Monographs on oceanographic methodology

Determination of photosynthetic pigments in sea-water

Unesco

Monographs on oceanographic methodology Monographs on oceanographic methodology
Monographs on oceanographic methodology Monographs on oceanographic methodology
Monographs on oceanographic methodology Monographs on oceanographic methodology
Monographs on oceanographic methodology Monographs on oceanographic methodology
Monographs on oceanographic methodology Monographs on oceanographic methodology
Monographs on oceanographic methodology Monographs on oceanographic methodology
Monographs on oceanographic methodology Monographs on oceanographic methodology
Monographs on oceanographic methodology Monographs on oceanographic methodology
Monographs on oceanographic methodology Monographs on oceanographic methodology
Monographs on oceanographic methodology Monographs on oceanographic methodology
Monographs on oceanographic methodology Monographs on oceanographic methodology
Monographs on oceanographic methodology Monographs on oceanographic methodology
Monographs on oceanographic methodology Monographs on oceanographic methodology
Monographs on oceanographic methodology Monographs on oceanographic methodology
Monographs on oceanographic methodology Monographs on oceanographic methodology
Monographs on oceanographic methodology Monographs on oceanographic methodology
Monographs on oceanographic methodology Monographs on oceanographic methodology
Monographs on oceanographic methodology Monographs on oceanographic methodology
Monographs on oceanographic methodology Monographs on oceanographic methodology
Monographs on oceanographic methodology Monographs on oceanographic methodology

Monographs on oceanographic methodology 1

Determination of photosynthetic pigments in sea-water

Unesco

First published in 1966 by the United Nations
Educational, Scientific and Cultural Organization
Place de Fontenoy, Paris - 7e
2nd impression 1969
Printed by Imprimerie Rolland-Paris

Printed in France
NS. 68/XVIII. 1a/A

Preface

Publication by Unesco of the series of monographs on oceanographic methodology follows a recommendation adopted by the Scientific Committee on Oceanic Research (SCOR) at its meeting in Halifax in 1963.

As a forerunner to the series, Unesco undertook to distribute to oceanographic laboratories of the world copies of the second and revised edition of *A Manual of Sea-Water Analysis* by Strickland and Parsons (Fisheries Research Board of Canada, 1964). The series was finally established by the compilation and printing of the present volume (No. 1). Further volumes in the series will be published following results of the current revision of various oceanographic methods being undertaken by several institutions and international bodies.

The present volume treats various approaches and recommendations for standardization of determinations of photosynthetic pigments, especially chlorophyll in phytoplankton.

Standardization of methods in biological oceanography entails more difficulty than in other fields. Regional differences in abundance and composition of marine communities call for quite a considerable variety of methods when one is measuring standing crop and specific composition as well as productivity. The measuring of primary productivity directly or through estimation of the amount of photosynthetic pigments in the phytoplankton of a given body of water is one of the primary objectives of biological oceanography. Problems of standardization do not appear to be insurmountable in this respect, and standardized methods for comparison over a wide range in space and season are of particular interest. If data for regional charts were comparable, such charts, with regional distribution of photosynthetic pigments and their seasonal variation in the ocean, would be most helpful for the mapping of the world ocean's productive areas. The storage and retrieval system for biological data, which is so urgently needed for further progress in our attempts to understand the ocean and its production of living resources, might also suitably include photosynthetic pigment data.

The International Council for the Exploration of the Sea (ICES) and the United States National Academy of Science's Committee on Oceanography have established small groups of experts to consider standardization of methods for determination of photosynthetic pigments in sea-water. In December 1963, the Scientific Committee on Oceanic Research and Unesco established a Joint Group

of Experts on Determination of Photosynthetic Pigments (SCOR Working Group No. 17). This latter group met in Paris from 4 to 6 June 1964 under Professor J. Krey's chairmanship and their report is given in the first part of the present volume. A very important background document for the June meeting was a survey of existing methods prepared by Dr. T. R. Parsons (at that time with Unesco) in his capacity as convener of the ICES Working Group on Methods for Measuring Photosynthetic Pigments in Sea-Water. Dr. Parsons' survey makes up the second part of this volume. Two Australian papers, published in the third and fourth parts of the volume, provide additional information on the methods and their limits. Although written after the group's meeting, these two papers relate closely to its deliberations.

The need for intercomparability of methods in oceanography has given strong impetus to critical analysis, improvement of accuracy, simplification of methods in use and invention of new methods. In recommending that Unesco publish these four contributions, SCOR was convinced that this would help to achieve world-wide intercomparison of data, provide a reference for intercalibration of other old and new methods, encourage further methodological studies, and give guidance to those laboratories and scientists who work in this field.

Any scientific opinions expressed in these papers are, naturally, those of individual scientists or groups of scientists, and should not be interpreted as the views of Unesco.

Contents

1

Determination of photosynthetic pigments

Report of SCOR-Unesco Working Group 17
which met from 4 to 6 June 1964,
Unesco, Paris

A mimeographed issue of this report has
been published in Sydney, Australia,
November 1964

I Introduction

In December 1963 SCOR and Unesco established a working group with the following terms of reference: to comment on experimental results on the following topics and to prepare a tentative standard method for pigment determination.

1. Type of filter for removing phytoplankton from sea-water.
2. Suction pressure to be applied to filter.
3. Necessity for grinding or sonification.
4. Extraction solvent.
5. Addition of basic material, e.g. $MgCO_3$ or dimethylaniline.
6. Desiccation of filters before extraction.
7. Steam treatment of filters.
8. Storage of filters.
9. Duration of extraction.
10. Removal of extracted residue by centrifugation or filtration.
11. Precision of chlorophyll *a* determination at 1.0, 0.1 and 0.01 μg levels under laboratory conditions.
12. Extinction coefficients of chlorophylls *a*, *b* and *c*.

Members of the working group were:

Professor J. Krey (Kiel), chairman;	Professor Ichimura (Tokyo);
Professor K. Banse (Seattle);	Dr. S. W. Jeffrey (Sydney);
Dr. G. F. Humphrey (Sydney);	Dr. L. P. Vernon (Ohio).

In subsequent discussion the extra topics listed below were added.

13. Optical means of measurement: band-width, interference filters, spectrophotometer type.
14. Blanks, corrections, equations.
15. Computer cards for pigment data.
16. Direct determination without solution processes.
17. Fluorescent methods.
18. Chromatographic methods.

Most of these topics were considered in correspondence and at the Paris meeting. Statements on some of these topics (numbered differently) are given in this report.

In addition to the results of some special experiments made in the laboratories of those present at the meeting, three main documents were available:

Parsons, T. R. 1963. The determination of photosynthetic pigments in sea-water. A survey of methods (mimeo NS/89J issued by Unesco).
(This survey was carried out on behalf of ICES Plankton Committee.)

Humphrey, G. F.; Wootton, M. 1964. Report to SCOR-Unesco Working Group 17: Determination of photosynthetic pigments (mimeo 1760 issued by CSIRO).
(These experiments were done for Working Group 17. A paper on these results will soon be submitted for publication.)

Ceccaldi, H. J.; Berland, Brigitte 1964. Extractions par quelques solvants organiques à diverses concentrations, des pigments photosynthétiques de cultures de la diatomée *Phaeodactylum tricornutum* (Bohlin), après rétention sur filtres Millipore, ou après lyophilisation directe (mimeo issued by Station Marine d'Endoume, Marseille).

Invitations to attend the Paris meeting were issued to international organizations and to national committees for oceanic research. Present at the meeting were:

Professor J. Krey (Kiel), chairman;
Professor K. Banse (Seattle), rapporteur;
Dr. H. J. Ceccaldi (Marseille);
Dr. V. K. Hansen (IOBC, Ernakulam);
Dr. G. F. Humphrey (Sydney);
Dr. S. W. Jeffrey (Sydney);
Dr. T. R. Parsons (Unesco, Paris);
Dr. L. P. Vernon (Ohio);
Dr. C. S. Yentsch (Woods Hole).

II Report

Preamble

Chlorophyll *a*, *b* and *c* concentrations in sea-water samples are used to estimate the biomass and the photosynthetic capacity of phytoplankton. Ratios between various plant pigments possibly indicate the taxonomic composition or the physiological state of the community.

The following recommendations aim at obtaining precise measurements of chlorophyll *a*, *b* and *c* in phytoplankton. The accuracy of such measurements cannot yet be stated since the recovery of known amounts of chlorophyll added to the plankton cannot be studied. Freeze-drying (lyophilisation) might be the most accurate way to prepare phytoplankton material for pigment measurements, and should be used in the future to evaluate modifications of pigment extraction methods.

1 *Type of filters for removing phytoplankton from sea-water*

Evidence was presented showing that neither paper nor glass-fibre filters retain all particulate matter containing chlorophyll, although their efficiency can be raised by covering them with powdered $MgCO_3$. It is recommended that:

Phytoplankton be concentrated by filtering sea-water samples through

cellulose or cellulose-derivative membrane filters of 0.45 to 0.65 μ pore size. Before filtration, the filters should be covered with sufficient finely powdered $MgCO_3$ to give about 10 mg/cm² of filter area. When the filters specified above clog with the particular water samples used, paper filters No. 575 of Schleicher & Schüll or equivalent, covered with $MgCO_3$ as above, may be used. In any case, pore sizes of filters should be recorded when reporting the data.

2 *Suction pressure to be applied to filter*

We know of no evidence that high suction pressure during filtration affects chlorophyll retention and detection. Since filtration is not materially hastened with the recommended filters by employing full vacuum, it is recommended that:

A suction of 2/3 atm be used.

3 *Necessity for grinding or sonification*

Data were presented showing that grinding the filters containing phytoplankton increases the amount of pigment recovered and reduces the time needed for extraction. It is recommended that:

Filters with the plankton be ground for 1 min with a pestle rotating at about 500 r.p.m. in the presence of the solvent.

It is further recommended that:

Since it appears that ultrasonic treatment of about 1 Mhz (1,000 kc) reduces the time needed for pigment extraction from marine plankton, experiments should be made to compare the effectiveness of grinding with that of ultrasonic destruction of cells in regard to recovery of plant pigments from natural plankton.

4 *Extraction solvent*

Although methanol is very efficient in extracting pigment from phytoplankton, 90 per cent acetone is favoured at present because (a) pure chlorophyll *a* is more stable in it; (b) the chlorophyll absorption band in the red is sharper in it; and (c) the extinction coefficient is higher in it. Therefore it is recommended that:

Ninety per cent acetone be used for extraction. Methanol as a solvent for plankton pigments should be further investigated in view of its superiority over acetone with *Scenedesmus*, a fresh-water green alga difficult to extract.

5 *Addition of basic material, e.g. $MgCO_3$ or dimethylaniline during extraction*

Addition of $MgCO_3$ as recommended under Section 1 promotes effective filtration and facilitates centrifugation. Its presence might prevent acidification of the extract and thus retard the formation of pheophytin.

Solutions of dimethylaniline become brown upon standing and might cause an erroneously high extinction. The usefulness of adding dimethylaniline or other basic substances to the solvent, to prevent possible breakdown of plant pigments, should be investigated.

6 *Desiccation of filters before extraction*

We know of no evidence showing that desiccation of filters is necessary before extraction of pigment. However, since salt affects the solubility of Millipore filters, filters should be sucked as dry as possible after filtration.

7 *Steam treatment of filters*

There is no evidence that steam treatment is necessary to stabilize pigments. Since heat facilitates isomerization and oxidation of chlorophyll, the over-all effect of steam treatment might be harmful. If it is used, its effect should be investigated and the results stated.

8 *Storage of filters*

There are data showing that dry filters containing $MgCO_3$ can be stored in the dark at $+1°C$ or less for two months without significant loss of pigment (the loss is probably less than 15 per cent). Highest results are obtained by extracting damp, unstored filters.

Experiments on very long-term (several months) storage of filters are desired.

9 *Duration of extraction*

Under Section 3, grinding of plankton before extraction is recommended. Ten minutes of subsequent standing might suffice for optimal extraction. Since different types of phytoplankton might react differently and conditions of mechanical destruction might be critical, it is recommended that:

The investigator should make checks as to the length of extraction required after grinding the plankton.

Although extracts can be stored for several hours at room temperature in the dark without significant loss of pigment, it is recommended that:

Extracts should not be stored overnight.

10 *Removal of extract rêsidue by centrifugation or filtration*

On the basis of evidence available it is recommended that:

Extracts should be cleared by centrifugation; probably 10 minutes at 4,000 to 5,000 *g* in a swing-out centrifuge are needed.

11 *Precision of chlorophyll* a *determination at 1.0, 0.1 and 0.01 μg levels under laboratory conditions*

We are not in a position to specify the precision of chlorophyll determinations on marine phytoplankton under laboratory conditions. It is unlikely that the precision with the present procedure will be such that differences of 0.05 μg/l will be significant when comparing oceanic samples, i.e. in the range 0 to 1 μg/l. This holds also for chlorophyll *b* and *c*. It is recommended that precision be determined by each analyst.

12 *Optical means of measurement — band width — interference filters — spectrophotometer type*

It is recommended that:

Measurements be made with spectrophotometers with a band width of at most 2 to 3 mμ, allowing extinctions to be read to ± 0.001 units. The wavelength setting should be calibrated frequently.

If only chlorophyll *a* is determined, instruments with interference filters with not more than 5 to 10 mμ half-band width may be used; working extinction values must be determined for each filter with known amounts of chlorophyll *a*.

13 *Extinction coefficients of chlorophyll* a, b *and* c

The only available values of extinction coefficients for crystalline chlorophyll *a* in 90 per cent acetone were from preparations whose coefficients at the peak in the red in ether were at least 10 per cent lower than the highest values in the literature; these higher values were not based on crystalline pigment. It was decided to use the mean (99.87 l/g cm) of the three highest available values in ether which are based on different methods of determining the pigment concentration, 102.1 (Zscheile and Comar, 1941), 100.9 (Smith and Benitez, 1955) and 96.6 (Strain *et al.*, 1963). The value 99.87 l/g cm (ether) was used to determine the purity (86.81 per cent) of the crystals used by Jeffrey (unpublished) in obtaining absorption curves in ether and 90 per cent acetone. From this work of Jeffrey the values given below for 90 per cent acetone were calculated. It is recommended that:

An extinction coefficient of 89.31 l/g cm at the maximum of extinction at 663 mμ is used for chlorophyll *a* in 90 per cent acetone.

The working group will ask Dr. H. Strain to prepare an extinction curve of chlorophyll *a* in acetone from the ultra-violet through the visible range into the infra-red.

To prepare trichromatic equations, the extinction coefficients of chlorophyll *a* in 90 per cent acetone at 645 and 630 mμ have been calculated as 19.32 and 14.40 l/g cm.

The data for chlorophyll *b* were treated similarly, a mean value of 60.2 l/g cm (ether) being used to show that Jeffrey's crystals were 94.19 per cent pure. It is recommended that:

An extinction coefficient of 52.14 l/g cm at the maximum of extinction at 645 mμ is used for chlorophyll *b* in 90 per cent acetone.

To prepare trichromatic equations, the extinction coefficients of chlorophyll *b* in 90 per cent acetone at 663 and 630 mμ have been calculated as 9.57 and 15.22 l/g cm.

The extinction coefficient for chlorophyll *c* at the peak in the red in 90 per cent acetone has been determined by Jeffrey (1963). It is recommended that:

An extinction coefficient of 19.44 l/g cm at the maximum of extinction at 630 mμ is used for chlorophyll *c*.

To prepare trichromatic equations, the extinction coefficients of chlorophyll *c*

in 90 per cent acetone at 663 and 645 mμ have been calculated as 0.47 and 3.48 l/g cm.

14 *Blanks, correction and equations*

On the basis of the available evidence it is recommended that:

Blanks be 90 per cent acetone. Readings should be taken at 750 mμ to correct for turbidity of the extract and must not exceed 0.005 per centimetre of light path. This reading should be subtracted from the readings at 663, 645 and 630 mμ.

The following trichromatic equations are recommended:

chl. $a = 11.64\ e_{663} - 2.16\ e_{645} + 0.10\ e_{630}$

chl. $b = -3.94\ e_{663} + 20.97\ e_{645} - 3.66\ e_{630}$

chl. $c = -5.53\ e_{663} - 14.81\ e_{645} + 54.22\ e_{630}$

where chl. *a* (*b* or *c*) is in μg/ml.

e_{663}, e_{645} and e_{630} are the extinctions

(optical densities, absorbances)/cm of light path at 663, 645, and 630 mμ after subtracting the 750 mμ reading.

The equations have been checked on mixtures of solutions with known amounts of the three chlorophylls. The results are shown below:

Chlorophyll *a*			Chlorophyll *b*			Chlorophyll *c*		
Found (μg/ml)	Added (μg/ml)	Recovery (%)	Found (μg/ml)	Added (μg/ml)	Recovery (%)	Found (μg/ml)	Added (μg/ml)	Recovery (%)
6.03	5.87	103	1.08	0.79	111	4.87	5.07	96
0.79	0.74	106	0.53	0.49	108	1.22	1.27	97
0.41	0.37	110	0.30	0.24	125	0.75	0.64	117

REFERENCES

JEFFREY, S. W. 1963. Purification and properties of chlorophyll *c* from *Sargassum flavicans*. *Biochem. J.*, **86** : 313-18.

SMITH, H. C.; BENITEZ, A. 1955. In: K. Paech and M. Tracey (eds.). *Modern methods of plant analysis*, vol. 4, p. 142-96. Heidelberg, Springer Verlag.

STRAIN, H. H.; THOMAS, Mary R.; KATZ, J. J. 1963. Spectral absorption properties of ordinary and fully deuteriated chlorophylls *a* and *b*. *Biochim. Biophys. Acta*, **75** : 306-11.

ZSCHEILE, F. P. Jr.; COMAR, C. L. 1941. Influence of preparative procedure on the purity of chlorophyll components as shown by absorption spectra. *Bot. Gaz.*, **102** : 463-81.

III Tentative standard method for determination of chlorophylls in samples of sea-water

Concentration of sample

Use a volume[1] of sea-water which contains about 1 μg chlorophyll *a*. Filter[2] through a filter[3] covered by a layer of $MgCO_3$.[4]

Storage

The filter can be stored in the dark over silica gel at 1°C or less for two months but it is preferable to extract the damp filter immediately and make the spectrophotometric measurement without delay.

Extraction

Fold the filter (plankton inside) and place it in a small (5 to 15 ml) glass, pestle-type, homogenizer. Add 2 to 3 ml 90 per cent acetone. Grind 1 minute at about 500 r.p.m. Transfer to a centrifuge tube and wash the pestle and homogenizer 2 or 3 times with 90 per cent acetone so that the total volume is 5 to 10 ml. Keep 10 min in the dark at room temperature. Centrifuge[5] for 10 min at 4,000 to 5,000 *g*.[6] Carefully pour into a graduated tube so the precipitate is not disturbed and if necessary dilute[7] to a convenient volume.[8]

Measurement

Use a spectrophotometer with a band-width of 3 mμ or less, and cells with a light-path of 4 to 10 cm.[9] Read the extinction (optical density, absorbance) at 750,[10] 663, 645, and 630 mμ against a 90 per cent acetone blank.

Calculation

Subtract the extinction at 750 mμ from the extinctions at 663, 645, and 630 mμ. Divide the answers by the light-path in centimetres of the cells. If these corrected extinctions are e_{663}, e_{645}, and e_{630} the concentrations of chlorophylls in the 90 per cent acetone extract as μg/ml are given by the following equations:

chl. $a = 11.64\ e_{663} - 2.16\ e_{645} + 0.10\ e_{630}$

chl. $b = -3.94\ e_{663} + 20.97\ e_{645} - 3.66\ e_{630}$

chl. $c = -5.53\ e_{663} - 14.81\ e_{645} + 54.22\ e_{630}$

If the values are multiplied by the volume of the extract in millilitres and divided by the volume of the sea-water sample in litres, the concentration of the chlorophylls in the sea-water is obtained as μg/l (= mg/m^3).

NOTES

1 The amount of chlorophyll *a* should be less than 10 μg, otherwise a second extraction with 90 per cent acetone might be necessary. With ocean water about 4 to 5 litres of sample should be used; with coastal and bay waters, sometimes one-tenth of this amount is sufficient.
2 Use no more than two-thirds of full vacuum.
3 Satisfactory filters include paper (Albet), cellulose (Cella 'grob'), and cellulose ester (0.45 to 0.65 μ pore size); the filter should be 30 to 60 mm diameter. If these filters clog with inorganic detritus, use Schleicher & Schüll 575.
4 Add about 10 mg $MgCO_3$/cm^2 filter surface, either as a powder or as a suspension in filtered sea-water.
5 A swing-out centrifuge gives better separation than an angle centrifuge.
6 If a stoppered, graduated centrifuge tube is used, the extract can be made up to volume and the supernatant carefully poured or pipetted into the spectrophotometer cell.
7 If turbid, try to clear by adding a little 100 per cent acetone or distilled water or by re-centrifuging.
8 This depends on the spectrophotometer cell used. The volume should be read to 0.1 ml.
9 Dilute with 90 per cent acetone if the extinction is bigger than 0.8.
10 If the 750 μ reading is greater than 0.005/cm light-path, reduce the turbidity as in Note 7.

IV Important subjects not discussed fully at the working group

Accuracy of the equations

1. Permissible ratios of pigments.
2. Permissible range of pigment concentration.
3. Accuracy of the equations using pure chlorophyll solutions.

Work on these is being carried out by Humphrey and Jeffrey.

Chromatographic methods

For the determination of the exact pigment composition of the sample, use should be made of available chromatographic methods for pigment separations: (a) small columns (Parsons); (b) thin layer methods (Madgwick); (c) paper (Jeffrey).

These methods have been adapted to pigment analyses in cultures where large quantities of pigments are present, and to sea-water samples with much lower concentrations of pigments. For quantitative analyses, pigments may be eluted and analysed with an 80 per cent recovery.

A paper chromatographic method has been used at sea; it is an adaptation of the 'chromatobox'. Samples can be run even in the most violent storm, since it is not necessary to have a stable horizontal base for the development of the solvent front.

Determination of carotenoids

Things which need to be done:

1. Search for a good simple method of separating chlorophylls as a group from carotenoids as a group in marine phytoplankton, so that a simple measure of total carotenoids may be made.
2. Accurate extinction values for fucoxanthin and peridinin.
3. A general extinction value for total carotenoids in marine algae.

Units

A plea is made to oceanographers to use ways of expressing and evaluating data that have meaning to the plant physiologist, so that important data obtained with cultures may have application when trying to explain and understand results of field experiments. For example, to express photosynthetic rates in the ocean as μ moles or μ litres CO_2/mg chlorophyll $a + c$, instead of counts C^{14}/m^3, which has no meaning in work with cultures.

Computer cards

Consideration of this should be left to Working Group 18: Biological Data. Such consideration would be helped if a member of WG 17 participated in WG18.

Direct determination

Fluorescent methods

2

The determination of photosynthetic pigments in sea-water

A survey of methods

T. R. Parsons

Office of Oceanography,
Unesco, place de Fontenoy,
Paris-7e, France

Present address:
Pacific Oceanographic Group,
Nanaimo, B.C.,
Canada

Manuscript received 2 May 1963

Preface

At the 1962 meeting of the International Council for the Exploration of the Sea (ICES), the Plankton Committee appointed convenors for four small working groups to study current methods in biological oceanography. One of these groups was to study current methods for the measurement of photosynthetic pigments in sea-water. As convenor for this group I have considered that some preparation is necessary in order to provide a working group with material with which to discuss the problem and eventually decide on a standard procedure. I anticipated, therefore, asking persons to take part in a meeting of a working group on this subject some time in 1964 when the preparatory work had been completed.

In organizing the first part of the preparatory work as described in the following presentation I am grateful to those persons who returned the Unesco questionnaire NS/9/114/89 and to the persons whose comments on the organization of this work are reported in Appendix II. Pending the acceptance of this report by the ICES, Dr. G. F. Humphrey, CSIRO, Cronulla, Australia, has agreed to supervise the type of experiments envisaged in the report.

Acknowledgement

The author wishes to acknowledge the assistance of Dr. W. S. Wooster in the preparation of this report.

I Introduction

Under the resolution adopted at the fiftieth statutory meeting of the ICES (C. Res. 1962/4(8)), the work on the determination of photosynthetic pigments in sea-water to be carried out may be summarized as follows:

1. A review of methods normally used.
2. An experimental examination of various procedural steps, leading to an eventual recommendation for a standard procedure for pigment analysis.

The following discussion pertains principally to the requirements in 1 above. In addition, however, an attempt has been made to identify the problems requiring further experimental work. Further, it has been assumed that the most immediate need is for a standard method to be used by oceanographers making synoptic surveys of large areas of ocean. In order to obtain comparable results from ships operating in different areas or at different times it is necessary to have a reliable universal procedure. Particular attention has been given, therefore, to procedures which are usable aboard ship, although they may not be so satisfactory for some purposes as more sophisticated methods which could be employed, for example, in a laboratory concerned with studies on phytoplankton cultures.

II Methods currently employed for the determination of photosynthetic pigments in sea-water

It is general experience that no analytical method, however well described, will be performed in exactly the same way by different analysts. Differences in technique which appear to be small may lead to significant differences in accuracy and precision of the measurement. Thus the following presentation places more emphasis on evaluating differences in individual techniques than on a review of the techniques themselves.

Two basic techniques have been employed; extractive spectrophotometry and extractive fluorimetry. Examples of the former technique as used by marine scientists are given by Krey (1939), and Richards with Thompson (1952), and of the latter technique, by Kalle (1951), and Yentsch and Menzel (1963). In addition, reviews on these and other techniques have been written (e.g. Krey, 1958;

Strickland, 1960). Since most workers employ the spectrophotometric method, comment on the fluorometric method is limited to the last portion of this report.

Information on present methodology was obtained by sending forty-four questionnaires to representative marine scientists in twelve countries. A summary of some thirty replies is given in Appendix I. For the most part the various differences in technique represent individual modifications of one or two methods —thus it seems desirable to establish which steps in the procedure are most sensitive to such modifications.

It should be noted that in some cases the only pigment being determined is chlorophyll *a*. Since the reported precision and accuracy for the determination of other pigments is lower than that for chlorophyll *a* (Richards with Thompson, 1953; Strickland and Parsons, 1960), and because chlorophyll *a* is the most widely used pigment for the estimation of standing crop or photosynthetic rate, the following section is devoted to a consideration of problems relating to the establishment of a standard method for chlorophyll *a* alone (at the end of the section, there is a suggestion for the determination of other pigments).

The summary of data which has been presented in Appendix I shows the amount of variation which has been introduced into the stepwise procedure for chlorophyll *a* analysis. No indication is given of which individual variations are most commonly employed and this has been omitted for two reasons. Firstly the most popular use of a piece of apparatus or procedure tends to be biased towards the country to which the largest proportion of questionnaires was sent. Secondly it would seem incorrect in trying to establish the most reliable procedure for chlorophyll *a* analysis to draw attention to a piece of apparatus or procedure most commonly used when it is the purpose of this investigation to obtain an objective appraisal of only what is best.

III A suggested procedure for the establishment of a standard method for the determination of chlorophyll *a* in sea-water[1]

A APPARATUS

1 *Spectrophotometers and colorimeters*

Since maximum sensitivity of the determination requires maximum extinction of light per unit weight of compound to be analysed, the use of optical equipment with broad wave-band widths, wide slit widths or wave-length settings which are difficult to adjust, should be discouraged. Some types of spectrophotometers meet these requirements to a greater or lesser degree, but colorimeters are of limited use in waters of low pigment concentration because the broad band-pass of the filter leads to reduced sensitivity. It is important that the wave-length setting of spectrophotometers be routinely checked (see suggestions of NASCO report).[2] In order to permit intercomparison of different spectrophotometers,

1 The following discussion is keyed to the information reported in Appendix I.
2 Excerpts from the NASCO report and the SCOR-Unesco intercalibration test are given at the end of this presentation.

the optical density at a given wave-length should be standardized in terms of the resolution of the instrument and the slit width through which the light passes.

2 *Light path of cuvettes*

The spectrophotometer employed should be capable of accommodating several different sizes of cuvettes. When pigment values are known to vary over a wide range, light path lengths of 1 cm and 10 cm, and possibly an intermediate length, are required for obtaining optical density readings in the most accurate portion of the scale.

3 *Type of filter for removing plankton from sea-water*

It may be seen in Appendix I that at present there is a tenfold range in the pore size of filters employed for removing plankton from sea-water. In addition, the material of which the filters are composed (not stated in every case) may be variably soluble in the extraction solvent and, in some cases, may have a deleterious effect on the light transmission of the solvent. The type of filter employed for removing phytoplankton from sea-water should be standardized therefore, and in addition made readily available to all oceanographers (cf. recommendation of the SCOR-Unesco intercalibration test and NASCO report).[1]

4 *Approximate suction pressure*

The use of high suction pressure has been found to damage phytoplankton during the course of filtration. A maximum suction pressure to be applied to plankton filters should be determined experimentally, taking into account the recommendations of the SCOR-Unesco intercalibration tests and the NASCO report.

5 *Sonification and grinding apparatus*

There is some evidence (Nelson, 1960; Laessøe and Hansen, 1961) that the use of sonification apparatus is necessary for the complete extraction of pigment from some species of phytoplankton. In addition it is noted in Appendix I that some persons employ grinding apparatus which, if found as effective as sonification, should be given prior recommendation on the basis of its lower cost. A thorough testing of the effect of sonification and grinding apparatus on a series of natural phytoplankton blooms and a standard minimum treatment (for sonification apparatus, in terms of period of treatment, frequency and energy of sonifier) should be determined if found necessary.

B REAGENTS

1 *Solvent with which cells are extracted*

Some evidence exists (Laessøe and Hansen, 1961; see also NASCO report) that methanol is a better solvent for the extraction of marine phytoplankton than

1 Excerpts from the NASCO report and the SCOR-Unesco intercalibration test are given at the end of this presentation.

90 per cent acetone. A comparison of these two solvents should be made on a series of natural phytoplankton blooms and the best solvent recommended for routine use.

2 *Addition of basic material during extraction*

For preventing the formation of pheo-pigments $MgCO_3$ is often added during extraction. This should be compared for effectiveness with dimethylaniline which has been reported to be a better additive for this purpose (Vallentyne, 1955; Patterson and Parsons, 1963).

C PROCEDURE

For discussions of volume of sea-water filtered (C.1) and chlorophyll concentrations encountered (C.2), see discussion on precision of chlorophyll *a* determinations (E.4) below.

3 *Desiccation of filters prior to extraction*

The need for desiccation prior to extraction should be demonstrated experimentally. A standard minimum treatment should be found if desiccation is shown to be necessary.

4 *Steam treatment of filters*

Steam treatment of samples has been employed by a number of scientists, presumably to prevent formation of chlorophyllide from chlorophyll by the action of chlorophyllase. Since chlorophyllide *a* has been reported to have the same spectrum and extinction coefficients as chlorophyll *a* (Holt and Jacobs, 1954), the use of steam would appear to be an unnecessary step. The effect if any should be demonstrated experimentally on natural populations and on *Skeletonema costatum* which has been reported to have a very high chlorophyllase activity (Patterson and Parsons, 1963; Jeffrey, 1963).

5 *Storage of filtered sample*

Together with C.3, covering the desiccation of samples, the preservation of plankton samples for different periods of time should be tested experimentally. A maximum storage period of three months would seem, if experimentally possible, sufficient for scientists on ships which do not have facilities for carrying out all parts of the procedure on board.

6 *Type of container used to carry out extraction*

The facility of extraction, centrifugation and volume adjustment in glass-stoppered graduated centrifuge tubes should be compared with other apparatus and a standard extraction vessel recommended. This consideration probably has little effect on the precision and accuracy of the method but for laboratories starting pigment work it is useful to know the best pieces of apparatus to order.

7 *Length of extraction time*

In combination with items C.9 and A.5, covering the use of apparatus employed to rupture cells, the minimum period of time required for an extraction, and the benefit if any of hot extractions, should be found experimentally with the use of natural populations. It is possible that a long extraction period without the use of apparatus to rupture cells may be found equivalent to a very short extraction with such apparatus. Equivalent extraction procedures should be recommended as alternative procedures.

8 *Volume for extraction solvent*

Discussed under E.4, Precision.

9 *Methods employed to rupture cells*

Discussed under C.7 and A.5.

10 *Removal of extracted material*

The use of filters compared with centrifugation for the removal of extracted material should be examined with attention being paid to the facility of operation and the efficiency of removal of extracted material.

11 *Blank employed of 0 optical density*

The choice of a suitable 'blank' for 0 optical density should be made experimentally between the use of the extraction solvent and the use of the solvent plus filter material and any additive to prevent peophytin formation.

12 *Wave-lengths at which measurements are made*

As Krey observed in 1958 (loc. cit.), the determination of chlorophyll *a* by trichromatic spectrophotometry is only slightly affected by chlorophylls *b* and *c*. If all three chlorophylls are present in equal amounts the maximum error in the estimation of chlorophyll *a* by a single 665 mμ reading in 90 per cent acetone is about 10 per cent. Since most of this error is contributed by chlorophyll *b* which is generally absent from oceanic sea-water samples, the actual error in making a chlorophyll *a* estimation uncorrected for other chlorophylls is not more than about 1 per cent. For chlorophyll *a* determinations alone, therefore, a single optical density reading might be recommended for the measurement of the pigment. A correction for turbidity should be introduced by making a measurement at 750 mμ and the establishment of a standard procedure for a 750 mμ correction should be considered along the lines recommended by the NASCO report and the SCOR-Unesco intercalibration test.

13 *Extraction performed*

Because of the limited time and space available on some ships for the completion of all parts of the procedure on board it is necessary that the final procedure be

written to give an indication of where it is possible to break off and complete the analysis on shore. It is recommended that this should be considered under C.5 and C.3.

D STANDARDIZATION

1 *Extinction coefficient employed for chlorophyll* a

The choice of a suitable extinction coefficient for chlorophyll *a* should be made from the large number of values quoted in the literature. For this purpose it is recommended that the value quoted by Smith and Benitez (1955) which agrees with the value of Zscheile and Comar (1941) of 102 l/g cm in ethyl ether at 662 mμ should be given primary consideration. This value is suggested by Smith and Benitez (1955) for use as a standard since in an extractive spectrophotometric procedure, chlorophyll *a* is not dried in the extracted state. Chlorophyll *a* which has been dried in the extracted state was found by Zscheile and Comar (1941) to give a lower specific absorption coefficient than undried chlorophyll. The specific absorption coefficient in 90 per cent acetone corresponding to the value quoted above in ethyl ether has been found by Vernon (1960) to be 91 l/g cm at 664 mμ. [1]

Following the choice of an extinction coefficient for chlorophyll *a* it should not be recommended that a commercial preparation of chlorophyll *a* be used as a primary standard. Some commercial preparation of chlorophyll *a*, or of a more stable derivative such as pheophytin, might be recommended, however, as a secondary standard with which to compare optical densities as described above (A.1).

2 *Extinction coefficients employed for other pigments estimated*

Discussed under F.1.

E CALCULATIONS OF RESULTS

1 *Turbidity correction*

Discussed under C.12.

2 *Correction made for other chlorophylls at wave-length for chlorophyll* a

Discussed under C.12.

3 *Correction for degradation products of chlorophyll* a

It would be very useful to have some measure of the amount of degradation products of chlorophyll *a* present in marine samples since if these are appreciable

1 If a decision is made to employ methanol as an extraction solvent the specific absorption coefficient of chlorophyll *a* in methanol will have to be determined in a manner similar to that employed by Vernon (1960) for the determination of the specific absorption coefficient of chlorophyll *a* in 90 per cent acetone.

they will cause an erroneous over-estimation of chlorophyll *a*. At present there appears to be no reliable quantitative method for such an estimation to be incorporated in a standard procedure for chlorophyll *a* analysis of sea-water samples. The introduction of some technique at a later date would seem advisable.

4 *Precision of the chlorophyll* a *determination*

The precision of chlorophyll *a* determinations is considered here in conjunction with the amount of sea-water filtered (C.1), the range of chlorophyll *a* values encountered (C.2), the volume of the extraction solvent (C.8) and the light path length of cuvettes (A.2).

The precisions of chlorophyll *a* determinations quoted in Appendix I (E.4) have been taken as representative of a number of values quoted by scientists, often in the absence of an explanation of what the precision quoted actually means in statistical terms. A more detailed description of precision in relation to volume of sea-water filtered, light path of cuvettes and volume of extraction solvent may be considered as follows.

The precision for chlorophyll *a* determination at the 5 μg level reported by Strickland and Parsons (1960) is approximately ± 5 per cent.[1] Employing the same extinction coefficient for chlorophyll *a* that was used in those calculations, the optical density reading for this amount of pigment in 10 ml of extract and using a 10 cm cuvette is about 0.33. If it may be assumed that optical density readings down to about 0.1 can be measured without introducing a decrease in the precision to more than about ± 10 per cent, then the lower limit of pigment detection at this order of precision, employing the extinction coefficient suggested in D.1 above, is about 1 mg/m³ if 1 litre of sea-water is filtered for extraction of the residue with 10 ml of solvent and for an extinction read in a 10 cm cuvette. The lower limit of pigment detection at the order of precision stated above can be decreased to 0.1 mg/m³ if 10 litres of sea-water are filtered. It is probable, therefore, that this value represents the lower limit of chlorophyll *a* values which should be quoted by persons using extractive spectrophotometry in order that all results may be considered to be comparable, that is, obtained with the same order of precision. Modifications such as reducing the extraction solvent to 5 ml (but maintaining a 10 cm light path) or filtering 20 litres of sea-water will almost certainly introduce difficulties and unnecessary delays in procedure for an increase in the limit of detection by a factor of only two. It might be considered advisable, therefore, that chlorophyll *a* values of less than 0.1 mg/m³ should be reported as < 0.1 mg/m³ and not as some actual value which would not be comparable with pigment values determined above the limit of detection quoted here. In the table below the lower limit of chlorophyll *a* detection, assuming about ± 10 per cent precision, is shown for various combinations of cell lengths and volumes of sea-water filtered and assuming 10 ml of solvent are employed for the extraction. The table emphasizes the necessity for the use of 10 cm light paths for the determination of pigments in the range 0.1 to 1.0 mg/m³.

1 See page 5 of reference quoted for an explanation of this value.

Suggested lower limit of chlorophyll *a* concentrations to be reported, expressed as a function of the light path length and the volume of sea-water filtered.

Cell length	Volume of sea-water filtered (litres)			
	10	5	2.5	1
	mg/m³	mg/m³	mg/m³	mg/m³
1 cm	1	2	4	10
5 cm	0.2	0.4	0.8	2
10 cm	0.1	0.2	0.4	1

In conclusion to this section, the final determination of the precision of chlorophyll *a* estimations will have to be made after the formulation of a standard procedure. The experiment should be designed to show the precision obtainable by a number of individuals and should further show whether the means of individual determinations fall within the limits of precision found or if inter-calibration factors are necessary because of the use of different apparatus (e.g. spectrophotometers).

Finally it is suggested that in reaching a conclusion on all the steps in the procedure for the determination of chlorophyll *a* described above, the most suitable piece of apparatus or procedure should be recommended in each case together with alternatives which are not found to cause significant variations in the determination of chlorophyll *a*. Procedures and any apparatus which do cause differences in the final results should also be listed as not being recommended. Thus it may be possible to establish a 'kit' for chlorophyll *a* determinations in sea-water and where apparatus or facilities for a certain part of the procedure are not available in some countries or on board some ships to supplement these by using an alternate recommendation.

F OTHER PIGMENTS AND METHODS

1 *Pigments determined other than chlorophyll* a

Some discussion has already been presented (see Section II) on the *a priori* need for a standard method for chlorophyll *a* analysis. The danger exists, however, that in the establishment of any standard method the limitations imposed by the standard procedure will distract from an elaboration and variation of a procedure which might eventually lead to a modification yielding more comprehensive results. It is not the intention, therefore, to suggest here that pigment measurements in sea-water should be confined to chlorophyll *a*. It does appear, however, to be more difficult to standardize the method for the measurement of other pigments. Chlorophyll *b* values in the oceans are so low, for example, that the values as calculated by trichromatic spectrophotometry may sometimes yield a negative amount and, when positive, the extremely small order of magnitude coupled with the lack of precision for such low values leaves doubt as to whether the pigment is actually present or not. Chlorophyll *c*, although known to be present in marine phytoplankton, is equally difficult to determine by trichro-

matic spectrophotometry on oceanic pigment samples. When one considers that the optical density at 630 mμ contributed by 1 μg of chlorophyll *c* in 10 ml of solvent using a 10 cm cuvette is only about 0.02, and that the value of 1 μg is probably more than will normally be encountered in ocean areas, it is not surprising that some extraordinary ratios of chlorophyll *c* : *a* have been reported for ocean areas (see accumulated values by Humphrey, 1961, for example) which have not been confirmed with determination on phytoplankton cultures. In the case of estimations of plant carotenoids, the difficulty recognized by Richards with Thompson (1952) of having to employ specific pigment unit is complicated further by the reported use of a different specific pigment unit (Appendix I, D.2) than that originally defined by Richards with Thompson (1952).

In view of these comments it would seem that the best way to obtain maximum benefit from the extracted pigments, other than for the estimation of chlorophyll *a*, is to read extinctions at certain other wave-lengths but not to interpret these readings in terms of absolute amounts of pigment. Thus it might be suggested that in addition to a reading at 750 mμ and 665 mμ for the estimation of chlorophyll *a*, additional optical densities should be read at 645, 630, 510 and 480 mμ. Further readings that may eventually prove useful would be at 505 and 430 mμ. Ratios of optical densities at these wave-lengths may prove more reliable than attempting to determine the pigments involved in absolute amounts. Measurement of the entire spectrum of pigment extracts would present the best solution to this problem but this is undoubtedly too tedious for routine analysis except when a specific study is being made.

2 *Use of a fluorometric technique for routine determinations*

For some oceanic areas (e.g. Sargasso Sea) the limit of chlorophyll *a* detection of 0.1 mg/m^3 (discussed above (E.4)) may not be low enough to show seasonal and spatial differences in chlorophyll *a* concentrations. In such areas it may be advisable to employ a fluorometric technique, since the limit of detection for fluorometric measurements of chlorophyll *a* is at least ten times lower than for spectrophotometric measurements. Fluorometric estimations include all chlorophylls, however, and thus the results are not strictly comparable to a spectrophotometric technique for chlorophyll *a* alone. On the other hand it has been found possible to give some measure of the proportion of chlorophyll degradation products by fluorimetry (Yentsch and Menzel, 1963) which has been mentioned here (E.3) as one desideratum for the spectrophotometric determination of chlorophyll *a*.

In reaching a conclusion on the desirability of using a fluorometric technique for chlorophyll determinations on a routine basis it may be advisable to suggest that a sufficient number of spectrophotometric measurements should be performed to characterize an area of low chlorophyll content (i.e. < 0.1 mg/m^3) and that a more detailed description could then be presented in terms of fluorimetric determinations. A suitable method for the fluorimetric determination of chlorophyll in sea-water has been reported by Kalle (1951) and another by Yentsch and Menzel (1963), which is a modification of Kalle's technique.

EXCERPTS AND REFERENCES

Excerpts from the NASCO report entitled 'Recommended Procedure for the Measurement of Phytoplankton Pigments prepared by the NAS/NRC Committee on Oceanography Working Group on Standardization and Intercalibration of Biological Measurements and Sampling Methods', 28 March 1963

With reference to:

Section A.1 'It is recommended that the spectrophotometer be calibrated frequently using narrow band-pass filters or Didymium glass (or equivalent).'

Section A.3 'The water samples collected for phytoplankton pigment analysis should be filtered through cellulose-type membrane filters (e.g. Millipore R Type HA or PH, or equivalent) or possibly fine glass-fiber filters (e.g. Whatman GF/C, Gelman glass filters, or equivalent).'

Section A.4 'The pressure reduction should not exceed 50 cm Hg.'

Section B.1 'Methanol and diethylether extract phytoplankton pigments better than acetone.'

Section C.12 'Following extraction, acetone solutions should be centrifuged so that the optical density at 750 mμ is less than 0.005/cm of path length of light, after the blank has been subtracted; the optical density at 750 mμ must be kept below 0.01.'

Excerpts from SCOR-Unesco intercalibration test entitled 'Circular Memorandum to National Committees, Indian Ocean Investigations', 9 January 1962

With reference to:

Section A.3 'Filters should be soluble in 90 per cent acetone, should have a pore size of no more than 0.8 μ, and should not be subjected to high vacuum during filtration.'

Section A.4 'The reduction in pressure should be about $^1/_2$ to $^1/_3$ of an atmosphere.'

Section C.12 'If the optical density at 750 mμ is greater than 0.005/cm path, recentrifuge, refilter, or dilute to reduce this reading.'

HOLT, A. S.; JACOBS, E. E. 1954. Spectroscopy of plant pigments. I Ethyl chlorophyllides A and B and their pheophorbides. *Amer. J. Bot.*, **41**.

HUMPHREY, G. F. 1961. Phytoplankton pigments in the Pacific Ocean. Preprint, *Symposium on algal productivity in the Pacific, 10th Pac. Sci. Congr.*, p. 16.

JEFFREY, S. W. 1963 (results to be published).

KALLE, K. 1951. Meereskundlich-chemische Untersuchungen mit Hilfe des Pulfrich-Photometers von Zeiss VII. Die Mikrobestimmung des Chlorophylls und der Eigenfluoreszens des Meerwassers. *Deutsch. Hydrogr. Zeitschr.*, **4**.

KREY, J. 1939. Die Bestimmung des Chlorophylls in Meerwasser-Schöpfproben, *J. du Cons.*, **14**.

——. 1958. Chemical methods of estimating standing crop of phytoplankton. *Rapp. et Proc.-Verb. Cons. internat. Explor. de la Mer*, **144**.

LAESSØE, A.; HANSEN, Vagn Kr. 1961. Ultrasonic and extraction of chlorophyll *a* from phytoplankton. Plankton Committee Report No. 143. *Cons. internat. Explor. de la Mer.*

NELSON, D. J. 1960. Improved chlorophyll extraction method. *Science*, **132**.

PATTERSON, J.; PARSONS, T. R. 1963. Distribution of chlorophyll *a* and degradation products in various marine samples. *Limnol. Oceanogr.* (in press).

RICHARDS, F. A. WITH THOMPSON, T. G. 1952. The estimation and characterization of plankton populations by pigment analysis. II. A spectrophotometric method for the estimation of plankton pigments. *J. Mar. Res.*, **11**.

SMITH, J. H. C.; BENITE, A. 1955. Chlorophylls. Analysis in plant materials. *Modern methods of plant analysis*, vol. IV, p. 143-96. Berlin, Springer-Verlag.

STRICKLAND, J. D. H. 1960. Measuring the production of marine phytoplankton. *Bulletin No. 122 Fish. Res. Bd. Canada*, 172 p.

——; PARSONS, T. R. 1960. A manual of sea-water analysis. *Bulletin No. 125 Fish. Res. Bd. Canada*, 185 p.

VALLENTYNE, J. R. 1955. Sedimentary chlorophyll determination as a paleobotanical method. *Can. J. Botany*, **33**.

VERNON, L. P. 1960. Spectrophotometric determination of chlorophylls and phaeophytins in plant extracts. *Anal. Chem.*, **32**.

YENTSCH, C. S.; MENZEL, D. W. 1963. A method for the determination of phytoplankton chlorophyll and phaeophytin by fluorescence (unpublished manuscript).

ZSCHEILE, F. P.; COMAR, C. L. 1941. Influence of the preparation procedure on the purity of chlorophyll components as shown by the absorption spectra. *Bot. Gaz.*, **102**.

Appendixes

I Estimation of photosynthetic pigments in sea-water [1]

A summary of routine procedures employed by scientists

A. APPARATUS

1 *Spectrophotometers and colorimeters*
Beckman D. U., DK II A and B; Unicam SP 500 and 600; Perkin Elmor 137 UV; Cary (model 14R); Zeiss (RPQ20A); Bausch and Lomb Spectronic 20 and 340; Elko II (Zeiss); Russian model S-F-4.

2 *Light path of cuvettes*
1, 2, 3, 4, 5, 10 and 12 cm; 0.5 inch.

3 *Type of filter for removing plankton from sea-water*
Schleicher and Schüll no. 1575; Millipore AA and HA; Whatman GF/C; Albet 242; Polypore; Cella; Reeve Angel and Co. Glass Fibre no. 984 H; Gelman Instrument Co. Type A Glass Fibre Filter; Membrane W5 covered by glass powder. Filter material: Cellulose nitrate; paper; fibre-glass.
Pore sizes: 0.45 μ; 1 μ; 3 μ; 0.3-0.6 μ; 0.8 μ; 5 μ.

4 *Approximate suction pressure**
(Negative) 10 cm; 15-30 cm; 35-50 cm; 60 cm.
(Positive) 12 lb per sq inch.

5 *Sonification and grinding apparatus*
None; electrically operated teflon tissue grinder; Ultrasonic disintegrator (type VSL G300, Schoeller & Co.); M.S.E./Mullard 20 kc disintegrator; Ultraschall-Gerat T200 (Fa. Lehfeld); mechanical fragmentation of fibre-glass filters; Sonicator, Tokyo-Riko 50-5 type.

B. REAGENTS

1 *Solvent with which cells are extracted*
Methanol; 90 per cent acetone; 85 per cent acetone; Acetone (+MeOH or EtOH); 90 per cent methanol.

2 *Addition of basic material during extraction*
None; $MgCO_3$.

C. PROCEDURE

1 *Range of sea-water volumes filtered* (litres)*
0.5-4; 1-11; 6-8; 4-5; 1-20; 0.05-1.

2 *Range of chlorophyll* a *concentrations encountered* (mg/m³)*
0-10; 0.2-30; 0.05-0.4; 0.02-20; 5-500.

3 *Desiccation of filters prior to extraction**
None; silica-gel in dark and freezer; in dark, alumina, — 20° C; *in vacuo*, dark, room temperature.

4 *Steam treatment of filters*
None; 20 sec; 30 sec.

5 *Storage of filtered samples**
None; 1-2 months; 6-10 weeks; 2-3 days; 3-4 weeks; 1-10 weeks.
Method of storage: see 3 above.

6 *Type of container used to carry out extraction**
Test tube; glass or plastic bottle; stoppered centrifuge tubes, glass or plastic; screw-cap centrifuge tube; stoppered 50 ml flask; graduated, stoppered centrifuge tube.

1 The asterisk (*) is employed in this appendix to indicate representative replies covering a range of values or procedures.

7 *Length of extraction time**
24 hours, cold; 1 min hot (boiling acetone) + 24 hours, cold; 14-18 hours; 24 hours, room temperature; 10 + 5 min, hot; 30 min, hot (40° C.); 3 × 3 min extraction, room temperature.

8 *Volume of extraction solvent** (ml)
5-10; 10-25; 4; 6; 3-4.

9 *Method employed to rupture cells*
None; hot extractions; see A.5 above.

10 *Removal of extracted material**
(i) Centrifugation: 6,000 *g*, 10 min; 4,300 *g*, 10 min; 2,000 *g*, 5 min; 500 *g*, 3 min; 2,500 *g*-min.
(ii) Filtration: Hard filter paper; Schleicher & Schüll No. 1575 double filter; paper filter, Toyo no. 101.

11 *Blank employed for 0 optical density or 100 per cent transmission*
Solvents, see B.1 above; solvent extract of filter material plus $MgCO_3$.

12 *Wave-lengths at which measurements are made or filter band-widths** (mμ)
665, 645, 630, 510, 480; 750, 665, 645, 630, 510, 480; Entire spectrum 700-400 and 700-500; 750, 665, 645, 630, 480; 750, 665, 645, 630, 510, 480, 430; 750, 665, 645, 630; 665, 505; 662; 665; 750, 663; 750, 665, 645, 630, 510, 480, 435, 410; 700, 667, 550; 662 mμ filter, 1/2 b.w., 5-10 mμ.

13 *Extraction performed*
At sea; on shore; both.

D STANDARDIZATION

1 *Extinction coefficient employed for chlorophyll* a (1/gm cm)
(i) 102, ether, 662 mμ; 89, 90 per cent acetone, 665 mμ; 80.5, 90 per cent acetone, 667 mμ; 66.7, 90 per cent acetone, 665 mμ; 28.7 methanol, 662 mμ filter.
(ii) Source of chlorophyll *a* if standard curve is employed: Sandoz, Basle, Switzerland.

2 *Extinction coefficients employed for other pigments estimated*
(a) Reference: RICHARDS, F. A. WITH THOMPSON, T. G. 1952. *J. mar. Res.*, **11** : 152-72.
(b) Optical densities recorded but no extinction coefficients applied to data.
(c) Chlorophyll *b* 54.0, 90 per cent acetone, 645 mμ (1/gm cm).
Chlorophyll *c* 19.5, 90 per cent acetone, 630 mμ (1/gm cm).
Carotenoids 100 or 200 1/SPU. cm, 90 per cent acetone, 480 mμ depending on species.

E CALCULATION OF RESULTS

1 *Turbidity correction**
None; 750 mμ optical density subtracted from all other optical densities; same but 480 — (1.5 × 750) and 510 — (2 × 750); same but 480 — (3 × 750) and 510 — (2 × 750); 700 mμ correction.

2 *Correction made for other chlorophylls at wave-length for chlorophyll* a
(a) Reference: RICHARDS, F. A. WITH THOMPSON, T. G. 1952. *J. mar. Res.*, **11** : 152-72.
(b) No correction.
(c) Reference: SMITH, J. H. C.; BENITEZ, A. 1955. *Modern methods of plant analysis*, vol. IV, p. 158.

3 *Correction for degradation products of chlorophyll* a
(a) No correction.
(b) Reference: ZSCHEILE, F. P.; COMAR, C. L. 1941. *Bot. Gaz.*, **102** : 463-81.

4 *Precision of chlorophyll* a *determination**
1-25 mg/m³ ± 10 per cent; 0.05-0.4 mg/m³ ± 22 per cent; 0.8-2.2 mg/m³ ± 14 per cent; 0.8 mg/m³ ± 13 per cent; 0.01-11 mg/m³ ± 10 per cent; 0.02-20 mg/m³ ± 0.02 mg/m³; 0.1-10 mg/m³ ± 10 per cent.

F OTHER PIGMENTS AND METHODS

1 *Pigments determined other than chlorophyll* a
None; chlorophyll *b* and *c*, total plant and animal carotenoids.

2 *Use of a fluorometric technique for routine determinations*
(i) KALLE, K. 1951. *Deutsch. Hydrog. Zeit.*, **4** : 92-6.
(ii) YENTSCH, C. S.; MENZEL, D. W. (unpublished manuscript).

3 *Routine use of a method not involving extraction*
None.

II Comments on the organization of work described in the preceding report

Copies of the preceding report, together with Appendix I, were sent to a small number of marine laboratories to obtain comments and further suggestions on the organization of the work described in the report. The following laboratories contributed to the comments shown below.

Department of Oceanography, University of Washington, U.S.A. (Dr. G. Anderson and Dr. K. Banse).

Water Research Laboratory, Faculty of Science, Nagoya, Japan (Dr. Y. Saijo).

Department of Oceanography, Johns Hopkins University, U.S.A. (Dr. J. Carpenter).

National Institute of Oceanography, United Kingdom (Mr. R. Currie).

Institute of Oceanology, Academy of Sciences of the U.S.S.R., Bakhrashin St., Moscow, U.S.S.R. (Dr. O. J. Koblentz-Mishke).

GENERAL COMMENT

Persons commenting on the report were generally in favour of the approach which had been adopted. It was suggested that some variations in the use of apparatus are due to financial limitations or to the unavailability of certain equipment in some countries. It was also suggested that any final procedure which came out of these studies should be called a recommended procedure rather than a standard procedure.

Comments on specific items in the report are given below following the titles given in Section III of the report and in Appendix I. Where no comment was made on a section it has been omitted. A comment by a different individual on the same section is distinguished from the preceding comment by (i), (ii), etc.

A APPARATUS

1 *Spectrophotometers and colorimeters*
Add Pulfrich photometer.

3 *Type of filter for removing plankton from sea-water*
(i) Certain commercial filters may be too expensive and/or not available in all countries.
(ii) The effect of dissolved membrane filters on the solubility of chlorophyll should be investigated.
(iii) Centrifugation may be a better method for separating plankton in tropical waters (cf. method employed by Richards with Thompson).

4 *Approximate suction pressure*
(i) With some filters, the suction pressure to which the plankton are exposed may be considerably less than that recorded by the pressure gauge. With some filters it is also important to note that there is an upper and lower surface which are not the same.
(ii) The speed of filtering may be an easier variable to standardize than the suction pressure which is not always directly measurable.

5 *Sonification and grinding apparatus*
(i) This is one of the most urgent problems to be studied.
(ii) We can strongly recommend sonification treatment in extraction. Complete extraction of every sample can be obtained with the treatment by sonification. In my laboratory all samples are treated by sonification.

B REAGENTS

1 *Solvent with which cells are extracted*
The possible use of methanol and the determination of specific absorption coefficients in methanol is an urgent problem.

2 *Addition of basic material during extraction*
According to our experiments $MgCO_3$ powder adsorbs a small amount of pigment which depends on the amount of $MgCO_3$ added. $BaCO_3$ (and powdered glass) do not adsorb any pigment.

C PROCEDURE

1 *Range of sea-water volumes filtered (litres)*
Some investigation is needed of the maxi-

mum permissible volume to be filtered. The concentration of chlorophyll decreases if the volume filtered exceeds 5 litres.

4 *Steam treatment of filters*
(i) The optical density of methanol extracts declines rapidly (of the order of $^1/_2$ in one week, stoppered glass in the refrigerator). Extracts of steamed plankton have been found to have the same extinction after a fortnight (Gessner, 1944, *Arch. Hydrobiol.*, **40** (3): 691). Steaming may be unnecessary if a short extraction period is employed.
(ii) Absorption spectra of pigments extracted from plankton change after treatment of concentrated plankton samples with steam.

5 *Storage of filtered samples*
(i) A maximum storage period of three months seems to be too long. In the International Indian Ocean Expedition we found lower values of some samples which were stored about three months. Therefore, it is necessary to store the filtered samples in a dark, dry and cool place (in refrigerator) and analyse within one or two months.
(ii) There is an initial noticeable decrease in pigment concentration on storage of filters which does not continue appreciably during storage. Too much attention to this point, however, might lead to a recognition that storage of filtered samples was wrong which would be an undesirable conclusion for many oceanographers.

7 *Length of extraction time*
Sentence in text starting 'It is possible...' is incorrect according to Laessøe and Hansen (1961).

11 *Blank employed for 0 optical density or 100 per cent transmission*
In determining the blank of the filter material plus solvent, the filter employed should be one *through which prefiltered sea-water has passed.*

12 *Wave-lengths at which measurements are made or filter band-widths* (mμ)
People working in estuaries may well encounter chlorophyll *b*. Therefore, *oceanic* should be underlined in the sixth line of the text.

D STANDARDIZATION

1 *Extinction coefficient employed for chlorophyll* a (1/g cm)
(i) Fundamental knowledge of the specific extinction coefficient in various solvents is required.
(ii) When the equations of Richards with Thompson, based on the values given by Zscheile and Comar, are used, chlorophyll *a* concentration data are about 1.5 times in excess. It is more appropriate to use Smith and Benitez values or those of Wettstein (*Exper. Cell Res.*, **12**, 1957) which are close enough to those of Smith and Benitez.

E CALCULATION OF RESULTS

1 *Turbidity correction*
Turbidity corrections are essential since in their absence errors of several hundred per cent can often be made.

4 *Precision of chlorophyll* a *determination*
(i) The table quoted in this section requires investigation with regard to the actual precision finally obtained for a recommended procedure.
(ii) Almost all the chlorophyll *a* values which we obtained in the Indian Ocean were in the range of 0.01 to 0.1 mg/m^3 and some distinct stratifications were observed. As can be seen from these results (results were shown for three Indian Ocean stations, all values less than 0.1 mg/m^3), if we report all of the chlorophyll *a* values of less than 0.1 mg/m^3 as < 0.1 mg/m^3, all of the vertical and horizontal changes in chlorophyll *a* will be erased (details of extraction volume, etc. given). From these values the deviation in chlorophyll determination is approximately $\pm$ 10 per cent at 0.1 mg/m^3, $\pm$ 20 per cent at 0.05 mg/m^3 and $\pm$ 50 per cent at 0.01 mg/m^3. Even if we permit these deviations in the chlorophyll values described above, we can recognize the vertical and horizontal change of chlorophyll *a*.

F OTHER PIGMENTS AND METHODS

1 *Pigments determined other than chlorophyll* a
The measurements of chlorophylls *b*, *c* and carotenoids is a quite interesting problem

in the biological study of the oceans. However, our experience shows that the values obtained by the Richards with Thompson method on these pigments are quite unreliable. Therefore, it will be better to restrict our pigment analysis to chlorophyll *a* until we have a new, reliable technique for the analysis of pigments other than chlorophyll *a*.

2 *Use of a fluorometric technique for routine determinations*

(i) An intercalibration of an absorption and fluorometric technique is shown in Krey (1958). The problem with a fluorometric method is standardization (see Rodhe *et al.* (1958). In: Buzzati Traverso (ed.) *Perspectives in marine biology*, p. 299-322, Univ. California Press).

(ii) When we consider the very small amount of chlorophyll *a* in the oceans and the precision of the usually employed spectrophotometric techniques for chlorophyll *a* determination, we should have a more sensitive technique for our routine study. Therefore we will adopt the fluorometric technique immediately if that technique is recognized as reliable and convenient for field study.

ADDITIONAL REFERENCES

VINBERG, G. G. *Primary production of the water basins*, Minsk, 1960. (In Professor Doty's laboratory in the University of Hawaii there is a full translation into English of this book.)

VINBERG, G. G.; SIVKO, T. N.; KOVALEVSKAIA, R. Z. Methods of chlorophyll concentration determination in plankton and some results of their application. In: Collection of papers, *Primary production of seas and inland waters*, p. 231-40. Minsk, 1961.

3

Comparison of the techniques used in the determination of phytoplankton pigments

G. F. Humphrey, and M. Wootton

Division of Fisheries and
Oceanography, CSIRO,
Sydney, Australia
(Reprint No. 584)

Manuscript received 15 June 1965

Summary

Several steps in the various modifications of the Richards-Thompson trichromatic spectrophotometric method for chlorophylls were compared. Cellulose-ester filters were better than paper or glass, because of their speed, retention, and solubility. Irrespective of the type of filter used, it was found that a layer of $MgCO_3$ added before filtration gave faster and more effective retention. High suction pressure had no harmful effect. Filters could be extracted damp or after storage and were ground to give maximal chlorophyll values. Turbid extracts could be used if the extinction at 750 mμ was subtracted from the other values. For the estimation of chlorophylls *a*, *b*, and *c*, in the ranges of concentration found in sea-water, coefficients of variation of 15, 40 and 40 per cent were obtained.

Acknowledgements

The authors' thanks are due to Mr. J. D. Kerr, CSIRO Division of Mathematical Statistics, for making the statistical analyses and to the Office of Oceanography, Unesco, Paris, for obtaining samples of the different filters used.

I Introduction

In 1952, Richards with Thompson published the details of a trichromatic spectrophotometric method for determining chlorophylls *a*, *b*, and *c*, and carotenoids in 90 per cent acetone extracts of the suspended material (largely phytoplankton) which could be centrifuged out of sea-water. In 1955, Creitz and Richards introduced the use of Millipore, cellulose-ester, filters for collecting the suspended material before extraction.

A number of modifications of the method are used in oceanographical laboratories and it is not known whether results obtained by these different techniques are comparable. Before the results obtained by the various laboratories can be compared or used together it is necessary to check each step in the procedure for which different modifications are used. These steps include type of filter, filtering pressure, conditions of filter storage, extraction procedure, and turbidity correction. In addition, it is necessary to know the precision of the results obtained.

In the present paper, results of tests of these modifications are given. No tests were made to discover which are the best equations or extinction coefficients to use for calculating amounts of chlorophylls.

II Methods

The analytical method was based on that of Richards with Thompson (1952). As modified it consisted of the following steps. A Millipore, cellulose-ester filter (HA, 47 mm, white, plain) was placed in a plastic holder (Humphrey, 1960) and covered with about 0.1 g $MgCO_3$. About 100 ml filtered sea-water was added and sucked through so as to distribute the $MgCO_3$ over the filter. Then the experimental material was added, either in one lot or over several minutes, applying a suction pressure of 20 in. The pressure was kept at 20 in or below by a suction, relief, valve. After filtration, the sides of the plastic holder were washed with filtered sea-water. The filter was then placed in a nylon or polythene centrifuge tube and 10 ml 90 per cent acetone added. The mixture was stirred and kept overnight in the dark in a closed container, at room temperature. After centrifuging for 10 min at 4,300 *g* in a swing-out centrifuge, the supernatant

was decanted into a 15-ml graduated tube. If the residue was still pigmented, a further extraction was made for a few minutes with 4 ml 90 per cent acetone. If the extract was turbid, 100 per cent acetone was added until a clear solution was obtained. The volume was then read to 0.1 ml and the extinction determined at 750, 663, 645 and 630 mμ in a 4-cm cell in a Unicam SP 600 spectrophotometer. Chlorophylls were calculated by the equations developed by Humphrey and Jeffrey (1965); two-component equations were used for the algal cultures because they contained only chlorophylls *a* and *b*, or *a* and *c*.

The method given above was used in all the tests. Changes made for the purpose of a particular comparison are given in the relevant section.

Algae were grown in the soil medium previously described (Humphrey, 1963). Diatom 4 (D4) was similar to *Nitzschia*, and measured 100 to 120 μ total length by 4 to 8 μ width. Phytoplankton were obtained from surface sea-water samples taken near the laboratory jetty in Port Hacking.

Irrespective of the amount of algal culture or sea-water sample used, concentrations were calculated as if the chlorophyll obtained had come from a volume of 5 litres. In this way, it was possible to emphasize that the amounts of chlorophyll handled were within the range found when analysing samples of ocean water or sea-water from the continental shelf, i.e. concentrations less than 5 μg/l (usually less than 1 μg/l).

When filters were ground, a glass homogenizer consisting of about 3 in of $^3/_8$ in internal diameter Pyrex tube fused onto 3 to 4 in of $^3/_4$ in tube was used. The smaller tube was sealed and rounded off, and a glass pestle ground to fit closely. The pestle was used at 500 r.p.m. for 1 min after adding 3 ml 90 per cent acetone. The homogenizer was washed twice with 4 ml 90 per cent acetone and the combined suspension kept 10 to 15 min in the dark before centrifugation.

III Results

A FILTERS

Filtration, rather than centrifugation, is usually used to concentrate the phytoplankton for analysis. Filters commonly used are Millipore cellulose-ester type HA, Schleicher & Schüll paper no. 1575 (S & S 1575) and Whatman glass-fibre grade GF/C. Table 1 shows the results of a comparison of these three filters and four types of Whatman papers often used in general laboratory work. The HA filter was used with a Millipore backing pad in the filter holder; the other filters were backed with an HA filter which was analysed separately. There was so little material on the backing HA filters that only chlorophyll *a* could be determined on them.

In this and similar experiments, Whatman 3 and GF/C filters were very fast, needing only a few minutes to filter 5 litres. Whatman 5, 32, and 42, and Millipore HA needed about 30 min S & S 1575 (pore size 1.5 μ) was very slow, needing 1 to 2 hours or clogging completely after 2 or 3 litres. Only with S & S 1575 did the total

chlorophyll *a* equal that recovered by a single HA. This suggests that there was incomplete extraction from the other filters or that these filters disintegrated the algae, thus allowing their contents to pass through.

TABLE 1. Comparison of paper, glass, and Millipore filters[1]

Filter	Chlorophyll (μg/l)				
	a	*b*	*c*	*a*[2]	Total *a*
Whatman 3	1.68	0.32	0.72	0.15	1.83
Whatman 5	1.89	0.17	0.52	0.16	2.05
Whatman 32	1.86	0.36	0.92	0.08	1.94
Whatman 42	1.76	0.23	0.58	0.10	1.86
Whatman GF/C	1.79	0.24	0.86	0.11	1.90
S & S 1575	2.02	0.23	0.67	0.16	2.18
Millipore HA	2.24	0.32	0.95	—	2.24

1 Sea-water was filtered through the filters covered with $MgCO_3$.
2 On backing HA filter.

TABLE 2. Comparison of S & S, GF/C, and HA filters[1]

Filter	*Gymnodinium*				*Diatom 4*			
	— $MgCO_3$		+ $MgCO_3$		— $MgCO_3$		+ $MgCO_3$	
	Chlorophyll		Chlorophyll		Chlorophyll		Chlorophyll	
	a	*c*	*a*	*c*	*a*	*c*	*a*	*c*
S & S	1.04	0.56	2.00	0.82	0.74	0.36	1.56	0.59
GF/C	1.66	1.06	1.56	1.10	0.82	0.44	1.00	0.56
HA	2.08	1.18	2.02	1.04	1.44	0.54	1.44	0.67

1 The concentration of the chlorophylls is in μg/l.

These questions were not investigated, but the three filters commonly used in marine work were tested for their ability to retain the delicate *Gymnodinium* and the hardy diatom, D4 (Table 2). Again HA was the best in speed and efficiency; $MgCO_3$ increased its speed slightly but not its efficiency. $MgCO_3$ raised the efficiency of S & S 1575 to that of HA but the speed was still far less. GF/C was the fastest but also the least efficient.

In early packings of Millipore filters, upper and lower surfaces were distinguished but this is no longer done. Table 3 shows that it is not necessary to distinguish these surfaces, similar results being obtained irrespective of the direction of filtration.

Although the most commonly used Millipore filter is HA (pore size 0.45 μ) other Millipore types have been used (RA, 1.2 μ; AA, 0.8 μ; DA, 0.65 μ; PH, 0.3 μ). These five types were compared using sea-water and algal cultures without finding any clear difference in retention; the larger the pores the greater was the speed, the RA being about four times as fast as PH. Table 4 shows one of the series of results obtained.

In the next group of experiments, filters used in different laboratories were compared to HA. Table 5 shows no significant difference between No. 5 Soviet cellulose-ester filters and HA. Because the Soviet filters are only 35-mm diameter

the HA filters were cut to this size before use. Filtering speeds were similar. Table 6 shows the results of a similar comparison between HA and Group 1 (= MF 100, pore size 0.8 μ) cellulose-ester filters from Membranfilter Gesellschaft, Gottingen. There was no consistent difference in retention or speed. This company also supplies an insoluble filter (the Cellafilter); type Grob with pore size 0.5-3.0 μ was used. It is distributed moist, in plastic wrapping and is slower than HA if $MgCO_3$ is not used. The results in Table 7 show that it has slightly higher retention than HA.

The Polypore cellulose-ester filter, type AM-6, pore size 0.45 μ, from Gelman Co., Michigan, is similar in design to HA. There was no consistent difference in speed or retention between them (Table 8).

The only cellulose filter used routinely for phytoplankton pigment determination is Albet no. 242 Papel de Filtro. Table 9 shows that it was equal in efficiency to HA with sea-water but that it was less efficient with the algae used.

B FILTERING PRESSURE

To get adequate filtering speed it is necessary to apply suction but there is always the possibility that organisms break under high, prolonged suction and then pass through the filter. In an attempt to demonstrate such an effect, Millipore filters were used at full suction (29 in) and at the usual 20 in. No difference was found even with a fragile organism such as *Gymnodinium* (Table 10).

With the very fast glass filters it is possible to use suction as low as 5 in (Ryther, personal communication). The use of such gentle suction does not improve the retention by GF/C; they are still at least 15 per cent less efficient than HA at 20 in (Table 11).

C FILTER STORAGE

The results in Tables 12, 13 and 14 show the effects of storing filters in the dark, over silica gel at 10°, 1° and — 10°C before analysis. At each temperature there is a 10 per cent or larger decrease after one day; this effect might be related more to the process of drying than to storage.

D EXTRACTION

Yentsch and Menzel (1963) used glass-fibre filters (Whatman GF/C and Gelman Type A) and stated that grinding 'facilitates immediate extraction'. Table 15 shows that grinding gives values higher than the usual method of allowing to stand overnight. This effect is partly due to the fact that grinding allows the determination to be made in less than an hour. If the suspensions are kept overnight after grinding, the values are reduced (Table 16).

E TURBIDITY OF EXTRACTS

The acetone extract is often slightly turbid after centrifuging off the cell debris, $MgCO_3$, and filter debris. In most cases, the turbidity can be removed by adding 90 or 100 per cent acetone. If the turbidity persists, the extinction at 750 mμ should be measured and subtracted from the extinctions at the other wave-lengths. The results in Table 17 show that this correction made discordant quadruplicates agree for chlorophyll *a* and *b*; the effect with chlorophyll *c* was variable. The turbidities in these quadruplicates were not produced by varying the steps in the method; they were otherwise normal estimations, but turbidity persisted even after adding extra acetone.

F PRECISION

The results in Tables 12, 13 and 14 were combined to calculate the standard deviation of the method. For chlorophyll *a* the SD, was 0.06; for chlorophyll *b*, 0.03; and for chlorophyll *c*, 0.08 μg/l. The corresponding coefficients of variation were 15, 40 and 40 per cent.

IV Discussion

A FILTERS

The results in Tables 1-9 show that with the exception of glass-fibre filters, all filters commonly used for determining chlorophyll give results within ± 20 per cent of each other for phytoplankton and for most of the cultures used, provided that $MgCO_3$ is added before filtration. The glass-fibre filters, both Whatman GF/C and Gelman type A, consistently gave low results (Tables 1, 3, 11, and unpublished), sometimes equal to only half those obtained with cellulose-ester filters. Because they filter quickly and dissolve in 90 per cent acetone, the cellulose-ester filters are easier to use than the other types which have equal retention.

Because $MgCO_3$ usually increases the speed and retention of many filters (Tables 2, 6-8, and 15-16) it should be added in sufficient quantity to cover the filter and before commencing filtration, not after filtration as is recommended by Strickland and Parsons (1960).

The results in Table 4 showing that the grades of Millipore filters with pore sizes 0.30, 0.45 and 1.2 μ have equal retention do not contradict those obtained by Lasker and Holmes (1957), Holmes (1958), Holmes and Anderson (1963) and Saijo (1964). These authors investigated the retention of particulate matter in sea-water and algal cultures, by nets and cellulose-ester filters after the sea-water or cultures had been incubated in artificial light for several hours with added radioactive bicarbonate; they did not use $MgCO_3$ on the filters. Holmes and his colleagues concluded that 0.30 μ filters retained more radioactivity than 0.45 μ

filters and these retained more than 0.80 μ filters, the differences in retention possibly being due to 'radioactive fragments and/or exudates resulting from mechanical fragmentation of cells during filtration' (Holmes and Anderson, 1963). Because the effect was not obtained with bacteria-free cultures (Lasker and Holmes, 1957) it is possible that the fragments or exudates are formed by bacterial action during incubation rather than during filtration or that the cells during incubation are brought into a condition causing such formation during filtration.

There is no evidence that similar fragments or exudates are formed when sea-water or algal cultures are filtered without such incubation. If they are formed, they do not contain chlorophyll (Table 4).

The experiments of Saijo (1964) showed that 0.45 μ filters were no better than 0.80 μ filters when samples of oceanic water from 50 m. were used, but that with surface samples, up to 37 per cent of the retainable radioactivity passed through the 0.80 μ filter. The difference in reaction of the surface and deep samples might have been due to the presence of different organisms or to the fact that the organisms were adapted to different light intensities before the incubation for 4 hours at 1,500 ft c.

B FILTERING PRESSURE

NASCO (1964) recommended, without quoting evidence, that 'pressure reduction should not exceed 50 cm' (20 in). Although 29 in is seldom if ever necessary, no evidence of harmful effects could be found (Table 10).

C FILTER STORAGE

Creitz and Richards (1955) stated that filters could be stored for three weeks in a desiccator in the refrigerator without loss of pigment. Strickland and Parsons (1960) stated that filters could be stored for up to six weeks over silica gel provided the temperature was — 20°C or less.

Tables 12, 13 and 14 give the losses sustained at temperatures corresponding to coolroom (10°C), refrigerator (1°C), and deep-freeze (— 10°C) storage and show that 1° and — 10°C are adequate for several weeks. They also show, contrary to the unsubstantiated statement 'filters must be thoroughly desiccated prior to extraction' (NASCO 1964), that highest results are obtained by extracting the damp, fresh filter.

D EXTRACTION

Richards with Thompson (1955) recommended at least 9 hours for extraction and stated that grinding was ineffective. For convenience, overnight extraction is used, either at room temperature (Humphrey, 1960) or in the refrigerator (Strickland and Parsons, 1960). There is no evidence for the statement 'During extraction, samples must be kept refrigerated' (NASCO, 1964). The technique of Yentsch and Menzel (1963) makes these questions unimportant because it

is now clear (Table 15) that mechanical grinding gives results higher than simple extraction. Hand grinding (with or without glass powder) was ineffective (unpublished work).

E TURBIDITY OF EXTRACTS

Creitz and Richards (1955) showed that Millipore AA and HA filters absorbed slightly at 750 mμ and lower wave-lengths, e.g. a 47 mm HA filter in 5 ml 90 per cent acetone had an extinction of 0.001 per centimetre light path at 750, 665, 645 and 630 mμ. They showed that filters had no significant effect on the absorption curves of phytoplankton extracts.

In the analyses reported by Holmes (1958) 'Turbidity corrections were made on the basis of the sample transmission at 750 mμ'. Presumably this means that the 750 mμ extinctions were subtracted; Strickland and Parsons (1960) make such a recommendation. Table 17 shows that such a correction is effective for chlorophylls *a* and *b* but not for chlorophyll *c*.

F PRECISION

Richards with Thompson (1952) gave the errors of chlorophyll *a* and *c* determinations as ± 14 and ± 43 per cent. Parsons and Strickland gave ± 0.26 μg for a single determination of 5 μg chlorophyll *a*, ± 0.21 μg for 0.5 μg chlorophyll *b*, and ± 0.16 μg for 1.5 μg chlorophyll *c*.

The values of the coefficient of variation in Section III.F of this paper are almost the same as the errors given by Richards with Thompson (1952). The accuracy of the method cannot be stated; the absolute amounts of the chlorophylls in algae or phytoplankton are unknown and the recovery of known, added amounts of chlorophylls cannot be accurately studied. Because of these considerations, the procedure giving the highest value is taken as giving the best value. The analytical errors of determination are probably small compared to errors in sampling phytoplankton in the sea.

V Conclusions

Cellulose-ester filters are preferable because of their speed, retention and solubility. The pore-size should be chosen according to the type of material to be filtered. Cella filters give slightly higher results but are less convenient. With all filters, $MgCO_3$ should be added before filtration. Any convenient suction pressure can be used. Although it is best to extract the damp filter immediately after filtration, filters can be stored for several weeks at 1°C or less. Grinding should always be used. Turbidity in the extracts should be reduced as much as possible; 750 mμ extinctions should be subtracted from those at other wave-lengths.

In combining results obtained by different methods, it is not necessary to reject results from paper filters. Results from glass filters should be rejected if other results are available; if not, 25 per cent should be added.

In addition to the tests made on various parts of the method, there still remains the question of the validity of the various extinction coefficients and equations proposed by different authors.

TABLE 3. Filtering surface of Millipore HA[1]

Filter surface	Chlorophyll		
	a	*b*	*c*
Upper	0.15	0.05	0.10
	0.12	0.04	0.07
	0.13	0.03	0.06
	0.11	0.04	0.08
	0.13	*0.04*	*0.08*
Lower	0.11	0.05	0.08
	0.13	0.04	0.10
	0.13	0.05	0.11
	0.12	0.09	0.21
	0.12	*0.06*	*0.13*

1 The filter surface near the lid of the box was called the upper surface. Quadruplicate estimations were done on sea-water. The values printed in italics are means (µg/l).

TABLE 4. Comparison of PH, HA, and RA Millipore filters[1]

PH (0.3 µ)			HA (0.45 µ)			RA (1.2 µ)		
Chlorophyll			Chlorophyll			Chlorophyll		
a	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
			Gymnodinium					
0.29		0.21	0.28		0.19	0.27		0.22
0.28		0.22	0.28		0.19	0.27		0.23
0.29		0.21	0.30		0.28	0.29		0.23
0.29		*0.21*	*0.29*		*0.22*	*0.28*		*0.23*
			Nannochloris					
0.17	0.03		0.22	0.05		0.21	0.05	
0.19	0.07		0.21	0.02		0.21	0.02	
0.19	0.02		0.21	0.05		0.22	0.02	
0.18	*0.04*		*0.21*	*0.04*		*0.21*	*0.03*	
			Sea-water					
0.33	0.05	0.20	0.31	0.03	0.13	0.32	0.07	0.22
0.33	0.05	0.17	0.33	0.10	0.22	0.32	0.05	0.16
0.33	0.04	0.16	0.33	0.04	0.21	0.34	0.05	0.24
0.33	*0.05*	*0.18*	*0.32*	*0.06*	*0.19*	*0.33*	*0.06*	*0.21*

1 Triplicate estimations using $MgCO_3$. Values printed in italics are means (µg/l).

TABLE 5. Comparison of Soviet No. 5 and Millipore HA cellulose ester filters[1]

$MgCO_3$	HA Chlorophyll *a*	HA Chlorophyll *b*	HA Chlorophyll *c*	Soviet Chlorophyll *a*	Soviet Chlorophyll *b*	Soviet Chlorophyll *c*
			Nannochloris			
—	1.92	0.79		1.58	0.69	
	1.60	0.70		1.61	0.74	
	1.56	0.72		1.59	0.67	
	1.63	0.74		1.57	0.69	
	1.68	*0.75*		*1.59*	*0.70*	
+	2.09	0.68		1.74	0.61	
	2.09	0.66		1.50	0.59	
	1.61	0.77		1.62	0.61	
	1.79	0.64		1.58	0.65	
	1.89	*0.69*		*1.61*	*0.62*	
			Dunaliella			
—	0.44	0.23		0.41	0.22	
	0.42	0.22		0.42	0.22	
	0.46	0.22		0.41	0.24	
	0.41	0.20		0.39	0.21	
	0.43	*0.22*		*0.41*	*0.22*	
+	0.54	0.22		0.49	0.23	
	0.51	0.22		0.44	0.20	
	0.52	0.24		0.46	0.19	
	0.54	0.21		0.44	0.25	
	0.53	*0.22*		*0.46*	*0.22*	
			Sea-water			
—	0.10	0.02	0.00	0.15	0.06	0.14
	0.12	0.04	0.07	0.11	0.04	0.09
	0.12	0.05	0.07	0.13	0.07	0.08
	0.12	0.05	0.09	0.09	0.05	0.06
	0.12	*0.04*	*0.06*	*0.12*	*0.06*	*0.09*
+	0.15	0.07	0.11	0.11	0.02	0.03
	0.15	0.05	0.07	0.11	0.06	0.15
	0.12	0.06	0.14	0.12	0.04	0.07
	0.11	0.04	0.09	0.09	0.04	0.09
	0.13	*0.06*	*0.10*	*0.10*	*0.04*	*0.09*
			Gymnodinium			
—	0.32		0.21	0.35		0.23
	0.31		0.26	0.33		0.22
	0.32		0.29	0.33		0.20
	0.33		0.28	0.33		0.26
	0.32		*0.26*	*0.34*		*0.23*
+	0.33		0.28	0.32		0.32
	0.30		0.19	0.33		0.26
	0.34		0.36	0.33		0.20
	0.33		0.30	0.31		0.21
	0.33		*0.28*	*0.32*		*0.25*

TABLE 5 *(continued)*

$MgCO_3$	HA			Soviet		
	Chlorophyll			Chlorophyll		
	a	*b*	*c*	*a*	*b*	*c*
			Nitzschia			
—	0.41		0.13	0.39		0.18
	0.41		0.11	0.34		0.15
	0.35		0.11	0.38		0.13
	0.36		0.13	0.37		0.18
	0.38		*0.12*	*0.37*		*0.16*
+	0.36		0.13	0.36		0.18
	0.38		0.21	0.38		0.17
	0.39		0.20	0.35		0.14
	0.39		0.13	0.36		0.22
	0.38		*0.17*	*0.36*		*0.18*
			Skeletonema			
—	0.74		0.37	0.73		0.32
	0.73		0.34	0.73		0.40
	0.75		0.35	0.75		0.43
	0.74		0.34	0.69		0.39
	0.74		*0.35*	*0.72*		*0.39*
+	0.80		0.28	0.74		0.41
	0.78		0.31	0.70		0.31
	0.78		0.31	0.72		0.28
	0.80		0.38	0.72		0.33
	0.79		*0.32*	*0.72*		*0.33*

1 The values printed in italics are means (µg/l).

TABLE 6. Comparison of Membran MF 100 and Millipore HA filters[1]

$MgCO_3$	HA			Membran		
	Chlorophyll			Chlorophyll		
	a	*b*	*c*	*a*	*b*	*c*
			Dunaliella			
—	0.47	0.16		0.53	0.18	
	0.42	0.17		0.44	0.17	
	0.48	0.19		0.48	0.18	
	0.48	0.18		0.51	0.19	
	0.46	*0.18*		*0.49*	*0.18*	
+	0.62	0.19		0.65	0.20	
	0.62	0.21		0.56	0.17	
	0.61	0.20		0.57	0.18	
	0.58	0.21		0.58	0.16	
	0.61	*0.20*		*0.59*	*0.18*	
			Skeletonema			
—	1.18		0.73	1.44		0.90
	1.28		0.82	1.48		0.76
	1.44		0.82	1.38		0.71
	1.43		0.61	1.35		0.77
	1.33		*0.75*	*1.41*		*0.79*
+	1.84		0.90	1.67		0.80
	1.76		0.95	1.88		1.01
	1.85		0.84	1.85		0.97
	1.82		0.91	1.87		0.96
	1.82		*0.90*	*1.82*		*0.94*
			Sea-water			
—	0.45	0.03	0.19	0.40	0.09	0.28
	0.52	0.05	0.25	0.33	0.09	0.30
	0.34	0.03	0.16	0.43	0.08	0.29
	0.50	0.09	0.21	0.39	0.04	0.20
	0.45	*0.05*	*0.20*	*0.39*	*0.08*	*0.27*
+	0.50	0.11	0.18	0.37	0.07	0.27
	0.51	0.03	0.17	0.40	0.09	0.28
	0.55	0.01	0.13	0.48	0.07	0.24
	0.55	0.07	0.22	0.47	0.08	0.26
	0.53	*0.06*	*0.18*	*0.43*	*0.08*	*0.26*

1 The values printed in italics are means (μg/l).

TABLE 7. Comparison of Cella Grob and Millipore HA filters[1]

$MgCO_3$	HA			Cella		
	Chlorophyll			Chlorophyll		
	a	*b*	*c*	*a*	*b*	*c*
			Dunaliella			
—	0.66	0.20		0.91	0.26	
	0.70	0.22		0.87	0.25	
	0.73	0.23		0.89	0.29	
	0.70	0.26		0.84	0.26	
	0.70	*0.23*		*0.88*	*0.27*	
+	0.65	0.20		0.86	0.26	
	0.71	0.23		0.87	0.28	
	0.62	0.19		0.83	0.28	
	0.68	0.21		0.83	0.30	
	0.67	*0.21*		*0.85*	*0.26*	
			Skeletonema			
—	0.50		0.20	0.37		0.22
	0.43		0.14	0.35		0.19
	0.50		0.15	0.32		0.17
	0.52		0.12	0.36		0.25
	0.49		*0.15*	*0.35*		*0.21*
+	0.47		0.25	0.46		0.15
	0.46		0.22	0.49		0.26
	0.50		0.17	0.49		0.24
	0.47		0.19	0.49		0.19
	0.48		*0.21*	*0.48*		*0.21*
			Sea-water			
—	0.45	0.03	0.19	0.63	0.15	0.35
	0.52	0.05	0.25	0.65	0.22	0.44
	0.34	0.03	0.16	0.59	0.14	0.34
	0.50	0.09	0.21	0.60	0.14	0.21
	0.45	*0.05*	*0.20*	*0.62*	*0.15*	*0.33*
+	0.50	0.11	0.18	0.62	0.01	0.03
	0.51	0.03	0.17	0.61	0.05	0.14
	0.55	0.01	0.13	0.60	0.06	0.08
	0.55	0.07	0.22	0.59	0.00	0.02
	0.53	*0.06*	*0.18*	*0.61*	*0.03*	*0.07*

1 The values printed in italics are means (µg/l).

TABLE 8. Comparison of Polypore AM-6 and Millipore HA filters[1]

$MgCO_3$	HA			Polypore		
	Chlorophyll			Chlorophyll		
	a	*b*	*c*	*a*	*b*	*c*
			Dunaliella			
—	0.66	0.20		0.63	0.24	
	0.70	0.22		0.65	0.26	
	0.73	0.23		0.69	0.22	
	0.70	0.26		0.79	0.24	
	0.70	*0.23*		*0.69*	*0.24*	
+	0.65	0.20		0.69	0.22	
	0.71	0.23		0.74	0.23	
	0.62	0.19		0.77	0.28	
	0.68	0.21		0.83	0.26	
	0.67	*0.21*		*0.76*	*0.25*	
			Skeletonema			
—	0.50		0.20	0.49		0.26
	0.43		0.14	0.43		0.22
	0.50		0.15	0.49		0.36
	0.52		0.12	0.47		0.39
	0.49		*0.15*	*0.47*		*0.31*
+	0.47		0.25	0.43		0.13
	0.46		0.22	0.56		0.23
	0.50		0.17	0.47		0.21
	0.47		0.19	0.43		0.20
	0.48		*0.21*	*0.47*		*0.19*
			Sea-water			
—	0.45	0.03	0.19	0.48	0.06	0.24
	0.52	0.05	0.25	0.49	0.04	0.22
	0.34	0.03	0.16	0.56	0.09	0.18
	0.50	0.09	0.21	0.39	0.08	0.09
	0.45	*0.05*	*0.20*	*0.48*	*0.07*	*0.18*
+	0.50	0.11	0.18	0.50	0.05	0.24
	0.51	0.03	0.17	0.52	0.02	0.17
	0.55	0.01	0.13	0.49	0.08	0.27
	0.55	0.07	0.22	0.59	0.04	0.23
	0.53	*0.06*	*0.18*	*0.53*	*0.05*	*0.23*

1 The values printed in italics are means (μg/l).

TABLE 9. Comparison of Albet 242 and Millipore HA filters[1]

$MgCO_3$	HA			Albet		
	Chlorophyll			Chlorophyll		
	a	*b*	*c*	*a*	*b*	*c*
			Dunaliella			
—	0.47	0.16		0.18	0.08	
	0.42	0.17		0.20	0.08	
	0.48	0.19		0.14	0.04	
	0.48	0.18		0.17	0.07	
	0.46	*0.18*		*0.17*	*0.07*	
+	0.62	0.19		0.27	0.13	
	0.62	0.21		0.29	0.15	
	0.61	0.20		0.29	0.08	
	0.58	0.21		0.29	0.09	
	0.61	*0.20*		*0.29*	*0.11*	
			Skeletonema			
—	0.50		0.20	0.16		0.19
	0.43		0.14	0.25		0.15
	0.50		0.15	0.28		0.23
	0.52		0.12	0.21		0.20
	0.49		*0.15*	*0.23*		*0.19*
+	0.47		0.25	0.38		0.15
	0.46		0.22	0.37		0.24
	0.50		0.17	0.41		0.23
	0.47		0.19	0.47		0.25
	0.48		*0.21*	*0.41*		*0.22*
			Sea-water			
—	0.45	0.03	0.19	0.45	0.09	0.15
	0.52	0.05	0.25	0.32	0.07	0.12
	0.34	0.03	0.16	0.42	0.12	0.13
	0.50	0.09	0.21	0.54	0.06	0.19
	0.45	*0.05*	*0.20*	*0.46*	*0.09*	*0.15*
+	0.50	0.11	0.18	0.52	0.08	0.31
	0.51	0.03	0.17	0.54	0.08	0.21
	0.55	0.01	0.13	0.51	0.03	0.15
	0.55	0.07	0.22	0.47	0.04	0.17
	0.53	*0.06*	*0.18*	*0.51*	*0.06*	*0.21*

1 The values printed in italics are means (µg/l).

TABLE 10. Suction pressure and Millipore filters[1]

Filter	20 in suction		29 in suction	
	Chlorophyll		Chlorophyll	
	a	*c*	*a*	*c*
AA	1.52	1.26	1.55	1.41
DA	1.38	1.58	1.42	1.04
HA	1.42	1.08	1.46	1.05
PH	1.43	1.48	1.46	1.21

1 *Gymnodinium* was filtered through filters covered with $MgCO_3$. Values are in µg/l.

TABLE 11. Suction pressure: Whatman GF/C and Millipore HA filters[1]

GF/C at 5 in			HA at 20 in		
Chlorophyll			Chlorophyll		
a	*b*	*c*	*a*	*b*	*c*
		Sea-water			
0.37	— 0.02	0.04	0.45	0.02	0.07
0.46	0.03	0.12	0.49	0.06	0.15
0.38	0.02	0.11	0.53	0.05	0.19
0.41	0.07	0.13	0.40	0.00	0.08
0.41	*0.03*	*0.10*	*0.47*	*0.03*	*0.12*
		Dunaliella			
2.76	0.77		3.19	1.00	
2.61	0.71		2.94	0.92	
2.49	0.67		2.84	0.89	
2.40	0.67		3.08	0.99	
2.57	*0.71*		*3.01*	*0.95*	
		Gymnodinium			
0.38		0.21	0.52		0.39
0.37		0.19	0.47		0.40
0.37		0.31	0.48		0.50
0.36		0.21	0.48		0.33
0.37		*0.23*	*0.49*		*0.41*
		Nannochloris			
0.20	0.05		0.24	0.08	
0.23	0.08		0.32	0.16	
0.26	0.08		0.34	0.13	
0.28	0.09		0.32	0.13	
0.24	*0.08*		*0.31*	*0.13*	
		Nitzschia			
0.25		0.03	0.42		0.14
0.23		0.03	0.40		0.17
0.25		0.02	0.40		0.18
0.24		0.06	0.39		0.16
0.24		*0.04*	*0.40*		*0.16*

1 The values printed in italics are means (µg/l). $MgCO_3$ was used.

TABLE 12. Filter storage at 10°C[1]

Days	Chlorophyll		
	a	*b*	*c*
0	0.65	0.08	0.37
	0.64	0.06	0.33
	0.62	0.04	0.36
	0.69	0.07	0.36
	0.65	*0.06*	*0.36*
1	0.49	0.05	0.22
	0.53	0.03	0.16
	0.56	0.07	0.24
	0.54	0.06	0.22
	0.53	*0.05*	*0.21*
7	0.53	0.02	0.18
	0.53	0.02	0.18
	0.55	0.05	0.30
	0.55	0.07	0.23
	0.54	*0.04*	*0.22*
14	0.49	0.03	0.26
	0.47	0.07	0.26
	0.50	0.05	0.03
	0.41	0.01	0.11
	0.47	*0.04*	*0.17*
21	0.43	0.04	0.14
	0.44	0.09	0.26
	0.45	0.05	0.14
	0.44	0.07	0.17
	0.44	*0.06*	*0.19*
28	0.42	0.02	0.24
	0.42	0.03	0.17
	0.46	0.05	0.27
	0.42	0.04	0.19
	0.43	*0.04*	*0.22*
42	0.40	0.11	0.36
	0.38	0.11	0.38
	0.39	0.08	0.26
	0.42	0.12	0.42
	0.40	*0.11*	*0.36*
56	0.42	0.12	0.35
	0.39	0.12	0.26
	0.37	0.12	0.16
	0.40	0.14	0.19
	0.40	*0.13*	*0.24*
84	0.38	0.11	0.27
	0.34	0.10	0.18
	0.40	0.12	0.29
	0.40	0.11	0.14
	0.38	*0.11*	*0.22*

1 110 litres sea-water was centrifuged at 5,000 *g* in a continuous centrifuge. The sediment was suspended in 15 litres sea-water and aerated overnight at 23°C and 400 f.c. Analyses were made in quadruplicate. Values printed in italics are means (µg/l).

TABLE 13. Filter storage at 1°C[1]

Days	Chlorophyll		
	a	*b*	*c*
0	0.34	0.10	0.26
	0.34	0.09	0.15
	0.33	0.09	0.24
	0.39	0.05	0.16
	0.35	*0.08*	*0.20*
1	0.31	0.09	0.22
	0.33	0.09	0.21
	0.29	0.16	0.12
	0.33	0.10	0.25
	0.32	*0.11*	*0.20*
7	0.33	0.07	0.13
	0.36	0.09	0.18
	0.39	0.10	0.21
	0.39	0.09	0.34
	0.37	*0.09*	*0.22*
14	0.35	0.08	0.15
	0.36	0.10	0.21
	0.35	0.09	0.16
	0.35	0.06	0.19
	0.35	*0.08*	*0.18*
28	0.32	0.07	0.24
	0.28	0.08	0.22
	0.31	0.07	0.22
	0.33	0.06	0.17
	0.31	*0.07*	*0.21*
56	0.33	0.12	0.23
	0.28	0.14	0.20
	0.28	0.11	0.24
	0.30	0.09	0.14
	0.30	*0.12*	*0.20*

1 64 litres sea-water was centrifuged at 5,000 *g* in a continuous centrifuge. The sediment was suspended in 10 litres sea-water. Analyses were made in quadruplicate. Values printed in italics are means (µg/l).

TABLE 14. Filter storage at — 10°C[1]

Days	Chlorophyll		
	a	*b*	*c*
0	0.31	0.06	0.27
	0.32	0.08	0.29
	0.29	0.07	0.23
	0.31	0.10	0.32
	0.31	*0.08*	*0.28*
1	0.30	0.06	0.26
	0.27	0.06	0.20
	0.28	0.05	0.18
	0.27	0.04	0.21
	0.28	*0.05*	*0.21*
7	0.30	0.06	0.17
	0.26	0.05	0.20
	0.28	0.06	0.20
	0.26	0.05	0.18
	0.28	*0.06*	*0.19*
14	0.30	0.06	0.18
	0.31	0.05	0.15
	0.29	0.06	0.13
	0.31	0.10	0.31
	0.30	*0.07*	*0.19*
28	0.28	0.08	0.19
	0.28	0.09	0.25
	0.28	0.06	0.21
	0.31	0.09	0.18
	0.29	*0.08*	*0.21*
56	0.26	0.08	0.18
	0.23	0.07	0.15
	0.26	0.07	0.20
	0.27	0.07	0.10
	0.26	*0.07*	*0.16*

1 64 litres sea-water was centrifuged at 5,000 *g* in a continuous centrifuge. The sediment was suspended in 10 litres sea-water. Analyses were made in quadruplicate. Values printed in italics are means (µg/l).

TABLE 15. Comparison of extraction procedures[1]

$MgCO_3$	Overnight extraction			Grinding		
	Chlorophyll			Chlorophyll		
	a	*b*	*c*	*a*	*b*	*c*
			Nannochloris			
—	0.27	0.11		0.34	0.13	
	0.24	0.07		0.32	0.13	
	0.35	0.15		0.36	0.17	
	0.32	0.12		0.36	0.15	
	0.27	*0.11*		*0.35*	*0.15*	
+	0.31	0.09		0.35	0.16	
	0.28	0.06		0.38	0.10	
	0.33	0.13		0.34	0.11	
	0.32	0.12		0.42	0.19	
	0.31	*0.10*		*0.37*	*0.14*	
			Dunaliella			
—	2.40	0.72		2.89	0.73	
	2.32	0.64		2.84	0.63	
	2.16	0.65		2.81	0.78	
	2.29	0.70		3.11	0.71	
	2.29	*0.68*		*2.91*	*0.71*	
+	2.17	0.64		2.90	0.80	
	2.09	0.65		2.76	0.80	
	2.14	0.63		2.83	0.82	
		lost		2.74	0.83	
	2.13	*0.64*		*2.81*	*0.81*	
			Skeletonema			
—	0.16		0.06	0.34		0.14
	0.17		0.10	0.32		0.13
	0.16		0.11	0.32		0.20
	0.17		0.15	0.36		0.13
	0.17		*0.11*	*0.34*		*0.15*
+	0.20		0.13	0.42		0.19
	0.19		0.11	0.40		0.17
	0.21		0.09	0.39		0.24
	0.15		0.02	0.39		0.31
	0.19		*0.09*	*0.40*		*0.23*
			Nitzschia			
—	0.63		0.20	0.93		0.29
	0.63		0.20	0.79		0.37
	0.62		0.29	0.77		0.20
	0.59		0.28	0.78		0.31
	0.62		*0.24*	*0.82*		*0.29*
+	1.03		0.46	1.59		0.66
	0.94		0.40	1.58		0.68
	1.02		0.52	1.53		0.57
	0.78		0.35	1.37		0.65
	0.94		*0.43*	*1.52*		*0.64*

TABLE 15 *(continued)*.

$MgCO_3$	Overnight extraction			Grinding		
	Chlorophyll			Chlorophyll		
	a	*b*	*c*	*a*	*b*	*c*
			Gymnodinium			
—	0.14		0.07	0.22		0.18
	0.12		0.06	0.23		0.14
	0.14		0.04	0.18		0.06
	0.14		0.01	0.18		0.13
	0.14		*0.05*	*0.20*		*0.13*
+	0.07		0.10	0.13		0.14
	0.07		0.08	0.10		0.05
	0.07		0.02	0.10		0.08
	0.08		0.02	0.09		0.01
	0.07		*0.06*	*0.11*		*0.07*

1 Gelman Type A glass filters were used at 5 in suction. Values printed in italics are means (µg/l).

TABLE 16. Effect of grinding and extraction time[1]

$MgCO_3$	Without grinding			With grinding					
	Extraction 18 hr			Extraction 10 min			Extraction 18 hr		
	Chlorophyll			Chlorophyll			Chlorophyll		
	a	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
				Dunaliella					
—	1.19	0.39		1.68	0.31		1.00	0.35	
	1.09	0.37		1.69	0.31		0.91	0.35	
	1.15	0.39		1.51	0.28		0.79	0.35	
	1.04	0.33		1.53	0.35		0.88	0.34	
	1.12	*0.37*		*1.60*	*0.31*		*0.90*	*0.35*	
+	1.21	0.36		1.50	0.38		0.88	0.35	
	1.19	0.34		1.59	0.52		0.89	0.37	
	1.03	0.33		1.50	0.44		0.98	0.36	
	1.06	0.32		1.45	0.38		0.79	0.30	
	1.12	*0.36*		*1.51*	*0.30*		*0.87*	*0.37*	
				Nitzschia					
—	0.59		0.30	0.72		0.31	0.56		0.18
	0.58		0.29	0.76		0.40	0.61		0.30
	0.56		0.20	0.73		0.33	0.55		0.19
	0.52		0.28	0.72		0.37	0.57		0.37
	0.56		*0.28*	*0.73*		*0.35*	*0.57*		*0.26*
+	0.54		0.32	0.70		0.39	0.56		0.41
	0.52		0.28	0.73		0.39	0.54		0.39
	0.52		0.37	0.71		0.40	0.56		0.26
	0.52		0.31	0.74		0.41	0.52		0.33
	0.53		*0.32*	*0.72*		*0.40*	*0.55*		*0.35*
				Sea-water					
—	0.61	0.05	0.07	0.70	0.00	0.08	0.67	0.02	0.03
	0.50	0.07	0.19	0.73	0.02	0.10	0.58	0.09	0.14
	0.50	0.06	0.11	0.60	0.04	0.08	0.46	0.11	0.13
	0.72	0.11	0.16	0.58	0.09	0.07	0.49	0.06	0.05
	0.58	*0.07*	*0.13*	*0.85*	*0.06*	*0.10*	*0.55*	*0.07*	*0.09*
+	0.67	0.14	0.37	0.65	0.07	0.08	0.74	0.06	0.12
	0.55	0.09	0.11	0.89	0.07	0.18	0.51	0.09	0.17
	0.49	0.08	0.10	0.74	0.08	0.13	0.53	0.07	0.17
	0.46	0.09	0.12	0.59	0.09	0.11	0.44	0.12	0.18
	0.54	*0.13*	*0.18*	*0.72*	*0.08*	*0.13*	*0.56*	*0.09*	*0.16*
— (*)	0.27	0.00	0.02	0.33	0.03	0.13	0.33	0.03	0.13
	0.28	0.00	0.00	0.36	0.00	0.11	0.34	0.00	0.02
	0.30	0.00	0.02	0.37	0.00	0.08	0.40	0.02	0.10
		lost		0.40	0.06	0.13	0.37	0.00	0.06
	0.29	*0.00*	*0.02*	*0.35*	*0.02*	*0.11*	*0.38*	*0.01*	*0.08*
+ (*)	1.58	0.06	0.23	1.74	0.00	0.16	1.67	0.11	0.23
	1.54	0.06	0.17	1.84	0.02	0.31	1.75	0.09	0.18
	1.50	0.10	0.20	1.76	0.00	0.13	1.68	0.00	0.28
	1.56	0.11	0.24	1.73	0.04	0.17	1.62	0.10	0.25
	1.54	*0.08*	*0.21*	*1.77*	*0.02*	*0.19*	*1.68*	*0.08*	*0.24*

TABLE 16 *(continued)*

$MgCO_3$	Without grinding			With grinding					
	Extraction 18 hr			Extraction 10 min			Extraction 18 hr		
	Chlorophyll			Chlorophyll			Chlorophyll		
	a	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
				Skeletonema					
—	0.48		0.35	0.83		0.38	0.56		0.37
	0.49		0.43	0.85		0.35	0.52		0.47
	0.55		0.44	0.83		0.35	0.53		0.47
	0.44		0.54	0.80		0.45	0.52		0.38
	0.49		*0.44*	*0.83*		*0.38*	*0.53*		*0.42*
+	0.52		0.44	0.94		0.57	0.55		0.40
	0.52		0.45	0.91		0.49	0.54		0.52
	0.51		0.45	0.83		0.38	0.56		0.40
	0.46		0.39	0.88		0.44	0.58		0.60
	0.50		*0.43*	*0.89*		*0.47*	*0.56*		*0.48*
				Nannochloris					
+	1.03	0.62		1.29	0.69		0.99	0.73	
	0.95	0.49		1.23	0.60		1.05	0.51	
	0.98	0.62		1.26	0.72		1.07	0.78	
	1.07	0.65		1.18	0.57		0.98	0.60	
	1.01	*0.60*		*1.24*	*0.65*		*1.02*	*0.66*	

1 Millipore HA filters except (*) where Gelman Type A used. Values printed in italics are means (µg/l).

TABLE 17. Turbidity correction[1]

$MgCO_3$	Organism	E_{750}	Chlorophyll *a*	Chlorophyll *b*	Chlorophyll *c*
		Gelman glass A filter			
—	*Nannochloris*	1	0.34	0.13	
		1	0.32	0.13	
		3	0.36	0.17	
		9	0.41(36)	0.23(15)	
+	*Skeletonema*	0	0.19		0.11
		7	0.25(21)		0.21(09)
		23	0.34(20)		0.56(13)
		25	0.44(15)		0.53(02)
+	*Skeletonema*	5	0.43(40)		0.28(17)
		6	0.43(39)		0.35(24)
		10	0.48(42)		0.41(19)
		31	0.58(39)		0.96(31)
—	*Skeletonema*	6	0.19(17)		0.24(15)
		7	0.20(16)		0.24(11)
		13	0.22(16)		0.26(06)
		15	0.24(17)		0.34(10)
+	*Nitzschia*	4	1.61(1.58)		0.78(68)
		9	1.66(1.59)		0.87(66)
		11	1.44(1.37)		0.90(65)
		22	1.67(1.53)		1.09(57)
		Millipore HA filter			
+	*Gymnodinium*	0	0.35		0.36
		10	0.42(35)		0.62(39)
		12	0.40(32)		0.51(27)
		13	0.44(36)		0.63(36)
—	*Skeletonema*	3	0.55(53)		0.54(47)
		14	0.60(52)		0.76(47)
		22	0.66(52)		0.84(38)
		28	0.73(56)		0.93(37)
+	*Skeletonema*	0	0.54		0.52
		4	0.57(55)		0.47(40)
		19	0.70(58)		1.00(60)
		21	0.69(56)		0.84(40)
+	Phytoplankton	3	0.16(15)	0.06(05)	0.14(10)
		10	0.16(12)	0.09(04)	0.20(07)
		15	0.17(11)	0.12(04)	0.28(08)
		31	0.26(13)	0.19(03)	0.47(06)
—	*Dunaliella*	4	0.46(44)	0.25(23)	
		9	0.45(42)	0.28(22)	
		10	0.49(46)	0.29(22)	
		14	0.46(41)	0.29(20)	
+	*Dunaliella*	3	0.53(52)	0.26(24)	
		13	0.56(51)	0.30(22)	
		18	0.61(54)	0.34(22)	
		19	0.61(54)	0.32(21)	
+	Phytoplankton	2	0.16(15)	0.08(07)	0.13(11)
		2	0.13(12)	0.07(06)	0.16(14)
		5	0.13(11)	0.06(04)	0.15(09)
		10	0.19(15)	0.10(05)	0.21(07)

TABLE 17 *(continued)*.

$MgCO_3$	Organism	E_{750}	Chlorophyll		
			a	*b*	*c*
		Millipore HA filter (continued)			
+	*Gymnodinium*	6	0.36(33)		0.40(30)
		9	0.37(33)		0.43(28)
		11	0.39(34)		0.53(36)
		20	0.39(30)		0.52(19)
—	*Nitzschia*	0	0.36		0.13
		1	0.42(41)		0.15(13)
		2	0.42(41)		0.15(11)
		19	0.41(35)		0.41(11)
—	*Skeletonema*	2	0.76(75)		0.38(35)
		4	0.76(74)		0.44(37)
		10	0.79(74)		0.50(34)
		20	0.83(73)		0.67(34)
+	*Skeletonema*	1	0.80(80)		0.40(38)
		9	0.83(78)		0.46(31)
		9	0.83(78)		0.46(31)
		16	0.88(80)		0.54(28)
		Soviet filter			
—	*Dunaliella*	1	0.41(41)	0.25(24)	
		3	0.42(41)	0.24(22)	
		10	0.43(39)	0.28(21)	
		24	0.51(42)	0.37(22)	
+	*Dunaliella*	4	0.51(49)	0.25(23)	
		6	0.46(44)	0.24(20)	
		12	0.48(44)	0.32(25)	
		13	0.51(46)	0.27(19)	
+	Phytoplankton	0	0.11	0.06	0.15
		0	0.09	0.04	0.09
		5	0.14(12)	0.07(04)	0.14(07)
		15	0.17(11)	0.10(02)	0.23(03)
+	*Gymnodinium*	5	0.35(33)		0.35(26)
		5	0.36(33)		0.29(20)
		8	0.36(32)		0.46(32)
		20	0.41(31)		0.55(21)
—	*Nitzschia*	1	0.38(38)		0.15(13)
		3	0.38(37)		0.23(18)
		4	0.41(39)		0.25(18)
		5	0.36(34)		0.23(15)
—	*Skeletonema*	1	0.73(73)		0.42(40)
		1	0.76(75)		0.44(43)
		7	0.72(69)		0.51(39)
		21	0.83(73)		0.67(32)
+	*Skeletonema*	2	0.75(74)		0.45(41)
		5	0.74(72)		0.41(33)
		6	0.72(70)		0.41(31)
		14	0.78(72)		0.51(28)

1 Chlorophyll concentrations are in µg/l. The values in parentheses were obtained when the E_{750} was subtracted. E_{750} is given in units of 0.001.

REFERENCES

CREITZ, G. I.; RICHARDS, F. A. 1955. The estimation and characterization of plankton populations by pigment analysis. III. A note on the use of 'Millipore' membrane filters in the estimation of plankton pigments. *J. Mar. Res.*, **14** : 211-6.

HOLMES, R. W. 1958. Size fractionation of photosynthesizing phytoplankton. *U.S. Dept. Interior. Special Scientific Rep., no.* **279** : 69-71.

——; ANDERSON, G. C. 1963. Size fractionation of C^{14}-labeled natural phytoplankton communities. In: Oppenheimer, C. H. (ed.). *Symposium on marine microbiology*, p. 241. Springfield, Illinois, Charles C. Thomas.

HUMPHREY, G. F. 1960. *The concentration of plankton pigments in Australian waters.* CSIRO. Aust. Div. Fish. Oceanogr. (Technical paper no. 9.)

——. 1963. Chlorophyll *a* and *c* in cultures of marine algae. *Aust. J. mar. freshw. Res.*, **14** : 148-54.

——; JEFFREY, S. W. 1965. In preparation.

LASKER, R.; HOLMES, R. W. 1957. Variability in retention of marine phytoplankton by membrane filters. *Nature*, **180** : 1295-6.

NASCO. 1964. *Recommended interim procedures for measurements in biological oceanography.* Washington, D.C., National Academy of Sciences Committee on Oceanography.

RICHARDS, T. A. WITH THOMPSON, T. G. 1952. The estimation and characterization of plankton populations by pigment analyses. II. A spectrophotometric method for the estimation of plankton pigments. *J. Mar. Res.*, **11** : 156-72.

SAIJO, Y. 1964. Size distribution of photosynthesizing phytoplankton in the Indian Ocean. *J. Oceanogr. Soc. Japan*, **19** : 187-9.

STRICKLAND, J. D. H.; PARSONS, T. R. 1960. A manual of sea-water analysis. *Bull. Fish. Res. Bd. Can.*, no. 125.

YENTSCH, C. S.; MENZEL, D. W. 1963. A method for the determination of phytoplankton chlorophyll and phaeophytin by fluorescence. *Deep-sea Res.*, **10** : 221-31.

4

Extraction of chlorophyll *a* from *Nitzschia closterium* by grinding

J. D. Kerr,
Division of Mathematical Statistics,
CSIRO, Cronulla, N.S.W.
(Reprint No. 582)

D. V. Subba Rao,
Division of Fisheries and
Oceanography, CSIRO,
Cronulla, N.S.W.
Present address:
Department of Zoology,
Andhra University,
Waltair, India

Manuscript received 15 June 1965

Summary

The amounts of chlorophyll extracted at various combinations of duration of grinding, extraction time, and temperature, were studied. In the ranges examined, 30 sec grinding followed by 10 min extraction at room temperature gave the highest chlorophyll values.

I Introduction

Yentsch and Menzel (1963) stated that determination of phytoplankton pigments by the method of Richards with Thompson (1952) and by similar methods was not accurate because extraction was incomplete. Yentsch and Menzel used a mechanical grinding technique to extract chlorophyll but did not include the results of any tests for the effects of duration of grinding, duration of extraction, and temperature at which extraction proceeded. In the present work, the chlorophyll *a* content of *Nitzschia closterium* was studied to find out the optimal conditions for the determination of pigments by the grinding technique.

II Methods

A MATERIAL

Cultures of *Nitzschia closterium* grown for four days in an artificial medium (Humphrey, 1963) at 1,100 f.c. and 25°C were used. Forty millilitres of culture were diluted to 1,000 ml with filtered sea-water and 100 ml aliquots were used to obtain amounts of pigment similar to those in 5 litres of ocean water, i.e. 0.5 to 2.5 μg chlorophyll *a*.

B CHEMICAL ANALYSIS

Filtration was carried out as described by Humphrey (1960) but at about one-sixth of full vacuum. The Millipore filter was then folded and pushed to the bottom of the homogenizer described by Humphrey and Wootton (1966). Three millilitres of 90 per cent acetone were added and the filter was ground. The contents of the homogenizer were then poured into a nylon centrifuge tube (15 mm × 70 mm). The pestle and homogenizer were carefully washed with 90 per cent acetone. The total volume was about 8 ml. The tube was stoppered with a nylon stopper and kept dark at the required temperature for a specified period. The extract was then centrifuged at 4,300 *g* for 10 min. The supernatant was decanted into a 15 ml graduated tube and made up to 10 ml with 90 per cent acetone.

The extinctions were determined at 750, 663 and 630 mμ in a 4 cm cell in a Unicam SP 600 spectrophotometer. The reading at 750 mμ was always less than 0.012 and was subtracted from the other extinctions to correct for turbidity. The following equation (Humphrey and Jeffrey, unpublished) was employed to calculate chlorophyll *a*

$$\text{chl. } a = 13.4\, e_{663} - 0.3\, e_{630}$$

where chl. *a* is in μg/ml acetone extract and e_{663} and e_{630} are the extinctions per centimetre of light path at 663 and 630 mμ after subtracting the 750 mμ reading.

Results were calculated as μg/l as if the chlorophyll in the acetone extract had come from 5 litres of algal suspension. The e_{630} value corrects for the extinction of chlorophyll *c* at 663 mμ.

C PROCEDURE

Two series of experiments were done, each consisting of 27 combinations of time of grinding, duration of extraction, and temperature at which extractions were done. A 3^3 factorial design was used. Each series of twenty-seven was subdivided into three groups of nine samples prepared under the same conditions, by partial confounding of the higher order interaction. In both series three levels of duration of grinding, i.e. 30 sec, 1 min 30 sec, and 4 min 30 sec, and extractions at 0°C, 20°C and 40°C were studied. In the first series variations in the duration of extraction were 10 min, 1 hr 15 min, and 6 hr 15 min, and in the second, 10 min, 2 hr and 16 hr.

III Results

The table below summarizes the analysis of variance of the fifty-four observations.

The mean values for chlorophyll *a* at the different grinding times were 0.45 μg/l at 30 sec, 0.44 at 1 min 30 sec, 0.39 at 4 min 30 sec, the yield decreasing as grinding time increased.

The mean values for chlorophyll *a* at the different extraction times were 0.46 μg/l at 10 min, 0.42 at 1 hr 15 min, 0.40 at 2 hr, 0.40 at 6 hr 15 min, 0.41 at 16 hr. The yield decreased approximately linearly as the logarithm of extraction time increased.

Any significant effect due to temperature alone was not shown.

Analysis of variance

Source	df	Sum of squares	Mean square	F
Grinding time (G)	2	0.03698	0.01849	4.17[1]
Extraction time (E)	4	0.03375	0.00844	1.90
—Regression on Log (E)	1		0.02741	6.17[1]
Extraction temperature (T)	2	0.00272	0.00136	<1
G × E interaction	8	0.01310	0.00164	<1
G × T interaction	4	0.02282	0.00570	1.29
E × T interaction	8	0.09143	0.01143	2.58
Blocks	5	0.21753	0.04350	9.80[1]
Error	20	0.08871	0.00444	

1 Probability <0.05.

IV Discussion

Humphrey (1962, Table 3) summarized the details of the differences in the analytical methods commonly used for pigment analyses. This summary shows that extractions are carried out in the dark, both at room temperature and in the cold, for varying periods up to 24 hr.

Yentsch and Menzel (1963), after grinding the filter for 1 min, centrifuged at once for 1 min and, after allowing the extract to stand for several minutes to reach room temperature, measured the fluorescence. This procedure of centrifuging immediately after grinding would not be suitable for a series of determinations because it would mean centrifuging the samples one by one. Therefore, in the present work, an extraction step was introduced so that a sample could be ground and left while other samples were being ground. Because the length of this extraction step did not significantly influence the chlorophyll value (see table) the extraction time is not critical and 10 min is recommended as a convenient period.

A grinding period of 30 sec gave the highest chlorophyll values (see Section III), and is therefore recommended. Because extraction temperature had no significant influence, room temperature is recommended as being the most convenient.

Although the experiments were done with Millipore filters covered with $MgCO_3$, it is probable that similar results would be obtained with other cellulose-ester filters. It is not certain that the conditions found optimal for *Nitzschia* would be the best for algae of all sizes and types of cell-wall.

V Conclusions

It is recommended that after collecting algae on $MgCO_3$ treated filters, the filters should be ground mechanically for 30 sec, allowed to stand for 10 min at room temperature and then centrifuged.

REFERENCES

HUMPHREY, G. F. 1960. *The concentration of plankton pigments in Australian waters.* (CSIRO Aust. Div. Fish. Oceanogr. Tech. Pap. no. 9.)

——. 1962. Phytoplankton pigments in the Pacific Ocean. In: *Proc. Conf. Primary Production Measurement, Marine and Freshwater, Hawaii*, p. 121-41.

——. 1963. Chlorophyll *a* and *c* in cultures of marine algae. *Aust. J. mar. freshw. Res.*, **14**: 148-54.

——; WOOTTON, M. 1966. *Comparison of techniques used in the determination of phytoplankton pigments.* Paris, Unesco. (Monographs on oceanographic methodology, no. 1.)

RICHARDS, F. A. WITH THOMPSON, T. G. 1952. The estimation and characterization of plankton populations by pigment analyses. II. A spectrophotometric method for the estimation of plankton pigments. *J. Mar. Res.*, **11**: 156-72.

YENTSCH, C. S.; MENZEL, D. W. 1963. A method for the determination of phytoplankton chlorophyll and phaeophytin by fluorescence. *Deep-sea Res.*, **10**: 221-31.