Institute of Freshwater Ecology

Chlorophyll a
SCA Method Revision

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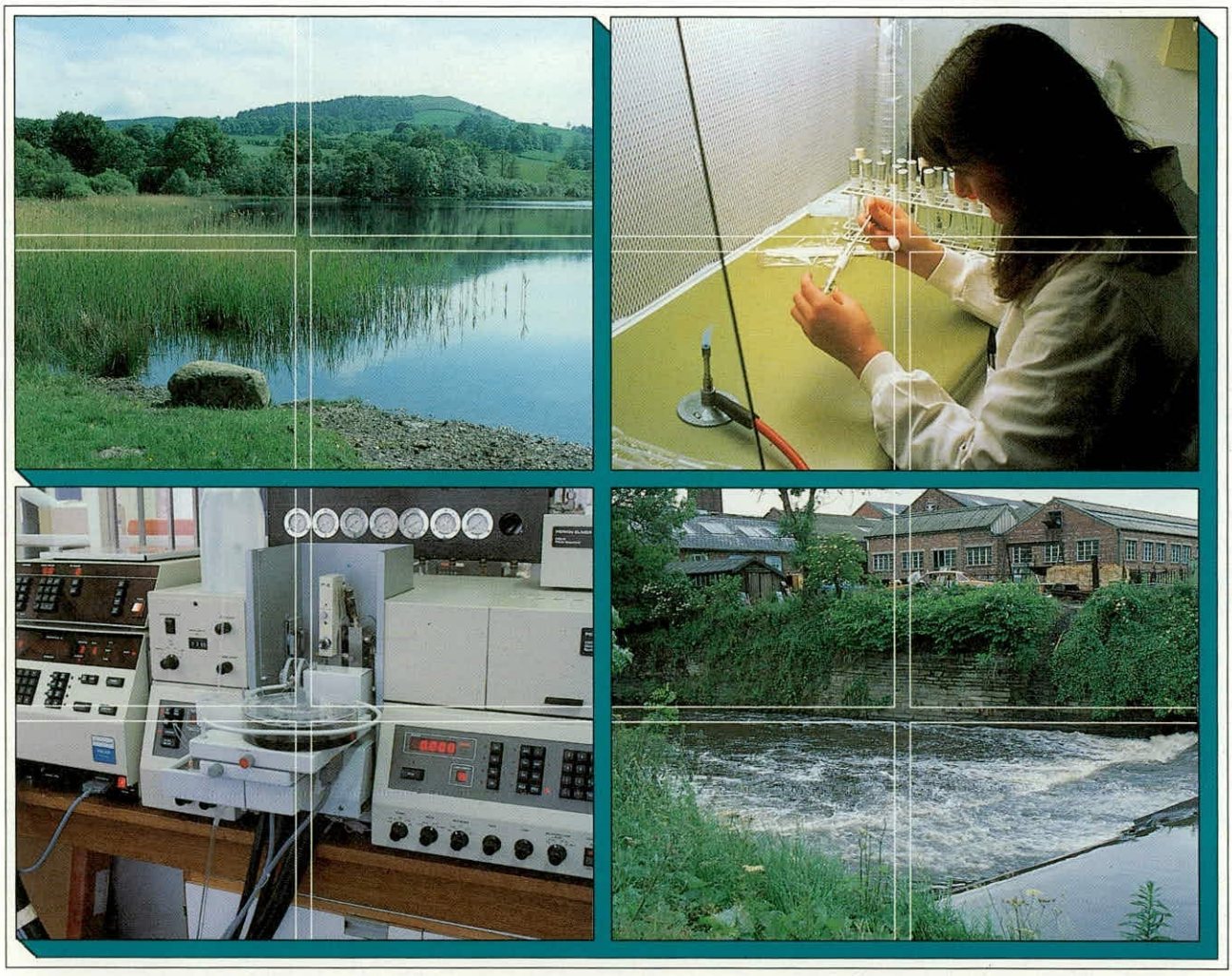

Natural Environment Research Council

Publisher
National Rivers Authority
Rivers House
Waterside Drive
Aztec West
Bristol
BS12 4UD

Tel: 0454 624400
Fax: 0454 624409

First Published 1994

Dissemination Status:
Internal: Limited Release
External: Restricted

Statement of Use:
The method will be implemented as a standard method within NRA Laboratories and be used as a basis for a revision of the Standing Committee of Analysts (SCA) Blue Book Method.

Research Contractor:
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PR 395/3/A

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ACKNOWLEDGMENTS

The smooth passage of this contract has been aided by several members of the Standing Committee of Analysts, particularly Mr L.R. Pittwell. Several algal experts have also been very helpful to me; Dr C.S. Reynolds, IFE Windermere, for advice on *in vivo* fluorometry; Dr R.F.C. Mantoura for advice on HPLC of plant pigments. Dr H. Rai, Max Planck, Plön, Germany and Dr B. Riemann, Denmark for the use of ethanol in Germany and Denmark for extracting pigments .

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 relevant literature is largely reviewed in the appropriate sections in the appendix but the most important parts are **repeated here.** The revision brings the primary methods up to date and greatly simplifies the spectrophotometer procedures. All laboratories will have the basic safety procedures in place so that only minor additions should be required for plant pigment analysis. The introduction of the COSHH regulations in January 1991 makes the use of methanol as the primary extraction solvent less desirable because there is a general requirement to use the least hazardous procedure possible.

2.1 *In-vitro* spectrophotometric methods

2.1.1 Sample Collection and Preservation

It must be emphasized that throughout these methods samples and sample extracts should be handled, as far as possible, in subdued light.

Phytoplankton can be patchily distributed both laterally and vertically in lakes and reservoirs. This must be taken into account when embarking on a sampling programme. This is not covered in this revision since the analysis of chlorophyll is concerned, primarily, with an analytical method.

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 Heinonen 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).

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

2.1.2 Filtration

Generally glass-fibre filters (1.2 µm porosity), e.g. Whatman grade GF/C or the equivalent, are used. But the retentive capacity of GF/C filters may need to be checked against membrane filters (Lenz and Fritsche 1980, Munawar *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).

2.1.3 Choice of Solvent

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

(i) the superiority of the alcohols (especially when hot) as extractants;
(ii) the better-known characteristics and greater stability of chlorophyll a solutions in acetone;
(iii) the ease of making a simple 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, although a more complex method, using methanol, is given for algae particularly resistant to extraction.

It should be particularly noted that in alcohols extractions occur best when the water content of the solvent approaches zero. This is in contrast to acetone where the addition of 10% water is preferred. Many earlier protocols erronously stipulate 90% methanol as the extractant although it has been known for more than twenty years that absolute alcohol was more effcient.

For further discussion and comparative data see Talling and Driver (1963), Marker (1972) and Jones (1977), Riemann (1978), Riemann and Ernst (1982), Jespersen and Christoffersen (1987). Other solvents have been used with apparent success, for example, dimethyl sulphoxide (Shoaf and Lium 1976, Palumbo *et al.* 1987), methanol-chloroform mixtures (Bowles *et al.* 1985, Wood 1985, Lloyd and Tucker 1988) and dimethylformamid (Neveux 1988). Since full QA/QC are not available in the literature these methods have not been included.

Ethanol is now recommended as the primary extractant in line 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 the formation of allomers and epimers of the chlorophylls (Mantoura and Llewellyn 1983). We must assume that similar transformations will also occur in other alcohols.

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.

2.1.4 Methods to Distinguish Undegraded and Degraded Pigments

Not all the procedures,listed in the appendix, distinguish between undegraded and degraded ("dead") forms of chlorophyll a. Section A offers simple methods of estimating of chlorophyll a only, with corrections for interference from phaeopigments. 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). Section B, although offering a rapid and sensitive *in vivo* method of measuring chlorophyll a, does not distinguish the breakdown products. Section C offers two methods of making a more rigourous separation, the first by improved but classical trichromatic equations (Jeffrey and Humphrey 1975), the second 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) and paper chromatography (Hallegraeff 1976, Jensen and Liaaen-Jensen 1959, Jensen and Sakshaug 1973, Eloranta 1986) have been widely used but quantitative analysis is more time consuming and are not listed here.

2.1.5 Checking the Accuracy of Analytical Results (Quality Control)

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.

Pure chlorophyll *a* is not available commercially. Material available from, for example, "Sigma Chemical Company Ltd." is spectroscopically pure but not gravimetrically pure. In other words there are no coloured compounds interfering with the visible spectrum. HPLC does not reveal any epimers, allomers nor any Mg- nor phytol-lacking derivatives. But, because the calculated molar extinction coefficient is significantly lower than that published by Jeffrey and Humphrey (1975) there must be either water or a colourless contaminant present. Standard methods of drying risk significant degradation. Therefore, the use of commercial chlorophyll is not generally recommended, unless a solution in 90% acetone is initially calibrated at 665 nm against published absorption coefficients (Jeffrey and Humphrey 1975). Since solutions are unstable and chlorophyll *a* costs £(UK)47 per mg, it is unlikely to be available to most users for routine purposes. The following protocol is recommended.

1. Always use matched spectrophotometer cuvettes.

2. Number these cuvettes on their base and always use them in the same location in the multiple cell holder and always in the same orientation.

3. Initially, check the variations between cuvettes by measuring the absorbance at 750 and 665 nm, filled with the standard solvent in normal use. These values will give the extent of cuvette to cuvette differences and must be carefully recorded in a "log book". This procedure must be used regularly, say every month, and changes will indicate deteriorating optical surfaces or accumulated deposits. For a reagent blank the absorbances at 665 and 750 nm should be the same, even if slightly different from zero. Consequently for a pigment extract, subtracting the absorbance at 750 nm from the absorbance at 665 nm corrects for both cuvette to cuvette differences as well as residual turbidity.

4. Although modern spectrophotometers have built-in wavelength checking mechanisms each time they are powered up, quality control demands that this must be independently checked. Standard didymion filters serve this purpose admirably and have two sharp absorption maxima at 573 and 585 nm (data provided with the filter).

5. In addition, the absolute accuracy of the absorbance reading, itself, can be checked using standard solutions of potassium dichromate ("standard solution 'Spectrosol' for calibration of spectrophotometers" --- from Merck).

6. The wavelength and absorbance checks (4 & 5) enable the specific absorption coefficients of chlorophyll *a* published in the scientific literature to be used. For 90% acetone the most accurate and recent of these is that of Jeffrey and Humphrey (1975) and is 89. The reciprocal (x1000) of this, 11.2, is the constant used in the equations in section A10 and supersedes earlier constant based on outdated literature. For ethanol we propose to adopt the constants used in Germany and Denmark, 12.2 (Wintermans and De Mots 1965) and for methanol the constant is 13.0.

7. The error associated with the final analysis (i.e. after extraction) is primarily dependant on the absorbance of the extract. Since this is partly controlled by the volume filtered,

the volume of the extractant and the path length of the cuvette, the analytical error is partially independent of phytoplankton concentration. The table, on the next page, shows a range of absorbance values with the associated analytical errors. These results were obtained from extracts in ethanol and a similar range would be obtained with 90% acetone. The use of hot alcohol barely affects the analytical error. The use of methanol as an extractant requires an additional neutralisation step which will slightly increase the associated errors.

Pigment	**Absorbance**	**MEAN**	**SD**	**%**	**SE**	**%**
Chlorophyll *a*	0.030	1.07	0.093	8.66	0.029	2.74
Phaeo-pigment *a*		0.56	0.123	21.78	0.039	6.89
Chlorophyll *a* (no correction)		1.40	0.053	3.74	0.017	1.18
Chlorophyll *a*	0.040	2.60	0.200	7.71	0.063	2.44
Phaeo-pigment *a*		2.35	0.313	13.25	0.099	4.22
Chlorophyll *a* (no correction)		3.98	0.162	4.07	0.051	1.29
Chlorophyll *a*	0.095	2.90	0.103	3.53	0.033	1.12
Phaeo-pigment *a*		0.91	0.142	15.65	0.045	4.95
Chlorophyll *a* (no correction)		3.44	0.121	3.50	0.038	1.11
Chlorophyll *a*	0.115	3.82	0.194	5.09	0.062	1.61
Phaeo-pigment *a*		0.78	0.122	15.69	0.039	4.96
Chlorophyll *a* (no correction)		4.28	0.141	3.29	0.045	1.04
Chlorophyll *a*	0.200	9.16	0.287	6.15	0.091	0.99
Phaeo-pigment *a*		4.74	0.304	6.41	0.096	2.03
Chlorophyll *a* (no correction)		11.90	0.334	8.81	0.106	0.89
Chlorophyll *a*	0.500	24.3	0.693	2.86	0.219	0.9
Phaeo-pigment *a*		10.0	0.579	5.79	0.183	1.83
Chlorophyll *a* (no correction)		30.1	0.681	2.26	0.216	0.72
Paired comparison						
Hot ethanol extraction						
Chlorophyll *a*	0.133	6.301	0.084	1.33	0.037	0.59
Phaeo-pigment *a*		2.218	0.161	7.24	0.072	3.24
Chlorophyll *a* (no correction)		7.600	0.121	1.59	0.054	0.71
Cold ethanol extraction						
Chlorophyll *a*	0.125	6.150	0.084	1.36	0.037	0.61
Phaeo-pigment *a*		2.178	0.183	8.40	0.818	3.76
Chlorophyll *a* (no correction)		7.430	0.080	1.08	0.358	0.48

2.2 *In vitro* Fluorometry

In vitro fluorometry is widely used in oligotrophic environments (e.g .Loftus and Carpenter 1971, Daley *et al.* 1973, Stainton *et al.* 1977, Neveux and Parnouse 1987, Coveney 1988) as a more sensitive alternative to absorption spectrophotometry. The sensitivity of the technique, as applied to an extract, is much greater than that of the corresponding spectrophotometric method and is, therefore, particularly useful in oligotrophic environments or any situations where algal concentrations are particularly low.

Detailed quality control data are not readily available at the moment and are best assessed by individual laboratories, tailored to their specific requirements. However, careful calibration of the fluorometer is essential. The original "Blue-Book" recommended calibration against an extract of phytoplankton of known chlorophyll *a* concentration (determined spectrophotometrically). It is equally valid to calibrate against commercial chlorophyll (see section 2.5) but, again, solutions must be calibrated initially by spectrophotometry.

2.3 *In vivo* Fluorometry

Direct in vivo measurement of fluorescence at ca 680 nm by excitation of the water sample at 430-450 nm (e.g. Loftus and Seliger 1975, Heaney 1978, Faust and Norris 1985, Ernst 1987) is a very valuable tool for the management of reservoirs.

In vivo readings, although related to chlorophyll concentration, are affected by a number of factors, in particular species composition, nutrient status, irradiance levels and the immediate past physiological history of the algae. 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. Consequently regular comparisons, using extractive spectrophotometry, are essential. Background fluorescence is a possible source of interference and a correction must be made. The frequency cannot be laid down, with any certainty, in a standard protocol. Operator experience and judgement is essential. Sampling homogeneous populations calibration may only be necessary every 30 samples. But depth profiles, revealing different population structures, or lateral sampling from open water into a bloom of Cyanobacteria will require more frequent calibration. Detailed QC is a complex matter, requiring the interfacing of a formal chemical method (calibration) with an essentially field biological method (*in vivo* fluorometry). The method is, therefore, not subject to the same type of quality control as a formal analytical method (A8-11). However it is an exceptionally valuable operational instrument for managers of reservoirs etc. Skilful use of these instruments can save hours of analytical laboratory time and this advantage should not be underestimated when operational protocols and quality control procedures are drawn up.

2.8 Trichromatic equations

High precision instrumentation is required to use the trichromatic equations of Jeffrey and Humphrey (1975). Detailed performance characteristics have not been published.

2.9 High performance liquid chromatography

This is certainly the accurate but also the slowest of the methods. Although now widely used for the accurate and rigorous of analyses, speed of analysis limits its use in most operational areas and detailed methodology is not given

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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

Appendix

THE DETERMINATION OF CHLOROPHYLL A IN AQUATIC ENVIRONMENTS

0 ABOUT THESE METHODS

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 Recommended Methods

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

a) Simple solvent extraction techniques (Section A) using either ethanol, methanol or acetone, followed by spectrophotometric or fluorometric evaluation of the extract.
b) In-vivo fluorometry (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 (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 simple method of estimating of chlorophyll a only, with corrections for interference from phaeopigments. Section B, although offering a rapid and sensitive *in vivo* method of measuring chlorophyll a but does not distinguish the breakdown products. Section C offers two methods of making a more rigourous separation, the first by improved but classical trichromatic equations (Jeffrey and Humphrey1975), the second 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, Hallegraeff & Jeffrey 1985, Jeffrey 1968, 1974, 1981. Gieskes *et al.* 1978) and paper chromatography (Hallegraeff 1976, Jensen and Liaaen-Jensen 1959, Jensen and Sakshaug 1973 and 1986) have been widely used but quantitative analysis is more time consuming.

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 a & b 1980, 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 an 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 realistic limit of detection of the fluorometric method is about 1 μg l^{-1} chlorophyll *a*, after filtering 2000 ml raw water and using 10 cm cuvettes. The realistic limit of detection of the fluorometric determination of extracts is between 20 and 100 ng l^{-1} chlorophyll *a*. For further information on quality assurance see A13.
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 spectrophoto- or fluoro-metric methods 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. Smaller numbers are disproportionately more time consuming.

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) 665 nm, the absorption maximum of chlorophyll a

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

A2.2 The choice between ethanol, methanol or 90% 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;

(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, although a method using methanol is given: for algae particularly resistant to extraction, hot methanol or ethanol is more effective then acetone. **It should be particularly noted that in alcohols extractions occur best when the water content of the solvent approaches zero.** This is in contrast to acetone where the addition of 10% water is preferred. For this reason, after filtration filters are allowed to dry partially (see sections A8.3 and A9.3). For further discussion and comparative data see Talling and Driver (1963), Marker (1972) and Jones (1977), Riemann (1978), Riemann and Ernst (1982), Jespersen and Christoffersen (1987). Other solvents have been used with apparent success, for example, dimethyl sulphoxide (Shoaf and Lium 1976, Palumbo *et al.* 1987), methanol-chloroform mixtures (Bowles *et al.* 1985, Wood 1985, Lloyd and Tucker 1988) and dimethylformamid (Neveux 1988). Since full QA/QC are not available in the literature these methods have not been included.

A2.3 Fluorometry (Loftus and Carpenter 1971, Daley *et al.* 1973, Stainton *et al.* 1977, Neveux and Parnouse 1987, 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 and is, therefore, particularly useful in oligotrophic environments or any situations where algal concentrations are particularly low.

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.

A4 Hazards

Normal laboratory safety precautions must be observed.
Standard laboratory procedures should be used. COSHH regulations impose constraints 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. Only small quantities should be left unattended (e.g. 250 ml) on the laboratory bench since unexpected hazards can easily arise (e.g., flash ignition from an unprotected spark source; sunlight falling on solvent in a close bottle or wash bottle is also a potential hazard. 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 or latex should also be used.

(iii) Methanol is **highly toxic** and should not be inhaled. All operations involving open vessels must 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 (e.g. using Dräger tubes).

(iv) Even with ethanol and acetone fume cupboards should be used wherever possible and certainly when large quantities are being dispensed. Spillage procedures should be clearly posted.

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. See Section A6.8 for the types of centrifuge tube that should be used.

Although it is preferable to shield or net all apparatus operated under reduced pressure to prevent injury to the operator in the event of an implosion, this is very unlikely to occur. The reduction of pressure by ⅓ atmosphere carries little risk using new "Buchner" flasks.

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. Although this is not a problem for most bonefide laboratories, some environmental analyses may be carried out at remote field stations, where facilities may be very restricted.

A5 Reagents

A5.1 Analytical reagent grade chemicals, and distilled or deionized water (18 MΩ cm) should normally be used throughout. For *in vitro* fluorometry, HPLC-grade solvents maybe required when concentrations of chlorophyll are low.

A5.2 Ethanol (Section A8)
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. The shelf life in a well-stoppered bottle should be at least one month.

A5.3 Methanol (Section A9)
90% v/v methanol aqueous (for dilution and reference cells in section A9)
Proceed as in A5.2 using methanol instead of ethanol. The shelf life in a well-stoppered bottle should be at least one month.

A5.4 Acetone (Section A10)
90% v/v acetone aqueous (for dilution and reference cells in section A10)
Proceed as in A5.2 using acetone instead of ethanol. The shelf life in a well-stoppered bottle should be at least one month.

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). There is some latitude allowed in the precise concentration. It is adequate to dilute 3 ml concentrated HCl (10 - 12 M) to 100 ml with distilled/ deionized water.

A5.6 Organic base (for neutralizing acid in A9 only, see A5.5)
3 x 10^{-1}M methanolic 2-phenylethylamine. Weigh out 3.63 g (3.78 ml) and dilute to 100 ml with absolute methanol. Keep cool and dark in a well stoppered bottle. Replace every two to three weeks.

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 at 685 nm. Many manufacturers (e.g. Turner Designs) supply specialized kits, specifically for chlorophyll analysis.

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 a fluorometer A6.2. Alternatively a suitable flow-through cell may be used.

A6.3 **Glass-fibre filters** Generally glass-fibre filters (1.2 μm porosity), e.g. Whatman grade GF/C or the equivalent, are used. But the retentive capacity of GF/C filters may need to be checked against membrane filters (Lenz and Fritsche 1980, Munewar *et al.* 1982, Venrick *et al.* 1987) or GF/F filters (Prepas *et al.* 1988) if picoplankton are likely to be important. Membrane filters should be 0.45 μm or 0.2 μm (porocity), but the latter may have a very low filtering capacity. The filtration rate through GF/F filters (0.8 μm porocity) will be faster but many of the picoplankton will be smaller and may pass through.

A6.3.1 Method: Filter a known volume of phytoplankton sample through a GF/C filter. Use the filtrate for a further filtration step with one of the finer porocity filters. Extract the chlorophyll from the two filters separately following the procedures outlined in sections A8 or A9.

Appendix

Let c_1 be the chlorophyll *a* retained during the first filtration step
Let c_2 be the chlorophyll *a* retained during the second filtration step
then the percentage retention by the GF/C filter will be:

$$\frac{C_1 \times 100}{C_1 + C_2}$$

Powdered $MgCO_3$ as a filtering aid is unnecessary (Lenz and Fritsche 1980, Lloyd and Tucker 1988).

A6.4 **Filter holder:** These were traditionally porcelain Hartley funnels to support the filter on a detachable porous support plate; they come with a choice of sizes and the support plate is generally either nylon or polypropylene. It is useful to use a a small circular spacer on the support plate, made of tygan mesh, say 1mm porocity. The Hartley funnel should have provision to operate under reduced pressure and should clamp the filter around its periphery.

A6.5 **Suction pump**: A small electric pump, or a water pump fitted with a non-return valve and an intermediate trap.

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

A6.7 **Simple laboratory centrifuge** Capable of 3000 rpm and taking 4 - 8 x 50ml centrifuge tubes.

A6.8 **Centrifuge tubes** 50 ml stoppered or capped centrifuge tubes. They may be borosilicate or polypropylene. The capped polypropylene variety minimizes breakages and spillages.

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

A6.10 **Vortex mixer** Used for dispersing pigment from algae on filters throughout the extraction solvent. Since the extraction tubes are stoppered, the vortex mixer does not have to be spark free.

A6.11 **Refrigerator** Spark-free, for keeping the extracting samples cool in the dark.

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 Heinonen 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).

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

Appendix

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 ⅔ 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) Residual water, retained on the filter, should weigh 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. (see A2.2)
A8.4(i)	Either, add a known volume of hot ethanol (70 °C), 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, heat to boiling and boil for 2-3 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 (e.g. overnight). Agitate the filter briefly from time to time during this period.	(g) Immersion of the tube in a water bath is effective; the water temperature should be just above the boiling point of ethanol (78.5°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 remain covered to prevent loss of ethanol.

A8.7 Allow the 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 (see A8.3) so that the final concentration is 10% water in ethanol (note h).

(h) If 20 ml 100% ethanol was used initially, a total of 2.22 ml of water will be required to make the final solution 90%. Take into account the residual water on the filter (A8.3) and then add the appropriate quantity of additional water required.

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

(i) Centrifugation for 7 minutes at 3000 rev/min is usually sufficient.

(j) Alternatively the supernatant may be siphoned off without disturbing the sediment.

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

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 k).

(k) 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 l). The calculations are susceptible to error from incorrect setting of the spectrophotometer wavelength. Check this setting regularly using a didymium filter (see A13(4)). If corrections are not required for the presence of phaeopigments, omit steps A8.17-19 inclusive. If corrections are

(l) 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 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

required omit steps A8.15 and A8.16 and proceed direct to step A8.17.

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

A8.16 The chlorophyll a content of the sample, expressed as $\mu g\ l^{-1}$ ($\equiv mg\ m^{-3}$):

$$\frac{12.2 \times A \times v}{d \times V}$$

(note n)

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

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

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

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

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

(note q)

The undegraded chlorophyll a content in $\mu g\ l^{-1}$ i.e. 0.020 units in a 40 mm pathlength cell.

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

(n) The factor 12.2 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), as 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.

(o) 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).

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

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

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

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

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

(Golterman and Clymo 1969).

$$= C_a$$

where:

$$C_a = \frac{12.2\ (2.43\ (A_n A_m))\ \times v}{D \times V}$$

and the phaeopigment content µg l^{-1} will be:

$$P_a = \frac{12.2 \times 1.7 \times (A_n - (2.43 \times (A_n - A_m))) \times v}{d \times V}$$

notes r and s

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

Appendix

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 ⅔ 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 residual water retained on the filter should weigh 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 (see A2.2).
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, heat to boiling and boil for 2-3 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 ($4^{o}C$) place for 12 hours (eg overnight). Agitate the filter briefly from time to time during this period.	(g) Immersion of the tube in a water bath is effective; the water temperature should be just above the boiling point of methanol (65.5 °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 remain covered to prevent loss of methanol.	

A9.7 Allow the 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 (see A9.3) so that the final concentration is 10% water in methanol (note h).

(h) If 20 ml 100% methanol was used initially; a total of 2.22 ml of water will be required to make the final solution 90%. Take into account the residual water on the filter (A9.3) and then add the appropriate quantity of additional water.

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 to prevent loss of methanol by volatilization, until a clear extract solution of pigment is obtained (note i). Let the total volume of this extract be *v* ml. Decant the clear extract without disturbing the sediment (note j).

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

(i) Centrifugation for 7 minutes at 3000 rev/min is usually sufficient.

(j) Alternatively the supernatant may be siphoned off 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 k).

(k) 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 l). 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 not** required for the presence of phaeopigments, omit steps A9.17-19 inclusive. If corrections **are** required omit steps A9.15 and A9.16 and proceed direct to step

(l) 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.

A9.17.

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

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

A9.16 The chlorophyll a content of the sample, expressed as $\mu g\ l^{-1}$ ($\equiv mg\ m^{-3}$)

$$= \frac{13.0 \times A \times v}{d \times V}$$

(note n)

(n) The factor 13.0 approximates to the reciprocal of the specific absorption coefficient at 665 nm for chlorophyll a in methanol (Marker *et al.* 1980).
Note again that this 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 o). Allow the acidified extract to stand for 5 minutes. Then add 0.1 ml 0.3M of the organic base (see section A5.6).

(o) 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 p). Note the values obtained.

(p) 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).

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

Then the degree of degradation

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

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

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

(note q)

The undegraded chlorophyll a content, in $\mu g\ l^{-1}$ (Golterman and Clymo 1969).

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

where:

$$C_a = \frac{13.0\ (3.0\ (A_n - A_m)) \times v}{d \times V}$$

and the phaeopigment content, P_a in µg l^{-1}, will be:

$$P_a = \frac{13.0 \times 1.6\ (A_n - (3.0\ (A_n - A_m))) \times v}{d \times V}$$

notes r and s

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

Appendix

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 ⅔ 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 less than 2.2 g for a sample requiring 20 ml 100% acetone, and proportionately more or less for different 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) If 20 ml 100% acetone was used initially; a total of 2.22 ml of water will be required to make the final solution 90%. Take into account the residual water on the filter (A10.3) and then add the appropriate quantity of additional water.
A10.5	Transfer to a graduated flask, add the appropriate volume of additional water (note g) and make up to volume with 90% acetone.	
A10.6	The tube, or volumatric flask must be stoppered 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 to prevent loss of acetone by volatilization, until a clear extract solution of pigment is obtained (note i). Let the total volume of this extract be v ml. Decant the clear extract without disturbing the sediment (note j).

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

(i) Centrifugation for 7 minutes at 3000 rev/min is usually sufficient.

(j) Alternatively, the supernatant may be siphoned off 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 k).

(k) 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 l). 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 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.

(l) 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.

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 m).

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

A10.15 The chlorophyll *a* content of the sample, in $\mu g\ l^{-1}$ ($\equiv mg\ m^{-3}$)

$$= \frac{11.2 \times A \times v}{d \times V}$$

(n) 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

(note n)

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

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 o). Allow the acidified extract to stand for 5 -30 minutes.

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

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_n (unacidified) and A_m (acidified).

Then the degree of degradation

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

(note q)

The undegraded chlorophyll a content, in $\mu g\ l^{-1}$, (Golterman and Clymo 1969).

$$= C_a$$

where:

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.

(o) 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).

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

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

(r) see Section A10.15 and Section A12 Sources of Error.

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

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

$$C_a = \frac{11.2\,(2.43\,(A_n - A_m)) \times v}{d \times V}$$

and the phaeopigment content, in μg l^{-1}, will be:

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

notes r and s

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

Appendix

A11 *In Vitro* Fluorometry

Calibration Procedure

A11.1 Either, prepare a chlorophyll *a* extract using either one of the procedures as given in Section A8, A9 or A10 (note a).
or, a chlorophyll *a* solution from a commercial preparation of chlorophyll (e.g. "Sigma Chemicals" --- see note b)

(a) Using ethanol, methanol or 90% acetone.

(b) This is spectroscopically pure but not gravimetrically pure and therefore still requires calibration.

A11.2 Determine the chlorophyll a concentration of the extract spectrophotometrically as given in either Sections A8, steps 12 to 19, or A9, steps 12 to 19, or A10, steps 11 to 18, using the 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 c).

(b) Take care to use the appropriate solvent and specific absorption coefficient.

A11.4 Using the fluorometer as directed by the manufacturer measure the fluorescence at 668 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 d) proceed as given in step A11.8. Otherwise dilute the extract with the appropriate solvent (note b) sufficiently to reduce the absorbance below 0.1 units per 10 mm pathlength (note e). Note the dilution factor used.

(d) either ethanol, methanol or acetone as used for the extraction procedure.

A11.8 Using the fluorometer as directed by the manufacturer measure the fluorescence at 668 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

(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.

appropriate calibration graph or factor (see Sections A11.1 to A11.5) to obtain the chlorophyll a content of the extract solution.

(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, in $\mu g\ l^{-1}$

$$\frac{C \times N \times v}{V}$$

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 (for sections A8 - A11)

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. See A13.
7. Spectrophotometer absorbance scale calibration errors. See A13.

A13 Checking the Accuracy of Analytical Results (Quality Control, for sections A8 -10 only)

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.

Pure chlorophyll *a* is not available commercially. Material available from, for example, "Sigma Chemical Company Ltd." is spectroscopically pure but not gravimetrically pure. In other words there are no coloured compounds interfering with the visible spectrum. HPLC does not reveal any epimers, allomers nor any Mg- nor phytol-lacking derivatives. But, because the calculated molar extinction coefficient is significantly lower than than that published by Jeffrey and Humphrey (1975) there must be either water or a colourless

contaminant present. Standard methods of drying risk significant degradation. Therefore, the use of commercial chlorophyll is not generally recommended, unless a solution in 90% acetone is initially calibrated at 665 nm against published absorption coefficients (Jeffrey and Humphrey 1975). Since solutions are unstable and chlorophyll *a* costs £(UK)47 per mg, it is unlikely to be available to most users for routine purposes. The following protocol is recommended.

1. Always use matched spectrophotometer cuvettes.

2. Number these cuvettes on their base and always use them in the same location in the multiple cell holder and always in the same orientation.

3. Initially, check the variations between cuvettes by measuring the absorbance at 750 and 665 nm, filled with the standard solvent in normal use. These values will give the extent of cuvette to cuvette differences and must be carefully recorded in a "log book". This procedure must be used regularly, say every month, and changes will indicate deteriorating optical surfaces or accumulated deposits. For a reagent blank the absorbances at 665 and 750 nm should be the same, even if slightly different from zero. Consequently for a pigment extract, subtracting the absorbance at 750 nm from the absorbance at 665 nm corrects for both cuvette to cuvette differences as well as residual turbidity.

4. Although modern spectrophotometers have built-in wavelength checking mechanisms each time they are powered up, quality control demands that this must be independently checked. Standard didymion filters serve this purpose admirably and have two sharp absorption maxima at 573 and 585 nm (data provided with the filter).

5. In addition, the absolute accuracy of the absorbance reading, itself, can be checked using standard solutions of potassium dichromate ("standard solution 'Spectrosol' for calibration of spectrophotometers" --- from Merck).

6. The wavelength and absorbance checks (4 & 5) enable the specific absorption coefficients of chlorophyll *a* published in the scientific literature to be used. For 90% acetone the most accurate and recent of these is that of Jeffrey and Humphrey (1975) and is 89. The reciprocal (x1000) of this, 11.2, is the constant used in the equations in section A10 and supercedes earlier constant based on outdated literature. For ethanol we propose to adopt the constants used in Germany and Denmark, 12.2 (Wintermans and De Mots 1965) and for methanol the constant is 13.0.

7. The error associated with the final analysis (i.e. after extraction) is primarily dependant on the absorbance of the extract. Since this is partly controlled by the volume filtered, the volume of the extractant and the path length of the cuvette, the analytical error is partially independant of phytoplankton concentration. Shown below are two extreme absorbance values, likely to be encountered with the associated errors.

Appendix

Pigment	Absorbance	MEAN	SD	%	SE	%
Chlorophyll *a*	0.500	24.3	0.693	2.86	0.219	0.9
Phaeo-pigment *a*		10.0	0.579	5.79	0.183	1.83
Chlorophyll *a* (no correction)		30.1	0.681	2.26	0.216	0.72
Chlorophyll *a*	0.030	1.07	0.093	8.66	0.029	2.74
Phaeo-pigment *a*		0.56	0.123	21.78	0.039	6.89
Chlorophyll *a* (no correction)		1.40	0.053	3.74	0.017	1.18

Appendix

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 $\mu g\ l^{-1}$ 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	5 - 100 ng chlorophyll *a* l^{-1}.
B1.8	Bias	see section B12
B1.9	Interferences	Degradation products of chlorophyll a and background fluorescence.
B1.10	Time required for analysis	*In vivo* field determinations take only one minute. However, travel to and from the field site plus boat time must be taken into account. For calibration, see sections A8 - 10.

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.

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

Unlike the *in vitro* methods, outlined in section A7, samples collected in the field are measured immediately, or as soon as possible, after collection. On no account should they be preserved.

Appendix

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 (e.g. Whatman GF/F).	
B8.6	Measure the fluorescence of the filtrate as described above for the first sub-sample in step B8.4 (note d).	(d) Background fluorescence is usually fairly 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 lakes, reservoirs and rivers.

B11 Sources of error

The chief sources of error other than instrument malfunction are due to, (1) the presence of other pigments and other fluorescent substances present in the sample, and (2) to decomposition or growth of the sample prior to analysis.

B12 Quality control

Detailed QC is a complex matter, requiring the interfacing of a formal chemical method (calibration) with an essentially field biological method (*in vivo* fluorometry). The method is, therefore, not subject to the same type of quality control as a formal analytical method (A8-11). However it is an exceptionally valuable operational instrument for managers of reservoirs etc. Skilful use of these instruments can save hours of analytical laboratory time and this advantage should not be underestimated when operational protocols and quality control procedures are drawn up.

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. Two methods (C1 and C2) are covered in outline only. Full QA/QC are not publically available.

C1 Determination of Chlorophyll a, b and c, using multiple equations

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 1972, 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.

C1.1 Principles

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

C1.2 Hazards See Section A4.

C1.3 Reagents See Section A5.

C1.4 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.

C1.5 Sampling and Sample Collection See Section A7.

C1.6 Analytical Procedure

Step	Procedure
C1.6.1	Obtain an acetone extract as given in Section A10.
C1.6.2	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 Let C_a be the concentration of chlorophyll *a* *v* be the total volume of extract (ml) *d* be the cell pathlength (cm) *V* be the sample volume (l)
C1.6.3	For higher plants and green algae containing chlorophylls *a* and *b* (solvent 90% acetone). $$C_a = \frac{(11.93A_q - 1.93A_p) \times v}{d \times V}$$ $$C_b = \frac{(20.36A_p - 5.50A_q) \times v}{d \times V}$$
C1.6.4	For diatoms, chrysomonads and brown algae containing chlorophylls *a*, c_1 and c_2 in equal proportions (solvent 90% acetone). $$C_a = \frac{(11.47A_q - 0.40A_o) \times v}{d \times V}$$ $$C_{c12} = \frac{(24.36A_o - 3.73A_q) \times v}{d \times V}$$
C1.6.5	For dinoflagellates and cryptomonads containing chlorophylls *a* and c_2 (in this case **the solvent is 100% acetone).**

$$C_a = \frac{(11.43A_r - 0.64A_o) \times v}{d \times V}$$

$$C_{c2} = \frac{(27.09A_o - 3.63A_r) \times v}{d \times V}$$

C1.6.6 For mixed phytoplankton populations.

$$C_a = \frac{(11.85A_q - 1.54A_p - 0.08A_o) \times v}{d \times V}$$

$$C_b = \frac{(-5.43A_q + 21.03A_p - 2.66A_o) \times v}{d \times V}$$

$$C_{c12} = \frac{(-0.67A_q - 7.60A_p + 24.52A_o) \times v}{d \times V}$$

C1.7 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. Multiple equations have also been developed for use with spectrofluorometers but these require very careful calibration with samples of the pure chlorophylls are their breakdown products (Neveux and Parnhouse 1987).

C2 General introduction to the separation of chlorophylls a, b and c and their breakdown products using high performance liquid chromatography (HPLC)

C2.1 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 HPLC experience. 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 and is not a detailed protocol.

C2.2 The apparatus requires a gradient elution system consisting of:

(i) Two high quality pumps
(ii) Three solvent reservoirs
(iii) One injection valve (e.g. "Rheodyne")
(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 generates peak height, peak area and elution time or similar output device

C2.3 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 (e.g. Waters C-18).

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.

C2.5 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.

C2.6 For further reading, refer to Mantoura and Llewellyn (1983), Wright and Shearer (1984), Gieskes and Kraay (1983, 1986 a & b), Murray *et al.* 1986, Zapata *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 1984) 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.

Dl.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 drained weight of shoots, by cutting or pulling fresh plant material. Store in an air-tight container. Remove algae and treat as in HMSO (1983).

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. Chlorophyceae and Cyanobacteria 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 100°C for 24 hours 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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