Draft Project Report R&D Project 395

Chlorophyll a --- SCA Method Revision

Institute of Freshwater Ecology

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EXECUTIVE SUMMARY

The SCA standard method for the analysis of chlorophyll a has been revised and supplied in this draft project report in draft form. Two points must be made clear. First, this draft will be submitted to the SCA for peer review and revision. Secondly the draft has been included as an appendix since the format and general layout conforms to SCA standards rather than those of the NRA.

The main method proposed remains the traditional solvent extraction method. The primary solvent recommended has been changed to ethanol to conform to European continental recommended methods and also to ease problems arising with COSHH regulations. Methods involving methanol and acetone are still included for those with specific requirements but the advantages/disadvantages are clearly shown. The section on correcting chlorophyll a estimates for pigment breakdown products has been absorbed into the main section. This greatly simplifies the whole document.

A new introductory section on high performance liquid chromatography has been included. No attempt has been made to give a completely detailed method. The equipment requires a high level of technical expertise and since it is considerably slower than the classical methods, it assumed that the method will not be used for most routine surveillance operations.

KEYWORDS

Chlorophyll, plant pigments, spectrophotometry, fluorometry, HPLC

GLOSSARY

Acronyms used in this document

COSHH	Control of substances hazardous to health
IMS	Industrial methylated spirits
HPLC	High performance liquid chromatography
IFE	Institute of Freshwater Ecology
NRA	National Rivers Authority
HMSO	Her Majesty's Stationary Office
DoE	Department of the Environment
SCA	Standing Committee of Analysts (component body of DoE)
WG7	Working Group 7 (biological), part of SCA

1. PROJECT DESCRIPTION

1.1 Background

The work reported here arose some time ago within the Biological Working Group (WG7) of the Standing Committee of Analysts (SCA) in the Department of the Environment (DoE). The standard method for the analysis of chlorophyll a was published twelve years ago (HMSO 1983) and derivations of the basic procedures set out in that publication have been widely used throughout the water industry. Over the intervening fourteen years, since most of the original drafting work took place between 1976 and 1978, there have been a number of developments which suggested to the Standing Committee that a revision was required. The National Rivers Authority agreed to support this revision.

1.2 Contractual Objectives

1.2.1 Overall Project Objective

To update the SCA method for the determination of chlorophyll a.

1.2.2 Specific Objectives

(i) To review developments in the analysis of chlorophyll a and other algal pigments.

(ii) To provide detailed methods for the determination of algal pigments covering the range of solvents currently in use and include relevant COSHH assessments.

(iii) To ensure that these methods represent European and international views.

(iv) To present the findings in the form of a revised SCA method, including the required analytical quality control.

2. SUMMARY OF THE REVISION

Chlorophyll a is widely used throughout the world as a primary variable describing the quantity of phytoplankton in a water body. Although chlorophyll concentration can be a poor estimate of biomass, the speed and, generally the universality of application has ensured its continuation as an analytical method in the water industry. The original SCA method was structured into a number of interlinked methods.

I Extractive methods involving acetone and methanol.
- (i) Absorption spectrophotometry,
- (ii) Fluorometry,
- (iii) "Degradation" studies,

II *In vivo* fluorometry,

III Applications to macrophytes and the benthic or periphytic algae.

The introduction of the COSHH regulations in January 1991 made the use of methanol as the primary extraction solvent undesirable. Ethanol is now recommended as the primary extractant inline with our European colleagues (see DIN 1986, DS 1986). Methanol is included as the second choice since there is still some discrepancy over the relative merits of the two solvents as extractants but much greater care must be exercised in its use (COSHH) and this will reduce the speed of analysis. 90% acetone is not recommended for most routine purposes since it is known to be a poor extractant of the Chlorophyceae and Cyanobacteria. Although careful grinding will overcome many of these problems, many of the small Chlorophyceae (eg *Chlorella spp*) will always present difficulties when time is a primary consideration. However, a method involving 90% acetone is still included for use when specific specialist analytical methods are used:

- (i) The senior/experienced analyst must be satisfied that extraction is sufficiently complete for their purposes; this will require skilled microscopy and/or fluorometry for detecting residual chloroplasts.
- (ii) The trichromatic method of estimating chlorophylls a, b and c concurrently has only been developed for 90% acetone (Jeffrey and Humphrey 1975).
- (iii) Extraction in 90% acetone is required for HPLC analysis, because extraction in methanol may lead to allomerization and epimerization of the chlorophylls (Mantoura and Llewellyn 1983). We must assume that similar transformations will also occur in other alcohols.

Methods have not been listed involving the use of other solvents (Bowles *et al.* 1985, Wood 1985, Palumbo *et al.* 1987).

Simple spectrophotometric methods of correcting for interference from phaeopigments have been included for each of the three solvents but written in such a way that the additional steps can be avoided if required (Lorenzen 1967, Marker *et al.* 1980, Marker and Jinks 1982, DIN 1986, DK 1986).

Various denatured forms of ethanol are available commercially but these have not been rigorously tested in this country:

(i) Industrial methylated spirits (IMS) is a somewhat impure product and contains some methanol. On no account should it be used.

(ii) In Germany 96% ethanol is denatured with methyl-ethyl-ketone (Otto Reichelt, Essen {Nusch, pers. comm.}) and the official standard there recommends rigorous tests with each new batch of alcohol. In Britain ethanol is denatured with "Bitrex" (dinatonium benzoate) and is sold as ethanol B. Pigment extracts have not been tested with this product for stability and so cannot be recommended at this stage.

Users of duty-free ethanol require a site-specific licence from HM Customs and Excise and the provision of a secure bonded store.

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4. APPENDIX

4.1 Preface

The SCA draft method has been put in an appendix because the notation does not conform to NRA requirements. To have had in circulation two virtually identical documents, one conforming to NRA standards, the other to SCA standards would have lead to unnecessary confusion.

4.2 SCA Draft Manuscript

The remainder of this document is the draft method which will be submitted to Working Group 7 of the Standing Committee of Analysts (Department of the Environment).

Appendix

THE DETERMINATION OF CHLOROPHYLL A IN AQUATIC ENVIRONMENTS

0 ABOUT THIS METHOD

01 Introduction

Analyses for pigments such as chlorophyll, and especially chlorophyll a, are widely used to assess the abundance of micro-algae present in suspension in natural waters, and - to a lesser extent - the 'attached' and benthic algae. Under favourable conditions chlorophyll determination is rapid, reproducible and reasonably specific for photosynthetic plant material. Chlorophyll analysis can also be applied to estimate the cover-density of larger plants (macrophytes). However, as the latter provide bulky samples and can be more readily separated from extraneous material, other methods of assessing biomass (eg. fresh weight, ash-free dry weight) are more commonly used. Care must be taken in the use of chlorophyll a as a measure of biomass (White *et al.* 1988)

Although it is often used to assess biomass the pigment content of different plant species can show wide variations. For instance, chlorophyll a may range between 0.4 and 4.0% on a dry weight basis. It may also be difficult to achieve a complete extraction of pigments from the cells of some species. Consequently determinations of pigment content may give rise to biomass values very different from those obtained by other methods and results must always be regarded as one parameter contributing to a series of other assessments of biomass rather than as a single definitive technique.

In selecting the most appropriate method for the measurement of chlorophyll a it is important to consider the objectives of the work being undertaken and, in particular, whether the results are required for immediate decisions on the management of a water body, or whether they are required for a deeper ecological study. For management purposes, speed of analysis will often be more important than high accuracy or precision, and the time saved may be used to obtain valuable ancillary information such as the examination and identification of the algae present. For more comprehensive studies, the accuracy and reproducibility of results together with information on other plant pigments present may be more valuable.

02 Actual Methods Given

This booklet describes several methods for the determination of chlorophyll a in plant material obtained from an aquatic environment:

a) Simple solvent extraction techniques (see Section A) using either ethanol, methanol or acetone, followed by spectrophotometric or fluorometric evaluation of the extract.
b) In-vivo fluorometry (see Section B).
c) Extraction in acetone followed by high precision spectrophotometry may be used to separate chlorophylls a, b and c. High performance liquid chromatography (HPLC) may be used for the most rigorous separation of the chlorophylls and their breakdown products (see section C).

Appendix

03 Methods to Distinguish Undegraded and Degraded Pigments

Not all the procedures distinguish between undegraded and degraded ("dead") forms of chlorophyll a. Section A offers a very simple separation of chlorophyll a only from phaeopigments. Section B, although offering a rapid and sensitive method of measuring chlorophyll a, does not distinguish the breakdown products. A rigorous distinction is possible only by HPLC (Mantoura and Llewellyn 1983, Wright and Shearer 1984, Bidigare *et al.* 1985 Gieskes and Kraay 1986a & b, Zapata *et al.* 1987), thin-layer chromatography (Daley *et al.* 1973, Jeffrey 1968, 1974, 1981. Gieskes *et al.* 1978) or paper chromatography (Hallegraeff 1976, Jensen and Liaaen-Jensen 1959, Jensen and Sakshaug 1973, Eloranta 1982 and 1986).

04 Extension of the Method to other Pigments

The determination may be extended to other pigments but only by the most rigorous of methods (see Section C).

05 General Literature References

For further general information concerning the determination of chlorophyll see literature Lorenzen 1967, Golterman and Clymo 1969, Strickland and Parsons 1973, Talling 1974, Wetzel and Westlake 1974, Loftus and Seliger 1975, Holm-Hansen and Riemann 1978, Moed and Hallegraeff 1978, Stainton *et al.* 1977, Rai 1980 a and b, Chang and Rossmann 1982, Jespersen and Christoffersen 1987).

THE DETERMINATION OF CHLOROPHYLL A IN PLANT MATERIAL (PHYTOPLANKTON) IN SUSPENSION IN WATER (SOLVENT EXTRACTION METHOD)

A1 Performance Characteristics of the Method

A1.1	Substance determined:	Chlorophyll a
A1.2	Type of sample:	Natural waters (phytoplankton), micro-plant growth on a substratum (microbenthos), periphyton and rarely larger aquatic plants (macrophytes).
A1.3	Basis of method:	Extraction of pigments into an organic solvent, followed by spectrophotometric or fluorometric determination.
A1.4	Range of application:	
A1.5	Calibration graph:	Standard absorption coefficients are applied to spectrophotometric measurements.

		Fluorometric methods are calibrated using a suitable chlorophyll a extract solution in which the concentration has been determined spectrophotometrically.
A1.6	Total standard deviation:	The ***precision*** of the method depends on the absorbance of the extract in the cuvette. For a extract that is 20% degraded with an absorbance between 0.25 and 0.60 the coefficient of variation for chlorophyll a would be ca 3% and for phaeopigments ca 6%. However, the ***accuracy*** is more difficult to quantify, see A1.9 and A1.10 below.
A1.7	Limit of detection:	The fluorometric determination of extracts is at least 100 x as sensitive as spectrophotometry although the precision may not be as good.
A1.8	Sensitivity:	
A1.9	Bias:	No information apart from that arising from the presence of interfering substances. Incomplete extraction will give low results.
A1.10	Interferences:	The major degradation products of chlorophyll a (phaeophorbide, phaeophytin) may be corrected for but other pigments may interfere (eg chlorophyllide and chlorophyll b).
A1.11	Time required for analysis:	1 hour for the spectrophotometric method if a batch of at least 20 samples is analyzed but much depends upon the experience of the operator and the equipment used in the laboratory.

A2 Principle

A2.1 Plant material such as plankton is obtained by filtration of the water sample. However, in the case of attached algae e.g. microbenthos or periphyton, the separation methods given in Section D may be more applicable.

Chlorophyll a is extracted from the plant material using either ethanol, methanol or acetone as appropriate (see Section A2.2) and its concentration in the extract (and hence in the sample) is determined spectrophotometrically by carrying out absorbance measurements at two wavelengths, i.e.:-

(i) at 665 nm, the absorption maximum of chlorophyll a

(ii) at 750 nm in order to compensate for "background turbidity".

A2.2 The choice between ethanol, methanol or acetone as the solvent for extraction is influenced by:

(i) the greater superiority of the alcohols (especially when hot) as extractants;

(ii) the better-known characteristics and greater stability of chlorophyll a solutions in acetone; and

(iii) the greater possibility of making some distinction between undegraded and degraded pigment in ethanol or acetone extracts. Thus if degradation products are likely to be abundant, extraction with ethanol or acetone followed by the appropriate measurements and calculations is recommended: otherwise, and especially with algae particularly resistant to extraction. hot methanol is simpler and effective. For further discussion and comparative data see Talling and Driver 1963, Marker 1972 and Jones 1977, Riemann 1978, Riemann and Ernst 1982, Bowles *et al.* 1985, Neveux and Parnhouse 1987, Palumbo *et al.* 1987, Lloyd and Tucker 1988.

A2.3 Fluorometry (Loftus and Carpenter 1971,Daley *et al.* 1973, Stainton *et al.* 1977, Coveney 1988) may be used as an alternative to absorption spectrophotometry to evaluate extracts since chlorophyll a exhibits a deep red fluorescence when excited by blue light. The sensitivity of the technique as applied to an extract is much greater than that of the corresponding spectrophotometric method.

A3 Interferences

A3.1 Other Pigments

If present in the sample of plant materials, chlorophylls b and c and other pigments such as carotenoids will be extracted by the solvent used and chlorophylls b and c will contribute to the absorbance of the extract, even at the wavelength selected for chlorophyll a. Thus the chlorophyll a content of the sample, as calculated in section A8, A9 and A10, may not be the true value. Bacterial chlorophylls will also interfere (Eloranta 1985).

A3.2 Degradation Products

A similar effect is obtained from the presence of degradation products of chlorophyll, which may be present in appreciable amounts. The effect may be a more serious interference than that of A3.1.

A3.3 Corrections for Interference Effects

A procedure to estimate chlorophylls b and c is given in section C.

Appendix

A4 Hazards

Normal laboratory safety precautions must be observed.

COSHH regulations impose considerable restraints on the use of organic solvents:

(i) The solvents used for the extraction of pigments are highly flammable and should be handled with extreme care. No more than 250 ml may be left unattended on the laboratory bench. Larger quantities must be stored in suitable storage cabinets or solvent stores. The liberal use of warning symbols is strongly advised.

(ii) Disposable vinyl gloves should also be used.

(iii) Methanol is highly toxic and should not be inhaled. All operations involving open vessels should be performed in a suitable fume cupboard. Cuvettes, used in spectrophotometry, must be of the sealed, stoppered variety. If necessary, vapour concentrations should be monitored using Dräger tubes.

(iv) Even with ethanol and acetone fume cupboards should be used wherever possible and certainly when large quantities are being dispensed. If spillages occur, mop up with absorbent tissue and leave these to dry off either in the fume cupboard or outside, in the open air.

Centrifuges must be mounted securely and should be shielded to protect the operator in the event of mechanical breakdown. Manufacturers instructions to balance the rotors must be strictly observed and the lid must not be opened whilst the centrifuge is operating. All modern centrifuges have automatic locking devices.

Apparatus operated under reduced pressure must be shielded to prevent injury to the operator in the event of an implosion.

If the excitation source in the fluorometer emits ultraviolet radiation care should be taken to avoid eye or skin exposure.

The use of duty-free ethanol requires a licence from HM Customs and Excise who will require the provision of a secure bonded store and the maintenance of detailed records,

A5 Reagents

A5.1 Analytical reagent grade chemicals, and distilled or deionized water should normally be used throughout.

A5.2 Ethanol.
90% v/v ethanol aqueous (for dilution and reference cells in section A8)
Add 10 ml of distilled water to 90 ml of ethanol. Mix well.

A5.3 Methanol.
90% v/v methanol aqueous (for dilution and reference cells in section A9)

Proceed as in A5.2 using methanol instead of ethanol.

A5.4 Acetone
90% v/v acetone aqueous (for dilution and reference cells in section A10)
Proceed as in A5.2 using acetone instead of ethanol.

A5.5 Hydrochloric acid (for acidifying pigment extracts in A8, A9 and A10)
3 x 10^{-1}M aqueous hydrochloric acid. The final concentration in the spectrophotometer cell is $3x10^{-3}$M. This is adequate for complete conversion of chlorophyll to phaeophytin but the reaction is sufficiently slow to require several minutes for the reaction to complete. If higher concentrations of mineral acid are used there is a danger of oxidation of epoxicarotenoids with subsequent interference at 650 and 750 nm (Holm-Hansen and Riemann 1978).

A5.6 Organic base (for neutralizing acid in A9 only, see A5.5)
3 x 10^{-1}M methanolic 2-phenylethylamine.

A6 Apparatus

A6.1 A spectrophotometer for use in the visible region of the spectrum and capable of accepting 1-cm and 4-cm pathlength cells. A double-beam, semi-automatic, PC-linked instrument can save considerable time.

Resolution at 665 nm should be 1-2 nm wavelength.

Matched cells with stoppers should be reserved for use in this method. Both sample and reference cells must be kept scrupulously clean and the same cells should be used for sample and reference solutions respectively. They should always be placed in the same position in the holder with the same face toward the light source.

A6.2 Fluorometer equipped with a high output excitation source at wavelengths in the region of 430 nm and fitted with:

1) A blue excitation filter e.g. Corning CS 5-60.

2) A red emission filter e.g. Corning CS 2-64.

3) A red sensitive photomultiplier tube having good response up to 685 nm.

The instrument must be used strictly in accordance with the manufacturers instructions. Care must be taken to avoid exposure to ultraviolet radiation.

A6.2.1 Optical cells, pathlength 10 mm compatible with fluorometer A6.1. Alternatively a cell of a suitable flow-through type may be used.

A6.3 **Glass-fibre filters**, fine porosity. e.g. Whatman grade GF/C or the equivalent. Filter diameters between 55 and 90 mm are most convenient. The retentive capacity of GF/C filters may need to be checked against 0.4 μm membrane filters (Lenz and Fritsche 1980, Munewar *et al.* 1982, Venrick *et al.* 1987) or GF/F filters (Prepas *et al.* 1988). Powdered $MgCO_3$ as a filtering aid is unnecessary (Lenz and Fritsche 1980, Lloyd and Tucker 1988).

A6.4 **Filter holder:** e.g. porcelain Hartley funnel, or a metallic sinter type to support the filter on a porous base. Either type should have provision to operate under reduced pressure and should clamp the filter around its periphery.

A6.5 **Suction pump**: water pump fitted to the mains-pressure water tap, or a small electric. pump

A6.6 **Test-tubes**, preferably stoppered, approximate capacity 20 to 50 ml.

A6.7 **Simple laboratory centrifuge**

A6.8 **Centrifuge tubes**

A6.9 **A homogenizer or grinder** (if required) for disintegrating algal cells when acetone is used as a solvent for extraction.

A7 Sample Collection and Preservation

It must be emphasized that throughout this method all samples and sample extracts should not be exposed to sunlight. Preferably they should be handled in subdued light and if storage is necessary this should be in darkness in an air-tight container.

Collect a suitable volume of water usually 1 litre, that contains algae (phytoplankton) in suspension using a surface dip sample, a self-closing bottle for samples taken at depth, or by using a weighted plastic tube to obtain vertically integrated samples (Mackereth *et al.* 1978).

If larger volume samples are required from treatment or filtration plants use techniques appropriate to the site.

For methods of sampling materials other than water see Section D.

Samples are best analyzed on the day of collection, or at most after overnight storage in darkness in a refrigerator or cool (<10°C) place (Herve and Heininen 1984). Avoid exposure to strong light or high temperatures in transit. Particulate material is sometimes stored for several weeks, frozen on filters after the filtration step, but when preceded by drying this treatment may lead to under-estimates (see Sand-Jensen 1976, Lenz and Fritsche 1980, Herve and Heinonen 1987).

Appendix

Storage of extracts overnight at about 4°C is permissible.

A8 Analytical Procedure

(i) Ethanol - recommended method

Step	Procedure	Notes
A8.1	Filter a measured volume V, (note a) of sample through a glass-fibre filter clamped in a suitable holder (A6.4) (note b). Discard the filtrate (note c).	(a) For most natural waters 1 litre is a suitable volume but this should be adjusted if the expected phytoplankton content is abnormally high or low.
A8.2	After filtration is completed the residual water content of the filter is reduced by allowing air to be drawn through for a short time, usually 30 seconds.	(b) Filtration is accelerated by applying slightly reduced pressure to the receiving vessel. i.e. reduction to 3 atmosphere (corresponding to reduction to 500 mm Hg or 66.6 K Pa). Do not reduce the partial pressure further or the cells may rupture on the filter.
		(c) The filtrate may be used for the analysis of nutrients and/or trace metals etc by appropriate methods given in other booklets in this series. Care must be taken that the method of filtration is suitable.
A8.3	Remove the filter paper from the holder and weigh. Allow to dry, partially, in the dark. Weigh the filter, then fold it three times (note d). Transfer the filter paper to a test tube. Choose, one only, of the three following methods (A8.4(i) or (ii) or (iii):	(d) The weight of water retained on the filter should be between 0.5 - 0.7 g for a 9 cm diameter filter, requiring 20 ml ethanol, and proportionately more or less for different diameter filters with differing solvent volumes.
A8.4(i)	Either, add a known volume of hot ethanol, usually 15 ml or 20 ml, sufficient to cover the filter and stopper (notes e and f).	(e) Carry out this procedure in the fume cupboard.
(ii)	Or, add a known volume of cold ethanol, usually 15 ml or 20 ml, sufficient to cover the filter and heat to boiling and boil for about 10 seconds. The tube should be covered to prevent loss of solvent (notes e and g).	(f) Heat the ethanol in a separate vessel. If necessary use a reflux system to prevent loss of solvent. The extraction procedures should all be carried out in subdued light and any contact with acid vapours avoided.
(iii)	Or, add a known volume of cold ethanol, usually 15 ml or 20 ml, sufficient to cover the filter and stopper. Place in the dark cold (4°C) place for 12 hours (eg overnight). Agitate the filter briefly from time to time during this period (note e).	(g) Immersion of the tube in a water bath is effective; the water temperature should be just above the boiling point of ethanol (80°C).
A8.5	Agitate the paper briefly with forceps to ensure	

that the paper is in complete contact with the solvent.

A8.6 The tube should be carefully covered to prevent loss of ethanol.

A8.7 Allow the warm solution to stand in the dark for at least 30 minutes, but preferably for about 12 - 24 hours (i.e. overnight).

A8.8 Add water accurately (note h).

(h) Use 100% ethanol initially; take into account the residual water on the filter and then add the appropriate quantity of additional water required to make the final concentration 90%.

A8.9 Still in dim light ensure the sample is well mixed using a vortex stirrer. Remove the filter paper from the ethanol with forceps. Squeeze the paper against the rim of the tube so that as much ethanol as possible drains back into the tube.

A8.10 Either, centrifuge the ethanol extract, in a stoppered tube (note i) to prevent loss of ethanol by volatilization, until a clear extract solution of pigment is obtained (note j). Let the total volume of this extract be v ml. Decant the clear extract without disturbing the sediment (note k).

Or, filter the extract through a small GF/C filter, held in a suitable holder, into a clean tube.

(i) A covering of stretch plastic film is usually adequate to prevent loss of ethanol by volatilization.

(j) Centrifugation for 7 minutes at 3500 rev/min is usually sufficient.

(k) A pipette, fitted with a low pressure suction device, may also be used to decant the supernatant without disturbing the sediment.

A8.11 Reserve this extract in a stoppered tube for the absorbance measurements.

A8.12 Fill a spectrophotometer stoppered cuvette (generally 10 mm or 40 mm pathlength) with the pigment extract solution. Let the pathlength of the cuvette used be d mm (note l).

(l) Commonly available 10 mm pathlength cuvettes require 3 ml of extract whereas 40 mm pathlength cuvettes require 10 ml.

Lesser volumes will require the use of narrow, semi-micro 40 mm pathlength cells provided that these are compatible with the spectrophotometer.

A8.13 If appropriate use 90% v/v aqueous ethanol as, used to extract the pigments, in the reference beam of the spectrophotometer.

A8.14 Measure the absorbance of the extract at wavelengths of 665 nm, and 750 nm (note m). The calculations are susceptible to error from

(m) Absorbance at 665 nm should fall within the range 0.050 to 0.700 units, otherwise adjust either the volume of sample, the

incorrect setting of the spectrophotometer wavelength. Check this setting regularly using the hydrogen line emitted by the deuterium lamp (ca 656 nm). If corrections are not required for the presence of phaeopigments, omit steps A8.17-19 inclusive. If corrections are required omit steps A8.15 and A8.16 and proceed direct to step A8.17.

volume of aqueous ethanol, or the pathlength of the cell, to meet these criteria.

Absorbance at 750 nm should not exceed 0.005 units per 10 mm of cell pathlength i.e. 0.020 units in a 40 mm pathlength cell.

A8.15 Subtract the absorbance value obtained at 750 nm from that obtained at 665 nm and let this be A (note n).

(n) This step is a correction for any turbidity present.

A8.16 The chlorophyll a content of the sample

$$= \frac{12.0 \times A \times v}{d \times V} \mu g\, l^{-1} (= mg\, m^{-3})$$

(note o)

Where A = absorbance
v = volume of solvent in ml
V = volume of initial filtered samples in litres and
d = cell pathlength in cm

(o) The factor 12.0 approximates to the reciprocal of the specific absorption coefficient at 665 nm for chlorophyll a in ethanol of ca 83 (Wintermans and De Mots 1965) and used in Germany (DIN 1986) and Denmark (Dansk Standardiseringsrau 1986).

Note again that this calculation makes no correction for the presence of degraded matter or of other pigments.

A8.17 Corrections for phaeopigments: do not remove the extract from the cuvette: to 10 ml of extract add 0.1 ml of 0.3M hydrochloric acid solution and mix well (note p). Allow the acidified extract to stand for 5 minutes.

(p) A micro-pipette should be used. Less than 5 minutes may give rise to incomplete conversion of chlorophyll to phaeophytin. Many samples may be left for up to 30 minutes without interference from epoxicarotenoids but this should be carefully checked (Holm-Hansen and Riemann (1978).

A8.18 Measure the absorbance of the acidified extract at 665 and 750 nm (note q). Note the values obtained.

(q) Cell faces must be cleaned and re-polished. The cell must always be placed the same way round in the spectrophotometer.

A8.19 For both the unacidified and acidified extracts subtract the absorbance at 750 nm from that at 665 nm. Let the corrected values be A_n (unacidified) and A_m (acidified).

Then the degree of degradation

$$= \frac{A_n}{A_m}$$

(r) Degradation absent: a value of approximately
1 .7
Degradation complete: value 1.0

(s) See note r, Section A8.16 and Sources of Error, A12.

(t) 2.43 is a factor derived from the absorbance of chlorophyll a at 665 nm

before and after acidification

(note r)

The undegraded chlorophyll a content(Golterman and Clymo 1969). $\left(\frac{A_n}{A_n - A_m} - 2.43\right)$

$- C_a$

where:

$$C_a = \frac{12.0\,(2.43\,(A_n - A_m)) \times V}{d \times V}\ \mu g\ l^{-1}$$

and the phaeopigment content will be:

$$P_a = \frac{12.0 \times 1.7\,(A_n - (2.43\,(A_n - A_m))) \times V}{d \times V}\ \mu g\ l^{-1}$$

notes s and t

where v = total volume of extract (ml)
d = cell pathlength (cm)
V = volume of sample taken (l)

A9 Analytical Procedure

(i) Methanol - alternative method

Step	Procedure	Notes
A9.1	Filter a measured volume V, (note a) of sample through a glass-fibre filter clamped in a suitable holder (A6.4) (note b). Discard the filtrate (note c).	(a) For most natural waters 1 litre is a suitable volume but this should be adjusted if the expected phytoplankton content is abnormally high or low.
A9.2	After filtration is completed the residual water content of the filter is reduced by allowing air to be drawn through for a short time, usually 30 seconds.	(b) Filtration is accelerated by applying slightly reduced pressure to the receiving vessel. i.e. reduction to 3 atmosphere (corresponding to reduction to 500 mm Hg or 66.6 K Pa). Do not reduce the partial pressure further or the cells may rupture on the filter.

(c) The filtrate may be used for the analysis of nutrients and/or trace metals etc by appropriate methods given in other booklets in this series. Care must be taken that the method of filtration is suitable.

A9.3 Remove the filter paper from the holder and weigh. Allow to dry, partially, in the dark. Weigh the filter, then fold it three times (note d). Transfer the filter paper to a test tube. Choose, one only, of the three following methods (A8.4(i) or (ii) or (iii):

(d) The weight of water retained on the filter should be between 0.5 - 0.7 g for a 9 cm diameter filter, requiring 20 ml ethanol, and proportionately more or less for different diameter filters with differing solvent volumes.

A9.4(i) Either, add a known volume of hot methanol, usually 15 ml or 20 ml, sufficient to cover the filter and stopper (notes e and f).

(e) Carry out this procedure in the fume cupboard.

(ii) Or, add a known volume of cold methanol, usually 15 ml or 20 ml, sufficient to cover the filter and heat to boiling and boil for about 10 seconds. The tube should be covered to prevent loss of solvent (notes e and g).

(f) Heat the methanol in a separate vessel. If necessary use a reflux system to prevent loss of solvent. The extraction procedures should all be carried out in subdued light and any contact with acid vapours avoided.

(iii) Or, add a known volume of cold methanol, usually 15 ml or 20 ml, sufficient to cover the filter and stopper. Place in the dark cold (4°C) place for 12 hours (eg overnight). Agitate the filter briefly from time to time during this period (note e).

(g) Immersion of the tube in a water bath is effective; the water temperature should be just above the boiling point of methanol (65-70°C).

A9.5 Agitate the paper briefly with forceps to ensure that the paper is in complete contact with the solvent.

A9.6 The tube should be carefully covered to prevent loss of methanol.

A9.7 Allow the warm solution to stand in the dark for at least 30 minutes, but preferably for about 12-24 hours (i.e. overnight).

A9.8 Add water accurately (note h).

(h) use 100% methanol initially; take into account the residual water on the filter and then add the appropriate quantity of additional water so that the final concentration is 90%.

A9.9 Still in dim light ensure the sample is well mixed using a vortex stirrer. Remove the filter paper from the methanol with forceps. Squeeze the paper against the rim of the tube so that as much methanol as possible drains back into the tube.

A9.10 Either, centrifuge the methanol extract, in a stoppered tube (note i) to prevent loss of methanol by volatilization, until a clear extract solution of pigment is obtained (note j). Let the total volume of this extract be v ml. Decant the clear extract without disturbing the sediment (note k).

Or, filter the extract through a small GF/C filter, held in a suitable holder, into a clean tube.

(i) A covering of stretch plastic film is usually adequate to prevent loss of methanol by volatilization.

(j) Centrifugation for 7 minutes at 3500 rev/min is usually sufficient.

(k) A pipette, fitted with a low pressure suction device, may also be used to decant the supernatant without disturbing the sediment.

A9.11 Reserve this extract in a stoppered tube for the absorbance measurements.

A9.12 Fill a spectrophotometer stoppered cuvette (generally 10 mm or 40 mm pathlength) with the pigment extract solution. Let the pathlength of the cuvette used be d mm (note l).

(l) Commonly available 10 mm pathlength cuvettes require 3 ml of extract whereas 40 mm pathlength cuvettes require 10 ml.

Lesser volumes will require the use of narrow, semi-micro 40 mm pathlength cells provided that these are compatible with the spectrophotometer.

A9.13 If appropriate use 90% v/v aqueous methanol as, used to extract the pigments, in the reference beam of the spectrophotometer.

A9.14 Measure the absorbance of the extract at wavelengths of 665 nm, and 750 nm (note m). The calculations are susceptible to error from incorrect setting of the spectrophotometer wavelength. Check this setting regularly using the hydrogen line emitted by the deuterium lamp (ca 656 nm). If corrections are <u>not</u> required for the presence of phaeopigments, omit steps A9.17-19 inclusive. If corrections <u>are</u> required omit steps A9.15 and A9.16 and proceed direct to step A9.17.

(m) Absorbance at 665 nm should fall within the range 0.050 to 0.700 units, otherwise adjust either the volume of sample, the volume of aqueous methanol, or the pathlength of the cell, to meet these criteria.

Absorbance at 750 nm should not exceed 0.005 units per 10 mm of cell pathlength i.e. 0.020 units in a 40 mm pathlength cell.

A9.15 Subtract the absorbance value obtained at 750 nm from that obtained at 665 nm and let this be A (note n).

(n) This step is a correction for any turbidity present.

A9.16 The chlorophyll <u>a</u> content of the sample

$$= \frac{13.0 \times A \times v}{d \times V} \ \mu g\, l^{-1} \ (= mg\, m^{-3})$$

(note o)

(o) The factor 13.0 approximates to the reciprocal of the specific absorption coefficient at 665 nm for chlorophyll <u>a</u> in methanol (Marker *et al.* 1980).

<u>Note again that this</u> calculation makes no correction for the presence of degraded matter or of other pigments.

Where A = absorbance
v = volume of solvent in ml
V = volume of initial filtered samples in litres
and
d = cellpath length in cm

A9.17 Corrections for phaeopigments: do not remove the extract from the cuvette: to 10 ml of extract add 0.1 ml of 0.3M hydrochloric acid solution and mix well (note p). Allow the acidified extract to stand for 5 minutes. Then add 0.1 ml 0.6M of the organic base (see section A5.6).

(p) A micro-pipette should be used. Less than 5 minutes may give rise to incomplete conversion of chlorophyll to phaeophytin. Many samples may be left for up to 30 minutes without interference from epoxicarotenoids but this should be carefully checked (Holm-Hansen and Riemann (1978).

A9.18 Measure the absorbance of the neutralized extract at 665 and 750 nm (note q). Note the values obtained.

(q) Cell faces must be cleaned and re-polished. The cell must always be placed the same way round in the spectrophotometer.

A9.19 For both the unacidified and acidified extracts subtract the absorbance at 750 nm from that at 665 nm. Let the corrected values be A_n (unacidified) and A_m (acidified).

(r) Degradation absent: a value of approximately
1.6
Degradation complete: value 1.0

Then the degree of degradation

$$= \frac{A_n}{A_m}$$

(s) See note r, Section A8.24 and Section D1.7 Sources of Error.

(t) 3.0 is a factor derived from the absorbance of chlorophyll a at 665 nm before and after acidification

$$\left(\frac{A_n}{A_n - A_m} = 3.0\right)$$

(note r)

The undegraded chlorophyll a content (Golterman and Clymo 1969).

$$= C_a$$

where:

$$C_a = \frac{13.0\,(3.0\,(A_n - A_m)) \times v}{d \times V} \ \mu g\, l^{-1}$$

and the phaeopigment content will be:

$$P_a = \frac{13.0 \times 1.6\ (A_n - (3.0\ (A_n - A_m))) \times v}{d \times V}\ \mu g\ l^{-1}$$

notes s and t

where v = total volume of extract (ml)
d = cell pathlength (cm)
V = volume of sample taken (l)

A10 Analytical Procedure

(i) Acetone - specialist preparatory method (see Section C)

Step	Procedure	Notes
A10.1	Filter a measured volume V, (note a) of sample through a glass-fibre filter clamped in a suitable holder (A6.4) (note b). Discard the filtrate (note c).	(a) For most natural waters 1 litre is a suitable volume but this should be adjusted if the expected phytoplankton content is abnormally high or low.
A10.2	After filtration is completed the residual water content of the filter is reduced by allowing air to be drawn through for a short time, usually 30 seconds.	(b) Filtration is accelerated by applying slightly reduced pressure to the receiving vessel. i.e. reduction to 3 atmosphere (corresponding to reduction to 500 mm Hg or 66.6 K Pa). Do not reduce the partial pressure further or the cells may rupture on the filter. (c) The filtrate may be used for the analysis of nutrients and/or trace metals etc by appropriate methods given in other booklets in this series. Care must be taken that the method of filtration is suitable.
A10.3	Remove the filter paper from the holder and weigh. Weigh the filter, then fold it three times (note d). Transfer the filter paper to an homogenizer.	(d) The weight of water retained on the filter should be between 0.5 - 0.7 g for a 9 cm diameter filter, requiring 20 ml ethanol, and proportionately more or less for different diameter filters with differing solvent volumes.
A10.4	Add a small volume of 100% acetone, usually <15 ml and grind vigorously for a few minutes (notes e and f).	(e) Use, either a motor-driven system or a hand held pestle and mortar. (f) Carry out this procedure in the fume cupboard.

(g) Use 100% acetone initially; take into account the residual water on the filter and then add the appropriate quantity of additional water so that the final concentration is 90%.

A10.5 Transfer to a graduated flask and make up to volume in 90% acetone (note g).

A10.6 The tube should be carefully covered to prevent loss of acetone.

A10.7 Allow the solution to stand in the dark for at least 30 minutes, but preferably for about 12-24 hours (i.e. overnight, see note h).

(h) For subsequent HPLC studies leave only for 30 minutes to 1 hour. Longer periods may lead to allomerization and epimerization, which does not interfere with the simpler spectrophotometric and fluorometric techniques.

A10.9 Either, centrifuge the acetone extract, in a stoppered tube (note i) to prevent loss of acetone by volatilization, until a clear extract solution of pigment is obtained (note j). Let the total volume of this extract be v ml. Decant the clear extract without disturbing the sediment (note k).

Or, filter the extract through a small GF/C filter, held in a suitable holder, into a clean tube.

(i) A covering of stretch plastic film is usually adequate to prevent loss of acetone by volatilization.

(j) Centrifugation for 7 minutes at 3500 rev/min is usually sufficient.

(k) A pipette, fitted with a low pressure suction device, may also be used to decant the supernatant without disturbing the sediment.

A10.10 Reserve this extract in a stoppered tube for the absorbance measurements.

A10.11 Fill a spectrophotometer stoppered cuvette (generally 10 mm or 40 mm pathlength) with the pigment extract solution. Let the pathlength of the cuvette used be d mm (note l).

(l) Commonly available 10 mm pathlength cuvettes require 3 ml of extract whereas 40 mm pathlength cuvettes require 10 ml.

Lesser volumes will require the use of narrow, semi-micro 40 mm pathlength cells provided that these are compatible with the spectrophotometer.

A10.12 If appropriate use 90% v/v aqueous acetone as, used to extract the pigments, in the reference beam of the spectrophotometer.

A10.13 Measure the absorbance of the extract at wavelengths of 665 nm, and 750 nm (note m). The calculations are susceptible to error from incorrect setting of the spectrophotometer wavelength. Check this setting regularly using the

(m) Absorbance at 665 nm should fall within the range 0.050 to 0.700 units, otherwise adjust either the volume of sample, the volume of aqueous acetone, or the pathlength of the cell, to meet these criteria.

hydrogen line emitted by the deuterium lamp (ca 656 nm). If corrections are not required for the presence of phaeopigments, omit steps A10.16-18 inclusive. If corrections are required omit steps A10.14 and A10.15 and proceed direct to step A10.16.

Absorbance at 750 nm should not exceed 0.005 units per 10 mm of cell pathlength i.e. 0.020 units in a 40 mm pathlength cell.

A10.14 Subtract the absorbance value obtained at 750 nm from that obtained at 665 nm and let this be A (note n).

(n) This step is a correction for any turbidity present.

A10.15 The chlorophyll a content of the sample

$$= \frac{11.2 \times A \times v}{d \times V} \mu g\, l^{-1} (= mg\, m^{-3})$$

(note o)

Where A = absorbance
v = volume of solvent in ml
V = volume of initial filtered samples in litres and
d = cellpath length in cm

(o) The factor 11.2 approximates to the reciprocal of the specific absorption coefficient at 665 nm for chlorophyll a in ethanol. This is based on the most recently determined specific absorption coefficients (Jeffrey and Humphrey 1975) and supercedes earlier constants (cf 11.9, see Talling and Driver 1963).

Note again that this calculation makes no correction for the presence of degraded matter or of other pigments.

A10.16 Corrections for phaeopigments: do not remove the extract from the cuvette: to 10 ml of extract add 0.1 ml of 0.3M hydrochloric acid solution and mix well (note p). Allow the acidified extract to stand for 5 -30 minutes.

(p) A micro-pipette should be used. Less than 5 minutes may give rise to incomplete conversion of chlorophyll to phaeophytin. Many samples may be left for up to 30 minutes without interference from epoxicarotenoids but this should be carefully checked (Holm-Hansen and Riemann (1978).

A10.17 Measure the absorbance of the acidified extract at 665 and 750 nm (note q). Note the values obtained.

(q) Cell faces must be cleaned and re-polished. The cell must always be placed the same way round in the spectrophotometer.

A10.18 For both the unacidified and acidified extracts subtract the absorbance at 750 nm from that at 665 nm. Let the corrected values be A_u (unacidified) and A_m (acidified).

Then the degree of degradation

(r) Degradation absent: a value of approximately
1 .7
Degradation complete: value 1.0

(s) See note r, Section A8.24 and Section D1.7 Sources of Error.

(t) 2.43 is a factor derived from the absorbance of chlorophyll a at 665 nm before and after acidification

$$- \frac{A_n}{A_m} \qquad \left(\frac{A_n}{A_n - A_m} - 2.43\right)$$

(note r)

The undegraded chlorophyll a content (Golterman and Clymo 1969).

$$- C_a$$

where:

$$C_a = \frac{11.2\,(2.43\,(A_n - A_m)) \times v}{d \times V}\ \mu g\, l^{-1}$$

and the phaeopigment content will be:

$$P_a = \frac{11.2 \times 1.7\,(A_n - (2.43\,(A_n - A_m))) \times v}{d \times V}\ \mu g\, l^{-1}$$

notes s and t

where v = total volume of extract (ml)
d = cell pathlength (cm)
V = volume of sample taken (l)

A11 *In Vitro* Fluorometry

Calibration Procedure

A11.1 Prepare a chlorophyll a extract using either one of the procedures as given in Section A8, A9 or A10 (note a).

(a) Using aqueous ethanol, methanol or acetone or methanol.

A11.2 Determine the chlorophyll a content of the extract spectrophotometrically as given in Section A8 steps 12 to 19, A9 steps 12 to 19 or A10 steps 11 to 18 using the reciprocal specific absorption coefficient appropriate to the chosen solvent.

A11.3 Prepare serial dilutions of the extract with the chosen extraction solvent to obtain chlorophyll a concentrations of approximately 2, 6, 20 and 60 $\mu g\ l^{-1}$ (note b).

(b) If aqueous acetone is used the dilution should be made with *90% v/v* acetone.

A11.4 Using the fluorometer as directed by the manufacturer measure the fluorescence at 663 nm (between 660 and 690 nm) of these solutions excited at about 430 nm, at a series of sensitivity settings. Note the fluorescence scale reading and the corresponding sensitivity setting each time.

A11.5 Use the values obtained in step A11.3 to derive calibration graphs, or factors, relating fluorescence measurements to the corresponding concentrations of chlorophyll a ($\mu g\ l^{-1}$).

Fluorescence Measurements

A11.6 Measure the absorbance of the solvent extract in a 10 mm pathlength cell at a wavelength of 430 nm.

A11.7 If the absorbance value is less than 0.1 units (note c) proceed as given in step A11.8. Otherwise dilute the extract with the appropriate solvent (note d) sufficiently to reduce the absorbance below 0.1 units per 10 mm pathlength (note e). Note the dilution factor used.

(c) aqueous acetone or methanol as used for the extraction procedure.

(d) 90% v/v aqueous acetone (A5.4) or methanol as used for the extraction procedure.

A11.8 Using the fluorometer as directed by the manufacturer measure the fluorescence at 663 nm of the extract solution (note f) excited at 430 nm. Note the fluorescence scale reading and the sensitivity setting used. Relate these values to the appropriate calibration graph or factor (see Sections A11.1 to A11.5) to obtain the chlorophyll a content of the extract solution.

(e) If the pathlength of the fluorometer cell differs from 10 mm the dilution of the extract must be adjusted accordingly in inverse proportion to the change in pathlength.

(f) This will have been obtained using either one of the extract procedures (a), (b), or (c) which may have subsequently been diluted (see step A11.7).

Calculation of Results

A11.9 The chlorophyll a content of the sample

$$= \frac{C \times N \times v}{V} \mu g\, l^{-1} (= mg\, m^{-3})$$

where C = chlorophyll a content of extract solution.
N = factor by which the original extract is diluted.
v = total volume of original extract (in ml).
V = volume of sample taken (in l).

A12 Sources of Error

1. The presence of chlorophyll b and c as well as Mg-containing porphyrins.
2. Failure to achieve complete extraction of chlorophyll.
3. Exposure of the sample or sample extract to light.
4. The stability of extract solutions.
5. Loss of solvent by evaporation during analytical procedures.
6. Spectrophotometer wavelength calibration scale errors. Wavelength scales should be checked frequently using a didymium or holmium filter. If the instrument is fitted with a hydrogen lamp a characteristic emission peak at 656.3 nm can be used.
7. Spectrophotometer absorbance scale calibration errors. Check as instructed by the manufacturer.

A13 Checking the Accuracy of Analytical Results

Once the method has been put into normal routine operation many factors may subsequently adversely affect the accuracy of analytical results. It is recommended that tests to check sources of inaccuracy should be made regularly as appropriate. Unfortunately, due to the nature of the sample, simple control chart procedures with a standard sample are not directly possible.

B IN VIVO FLUOROMETRIC DETERMINATION OF CHLOROPHYLL A

B1 Performance Characteristics of the Method

Step	Procedure	Notes
B1	Substance determined	Chlorophyll a.
B1.2	Type of sample	Aqueous suspension of algae or phytoplankton.
B1.3	Basis of method	Direct measurement of fluorescence *in vivo*.

B1.4	Range of application	1 to 100 μg l^{-1} chlorophyll a; but is extremely variable depending upon the type of algae.
B1.5	Calibration graph	Method must be calibrated for each type of alga or phytoplankton community of interest with reference to an absolute extractive spectrophotometric method (see Section A).
B1.6	Total standard deviation	Highly dependent upon the type and physiological state of the algae or phytoplankton present and upon the amount of degraded matter present, and background fluorescence. See also method D.
B1.7	Limit of detection	
B1.8	Bias	
B1.9	Interferences	Degradation products of chlorophyll a and background fluorescence.
B1.10	Time required for analysis	< 5 minutes per determination excluding calibration.

B2 Principle

Direct in vivo measurement of fluorescence at above 650 nm by excitation of the water sample at 430-450 nm (Daley et al. 1973, Loftus and Carpenter 1971, Stainton et al. 1977, Loftus and Seliger 1975, Heaney 1978, Faust and Norris 1985, Ernst 1987).

B3 Interferences

The degradation products of chlorophyll, such as phaeopigments, may be present in appreciable amounts and are difficult to distinguish adequately leading to an overestimation of the true chlorophyll a content.

Background fluorescence is a possible source of interference and a correction must be made.

B4 Hazards

See section A4.

B5 Reagents

See Section A5.

Appendix

B6 Apparatus

B6.1 Fluorometer equipped with a high excitation source at wavelengths in the region of 430 nm and fitted with:

1. A blue excitation filter e.g. Corning CS 5-60
2. A red fluorescence filter e.g. Corning CS 2-64
3. A red sensitive photomultiplier tube having good response at 685 nm.

The instrument must be used strictly in accordance with the manufacturers instructions.

B6.2 Optical cells, pathlength 10 mm compatible with fluorometer B6.1. Alternatively a cell of a suitable flow-through type may be used.

B6.3 Sample mixer

e.g. "micro Standard Silverson Laboratory Mixer", Silverson Machines Ltd., Waterside, Chesham, Bucks.

B6.4 Filtration apparatus capable of accommodating glass fibre filter papers.

B7 Sample Collection and Preservation

B8 Analytical Procedure

Step	Procedure	Notes
B8.1	Divide the sample into two representative subsamples.	
B8.2	Using the mixer (B6.3) mix one subsample for 120 ± 10 seconds and place in the dark for between 10 and 60 minutes (note a).	(a) This procedure eliminates the possible depression of fluorescence from previous illumination.
B8.3	Mix the sub-sample well by shaking and transfer a suitable volume to a fluorometer cell (note b).	(b) Flow-through type cells may be used.
B8.4	Measure the fluorescence with equipment as described in B6.1. Note the fluorescence scale reading and the sensitivity setting used (note c).	(c) The appropriate sensitivity setting must be chosen as directed by the instrument manufacturer appropriate to the level of fluorescence to be measured.
	"Background fluorescence" determination	
B8.5	Filter the second sub-sample through a glass fibre filter.	
B8.6	Measure the fluorescence of the filtrate as	(d) Background fluorescence is usually fairly

	described above for the first sub-sample in step B8.4 (note d).	constant for similar samples taken over a short period of time eg. one day's samples from one reservoir.
	Calculation	
B8.7	Subtract the "background fluorescence from that of the sample. Relate this corrected fluorescence to an appropriate calibration graph prepared as given in section B9 to obtain the concentration of chlorophyll a present.	

B9 Calibration Procedure

Step	Procedure	Notes
B9.1	Obtain a representative sample of algae or phytoplankton from the community of interest (note a).	(a) This calibration procedure must be carried out for each particular community of interest since the slope of the calibration graph depends upon the types of algae present, the physiological state of the algae, and the mode of operation of the fluorometer (Heaney 1978).
B9.2	Determine the chlorophyll a content of the sample by one of the solvent extraction methods (a), (b) or (c) as given in Section A8, A9 or A10.	
B9.3	Proceed as given in Section A11 steps 3 to 5 (note b).	(b) Using an optical pathlength of 10 mm in the fluorometer a typical calibration curve is usually linear over the range 1 to approximately 100 $\mu g\ l^{-1}$ chlorophyll a.

B10 Submersible fluorometers

Submersible *in-vivo* fluorometers are commercially available (e.g. 'Aquatracker II' from Chelsea Instruments, London) for detecting *in-situ* changes in signal, alluding to concentration differences in chlorophyll with high resolution. This equipment is particularly useful for detecting small scale spatial patterns of chlorophyll distribution and for monitoring temporal changes in concentration. It is highly sensitive and, although developed primarily for oceanic work, is also well suited to lake, reservoir and river work.

B11 Sources of Error

The chief sources of error other than instrument malfunction are due to the presence of other pigments and other fluorescent substances present in the sample, and to decomposition or growth of the sample prior to analysis.

Appendix

C DETERMINATION OF PIGMENT MIXTURES. ESPECIALLY CHLOROPHYLLS a, b AND c AND THEIR BREAKDOWN PRODUCTS.

C0 Introduction

In addition to chlorophyll a, which is present in all green plants, algae and Cyanobacteria, chlorophyll b is present in the Chlorophyceae and all higher plants while chlorophyll c is present in a wide range of 'brown' algae (eg Bacillariophyceae, Phaeophyceae, Dinoflagellata, Cryptomonads etc.

C1 Determination of Chlorophyll a, b and c

The traditional method of estimating the minor chlorophylls b and c involves the use of 'trichromatic equations' (Richards with Thompson 1952, Parsons and Strickland 1963, Strickland and Parsons 1973, Chang and Rossmann 1981, Jeffrey and Humphrey 1975). However, this procedure is particularly susceptible to errors and requires the use of top quality instrumentation (Marker *et al.* 1980). Moreover, if degradation products are present, the equations cannot work, even on theoretical grounds.

C2.1 Principles

The method is taken from Jeffrey and Humphrey (1975). Spectrophotometric measurement of the absorbance of acetone or methanol extracts of plant material at wavelengths of 630, 647, 664 and 663 nm.

C2.2 Hazards

See Section A4.

C2.3 Reagents

See Section A5.

C2.4 Apparatus

See Section A6.

Note, however, that an exceptionally well maintained, top quality spectrophotometer together with top quality accessories is required which will record absorbances to four or five decimal places (Jeffrey and Humphrey 1975). The wavelength setting must be checked before each analysis. The standard analytical spectrophotometer is unlikely to be adequate.

C2.5 Sampling and Sample Collection

See Section A7.

Appendix

C2.6 Analytical Procedure

Step	Procedure
C2.6.1	Obtain an acetone extract as given in Section A10.
C2.6.2	Measure the absorbance of the extract at wavelengths of 630 nm, 647 nm, 664 nm, 663 nm and 750 nm. Subtract the absorbance at 750 nm from that of each of the others. Let these values be Ao, Ap, Aq and Ar, respectively.
	Calculations
C2.6.3	For higher plants and green algae containing chlorophylls a and b (solvent 90% acetone).

$$C_a = \frac{(11.93A_q - 1.93A_p) \times v}{d \times V} \ \mu g\, l^{-1}$$

$$C_b = \frac{(20.36A_p - 5.50A_q) \times v}{d \times V} \ \mu g\, l^{-1}$$

C2.6.4 For diatoms, chrysomonads and brown algae containing chlorophylls a, c_1 and c_2 in equal proportions (solvent 90% acetone).

$$C_a = \frac{(11.47A_q - 0.40A_o) \times v}{d \times V} \ \mu g\, l^{-1}$$

$$C_{c12} = \frac{(24.36A_o - 3.73A_q) \times v}{d \times V} \ \mu g\, l^{-1}$$

C2.6.5 For dinoflagellates and cryptomonads containing chlorophylls a and c_2 (in this case the solvent is 100% acetone).

$$C_a = \frac{(11.43A_r - 0.64A_o) \times v}{d \times V} \ \mu g\, l^{-1}$$

$$C_{c2} = \frac{(27.09A_o - 3.63A_r) \times v}{d \times V} \ \mu g\, l^{-1}$$

C2.6.6 For mixed phytoplankton populations.

$$C_a = \frac{(11.85A_q - 1.54A_p - 0.08A_o) \times v}{d \times V} \ \mu g\, l^{-1}$$

$$C_b = \frac{(-5.43A_q + 21.03A_p - 2.66A_o) \times v}{d \times V} \ \mu g\, l^{-1}$$

$$C_{c12} = \frac{(-0.67A_q - 7.60A_p + 24.52A_o) \times v}{d \times V} \ \mu g\, l^{-1}$$

where v = total volume of extract (ml)
d = cell pathlength (cm)
V = sample volume (l)

C3 Sources of Error See Section A12

These equations make no allowance for chlorophyll breakdown products. The method must not be attempted when these are present (>5%) since they lead to very misleading results.

C4 Separation of chlorophylls a, b and c and their breakdown products using high performance liquid chromatography (HPLC).

C4.1 Introduction. This is a very accurate, quantitative method of separating:

(i) the primary chlorophylls (a, b and c),
(ii) the chlorophyllides (phytol chain missing),
(iii) the phaeophytins (Mg missing) and the phaeophorbides (both phytol and Mg

missing).

The method is elaborate and requires extensive expertise and should not be attempted without detailed methodological preparation or substantial in-house expertise. In addition there should be clearly established objectives showing why such elaborate methodology is necessary. For these reasons only an outline of the procedure is given below.

C4.2 The apparatus requires a gradient elution system consisting of:

(i) Two high quality pumps,
(ii) Three solvent reservoirs,
(iii) One rheodyne injection valve,
(iv) Solvent switching mechanism, either manual or automatic,
(v) A column suitable for reverse-phase chromatography (eg Shandon Hypersil ODS, Mantoura and Llewellyn 1983),
(vi) A very sensitive fluorometer detection system using excitation at 440nm (ie blocked above 480 nm) and an emission filter blocking output below 600 nm.
(vii) An integrating chart recorder which generate peak height, peak area and elution time.

C4.3 Samples and standards. Samples must be extracted in 90% acetone using grinding to rupture cell walls. Prolonged extraction is not advised and extraction in alcohol readily leads to allomerization and epimerization of the native chlorophylls. These products have significantly different elution times and therefore adversely affect the accuracy of the results. Samples may be concentrated using Sep-Pak cartridges.

Chlorophylls a and b are available commercially (Sigma) but must be checked for purity and then calibrated. Purity is established chromatographically. Calibration is performed by dissolving the standard in 90% acetone and estimating the concentration spectrophotometrically using the specific absorption coefficients of Jeffrey and Humphrey (1975). The corresponding phaeophytins may be prepared from the standard chlorophylls by mild acidification. The calibration of chlorophyll c is under review (Mantoura pers. comm.) and should be available shortly.

C4.5 Solvent systems. There are numerous solvent systems that can be used but that of Mantoura and Llewellyn (1983) is well established. The first solvent system contains an ion pairing reagent which aids the separation of the more acidic chlorophyllides and phaeophorbides from chlorophyll c.

C5.5 For further reading, refer to Mantoura and Llewellyn (1983), Wright and Shearer (1984), Gieskes and Kraay (1983, 1986 a & b), Murray *et al.* 1986, Zepata et al. (1987) and Yacobi *et al.* (1991).

Appendix

D METHODS OF COLLECTING AND EXTRACTING SURFACE LIVING ATTACHED ALGAE (MICROBENTHOS AND PERIPHYTON)

D1 Sample Collection

D1.1 From Submerged Surfaces

Use methods described in another booklet in this series (HMSO 1983) to remove attached algae from submerged surfaces.

D1.2 From soft benthic sediments, e.g. mud and silt.

The algae removed from these sources will usually be obtained in an aqueous suspension and this should be filtered as given in Section A.

D1.3 From gravel and small stones

Immerse a representative sample of substratum directly in a suitable volume of the chosen solvent (see sections A8 and A9). Because accurate sub-sampling is difficult extraction of part of the sample only should be avoided.

D1.4 From Larger Aquatic Plants (Macrophytes)

Obtain a sample, of the order of 25 g fresh weight of shoots, by cutting or pulling fresh plant material. Store in an air-tight container.

D2 Sample Preservation

Observe the precautions given in Section A7.

D3 Sample Extraction

D3.1 Gravel and Small Stones

The pigments from gravel and small stones may be extracted into ethanol or methanol by placing the stones in a suitable volume of solvent contained in a wide-mouthed vessel with a tight fitting screw lid. Since many encrusted populations (Chlorophyceae and Cyanobacteria) are very difficult to extract and grinding is impractical, it may be necessary to use methanol (A9). Due to the large volumes of methanol required, particular attention must be given to safety hazards.

D3.2 Sediments

Grinding to aid extraction is only possible when dealing with the finest of sediments. Pigments can be extracted from diatoms into 90% v/v acetone during 24 h in the dark at about 4°C without grinding. Green and blue algae in the periphyton are particularly

resistant to extraction and it is essential to use ethanol or methanol, rather than acetone, for extraction purposes.

D3.3 Extraction of Larger Aquatic Plants (Westlake 1974)

Grind and homogenize 25 g of sample D1.4 and extract into a suitable known volume of solvent, typically 250 ml. Centrifuge to obtain a clear solution for either spectrophotometric evaluation as given in Section A8, A9 or A10.

D3.3.1 Allowance must be made for the water content of the plant material when carrying out the procedures. For this, determine the percentage loss in weight on drying at 80°C using replicate samples.

D4 Analytical Procedures

The absorbances of the extracts are measured using the procedures described in Section A8, A9 or A10 of this booklet as appropriate. Note, however, that a modified calculation procedure may be necessary since most of the samples described in this section are taken by weight and not by volume.

D5 Degradation Studies See Section A8 and A9.

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