynthetic process. Chlorophyll has been used as a biomass indicator for aquatic microalgae, and is probably the most frequently measured biochemical parameter in oceanography (Jeffrey *et al*., 1997).

The determination of algal pigments relies mainly on the pigments' spectroscopic characteristics. In discrete samples, photosynthetic pigments can be measured either by traditional non-separative spectroscopic methods, or, after chromatographic separation, using high performance liquid chromatography (HPLC) coupled to various detectors such as an Ultra Violet (UV)/Visible detector, Mass Spectrometer (MS), or Diode Array Detector (DAD). In addition, detection by remote sensing devices, such as satellites or *in situ* sensors, are increasingly used. The measurements of these remote sensors are based on the relationship between chlorophyll concentration and the optical absorption/fluorescence properties of living cells at wavelengths matching chlorophyll adsorption/fluorescence *in vivo*. These devices have been used for a number of years, but still require further development, especially in regard to validation with discrete chemical measurements of samples at a frequency appropriate to local conditions. The comparability of such datasets was reviewed as part of EU funded projects, such as the chlorophyll case study in the Joint Monitoring Programme for the North Sea and Celtic Sea (JMP NS/CS) project (Baretta-Bekker *et al*., 2015).

Chlorophyll determination is complex but non-specific, making the establishment of traceability and quality controls difficult. Quality Assurance (QA) relies strongly on the rigorous application, at each step, of recommended protocols that have been checked by specialists, and a good appraisal of the validity limits of the method. Specific publications from international bodies have reviewed the procedures, and/or proposed guidelines and recommendations [Science Committee on oceanic Research (SCOR)-UNESCO, 1966; Edler, 1979). 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). That book contains methodological developments and recommendations for pigment studies. A follow-up book (Roy *et al*., 2011) covers new algal classes and pigments discovered since the publication of the work by SCOR Working Group 78, and documents the advances in analytical techniques developed for characterizing and quantifying them.

The aim of this report is to provide advice to those interested in undertaking chlorophyll analysis in seawater samples. This report does not to provide a detailed background, which can already be found in publications such as Jeffrey *et al*. (1997) and Roy *et al.* (2011). This report does not recommend best approaches or best use, because different regions have their own requirements. This guideline updates and replaces the previously published ICES Techniques in Marine Environmental Sciences Vol. 30 (Aminot and Rey, 2001), drawing attention to critical points and issues associated with the different analytical techniques used within the ICES region. Finally, this report draws heavily on the UNESCO monograph (Jeffrey *et al*., 1997) and

several other sources for adequate QA/Quality Control (QC) practices (see references). More complex methods can be used, but they are out of the scope of this document.

2 Sample and sample handling

2.1 Sampling

Water samples can be collected using a number of techniques, depending on the site and operating platform. The depth of sampling will depend on project requirements, but for monitoring purposes, samples should be collected from the photic zone, where phytoplankton are found. Suitable samplers include Niskin bottles or equivalent (either operated manually or mounted on Rosette samplers), Lund tubes, flow through systems, and Knudsen reverser bottles. Chlorophyll pigments are susceptible to degradation in sunlight, so sampling devices should be opaque and made of a non-toxic material. Subsamples should preferably be collected in opaque bottles, protected from heat and light, and filtered as soon as possible (see Section 2.2).

Zooplankton may contain chlorophyll pigments, and their presence can thus induce variability in the results. Pre-filtering through a nylon net with a 100–150 μm mesh size can eliminate the majority of this problem, but it cannot be applied if large or colonial phytoplankton dominate. In this latter case, subsequent removal of large zooplankton from the filter using flat tipped forceps is suggested.

The sample volume should take into account the method of detection, including the limit and range of detection, to ensure there is a sufficient pigment concentration for reliable measurement, and to avoid the need for additional dilution during analysis. The ideal sample volume should be determined as part of the method validation process. To account for variability in sampling and potential issues with the analysis, duplicate samples should be collected as a minimum, especially when the filtration volume is small.

2.2 Filtration

All photosynthetic pigments are light and temperature sensitive. Therefore, to prevent chlorophyll degradation, it is recommended to filter in subdued light, and in the absence of direct sunlight and excessive heat. Ideally, the sample should be filtered immediately after collection, as algal populations can change quickly. If this is not possible due to practical constraints, the sample should be stored chilled and in the dark, and filtered as soon as possible, ideally within 4 h. Prior to filtration, the sample should be thoroughly but gently mixed to resuspend any large cells that may have settled.

The duration between sample collection and filtration should be considered during the method validation process.

2.2.1 Filtration apparatus

Diagrams of the filtration apparatus can be found in Jeffrey *et al*. (1997). The apparatus consists of (i) a vacuum pump that connects to a filter funnel, (ii) a glass, plastic, or metal fritted support base, and (iii) a vessel for collecting the filtrate. Other required equipment are filters, flat-tipped forceps, a measuring cylinder, and a rinse bottle. A manifold filtration system is strongly recommended for filtering multiple samples.

2.2.2 Equipment cleaning procedure

Filtration equipment should be clean prior to use. This can be achieved by (i) rinsing the fritted support base with 0.1 M hydrochloric acid after use, which will remove biological material; and

(ii) thoroughly rinsing with distilled/deionised water to remove the acid, which could degrade the chlorophyll present in the next sample.

In addition, the filtration apparatus should be washed down with distilled/deionised water between samples.

2.2.3 Filters

Two types of filter are commonly used: membrane and glass-fibre filters.

Membrane filters are widely used for spectrophotometric and fluorometric methods, and when undertaking size fractionated chlorophyll measurements of phytoplankton size classes [pico- (< 2 μm), nano- (2–20 μm), and micro-phytoplankton (> 20 μm); National Oceanographic and Atmospheric Administration (NOAA, US) Great lakes Environmental Research Laboratory; Knefelkamp, 2007). However, they are not suitable for chlorophyll analysis using chromatographic methods. Compared to glassfibre filters, membrane filters have lower flow rates and filtration capacity, are more expensive, may dissolve in the extraction solvent resulting in the blockage of HPLC columns, may cause some cells to break down, and may contain extractable dyes which elute near some of the pigments detected by HPLC.

Glassfibre filters are extensively used for chromatographic methods. Glassfibre filters retain particles on their surface and throughout the internal spaces of the filter. The most widely used glassfibre filters are the GF/F (0.7 μm nominal retention rate (NRR); Whatman, Merck KGaA, Germany), GF/C (1.2 μm NRR; Whatman), and the Gelman A/E (1 μm NRR; Gelman, Hach, CO, USA). The retention characteristics of various glassfibre filters have been evaluated (Jeffrey *et al*., 1997), and it was found that GF/F filters (0.7 μm NRR) consistently yielded the highest concentrations of chlorophyll *a*, with > 94% of picoplankton being recovered.

Filters may become contaminated during transport and storage. This should be accounted for in the analytical process, by undertaking a procedural blank (see Section 6).

The pore size or NRR should be sufficiently small to prevent the loss of picoplankton (0.2–2 μm), especially in the open ocean. The choice of filter type should consider local conditions, and be accounted for during the method validation process.

2.2.4 Vacuum pressure

To avoid damaging cells during filtration, vacuum pressure should be limited to around 500 hPa (380 mmHg; Jeffrey *et al*., 1997). Vacuum pressures greater than 700 hPa (530 mmHg) are to be avoided.

2.2.5 Filtration process

To prevent contamination, filters should only be handled with forceps. It is recommended that filters are only placed on the apparatus when they are required. If this is not possible due to practical reasons, filters can be placed on the apparatus in advance (maximum 4 h) and protected from dust with a cover.

The volume of sample to be filtered should be determined on an individual basis, and will depend on the detection method employed and on the amount of algae in the sample, which, in turn, will mainly depend on the location of the sampling station, the depth from which the sample was obtained, the time of year, and the amount of extraction solvent used. The volume of seawater filtered must be recorded for calculation purposes. To ensure planktonic material is not stuck on the filtration apparatus, it may be necessary to rinse the sides down with either pre-filtered seawater or synthetic seawater of an appropriate salinity. The use of deionised water for this purpose should be avoided as planktonic cells may rupture.

After filtration, the filter should be removed immediately from the filtration apparatus, using forceps, and folded at least once with the algae inside. The folded filter is then blotted on absorbent paper to remove excess water, and wrapped in aluminium foil and/or placed in a screw top test tube or cryo-vial. In some instances, the sample is placed directly into the extraction solvent, to prevent cell breakdown due to contact with oxygen. If prolonged storage is required of sample filters placed directly into the extraction solvent, the filter should be removed after the extraction period. In all instances the sample should be stored appropriately (see Section 2.3), and protected from light and heat exposure during transport and storage.

2.3 Sample preservation and storage

Storage of filtered samples in either liquid nitrogen (−196 °C) or ultra-cold freezers (−80 °C) is widely used. Pigments remain stable for periods of up to 1 year at both these temperatures (Roy *et al*., 2011). Both liquid nitrogen and ultra-cold freezers are suitable for the storage of samples which have been flash-frozen immediately after filtration. Flash-freezing of samples immediately following filtration prevents the alteration of pigments due to rapid xanthophyll cycling (Roy *et al*., 2011).

Dry ice (−78 °C) can be used to store samples for < 1 month when access to liquid nitrogen or ultra-cold freezers is limited. Care must be taken to minimize exposure to carbon dioxide gas, which is produced as dry ice evaporates and creates acidic conditions which can rapidly transform and/or degrade pigments (Roy *et al*., 2011).

3 Analytical methods and difficulties with chlorophyll determination

There is no single standard extraction or detection technique recommended for the determination of chlorophyll and marine algal pigments. The revised OSPAR Joint Assessment Monitoring Programme (JAMP) eutrophication monitoring guidelines (OSPAR, 2012) lists the standard procedures for chlorophyll analysis from Strickland and Parsons (1968), UNESCO (1994), HELCOM (1988), ISO 10260 (1992), and Wright *et al*. (1991), and state the importance of recording the method used when reporting data.

3.1 Extraction techniques and solvents

Standard procedures use a range of techniques for extracting chlorophylls from filters, including soaking, grinding, or sonicating the filter in the presence of a solvent such as acetone, ethanol, dimethyl sulfoxide (DMSO), or methanol. Extraction methods and solvents are comprehensively described in Jeffrey *et al.* (1997) and Roy *et al.* (2011), and only a synthesis will be presented here.

The choice of solvent for extraction depends on a number of factors, including the solvents ability to extract pigments from algae, the stability of pigments in that solvent, and potential health and safety issues. For the latter reason alone, DMSO is not recommended as an extraction solvent. Recent studies undertaken by QUASIMEME (Quality assurance of information for marine environmental monitoring in Europe) considered the choice of solvent and extraction process for a limited number of cell cultures and natural seawater samples (see details in Annex 1). They concluded that chlorophyll pigment recovery was poor for some individual algae species when extracted with acetone, and increased when extracted with ethanol. However, this conclusion did not translate to the natural seawater samples tested, where there was no difference in chlorophyll pigment recovery between the two solvents. This indicates that an understanding of the algal species present in a sample is advantageous when selecting the solvent to use for extraction. It should also be noted that for sample storage, acetone provides better chlorophyll stability.

The choice of extraction mechanism, and the duration of the extraction, should also be considered. The recent QUASIMEME study (Annex 1) noted that if the solvent volume was < 10 ml and extraction times were < 1 min significant differences in results were observed. Any extraction process that results in an increase in sample temperature can result in degradation of chlorophyll pigments. Smith *et al*. (2007) found that grinding processes, such as sonication or homogenization, created heat that could result in the degradation of chlorophylls, and gave inconsistent results when compared with extraction by soaking alone. This problem can be reduced by appropriate cooling, such as undertaking the process with the sample tube/cryovial placed in an ice bucket. QUASIMEME reported that samples extracted by sonication alone resulted in an underestimation of chlorophyll concentrations, but it is not clear whether the participating laboratories used mild bath sonication or the more vigorous high-powered tip sonication. Smith *et al*. (2010) found that the most efficient extraction procedure for HPLC analysis was a combination of vigorous tip-sonication followed by overnight soaking.

Following extraction by grinding or sonication, the sample supernatant should be clarified, either by centrifugation or filtering, to ensure fibres are not transferred into the optical cuvette or HPLC sampler vial. If the sample is centrifuged, it should be undertaken in a refrigerated centrifuge to prevent sample degradation prior to analysis. If the sample is filtered, suitable filters include 0.45 μm PTFE syringe filters.

3.2 Procedural blank

The extraction and analysis of a procedural blank corrects for background absorption and/or turbidity produced by the filters and any particulate matter and should be treated as described in Section 6. The procedural blank should be checked for stability over the time required for measuring the sample.

3.3 Interfering chlorophylls and degradation products

Chlorophyll exists in many different forms (*a*, *b*, *c*, *d*, *e,* and *f*), with at least two known subtypes of chlorophyll *a*, two of chlorophyll *b*, and eight of chlorophyll *c* (Zapata *et al.*, 2000). All photosynthetic algae and higher plants contain chlorophyll *a* as the principal pigment. As a result, chlorophyll *a* has been the primary pigment of interest in marine monitoring programmes, and has been used as a proxy to estimate phytoplankton biomass. Table 3.1 shows the natural occurrence of the main pigments.

Table 3.1. Natural occurrence of the main pigments of the chlorophyll group.

Pigment	Occurrence
Chlorophyll *a*	All photosynthetic algae and higher plants
Chlorophyll *b*	Higher plants, green algae, and prochlorophytes
Chlorophyll *c*	Chromophyte algae and brown seaweeds
Chlorophyllide *a*	Biologically aged tissue, damaged centric diatoms, and zooplankton faecal pellets
Chlorophyllide *b*	Biologically aged tissue and zooplankton faecal pellets
Phaeophytin *a*	Photosynthetic reaction centres of higher plants and plant and algal detritus
Phaeophytin *b*	Terrestrial plant detritus, and protozoan faecal pellets
Phaeophorbide *a*	Marine detritus, zooplankton, and protozoan faecal pellets
Phaeophorbide *b*	Terrestrial plant detritus, and protozoan faecal pellets

Chlorophyll may be altered and degraded by chemical, photochemical and biological processes (Jeffrey *et al*., 1997). Under chemical or photochemical conditions, chlorophylls may be altered by processes such as oxidation, dephytylation, and halogenation.

Several pigments and degradation products may be found simultaneously in a sample. An analysis of the detailed pigment composition can significantly improve knowledge of phytoplankton composition and physiology. This analysis is not necessary for routine monitoring using chlorophyll *a* as a biomass indicator, but it is recommended that laboratories consider interference and degradation when developing methods and undertaking routine analysis. It is also recommended that laboratories are clear regarding what type of chlorophyll data they are reporting, and follow the NASA Technical Memorandum 2012-217503 (Hooker *et al*, 2012) which defines total chlorophyll *a*, *b*, and *c,* and total chlorophyll as follows:

- Total chlorophyll a = chlorophyllide *a* + divinyl chlorophyll *a* + chlorophyll *a*
- Total chlorophyll b = divinyl chlorophyll *b* + chlorophyll *b*
- Total chlorophyll *c* = chlorophyll *c1* + chlorophyll *c2* + chlorophyll *c3*
- Total chlorophyll = total chlorophyll *a* + total chlorophyll *b* + total chlorophyll *c*

4 Instrumental analysis

As with the extraction process, there is no single standard analysis technique recommended for the determination of chlorophyll and algal pigments. The choice of instrument used depends on the purpose, cost, and the laboratories preference. Historically, spectrophotometry and fluorometry were the instruments of choice for chlorophyll analysis due to their relatively low cost, ease of use, and high sample throughput. However, both are limited by interferences from compounds which can be detected at the same wavelength as chlorophyll *a* (Arar, 1994). More recently, the use of HPLC, with either an UV/visible detector, diode array detector (DAD) or mass spectrometry (MS) detection, have become more popular. HPLC methods have the advantage over spectrophotometry and fluorometry methods in that interfering pigments are separated from chlorophyll *a*, resulting in an accurate determination of concentration (Smith *et al*., 2010). HPLC methods are also more sensitive, robust, and reliable, and can provide valuable information about the contribution of different functional groups to the biomass of the phytoplankton community. However, HPLC analysis can be much more time consuming, requires experienced analytical staff, is expensive relative to fluorometric analysis, and may not be necessary for routine monitoring of biomass.

4.1 Spectroscopic measurements overview

Spectrophotometry and fluorometry can both be used for the detection of chlorophyll and any interfering magnesium free chlorophyll derivatives (phaeophytins and phaeophorbides, collectively known as phaeopigments). Both spectroscopic and fluorometric methods are suitable when using cholophyll *a* as a biomass indicator of marine microalgae, with fluorometric methods being the more sensitive (Yentsch *et al*., 1963; Holm-Hansen *et al*., 1965; Strickland and Parsons, 1968). Fluorometry is the preferred method when the available volume of water is insufficient for a reliable absorbance measurement. It should be noted that, unlike spectrophotometers, fluorometers have to be calibrated with chlorophyll standards.

There are two main 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. They can potentially overestimate chlorophyll as a consequence of spectroscopic interference (Jeffrey *et al*., 1997). Absorbance must be measured at the three maximum wavelengths of the three chlorophylls, plus a wavelength where chlorophylls do not absorb. A set of three equations is then used to calculate the chlorophyll concentrations (Section 4.1.1.2). Monochromatic methods have been developed to correct chlorophyll *a* for phaeopigment *a*. Absorbances are measured at the red maximum 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 after acidification allows both chlorophyll *a* and phaeopigment *a* to be calculated.

The classic fluorometric method for routine determination of chlorophyll concentrations is similar to the spectrophotometric monochromatic method. The fluorescence of the extract is measured at a unique wavelength before and after acidification, allowing the calculation of chlorophyll *a* and phaeopigment *a* concentrations.

Acidification as means of correcting for phaeopigments is no longer recommended for data which are to be reported as part of the OSPAR JAMP monitoring process (OSPAR, 2012), although it still included in the HELCOM guidelines for measuring chlorophyll (HELCOM, 2019). Therefore, it is important to note the requirements for the region before commencing

analysis. The presence after acidification of chlorophylls *b* and *c* and, especially, their derivatives, can significantly interfere with chlorophyll *a* measurements, depending on the amount present. If chlorophyll *b* is present in the sample, it will result in an underestimation of chlorophyll *a* and an overestimation of phaeophytin *a*. The degree of interference depends upon the ratio of chlorophyll *a* to chlorophyll *b*. The presence of chlorophyll *c* also causes the underestimation of phaeophytin *a*, although the effects are not as severe as for chlorophyll *b* (Arar and Collins, 1997). The acidification process is not covered in this report.

None of the spectroscopic methods correct for chlorophyllide *a*.

Overestimation of chlorophyll by trichromatic equations due to the presence of phaeopigments, is a particular problem in coastal and estuarine waters.

4.1.1 Specrophotometry

4.1.1.1 Instrumental characteristics

The determination of chlorophyll relies on absolute absorbance values, with no calibration required for high-performance spectrophotometers. Wavelength accuracy and sensitivity should be checked using certified reference standards, or as part of an annual instrument service/calibration. Individual chlorophyll extracts should be measured using light absorption at 663–665 nm with the bandwidth ≤ 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 due to converging beams.

4.1.1.2 Trichromatic method

Four sets of trichromatic equations have been published, following improvements in the values of maximum absorption wavelengths and extinction coefficients. Published extinction coefficients were determined for pigments dissolved in 90% acetone, and concentrations are in mg m^{-3}. 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; equations 1–3) are the only ones recommended for calculating the concentration of the three chlorophylls in mixed phytoplankton populations.

$$\text{Chlorophyll } a \ (\mu g\ ml^{-1}) = [11.85(E_{664} - E_{750}) - 1.54(E_{647} - E_{750}) - 0.08(E_{630} - E_{750})]\ V_e / LV_f \quad (1)$$

$$\text{Chlorophyll } b \ (\mu g\ ml^{-1}) = [-5.43(E_{664} - E_{750}) + 21.03(E_{647} - E_{750}) - 2.66(E_{630} - E_{750})]\ V_e / LV_f \quad (2)$$

$$\text{Chlorophyll } c1 + c2 \ (\mu g\ ml^{-1}) = (-1.67(E_{664} - E_{750}) - 7.60(E_{647} - E_{750}) + 24.52(E_{630} - E_{750}))\ V_e / LV_f \quad (3)$$

Where:

L: Cuvette light-path in centimetres.

V_e : Extraction volume in millilitres.

V_f : Filtered volume in litres.

E_{xxx} : Absorbance at wavelength xxx in nm.

Note: Concentrations are obtained in μg ml^{-1}, while coefficients are expressed in mg m^{-3}.

4.1.2 Monochromatic method

The original monochromatic method, described by Lorenzen (1967), used an extinction coefficient of 91.11 g^{-1} cm^{-1} in 90% acetone, which results in chlorophyll concentrations approximately 4% lower than those calculated by the recommended extinction coefficient for the trichromatic method (Turner Designs application note 998-6000). The revised Lorenzen equations are accurate if the unknown sample contains phaeophytin *a* as the only degradation product of chlorophyll *a* (which is seldom the case). The calculated weight of phaeopigments can be overestimated by a factor of 1.51 if the unknown sample contains phaeophorbide *a* rather than phaeophytin *a* (Jeffrey *et al*., 1997). Phaeophytin *a* is found in zooplankton faecal pellets and sediments, whereas phaeophorbide *a* is found in protozoan faecal pellets. Any chlorophyll *b* present in the sample will also be degraded by the acidification process, and will be incorrectly detected as a phaeopigment. For most samples from the euphotic zone, the mass of chlorophyll *a* greatly outweighs that of phaeopigments, and calculated concentrations of chlorophyll *a* can be considered accurate, provided caution is exercised in judging the accuracy of the phaeopigment concentration.

4.1.2.1 Specific points of the spectrophotometric procedure

The extraction and analysis of the procedural blank corrects for background absorption. It is important to note that the extracted sample should be clarified by secondary filtration or centrifugation to remove particles of filter material present which may scatter light and affect absorption. The procedural blank should be checked for stability over the time required for measuring the sample.

4.1.3 Fluorometry

Fluorescence assays are one to two orders of magnitude more sensitive than spectrophotometric methods. This makes fluorometry particularly attractive when analysing samples in oligotrophic areas.

Fluorometric measurements are typically based on the methods of Holm *et al*. (1965) and Lorenzen (1967), using excitation in the range 430–485 nm, and emission in the range 630–685 nm, dependant on the extraction solvent used and the chlorophylls present. Some instruments, such as the fluorometers from Turner Designs Inc. (CA, USA), provide optical kits that have been designed specifically for the analysis of chlorophyll in marine waters. When using these optical kits, it is important that the user is aware of the wavelength of the filters, as these are designed for optimal measurement of different chlorophyll pigments.

An alternative to traditional fluorometers is the Pulse-Amplitude-Modulation (PAM) fluorometer. PAM fluorometers work by emitting low intensity fluorescence light pulses in short repetitive bursts (μS-range) that are synchronized to an amplifier, permitting quantum yield determinations. To date, these fluorometers have not been widely utilized in the marine environment, and will not be discussed further in this report.

4.1.3.1 Fluorometer characteristics and calculations

It is important to note that when the fluorometer settings, lamp, filters, or optical kit are changed the instrument must be recalibrated.

Equations for chlorophyll *a* and phaeopigments *a* have been proposed by Holm-Hansen *et al.* (1965). As the acidification procedure is no longer recommended, the equation for calculating chlorophyll *a* concentration is simplified to:

$$\text{Chlorophyll } a \text{ concentration} = K \times Ve \times F / V_f \quad (4)$$

Where:

K: Calibration coefficient = μg Chlorophyll *a* per ml 90% acetone and per instrument fluorescence unit.

F: Sample fluorescence.

V_e : Extraction volume in millilitres.

V_f : Filtered volume in litres.

Note: Concentrations are obtained in μg L^{-1}, equivalent to and expressed in mg m^{-3}.

4.1.3.2 Specific points of note for the fluorometric procedure

Problems with the procedural blank are similar to those encountered in spectrophotometry.

Quenching effects may be observed at high chlorophyll concentrations or in the presence of carotenoids. To prevent this, samples with high chlorophyll concentrations should be diluted.

Due to lamp deterioration over time, it is recommended that the analysis of an instruments specific solid secondary standard, along with a Laboratory Reference Material (LRM) sample, is performed on each day of analysis (see Section 6).

Instruments should be calibrated after each maintenance procedure occurs, such as replacing a lamp, or at least every six months. Chlorophyll *a* standards are commercially available from suppliers such as Merck Life Science Limited (UK) and DHI (Denmark). Chlorophyll *a* standards from spinach may contain high concentrations of chlorophyll *b*, and are no longer recommended for calibration purposes. Standards sourced from algae are preferred, such as chlorophyll *a* from *Anacystis nidulans* algae (Merck Life Science Limited, UK).

The instrument must be calibrated using known concentrations of a chlorophyll *a* standard, determined by spectrophotometry, as the weights provided on the standard vial may not be accurate. The calibration should be carried out at concentrations covering the range found naturally in the waters to be analysed. It is also possible to calibrate with algal extracts if the 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.

Fluorometric determinations are temperature dependent, and, therefore, calibration and analysis of samples, blanks, and LRMs should be undertaken in temperature-controlled laboratory conditions, where temperature variation remains within the specifications of the fluorometer model used.

4.2 High performance liquid chromatography with UV/visible detector, diode array detector, or mass spectrometer

HPLC is an alternative method to spectroscopic methods for characterizing phytoplankton. It is used in conjunction with detectors such as UV/Visible, DAD or MS. It can separate, identify, and quantify over 50 chlorophylls, carotenoids, and their derivatives and isomers in marine phytoplankton. The data obtained provide valuable information about the contribution of different functional groups to the biomass of the phytoplankton community. As many algal classes share the same pigments, HPLC data should be supported by microscopic verification. Silva *et al*. (2008), reported a HPLC-DAD method which uses a chemotaxonomic approach to compare major phytoplankton groups, based on HPLC pigment analysis and cell counting by

inverted microscopy, in order to study the seasonal variability of the phytoplankton community in Lisbon Bay, Portugal. This method quantifies chlorophylls *a*, *b*, *c2* and *c3*, peridinin, fucoxanthin, diadinoxanthin, diatoxanthin, 19-hexanoyloxyfucoxanthin, neoxanthin, prasinoxanthin, violaxanthin, alloxanthin, 19-butanoyloxyfucoxanthin, and zeaxanthin, using commercial standards from DHI Laboratory Products (Denmark).

The HPLC method is typically more accurate than spectroscopic methods, as it can distinguish between compounds without the need for approximate calculations, is subject to virtually no interference, and is able to offer accurate values for all pigment types. The chlorophyll *a* detection limit for all HPLC detectors is significantly lower than for spectroscopic methods.

4.2.1 Columns and mobile phase choice

The most widely used methods for HPLC analysis of chlorophyll and pigments are the Van Heukelem and Thomas method (VHT; Van Heukelem and Thomas, 2001), which uses a methanol-based gradient solvent system; and the method from Zapata *et al*. (2000), which uses a mobile phase containing pyridine. Prepared solvents should be ultrasonicated prior to use, as an initial means of degassing, followed by an in-line instrument degassing. Both methods use C8 columns. Further details on these methods can be found in the NASA Technical Memorandum 2012-217503 (Hooker *et al.,* 2012).

4.2.2 HPLC detectors

Automated HPLC methods for the routine determination of chlorophylls, carotenoids, and their degradation products were first developed in the late 1970s and early 1980s, and have continually been improved upon (Wright *et al.,* 1991). Publications on early pigment HPLC methods describe the separation of chlorophylls, carotenoids, and their degradation products, but only provide qualitative information, as a consequence of the limited availability of standards (Jeffrey *et al*., 1997). The US Environmental Protection Agency Method 447 (Arar, 1997) describes the quantification of chlorophylls *a* and *b*, and the identification of the other pigments of interest, using HPLC-UV/Visible. In 2002, QUASIMEME held a workshop to discuss the analysis of chlorophyll *a* (QUASIMEME, 2002). During this workshop, a subgroup of participants, undertaking chlorophyll analysis by HPLC, discussed the various detection methods used for routine analysis of chlorophyll and algal pigments. Participants used both UV/Visible and DAD for detection of chlorophyll *a.* There was no information given on the quantification of the other pigments of interest, and no conclusions were made regarding detector suitability.

Pigment analysis by UV/Visible detection is made primarily on the basis of retention time. This might result in misidentification of pigments in the complex area of the chromatogram. DAD detectors produce a full spectrum for each pigment peak, without stopping the flow. This greatly facilitates the identification of common chlorophylls and carotenoids, as the presence of a particular pigment can be confirmed or refuted by comparing the sample spectrum with that of standards.

Improvements in HPLC methods have led to the detection of pigments which are unidentified but spectrally related to known compounds. The characterization of these novel compounds involves preparative isolation followed by rigorous chemical and analytical techniques (Roy *et al* 2011). The use of HPLC-MS permits acquisition of structural data during the chromatographic run. Molecular mass information, when used in conjunction with spectra from a DAD, is often sufficient for the identification of compounds. HPLC-MS is not currently used for the analysis of chlorophyll and algal pigments in routine monitoring samples.

4.2.3 Specific points of the HPLC procedure

The pigment extracts from the sample need to be mixed with water or buffer immediately prior to injection. This ensures that even the most polar pigments are adsorbed onto a narrow zone on top of the HPLC column (Jeffrey *et al.*, 1997). The omission of this step results in poor peak shape, and poor resolution of the most polar pigments (Smith *et al.*, 2007). It is important that the extract is not diluted until immediately prior to injection, as highly aqueous extracts are not stable, and pigments (particularly hydrophobic pigments) will be lost within 1 h through adsorption onto the vial walls or precipitation. An autosampler that can effectively add water or buffer to the samples and mix is recommended for HPLC analysis.

When using an HPLC system with a UV/Visible detector it is important to minimize the introduction of air, and in particular carbon dioxide, to prevent alteration of the instrument signal and retention time shift. Therefore, it is important to ensure that all solvents have been thoroughly degassed by means of ultrasonication and in-line HPLC system degassers.

The use of an internal standard is strongly recommended. An internal standard should be stable, have spectral properties similar to the pigments analysed, and should not interfere with pigments on the chromatogram. A number of internal standards, suitable for use, have been reported (NASA Technical Memorandum 2012-217503, Hooker *et al.*, 2012), including Canthaxanthin, trans-β-apo-8'-carotenal, and, the most widely used, Vitamin E acetate. The extraction solvent and internal standard can be combined together in a mixture prior to addition to the sample (NASA Technical Memorandum 2012-217503, Hooker *et al.*, 2012).

Sample blanks must contain the same extraction solvent and internal standard mixture as the samples being analysed.

Samples must be kept chilled prior to analysis to prevent sample degradation. The use of a refrigerated auto-sampler is recommended.

Laboratory temperature variation may lead to retention time shift. The use of a column oven to maintain a constant temperature is recommended.

Details of the equations used for calculating chlorophyll and algal pigments by HPLC are detailed in Chapter 1 of NASA Technical Memorandum 2012-217503 (Hooker *et al.*, 2012) and are not recreated here.

5 Remote Sensors/Field Fluorometers

Remote sensing/field fluorometer devices are increasingly being used in the determination of chlorophyll for both biomass and eutrophication monitoring purposes. The aim of this section is not to recommend a remote sensing/field fluorometer format but to highlight this technology and calibration requirements.

Field fluorometers are often mounted on conductivity, temperature, depth (CTD) devices on oceanographic buoys, profiling devices including autonomous gliders, or in FerryBox systems on ships of opportunity, e.g. ferries, cargo ships, or research vessels. In shallow or coastal waters hand-held devices may also be used, although the accuracy of such devices should be determined before use. Field sensor/fluorometers have the advantage of providing measurements at a high frequency, which would be impossible to obtain with discrete sampling methods. However, it should be noted that during long-term deployments biofouling may affect sensors and interim maintenance is advisable.

Remote sensing of ocean colour by satellites gives the opportunity to cover large sea areas during daylight and under cloud-free conditions. There are currently several suitable satellites available, namely the

- NASA satellites Aqua and Terra, with the Moderate-Resolution Imaging Spectroradiometer (MODIS) sensor, Landsat 8, the National Polar-orbiting Operational Environmental Satellite System Preparatory Project (NPP), with the Visible Infrared Imaging Radiometer Suite (VIIRS) sensor,
- The European Space Agency (ESA) satellites Sentinel 2 and, particularly, Sentinel 3, with The Ocean and Land Colour Instrument and Medium Resolution Imaging Spectrometer.

Satellites are limited by the depth in the water column they can penetrate. In open ocean waters their reach can be greater than 20 m depth, while in highly productive waters it can be limited to only 1–2 m. Additional known problems associated with estimating chlorophyll using satellite remote sensing include: cloud cover, non-phytoplankton particles, and influence from suspended matter. Coastal areas and areas with a high coloured dissolved organic matter (CDOM) can be particularly problematic

5.1 Calibration of sensors

Earp *et al*. (2011) reviewed materials for the laboratory standardisation of the output from *in situ* fluorometers, so that sensor stability can be monitored over time, and the fluorescence output between different sensors and sensor manufacturers can be compared. Fluorescein was found to be suitable for the calibration of chlorophyll *a* measurements, as its emission spectra overlaps with that of phytoplankton, and it produces a consistent linear fluorescence response with increasing concentration. In addition, a solid-state standard to check the fluorometer output before and after use, can be used to check for instrument drift, which is common in optical sensors (Earp *et al*., 2011).

The *in vivo* chlorophyll fluorescence ratio to extracted Chlorophyll *a* varies widely due to differences including phytoplankton taxonomy and size, pigment composition, cell size, non-photochemical quenching and environmental conditions (Bricaud *et al*., 1995; Carberry *et al*., 2019; Falkowski *et al*., 2011). All *in vivo* sensors require regular calibration against *in vitro* analyses of discrete water samples collected at the sensor location. Currently, no analytical technique has been recommended for the calibration of sensors using fluorescence detectors.

The temporal and spatial variability in the phytoplankton community composition at the deployment site should be well characterized to determine its the influence on fluorescence measurements. Additionally, the variability of key environmental parameters at the deployment site, such as nutrients, temperature, light, salinity, and turbidity, should be determined and taken into account.

6 Quality control

6.1 Blanks

To ensure Quality Assurance (QA) of a method, procedural blanks are performed to account for any contamination during transport and storage. During the method validation process, laboratories should consider whether a sampling procedural blank is necessary. To obtain a sampling procedural blank, a blank filter should be folded and stored along with the samples at the time of sample collection, as per Section 2.3. Note that deionised water should not be passed through the filter before it is folded. The sampling procedural blank should subsequently be analysed as a sample.

An analytical procedural blank should be analysed at least once with each sample analysis batch. The blank should include a filter, and should be prepared simultaneously to the samples, using the same chemicals and solvents. The measurement of a chlorophyll concentration above the method detection limit in the analytical procedural blank indicates a contamination, and the source of the contamination should be determined.

The chlorophyll concentration should be corrected for the analytical procedural blank in both samples and LRMs. If a correction is also required to account for the sampling procedural blank, this should be undertaken after the correction for the analytical procedural blank.

6.2 System suitability checks

Instrument performance may deteriorate over time as a result of the ageing of lamps, monochromators, and HPLC columns, or of maintenance issues, such as column blockages. To identify any such changes, a system suitability check (SSC) should be carried out prior to analysing the samples, to ensure that the system is working satisfactorily. The SSC should contain, as a minimum, a standard solution of chlorophyll *a*.

DHI (Denmark) supply mixed pigment standards for HPLC analysis. These can be used to monitor HPLC performance and precision, determine elution order, and identify chromatographic peaks.

Criteria for passing the SSC should be set by individual laboratories, and ideally SSC results should be monitored on control charts to identify any long-term trends.

6.3 Precision and accuracy

There is currently no certified reference material (CRM) available for chlorophyll analysis, although reference materials with different origins can be purchased upon request from the external proficiency test provider WEPAL-QUASIMEME[1].

LRM's should therefore be included in every analysis, with at least one LRM sample for each batch of identically prepared samples. The LRM must be homogeneous, well characterized for chlorophyll concentration, and must be proven to produce consistent results in stability tests over time. The LRM determinand concentrations must be in a comparable range to those of the samples. If this is not possible, e.g. if the range of determinand concentrations in the sample is

[1] https://www.wur.nl/en/article/proficiency-testing-wepalquasimeme.htm. Last accessed 18/11/2022.

large, at least two different LRMs should be included in each batch of analyses to cover the lower and upper concentrations present in the sample. A quality control chart should be used to monitor LRM performance.

6.4 Preparation of in-house laboratory reference material

LRMs can be prepared from natural samples, commercial cultures, or commercial marine microalgae mixes (food for filter-feeders such as Phyto Feast[2]). Commercial cultures or Phyto Feast should be diluted to the appropriate concentration with autoclaved low-chlorophyll seawater. It is important to gently mix the sample (use a combined gentle swirl and up-and-down movement) before each subsample is taken for filtration, to ensure homogeneity and uniform mixing of the pigments. After mixing, the required volume should be filtered as described in Section 2.2.5. The filter is then folded once with the algae inside and stored in labelled cryovials. To prevent degradation, samples should ideally be flash-frozen in liquid nitrogen before storage. To ensure that degradation did not occur during preparation, and to determine the concentration of the unknown pigments in the prepared LRM, at least seven filters, from different stages of LRM preparation process, should be randomly selected for immediate analysis. The results should be monitored on quality control charts.

It is recommended to prepare a large batch of LRM, and store it in an ultracold freezer (−80°C). LRM will be stable for up to one year (see Section 2.3). Ideally, a new batch of LRM should be prepared before the old one is finished, and a parallel check of LRM batches should be performed to demonstrate the suitability of the new batch.

6.5 Laboratory performance

The participation on a regular basis in interlaboratory comparison studies or proficiency testing schemes such as QUASIMEME, preferably at an international level, is of great help to evaluate and improve laboratory performance. In addition, laboratories should ensure their analysis is robust, repeatable, and auditable, ideally through method accreditation to the international testing and calibration standard ISO 17025[3].

[2] https://reefnutrition.com/product_phyto_feast.php; last accessed 16/01/2020

[3] https://www.iso.org/ISO-IEC-17025-testing-and-calibration-laboratories.html; last accessed 27/10/2021

7 Metadata

Laboratories should maintain metadata records when collecting and performing chlorophyll analysis, for use in assessments, if required. The metadata associated with the process (e.g. filters, extraction solvent, and extraction method used) should be provided to datacentres for assessment purposes.

The minimum recommended metadata should include:

- Sampling location
- Sampler type
- Date
- Time of sampling (check in advance the format required by relevant databases for submission)
- Sampling depth
- Time of filtration
- Sample volume filtered
- Filter type
- Filter pore size or NRR
- Storage conditions
- Storage duration
- Extraction type (e.g. sonication, soaking, or grinding)
- Extraction conditions (e.g. temperature and duration)
- Extraction solvent
- Instrument and detector (e.g. HPLC with UV/Visible, or HPLC with DAD)
- Detector conditions (e.g. absorption/emission and wavelengths used)
- Standards for calibration
- ISO 17025 accreditation status (if applicable)

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Annex 2: List of abbreviations

CDOM	Coloured dissolved organic matter
CRM	Certified reference material
CTD	Conductivity, temperature, and depth device
DAD	Diode array detector
DMSO	Dimethyl sulfoxide
ESA	European Space Agency
HPLC	High pressure liquid chromatography
JAMP	OSPAR Joint assessment monitoring programme
PAM	Pulse-Amplitude-Modulation
QUASIMEME	Quality assurance of information for marine environmental monitoring in Europe
LRM	Laboratory reference material
MODIS	Moderate-resolution imaging spectroradiometer
MS	Mass spectrometer
NASA	National Aeronautics and Space Administration, US
NOAA	National Oceanographic and Atmospheric Administration, US
NPP	National Polar-orbiting Operational Environmental Satellite System Preparatory Project
NRR	Nominal retention rate
QA	Quality assurance
QC	Quality control
SCOR	UNESCO Science Committee on Oceanic Research
SSC	System suitability check
TIMES	ICES Techniques in Marine Environmental Sciences
UV	Ultraviolet
VHT	Van Heukelem and Thomas method
VIRIS	Visible Infrared Imaging Radiometer Suite

Annex 3: QUASIMEME case study - results from different extraction and analysis methods

QUASIMEME are a proficiency testing scheme provider originating in the 1980's, designed as a holistic quality assurance programme for marine environmental monitoring information. QUASIMEME was created blending experience with quality assurance of the former EC Community Bureau of reference (BCR) and ICES Marine Chemistry Working Group. Hosted at the Wageningen University & Research Centre in the Netherlands, in 2011 QUASIMEME merged with WEPAL proficiency testing scheme provider for terrestrial environment[4].

A QUASIMEME chlorophyll and nutrients workshop held in 2014 highlighted the lack of improvement in the performance of chlorophyll exercises, in contrast to other exercises, such as the determination of nutrient concentrations. QUASIMEME initiated an internal investigation into the methods used by participants for reporting chlorophyll concentrations.

Introduction and method

The QUASIMEME proficiency testing scheme undertook a preliminary experiment in 2013 to determine which method-related differences were responsible for differences found in chlorophyll *a* analysis results. Three different types of filter samples were sent to the QUASIMEME participants: a natural seawater filter sample, a *Nannochlorpsis* sp. sample, and a sample from a mixture of three different algae (*Pyramimonas* sp., *Chaetocheros* sp., and *Isochrysis* sp.). A total of 36 laboratories joined this preliminary study, and provided QUASIMEME information on the method used together with the chlorophyll *a* analysis results. Initial results indicated that differences in chlorophyll *a* concentrations resulted from differences in the types of extraction solvent used, and differences in the detection methods. Other differences in the methods of extraction, such as extraction time, were not as important.

Following up on this initial experiment, QUASIMEME asked a selection of laboratories involved in their proficiency testing program, to analyse the chlorophyll *a* in two different types of samples: a natural seawater sample, and a sample prepared from *Nannochloropsis* sp. The laboratories were provided with these samples on GF/F filters (Whatman). In addition to these filter samples, QUASIMEME provided the laboratories with extracts from these samples, prepared by QUASIMEME by ethanol extraction of the GF/F filters, followed by filtration through filters with an 0.45 μm pore-size (type Spartan 13; Whatman). QUASIMEME provided the extracted sample to exclude the potential effect of differences in the individual laboratories' treatment procedures (e.g. solvents). The laboratories were instructed to use their own methods, including correction for blanks or standard recoveries if needed. The results were tested for significance with two-sided T-tests.

These initial studies were followed in 2019 by a further study where participants were requested to analyse chlorophyll concentrations from two cultured algae samples prepared by QUASIMEME. The aim of this was to determine if the extraction technique or the detection method influenced the concentrations obtained. The first of the samples was a previously analysed proficiency test sample and the second an acetone extract of this sample.

[4] https://www.wepal.nl/en/wepal/About-us.htm

Results

The outcome of the preliminary experiment in 2013, in which 36 laboratories analysed the three species, is detailed in Table A3.1. Extraction with acetone was found to give poor results for some of the samples provided, especially for the algae *Nannochloropsis* sp. However, sample storage is most stable if samples are stored in acetone. The extraction time, extraction volume, and the method of filter extraction, had limited effect on the data, with only sonication resulting in lower chlorophyll *a* concentrations. However, the four labs that undertook sonication also extracted with acetone.

Table A3.1. Average chlorophyll *a* concentrations for laboratories participating in preliminary experiment. Concentrations are in µg L^{-1} chlorophyll *a*.

Processing step	*Nannochloropsis* sp.	Algae mix	Seawater
Extraction liquid			
Acetone	3.00 ± 2.21	1.30 ± 0.47	5.04 ± 1.41
Ethanol	9.84 ± 3.15	1.62 ± 0.31	5.29 ± 1.22
Methanol	9.30 ± 2.07	1.49 ± 0.18	4.46 ± 1.15
Analysis/Detection			
Spectroscopy	6.99 ± 4.10	1.57 ± 0.39	5.54 ± 1.26
Fluorimetry	3.73 ± 3.61	1.29 ± 0.48	4.82 ± 1.40
HPLC	3.62 ± 2.79	1.19 ± 0.17	3.99 ± 0.63
Extraction volume			
< 10 ml	5.07 ± 4.08	1.27 ± 0.32	4.85 ± 1.09
10 ml	5.88 ± 4.07	1.76 ± 1.08	5.09 ± 1.24
> 10 ml	3.59 ± 3.84	1.29 ± 0.47	4.57 ± 1.35
Extraction time			
< 1 min	4.75 ± 2.01	1.25 ± 0.13	4.32 ± 0.93
1–60 min	7.12 ± 4.15	1.44 ± 0.39	5.39 ± 1.88
> 60 min	4.77 ± 4.18	1.42 ± 0.48	5.05 ± 1.26
Extraction type			
Homogenization	5.94 ± 3.60	1.50 ± 0.48	5.16 ± 1.08
Sonication	1.49 ± 0.88	1.24 ± 0.20	4.60 ± 0.77
Soaking	4.74 ± 4.03	1.34 ± 0.45	4.85 ± 1.33

The results of the follow-up experiment, undertaken by a selection of laboratories involved in the proficiency tested scheme, are presented in figures A3.1 (*Nannochloropsis* sp.) and A3.2 (seawater). *Nannochloropsis* sp. filter results for the different laboratories, were found to be very variable. Concentrations of chlorophyll *a* were lowest for laboratories which had used acetone as the extraction solvent (Figure A3.1, panel A). Only one laboratory used methanol as the extraction solvent, so no conclusions could be drawn. There were no clear differences observed for the natural seawater filter sample related to solvent extraction (Figure A3.2, panel A), indicating that extraction from natural algae was easier than from cultured samples. The detection method used for these samples had very limited effect the chlorophyll *a* concentration obtained (Figure A3.2, panel B).

In 2019, QUASIMEME supplied two additional samples for chlorophyll analysis (QCH096SW and QCH097SW). QCH096SW had previously been analysed as QCH093SW in 2018.

QCH097SW was provided to participants as an acetone extract prepared from the QCH096SW sample. Participant results were variable and no assigned value could be calculated for QCH096SW or QCH097SW. This indicates that, for this particular sample produced from cultured algae (*Nannochloropsis* spec), variability of chlorophyll *a* concentrations is caused by differences in the detection methods used, and is not linked to the extraction solvent or extraction method. This hypothesis is supported by the Youden plot of QCH096SW vs. QCH097SW (Figure A3.3).

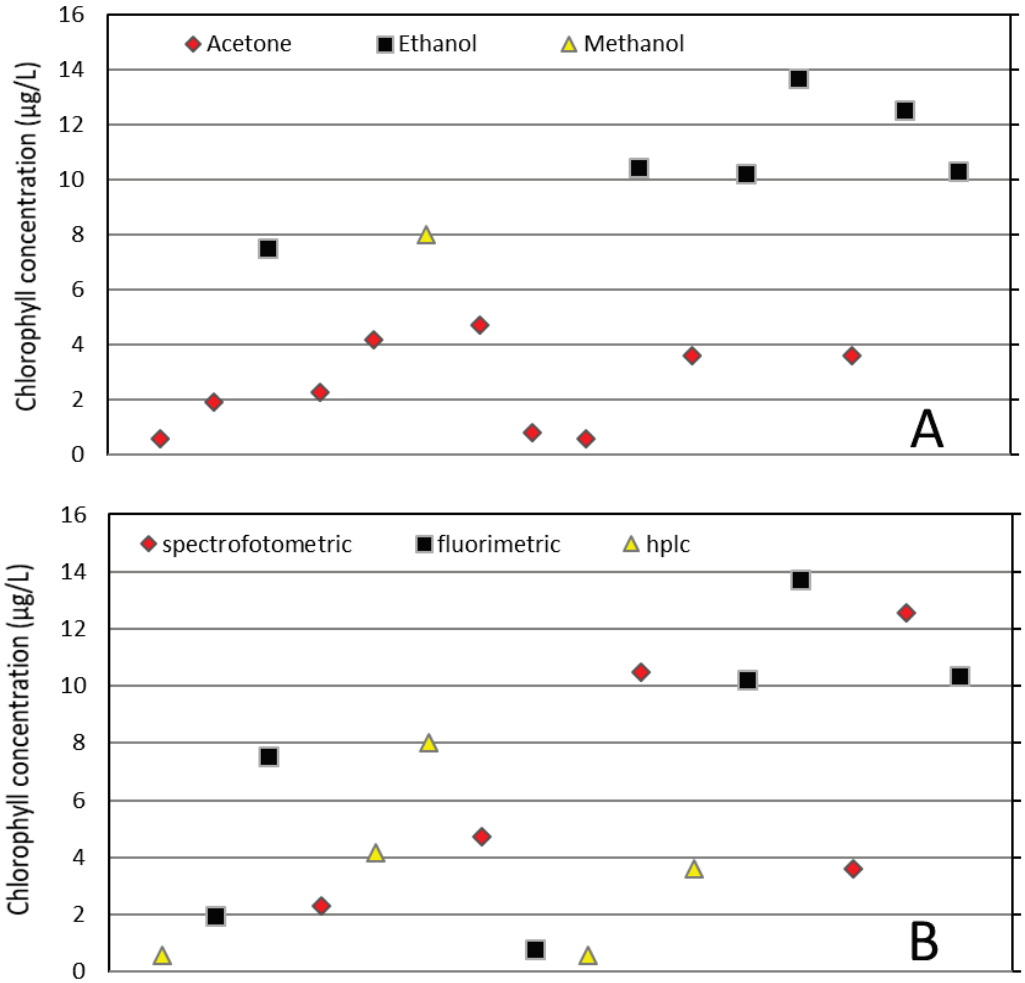

Figure A3.7.1. Analysis of chlorophyll a from a *Nannochloropsis* spec. filter sample in relation to the extraction solvent used (A); in relation to the method of detection used (B).

Discussion and conclusions

The analysis of the different steps within chlorophyll extraction and quantification methods indicated that acetone may not be suitable for all algae species. Recovery rates were particularly poor for *Nannochloropsis* sp., when compared to the natural seawater samples that contained a mixture of algal species, indicating that the extraction and detection efficiency may be dependent on the algal species present.

HPLC measurements also might not be directly comparable with the conventional spectrophotometric and fluorometric quantification methods. The differences in chlorophyll *a* concentrations reported seem to be dependent on the pigment composition. No significant difference in measured chlorophyll *a* concentrations for the *Nannochloropsis* sp. sample was

found when using HPLC and the conventional spectroscopy and fluorometry methods. Therefore, an understanding of the algal species present is recommended when undertaking chlorophyll analysis.

No significant differences in participant results were observed when using differing extraction times or solvent volumes. However, using an extraction volume lower than 10 ml and/or an extraction time lower than 1 min may give poorer chlorophyll *a* recovery rates. In addition, recovery rates were dependent on the method of extraction (sonication or soaking) and the extraction solvents used. Ethanol seems to be the better solvent for extracting chlorophylls. Samples extracted by sonication alone resulted in an underestimation of chlorophyll concentrations. A clear difference was observed between participants reporting chlorophylls as chlorophyll *a* by either fluorometric or photometric methods, and those using HPLC. As indicated within this report, the standard photometric and fluorometric methods for determining chlorophylls do not completely separate the different chlorophyll pigments, while HPLC does. Therefore, HPLC will provide lower but accurate concentrations of chlorophyll *a*.

Future QUASIMEME exercises will assess the data from HPLC and fluorometric methods separately, and the exercise may be expanded to include additional pigments. The last workshop in 2014, and subsequent experiments, highlighted the need to harmonize the methods used for the analysis of chlorophylls in marine waters.

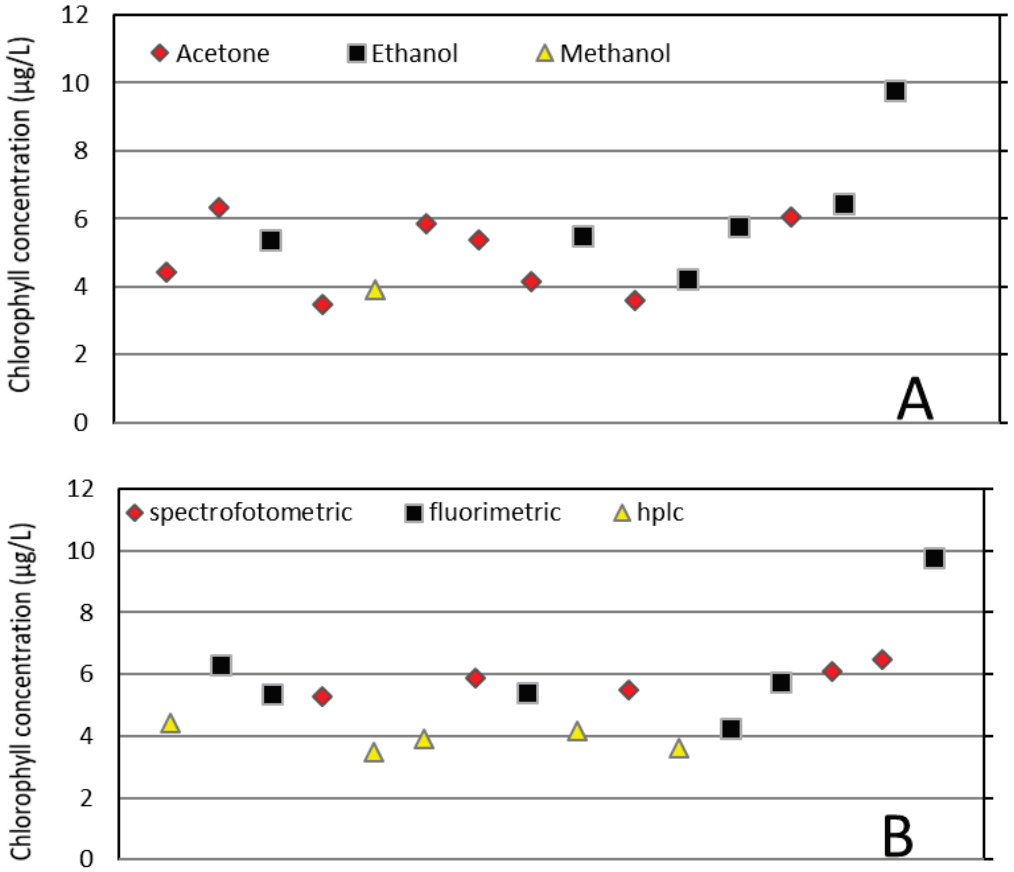

Figure A3.7.2. Analysis results of chlorophyll a from a seawater filter sample in relation to the extraction solvent used (A); in relation to the method of detection used (B).

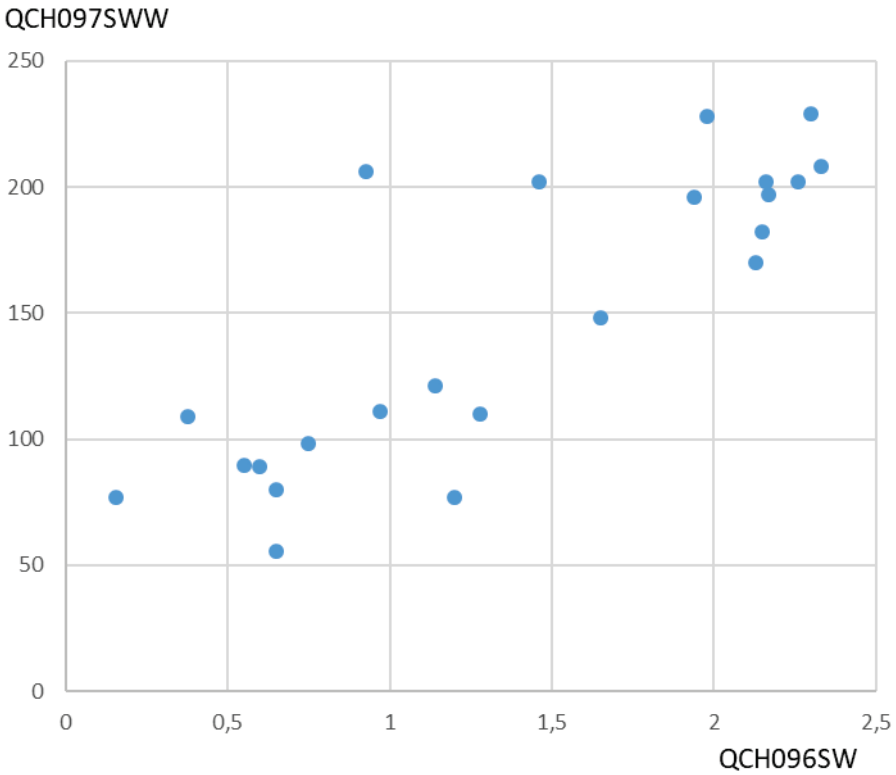

Figure A3.3. Youden plot of chlorophyll a concentrations of sample QCH096SW (supplied on filter for participant extraction) and QCH097SW (acetone extract of QCH096SW for participant detection)