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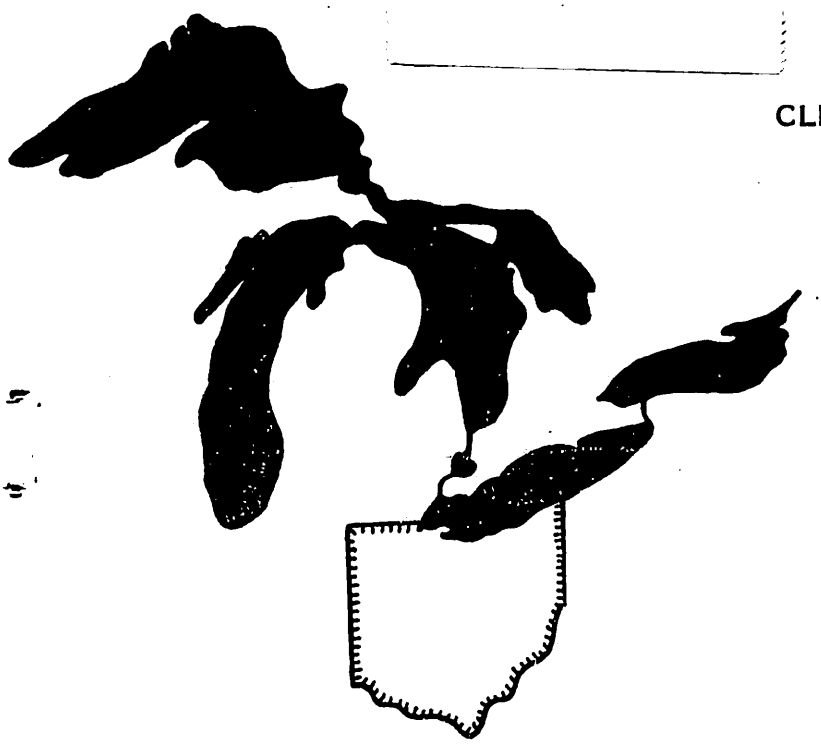

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The Status of Methods for the Analysis of Chlorophyll in Periphyton and Plankton

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INTRODUCTION

The development of methods to measure the concentration of chlorophyll in the algae of surface waters spans a period of approximately five decades. The first reported measurments of phytoplankton pigments were made in the 1930°s and 1940°s by oceanographers and limnologists (1-6) who were searching for a substitute for counting techniques to estimate phytoplankton biomass.

The measurement of chlorophyll a concentrations in periphyton and plankton is now widely used to estimate algal standing crops and photosynthetic rates, the trophic status of surface waters, and the effects of effluents. The relative abundance of chlorophyll a, b and c is characteristic of the various major groups of algae and provides information on the taxonomic composition of the algal community. Chlorophyll analyses are far less time consuming than measurements of many other properties of periphyton and phytoplankton communities related to standing crop, community structure and function, and has been correlated with parameters such as temperature (7), total phosphorus (8-10), cell number (11-13), cell volume (12-18), cell surface area (13,16,19), carbon content (20-22), primary production (12,16,20,23-27), biomass (28-38), community structure (39) and diversity (40-48).

The use of chlorophyll to estimate the biomass and productivity of periphyton began in the 1950°s, and a very extensive literature now exists on this subject (49-107). Examples of the use of periphyton chlorophyll measurements in water quality monitoring can be found in the chlorophyll-biomass relationship, now called the Autotrophic Index, which was first used by the Tennessee Valley Authority (108-110) in measuring the effects of heated discharges on receiving waters in the Tennessee River Basin. Weber (39), working with the effects of domestic waste on periphyton in the Ohio River, proposed the use of standardized units to calculate this index. Because of its potentially wide application in measurements of the effects of pollution on periphyton communities, it has been incorporated into the Model State Water Monitoring Program and Basic Water Monitoring Program of the U.S. Environmental Protection Agency (111-112).

CHLOROPHYLL STRUCTURE AND PROPERTIES

Although the term,"chlorophyll," credited to Pelletier and Caventou (113), has existed since 1818, characterization of the chlorophylls began first with Tswett (114) in 1906, who used chromotography to separate plant pigments into their components. He observed two principle pigments, which he named "alpha" and "beta" (now known as chlorophyll a and b, respectively). Most of the historical literature on chlorophyll arose from laboratory studies of vascular plant pigment chemistry, and was concerned with subjects such as separation techniques, and absorption peaks and coefficients (115-126).

Chlorophyll a is the primary algal pigment required for photosynthesis and is found in all algae in similar concentrations, usually constituting

1-2% of the dry weight of organic matter (39). However, in addition to chlorophyll a, algae may contain appreciable amounts of the accessory pigments, chlorophyll b and chlorophyll c.

The various taxonomic groups of algae differ greatly in their content of chlorophylls b and c. The green algae (Chlorophyta) contain chlorophylls a and b in a ratio of approximately 2:1, but lack chlorophyll b. The diatoms (and other Chrysophyta) contain chlorophylls a, and c, but lack chlorophyll b. The blue-green algae (Cyanophyta) lack chlorophylls b and c (Meeks, 127; Jeffrey,128) (Table 1).

Chlorophylls a and b consist of a water soluble porphyrin ring with a lipid-soluble, unsaturated, hydrocarbon (phytol) side chain (Figure 1A). Chlorophyll c lacks the phytol chain and would be more correctly called a chlorophyllide. It reportedly consists of two closely related compounds (c*1 and c*2) (Figure 1B).

The chlorophylls absorb light strongly in the red and blue regions of the visible spectrum, which is responsible for their green color. Generally, only the red absorption peaks are used to measure these pigments. Vernon (129) and Jeffrey and Humphrey (130) reviewed the published data on the position of the absorption peaks of chlorophyll a, b, and c in different solvents. In 90% acetone, these peaks occur at 664nm, 647nm, and 630nm for chlorophylls a, b, and c, respectively (130). Over the years, periodic modifications have been made in the wave lengths and absorption coefficients of the chlorophylls, largely as the result of improvements in spectrophotometers and the use of more highly purified extracts and solvents.

DEGRADATION PRODUCTS

Many chlorophyll degradation products, such as the pheophytins, pheophorbides and chlorophyllides, absorb light in the same region of the spectrum as the chlorophylls and may, if present in significant amounts, cause serious errors in the estimates of chlorophyll concentrations in the algae (131). Chlorophyllides a and b are the most well known, and both exhibit absorption curves similar to those of their parent chlorophylls (132,133). Chlorophyllides lack the phytol chain, and can be formed by hydrolysis of chlorophyll. Hydrolysis of the phytol chain is catalyzed by the enzyme, chlorophyllase, present in plant tissue.

Pheophytins lack the magnesium atom at the center of the porphyrin ring, and can be formed "in vitro" by acidifying a chlorophyll extract. Pheophorbides result from the removal of magnesium from chlorophyllides or the phytol chain from pheophytins. Treatment of chlorophylls with weak acids will produce pheohytins, whereas the use of strong acids will result in the formation of pheophorbides (129,134).

Chlorophll-------Pheophytin----------Pheophorbide
-Mg -phytol

Chlorophyll-------Chlorophyllide--------Pheophorbide
-phytol -Mg

Although chlorophyll degradation products may occur in significant amounts in periphyton and plankton pigment extracts, their importance in chlorophyll measurements was discovered only recently by aquatic biologists. Work with chlorophyll degradation products was reported as early as 1926 by Spoehr (135), who cited Willstatter and Mieg as the originators of a method of pigment seperation which differentiates chlorophyll derivatives by means of a "hydrochloric acid number". This number is the concentration of hydrochloric acid necessary to extract the pigment from an ether solution. As mentioned above, mild acidification of pigment extracts removes the magnesium from the porphyrin ring, producing pheopigments, whereas strong acidification produces pheophorbides. Either treatment alters the solubility and light absorption properties of the compounds. In both cases, the absorbance of the product is lower and the absorption peaks occur at wavelengths different from those of the parent compounds (Fig 2). Mild acidification of pure chlorophyll *a* yields pheophytin *a* which in 90% aqueous acetone has an absorption peak of 665 nm and a maximum absorbance approximately 60% that of chlorophyll *a*.

The importance of the measurement of degredation products in algae was not emphasized until studies of the origin and composition of pigments in sediments (136) were extended into the water column (137). The ratio of the absorbance maxima before and after acidification (b/a ratio) was used to correct the chlorophyll *a* concentration for the presence of pheophytin *a*. Yentsch and Menzel (137), using a flourometer, found B/A ratios of 1.5-1.8 for plankton cultures, and a minimum B/A ratio of 1.0 for net tows containing large numbers of zooplankton. Lorenzen (138) stated that a fluorescence ratio of 1.8 was indicative of pure chlorophyll *a* (the absence of pheopigments) and was likely to be found in surface waters at inshore stations, whereas B/A ratios of 0.95 (minimum ratio) were likely to be found below the thermocline (aphotic zone).

Equations to correct spectrophotometric chlorophyll *a* measurements for pheopigments (pheophytin and pheophorbide), were devised by Lorenzen in 1967 (131), but no correction was made for chlorophyllide *a*, which if present is included in the estimate of chlorophyll *a*. Lorenzen°s equations were later simplified by Strickland and Parsons (139), and have been widely used.

Measurements of the absorption of pigment extracts before and after acidification ahould be carried out routinely to correct for the presence of pheopigments in chlorophyll *a* measurements. If the results indicate that pheopigments are absent, the trichromatic equations may then be used to estimate chlorophylls *a*, *b* and *c*.

SAMPLE COLLECTION AND PREPARATION

The techniques used for the preparation and analysis of periphyton and plankton samples for chlorophyll content are very similar. Periphyton samples are collected from natural or artificial substrates and immediately iced or frozen, or are placed directly into 90% aqueous acetone in the field, and are held in the dark until analyzed. After removal from the substrate, the periphyton is homogenized in a tissue grinder and steeped in 90% aqueous acetone for 18-24 hrs (140). The extract is then clarified by centrifugation and analyzed by spectrophometric, flourometric or chromatographic methods.

The plankton is generally concentrated from surface water grab samples by filtration or centrifugation, and treated in the same manner as the periphyton.

Centrifugation and Filtration

Early plankton pigment work was done with samples collected with fine bolting cloth (70 um mesh), which allowed the small cells to escape. The errors inherent in the use of nets were recognized by Kofoid (141) and Lohmann (142,143). Lohmann centrifuged sea water and found small flagellates and diatoms not observed in netted samples taken from the same water. He also noted that the weight of the plankton recovered by the centrifuge surpassed that of the netted material. Saunders (144), in a study of Western Lake Erie, estimated that nannoplankton compromised 75% of the total phytoplankton. Because of the potential loss of such a large percentage of nannoplankton, it is obvious that nets should not be used to collect samples for chlorophyll analyses when quantitative data are required.

The discovery of the relative importance of the nannoplankton, and the improved retention of the plankton by centrifugation, led to the introduction of the Foerst centrifuge (145), which became widely used to concentrate the plankton in surface waters.

Creitz and Richards (146) compared the efficiency of the retention of phytoplankton by the newly-marketed membrane (Millipore) filters with that of the Foerst plankton centrifuge and found that the centrifuge recovered only 69%-86% of the pigment retained on the AA Millipore filter (0.22 micrometer pore diameter). As a result, membrane filtration became the standard method of concentrating phytoplankton. Membrane filters quantitatively retain the phytoplankton and are soluble in the polar solvents used to extract the chlorophyll from the algae. However, there are some serious disadvantages in their use. They clog readily, cause interferences in chromatographic separations (147,148), and they may precipitate from the solvent if acid is added to the extract to obtain the pheophytin correction or if the salt is not washed from the filter (when used to concentrate marine algae). Coating the filters with $MgCO_3$ was found to delay clogging and increase throughput.

Upon the introduction of glass fiber filters, conflicting reports emerged on their relative efficacy, compared to that of membrane filters. Garside and Riley (147) reported that chlorophyll recoveries with the Whatman glass fiber GF/C filters were usually 5-10% lower than those obtained with other filters. The error was reduced, however, by the addition of a 1-2 mm layer of magnesium carbonate. Humphrey and Wootton (149) observed that GF/C filters retained only 75% of the chlorophyll recovered by the HA Millipore filter. Parsons and Strickland (150) found that GF/C filters were comparable to Millipore filters. Futhermore, Long and Cooke (151), compared the performance of the GF/A, GF/C and HA Millipore filters, and found that the two types of glass-fiber filters gave comparable results, and were superior to membrane filters in the following ways: (1) their filtering rates were 10 times greater, (2) they cost only one-fourth as much, and (3) they yielded 6-18% more pigment than the membrane filters.

Because of the apparent advantage of glass fiber filters, use of GF/A or

GF/C filters, with a coating of magnesium carbonate, was adopted by International Field Year for the Great Lakes (152). The magnesium carbonate was added to the plankton sample prior to filtration to increase the efficiency (retention) of the filter and protect the chlorophyll from degradation. (The buffering role of magnesium carbonate is discussed in detail later in this report).

Holm-Hansen and Reimann (153) recently compared the retention of plankton by the micro-fine glass fiber filters (Reeve Angel 984H) and GF/C filters and found no significant difference between them.

Results similar to those of Long and Cooke were obtained in the EMSL-USEPA laboratory using plankton samples from the Ohio River. It was observed that the use of glass fiber filters resulted in the recovery of 27% more pigment than was obtained with membrane filters (Figure 3), an effect which may be due to the greater abrasiveness of the glass fibers when the sample is macerated in the tissue grinder.

Use of a filter in processing periphyton samples is optional. The periphyton may be scraped directly into a tissue grinder or onto a filter (preferably a glass fiber filter), or may be dewatered by centrifugation, and then transferred to the tissue grinder. However, care should be taken to keep the acetone-water ratio in the extract at the nominal 9:1 level.

Chlorophyll Stability

Chlorophyll is relatively labile and its stability after sample collection depends upon the conditions under which the samples are handled. The factors of greatest importance in chlorophyll stability during storage and subsequent analysis are light, temperature and acidity. Sample handling procedures which minimize chlorophyll degradation should be rigidly followed.

In many cases it is inconvenient or impossible to begin processing periphyton or plankton samples immediately after collection. Rather than forego completely the collection of samples under these circumstances, it may be desirable to store the samples for several hours or days before the pigments are extracted and analyzed.

Periphyton samples, in the form of colonized glass slides or scrappings from natural or other artificial substrates, may be placed immediately in 90% aqueous acetone in the dark at 4C when collected in the field, or may be chilled and then frozen (at -20 C) and stored for later analysis.

The phytoplankton may be concentrated from grab samples in the field, or whole water samples may be returned to the laboratory for preparation and analysis.

Plankton Grab Samples. The effects of storage on the concentration of chlorophyll in plankton grab samples were studied in the EMSL-USEPA

laboratory by examining the chlorophyll content of surface water grab samples stored in the dark at room temperature (20 C) and in the refrigerator (4 C). The concentration of chlorophyll *a* in the refrigerated samples remained relatively unchanged for 18 days, whereas the concentration of chlorophyll *a* in the samples stored at room temperature fell to 50% of its original value in 5 days (Figure 4).

Plankton Stored on Filters. Phytoplankton concentrates on filters are generally placed in glass vials, sealed plastic petri dishes (9), or in glassine envelopes (154) and refrigerated or frozen. The effects of storage of algae on filters has been the subject of numerous studies. Creitz and Richards (146) reported that plankton could be stored dry, in the dark, in a refrigerated vacuum desicator for at least 3 weeks without change. Yentsch and Menzel (137) found no change in the fluorescence of frozen samples after 2 days, but after 5 days they noted that the flourscence and optical density had declined 20%. Members of the SCOR/UNESCO Working Group No. 17 (155) obtained their highest chlorophyll values with immediate extraction of filters, but noted that dry filters containing MgCO3 could be stored in the dark at 1 C for 2 months without losing more than 15% of the pigment content. Robertson *et al.* (156) found that samples (collected in triplicate) that had already been stored for 31 days lost only 9% of their chlorophyll if stored an additional 60-70 days (Table 2). Daley *et al.* (148) found no loss of chlorophyll or formation of chlorophyll degradation products in algae stored on filters for 2 months at -20 C. Holm-Hansen and Reimann (153) found no detectable loss of chlorophyll in extracts or wet filters stored at -20 C for 3 weeks. In a study of chlorophyll degradation kinetics, Wyeth and Lorfice (157) found rates of chlorophyll *a* degradation at -15 C, 0 C and 20 C of 0.16%, 0.51% and 3.1% per day, respectively. If these decay rates are found to be generally applicable, it may be possible to use them to correct for chlorophyll *a* degradation during sample storage.

A study of the stability of plankton concentrate on filters carried out at the EMSL-USEPA laboratory indicated that algae can be stored on filters at least 3 1/2 weeks at -20 C without a measureable loss of chlorophyll *a* (Figure 3).

Buffering.

The currently widespread use of buffering agents in chlorophyll extracts to protect the pigments from degradation can be traced at least as far back as the work of Kozminski (3), who added calcium carbonate to the solvent to neutralize plant acids that might be liberated during pigment extraction. Magnesium carbonate was used by Harris and Zscheile (117), and was also recommended by Richards and Thompson (158) and Creitz and Richards (146). As mentioned earlier, magnesium carbonate has also been used with plankton samples to increase filter throughput and enhance algal retention. Several methods of application of MgCO3 have been suggested: (a) coating of the filter pad with approximately 10 mg finely powered MgCO3 / cm squared of filter surface prior to filtration (131,155); (b) adding several drops of saturated MgCO3 to the last 100 ml of water sample being filtered (150); (c)

adding 0.1 g MgCO3 to the acetone solvent during extraction (158); and (d) adding 5 ml of 3% magnesium carbonate slurry to the sample prior to filtration (148). The IFYGL Program (152) recommended addition of MgCO3 prior to filtration unless filter clogging was a problem, in which case it was to be added upon completion of filtratiion. (Assuming the use of a 47mm diameter filter, with an effective filtering area of approximately 10 cm (squared), a coating of 10 mg MgCO3/cm (squared) would result in a total load of 100 mg MgCO3 on the filter. This entire amount would be transferred to the extraction tube and would be present with the algae when it is treated in a tissue grinder and steeped.) The final volume of extract is usually 10-15 ml.

Rai (159) found no breakdown of chlorophyll in extracts lacking MgCO3 when stored in the dark in the cold for 24 hours. Daley et al. (148) observed that chlorophyllide a and pheophorbide a were strongly absorbed by MgCO3 powder used to coat filters, and concluded that the potential error from the adsorption of chlorphyll c by MgCO3 was greater than the error from incomplete retention of algae on the filter when MgCO3 was not used. However, they recommended its addition to the extraction solvent.

Lium and Shoaf (160) prepared samples with and without the use of MgCO3 and found no difference either in the retentiion of algae or in the stability of chlorophyll. Humphrey and Wootton (155), however, found that the use of MgCO3 increased the recovery of chlorophyll c in 37 of 48 samples extracted with and without the use of MgCO3.

The possible adsorption of chlorophylls a, b and c on the MgCO3 particles used to coat filters was examined in the EMSL-USEPA laboratory. MgCO3 was added as a slurry to a 90% aqueous acetone extract of a natural periphyton community in amounts ranging from 0.5 mg/15 ml of extract to 150 mg/15 ml of extract. Samples were prepared in triplicate and analyzed after 15 minutes and 24 hours with a Beckman ACTA-V, UV-VIS Spectrophotometer.

The results (Table 3) indicated little if any effect in samples containing 10mg MgCO3 or less per 15 ml of extract. At larger concentrations of MgCO3, however, a signigicant decrease in the concentration of the chlorophylls was noted after 15 minutes, and an even greater decrease occurred in 24 hours. The reduction in chlorophyll concentrations was due, presumably, to adsorption of the pigments on the MgCO3. At the levels of MgCO3 normally used in samples (100 mg MgCO3/15 ml), chlorophyll b concentrations showed the greatest short-term decline (17%). After 24 hours, the concentrations of chlorphylls b and c showed similar declines (21%).

Extraction Solvents

Chlorphyll solvents must be polar, lipid-soluble and miscible in water. The solvents found to be most suitable for extraction of the chlorophylls from periphyton and plankton are pyridine, methanol, ethanol, acetone, acetone and ethyl acetate, and DMSO. The most frequently used solvents are aqueous acetone and methanol (Table 4).

Rai (159) compared the results of three sets of trichromatic equations - Richards and Thompson (158), Parsons and Strickland (150) and UNESCO (155) - using three different solvents - acetone-methanol (1:1), 90% acetone and methanol - and concluded that methanol was by far the most effective solvent, followed by the acetone:methanol (1:1) mixture (Table 5). Daley et al. (148) made a similar comparison between 90% acetone, 90% methanol and various mixtures of acetone and methanol and reported that a combination of 80% acetone/15% methanol/15% water was a superior extractant for pigments in lacustrine algae. Holm-Hansen and Reimann (153) found that methanol was a more effective extractant of chlorophyll than was acetone.

Although methanol is much more effective than acetone in removing the pigments from algae that resist extraction, the use of acetone has several advantages which tend to outweigh those of methanol. Acetone solutions of chlorophyll have sharper absorption peaks, higher specific absorption coefficients and have been reported more stable than methanol solutions (147,155). For these reasons, 90% aqueous acetone continues to be the preferred solvent.

The spectropscopic and fluorometric properties of the chlorophylls are associated with the solvent used to extract the chlorophyll from the plant tissue. Harris and Zscheile (117) compared the refractive index (RI) and the red and blue maxima of several solvents and found that the absorption peaks of the pigments occurred at wavelengths less than 670nm only with solvents that have RI's less then 1.5. Water has been found to be an important component of the solvent (148,153). Water reduces the nucleophilic reactivity of the pigment and stablilizes the chlorophyll molecue. Delaporte and Laval-Martin (168) found the smallest pheophytinization rates in acetone solutions containing 16% water. As mentioned above, methanol is highly efficient in pigment extraction, but has disadvantages that outweigh its advantages. Dimethyl sulfoxide (Shoaf and Lium, 164) is also very effective in pigment extraction, but is viscous, and difficult to use.

Species Composition. The ease with which pigments are extracted depends on the age and physiological condition of the cells, the species and even the strain of algae used, and the nature of the solvent employed (148). Frequently, the pigments can be quantitatively extracted from the algae simply by steeping them briefly in 90% aqueous acetone (Strickland and Parsons, 139). The extraction of pigments by steeping alone, however is often incomplete. Reports in the literature mentioning difficulties with the extraction of pigments from algae include the following:

Reports	Organisms Mentioned
1. Odum et al.(163)	Chlorella
	Cladophora
	Diplanthera
	Enteromorpha
	Phormidium
	Prasiola
	Thalassia
	Ulva
2. Steemann Nielsen (169)	Chlorella vulgaris
3. UNESCO (155)	Scenedesmus
4. Garside and Riley (147)	Chlorella salina
5. Subba Rao and Platt (170)	Chaetoceros curvisetus
	Fragilaria sp.
	Phaeodactylum tricornutum
6. Marker (87)	Chlorela pyrenoidosa
	Cladophora
	Ulvella frequens
	Vaucheria
	Verrucaria elaeomelaena
7. Rai (159)	Scenedesmus quadricauda
8. Shoaf and Lium (167)	Ankistrodesmus braunii
	Chlorella pyrenoidosa
	Oocystis marssonii
	Scenedesmus quadricauda
	Selenastrum capricornutum
	Tetraedron bitridens
9. Holm-Hansen and Reimann (153)	Aphanizomenon flos aqua

The difficulty in extracting the pigments from some green and blue-green algae may be related to the structure of the cell wall. The green algae may be divided into three subgroups based on the composition and organization of the microfibrils of the cell walls. The walls of the first subgroup are composed mainly of cellulose microfibrils and amorphous material - Cladophora and Chaetomorpha are representative of this group. The walls of the second group have a less distinct organization of cellulose and have more amorphous material. This group contains several of the algae that are most difficult to extract (Scenedesmus, Chlorella and Ankistrodesmus). Atkinson et al. (171) found that half the species of Chlorella, Scenedesmus and Prototheca had a 14 nm thick, trilaminar layer outside the cell wall proper. This layer

is extremely resistant to solvents and is believed to consist of polymerized caroteniod material similar to sporopollenin. The sheath contains the only known organic component of plant cell walls that can withstand acetolysis (treatment with concentrated sulfuric acid and acetic anhydride at 95 C (Pickett-Heaps 172)). Sporopollenin is typically found in spores and pollen of higher plants.

The walls of the third group are based on polymers of xylan and mannan (a structural polysaccharide) and amorphous material rather than on cellulose microfibrils. Algae in this group were formerly of the Siphonales i.e. Chamydomonas (Dodge, 173).

Electron microscope studies have shown that the cell walls of the blue-green algae are 35-50 nm thick and are multi-layered. Two distinct types of cells, heterocysts and akinetes, have more extensive thickening of their cell walls. Frank et al. (174) were the first to present evidence that murein, which is responsible for the rigidity of the cell wall, is similar to peptidoglycan, glycopeptide and mucopeptide, and contributes up to 50 % of the dry weight of the cell walls of blue-green algae. Mannose and lipopolysaccarides are also present in the cell wall. Gelatinuous sheaths of a polysaccaharide nature provide a thick solid outer cell envelope.

Chlorophyll is extracted relatively easily from two groups of algae. One group, the Chrysophyceae, has incomplete cellulosic cell walls called loricas. Dodge (173) reported that the loricas could be removed by merely washing with water. The other group, the Bacillariophyceae (diatoms), have outer walls of silica.

Cell Disruption

Many conflicting results of steeping, grinding and sonifying may be found in the literature. Satisfactory extraction of pigments from most samples can be achieved with 90 % aqueous acetone if the cells are disrupted with a suitable apparatus (170). Tissue grinders are the most commonly used devices for cell disruption, but the literature contains some reports that sonifiers are also effective (148,175).

Yentsch and Menzel (137) found that 10-15 minutes of hand grinding was required in most cases to free all pigmets. Kerr and Subba Rao (176) reported that with the diatom, Nitzschia closterium, mechanical grinding was 80 % efficient in extracting pigments, and resulted in only a 5 % loss due to degradation. Optimum grinding and extraction times and temperatures for five species of marine phytoplankton were reported by Subba Rao and Platt (170).

Garside and Riley (147) obtained 100 % extraction without the degradation of pigments when an antioxidant (absorbic acid) was added. Daley et al. (148) recommended sonication for ten minutes at low temperatures to minimize isomerization. Suggestions in the literature for the addition of antioxidants to reduce chemical and enzymic oxidation, or bases to prevent pheopigment formation include N2, H2S ascorbate (Kim,177) or ethoxyquin (Nelson and Livingston, 178), and MgCO3 or dimethylaniline (Vallentyne, 136). Daley et al. (148) reported that none of the above antioxidants or antaccids

were necessary to prevent degradation of pigments in the species of algae examined. However, they decided to continue use of antioxidants because of the variability of enzymatic activities and organic acid levels from species to species, and found that of all the antioxidants suggested, ascorbate was least troublesome to use.

A comparison of the recovery of chlorophyll a from periphyton extracted with and without grinding was carried out in the EMSL-USEPA laboratory using a series of 11 sets of samples collected on glass slides and exposed for 4 weeks in a floating sampler (Weber, 179,180) in a small stream near Cincinnati, Ohio. Four replicate slides were used for each treatment. The chlorophyll was extracted by (1) placing the colonized slides directly in 100 ml of 90 % acetone in a darkened container in the field and allowing them to steep, or (2) icing the slides until returning to the lab (2-3 hrs), scraping the periphyton slides, homogenizing the scrapings in 3-5 ml of 90 % acetone in a glass-to-glass Kontes type C tissue grinder, and allowing them to steep 15-30 minutes. The optical density of the chlorophyll solutions was measured using a 1 cm cuvette in a Perkin-Elmer Model 124 spectrophotometer. When the extracts were analyzed on the day of sample collection, steeping alone yielded from 72 % to 103 % as much pigment as grinding, but averaged only 90 % as efficient. When steeped an additional 24 hrs in the dark, both types of samples generally yielded a small (1 %-5 %) additional amount of chlorophyll a. These results do not support the recommendations of the UNESCO chlorophyll monograph, (155) which warns against storing extracts "overnight". We have also examined sonication as a cell disruption technique and found it to be ineffective for coccoid green algae and other extraction-resistant forms.

In current practice, pigment is generally extracted from periphyton and plankton samples by grinding in 90 % aqueous acetone in a tissue grinder for 1 minute at 500 rpm. A glass pestle is used for periphyton scrapings and for plankton samples concentrated by centrifugation or on membrane filters. A TEFLON pestle is used for samples on glass-fiber filters. The samples are then steeped overnight (18-24 hrs), clarified by centrifuging 10 minuutes at 1000 Xg, and analyzed.

Solubility of Chlorophyll in Aqueous Acetone

Creitz and Richards (146) inferred that deviations from Beer's Law occurred in chlorophyll solutions in which the OD663 exceeded 0.8, and they suggested that the concentration of pigment in extracts be adjusted routinely to keep the OD663 within the range 0.2-0.8. In the absence of published data on the solubility of chlorophyll in 90 percent aqueous acetone,the validity of their report was examined in the EMSL-USEPA laboratory by analyzing a series of 11 dilutions of a highly concentrated chlorophyll extract obtained from a natural periphyton community. The dilutions were prepared to provide 1 cm OD663 readings evenly spaced over a range of 0.0 to 2.0 absorbance units, which was the operating range of the Perkin-Elmer (Coleman) Model 124 spectrophotometer employed.

A linear relationship was obtained between the observed and calculated chlorophyll a concentrations (Figure 5), indicating compliance with Beer's

Law over the entire range of chlorophyll a concentrations tested (1.21 mg/l to 20.39 mg/l). The chlorophyll c concentrations, calculated using the UNESCO (155) trichromatic equations, were also linear over the full range of dilutions studied, and the chlorophyll a/c ratio was nearly constant (mean = 1.26). The chlorophyll b concentration in the parent solution was very low (1.16 mg/l) and, as a result, appeared somewhat erratic in the dilutions. The proportion of this pigment increased significantly, however, where the calculated concentration dropped below 0.0004 mM, indicating either an anomoly in the UNESCO trichromatic equations or, perhaps, the presence of dimers.

Photodecomposition of Chlorophyll

The susceptibility of chlorophyll solutions to photodecomposition is well known (181-184), and reports dealing with chlorophyll methodology generally warn the reader to handle extracts in subdued light.

A study of the photodecomposition of chlorophyll was carried out in the EMSL-USEPA laboratory to determine the rates of chlorophyll breakdown at different levels of illumination. Aliquots of a 90 % aqueous acetone periphyton extract were exposed to 8 different levels of artificial (daylight fluorescent) and natural illumination ranging in intensity from 12 to 12,000 foot candles (fc), and the solutions were analyzed periodically by the UNESCO (155) spectrophotometric method. The intensity of light falling on the samples was measured with a Weston Model 756 light meter.

At full sunlight (12,000 fc), the chlorophyll concentration fell very rapidly, dropping 50 percent in only 4 minutes (Figure 6). Even at a light intensity as low as 12 fc, the decline in the OD663 was 3 per cent per hour (Table 6). These data indicate that it is essential to carry out chlorophyll extractions in dim light, and to cover vessels containing chlorophyll solutions to protect them from light, even at low ambient levels of illumination.

Long-term Stability of Pigment Extracts Stored in the Dark

A long-term study of the stability of chlorophyll extracts was carried out in the EMSL-USEPA laboratory to determine the feasibility of preparing a reference sample for an interlaboratory study of chlorophyll methodology and for quality control purposes. Chlorophyll solutions were sealed under air in 20 ml glass ampuls, wrapped in aluminum foil and stored at each of the following four tempertures: -20 C, 4 C, ambient laboratory temperatures (20 C to 30 C); and 37 C. Three ampuls were removed periodically from each batch and analyzed according to the UNESCO (155) trichromatic method (Figure 7). The changes observed in the chlorophyll a content of the ampuls during 11 months of storage are summarized in Table 7. The loss of chlorophyll in the sample stored at -20 C was less than 1 percent over the full term of the study.

MEASUREMENT OF CHLOROPHYLL

The earliest quantitative method of estimating the amount of chlorophyll in phytoplankton, developed by Harvey (2), was based on the visual comparison of the color of acetone extracts of phytoplankton chlorophyll with color standards consisting of graded solutions of potassium chromate and nickel sulfate. In his system, one unit of pigment (Harvey Unit) was equivalent to 25 ug K_2CrO_4 and 425 ug $NiSO_4.6H_2O$.

Chlorophyll can be quantified within the living cell (in vivo) or after extraction with a suitable solvent (in vitro). Each approach has advantages and limitations. (In vivo) chlorophyll techniques are generally less time consuming, but currently are limited to the measurement of chlorophyll a. This approach includes direct, laboratory, fluorometric analyses of aliquots of "raw" (unpreserved) plankton and periphyton samples, and remote sensing. (In vitro) measurements offer much more flexibility and accuracy in the separation and quantification of the chlorophylls and other pigments, and currently include the use of spectrophotometry, fluorometry, and chromatogaphy.

Spectrophotometric Methods

Visual comparisons in chlorophyll analyses (2) were soon replaced by colorimeters (3,11), and with the advent of the spectrophotometer, the absorption coefficients of chlorophyll a, b and c were determined with sufficient accuracy to permit the development of equations for the simultaneous determination of the concentrations of mixtures of these pigments in the extracting solvent.

The spectrophotometric methods of chlorophyll analysis currently employed involve the measurement of the absorbance of chlorophyll extracts at 750nm, and at the presumed absorption maxima for chlorophyll a (663nm, 664nm, or 665nm), b (645nm, 647nm), and c (630nm, 665nm,) depending upon the equations used to convert absorbance to chlorophyll concentration. The OD at 750nm is used as a correction for turbidity in the extract. Several trichromatic, dichromatic and monochromtic equations are available for use

with marine and freshwater samples.

Effect of Spectrophotometer Resolution The accuracy of the estimates of the concentration of chlorophyll in extracts depends in large measure on the resolution of the spectrophotometer employed in the analysis. Broad band pass instruments similar to the Bausch and Lomb Spectronic "20" and Beckman B average the optical densities over a wide interval of the absorption curve and significantly underestimate the chlorophyll a concentration. For example, the Beckman B single-beam spectrophotometer, with an advertised half band width of 8nm, provided estimates of chlorophyll a that were 28-35 % lower than values obtained with the Coleman Model 124, which has a half band width of approximately 1 nm. (Figure 8).

Computer simulations of the absorption curves of the chlorophyll extract as they would appear at different instrument resolutions, were prepared by the EMSL-USEPA laboratory to demonstrate the effect of spectrophotometer resolution on the results of chlorophyll analyses. The basic data employed in the simulation consisted of the optical densities (OD's) of a 90 % aqueous acetone solution of (Sigma) purified chlorophyll a obtained at 1 nm intervals over the range 600 nm to 720 nm, using a Beckman ACTA V Spectrophotometer which has a resolution better than 1 nm. The ACTA V OD's were averaged successively, by computer, over a broader and broader wave-length interval (2nm, 3nm, 4nm, ...etc.) to a maximum interval of 40 nm, to simulate resolution over the full range of instruments currently in use. The absorption curves were plotted (Figure 9) and the apparent concentration of chlorophyll a was calculated at each simulated level of resolution using the SCOR-UNESCO (155) trichromatic equations. The concentrations were expressed as the percent recovery, compared to the concentration obtained with the original data. The results were as follows:

Trichromatic Method. Trichromatic equations for the simultaneous determination of the concentrtions of chlorophyll a, b, c were first developed by Richards and Thompson (158) using specific absorption coefficients obtained in their laboratory (185) and data published by Zschiele et al. (126). Their equations are as follows:

$$\text{Chl } a \text{ (mg/liter)} = 15.6(OD665) - 2.0(OD645) - 0.8(OD630)$$
$$\text{Chl } b \text{ " " } = 25.4(OD645) - 4.4(OD665) - 10.3(OD630)$$
$$\text{Chl } c \text{ " " } = 109.0(OD630) - 12.5(OD665) - 28.7(OD645)$$

These equations were revised by Parsons and Strickland (150) to bring them into agreement with later, more accurate determinations of the specific absorbances of the chlorophylls (129,186), which were found to be much higher than previously reported. The revision resulted in a reduction of values of the coefficients in the equations, which lowered the estimates of the chlorophyll concentrations in the extracts by the following approximate amounts : chlorophyll *a*, 25%; chlorophyll *b*, 15%; chlorophyll *c*, 50%:

Chl *a* (mg/liter) = 11.6(OD665) - 1.31(OD645) - 0.14(OD630)
Chl *b* " " = 20.7(OD645) - 4.34(OD665) - 4.42(OD630)
Chl *c* " " = 55.0(OD630) - 4.64(OD664) - 16.30(OD645)

Slight modifications were made in the Parsons and Strickland equations by the Scientific Committee on Oceanographic Research (SCORI) (155) by shifting the wavelength of the absorption peak of chlorophyll *a* in 90 % aqueous acetone from 665nm to 663nm, and adjusting the coefficients to reflect more recent estimates of the specific absorbances of the pigments:

Chl *a* (mg/liter) = 11.64(OD663) - 2.16(OD630) - 0.10(OD630)
Chl *b* " " = 20.97(OD645) - 3.94(OD663) - 3.66(OD630)
Chl *c* " " = 54.22(OD630) - 5.53(OD663) - 14.81(OD645)

Richards and Thompson's (158) equations have been generally claimed to overestimate the amount of chlorophyll by approximately 20 % to 25 %. However, close agreement is seen for chlorophyll *a* values obtained by Parsons and Stricklad's equations (150) and those recommended by SCOR/UNESCO (155) (Table 5). Banse and Anderson (187) also compared the results by applying the Parsons and Strickland and UNESCO equations to their data, and concluded that the differences were generally small. All three sets of trichromatic equations were listed by Strickland and Parsons (139), with no comment regarding their relative merit.

The most recent modifications of the trichromatic equations are those of Jeffrey and Humphrey (130), who reduced the constant for chlorophyll *c* approximately 50 %, changed the wave lengths for the absorption maxima of chlorophyll *a* and *b* to 664 nm and 647 nm, respectively, and made slight adjustments in the other coefficients. This set of equations represents the state-of-the-art in trichromatic analyses.

Chl *a* (mg/l) = 11.85(OD664) - 1.54(OD647) - 0.08(OD630)
Chl *b* " " = 21.03(OD647) - 5.43(OD664) - 2.66(OD630)
Chl *c* " " = 24.52(OD630) - 1.67(OD664) - 7.60(OD647)

A set of equations for the interconversion of data from th various trichromatic equations was prepared by Wartenberg (188). The comparison of pre-spectrophotometric with current chlorophyll data is difficult, but Vinberg (189) estimated that one "Harvey Unit" is equivalent to 0.30 mg/l chlorophyll *a*.

Dichromatic Equations. The most widely used dichromatic equations are those of Arnon (161) for the determination of chlorophyll a and b in 80% aqueous acetone. These equations are employed largely for work with higher plants, and are as follows:

Chl a (mg/l) = 12.7(OD663) - 2.69(OD645)
Chl b " " = 22.9(OD645) - 4.68(OD663)

A set of dichromatic equations is also available for the determination of chlorophylls a and b in 96 % ethanol (Wintermans and DeMots, 190):

Chl a (mg/l) = 13.70(OD665) - 5.76(OD649)
Chl b " " = 25.80(OD649) - 7.60(OD665)

Dichromatic equations usually have not been employed for the determination of chlorophylls in periphyton and plankton samples because they do not provide the chlorophyll c concentrations. However 3 sets of dichromatic equations are available for the analysis of extracts of algae containing only chlorophylls a and b, or a and c (c1 and c2) in 90 % or 100 % acetone (130), as follows:

1. For green algae containing only chlorophylls a and b (and for higher plants) (using 90 % aqueous acetone):

   Chl a (mg/l) = 11.93(OD664) - 1.93(OD647)
   Chl b " " = 20.36(OD647) - 5.50(OD664)

2. For diatoms, chrysomonads and brown algae containing chlorophylls a and c (c1 and c2) (using 90 % aqueous acetone):

   Chl a (mg/l) = 11.47(OD664) - 0.40(OD630)
   Chl c " " = 24.36(OD630) - 3.73(OD664)

3. For dinoflagellates and cryptomonads containing chlorophylls a and c2 (using 100 % acetone):

   Chl a (mg/l) = 11.43(OD664) - 0.64(OD630)
   Chl c2 " " = 27.09(OD630) - 3.63(OD664)

Monochromatic Equations. A monochromatic equation was proposed by Odum et al. (163) for the determination of chlorophyll a in 90 % aqueous acetone, using a moderately narrow band spectrophotometer (Beckman DU):

Chl a (mg/l) = 13.4(OD665)

This equation represents a simplification of the now obsolete Richards and Thompson equation for chlorophyll a, and is not recommended.

A monochromatic method for the microdetermination of chlorophyll c was developed by Parsons (191) for use with chlorophyll extracts containing very

low pigment concentrations. In this method, the chlorophyll c is separated from the other pigments by liquid-liquid partitioning, and the OD450 is determined before and after acidification. Acidification results in a decrease in the OD450 which is proportional to the amount of chlorophyll c in the extract.

$$\text{Chl } c \text{ (ug/10 ml of extract)} = D\ OD450 \times 17.5$$

This method has several advantages over the UNESCO trichromatic method: (a) the sensitivity of the determination of chlorophyll c is increased by a factor of approximately four over that of the trichromatic method, (b) the measurement is highly specific for chlorophyll c, (c) the precision of the individual measurements is much greater than that of the trichromatic method, and (d) the interference by chlorophyllides can be reduced by a special correction procedure.

Pheophytin correction. The concentrations of chlorophyll a and pheophytin a can be determined independently in the same solution by measuring the OD663 before and after acidification (Lorenzen, 131). As discussed above, acidification removes the magnesium ion from chlorophyll a converting it to pheophytin a in 90 % aqueous acetone and has a specific absorption coefficient approximately 60 % that of chlorophyll a and has an absorption peak at 665nm. The before/after ratios of algal extracts generally fall in the range of 1.00 - 1.70, but values outside of the theoretical limits are occasionally seen.

The following equations are used to calculate the concentrations of chlorophyll a corrected for pheophytin a, (modified from Strickland and Parsons, 139):

$$\text{Chl } a \text{ (mg/l)} = \frac{26.7(OD663b - OD665a) \times E}{S \times C}$$

$$\text{Pheo } a \text{ (mg/l)} = \frac{26.7((1.7(OD665a) - OD663b)) \times E}{S \times C}$$

Where:
E = Volume of extract in milliliters
S = Volume of samples in liters
C = Light path of cuvette in centimeters

Because of reports of anomylous B/A ratios received from persons working in Federal and state water pollution programs, and because of our own observations, the optical properties of acetone solutions of purified

chlorophyll a, b and c, prepared separately and in combination, were examined by the EMSL-USEPA laboratory. It was observed that when the pigments were prepared in separate solutions, the wave lengths at the absorption maxima before and after acidification did not vary appreciably over an acetone concentration range of 80 % to 100 %. The before/after acidification peaks were as follows : (a) Chlorophyll a -663nm/665nm, (b) Chlorophyll b - 647nm/653nm, and (c) Chlorophyll c - 630nm/(no peak) Figure 10). Although the chlorophyll c peak at 630 nm disappeared upon acidification, new peaks appeared at 574.5 nm and 595 nm, as described by Jeffrey and Shibata (192).

In solutions containing both chlorophylls a and b, however, the B/A ratios varied significantly with the acetone:water proportions. In 100 % and 90 % acetone, the absorption peaks of these solutions after acidification decreased to that of pheophytin b (653 nm - 654 nm), even at the lowest ratio (24:1) of chlorophyll a and b concentrations examined (Table 8, Figure 11a). It was also noted that the absorption maxima of the extracts prior to acidification were significantly depressed at the higher ratios of chlorophyll b and higher concentrations of acetone. As the proportion of acetone decreased, the "before acidification" peak increased to 663 nm and the "after acidification" peak increased to 665 nm.

The effect of different acetone:water ratios on the absorption curves of periphyton pigment extracts was also examined, with similar results (Figure 11b).

At the higher proportions of acetone, the purified chlorophyll and periphytin chlorophyll solutions both gave excessively high 663 nm/665 nm before/after ratios. When these ratios were used to calculate the corrected chlorophyll a and pheophytin a concentrations, as they normally would be in routine chlorophyll work, their use resulted in inflated corrected chlorophyll a values and depressed pheophytin a values (Tables 9 and 10).

The cause of this phenomenon is unknown, but it appears to result from an interaction between chlorophyll a and b, and indicates that strict adherence ot the 9 : 1 acetone-water ratio must be maintained to assure accurate pheopigment corrections.

Fluorometric Methods

Chlorophyll solutions fluoresce strongly in the red region of the spectrum. Brewster (193) was the first to recognize the fluorescent properties of chlorophyll, which he termed opalescence. Dhere (194) and Wilsche (195) contributed the first photographs of the fluorescence spectrum, but the technique was not suitable for quantitative analysis. The use of fluorescence to measuure chlorophyll was also proposed by Krey (5) and Kalle (196).

Current fluorometric analysis are based on the work of Yentsch and Menzel (137), who employed a Turner Model 111 fluorometer equipped with a R-136 (red-sensitive) photomultiplier tube and Corning CS-5-60(blue)

excitation and CS-2-60 (red) emission filters. Holm-Hansen et al. (197) later reported that greater selectivity for chlorophyll a is obtained with a Corning CS-2-60 emission filter.

The fluorometric method of measuring chlorophyll a is relatively simple and is far more (10-100 times more) sensitive than the spectrophotometric method (137,197). The instruments are relatively stable and the readings are repeatable. Yentsch and Menzel (137) observed a consant "blank" calibration of fluorescence over long periods of time, with an instrument reading repeatability of ± 3 %. A solution of quinine sulfate in 0.1N H2S04 (Loftus et al.,198; Boto and Bunt, 199) is commonly used as a reference material to monitor the sensitivity of the fluorometer.

The concentration of chlorophyll a alone, or mixture of chlorophylls a, b and c, can be measured depending upon the filters used (Holm-Hansen, et al.,197; Loftus and Carpenter, 198). A fluorometric method for determining chlorophyll a: chlorophyll b ratios in ethanol or diethyl ether is also available (Boardman and Thorne, 200). However, multi-filter fluorometric methods for the simultaneous measurement of more than one chlorophyll in extracts are too cumbersome for routine use in pigment analysis.

A major disadvantage of the fluorometric method is the necessity to calibrate the fluorometer with a chlorophyll solution of known concentration. The calibration is ordinarily carried out with a pigment extract previously analyzed with a spectrophotometer. However, chlorophyll reference solutions of known concentration now available from the EMSL-USEPA laboratory permit the direct calibration of fluorometers.

Other problems not easily circumvented are the quenching of chlorophyll a fluorescence by b-carotene and other accessory pigments in algal extracts (Murty and Rabinowitch,201), the varying relationship between the extract fluorescence and the chlorophyll concentration, depending upon the species conposition of the phytoplankton (Holm-Hansen et al.,197), and dependence of fluorescence on temperature. The relationship between fluorescence and chlorophyll concentration must be checked for each sampling run. Chlorophyll fluorescence changes approximately 0.3 %/ C (137). Extract temperatures in the fluorometer, therefore, should be maintained within a range of ± 3 C to avoid significant errors.

Pheopigment Correction. Fluorometric determinations of the concentration of pheopigments in algal extracts are accomplished by measuring the fluorescence before and after acidification, as in the spectrophotometric methods (137,197). The equations employed for the calculation of chlorophyll a corrected for pheopigments, and for pheophytin a, are as follows (Strickland and Parsons, 139):

$$\text{Chl } a \text{ (mg/l)} = F\frac{Q}{(Q - 1)}(Rb - Ra)$$

$$\text{Pheo } \underline{a} \text{ (mg/l)} = F\frac{Q}{(Q-1)}(QRa - Rb)$$

Where:

$$F = \frac{\text{Concentration of Chlorophyll } \underline{a} \text{ mg/l)}}{\text{Fluorometer reading}}$$

Q = Ratio of fluorescence before and after acidification using an extract free of pheopigments.

Rb = Fluorometer reading of the above extract before acidification.

Ra = Fluorometer reading of the above extract after acidification.

The fluorescence of pheophytin a is 40% that of chlorophyll a, and the acid factor (Q), therefore , is approximately 2.5. The value of Q, however, varies with the instrument used, the composition of the pigment (197) and the excitation wavelength (Saijo and Mishizawa, 202). The presence of significant amounts of chlorophyll c in algae extracts may result in acidification ratios in excess of 2.5, which would yield negative values for pheophytin a. Holm-Hansen et al. suggested the use of a value for Q of 3.0 as repesentative of the upper limit of acid factors obtained from samples of healthy phytoplankton in marine waters. The validity of this approach, however, has not been established, and the problem of accessory pigment interferences in the determination of chlorophyll a in extracts of freshwater phytoplankton and periphyton also has not been addressed.

In Vivo Fluorometric Method. The fluorescence of chlorophyll in living periphyton and phytoplankton in vivo can be employed to quantitate the chlorophyll in much the same manner as described for the fluorometric in vivtro analysis (Lorenzen, 203). This technique is the most direct approach to the measurement of algal chlorophyll and has been widely used (29,204-221).

The method suffers from two serious disadvantages: (1) it is only one-tenth as sensitive as the in vitro method (221), and (2) the amount of in vivo fluorescence varies with the availability of nutrients, the presence of toxic substances and the species composition of the algae (29,205,212,216,222). The effects of nutrients, toxicants and taxonomic composition can now be abrogated by the addition of small quantities of 3-(3,4 dichlorophyenyl)-1, 1-diemthylurea (DCMU), which blocks the photosynthetic electron transport system and results in a fluorescence yield which is proportional to the amount of chlorophyll present, regardless of

taxonomic composition or nutritional conditions (Slovacek and Hannan, 223). This approach, based on the earlier work of Duysens and Sweers (224), Bannister (225), Papageorgiou and Govindjee (226) and Vredenburg (227), involves the addition of DCMU at concentrations of 3uM - 10uM. The DCMU penetrates the cells rapidlly, and the maximum fluorescence yield were obtained in as little as 2.5 minutes (Slovacek and Hannan, 223). Changes in in vivo chlorophyll fluorescence mitigated by DCMU also were found to be useful in determining the photosynthetic capacity of algae (Samuelsson and Oquist, 228).

Chromatographic Methods

Numerous column (229-233). paper (224-237), thin-layer (231,232 238-246), liquid (247), high-pressure liquid (248-251), and gas (252-253) chromaatographic methods are available for algal pigment separation and characterization, but most of them are too time consuming for routine use, especially in the field. Jeffrey and Allen (237) proposed a paper chromatographic method for shipboard use, but recommended it as a special tool to obtain detailed information about the pigment content of the algae and the occurrence and types of degradation products, rather than as a method for routine pigment survey work. Recently-published high-pressure liquid chromatographic methods for identification and quantification of chlorophylls a and b and pheophytins a and b (Jacobsen,250; Shoaf, 251) are the most amenable to use in survey work because they can be automated and can provide high rates of sample throughput, but are seriously limited in their application because they can not measure chlorophyll c, which is a taxonomically important component of phytoplankton and periphyton pigments.

Remote Sensing

The geographic scope of pigment surveys traditionally has been limited by the logistics of sample collection and analysis. However, recent developments in remote sensing technology (254-270), involving the use of spectral analysis of reflected light and laser-activated chlorophyll fluorescence by instruments aboard low-flying aircraft and space vehicles, may soon provide rapid and useful estimates of the concentration of chlorophyll in surface waters on a broad geographic and perhaps even global scale. Because of the limitations in these approaches, however, traditional methods of chlorophyll analysis will still be required in most studies.

ASPECTS OF CHLOROPHYLL METHODOLOGY THAT NEED ADDITIONAL STUDY

Chlorophyll methodology is widely used and is approaching a relatively high degree of standardization. Many of the facets of chlorophyll measurements have been thoroughly examined and are well understood, but questions still remain regarding the effects that the many small but important steps in sample collection, preparation and analysis, and possible interferences between pigments may have on the data obtained. Areas which still require study include:

1. The stability of chlorophyll in periphyton and plankton samples during the period between collection and processing.

2. The efficacy of filters and centrifugation in the recovery of phytoplankton from water samples.

3. The stability and photosensitivity of chlorophyll in algal concentrates on filters.

4. The efficacy of pigment extraction methods (solvents, grinding, steeping, etc.).

5. Interferences in chlorophyll *a* determinations caused by degradation products and accessory pigments.

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TABLES

Table 1. Pigment Composition of Major Groups of Algae (From Jeffrey, 128)

	CHL A	CHL B	CHL C	CHL C
CYANOPHYTA	+			
CHLOROPHYTA	+	+		
EUGLENOPHYTA	+	+		
CHRYSOPHYTA	+		+	+
CRYPTOPHYTA	+		-	+
PYRROPHYTA				
Peridinin-containing	+		-	+
Fucoxanthin-containing	+		+	+
RHODOPHYTA	+			
PHEOPHYTA	+		+	+

Table 2. Effects of storage time on the chlorophyll concentration in plankton samples.

Concentrations (mg/m3)

		Chlorophyll a		Chlorophyll b		Chlorophyll c	
Station	Depth(m)	6 weeks	14 weeks	6 weeks	14 weeks	6 weeks	14 weeks
C-3	0-25	1.209	1.129	0.032	0.107	0.251	0.246
	25	1.574	1.392	0.025	-0.086	0.054	0.277
C-5	0-25	1.209	1.103	0.037	-0.038	0.190	0.101
	25	0.857	0.737	0.029	0.016	0.504	-0.026
C-7	0-25	1.549	1.440	-0.008	0.460	0.327	0.528
	25	3.395	3.177	0.163	0.404	1.091	1.273

Table 3. Effect of MgCO3 on chlorophyll recovery.

Pigment (mg/l)

MgCO3 (mg/15ml)	CHL A 15 min Conc	%Loss	CHL A 24 hr Conc	%Loss	CHL B 15 min Conc	%Loss	CHL B 24 hr Conc	%Loss	CHL C 15 min Conc	%Loss	CHL C 24 hr Conc	%Loss
0.0	7.16	0	7.16	0	2.23	0	2.23	0	1.65	0	1.65	0
0.5	7.15	0	7.16	0	2.23	0	2.24	0	1.57	0	1.71	0
1.0	7.15	0	7.15	0	2.34	0	2.23	0	1.57	0	1.60	3
5.0	7.13	0	7.11	0	2.25	0	2.19	2	1.67	0	1.55	6
10.0	7.08	1	7.03	2	2.16	3	2.12	5	1.56	5	1.55	6
50.0	6.88	4	6.75	6	1.98	11	1.91	14	(a)		1.57	5
100.0(b)	6.55	9	6.40	11	1.85	17	1.76	21	1.52	8	1.30	21
150.0	6.28	12	6.06	15	1.76	21	1.59	29	1.47	11	1.13	32

(a) Erratic value.
(b) Determined by graphical interpolation.

Table 4. Solvents used in chlorophyll analyses (taken in part from 159).

Solvent	Investigator
80% Acetone	Mackinney (121)
	Arnon (161)
	Vernon (129)
	Bruinsma (162)
85% Acetone	Yentsch and Menzel (137)
90% Acetone	Richards and Thompson (158)
	Odum et al. (163)
	Humphrey and Wootton (155)
	Talling and Driver (164)
	Parsons and Strickland (150)
	UNESCO (155)
	Lorenzen (131)
	Jeffrey and Humphrey (130)
100% Acetone	Jeffrey and Humphrey (130)
	Holm (165)
Methanol	Marker (87, 99, 101)
	Tett et al. (96, 103, 104)
	Holm-Hansen and Reimann (153)
	Talling and Driver (164)
	Livingstone et al. (166)
DMSO:90% Acetone	Shoaf and Lium (164)

Table 5. Comparison of chlorophyll concentrations obtained using different solvents and trichromatic equations.

Trichromatic Equations								
Richards			Thompson (158)			Parsons	Strickland (139)	
acetone-methanol	acetone 90%	methanol	acetone-methanol	acetone 90%	methanol	acetone-methanol	acetone 90%	methanol
61.5	21.1	80.1	46.4	15.7	60.4	45.7	15.7	59.4
61.5	21.6	80.0	46.4	16.1	60.4	45.6	16.0	59.3
61.9	21.2	80.0	46.7	15.8	60.4	46.0	15.7	59.3
61.8	20.7	80.0	46.6	15.4	60.7	45.9	15.3	59.8
61.1	21.6	80.1	46.1	16.1	60.4	45.4	16.0	59.4

Table 6. Rates of photodecomposition of chlorophyll in a 90% aqueous acetone extract of periphyton pigments.

Intensity of Incident Illumination (fc)	Initial rates of decline in OD663 (percent/hour)	Time required for 50% reduction in OD663
12	3.2	24 hours
25	3.8	18 hours
50	4.7	14 hours
100	5.6	10 hours
200	11.3	4 hours
450	22.0	2 hours
900	50.0	1 hour
12,000	(12.5%/min)	4 minutes

Table 7. Effect of long-term storage (11 months) on the chlorophyll *a* concentration in a 90% aqueous extract of periphyton pigments.

Storage Temperature (C)	Decline in OD663 (Percent)
37	27
Ambient	18
4	8
-20	< 1

Table 8. Effect of spectrophotometer resolution on the apparent concentration of chlorophyll *a* in pigment extracts.

Resolution (nm) (Half-band Width)	Recovery of Chl *a* (%)
0.1	100.0
1	99.6
2	98.8
5	93.4
10	78.6
20	48.5

Table 9. Effect of the acetone:water ratio on the absorption peaks of chlorophyll solutions obtained before and after acidification.

	Chl A:Chl B				
	2:1	3:1	6:1	12:1	24:1
Percent Acetone					
100% B/A*	661/653	662/654	662/654	662/654	663/654
98% B/A	661/653	662/654	662/654	663/654	663/654
96% B/A	662/656	663/658	663/658	663/660	663/662
94% B/A	662/658	663/662	663/664	663/664	663/664
92% B/A	662/664	663/664	663/664	663/665	663/665
90% B/A	662/665	663/664	663/665	663/665	663/665
88% B/A	662/665	663/664	663/665	663/665	663/665
86% B/A	662/665	663/664	663/665	663/665	663/665
84% B/A	662/665	663/664	663/665	663/665	663/665
82% B/A	662/665	663/664	663/665	663/665	663/665
80% B/A	663/665	663/665	663/665	663/665	663/665

*Wave Length (nm) of absorption peaks before (B) and after (A) acidification

Table 10. Effect of the acetone:water ratio on the B/A ratios and concentrations of (corrected) chlorophyll *a* and pheophytin *a* in solutions containing purified chlorophyll *a* and *b*.

ACETONE (%)	OD663/OD665	CHL A (MG/L)*	PHEO A (MG/L)*
100	3.19	9.28	- 6.32
98	3.11	9.16	- 6.13
96	2.39	7.85	- 3.91
94	2.06	6.94	- 2.36
92	1.90	6.39	- 1.41
90	1.80	5.99	- 0.73
88	1.72	5.64	- 0.14
86	1.70	5.56	0.00
84	1.71	5.61	- 0.09
82	1.70	5.56	0.00
80	1.72	5.64	- 0.14

* Concentration in the extract
(Chl a/ Chl b = 2:1)

Table 11. Effect of the acetone:water ratio on the B/A ratios and concentrations of (corrected) chlorophyll *a* and pheophytin *a* in a periphyton pigment extract.

ACETONE (%)	OD663/OD665	CHL A (MG/L)*	PHEO A (MG/L)*
100	1.68	5.67	0.152
98	1.64	5.45	0.52
96	1.48	4.54	2.06
94	1.40	3.98	3.02
92	1.35	3.64	3.61
90	1.35	3.64	3.61
88	1.37	3.77	3.38
86	1.39	3.93	3.11
84	1.40	3.98	3.02
82	1.41	4.06	2.88
80	1.43	4.20	2.65

*Concentration in the extract

FIGURES

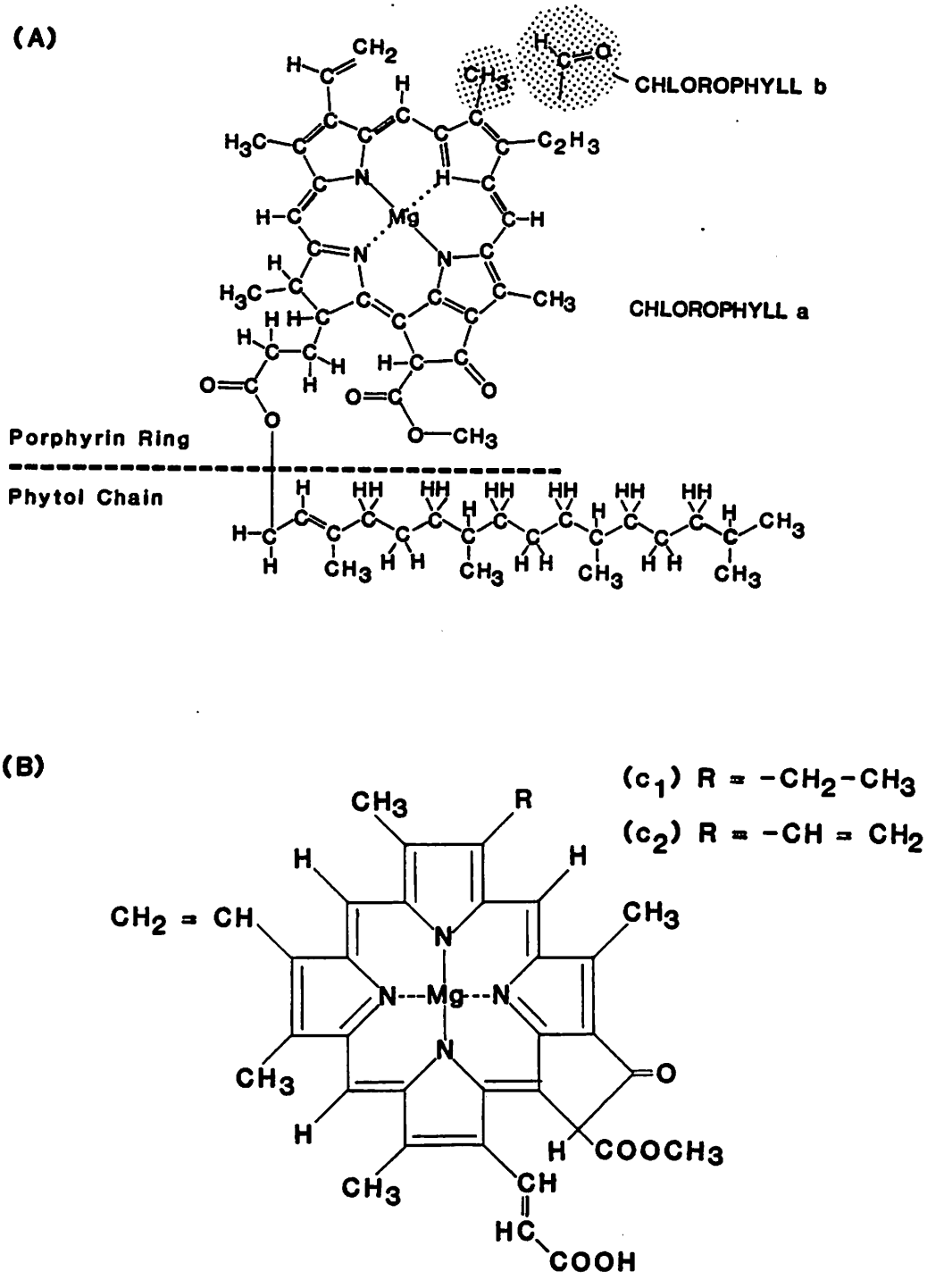

Figure 1. Structure of chlorophylls: (A) chlorophylls a and b; (B) chlorophylls c_1 and c_2.

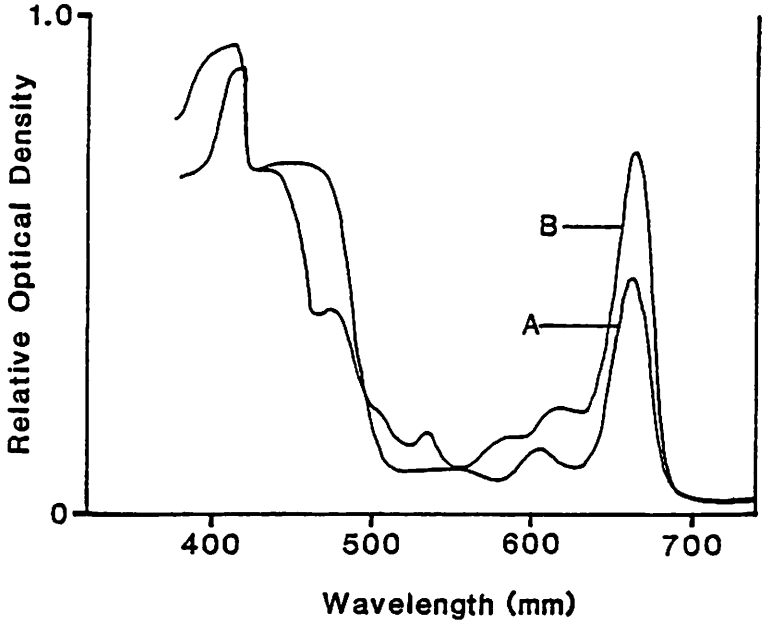

Figure 2. Absorption spectra of a solution of chlorophyll in 90% aqueous acetone before (B) and after (A) acidification (Weber, 39).

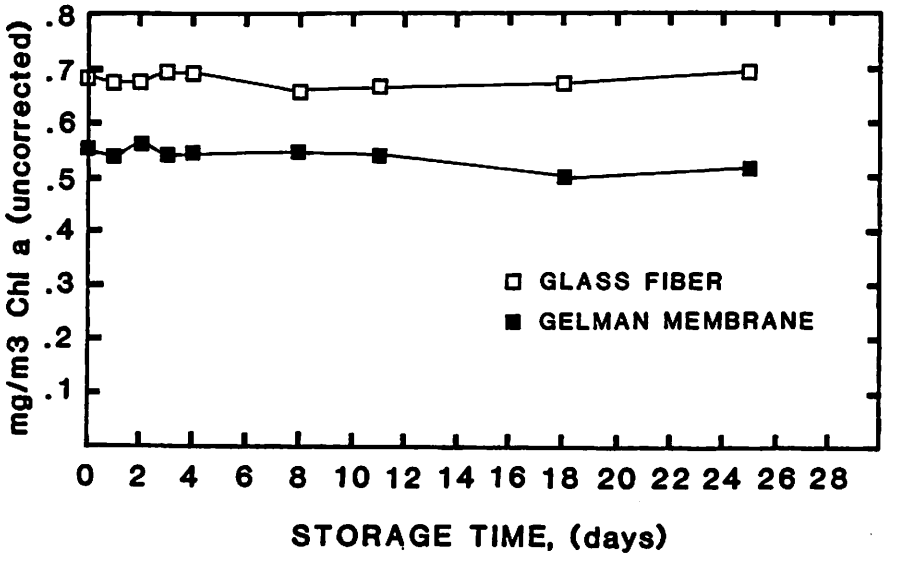

Figure 3. Effect of storage on the chlorophyll a content of phytoplankton on filters held in the dark at -20°C.

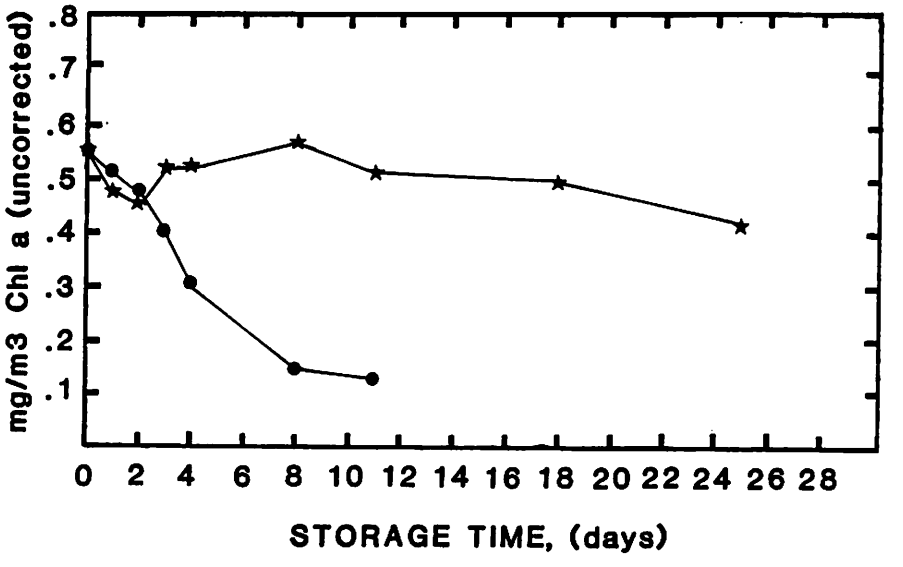

Figure 4. Effect of storage on the chlorophyll a concentration in plankton grab samples held at 4°C (★) and at 20°C (●).

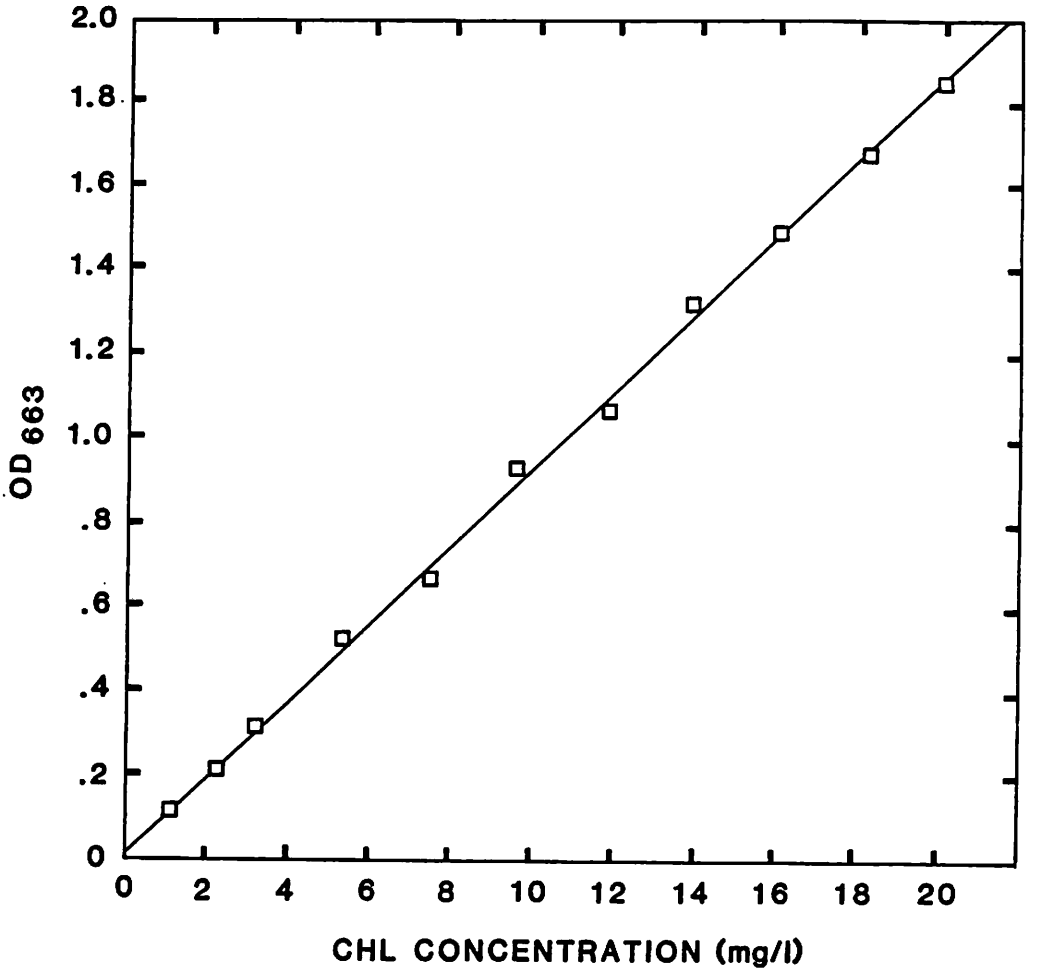

Figure 5. Relationship between the OD663 and the concentration of chlorophyll a in a 90% aqueous acetone algal pigment extract.

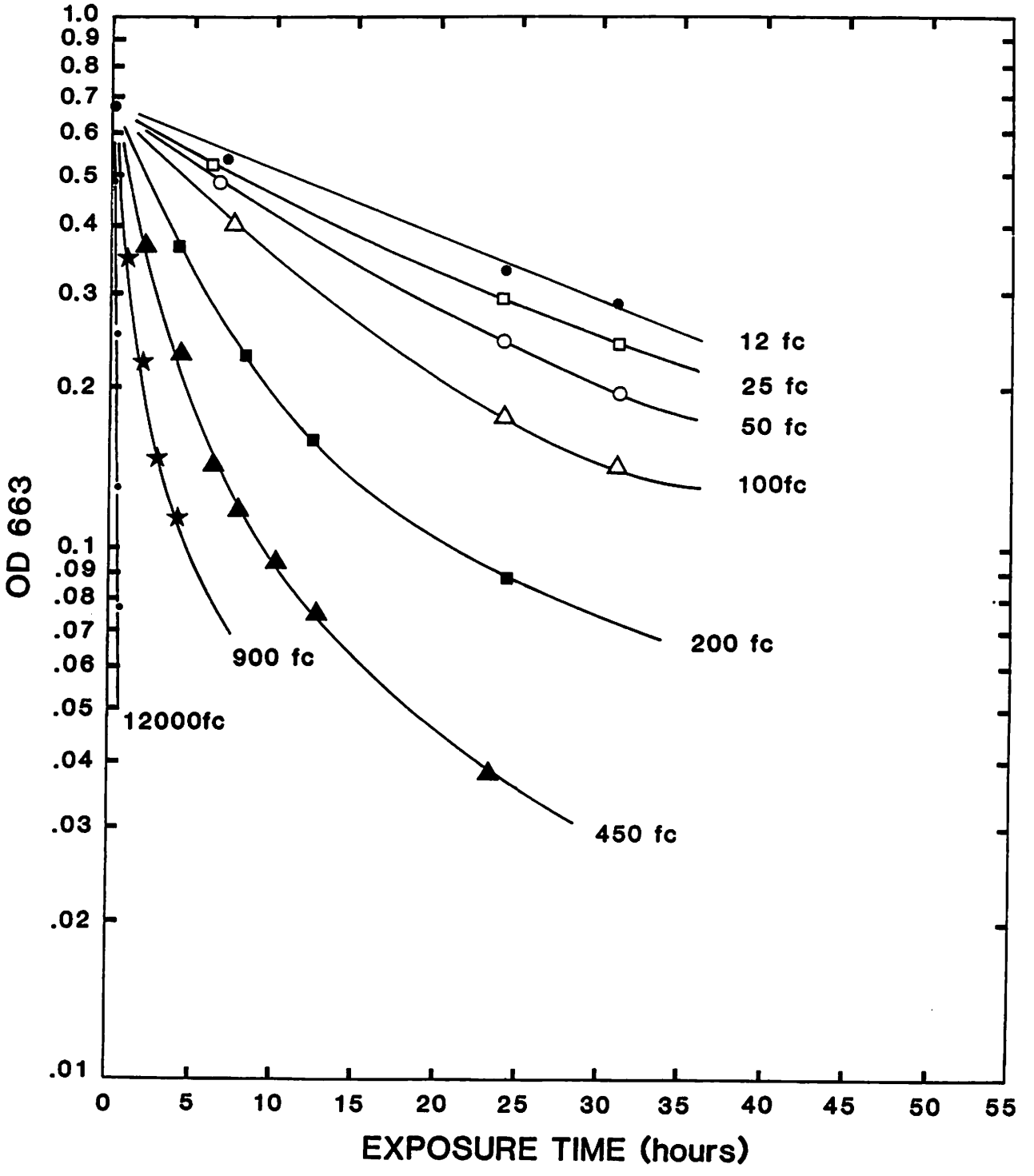

Figure 6. Effect of light on the concentration of chlorophyll a (OD663) in 90% aqueous acetone

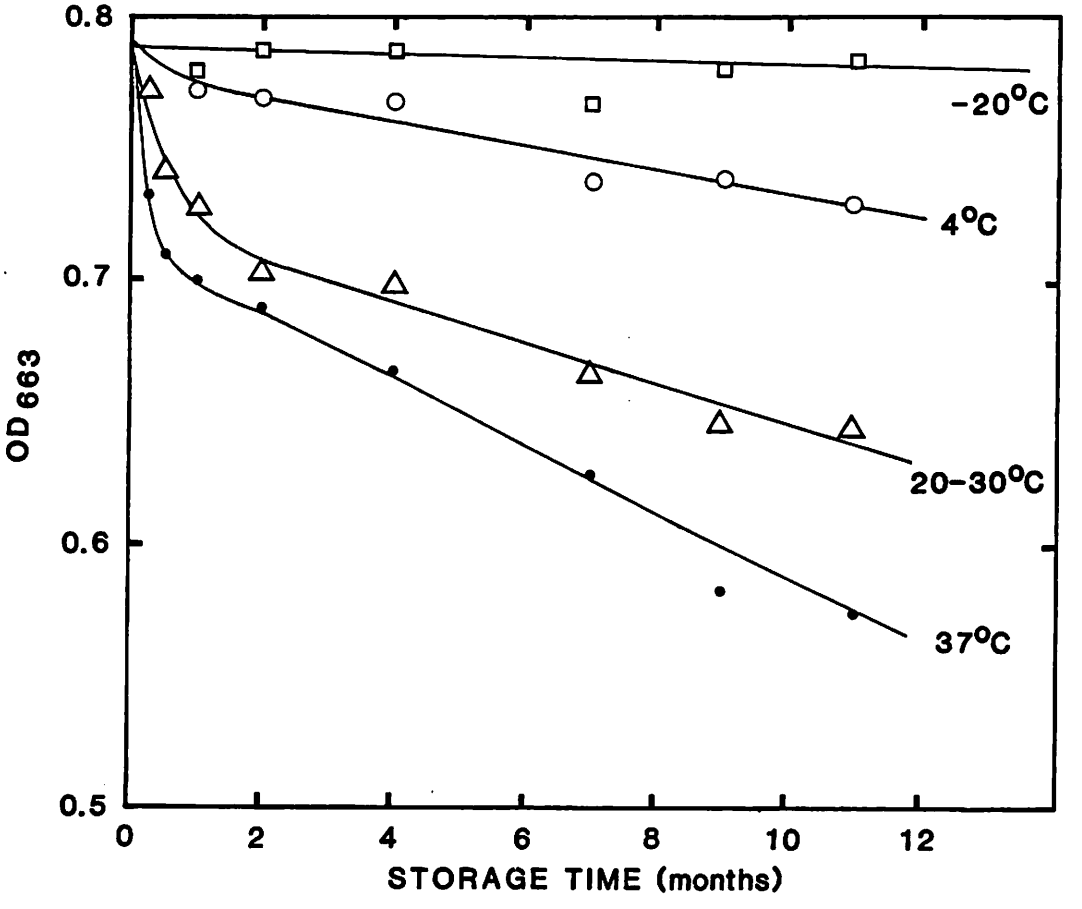

Figure 7. Effect of prolonged storage on the concentration of chlorophyll a (OD 663) in 90 % aqueous acetone.

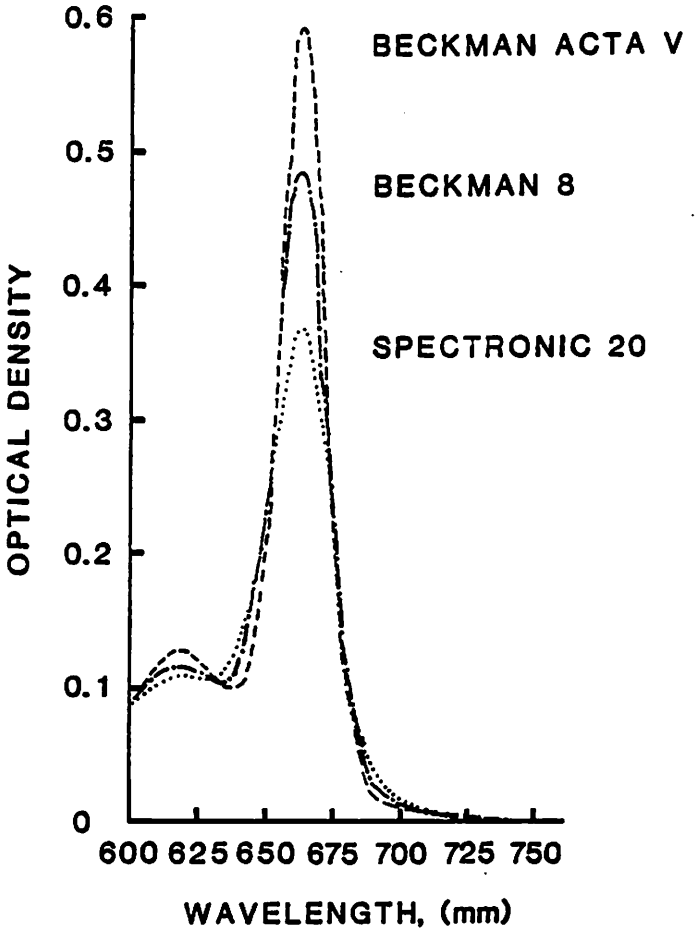

Figure 8. Chlorophyll absorption curves of the same pigment extract obtained with spectrophotometers of differing resolutions.

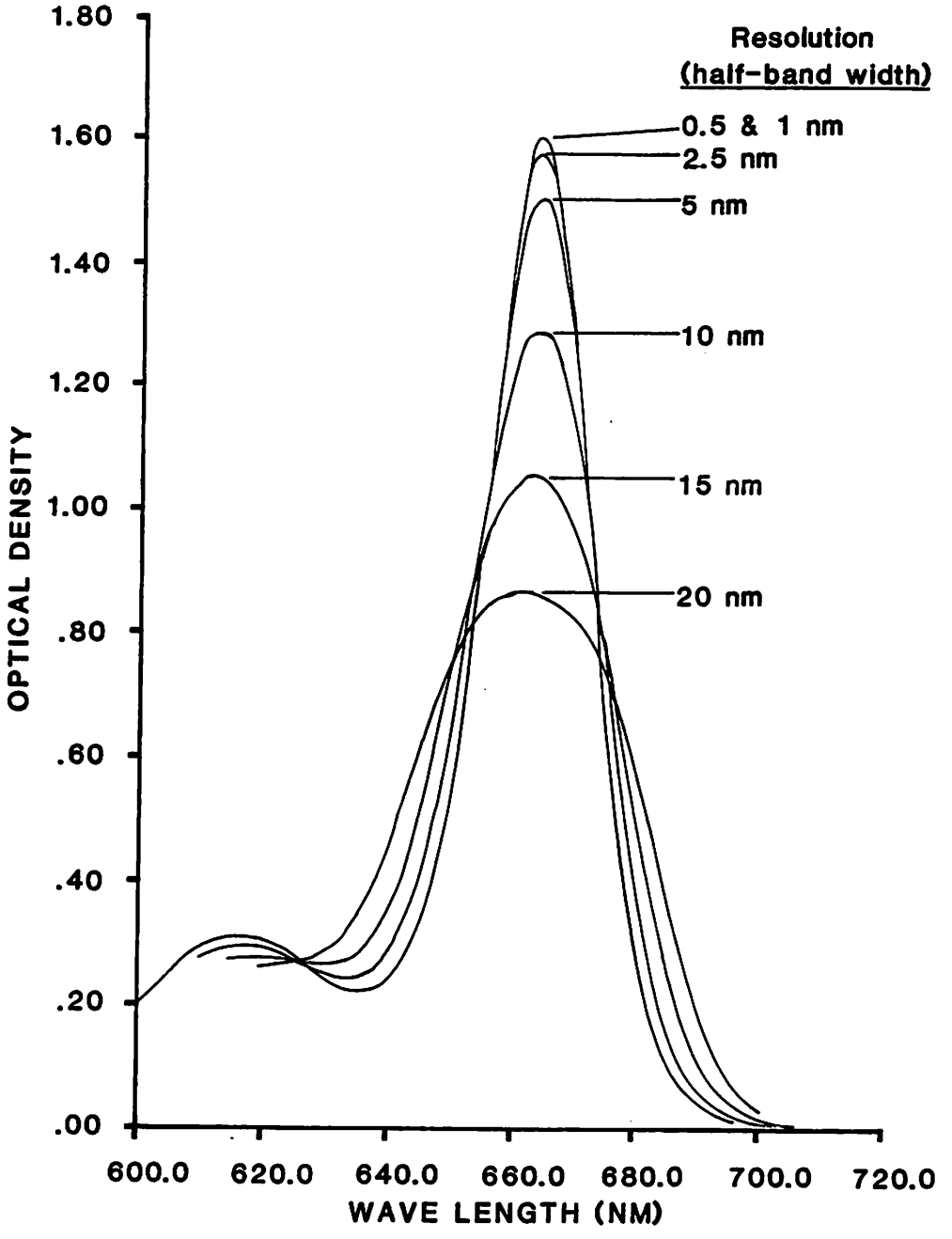

Figure 9. Computer simulation of the effect of spectrophotometer resolution on the chlorophyll absorption curve.

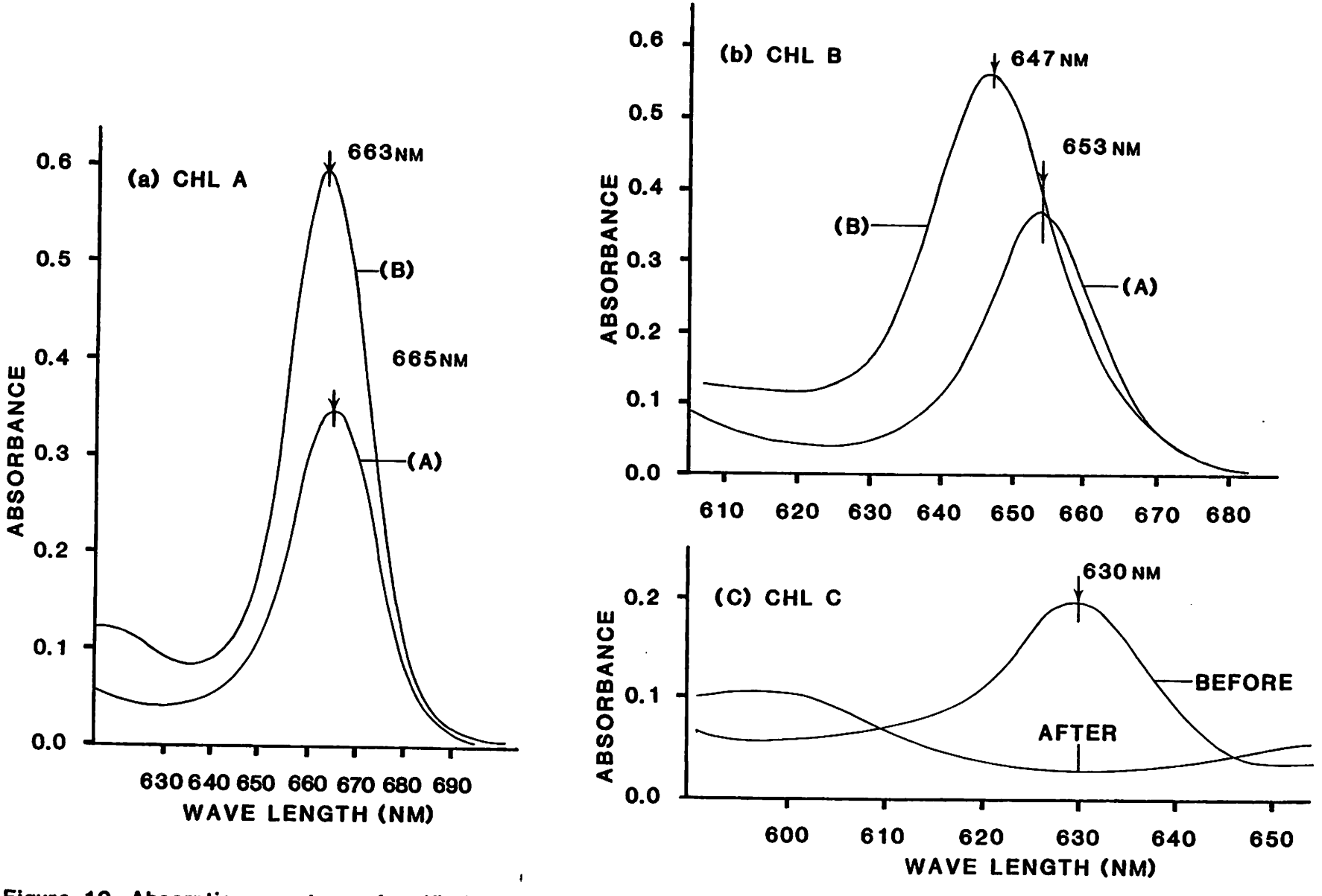

Figure 10. Absorption spectrum of purified chlorophylls a, b, and c, before (B) and after (A) acidification: (a) Chlorophyll a, (b) chlorophyll b, and (c), chlorophyll c.

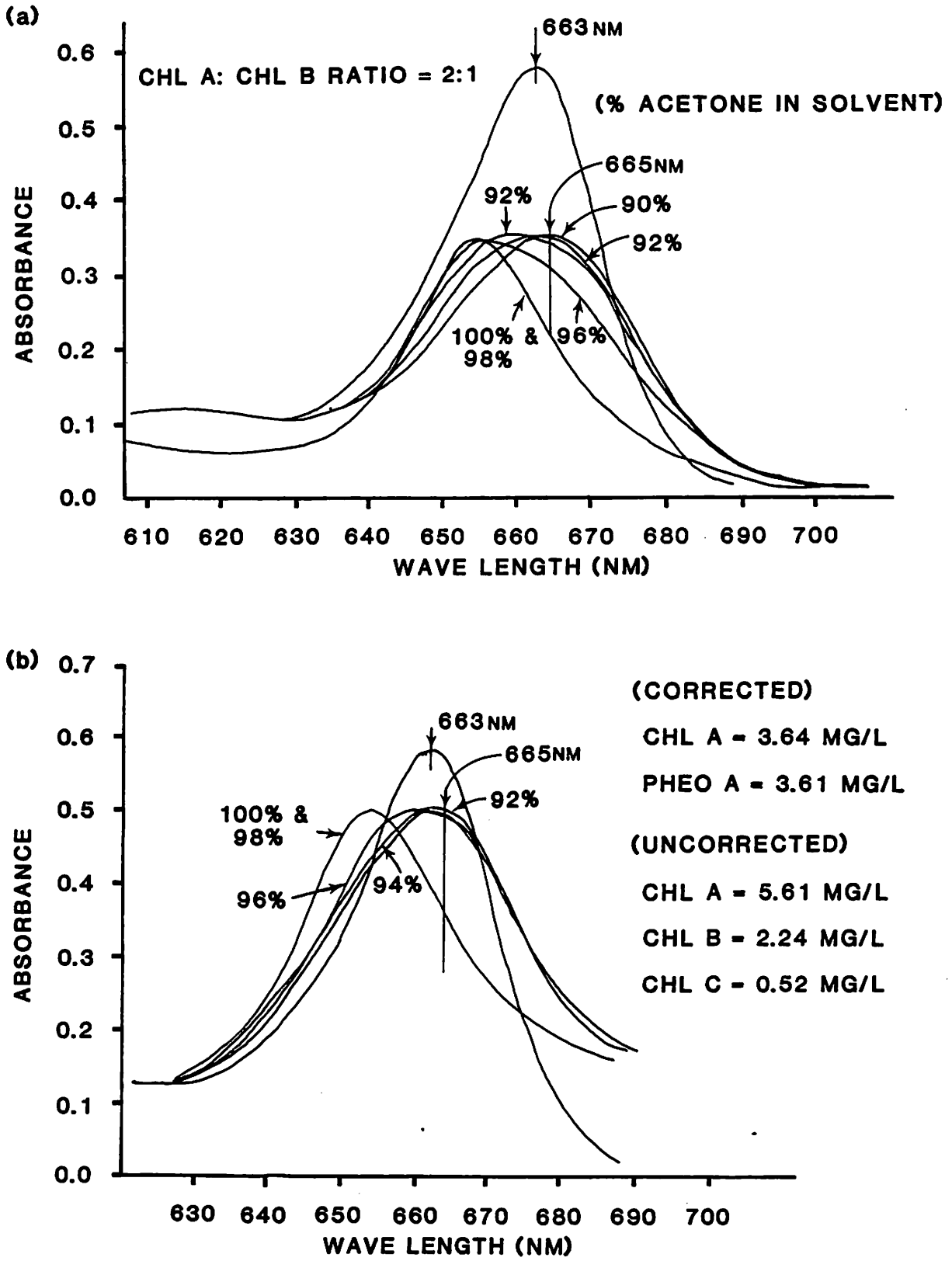

Figure 11. Effect of the acetone:water ratio on the absorbance peaks of 90% aqueous acetone solutions of chlorophylls a and b after acidification: (a) solution containing purified chlorophyll a and b, and (b) periphyton pigment extract containing chlorophyll a and b.