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A method for the determination of phytoplankton chlorophyll and phaeophytin by fluorescence*

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Abstract—The concentration of chlorophyll, principally chlorophyll *a*, and after acidification phaeophytin, is measured in 85% acetone extracts using a sensitive fluorometer. The method is very sensitive in that 10 fluorescence units are equal to 0·001 O.D.$^{10\ cm}_{665\ m\mu}$. The maximum variation is 15%. The method is highly suitable for routine analysis at sea or in the laboratory.

INTRODUCTION

IN THE open ocean one routinely encounters chlorophyll concentrations of less than 1 μg per l. Such small amounts of pigment may be measured by absorption spectrophotometry by using light paths of 10 cm; however, the particulate matter has to be concentrated from 4 to 6 l. of sea water and the extract volume maintained below 10 ml. The alignment of small volume, long light path cuvettes in the centre of the light beam is difficult in convectional spectrophotometers such as the Beckman DU, and any scattering by the cuvette or particles within the cuvette leads to serious errors.

In investigating the seasonal sequences of phytoplankton production in tropical waters it became obvious that a method was needed with considerably more sensitivity. It seemed unlikely that larger volumes of water could be easily and conveniently handled aboard ship; furthermore, water for the measurement of other parameters of the phytoplankton community is needed from the same water sample.

The use of a sensitive photomultiplier for the detection of long wavelength light fluoresced from pigment extracts irradiated with short wavelengths seemed a logical approach. Previous methods for the quantitative determination of chlorophyll by fluorescence had been proposed by KALLE (1937), KREY (1957), and GOODWIN (1947). The method presented in this report uses the highly sensitive optical system of the Turner fluorometer.

Concentration and extraction of chloroplastic pigments

Three changes had been made to the method introduced originally by CREITZ and RICHARDS (1955). (1) Glass fibre filters are used in place of membrane filters, (2) 85% acetone was used in place of 90%, and (3) the filter and particulate matter are ground in a convectional tissue grinder which facilitates immediate extraction.

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Filters may be purchased at W. and R. Balston Ltd., England. 'Whatman Glass paper (GF/C)' or 'Glass Fibre Paper Filter 47 mm, type A,' is made by Gelman Instrument Co., Chelsea, Michigan. These filters are insoluble in acetone, hence one avoids troublesome cloudy precipitates encountered using membrane filters. The speed of filtration is higher using the glass filter as opposed to the membrane filter; however, the exact pore diameter of the glass fibre filter is not known. Tests in our laboratory show that the smallest phytoplankters (3-4 μ) are retained. The grinding pestle and vessel may be purchased from Arthur H. Thomas, # 4288-B, size B, or the Kontes Glass Co., Vineland, New Jersey, # K-88600, size C. Our grinding motor consists of an ordinary hand drill with $\frac{1}{4}$" chuck and the drill press adaptor. Both may be purchased at most hardware stores.

The complete procedure for concentrating an extraction of chloroplastic pigments is as follows : A measured portion of sea water is filtered under vacuum through a glass fibre filter which is then pushed into the bottom of a tissue grinding tube. 2 ml of 85% acetone and 0·1 g of magnesium carbonate are added to the grinding tube and the pestle is inserted. The grinding motor (a conventional hand drill) is turned on and the contents are thoroughly ground for one minute. The data (TABLE 1) show that this is sufficient time for total extraction. The contents of the grinding tube are carefully washed with 5 ml of 85% acetone into a screw-cap centrifuge tube and centrifuged for one minute. The tubes are removed and allowed to stand in darkness for several minutes until they come to room temperature. The contents of the tube are carefully poured from the centrifuge tube into the fluorescence cuvette. The fluorescence cuvette should contain exactly 5 ml of extract.

Table 1. *Fluorescence of* 85% *acetone extract of natural phytoplankton chlorophyll one minute after grinding and* 18 *hr later. Filter material and particulate debris were centrifuged to the bottom of the tube.*

Sample	*Fluorescence*	
	1 *min after grinding*	18 *hr after grinding*
1	65	63
2	10	10
3	50	51
4	23	22

Excitation and emission spectra of pigment extracts of phytoplankton

Extracts of chloroplastic pigments of phytoplankton cultures were prepared. The cultures were grown in cotton-stoppered flasks at 20°C under daylight fluorescent lamps at an intensity of 500 foot-candles. The culture medium was enriched sea water described by YENTSCH and VACCARO (1958). Approximately 50 ml of each culture were filtered and the final volume of 85% acetone extract was 10 ml.

Fluorescent emission and excitation characteristics of the extracts were recorded using the Aminco-Brown spectrophotofluorometer (No. 4–8106). By manually manipulating the monochrometer the wavelength of maximum emission excitation were located in each of the extracts. The wavelength of maximum excitation was then fixed and the emission spectrum recorded (FIG. 1). Maximum emission occurred in all extracts between 650 and 675 mμ. The wavelength accuracy of the

instrument used by the present authors is not known. Maximum excitation occurred between 430 and 450 mμ. An emission peak, 625–630 mμ, of approximately one quarter of the intensity of the principal peak is apparent in the diatom extracts and is undoubtedly due to the presence of chlorophyll *c*.

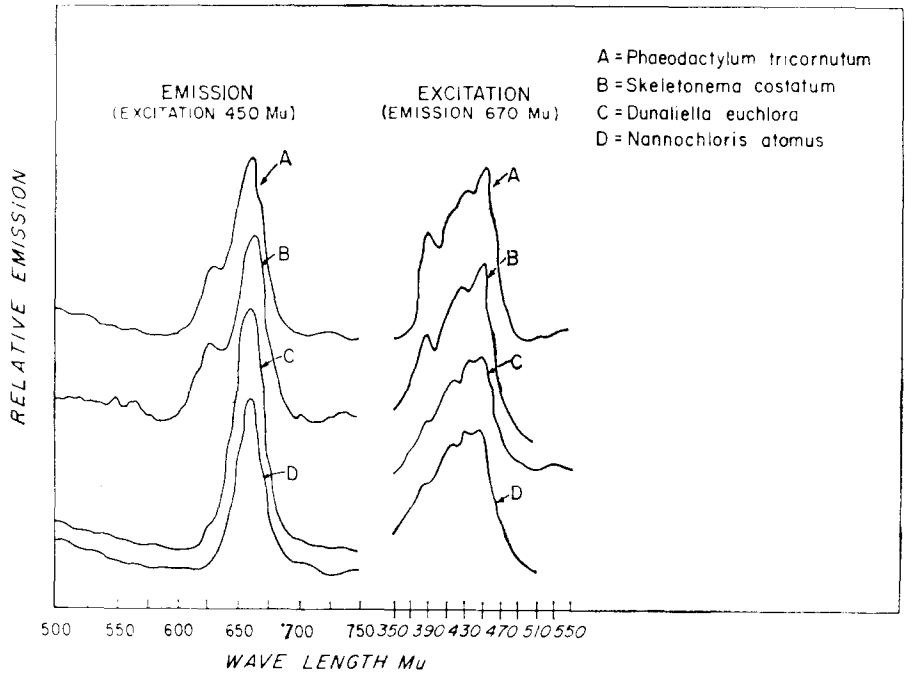

FIG. 1. Excitation and emission spectra for several cultures of marine phytoplankton.

Quantitative comparison of chlorophyll absorption of light to fluorescence

From the foregoing information concerning wavelengths of maximum excitation and emission, suitable coloured filters were considered for use in simple fluorometers such as the type made by G. K. Turner Associates, Palo Alto, California. For excitation wavelengths, the Corning CS-5-60, # 5543 2-in^2, 4·9 mm thick polished glass filter or the Kodak Wratten gelatin filter No. 76 is used. For emission wavelengths Corning CS-2-60, # 2408 2-in^2, 3·0 mm thick polished glass filter or the Kodak Wratten filter No. 26 is used. Interference filters may be used, but due to reduced light transmission there is a considerable loss in sensitivity.

The Turner fluorometer is sold with a general purpose excitation light source (G. E. No. F4T4/BL). Much greater excitation energy is obtained in the region 430 mμ–450 mμ using the ' blue lamp,' Turner No. 110–853. Excitation from the source may be regulated by a sliding window with orifices sizes 1 X, 3 X, 10 X and 30 X. For a complete description of the operation of the fluorometer the reader is referred to the 'Operation and Service Manual' Turner fluorometer Model 110.

The intensity of fluorescence has been compared to the optical density at 665 mμ of acetone extracts of natural and cultured phytoplankton (FIGS. 2, 3, 4 and TABLE 2). In FIG. 2 the relationship is shown for natural phytoplankton in the Sargasso Sea.

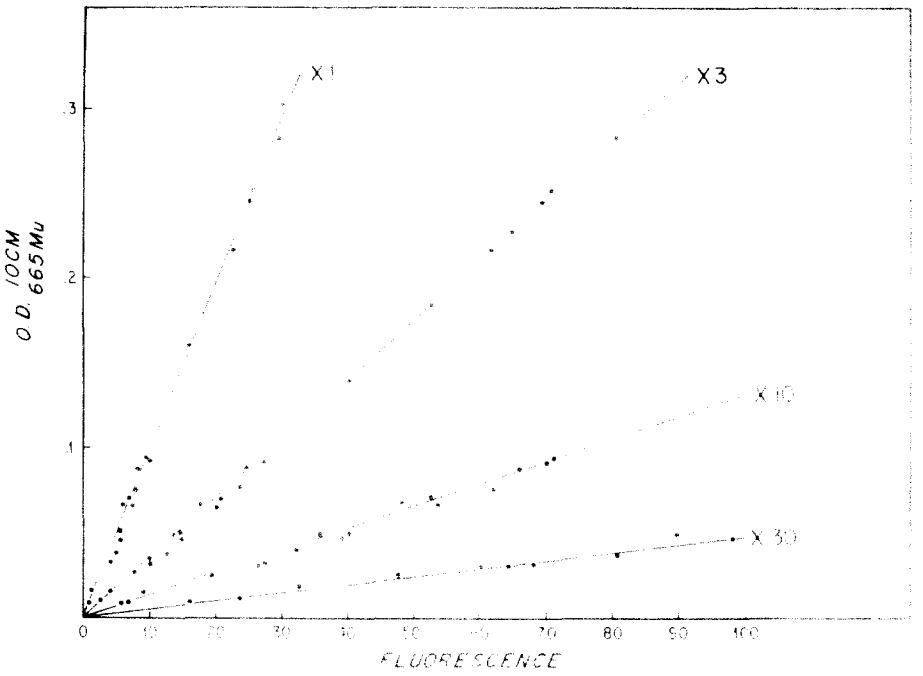

FIG. 2. Relationship between fluorescence and optical density, in 85% acetone extracts of natural Sargasso Sea phytoplankton, using the " general purpose " excitation source.

Table 2. Comparison between fluorescence and optical density in different species of cultured phytoplankton.

Organism	*A* *Optical density* 665 mμ 10 *cm light path*	*B* *Fluorescence**	*Ratio* *A* : *B*
Skeletonema costatum	0·110	94	$1{\cdot}18 \times 10^{-3}$
Phaeodactylum tricornutum	0·080	67	$1{\cdot}20 \times 10^{-3}$
Dunaliella euchlora	0·109	94	$1{\cdot}17 \times 10^{-3}$
Nannochloris atomus	0·076	62	$1{\cdot}22 \times 10^{-3}$

*Excitation 30X, with neutral density filter 1·00.

In this case the ' general purpose ' excitation source was used. In FIG. 3 the relationship is for natural phytoplankton from Woods Hole waters. In this case the Turner ' blue lamp ' was used for the excitation source. The greater sensitivity of the latter lamp is evident from the graphs.

The fluorometer scale is calibrated by directly relating optical density at 665 mμ in 85% acetone extracts of natural phytoplankton populations from Woods Hole Waters (FIG. 3). In the case of the 10 X and 30 X scales a neutral density filter was placed over the emission filter, redoing the sensitivity of the fluorescence meter to a region where accurate optical density measurements can be made simultaneously with the spectrophotometer. The linear relationship obtained in this region has been extrapolated to zero in FIG. 3. With either lamp the relationships shown are linear.

Since self quenching was observed when using either lamp above 30 fluorescence units on the 1X scale, use of the latter should be avoided. TABLE 2 shows the results of an experiment designed to ascertain if measurable differences between the relationship of optical density to fluorescence occur as the result of the species of algae

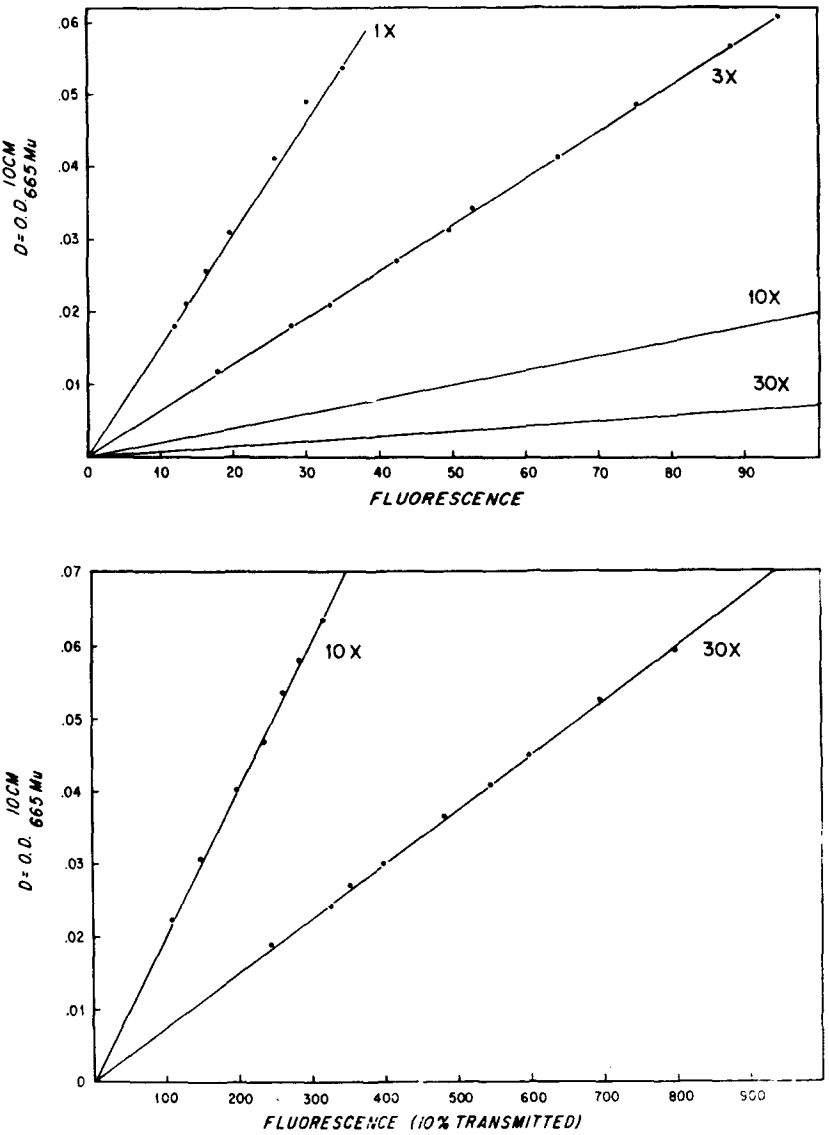

FIG. 3. Relationship between fluorescence and optical density in 85% acetone extracts of natural phytoplankton from Woods Hole waters, using the special ' blue lamp ' excitation source. Top graph, 1X and 3X scales without neutral density filter No. 1·0. Bottom graph, 10X and 30X scales with neutral density filter No. 10.

extracted. The results indicate no significant differences; hence the presence of different types of accessory fluorescing pigments appears to be minimal compared to emission by the principal pigment, chlorophyll *a*. FIG. 4 compares optical density and fluores-

cence in naturally occurring populations between the slope waters off Montauk, Long Island and the north central Sargasso Sea. Highest values for fluorescence and optical density occurred in the slope waters while lowest values are typical of open ocean situations. Throughout the two-fold range in optical density the agreement is quite good. It is felt that a major part of the slight variation is due to the spectrophotometry.

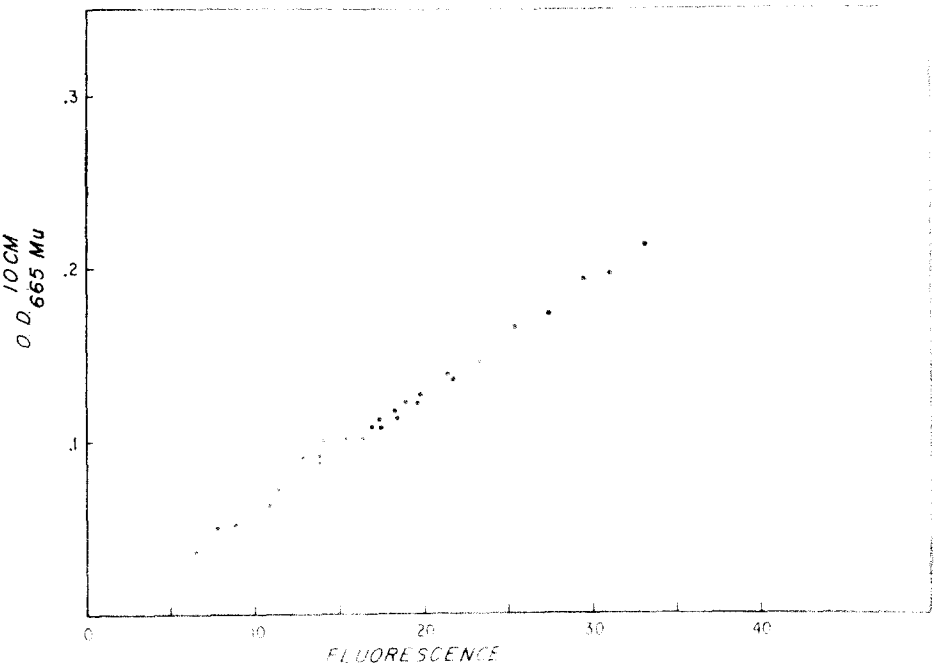

FIG. 4. Relationship between fluorescence and optical density in 85% acetone extracts of natural phytoplankton populations between coastal waters off Montauk Pt., Long Island, to the north central Sargasso Sea.

By using the relationship between optical density and fluorescence, chlorophyll concentrations can be calculated by the following relationship :

$$(1) \qquad \text{chlorophyll mg/m}^3 \text{ or } \mu\text{g/l} = \frac{D}{10} \cdot \frac{k}{1000} \cdot \frac{\text{Vol. ext. (cm}^3)}{\text{Vol. filt. (l)}}$$

where the D value is taken from either FIG. 2 or 3 depending on the specific machine used. The value k is the specific absorption coefficient which is the relationship between the optical density and the weight of pigment in the extract (TABLE 3).

*Determination of phaeophytin**

Solvent extracts of plant material may at times contain decomposition products of chloroplastic pigments, which further complicates the choice of a specific absorption value. An estimate of phaeophytin concentration may be made by measuring

*We are aware that the decomposition products of chloroplastic pigments include phaeophorbides as well as a variety of unknown decomposition products. The use of the term " phaeophytin " in the present case is designated to include all these forms.

Table 3. Specific absorption values (k) *for chlorophyll* a *in aqueous acetone.*

Reference		*Specific absorption* (k)* *for wavelength*		*Aqueous acetone*
		665 mμ	664–663 mμ	
Zscheile	1934	65·0	68·5	90%
MacKinney	1940	84·0	—	100%
MacKinney	1941	76·0	82·0	80%
Zscheile, *et al.*	1942	—	82·0	80%
Richards	1952	66·7	71·0	90%
Vernon	1960	90·8	92·6	100%
			91·1	90%

*The specific absorption value $k = l/l{\cdot}c \log_{10} I_0/I_1$ where l is the light path in centimeters and c concentration in gms/l.

the decrease in fluorescence upon acidification. The procedure is as follows : Exactly 5 ml of the extract are poured into the fluorescence cuvette and the fluorescence measured. Directly to this extract in the cuvette is added exactly 0·2 ml of 85% acetone saturated with oxalic acid. After three minutes the fluorescence value is again read. TABLE 4 gives some values for the ratio of fluorescence values of unacidified to acidified extracts from various sources. Chromatographed chlorophyll

Table 4. Changes in chlorophyll fluorescence of 85% *acetone extracts after acidification from different sources.*

Source	*Unacidified extract acidified extract*
CHROMATOGRAPHED CHLOROPHYLL *a* from *Skeletonemena costatum*	1·70–1·78
Healthy growing cultures	
Skeletomena costatum	1·6–1·7
Dunaliella euchlora	1·4–1·7
NATURAL POPULATIONS	
Woods Hole Harbour 9/2/62	1·8–1·6
Vineyard South 9/15/62	1·3
Buzzards Bay 9/20/62	1·5
Buzzards Bay Plankton tow # 2 net	0·9
Buzzards Bay Plankton tow # 10 net	1·2
Buzzards Bay Plankton tow # 20 net	1·4
MUD FROM CARIACO TRENCH	
800 meters, lat. 11° 56′N long. 72° 28·2′W	0·9–1·0
NORTH CENTRAL SARGASSO SEA 9/27/62	
0–75 meters	1·3–1·4
100–1000 meters	1·1–1·0

a gives values of 1·6–1·8. Healthy marine phytoplankton cultures give values of 1·5–1·8. In contrast, extracts of anaerobic marine muds and crustaceans yield values of around 1·0. Values in the natural marine environment are varied; net tows containing large concentrations of grazing herbivorous crustaceans consistently yield values close to 1·0. Offshore studies show that this ratio is highest throughout the euphotic zone; below this zone practically all the ratio is 1·0.

With the assumption that an acidified : unacidified ratio of 1·7 or greater is indicative of pure chlorophyll with no phaeophytin the amount of fluorescence due to pure chlorophyll (F_{chl}) may be computed by the following relationship :

$$F_{chl} = F_0 \frac{[(F_0/F_a) - 1{\cdot}0]}{0{\cdot}7} \quad (2)$$

where F_0 is the original fluorescence prior to acidification and F_a fluorescence after acidification. The value (F_{chl}) may then be used in equation (1) with a chosen specific absorption value. It also follows that the fluorescence by phaeophytin alone will be equal to the total fluorescence minus that by pure chlorophyll. Computation of phaeophytin is made by using equation (1) and the specific absorption value of 56·6 given by VERNON (1960).

Sensitivity

The maximum sensitivity of the fluorescence method was compared to the light absorption technique in the following manner. Small quantities of chlorophyll extract were added by micropipette to an optical cuvette, which was then filled with 85% acetone. The optical density of this mixture was then measured at 665 and 750 millimicrons (TABLE 5). The contents of this cuvette were poured into a fluorescence

Table 5. Comparison of optical density and fluorescence after additions of 85% acetone extracts of chlorophyll.

Addition	$O.D._{665\ m\mu}^{10\ cm} - O.D._{750\ m\mu}^{10\ cm}$	*Fluorescence* (30 X *blue lamp*)
1	0·000	10
2	0·000	20
3	0·000	31
4	0·004	41

cuvette and the intensity of the fluorescence measured. This procedure was repeated four times; each time more chlorophyll was introduced. No significant optical density reading was obtained until the fourth addition. At this time the fluorometer registered 41 units. The high sensitivity of the fluorescence technique markedly decreases the volume needed for filtration and hence the size of sampling devices. A maximum of 1 l. of water is needed even in the most oligotropic situations. In coastal waters, similar to those at Woods Hole, 50–100 ml suffice.

Accuracy

The overall accuracy of the pigment determination has always been complicated by a wide divergence in values for specific absorption. Because of the wide use of the RICHARDS with THOMPSON (1952) method one might prefer to use this value. In doing so it must be realized that the values will be approximately 20% higher than those of a worker using some of the higher specific absorption values. There are reasons concerning the procedure for chromatographic separation that might lead one to prefer some of the higher values for specific absorption; still it would appear that this problem of standardization will not be clearly resolved for some time, and

as a result workers should indicate the specific absorption value used when quoting chlorophyll results. In TABLE 3 we listed specific absorption coefficients from various sources for two wavelengths and acetone with various contents of water. This is because in most spectrophotometers the wavelength accuracy is not much better than $\pm$ 1–2%. Differences in the water content of solvents can not be expected to produce great errors (see ODUM *et al.* 1958).

The accuracy of the fluorescence method proposed here is also complicated by the fact that fluorescence is not entirely from purely chlorophyll *a*. Initially in attempting the calibration we applied the equation of RICHARDS with THOMPSON (1952) in comparison with chlorophyll *a* fluorescence. The comparison gave much more variability than a comparison to the optical density at 665 mμ alone. The increased variability of specific chlorophyll *a* measurements occurred even on extracts from the same type of organisms, which led us to conclude that the variation was due to inaccuracies added by measurements at wavelengths other than 665 mμ.

In an attempt to analyze the ' State of the Pigment ' we have proposed the simple determination of phaeophytin which involves a comparison of the fluorescence before and after acidification. Similar techniques, using absorption spectrophotometry have been proposed by VERNON (1960) and ORR and GRADY (1957). This step should permit a more sensible choice of the specific absorption coefficient and should be welcomed by ecologists who have frequently claimed that pigment extracts are contaminated by ' dead pigment fractions.'

Precision

The precision of the RICHARDS with THOMPSON (1952) and other similar methods is hampered by incomplete extraction. A number of workers have stressed this point (NELSON (1960), LAESSØE and VAGN KR. HANSEN (1961)) and have proposed sonification. Repeated attempts to sonify cells such as *Nannochloris atomus* have resulted in only partial success and were accompanied by considerable heating. As a result we abandoned this technique for conventional grinding in a tissue homogenizer We found that 10–15 minutes of grinding by hand was necessary to free all pigment in most cases; however, when mechanically ground, less than one minute was required and very little heat was developed. The importance of grinding samples is shown in FIG. 5. The samples were extracted for 18 hr in darkness under refrigeration, after which the fluorescence was read. The filters were then ground for one minute and the fluorescence read again. These samples show that fluorescence is increased by grinding by 5–60%. A shocking comparison can be made by measuring the fluorescence of an extract of *Nonochloris atomus* before and after grinding.

Our experience is that the fluorometer is extremely stable even during rough periods at sea. The entire method is easily performed aboard ship. For the convenience of doing samples at the shore laboratory, thoroughly dried filters may be stored following the procedure of CREITZ and RICHARDS (1955) for at least 30 days. Prolonged storage of extracts under refrigeration and darkness should be avoided. In extracts stored in this manner we have observed no change in fluorescence or optical density after two days; however, by five days both had decreased 20%. Fluorescence of extracts kept at room temperature and in light are stable for at least ten hours. Variations in fluorescence will occur as a function of the temperature of the extract. Fluorescence intensity increases, with decreasing temperature. Fluores-

cence of extracts held at 1–2°C is approximately 10% higher than those at room temperature.

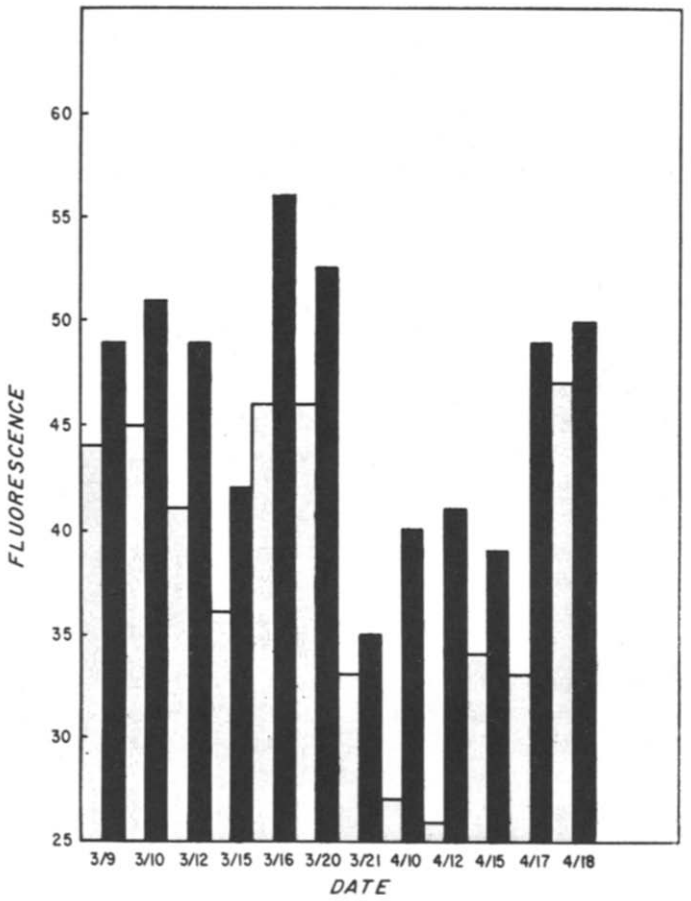

FIG. 5. Comparison of fluorescence in 85% acetone extracts of natural phytoplankton in Woods Hole waters before (stipped bar) and after (solid bar) grinding.

Table 6. The results of ten 500 ml samples filtered from a single container at Woods Hole Harbour water, 10/2/62.

Sample	*Fluorescence unacidified*	*Fluorescence acidified*
1	31·0	16·5
2	28·0	16·0
3	30·0	16·5
4	27·5	16·0
5	28·0	16·0
6	27·0	15·5
7	27·0	15·0
8	28·5	16·0
9	27·5	15·5
10	30·0	16·0
Mean deviation	1·78	0·19
Standard deviation	1·33	0·44

The precision of the present method using natural populations shows for ten samples a maximum variation of 15%. Deviation from the mean and standard deviation for the data are also given (TABLE 6). The ' blank ' calibration of the fluorometer is constant for long periods and instrument readings are repeatable within $\pm 3\%$.

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