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# THE ESTIMATION AND CHARACTERIZATION OF PLANKTON POPULATIONS BY PIGMENT ANALYSES

## II. A SPECTROPHOTOMETRIC METHOD FOR THE ESTI- MATION OF PLANKTON PIGMENTS<sup>1</sup>

By

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### ABSTRACT

A semimicro spectrophotometric method is described for the estimation of chlorophyll *a*, *b*, and *c* and of astacin and nonastacin 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 ship-board use. Methods of collecting, preparing and extracting plankton samples, spectrophotometric measurements, computation of results, and errors are discussed.

### INTRODUCTION

A semimicro method has been developed for the simultaneous spectrophotometric determination of chlorophylls *a*, *b*, and *c* and of two types of carotenoids in 90% acetone extracts of plant and animal material. This method, which avoids the separation and individual determination of the several components, results in minimum 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) a measure of the potential of the plankton for absorbing radiant energy for photosynthesis, (b) some measure of the extent and stage of development of the phytoplankton, and (c) a possible measure of the presence of animals grazing on the crop. The method can be considered as a logical development of "pigment unit" determinations by Harvey (5) and of chlorophyll determinations by Graham, Kozminski, Krey, Riley, Tucker, and others (4, 7, 8, 14, 15, 20).

The method meets the practical requirements necessary for the

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determination of pigments in plankton on shipboard. It permits the use of relatively small water samples rather than net catches; it also enhances the value of surface samples and microplankton, and it aids in 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% Acetone: Reagent grade acetone is distilled over aqueous  $\text{Na}_2\text{CO}_3$  and  $\text{Na}_2\text{SO}_3$  solution through an 18 inch fractionating column. 100 ml of water are pipetted into a one liter flask and made up to the mark with acetone, B.p. 56.5° C.

Magnesium carbonate: Reagent grade powdered magnesium carbonate.

*Procedure.* About 0.1 g 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<sup>3</sup> 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 one liter flows through the centrifuge in about seven minutes. The centrifuge is turned on before the flow is started and is left on until the aspirator bottle is completely rinsed and the centrifuge 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 is removed.

The particulate matter in the cup is scrubbed down with a rubber policeman and is rinsed into a 16 or 20 by 125 mm screw-cap test tube; the cup is then rerinsed. The material is then centrifuged at high speed in a clinical centrifuge for three minutes, the water is poured off, and the particulate matter is allowed to drain in the tube. The tube is placed in a vacuum desiccator and dried.

When the tube is dry, a definite volume of 90% acetone is pipetted into it and is 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, tightly capped, is then placed in the dark for 18-24 hours, after which time the material is again mixed; the tube is then recapped and the material is 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 absorbencies at 665, 645, 630,

<sup>3</sup> A large leveling bulb has been found more convenient than an aspirator bottle.





510, and 480  $m\mu$  are determined on a Beckman Model DU quartz spectrophotometer (2, 3). The red-sensitive tube is used for readings at 665, 645, 630  $m\mu$ , the blue-sensitive tube at 510 and 480  $m\mu$ . The tungsten filament light source is 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 absorbencies ( $\log I_0/I$ ) at 665, 645 and 630  $m\mu$  respectively and are determined directly with the spectrophotometer.  $C_a$ ,  $C_b$  and  $C_c$  are concentrations of chlorophylls  $a$ ,  $b$ , and  $c$  in milligrams per liter. Solving these equations:

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

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

$$C_c(\text{MSPU/L})^4 = 109 D_{630} - 12.5 D_{665} - 28.7 D_{645} .$$

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

The concentrations of two carotenoid components are calculated from absorbencies at 510 and 480  $m\mu$ . The contributions of the chlorophylls to these absorbencies are computed and subtracted, leaving residual absorbencies ( $D_{res}$ ) 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 nonastacin type and astacin type carotenoids.

Solving:

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

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

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

$$D_{665} = .068, D_{645} = .020, D_{630} = .0185, D_{510} = .127 \text{ and } D_{480} = .325 ;$$

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

$$= 1.01 \text{ mg/L} ;$$

$$\text{Chlorophyll } b, \text{ mg/L} = 25.4 \times .020 - 4.4 \times .068 - 10.3 \times .0185$$

$$= 0.02 \text{ mg/L} ;$$

$$\text{Chlorophyll } c, (\text{MSPU/L}) = 109 \times .018 - 12.5 \times .068 - 28.7 \times .020$$

$$= 0.59 \text{ MSPU/L} ;$$

<sup>4</sup>For definition and explanation of MSPU, see page 160.



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

$$= .123 ;$$

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

$$= .320 ;$$

$$\text{Astacin type carotenoids, MSPU/L} = 2(4.45 \times .123 = .320)$$

$$= 0.45 \text{ MSPU/L} ;$$

$$\text{Nonastacin carotenoids, MSPU/L} = 7.6 (.320 - 1.49 \times .123)$$

$$= 1.04 \text{ MSPU/L} .$$

The concentrations in a known volume of the extract can be converted to concentrations of pigments in the original sea water by multiplying by the appropriate factor according to the volume of sea water used, the final extraction volume, and the units in which the concentrations are expressed. For example, if 1.50 l of sea water were used and if the final volume of the acetone extract prior to spectrophotometric analysis were 5.0 ml, multiplying by the factor 5.0/1.50 would give the concentration in mg/M<sup>3</sup> for chlorophylls *a* and *b* or MSPU/M<sup>3</sup> in the case of chlorophyll *c*, astacin and nonastacin carotenoids.

The concentrations of the pigments in the extracts in the example above can be converted to concentrations in sea water as follows:

$$\text{Chlorophyll } a = 1.01 \times 5.0/1.50 = 3.37 \text{ mg/M}^3 ;$$

$$\text{Chlorophyll } b = 0.02 \times 5.0/1.50 = 0.07 \text{ mg/M}^3 ;$$

$$\text{Chlorophyll } c = 0.59 \times 5.0/1.50 = 1.97 \text{ MSPU/M}^3 ;$$

$$\text{Astacin type carotenoids} = 0.45 \times 5.0/1.50 = 1.50 \text{ MSPU/M}^3 ;$$

$$\text{Nonastacin type carotenoids} = 1.04 \times 5.0/1.50 = 3.47 \text{ MSPU/M}^3 .$$

The individual calculations of the several components of the carotenoid mixture were 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, which depends on the phylogenetic groups represented in the plankton (17) is unknown.

## DISCUSSION OF PROCEDURE AND CALCULATIONS

*Removal of Plankton from Water Samples.* The most satisfactory apparatus for the removal of plankton from water samples is the Foerst plankton centrifuge. Various filters—paper, sintered glass, and Caldwell type, were used both with and without freshly precipitated barium sulfate, but they were rejected because of inadequate speed or retention and because of the presence of extraneous matter in the

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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 was found to be inadequate, although other workers have used much shorter periods (4, 5, 7, 14). After 24 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, absorbencies below 0.8 were directly proportional to concentrations, conforming to Beer's Law. It was necessary to dilute extracts having absorbencies over 0.8.

*Calculation of Results.* Absorbencies of some of the mixtures of pigments obtained by acetone extractions of plankton samples were determined over the spectral range 320–700  $m\mu$  (Fig. 1A, B). The concentrations of the pigments can be computed from these spectra, since the absorbency 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 each should be chosen where the absorption of one of the compounds is large. In the spectral range 320–430  $m\mu$ , substances other than the chlorophylls and carotenoids frequently give high absorbencies, eliminating this range from the useful portion of the spectrum.

*Specific absorption coefficients* of 90% acetone solutions of chlorophylls *a* and *b* and beta carotene are reported in the literature (13, 21), but they are unknown for chlorophyll *c* and for 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% acetone has, at the wave length of maximum absorption, the absorbency shown in Table I. These absorbencies 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 undetermined 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  $E_{lcm}^{lgm}$  and  $E_{lcm}^{SPU}$  respectively.

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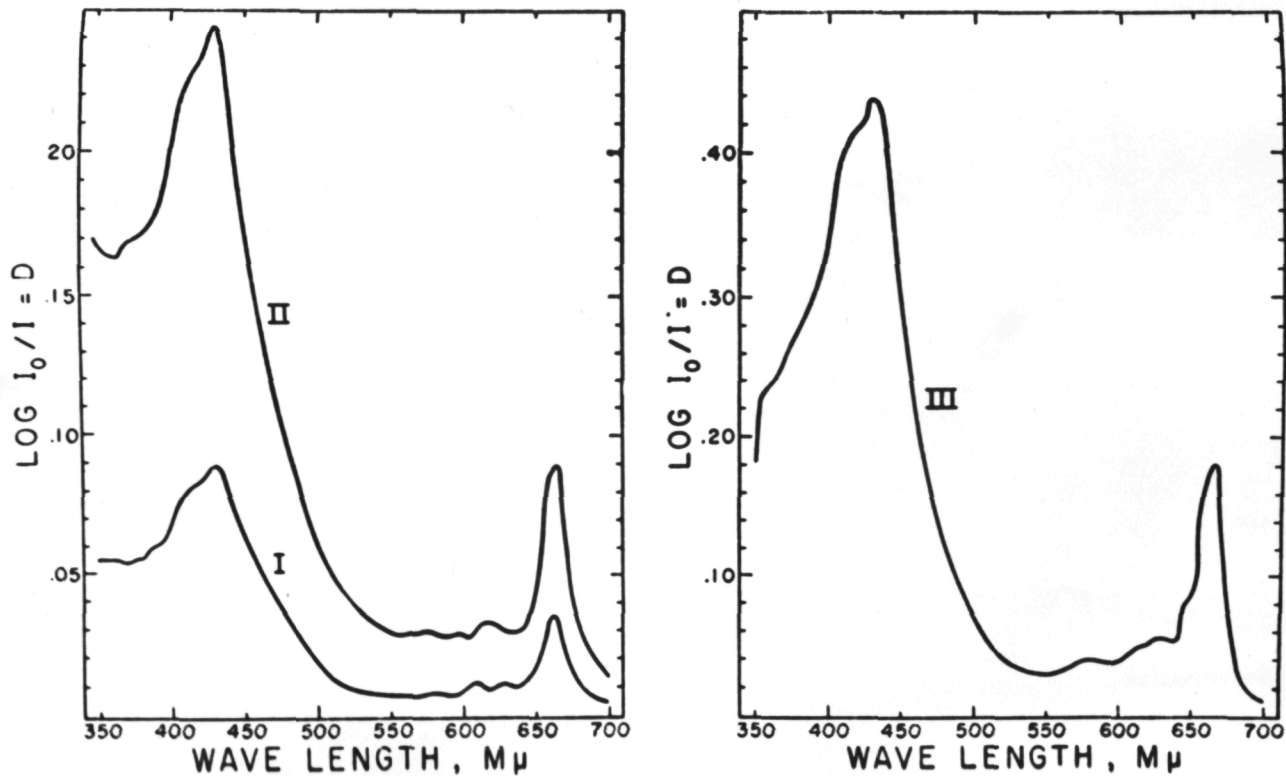


Figure 1. A (left) B (right). Absorption spectra of 90% acetone extracts of natural plankton collections.

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Concentrations of chlorophyll *c* and the xanthophylls in Table I can thus be expressed in terms of SPU or in thousandths thereof (MSPU).

Table II shows specific and specified absorption coefficients for a group of pigments to be found in plankton. These were calculated from absorption spectra (13) and according to the definition of Specified Pigment Units given above. Using them, the concentrations 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

TABLE I. ABSORBENCIES OF SOLUTIONS CONTAINING ONE SPECIFIED PIGMENT UNIT PER LITER OF 90% ACETONE AT WAVE LENGTH OF ABSORPTION MAXIMUM

Compound	Wave Length of Absorption Maximum, $m\mu$	Specified
		Absorbency ( $E_{1cm}^{SPU}$ ) of a one cm layer
Chlorophyll <i>c</i>	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

necessary to group together those carotenoids with absorption maxima close to 450  $m\mu$  and to calculate the concentration of this group as a whole.

Simplifications tacit in 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, diatloxanthin, diadinoxanthin, and pigments of very similar absorption spectra. These were found by Strain, Manning and Hardin (19) and by Richards (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 carotenoids of dinoflagellates, and of neoperidinin are similar to that of fucoxanthin, although chromatographically they are separable. If the above carotenoids occur in a fixed ratio in the plankton, then they can be grouped together, and absorption coefficients for the average mixture can be calculated.

Pace (11) has reported quantitative analyses of the pigments that occur in cultures of the marine diatom *Nitzschia Closterium*, and these ratios can be used to compute average absorption coefficients. He found five xanthophyll fractions which, on the basis of recent work by



TABLE II. SPECIFIC AND SPECIFIED ABSORPTION COEFFICIENTS OF SOME PLANKTON PIGMENTS IN 90% ACETONE SOLUTION\*

Wave Length $M_{\mu}$	Chlorophyll a E 1gm 1cm	Chlorophyll b E 1gm 1cm	Chlorophyll c SPU E 1cm	Beta Carotene E 1gm 1cm	Neofucozanthin A SPU E 1cm	Neofucozanthin B SPU E 1cm	Fucozanthin SPU E 1cm	Diadinozanthin SPU E 1cm	Diatoxanthin SPU E 1cm	Asclacin-type pigment SPU E 1cm	Nonastacin type Carotenoids in average mixture (p. 158) SPU E 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	3.5†	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 (13), except chlorophyll b values, which are from Zecheile, Comar, and Mackinney (21).

† Estimated.

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Strain, et al. (19) and by Richards (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 that these pigments occur in the following averaged ratios:

<i>Pace's Identification</i>	<i>mg/100 gm Dry Weight</i>	<i>Fraction of Total</i>	<i>Revised Identification</i>
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
	616.4		

Fraction Y (fucoxanthin) is by far the most important contributor to the mixture.<sup>5</sup> The other less abundant pigments probably occur in a more or less fixed ratio to fucoxanthin.

From the foregoing ratios as well as from 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 I. Averaged coefficients were used for neofucoxanthin *A* plus *B* and for diatoxanthin plus diadinoxanthin.

At 420  $m\mu$  E  $\frac{\text{SPU}}{1\text{cm}}$ , total nonastacin carotenoids = 171 ;

450  $m\mu$  E  $\frac{\text{SPU}}{1\text{cm}}$ , total nonastacin carotenoids = 246 ;

480  $m\mu$  E  $\frac{\text{SPU}}{1\text{cm}}$ , total nonastacin carotenoids = 203 ;

510  $m\mu$  E  $\frac{\text{SPU}}{1\text{cm}}$ , total nonastacin carotenoids = 45 .

These are the coefficients used in the equations on page 158 for the calculation of carotenoid concentrations.

Carotenoids of the astacin<sup>6</sup> type (Kuhn and Lederer, 9), presumably

<sup>5</sup> 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.

<sup>6</sup> The absorption maxima of carotenoids having 11 conjugated double bonds occur around 450  $m\mu$  in acetone solution, whereas the maxima in the spectra of those carotenoids having 13 conjugated double bonds (e. g., astacin) should occur at 25-30  $m\mu$  closer to the red.

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TABLE III. RELATIVE ABSORPTION SPECTRUM OF A 90% ACETONE SOLUTION OF ASTACIN-TYPE PIGMENT EXTRACTED FROM CRUSTACEANS, CORRECTED FOR CHLOROPHYLL *a* ABSORPTION

Wave Length	$E_{1cm}$	Wave Length	$E_{1cm}$	Wave Length	$E_{1cm}$	Wave Length	$E_{1cm}$
$M_\mu$		$M_\mu$		$M_\mu$		$M_\mu$	
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				

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% acetone extracts of this material showed that one contained very small amounts of chlorophylls, the other appreciable concentrations. Absorption spectra of these extracts, corrected for the chlorophyll content, are shown in Fig. 2 and Table III. These spectra, very similar to those of pigment extracts from shrimp (Brown, 1) and a chloroform extract from lobster blood (Redfield, 12), can be tentatively identified as 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  $m_\mu$ , and since the absorption maximum of astacin in acetone solution should occur at wave lengths 25–30  $m_\mu$  nearer the blue (16), it is concluded that the spectra represent a pigment (or pigments) of the astacin type which occurs in naturally growing crustaceans. Following the practice established on page 160, a specified pigment unit of the astacin-type carotenoid was defined so that the absorbcency, as in Table I, is 251 at 474  $m_\mu$ . Specified absorption coefficients computed on this basis are shown in Table II.

*Instrument Errors.* At low absorbcencies, errors in reading the spectrophotometer might become important, but at these densities the scale divisions are large and readings are reproducible within  $\pm .0015$  absorbcency (log  $I_0/I$  units). The concentrations of the five components at which these instrumental errors cause 5, 10 and 20% errors in concentrations are shown in Table IV. An absorption cell, such as

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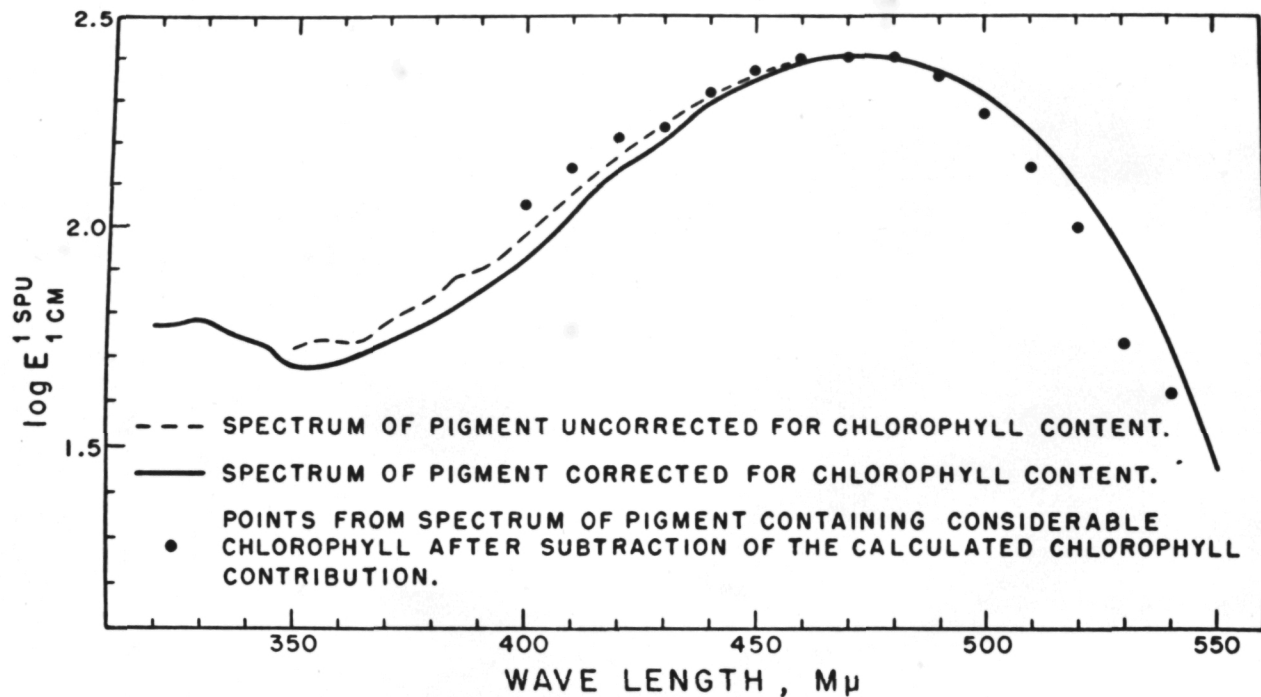


Figure 2. Specified absorption spectrum of astacin-type carotenoids.

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that described by Kirk, et al. (6), in which the length to volume ratio is increased, should permit greater precision in the determination of the absorbencies 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.

TABLE IV. CONCENTRATIONS OF PIGMENTS AND CORRESPONDING INSTRUMENTAL ERRORS

Compound	±20% or over	±10%	±5%
Chlorophyll <i>a</i> , mg/L	.16	.32	.64 to 12.8*
Chlorophyll <i>b</i> , mg/L	.25	.50	1.00 to 20.0*
Chlorophyll <i>c</i> , MSPU/L	1.09	2.18	4.36 to 87.2*
Nonastacin 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.

*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 IV) would frequently be very large. However, both chlorophyll *c* and carotenoid concentrations can be checked by computing them from the absorbencies at 450  $m\mu$ , where the nonchlorophyll absorption is represented by

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

From the large chlorophyll *c* coefficient (this is within 5  $m\mu$  of its absorption maximum), it can be seen that errors in its estimation would result in inordinately large errors in  $D_{rea,450}$ . The good checks ( $\pm 5\%$ ), which were found between carotenoid values calculated from absorbencies at this wave length and at 480  $m\mu$ , afford verification of both chlorophyll *c* and carotenoid concentrations.

The chlorophyll *c* values shown in Fig. 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/100 g dry weight) as follows:

100

100

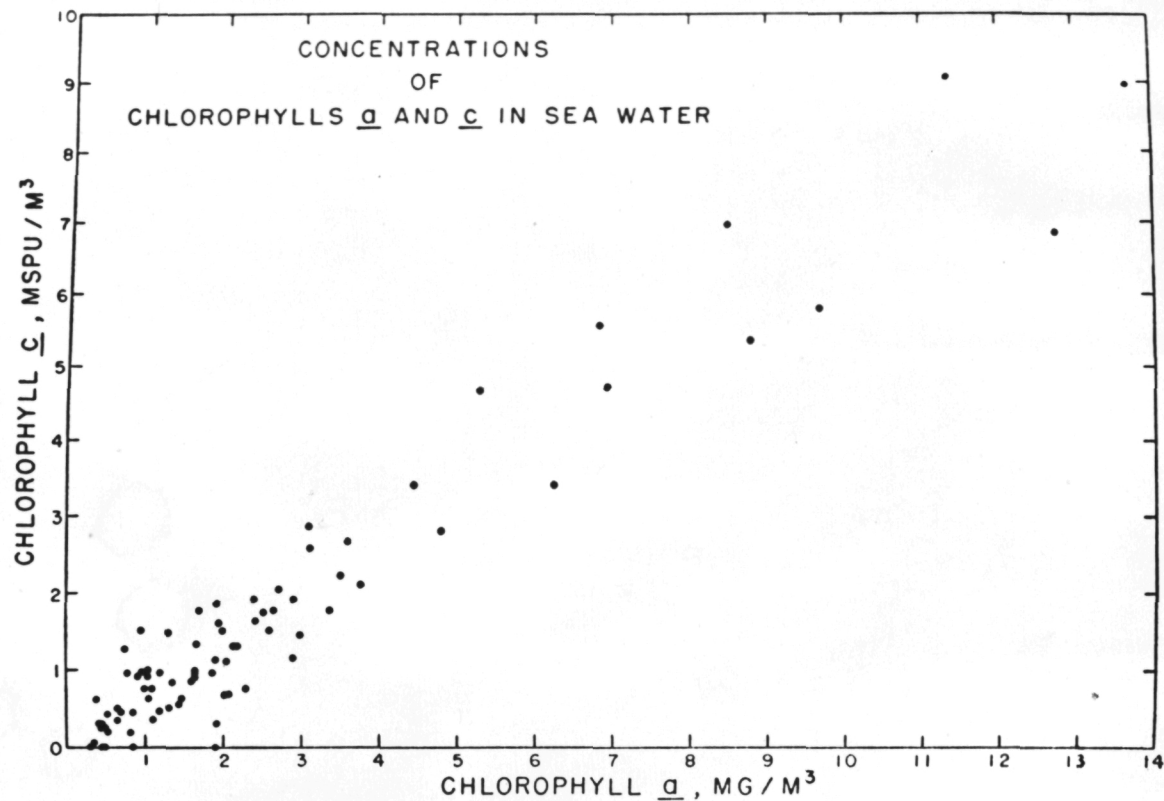
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100

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100

Figure 3. Chlorophyll  $\underline{a}$  and  $\underline{c}$  concentrations found in sea water samples.

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<i>a</i>	<i>b</i>
2310	+
2000	95
1850	130
2530	240

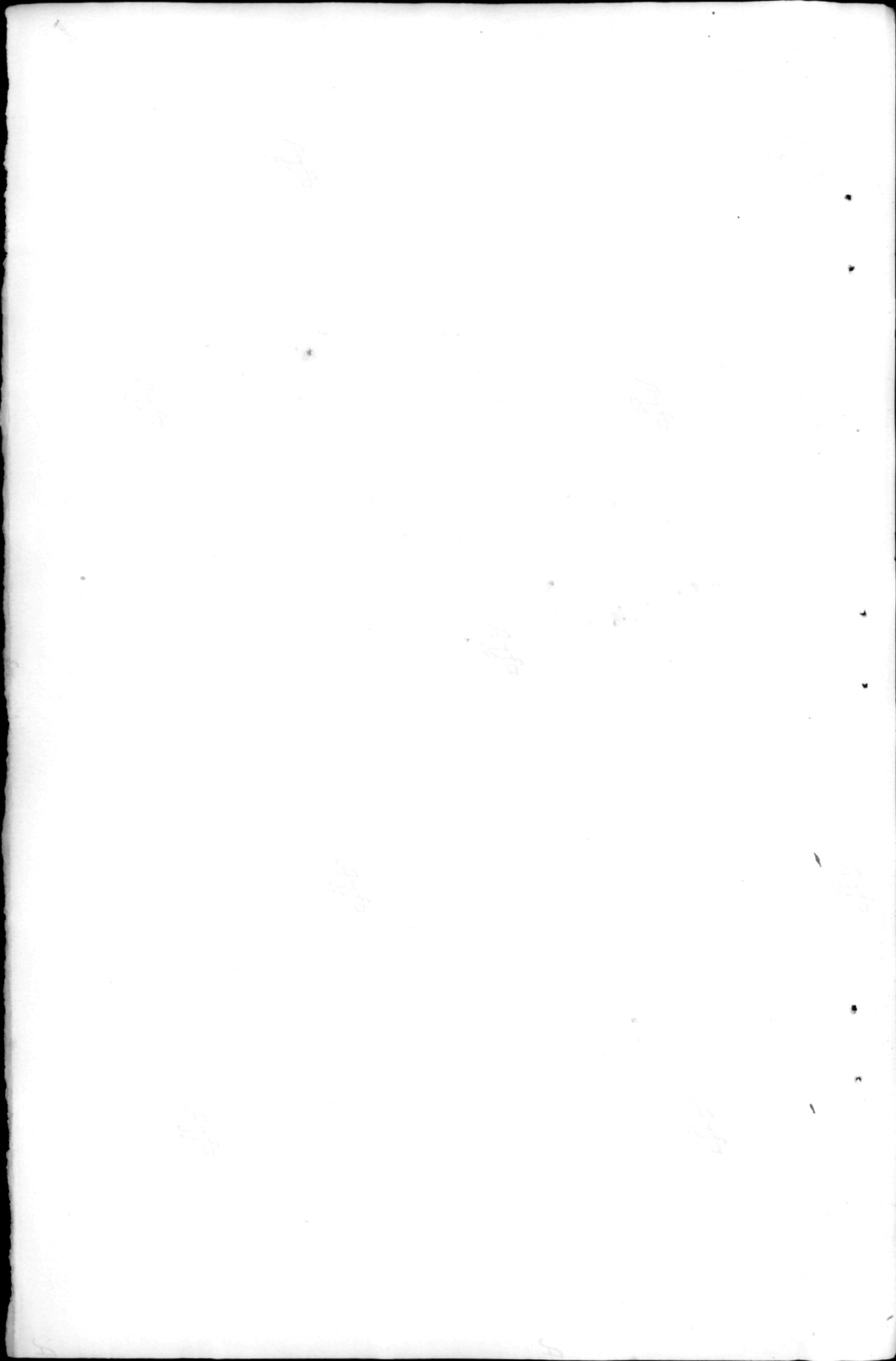
If *N. Closterium* contains chlorophyll *c* instead of *b*, as is true of other diatoms (13, 18), then Pace's values (assuming chlorophyll *b*) should be recalculated. His determinations were based on a calibration curve prepared with a chlorophyll *b* standard, which would have a much greater absorbency in the region measured than would an equal concentration of chlorophyll *c*. This difference would be greater than the ratio of the spectral maxima, because both peaks are broad for their height, and since a filter photometer with a relatively wide transmission was used, the area under the absorption curve was involved. His filter would eliminate about half of the chlorophyll *c* absorption, but it includes 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 (nonchlorophyll *a*) chlorophyll component are too low, perhaps by a factor of 10 to 20. Therefore, Pace's small chlorophyll *b* values would represent rather large amounts of chlorophyll *c*.

Perhaps a more important factor in the apparent concentration of chlorophyll *c* is the method used to prepare the material from which the coefficients in Table II were determined. Strain and Manning (18) observed that at 630  $m\mu$  the characteristic absorption coefficient of chlorophyll *c* (in methanol solution) prepared by solvent partition is about 0.1 unit larger than that prepared by chromatographic absorption. This would make the specified absorption coefficient at this wave length about 25% greater than that of chromatographed material. If this is also true of acetone solutions, the specified adsorption coefficient of unchromatographed material should be, at 630  $m\mu$ , 13.1 instead of 10.4 as shown in Table II, and the equations for computing the chlorophyll concentrations would be:

$$\begin{aligned} C_a \text{ (mg/L)} &= 15.7 D_{665} - 2.07 D_{645} - 0.64 D_{630} ; \\ C_b \text{ (mg/L)} &= 25.1 D_{645} - 4.75 D_{665} - 8.02 D_{630} ; \\ C_c \text{ (MSPU/L)} &= 85.6 D_{630} - 9.80 D_{665} - 2.25 D_{645} . \end{aligned}$$

*Carotenoids.* Calculations of nonastacin carotenoid concentrations from independent measurements at 450 and 480  $m\mu$  gave good checks ( $\pm 5\%$ ). Values calculated from absorbencies at 420  $m\mu$ ,

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





were found to be high because of invisible impurities, as expected.<sup>7</sup> Density readings at 510  $m\mu$  are generally low; when astacin-type carotenoids are less than 8–10% of the total pigments, the calculated concentrations are probably unreliable.

*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, etc. They are shown in Table V; 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.

TABLE V. INSTRUMENTAL AND OTHER ERRORS FOUND IN OVER-ALL ANALYTICAL PROCEDURE

<i>Pigment and Concentration</i>	<i>Maximum Deviation</i>	<i>Expected</i>	<i>Other Errors</i>
		<i>Instrumental Error</i>	
Chlorophyll <i>a</i> 1.0 mg/L	14%	$\pm 5\%$	9%
Chlorophyll <i>c</i> .75 MSPU/L	43%	Ca. $\pm 30\%$	Ca. $\pm 13\%$
Astacin-type Carotenoids 1.17 MSPU/L	20%	$\pm 5\%$	15%
.40 MSPU/L	10%	10%	—
Nonastacin-type Carotenoids 1.0 MSPU/L	6%	5%	1%

## SUMMARY

A semimicro spectrophotometric method is presented for the estimation of chlorophylls *a*, *b* and *c* as well as astacin and nonastacin 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.

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<sup>7</sup> This observation, and the frequently observed very high absorbencies in the spectral regions 320–400  $m\mu$ , appears consistent with investigations by Lane (10) of a "plankton oil" extracted from zooplankton. The material Lane describes as a noncarotene provitamin A has a very high absorption maximum near 310  $m\mu$  and appreciable absorption at 400 and 450  $m\mu$ , dropping to negligible values at 500  $m\mu$ .

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