Fluorometric Determination of Chlorophyll

By

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The concentration of chlorophyll in laboratory grown cultures of marine phytoplankton and in oceanic samples has been determined both by measurement of fluorescence and by measurement of light absorption. The lower limit for detection of chlorophyll by fluorescence with the instrumentation described is about 0·01 µg chlorophyll *a*, which is about 5% that required for a spectrophotometric determination. Through choice of appropriate filters, the amount of fluorescence reflects either the chlorophyll *a* concentration or the sum of chlorophylls *a* and *c*. By measurement of fluorescence before and after acidification, the ratio of chlorophyll to phaeophytin can be readily determined. Dilute HCl is superior to oxalic acid for acidification of pigment extracts. As the fluorometric determination of chlorophyll and phaeophytin is fast, reliable and sensitive, it will be very useful in field studies of productivity.

Introduction*

Determination of phytoplankton in any body of water is most commonly done by measuring the chlorophyll *a* content. The phytoplankton carbon may be estimated by applying appropriate arithmetic factors. Chlorophyll *a* and other pigments are usually determined by extracting them into an organic solvent and determining the absorption values at specific wavelengths; these values are then used in empirically derived formulae (PARSONS and STRICKLAND, 1963). Another method of measuring chlorophyll, which depends upon measurement of fluorescence, has been described by KALLE (1951) and more recently by YENTSCH and MENZEL (1963). This method has several important advantages over light absorption methods. Firstly, it is far more sensitive, permitting chlorophyll determinations to be made on samples of one litre or

*) Abbreviations: F, conversion factors which equate amount of fluorescence to concentration of the pigment as determined by light absorption values; O.D., optical density; R_b, amount of fluoresced light before acidification of a pigment extract; R_a, amount of fluoresced light after acidification; R_b/R_a, value of the acid factor.

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less of open-ocean water, compared with the 5–10 l required for a light absorption measurement in many such areas. Secondly, measurement of fluorescence is much quicker than determining extinction values at several wavelengths. Thirdly, the instrument required for measuring fluorescence does not depend upon critical wavelength alignment as does a spectrophotometer.

In our initial studies, however, the concentration of chlorophyll *a* as determined by fluorescence, showed considerable variation when compared with values obtained with a spectrophotometer. The factors for converting units of fluorescence to corresponding optical density values varied by as much as 50%. Both laboratory-grown cultures of several different species of phytoplankton and ocean samples were used in these determinations. The fluctuations seemed to be caused by differences in the biological material and were not due to technique of the operator, as almost identical results were obtained by three different operators. Since such deviations between fluorescence and light absorption measurements were in contrast to the results of YENTSCH and MENZEL (1963), the fluorescence method was studied in respect to factors which might influence the ratio of fluorescence to light absorption by the algal pigments. The results of these investigations are described below.

Materials and Methods

Pigments were extracted from phytoplankton collected from both coastal and open-ocean water in the Pacific and also from cultures grown in the laboratory. The phytoplankton was harvested by filtering through glass paper filters (Whatman GF/C, 4·25 cm), one ml of a 1% suspension of $MgCO_3$ in distilled water being added to each suspension as it was being filtered. The filter was then ground in a Potter-Elvehjem tissue grinder for one minute at room temperature with 4 or 5 ml of 90% acetone (STRICKLAND and PARSONS, 1965). The suspension was transferred to a centrifuge tube and the volume taken to 12·0 ml with 90% acetone. After shaking, the tube was placed in the dark for 10–30 minutes to allow complete extraction of the pigments. The suspension was then centrifuged at 15,000 × g for five minutes and the supernatant carefully decanted into 10 cm pathlength cells for optical density measurements in a Beckman DU spectrophotometer. When optical density values at 665 mμ were 0·15 or less, the extract was poured directly into small tubes for determination of fluorescence on the fluorometer; with O.D. values greater than 0·15, the extract was first diluted with 90% acetone.

The amount of chlorophyll *a* in the extract was calculated from the extinction values obtained on the spectrophotometer, using the formula given by STRICKLAND and PARSONS (1965). The amount of chlorophyll *c* separated from the hexane layer was calculated from the formula of PARSONS (1963). The following equation was used to relate chlorophyll *a* concentration to fluorescence:

$$\frac{\text{chlorophyll } a \text{ (μg)}}{\text{dilution factor}} = (F) \times (R_b),$$

where F is the conversion factor for any particular slit setting on the Turner fluorometer and R_b is the reading on the dial which indicates the amount of light received by the phototube. An estimate of the amount of phaeophytin present was obtained by measuring the amount of fluorescence before and after

Table 1

Conversion factors and acidification values for determination of chlorophyll *a* by light absorption or by fluorescence using both laboratory cultures and natural populations (See text for details)

Organism	Depth of sample in metres	Conversion factor (F) for slit 3 ($\times 10^{-5}$)	Average factor ($\times 10^{-5}$)	Average acid factor
Thalassiosira rotula.....	–	1·7, 2·3, 2·4, 2·5	2·2	2·9
Thalassiosira fluviatilis....	–	2·5, 2·7	2·6	2·6
Coccolithus huxleyi.....	–	2·0, 2·0	2·0	3·0
Amphidinium carteri.....	–	2·0	2·0	3·1
Syracosphaera elongata...	–	2·2, 2·2	2·2	2·2
Skeletonema costatum....	–	2·5, 2·9, 3·1, 3·1	2·9	1·6
Monochrysis sp.....	–	3·0, 3·0, 3·1, 3·1	3·0	2·5
Chlorella pyrenoidosa.....	–	2·9, 2·9	2·9	1·8
Ocean sample.....	0	2·3	2·3	–
Ocean sample.....	10	–	–	2·3
Ocean sample.....	15	2·6	2·6	2·3
Ocean sample.....	0	2·5	2·5	2·3
	80	2·1	2·1	1·7
Ocean sample.....	0	2·9	2·9	2·0
	10	2·9	2·9	2·0
	70	2·7	2·7	1·7
	90	3·8	3·8	1·2
Ocean sample.....	10	2·4	2·4	2·2
	25	2·5	2·5	2·1
	50	2·5	2·5	2·2
Ocean sample.....	2500	–	–	0·9

Table 2

Comparison of light absorption and fluorescence of chlorophylls *a* and *c*. Fluorescence data obtained from Turner with standard lamp, high sensitivity door, and the red 25 filter (Other details in text)

Pigment	Organism from which isolated	Conversion factor for slit 3	Acid factor
Chlorophyll *a*	*Macrocystis* sp.	$3·6 \times 10^{-2}$	2·4
Chlorophyll *a* and carotenoids	*Thalassiosira rotula*	$3·5 \times 10^{-2}$	2·3
Chlorophyll *c*	*Macrocystis* sp.	$4·1 \times 10^{-2}$	6·1
	T. rotula	$4·1 \times 10^{-2}$	5·6
Chlorophylls *a* (1·52 μg) and *c* (0·22 μg)	*Macrocystis*	$2·6 \times 10^{-2}$	2·9
Faecal pellet extract	*Calanus helgolandicus*	–	1·05

Table 3

Fluorescence before and after acidification of chlorophylls *a* and *c* when mixed in various proportions. Data obtained from Turner fluorometer with high-output lamp, standard door and slit 3 (Details in text)

Chlorophyll *a* (μg)	Chlorophyll *c* (μg)	Σ Chlorophyll (μg)	With filter R_b	CS-2-60 R_b/R_a	With filter R_b	CS-2-64 R_b/R_a
0·364	0	0·364	71·0	2·1	55·0	2·1
0·337	0·038	0·375	71·0	2·2	50·0	2·0
0·291	0·100	0·391	71·0	2·4	45·5	2·1
0·255	0·150	0·405	72·0	2·7	42·5	2·1
0·218	0·200	0·418	71·5	3·0	37·5	2·2
0·109	0·350	0·459	72·0	4·0	27·0	2·2
0	0·501	0·501	74·0	6·4	16·5	2·4

adding acid. Acidification was achieved by adding 5 drops (or 0·2 ml) of 85% acetone saturated with oxalic acid to the extract in the fluorometer tube or two drops of dilute HCl (1·0 N or 0·5 N). After shaking to affect complete mixing, readings were taken at short intervals up to 15 minutes.

The data in Tables 2 and 3 were obtained by extracting pigments from a freshly picked frond of the Brown alga *Macrocystis pyrifera*, from a laboratory-grown culture of *Thalassiosira rotula*, or from copepod faecal pellets. Chlorophyll *c* was separated from other photosynthetic pigments by hexane/acetone fractionation as described by PARSONS (1963). The hexane layer, which contained chlorophyll *a* plus carotenoids, was washed with 90% methanol and taken to dryness with a stream of nitrogen. It was then redissolved in hexane containing 0·5% n-propanol and run through a powdered sugar column, using the same solvent as the eluting agent. The fraction of eluate containing chlorophyll *a* was taken to dryness with nitrogen and redissolved in 90% acetone. The column chromatography was omitted for the chlorophyll-carotenoid containing hexane layer obtained from the extract of *T. rotula*. It was simply taken to dryness with nitrogen and redissolved in 90% acetone. The faecal pellets were from the copepod *Calanus helgolandicus* which had been reared in the laboratory on a diet of *T. fluviatilis* and *Dunaliella tertiolecta*. The pellets were separated from the culture medium by filtration through a phytoplankton net of 50 micron mesh size, and rinsed several times with millipore filtered sea water. The pellets were extracted by 90% acetone; no attempts were made to separate the pigments in this extract. The purity of the isolated pigments was checked by comparing the absorption curves obtained on a Bausch and Lomb 505 recording spectrophotometer (400 to 700 mμ) with published absorption curves of the individual pigments. There appeared to be little or no contaminating pigments in the separated solutions of chlorophylls *a* or *c*.

The spectrophotometric determination of chlorophyll is critically dependent upon proper wavelength alignment of the instrument. Proper alignment of the DU was achieved by setting the wavelength reading to the 6563 Å line emitted from a hydrogen lamp. Most of the fluorometric determinations were made in a Turner fluorometer equipped with a F4T4-BL lamp, a high sensitivity door, a Wratten 47B blue filter for the excitation light, and a Wratten red 25 filter to screen emitted light. Some results were obtained with a Turner fluorometer equipped as described by YENTSCH and MENZEL (1963); it had the high-output lamp No. 110–853, the standard door, and Corning filter CS-5-60 for light excitation and either CS-2-60 or CS-2-64 for light screening. The spectral transmission characteristics of the five filters mentioned above were determined in the Bausch and Lomb 505 recording spectrophotometer. Emission and excitation spectra of the extracted pigments were obtained in an Aminco-Bowman recording spectrofluorometer.

Results

Preliminary work indicated that readings obtained on the Turner fluorometer are very reproducible, and that dilutions of any one solution yield a straight line when the O.D. values at 665 mμ are plotted against fluorescent units (see also YENTSCH and MENZEL, 1963). There did not, however, seem to be great consistency between the conversion factors (*F* values) when extracts of different

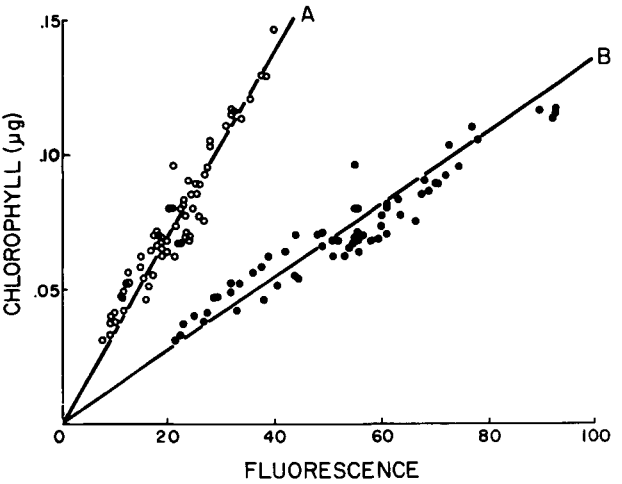

Figure 1. Relationship between chlorophyll *a* concentration as determined by light absorption and by fluorescence. All surface samples from tropical Pacific taken between 11°N–10°S and 112°W–137°W. See text for details. Curve A, fluorescent readings with slit 3; curve B, readings with slit 10.

species were compared. Data in Table 1 show both the conversion factors and the acidification ratios for different species of phytoplankton as well as for various natural populations. With a few exceptions, the value of the conversion factor for slit 3 on a Turner fitted with the high sensitivity door was between 2×10^{-2} and 3×10^{-2}. The acid factors varied from 0·9 in the ocean sample from 2,500 m to 3·0 in a laboratory culture of *Coccolithus huxleyi*.

Figure 1 shows the relationships between chlorophyll *a* concentrations determined on the spectrophotometer and readings of fluorescence obtained on a large number of surface samples collected in the eastern tropical Pacific between 11°N–10°S and 112°W–137°W. The fluorometer used for this work was equipped with the high-output lamp and filter CS-2-60; its sensitivity was about 5 times greater than the instrument fitted with the high sensitivity door. It can be seen that there was about a 15–20% variation above and below the lines drawn for slit 3 and slit 10. This variation is just a little less than that found in the laboratory work summarized in Table 1. It should be noted that when different slits are used with any one solution, the amount of fluoresced light is not necessarily in the ratios of 1, 3, 10, and 30 as would be expected from the nominal slit sizes. The exact relationships must be determined with any particular instrument.

The various conversion factors obtained (Table 1) and the scatter about the lines in Figure 1 might be reflecting different quantum yields for fluorescence of the various photosynthetic pigments. The fluorescence of isolated pigments was therefore examined. The values obtained for light absorption and for fluorescence of solutions of chlorophylls *a* and *c* in 90% acetone are shown in Table 2. It may be seen that the amount of light fluoresced by chlorophyll *c* is only 88% of that emitted by chlorophyll *a* per unit dry weight of each pigment (fluorescent intensities are inversely proportional to conversion factors). When chlorophylls *a* and *c* were mixed in the ratio of 7 to 1 (by dry weight), the

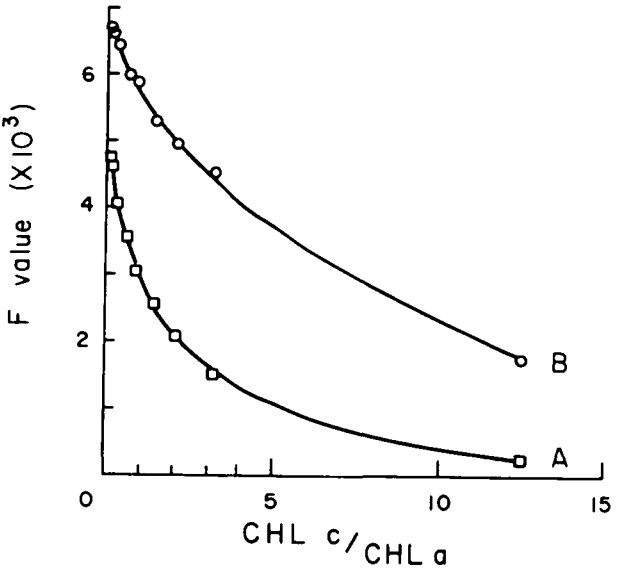

Figure 2. Relation of conversion factors (F) to the ratio of chlorophyll *c* to chlorophyll *a*. Fluorometer equipped with high-output lamp, standard door and slit 3. Curve A, with CS-2-60 filter; curve B, with CS-2-64 filter.

conversion factor was only $2{\cdot}6 \times 10^{-2}$. The fact that this value is lower than that for chlorophyll *a* ($3{\cdot}6 \times 10^{-2}$) and for chlorophyll *c* ($4{\cdot}1 \times 10^{-2}$) is because the fluorescence reading is the sum of the light emitted from both chlorophyll *a* and chlorophyll *c*, but in calculating the value for *F* only the extinction values relating to chlorophyll *a* were used.

Additional data concerning the dependence of fluorescence upon the ratio of chlorophyll *a* to chlorophyll *c* in the extract are shown in Table 3 and Figure 2. Table 3 shows the actual amounts of chlorophylls *a* and *c* in solutions prepared by mixing samples of the isolated pigments and the amount of fluorescence when using either the CS-2-60 or CS-2-64 filter. The *F* values obtained from these data are shown in Figure 2. It is seen that both lines A and B have negative slopes which are steepest in the region where the ratio of chlorophyll *c* to chlorophyll *a* is between 0 and 1. The variation in *F* values in this region is, however, far less for filter CS-2-64 (curve B) than for filter CS-2-60 (curve A).

It is possible to determine the ratio of chlorophyll *a* to phaeophytin *a* by fluorescence measurements as the fluorescence of phaeophytin *a* is only about 42% that of the same concentration of chlorophyll *a*. Chlorophyll is readily converted to phaeophytin by dilute acids. YENTSCH and MENZEL (1963) used oxalic acid-saturated acetone for acidification of chlorophyll solutions but the rate of conversion of chlorophyll to phaeophytin is low with this acid. Dilute hydrochloric acid, however, resulted in a very rapid and reproducible decrease in fluorescence. Typical results showing the effects of these two acids are shown in Figure 3. It is seen that an acid factor of 2·0 was obtained with oxalic acid only after 5–10 minutes, whereas with addition of HCl an acid factor of 2·4

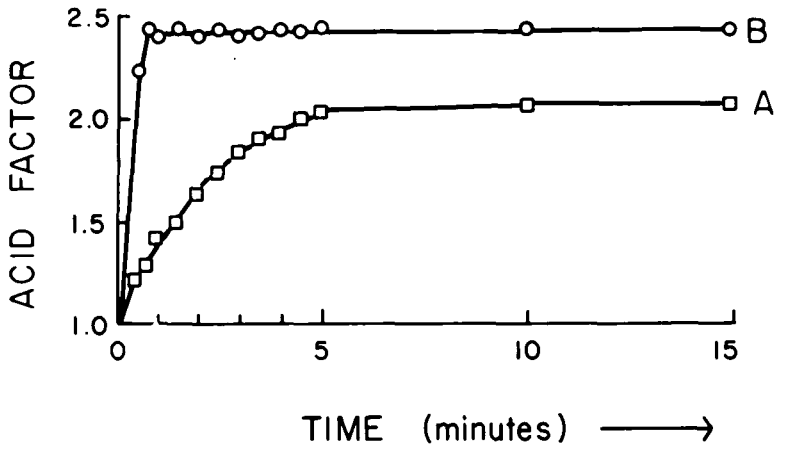

Figure 3. Time curves showing decrease of fluorescence upon acidification of chlorophyll solutions. Five ml of a 90% acetone extract of *Chaetoceros* sp. treated with 0·2 ml of 85% acetone saturated with oxalic acid (curve A) or with 2 drops of 1N HCl (curve B). Measurements made with fluorometer with the high-output lamp and filter CS-2-60.

was obtained within 1 minute. Neither the concentration nor the amount of HCl added to the solutions seem to be very critical. For general work, two drops of 0·5 N HCl gave excellent results.

The acidification factors obtained on various cultures and in ocean samples ranged from 0·9 to 3·1 (Table 1). As YENTSCH and MENZEL (1963) used a value of 1·7 to indicate pure chlorophyll, it was surprising to get values so much higher than 1·7. It is seen from Table 2, however, that chlorophyll *c* isolated from *Macrocystis* and from *T. rotula* has acid factors of 6·1 and 5·6, respectively. The acid factors for chlorophyll *a* isolated from the same organisms were 2·4 and 2·3, respectively. When chlorophylls *a* and *c* were mixed in the ratio of 7 to 1, which corresponds to a ratio that might be found in nature, an acidification ratio of 2·9 was obtained. Copepod faecal pellets yielded an acetone extract which had an acid factor of 1·05. The absorption spectrum of this extract was very similar to that of pure phaeophytin *a* (SMITH and BENITEZ, 1955).

Similar results are shown by the acid factors in Table 3. When using the CS-2-60 filter, the acid factors ranged from 2·1 (all chlorophyll *a*) to 6·4 (all chlorophyll *c*). With the CS-2-64 filter, the range in value of the acid factor was 2·1 (all chlorophyll *a*) to 2·4 (all chlorophyll *c*).

Figure 4 shows the emission spectra of chlorophylls *a* and *c* when excited with light of 420 mμ, as well as the absorption curves for the five different filters used in the present investigation. It is seen that the blue filters should be equally good for this work as they both transmit maximally in the region of 420 mμ. The three red filters differ appreciably, however, in the extent to which they absorb the light fluoresced by chlorophyll *c*. At the peak of chlorophyll *c* fluorescence (about 632 mμ), filters 25, CS-2-60, and CS-2-64 transmit about 80%, 40% and $< 1\%$ respectively, of the incident light.

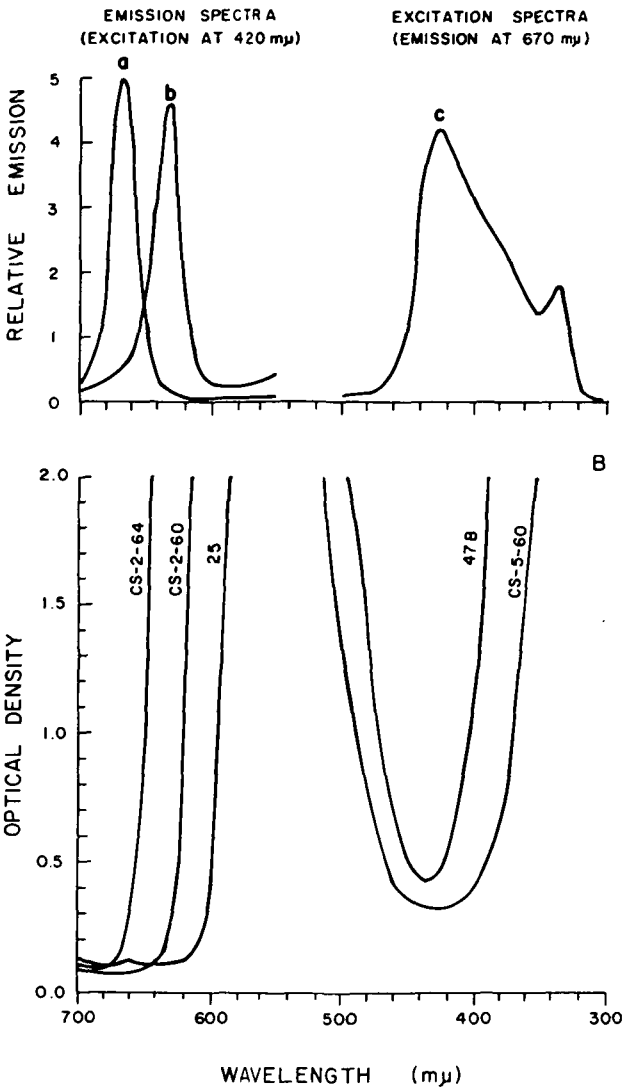

Figure 4.
(A) Emission and excitation spectra of chlorophylls *a* and *c* extracted from *Macrocystis* sp. Curve a, chlorophyll *a*; curve b, chlorophyll *c*; curve c, mixture of chlorophylls *a* and *c*.
(B) Absorption spectra of the red and blue filters used in the fluorometers.

Discussion

The value of the fluorescence method of chlorophyll determination is that it enables one to get a quick and sensitive estimate of the concentration of both chlorophyll and phaeophytin. In order to estimate these pigments, it is necessary to know (1) the conversion factor for equating a unit of fluoresced light to a specific amount of chlorophyll, (2) the ratio of the fluorescence of chlorophyll to that of an equivalent weight of phaeophytin. If one were working with pure, isolated pigments, the values for the above factors would be constant. In acetone extracts of phytoplankton, however, there is a mixture of chlorophylls and carotenoids, all of which can affect the magnitude of the factors mentioned above. These effects are discussed later in this section.

The conversion factor which is used in the calculations below ($2{\cdot}5 \times 10^{-2}$ for slit 3 of the Turner equipped with Lamp No. F4T4-BL and high sensitivity door) was obtained by taking the average value of conversion factors obtained from laboratory-grown cultures and also from surface or near-surface samples of oceanic phytoplankton. It is seen from the data that many factors were obtained which were larger than $2{\cdot}5 \times 10^{-2}$, but these can be correlated with the presence of phaeophytin. When a solution of chlorophyll *a* is acidified the extinction value of 665 mμ decreases by about 40%, while the amount of light fluoresced decreases by about 58%. A large amount of phaeophytin in a sample (before acidification) will therefore be associated with less fluorescence than would be expected on the basis of light absorption at 665 mμ. As the conversion factor is obtained by dividing the amount of chlorophyll as determined by light absorption by the amount of fluorescence, a greater proportionate decrease in the divisor will result in high conversion factors.

The acidification ratios of phytoplankton extracts ranged from 0·9 to 3·1 (see Table 1), while for pure chlorophyll *a* and *c* they were about 2·4 and 6·1, respectively. As pure phaeophytin would yield a ratio of 1·0, the low ratios obtained in natural populations must be associated with a large ratio of phaeophytin to chlorophyll. Ratios between 2·5 and 3·1 indicate the presence of significant amounts of chlorophyll *c* or some other pigment which shows a high acidification number. As natural populations will often contain considerable amounts of accessory chlorophylls, the acid factor for chlorophyll *a* (2·4) can not be used. The value used (3·0) in the calculations below was chosen as it represents the higher limit of acid factors obtained from healthy samples of phytoplankton; it also is in close agreement to the value of 2·9 obtained when chlorophylls *a* and *c* were mixed in the ratio of 7 to 1 (Table 2).

Using the above values, the equations for obtaining the concentrations of chlorophyll *a* and phaeophytin *a* are as follows:

$$\text{Chlorophyll } a = (1{\cdot}5) \times (F) \times (R_b - R_a) \quad (1)$$

$$\text{Phaeophytin } a = (1{\cdot}5) \times (F) \times [3{\cdot}0\,(R_a) - R_b] \quad (2)$$

It should be noted that if a sample shows an acidification ratio of greater than 3·0, then equation (2) would yield a negative value and equation (1) would give too high a value for chlorophyll *a*. This merely reflects the fact that the value used for the upper limit of the acidification ratio will be dependent upon the ratio of chlorophyll *a* to accessory chlorophylls. Occasionally one may obtain

a sample which is unusually high in chlorophyll *c* relative to chlorophyll *a*, which would lead to the negative value for phaeophytin when calculated as in equation (2). If such samples are encountered in nature it would be of interest to determine the pigment composition of the algae to verify that they do indeed have a low amount of chlorophyll *a* relative to chlorophyll *c*, or that they might have other pigments which are fluorescing red light.

In their equation for estimation of phaeophytin, YENTSCH and MENZEL (1963) used an acidification ratio of 1·7. This was obtained by acidifying with oxalic acid-saturated acetone (85%) and reading the fluorescence after 3 minutes. From Figure 3 it is seen that a similar number was obtained when we repeated their method. However, when dilute HCl was used, phaeophytinization was complete within a minute. If one waits 10–15 minutes before reading the fluorescence on the oxalic acid treated sample, it is significantly higher than after 3 minutes. Figure 3 shows that the final value is about 83% of that obtained in the sample treated with HCl. For most samples of natural phytoplankton, the final values obtained (after 10–15 minutes) with oxalic acid or HCl have agreed within 10%. In all these cases, however, the value obtained with oxalic acid after only three minutes was significantly lower. While dilute acids remove the Mg from chlorophyll (to form phaeophytin), strongly acidic conditions can degrade the phaeophytin to phaeophorbide by removal of the phytol group. The concentration of HCl added to our samples is not, however, sufficient to cause removal of the phytol group. It therefore seems that HCl is preferable to oxalic acid in this method as it produces a reliable and rapid conversion of chlorophyll to phaeophytin.

It should be recognized that photosynthetic pigments are not the only biological materials which will emit red light upon absorption of blue light. The dihydroporphyrin compounds (chlorophyll derivatives) are the most active porphins in regard to fluorescing light of wavelengths greater than 580 mμ, but many other porphyrins and metalloporphyrins also can fluoresce in this region upon excitation with blue light (FALK, 1964). In most field work, the concentration of such porphyrins will be insignificant in the amounts of water filtered for a chlorophyll determination. When 16 l of water from a depth of 2500 m was filtered, however, there was no detectable absorption at 665 mμ, but the fluorometer gave a reading of 52 with slit 30. The acidification ratio of this sample was 0·9; that is, fluorescence increased upon adding acid. Without a spectral analysis of the pigment(s) responsible for this fluorescence, it is not possible to ascribe the fluorescing material to plant or animal origin. As phaeophytins *a* and *b* and phaeophorbides *a* and *b* have been detected in marine sediments and fossil fuels (VALLENTYNE, 1960) it seems likely that the observed fluorescence is due to degradation products of chlorophyll, though compounds of animal origin can not be ruled out at the present time.

There are various sources of errors inherent in the fluorescence method of chlorophyll determination. (1) Reabsorption of fluoresced light by one or more of the photosynthetic pigments will result in low estimates of chlorophyll *a*. This effect would be most pronounced in pigment-rich extracts, and apparently is the reason why readings with slit 1 of the fluorometer are considered to be unreliable (YENTSCH and MENZEL, 1963). (2) MURTY and RABINOWITCH (1964) showed that when chlorophyll *a* is excited by blue light, the presence of B-carotene quenches the red fluorescence of chlorophyll. This effect, which was

not present when the chlorophyll was excited with light at 660 mμ, was attributed to intermolecular quenching of the second excited state of chlorophyll *a*. (3) Allomerization of chlorophyll (oxidation of carbon no. 10 in the cyclopentanone ring) can occur when chlorophyll is in solution and exposed to oxygen, and is accelerated by the presence of certain inorganic ions or oxidizing agents (RABINOWITCH, 1951, p. 613). The effect of allomerization on fluorescence differs in magnitude for the various chlorophylls (RABINOWITCH, 1951, p. 754), and hence might be responsible for changing the amount of fluorescence per unit chlorophyll. (4) As the conversion factors were obtained by relating fluorescence to amount of chlorophyll *a*, the presence of any other pigments which absorb blue light and fluoresce red light would result in artificially high values for chlorophyll *a*. These would include the chlorophylls other than chlorophyll *a*, the phycobilin pigments, and various porphyrins (FALK, 1964).

As the fluorescent method of chlorophyll determination will most likely find increasing use in oceanographic research, it is well to consider the significance and magnitude of the errors inherent in the method. As presently used, the fluorescent method integrates all fluoresced light which passes through the emission filter. When using the red 25 filter, one can not distinguish between chlorophylls *a*, *b*, or *c*. One way in which one could measure the fluorescence of the individual pigments would be to use filters which transmit just a narrow band at the peak of fluorescence of each pigment. This would, however, greatly reduce the sensitivity of the method, and it would also be limited by the fact that the fluorescence bands of the various chlorophylls overlap somewhat, just as do the absorption spectra. The choice of emission filter will be dictated by whether one wants a value for total chlorophyll or only a value for chlorophyll *a*. If the former is desired, then the red 25 filter would be appropriate. The use of this filter involves, however, the errors introduced by different F values and acid factors for the different chlorophylls. If one wanted to know only the amount of chlorophyll *a*, filter CS-2-64 would be the best of the three red filters we had available for the present investigation. This filter absorbs only about 10% of the light fluoresced by chlorophyll *a* but over 80% of the light fluoresced by chlorophyll *c*. The use of this filter thus greatly reduces the error in the determination of chlorophyll *a* introduced by the presence of chlorophyll *c*.

The amount of phaeophytin in laboratory grown cultures is very small and often can not be detected (PATTERSON and PARSONS, 1963). LORENZEN has shown (unpublished) that the ratio of phaeophytin to chlorophyll, as determined by fluorescence before and after acidification, does not vary significantly in cultures of *Chaetoceros* sp. which have been standing for 60 days. It would appear, therefore, that a large amount of phaeophytin can best be correlated with grazing by zooplankton (CURRIE, 1962). The faecal pellets mentioned in Table 2 showed an acid factor (1·05) characteristic for phaeophytin, and also had an absorption curve from 400 to 700 mμ that resembled published spectra of phaeophytin. The decrease in acid ratios as one increases in depth (Table 1) is thus most likely an indication of increasing accumulation, near and below the pycnocline, of faecal material.

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Summary

1. The concentration of chlorophyll *a* in extracts of natural populations and in laboratory cultures was determined both by fluorescence and by light absorption measurements. The chlorophyll *a* concentration as determined by fluorescence varied by about $\pm$ 20% of the value as determined spectrophotometrically.

2. Much of the variation in chlorophyll *a* concentration as determined by fluorescence is due to algal pigments other than chlorophyll *a* which can fluoresce red light when excited with blue light. Through the use of different filters, the error introduced by these accessory pigments (such as chlorophyll *c*) can be largely eliminated. This, however, greatly decreases the sensitivity of the chlorophyll *a* determination.

3. Equations are given for the calculation of the amount of chlorophyll *a* or phaeophytin *a* from measurements of fluorescence before and after acidification.

4. The use of dilute HCl is preferable to oxalic acid in converting chlorophyll to phaeophytin, as HCl causes complete phaeophytinization within one minute.

5. The determination of phaeophytin *a* is complicated by the fact that the different chlorophylls may show very different acid factors. Thus, pure phaeophytin *a* fluoresced about 42% as much light as an equivalent weight of chlorophyll *a*, while pure phaeophytin *c* fluoresced only about 16% as much as an equivalent amount of chlorophyll *c*.

6. The acid factor (using HCl) of natural populations decreases with depth. The factor of surface samples averages about 2·3, and decreases to about 1·0 in deep samples. An acid factor of 1·0 indicates all phaeophytin (or possibly other degradation products of chlorophyll) and little or no chlorophyll, and is suggestive of grazing by zooplankton.

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