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Chlorophyll a determination: improvements in methodology

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Chlorophyll and phaeopigment determinations on fresh water and marine samples of phytoplankton have shown that the following considerations are important in routine pigment analyses. (a) Methanol should be used as extraction solvent instead of acetone due to better extraction efficiency, shorter extraction time, and elimination of the need to boil or homogenize the samples. (b) For spectrophotometric determinations of phaeopigments, the methanol extracts should be acidified with HCl to a final concentration of 3×10^{-3} M. (c) When a spectrophotometer is used for phaeopigment determination, subsequent neutralization of the acidified methanol extracts to the original pH is required; fluorometric determinations do not require this neutralization. (d) The addition of $MgCO_3$ to filters or to extracts is not necessary. (e) There is no detectable loss of chlorophyll when either sample extracts or wet filters are stored at $-20^\circ C$ for three weeks and then extracted with either acetone or methanol. (f) Glass fiber filters are effective in retaining all phytoplankton and have some advantages over membrane filters.

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Определения содержания хлорофилла и феопигмента в пробах пресноводного и морского фитопланктона показали, что в системе анализа пигмента имеют значение следующие соображения: а/ для экстракции следует использовать вместо ацетона метанол, обладающий более высокой эффективностью экстракции, более короткой продолжительностью экстракции, не требующей кипячения или гомогенизации проб; б/ для спектрофотометрических определений феопигментов метаноловые экстракты должны быть подкислены соляной кислотой до окончательной концентрации 3×10^{-3} М; в/ при использовании спектрофотометра для определения феофермента необходима последовательная нейтрализация подкисленных метаноловых экстрактов до исходной pH. Флуориметрические определения не требуют нейтрализации; г/ добавление $MgCO_3$ к фильтру или экстракту не требуется; д/ при хранении экстрактов проб или влажных фильтров при -20° в течение 3–х недель с последующей экстракцией ацетоном или метанолом заметных потерь хлорофилла не установлено; е/ фильтры из стеклянного волокна эффективно удерживают весь фитопланктон и имеют преимущество перед мембранными фильтрами.

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1. Introduction

For determination of chlorophyll in sea water or fresh water samples most investigators use the procedures outlined by Strickland and Parsons (1968) or in the UNESCO report (1966). These procedures involve the use of $MgCO_3$ on membrane filters, grinding of samples in distilled acetone, and measurement of the extracted chlorophyll after short storage periods. We used these same procedures originally, but during the course of some years we have modified and improved many aspects of the above methodology. The changes we have incorporated into our procedures involve (a) type of filter used, (b) nature of the extraction medium, (c) elimination of $MgCO_3$ (d) elimination of homogenization of samples, (e) treatment of the pigment extract by either fluorometric or spectrophotometric methods, and (f) storage time of frozen filters. Our present procedure, as described in this paper, represents a more efficient and reliable methodology for determination of chlorophyll a and phaeopigment a in natural water samples.

2. Methods and materials

The sea water samples were obtained with alcohol-scrubbed 8.0-l PVC Van Dorn bottles, and the fresh water samples with 2-l polythene bottles. Replicate aliquots of water were filtered at a vacuum of 25 cm Hg through either a 2.5 cm microfibre glass fiber filter (Reeve Angel 984H), a 2.5 cm GF/C glass fiber filter (Whatman), or an HA membrane filter (Millipore) with a pore size of 0.45 μm . When $MgCO_3$ was added to the sample, 1.0 ml of a 1% suspension was added either to the last few hundred ml of the sample, or added to the filter before addition of the water sample. Filters were either extracted immediately as outlined below, or were placed in 15 ml glass-stoppered test tubes and stored at $-20^\circ C$ until time of extraction.

Acetone extractions: Immediately upon completion of filtration, the filter was folded in half and either (a) placed in a glass-stoppered centrifuge tube, to which was added 10 ml of 90% acetone, or (b) ground with 90% acetone in a Potter-Elvehjem homogenizer or in a Braun Melsungen (Type 853202) watercooled homogenizer, after which the slurry was transferred to glass-stoppered centrifuge tubes and taken to a final volume of 10 ml with 90% acetone. Samples were stored in complete darkness at room temperature during the extraction period, which varied from 10 min to many hours (details in text). At the end of the extraction period, the tubes were shaken by hand, centrifuged at $1000 \times g$ for 5 min at $4^\circ C$, and then taken to room temperature by placing in a water bath for 10 min. The acetone used in these extractions was reagent grade, but it was neither distilled nor stored with $MgCO_3$ as suggested by Strickland and Parsons (1968). Years of experience and many test extractions have demonstrated

to us that distillation of the acetone and the use of $MgCO_3$ are not necessary if high quality acetone is used.

Methanol extractions: After filtration the filter was folded and either treated as above (except that absolute methanol was used instead of acetone), or the filter was placed in 3 ml of absolute methanol. For the samples in 3 ml methanol, after one hour of extraction the filter was wrung against the sides of the glass vessel using a glass roller and then rinsed twice in methanol; the final solution was then filtered through a Whatman glass fiber filter to obtain a clear solution free from filter residues and cell debris. To avoid oxidation and allomerization of the pigments during the period of extraction, the methanol was saturated with hydrogen sulphide before use (Jensen and Sakshaug 1973).

The concentration of chlorophyll a and phaeopigment a in the extracts was determined either fluorometrically in a Turner model No. 111 (Holm-Hansen et al. 1965) or spectrophotometrically with a Beckman DB-GT spectrophotometer. The fluorometer was equipped with a Corning CS2-64 filter for the excitation light and a Corning C52-64 filter for the emitted light. Calculations of amount of chlorophyll a and phaeopigment a followed Lorenzen (1967) and Moss (1967a, b). Absorption coefficients for pigments in methanol are cited in the text.

The emission spectra of pigment fluorescence were measured in a Turner spectrofluorometer (model 430-025) with an activation wavelength of 440 nm. It should be noted that this instrument is an "uncorrected" spectrofluorometer, and hence the spectra obtained with it are not corrected for non-linearity associated with the photodetector as well as for other instrumental artifacts. The spectra shown in this paper (Figs 2 and 4) may thus show minor differences with other data in the literature, but they are sufficiently reliable and valid to support our conclusions.

Paper chromatographic separations of chlorophylls a and b followed Jensen and Liaaen-Jensen (1959) and Jensen and Sakshaug (1973).

3. Results and discussion

3.1. Choice of extraction solvent

Most researchers in oceanography and limnology, as well as terrestrial ecology (Linder 1974), still use the traditional acetone for pigment extraction. It has been well known, however, that very low acetone extraction efficiency is obtained when common algae belonging to the Chlorophyceae or Cyanophyceae are extracted and compared to methanol extraction (Steemann Nielsen 1961, UNESCO 1966, Golterman 1969, Rai 1973, Riemann 1976, Sand-Jensen 1976). Although the use of methanol is common in laboratories studying the biochemistry and physiology of algae, there has been great apathy by aquatic ecologists to changing their extraction methodology. The data we present below show that methanol extracts pigments from phytoplankton faster

Tab. 1. Efficiency of 90% acetone and absolute methanol for extraction of chlorophyll a from diatoms and blue-green algae from Lake Mossø. Samples were not homogenized. Standard error is based on five replicate samples. Pigment levels were determined spectrophotometrically according to Moss (1967a) and Riemann (1976).

Dominant phytoplankton	Extraction time (h)	µg chlorophyll a/sample	
		Acetone	Methanol
Diatoms	1	34.2 ± 0.4	37.0 ± 0.6
	6	35.1 ± 0.8	—
	12	36.2 ± 0.7	—
	20	36.8 ± 0.6	—
Blue-green algae	1	28.6 ± 0.2	34.1 ± 0.4
	6	29.4 ± 0.4	—
	12	29.8 ± 0.8	—
	20	30.0 ± 0.5	—

and more completely than does acetone, and also that there are no inherent problems associated with the use of methanol for either spectrophotometric or fluorometric determinations of pigment concentration.

The extraction efficiencies of acetone and methanol were studied with fresh water samples from eutrophic Lake Mossø, Denmark, which were dominated either by diatoms or by blue-green algae (mostly *Aphanizomonon flos aquae* Ralfs). The results (Tab. 1) show that after one hour there was complete extraction in all methanol samples, but incomplete with the acetone extracts. After 20 h extraction, the diatom samples were close to 100% extraction, but the blue-green samples were only 88% extracted as compared to the methanol samples.

Many tests with coastal marine phytoplankton have yielded results similar to the diatom data in Tab. 1. That is, if samples are ground and then extracted for a few hours, comparable results are obtained with either acetone or methanol. In all these tests, however, there were no detectable blue-green or green algae present in the water samples. Recent data indicate that freeliving and symbiotic blue-green algae are, however, found in significant numbers in the oligotrophic waters of the north and south Pacific gyres (Weare et al. 1974, Mague et al. 1974). The widespread distribution of blue-green algae has been previously reported in intertidal environments and in most ocean water with the exception of the polar regions (Fogg 1973). It is likely therefore that, even in marine studies, one is apt to encounter blue-green algae which show poor extraction characteristics with acetone. Other algal groups may also show poor extraction into acetone, as is seen in the data in Fig. 1. Symbiotic dinoflagellates were isolated from the coral *Pocillopora capitata* Verrill (Fig. 1A) and from the giant clam *Tridacna gigas* L. (Fig. 1B) and extracted with acetone or methanol, both with and without grinding in a Potter-Elvehjem homogenizer. The coral dinoflagellates extracted quite rapidly in acetone, but the dinoflagel-

lates from the clam extracted very poorly in acetone. Complete extraction from the clam dinoflagellates was obtained within 45 min (the shortest time used) in methanol, either with or without grinding; with acetone, however, extraction was only about 20% without grinding, and even with grinding it required 2.5 h for complete extraction.

The difficulty of extracting pigments from these zooxanthellae has been previously described by Jeffrey and Haxo (1968), who used a combination of freezing followed by methanol extraction. The yield of fluorescence as measured in our instrument is less per unit chlorophyll when methanol is used as compared to acetone. When methanol extracts are used, a larger factor must therefore be employed in the equation to equate fluorescence units to chlorophyll concentrations. The slight drop in fluorescence seen in Fig. 1B between 45 and 75 min most likely reflects changes in instrument sensitivity due to either incomplete warming up or to voltage fluctuations on board the research vessel, as the slight decrease was seen in both the acetone and methanol extracts.

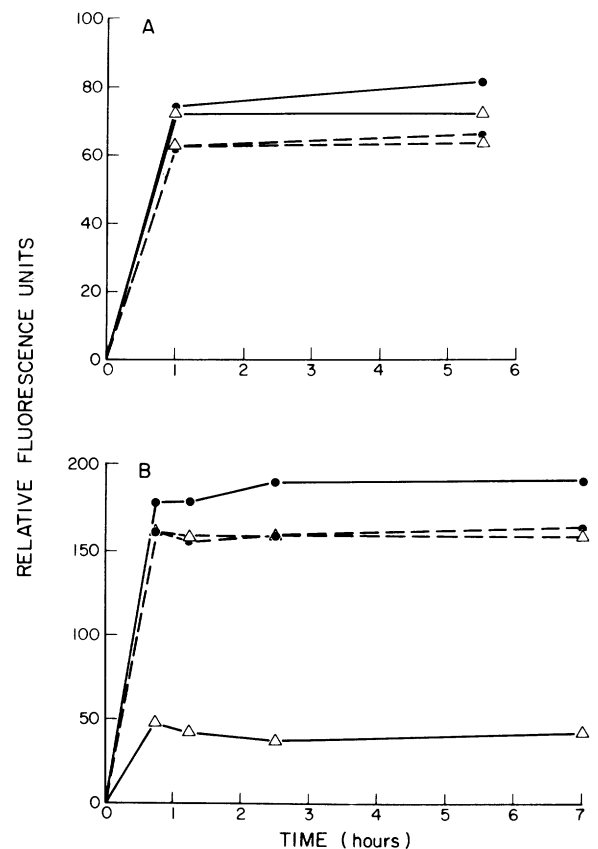


Fig. 1. Efficiency of extraction with time of symbiotic dinoflagellates from the coral *Pocillopora capitata* (A) and from the giant clam *Tridacna gigas* (B). Extraction solvents were 90% acetone (—) and absolute methanol (-----), both with (●) and without (△) grinding of the sample.

Previous studies have reported similar low acetone extraction efficiency using common algae like *Chlorella vulgaris* (Cambridge Culture Collection 211/11h) (Stemann Nielsen 1961), *Scenedesmus quadricauda* Brebisson (Rai 1973), or the filamentous *Cladophora* and *Vaucheria*, which can only be extracted completely in methanol (Marker 1972). When methanol is used to extract such cells it is not necessary to homogenize or to boil the filters, which results frequently in significant losses of chlorophyll a and increased amounts of phaeopigment a (Riemann 1976).

Specific absorption coefficients for chlorophyll a in methanol are given as $74.5 \text{ l g}^{-1} \text{ cm}^{-1}$ (Mackinney 1941, Seely and Jensen 1965) or as $75 \text{ l g}^{-1} \text{ cm}^{-1}$ (Lenz and Zeitzschel 1968). Using an acid factor of 1.5 for chlorophyll a in methanol, Marker (1972) gives a specific absorption coefficient for phaeophytin a in methanol between 49.6 to $50.0 \text{ l g}^{-1} \text{ cm}^{-1}$, depending on the choice of absorption coefficient for chlorophyll a.

On the basis of the above data, it is suggested that absolute methanol should be used rather than acetone because of 1) shorter extraction times, and 2) elimination of the requirement for homogenization or boiling. Further reduction of the likelihood of oxidation of the pigments during extraction may be afforded by using methanol saturated with hydrogen sulphide (Jensen and Sakshaug 1973). We are not aware of any problems or drawbacks associated with the use of methanol as compared to acetone for pigment determinations.

3.2. Photometric and fluorometric measurements of pigments extracted in methanol

3.2.1. Acidification procedure

The spectrophotometric (Lorenzen 1967, Moss 1967a, b) and the fluorometric (Holm-Hansen et al. 1965, Loftus and Carpenter 1971) determinations of chlorophyll a and phaeopigment a involve acid treatment of the extracted pigments. A number of chemical changes occur when chlorophyll extracts are treated with acid. These changes vary in different extraction solvents and depend upon the nature of the pigments, the amount of acid added, and the period of time the acid reacts with the extract.

The acidification step was studied in a culture of *Dunaliella tertiolecta* Butcher (containing both chlorophyll a and b) extracted in methanol. Fig. 2 shows the absorption and emission spectra before and after addition of HCl to different final molarities. The red absorption maximum of the phaeopigments resulting from acidification depends upon the concentration of HCl in the final acidified solution (Fig. 2A). Higher molarity of the HCl reduces the readings at 665 nm due to changes in the absorption spectrum.

The wavelength of the maximum emission peak did not change significantly with different acid concentrations (Fig. 2B); increased emission was observed, however, when acidified to 3×10^{-2} and 1×10^{-1} M re-

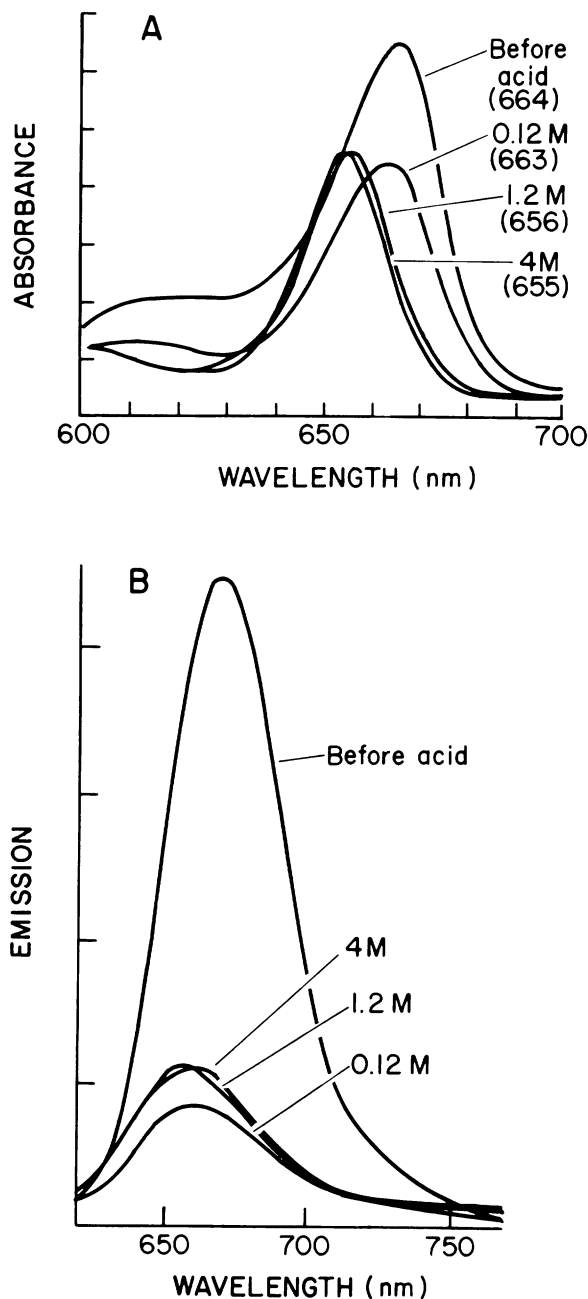


Fig. 2. Absorption (A) and emission (B) spectra of pigments extracted in methanol from a culture of *Dunaliella tertiolecta*. To acidify extracts, 0.1 ml of various HCL solutions (0.12, 1.2, and 4.0 M) were added to the 4 ml of methanol extract.

spectively. These changes were probably due to further degradation of chlorophyll b forming phaeophytin b and resulting in increased emission (see below and Fig. 4D). Acidification to 3×10^{-3} M degraded only chlorophyll a to phaeophytin a. This is in accordance with Joslyn and Mackinney (1938) and Schanderl et al. (1962) who reported that degradation of chlorophyll b

to phaeophytin b was 5.5 to 9 times slower than degradation of chlorophyll a to phaeophytin a, and the rate of degradation of chlorophyll b depended on the molarity of acid in the acidified extract.

The absorption spectra in Fig. 2A include also interference of phaeophytin b, when acidified to 3×10^{-2} or 1×10^{-1} M HCl. The absorption spectrum of phaeophytin b is, however, close to the spectrum of chlorophyll b in the red region, making the difference before and after acidification insignificant. Here the observed changes upon acidification are probably caused by spectral changes of phaeophytin a due to changes in pH, like those reported by Livingstone et al. (1953) and Marker (1972), thus requiring neutralization of the acidified extracts to the pH found in the original extract. Subsequent neutralization of the acidified extracts to the pH found in the solution before acidification (pH 7.7) changed all the spectra of acidified extracts to one identical spectrum with an absorption maximum close to 665 nm, thus indicating that the spectral changes of the phaeopigment a was due to pH changes in the extracts.

Spectrophotometric and fluorometric drift in the acidified extracts may be caused by (1) incomplete conversion of chlorophyll a to phaeophytin a, (2) phaeophytinization of other chlorophylls than chlorophyll a, and (3) further chemical reactions. Both spectrophotometric and fluorometric drift was observed when chlorophyll extracts were acidified. Using very dilute HCl (3×10^{-3} M in the extract), drifting practically stopped within three minutes when reading the absorption at 665 nm. In contrast to this, 1×10^{-1} M HCl or stronger in the acidified extract increased drifting in an anomalous way, indicating further chemical reactions. Concomitant with this drifting, a marked increase in absorption at 750 nm was also noted. Absorption at 750 nm is normally used to correct for absorption of other components than chlorophylls and should not exceed 0.005. In the fluorometer, drifting normally stopped within three minutes when acidified to 3×10^{-3} M; further addition of HCl altered the emission as shown by the data in Fig. 2B.

When a spectrophotometer is used neutralization of the acidified pigments is required to (1) convert the acidified phaeophytin a spectrum to the neutral form, (2) stop further drifting at other wavelengths.

The rate of neutralization of acidified extracts was studied in methanol extracts by measuring the "apparent pH" (Fig. 3). When methanol extracts are made 1.2×10^{-2} M with HCl, neutralization by addition of MgCO_3 requires more than one hour; if the concentration of HCl is 1.2 mM, however, the extract can be neutralized by MgCO_3 within 10 min. Consequently when acidification is made in methanol, very dilute HCl is required (final concentration in the extract should be about 1 mM) to facilitate the subsequent neutralization step and also to minimize drifting. Riemann (1976) has followed the phaeophytinization of chlorophyll a by pa-

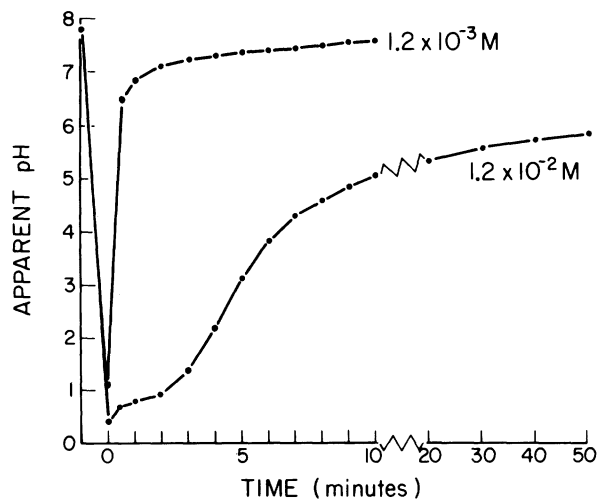


Fig. 3. Rates of neutralization in absolute methanol acidified with HCl and neutralized with 25 mg MgCO_3 per ml methanol. The molarity of the HCl in the acidified extract is indicated on the figure.

per chromatography and reported that complete phaeophytinization occurred when the concentration of HCl in the acidified extract was as low as 4×10^{-4} M. The concentration of acid needed to affect this chemical reaction is far less than many procedures recommended in the literature, which range from 0.01 to 0.2 M (Lorenzen 1967, Moss 1967a, b, Strickland and Parsons 1968, Marker 1972).

In view of the above results, the following acidification procedures are proposed when pigments are extracted in absolute methanol.

Spectrophotometric determinations: the molarity of the HCl should be about 3×10^{-3} M in the acidified extract. Reaction time is three minutes. Neutralization is accomplished by addition of 25 mg MgCO_3 per ml extract, and takes about 10 min with slow stirring. Subsequent filtration through a Whatman glass fiber filter produces a clear solution.

Fluorometric determinations: the molarity of the HCl should be about 3×10^{-3} M in the acidified extract. Reaction time is less than three minutes, and subsequent neutralization is not required.

3.2.2. Influence of chlorophyll b on the determination of other pigments

In many eutrophic waters green algae, which contain both chlorophylls a and b, often constitute a significant proportion of the phytoplankton. As chlorophyll b can interfere with the determination of phaeophytin a, both chlorophylls a and b were separated from species of the grass *Poa* by paper chromatographic procedures (Jensen and Liaaen-Jensen 1959, Jensen and Sakshaug 1973) and their absorption and emission spectra determined in absolute methanol. After acidification of these solutions, aliquots were chromatographed and the

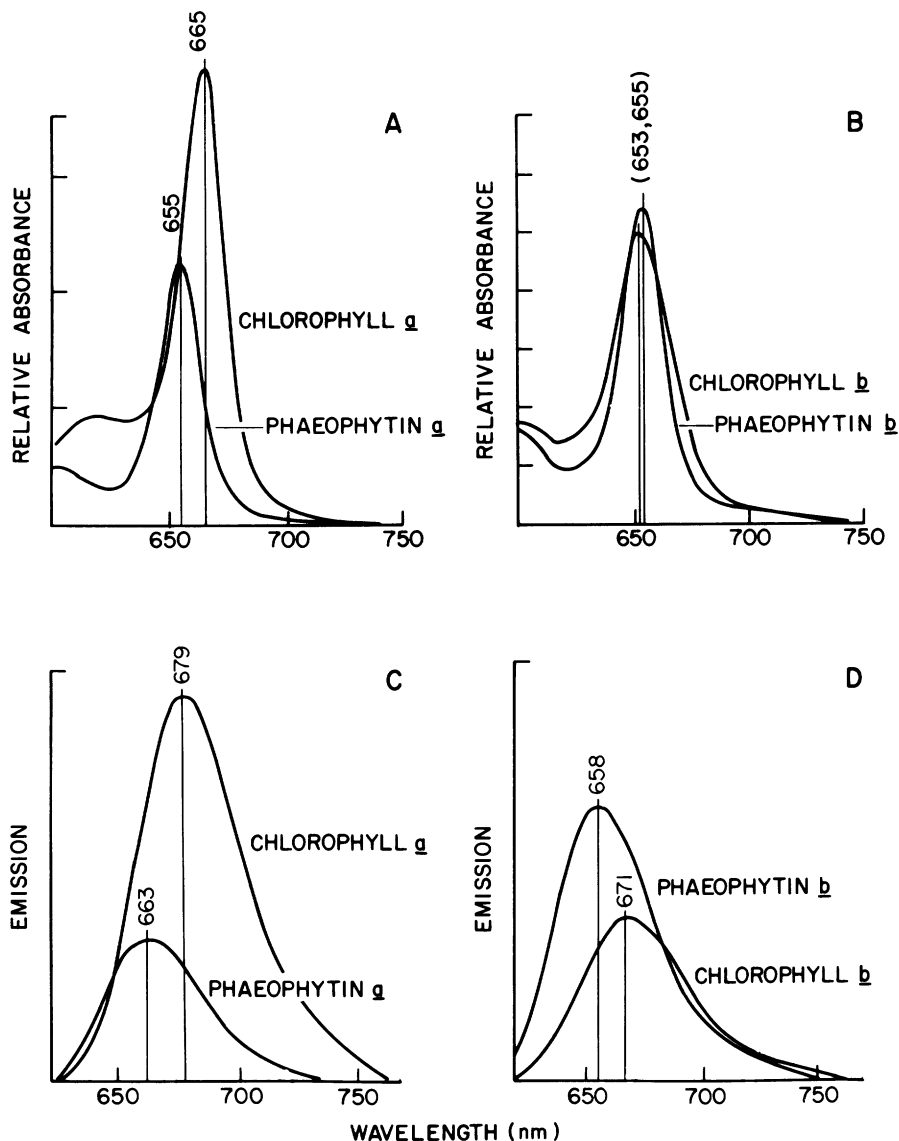


Fig. 4. Absorption (A and B) and emission (C and D) spectra for chlorophylls a and b and phaeophytins a and b in methanol. Pigments were isolated from species of the grass *Poa* by paper chromatographic techniques. Acidification was done by adding 0.1 ml of 1.2 M HCl to 4 ml of methanol extract.

phaeophytins a and b were isolated and used for analysis of their absorption and emission spectra. Fig. 4 shows the absorption and emission spectra for all four of these pigment components in methanol. Both the absorption and emission of phaeophytin a decrease compared with chlorophyll a, but the peaks also changed to shorter wavelengths (Figs 4A and 4C). The absorption of phaeophytin b in the red region is nearly the same as the absorption of chlorophyll b, although a small decrease was found upon acidification of chlorophyll b. The fluorescence of phaeophytin b, however, was markedly different from that of chlorophyll b; upon acidification of chlorophyll b, the emission increased significantly and the peak decreased from 671 nm to 658 nm.

Loftus and Carpenter (1971) showed the emission spectra of chlorophylls a and b and their respective phaeophytins in acetone. Their spectra are fairly simi-

lar to those we show in Fig. 4 in methanol, in that both absorption and emission of phaeophytin a decreased as compared to chlorophyll a, while the emission of phaeophytin b increased dramatically as compared to chlorophyll b. These data indicate that the calculations of phaeophytin a upon acidification in methanol or acetone extracts will be very much dependant upon the ratio of the phaeophytins a and b.

The absorption spectra in the red region of chlorophyll b and phaeophytin b are nearly the same (Fig. 4B). Consequently the determination of chlorophyll a and phaeophytin a will be complicated by the presence of chlorophyll b. When the concentration of chlorophyll b is small relative to chlorophyll a, the effect on chlorophyll a determinations will be minor and can be ignored. When the ratio of chlorophyll b/chlorophyll a exceeds ~ 0.4 , however, the optical density of the acidified solu-

tion will be high due to the absorption of phaeophytin b. In this way the amount of phaeopigment a will be over-estimated when the spectrophotometric equations are used.

The influence of chlorophyll b on the determination of chlorophyll a by fluorometry or spectrophotometry are discussed by Loftus and Carpenter (1971). The most direct way to discern if the calculated amount of phaeopigment a represents an increase in the ratio of chlorophyll b/a, however, is to separate the pigments by chromatography. Jeffrey (1976) has also discussed the advantages of chromatographic separations of pigments prior to their analysis.

3.2.3. Influence of chlorophyll a on the determination of other pigments

Chlorophyll c is found in common groups of plankters like diatoms and dinoflagellates. Ratios of chlorophyll c/a normally range between 0.5 in dinoflagellates (Madgwick 1966) up to 1.0 in an *Amphidinium* sp. culture (Jeffrey 1968).

No attempt was made in this study to isolate chlorophyll c. Although the developed zone of chlorophyll c can be seen, it is rather close to the center of the chromatogram, where other pigments like chlorophyllide a and phaeophorbide a interfere. The same problem is found, when sucrose thin-layer chromatograms are made (Jeffrey 1976), and additional problems arise when elutions from the chromatograms are made, due to the fact that chlorophyll c is reported to occur in two forms: chlorophyll c_1 and c_2 (Dougherty et al. 1966, Jeffrey 1968).

Loftus and Carpenter (1971) isolated chlorophyll c using the n-hexane-90%-acetone phase separation described by Parsons (1963) and they reported an over-estimation of chlorophyll a by as much as 10% when the ratio c/a was 1.0.

Further studies on the absorption spectra and emission spectra of chlorophyll c and phaeophytin c in methanol are needed before an exact evaluation of the influence of these two components can be done. From the present knowledge the occurrence of chlorophyll c seems to have a minor influence on both the fluorometric and spectrophotometric determinations of chlorophyll a and phaeopigment a. (Holm-Hansen et al. 1965, Loftus and Carpenter 1971). In regard to the fluorometric determinations, this assumes that the instrument is equipped with the proper filters as discussed in the references above.

3.3. Methodological variations applicable to both methanol and acetone

3.3.1. Choice of filter

There has been some reluctance by oceanographers and limnologists to use glass fiber filters instead of membrane filters for pigment studies. Part of this reluctance has undoubtedly been due to the fact that glass fiber filters

do not have an advertised "pore size", and this has given rise to apprehensions regarding the efficiency of the glass fiber filters to retain small cells. Glass fiber filters were suggested by Vollenweider (1969), with a warning that such filters may not retain as many small cells as the membrane filters. Sheldon (1972) has provided data that minimizes this possible objection to the use of glass fiber filters. By the use of an electronic particle counter, Sheldon has shown that the median retention sized for HA Millipore filters, GF/C glass fiber filters, and 984H glass fiber filters are close to 0.5, 0.7 and 0.5 μm respectively. The 984H glass fiber filters are very effective in retaining most bacterial cells we have tested in the laboratory, and hence it can safely be assumed that they will also retain essentially all phytoplankton cells in natural populations. We have frequently filtered phytoplankton samples from diverse aquatic environments (eutrophic and oligotrophic waters, coral reefs, polar waters, etc.) through membrane filters (0.22 and 0.45 μm pore size) and through Whatman GF/C glass fiber filters; in all tests the amount of chlorophyll recovered from the glass fiber filters was equal to or greater than the membrane filters. These observations are supported by data of Long and Cooke (1971), who have shown that the use of glass fiber filters results in slightly higher chlorophyll values as compared with membrane filters.

There are some claims (Farooq Azam pers. comm.) that micro-fine glass fiber filters (Reeve Angel 984H) are more efficient for chlorophyll retention than are GF/C filters. We tested these two filters with tropical lagoon waters which were rich in small green flagellated cells. Six replicate samples with GF/C filters gave a chlorophyll concentration of 4.38 $\mu\text{g l}^{-1}$, while six samples with 984H glass fiber filters gave 4.43 $\mu\text{g chlorophyll l}^{-1}$. As there was no significant loss of chlorophyll through the GF/C glass fiber filter, it apparently is not necessary to use the micro-fine glass fiber filters in chlorophyll determinations.

The use of glass fiber filters offers the following advantages when compared to the use of membrane filters. (1) The glass fiber filters filter much faster and do not clog as rapidly. (2) After centrifugation there is no turbidity in the acetone or methanol extracts when using glass fiber filters. (3) If one wants to rupture cells by grinding, the glass fibers are excellent abrasive material for aid in cell breakage. Even though this may not be essential for pigment work, some investigators use the same procedure for rupturing cells for enzymatic studies (Packard et al. 1971). (4) In most field studies it is desirable to have data on various chemical parameters on the same water sample. For such comparisons it is essential that all filters used for the various determinations retain the same size fraction of particulate materials. Although older methods for particulate organic nitrogen and phosphorus utilized membrane filters (Strickland and Parsons 1965), most investigators now use glass fiber filters for these determination as well as

for particulate organic carbon. It is therefore appropriate that pigment data also should be obtained by the use of glass fiber filters.

3.3.2. Use of $MgCO_3$ during filtration

Fig. 5 and Tab. 2 show the effect of addition of $MgCO_3$ to the filter or to the sample before filtration in test samples containing varying amounts of chlorophyll and phaeopigments. In the sea water samples from Scripps pier (Fig. 5 upper figure) no significant effect on concentrations of chlorophyll a or phaeopigment a was found. In the fresh water samples shown in Fig. 5 (lower figure), although not significant the concentrations of phaeopigment a are slightly higher in 3 of the 5 samples without addition of $MgCO_3$. This tendency is significant in Tab. 2 in the test samples from July. Here 8% more phaeopigment a is found in the samples without $MgCO_3$ than with it.

Both Patterson and Parsons (1963) and Daley et al. (1973) reported reduction of both phaeophytin a (up to 100%) and phaeophorbide a (up to 70%) when either $MgCO_3$ or dimethylaniline was added to the filter. This phenomenon could explain the increase in the amount of phaeopigments found in our samples without $MgCO_3$. In the samples with $MgCO_3$ some of the phaeopigments were probably adsorbed by the $MgCO_3$ and thus not detected by subsequent analyses. In the marine samples from Scripps pier and in the samples from March (Tab. 2), where no differences were found, the actual amount of phaeopigments were so small that adsorption of some of the phaeopigments by $MgCO_3$ would not alter the results.

As no significant loss of chlorophyll a was found in any of the samples without $MgCO_3$, and as the addition of $MgCO_3$ slows down the filtration rate appreciably, and reduces the amount of phaeopigments in the sample, it is suggested that the use of $MgCO_3$ be discontinued. Difficulties associated with the use of $MgCO_3$ have also been described by Sand-Jensen (1976), who attributes the loss of chlorophyll a during storage to forma-

Tab. 2. The effect of addition of 1 ml 1% $MgCO_3$ to the filter, before filtration, on the amounts of chlorophyll a and phaeopigment a extracted from different phytoplankton populations in Lake Mossø. Diatoms were dominant in March and bluegreen algae were most abundant in July. Standard error is given for $n = 10$. Chlorophyll a and phaeopigment a in μg . Pigment levels were determined spectrophotometrically according to Moss (1967a, b) and Riemann (1976).

Samples from March			
+ $MgCO_3$		- $MgCO_3$	
Chl. a	Phaeo. a	Chl. a	Phaeo. a
70.7 ± 1.1	10.5 ± 0.3	71.3 ± 0.8	10.0 ± 0.2
Samples from July			
+ $MgCO_3$		- $MgCO_3$	
Chl. a	Phaeo. a	Chl. a	Phaeo. a
72.5 ± 0.9	40.8 ± 0.7	69.3 ± 1.1	44.1 ± 0.5

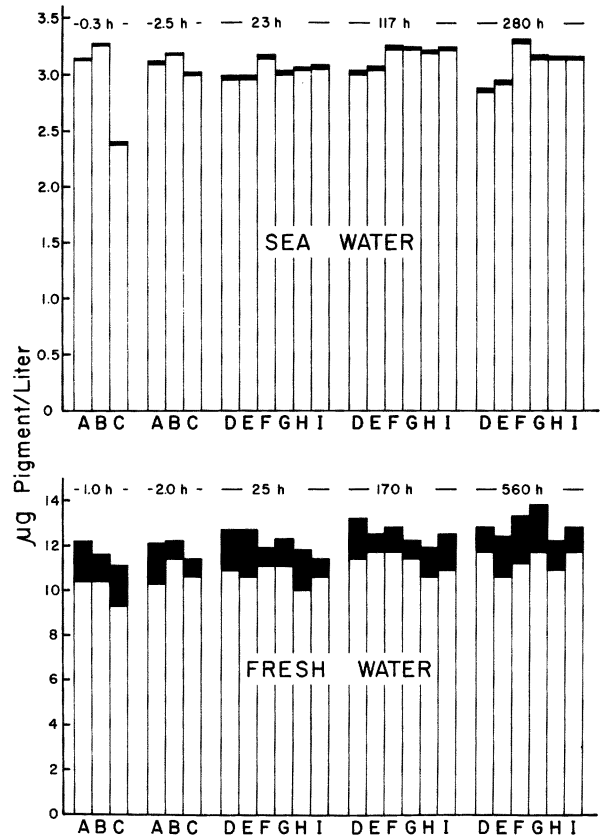


Fig. 5. Recovery of chlorophyll a (clear vertical bar) and phaeopigment a (solid bar) from phytoplankton as a function of length of extraction period, storage conditions, presence or absence of $MgCO_3$, and whether or not sample was ground. Whatman GF/C Glass fiber filters were used for all samples. The times shown in the figures refer to the length of the period between filtration of the sample and measurement of pigment fluorescence. All measurements were done by fluorometry as described by Holm-Hansen et al. (1965). Upper figure: coastal water a short distance from Scripps Institution of Oceanography. Lower figure: fresh water sample from a pond in the coastal mountains east of San Diego.

- A - Without $MgCO_3$, ground in acetone, room temperature.
- B - With $MgCO_3$, ground in acetone, room temperature.
- C - With $MgCO_3$, acetone added but filter not ground, room temperature.
- D - Without $MgCO_3$, filter stored frozen, then ground in acetone.
- E - With $MgCO_3$, filter stored frozen, then ground in acetone.
- F - Without $MgCO_3$, ground in acetone, stored frozen.
- G - With $MgCO_3$, ground in acetone, stored frozen.
- H - Without $MgCO_3$, acetone added but filter not ground, stored frozen.
- I - With $MgCO_3$, acetone added but filter not ground, stored frozen.

tion of aggregates of algae and $MgCO_3$; these aggregates supposedly retain chlorophyll during subsequent extraction attempts, causing a significant loss of chlorophyll. Such algae- $MgCO_3$ aggregates may be species-specific, as our studies with marine and freshwater

plankton (Fig. 5) did not show any loss during storage associated with addition of $MgCO_3$ to the samples.

3.3.3. Grinding and storage of filters

In some of our field studies we have frozen samples filtered on glass fiber filters for 2–3 wk ($-20^\circ C$) before extraction with acetone (Holm-Hansen 1969, Holm-Hansen et al. 1970). There is a question of the reliability of pigment concentrations when samples have been stored in this manner, as Strickland and Parsons (1968) state that filters should be extracted without delay if at all possible as storage always leads to low results.

Data in Fig. 5 show our observations on measurements of chlorophyll a and phaeopigment a in both fresh water and marine samples extracted in 90% acetone with respect to the effects of grinding and storage at $-20^\circ C$. Both sets of data in Fig. 5 support the following conclusions. (a) There is neither loss of pigment nor any significant change in the chlorophyll/phaeopigment ratio when extracted samples are stored three weeks at $-20^\circ C$. It thus seems safe to store extracts at $-20^\circ C$ during field studies and to analyze them at one time in the base laboratory, even if the sampling program extends over several weeks. (b) There is no loss of chlorophyll in samples which have been frozen in the wet state, and which are subsequently extracted with acetone prior to determination of pigment content. This is an advantage in field work, as one does not have to have a supply of acetone and it also eliminates the possibility of loss of extracted pigment during transport of samples. (c) If samples are to be analyzed within a few hours after filtration, it appears that it is necessary to grind filters in the homogenization tube. When the extraction time exceeds a few hours, the data in Fig. 5 indicate that there is no difference between samples which have been extracted with or without grinding.

Subsequent studies have shown that no detectable differences in the amount of chlorophyll were found when filters were extracted in absolute methanol after three weeks of storage at $-20^\circ C$.

Fig. 6 shows data on the effect of grinding of filters which have been extracted 24 h in 90% acetone (A) or 1 h in absolute methanol (B). Samples were from the eutrophic Lake Mossø, with the phytoplankton consisting mostly of diatoms and some blue-green algae. In both extraction solvents no significant effect of homogenization was found when ground samples were compared to the unhomogenized ones. Why the amount of phaeopigments were higher in the acetone extract than in the methanol extract seems obscure, because no corresponding differences were found in the amount of chlorophyll a in the two solvents. Further experiments in Lake Mossø with phytoplankton populations dominated by blue-green algae showed no decrease in chlorophyll or phaeopigment concentration in methanol as compared to acetone.

It should be noted, however, that some investigators

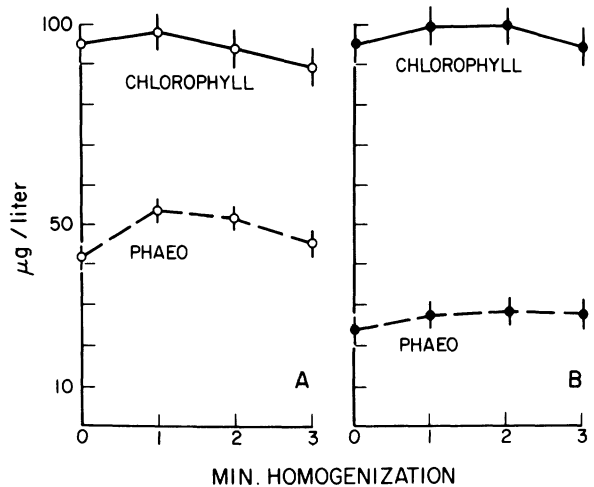


Fig. 6. Effect of homogenization on concentration of chlorophyll a (—) and phaeopigment a (----) Water samples from Lake Mossø were filtered and extracted in either 90% acetone for 24 h (A) or in absolute methanol for 1 hour (B), both with and without homogenization. Bars indicate the 95% confidence limits.

(Yentsch and Menzel 1963) have published data indicating that grinding of samples results in significantly higher chlorophyll concentrations. These studies utilized acetone as the extractant, however, and hence merely confirm our data (Fig. 5) that for some environmental samples, grinding does enhance the rate of chlorophyll extraction when acetone is used as the extractant. As far as we are aware, there are no published reports showing the beneficial effect of grinding when absolute methanol is used as the extractant. The investigator must be aware, however, that some samples of phytoplankton may possibly show slow extraction even when methanol is used. An example of this is the report by Jeffrey and Haxo (1968) in which they used an initial freezing procedure to facilitate subsequent extraction of pigments from zooxanthellae into methanol. Although we have never obtained any beneficial effect of grinding when methanol is the extractant, it would behoove the investigator to verify this for whatever algal samples are being studied. Our conclusions are that grinding sometimes may be important when extracting with acetone, but is superfluous when using methanol.

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