Determination of Chlorophyll and Pheo-Pigments: Spectrophotometric Equations[1]

It has been shown that chlorophyll degradation products may at times constitute a significant fraction of the total green pigments present in seawater (Yentsch and Menzel 1963; Lorenzen 1965; Yentsch 1965). These degraded forms, or inactive chlorophyll, absorb light in the red part of the spectrum; if they are present in concentrations significant relative to chlorophyll *a*, a serious error may be introduced into chlorophyll data using the present spectrophotometric techniques (Richards with Thompson 1952; Parsons and Strickland 1963), because the absorption of light by the degraded forms is not distinguished from that absorbed by active chlorophyll.

Chlorophyll *a* can readily be converted to pheophytin simply by the addition of a weak or dilute acid, either oxalic acid or 1 N HCl, and when the reaction is carried out on a specific sample the absorbancy of the solution is reduced (Vernon 1960). The pheo forms of chlorophyll, both pheophytin and pheophorbide, do not show a reduction in absorbancy when treated with an acid, although the chlorophyllide apparently does (unpublished data). This reduction in absorbancy by acid is probably the result of the removal of the bound Mg atom in the porphyrin ring.

This simple property, brought about by the conversion of chlorophyll to pheophytin, could be used in a method for the determination of chlorophyll *a* in samples containing pheo-pigments. The fluorometric technique for pigment determination (Yentsch and Menzel 1963; Holm-Hansen et al. 1965) has been used extensively in this laboratory, and its precision and the recovery of both chlorophyll and pheophytin from prepared solution is excellent; but the presence of chlorophyll *b* may interfere with the determination since, unlike the other two chlorophylls, *a* and *c*, *b* shows an increase in fluorescence upon acidification. Algae containing chlorophyll *b* generally have an acid factor slightly less than those without chlorophyll *b*, which will result in a slight underestimation of chlorophyll *a* and a slight overestimation of pheopigments. In taxonomically diverse populations this would not be a serious problem. On the other hand, all three chlorophylls show a reduction in absorbancy upon acidification; that is, the pheophytin absorbs less light per unit weight than its respective chlorophyll.

The method proposed is similar to that described by Vernon (1960) and others reviewed by Holden (1965), but absorbancies are measured at only two wavelengths, 750 and 665 mμ, before and after acidification of the sample, and only chlorophyll *a* and pheo-pigments are calculated. An attempt to measure all three chlorophylls and their pheo forms was deemed unwarranted for routine measurements because eight absorbancies would have to be determined on each sample, and the uncertainty of the calculated values derived for chlorophyll *b* and *c* and their degraded forms would be too high.

This method may find application in freshwater, estuarine, and coastal environments where a relatively small quantity of water can be filtered in a short time yielding samples with an absorbancy at 665 mμ of 0.2 or greater.

EXPERIMENTAL

Chlorophylls were purified in three ways from *Dunaliella* sp., *Chaetoceros* sp., *Macrocystis* sp., and English Ivy. The latter two plants were used when a large quantity of pigment was desired.

The first purification method was two-dimensional paper chromatography according to the method of Jeffrey (1961) using 4% *n*-propanol in ligroine (bp: 65–90C) for the first dimension and 30% chloroform in

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TABLE 1. *Change in absorbancy of chlorophyll* a, b, *and* c *at the wavelengths specified when treated with 1 N HCl. Readings expressed as ratio of absorbance before divided by absorbance after the addition of acid*

	665 mμ	645 mμ	630 mμ
Chlorophyll *a*	1.7	1.4	2.0
Chlorophyll *b*	1.0	2.3	2.0
Chlorophyll *c*	1.0	2.0	3.1
Chaetoceros sp.	1.67	1.91	3.57
Dunaliella sp.	1.71	2.14	3.45
Gymnodinium sp.	1.66	2.03	3.00

ligroine for the second dimension. Pigments were identified by their movement relative to the solvent front and each other, and R_f factors usually corresponded to published factors. Chromatograms were run simultaneously to yield enough pigment for absorption spectra (obtained with a Bausch and Lomb 505 recording spectrophotometer), and these were compared with published absorption spectra.

Larger quantities of pigments were handled either by column chromatography or partition chromatography. Pigments were separated on sugar columns (Strain 1958) using a variety of solvents, but hexane was used most frequently. A good separation of chlorophyll *a* and *c* was obtained by the method of Parsons (1963), although this did not provide a good separation of chlorophyll *c* and chlorophyllide *a*. Chlorophyll *a* and carotenoids were not separated by partition chromatography. At times a combination of all three techniques was used, but the paper chromatography was usually used as a criterion of purity.

The pheo form of the pigment was either prepared from the chlorophyll by acidification, or obtained from fecal pellets of crustaceans feeding on algal suspensions. The former methol produced pheophytin, but if chlorophyllide *a* was acidified, the pheophorbide was produced. Fecal pellets yielded pheophorbide rather than pheophytin.

RESULTS

Pigments were always transferred to 90% acetone before spectrophotometric measurements. Absorption spectra were obtained for chlorophyll *a*, *b*, and *c*, and for their respective pheophytins. Pheophorbide was spectrophotometrically indistinguishable from pheophytin *a* but was readily identified by chromatography. It was not possible to obtain enough pure chlorophyllide *a* to measure an absorption spectrum, and it was uncertain if the chlorophyllide or pheophorbide of the other two chlorophylls were observed.

The red absorption maximum of chlorophyll *a* and *b* shifted to longer wavelengths upon acidification, and the red peak for chlorophyll *c* virtually disappeared when acidified in acetone. These pigments behave similarly in ether (Smith and Benitez 1954), and have been observed to behave in this manner in acetone by other investigators (Vernon 1960; Parsons 1963).

The reduction in absorbance observed upon acidification of chlorophyll extracts showed some variation which seemed to be due to the handling technique. Fresh extracts, and those that were not dried when transferring from one solvent to another, showed a reduction in absorbance at 665 mμ of 0.58–0.61 of the initial value. Other extracts showed a much smaller reduction, sometimes only 0.8 of the original. For the fresh extracts, the ratio before acidification to that after acidification (corrected for the 750 mμ reading), $665_0 : 665_a$, was 1.7. Chlorophyll *a* showed a similar change at 665 mμ, while both chlorophyll *b* and *c* showed essentially no change. All three chlorophylls showed changes at the other two wavelengths normally used for pigment estimation, 645 and 630 mμ. The magnitude of these changes is shown in Table 1.

PROPOSED METHOD

The sample should be handled as follows: A volume of water is filtered through a Whatman GF/C glass paper filter. These filters have a slightly irregular effective pore size, so a small quantity of $MgCO_3$ suspension is placed on the filter and drawn through before the water sample is filtered. After the sample has passed through the filter, the filter is ground in a Teflon tissue grinder with acetone until it is thoroughly macerated. The time will vary with the

motor used, but a regular hand drill with about 1,000 rpm is satisfactory and will macerate the filter in about 1 min. The macerated sample is placed in a centrifuge tube with the required rinses of the mortar and pestle, and the final volume is made up to 10 ml plus the volume of the filter. The pigments are eluted for 30–60 min and then centrifuged. The centrifuge tubes can be shaken once or twice during extraction. The absorbance is read at 750 and 665 mμ in a spectrophotometer before and after acidification with 2 drops of 1 N HCl. The absorbance can be controlled to some extent by adjusting the amount of water filtered, volume of acetone used to extract the sample, and the length of the cuvette. The greater the first and last of these factors, and the smaller the volume of acetone used, the greater the absorbance will be. It is important to keep the absorbance greater than 0.2 for the initial reading, but it is probably not necessary for it to be above 0.5. The cuvette should be shaken after the acid is added. Most cuvettes, including the 1-cm cell, hold at least 4 ml and the quantity of acid added does not appreciably alter the final readings.

The readings obtained at 665 mμ before and after acidification, corrected for the 750 mμ reading and cell to cell differences, are entered in the following equations.

$$\text{Chl } a \text{ (mg/m}^3) = \frac{A \times K \times (665_0 - 665_a) \times v}{V_f \times l},$$

$$\text{pheo (mg/m}^3) = \frac{A \times K(R[665_a] - 665_0) \times v}{V_f \times l},$$

where

- A absorption coefficient of chlorophyll $a = 11.0$,
- K factor to equate the reduction in absorbancy to initial chlorophyll concentration, 1.7 : 0.7, or 2.43,
- 665_0 absorbance before acidification,
- 665_a absorbance after acidification,
- v volume of acetone used for extraction (ml),
- V_f liters of water filtered,
- l path length of cuvette (cm)
- R maximum ratio of $665_0 : 665_a$ in the absence of pheo-pigments, 1.7.

TABLE 2. *Recovery of chlorophyll* a *and pheo-pigment from known solution using the proposed equations. Concentrations of pigments expressed in μg/10 ml of 90% acetone solution. Also shown are the values of chlorophyll* a *that would have been calculated if the sample had not been acidified and a second reading taken, and the ratio of absorbance before and after acidification,* $665_0 : 665_a$

Chlorophyll		Chloro-phyll without correction	Pheo-pigment		$665_0 : 665_a$
Added	Recovered		Added	Recovered	
1.70	1.70	1.69	0.00	0.03	1.69
1.57	1.59	1.58	0.15	0.06	1.67
1.42	1.36	1.55	0.30	0.39	1.54
1.29	1.32	1.53	0.45	0.44	1.52
1.16	1.14	1.49	0.60	0.67	1.44
1.02	0.92	1.42	0.75	0.92	1.35
0.88	0.88	1.41	1.02	0.97	1.33
0.75	0.79	1.37	1.05	1.04	1.30
0.61	0.67	1.33	1.20	1.19	1.26
0.47	0.55	1.30	1.35	1.33	1.20
0.00	0.09	1.16	1.88	1.88	1.02

CONCLUSIONS

The proposed method is intended to enable one to estimate both chlorophyll *a* and pheo-pigments. Since the change in absorbance brought about by treatment with 1 N HCl is measured, it in fact probably discriminates between chlorophyllous compounds containing Mg atoms and those which are Mg-free. Chlorophyll *a* and chlorophyllide *a*, if present, are measured as "chlorophyll *a*" and both pheophytin and pheophorbide are measured together as "pheo-pigments." The calculation of the pheo-pigments assumes that all this pigment is pheophytin, which probably is not the case (Patterson and Parsons 1963), but the absorption coefficient of pheophorbide is unknown.

An estimate of the recovery of both chlorophyll *a* and pheo-pigments was made by mixing a known solution of chlorophyll *a* and pheophytin in varying proportions (Table 2). The amount of chlorophyll *a* that would have been calculated if no acid was added and the ratio $665_0 : 665_a$ are included in Table 2. The recovery of either chlorophyll *a* or pheo-pigment is high if the concentration is high, and the error of estimation increases when the concentration decreases. The difference

between the value calculated for chlorophyll without adding acid and that calculated using the above equation is small when the pheo-pigment concentration is low, but it increases with increasing concentrations of pheo-pigments. This is the case when one samples at increasing depths in the open ocean (Lorenzen 1965) and in coastal areas where wave action may either resuspend particles from the bottom or keep particles in suspension (unpublished data). In the Sacramento River delta, chlorophyll samples usually show a rather low acid ratio at 665 mμ, which would indicate that pheo-pigments frequently form a large fraction of the green pigments absorbing light at 665 mμ (T. E. Bailey, personal communication).

This method may be useful if high contamination of samples with pigments other than chlorophyll is suspected. This could be determined by acidifying a few of the regular samples after the normal procedure and again observing the absorbancies at 665 and 750 mμ. If the ratio of the corrected readings at 665 mμ is less than 1.6, the above procedure should be followed.

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THE USE OF FILAMENT TAPE IN RAISING LONG CORES FROM SOFT SEDIMENT

This note describes the advantages of using filament tape to line the inside of a sediment coring tube. Such tape has been used with a hand-driven aluminum sampler (Livingstone 1955) weighing 10 or 20 kg and suitable for use by one man from a skiff in 10 or 20 m of water. It should be equally valuable when used with any of the variety of heavier piston samplers suitable for work in soft sediment in water of any depth (Wright, Cushing, and Livingstone 1965).

It is desirable for cores from the soft sediments of lakes or the ocean to be long, complete, and undisturbed. The principal barrier to obtaining such cores is the fric-