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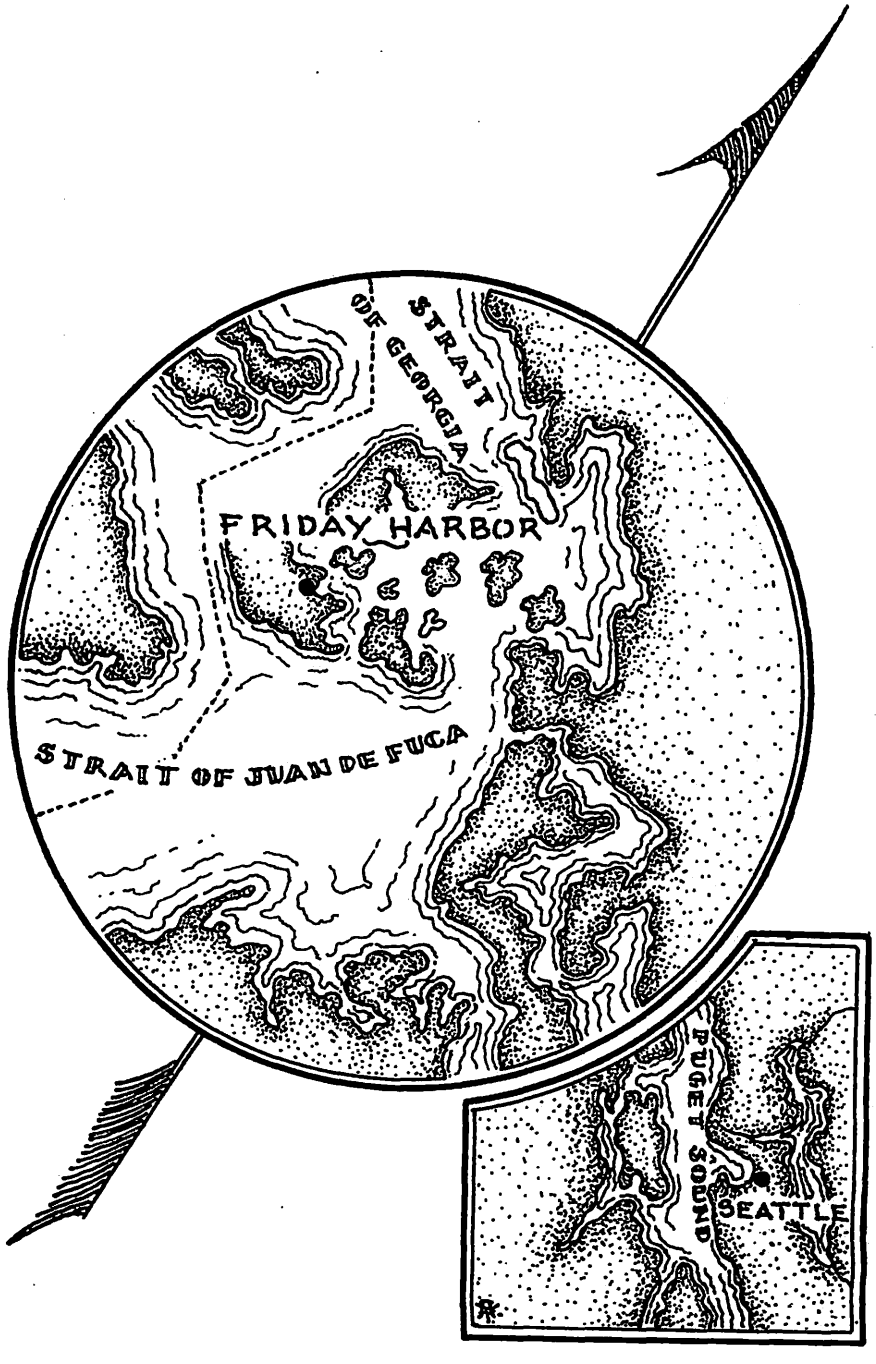
Technical Report No. 5



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by

Francis A. Richards with Thomas G. Thompson

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ABSTRACT

A semi-micro spectrophotometric method is described for the estimation of chlorophyll a, b, and c, astacin type carotenoids and non-astacin type carotenoids in acetone extracts of plant and animal material. Developed specifically for use in estimating and characterizing plankton populations, the method is highly sensitive and practical for shipboard use. Methods of collecting, preparing and extracting plankton samples, spectrophotometric measurements, computation of results, and errors are discussed.

ACKNOWLEDGMENT

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A SPECTROPHOTOMETRIC METHOD FOR THE ESTIMATION OF PLANKTON PIGMENTS

INTRODUCTION

A semi-micro method for the simultaneous spectrophotometric determination of chlorophylls a, b, and c and two types of carotenoids in 90 per cent acetone extracts of plant and animal material has been developed. This method avoids the separation and individual determination of the several components, resulting in the minimum of manipulation, loss, and contamination of the sample.

The method was developed specifically for the examination of plankton, since pigment analyses, extended to include the various chlorophylls and carotenoids, should give a measure of the potential of the plankton for absorbing radiant energy for photosynthesis, some measure of the extent and stage of development of the phytoplankton, and a possible measure of the presence of animals grazing on the crop. The method can be considered as a logical development of Harvey's (5) "pigment unit" determinations, and the determinations of chlorophyll made by Graham, Kozminski, Krey, Riley, and others (4, 6, 8, 14).

The method meets the practical requirements of shipboard use necessary for the determination of the pigments in plankton. It permits the use of relatively small water samples rather than net catches, thus facilitating the taking of subsurface samples, the capture of microplankton, and definitely fixing the origin of the sample.

One to two liters of sea water provide an adequate sample except in cases of very sparse plankton. Net tows readily give sufficient material for analysis.

METHOD OF ESTIMATION OF PLANKTON PIGMENTS

Reagents: 90 per cent Acetone:

Reagent grade acetone is distilled over aqueous Na_2CO_3 and Na_2SO_3 solution, through an 18-inch fractionating column. B.p. 56.5°C. 100 ml of water are pipetted into a one-liter flask and made up to the mark with acetone.

Magnesium carbonate - Reagent grade powdered magnesium carbonate.

Procedure:

About one-tenth gram of powdered magnesium carbonate (to prevent acidity and subsequent pheophytin formation) is added to a one to two liter water sample. After shaking, the sample is poured into an aspirator bottle from which a rubber tube fitted with a stopcock leads to the intake of a Foerst Plankton Centrifuge (Foerst Mechanical & Specialties Co.). The stopcock is adjusted so that 1 liter flows through the centrifuge in about seven minutes. The centrifuge is turned on before the flow is started and left on until rinsing of the aspirator bottle is complete and the centrifuge has drained. When the last of the sample has flowed through, the bottle is rinsed three times with a strong stream of distilled water. The centrifuge is then stopped and the cup removed.

The particulate matter in the cup is scrubbed down with a rubber policeman and rinsed into a 15 ml centrifuge tube and the cup re-rinsed. The material is then centrifuged at high speed in a clinical centrifuge for three minutes, the water poured off and the particulate matter allowed to drain in the tube. The tube is placed in a vacuum desiccator and dried.

When dry, a definite volume of 90 per cent acetone is pipetted into the tube and stirred with the particulate matter and magnesium carbonate. The volume of acetone depends on the amount of plankton present; 5 ml has been used in most cases. The tube is then tightly corked and placed in the dark for 18-24 hours, after which time the material is again mixed, the tube re-stoppered and centrifuged in the clinical centrifuge for three minutes at high speed.

The extract is decanted into a 1 cm properly calibrated glass-stoppered Corex absorption cell, and the optical densities at 665, 645, 630, 510, and 480 $m\mu$ determined on a Beckman Model DU quartz spectrophotometer (2, 4). The red-sensitive tube is used for readings at 665, 645, and 630 $m\mu$; the blue-sensitive tube at 510 and 480 $m\mu$. The tungsten filament light source and 0.04 mm slit width are used at these wave lengths.

Calculation of Results:

The equations for the calculation of chlorophylls a, b, and c are:

$$D_{665} = .0667\ C_a + .0065\ C_b + .0011\ C_c$$

$$D_{645} = .0164\ C_a + .0456\ C_b + .0044\ C_c$$

$$D_{630} = .0119\ C_a + .0127\ C_b + .0104\ C_c$$

D_{665}, D_{645}, and D_{630} are the observed optical densities (log I_o/I), at 665, 645 and 630 mμ respectively, and are determined directly with the spectrophotometer. C_a, C_b, and C_c are concentrations of chlorophylls a, b, and c. Solving these equations gives:

$$C_a(\text{milligrams/L}) = 15.6\ D_{665} - 2.0\ D_{645} - 0.8\ D_{630}$$

$$C_b(\text{milligrams/L}) = 25.4\ D_{645} - 4.4\ D_{665} - 10.3\ D_{630}$$

$$C_c(\text{MSPU*/L}) = 109\ D_{630} - 12.5\ D_{665} - 28.7\ D_{645}$$

It is evident from their spectra that the carotenoids make no contribution to the optical density at these wave lengths.

The concentrations of two carotenoid components were calculated from optical densities at 510 and 480 mμ. The contributions of the chlorophylls to these optical densities were computed and

*Specified Pigment Units (SPU) represent a specific but unknown exact weight of the pigment which however approximates one gram. (See page 9.) 0.001 SPU = 1MSPU (one thousandth of a specified pigment unit).

subtracted, leaving residual densities (D_{res}) which are the result of carotenoid absorption. The equations for these calculations are:

$D_{res,510} = D_{510} - .0026\ C_a - .0035\ C_b - .0021\ C_c = .045\ C_{nac} + .169\ C_{ac}$

$D_{res,480} = D_{480} - .0019\ C_a - .0136\ C_b - .0054\ C_c = .203\ C_{nac} + .249\ C_{ac}$

where C_{nac} and C_{ac} are the respective concentrations of non-astacin type and astacin type carotenoids.

Solving,

Astacin type carotenoids (MSPU/L) $= 2(4.45\ D_{res,510} - D_{res,480})$

Non-Astacin type carotenoids (MSPU/L) $= 7.6(D_{res,480} - 1.49\ D_{res,510})$

The individual calculation of the several components of the carotenoid mixture was found impracticable, because in such calculations it is necessary to solve a number of simultaneous equations equal to the number of components present and to make measurements at an equal number of wave lengths. Errors in such calculations are cumulative, and small errors in density measurements become magnified. Furthermore, the exact number of components is unknown, depending on the phylogenetic groups represented in the plankton (17).

To illustrate these computations, the pigment concentrations in a plankton extract were calculated from the observed optical densities:

$D_{665} = .068$, $D_{645} = .020$, $D_{630} = .018_5$, $D_{510} = .127$ and $D_{480} = .325$

Chlorophyll a, mg/L $= 15.6 \times .068 - 2 \times .020 - 0.8 \times .0185$

$= 1.01$ mg/L

Chlorophyll b, (mg/L) = 25.4 x .020 - 4.4 x .068 - 10.3 x $.018_5$

= 0.02 mg/L

Chlorophyll c, (MSPU/L) = 109 x $.018_5$ - 12.5 x .068 - 28.7 x .020

= 0.59 MSPU/L

$D_{res,510}$ = .127 - 2.6 x .00101 - 3.5 x .00002 - 2.1 x .00059

= .125

$D_{res,480}$ = .325 - 1.9 x .00101 - 13.6 x .00002 - 5.4 x .00059

= .320

Astacin type carotenoids, SPU/L = 2 (4.45 x .123 - .320)

= 0.45 MSPU/L

Non-astacin carotenoids, SPU/L = 7.6 (.320 - 1.49 x .123)

= 1.04 MSPU/L

DISCUSSION OF PROCEDURE AND CALCULATIONS

Removal of Plankton from Water Samples:

The most satisfactory method for the removal of plankton from water samples was the Foerst plankton centrifuge (Foerst Mechanical and Specialties Company). Various paper, sintered glass, and Caldwell type filters, alone and coated with freshly precipitated barium sulfate, were investigated and rejected because of inadequate speed or retention and the presence of extraneous matter in the separated plankton. With the Foerst centrifuge, the plankton can be collected from one liter of water in about seven minutes.

Extraction Methods:

Less than nine hours of steeping were found to be inadequate, although other workers have used much shorter periods (4, 5, 8, 14). After twenty-four hours, there was no spectrophotometric evidence of decomposition of pigments. Neither grinding nor re-extracting the cells with fresh portions of solvent materially increased the amount of pigment extracted; evidently the excess of solvent was so great that the equilibrium distribution of pigment was greatly in favor of the solution instead of the particulate phase.

Beer's Law:

Dilution experiments showed that at the wave lengths used for calculating pigment concentrations, optical densities below 0.8 were directly proportional to concentrations, according to Beer's Law. It was necessary to dilute extracts having optical densities over 0.8.

Calculation of Results:

Optical densities ($\log I_0/I$) of some of the mixtures of pigments obtained by acetone extraction of plankton samples were determined over the spectral range 320-700 mμ (Fig. 1A, B). The concentrations of the pigments can be computed from these spectra, since the optical density is directly proportional to concentration. However, the computations require knowledge of the absorption coefficients of each component at various wave lengths. The number of wave lengths must equal the number of components to be computed, and

FIGURE 1: A, B

Absorption spectra of 90 per cent acetone extracts of natural plankton collections.

LOG $I_0/I = D$
.05
.10
.15
.20
350
400
450
500
550
600
650
700
WAVE LENGTH, mμ
I
II

LOG $I_0/I = D$
10
20
30
40
350
400
450
500
550
600
650
700
WAVE LENGTH mμ
III

each should be chosen where the absorption of one of the compounds is large. In the spectral range 320-430 mμ, substances other than the chlorophylls and carotenoids frequently give high optical densities, eliminating this range from the useful portion of the spectrum.

Specific absorption coefficients of 90 per cent acetone solutions of chlorophylls a and b and beta carotene have been reported in the literature (13, 20), but are unknown for chlorophyll c and the xanthophylls expected in the plankton. In order to express relative concentrations of the latter compounds, Specified Pigment Units (SPU) are used. These units are defined so that one such unit in a liter of 90 per cent acetone, has at the wave length of maximum absorption, the optical density shown in Table 1. These optical densities were chosen equal (or nearly so) to the specific absorption coefficients of corresponding maxima in the spectra of related compounds, and thus the SPU represents a specific but unknown weight of the pigment which should be about one gram. For those pigments whose Specific Absorption Coefficients are unknown, the SPU is used instead of the gram in calculating Specified Absorption Coefficients. These are symbolized by E^{1gm}_{1cm} and E^{SPU}_{1cm} respectively. Concentrations of chlorophyll c and the xanthophylls in Table 1 can now be expressed in terms of SPU or thousandths thereof (MSPU).

Table 2 shows specific and specified absorption coefficients of a group of pigments to be found in plankton. These were calculated from previously reported absorption spectra (13) and the

Table 1

Optical Densities of Solutions Containing one Specified Pigment Unit per liter of 90% Acetone at Wave Length of Absorption Maximum

Compound	Wave Length of Absorption Maximum mμ	Specified Optical Density (E^{SPU}_{1cm}) of a one cm layer
Chlorophyll c	445	83.5
Neofucoxanthin A	447-8	251
Neofucoxanthin B	446-8	251
Fucoxanthin	448-9	251
Diatoxanthin	451	251
Diadinoxanthin	444-5	251
Astacin-type pigment	475	251

Table 2

Specific and Specified Absorption Coefficients of Some Plankton Pigments in 90% Acetone Solution*

Wave Length, mμ	Chlorophyll a E^{1gm}_{1cm}	Chlorophyll b E^{1gm}_{1cm}	Chlorophyll c E^{SPU}_{1cm}	Beta Carotene E^{1gm}_{1cm}	Neofucoxanthin A E^{SPU}_{1cm}	Neofucoxanthin B E^{SPU}_{1cm}	Fucoxanthin E^{SPU}_{1cm}	Diadinoxanthin E^{SPU}_{1cm}	Diatoxanthin E^{SPU}_{1cm}	Astacin type pigment E^{SPU}_{1cm}	Non-astacin type Carotenoids in average mixture (p.9) E^{SPU}_{1cm}
665	66.7	6.5	1.1	0	0	0	0	0	0	0	0
645	16.4	45.6	4.4	0	0	0	0	0	0	0	0
630	11.9	12.7	10.4	0	0	0	0	0	0	0	0
510	2.6	--	2.1	45.5	62.0	51.3	56.6	9.0	33.7	169	45
480	1.9	13.6	5.4	223	205	190	203	186	210	249	203
450	8.9	54.0	78.5	244	249	248	249	239	250	221	246
420	70.7	26.8	37.3	148	190	192	169	181	184	147	171

* Values from Richards and Thompson (13) except chlorophyll b values, which are from Zscheile, Comar, and Mackinney (20).

definition of Specified Pigment Units given above. Using them, the concentration in grams or SPU of the pigments in a mixture can be calculated from the total absorption at these wave lengths. In practice, it has been found necessary to group those carotenoids having absorption maxima close to 450 mμ, and calculate the concentration of this group as a whole.

Considerations which led to simplifications enabling the above calculation of the two types of carotenoids follow:

The principal carotenoids of diatoms and dinoflagellates are beta carotene, fucoxanthin, neofucoxanthin A and B, diatoxanthin, diadinoxanthin, and pigments of very similar absorption spectra. These were found by Strain, Manning and Hardin (18) and Richards and Thompson (13) in a variety of diatomaceous and brown algal materials examined by chromatographic and spectrophotometric methods. The absorption spectra of peridinin, reported by Strain, et al. as the principal carotenoid of dinoflagellates, and of neoperidinin are very similar to that of fucoxanthin, although the two are chromatographically separable. If the above carotenoids occur in a fixed ratio in the plankton, they can be grouped together and absorption coefficients for the average mixture calculated.

Pace (11) has reported quantitative analyses of the pigments occurring in cultures of the marine diatom, Nitzschia Closterium, which can be used to compute average absorption coefficients. He found five xanthophyll fractions which, on the basis of the work of Strain, et al.

(18) and the writers (13) are assumed to be neofucoxanthin A and B, fucoxanthin, diadinoxanthin, and diatoxanthin. Revising Pace's identifications in the light of the more recent work, he found these pigments occurring in the following averaged ratios:

Pace's Identification	mg/100 gms dry weight	Fraction of Total	Revised Identification
Beta carotene	65.9	.107	
Cryptoxanthin	11.1	.018	[Neofucoxanthin A or B]
Lutein	87.9	.142	[Diadino- or Diatoxanthin]
Isolutein	22.2	.036	[Neofucoxanthin A or B]
Fraction Y	339.1	.549	[Fucoxanthin]
Fraction Z	90.2	.146	[Diadino- or Diatoxanthin]
Total	616.4		

Fraction Y [fucoxanthin] is by far the most important contributor to the mixture.* The other less abundant pigments probably occur in a more or less fixed ratio to fucoxanthin.

From the foregoing ratios and the absorption coefficients of the individual carotenoids, average specified absorption coefficients for these carotenoids were calculated. These coefficients are the sum of the products of the ratios of the pigments found by Pace and the absorption coefficients shown in Table 1. Averaged coefficients were

*Strain, et al. and the present writers have found this to be the case in other diatoms as well as brown algae. Strain (16) also reports that in leaf plants, the ratio in which the xanthophylls occur is rather constant until autumnal yellowing begins.

used for Neofucoxanthin A plus B and for diatoxanthin plus diadinoxanthin.

At 430 mμ E^{SPU}_{1cm}, total non-astacin carotenoids = 171

450 mμ E^{SPU}_{1cm}, total non-astacin carotenoids = 246

480 mμ E^{SPU}_{1cm}, total non-astacin carotenoids = 203

510 mμ E^{SPU}_{1cm}, total non-astacin carotenoids = 45

These are the coefficients used in the equations on page 5 for the calculation of carotenoid concentrations.

Carotenoids of the astacin* type (Kuhn and Lederer, 9), presumably characteristic of the crustaceans, can also be estimated when they become an important part of the plankton pigments. Samples of this pigment were obtained from two plankton tows consisting mainly of crustaceans (copepods, other crustaceans, crustacean larvae) and a few diatoms. The spectra of 90 per cent acetone extracts of this material showed that one contained very small amounts, the other appreciable concentrations of chlorophylls. Absorption spectra of these extracts, corrected for the chlorophyll content, are shown in Fig. 2 and Table 3. These spectra are very similar to those of pigment extracts from shrimp (Brown, 1) and a chloroform extract of lobster blood (Redfield, 12) and can be tentatively identified as

*The absorption maxima of carotenoids having 11 conjugated double bonds occur around 450 mμ in acetone solution, whereas the maxima in the spectra of those carotenoids having 13 conjugated double bonds (e.g., astacin) should occur 25-30 mμ closer to the red.

FIGURE 2

Specified absorption spectrum

of astacin type carotenoids.

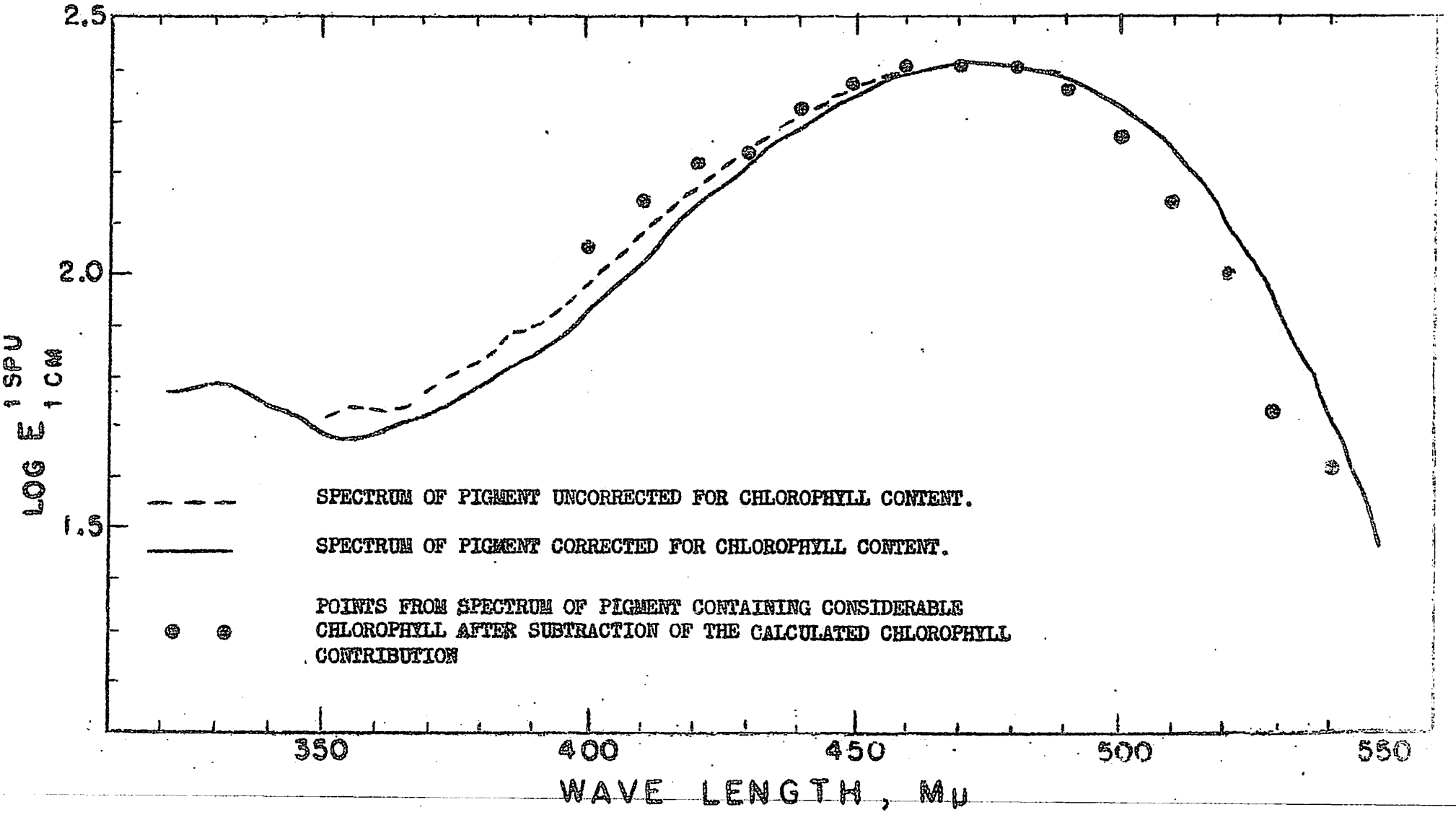

2.5
2.0
1.5
LOG E 1 SPU 1 CM
350
400
450
500
550
WAVE LENGTH, Mμ
SPECTRUM OF PIGMENT UNCORRECTED FOR CHLOROPHYLL CONTENT.
SPECTRUM OF PIGMENT CORRECTED FOR CHLOROPHYLL CONTENT.
POINTS FROM SPECTRUM OF PIGMENT CONTAINING CONSIDERABLE CHLOROPHYLL AFTER SUBTRACTION OF THE CALCULATED CHLOROPHYLL CONTRIBUTION

Table 3

Relative Absorption Spectrum of a 90% Acetone Solution of Astacin-type pigment extracted from Crustaceans, corrected for Chlorophyll a absorption.

Wave Length mμ	E_{1cm}	Wave Length mμ	E_{1cm}	Wave Length mμ	E_{1cm}	Wave Length mμ	E_{1cm}
350	47.2	400	84.3	450	220.9	500	204.2
355	48.9	405	92.7	455	236.0	510	168.7
360	47.2	410	106.2	460	236.0	520	126.5
365	50.6	415	121.3	465	246.0	530	86.1
370	52.2	420	134.9	470	251.2	540	50.5
380	59.0	425	146.9	475	251.2	550	28.6
390	69.0	430	161.8	480	249.5	560	16.9
		435	177.0	490	227.5		
		440	195.4				
		445	208.9				

astacin, which Kuhn and Lederer (9) prepared from the Norwegian lobster, Astacus gammarus. Since the absorption maximum of astacin in carbon disulfide and pyridine occurs at 500 mμ, and in acetone solution should occur at wave lengths 25-30 mμ nearer the blue (16), it is concluded that the spectra represent a pigment or pigments of the astacin type occurring in naturally growing crustaceans. Following the practice established on page 8, a specified pigment unit of the astacin-type carotenoid was defined so that the optical density as in Table 1 is 251 at 475 mμ. Specified absorption coefficients computed on this basis are shown in Table 2.

Instrumental Errors:

At low optical densities, errors in reading the spectrophotometer might become important, but at these densities the scale divisions are large and readings are reproducible within ± .0015 density (log I_o/I) units. The concentrations of the five components at which these instrumental errors cause 5 per cent, 10 per cent and 20 per cent errors in concentrations are shown in Table 4. An absorption cell such as described by Kirk, et al. (7) in which the length to volume ratio is increased, should permit greater precision in the determination of the optical densities of the more dilute solutions.

In plankton extracts, chlorophyll b concentrations have been found to be very small or absent; calculations frequently lead to small negative values which are within the instrumental error.

Chlorophyll c:

Large errors in the determination of chlorophyll c might be expected because of its small absorption in the red spectral range. In general these errors are difficult to estimate, particularly in view of the difficulty in preparing the pure material for study.

At concentrations of chlorophyll c frequently found in collections of natural plankton, the instrumental error (Table 3) would frequently be very large. However, both chlorophyll c and carotenoid concentrations can be checked by computing them from the optical density at 450 mμ, where the non-chlorophyll absorption is represented by

$$D_{res,450} = D_{450} - .0089\ C_a - .054\ C_b - .0785\ C_c$$

Table 4

Compound	Concentrations of Pigments and Corresponding Instrumental Errors		
	± 20% or over	± 10%	± 5%
Chlorophyll a, mg/L	≤ .16	.32 mg/L	.64 to 12.8*
Chlorophyll b, mg/L	≤ .25	.50	1.00 to 20.0*
Chlorophyll c, MSPU/L	≤ 1.09	2.18	4.36 to 87.2*
Non-astacin Carotenoids MSPU/L	≤ .105	.210	.420 to 7.12*
Astacin type Carotenoids MSPU/L	≤ .190	.380	.760 to 6.08*

* At higher concentrations there is a deviation from Beer's Law and extracts should be diluted.

From the large chlorophyll c coefficient (this is within 5 mμ of its absorption maximum), it can be seen that errors in its estimation would result in inordinately large errors in $D_{res,450}$.

The good checks (± 5%) which were found between carotenoid values calculated from optical densities at this wave length and at 480 mμ, afford verification of both chlorophyll c and carotenoid concentrations.

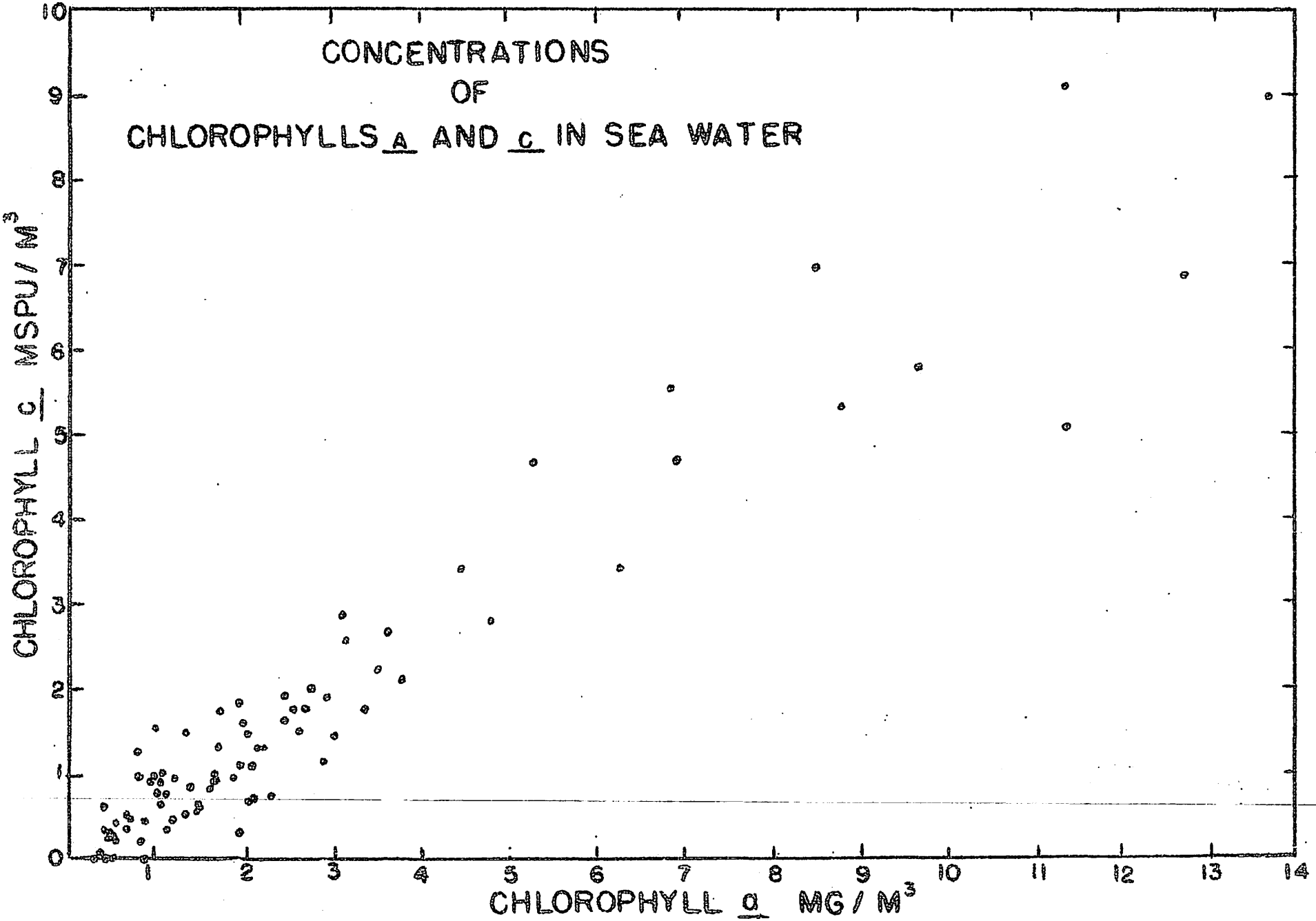
The chlorophyll c values shown in Figure 3 may appear high (even considering the errors discussed above) for a pigment which so many workers in the field have disregarded as unimportant. A search has failed to reveal reports of other determinations of chlorophyll c in phytoplankton. However, Pace determined chlorophyll a and b in extracts of Nitzschia Closterium (mg per 100 gm dry weight) as follows:

a	b
2310	4
2000	95
1850	130
2530	240

If N. Closterium contains chlorophyll c instead of b as is true of other diatoms (13,18), then Pace's values (assuming chlorophyll b) should be recalculated. His determinations were based on a calibration curve prepared using a chlorophyll b standard, which would have a much greater optical density in the region measured than an equal concentration of chlorophyll c. This difference would be greater than the ratio of the spectral maxima because both peaks are broad

FIGURE 3

Chlorophyll a and c concentrations found in sea water samples.


CONCENTRATIONS
OF
CHLOROPHYLLS A AND C IN SEA WATER
CHLOROPHYLL c MSPU / M³
CHLOROPHYLL a MG / M³
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14

for their height and a filter photometer with a relatively wide transmission was used, thus involving the area under the absorption curve. His filter would eliminate about one half of the chlorophyll c absorption, but include most of the peak of the chlorophyll b used for the construction of the calibration curve. It is estimated that the values given by Pace for the second (non-chlorophyll a) chlorophyll component are too low, perhaps by a factor of ten to twenty. Therefore Pace's small chlorophyll b values would represent rather large amounts of chlorophyll c.

Carotenoids:

Calculations of non-astacin carotenoid concentrations from independent measurements at 450 and 486 mμ gave good checks (± 5%). Values calculated from optical densities at 420 mμ

$$(D_{res,420} = D_{420} - .0707 \times C_a - .0268 \times C_b - .0373 \times C_c)$$

were found to be high because of invisible impurities, as expected*. Density readings at 510 mμ are generally low; when astacin-type carotenoids are less than 8-10 per cent of the total pigments, the calculated concentrations are probably unreliable.

*This observation, and the frequently observed very high optical densities in the spectral regions 320-400 mμ, appears consistent with investigations by Lane (10) of a "plankton oil" extracted from zooplankton. The material Lane describes as a non-carotene provitamin A has a very high absorption maximum near 310 mμ and appreciable absorption at 400 and 450 mμ, dropping to negligible values at 500 mμ.

Over-all Errors:

A series of analyses made on as nearly identical samples as could be prepared showed good reproducibility of results. The over-all errors would include errors in preparing identical samples, in the operation of the centrifuge, measuring of extractant, et cetera. They are shown in Table 5 and reproducibility of the same order has been achieved by students using the method in classroom and field work. Experience in using the method should afford increased reproducibility.

Summary:

A semi-micro spectrophotometric method for the estimation of chlorophylls a, b, and c, astacin type, and non-astacin type carotenoids in acetone extracts of plant and animal material is presented. Developed specifically for use in estimating and characterizing plankton populations, the method is highly sensitive and practical for shipboard use. Methods of collecting, preparing and extracting plankton samples, spectrophotometric measurements, computation of results, and errors are discussed.

Table 5

Instrumental and Other Errors Found in Over-all Analytical Procedure

Pigment and Concentration	Maximum Deviation	Expected Instrumental Error	Other Errors
Chlorophyll a 1.0 mg/L	14%	± 5%	9%
Chlorophyll c .75 SPU/L	43%	Ca. ± 30%	ca. ± 13%
Astacin-type Carotenoids 1.17 SPU/L	20%	± 5%	15%
.40 SPU/L	10%	± 10%	--
Non-astacin type Carotenoids 1.0 SPU/L	6%	5%	1%

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