A Fluorometric Method for Determining Chlorophylls a, b, *and* c[1]

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ABSTRACT

A method for the determination of chlorophylls *a*, *b*, and *c* by fluorometric techniques is presented. The effects of accessory carotenoids and chlorophyll derivatives on the accuracy of this method are analyzed. Good agreement between the results derived with this technique and those derived with the spectrophotometric method has been demonstrated in the absence of pheopigments in 90%-acetone extracts. An inherent error in the current fluorometric methods for the determination of chlorophyll *a* and pheophytin *a* is demonstrated, and a method for improving the estimation of pheophytin *a* is given.

1. *Introduction.* The estimated concentration of chlorophylls and their derivatives in phytoplankton samples is widely used in descriptions of plankton populations in natural waters. Pigment concentrations have been related to primary productivity (Ryther 1956, Ryther and Yentsch 1957, Ichimara et al. 1962, Curl and Small 1965), to microbial biomass (Holm-Hansen 1969), and to phytoplankton composition (Margalef 1961). Presently used methods of analysis have been reported in SCOR-Unesco (1966), and by Parsons and Strickland (1963), who employed spectrophotometric techniques, and by Yentsch and Mensel (1963) and Holm-Hansen et al. (1965), who employed fluorometric techniques.

The trichromatic equations used in spectrophotometric methods permit determination of chlorophylls *a*, *b*, and *c* in a pigment mixture, but this method requires the assumption that the absorbancy at the three wavelengths used is due to only chlorophylls. Vernon (1960), Lorenzen (1967), and Moss (1967a, 1967b), who have recognized that such an assumption may be invalid, have presented methods that make possible the quantitative estimates of pheopig-

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ments in 90%-acetone extracts, thus improving the chlorophyll-concentration estimates when chlorophyll degradation products occur in samples.

The fluorometric methods have the attractive features of rapidity and sensitivity when compared with spectrophotometric techniques. With the proper selection of excitation and emission filters, measurements can be made partially selective for chlorophyll-*a* fluorescence (Yentsch and Menzel 1963, Holm-Hansen et al. 1965). It has been recognized that other pigments that occur naturally in extracts can be a source of error with fluorometric techniques, as with the spectrophotometric methods. An extension of a fluorometric method that includes quantitative analyses of other pigments in extracts, with a limited sacrifice of sensitivity, has been suggested (Holm-Hansen et al. 1965). Methods for such an extension and an evaluation of these new techniques are the topic of this paper.

2. *General Considerations.* Compounds that fluoresce with a red color when they are excited by blue light and those that may be found in 90%-acetone extracts of phytoplankton material from natural waters are: chlorophylls *a*, *b*, and *c*, chlorophyllides *a* and *b*, pheophytins *a*, *b*, and *c*, pheophorbides *a* and *b*, and several porphyrins and metalloporphyrins that have not been well described (Falk 1964, Smith and Benitez 1955).[2] An accurate analysis of such a mixture appears to require chromatographic separation of the individual pigments prior to a fluorescence assay. However, a useful approximate analysis might be based on the following considerations: (i) the several porphyrins and metalloporphyrins that possibly interfere have not been observed in significant concentrations in natural samples (Holm-Hansen et al. 1965, Patterson and Parsons 1963), (ii) the absorption spectra of the chlorophylls and the respective chlorophyllides are similar (Patterson and Parsons 1963), and the fluorescence spectra might be expected to be similar (Rabinowitch 1956), (iii) the absorption spectra and the fluorescence of the pheophytins and the respective pheophorbides might be expected to be similar, and (iv) the fluorescence of a mixture is the sum of the fluorescence of the individual components without interaction of each with the others in a dilute solution (Goodwin 1947, Falk 1964). The extracts can thus be treated as a six-component mixture: chlorophyllous pigments *a*, *b*, and *c* and pheopigments *a*, *b*, and *c*.

When such a mixture is acidified, the existing chlorophyllous materials are converted to their pheopigment derivatives, producing a change in the fluorescence. As may be seen in Fig. 1, the spectral overlap in the fluorescent emission of the pigments and the transmission of the employed filters do not permit complete segregation of light fluoresced by any one component. However, measurements before and after acidification with three different filters provide

2. The structure of chlorophyll *c* has been determined recently (Dougherty et al. 1966) to be that of a chlorophyllide, but the commonly used term chlorophyll *c* will be used in this paper. Similarly, the magnesium-free compound is properly pheophorbide *c*, but it will be termed pheophytin *c* here.

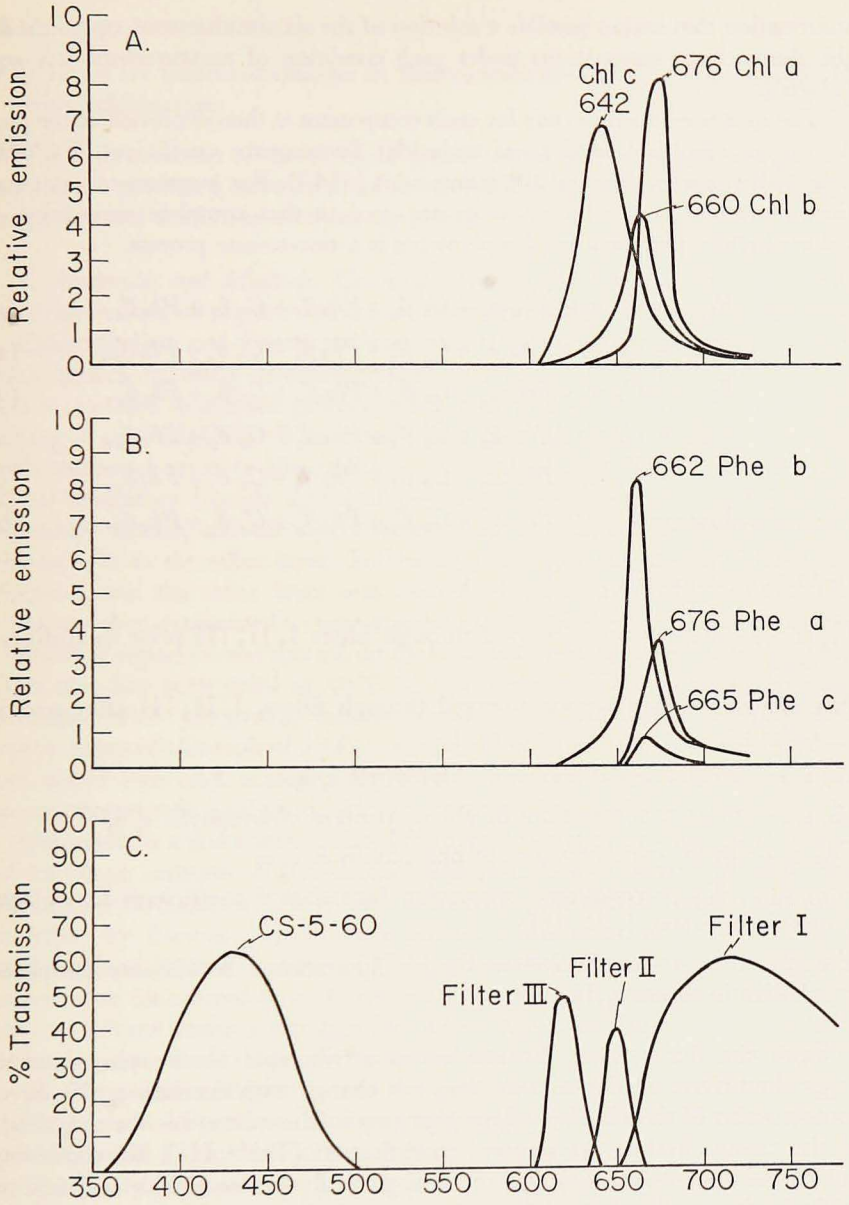

Figure 1. A, B. The relative emission spectra of equimolar concentrations of chlorophylls and pheophytins in 90% acetone, with excitation light provided by a mercury-vapor lamp and transmitted through Corning-glass filter CS-5-60 used in fluorometer. The measurements were made with a quarter-meter spectrometer. The corrections for photocathode sensitivity with wavelength were applied. — C. The transmission spectra of the excitation filter (CS-5-60, above) and emission filters I, II, and III described for this analysis.

information that makes possible a solution of the six simultaneous equations for the fluorescence summations under each condition of measurement [see eqs. (1)–(6)].

The measured fluorescence for each component is then expressed as the product of an empirical conditional molecular fluorescence coefficient (F.l./nM) and of the concentration of the component (nM/l). For purposes of computation, molecular concentration units are used so that complete conversion of chlorophyllous pigments to pheopigments is a one-to-one process.

$$F_I = C_a\,\alpha_1 + Ph_a\,\alpha'_1 + C_b\,\beta_1 + Ph_b\beta'_1 + C_c\,\delta_1 + Ph_c\delta'_1, \quad (1)$$

$$F_{IA} = C'_a\,\alpha'_1 + Ph_a\,\alpha'_1 + C'_b\,\beta'_1 + Ph_b\beta'_1 + C'_c\,\delta'_1 + Ph_c\delta'_1, \quad (2)$$

$$F_{II} = C_a\,\alpha_2 + Ph_a\,\alpha'_2 + C_b\,\beta_2 + Ph_b\beta'_2 + C_c\,\delta_2 + Ph_c\delta'_2, \quad (3)$$

$$F_{IIA} = C'_a\,\alpha'_2 + Ph_a\,\alpha'_2 + C'_b\,\beta'_2 + Ph_b\beta'_2 + C'_c\,\delta'_2 + Ph_c\delta'_2, \quad (4)$$

$$F_{III} = C_a\,\alpha_3 + Ph_a\,\alpha'_3 + C_b\,\beta_3 + Ph_b\beta'_3 + C_c\,\delta_3 + Ph_c\delta'_3, \quad (5)$$

$$F_{IIIA} = C'_a\,\alpha'_3 + Ph_a\,\alpha'_3 + C'_b\,\beta'_3 + Ph_b\beta'_3 + C'_c\,\delta'_3 + Ph_c\delta'_3. \quad (6)$$

In these equations:

$F_{I,\,II,\,III}$ = fluorescence observed through filters I, II, III prior to acidification;

$F_{IA,\,IIA,\,IIIA}$ = fluorescence observed through filters, I, II, III after acidification;

$C_{a,\,b,\,c}$ = molar concentrations of chlorophyll *a*, *b*, *c*;

$C'_{a,\,b,\,c}$ = molar concentrations of pheophytinized chlorophylls *a*, *b*, *c*;

$Ph_{a,\,b,\,c}$ = molar concentrations of pheophytin *a*, *b*, *c*;

$\alpha_{1\,2\,3}\ \beta_{1\,2\,3}\ \delta_{1\,2\,3}$ = conditional molecular fluorescence coefficients for chlorophylls in filters, I, II, III;

$\alpha'_{1\,2\,3}\ \beta'_{1\,2\,3}\ \delta'_{1\,2\,3}$ = conditional molecular fluorescence coefficients for pheophytins in filters I, II, III.

Since the fluorescence through filters attributable to the pheopigments originally present in the mixture does not change with decreasing pH, direct measurement of the chlorophyllous pigments in the mixture is the magnitude of the change in fluorescence with acidification (Table III). By combining (1)–(6) to produce eqs. (7)–(9), the change in fluorescence with acidification is obtained:

$$\Delta F_I = C_a(\alpha_1 - \alpha'_1) + C_b(\beta_1 - \beta'_1) + C_c(\delta_1 - \delta'_1), \quad (7)$$

$$\Delta F_{II} = C_a(\alpha_2 - \alpha'_2) + C_b(\beta_2 - \beta'_2) + C_c(\delta_2 - \delta'_2), \quad (8)$$

$$\Delta F_{III} = C_a(\alpha_3 - \alpha'_3) + C_b(\beta_3 - \beta'_3) + C_c(\delta_3 - \delta'_3). \quad (9)$$

Here:

$\Delta F_{I, II, III}$ are measured changes in fluorescence through filters I, II, and III with acidification.

The determination of the 18 conditional molecular fluorescence coefficients then makes possible a calculation of the coefficient for each of the chlorophylls in (7)–(9) and a solution of those equations.

3. *Materials and Methods.* Conditional molecular fluorescence coefficients were determined on pigments that have been isolated by the following methods:

Chlorophylls *a* and *b* were isolated from either a variety of grasses or from fresh spinach by using chromatographic techniques. The source material was first mascerated in a tissue grinder, using 5 to 7 ml of absolute methanol containing 1 gm/l $MgCO_3$. The extract was then cleared and made to 10 ml with methanol prior to the addition of 10 ml of 10% NaCl (aqueous) in a 60-ml separatory funnel; 10 ml of petroleum ether were then added to the separatory funnel, and the layers were mixed to facilitate the transfer of the chlorophylls to the ether layer. Following separation, the methanol layer was discarded and the ether layer was cleared of water by centrifugation. The ether was then evaporated to near dryness under a nitrogen jet. The remaining solution of pigments was spotted on an Eastman 6061 silica-gel chromatogram sheet that had been dried at 50°C for 30 minutes. The chromatogram was developed with 58:30:12, hexane: ethylacetate: dimethylamine (Gebelein 1967). Spots of chlorophyll *a* (R_f 7.4) and chlorophyll *b* (R_f 7.1) were cut out and eluted into 90% acetone; this solution was stored at −20°C until the spectrophotometric analysis could be performed.

Chlorophylls *a* and *c* were isolated from 90% acetone (1 g $MgCO_3$/l) extracts of log-phase cultures of *Goniaulax polyhedra*, *Gymnodinium splendens*, and *Pyrodinium bahamense*, using the n-Hexane-90%-acetone-phase separation described by Parsons (1963). Any accessory pigments in the chlorophyll-*c* acetone phase were not removed. The chlorophyll *a* plus the accessory pigments were transferred to 90% acetone following evaporation of the hexane: acetone solvent under a nitrogen jet. These pigments were stored at −20°C in 90% acetone until they were used. Isolation techniques were carried out in a glove box, with nitrogen flushed through it and with subdued light at 23°–25°C. Extracts of purified chlorophylls were generally analyzed spectrophotometrically and fluorometrically within 12 hours of their purification.

The purity and concentration of the isolated chlorophylls were determined by using spectrophotometric methods. A Beckman DK II ratio-recording spectrophotometer with optically matched 2-cm cuvettes was used for absorption measurements. The absorption spectra of the purified pigments were recorded between 400 nm and 750 nm at slow speed, with the sensitivity set such that the slit width was 0.015 mm at 650 nm and less than 0.01 mm

Table I. The wavelengths of the red maximum absorbances and mean acid ratios of N determinations for purified chlorophylls and their pheophytin derivatives in 90%-acetone.

Pigment	nm red chlorophylls	Max. O.D. pheophytins	N	Red max. acid ratio
a.....	663–665	667–669	8	1.71 ± 0.042
b.....	647–649	653–655	7	1.49 ± 0.036
c.....	630–633	595–597	5	1.80 ± 0.070

below 600 nm. The absorbancy at the red and blue maxima were checked manually. Aliquots of the extracts were acidified with 0.1 ml of 1.0 N HCl, and the absorption spectra of the pheophytinized extracts were then measured.

The criterion for purity of the pigment was the ratio of the red absorption peaks before and after acidification of each pigment. These mean ratios for several purifications of each pigment, shown in Table I, are in good agreement with those reported in, or derived from, the literature (Lorenzen 1967, Vernon 1960, Jeffreys 1963, Falk 1964). Table II shows the molecular weights and specific absorption coefficients used in determining the pigment concentrations in the calibration solutions. These solutions were diluted volumetrically to prepare standards for the fluorometric readings.

The conditional molecular fluorescence coefficients were determined by equipping a Turner 111 fluorometer with a G4 T4-1 lamp and a high-sensitivity door that held a 30-ml pyrex test tube. The excitation light was passed through a 5-cm² Corning glass CS-5-60 filter, and the fluorescence was monitored through three combination filters:

I. Filters: Corning-glass filter CS-2-64 (5-cm²) with the Wratten gelatin filter # 70 protected by a (5-cm²) glass.

II. Filters: Corning-glass filter CS-2-59 (5-cm²) with a Corion Instrument Company interference filter # 6509 (0.5 bandwidth 12.8 nm).

III. Filters: Corning-glass filter CS-2-62 (5 cm²) with a Corion Instrument Company interference filter # 6217 (0.5 bandwidth 12.2 nm).

The fluorescent light transmitted by filter I is primarily that obtained from chlorophyll *a*, by filter II that obtained from chlorophyll *b*, and by filter III that obtained from chlorophyll *c*. Filter-transmission characteristics and chlorophyll-emission spectra are shown in Fig. 1.

Measurements obtained with the various sensitivity settings on the fluorometer were converted to equivalent units on the most sensitive scale (30 scale), and the coefficients were then expressed in these units.

The conditional molecular fluorescence coefficients were determined for each chlorophyll and pheopigment by adding 0.01 ml to 0.1 ml of the preparation to 25 ml of 90% acetone in the fluorometer tube. The concentration in the tube was then related to the number of "30-scale" equivalent units regis-

tered by the fluorometer, and the conditional molecular fluorescence coefficient was calculated as $(R_o - R_b)/C_p = Km$, where C_p = the concentration in nM/l, R_o = the solution reading corrected to "30-scale" units, R_b = the blank reading, and K_m = the conditional molecular fluorescence coefficient.

The solution was then acidified by adding several drops of 1.0 N HCl, and the fluorescence was determined following pheophytinization. The pheopigment coefficient was calculated as $(R_a - R_b)/C_{ph} = K'_m$, where R_a is the reading for the acidified solution (30-scale units) and K'_m is the pheopigment conditional molecular fluorescence coefficient.

By varying the pigment concentration in the assay tube, a linear relationship was found for each pigment in a concentration range from 1 nM to 60 nM. The mean values K_m and K'_m were therefore used to solve (7)–(9) for chlorophylls *a*, *b*, and *c* to produce eqs. (10)–(12) in terms of measured changes in fluorescence through each filter. Throughout the calibration and in later tests, the stability of the fluorometer was monitored, using quinine sulfate in 0.1 N H_2SO; the fluorescence was read through filter III.

Solutions of (7)–(9) and the transformation to weight units result in (10)–(12) for our instrument:

$$C_a\,\mu g/l = 0.110\,\Delta F_I - 0.109\,\Delta F_{II} + 0.110\,\Delta F_{III} \quad (10)$$

$$C_b\,\mu g/l = 0.052\,\Delta F_I - 0.286\,\Delta F_{II} + 0.310\,\Delta F_{III} \quad (11)$$

$$C_c\,\mu g/l = -0.012\,\Delta F_I + 0.036\,\Delta F_{II} + 0.265\,\Delta F_{III} \quad (12)$$

Table II. Molecular weights, specific absorption coefficients, and calculated molecular extinction coefficients for chlorophylls and pheophytins at red maximum wavelengths.

Pigment	Mol. *g*	Reference	Spec. absorp. coef. α $l.gm^{-1}cm^{-1}$	Reference	Mol. extinc. coef. ε $l.mol.^{-1}cm^{-1}$	Red max. λ (nm)
Chl a.....	893.48	Smith & Benitez (1955)	91.1	Vernon (1960)	8.139×10^4	665
Phe a.....	871.18	"	–		4.7626×10^4	667
Chl b.....	907.46	"	52.14	SCOR-Unesco (1966)	4.7295×10^4	645
Phe b.....	885.16	"	–	–	3.0982×10^4	655
Chl c.....	1052	Jeffreys (1963)	19.44	SCOR-Unesco (1966)	2.0451×10^4	630
Phe c.....	1028*	–	–	–	1.1355×10^4	595

* The molecular weight of pheophytin *c* is estimated by assuming that there is 1 magnesium atom per molecule lost with acidification.

Table III. The mean conditional molecular fluorescence coefficients with S.E. for 10 samples of Chlorophylls and Pheopigments determined for Turner Model 111 fluorometer with emission filter I, II, and III.

Pigments	Filters I	II	III
Chl a	14.75 ± 0.780	2.70 ± 0.145	0.19 ± 0.025
Phe a	4.80 ± 0.302	0.73 ± 0.080	0.04 ± 0.003
Chl b	1.90 ± 0.097	3.92 ± 0.179	0.63 ± 0.055
Phe b	5.75 ± 0.293	7.46 ± 0.196	0.31 ± 0.026
Chl c	2.29 ± 0.229	4.93 ± 0.247	3.75 ± 0.605
Phe c	1.22 ± 0.085	0.36 ± 0.025	0.27 ± 0.020

4. *Results.* The consistency in determining the conditional molecular fluorescence coefficient is shown in Table III. The coefficients, from at least three separate preparations of spectrophotometrically analyzed material, are the mean values of 10 determinations. With the assumption that the experimental values are normally distributed about the mean, the standard error of the estimated coefficients is generally less than ± 10% of the mean value, with a greater variation associated with a low fluorescent yield through a particular filter.

In determining the pheopigment coefficients, we found that the interval between the time of acidification and the reading of the fluorescence value was critical. The fluorescence change with pheophytinization is shown in Fig. 2. Stability for all three pigments was reached three minutes after acidification, so that a minimum delay of three minutes before making the reading appears to be necessary. The acid factors for each pigment with each filter are presented in Table IV; the values reported are dissimilar to those observed by Holm-Hansen et al. (1965), who used a filter whose transmission characteristics are similar to filter I. The difference in the acid factors for chlorophylls *a* and *c* (the Holm-Hansen values 2.1 and 2.4, our values 3.07 and 1.88) is probably due to differences in the instrumentation, in the emission, and in the excitation filters used (Saijo and Nishizawa 1969). These differences clearly indicate the necessity of individual instrument calibration by each investigator employing a fluorometric method.

With acidification, the fluorescence of the purified chlorophylls *a* and *c* decreased through all filters while the fluorescence of the purified chlorophyll *b* increased through filters I and II but decreased through filter III. The effect of this behavior of the chlorophylls upon the observed change in fluorescence (ΔF) in pigment mixtures can be calculated by using the values in Table III and eqs. (7)–(9). In Fig. 3, the calculated ΔF values for solutions containing chlorophyll *a* alone or in combination with chlorophylls *b* or *c* are compared with the ΔF values for culture extracts of *G. splendens* and *Dunaliella tertiolecta* in 90% acetone, using filter I. The range of the c/a ratio for *G. splendens* was

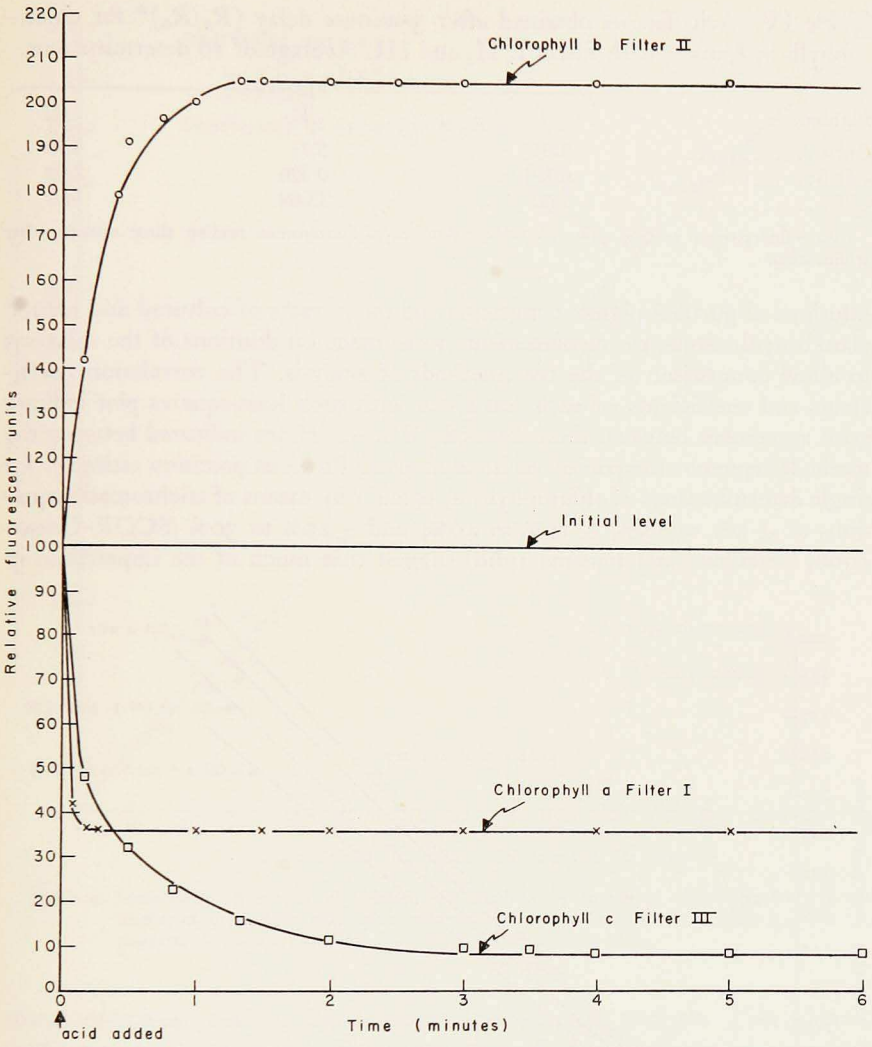

Figure 2. The fluorescent changes occurring with acidification of pure chlorophylls. The change in chlorophyll *a* fluorescence is shown for filter I, the change in chlorophyll *b* is shown for filter II, and the change in chlorophyll *c* is shown for filter III. 100 represents the level of fluorescence prior to acidification.

0.4–0.52 and the range of the b/a ratio for *D. tertiolecta* was 0.21–0.35. There is generally good agreement between the hypothetical and experimental values when accessory chlorophyll is present in addition to chlorophyll *a*.

Figs. 4–6 show a comparison of the spectrophotometric and fluorometric methods for chlorophylls *a*, *b*, and *c*. Absorbancy measurements were made on

Table IV. Acid factors obtained after 3-minute delay $(R_o/R_a)^*$ for chlorophylls *a*, *b*, and *c* with filters I, II, and III. Average of 10 determinations.

Pigment	Filters I	II	III
Chl a.....	3.07	3.71	4.76
Chl b.....	0.321	0.526	2.03
Chl c.....	1.88	13.69	14.1

R_0 = fluorescence reading prior to acidification, R_a = fluorescence reading three minutes after acidification.

solutions of purified-pigment mixtures and on extracts of cultured and natural plankton; fluorometric measurements were made on dilutions of the solutions to allow comparison of the two methods of analysis. The correlation coefficients and coefficients of variation given with each least-squares plot indicate good agreement between these methods. The variations indicated between the methods represent the pooled variance in each. Previous precision estimates for single determinations of chlorophylls *a*, *b*, and *c* by means of trichromatic equations of ± 5% to 15%, ± 10% to 40%, and ± 10% to 30% (SCOR-Unesco 1966, Strickland and Parsons 1968) suggest that much of the imprecision in

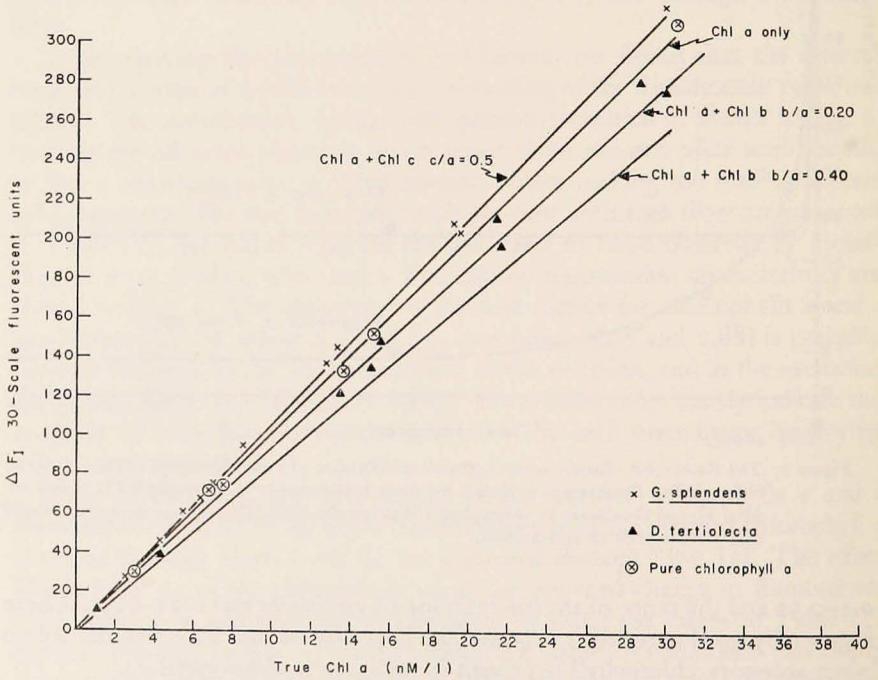

Figure 3. Calculated ΔF_I curves based on results from use of several c/a and b/a pigment ratios compared with observed ΔF_I values derived from culture extracts of *G. splendens* (c/a 0.40–0.52) and *D. tertiolecta* (b/a 0.21–0.35).

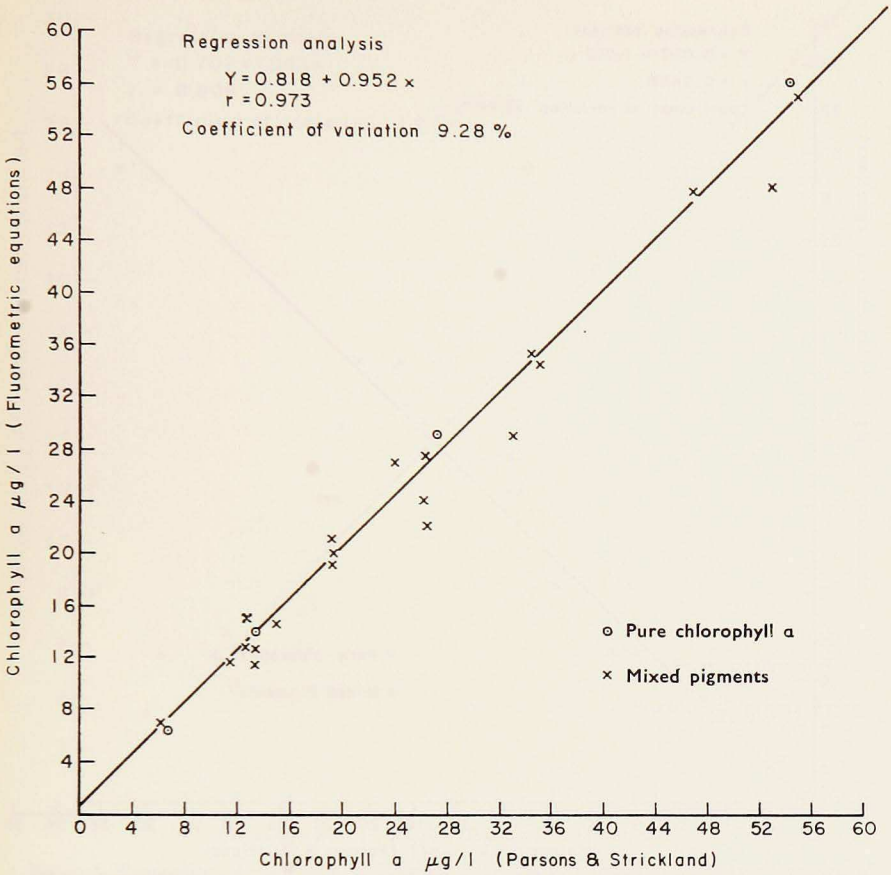

Figure 4. Comparison of results of chlorophyll-*a* analysis by means of spectrophotometric and fluorometric methods when pure chlorophyll *a* and chlorophyll *a* in mixes (natural and prepared) is present throughout a wide range of concentrations.

this comparison may be due to spectrophotometric analysis. This view is substantiated by estimates of the precision of the fluorometric equation: ±7.3% for chlorophyll *a*, ±7.8% for chlorophyll *b*, and ±10.7% for chlorophyll *c* in test series where dilution factors are used to predict pigment concentrations between 60 and 10 µg Chl/l. The precision of estimates for chlorophyll *a* by this fluorometric method in pigment mixtures is comparable to the trichromatic analysis whereas estimates of chlorophylls *b* and *c* can be more precisely determined by this fluorometric method.

Several experiments were performed to test the effects of other pigments on the fluorescent yield of chlorophyll *a* by using chlorophyll *a* and accessory pigments chromatographically separated from extracts of *P. bahamense* cultures.

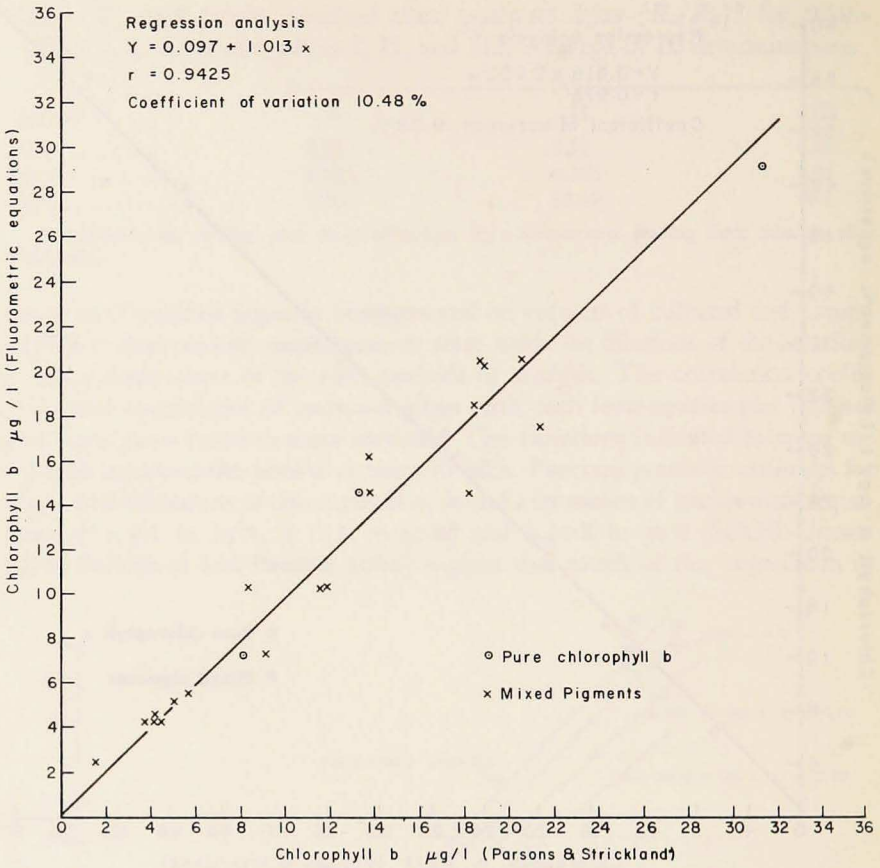

Figure 5. Comparison of results of chlorophyll-*b* analysis by means of spectrophotometric and fluorometric methods when pure chlorophyll *b* and chlorophyll *b* in mixes (natural and prepared) is present throughout a wide range of concentrations.

When aliquots of the acetone eluant of the carotene spot (R_f 10.0) were added to pure chlorophyll *a* (14 µg/l) such that the carotene: chlorophyll-*a* ratios were approximately 1.0, the fluorescence reading through filter I decreased by 2.4%. When the carotene concentration was doubled, the decrease was 7.6%. However, the decrease in fluorescence upon acidification in both cases was not significantly effected by the presence of the carotenes. The addition of other carotenoids and neutralized pheopigments likewise did not significantly effect the magnitude of the decrease in chlorophyll-*a* fluorescence. Since the carotenoid-pigment concentrations tested were far greater relative to chlorophyll *a* than would normally be experienced in algal extracts (Jeffreys 1968) and since the ΔF_I values were not effected by nonred-fluorescing pigments, significant interference from accessory carotenoids in extracts would not be expected. The

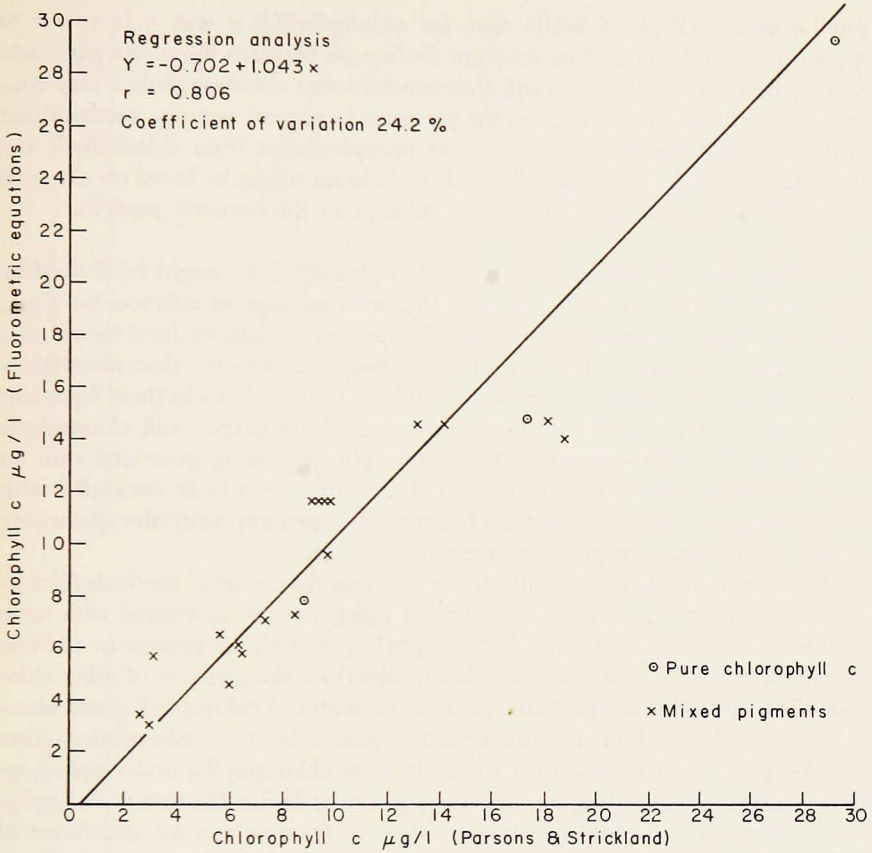

Figure 6. Comparison of the results of chlorophyll-*c* analysis by means of spectrophotometric and fluorometric methods when pure chlorophyll *c* and chlorophyll *c* in mixes (natural and prepared) is present throughout a wide range of concentrations.

determination of the fluorescence constants with chlorophyll *a* from *P. bahamense* with accessory carotenoids gave values for $\alpha_{1\,2\,3}$ and $\alpha'_{1\,2\,3}$ that are not significantly different from those obtained with spinach chlorophyll *a*, where accessory carotenoids and chlorophyll *b* were removed.

Chlorophyllide *a* and pheophorbide *a* were prepared from cultures of *Skeletonema costatum*, using methods described by Barrett and Jeffreys (1964). A conversion greater than 96% was indicated by the lack of solubility in hexane. Little pheophorbide *a* was present, since the absorption acid factor was 1.69; this is essentially identical to the absorption acid factor of chlorophyll *a* (Table I). The fluorescence acid factor for chlorophyllide *a* in filter I was found to be 3.1 in one preparation and 2.5 in a second, indicating a fluorescent behavior that is similar to that of chlorophyll *a*. With filter I, the O.D. $_{665}$/Fl. for chloro-

phyll *a* was 5.48×10^{-6} while that for chlorophyllide *a* was 5.49×10^{-6} to 5.76×10^{-6}; so the procedure does not distinguish between these two pigments. Since Patterson and Parsons (1963) determined that chlorophyllide *a* may constitute a significant percentage of the pigments in natural samples, methods that will distinguish the chlorophyllides and pheophorbides from chlorophylls and pheophytins would be useful, and such techniques might be based on the 90% acetone:hexane phase separation with subsequent fluorometric analysis.

5. *Discussion.* Equations for each of the pheopigments might be derived by substituting (10)–(12) in (2), (4), and (6), with subsequent solution for C_{pha}, C_{phb}, and C_{phc}. Attempts to use these pheopigment equations have shown that the resultant estimates suffer from such great inaccuracies that uncertainty precludes their usefulness. The use of Table III coefficients in these equations for mixtures of prepared solutions containing pheopigments and chlorophylls has resulted in poor estimates of all three pheopigments, generally with an underestimation of the amount present. The errors seem to be associated with the variance in the individual coefficients and perhaps with the quenching effects of the accessory pigments present.

We have found that the results of the previous fluorometric methods (Holm-Hansen et al. 1965, Yentsch and Menzel 1963) have to be viewed with some qualification when chlorophyll *b*, chlorophyll *c*, or both are present in addition to chlorophyll *a*. The interference that results from the presence of other chlorophylls may lead to a significant error in estimates of chlorophyll *a* and pheopigment *a* when all of the fluorescence passed by the red-emission filter (CS-2-64, CS-2-60) is assumed to result from chlorophyll *a* and its pheopigment derivatives. By using the equations given by Holm-Hansen et al. (1965) in combination with our conditional molar coefficients and an acid factor of 3.07 for filter I (Tables III, IV), we obtained the following:

$$\text{Chlorophyll } a \text{ (nM/l} = (1.48)\,(0.067)\,(R_o - R_a), \tag{13}$$

$$\text{Pheophytin } a \text{ (nM/l)} = (1.48)\,(0.067)\,(3.07\,R_a - R_o). \tag{14}$$

In the absence of pheopigments and in the presence of chlorophyll *a* and either chlorophyll *b* or *c*, the following equations can be derived from the Table III coefficients:

$$R_o = 14.75\,C_a + 1.90\,C_b \text{ (or } 2.29\,C_c), \tag{15}$$

$$R_a = 4.80\,C_a + 5.75\,C_b \text{ (or } 1.22\,C_c). \tag{16}$$

A range of assumed values for the chlorophyll and pheophytin concentrations was used in these equations to calculate the readings; then these calculated readings were substituted in (13) and (14) to produce computed apparent chlorophyll-*a* and pheophytin-*a* concentrations. Fig. 7 shows the results of these

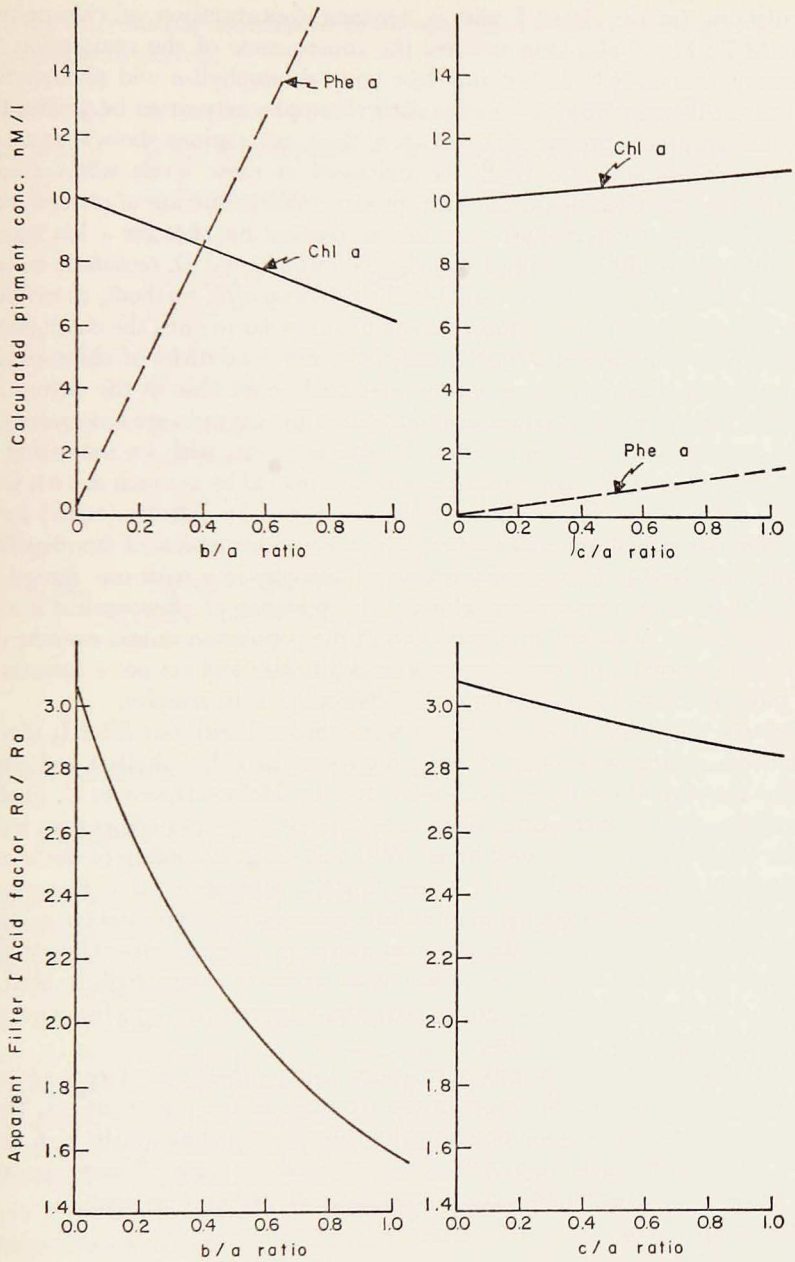

Figure 7. Apparent pigment concentrations that result from computations using equations (such as those of Strickland and Parsons 1968) with an assumption of no effect of chlorophylls *b* and *c* when these pigments are present. A true concentration of 10 nM/l chlorophyll *a* assumed, with no pheopigments. Variation in acid factors also shown.

calculations for our filter I with a constant concentration of chlorophyll *a* (10 nM/l); Fig. 7 also demonstrates the consequence of the assumption that accessory chlorophylls do not interfere with chlorophyll-*a* and pheophytin-*a* estimates. Although the b/a ratios in natural samples may never be greater than 0.4, and are rarely greater than 0.1–0.2, these calculations show that a large quantity of pheophytin *a* would be estimated at these levels when none is actually present. Commensurate with the erroneous prediction of pheophytin *a*, concentrations of chlorophyll *a* are underestimated by 16% for a b/a ratio of 0.4. Jeffreys (1968) determined that the b/a ratio for old *D. tertiolecta* cultures is 0.89. If such a sample were analyzed by fluorometric methods, as reviewed by Strickland and Parsons (1968), using a filter similar to ours, the chlorophyll *a* would be underestimated by 35%, and, for every 10.0 nM/l of chlorophyll *a*, 19.5 nM/l of pheophytin *a* would be predicted above that in the culture.

The errors in the predictions as a function of the c/a ratio are less severe than those associated with the b/a ratios. In this instance, with an increasing c/a ratio, the chlorophyll-*a* concentration is overestimated by as much as 10% when the c/a ratio is 1.0. Ratios near 1.0 were obtained by Jeffreys (1968) for an *Amphidinium* sp. culture, and ratios of 0.5 or more are typical of dinoflagellates (Madgwick 1966). The overestimation of pheophytin *a* with our filter I indicates that those investigators who use the presence of pheopigment *a* as an indicator of predation and/or a condition of the population should exercise care to see that estimates of these pigments are legitimate and are not a function of the ratio of the accessory chlorophylls: chlorophyll *a* in samples.

Because the results shown in Fig. 7 were obtained with our filter I, there is excluded a greater percentage of light fluoresced by chlorophylls *b* and *c* than by the Corning CS-2-64 and CS-2-60 used by Holm-Hansen et al. (1965). Therefore, the use of the latter filters would result in greater errors than would be the case with the filters used by us. With any filter, according to the recommendations of Strickland and Parsons (1968), calibration of a fluorometer when using mixed phytoplankton or culture material results in positive or negative errors as a function of the ratios of accessory chlorophylls-chlorophyll *a*. Without knowledge of the concentrations of accessory chlorophylls in both the sample and the calibration solution, accurate determination of pheopigment *a* and chlorophyll *a* are impossible.

As indicated above, the direct solutions using (1)–(6) produced 18 term equations and unacceptable errors in the estimates of the pheopigments. However, pheopigment *a* may be estimated by determining chlorophylls *a*, *b*, and *c* and by then using these results in an equation where these estimates are used as single-term corrections for the fluorescence contributed by each.

Equation:

$$\text{pheopigment-}a\ \mu\text{g/l} = k_1(\alpha_1)^{-1}K_a[(k_2(R'_a - \beta'_1 K'_b W_b C_b - \delta'_1 K'_c W_c C_c)] + [(\beta_1 K_b C_b + \delta_1 K_c C_c - R'_0)].$$

Here the fluorescence contributed by pheopigments *b* and *c*, which were present originally, is neglected:

$(\beta_1' K_b' W_b C_b)$	is the fluorescence of pheophytinized chlorophyll *b*;
$(\delta_1' K_c' W_c C_c)$	is the fluorescence of pheophytinized chlorophyll *c*;
$(\beta_1 K_b C_b)$	is the fluorescence of chlorophyll *b* prior to acidification;
$(\delta_1 K_c C_c)$	is the fluorescence of chlorophyll *c* prior to acidification;
$K_{abb'cc'}$	are factors that convert molar fluorescence coefficients to weight coefficients;
W_{bc}	are factors that convert weights of pheopigments to weights of chlorophyll;
$\alpha_1, \beta_1^{(\prime)}, \delta_1^{(\prime)}$	are conditional molar fluorescence coefficients for chlorophylls and pheopigment filter I;
R_o'	is the reading before acidification for mixed pigment sample;
R_a'	is the reading after acidification of the above mixture;
$k_2 = R_o/R_a$	represent the ratio of readings before and after acidification of pure chlorophyll *a* through filter I;

$$k_1 = \frac{k_2}{k_2 - 1}.$$

For our instrument, the above reduces to:

$$\text{pheopigment } a\ \mu\text{g/l} = 0.344\ R_a' - 0.112\ R_o' - 2.06\ C_b - 0.174\ C_c.$$

Equations for pheopigments *b* and *c* could be derived similarly; however, the uncertainty in the estimates of chlorophyll *a* might be as great as the concentration of these pigments; this would therefore lead to a high degree of uncertainty in their estimation.

It is important to note that the methods developed here for the analysis of chlorophylls *a*, *b*, and *c* and of pheophytin *a* require that the equations for pigment calculation *must be derived for each instrument*. The filters, the instrument geometry, and the electronics influence the specificity and sensitivity for the several components in the analysis. Since both fluoresced and scattered light are incident on the emission filter, the use of cutoff filters with interference filters is necessary to maintain transmission integrity. Slight variations in the transmission characteristics within the commercial specifications can limit the usefulness of a particular filter.

The sensitivity of the Turner 111 fluorometer is controlled by (i) the spectral emission and intensity of the exciting lamp, (ii) the transmission charac-

teristics of the emission and excitation filter, (iii) the geometry of the tube door, (iv) the spectral sensitivity of the photomultiplier, and (v) the size of the excitation slit. Each instrument must be calibrated for a particular set of these parameters, and recalibration is necessary if any parameter is changed.

Since the initial calibration is somewhat tedious and time consuming, the level of sensitivity desired should be determined before the calibration is performed. For our purposes, the maximum level of sensitivity was not required, since chlorophyll concentrations in estuarine and coastal waters generally range above 1 μg/l. We have therefore used the general purpose U V lamp, the P-121 photomultiplier, and a mirrored tube door for tubes containing 30 ml and having a pathlength of 2.2 cm. By using the same filters and door, we found that, with a red-sensitive photomultiplier, an increase in sensitivity by a factor of 5–10 can be obtained. This level of sensitivity may be required for oceanic studies. Since numerous modifications are possible with the Turner 111 instrument, each investigator should determine the effects of the accessory pigments on his procedure. Goodwin (1947) has shown that the quenching of chlorophyll fluorescence varies with the spectral character of the exciting light, and it is likely that the calibration values for each pigment will show some variation, depending upon all the parameters previously mentioned.

After the calibration has been achieved, the integrity of the method with time relies upon the stability of the instrument. This stability has a twofold character: (i) linearity in response to concentration, and (ii) repetitive response to solutions of standard fluorescence. We have used standard solutions of quinine sulfate in 0.1 N H_2SO_4 to check both of these features of the instrument's stability. Over the nine months of use, our fluorometer has shown good repetitive and linear stability with response to standard solutions that have varied less than 4%. Preparations of quinine sulfate in 0.1 N H_2SO_4, in concentrations of 5 and 10 mg/l, have served to monitor both features of stability. These concentrations have given readings that are near the middle and full scale with our filter III when the excitation slit is set at "30". At higher concentrations, quenching is observed and sensitivity to change is poor. The use of similar standardizing procedures is advised if long-term precision is desired.

6. *Conclusions.* The methods presented have several advantages over currently used techniques. When compared with the trichromatic methods, this technique requires no assumption of the absence of pheopigment. The three chlorophylls may be determined at a faster rate than otherwise, since the sensitivity of the fluorometer requires only a fraction of the sample volume necessary for the spectrophotometric methods. These improvements are accompanied by no loss (and perhaps significant gain) in the precision for estimates of the three chlorophylls.

We have shown that the presence of accessory chlorophylls can cause not only serious overestimation of pheophytin *a* but errors in the estimates of

chlorophyll *a* dependent upon the ratio of c/a and/or b/a when single-filter methods of fluorometric analysis are used. The multiple-filter method described here permits measurement of pheopigment *a* with an accuracy such that the quantities might be useful indicators of community conditions and interaction. We therefore feel that this multifilter method constitutes an improvement over current methods of chlorophyll analysis.

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