Discussion of Spectrophotometric Determination of Marine-plant Pigments, with Revised Equations for Ascertaining Chlorophylls and Carotenoids

T. R. Parsons[1] and J. D. H. Strickland[2]

Fisheries Research Board of Canada
Pacific Oceanographic Group, Nanaimo, B.C.

ABSTRACT

A number of discrepancies in the spectrophotometric determination of plant pigments with the Richards with Thompson method have been reported. A revised set of equations for the determination of plant chlorophylls and a new equation for the approximate estimation of plant carotenoids are presented.

Introduction. The most widely employed method for the determination of plankton pigments in sea water by spectrophotometric analysis is that described by Richards with Thompson (1952), and modified by Creitz and Richards (1955) for the use of Millipore membrane filters for the collection of plankton samples. Certain refinements of that method and full working instructions for it have been given by Strickland and Parsons (1960). Recent work in our own and other laboratories has indicated various discrepancies in the Richards with Thompson equations for the determination of chlorophylls *a*, *b*, and *c*, and total carotenoids; these discrepancies may be placed in the following categories:

1. The specific absorption coefficients for chlorophyll *a* and *b* employed by Richards with Thompson are too low. This indicates that these pigments have been overestimated by persons using their method.

2. Values obtained for the chlorophyll *c* concentration in oceanic seawater samples have given rise to the impression that this is the major chlorophyll in many parts of the oceans (Currie, 1958; Humprey, 1960; McAllister *et al.*,

[1] Present address: Office of Oceanography, UNESCO, Place de Fontenoy, Paris 7, France.
[2] Present address: Institute of Marine Resources, University of California, P.O. Box 109, La Jolla, California, U.S.A.

1960). This error is largely independent of the arbitrary extinction of the specific pigment unit employed by Richards with Thompson.

3. The specific pigment unit assumed for the determination of total carotenoids is likely to be much greater than one gram due to the low specific absorption coefficients of peridinin and fucoxanthin. Thus total carotenoids have tended to be underestimated with the method of Richards with Thompson if one assumes their statement that the specific pigment unit approximates a gram of pigment.

4. The plant xanthophylls—peridinin and fucoxanthin—both give positive animal carotenoid values when determined by the Richards with Thompson method for total animal carotenoids.

The revised equations given on p. 162 are not intended to be definitive and it may be that a multichromatic approach to the determination of marine pigments following Millipore filtration is an unnecessary refinement for many field experiments. Results should always be interpreted with caution, especially when low extinction values are obtained. However, if the Richards with Thompson technique is used, it is believed that the new formulations given here represent a significant methodological improvement.

Experimental

PERIDININ AND FUCOXANTHIN were prepared from 90% acetone extracts of the dinoflagellate *Amphidinium carteri* and the seaweed *Sargassum muticum*, respectively. The dinoflagellate was grown in mass culture in the laboratory as described previously (Parsons *et al.*, 1961) and the seaweed was collected from Departure Bay, Nanaimo. The former required no treatment prior to extraction while the latter was ground in a conventional meat grinder. The procedure for the isolation of each of the two pigments was essentially the same, but only the method employed for extracting fucoxanthin is described here in detail.

A 60% (approx.) acetone extract of *S. muticum* was placed in a separatory funnel, and an equal volume of hexane was added. The xanthophylls and chlorophyll *a* were transferred to the hexane layer by shaking, and the hexane extract was taken to dryness under reduced pressure in an atmosphere of nitrogen. The pigments were taken up in ethyl ether, and an equal volume of ligroine (b.p. 20–40°C) was added. The mixture was evaporated to approximately half its volume and cooled to −20°C. The heavy, orange-red precipitate that formed on cooling was filtered off and redissolved in a small amount of ethyl ether. To the ethyl ether extract an equal volume of hexane was added, and the mixture was then poured onto a column of confectioner's sugar 5 cm in diameter and 30 cm in length. The column was developed with 100 ml of hexane followed by the addition of progressively stronger solutions of n-propanol in hexane; the first additions, 0.1% and upward, removed traces of chlorophyll *a* as well as other carotenoids, while the final additions, 0.5%,

separated fucoxanthin from its isomers. The fucoxanthin eluted from the column was taken to dryness under reduced pressure in an atmosphere of nitrogen and redissolved in ethyl ether. To the ethyl ether an equal volume of ligroine was added, and the volume was reduced to about half. On cooling to −20°C, an orange-red precipitate of fucoxanthin separated out; this was redissolved and reprecipitated several times by the above procedure, and the supernatant liquid was discarded each time. By slow evaporation at room temperature of the final preparation, which consisted of the pigment dissolved in a mixture of ethyl ether and ligroine, crystals of fucoxanthin were obtained.

Approximately 50 mg of crystalline fucoxanthin were obtained from about 5 kg (wet weight) of *S. muticum*. The crystals had a melting point of 146–147°C and contained no ash following incineration at 500°C for three hours. Approximately 2 mg of peridinin were obtained from about 1.0 g (dry weight) of *A. carteri*. No additional tests for purity were performed on the small amount of material obtained.

The two pigments were dried in a vacuum dessicator to constant weight and dissolved in 90% acetone. The specific absorption coefficients were determined at various wavelengths using a Beckman DU spectrophotometer. The results for peridinin and fucoxanthin are shown in Table 1.

TABLE I. Specific Absorption Coefficients (L/G CM) of Pigments in 90% Acetone.

Wavelength (mμ)	Chlorophyll *a*	Chlorophyll *b*	Chlorophyll *c*	Peridinin	Fucoxanthin
430	*	*	*	69.0	73.1
450	*	*	*	77.0	88.0
480	1.2	28.0	4.7	83.8	72.5
510	2.1	3.1	1.8	49.1	19.9
580	10.4	9.3	11.5	0.8	–
630	13.9	16.4	19.5	–	–
645	21.8	54.0	4.3	–	–
665	89.0	6.3	0.7	–	–

* Indicates values not determined.
– Indicates no absorption at these wavelengths.

Chlorophyll *a* and *b* were prepared by chromatographic separation of a 90% acetone extract derived from a mixture of grasses and clover; the method was similar to that described by Smith and Benitez (1955: 143–196). The two principal absorption maxima for the preparations in ethyl ether, and the ratios of the blue-to-red peak heights were: 430 and 661 mμ, and 1.31 for chlorophyll *a*; 455 and 643 mμ, and 2.85 for chlorophyll *b*. The ratio of the absorptions at various wavelengths in ether and 90% acetone was determined by using small aliquots of the same ether solution diluted to a known volume with the two solvents. This technique was employed by Vernon (1960) to determine the specific absorption coefficients of chlorophylls *a* and *b* in 80,

90, and 100% acetone from the specific absorption coefficients of chlorophylls *a* and *b* in ethyl ether given by Smith and Benitez.

Discussion

Chlorophyll *a*. Vernon (1960) has reported a value of 91.1 l/g cm for the specific absorption coefficient for chlorophyll *a* in 90% acetone at a wavelength of 664 mμ. With the same technique, the value obtained in our laboratory was 92 l/g cm. The difference between these values is sufficiently small to be accountable in terms of instrumental errors; the value 91.1 l/g cm at 664 mμ has therefore been accepted, since it is an average obtained from a greater number of preparations than were employed in our own experiments.

The specific absorption coefficient of chlorophyll *a* in ethyl ether, on which both Vernon's values and our own are based, was obtained by Smith and Benitez without drying their preparation; this, according to Smith and Benitez, gave a different value from that obtained when the preparation was completely dried. The value obtained by Vernon (1960) and by us for the specific absorption coefficient in 90% acetone at 664 mμ similarly was obtained without drying the chlorophyll preparation. Considering that the routine Richards with Thompson estimation of pigments does not involve drying the extracted pigment, it appears best to adopt the values obtained from undried material. Specific absorption coefficients at the wavelengths reported in Table 1, which are not quoted by Vernon (1960), have been determined from the chlorophyll *a* absorption spectrum obtained by us, standardized on the 664-mμ absorption coefficient. These values, used to recalculate the constants in equations given by Richards with Thompson, are shown on p. 162 as eqs. (1) to (6).

Values employed by Richards with Thompson for the specific absorption coefficients of chlorophyll *a* in 90% acetone were obtained from Zscheile (1934). Values obtained more recently by Zscheile *et al.* (1942) for chlorophyll *a* in 80% acetone are appreciably higher than those obtained by Richards with Thompson. The difference in absorption coefficients in 80 and 90% acetone is about 1% (Vernon, 1960), which does not account for the low values reported by Zscheile. Our rejection of the widely accepted 1942 values by Zscheile *et al.* is based on the observation already mentioned—that the absorption coefficient from dried preparations of chlorophyll *a* differs from those obtained from undried preparations. The values shown in Table 1 are approximately 25% higher than values given by Zscheile and 10% higher than values given by Zscheile *et al.* Thus the incorporation of these values from Table 1 into eqs. (1), (2), and (3) has resulted in a lower factor for the determination of chlorophyll *a* in eq. (4).

Chlorophyll *b*. The specific absorption coefficients of chlorophyll *b* employed by Richards with Thompson were taken from Zscheile *et al.* Values taken by Richards with Thompson for the specific absorption coefficients at

665, 645, and 630 mμ are for chlorophyll *b* in 80% acetone. The values for chlorophyll *b* reported in Table 1 have been estimated from Vernon's figure of 52.2 l/g cm for the specific absorption coefficient at 648 mμ in 90% acetone. The value obtained under the same conditions in our laboratory was 53.5 l/g cm. Following the same reasons as those presented in the discussion on chlorophyll *a*, the value 52.5 l/g cm for chlorophyll *b* at 648 mμ has been used to standardize the values in Table 1. The values taken from Table 1 for use in eqs. (1), (2), and (3) are about 15% higher than the values employed by Richards with Thompson, which has resulted in a lower factor for the determination of chlorophyll *b* in eq. (5).

Chlorophyll *c*. The values for the specific absorption coefficient of crystalline chlorophyll *c* in 100% and 90% acetone at 630 mμ have recently been found by Jeffrey to be 15.8 and 19.5 l/g cm, respectively (Jeffrey, 1962, 1963). The values obtained by Jeffrey (1963) at different wavelengths, shown in Table 1, have been used for the development of eqs. (1), (2), and (3). The weight of chlorophyll *c* as determined by use of eq. (6) is about half the specified pigment unit obtained by use of the Richards with Thompson equation.

A second error in the estimation of chlorophyll *c* by spectrophotometric measurements at 630 mμ, shown to be largely independent of the specified absorption coefficient employed, has resulted in the impression that chlorophyll *c* is the major chlorophyll in many oceanic seawater samples (Currie, 1958; Humphrey, 1960; McAllister *et al.*, 1960). A simulation of the type of error involved is shown in Table II. In this experiment the turbidity blanks on a 10-cm lightpath were altered by the addition of 1, 2, or 3 Millipore filters to 10 ml of 90% acetone extract. At the same time measurements were made on different concentrations of pigment from the same culture of *A. carteri*.

TABLE II. The Effect of Turbidity on the Spectrophotometric Estimation of Chlorophylls *a* and *c*.

Sample No.	*Amphidinium* Culture (ml)	Optical Density Readings 750	665	645	630	Chlorophylls *a*	*c*	*c*/*a* Ratio
		(10-cm lightpath)				(μSPU/10 ml acetone)		
1	1.0	.032	.096	.063	.065	0.91	1.91	2.1
2	1.0	.068	.148	.112	.116	1.12	2.96	2.6
3	2.0	.046	.185	.103	.106	2.01	3.19	1.6
4	2.0	.053	.188	.107	.114	1.94	3.38	1.7
5	2.0	.060	.197	.116	.122	1.98	3.64	1.8
6	10.0	.100	.775	.313	.332	9.9	10.8	1.1
7	10.0	.112	.770	.322	.350	9.7	11.8	1.2
		(1-cm lightpath)						
8	10.0	.000	.072	.0235	.0240	10.5	10.5	1.0

The values for chlorophyll *a* and *c* have been obtained by use of the Richards with Thompson equations, with the modification (Strickland and Parsons, 1960) of subtracting the 750-mμ extinction from the readings at 665, 645, and 630 mμ in order to correct for turbidity when using a 10-cm lightpath.

The results show that the greatest chlorophyll *c*-to-*a* ratio is obtained with a combination of the lowest extinctions at 665, 645, and 630 mμ and a relatively high turbidity reading at 750 mμ (sample 2). The effect of correcting for the turbidity readings at 750 mμ (all of which are representative of those encountered in practice after centrifuging natural populations) is apparent throughout the three ranges of pigment concentration. The effect is least in the most concentrated extracts with the smallest blank (sample 6). The use of a 1-cm lightpath, which gave a turbidity blank of zero, showed the lowest ratio of chlorophyll *c*-to-*a*. If no 750-mμ correction is made in the 665, 645, and 630-mμ extinctions, the apparent chlorophyll *c*-to-*a* ratios are even greater.

The use of the equations on p. 162 instead of the Richards with Thompson equations still do not remove the artifact of high ratios of chlorophyll *c* to *a* when dealing with small extinctions and large blanks. Attempts to remedy this situation by prolonged centrifugation or complete dessication of pigment samples prior to extraction were unsuccessful. A consideration of the optical density units that are being measured, however, illustrates why trichromatic readings in the 600-mμ region for the determination of chlorophyll *c* can be unreliable; if, for example, it is assumed that the value obtained from the extinctions read on the 1-cm cell are correct (sample 8), then we may assume that 1.05 μ SPU of chlorophyll *c* are present in the 1 ml of culture (sample 2). If the Richards with Thompson specific absorption coefficient of 10.4 l/SPU cm at 630 mμ is assumed, then the contribution of chlorophyll *c* to the 630-mμ extinction in sample 2 is approximately 0.01. This value is considerably smaller than the turbidity blank with which the 630-mμ reading is corrected, and further, it is only one fifth of the total extinction at 630 mμ. Any error in the final figure grossly exaggerates the absolute amount of chlorophyll *c* when the factor of approximately 100 is used to evaluate the amount present. In the following paper, a second method has been developed as a solution to the problem of determining chlorophyll *c* at low concentrations (Parsons, 1963).

The cause of the high 750-mμ extinctions in acetone extracts of natural populations has been found to be largely attributable to the amount of salt that is carried over by the Millipore filter following filtration of a seawater sample. The complete dissolution of the Millipore filter, composed of high polymer cellulose nitrate, is very susceptible to the ionic concentration in the 90% acetone, and small differences in the amount of salt adhering to the Millipore filter can cause large differences in the 750-mμ and other extinctions.

Plant Carotenoids. The specific absorption coefficients of fucoxanthin and peridinin in Table 1 are approcimately 35% and 40%, respectively, of

the value for β-carotene measured at 480 mμ. Thus the use of an average specific absorption coefficient based on β-carotene, which is the basis of the specific pigment unit employed by Richards with Thompson, is undesirably high in view of the predominance of fucoxanthin among members of the Chrysophyceae and Bacillariophyceae, and of peridinin among the Dinophyceae (Jeffrey, 1961; Parsons, 1961). Due to the large difference between the specific absorption coefficients of fucoxanthin and peridinin as compared with other carotenoids quoted by Goodwin (1955: 272–311), there appears to be no really satisfactory method for estimating the total carotenoids without separating them chromatographically, as suggested by Humphrey (1961). If an approximate measure of the amount of total carotenoids in a seawater sample is required, however, then a re-evaluation of the specific pigment unit is desirable. The new unit may be defined so that one such unit in one liter of 90% acetone has, at a wavelength of 480 mμ, an absorbency of 100. This unit will only approximate 1 g if the predominant carotenoid in a seawater sample is fucoxanthin or peridinin, or a mixture of both. In the event that a phytoplanktonic crop may be predominantly composed of classes of organisms such as the Chlorophyceae and Cyanophyceae, which lack these pigments, then the specific pigment unit as defined by Richards with Thompson would be a closer approximation to 1 g of pigment. Corrections applied at 480 mμ for chlorophyll absorption as described by Richards with Thompson are without significance in view of the approximate nature of the absorption coefficient suggested above.

Animal Carotenoids. If the Richards with Thompson equation for the estimation of animal carotenoid is applied to the specific absorption coefficients for peridinin and fucoxanthin in Table 1, then both pigments give positive animal carotenoids. In our own experience, it has been found better to neglect the animal carotenoid estimation as being largely an artifact. Furthermore, if phytoplankton samples are filtered through a coarse net prior to filtration onto a membrane filter, most of the larger zooplankton are removed and the contribution of astacin-type carotenoids can be considered a second order correction in the estimation of plant carotenoids.

Equation for Estimating Plant Carotenoids. For the routine spectrophotometric determination of plant carotenoids, extinctions should be measured at 750 and 480 mμ. The 480-mμ extinction must be corrected for the 750-mμ extinction multiplied by three to allow for the increased light scattering in blue compared with red light (Strickland and Parsons, 1960). The corrected extinction at 480 mμ can be used to determine the millispecific pigment units of carotenoids in sea water by the equation:

$$\mathrm{MSPU/m^3} = \frac{10\,(D_{480} - 3\,D_{750})\,v}{l \times V},$$

where D is the optical density at the wavelength indicated, l the lightpath of the cuvette in centimeters, v the volume of the acetone extract in milliliters, and V the volume of seawater filtered in liters. For phytoplanktonic crops that are dominated by members of the Chlorophyceae or Cyanophyceae, the amount of carotenoid given by the above equation should be divided by 2.5.

Equations for Estimating Chlorophylls *a*, *b*, and *c*. From the previous discussion it is apparent that the equations for the estimation of chlorophylls *a*, *b*, and *c*, originally published by Richards with Thompson, require revision to include the specific absorption coefficients shown in Table 1. Thus the absorption contributing to optical density measurements at 665, 645, and 630 mμ may be expressed as follows:

$$D_{665} = 0.089\ C\ a + 0.0063\ C\ b + 0.0007\ C\ c, \quad (1)$$
$$D_{645} = 0.0218\ C\ a + 0.054\ C\ b + 0.0043\ C\ c, \quad (2)$$
$$D_{630} = 0.0139\ C\ a + 0.0164\ C\ b + 0.0195\ C\ c, \quad (3)$$

where D is the optical density in 1-cm pathlength cell at the wavelength shown, and where C *a*, C *b*, and C *c* are the concentrations of chlorophylls *a*, *b*, and *c* in mg/l of 90% acetone, respectively. Rearranging (1) to (3), the following equations are derived, with the Richards with Thompson factors in parentheses:

$$C\ a = 11.6\ D_{665}\ (15.6) - 0.14\ D_{630}\ (0.8) - 1.31\ D_{645}\ (2.0), \quad (4)$$
$$C\ b = 20.7\ D_{645}\ (25.4) - 4.34\ D_{665}\ (4.4) - 4.42\ D_{630}\ (10.3), \quad (5)$$
$$C\ c = 55\ D_{630}\ (109) - 16.3\ D_{645}\ (28.7) - 4.64\ D_{665}\ (12.5). \quad (6)$$

The use of eqs. (4), (5), and (6) for the determination of chlorophylls *a*, *b*, and *c* is the same as that in an example determination shown by Richards with Thompson. In addition to the wavelengths for optical density measurements reported above, a reading should also be made at 750 mμ and this value subtracted from the optical densities at 665, 645, and 630 mμ. The use of the 750 mμ correction may eliminate turbidity errors of up to 50% over estimation of pigments when using cells of 10-cm lightpath and low optical density values.

To find the concentration of chlorophylls in a seawater sample of liter volume, V, using acetone extracts of milliliter volume, v, in a cuvette of centimeter pathlength, l, use the equation:

$$\text{mg chlorophyll } (a, b, \text{ or } c)/\text{m}^3 \equiv \frac{C_{(a \cdot b \cdot c)} \times v}{l \times V},$$

where $C_{(a \cdot b \cdot c)}$ is the concentration of the respective chlorophyll determined from eqs. (4), (5), and (6).

REFERENCES

CREITZ, G. I., AND F. A. RICHARDS

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*: 211–215.

CURRIE, R. I.

1958. Some observations on organic production in the North East Atlantic. Rapp. Cons. int. Explor. Mer, *144*: 96–102.

GOODWIN, T. W.

1955. Carotenoids. Modern Methods of Plant Analysis, *3*. Springer-Verlag, Berlin.

HUMPHREY, G. F.

1960. Concentration of plankton pigments in Australian waters. C.S.I.R.O. Aust. Fish. Oceanogr. Tech. Pap., *9*; 27 pp.

1961. Phytoplankton pigments in the Pacific Ocean. Preprint in Symposium on algal productivity in the Pacific. 10th Pacif. Sci. Congr.; 16 pp.

JEFFREY, S. W.

1961. Paper-chromatographic separation of chlorophylls and carotenoids from marine algae. Biochem. J., *80*: 336–342.

1962. Purification of chlorophyll *c* from *Sargassum flavicans*. Nature, London, *194*: 600.

1963. Purification and properties of chlorophyll *c* from *Sargassum flavicans*. Biochem. J., *86*: 313–318.

MCALLISTER, C. D., T. R. PARSONS, AND J. D. H. STRICKLAND

1960. Primary productivity at Station "P" in the North East Pacific Ocean. J. Cons. int. Explor. Mer, *25*: 240–259.

PARSONS, T. R.

1961. On the pigment composition of eleven species of marine phytoplankters. J. Fish. Res. Bd. Canada, *18*: 1017–1025.

1963. A method for the microdetermination of chlorophyll *c* in sea water. J. Mar. Res., *21* (3): 164–171.

PARSONS, T. R., K. STEPHENS, AND J. D. H. STRICKLAND

1961. On the chemical composition of eleven species of marine phytoplankters. J. Fish. Res. Bd. Canada, *18*: 1001–1016.

RICHARDS, F. A., WITH T. G. THOMPSON

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*: 156–172.

SMITH, J. H. C., AND A. BENITEZ

1955. Chlorophylls: Analysis in plant materials, vol. 4, *in* Modern methods of plant analysis. Springer-Verlag, Berlin.

STRICKLAND, J. D. H., AND T. R. PARSONS

1960. A manual of sea water analysis. Bull. Fish. Res. Bd. Canada, 125; 185 pp.

VERNON, L. P.

1960. Spectrophotometric determination of chlorophylls and pheophytins in plant extracts. Anal. Chem., *32*: 1144–1150.

ZSCHEILE, F. P., JR.

1934. A quantitative spectrophotoelectric analytical method applied to solutions of chlorophylls *a* and *b*. J. phys. Chem., *38*: 95–102.

ZSCHEILE, F. P., JR., C. L. COMAR, AND G. MACKINNEY

1942. Inter-laboratory comparison of absorption spectra by the photoelectric spectrophotometric method. — Determinations on chlorophyll and Weigert's solution. Plant Physiol., *17*: 666–670.