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A REVIEW OF ANALYTICAL METHODOLOGY USED TO DETERMINE PHYTOPLANKTON PIGMENTS IN THE MARINE ENVIRONMENT AND THE DEVELOPMENT OF AN ANALYTICAL METHOD TO DETERMINE UNCORRECTED CHLOROPHYLL A, CORRECTED CHLOROPHYLL A AND PHAEOPHYTIN A IN MARINE PHYTOPLANKTON

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SUMMARY

1. Chlorophyll measurements are made to estimate the phytoplankton biomass in the marine environment and are probably the most frequently measured biochemical parameter in oceanography.

2. FRS monitors conditions at two Scottish coastal sites and at key points in the offshore and oceanic waters around Scotland. The results from these monitoring programmes are presented regularly in the FRS 'Scottish Annual Ocean Climate Status Summary' reports and the 'ICES Annual Ocean Climate Status Summary'. Chlorophyll samples are collected from aquaculture hotspot areas three times a year as part of the OSPAR Comprehensive Procedure for assessment of eutrophication status.

3. Chlorophyll *a* is the primary pigment of interest in monitoring programmes, but several pigments and degradation products may be found at any one time in a given sample. Due to spectral overlap between chlorophylls, cartenoids and degradation products, under or over estimation of chlorophyll *a* can occur.

4. Fluorometry is the standard method for measuring chlorophyll *a* in international monitoring programmes and there are long national and international time series data using this method, which FRS have to contribute to. An extractive fluorometric method was developed and validated on both the Turner 10-AU and Turner TD-700 fluorometers. The pigments were extracted from the filter paper by soaking in 90% buffered acetone for 16 to 30 hours. Measurements were made both before (Rb) and after (Ra) acidification.

5. Both uncorrected chlorophyll *a* and corrected chlorophyll *a* have been reported. Corrected chlorophyll *a* is chlorophyll *a* corrected for the presence of phaeopigments. However, it is widely reported that the fluorometric equations used to calculate corrected chlorophyll *a* and phaeophytin *a* are inaccurate when significant amounts of chlorophylls *b* and *c* are present in the samples. Phaeophytin *a* concentrations were calculated using the fluorometric equations. There are no commercially available standards for phaeophytin *a* therefore separate validation data could not be generated. Highly erratic data was generated for phaeophytin *a* using the fluorometric equations, with negative concentrations calculated and in some instances coefficients of variation as high as 300%. This is due to error associated with the analytical method and the fluorometric equations.

6. Replicate analysis for high standards and samples and low standards and samples on the same day gave %CV of <15% for both uncorrected chlorophyll *a* and corrected chlorophyll *a* on both instruments. Replicate analysis for high standards and samples and low standards and samples on different days gave %CV of <15% for both uncorrected chlorophyll *a* and corrected chlorophyll *a* on both instruments.

7. The limit of detection (LOD) was calculated for uncorrected chlorophyll *a* and corrected chlorophyll *a* using typical sample volumes filtered (0.2 – 2 l) and sample extracts volume (15 ml). The LODs for both uncorrected chlorophyll *a* and corrected chlorophyll *a* were found to be 0.007 to 0.07 µg l^{-1} on the 10-AU and 0.008 to 0.08 µg l^{-1} on the TD-700.

8. Recovery for both uncorrected chlorophyll *a* and corrected chlorophyll *a* on both instruments was found to be ~ 73%, with a %CV of <15%.

9. It is widely reported in the literature that the fluorometric equations used for calculating chlorophyll *a* and phaeophytin *a* are accurate when these are the only two components present in the sample, which is not the case in algae samples collected from the marine environment. The before – to –after acidification response ratio (r) also influences the accuracy of corrected chlorophyll *a* and phaeophytin *a* determinations. It is suggested in the literature that the acidification to correct for phaeophytin *a* impairs the chlorophyll *a* measurement rather than improve it and it is recommended that this step should be abandoned. The use of Welschmeyer's non-acidification fluorescence method or an HPLC method should be considered as these give a more accurate result for determination of chlorophyll *a*.

INTRODUCTION

Phytoplankton, algal blooms and eutrophication

Chlorophyll is the biological compound which plants and algae use to produce food from sunlight in a process known as photosynthesis[1]. Phytoplankton are small single celled organisms at the base of the marine food web. Most contain chlorophyll *a* which they use to harvest light energy from the sun. This energy is then passed up the marine food web to higher trophic levels.

There are tens of thousands of species of phytoplankton. Most, but not all, contain chlorophyll *a*. Because of their evolutionary history different functional groups, e.g. diatoms, dinoflagellates, cyanobacteria, contain different and sometimes unique pigments in their chloroplasts. The most routinely observed phytoplankton groups in Scottish waters are diatoms, dinoflagellates and microflagellates[2]. Different functional groups dominate the phytoplankton population at different times of year depending on a combination of temperature, nutrient availability, light availability and grazing pressure from zooplankton. Of particular importance is the spring diatom bloom which begins early in springtime when light and temperature become favourable after the winter. In some instances conditions can favour the growth and accumulation of particular phytoplankton species. These can achieve very high cell densities in the water column and form what is known as an algal bloom. The majority of algal blooms are natural events and are part of the annual biological cycle in the marine environment. In some instances blooms can have an adverse effect on marine life or pose a threat to human health. These have been given the name harmful algal blooms[3]. These algal blooms may be harmful for a variety of reasons;

- Commercially important farmed fish species can be killed due to the action of icthyotoxins causing clogging/irritation of the gills or increased oxygen demand during night time due to respiratory demand of high biomass blooms, particularly if cells are scenescent and undergoing decay.

- The abundance of decaying algal material from high biomass blooms falling to the seabed severely reduces the oxygen concentration in bottom waters, killing benthic animals

- Some algal blooms (e.g. members of the genus *Phaeocystis)* can also form unsightly foam, which when stranded on the shore, can have an adverse effect on tourism.

Some genera produce compounds termed algal toxins. When shellfish eat these algae they accumulate these compounds in their tissues. While these compounds do not affect the shellfish they pose a risk to human health when consumed. Rigorous monitoring programmes are enforced by the European Community to ensure the safety of shellfish products under directives EC 2073/2005, EC2074/2005 and EC854/2004.

Light and inorganic nutrients are the environmental parameters that mainly control phytoplankton growth[4]. In marine ecosystems, nutrients, which are normally replenished from oceanic reserves and through recycling, can also be introduced to the marine environment from a variety of sources, including decaying organic matter, sewage waste and agricultural run-off. In certain circumstances an excess of nutrients can enter the sea. This effect is known as eutrophication. Evidence of eutrophication is most often found within some coastal areas, such as embayments and estuaries[5]. The problems caused by excess nutrients in coastal areas may also have effects on marine ecosystems outside the immediate areas concerned. Currently a number of Scottish Executive drivers are directly concerned with identifying and remediation of the effects of eutrophication on the marine environment e.g. The Urban Waste Water Treatment Directive (UWWTD), The Water Framework Directive (WFD), the Oslo Paris Commission (OSPAR) and the forthcoming EU Marine Strategy.

Why measure chlorophyll in the marine environment?

Phytoplankton biomass represents the majority of carbon that is being fixed in the marine environment and is available for consumption by higher trophic levels in the food web. The phytoplankton community at any one time can comprise of a variety of species that can vary in size from >2 µm diameter to > 100 µm diameter. Many species possess complex three dimensional shapes and determining the biovolume and deriving a carbon content for each species in a sample is highly time consuming and often not possible as part of routine phytoplankton analysis. Reporting phytoplankton cell numbers may not give an accurate indication of the biomass present e.g. a community can be dominated by high numbers of small species. This may actually represent a lower biomass then a community that is dominated by lower numbers of larger species. The great majority of phytoplankton species contain chlorophyll *a*. Assuming that larger cells contain more chlorophyll than smaller cells, measuring the concentration of chlorophyll *a* in the water column is a crude way of estimating the biomass present.

Fisheries Research Services (FRS) collects samples for chlorophyll analysis from two Scottish ecosystem monitoring sites[6], at key points in the offshore and oceanic waters around Scotland[2] and from sea lochs and voes, some of which are associated with aquaculture[7].

Field Studies

Stonehaven and Loch Ewe ecosystem monitoring

FRS has established two Scottish coastal ecosystem monitoring sites; one on the east coast and one on the west coast. Since 1997 physical and chemical (temperature, salinity and nutrients) samples have been collected using water bottles at the surface and a depth of approximately 45 m from a site on the east coast, 3 km offshore from the town of Stonehaven (56º57.8'N, 02º06.2'W). The site on the west coast, is at Loch Ewe (57º50.99'N, 05º38.97'W), where samples have been collected at surface and from a depth of 30 m, since 2002[6]. Samples for chlorophyll determination and species community phytoplankton analysis are taken from a 10 m integrated tube sampler (Lund sampler), zooplankton samples are also taken using 200 µm mesh bongo nets. These samples are collected on a weekly basis from both sites, weather permitting.

These coastal time series provide indications of the state of these ecosystems in relation to background seasonal variability, as well as larger field processes such as ocean climate changes in the seas surrounding Scotland and in the North Atlantic.

Offshore and oceanic waters

FRS also monitors conditions at key points in the offshore and oceanic waters around Scotland at least three times each year using the Fisheries Research Vessel (FRV) *Scotia*[2]. In particular, samples are collected from the northern North Sea, along a section north east of Orkney (59º 17'N, 2º 14'W) to the centre of the North Sea (59º 17'N, 0º 10'W), known as the JONSIS line and along sections of the deep water channel separating Scotland from the Faroe Islands (Fair Isle – Munken and Nolso – Flugga survey lines).

The results from these monitoring programmes are presented regularly in the FRS 'Scottish Annual Ocean Climate Status Summary' reports[8]. These reports give information on the weather in Scotland, and details of conditions in coastal, offshore and oceanic waters for the period of the report. A similar report is prepared each year by a group of researchers from countries across the North Atlantic Region. These reports are published by the International Council for the Exploration of the Sea (ICES) as the 'ICES Annual Ocean Climate Status Summary'[9].

Aquaculture hotspots

The aquaculture industry in Scotland is dominated by Atlantic salmon (*Salmo salar*), but other species are produced, e.g. trout, halibut, cod and shellfish including mussels and oysters. Most production is located in semi-enclosed water bodies on the west coast mainland of Scotland and the western and northern isles. Finfish diets contain pellets of fish meal which contribute a major source of nitrogen in fish feed. Nitrogen is excreted by the fish and released as soluble nitrogen into the receiving waters. Particulate nitrogen is also released into the receiving waters from waste feed pellets or faecal material. Nitrogen inputs from salmon farms have been blamed for producing harmful algal blooms in coastal waters and eutrophication[7].

As a result, nutrient inputs to coastal waters from fish farms are being increasingly monitored both nationally (e.g. Petition PE96) and internationally (OSPAR Comprehensive Procedure for assessment of eutrophication status). As part of the OSPAR Strategy to Combat Eutrophication[10], OSPAR has agreed a detailed Comprehensive Procedure[10] for assessing the eutrophication status of marine areas. Category II, Parameter I of the Comprehensive Procedure requires determination of the maximum and mean chlorophyll *a* concentration.

Eutrophication assessment of a selection of these water bodies, where the expected level of nutrient enhancement from aquaculture discharges was relatively high in comparison to other areas ('hotspots') was undertaken in 2003[7]. 110 water bodies supporting aquaculture in Scottish waters were assessed for maximum and mean chlorophyll *a* concentration. For assessment of chlorophyll *a* levels under the Comprehensive Procedure, a criteria of >50% above spatial (offshore) background concentrations is applied. Area averaged chlorophyll *a* levels did not significantly exceed the assessment criteria of any of the areas surveyed. At some locations within Shetland voes and sounds, chlorophyll *a* levels did exceed the assessment criteria, but chlorophyll *a* is very spatially and temporally variable and occasional exceedance of monthly, time-averaged background values by spot samples (e.g. during periods of high productivity in spring) is to be expected. Work is continuing as the OSPAR Eutrophication Committee will repeat the assessment of inputs from aquaculture as part of the reapplication of the Comprehensive Procedure during 2007.

Physiological Studies

The pigment composition of phytoplankton is a reflection of their physiological state and the environmental conditions in which they are grown. The photosynthetic apparatus of phytoplankton contains a complex suite of light harvesting, photo protective and accessory pigments. The majority, but not all, phytoplankton contain chlorophyll *a*. Many contain additional chlorophylls e.g., chlorophyll *b* or chlorophyll *c* as well as a variety of carotenoid pigments. This complex apparatus has evolved to allow phytoplankton to adapt to a variety of different habitats and light regimes. An accurate analysis of the phytoplankton pigment concentrations and ratios under different light, nutrient and temperature regimes will increase the capacity to assess the physiological response of phytoplankton to variations in environmental parameters.

Chlorophyll Chemistry

Three types of chlorophyll compounds have been identified in the marine environment; chlorophyll *a*, chlorophyll *b* and chlorophyll *c.* All photosynthetic algae and higher plants contain chlorophyll *a* as the principal pigment. Chlorophyll *b* is found in higher plants, green algae and symbiotic prochlorophytes. Chlorophyll *c* is found in chromophyte algae and brown seaweeds, and there are six known types[11].

The chemical structures of chlorophyll *a,* chlorophyll *b,* chlorophyll c_1 and chlorophyll c_2 are given in Figure 1[12]. Chlorophylls *c* do not contain the phytol side chain. The other 4 chlorophyll *c* compounds [chlorophyll c_3, chlorophyll $c_{cs\text{-}170}$, MgDVP (Mg-3,8-divinylphytoporphyrin-13^2-methylcarboxylate) and protochlorophyllide] have a similar chemical structure to chlorophylls c_1 and c_2, but with slight modifications to the branches and saturation[13]. Treatment of chlorophyll *a* with acid removes the central magnesium ion replacing it with two hydrogen ions to produce phaeophytin *a.* Phaeophytin *a* is also a natural degradation product of chlorophyll *a* produced during photosynthesis[13] and occurs in the marine environment when phytoplankton cells are grazed upon by zooplankton and egested in feacal material. Hydrolysis of phaeophytin *a* splits off the phytol side chain to produce phaeophorbide *a*. Treatment of chlorophyll *a* with a base removes the phytol side chain to produce chlorophyllide *a*. Similar compounds are obtained with chlorophyll *b*[14]. Chlorophyll *a* is the primary pigment of interest in monitoring programmes as it is the only pigment present in all marine planktonic algae (with the exception of prochlorophytes, however the chlorophyll *a* derivative found in prochlorophytes is measured as chlorophyll *a*[11]).

Several pigments and degradation products are present at any one time in a given sample; the relative amounts present being dependant on the taxonomic composition and

physiological condition of the phytoplankton. When a sample is filtered and extracted for chlorophyll analysis it therefore contains a number of pigments other than chlorophyll *a*. Due to the spectral overlap of the different chlorophyll compounds and phaeophytin *a* under or over estimation of the pigments is inevitable. Chlorophylls *b* and *c* may significantly interfere with chlorophyll *a* measurements depending on the amount present. If chlorophyll *b* is present in the sample this will result in an underestimation of chlorophyll *a* along with an overestimation of phaeophytin *a*. The degree of interference depends upon the ratio of chlorophyll *a* : chlorophyll *b*. The presence of chlorophyll *c* also causes the underestimation of phaeophytin *a*, although not as severe as the effects of chlorophyll *b*[15]. Most analytical methods address specific interferences but often ignore others. Knowledge of the taxonomy of the algae under consideration is required to determine which analytical method produces the most appropriate information.

This report will describe the extractive fluorescence method routinely used by FRS to determine uncorrected chlorophyll *a*, corrected chlorophyll *a* and phaeophytin *a* in marine samples. The validation data will be presented for both the Turner 10-AU digital fluorometer and the Turner TD-700 laboratory fluorometer. A discussion of other analytical methods used to determine chlorophyll *a* in marine samples will also be given.

EXPERIMENTAL

Reagents

Acetone and water were both HPLC grade and purchased from Rathburn Chemicals, Walkerburn, Scotland, UK. Concentrated hydrochloric acid 'AnalR' sp. gr. 1.16 was purchased from VWR International Ltd., Leicstershire, UK. Magnesium carbonate ($MgCO_3$) was purchased from Sigma-Aldrich Company Ltd., Dorset, UK.

90% buffered acetone was used as the extraction solvent. This was prepared by mixing the powder free solution from a saturated magnesium carbonate solution with acetone.

Equipment

A digital fluorometer (Turner 10-AU) and a laboratory fluorometer (Turner TD-700) were used to measure uncorrected chlorophyll *a*, corrected chlorophyll *a* and phaeophytin *a* in the standards and samples. Both fluorometers are fitted with an excitation filter (part number 10-050R, colour specification 5-60) and an emission filter (part number 10-051R, colour specification 2-64). The 10-AU fluorometer also uses a reference filter [part number 10-032 1 neutral density (1 ND)]. The excitation filter is a bandpass filter which allows light to pass from 340 nm to 500 nm. The emission filter is a cutoff filter which allows light to pass at >665 nm. In combination with a light source (daylight white lamp, part number 10-045), the excitation filter allows only light which excites the molecule of interest to strike the sample. The emission filter allows the fluorescence from the sample to pass to the detector and blocks stray light from the light source or interfering components in the sample. The reference filter is used in the reference path of the 10-AU fluorometer and is a factor in determining the basic operating level of the instrument[16]. The 10-AU fluorometer can be used in the field and has greater sensitivity than the TD-700 fluorometer.

Fluorescence is temperature dependant with higher sensitivity occurring at lower temperatures. Arar & Collins[15] state that 'ambient temperature should not fluctuate more than ± 3ºC between calibrations or recalibration of the fluorometer will be necessary'. This was avoided by keeping the fluorometers in a temperature controlled laboratory at approximately 19ºC ± 3ºC.

Calibration and Quality Control

A stock standard solution was prepared from chlorophyll *a* from *Anacystis nidulans* algae (purchased from Sigma-Aldrich Company Ltd., Dorset, UK) in 90% buffered acetone. The concentration of chlorophyll *a* in this solution was determined using the monochromatic spectroscopic method and the revised Lorenzen equations[11,13,17] (the revised Lorenzen equations are given in Appendix 1). An ultraviolet (UV)/visible spectrophotometer (Ultraspec 3300 *pro,* Amersham Pharmacia Biotech) was used to make the measurements. A series of diluted standard solutions were then prepared from the stock standard solution covering the range 1 µg l^{-1} to 200 µg l^{-1} chlorophyll *a*.

A one-point calibration was performed on both fluorometers using a standard solution at approximately 80% of the highest concentration of the samples to be measured; in this case the 150 µg l^{-1} l chlorophyll *a* diluted standard solution was used. After calibration, each diluted standard solution was measured before and after acidification, and the before – to – after acidification response ratio (r) was calculated. This was found to be 1.788 for the 10-AU fluorometer and 1.877 for the TD-700 fluorometer. Both fluorometers give a direct readout of the concentration of the samples and standards without the need for compensation for the various sensitivity settings. Turner Designs state that when the fluorometers are calibrated with a standard of known concentration then F_s (response factor for the sensitivity setting used) will equal 1, as the instrument automatically performs the calculations for the range and sensitivity settings. This was checked by plotting a graph of the chlorophyll *a* concentration of the diluted standard solutions calculated from the monochromatic method against the corrected chlorophyll *a* concentration of the diluted standard solutions measured using the fluorometer. The correlation coefficient is equivalent to F_s. This was calculated to be 0.9995 for the 10-AU fluorometer and 0.9982 for the TD-700 fluorometer, and is shown in Figures 2a and 2b. These values were accepted as they are ≥ 0.995.

The calibration was then checked by measuring two ready-to-use liquid fluorometric standards, one high-level concentration and one low-level concentration (part number 10-850, purchased from RSAqua Ltd, Hampshire, UK). The measured concentrations were within ± 10% of the concentration given on the certificate of analysis.

The red solid secondary standard (10-AU part number 10-AU-904; TD-700 part number 7000-994 purchased from RSAqua Ltd, Hampshire, UK) was measured daily as a system suitability check. The solid secondary standard is a simple, stable and less expensive alternative to multiple calibrations with a primary standard.

The fluorometers were calibrated every 6 months, or when an adjustment had made to the instrument, such as replacing the lamp or filters, as recommended by Turner Designs.

Chlorophyll *a* is extremely light and temperature sensitive; therefore, standard solutions were prepared in amber volumetric flasks wrapped in aluminium foil and kept at the same temperature as the laboratory during measurement. The lighting in the laboratory was subdued and the standards were measured on the same day as preparation.

A laboratory reference material (LRM) was prepared by growing in culture a species, *Thalassiosira* sp. (purchased from Culture Collection of Algae and Protozoa, Argyll, Scotland, UK). This species is commonly observed in the phytoplankton community in Scottish waters. A series of filter papers were prepared by filtering a known volume of culture through a Whatman GF/F filter paper. The filter papers were preserved by 'flash freezing' in liquid nitrogen and stored in a cryogenic freezer. An LRM sample was analysed

with every 20 samples, and the concentration of uncorrected chlorophyll *a*, corrected chlorophyll *a* and phaeophytin *a* determined. The data obtained from the LRM was plotted on Shewhart control charts with warning and action limits drawn at ± 2 x and ± 3 x the standard deviation of the mean. Further quality assurance was provided through successful participation in the chlorophyll programme of QUASIMEME (Quality Assurance of Information for Marine Environmental Monitoring in Europe).

Sampling

Seawater is collected using pump or discrete water bottle sampling from specific depths or more routinely, samples are collected using a Lund 0 – 10 m integrating hose sampler deployed vertically on station alongside a CTD (conductivity, temperature, depth) sampler. Some samples are collected from non toxic seawater supplies on board the ships. The CTD samplers are usually equipped with fluorescence profilers and sometimes other sensors. Often the chlorophyll samples collected from discrete depths are used to calibrate the fluorescence profilers on the CTD casts. The seawater collected is decanted into clean 1, 2 or 5 litre polyethylene bottles. Samples collected aboard the research vessels FRV *Scotia* and FRV *Clupea* are filtered on board, whereas samples collected from inshore sites, i.e. Stonehaven and Loch Ewe, are kept cool and protected from light. These are then filtered on return to the laboratory within 2 hours of collection as algal populations change quickly due to photodecomposition. If there is a delay in filtering the sample then it is stored in the dark in a refrigerator and filtered as soon as possible. Prior to sub-sampling the polyethylene bottle containing the sample is gently agitated to re-suspend any large cells that may have settled. The sample is filtered through a Whatman GF/F 47 mm filter paper (0.7 µm retention), using a low vacuum of approximately 0.5 atm to avoid damaging the cells. Usually a standard volume to be filtered is specified for each investigation, in the range 200 – 2000 ml. Where filters clog then a note is made of the precise volume filtered, if not then of the standard volume used. The filter paper is removed from the fritted base using tweezers, avoiding disturbing the filtrate, and folded once (algae inside). The folded filter paper is blotted gently with tissue to remove excess moisture and placed in a pre-numbered centrifuge tube. The centrifuge tubes containing the samples are protected from light either by wrapping the centrifuge tube in aluminium foil or by storing in a light tight insulated container and frozen immediately at -20ºC. A record is made of the sample number, sample type, date, time, depth, position, and the volume filtered.

Sample Preservation

Filter papers were stored in a laboratory freezer held between -10ºC and -40ºC aboard the research vessels. Samples were transported back to the laboratory frozen in coolboxes, where they were stored in a cryogenic freezer held between -60ºC and -90ºC prior to analysis.

Extraction of the Samples

Samples were extracted in one of two ways.

Extraction by grinding

Samples were extracted in 90% acetone with a mechanical tissue grinder. The sample was ground for less than 1 minute so as not to overheat the sample and cause degradation of the pigments. The resultant filter slurry was then transferred to a 15 ml polypropylene centrifuge tube and centrifuged at 3000 rpm for 15 minutes.

Extraction by soaking

Samples were extracted by adding 15 ml 90% buffered acetone to a 15 ml polypropylene centrifuge tube containing the filter paper. The samples were then allowed to soak in a laboratory refrigerator held between 0ºC and 8ºC for between 16 to 30 hours. This range was chosen as it was convenient for a typical laboratory working day.

Sample Measurement

The fluorescence of the sample extract was measured and recorded (Rb) on the 10-AU and/ or TD-700 fluorometers. Hydrochloric acid (0.1M, 100 µl) was then added to the cuvette containing the sample extract, to convert chlorophyll *a* to phaeophytin *a*. The acidified extract was mixed by inversion and the fluorescence was re-measured after 90 seconds (Ra). The concentration of uncorrected chlorophyll *a*, corrected chlorophyll *a* and phaeophytin *a* in the sample extract was then calculated using the fluorometric equations[15].

RESULTS AND DISCUSSION

Spectrophotometry

The concentration of the standard solutions used to calibrate the fluorometers must be determined spectrophotometrically using a multiwavelength spectrophotometer[15].

Chlorophyll compounds exhibit two absorbance maxima; one on the blue side of the visible spectrum (< 460 nm) and one on the red side (630 – 670 nm). Cartenoids, which are co-extracted with chlorophylls, exhibit strong absorption maxima on the blue side, therefore, chlorophyll spectrophotometric measurements are made on the red side. The main absorption bands of the chlorophyll compounds overlap in the range 630 – 670 nm. Several spectrophotometric methods have been described for the estimation of chlorophylls *a*, *b* and *c* in phytoplankton extracts and are known as trichromatic methods[11]. They all use extinction coefficients determined for pigments dissolved in 90% acetone, to calculate the concentrations of chlorophylls *a*, *b* and *c*.

The first trichromatic method was described by Richards and Thompson in 1952[18]. This method overestimates chlorophyll *a*, as a result of low extinction coefficients, particularly if chlorophyll degradation products are present in the sample[13]. Extinction coefficients for chlorophyll *c* were unavailable at the time, and so chlorophyll *c* concentrations were expressed in 'millispecific pigment units'. This method is now obsolete and should not be used[11]. Improvements in the trichromatic method were published in the next decade[19, 20, 21]. These methods all accurately determine chlorophyll *a*, but only those of Jeffrey & Humphrey [19] are accurate for chlorophylls *b* and *c*.

A serious error is introduced into the data when using the trichromatic methods when degradation products are present in the sample at significant concentrations relative to chlorophyll *a,* as they cannot be readily distinguished. Degradation products often dominate in deep waters, tidal estuaries, sediment traps and samples of, or contaminated by, zooplankton faeces[13]. The presence of phaeopigments results in an overestimation of chlorophyll *a*[13]. Chlorophyll *a* can be readily converted to its phaeo forms (phaeophytin *a* and phaeophorbide *a*) by the addition of weak acid. This results in a decrease in absorbance which is caused by the removal of the bound magnesium ion in the porphyrin ring. This method, which is commonly referred to as the monochromatic method, is used to determine chlorophyll *a* in samples containing phaeopigments. Chlorophyll *a* and chlorophyllide *a*, if present, are measured as 'chlorophyll *a*' and both phaeophytin *a* and

phaeophorbide *a* are measured together as 'phaeopigments'. The calculation of the phaeopigments assumes that all of this pigment is phaeophytin *a*, which is probably not the case[17], but the absorption coefficient of phaeophorbide is unknown. Lorenzen[17] determined that the absorbance at 665 nm was reduced by a factor of 1.7 when pure chlorophyll *a* in 90% acetone was treated by a weak acid, defining the 'acid ratio' for pure chlorophyll *a*. The value of the acid ratio for any unknown sample will range from 1.0 (pure phaeophytin *a*) to 1.7 (pure chlorophyll *a*).

The original monochromatic method described by Lorenzen[17] used an extinction coefficient of 91.11 g^{-1} cm^{-1} in 90% acetone which will give chlorophyll concentrations about 4% lower than those calculated by the recommended extinction coefficient of the trichromatic method[16]. In order to maintain consistency between the trichromatic and monochromatic spectroscopic methods and the fluorometric methods, described below, the corresponding factor of Lorenzen's equation was set to 11.4 rather than 11.0 given in the original method[11]. The monochromatic method and the revised Lorenzen equations are used to calculate the concentration of the stock standard solution prepared as part of the calibration procedure for the FRS method to determine uncorrected chlorophyll *a*, corrected chlorophyll *a* and phaeophytin *a* in marine phytoplankton using fluorometry.

These equations are particularly accurate if the unknown sample contains phaeophytin *a* as the only degradation product of chlorophyll *a* (which is seldom the case). However, the calculated weight of 'phaeopigment' can be overestimated by a factor of 1.51 if the unknown sample contains phaeophorbide *a* rather than phaeophytin *a*[13]. Phaeophytin *a* is found in zooplankton faecal pellets and sediments whereas phaeophorbide *a* is found in protozoan faecal pellets[13]. Any chlorophyll *b* present in the sample is also degraded by the acidification and is incorrectly expressed as 'phaeopigment'. For most samples from the euphotic zone the mass of chlorophyll *a* greatly outweighs that of phaeopigments, and so calculated concentrations of chlorophyll *a* should be accurate for these samples, provided caution is exercised in judging the accuracy of the phaeopigment concentration. Samples collected by FRS for the various research and monitoring programmes described above are mainly collected from the euphotic zone.

Fluorometric Methods

Fluorometric measurement of chlorophyll *a* is up to 50 times more sensitive than spectrophotometry, allowing the analyses of much smaller seawater samples and detection at lower levels[13]. Therefore, this technique is widely used by oceanographers as environmental chlorophyll *a* concentrations vary considerably with season and between locations, and are often low (< 0.1 µg l^{-1}). Fluorometer measurements are now routinely made by instruments deployed in the field alongside CTD profilers and other environmental sensors. Measurements of chlorophyll *a* and other pigments are also increasingly being used to ground truth satellite observations and estimates of phytoplankton biomass and distributions.

The fluorescence acidification techniques, which correct for the presence of phaeopigments, have been routinely used in oceanography for over 40 years[13, 22], and are similar to the monochromatic spectroscopic method. It is assumed that:

- When chlorophyll *a* is treated with weak acid it will be converted to phaeophytin *a* with 100% molar stoichiometry.

- The molar fluorescence response of phaeophytin *a* is lower than that for chlorophyll *a*, yielding a constant relative ratio of fluorescence responses for the two pigments.

Fluorometers are fitted with specific optical kits for the application of interest, which result in wavelength dependant response characteristics that are unique to each instrument. Unlike spectrophotometric methods, the sensitivity coefficient and the maximum fluorescence: acid ratio must be determined with a standard of pure chlorophyll *a* that is free from both phaeopigments and chlorophyll *b*. The concentration of the chlorophyll *a* calibration solutions are determined using the monochromatic spectroscopic method described above.

FRS has used an extractive fluorometric method for many years, which uses an acidification step and the fluorometric equations to estimate the corrected chlorophyll *a* and phaeophytin *a* concentrations in marine samples. This method is also the standard method for measuring chlorophyll *a* in international marine monitoring programmes and there are long national and international time series data which have to be related to and contributed to. This method was validated on both the Turner 10-AU and Turner TD-700 fluorometers.

Comparison of extraction methods

At FRS, prior to May 2006, the pigments were extracted from the filter paper by grinding. A motor-driven Teflon pestle was used to convert the sample into a slurry in a glass homogenisation tube. This is a time consuming process which involved the awkward operation of transferring the slurry into a centrifuge tube which can sometimes result in loss of sample. Initial experiments carried out in 2004 at FRS showed that the grinding process created heat that could result in the degradation of chlorophyll *a* and gave a more variable set of results when compared with extraction by soaking. Extraction by soaking alone is the most common method currently used by laboratories. It is simple and suitable for preparing large numbers of samples for analysis. Extraction by soaking requires extended extraction times. Soaking overnight in acetone, in a dark refrigerator (approximately 5ºC), has been shown to be sufficient for the complete extraction of pigments[13].

Acetone (90%) has been validated as an extraction solvent for chlorophyll *a* and phaeopigments *a* as biomarkers in routine field work[11]. Spectral data for chlorophylls *a*, *b* and *c* in 90% acetone are well known[13] and accurate spectrophotometric equations have been derived[11, 13, 19]. This extraction solvent is also used in the United States Environmental Protection Agency Method 445.0[15] for determination of chlorophyll *a* and phaeophytin *a* by fluorescence. Buffering the acetone with magnesium carbonate solution helps to prevent premature degradation of chlorophyll *a* to phaeophytin *a*, and is recommended by Turner Designs.

Samples were prepared from an *Alexandrium* sp. culture grown at FRS at approximately 10% and 90% of the analytical working range of the fluorometer [i.e. Rb values of 20 µg l^{-1} and 180 µg l^{-1} chlorophyll *a* (where Rb is the fluorescence reading before acidification)]. Ten replicate samples of both concentrations (~10% and 90%) were extracted by grinding. This was repeated using the soaking method.

An aliquot of each sample extract was measured on both the 10-AU and TD-700 fluorometers and the concentration of uncorrected chlorophyll *a*, corrected chlorophyll *a* and phaeophytin *a* was determined. Tables 1a and 1b summarise the results for uncorrected chlorophyll *a* and corrected chlorophyll *a* respectively. Results for soaked samples which were mixed by centrifugation were rejected as the calculated concentrations were highly erratic when compared with the results for soaked samples that had been mixed by inversion, showing that centrifugation alone did not adequately mix the samples. A 2 sample t-test at significance level of 5% was performed using MINITAB® statistical software version 14, to detect any significant statistical differences between samples which were extracted by soaking and samples which were extracted by grinding.

For both uncorrected chlorophyll *a* and corrected chlorophyll *a* higher means were achieved when both the 10% and 90% samples were extracted by soaking rather than grinding. Although there were no significant differences between the two extraction methods for the 10% samples (uncorrected chlorophyll *a*: 10-AU p-value of 0.141, TD-700 p-value of 0.224 corrected chlorophyll *a*: 10-AU p-value of 0.162, TD-700 p-value of 0.220), there were significant differences found between extraction by soaking and grinding for the 90% samples (uncorrected chlorophyll *a*: 10-AU p-value of 0.002, TD-700 p-value of 0.000 corrected chlorophyll *a*: 10-AU p-value of 0.029, TD-700 p-value of 0.001).

For the determination of phaeophytin *a* in both the 10 and 90% samples negative values were calculated in 7 out of the 64 measurements made. Negative values were obtained when the samples were extracted both by grinding and soaking. The extinction coefficient of phaeophytin *a* is less than chlorophyll *a*, so a formula was generated for calculating both[22] . However, this formula is valid when the only components in the sample are chlorophyll *a* and phaeophytin *a,* which is rarely the case in samples collected from the marine environment. The samples analysed here were prepared from an *Alexandrium* sp. culture and may contain phaeophytin *a*, and a range of other pigments and degradation products as well as chlorophyll *a*. High concentrations of chlorophyll *c* can result in a slight overestimation of chlorophyll *a* and an underestimation of phaeophytin *a* which can sometimes result in negative phaeophytin readings[23]. There is a tendency to forget these values and give them a value of zero. However this is not recommended as a negative phaeophytin *a* appears as positive chlorophyll *a*[24], i.e.; the lower the value for phaeophytin *a*, the higher the value for corrected chlorophyll. Negative phaeophytin *a* concentrations may also indicate the presence of chlorophyll *c* in the samples[23].

Extraction by soaking will be used in all future FRS analyses as this produced higher mean concentrations for both uncorrected chlorophyll *a* and corrected chlorophyll *a* when compared to extraction by grinding. Extraction by soaking is also much less time consuming than extraction by grinding and there are no sample transfer or sample heating issues involved. This method was optimised and validated as discussed below.

Varying the soaking time

The period of time that the samples could be left to soak for was determined. Twenty-four hours was taken to be the optimum soaking time as this was used in the initial tests carried out in 2004 at FRS. Other time points tested were 16, 20, 28 and 30 hours as these were convenient for a typical laboratory working day.

Samples were prepared from a *Chaetoceros* sp. culture grown at FRS at approximately 50% of the analytical working range of the fluorometer (i.e. Rb value of 100 µg l^{-1} chlorophyll *a*). Seven samples were soaked for 16, 20, 24, 28 and 30 hours in a laboratory refrigerator. The data was analysed using MINITAB® statistical software version 14. A 2 sample t-test at significance level of 5% was used to detect statistical differences between the extraction times. As 24 hours was regarded as the optimum soaking time the other time points were tested against this.

Tables 2a and 2b summarise the data when the soaking time was varied for uncorrected chlorophyll *a* and corrected chlorophyll *a*.

For the samples analysed on the TD-700 fluorometer the mean corrected chlorophyll *a* concentration increased with an increase in soaking time. This trend was not observed with the data collected using the 10-AU fluorometer for corrected chlorophyll *a*. Only 2 significant statistical differences were found; for uncorrected chlorophyll *a* determined on the 10-AU when the 20 hour soak was compared with the 24 hour soak (p-value of 0.020) and for

corrected chlorophyll *a* determined on the 10-AU when the 20 hour soak was compared with the 24 hour soak (p-value of 0.013).

The test data showed that optimally the samples should be soaked for at least 16 and up to 30 hours. Soaking times out with this period were not tested as they were not convenient for a typical working day in the laboratory. The sample extracts were always measured within this time window. We note too, that Wasmund *et al.*[25] have shown that extracts can be stored for 3 months at -20ºC without significant loss of chlorophyll *a*.

Method validation

The extraction by soaking method using 90% buffered acetone as the extraction solvent and 16 – 30 hours soaking in a laboratory refrigerator was fully validated on both the Turner 10-AU digital fluorometer and the Turner TD-700 laboratory fluorometer. The following was investigated:

- Standard and sample repeatability (within batch)
- Standard and sample reproducibility (between batch)
- Limit of detection (LOD)
- Bias / Recovery

The linearity was checked as part of the instrument calibration and is discussed above (Calibration and Quality Control).

The validated method is given in Appendix II.

Standard and sample repeatability

A stock chlorophyll *a* standard solution was prepared from *Anacystis nidulans* algae (purchased from Sigma-Aldrich Company Ltd., Dorset, UK) in 90% buffered acetone at a concentration of ~ 10,000 µg l^{-1} chlorophyll *a*. The actual concentration of the stock standard solution was determined using the monochromatic spectroscopic method and the revised Lorenzen equations (the equations are given in Appendix I)[11, 13]. The stock standard solution was diluted to give standards at approximately 10% and 90% of the analytical working range of the fluorometer (i.e. Rb values of 20 and 180 µg/l chlorophyll *a*). Samples were prepared from a *Thalassiosira* sp. culture grown at FRS Marine Laboratory at approximately 10% and 90% of the analytical working range of the fluorometer (i.e. Rb values 20 µg l^{-1} and 180 µg l^{-1} chlorophyll *a*). The standard and sample repeatability was investigated by analysing 7 replicates on the same day.

Tables 3a and 3b summarise the data for the standard and sample repeatability for uncorrected chlorophyll *a* and corrected chlorophyll *a*.

% CV for both uncorrected chlorophyll *a* and corrected chlorophyll *a* on both instruments was <12% for the high standards, <9% for the low standards, <10% for the high samples and <15% for the low samples. The calculated phaeophytin *a* concentrations were highly erratic with calculated concentrations ranging from -44 to 30 µg l^{-1}. This erratic data was observed in both the standards and the samples, but was more pronounced in the standards. In theory there should be no phaeophytin *a* present in the standards as these are prepared from pure chlorophyll *a* so any errors associated with the data are a result of errors with the analytical method and inaccuracies with the fluorometric equations.

Each individual fluorometer requires to be calibrated with a pure chlorophyll *a* standard to determine instrument specific calibration constants, which includes the before – to – after acidification response ratio (r). This was calculated to be 1.788 for the 10-AU fluorometer and 1.877 for the TD-700 fluorometer. It has been shown in the literature that r values can range from 1 to 11.5 depending upon the excitation wavelength[26]. The value for r influences the accuracy of chlorophyll determinations. Baker[27] has shown that the use of a lamp which gives an r value of 2.0 or greater will cause no amplification of error in the measurement of chlorophyll *a* and phaeophytin *a.* The values for r that have been calculated are less than 2.0. The lamp used is purchased from Turner Designs and is that recommended for the determination of chlorophyll *a* using an extractive fluorometeric method. The standard solutions were freshly prepared on the day of calibration and are protected from the effects of heat and light so it is highly unlikely that they will have degraded in the period between preparation and measurement.

Arar & Collins[15] state that for a set of 10 natural samples the %CV for uncorrected chlorophyll *a* should not exceed 15% for samples that are approximately 10 x the instrument detection limit. Arar & Collins[15] also state that the %CV for phaeophytin *a* might typically range from 10 to 50% They suggest highly erratic phaeophytin *a* concentrations may be due to poor mixing of the acidified sample when determining corrected chlorophyll *a*. If this were the case there would be poor agreement between corrected chlorophyll *a* and uncorrected chlorophyll *a*[15]. A 2 sample t-test using MINITAB® statistical software version 14 was performed to assess if there were significant statistical differences between the uncorrected chlorophyll *a* results and the corrected chlorophyll *a* results for each data set. No significant statistical differences were found at the 5% significance level when comparing the uncorrected chlorophyll *a* data with the corrected chlorophyll *a* data indicating that the variable phaeophytin *a* concentrations are not a result of inadequate mixing after acidification.

The 10% and 90% samples were prepared from a *Thalassiosira* sp. culture. *Thalassiosira* sp. is a diatom culture which will contain levels of chlorophyll *c*. As discussed above high concentrations of chlorophyll *c* can result in a slight overestimation of chlorophyll *a* and an underestimation of phaeophytin *a,* which can sometimes result in negative phaeophytin readings[23].

Stich & Brinker[28] compared chlorophyll *a* concentrations determined using a monochromatic spectrophotometric method in non-acidified extracts (uncorrected chlorophyll *a* concentrations) and acidified extracts (corrected chlorophyll *a* concentrations) with concentrations determined by high performance liquid chromatography (HPLC) in non-acidified extracts. They found that the spectrophotometric determinations of chlorophyll *a* in the non-acidified and acidified extracts produced significantly different results. However, the concentration of chlorophyll *a*, determined using the spectrophotometric method, in the non-acidified extracts did not differ significantly from the concentration determined using the HPLC method, but the spectrophotometric acidified extracts did. The authors suggested that the 'phaeophytin correction' by acidification impairs the chlorophyll *a* measurement rather than improve it and recommended that the acidification step should be abandoned, as the results from non-acidified extracts are more accurate than the results from acidified extracts. Furthermore, it is well documented that the fluorometric acidification technique is inaccurate when chlorophyll *b* is present, grossly overestimating the phaeopigment component, as a result of spectral overlap of phaeophytin *b* with chlorophyll *a* [15, 26, 29].

Welschmeyer described a non-acidification method that accurately determines chlorophyll *a* in the presence of phaeopigments and chlorophyll *b*[30]. This method uses a different lamp and filters from the acidification method described above, which provides maximum sensitivity to chlorophyll *a* while maintaining desensitised responses to chlorophyll *b* and

phaeophytin *a*. The calibration procedure is reduced to determining the response factor for sensitivity setting (S) and reduces the requirement for pure chlorophyll *a* standards; pigment extracts from natural sources can be used as calibration solutions as there is some tolerance for the presence of accessory chlorophylls and phaeopigments. However, like the other spectrophotometeric and fluorescence methods described, this method does not distinguish chlorophyllide *a* from chlorophyll *a* because the two pigments have identical spectral properties. In addition this method does not provide information on phaeopigment concentrations.

Therefore, the extractive fluorometric method for the determination of phaeophytin *a* in marine algae should only be used to give indicative concentrations and cannot be considered to be a quantitative method. For the accurate quantitative determination of the individual pigment components found in marine algae an HPLC method is recommended[13, 29]. A review of some of the many HPLC methods cited in the literature is given later in this report.

Standard and Sample Reproducibility

The 90 and 10% standards and samples prepared as above were also analysed on different days to give the between batch precision with one standard and sample being analysed on each of 7 days.

Tables 4a and 4b summarise the data for the standard and sample reproducibility for uncorrected chlorophyll *a* and corrected chlorophyll *a*. The data for the standard reproducibility are expressed as a percentage of the theoretical concentration of the standard solution.

% CV for both uncorrected chlorophyll *a* and corrected chlorophyll *a* on both instruments was <8% for the high standards, <14% for the low standards, <11% for the high samples and <11% for the low samples.

Limit of Detection

The limit of detection (LOD) was determined based on the response of the lowest calibration standard (1 µg l^{-1}); as the response from the blank filter was much lower than a typical sample. The LOD was calculated for uncorrected chlorophyll *a* and corrected chlorophyll *a* for both the 10-AU and TD-700 fluorometers using typical sample volumes filtered (0.2 – 2 l) and sample extract volume (15 ml). The LOD for each sample volume is summarised in Tables 5a (10-AU) and 5b (TD-700).

Bias / Recovery

The recovery samples were prepared by spiking 250 ml of *Thalassiosira* sp. culture with 10 ml of chlorophyll *a* standard. The actual concentration of the stock standard solution was determined using the monochromatic spectroscopic method and the revised Lorenzen equations (the equations are given in Appendix I)[11, 13] and calculated to be 10305.144 µg l^{-1}. Aliquots (10 ml) of both the unspiked culture and the spiked culture were filtered onto Whatman GF/F filter papers. The filter papers were preserved by 'flash freezing' in liquid nitrogen and stored in a cryogenic freezer until the day of analysis. One spiked filter paper was analysed each day for 7 days.

Tables 6a and 6b summarise the recovery data for uncorrected chlorophyll *a* and corrected chlorophyll *a* on both the 10-AU and TD-700 fluorometers. For both instruments the mean

% recovery is ~73% for uncorrected chlorophyll *a* and corrected chlorophyll *a* and the %CV is <15%.

Arar & Collins[15] reported the results from a multi laboratory validation and comparison study of the United States Environmental Protection Agency method for 'In Vitro Determination of Chlorophyll a and Phaeophytin a in Marine and Freshwater Algae by Fluorescence'. To address bias in chlorophyll *a* determination, three pure algal cultures (*Amphidinium*, *Dunaliella* and *Phaeodactylum*) were grown and 4 different concentrations of each were prepared. Considerable lab-to-lab variation was found for the bias determination and the median was found to be a better measure of central tendency rather than the mean. The median % recoveries for *Amphidinium* ranged from 107 to 111% for the 4 concentration levels, from 210 to 250% for *Dunaliella* and 254 to 296% for *Phaeodactylum.* One reason for the high recoveries for *Phaeodactylum* was that it contained significant amounts of chlorophyllide *a* which is determined as chlorophyll *a* by fluorescence. No explanation was given for the high recoveries for *Dunaliella*.

High Performance Liquid Chromatography (HPLC)

It is evident that complex mixtures of pigments, such as those found in the marine environment cannot be assayed accurately using simple spectroscopic or fluorometric methods for chlorophyll *a*. Separation techniques were developed to allow for a more accurate assessment of chlorophyll compounds in the late 1970's and early 1980's, and subsequently HPLC methods of increasing complexity have been published using both reverse-phase and normal phase systems[13]. Generally normal phase HPLC systems are less selective for algal pigments than reverse-phase systems. There is no one ideal method for separating and analysing the many chlorophyll compounds, cartenoids and degradation products found in aquatic systems. Until 1988, the best methods were that of Mantoura & Llewellyn[31] for separation of chlorophyll compounds and their derivatives and Wright & Shearer[32] for carotenoid resolution.

Mantoura & Llewellyn[31] used a gradient HPLC system from 100% solution A [10 : 10 : 80 (v/v/v) mixture of ion-pairing reagent, water and methanol] to 100% solution B [20: 80 (v/v) mixture of acetone and methanol] using a 25 x 0.5 cm column packed with octadecyl-silane bonded 5 µm ODS-Hypersil. Fluorescence and absorbance detectors were used to quantify fourteen chlorophyll compounds and their breakdown products and seventeen carotenoids respectively. Detection limits of 0.01 – 0.2 ng on column (20 µl injection) for the chloropigments and 2 – 6 ng on column (20 µl injection) for the cartenoids were found. Chlorophyll *a* concentrations of 0.1 ng l^{-1} were detected in seawater samples.

Wright & Shearer[32] used a gradient HPLC system from 90% acetonitrile to 100% ethyl acetate using two octadecyl silica columns connected in series (Rad-Pak A, 5 µm particle size). The first column was protected by a guard column (RCSS Guard-PAK) and a precolumn filter. The pigments were detected using the sum of absorbances at 405 nm and 436 nm from a Waters 440 two-channel absorbance detector. Forty-four (44) carotenes, xanthophylls, chlorophylls and their degradation products were separated with greater resolution than previously reported.

The Wright & Shearer method[32] does not resolve the polar chlorophylls c_1, c_2, c_3 and Mg-2,4 divinyl phaeoporphyrin a_5 mono methyl ester (MgDVP) or adequately resolve the chlorophyllides *a* and *b*. A single method which was suitable for determining the full range of important chlorophyll compounds, their breakdown products and taxonomically significant carotenoids found in phytoplankton was urgently required, particularly for international oceanographic programs looking at climate change. One of the objectives of the Scientific Committee on Oceanic Research Working Group 78 (SCOR WG 78) was to evaluate gradient HPLC systems for phytoplankton pigments[13, 33].

The SCOR WG 78 members developed a significantly improved HPLC system which separated more than 50 chlorophylls, cartenoids, their derivatives and isomers from marine phytoplankton in one ternary gradient[13, 33]. The solvent system of Wright & Shearer[32] was combined with the solvent system used by Welschmeyer for separating polar chlorophylls. Spherisorb ODS2 25 cm x 4.6 mm ID, 5 µm particle size columns packed by Australian Government Analytical Laboratories, Melbourne were used. Similar resolution was obtained with commercial C_{18} columns, although some were not as efficient, particularly in resolving lutein and zeaxanthin. Lutein and zeaxanthin are xanthophylls, which are oxidation products of carotenes. Lutein is a major pigment (>10%) found in *Chlorophyceae* algae and a minor (1-10%) pigment found in *Prasinophyceae* algae[13]. Zeaxanthin is a major pigment found in *Cyanophyta*, *Prochlorophyta* and *Rhodophyta* algae, a minor pigment found in *Prasinophyceae* algae and a trace pigment (<1%) found in *Eustigmatophyta* algae[13]. Pigment detection was at 436 nm for all chlorophylls and carotenoids and 405 nm for phaeophytin *a* and phaeophorbide *a.* This method gave better resolution of the polar chlorophylls and carotenoids whilst retaining the resolution of late – eluting pigments. A further advantage of this method is that no ion-pairing reagent is necessary as in the method of Mantoura & Llewellyn[31].

The range of compounds separated using this improved method is comprehensive, but it does exclude the *Prochlorophyceae* algal class which is widely distributed in the world's oceans. This was omitted as no culture of an isolated representative was available at the time. Prochlorophytes contain chlorophyll *a* - like and chlorophyll *b* – like pigments which have now been identified as 8-desethyl-8-vinyl chlorophyll *a* and *b* (or divinyl chlorophylls *a* and *b*). New advances in HPLC methods now allow both divinyl chlorophylls *a* and *b* to be separated from chlorophylls *a* and *b* in C_8 reverse phase systems, and these are recommended for use when prochlorophytes are of significance in field samples[13, 34, 35]. Chlorophyll c_3 was clearly resolved from other chlorophyll c pigments, but chlorophylls c_1, c_2 and MgDVP could not be resolved from each other which is a common problem to other HPLC systems using C_{18} columns[13, 33]. Since the SCOR WG 78 HPLC methods have been published which separate chlorophylls c_1, c_2, c_3 and MgDVP[36, 37, 38]. Two important fucoxanthin derivatives – 19'-hexanoyloxyfucoxanthin and 19'-butanoyloxyfucoxanthin – were not chromatographically separated from 9'-*cis* neoxanthin and siphonaxanthin respectively. These fucoxanthin derivatives are important indicators of particular prymnesiophytes, crysophytes and dinoflagellates. Van Heukelem *et al.*[39] have since used polymeric columns to separate several difficult cartenoid pairs including 19'-hexanoyloxyfucoxanthin and 9'-*cis* neoxanthin. HPLC methodology for the separation and accurate determination of the full suite of chlorophylls, cartenoids and degradation products is steadily improving with the discovery of new pigments and the improved resolution of 'old ones'. This added information can give valuable information about the contribution of different functional groups to the biomass of the phytoplankton community.

In marine ecology an accurate knowledge of the chlorophyll *a* concentration is often all that is required. Two simple but efficient HPLC systems were also developed during the SCOR WG 78. The first is an isocratic HPLC method for rapidly separating chlorophyll *a* from other pigments and the second is a step-isocratic (two solvents) method for separating and detecting chlorophylls, *a*, *b* and c_{1+2} and ten additional chlorophyllides and phaeopigments[13]. The analytical column used for both methods was a Perkin Elmer 3 µm Pecosphere C_{18}. The HPLC system was based on the Perkin Elmer (PE) system. The separated pigments were serially detected using a spectrophotometer with a wavelength of 440 nm followed by a LS-40 fluorescence detector with variable excitation and emission options. For the isocratic method the optimum separation of chlorophyll *a* was obtained with a 90: 10 (v/v) mixture of methanol and acetone. Chlorophyll *a* was eluted quickly, retention time of 2.4 minutes, and was fully resolved from the neighbouring chlorophyll *a*-allomer and chlorophyll *b*. Phaeophytin *a* and pyrophaeophytin *a* were also resolved with retention times of 6.5 minutes

and 11 minutes respectively, although these were deemed impractically long. Pigments more polar than chlorophyll *b* (e.g. chlorophyll *c*, chlorophyllide *a* and phaeophorbide *a*) co-eluted as a broad peak just after the solvent front, but were well resolved from chlorophyll *a*. The analytical performance of this method was compared to other published isocratic and step-isocratic methods, and was found to significantly improve the analysis time of chlorophyll *a* and solvent consumption[13]. An 8-minute elution cycle was adopted. The HPLC column was flushed periodically with acetone to avoid possible co-elution errors between chlorophyll *a* and pyrophaeophytins from previous samples.

The addition of a two-way valve to the inlet of a basic HPLC pump is an inexpensive way of transforming an isocratic HPLC into a two-eluant step-isocratic HPLC. The choice of the first eluant [80 : 20 (v/v) mixture of methanol and ammonium acetate] was based on the success of methanol based solvent separating the more polar chlorophylls in many HPLC systems[31, 33]. The second, less polar, eluant was the same as that used in the isocratic method described above. This was chosen to eluate the hydrophobic pigments (e.g. chlorophyll *a*, β,β-carotene). The three principal chlorophylls *a*, *b*, and c_1+c_2 and ten other chlorophyllides, phaeopigments and pyrophaeophytin *a* were reproducibly separated in 20 minutes using this system[13].

As discussed above, divinyl chlorophylls *a* and *b* co-elute with chlorophylls *a* and *b* when using C18 reverse-phase columns. In samples containing equal amounts of chlorophyll *a* and divinyl chlorophyll *a*, these two pigments would be overestimated by approximately 5% using both the isocratic and step-isocratic methods described. If it is necessary to separate divinyl chlorophyll *a* from chlorophyll *a* then the method of Gorericke & Repeta[34] should be used. Divinyl chlorophyll *a* and divinyl chlorophyll *b* are major pigments found in *Prochlorophyta* algae[13].

The United States Environment Protection Agency (EPA) has also published a reversed-phase HPLC method to quantify chlorophyll *a* and chlorophyll *b* in marine and freshwater phytoplankton, and identify other pigments of interest[40]. Unlike other published HPLC methods, this method includes quality assurance / quality control procedures and sample collection and extraction procedures.

CONCLUSIONS

1. Initial experiments carried out at FRS Marine Laboratory in 2004 showed that extraction by grinding degraded the sample and gave a more variable set of results when compared with extraction by soaking. Extraction by soaking alone is the most common method as it is suitable for preparing large numbers of samples for fluorometry. Further comparisons between the two extraction methods were carried out and the data showed that extraction by soaking gave higher mean concentrations when compared with extraction by grinding. Extraction by soaking also had the advantage of being simpler and less time consuming.

2. The extraction by soaking method was optimised. Samples were extracted by soaking in 90% buffered acetone in a refrigerator for a period of 16 to 30 hours.

3. The method was validated on both the Turner 10-AU digital fluorometer and the Turner TD-700 laboratory fluorometer. This method gives a direct reading of the uncorrected chlorophyll *a* concentrations. Acidification of the extracts converts chlorophyll *a* to phaeophytin *a*, and the corrected chlorophyll *a* concentration and phaeophytin *a* concentration were calculated using the fluorometric equations.

4. Replicate analysis of high (90%) and low (10%) standards on the same day gave a %CV of <12% for both uncorrected chlorophyll *a* and corrected chlorophyll *a*. Replicate analysis of high (90%) and low (10%) samples on the same day gave a %CV of <15% for both uncorrected chlorophyll *a* and corrected chlorophyll *a*. Replicate analysis of high (90%) and low (10%) standards on the different days gave a %CV of <14% for both uncorrected chlorophyll *a* and corrected chlorophyll *a*. Replicate analysis of high (90%) and low (10%) samples on different days gave a %CV of <11% for both uncorrected chlorophyll *a* and corrected chlorophyll *a*.

5. The limit of detection (LOD) was calculated for uncorrected chlorophyll *a* and corrected chlorophyll *a* using typical sample volumes filtered (0.2 – 2 l) and sample extract volume (15 ml). The LODs for both uncorrected chlorophyll *a* and corrected chlorophyll *a* were found to be 0.007 to 0.07 µg l^{-1} on the 10-AU fluorometer and 0.008 to 0.08 µg l^{-1} on the TD-700 fluorometer. The mean % recovery was ~73% for both uncorrected chlorophyll *a* and corrected chlorophyll *a*, with %CV of <15%. The method was therefore acceptable for the determination of uncorrected chlorophyll *a* and corrected chlorophyll *a*.

6. Highly erratic data was generated for the determination of phaeophytin *a*, with negative concentrations calculated. This was due to the error in the analytical method and the flurometric equations used to calculate the phaeophytin *a* concentration. The formula for calculating phaeophytin *a* is valid when the only components in the sample are chlorophyll *a* and phaeophytin *a*. However, in algae samples collected from the marine environment a wide variety of chlorophyll compounds and degradation products can be found in any one sample at a time. It is widely reported that the fluorometric acidification technique is inaccurate when chlorophyll *b* and/or chlorophyll *c* are present in the sample and that the accuracy of the phaeopigment concentration is questionable. Several researchers also recommend that the acidification step should be abandoned as the results are inaccurate.

7. The only way to accurately assess all chlorophylls in the presence of degradation products is to use a separation technique such as high performance liquid chromatography (HPLC). The Scientific Committee on Oceanic Research Working Group 78 (SCOR WG 78) developed a significantly improved HPLC system which separated more than 50 chlorophylls, cartenoids, their derivatives and isomers from marine phytoplankton in one ternary gradient. Since then the HPLC methodology for the separation and accurate determination of the full suite of chlorophylls, cartenoids and degradation products is steadily improving. The SCOR WG 78 also developed two simple HPLC systems; the first was an isocratic method for separating chlorophyll *a* from other pigments and the second was a step-isocratic method for separating chlorophylls *a, b* and c_{1+2} plus ten additional chlorophyllides and phaeopigments.

RECOMMENDATIONS

1. Fluorometric measurement of chlorophyll *a* is seen as standard in the international scientific community and as such for continuation of existing time series, harmonisation with other institutions and submission to international databanks this measurement should for the moment continue within Fisheries Research Services (FRS). Development of HPLC methods for the analysis of phytoplankton pigments has the potential to provide an improved measurement of chlorophyll *a*, additional

information about the composition of the phytoplankton community and increase the capacity for phytoplankton physiological studies in FRS.

2. It is widely recognised that there are a number of issues with the fluorometric methods for the determination of phaeophytin *a*. For most samples collected from the euphotic zone the mass of chlorophyll *a* greatly outweighs that of phaeopigments. Calculated concentrations of chlorophyll *a* should be accurate for these samples but caution should be exercised in judging the accuracy of the phaeophytin *a*.

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TABLE 1a

Summary of uncorrected chlorophyll *a* (µg l^{-1}) data for comparison of extraction by grinding with extraction by soaking for samples prepared from an *Alexandrium* sp. culture at 90% and 10% of the analytical range.

Sample	10-AU 10% Grind	10-AU 10% Soak	TD-700 10% Grind	TD-700 10% Soak	10-AU 90% Grind	10-AU 90% Soak	TD-700 90% Grind	TD-700 90% Soak
1	0.264	0.296	0.236	0.293	3.000	3.000	2.876	3.129
2	0.365	0.446	0.377	0.402	2.925	3.270	2.958	3.402
3	0.354	0.432	0.375	0.437	2.970	3.390	2.861	3.504
4	0.380	0.404	0.386	0.393	30.90	3.210	2.922	3.255
5	0.365	0.432	0.363	0.452	2.325	3.660	2.375	3.765
6	0.323		0.317		2.325	3.390	2.435	3.381
7	0.350		0.363		3.045		3.315	
8	0.315		0.320		2.910		2.907	
9	0.366		0.387		2.610		2.567	
10	0.411		0.398		3.060		2.874	
Mean	0.349	0.402	0.352	0.395	2.826	3.320	2.809	3.406
Standard deviation	0.040	0.061	0.049	0.062	0.296	0.220	0.279	0.218
% CV	11.560	15.263	13.946	15.742	10.477	6.632	9.927	6.414

TABLE 1b

Summary of corrected chlorophyll *a* (µg l^{-1}) data for comparison of extraction by grinding with extraction by soaking for samples prepared from an *Alexandrium* sp. culture at 90% and 10% of the analytical range.

Sample	10-AU 10% Grind	10-AU 10% Soak	TD-700 10% Grind	TD-700 10% Soak	10-AU 90% Grind	10-AU 90% Soak	TD-700 90% Grind	TD-700 90% Soak
1	0.269	0.272	0.210	0.293	2.886	2.748	2.546	3.049
2	0.351	0.446	0.330	0.361	2.689	2.669	2.490	3.345
3	0.331	0.407	0.367	0.380	2.722	3.260	2.521	3.388
4	0.354	0.387	0.333	0.321	2.853	3.069	2.530	3.302
5	0.321	0.410	0.321	0.444	1.918	3.929	2.058	3.073
6	0.328		0.275		2.374	2.984	2.141	4.271
7	0.318		0.333		2.755	2.925	3.049	3.110
8	0.298		0.290		2.689		2.502	
9	0.341		0.373		2.197		2.367	
10	0.410		0.358		2.951		2.283	
Mean	0.332	0.384	0.319	0.360	2.603	3.084	2.449	3.362
Standard deviation	0.037	0.066	0.050	0.058	0.334	0.421	0.272	0.423
% CV	11.188	17.228	15.515	16.143	12.813	13.660	11.115	12.588

TABLE 2a

Summary data for uncorrected chlorophyll *a* (µg l^{-1}) extracted from a *Chaetoceros* sp. culture following extraction by soaking for 16, 20, 24, 28 and 30 hours.

Sample	10-AU 16 hours	10-AU 20 hours	10-AU 24 hours	10-AU 28 hours	10-AU 30 hours	TD-700 16 hours	TD-700 20 hours	TD-700 24 hours	TD-700 28 hours	TD-700 30 hours
1	1.665	1.620	1.635	1.890	1.560	1.475	1.656	1.569	1.745	1.617
2	1.650	1.650	1.725	1.620	1.665	1.508	1.644	1.617	1.574	1.643
3	1.785	1.560	1.860	1.560	1.785	1.749	1.502	1.857	1.598	1.706
4	1.695	1.710	1.740	1.680	1.665	1.559	1.632	1.743	1.677	1.655
5	1.740	1.605	1.620	1.650	1.710	1.629	1.631	1.563	1.607	1.775
6	1.725	1.635	1.800	1.710	1.740	1.652	1.566	1.704	1.613	1.806
7	1.695	1.605	1.725	1.680	1.620	1.584	1.550	1.694	1.734	1.593
Mean	1.708	1.626	1.729	1.684	1.678	1.593	1.597	1.678	1.649	1.685
Standard deviation	0.046	0.047	0.085	0.103	0.075	0.093	0.058	0.105	0.069	0.081
%CV	2.704	2.860	4.900	6.121	4.483	5.824	3.649	6.251	4.187	4.779

TABLE 2b

Summary data for corrected chlorophyll *a* (µg l^{-1}) extracted from a *Chaetoceros* sp. culture following extraction by soaking for 16, 20, 24, 28 and 30 hours.

Sample	10-AU 16 hours	10-AU 20 hours	10-AU 24 hours	10-AU 28 hours	10-AU 30 hours	TD-700 16 hours	TD-700 20 hours	TD-700 24 hours	TD-700 28 hours	TD-700 30 hours
1	1.325	1.367	1.345	1.689	1.308	1.074	1.422	1.176	1.376	1.401
2	1.367	1.266	1.417	1.358	1.404	1.142	1.342	1.123	1.262	1.330
3	1.466	1.312	1.568	1.338	1.502	1.518	1.188	1.401	1.247	1.342
4	1.394	1.433	1.449	1.220	1.223	1.197	1.287	1.460	1.422	1.379
5	1.430	1.220	1.361	1.387	1.345	1.262	1.342	1.222	1.262	1.497
6	1.440	1.351	1.512	1.374	1.440	1.299	1.185	1.324	1.262	1.691
7	1.423	1.318	1.443	1.384	1.230	1.166	1.176	1.345	1.392	1.213
Mean	1.406	1.324	1.442	1.393	1.350	1.237	1.277	1.293	1.318	1.407
Standard deviation	0.048	0.069	0.079	0.143	0.105	0.145	0.097	0.123	0.076	0.151
%CV	3.410	5.244	5.475	10.248	7.785	11.707	7.594	9.511	5.732	10.749

TABLE 3a

Summary data for uncorrected chlorophyll *a* (µg l^{-1}) in standard solutions and samples prepared from a *Thalassiosira* sp. culture at 90% and 10% of the analytical range analysed on the same day.

Sample	10-AU 90% Standard	10-AU 10% Standard	10-AU 90% Sample	10-AU 10% Sample	TD-700 90% Standard	TD-700 10% Standard	TD-700 90% Sample	TD-700 10% Sample
1	172.000	19.600	2.700	0.239	179.600	22.400	2.771	0.300
2	173.000	20.900	2.820	0.287	178.700	22.700	2.959	0.327
3	181.000	20.100	2.775	0.240	178.700	21.200	2.769	0.315
4	175.000	20.400	2.760	0.294	190.700	21.000	2.827	0.302
5	178.000	20.300	2.805	0.234	181.700	21.700	2.798	0.308
6	194.000	20.400	2.820	0.284	187.800	21.300	2.587	0.291
7	182.000	22.000	2.460	0.305	191.300	23.200	2.460	0.273
Mean	179.286	20.529	2.734	0.269	184.071	21.929	2.739	0.302
Standard Deviation	7.521	0.757	0.128	0.030	5.678	0.844	0.165	0.017
%CV	4.195	3.685	4.684	11.162	3.084	3.849	6.007	5.717

TABLE 3b

Summary data for corrected chlorophyll *a* (µg l^{-1}) in standard solutions and samples prepared from a *Thalassiosira* sp. culture at 90% and 10% of the analytical range analysed on the same day.

Sample	10-AU 90% Standard	10-AU 10% Standard	10-AU 90% Sample	10-AU 10% Sample	TD-700 90% Standard	TD-700 10% Standard	TD-700 90% Sample	TD-700 10% Sample
1	158.832	19.741	2.733	0.210	178.069	24.613	2.407	0.299
2	158.832	22.463	2.689	0.276	173.146	23.543	3.035	0.299
3	194.229	21.102	2.723	0.228	167.368	19.476	2.631	0.292
4	183.111	21.329	2.519	0.279	197.331	20.974	2.752	0.286
5	184.699	19.741	2.723	0.230	177.213	22.259	2.473	0.283
6	218.735	21.329	2.791	0.289	192.195	20.760	2.396	0.266
7	189.238	23.825	2.345	0.313	175.501	23.329	2.448	0.241
Mean	183.954	21.361	2.646	0.261	180.117	22.136	2.592	0.281
Standard Deviation	20.843	1.450	0.158	0.038	10.703	1.820	0.235	0.021
%CV	11.331	6.786	5.956	14.587	5.942	8.220	9.057	7.408

TABLE 4a

Summary data for uncorrected chlorophyll *a* in standard solutions and samples prepared from a *Thalassiosira* sp. culture at 90% and 10% of the analytical range analysed on different days. The data for the standard solutions is expressed as a percentage of the theoretical concentration of the standard. The data for the samples is given as µg l^{-1} uncorrected chlorophyll *a*.

Sample	10-AU 90% Standard	10-AU 10% Standard	10-AU 90% Sample	10-AU 10% Sample	TD-700 90% Standard	TD-700 10% Standard	TD-700 90% Sample	TD-700 10% Sample
1	97.592	98.933	2.805	0.275	105.904	105.376	2.382	0.294
2	106.580	103.303	2.985	0.281	112.193	112.385	2.658	0.281
3	107.777	100.040	2.340	0.308	107.277	94.592	2.651	0.267
4	97.173	103.725	2.670	0.260	102.892	102.229	2.580	0.248
5	109.237	102.304	2.685	0.264	106.785	111.073	2.439	0.293
6	102.361	105.327	2.625	0.273	104.103	107.847	2.858	0.305
7	104.659	110.105	2.370	0.261	107.721	111.106	2.529	0.263
Mean	103.626	103.391	2.640	0.274	106.696	106.373	2.585	0.278
Standard Deviation	4.799	3.679	0.228	0.017	2.984	6.321	0.158	0.020
%CV	4.631	3.559	8.642	6.036	2.797	5.943	6.107	7.282

TABLE 4b

Summary data for corrected chlorophyll *a* in standard solutions and samples prepared from a *Thalassiosira* sp. culture at 90% and 10% of the analytical range analysed on different days. The data for the standard solutions is expressed as a percentage of the theoretical concentration of the standard. The data for the samples is given as µg l^{-1} corrected chlorophyll *a*.

Sample	10-AU 90% Standard	10-AU 10% Standard	10-AU 90% Sample	10-AU 10% Sample	TD-700 90% Standard	TD-700 10% Standard	TD-700 90% Sample	TD-700 10% Sample
1	100.743	102.934	2.689	0.262	108.453	97.092	2.478	0.295
2	98.737	85.002	3.131	0.262	115.269	106.903	2.825	0.257
3	104.627	100.012	2.413	0.323	103.088	72.256	2.421	0.254
4	95.881	97.311	2.658	0.261	105.295	96.057	2.453	0.257
5	97.375	99.485	2.655	0.261	97.097	118.862	2.328	0.247
6	101.305	110.919	2.638	0.265	93.574	101.388	2.931	0.305
7	96.001	102.204	2.406	0.235	99.964	103.901	2.244	0.222
Mean	99.238	99.695	2.656	0.267	103.249	99.923	2.526	0.262
Standard Deviation	3.190	7.794	0.241	0.027	7.280	13.271	0.255	0.029
%CV	3.214	7.818	9.077	10.056	7.051	13.282	10.110	10.939

TABLE 5a

Limit of Detection (LOD) summary data for the Turner 10-AU Fluorometer. This was calculated based on the response of the lowest calibration standard (1µg l^{-1}) and typical volumes of sample filtered and sample extract.

Determinand	Concentration in 1 µg l^{-1} calibration standard	Volume filtered (l)	Volume extracted (ml)	LOD (µg l^{-1})
Uncorrected chlorophyll a	0.964	-	-	-
Uncorrected chlorophyll a	0.964	0.2	15	0.072
Uncorrected chlorophyll a	0.964	0.5	15	0.029
Uncorrected chlorophyll a	0.964	1	15	0.014
Uncorrected chlorophyll a	0.964	2	15	0.007
Corrected chlorophyll a	0.944	-	-	-
Corrected chlorophyll a	0.944	0.2	15	0.071
Corrected chlorophyll a	0.944	0.5	15	0.028
Corrected chlorophyll a	0.944	1	15	0.014
Corrected chlorophyll a	0.944	2	15	0.007

TABLE 5b

Limit of Detection (LOD) summary data for the Turner TD-700 Fluorometer. This was calculated based on the typical response of a 1µg $l^{-1}$and typical volumes of sample filtered and sample extracted.

Determinand	Concentration in 1 µg l^{-1} calibration standard	Volume filtered (l)	Volume extracted (ml)	LOD (µg l^{-1})
Uncorrected chlorophyll a	1.000	-	-	-
Uncorrected chlorophyll a	1.000	0.2	15	0.075
Uncorrected chlorophyll a	1.000	0.5	15	0.030
Uncorrected chlorophyll a	1.000	1	15	0.015
Uncorrected chlorophyll a	1.000	2	15	0.008
Corrected chlorophyll a	1.070	-	-	-
Corrected chlorophyll a	1.070	0.2	15	0.080
Corrected chlorophyll a	1.070	0.5	15	0.032
Corrected chlorophyll a	1.070	1	15	0.016
Corrected chlorophyll a	1.070	2	15	0.008

TABLE 6a

Summary data for uncorrected chlorophyll *a* (%) in recovery samples prepared from a *Thalassiosira* sp. culture analysed on different days.

Sample	10-AU	TD-700
1	81.076	83.587
2	87.626	74.126
3	67.976	70.705
4	71.615	75.290
5	67.976	59.788
6	62.881	76.309
7	67.976	73.034
Mean	72.446	73.263
Standard deviation	8.728	7.175
%CV	12.047	9.794

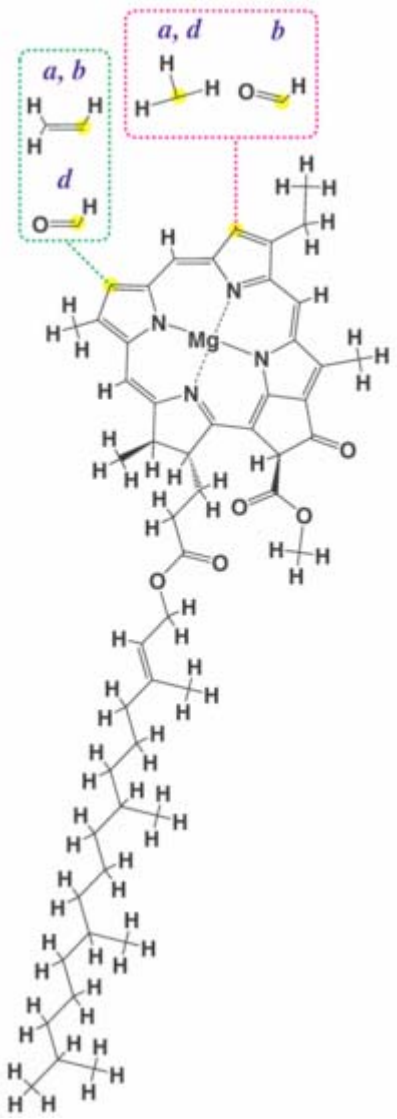
TABLE 6b

Summary data for corrected chlorophyll *a* (%) in recovery samples prepared from a *Thalassiosira* sp. culture analysed on different days.

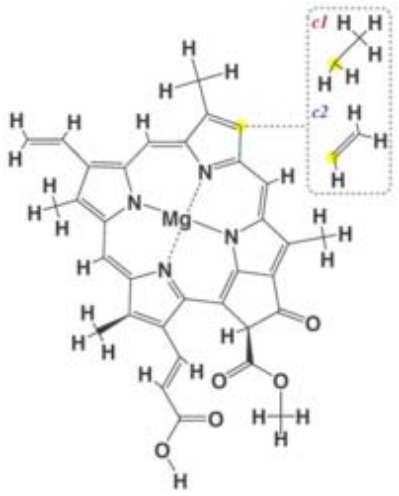
Sample	10-AU	TD-700
1	81.744	81.855
2	84.386	60.671
3	67.542	68.926
4	79.432	77.805
5	70.514	57.244
6	61.432	83.413
7	69.854	80.609
Mean	73.557	72.932
Standard deviation	8.416	10.682
%CV	11.441	14.647

Figure 1: Chemical structures of chlorophylls *a, b, c_1 c_2 and d*. Chlorophyll *d* is found in cyanobacteria and not applicable to this report Treatment of chlorophyll *a* with acid removes the central magnesium ion replacing it with two hydrogen ions to produce phaeophytin *a*. Phaeophytin *a* is a natural degradation product produced during photosynthesis. Chlorophylls *c* do not contain the phytol side chain.

Chemical structure of chlorophylls *a*, *b* and *d*.



Chemical structure of chlorophyll c_1 and c_2.



	Chlorophyll *a*	**Chlorophyll *b***	**Chlorophyll c_1**	**Chlorophyll c_2**
Molecular formula	$C_{55}H_{72}O_5N_4Mg$	$C_{55}H_{70}O_6N_4Mg$	$C_{35}H_{30}O_5N_4Mg$	$C_{35}H_{28}O_5N_4Mg$
C3 group	$-CH=CH_2$	$-CH=CH_2$	$-CH=CH_2$	$-CH=CH_2$
C7 group	$-CH_3$	-CHO	$-CH_3$	$-CH_3$
C8 group	$-CH_2CH_3$	$-CH_2CH_3$	$-CH_2CH_3$	$-CH=CH_2$
C17 group	$-CH_2CH_2COO-$ Phytyl	$-CH_2CH_2COO-$ Phytyl	-CH=CHCOOH	-CH=CHCOOH
C17-C18 bond	Single	Single	Double	Double

Figure 2a: Calibration curve for the determination of the response factor for sensitivity setting used (Fs) for chlorophyll a analysis using the 10-AU fluorometer

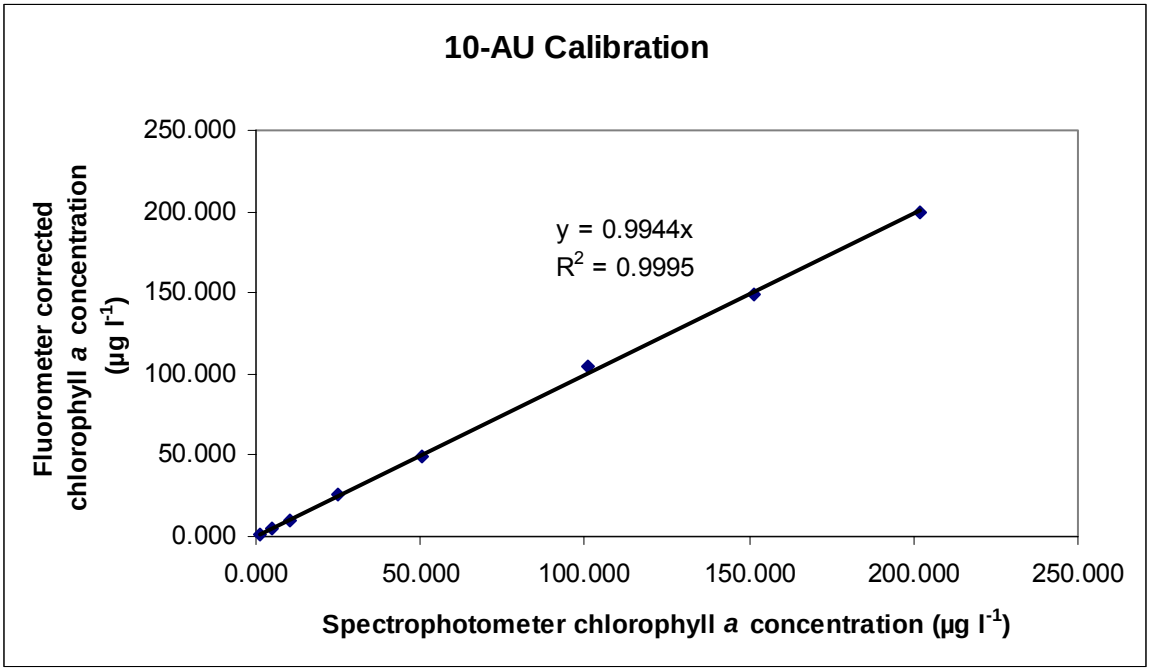
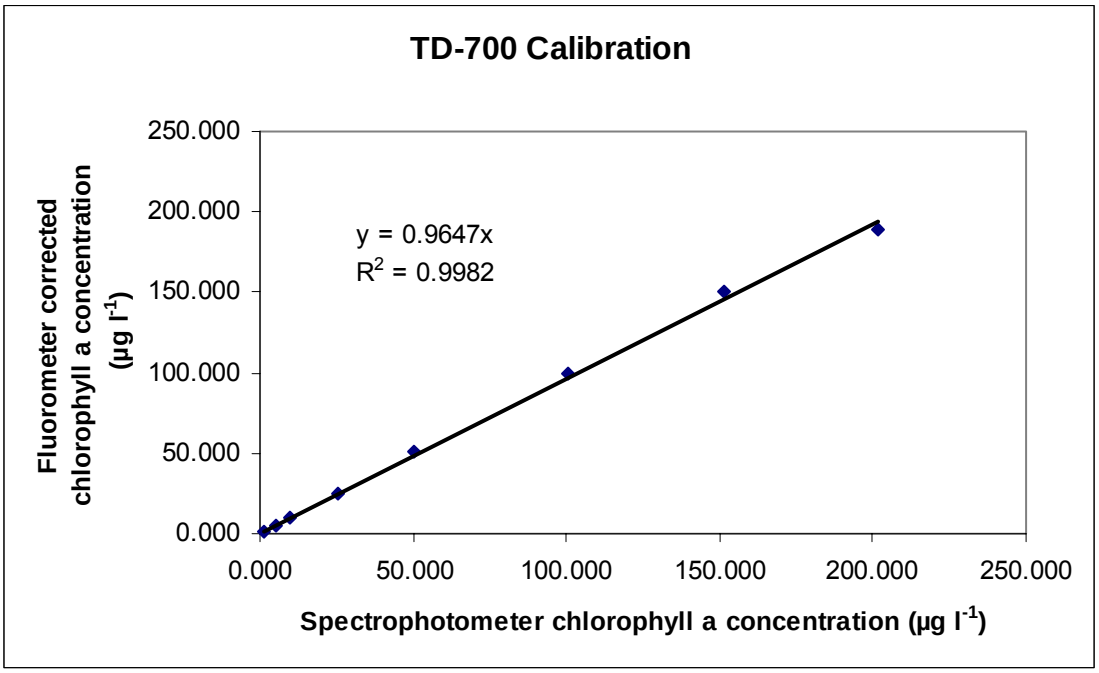


Figure 2b: Calibration curve for the determination of the response factor for sensitivity setting used (Fs) for chlorophyll a analysis using the TD-700 fluorometer



APPENDIX 1

THE REVISED LORENZEN EQUATIONS

$$Chlorophyll\ a\ (\mu g / l) = \left(\frac{11.4 \times 2.43 \times \left((R_{665b} - R_{750b}) - (R_{665a} - R_{750a}) \right)}{l} \right) \times 1000$$

Where:

- 11.4 is the absorption coefficient of chlorophyll *a*. See discussion in Aminot and Rey (2001)
- 2.43 is r/(r-1)
- R_{665b} is the absorbance of the standard at 665nm before acidification
- R_{750b} is the absorbance of the standard at 750nm before acidification
- R_{665a} is the absorbance of the standard at 665nm after acidification
- R_{750a} is the absorbance of the standard at 750nm after acidification
- l is the path length of the cuvette in centimetres

APPENDIX 2

Methods and Standard Operating Procedures (SOP) for the determination of uncorrected chlorophyll *a*, corrected chlorophyll *a* and phaeophytin *a* in marine phytoplankton using fluorometry

1. Introduction and scope

This method describes the procedure to be followed for determination of uncorrected chlorophyll *a*, corrected chlorophyll *a* and phaeophytin *a* in marine phytoplankton. **Note that this method is not accredited for phaeophytin *a*.** This method describes the use of both the Turner 10-AU fluorometer and the Turner TD-700 fluorometer. The Turner 10-AU fluorometer is the instrument of choice with the Turner TD-700 being used as a back-up. Typical concentration range is LOD – 200 µg l^{-1} uncorrected chlorophyll *a* and LOD – 200 µg l^{-1} corrected chlorophyll *a*.

2. Principle of the method

The degradation products of chlorophyll may constitute a significant fraction of the total green pigments present in seawater. These degradation products absorb light in the same part of the red spectrum as chlorophyll *a*. Chlorophyll *a* is readily converted to phaeophytin *a* by the addition of dilute hydrochloric acid. The reaction results in a decrease in absorbance as a result of the loss of the magnesium ion.

Chlorophyll containing phytoplankton in a measured volume of seawater is concentrated by filtering at low vacuum through a glass fibre filter paper. The pigments are extracted from the phytoplankton by soaking the filter paper in 90% buffered acetone overnight in a centrifuge tube. The centrifuge tube containing the extract is mixed by inversion before an aliquot is transferred to a glass cuvette and fluorescence is measured before and after acidification. Both fluorometers have been previously calibrated with a solution of known concentration of pure chlorophyll *a* (Turner 10-AU fluorometer SOP 3010; Turner TD-700 fluorometer SOP 3020), and therefore the concentration of uncorrected chlorophyll *a,* corrected chlorophyll *a* and phaeophytin *a* in the sample extract is calculated.

3. Reference material

The solid standard (Turner 10-AU fluorometer P/N: 10-AU-904; Turner TD-700 fluorometer P/N: 7000-994; Supplier: R.S. Aqua Ltd) is used as a system suitability check.

The LRM is prepared as directed in SOP 3030 and is used to maintain Shewhart control charts. A culture which is a common member of the phytoplankton community in Scottish waters is grown. A series of filter papers are prepared by filtering a known volume of this culture through a Whatman GF/F filter paper. The filter papers are preserved by 'flash freezing' in liquid nitrogen and storage in a cryogenic freezer. The concentration of uncorrected chlorophyll *a,* corrected chlorophyll *a* and phaeophytin *a* are calculated in the same way as the samples, but the volume of seawater filtered will be equal to 1 litre.

4. Reagents

- Acetone (Supplier: Rathburn Grade: HPLC or equivalent)
- Concetrated hydrochloric acid (Supplier: BDH Grade: AnalR or equivalent)
- Magnesium carbonate (Supplier: Sigma Catalogue number: M7179-500G or equivalent)
- Water (Supplier: Rathburn Grade: HPLC or equivalent)

5. Equipment

- 1 cm fluorescence cell (Supplier: Fischer Scientific Catalogue number: CXA-145-155Y)
- 1000 ml conical flask
- 1000 ml measuring cylinder
- 1000 ml volumetric flask
- 10-100 µl calibrated pipette
- 100-1000 µl calibrated pipette
- 15 ml centrifuge tubes, polypropylene (Supplier: VWR Catalogue number: 525-0118)
- Beakers
- Black box for transporting and storing samples in
- Calibrated bottle top dispenser
- Calibrated minimum / maximum thermometer (T197 or equivalent)
- Calibrated timer
- Calibrated top pan balance
- Centrifuge tube racks
- Disposable gloves
- Disposable measuring cups
- Laboratory refrigerator
- Pasteur pipettes
- Reagent bottles
- Tissues
- Turner 10-AU fluorometer (EN1277) or Turner TD-700 laboratory fluorometer (EN1300)
- Tweezers
- Whatman GF/F filter papers, 47 mm or 25mm

6. Environmental control

Chlorophyll *a* is extremely light sensitive. Avoid exposing the samples to strong light, particularly sunlight at all times. Use the black box to transport the samples and for storing the centrifuge tubes with the extracts prior to measuring on the fluorometer. Where the sample must be exposed, then work as quickly as possible.

Fluorescence is temperature dependant with higher sensitivity occurring at lower temperatures. Arar & Collins (1997) recommend that the fluorometer should be recalibrated if the ambient temperature fluctuates by more than ±3ºC. The fluorometers are kept in a temperature controlled laboratory. The temperature at calibration is noted on B 611. A maximum / minimum thermometer is used to note the temperature variation in the laboratory during analysis of the samples. If the temperature fluctuates by more than temperature on day of calibration ±3ºC discuss with technical management. The sample extracts should be stored in a cryogenic freezer and the instrument recalibrated before proceeding.

Fridge, freezer and cryogenic freezer temperatures are set and monitored as in SOP 0280.

7. Interferences

There are three types of chlorophyll; *a*, *b* and *c,* which are converted to phaeophytin *a, b* and *c* respectively when the magnesium ion is lost. Chlorophylls *b* and *c* may

significantly interfere with chlorophyll *a* measurements depending on the taxonomic composition of the phytoplankton. When chlorophyll *b* is present in the sample, there is a spectral overlap of chlorophyll *b* with chlorophyll *a* and phaeophytin *a*, which results in an underestimation of chlorophyll *a* accompanied by overestimation of phaeophytin *a*. The degree of interference depends upon the ratio of chlorophyll *a* :chlorophyll *b*. The presence of chlorophyll *c* causes the underestimation of phaeophytin *a*, but the effect is not as severe as the effects caused by chlorophyll *b*. This method can only be used to determine chlorophyll *a* and phaeophytin *a*. A trichromatic spectrophotometeric or HPLC method would need to be used to quantify chlorophylls *b* and *c* .

8. Sampling and sample preparation

The sampling and sample preparation is carried out by the client.

8.1 Collect the water sample using a water sampling bottle closed by messenger or electronic command at the sampling depth, by pump sampling from a specific depth or by using a depth integrating hose sampler. Most often water samplers are deployed alongside a conductivity/temperature/depth (CTD) sampler and electronic sensors for chlorophyll fluorescence etc.

8.2 Gently decant the seawater into a clean 1, 2 or 5 litre polyethylene bottle, preferably keeping the sample in the dark or at least out of sunshine and chilled in dim light.

8.3 Samples collected aboard the research vessels FRV *Scotia* and FRV *Clupea* are filtered on board, whereas samples collected from inshore sites are filtered upon return to the laboratory.

8.4 A known volume of the seawater sample should be filtered within 2 hours of collection as algal populations change quickly. If there is a delay in filtering the sample then it should be stored in the dark in a refrigerator and filtered as soon as possible.

8.5 Sampled volume should usually be standardised by reference to previous data or local considerations. Prior to filtration, gently agitate the bottle containing the sample, to resuspend any large cells that may have settled.

8.6 The sample is filtered through a Whatman GF/F filter paper (glass fibre, nominal porosity 0.7 µm), with a gentle vacuum, generally not more than 0.5 atm to avoid cell damage and loss of material through the filter. Sufficient seawater has been filtered when there is colour visible to the naked eye on the filter paper. In the event that the filtration slows strongly and is terminated before completeing the standard volume, ensure that the actual volume filtered is recorded.

8.7 Remove the filter paper from the fritted base using tweezers, avoiding disturbance of the filtered material and fold once (algae inside). Blot with tissue paper to remove excess moisture and place in a numbered centrifuge tube. The centrifuge tubes containing the samples are protected from light and frozen immediately. The centrifuge tubes containing the samples can be stored frozen at -20^{o}C for 1 month, or for at least 60 days in a cryogenic freezer.

8.8 Make a record of the sample number and the volume filtered on B 614.

8.9 Samples are logged into the laboratory according to SOP 0060.

9. Analytical procedure

9.1 Preparation of 90% buffered acetone solution

9.1.1 Preparation of saturated magnesium carbonate solution

Weigh 10 g ± 0.1g magnesium carbonate into a 1000 ml conical flask. Add 1000 ml water and mix thoroughly. The solution is allowed to settle for a minimum of 24 hours. **Note only the clear 'powder free' solution is used during subsequent steps.** This can be easily done by decanting the 'powder free'solution into a 1000 ml reagent bottle. Label the bottle with the following information:

- The contents of the bottle
- Analyst initials
- Preparation date
- Expiry date (1 month from the date of preparation)

9.1.2 Measure 100 ml ± 10 ml of 'powder free' saturated magnesium carbonate solution into the 1000 ml measuring cylinder. Transfer to a reagent bottle. Measure 900 ml ± 10 ml of acetone into the 1000 ml measuring cylinder and transfer to the reagent bottle. Mix thoroughly. Label the bottle with the following information:

- The contents of the bottle
- Analyst initials
- Preparation date

90% buffered acetone solution is prepared fresh each day. If there is any left from a previous day then this can be used for rinsing the cuvette. (Note: other volumes can be prepared from that stated above by adjusting the volumes accordingly).

9.1.3 The manufacturer and batch number of the magnesium carbonate, acetone and water used to prepare the 90% buffered acetone should be recorded in the chlorophyll laboratory book, along with the balance EN number, weights and volumes used.

9.2 Preparation of 0.1M hydrochloric acid

Fill a 1000 ml volumetric flask with approximately 500 ml of water. Measure 8.5 ml ± 0.2 ml concentrated hydrochloric acid into a 10 ml measuring cylinder. Transfer to the volumetric flask. Dilute to 1000 ml with water. Transfer to a reagent bottle and label with the following information:

- The contents of the bottle
- Analyst initials
- Preparation date
- Expiry date (6 months from the date of preparation)

The manufacturer and batch number of the hydrochloric acid and water used should be recorded in the chlorophyll laboratory book, along with volumes used.

9.3 Extraction of the pigments

9.3.1 A method blank is run at the beginning, after every 20th sample and end of each run using the same chemicals and solvents as the samples. The method blank values are used to assess contamination from the laboratory environment. If the value for the method blank exceeds the limit of detection then this indicates contamination and the source of contamination must be investigated. A blank piece of filter paper, the same size as that used for the samples, is folded using tweezers and inserted into a clean empty 15 ml centrifuge tube.

9.3.2 Transfer the LRM from the cryovial, removing the foil, to a clean empty 15 ml centrifuge tube. Record the LRM number, written on the cryovial, on the writing area of the 15 ml centrifuge tube.

9.3.3 The centrifuge tubes containing the method blanks, LRMs and samples are placed in a centrifuge rack and then wrapped in tin foil or in the black box to avoid exposure to light.

9.3.4 90% buffered acetone is added to the centrifuge tubes containing the blanks, LRMs and samples using the calibrated bottle top dispenser. If the sample was collected on a 25 mm filter paper then add 10 ml of 90% buffered acetone. If the sample was collected on a 47 mm filter paper then add 15 ml of 90% buffered acetone. Replace the cap.

9.3.5 The centrifuge rack wrapped in tin foil or the black box containing the samples are then placed in a spark-free laboratory refrigerator for between 16 to 30 hours in order to extract the chlorophyll *a* from the filter paper. Record the date and time that the samples were placed in the refrigerator in the chlorophyll laboratory book.

9.4 Analysis of the samples

Both the Turner 10-AU and TD-700 fluorometers have been validated for this method. The Turner 10-AU fluorometer is the instrument of choice with the Turner TD-700 fluorometer used as a back-up.

9.4.1 Remove the samples from the refrigerator. Record the date and time in the chlorophyll laboratory book. The samples must be allowed to come to room temperature prior to analysis, this will take at least 30 minutes. Where the samples cannot be analysed after 30 hours of soaking in the refrigerator, then discuss with technical management. The centrifuge tubes containing the extracts should be placed in a cryogenic freezer until they can be analysed.

9.4.2 Analysis of the samples using the Turner 10-AU fluorometer

9.4.2.1 Reset the minimum / maximum thermometer. For T197, press the 'MEMORY CLEAR'.

9.4.2.2 Turn on the fluorometer and allow it to warm up for a minimum of 10 minutes. The HOME screen will be displayed when the instrument is switched on. The concentration range on the HOME screen should be set to auto-ranging (AUTO) and µg l^{-1} units will be selected. If these parameters are not displayed then proceed to step 9.4.2.3, otherwise carry out the system suitability check as described in steps 9.4.2.4.

9.4.2.3 To access the HOME screen press <HOME>. To set the concentration range to auto-ranging (AUTO), access the calibration menu (screen 2.0). From the HOME

screen press<ENT> for the Main Menu, then <2> for screen 2.0. From screen 2.0 press <4> to bring up screen 2.4, then <3> to bring up screen 2.43 (set conc. control range), and press <ENT> to toggle. If the units need to be changed then return to the Main Menu by pressing <ESC>, then press <1>, followed by <2> for HOME display options, followed by <2> for units of measurement and press <ENT> to toggle.

9.4.2.4 The 10-AU fluorometer solid standard is used to monitor the performance of the instrument between calibration periods. Facing the fluorometer, place the solid standard in the sample chamber with the letter 'L' (etched on top of the standard holder) on your left hand side. Make sure that the metal pin is completely seated in the notches of the 13mm round cuvette adaptor. Once the reading appears to have stabilised press <*>. This takes 10 readings over 1 second intervals and averages them. When 'DONE' appears on the screen, note the reading (L) on B 613. Remove the standard and rotate 180 degrees, and reinsert into the fluorometer, making sure that the metal pin is completely seated in the notches of the 13mm round cuvette adaptor. Once the reading appears to have stabilised press <*>. When 'DONE' appears on the screen, note the reading (H) on B 613. The system suitability check is satisfactory if the readings recorded above are within ±10% of the value recorded on the day of calibration. This is recorded on B 613. If the system suitability check is satisfactory then proceed to measuring the samples as directed below. If the system suitability check is not satisfactory then check that the glass bead/rod is clean, free of dust and any residue. When placing the solid standard into the fluorometer ensure that it is properly aligned within the aperature. It may be necessary to 'tweak' the solid standard when it is placed in the fluorometer until a satisfactory reading is achieved. If after doing all of the above the system suitability check is still not satisfactory then the instrument may need to be recalibrated or the filters may require to be changed. See SOP 3010.

9.4.2.5 Rinse the cuvette thoroughly with 90% buffered acetone. Invert the centrifuge tube containing the method blank thoroughly to mix the sample and fill the cuvette, drying the sides with a clean tissue. Insert the cuvette into the fluorometer. Once the reading appears to have stabilised press <*>. When 'DONE' appears on the screen, note the reading (Rb) on B 610.

9.4.2.6 Remove the cuvette from the fluorometer. Using a calibrated pipette add 100µl of 0.1M HCl. Thoroughly mix by inversion with your thumb over the top and return to the fluorometer. Using a calibrated timer, wait 90 seconds and then press <*>. When 'DONE' appears on the screen, note the reading (Ra) on B 610.

9.4.2.7 The LRM is run after the method blank to ensure that the method is performing satisfactorily. Do not run any samples until the LRM is satisfactory as this is a destructive technique which uses the whole sample. Subsequent LRMs should be run after every 20th sample. Rinse the cuvette thoroughly with 90% buffered acetone. Invert the centrifuge tube containing the LRM thoroughly to mix the sample and fill the cuvette, drying the sides with a clean tissue. Insert the cuvette into the fluorometer. Once the reading appears to have stabilised press <*>. When 'DONE' appears on the screen, note the reading (Rb) on B 610.

9.4.2.8 Repeat step 9.4.2.6. Once the Rb and Ra readings for the LRM are obtained, calculate the concentration of uncorrected chlorophyll *a*, corrected chlorophyll *a* and phaeophytin *a* as directed in section 10. LRM data is monitored using Shewhart charts as described in SOP 1380.

9.4.2.9 Rinse the cuvette thoroughly with 90% buffered acetone. Invert the centrifuge tube containing the sample thoroughly to mix the sample and fill the cuvette, drying the sides with a clean tissue. Insert the cuvette into the fluorometer. Once the reading appears to have stabilised press <*>. When 'DONE' appears on the screen, note the reading (Rb) on B 610. If the fluorometer displays a value greater than 200 or OVER then the concentration exceeds the maximum limit of detectability for the instrument. Using a calibrated pipette and a clean centrifuge tube, dilute the sample with 90% buffered acetone. Start with a 1 : 1 dilution, i.e. 2 ml sample + 2 ml 90% buffered acetone. Continue diluting the sample until an on-scale reading is obtained, i.e. 1 : 2 dilution, 1 : 4 dilution, 1 : 6 dilution etc. Record the dilution on B 610. Measure the diluted sample on the fluorometer as instructed above.

9.4.2.10 Repeat step 9.4.2.6.

9.4.2.11 Using the calibrated minimum / maximum thermometer, record the minimum and maximum temperatures of the laboratory environment on B 610. This should be done for each page of B 610 used, i.e. the minimum and maximum temperatures are noted every 22 samples (including method blanks and LRMs) at most. Compare the temperatures recorded with that obtained on the day of calibration. This is recorded on B 611 and is held within folders in the chlorophyll laboratory. If the temperatures recorded are not within ±3ºC of that recorded on the day of calibration then discuss with technical management.

9.4.2.12 When all of the method blanks, LRMs and samples have been analysed, calculate the concentrations of uncorrected chlorophyll *a*, corrected chlorophyll *a* and phaeophytin *a* as directed in section 10.

9.4.3 Analysis of the samples using the Turner TD-700 laboratory fluorometer

9.4.3.1 Reset the minimum / maximum thermometer. For T197, press the 'MEMORY CLEAR'.

9.4.3.2 Turn on the fluorometer. A screen appears showing the title, software version, and a 600-second countdown. The countdown allows the instrument to warm up adequately before measurements are made. After the countdown period the HOME screen is displayed. The HOME screen is where samples are read.

9.4.3.3 The TD-700 fluorometer solid standard is used to monitor the performance of the instrument between calibration periods. Facing the fluorometer, place the solid standard in the sample chamber with the letter 'L' (etched on top of the standard holder) on your left hand side. Once the reading appears to have stabilised press <*>. This averages the reading of the sample for 12 seconds. When 'END' appears on the screen, note the reading (L) on B 613. Pull the solid standard out of the filter cylinder and rotate 180 degrees, and reinsert into the fluorometer. Once the reading appears to have stabilised press <*>. When 'END' appears on the screen, note the reading (H) on B 613. The system suitability check is satisfactory if the readings recorded above are within ±10% of the value recorded on the day of calibration. This is recorded on B 613. If the system suitability check is satisfactory then proceed to measuring the samples as directed below. If the system suitability check is not satisfactory then check that the glass bead/rod is clean, free of dust and any residue. When placing the solid standard into the fluorometer ensure that it is properly aligned within the aperature. It may be necessary to 'tweak' the solid standard when it is placed in the fluorometer until a satisfactory reading is achieved. If after doing all of

the above the system suitability check is still not satisfactory then the instrument may need to be recalibrated or the filters may require to be changed. See SOP 3020.

9.4.3.4 Rinse the cuvette thoroughly with 90% buffered acetone. Invert the centrifuge tube containing the method blank thoroughly to mix the sample and fill the cuvette, drying the sides with a clean tissue. Insert the cuvette into the fluorometer. Once the reading appears to have stabilised press <*>. When 'END' appears on the screen, note the reading (Rb) on B 610.

9.4.3.5 Remove the cuvette from the fluorometer. Using a calibrated pipette add 100µl of 0.1M HCl. Thoroughly mix by inversion with your thumb over the top and return to the fluorometer. Using a calibrated timer, wait 90 seconds and then press <*>. When 'END' appears on the screen, note the reading (Ra) on B 610.

9.4.3.6 The LRM is run after the method blank to ensure that the method is performing satisfactorily. Do not run any samples until the LRM is satisfactory as this is a destructive technique which uses the whole sample. Subsequent LRMs should be run after every 20th sample. Rinse the cuvette thoroughly with 90% buffered acetone. Invert the centrifuge tube containing the LRM thoroughly to mix the sample and fill the cuvette, drying the sides with a clean tissue. Insert the cuvette into the fluorometer. Once the reading appears to have stabilised press <*>. When 'END' appears on the screen, note the reading (Rb) on B 610.

9.4.3.7 Repeat step 9.4.3.5. Once the Rb and Ra readings for the LRM are obtained, calculate the concentration of uncorrected chlorophyll *a*, corrected chlorophyll *a* and phaeophytin *a* as directed in section 10. LRM data is monitored using Shewhart charts as described in SOP 1380.

9.4.3.8 Rinse the cuvette thoroughly with 90% buffered acetone. Invert the centrifuge tube containing the sample thoroughly to mix the sample and fill the cuvette, drying the sides with a clean tissue. Insert the cuvette into the fluorometer. Once the reading appears to have stabilised press <*>. When 'END' appears on the screen, note the reading (Rb) on B 610. If the fluorometer displays a value greater than 200 or OVER then the concentration exceeds the maximum limit of detectability for the instrument. Using a calibrated pipette and a clean centrifuge tube, dilute the sample with 90% buffered acetone. Start with a 1 : 1 dilution, i.e. 2 ml sample + 2 ml 90% buffered acetone. Continue diluting the sample until an on-scale reading is obtained, i.e. 1 : 2 dilution, 1 : 4 dilution, 1 : 6 dilution etc. Record the dilution on B 610. Measure the diluted sample on the fluorometer as instructed above.

9.4.3.9 Repeat step 9.4.3.5.

9.4.3.10 Using the calibrated minimum / maximum thermometer, record the minimum and maximum temperatures of the laboratory environment on B 610. This should be done for each page of B 610 used, i.e. the minimum and maximum temperatures are noted every 22 samples (including method blanks and LRMs) at most. Compare the temperatures recorded with that obtained on the day of calibration. This is recorded on B 611 and is held within folders in the chlorophyll laboratory. If the temperatures recorded are not within ±3ºC of that recorded on the day of calibration then discuss with technical management.

9.4.3.11 When all of the method blanks, LRMs and samples have been analysed, calculate the concentrations of uncorrected chlorophyll *a*, corrected chlorophyll *a* and phaeophytin *a* as directed in section 10.

10 Calculation of results

Calculate the concentration of uncorrected chlorophyll *a*, corrected chlorophyll *a* and phaeophytin *a* using B 616.

10.1 Calculation of uncorrected chlorophyll *a* concentration

$$Uncorrected\ chlorophyll\ a\ (\mu g/l) = Rb \times \left(\frac{Extract\ volume}{Volume\ of\ seawater\ filtered} \right) \times DF$$

Where:

- Rb is the fluorometer reading before acidification
- Extract volume in litres
- Volume of seawater filtered in litres (1 litre for method blanks and LRMs)
- DF is the dilution factor

10.2 Calculation of corrected chlorophyll *a* concentration

$$Corrected\ chlorophyll\ a\ (\mu g/l) = \left(\frac{r}{r-1} \right) \times (Rb - Ra) \times \left(\frac{Extract\ volume}{Volume\ of\ seawater\ filtered} \right) \times DF$$

Where:

- r is the before-to-after acidification ratio of chlorophyll *a* standard solutions. r is determined as part of the calibration procedure and is recorded on B 611.
- Rb is the fluorometer reading before acidification
- Ra is the fluorometer reading after acidification
- Extract volume in litres
- Volume of seawater filtered in litres (1 litre for method blanks and LRMs)
- DF is the dilution factor

10.3 Calculation of phaeophytin *a* concentration

$$Pheophytin\ a\ (\mu g/l) = \left(\frac{r}{r-1} \right) \times (rRa - Rb) \times \left(\frac{Extract\ volume}{Volume\ of\ seawater\ filtered} \right) \times DF$$

Where:

- r is the before-to-after acidification ratio of chlorophyll *a* standard solutions. r is determined as part of the calibration procedure and is recorded on B 611.
- Rb is the fluorometer reading before acidification
- Ra is the fluorometer reading after acidification
- Extract volume in litres
- Volume of seawater filtered in litres (1 litre for method blanks and LRMs)
- DF is the dilution factor

11 Precision, accuracy and practical detection limits

See validation plan on nts5/method validation/chlorophyll.

11.1 Summary of validation data for uncorrected chlorophyll *a* determined on the Turner 10-AU fluorometer.

	90% Sample Repeatability	10% Sample Repeatability	90% Standard Repeatability	10% Standard Repeatability
Number of observations	7	7	7	7
Mean	2.734 µg l^{-1}	0.269 µg l^{-1}	179.286 µg l^{-1}	20.529 µg l^{-1}
Standard Deviation	0.128	0.030	7.521	0.757
% CV	4.7	11.2	4.2	3.7

	90% Sample Reproducibility	10% Sample Reproducibility	90% Standard Reproducibility	10% Standard Reproducibility
Number of observations	7	7	7	7
Mean	2.640 µg l^{-1}	0.274 µg l^{-1}	103.626% *	103.391% *
Standard Deviation	0.228	0.017	4.799	3.679
% CV	8.6	6.0	4.6	3.6

* expressed as a percentage of the theoretical concentration which was determined using the monochromatic spectroscopic method.

	LRM 151	Bias/ Recovery	Linearity
Number of observations	11	7	
Mean	2.011 µg l^{-1}	72.446 %	
Standard Deviation	0.147	8.728	
% CV	7.3	12.0	
Correlation Coefficient			0.9998

Volume filtered (l)	Volume extracted (ml)	LOD (µg l^{-1}) **
0.2	15	0.072
0.5	15	0.029
1	15	0.014
2	15	0.007

** LOD is based on a typical response of a 1 µg l^{-1} calibration check standard.

11.2 Summary of validation data for corrected chlorophyll *a* determined on the Turner 10-AU fluorometer.

	90% Sample Repeatability	10% Sample Repeatability	90% Standard Repeatability	10% Standard Repeatability
Number of observations	7	7	7	7
Mean	2.646 µg l^{-1}	0.261 µg l^{-1}	183.954 µg l^{-1}	21.361 µg l^{-1}
Standard Deviation	0.158	0.038	20.843	1.450
% CV	6.0	14.6	11.3	6.8

	90% Sample Reproducibility	10% Sample Reproducibility	90% Standard Reproducibility	10% Standard Reproducibility
Number of observations	7	7	7	7
Mean	2.656 µg l^{-1}	0.267 µg l^{-1}	99.238%*	99.695% *
Standard Deviation	0.241	0.027	3.190	7.794
% CV	9.1	10.1	3.2	7.8

* expressed as a percentage of the theoretical concentration which was determined using the monochromatic spectroscopic method.

	LRM 151	Bias/ Recovery	Linearity
Number of observations	11	7	
Mean	2.023 µg l^{-1}	73.557 %	
Standard Deviation	0.125	8.416	
% CV	6.2	11.4	
Correlation Coefficient			0.9995

Volume filtered (l)	Volume extracted (ml)	LOD (µg l^{-1}) **
0.2	15	0.071
0.5	15	0.028
1	15	0.014
2	15	0.007

** LOD is based on a typical response of a 1 µg l^{-1} calibration check standard.

11.3 Summary of validation data for uncorrected chlorophyll *a* determined on the Turner TD700 fluorometer.

	90% Sample Repeatability	10% Sample Repeatability	90% Standard Repeatability	10% Standard Repeatability
Number of observations	7	7	7	7
Mean	2.739 µg l^{-1}	0.302 µg l^{-1}	184.071 µg l^{-1}	21.929 µg l^{-1}
Standard Deviation	0.165	0.017	5.678	0.844
% CV	6.0	5.7	3.1	3.8

	90% Sample Reproducibility	10% Sample Reproducibility	90% Standard Reproducibility	10% Standard Reproducibility
Number of observations	7	7	7	7
Mean	2.585 µg l^{-1}	0.278 µg l^{-1}	106.696 %*	106.373% *
Standard Deviation	0.158	0.020	2.984	6.321
% CV	6.1	7.3	2.8	5.9

* expressed as a percentage of the theoretical concentration which was determined using the monochromatic spectroscopic method.

	LRM 151	Bias/ Recovery	Linearity
Number of observations	11	7	
Mean	2.153 µg l^{-1}	73.263 %	
Standard Deviation	0.187	7.175	
% CV	8.7	9.8	
Correlation Coefficient			0.9991

Volume filtered (l)	Volume extracted (ml)	LOD (µg l^{-1}) **
0.2	15	0.075
0.5	15	0.030
1	15	0.015
2	15	0.008

** LOD is based on a typical response of a 1 µg l^{-1} calibration check standard.

11.4 Summary of validation data for corrected chlorophyll *a* determined on the Turner TD-700 fluorometer.

	90% Sample Repeatability	10% Sample Repeatability	90% Standard Repeatability	10% Standard Repeatability
Number of observations	7	7	7	7
Mean	2.592 µg l^{-1}	0.281 µg l^{-1}	180.117 µg l^{-1}	22.136 µg l^{-1}
Standard Deviation	0.235	0.021	10.703	1.820
% CV	9.1	7.4	5.9	8.2

	90% Sample Reproducibility	10% Sample Reproducibility	90% Standard Reproducibility	10% Standard Reproducibility
Number of observations	7	7	7	7
Mean	2.526 µg l^{-1}	0.262 µg l^{-1}	103.249 %*	99.923% *
Standard Deviation	0.255	0.029	7.280	13.271
% CV	10.1	10.9	7.1	13.3

* expressed as a percentage of the theoretical concentration which was determined using the monochromatic spectroscopic method.

	LRM 151	Bias/ Recovery	Linearity
Number of observations	11	7	
Mean	2.081 µg l^{-1}	72.932 %	
Standard Deviation	0.259	10.682	
% CV	12.4	14.6	
Correlation Coefficient			0.9982

Volume filtered (l)	Volume extracted (ml)	LOD (µg l^{-1}) **
0.2	15	0.080
0.5	15	0.032
1	15	0.016
2	15	0.008

** LOD is based on a typical response of a 1 µg l^{-1} calibration check standard.

12 Reports

The calculated results for uncorrected chlorophyll *a*, corrected chlorophyll *a* and phaeophytin *a* are transferred onto B 617. A hardcopy of B 616 along with all other relevant documentation (SOP 1350) are submitted to the Technical Manager. Batches of results and test reports are archived in Chemdat/Chlorophyll/Batches.

13 Safety

Refer to procedure risk assessment AI154.

14 Literature references

Arar, E.J. & Collins, G.B., 1997, 'In vitro determination of chlorophyll a and phaeophytin a in marine and freshwater algae by fluorescence', Method 445.0 (reference only)

Aminot, A. & Rey, F., 2001, 'Chlorophyll a: Determination by spectroscopic methods', *ICES Techniques in Marine Environmental Sciences*, 30 (reference only).

Lorenzen, C.J., 1967, 'Determination of chlorophyll and pheopigments: spectroscopic equations', *Limnology and Oceanography*, 12, 343-346 (reference only).

Turner Designs Model 10-AU-005 Field Fluorometer User's Manual (controlled)

Turner Designs TD-700 Laboratory Fluorometer Operating Manual (controlled)

15 Uncertainty of measurement

Sources of uncertainty:

- **Sampling:** Samples are analysed and results reported on the samples as received – outwith uncertainty calculations.
- **Sub-sampling:** The filter is the whole sample**,** not applicable.
- **Storage conditions:** Samples are stored frozen in the dark. Samples are not stored for longer than 1 month at -20ºC and 60 days in a cryofreezer – negligible contribution to uncertainty.
- **Reagent purity:** All reagents used are AnalR or HPLC grade quality – uncertainty accounted for in validation data.
- **Instrument effects:** The cuvette is rinsed with 90% buffered acetone in between samples – uncertainty accounted for in validation data.
- **Weight:** Not applicable
- **Volume:** Pipettes, used to prepare calibration standards and to acidify the calibration standards and samples, are calibrated to <1%. Dispenser, used to dispense 90% buffered acetone for extracting the samples, are calibrated to <2% - uncertainty accounted for in validation data.
- **Time:** Timers used to time acidification reaction are calibrated against the BT speaking clock to an accuracy of ±1 second – uncertainty accounted for in validation data.
- **Fluorometer:** The flurometer is calibrated using a standard of known chlorophyll a concentration. Once it has been calibrated a series of standards are run as samples and a graph plotted of theoretical concentration against concentration calculated from fluorometer readings for corrected chlorophyll *a*, r^2 must be greater than 0.995. System suitability check is done between calibration periods – uncertainty accounted for in validation
- **Spectrophotometer:** Other than a self-calibration at beginning no other checks carried out. Minimal contribution to uncertainity.
- **Computational effects:** Concentrations are calculated using a Microsoft Excel spreadsheet. Manual check of calculation has been carried out and found to be acceptable – negligible contribution to uncertainty.
- **Blank correction:** Method blanks are analysed at the beginning, after every 20^{th} sample and at the end of each batch of samples. Concentrations are typically found to be less than 0.002 µg l^{-1} uncorrected and corrected chlorophyll *a*.

- **Environment conditions:** Contamination is minimised by the use of a dedicated laboratory and equipment – uncertainty accounted for in validation data.
- **Operator effects:** All measurement methods are described in fully documented standard operating procedures to limit inconsistencies between operators. Only trained personnel may perform method unsupervised. Variations between operators are accounted for by control chart data. Uncertainty accounted for in validation data.
- **Random effects:** These will be accounted for by validation and control chart data.

Summary of Validation Data

Instrument	Determinand	Precision of LRM (%)	Recovery of spiked sample (%)
10-AU	Uncorrected chlorophyll *a*	7.3	72.446
10-AU	Corrected chlorophyll *a*	6.2	73.557
TD-700	Uncorrected chlorophyll *a*	8.7	73.263
TD-700	Corrected chlorophyll *a*	12.4	72.932

Instrument	Determinand	Systematic Component (C_s)	Random Component (C_r)	Combined Uncertainity (%)	Expanded Uncertainity (%)
10-AU	Uncorrected chlorophyll *a*	13.777	7.3	15.6	31.2
10-AU	Corrected chlorophyll *a*	13.222	6.2	14.6	29.2
TD-700	Uncorrected chlorophyll *a*	13.369	8.7	16.0	31.9
TD-700	Corrected chlorophyll *a*	13.534	12.4	18.4	36.7

Where:

- Systematic component C_s = (100 - % recovery)/2
- Random component C_r = precision of LRM

Assume linear summation and a value of k = 2:

- Combined standard uncertainity = $(C_s^{\,2} + C_r^{\,2})^{0.5}$ = X %
- Expanded uncertainity = 2 * $(C_s^{\,2} + C_r^{\,2})^{0.5}$ = X %

The reported expanded uncertainity is based on an uncertainity multiplied by a coverage factor of k = 2, providing a level of confidence of approximately 95%.