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The determination of photosynthetic pigments in sea-water

A survey of methods

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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.

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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.'

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