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Fluorometric analysis of chlorophyll *a* in the presence of chlorophyll *b* and pheopigments

Abstract—A fluorometric method is described which provides sensitive measurements of extracted chlorophyll *a* free from the errors associated with conventional acidification techniques. Fluorometric optical configurations were optimized to produce maximum sensitivity to Chl *a* while maintaining desensitized responses from both Chl *b* and pheopigments. Under the most extreme Chl *b*:Chl *a* ratio likely to occur in nature (1 : 1 molar), the new method results in only a 10% overestimate of the true Chl *a* value, while estimates from older acidification methods are 2.5-fold low. Under conditions of high pheopigment concentrations (pheo *a* : Chl *a* = 1 : 1 molar), the new method provides Chl *a* estimates that are equivalent to those determined from the acidification technique. The new simple method requires a single fluorescence determination and provides adequate sensitivity for small sample sizes (<200 ml) even in the most oligotrophic marine and freshwater environments.

Fluorescence analysis of chlorophyll *a* (Chl *a*) remains one of the most widely used and sensitive methods for studying the distribution of phytoplankton in nature (Holm-Hansen et al. 1965). The method has been widely accepted in marine research, where low algal biomass demands the most sensitive techniques. However, potential errors in the determination of Chl *a* by the fluorescence acidification technique, especially when Chl *b* is present, are well recognized (Gibbs 1979; Lorenzen 1981; Trees et al. 1985). The obvious presence of Chl *b*-containing chlorophytes in freshwater ecosystems has understandably limited the popularity of general fluorescence methods in limnologic work. On the contrary, it has often been assumed that concentrations of Chl *b* relative to Chl *a* were low in marine systems (Lorenzen 1981), therefore promoting the general acceptance of fluorescence Chl *a* determinations in marine research. However, our understanding of that situation has changed. Pigment analyses by high pressure liquid chromatography (HPLC) have shown that Chl *b* can be quite common in marine systems (Bidigare et al. 1986; Gieskes and Kraay 1986). The recent recognition of prochlorophytes in the oligotrophic ocean (Chisholm et al. 1988) raises particular concern over the use of conventional fluorescence acidification techniques to determine Chl *a* (including divinyl Chl *a*), since prochlorophytes are characterized by unusually high ratios of divinyl Chl *b* to divinyl Chl *a* (~1 : 1, Chisholm et al. 1988; Goericke and Repeta 1992). This observation has contributed to the increased popularity of HPLC techniques, which provide accurate measures of Chl *a* regardless of the relative abundances of accessory chlorophylls, degradation products, and carotenoids (Wright et al. 1991). However, although HPLC instrumentation is increasingly common, it still presents prohibitive initial costs to some laboratories, and generally high costs are associated with routine sample processing, calibration, and instrument maintenance.

Thus, there is a need for a simple, inexpensive assay for Chl *a* in both marine and freshwater research that adequately deals with the now ubiquitous Chl *b* problem. This note describes a modification of the optical characteristics of commonly used filter fluorometers that results in a simple method of determining Chl *a* in the presence of Chl *b* and pheopigments.

The general fluorescence characteristics of Chl *a*, *b*, and c_2 and their respective pheopigments are given in Fig. 1. Chl c_2, which is present in all chromophytic algae and common in both marine and freshwater systems, has fluorescence emission characteristics that can easily be discriminated against when conventional colored filters are used as described in standard fluorescence methods (Loftus and Carpenter 1971). Interference from pheophorbide c_2 can also be ignored, even though its emission spectrum overlaps somewhat with that of Chl *a*, because the mole-specific fluo-

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Table 1. Chromatographic conditions and instrumentation used to prepare pure Chl *a* and Chl *b*. Timing of the solvent gradient is given below, where A is 85:15 MeOH:0.5 M ammonium acetate (aqueous), B is 100% acetonitrile, and C is 100% acetone. Solvents were delivered at 1.5 ml min^{-1} (Varian 5060 pump) with a linear gradient between each time step. Eluting pigments were detected at 440 nm on a Linear Instruments model 200 VIS absorbance detector; samples were injected with a Gilson 203 autosampler onto a 25-cm C_{18} column (5-μm particles, Rainin Microsorb).

Time (min)	Solvent A	Solvent B	Solvent C
		(%)	
0	100	0	0
8	0	100	0
13	0	70	30
17	0	20	80
27	0	20	80
28	100	0	0

rescence signal from acid-produced pheophorbide c_2 is at least 10-fold lower than that of its parent Chl c_2. (Note that the data in Fig. 1 were normalized to unity and are therefore concentration-independent.) However, fluorescence characteristics of Chl *b*- and Chl *a*-derived pheopigments overlap with those of Chl *a*, and their fluorescent signals are strong enough to cause well-known analytical bias when broadbanded excitation-emission filters are used. The overall objective of this study was to select an optimum lamp and filter combination for existing fluorescence instrumentation that provided maximum sensitivity to Chl *a* while maintaining desensitized responses to Chl *b* and pheophytin *a* (phytin *a*). Similar attempts have been made with wavelength-selectable spectrofluorometers (Bazzaz and Rebeiz 1979) and also with multiple filter sets on simple filter fluorometers (Loftus and Carpenter 1971). However, these techniques have been somewhat less popular because they require multiple wavelength readings, cumbersome calibration procedures, and, in the case of spectrofluorometers, costly instrumentation.

In this study, pure preparations of pigments were quantitatively combined in mixtures to evaluate instrument sensitivity to Chl *a* under various fluorometric optical configurations. Pure preparations of Chl *a*, Chl *b*, phytin *a*, and phytin *b* were made in the laboratory from crude extracts of algal cultures or fresh spinach. Chl *a* and Chl *b* were isolated with a semipreparative chromatographic technique similar to that described by Welschmeyer et al. (1991). For each purification, 500 μl of dark-green crude extract in 90% acetone was injected on an HPLC system and separated under the conditions listed in Table 1. The center of each eluting peak of interest was collected without contamination from neighboring peaks and repurified with disposable C_{18} solid-phase extraction columns (Baker SPE, 100 mg). The extraction columns were tempered with two volumes of methanol (~2 ml), followed by two volumes of 100% acetone and one final volume of Nanopure water (the final water layer was drawn down by vacuum to ~5 mm above the solid packing and the vacuum released). The fraction-collected pigments in HPLC eluent (Chl *a* or Chl *b*) were diluted with an equivalent volume of water to reduce solvent strength (1:1, HPLC eluent:H_2O); the mixture was loaded on the disposable column and drawn through the solid phase under weak vacuum (~100 mm of Hg). The pure pigment retained on the column was rinsed with 2 ml of 50% acetone and eluted with 0.5 ml of 100% acetone. The resulting purified pigment was diluted to yield a final 90% acetone solution.

Phytin *a* was produced by acidifying a solution of purified Chl *a* prepared as above: 1 ml of Chl *a* solution + 20 μl of 1 N HCl. After 5 min, the acid was removed by repeating the solid-phase washing protocol above, including one column-volume of water in the wash procedure just before the final elution with 100% acetone. The purified phytin *a* was diluted to yield a 90% acetone solution. Removal of the acid was necessary to prevent pheophytinization of pure Chl *a* when test mixtures were combined. Phytin *a* was considered an adequate representative of most Chl *a*-derived "pheopigments," because the fluorescence excitation-emission spectra for pheophytin *a*, pheophorbide *a*, and many of their derivatives are indistinguishable (Lorenzen and Newton Downs 1986).

Pheophytin *b* (phytin *b*) was produced from pure Chl *b* by acidification; it was assumed that the conversion was stoichiometric and therefore that the molar concentration of phytin *b* was known. The reaction rate for acid pheophytinization of Chl *b* to phytin *b* is lower than for Chl *a* (Schanderl et al. 1962). Therefore,

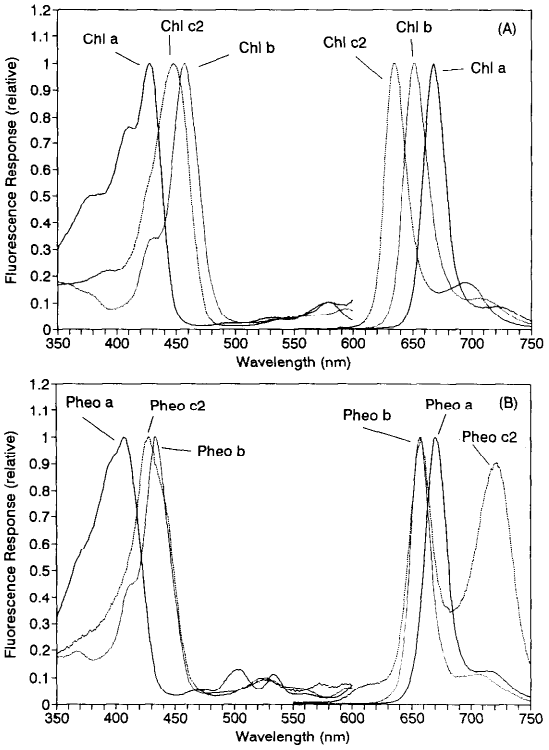

Fig. 1. Photon-corrected excitation-emission spectra of chromatographically purified solutions of (A) chlorophylls *a, b,* and c_2 and (B) their acid-produced pheopigments (all in 90% acetone). Fluorescence responses were measured at the peak excitation-emission wavelength for each pigment. Results were normalized to unity, yielding concentration-independent spectra.

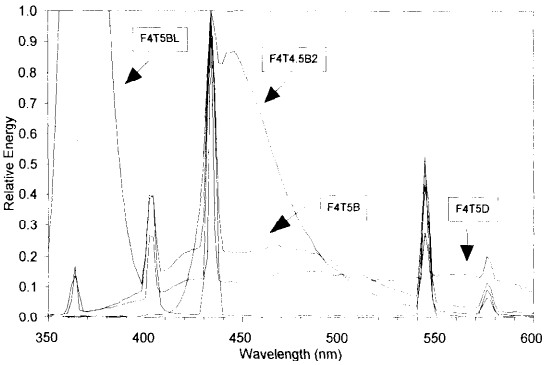

Fig. 2. Lamp energy spectra for the original lamp specified in conventional acidification methods (F4T5B), for substitute lamps (F4T5D and F4T5BL), and for the new lamp used in proposed method (F4T4½B2). Spectra were obtained by directing lamp output to the entrance slit of a monochromator equipped with photon-corrected photomultiplier (Spex Fluorolog 111A). Spectrum for the "cool-white" lamp (F4T5CW), which was similar to F4T5D, was omitted for clarity.

to ensure complete acid conversion of Chl *b* to phytin *b,* five times more HCl (1 N) was used and the acid mixture was allowed to sit in the dark for at least 15 min before making measurements. Phytin *b* is present in low concentrations in natural water-column samples and is not anticipated to present problems in the analysis of natural Chl *a* samples. However, the production of phytin *b* from ambient Chl *b* in the conventional fluorescence acidification technique is the source of the error in underestimating Chl *a* and overestimating pheopigments (Riemann 1978; Gibbs 1979; Trees et al. 1985). Data on relative molar fluorescence responses of phytin *b* are included in the results presented here, but mixtures of phytin *b* and Chl *a* were not prepared; hence, there was no need to produce acid-free phytin *b*.

The purified solutions of Chl *a,* Chl *b,* and phytin *a* were quantified from absorbances measured on an HP-8452 diode array spectrophotometer with absorption coefficients of 87.67, 51.36, and 49.50 liters g^{-1} cm^{-1}, respectively (Jeffrey and Humphrey 1975; Lorenzen and Newton Downs 1986); concentrations of phytin *b* were calculated from acidification stoichiometry. For the initial survey work in assaying the selectivity of various fluorometric optical configurations, the concentrations of the four pure pigments were adjusted with 90% acetone to yield separate equimolar solutions.

The selection of fluorescent lamps available for commonly used filter fluorometers (Turner 111, Sequoia Turner 112, Turner Designs model 10 and model 10AU) is limited. The original lamp specified in the earliest methods (F4T5B) is no longer commercially available; the daylight lamp (F4T5D) is now the most common replacement (Baker et al. 1983). A selection of lamps with adequate blue excitation energy was gathered for testing in this study: F4T5D (daylight), F4T5CW ("cool-white"), F4T5BL (blacklight), and F4T4½B2 (blue, custom color, Sequoia Turner). The relative energy spectrum for each lamp was measured by directing the lamp output to the entrance of the emission monochromator on a

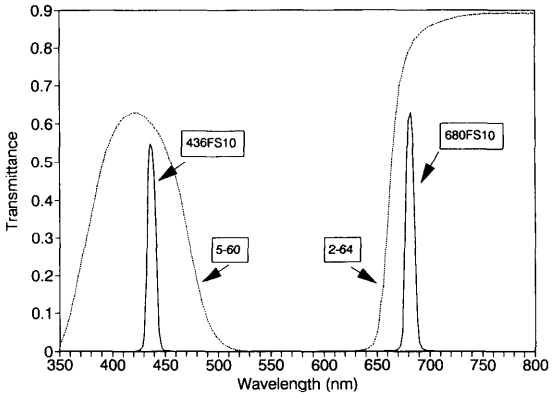

Fig. 3. Transmittance characteristics of excitation-emission filters used in conventional fluorometric acidification technique (Corning 5-60/Corning 2-64) and in the newly proposed method (436FS10/680FS10 interference filters, Andover Corp.).

Spex Fluorolog 111A scanning spectrofluorometer. The resulting spectral scans were normalized to the wavelength of greatest blue energy output for each lamp (Fig. 2). As seen in Fig. 2, all lamps share similar wavelengths of peak energy in the blue (436 nm), which makes all of them potentially useful in Chl *a* analysis. However, one lamp in particular, F4T4½B2, is unique in that it is missing the strong energy line at 408 nm; this wavelength corresponds to the excitation maximum for phytin *a* (Fig. 1B).

The spectral qualities of the conventional filters used in the standard fluorescence method are given in Fig. 3 (excitation, Corning 5-60; emission, Corning 2-64). The broad spectral nature of these filters leads to high sensitivity but also elicits fluorescence response from all pigments derived from Chl *a* and Chl *b* (hence, the nature of the analytical problem). (Chl c_1 + c_2 have emission maxima near 636 nm, which is low enough to present minimum band overlap in the standard filter combination). The following narrowband interference filters were evaluated in the selection of an optimized optical configuration for Chl *a* analysis: excitation, 430, 434, and 436 nm; emission, 665, 671, 680, and 690 nm (all filters were 25-mm diam, 10-nm half-bandwidth, Andover Corp.). The original Corning filters were also included for comparison.

The criteria for selecting the best lamp and filter combination for Chl *a* determination were based on balancing selectivity against overall sensitivity. The most important criterion was discrimination against contaminating pigments other than Chl *a*. The optimum method should ideally yield the highest fluorescence response for Chl *a* while maintaining minimum response from Chl *b*, phytin *a*, and phytin *b*; overall sensitivity was of secondary importance in this evaluation.

Fluorescence was measured on a Turner Designs 10AU fluorometer with automatic sensitivity adjustments and digital readout. Measurements were made on each of the separate equimolar solutions of pure Chl *a*, Chl *b*, phytin *a*, and phytin *b* with 10-mm-diameter disposable glass cuvets. The ratio of Chl *a* fluorescence, relative to each of the other pigments, was used as a quantitative scalar in evaluating selectivity. A semisystematic search through 25 lamp/filter combinations was made to find the optimum optical configuration; certain combinations could be ruled out, making it unnecessary to test every possible lamp and filter combination available. The relative sensitivity of each excitation lamp was measured after the optimum filter combination was selected (*see below*). The Turner Designs 10AU fluorometer is optically referenced to cancel excitation source fluctuations; the same is true for older Turner models 111 and 112. The reference signal is thus also subject to color variations in the excitation lamp; for this reason, the raw fluorescence readout after making lamp changes is not a suitable indicator of relative sensitivities. A nonreferenced, voltage-stabilized filter fluorometer (Kratos 950) was fit with the same filters and photomultiplier (Hamamatsu R446) used in the Turner 10AU and used to measure the relative lamp sensitivities to Chl *a*.

Table 2 lists the selectivity response ratios of Chl *a* fluorescence to fluorescence from Chl *b*, phytin *a*, and phytin *b* for selected optical combinations; relative lamp sensitivities to Chl *a* per se are also listed for the 436/680-nm filter combination. The selectivity response ratios for the standard optical configuration (F4T5D/5-60/2-64) were low. The greatest discrimination against Chl *b*, phytin *a*, and phytin *b* (and hence, highest selectivity ratios) resulted from the use of interference filters, particularly

Table 2. Fluorescence response of selected lamp and filter combinations to equimolar solutions of purified Chl *a*, Chl *b*, phytin *a*, and phytin *b*. The same Turner Designs model 10AU fluorometer was used for all measurements; no changes in electronic sensitivity were made between optical changes. Lamps and filters are described in text. Overall sensitivity to Chl *a* was normalized to 100 for the recommended lamp/filter combination F4T4½B2/436/680; data for this configuration were repeated in the table to facilitate comparisons. The last three columns give selectivity ratios expressed as the Chl *a* fluorescence divided by fluorescence of an equimolar solution of interfering pigment: $1-F_{Chla}:F_{Chlb}$; $2-F_{Chla}:F_{phyta}$; $3-F_{Chla}:F_{phytb}$.

Lamp	Excitation filter	Emission filter	Chl *a* sensitivity	1	2	3
F4T5D	5-60	2-64	—	3.22	1.71	1.48
F4T4½B2	436	680	100.0	8.60	6.93	2.99
F4T5D	436	680	60.6	9.15	5.50	3.33
F4T5CW	436	680	50.2	9.30	5.89	3.03
F4T5BL	436	680	40.3	8.69	7.50	2.84
F4T4½B2	436	680	100.0	8.60	6.93	2.99
F4T4½B2	434	680	67.5	9.66	5.70	2.92
F4T4½B2	430	680	49.3	10.58	3.81	3.46
F4T4½B2	436	680	100.0	8.60	6.93	2.99
F4T4½B2	436	671	166.0	6.69	8.40	1.75

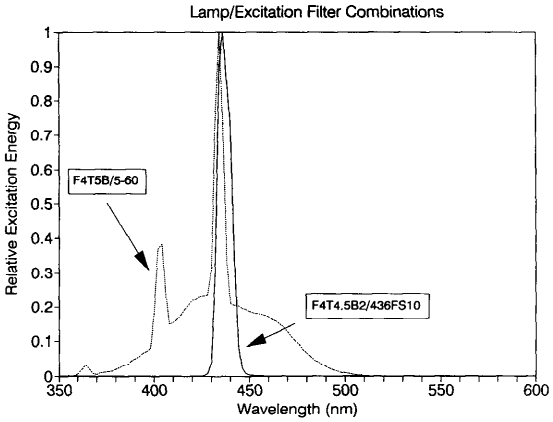

Fig. 4. Resultant energy spectra for combinations of lamp and excitation filter specified in conventional acidification methods (F4T5B blue lamp + Corning 5-60 glass filter) and for the new lamp-filter combination described here (F4T4½B2 blue lamp + 436FS10 interference filter). Spectra were obtained as in Fig. 2; relative energies were normalized to 1.0 at peak wavelength.

the 436/680 excitation-emission combination. Relatively minor differences in the selectivity ratios were noted among the four tested lamps when this filter pair was used (Table 2). Selection of the 680-nm emission filter resulted in a 1.66-fold loss in overall sensitivity relative to the 671-nm filter, which lies nearer to the emission maximum for Chl *a* (Table 2; Fig. 1A). However, the 680-nm filter increased the selectivity response to Chl *a* relative to the interfering pigments and this was the more desirable result. Although the 436/680 filter pair provided optimal selectivity for all lamps, overall sensitivity among the lamps tested ranged over a factor of ~2, with F4T4½B2 the most sensitive and F4T5BL the least sensitive (Table 2). The optimum lamp/filter combination was F4T4½B2/436/680, although similar selectivities with about half the sensitivity could be obtained with any of the other lamps.

A comparison of the energy spectrum of light emitted from the new lamp/filter combination (F4T4½B2/436) to that of the original configuration (F4T5B/5-60) is given in Fig. 4. The new F4T4½B2/436 excitation combination has essentially no energy in the 408-nm band compared to the originally recommended excitation set. For this reason, discrimination against Chl *a*-derived pheopigments is optimized, and acidification protocols to correct for pheopigments are unreliable. Measurements of F_a begin to approximate a blank reading, causing $F_o:F_a$ from pure Chl *a* to exceed 10; the $F_o:F_a$ ratio has high variance in the new optical arrangement. However, the acidification protocol is unnecessary when the new method is used, because even under worst-case conditions of equimolar Chl *a* and phytin *a* (rare in the water column), the fluorescence response is 6.9 times more sensitive to Chl *a* than to phytin *a* (Table 2), resulting in only a 13% overestimate of the true Chl *a* concentration.

The new lamp/filter configuration was tested under a range of mixtures of Chl *b*: Chl *a* and phytin *a*: Chl *a*. For comparison, measurements were included for the original optical configuration (F4T5B/5-60/2-64) and also for the currently used daylight lamp substitution (F4T5D/5-60/2-64). The maximum Chl *b*: Chl *a* ratio assayed was 1.0 (mol mol^{-1}). This represents the worst-case situation that would be experienced, for example, in analyzing a pure culture of prochlorophytes (Chisholm et al. 1988; Goericke and Repeta 1992). Typical Chl *b*: Chl *a* ratios in green algae range from 0.1

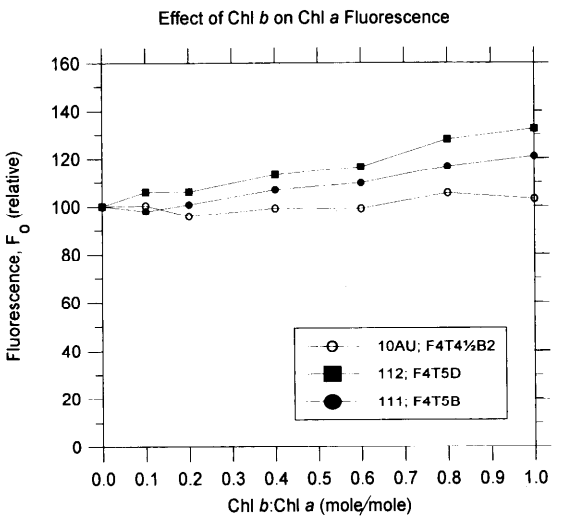

Fig. 5. Fluorescence response (F_o) under increasing Chl *b*:*Chl a* ratios. Chl *a* concentration was constant in all treatments. The new method (○) is relatively insensitive to Chl *b*, while conventional lamp-filter combinations on Turner 111 and 112 instruments show ~25% increases in F_o when Chl *b*:Chl *a* = 1.0 (molar). F4T5D—daylight lamp; F4T5B—blue lamp.

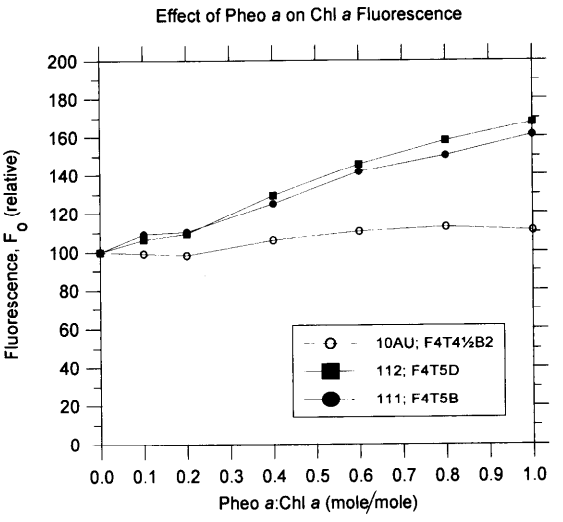

Fig. 6. Fluorescence response (F_o) under increasing phytin *a*:Chl *a* ratios. Note that the new lamp-filter combination is relatively insensitive to the presence of phytin *a* (○), while the older method results in 1.75-fold increases in F_o due to the presence of phytin *a*. Chl *a* concentration was constant in all treatments.

to 0.4 (Jeffrey 1980), so it is unlikely that a molar Chl *b*:Chl *a* ratio of 1.0 would ever be encountered in nature. The same molar constraints were used in testing the new technique for discrimination against phytin *a*.

Figure 5 shows that almost no interference from Chl *b* could be detected when the new method was used, whereas worst-case conditions for the older optical configurations showed ~25% higher fluorescense response when Chl *b* contamination was high (Chl *a*:Chl *b* = 1.0). For comparative purposes, this test focused only on the unacidified fluorescence readings (F_o) (*see below*). For pheopigments, the older optical configuration yields F_o readings that are 1.75-fold too high when the ratio of phytin *a*:Chl *a* is 1.0, while the new method shows only a 10% increase in F_o under the same conditions (Fig. 6).

The standard measurement of Chl *a* is most susceptible to error when acidification protocols are applied in the presence of Chl *b* (Holm-Hansen and Riemann 1978; Gibbs 1979; Trees et al. 1985). This effect is shown in Fig. 7, where the new method and the conventional acidification technique are compared. The two instruments used in this test were cross-calibrated to give identical results when assaying pure solutions of Chl *a*. For the new method, the calibration essentially reduces to determining a single-point calibration coefficient as long as the instrument is properly blanked and sample concentrations are maintained within its linear response range. For the older acidification method, calibration involves determination of both the sensitivity coefficient and the maximum acid ratio ($F_m = F_o : F_a$) for pure Chl *a* (free of phytin *a* and Chl *b*). Figure 7 again shows that the new method yielded an overestimate of only ~10% of Chl *a* under the worst-case interference from Chl *b* (Chl *b*:Chl *a* = 1.0). However, the corresponding results obtained from conventional acidification protocol yielded an estimate of Chl *a* that was 2.5-fold low; the well-known false calculation of phytin *a* was readily apparent (Fig. 7).

A similar comparison of the new method and the acidification method under varying conditions of phytin *a* contamination is given in Fig. 8. Here, both methods work well in discriminating against pheopigments; the assay for Chl *a* is within 15% of the true Chl *a* concentration in both techniques.

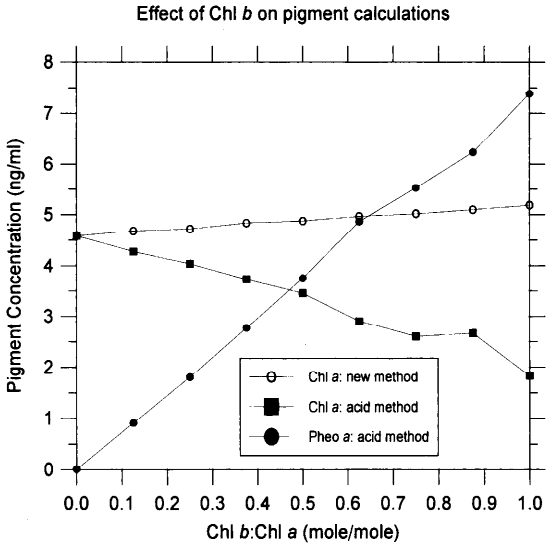

Fig. 7. Calculated Chl *a* concentrations made under increasing concentrations of Chl *b*. The true Chl *a* concentration was the same in all treatments (e.g. 4.8 ng ml^{-1} solvent). Acidification measurements were made on a Turner 112 with daylight lamp (F4T5D) and 5-60/2-64 filters; a Turner Designs model 10AU fluorometer with F4T4½B2/436/680 lamp-filter combination was used for the single-step fluorescence measurements. Note that at the highest Chl *b*:Chl *a* ratio, Chl *a* is underestimated by 2.5-fold and false concentrations of pheopigment are calculated when the conventional acidification technique (solid symbols) it used. The new method yields Chl *a* concentrations with ~10% error at the worst case.

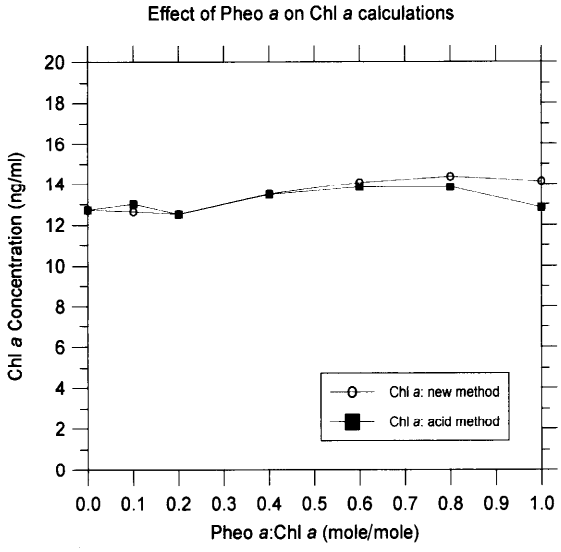

Fig. 8. Calculations of Chl *a* made under increasing pheophytic *a* concentrations. The true Chl *a* concentration (12.7 ng ml^{-1}) was held constant in all treatments. Acidification technique—■; new single-step Chl *a* method—○. Both methods work well in discriminating against phytin *a* interference.

The new method uses narrowbandwidth optical characteristics, which might create problems if excitation-emission spectra for Chl *a* shift as a function of solvent composition due, for instance, to variable water content (Seely and Jensen 1965; Holm-Hansen and Riemann 1978). However, no detectable change in fluorescence response could be measured for equimolar solutions of Chl *a* prepared in 80–100% acetone; the solvent composition was varied in 2% intervals for this test. Furthermore, since no acidification is required, the effects of final acid molarity and pH on spectral characteristics are not a concern in the present method (cf. Holm-Hansen and Riemann 1978).

The proposed method for Chl *a* analysis makes use of reasonably inexpensive optical modifications to filter fluorometers that may be readily available to researchers. There is some uncertainty about the future availability of the most sensitive lamp tested here (F4T4½B2); however, a blue lamp (type 9005) with identical spectral characteristics is now available from Turner Designs (No. 10-089). Other readily available lamps, such as the F4T4D daylight lamp, can provide similar selectivity with only about a 2-fold reduction in sensitivity. The new optical method, utilizing interference filters, yields a net sensitivity for Chl *a* which is about a fifth that of the original broadband filter method (cf. Holm-Hansen et al. 1965). However, this still provides more than enough sensitivity to assay small-volume samples (<200 ml) in any euphotic zone. As with all previous spectrophotometric and fluorometric assays, the new method will not discriminate chlorophyllide *a* from Chl *a* because the two pigments have identical spectral properties (Lorenzen and Newton Downs 1986). The unsettled issue of chlorophyllide *a* production from extraction artifacts will have to be resolved with chromatographic analyses.

The method proposed here for measuring Chl *a* is simple. For instruments with automatic digital sensitivity adjustments, such as the Turner Designs model 10AU, the method

reduces to a single-step measurement of F_o, with simple conversion to actual concentration units via a single calibration coefficient. Minimum effort is required for proper calibrations, and the required purity of the Chl *a* calibration solution is less stringent (i.e. there is some tolerance for the presence of accessory chlorophylls and pheopigments). Chl *a* standards, either as pure solutions or as pigment extracts from various natural sources (including green algae and higher plant tissue), can be used as calibration sources when referenced to spectrophotometric optical density measurements ($\epsilon = 87.67$ liters g^{-1} cm^{-1} at 664 nm for Chl *a*).

The new method provides no information on pheopigment concentrations or accessory chlorophylls. Arguably, these pigments are most accurately analyzed with the HPLC technique, where complete separations can be achieved. However, if a simple, sensitive method for determining Chl *a* (+ chlorophyllide *a*) is desired, the proposed modifications should be suitable. I anticipate that the technique will find wide acceptance in freshwater research, especially in oligotrophic systems, where a sensitive Chl *a* method free from Chl *b* interference has been needed for some time.

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