

The Determination of Chlorophyll a in Aquatic Environments 1980

Methods for the Examination of Waters and Associated Materials

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Warning to users

The analytical procedures given in this booklet should only be carried out by competent trained persons, with adequate supervision when necessary. Local Safety Regulations must be observed. Laboratory procedures should be carried out only in properly equipped laboratories. Field operations should be conducted with due regard to possible local hazards, and portable safety equipment should be carried. Care should be taken against creating hazards. Lone working, whether in the laboratory or field, should be discouraged. Reagents of adequate purity must be used, along with properly maintained apparatus and equipment of correct specification. Specifications for reagents, apparatus and equipment are given in manufacturers' catalogues and various published standards. If contamination is suspected, reagent purity should be checked before use.

There are numerous handbooks on first aid and laboratory safety. Among such publications are: 'Code of Practice for Chemical Laboratories' and 'Hazards in the Chemical Laboratory' issued by the Royal Society of Chemistry, London; 'Safety in Biological Laboratories' (Editors Hartree and Booth), Biochemical Society Special Publication No 5, The Biochemical Society, London, which includes biological hazards; and 'The Prevention of Laboratory Acquired Infection' Public Health Laboratory Service Monograph 6, HMSO, London.

Where the Committee have considered that a special unusual hazard exists, attention has been drawn to this in the text so that additional care might be taken beyond that which should be exercised at all times when carrying out analytical procedures. It cannot be

too strongly emphasised that prompt first aid, decontamination, or administration of the correct antidote can save life; but that incorrect treatment can make matters worse. It is suggested that both supervisors and operators be familiar with emergency procedures before starting even a slightly hazardous operation, and that doctors consulted after any accident involving chemical contamination, ingestion, or inhalation, be made familiar with the chemical nature of the injury, as some chemical injuries require specialist treatment not normally encountered by most doctors. Similar warning should be given if a biological or radio chemical injury is suspected. Some very unusual parasites, viruses and other micro-organisms are occasionally encountered in samples and when sampling in the field. In the latter case, all equipment including footwear should be disinfected by appropriate methods if contamination is suspected.

The best safeguard is a thorough consideration of hazards and the consequent safety precautions and remedies well in advance. Without intending to give a complete checklist, points that experience has shown are often forgotten include: laboratory tidiness, stray radiation leaks (including ultra violet), use of correct protective clothing and goggles, removal of toxic fumes and wastes, containment in the event of breakage, access to taps, escape routes, and the accessibility of the correct and properly maintained first-aid, fire-fighting, and rescue equipment. If in doubt, it is safer to assume that the hazard may exist and take reasonable precautions, rather than to assume that no hazard exists until proved otherwise.

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About this series

This booklet is part of a series intended to provide recommended methods for the determination of water quality. In the past, the Department of the Environment and its predecessors, in collaboration with various learned societies, has issued volumes of methods for the analysis of water and sewage culminating in 'Analysis of Raw, Potable and Waste Waters'. These volumes inevitably took some years to prepare, so that they were often partially out of date before they appeared in print. The present series will be published as individual methods, thus allowing for the replacement or addition of methods as quickly as possible without need of waiting for the next edition. The rate of publication will also be related to the urgency of requirement for that particular method, tentative methods being issued when necessary. The aim is to provide as complete and up to date a collection of methods and reviews as is practicable, which will, as far as possible, take into account the analytical facilities available in different parts of the Kingdom, and the quality criteria of interest to those responsible for the various aspects of the water cycle. Because both needs and equipment vary widely, where necessary, a selection of methods may be recommended for a single determinand. It will be the responsibility of the users — the senior analytical chemist, biologist, bacteriologist etc, to decide which of these methods to use for the determination in hand. Whilst the attention of the user is drawn to any special known hazards which may occur with the use of any particular method, responsibility for proper supervision and the provision of safe working conditions must remain with the user.

The preparation of this series and its continuous revision is the responsibility of the Standing Committee of Analysts (to review Standards Methods for Quality Control of the Water Cycle). The Standing Committee of Analysts is one of the joint technical committees of the Department of the Environment and the National

Water Council. It now has seven Working Groups, each responsible for one aspect of water cycle quality analysis. They are as follows:

- 1.0 General principles of sampling and accuracy of results
- 3.0 Empirical and physical methods
- 4.0 Metals and metalloids
- 5.0 General nonmetallic substances
- 6.0 Organic impurities
- 7.0 Biological methods
- 9.0 Radiochemical methods

The actual methods etc are produced by small panels of experts in the appropriate field, under the overall supervision of the appropriate working group and the main committee. The names of those associated with this method are listed inside the back cover.

Publication of new or revised methods will be notified to the technical press, whilst a list of Methods in Print is given in the current HMSO Sectional Publication List No 5, Corrections and Additions to already published methods will be issued in special compendia as part of this series.

T A DICK
Chairman

L R PITTWELL
Secretary

30 November 1982

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.

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 two types of method for the determination of chlorophyll a in plant material obtained from an aquatic environment:

- a) A solvent extraction technique (see Section A) using either acetone or methanol, followed by spectrophotometric or fluorometric evaluation of the extract.
- b) In-vivo fluorometry (see Section B).

Note: Fluorometry is the more sensitive technique and in-vivo fluorometry can be used for continuous monitoring purposes.

03 Methods to Distinguish Undegraded and Degraded Pigments

Not all the procedures distinguish between undegraded and degraded (“dead”) forms of chlorophyll a. A rigorous distinction is possible only by chromatographic methods^(1,2,3,28,29,30,31) but the change in absorbance brought about by the mild acidification of a solvent extract may give an indication of the original degree of degradation (see Section D).

04 Extension of the Methods of Other Pigments

The determination may be extended to other pigments e.g. to chlorophylls b and c, and to carotenoids but these are neither so universally present, nor do they possess such distinctive absorption characteristics as chlorophyll a and their estimation in mixtures is therefore less reliable (see Section E).

05 General Literature References

For further general information concerning the determination of chlorophyll see literature references 4 to 9, 12, 16 and 32.

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 <u>a</u>
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:	
A1.4 Calibration graph:	Standard absorption coefficients are applied to spectrophotometric measurements. Fluorometric methods are calibrated using a suitable chlorophyll <u>a</u> extract solution in which the concentration has been determined spectrophotometrically.
A1.6 Total standard deviation:	Difficult to quantify. The fluorometric determination of extracts is at least $100 \times$ as sensitive as spectrophotometry although the precision may not be as good.
A1.7 Limit of detection:	
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:	Degradation products of chlorophyll <u>a</u> (phaeophorbide, phaeophytin) and other pigments may interfere.
A1.11 Time required for analysis:	40 minutes for the spectrophotometric method exclusive of calibration.

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 C may be more applicable.

Chlorophyll a is extracted from the plant material using either acetone or methanol 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.:-

- 1) at 665 nm, the absorption maximum of chlorophyll a
- b) at 750 nm in order to compensate for "background turbidity".

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

- (i) the greater superiority of methanol (especially when hot) as extractant;
- (ii) the better-known characteristics and greater stability of chlorophyll a solutions in acetone; and

(iii) the greater possibility (see Section D) of making some distinction between undegraded and degraded pigment in acetone extracts. Thus if degradation products are likely to be abundant, extraction with acetone followed by the appropriate measurements and calculations (see Section D.1) is recommended: otherwise, and especially with algae resistant to extraction, hot methanol is simpler and effective. For further discussion and comparative data see references 10 and 11.

A2.3 Fluorimetry^(1,7,12) 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 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 A8.24, may not be the true value.

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

Although the methods lack precision it is possible to correct the chlorophyll a content of the sample obtained as given in section A8 step 24 for the effect of other substances by submitting the extract to extended absorbance measurements.

Procedures to estimate degradation products are based upon differences between the absorbances of unacidified and acidified extracts and are given in Section D.

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

If such corrections are contemplated the analyst should take especial note of the comments in the introductions to section D and E.

A4 Hazards

Normal laboratory safety precautions must be observed.

The solvents used for the extraction of pigments are highly flammable and should be handled with extreme care. Methanol is highly toxic and should not be inhaled. All operations involving the use of methanol should be performed in a suitable fume cupboard.

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 should not be opened whilst the centrifuge is operating.

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.

A5 Reagents

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

A5.2 **Acetone.**

A5.3 **Methanol.**

A5.4 **Acetone solution (aqueous)**

The water content of acetone used to extract the sample pigments must be adjusted to obtain a final concentration of 90% v/v acetone after contact with the moist sample filter. First determine M ml (the water retained by a filter) as given in section A6.3.1.

Let the required acetone content of the acetone solution to be added to the damp filter be $S\%$ v/v.

Let the volume of this acetone solution to be used be y ml.

Let the final volume of 90% acetone extract obtained be v ml.

Calculate S from the equation

$$S = \frac{10(10y - v + 10M)}{y} \%v/v$$

Pipette $(100-S) \pm 0.05$ ml of water into a 100 ml calibrated flask. Dilute to volume with acetone and mix well.

e.g. 1. A 70 mm diameter filter retained 1 ml of water. It requires the addition of 14 ml of 96.4% v/v acetone solution for complete immersion and to obtain a final acetone concentration of 90% v/v in 15 ml.

e.g. 2. Similarly a 55 mm filter retained 0.36 ml water; 7 ml of 94.6% v/v acetone solution added; final acetone concentration 90% v/v in 7.4 ml.

A5.4.1 *90% v/v acetone aqueous* (for dilution and reference cells Steps A8.21, A8.26 note v and A9.3)

Take a 100 ml measuring cylinder, add 10 ml of distilled water and make up to 100 ml with acetone. Mix well.

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.

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

Matched cells with lids 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 fluorescence 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.

A6.3.1 *Determination of the water retained by a filter*

The water retained by a filter must be determined so that the water content of acetone solution (A5.4) can be adjusted to obtain a final acetone concentration of 90% v/v in the sample pigment extract (see Section A8 steps 8.4 to A8.12).

Select a filter from the batch to be used to filter samples and weigh to ± 0.005 g.

Clamp the filter in a holder. Draw about 100 ml of water through it and reduce the residual water content using the same conditions as given for the sample in Section A8 steps 1 and 1a. Remove the filter from the holder and reweigh to ± 0.005 g.

The difference between the two weighings equals the mass of water retained by the filter. Let this be pg

$$pg \text{ of water} \equiv M \text{ ml}$$

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

A6.6 Test-tubes, preferably stoppered, approximate capacity 10 or 20 ml.

A6.7 Simple laboratory centrifuge.

A6.8 Centrifuge tubes, 10 ml graduated.

A6.9 A homogenizer (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 surface dip, self closing bottle for samples taken at depth, or by using a weighted plastic tube to obtain vertically integrated samples⁽¹³⁾.

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 C.

Samples are best analysed on the day of collection, or at most after overnight storage in darkness in a refrigerator or cool (<10°C) place. 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⁽¹⁴⁾.

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

A8 Analytical Procedure

Step	Procedure	Notes
A8.1	Filter a measured volume $V \pm 10$ ml, (note a) of sample through a glass-fibre filter clamped in a suitable holder (A6.4) (notes b and d). Discard the filtrate (note d).	(a) For most natural waters 1 litre is a suitable volume but this should be adjusted if the expected content of plant material 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 5 minutes (note c).	(b) Filtration may be accelerated by applying slightly reduced pressure to the receiving vessel. i.e. reduction to $\frac{2}{3}$ atmosphere (corresponding to reduction to 500 mm Hg or 66.6 K Pa). (c) Ensure that the water retention volume of the filter has been determined (see A6.3.1) under the same conditions used here. (d) 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.
A8.3	Remove the filter paper from the holder and roll it up carefully.	
Pigment Extraction Procedures		
(a)	Rapid acetone method steps A8.4 to A8.8 (note e)	(e) This is the preferred acetone extraction method.
(b)	Alternative slow acetone method steps A8.9 to A8.12.	
(c)	Methanol extraction method steps A8.13 to A8.19.	

Step	Procedure	Notes
	(a) <i>Rapid Acetone Extraction Method:</i>	
A8.4	Transfer the filter paper to a homogenizer and add a small volume of the aqueous acetone (notes f and g) usually 3–5 ml.	(f) The extraction of pigment into solvent should be carried out in subdued light and any contact with acid vapours must be avoided.
A8.5	Operate the homogenizer to obtain a finely divided suspension of paper in aqueous acetone.	(g) The aqueous acetone solution will have been prepared as given in section A5.4 such that the final concentration of acetone present after the extraction of pigment from the filter is 90% v/v.
A8.6	Dilute the suspension to known volume, usually 15.0 ± 0.1 ml with aqueous acetone and mix well (note h).	(h) The volume of aqueous acetone required will depend upon the size of filter used and the choice of 10 mm or 40 mm pathlength cells for subsequent absorbance measurements.
A8.7	Centrifuge the suspension, in a stoppered tube (note i), until a clear 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.	(i) A covering of stretched plastic film is usually adequate to prevent loss of acetone by volatilization. (j) Centrifugation for 7 minutes at 3500 rev/min is usually sufficient.
A8.8	Reserve this solution in a stoppered tube for the absorbance measurements given in steps A8.20 to A8.23 or for fluorometry as given in steps A8.25 to A8.28.	
	(b) <i>Slow Acetone Extraction Method</i>	
A8.9	Transfer the filter paper to a test tube and add a known volume of aqueous acetone, usually 14 ml (notes f, g and h) sufficient to cover the paper.	
A8.10	Agitate the paper briefly with forceps to ensure that the paper is in complete contact with the solvent (note f). Stopper the tube and place in the dark in a refrigerator at $4 \pm 0.1^\circ\text{C}$ for 24 hours.	
A8.11	Centrifuge. Decant the clear solution without disturbing the sediment (note k) and store in a stoppered tube for the absorbance measurements given in steps A8.20 to A8.23 or for fluorometry as given in steps A8.25 to A8.28. Let the total volume of this extract be v ml (note l).	(k) A pipette fitted with a low pressure suction device may be used and if necessary the separated solution may be centrifuged as given in section A8.7 to obtain a clear solution. (l) This comprises added aqueous acetone plus water contributed by the filter.
A8.12	Reserve this extract in a stoppered tube for absorbance measurements as given in steps A8.20 to A8.23 or for fluorometry as given in steps A8.25 to A8.28.	
	(c) <i>Methanol Extraction Method</i>	
A8.13	Transfer the filter paper to a test tube and add a known volume of methanol usually 14 ml, sufficient to cover the paper.	
A8.14	Agitate the paper briefly with forceps to ensure that the paper is in complete contact with the solvent.	
A8.15	Heat the methanol to boiling and boil for about 10 seconds. The tube should be carefully covered to prevent loss of methanol (notes m and n).	(m) Carry out this procedure inside a fume cupboard.
A8.16	Remove the tube from the source of heat, stopper and allow the warm solution to stand in the dark for about 5 minutes.	(n) Immersion of the tube in a water bath is effective; the water temperature should be just above the boiling point of methanol ($65\text{--}70^\circ\text{C}$).

Step	Procedure	Notes
A8.17	Still in the dark, cool the tube to room temperature. 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.	
A8.18	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).	
A8.19	Reserve this extract in a stoppered tube for the absorbance measurements given in steps A8.20 to A8.23 or for fluorometry as given in steps A8.25 to A8.28.	
Absorbance Measurements		
A8.20	Fill a 40 mm pathlength stoppered cell with the pigment extract solution obtained using either of the extraction methods (a), (b) or (c). Let the pathlength of the cell used be d mm (note o).	(o) Commonly available 40 mm pathlength cuvettes require a minimum extract volume of 11 ml. Lesser volumes will require the use of narrow, semi-micro 40 mm pathlength cells provided that these are compatible with the spectrophotometer.
A8.21	If appropriate use 90% v/v aqueous acetone (A5.4.1), as used to extract the pigments, in the reference beam of the spectrophotometer.	
A8.22	Measure the absorbance of the extract at wavelengths of 665 nm, and 750 nm (note p). As the calculations are susceptible to error from incorrect setting of the spectrophotometer wavelength. Check this setting before taking each reading. (Marker ⁽¹⁰⁾ has suggested that at 664 nm the factor of 11.9 in step A8.24 and note r would then be 10.8). If necessary reserve this solution for further measurements as given in sections D and E.	(p) 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.
A8.23	Subtract the absorbance value obtained at 750 nm from that obtained at 665 nm and let this be A (note q).	(q) This step is a correction for any turbidity present.
Calculation of Results		
(a) <i>Using acetone extract</i>		
A8.24	The chlorophyll <u>a</u> content of the sample $= \frac{11.9 \times A \times v}{d \times V} \mu\text{g/l} (\equiv \text{mg/M}^3)$	(r) The factor 11.9 approximates to the reciprocal of the specific absorption coefficient at 665 nm for chlorophyll a in acetone. It includes an arbitrary assessment of absorbance contributed by other pigments.
(b) <i>Using methanol extraction</i>		
	$= \frac{13.9 \times A \times v}{d \times V} \mu\text{g/l}$ (note s)	(s) The factor 13.9 approximates to the reciprocal of the specific absorption coefficient at 665 nm for chlorophyll <u>a</u> in methanol ^(5,15) .
Where A = absorbance v = volume of solvent in mls V = volume of initial filtered samples in litres and d = cellpath length in cms		
		(t) The calculation makes no correction for the presence of degraded matter or of other pigments. See Sections D and E if corrections are necessary.

Step	Procedure	Notes
Fluorescence Measurements		
A8.25	Measure the absorbance of the solvent extract in a 10 mm pathlength cell at a wavelength of 430 nm.	
A8.26	If the absorbance value is less than 0.1 units (note u) proceed as given in step A8.27. Otherwise dilute the extract with the appropriate solvent (note v) sufficiently to reduce the absorbance below 0.1 units per 10 mm pathlength (note w). Note the dilution factor used.	(u) aqueous acetone or methanol as used for the extraction procedure. (v) 90% v/v aqueous acetone (A5.4.1) or methanol as used for the extraction procedure.
A8.27	Using the fluorometer as directed by the manufacturer measure the fluorescence at 663 nm of the extract solution (note x) 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 Section A9) to obtain the chlorophyll <u>a</u> content of the extract solution. Calculation of Results	(w) 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. (x) This will have been obtained using either one of the extract procedures (a), (b), or (c) which may have subsequently been diluted (see step A8.26).
A8.28	The chlorophyll <u>a</u> content of the sample $= \frac{C \times N \times v}{V} \mu\text{g/l} (= \text{mg/M}^3)$ <p>where C = chlorophyll <u>a</u> content of extract solution (A8.27). N = factor by which the original extract is diluted (A8.26). v = total volume of original extract (in ml). V = volume of sample taken (in l).</p>	

A9 Fluorometric Calibration Procedure

Step	Procedure	Notes
A9.1	Prepare a chlorophyll <u>a</u> extract using either one of the procedures (a), (b) or (c) as given in Section A8 (note a).	(a) Using aqueous acetone or methanol as appropriate.
A9.2	Determine the chlorophyll <u>a</u> content of the extract spectrophotometrically as given in Section A8 steps 20 to 23 using the reciprocal specific absorption coefficient appropriate to the chosen solvent in calculation step A8.24.	
A9.3	Prepare serial dilutions of the extract with the chosen extraction solvent to obtain chlorophyll <u>a</u> concentrations of approximately 2, 6, 20 and 60 $\mu\text{g/l}$. (note b)	(b) If aqueous acetone is used the dilution should be made with 90% v/v acetone, A5.4.1, not with the extraction acetone A5.4.
A9.4	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.	
A9.5	Use the values obtained in step A9.4 to derive calibration graphs, or factors, relating fluorescence measurements to the corresponding concentrations of chlorophyll <u>a</u> ($\mu\text{g/l}$).	

- A10 Sources of Error**
1. The presence of pigments other than chlorophyll a.
 2. The presence of chlorophyll degradation products.
 3. Failure to achieve complete extraction of chlorophyll a.
 4. Exposure of the sample or sample extract to light.
 5. The stability of extract solutions.
 6. Loss of solvent by evaporation during analytical procedures.
 7. 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.
 8. Spectrophotometer absorbance scale calibration errors. Check as instructed by the manufacturer.

A11 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
B.1	Substance determinand	Chlorophyll <u>a</u> .
B1.2	Type of sample	Aqueous suspension of algae or photoplankton.
B1.3	Basis of the method	Direct measurement of fluorescence in vivo.
B1.4	Range of application	1 to 100 $\mu\text{g/l}$ chlorophyll <u>a</u> ; but is extremely variable depending upon the type of algae or phytoplankton ⁽¹⁾ .
B1.5	Calibration graph	Method must be calibrated for each type of algae 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 <u>a</u> 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^(1,7,12,16)
- 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 See Section A7.

B8 Analytical Procedure

Step	Procedure	Notes
B8.1	Divide the sample into two representative sub-samples. 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 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 ⁽¹⁷⁾ .
B9.2	Determine the chlorophyll <u>a</u> content of the sample by one of the solvent extraction methods (a), (b) or (c) as given in Section A8.	
B9.3	Proceed as given in Section A9 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 $\mu\text{g/l}$ chlorophyll <u>a</u> .

- B10 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.

C. Methods of Collecting and Extracting Surface Living Attached Algae (Microbenthos and Periphyton)

C1 Sample Collection C1.1. From Submerged Surfaces

Use methods described in another booklet in this series⁽¹⁸⁾ to remove attached algae from submerged surfaces.

C1.2 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.

C1.3 From gravel and small stones

Immerse a representative sample of substratum directly in a suitable volume of the chosen solvent (see sections A8 and C3). Because accurate sub-sampling is difficult extraction of part of the sample only should be avoided.

C1.4 From Larger Aquatic Plants (Macrophytes)

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

Observe the precautions given in Section A.7.

C2 Sample Preservation

C3 Sample Extraction C3.1 Gravel and Small Stones

The pigments from gravel and small stones may be extracted into 90% v/v acetone solution, or into 90% v/v or 100% v/v methanol by placing the stones in a suitable volume of solvent contained in a wide-mouthed vessel with a tight fitting screw lid.

C3.2 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 methanol, rather than acetone, for extraction purposes.

C3.3 Extraction of Larger Aquatic Plants ⁽¹⁹⁾

Grind and homogenize 25 g of sample C1.4 and extract into a suitable known volume of solvent, typically 250 ml. Centrifuge to obtain a clear solution for either spectrophotometric or fluorometric evaluation as given in Section A.

C3.3.1 Allowance must be made for the water content of the plant material when carrying out the procedures in A5.3.4 and A6.3.1. For this, determine the percentage loss in weight on drying at 80°C using replicate samples.

C4 Analytical Procedures

The absorbances of the extracts are measured using the procedures described in Section A, B, D or E 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.

C5 Degradation Studies

See Section D8.

D. Estimation of Chlorophyll a in the presence of phaeopigments

D0 Introduction

The solvent extraction procedure given in Section A of this booklet does not distinguish between degraded and undegraded forms of chlorophyll pigment and in the presence of degraded matter the results obtained for chlorophyll a will be spuriously high.

However, by acidifying the solvent extracts obtained by following the procedures given in Section A and carrying out additional absorbance measurements, it may be possible to determine the concentration of degraded matter present, often expressed as the degree of degradation. Acetone extracts are more suited to this extended treatment than methanol extracts. The analyst must satisfy himself from considerations of sample type and condition, the degree of precision required, and the time required for an increased number of analytical steps, that the procedures given in this section will provide meaningful information for a particular population of interest. A rapid test using differences in absorption of the methanolic extract at two wavelengths is also included in this Section.

Algal populations growing on firm surfaces, e.g. gravel, do not often show significant pigment degradation and in routine work an extended analysis of an extract may not provide any worthwhile information. However, the breakdown of chlorophyll in soft sediments can be quite pronounced (sometimes 80%) and a correction for degradation should always be made.

D1 Degradation Studies Using Extraction with Acetone

D1.1 Principle

The absorbance of the extract is measured before and after acidification⁽⁶⁾ to convert chlorophyll to phaeophytin.

D1.2 Hazards

See Section A4.

D1.3 Reagents

See Section A5.

D1.3.1 (1% V/V) Hydrochloric acid solution.

Dilute 10 ± 0.5 ml of hydrochloric acid ($d_{20}1.18$) to 1000 ± 10 ml with water in a measuring cylinder and mix well.

D1.4 Apparatus

See Section A6.

D1.5 Sample Collection and Preservation

See Section A7.

D1.6 Analytical Procedure

Step	Procedure	Notes
D1.6.1	Obtain an acetone extract of the sample as given in Section A.	
D1.6.2	Measure the absorbance of this extract at wavelengths of 665 nm and 750 nm as given in Section A8 step 22 and note these values.	
D1.6.3	To 10 ± 0.1 ml of acetone extract add 0.03 ml of 1% V/V hydrochloric acid solution and mix well (note a). Allow the acidified extract to stand for 5 minutes.	(a) A pipette or syringe may be used.
D1.6.4	Measure the absorbance of the acidified extract as given above in Section D1.6.2 using the same cell (note b). Note the values obtained	(b) Cell faces must be cleaned and polished. The cell must always be placed the same way round in the spectrophotometer.
D1.6.5	<p>Calculation</p> <p>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).</p> <p>Then the degree of degradation</p> $= \frac{A_n}{A_m} \quad (\text{note c})$ <p>The undegraded chlorophyll <u>a</u> content⁽⁸⁾ (note d)</p> $= C_u$ <p>where</p> $C_u = \frac{11.9[2.43(A_n - A_m)] \times v}{d \times V} \quad \mu\text{g/l} \quad (\text{notes e and f})$ <p>where v = total volume of extract (ml) d = cell pathlength (cm) V = volume of sample taken (l)</p>	<p>(c) Degradation absent: a value of approximately 1.7 Degradation complete: value 1.0</p> <p>(d) This calculation is only an approximate estimate of chlorophyll <u>a</u> concentration; but is more accurate when breakdown products are present than A8.24.</p> <p>(e) See note r, Section A8.24 and Section D1.7 Sources of Error.</p> <p>(f) 2.43 is a factor derived from the absorbance of chlorophyll <u>a</u> at 665 nm before and after acidification</p> $\left(\frac{A_n}{A_n - A_m} = 2.43 \right)$

D1.7 Sources of Error The problem of obtaining representative samples of complex, labile, heterogenous materials.

The factor 2.43 used in the calculation of undegraded chlorophyll a is easily invalidated by errors in spectrophotometer wavelength calibration in the region of 665 nm.

D2 Degradation Studies Using Extraction with Methanol

D2.0 Introduction

A rapid qualitative test and two quantitative determinations are given. Of the two quantitative determinations D2.3 is less time consuming than method D3 but is less accurate and less tested, although there is scope for further refinements of technique.^(9,10,20,21,22)

D2.1 Rapid Qualitative Test
D2.1.1 Principle The absorbance of a methanol extract of pigment is measured at wavelengths of 430 and 410 nm. The ratio of these absorbance values provides an approximate estimate of the degree of degradation.

D2.1.2 Hazards See Section A4.

D2.1.3 Reagents See Section A5.

D2.1.4 Apparatus See Section A6.

D2.1.5 Sample Collection and Preservation See Section A7.

D2.1.6 Analytical Procedure

Step	Procedure	Notes
D2.1.6.1	Obtain a methanol extract from the sample as given in section A of this booklet.	
D2.1.6.2	Measure the absorbance of this extract at wavelengths of 420 nm and 410 nm. Let these values be A_E and A_F respectively.	
	Calculation	
D2.1.6.2	Calculate the ratio $\frac{A_E}{A_F}$ (note a)	(a) If $\frac{A_E}{A_F} > 1$ degradation is probably <50% If $\frac{A_E}{A_F} < 1$ degradation is >50% i.e. more severe

D2.2 Quantitative Determination of Degradation (Methanol Extract Method)

D2.2.1 Principle The absorbance of the extract is measured before and after acidification to convert chlorophyll to phaeophytin. The phaeophytin molecule itself also dissociates in more concentrated acid solutions and consequently the form of the spectrum is affected. The red absorption maxima of phaeophytin a that occurs at a wavelength of 666–7 nm in dilute acid solution shifts towards 654 nm in more concentrated acid. However, in methanol solution this shift takes place in less concentrated acid solutions. Therefore after acidification of the extract to produce phaeophytin the extract is neutralised before making absorbance measurements. Magnesium carbonate has been widely used for this purpose but investigations suggest that other degradation products e.g. chlorophyllide and phaeophorbide become absorbed.⁽¹⁾ Preferably organic bases such as dimethylaniline⁽¹⁰⁾ or diphenyl ethylamine^(23,24) should be used.

D2.2.2 Hazards See Section A4
 Also the organic bases used for neutralization should not be allowed to come into contact with the skin.

D2.2.3 Reagents See Section A5

D2.2.3.1 Hydrochloric acid solution (0.3M)

Dilute 30 ± 0.1 ml of hydrochloric acid ($d_{20}1.18$) with water to volume in a 1 litre calibrated flask. Mix well.

D2.2.3.2 Organic base solution (0.3M)

Dissolve 9.1 ± 0.05 g of dimethyl aniline, or alternatively 14.8 ± 0.05 g of diphenylethylamine in about 200 ml of methanol. Dilute to volume with methanol in a 250 ml calibrated flask and mix well.

D2.2.4 Apparatus See Section A6

D2.2.5 Sampling and Sample Preservation See Section A7

D2.2.6 Analytical Procedure

Step	Procedure	Notes
D2.2.6.1	Obtain more than 15 ml of a methanol extract from the sample as given in Section A of this booklet.	
D2.2.6.2	Measure the absorbance of the extract at wavelengths of 665 nm and 750 nm. Subtract the absorbance at 750 nm from that at 655 nm and let this value be A_H .	
D2.2.6.3	To 15.0 ± 0.05 ml of methanol extract add 0.15 ± 0.02 ml (note a) of hydrochloric acid solution (0.3M) and mix well. Allow to stand for 5 minutes.	
D2.2.6.4	Add 0.15 ± 0.02 ml (note a) of organic base solution (0.3M) and mix well.	(a) A pipette or dispenser may be used.
D2.2.6.5	Within 5 minutes repeat the absorbance measurements at 665 nm and 750 nm. Subtract the absorbance at 750 nm from that at 665 nm and let this value be A_I . Correct A_I for the dilution effect of the acid and organic base and let this value be A_J (note b). Calculation	(b) $A_J = \frac{(v + 0.3) \times A_I}{v}$ where v = volume of acidified extract in ml.
D2.2.6.6	The undegraded chlorophyll <u>a</u> content of the sample. $\frac{13.9 [3.0 (A_H - A_J)] \times v}{d \times V} \mu\text{g/l}$ (Note c) where v = initial volume of extract taken (in ml) d = cell pathlength (in cm) V = sample volume (in l)	(c) 3.0 is a factor of similar derivation to that of 2.43 used in Section D1.6.5 except that in methanol the absorbance ratio $\frac{A_H}{A_J} = 1.5$ and $\frac{A_H}{A_H - A_J} = 3.0$ whereas the corresponding ratio in 90% acetone is $\frac{A_H}{A_J} = c.1.6$ and $\frac{A_H}{A_H - A_J} = c.2.667$

D2.2.7 Sources of error See Section A10.

D3 Quantitative Determination of Degradation (Extraction Solvent Exchange Method)

D3.1 Principle

A methanol extract solution of pigments is evaporated to dryness. The pigments are redissolved in 90% v/v aqueous acetone and the degree of degradation and chlorophyll a content are determined spectrophotometrically as given in Section D1.⁽¹⁰⁾

D3.2 Hazards

See Section A4

Apparatus used at reduced pressures must be shielded from the operator using a safety screen.

D3.3 Reagents

See Section A5

D3.3.1 90% v/v acetone solution

Dilute 90 ± 1 ml of acetone to 100 ± 1 ml with water in a measuring cylinder and mix well.

D3.4 Apparatus

D3.5 Sampling and Sample Preservation

See Section A7

D3.6 Analytical Procedure

Step	Procedure	Notes
D3.6.1	Transfer between 2 and 15 ml of a methanol extract obtained as given in Section A (note a) to the flask of a rotary evaporator.	(a) Choose an extract volume appropriate to the level of absorbance to be measured following the transfer of the pigments to acetone solution.
D3.6.2	Evaporate the extract to dryness in the dark using reduced pressure and a temperature not more than 15°C above ambient temperature (note b)	(b) Usually this step takes only a few minutes using a rotary evaporator at 35°C.
D3.6.3	Redissolve the pigments in a suitable volume of 90% v/v acetone solution (note c)	(c) 5 ml if a 10 mm path length cell is to be used for absorbance measurements, 15 ml if a 40 mm path length cell is to be used.
D3.6.4	Measure the absorbances of the solution as given in section D1 steps D1.6.2 to D1.6.4	
	Calculation	
D3.6.5	See Section D1 step D1.6.5	

D3.7 Sources of error

See Section A10.

E Determination of Other Pigments, Especially Chlorophylls b and c and the Determination of Chlorophyll a in the Presence of Other Pigments.

E0 Introduction

Where present in plant material chlorophylls b and c, and other pigments such as carotenoids will be extracted into acetone or methanol, together with chlorophyll a. However, their presence is not as universal as chlorophyll a and they do not possess such distinctive absorption characteristics. Determination in admixture is much less reliable than the determination of chlorophyll a.

E1 Determination of Chlorophylls b and c and related compounds

The traditional method for estimating the minor chlorophylls b and c involves the use of 'trichromatic equations' (see References 10, 11, 25, 26, 27). However, this procedure is particularly susceptible to errors and requires the use of top quality instrumentation. Moreover, if degradation products are present, the equations cannot even work on theoretical grounds. It is not therefore possible at present to recommend the use of trichromatic equations. More accurate methods, using paper chromatography^(28,29) or thin-layer chromatography^(2,30,31) are available but these methods are relatively slow and time-consuming.

E2 Determination of Chlorophyll a with correction for chlorophylls b and c

E2.1 Principle

Spectrophotometric measurement of the absorbance of acetone or methanol extracts of plant material at wavelengths of 630, 645, and 665 nm.

E2.2 Hazards

See Section A4.

E2.3 Reagents

See Section A5

E2.4 Apparatus

See Section A6.

Note, however, that an exceptionally well maintained, top quality spectrophotometer together with top quality accessories is required.

E2.5 Sampling and Sample Collection

See Section A7.

E2.6 Analytical Procedure

Step	Procedure	Notes
E2.6.1	Obtain an acetone or methanol extract as given in Section A.	
E2.6.2	Measure the absorbance of the extract at wavelengths of 630 nm, 645 nm (note a) 665 nm and 750 nm. Subtract the absorbance at 750 nm from that of each of the others. Let these values be A_o , A_p and A_q respectively.	(a) These measurements are required in addition to those of section A8.
	Calculation	
E2.6.3	The chlorophyll <u>a</u> content of the sample $= \frac{(11.6A_q - 1.31A_p - 0.14A_o) \times v}{d \times V} \mu\text{g/l}$	
	where v = total volume of extract (ml) d = cell pathlength (cm) V = sample volume (l)	

E3 Sources of Error

See Section A10

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The Standing Committee of Analysts
The Department of the Environment
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