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Universal chlorophyll equations for estimating chlorophylls *a*, *b*, *c*, and *d* and total chlorophylls in natural assemblages of photosynthetic organisms using acetone, methanol, or ethanol solvents

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Abstract

A universal set of equations for determining chlorophyll (Chl) *a*, accessory Chl *b*, *c*, and *d*, and total Chl have been developed for 90 % acetone, 100 % methanol, and ethanol solvents suitable for estimating Chl in extracts from natural assemblages of algae. The presence of phaeophytin (Ph) *a* not only interferes with estimates of Chl *a* but also with Chl *b* and *c* determinations. The universal algorithms can hence be misleading if used on natural collections containing large amounts of Ph. The methanol algorithms are severely affected by the presence of Ph and so are not recommended. The algorithms were tested on representative mixtures of Chls prepared from extracts of algae with known Chl composition. The limits of detection (and inherent error, ±95 % confidence limit) for all the Chl equations were less than 0.03 g m^{-3}. The algorithms are both accurate and precise for Chl *a* and *d* but less accurate for Chl *b* and *c*. With caution the algorithms can be used to calculate a Chl profile of natural assemblages of algae. The relative error of measurements of Chls increases hyperbolically in diluted extracts. For safety reasons, efficient extraction of Chls and the convenience of being able to use polystyrene cuvettes, the algorithms for ethanol are recommended for routine assays of Chls in natural assemblages of aquatic plants.

Additional key words: *Acaryochloris*; algorithms; error structure; *Phaeodactylum*; phaeophytin; *Rhodomonas*; spectrophotometric determination; *Synechococcus.*

Introduction

I present a consistent set of universal algorithms for the routine assays of chlorophyll (Chl) *a*, *b*, *c*, *d* and total Chl contents in acetone, methanol, and ethanol. The algorithms are intended for use on natural assemblages of algae rather than uni-algal cultures which have a known Chl composition. Chl *a* (or sometimes total Chl) are routinely used as the bases for the calculation of photosynthetic and respiratory rates, the metabolically active biomass, and the productivity of terrestrial and aquatic ecosystems (Šesták 1971). The relative amounts of secondary Chls in environmental samples of phytoplankton and algal mats give an important insight into the types of photosynthetic organisms in an algal community (MacLulich 1986a,b, 1987, Jeffrey and Vesk 1997, Thompson *et al.* 1999, Murphy *et al.* 2005). The relative abundance of secondary Chls, particularly Chl *b* and the various types of Chl *c*, vary with both irradiance and the respective spectral quality (Tandeau de Marsac and Houmand 1988, Atwell *et al.* 1999).

Chl *c* refers here to all forms of chlorophyll *c* (c_1 and c_2) because in a solvent extract from a natural assemblage of algae it is not possible to distinguish between these various forms spectrophotometrically (Svec 1991). My previously published algorithms for estimation of Chls using different solvents can be used for more accurate estimates of Chls in uni-algal cultures (Ritchie 2006). The universal algorithms would also be useful for screening cultures for photosynthetic contaminants.

Acetone solvent gives very sharp Chl absorption peaks and so is the solvent of choice for Chl assays (see Arnon 1949, Šesták 1971, Jeffrey and Humphrey 1975, Jeffrey *et al.* 1997, Humphrey and Jeffrey 1997, Porra *et al.* 1989, Porra 1991, 2002, Wright *et al.* 1997, Ritchie 2006) but acetone is sometimes a poor extractant of Chl from many vascular plants and some algae, particularly green algae (see Scheer 1991 for extraction methods).

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Complete extraction of all Chls is a very important consideration in determining Chl content of natural assemblages of algae, for example phytoplankton samples, algal mats, and scrapings from substrates such as intertidal rocks, coral rock, sandstones, limestones, large kelps *etc*. particularly where the aim is to try and use the ratios of Chls as signatures of what classes of algae are present (MacLulich 1986a,b, 1987, Jeffrey and Vesk 1997, Thompson *et al.* 1999, Murphy *et al.* 2005). Methanol and ethanol are often more efficient extractants (see Scheer 1991, Ritchie 2006), are much easier to transport, and easier to handle in the field. Neutralised methanol and ethanol need to be used to avoid formation of phaeophytin (Ph) and allomerization products of Chls, which are spectrally different. Unfortunately, the Chl red peaks (Q_y) are generally broader and lower in methanol and ethanol. The peaks for Chl *b*, Chl c_1+c_2, and Chl c_2 and Chl *d* are not only lower and broader in methanol and ethanol: the widened peak of Chl *a* in these solvents tends to interfere more strongly with the absorbance of the other Chls (Ritchie 2006).

Acetone can be an impractical solvent to use outside a research laboratory. Acetone is very volatile, highly flammable, causes headache, is narcotic in high concentrations, and is a skin irritant (erythema). Acetone is particularly unsuited for fieldwork because the combination of its flammability, propensity for leaking out of containers, volatility, and security concerns make it problematic to transport particularly by air. Plastic laboratory-ware is more suited to fieldwork but acetone attacks polystyrene and polymethylacrylates (PMMA) and many other types of plastic.

Methanol (particularly hot methanol at 60 °C) is a very efficient extractant for Chls, particularly from recalcitrant vascular plants and algae but it is an insidious and notoriously toxic solvent (Porra *et al.* 1989, Porra 1990, 1991, 2002, Thompson *et al.* 1999, Ritchie 2006). Methanol attacks some, but not all, types of plastic commonly used to make plastic laboratory ware.

Ethanol is a much safer solvent than either acetone or methanol and a full set of Chl equations are now available (Ritchie 2006). Although flammable it is not very toxic. Ethanol does not attack polystyrene and so polystyrene plastic spectrophotometer cuvettes can be used for Chl assays and polyethylene and polystyrene containers can be used to store and transport field extracts. Like methanol, hot ethanol (60 °C) is an efficient extractant of Chls even from very resistant material. There are considerable practical, safety, and economic advantages in using ethanol as the solvent for Chl assay.

The present study presents universal algorithms for determining Chls in the mentioned solvents. Blank-corrected absorbances measured at four wavelengths (quadrichroic) are used. Simpler trichroic algorithms for determining Chls in material not containing Chl *d* are also given. Some of the limitations of using the algorithms to profile the relative abundances of algae with different pigment compositions are assessed including the effects of the natural presence of Ph *a* in samples and inadvertent conversion of Chl *a* to Ph *a* during extraction and storage.

Materials and methods

Synechococcus R-2 (PCC 7942) originating from the Pasteur Culture Collection was used as an example of a cyanobacterium with only Chl *a*. It was grown in BG-11 medium (Allen 1973). English spinach (*Spinacia oleracea* L., Chenopodiaceae) was used as an example of a vascular plant with Chl *a* and *b*. Hydroponically-grown spinach was usually used fresh from a local supermarket and had a Chl *b/a* ratio of about 0.35 to 0.25, consistent with being grown in bright irradiance. The unusual chlorophyte, *Ostreobium quekettii* (Sammlung von Algenkulturen, Universität Gottingen, Germany) was included in the study because it has a Chl *b/a* ratio of about 1 and so could be used as a source of a Chl extract with a very high proportion of Chl *b*. The marine diatom, *Phaeodactylum* sp. (Sydney University Teaching Collection) was used as a source of Chl *a* with Chl c_1+c_2 as minor Chl pigments. *Rhodomonas* sp. N23 (Sydney University Teaching Collection) was used as the standard source of Chl *a* and c_2. *Acaryochloris marina* was a kind gift from Dr Min Chen (Sydney University); it is a marine oxyphotobacterium with Chl *d* as its major photosynthetic pigment with some Chl *a* (Miyashita *et al.* 1997, 2003, Akiyama *et al.* 2001, Kuhl *et al.* 2005). *Ostreobium*, *Phaeodactylum*, *Rhodomonas*, and *Acaryochloris* were mostly grown in enriched f-2 seawater (McLachlan 1973) as described previously (Ritchie 2006) but it was found in the later stages of the study that *Acaryochloris* and *Phaeodactylum* grew better in MBIC Medium No. 8 (MBIC 2006). *Synechococcus*, *Ostreobium*, *Phaeodactylum*, and *Rhodomonas* were grown on an orbital shaker (≈80 rpm) fitted with overhead fluorescent lights (*Sylvania Gro-Lux*). The irradiance was approximately 80 µmol(photon) m^{-2} s^{-1} (PAR, using a *Li-Cor* photon flux meter *LI-189*). *Acaryochloris* consistently grew better on the edge of the shaker where the irradiance was lower (≈40 µmol m^{-2} s^{-1}).

Laboratory procedures were performed in a naturally low-lighted laboratory with the fluorescent lights off. The normal lighting in the laboratory under such conditions was about 2 µmol m^{-2} s^{-1} (400–700 nm PAR) (*Li-Cor* quantum photometer *LI-189*). Exposure of Chl extracts to radiant energy was avoided.

Analytical grade acetone, methanol, and ethanol were from *Mallinckrodt Baker BV*, Deventer, The Netherlands. 99.5 % ethyl alcohol (*Chem-Supply*, Gillman, SA, Australia), denatured with 0.00066 % denatonium

benzoate, 0.0001 % fluorescein, and 0.25 % methyl isobutyl ketone, was free of spectroscopic contaminants in the visible range.

Commercial acetone and alcohols are often highly acidic leading to phaeophytinisation of Chls (Jeffrey 1981). 90 % acetone was made up using a saturated solution of magnesium carbonate hydroxide to remove any acid present. To ensure that 100 % methanol, 100 % ethanol, and denatured 99.5 % ethanol were acid-free, a small amount of magnesium carbonate was added, and then the suspension was clarified by filtration through filter paper. Solvents were kept at 4 °C.

Microalgae were collected by first centrifuging them at 3 000×*g* for 10 min, then re-suspending in deionised water, and pelleting a second time. After decanting and re-suspending the hard pellet, the pigments were extracted in a 1 : 1 : 1 mixture of neutralised 90 % acetone, 100 % methanol, and 100 % (99.5 %) ethanol, cleared by centrifugation, then stored at –20 °C (Ritchie 2006). All concentrated extracts were made up to about 6 cm^3 and stored in the dark in a freezer at –20 °C for no more than 7 d.

Extraction of Chl by soaking algae or vascular plants in solvents overnight was not employed because it provides an opportunity for chlorophyllase to convert Chls to chlorophyllides. Extraction using hot methanol or ethanol (60 °C) was not necessary for the algae used in the present study (see Šesták 1971, Porra 1991, and Svec 1991 for some extraction methods to use on difficult materials).

Spectrophotometric readings were made using a *Shimadzu UV-2550* UV-visible spectrophotometer using standard scanning settings and a 1 nm bandwidth and 1 nm sampling interval (Ritchie 2006). Quartz cuvettes were used unless otherwise stated. 50 mm^3 of pigment extract was diluted with assay solvent to make up to 1.0 cm^3 of assay mixture. Where mixtures of Chl extracts were being assayed it was ensured that the diluted sample was not contaminated with more than 6.7 % of foreign solvents. All Chl assays on the concentrated extracts were run in acetone, methanol, or ethanol so that direct cross-comparisons of Chl assays using the three solvent systems could be made.

Chls are converted to Phs by bleaching under strong irradiation and by dilute acid conditions during extraction and storage and also occur naturally, particularly in old algal blooms (Jeffrey 1981). Inadvertent conversion of Chls to Phs or the unsuspected presence of large amounts of Phs in natural collections is hence a common source of error in Chl determinations (Šesták 1971, Jeffrey 1981). Chl *a* was prepared using standard techniques from *Synechococcus* in 1 : 1 : 1 neutralised 90 % acetone/ 100 % methanol/100 % ethanol. Half the preparation was converted to Ph *a* by adding HCl to a final concentration of 10 mol m^{-3} HCl. Aliquots (n = 12) of the Chl *a* and Ph *a* preparations (50 mm^3 cm^{-3}) were scanned in the spectrophotometer at the appropriate wavelengths for assaying Chls in 90 % acetone, methanol, and ethanol (Ritchie 2006). The Ph *a* peaks were at 665 nm in acetone, 660 nm in methanol, and 666 nm in ethanol and so are very similar to its parent Chl *a*. In methanol the red peak was conspicuously shifted to 660 nm from 665 nm in the case of Chl *a*. In all three solvents the red peak of Ph *a* is broader and flatter than for Chl *a*. This flattening and broadening is most pronounced in methanol solvent. Hence, Ph *a* strongly interferes with determinations of all types of Chl but most severely in methanol solvent.

All error-bars are ±95 % confidence limits (CL) with the number of replicates in brackets. All Chl algorithms have been worked out for 1-cm light path cuvettes. Absorbance readings have dimensions A cm^{-1} and hence the absorbance coefficients have dimensions g m^{-3} cm A^{-1}.

Spectrophotometry theory

Arnon (1949), French (1960), Šesták (1971), Porra (1991, 2002), and Jeffrey and Welschmeyer (1997) give general outlines of the simultaneous equation approach to estimating separately the Chl in mixtures. It is customary to zero spectrophotometers at 750 nm to correct for turbidity and contaminating coloured compounds.

Algorithms for resolving two Chls in a mixture have the general form, $Z = Ax + By$ (Ritchie 2006). More complex algorithms using measurements at three wavelengths (trichroic equations) have been developed. The trichroic SCOR-UNESCO Publ. Chl equations used inaccurate extinction coefficients (see Porra 1991, Jeffrey *et al.* 1997). The trichroic equations developed by Jeffrey and Humphrey (1975) used more accurate extinction coefficients and have been used to determine Chl *a*, *b*, and *c* (c_1+c_2) in phytoplankton collections, mats of algae, scrapings from rocky substrates (MacLulich 1986a,b, 1987, Jeffrey and Vesk 1997, Thompson *et al.* 1999, Murphy *et al.* 2005), and the Chl composition of *Prochloron* which contains Chl *a*, *b*, and Mg-DVP (Larkum *et al.* 1994). Jeffrey and Humphrey (1975) did not publish estimates of the errors of their absorbance coefficients; here they have been assumed to be about ±0.5 % and the errors of their algorithms have been calculated as described in the Appendix (Ritchie 2006).

In general, algorithms resolving two or more types of Chl have a positive absorbance coefficient at the red peak for the Chl in question and the absorbance coefficients at the other wavelengths are negative, however, extinction coefficients are always positive. The trichroic algorithm for Chl *c* (more accurately c_1+c_2) has inherent limitations because the respective coefficients are slightly different (Jeffrey and Humphrey 1975) and the extinction coefficient of Mg-VP is not well documented (Jeffrey *et al.* 1997). Tests of this set of equations with mixtures of pure Chls show that they give good estimates of Chl *a* and *b* but assays of Chl *c* are inherently inaccurate (Svec 1991, Humphrey and Jeffrey 1997, Jeffrey and Welschmeyer 1997). Hence Chl *c/a* ratios calculated from such equations need to be interpreted with caution.

With the single known exception of *A. marina*, Chl *a* is the predominant Chl in oxygenic photosynthetic organisms. Absorption by Chl *a* over most of the red part of the spectrum interferes with determinations of Chl *b* and *c* (Mg-DVP, c_1+c_2, and c_2) and natural assemblages containing Chl *d*. Hence, algorithms to determine Chl *a* are usually both accurate and precise but the equations for the other Chls will be less reliable (Ritchie 2006).

Spectrophotometric assays of Chl *c* compounds present particular problems. The red peak (Q_y) for Chl *c* (Mg-DVP, c_1 and c_2, and related Chls) is much lower than for equimolar amounts of Chl *a* (Jeffrey *et al.* 1997). Furthermore, Chl *c* compounds normally represent less than 20 % of the total Chl of algal cells containing Chl *c* compounds and hence they are difficult to assay in Chl mixtures. Absorption by Chl *a* (and Ph *a*) at the red peak for Chl *c* compounds tends to drown out the signal from Chl *c* (Jeffrey and Humphrey 1975). In natural assemblages of algae, any significant Chl *b* present will also interfere with determinations of Chl *c*. Formulae to calculate Chl *c* in extracts where the proportions of Chl c_1, c_2, and Mg-DVP are not known have an inherently limited accuracy, particularly if only small amounts of Chl *c* are present (Svec 1991, Humphrey and Jeffrey 1997, Jeffrey and Welschmeyer 1997).

Chl *d* was originally described as an accessory Chl in extracts from rhodophyte algae (Manning and Strain 1943). Chl *a* and *d* algorithms have recently been published (Ritchie 2006). *Acaryochloris* contains very little Chl *a*, about 95 % or more of its total Chl is Chl *d* (Miyashita *et al.* 1996, 1997). In *Acaryochloris* it is difficult to estimate Chl *a* spectrophotometrically because the absorption of Chl *d* obscures the contribution of Chl *a*.

Chl spectrophotometric algorithms for mixtures of Chls have an inherent limitation. The more complex the algorithm, the more difficult it is to fit to a data set and the larger the inherent error (Appendix A). Hence, the least complex algorithm, consistent with a good fit to the data, should be adopted. For uni-algal cultures with known pigment composition the recently published dichroic (two-wavelength) algorithms would be appropriate (Ritchie 2006); for mixed algal populations where there is no evidence for the presence of Chl *d* a trichroic algorithm to determine Chl *a*, *b*, and *c* would appear to be appropriate but for material of unknown Chl composition quadrichroic algorithms should be used. The error is a constant, independent of the magnitude of the absorbance readings but a consequence of this is that the relative error increases as the abundance of a Chl decreases (Ritchie 2006).

Algorithms for determination of all the Chls in mixtures (from *Synechococcus*), Chl *a* and *b* (from spinach and *Ostreobium*), Chl *a* and c_2 (*Rhodomonas*), and Chl *a* and (c_1+c_2) (*Phaeodactylum*) were determined using non linear least squares fitting methods (Johnson and Faunt 1992, Straume and Johnson 1992) using the *SOLVER* software tool of *Microsoft EXCEL X* for Mac. In each algorithm, the absorbance coefficient at the red absorbance peak (Soret band) for the Chl in question was constrained to be positive, the others were unconstrained. Estimates of the Chls in preparations from the above organisms with known pigment composition in acetone, methanol, and ethanol were determined using the dichroic (two wavelength) equations presented by Ritchie (2006). The entire set of Chl determinations in acetone was then used to determine least squares fitted algorithms (trichroic and quadrichroic, 3 or 4 wavelengths) of the form Z = Av + Bw + Cx and Z = Av + Bw + Cx + Dy, respectively. Sets of Chl trichroic and quadrichroic equations for acetone, methanol, and ethanol determined in this way should yield estimates of Chl content of a concentrated extract that are similar to each other.

Mean square residuals (MSR) were then calculated for the fits. Sums of the squares (SS) of the absorbance readings were used to set up 3 X 3, or 4 X 4 matrices [M]. The matrix inversion software of *EXCEL* was used to invert these matrices ($[M]^{-1}$) to obtain estimates of the variances (var) associated with each of the fitted estimates of the absorbance coefficients, $E_{\lambda 1}$, $E_{\lambda 2}$, $E_{\lambda 3}$, $E_{\lambda 4}$ (see Appendix B). The ±95 % confidence limits of the coefficients could then be calculated. Using the calculation of the error of coefficient $A_{\lambda 1}$ as a worked example,

$$\Delta E_{\lambda 1} \approx t_{0.05,df,2} \sqrt{\frac{MSR.varE_{\lambda 1}}{n}} \qquad (1)$$

where $\Delta E_{\lambda 1}$ is the asymptotic error of the absorbance coefficient (E) for absorbance readings at wavelength (λ1), *t* is Students *t* for *p*=0.05, with degrees of freedom (df) determined by the number of sets of spectrophotometer readings (n) minus the number of absorbance readings used for the algorithm (3 or 4), two-tailed, MSR is the mean square residual of the fit $(\frac{SS}{df})$, $varE_{\lambda 1}$ is the variance estimate from the inverse matrix for the absorbance coefficient $E_{\lambda 1}$.

Results

Table 1 shows the algorithms for assay of Chl *a*, *b*, *c*, and *d* in acetone, methanol, and ethanol. The ±95 % confidence limits for each absorbance coefficient were calculated as described in the Theory and Appendix B. The inherent error of each spectrophotometric equation was then calculated as described in Appendix A.

In the development of the algorithms for 90 % acetone, 100 % methanol, and 100 % ethanol, 336 sets of spectrophotometric readings were made on Chl *a* from *Synechococcus*, 336 sets containing Chl *a*+*b* (from spinach and *Ostreobium*), 336 sets containing Chl *a*+c_2 (from *Rhodomonas*), 404 sets containing Chl *a*+Chl (c_1+c_2)

(*Phaeodactylum*), and 336 sets on Chl *a*+*d* extracted from *Acaryochloris*. The algorithms are therefore based on a data set of 1 748 sets of spectrophotometric readings. Measurements were usually made in sets of 12 or 4 on any single solvent extract. For example, the 336 spectrophotometric scans of Chl *a* from *Synechococcus* are based upon a total of 60 solvent extracts. Seven Chl extracts were used to prepare sets of 12 replicate diluted samples and 48 were used to prepare sets of 4 diluted Chl extracts.

Table 1. Absorbance coefficients (E_λ) for spectrophotometric equations for chlorophylls *a*, *b*, *c*, and *d* in 90 % acetone (Ac), methanol (Me), and ethanol (Et). The absorbance coefficients have the dimensions g m^{-3} cm A^{-1} because the absorbance readings have units of A cm^{-1}. The errors of the absorbance coefficients were calculated as described in the Theory (Eq. 4) and Appendix B. The total errors of the algorithms were calculated as described in Appendix A. The last column gives detection limit.

	Chlorophyll	$E_{630\ nm}$	$E_{647\ nm}$	$E_{664\ nm}$	$E_{691\ nm}$	±95 % CL
90 % Ac	Chl *a*	–0.3319±0.0019	–1.7485±0.0005	11.9442±0.0003	–1.4306±0.0002	0.00200
	Chl *b*	–1.2825±0.0072	19.8839±0.0021	–4.8860±0.0012	–2.3416±0.0007	0.00759
	Chl *c* (all forms)	23.5902±0.0070	–7.8516±0.0020	–1.5214±0.0012	–1.7443±0.0007	0.00745
	Chl *d*	–0.5881±0.0029	0.0902±0.0008	–0.1564±0.0005	11.0473±0.0003	0.00304
	Total Chl	21.3877±0.0053	10.3739±0.0015	5.3805±0.0009	5.5309±0.0005	0.00560
		$E_{632\ nm}$	$E_{652\ nm}$	$E_{665\ nm}$	$E_{696\ nm}$	
Me	Chl *a*	–2.0780±0.0065	–6.5079±0.0021	16.2127±0.0013	–2.1372±0.0006	0.00700
	Chl *b*	–2.9450±0.0197	32.1228±0.0064	–13.8255±0.0040	–3.0097±0.0018	0.02120
	Chl *c* (all forms)	34.0115±0.0112	–12.7873±0.0036	–1.4489±0.0023	–2.5812±0.0010	0.01200
	Chl *d*	–0.3411±0.0028	0.1129±0.0009	–0.2538±0.0006	12.9508±0.0003	0.00306
	Total Chl	28.6473±0.0130	12.9405±0.0042	0.6845±0.0026	5.2230±0.0012	0.0140
		$E_{632\ nm}$	$E_{649\ nm}$	$E_{665\ nm}$	$E_{696\ nm}$	
Et	Chl *a*	0.0604±0.0050	–4.5224±0.0015	13.2969±0.0009	–1.7453±0.0004	0.00530
	Chl *b*	–4.1982±0.0134	25.7205±0.0040	–7.4096±0.0023	–2.7418±0.0011	0.01420
	Chl *c* (all forms)	28.4593±0.0080	–9.9944±0.0023	–1.9344±0.0014	–1.8093±0.0007	0.00843
	Chl *d*	–0.2007±0.0022	0.0848±0.0006	–0.1909±0.0004	12.1302±0.0002	0.00234
	Total Chl	24.1209±0.0128	11.2884±0.0038	3.7620±0.0022	5.8338±0.0011	0.01350

Taking the values from Table 1, the quadrichroic equations for Chl *a* and *b* [g m^{-3}] in 90 % acetone would be (Eqs. 2a–e):

$$\text{Chl } a = -0.3319\ A_{630} - 1.7485\ A_{647} + 11.9442\ A_{664} - 1.4306\ A_{691}\ (\pm 0.0020) \quad (2a)$$

$$\text{Chl } b = -1.2825\ A_{630} - 19.8839\ A_{647} - 4.8860\ A_{664} - 2.3416\ A_{691}\ (\pm 0.0076) \quad (2b)$$

$$\text{Chl } c = 23.5902\ A_{630} - 7.8516\ A_{647} - 1.5214\ A_{664} - 1.7443\ A_{691}\ (\pm 0.0075) \quad (2c)$$

$$\text{Chl } d = -0.5881\ A_{630} + 0.0902\ A_{647} - 0.1564\ A_{664} - 11.0473\ A_{691}\ (\pm 0.0030) \quad (2d)$$

$$\text{Total Chl} = 21.3877\ A_{630} + 10.3739\ A_{647} + 5.3805\ A_{664} + 5.5309\ A_{691}\ (\pm 0.0056) \quad (2e)$$

The inherent errors of the spectrophotometric equations for all the Chls and the total Chl are all less than 0.01 g m^{-3} (μg cm^{-3}). These errors can be taken as the lower detection limits for both Chls in solvent extracts containing a mixture of Chls from various organisms. Equations for all the Chls in the different solvents can be written out from the data in Table 1 as for Eqs. 2a–e.

Trichroic equations for systems containing no Chl *d* were calculated by eliminating the *Acaryochloris* data from the data set leaving a total of 1 412 sets of spectrophotometric readings for 90 % acetone, 100 % methanol, and 100 % ethanol. Trichroic formulae of the form Z = Ax + By + Cv were fitted using *SOLVER* in *Excel* and the errors of the fitted absorbance coefficients calculated from solving a 3X3 matrix (Table 2a–c). Note that the resulting trichroic algorithms are not simply truncated forms of the four-term algorithms.

Table 3 presents some Chl assays (n = 12) on extracts from *Synechococcus* (Chl *a* only), spinach (Chl *a* and *b*), *Rhodomonas* (Chl *a* and c_2), *Phaeodactylum* [Chl *a* and (c_1+c_2)], and *Acaryochloris* (Chl *a* and *d*) using the algorithms developed in the present study compared to previously published formulae. Chls were assayed using the dichroic formulae previously published (Ritchie 2006), in the case of 90 % acetone solvent, the trichroic formulae of Jeffrey and Humphrey (1975), the trichroic formulae set out in Table 2, and the quadrichroic formulae set out in Table 1.

Table 3 shows that all the formulae give very similar assays for Chl *a* where it is the only Chl present in 90 % acetone, methanol, and ethanol. If dichroic, trichroic, and quadrichroic algorithms for Chls were perfectly accurate they would give a zero value for Chls *b*, *c*, and *d* in *Synechococcus*. The trichroic and quadrichroic formulae all gave small spurious values for the other Chls.

Table 2. Absorbance coefficients (E_λ) for spectrophotometric equations for chlorophylls *a*, *b*, and *c*. The absorbance coefficients have the dimensions g m^{-3} cm A^{-1} because the absorbance readings have units of A cm^{-1}. The errors of the absorbance coefficients were calculated as described in the Theory (Eq. 4) and Appendix B. The total errors of the algorithms were calculated as described in Appendix A.

	Chlorophyll	$E_{630\ nm}$	$E_{647\ nm}$	$E_{664\ nm}$	±95 % CL
90 % acetone	Chl *a*	–0.3002±0.0008	–1.7538±0.0002	11.9092± 0.0001	0.0009
	Chl *b*	–1.2942±0.0089	19.8952±0.0026	–4.9401± 0.0015	0.0094
	Chl *c* (all forms)	23.6723±0.0074	–7.9057±0.0021	–1.5467± 0.0013	0.0079
	Total Chl	22.0780±0.0067	10.2357±0.0019	5.4224± 0.0011	0.0071
		$E_{632\ nm}$	$E_{652\ nm}$	$E_{665\ nm}$	
Methanol	Chl *a*	–3.2416±0.0081	–6.4151±0.0026	16.4351± 0.0017	0.0087
	Chl *b*	–3.0228±0.0264	32.1478±0.0079	–13.8844±0.0050	0.0261
	Chl *c* (all forms)	34.2247±0.0129	–12.8087±0.0042	–1.5492± 0.0026	0.0139
	Total Chl	27.9603±0.0154	12.9241±0.0050	1.0015± 0.0031	0.0165
		$E_{632\ nm}$	$E_{649\ nm}$	$E_{665\ nm}$	
Ethanol	Chl *a*	–0.9394±0.0085	–4.2774±0.0025	13.3914± 0.0015	0.0090
	Chl *b*	–4.0937±0.0162	25.6865±0.0048	–7.3430± 0.0029	0.0171
	Chl *c* (all forms)	28.5073±0.0091	–9.9940±0.0027	–1.9749± 0.0016	0.0096
	Total Chl	23.4742±0.0166	11.4096±0.0049	4.0735± 0.0029	0.0175

Table 3 presents the results on a Chl extract from spinach (Chl *a* and *b*). Assays for Chl *a* and Chl *b* were all similar using the various formulae in 90 % acetone, methanol, and ethanol, however, the apparent Chl *b* content according to the methanol and ethanol formulae were consistently slightly lower than in the case of the 90 % acetone formulae. All the trichroic and quadrichroic formulae gave an apparent Chl *c* content near zero.

Table 3 presents the Chl assay results on Chls extracted from *Rhodomonas* (Chl *a* and c_2). The Chl *a* assay using all the formulae in the three different solvent systems are very similar. The extinction coefficients of Chl c_1 and c_2 are slightly different and so it would be expected that generalised Chl *c* formulae would have inherent inaccuracy. Table 3 shows that the dichroic formulae worked out specifically for algae known to have only Chl c_2 give comparable estimates of Chl *c*. The trichroic formulae of Jeffrey and Humphrey (1975) gave estimates of Chl *c* comparable to the dichroic formulae and a Chl *b* value near to zero but with a large inherent error. Unfortunately, the trichroic and quadrichroic formulae developed in this study produced high spurious Chl *b* values for old *Rhodomonas* cultures.

Table 3 shows the Chl assay results for Chl extracted from a diatom (*Phaeodactylum*, Chl *a* and c_1+c_2). The dichroic formulae gave similar estimates of Chl *a* and c_1+c_2 in the three solvents. As in the case of the results shown in Table 3, all the formulae gave similar estimates of Chl *a*. The Jeffrey-Humphrey trichroic formulae gave a good estimate for Chl *c* but a large spurious negative value for Chl *b*. The trichroic and quadrichroic formulae developed in the present study all gave similar Chl *c* values for the diatom and small spurious values for other Chls.

Chl assays *Acaryochloris* are shown in Table 3. The *Acaryochloris* material used for this test of the Chl formulae had been grown under low irradiance (<10 µmol m^{-2} s^{-1}, PAR) and so had a very low Chl *a* content (Ritchie 2006). The dichroic formulae all gave similar estimates of Chl *a* and Chl *d* in the three solvents. The trichroic formulae all gave incorrect high estimates of Chl *a*, *b*, and *c* and so the trichroic formulae cannot even be used to estimate Chl *a* in a Chl extract containing large amounts of Chl *d*. The quadrichroic formulae on the other hand all gave good estimates of Chl *a* and Chl *d* and estimates of Chl *b* and *c* that were close to zero.

Table 4 compares the performance of Chl formulae on Chl *a* from *Synechococcus* to a preparation converted to Ph *a*. These results are similar to those shown in Table 3. Conversion to Ph *a* led to an underestimation of Chl *a* of about 39±0.6 %, 52±0.9 %, and 36±0.5 % in acetone, methanol, and ethanol, respectively (Table 4). Hence, the most severe errors were where methanol was used. This was due to a combination of flattening and spectral shift in the red peak of Ph *a* compared to Chl *a*.

Application of the Chl formulae to the Ph *a* preparation gives consistently low estimates for Chl *a* (Table 4). As in Table 4, the trichroic and quadrichroic formulae for acetone and ethanol give spurious but small negative estimates of Chl *b*, *c*, and *d* but the apparent Chl *a* values are consistent with each other regardless of the Chl formulae used. Phaeophytinisation has a very severe effect on Chl estimates in methanol solvent. Not only does methanol solvent give the lowest estimate of Chl *a* but the large spectral shift leads to large spurious Chl *b* values (Table 4). There was also a large negative Chl *c* estimate (Table 4). Thus phaeophytinisation not only causes underestimation of Chl *a* content in all three solvents but Ph *a* interferes with estimates of Chl *b* and *c*. The worst effects of phaeophytinisation are seen in

methanol solvent. These spurious values would lead to overestimation of Chl *b* and underestimation of Chl *c* in Chl extracts from material containing a mixture of Chls contaminated with Ph *a*.

Discussion

As pointed out in the Theory section, multiple linear equations for Chls have the inherent limitation that the inherent errors of Chls are constant, independent of the magnitude of the absorbance readings. The more dilute the Chl solution the greater the relative error: this effect is most pronounced for the accessory Chl *b* and *c*. Tables 3 and 4 show that the relative errors of minor Chls are always much larger than for Chl *a* in typical oxygenic phototrophs. *Acaryochloris* is an exception: Chl *d* is the major pigment in *Acaryochloris* and so its relative error is small compared to that of Chl *a*. The statistical errors for Chls using the quadrichroic equations are greater than using the trichroic equations because they are less complex algorithms. However, use of these equations on Chl extracts of known composition show that unsuspected presence of Chl *d* renders the Chl *a*+*b*+*c* algorithms misleading and incorrect for Chl assay for all of these Chl not just Chl *a* (Table 3). Ph *a* severely interferes with all Chl determinations (Šesták 1971) but most severely interferes with Chl assays in methanol.

In general, my trichroic and quadrichroic spectrophotometric formulae give excellent estimates of Chl *a* in solvent extracts containing a mixture of Chls (Tables 3 and 4) but the trichroic equations must not be used on inappropriate material. One reason why the algorithms for methanol are less satisfactory than those developed for acetone and ethanol is because they are much more sensitive to the presence of Ph *a* (Table 4). This is caused by the large spectral shift towards the Chl *b* absorption peak of the red absorbance peak of Ph *a* compared to Chl *a* when measured in methanol. Gross errors can be made in assays of Chl *a* where a trichroic formula (the equation of Jeffrey and Humphrey 1975 or the algorithm set out in Table 2) is used to assay Chl *a* in extracts where large amounts of Ph *a* are present. The other circumstance where misleading errors can be made in estimates of Chl *a* are where a trichroic formula is used to assay Chl *a* in a mixture containing large amounts of Chl *d* (see Table 3) and where there is very little Chl *b* and Chl c_1+c_2 and a preponderance of Chl *a* (Jeffrey and Humphrey 1975).

Chl *b* only occurs in archegoniophytes (land plants), chlorophytes, and *Prochloron*. No Chl *b* should have been found using the trichroic and quadrichroic formulae on Chl extracts from *Synechococcus*, *Rhodomonas*, *Phaeodactylum*, or *Acaryochloris.* Chl *b* should have only been found in extracts from spinach. Table 3 shows that the quadrichroic equations gave small spurious Chl *b* values near zero in extracts from most of the organisms known to contain no Chl *b*. Use of the quadrichroic equations on extracts from *Rhodomonas* (Table 3) were less satisfactory, giving a spurious estimate of Chl *b* using the methanol equations.

The trichroic equations for acetone (Jeffrey and Humphrey 1975), methanol and ethanol (Table 2) all gave good estimates of Chl *b* and very small spurious Chl *c* values in spinach (Table 3). In *Synechococcus* and *Phaeodactylum* the Jeffrey-Humphrey formulae give a spurious but small negative value for Chl *b* (Table 3). The trichroic equations were less satisfactory for *Rhodomonas*: the Jeffrey-Humphrey equations for acetone gave a spurious negative Chl *b* value but the algorithms worked out in the present study gave values nearer to zero (Table 3). The presence of Chl *d* in a Chl extract (*Acaryochloris*, Table 3) results in the trichroic algorithms giving a false high estimate of Chl *b*, particularly where the algorithms for methanol are used.

Table 4 shows that phaeophytinisation is also a major source of error in estimations of Chl *b* in methanol solvent, giving spurious high values using both the trichroic and quadrichroic algorithms. Spurious high Chl *b* estimates were found in old cultures of *Rhodomonas* using both trichroic and quadrichroic equations. The effect was most severe for the methanol equations. This leads to the conclusion that these spurious Chl *b* values are the result of large amounts of Ph *a* being present in *Rhodomonas* cells that are no longer growing exponentially.

Jeffrey and Humphrey (1975) pointed out in their original paper that their equations for Chl c_2, Chl c_1+c_2 and their trichroic algorithm had increasing error as the abundance of the Chl *c* compounds decreased with reference to Chl *a*. Humphrey and Jeffrey (1997) tested their Chl equations, originally published in Jeffrey and Humphrey (1975), on a wide range of mixtures of chromatographically pure Chls. They confirmed that the Chl *a* and *b* equations were highly accurate for both Chl *a* and *b* but the Chl *c* formula overestimated the Chl *c* compounds when their abundances were low. Similar conclusions were drawn from comparisons of HPLC and spectroscopic studies on Chl *a*, *b*, c_2, and c_1+c_2 mixtures by Jeffrey and Wright (1997) and Mantoura *et al.* (1997). Unfortunately, one of the major aims in using these equations is to use the Chl *c* value as an index of the proportion of Chl *c*-containing organisms in a natural collection of algae (MacLulich 1986a,b, 1987, Tandeau de Marsac and Houmand 1988, Jeffrey and Vesk 1997, Murphy *et al.* 2005).

The results of the tests of the trichroic and quadrichroic algorithms for Chl *c* in the present study confirm the conclusions of Jeffrey and Humphrey (1997) and Mantoura *et al.* (1997) that spectrophotometric determinations of Chl *c* compounds need to be treated

Table 3. Comparison of chlorophyll (Chl) assay [g m^{-3}] algorithms used in analysis of *Synechococcus* (Chl *a* only), *Spinacia* (Chl *a*+*b*), *Rhodomonas* (Chl $a+c_2$), *Phaeodactylum* (Chl $a+c_1+c_2$), and *Acaryochloris* (Chl *a*+*d*). The error-bars are ±95 % confidence limits that include the error calculated between replicate samples (n = 12) and the inherent errors of the chlorophyll algorithms (Tables 1 and 2, see Appendix A). Sources of algorithms: R6 = Ritchie (2006), R8 = present study, JH = Jeffrey and Humphrey (1975).

		Reference source of Chl algorithm	Chl *a*	Chl *b*	Chl *c*	Chl *d*	ΣChl
Synechococcus	Ac	R6 – Chl *a*	3.430±0.009	–	–	–	3.430±0.011
		JH – trichroic formulae	3.444±0.060	–0.185±0.110	0.079±0.129	–	3.337±0.128
		Chl *abc* – R8, Table 2	3.436±0.009	–0.059±0.015	0.053±0.012	–	3.430±0.021
		Chl *abcd* – R8, Table 1	3.435±0.010	–0.060±0.013	0.048± 0.011	0.011±0.006	3.435±0.023
	Me	R6 – Chl *a*	3.461±0.023	–	–	–	3.461±0.023
		Chl *abc* – R8, Table 2	3.471±0.024	–0.174±0.038	0.038±0.021	–	3.347±0.038
		Chl *abcd* – R8, Table 1	3.453±0.029	–0.176±0.023	0.039±0.015	0.008±0.007	3.324±0.046
	Et	R6 – Chl *a*	3.388±0.015	–	–	–	3.388±0.015
		Chl *ab* – R8, Table 2	3.392±0.063	–0.112±0.048	0.046±0.055	–	3.363±0.092
		Chl *abcd* – R8, Table 1	3.387±0.018	–0.111±0.018	0.047±0.015	0.003±0.008	3.326±0.043
Spinacia	Ac	R6	4.433±0.056	1.519±0.021	–	–	5.952±0.074
		JH – trichroic formulae	4.467±0.082	1.417±0.110	0.099±0.129	–	5.983±0.147
		Chl *abc* – R8, Table 2	4.433±0.056	1.527±0.021	0.019±0.010	–	5.978±0.076
		Chl *abcd* – R8, Table 1	4.431±0.057	1.525±0.020	0.015±0.009	0.007±0.004	5.978±0.077
	Me	R6 – Chl *a*	4.282±0.029	1.320±0.012	–	–	5.602±0.037
		Chl *abc* – R8, Table 2	4.298±0.031	1.334±0.039	–0.039±0.022	–	5.593±0.048
		Chl *abcd* – R8, Table 1	4.291±0.040	1.336±0.026	–0.036±0.017	–0.010±0.005	5.582±0.059
	Et	R6 – Chl *a*	4.339±0.047	1.362±0.029	–	–	5.702±0.062
		Chl *abc* – R8, Table 2	4.351±0.035	1.390±0.062	–0.026±0.050	–	5.757±0.120
		Chl *abcd* – R8, Table 1	4.356±0.046	1.393±0.022	–0.031±0.014	–0.018±0.006	5.700±0.065
Rhodomonas	Ac	R6 – Chl *a*	4.586±0.035	–	1.243±0.017	–	5.829±0.048
		JH – trichroic formulae	4.623±0.069	–0.278±0.109	1.304±0.130	–	5.649±0.135
		Chl *abc* – R8, Table 2	4.599±0.035	–0.045±0.012	1.226±0.015	–	5.780±0.050
		Chl *abcd* – R8, Table 1	4.596±0.036	–0.047±0.011	1.215±0.015	–0.001±0.004	5.755±0.051
	Me	R6 – *Chl a*	4.627±0.036	–	1.104±0.009	–	5.730±0.041
		Chl *abc* – R8, Table 2	4.556±0.045	0.106±0.057	1.021±0.027	–	5.683±0.037
		Chl *abcd* – R8, Table 1	4.570±0.061	0.107±0.063	1.017±0.029	–0.007±0.004	5.688±0.042
	Et	R6 – Chl *a*	4.667±0.040	–	0.974±0.017	–	5.641±0.046
		Chl *abc* – R8, Table 2	4.706±0.111	0.006±0.085	0.883±0.063	–	5.619±0.113
		Chl *abcd* – R8, Table 1	4.685±0.056	–0.002±0.034	0.911±0.020	–0.010±0.004	5.584±0.058
Phaeodactylum	Ac	R6	3.636±0.016	–	0.406±0.011	–	4.041±0.019
		JH – trichroic formulae	3.651±0.062	–0.208±0.109	0.464±0.129	–	3.907±0.127
		Chl *abc* – R8, Table 2	3.638±0.016	–0.053±0.011	0.423±0.009	–	4.008±0.019
		Chl *abcd* – R8, Table 1	3.638±0.017	–0.052±0.009	0.419±0.009	–0.008±0.004	3.996±0.019
	Me	R6 – Chl *a*	3.399±0.027	–	0.368±0.017	–	3.767±0.041
		Chl *abc* – R8, Table 2	3.421±0.028	–0.037±0.041	0.380±0.024	–	3.763±0.051
		Chl *abcd* – R8, Table 1	3.419±0.035	–0.034±0.031	0.381±0.022	–0.012±0.007	3.754±0.066
	Et	R6 – Chl *a*	3.489±0.082	–	0.300±0.014	–	3.789±0.028
		Chl *abc* – R8, Table 2	3.492±0.068	–0.054±0.053	0.327±0.053	–	3.794±0.096
		Chl *abcd* – R8, Table 1	3.489±0.021	–0.053±0.018	0.332±0.015	–0.010±0.008	3.757±0.047
Acaryochloris	Ac	R6	0.285±0.006	–	–	8.820±0.062	9.859±0.071
		JH – trichroic formulae	1.478±0.061	1.802±0.110	1.456±0.130	–	4.735±0.132
		Chl *abc* – R8, Table 2	1.433±0.012	1.869±0.019	1.337±0.014	–	4.638±0.039
		Chl *abcd* – R8, Table 1	0.282±0.006	–0.010±0.010	–0.066±0.009	8.824±0.062	9.030±0.070
	Me	R6 – *Chl a*	0.275±0.006	–	–	8.961±0.049	9.236±0.048
		Chl *abc* – R8, Table 2	1.705±0.014	2.017±0.038	1.748±0.024	–	5.471±0.037
		Chl *abcd* – R8, Table 1	0.296±0.008	–0.065±0.024	–0.055±0.019	8.961±0.060	9.137±0.062
	Et	R6 – Chl *a*	0.289±0.011	–	–	8.798±0.060	9.087±0.066
		Chl *abc* – R8, Table 2	1.516±0.057	1.954±0.070	1.315±0.071	–	4.785±0.112
		Chl *abcd* – R8, Table 1	0.301±0.010	–0.062±0.019	–0.009±0.020	8.798±0.060	9.036±0.082

with caution. The trichroic formulae and quadrichroic formulae for acetone, methanol, and ethanol give apparent Chl *c* contents in *Synechococcus* (Chl *a* only) and spinach (Chl *a*+*b*) which are near to zero. As in the case for the Chl *b* formulae, the trichroic formulae, if used on an organism containing Chl *d*, give a spurious very high apparent Chl *c* content (Table 3). Comparison in Table 4 shows that Ph *a* gives a spurious very negative value for Chl *c* using the trichroic formulae, particularly if the methanol formula is used. Thus, Chl extracts unknowingly containing large amounts of Chl *d* will result in a severe overestimation of Chl *c* in a sample whereas substantial amounts of Ph *a* will mask the presence of Chl *c* giving a false low value. The trichroic formulae for acetone and ethanol are superior to the trichroic formulae for methanol which are severely affected by the presence of Chl *d* (Table 3) or Ph *a* (Table 4). The finding, that Chl *d* can lead to very serious errors in assays of Chl *a*, *b*, and *c*, has not been reported before. It is therefore important to check for the possible presence of Chl *d* by looking for its absorption peak in the far-red region of the spectrum in Chl extracts from environmental samples such as scrapings from rocks, algae from dark habitats and unusual environments. If the quadrichroic formulae indicate that substantial amounts of Chl *d* are present the trichroic algorithms should not be used. As pointed out by Ritchie (2006) the past habit of ignoring Chl *d* as merely an artefact of extraction of Chl from algae means that it is likely that Chl *d* containing organisms are more widespread than presently suspected (Murakami *et al*. 2004, Miller *et al*. 2005).

Table 4. Effect of phaeophytinisation [g m^{-3}]. The error-bars are ±95 % confidence limits that include the error calculated between replicate samples (n = 12) and the inherent errors of the chlorophyll (Chl) algorithms (Tables 1 and 2, see Appendix A). For abbreviations see Table 3.

	Solvent	Reference source of Chl algorithm	Chl *a*	Chl *b*	Chl *c*	Chl *d*	ΣChl
Unmodified	Acetone	R6 – Chl *a*	4.841±0.042	–	–	–	4.841±0.042
		JH – trichroic formulae	4.862±0.073	–0.263±0.109	0.092±0.129	–	4.691±0.133
		Chl *abc* – R8, Table 2	4.850±0.042	–0.197±0.008	–0.059±0.008	–	4.514±0.042
		Chl *abcd* – R8, Table 1	4.851±0.042	–0.082±0.008	0.054±0.009	–0.004±0.004	4.818±0.044
	Methanol	R6 – Chl *a*	4.896±0.056	–	–	–	4.896±0.056
		Chl *abc* – R8, Table 2	4.960±0.049	–0.316±0.029	–0.091±0.019	–	4.498±0.032
		Chl *abcd* – R8, Table 1	4.937±0.048	–0.194±0.025	0.071±0.021	–0.003±0.009	4.811±0.048
	Ethanol	R6 – Chl *a*	4.885±0.028	–	–	–	4.885±0.028
		Chl *abc* – R8, Table 2	4.917±0.029	–0.191±0.018	–0.031±0.010	–	4.647±0.031
		Chl *abcd* – R8, Table 1	4.912±0.028	–0.155±0.016	0.068±0.012	–0.013±0.006	4.812±0.026
Phaeophytinised	Acetone	R6 – Chl *a*	2.951±0.016	–	–	–	2.951±0.016
		JH – trichroic formulae	2.982±0.062	–0.355±0.109	–0.205±0.129	–	2.422±0.127
		Chl *abc* – R8, Table 2	2.980±0.016	–0.351±0.010	–0.313±0.008	–	2.247±0.016
		Chl *abcd* – R8, Table 1	2.980±0.016	–0.256±0.008	–0.217±0.008	0.029±0.004	2.536±0.014
	Methanol	R6 – Chl *a*	2.331±0.038	–	–	–	2.331±0.038
		Chl *abc* – R8, Table 2	1.882±0.019	2.458±0.045	–1.344±0.020	–	2.983±0.033
		Chl *abcd* – R8, Table 1	1.857±0.018	2.490±0.041	–1.295±0.019	–0.013±0.005	3.039±0.036
	Ethanol	R6 – Chl *a*	3.105±0.018	–	–	–	3.105±0.018
		Chl *abc* – R8, Table 2	3.190±0.018	–0.301±0.017	–0.515±0.011	–	2.346±0.024
		Chl abcd – R8, Table 1	3.169±0.016	–0.279±0.015	–0.455±0.012	–0.008±0.005	2.426±0.026

Ritchie (2006) suggested that assays of Chls using methanol had some value because the methanol algorithms would be convenient for assays associated with HPLC work even though they are less accurate than those for acetone and ethanol. Unfortunately, the present study shows that all the methanol equations are severely affected by any Ph *a* and any Chl *d* present and so are the least reliable Chl equations to use.

Ethanol-based Chl extraction and assay is much more suited to the teaching laboratory and is particularly suited to field extraction and determination of Chls in remote locations because it poses no significant solvent disposal problems (Ritchie 2006). The use of ethanol also offers the convenience of being able to use polystyrene cuvettes and laboratory plastic-ware. Allomerization of Chls in 100 % ethanol is not a major problem for dilute solutions of Chls. However, ethanol solvent is not a strong inhibitor of chlorophyllase activity: substantial activity can occur in even 95 % ethanol forming ethyl chlorophyllides (Hynninen 1991, Matile *et al.* 1999). Chlorophyllase can easily be removed by filtration or centrifugation because it is not soluble in alcohol. Chlorophyllase is also easily denatured by heating above 60 °C (BRENDA 2005). Soaking of plants in solvents for long periods as an extraction technique should be avoided unless some treatment to inactivate chlorophyllases has been used such as a short heat treatment.

MacLulich (1986a,b, 1987) used Chl *c/a* and Chl *b/a*

ratios calculated from the trichroic formulae of Jeffrey and Humphrey (1975) as indices of the relative importance of cyanobacteria, green algae, and chromophytes (mainly diatoms and sporelings of pheophytic algae) in the biofilm populations of algae on intertidal rock platforms. Algae were either scraped off in the field and scrapings extracted in acetone in the laboratory or directly extracted by brushing rocks with acetone solvent. It seems likely that both methods would have resulted in substantial amounts of Phs being formed, particularly during solvent extraction directly from rock surfaces in daylight. It is likely that the Chl assays were severely compromised by the presence of Ph breakdown products of Chls. The relative amounts of Chl *a*, *b*, and *c* in Chl extracts have long been used to monitor cyanobacterial, chlorophyte, and chromophyte (Chl *c*-containing organisms) in phytoplankton populations (Jeffrey and Vesk 1997) but the present study shows that HPLC rather than spectrophotometric methods would be better for such studies (Mantoura *et al.* 1997). Again unintentional conversion of Chl *a* to Ph *a* not only leads to underestimation of Chl *a* but interferes with Chl *b* and *c* determinations. Phs are sometimes naturally abundant in phytoplankton extracts such as samples taken during or after algal blooms (Jeffrey 1981, Svec 1991).

In conclusion, the quadrichroic Chl formulae should be used as the default equations on Chl extracts of unknown composition. The acetone and ethanol quadrichroic formulae give very reliable estimates of Chl *a* and Chl *d* and so can be used for searching for habitats with unsuspected Chl *d*-containing organisms. The quadrichroic Chl *b* equations for acetone and ethanol solvents are more reliable than the methanol formulae but both give spurious, but low Chl *b* values, in Chl extracts containing no Chl *b*. The methanol algorithms for Chl *b* are very adversely affected by any Ph *a* present. The quadrichroic Chl *c* equations for acetone and ethanol solvents are more reliable for organisms containing Chl c_1+c_2 than for those with only Chl c_2. The trichroic formulae (Table 2) are simpler than the quadrichroic formulae but should only be used where a Chl extract has no significant Ph *a* or Chl *d* content.

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Appendix A: Asymptotic Errors

For a multiple linear equation of the form,

$$Z = Av + Bw + Cx + Dy,$$

where the absorbance coefficient constants A,B,C, and D all have measurable errors ΔA, ΔB, ΔC, and ΔD, the asymptotic error (ΔZ) is

$$\Delta Z^2 \approx \left(\frac{dZ}{dv}\right)^2 \Delta A^2 + \left(\frac{dZ}{dw}\right)^2 \Delta B^2 + \left(\frac{dZ}{dx}\right)^2 \Delta C^2 + \left(\frac{dZ}{dy}\right)^2 \Delta D^2$$

since $\frac{dZ}{dv}, \frac{dZ}{dw}, \frac{dZ}{dx}$, and $\frac{dZ}{dy} = 1$,

$\Delta Z \approx \sqrt{\Delta A^2 + \Delta B^2 + \Delta C^2 + \Delta D^2}$. (Note that the error is independent of v, w, x, or y).

For example, for a spectrophotometric equation for Chl *a* using absorbances at four wavelengths, A_{630}, A_{647}, A_{664}, and $A_{691}$and calculated absorbance coefficients E_{630}, E_{647}, E_{664}, and E_{691},

$$\text{Chl } a\,[\mu g\,cm^{-3}] = A_{630}\,E_{630} + A_{647}\,E_{647} + A_{664}\,E_{664} + A_{691}\,E_{691},$$
$$\Delta\,\text{Chl } a\,[\mu g\,cm^{-3}] = \approx \sqrt{\Delta E_{630}{}^2 + \Delta E_{647}{}^2 + \Delta E_{664}{}^2 + \Delta E_{691}{}^2}.$$

The asymptotic error of a chlorophyll ratio can be calculated in a similar fashion.

For $Z = \frac{B}{A}$, where B and A have errors ΔB and ΔA, the error is approximately

$\Delta Z \approx \sqrt{\frac{dZ}{dA}\,\Delta A + \frac{dZ}{dB}\,\Delta B}$, which simplifies to $\Delta Z \approx Z\sqrt{\left(\frac{\Delta A}{A}\right)^2 + \left(\frac{\Delta B}{B}\right)^2}$.

For example, a Chl *a*/ΣChl (*abcd*) ratio or index can therefore be expressed as

$$\frac{\text{Chl } a}{\Sigma\text{Chl }(abcd)} \pm \left(\left(\frac{\text{Chl } a}{\Sigma\text{Chl }(abcd)} \right) \sqrt{\left(\frac{\Delta\text{Chl } a}{\text{Chl } a} \right)^2 + \left(\frac{\Delta\Sigma\text{Chl }(abcd)}{\Sigma\text{Chl }(abcd)} \right)^2} \right).$$

Appendix B: Matrix Algebra

The examples shown are four sets of spectrophotometric readings in acetone solvent where absorbances are measured at 630, 647, 664, and 691 nm which are the Q_y values for Chl c_2 and Chl c_1+c_2, Chl *b*, Chl *a*, and Chl *d*, respectively. All the Chl equations (Chl *a*, Chl *b*, Chl *c*, Chl *d*, and ΣChl (*a*+*b*+*c*+*d*) have the same solution matrix.

For a chlorophyll algorithm using three absorbance readings where there is no Chl *d* present,

$$\text{Chl} = E_{630}\,A_{630} + E_{647}\,A_{647} + E_{664}\,A_{664}$$

$$\begin{bmatrix} \sum_{i=1}^{N} \frac{dChl}{dA_{630}}^2 & \sum_{i=1}^{N} \frac{dChl}{dA_{630}}\frac{dChl}{dA_{647}} & \sum_{i=1}^{N} \frac{dChl}{dA_{630}}\frac{dChl}{dA_{664}} \\ \sum_{i=1}^{N} \frac{dChl}{dA_{647}}\frac{dChl}{dA_{630}} & \sum_{i=1}^{N} \frac{dChl}{dA_{647}}^2 & \sum_{i=1}^{N} \frac{dChl}{dA_{647}}\frac{dChl}{dA_{664}} \\ \sum_{i=1}^{N} \frac{dChl}{dA_{664}}\frac{dChl}{dA_{630}} & \sum_{i=1}^{N} \frac{dChl}{dA_{664}}\frac{dChl}{dA_{647}} & \sum_{i=1}^{N} \frac{dChl}{dA_{664}}^2 \end{bmatrix}^{-1} = \begin{bmatrix} \text{var } E_{630} & \text{cov } E_{630}\,E_{647} & \text{cov } E_{630}\,E_{664} \\ \text{cov } E_{630}\,E_{647} & \text{var } E_{647} & \text{cov } E_{647}\,E_{664} \\ \text{cov } E_{630}\,E_{664} & \text{cov } E_{647}.E_{664} & \text{var } E_{664} \end{bmatrix}$$

For a chlorophyll algorithm using four absorbance readings,

$$\text{Chl} = E_{630}\,A_{630} + E_{647}\,A_{647} + E_{664}\,A_{664} + E_{691}\,A_{691}$$

$$\begin{bmatrix} \sum_{i=1}^{N} \frac{dChl}{dA_{630}}^2 & \sum_{i=1}^{N} \frac{dChl}{dA_{630}}\frac{dChl}{dA_{647}} & \sum_{i=1}^{N} \frac{dChl}{dA_{630}}\frac{dChl}{dA_{664}} & \sum_{i=1}^{N} \frac{dChl}{dA_{630}}\frac{dChl}{dA_{691}} \\ \sum_{i=1}^{N} \frac{dChl}{dA_{647}}\frac{dChl}{dA_{630}} & \sum_{i=1}^{N} \frac{dChl}{dA_{647}}^2 & \sum_{i=1}^{N} \frac{dChl}{dA_{647}}\frac{dChl}{dA_{664}} & \sum_{i=1}^{N} \frac{dChl}{dA_{647}}\frac{dChl}{dA_{691}} \\ \sum_{i=1}^{N} \frac{dChl}{dA_{664}}\frac{dChl}{dA_{630}} & \sum_{i=1}^{N} \frac{dChl}{dA_{664}}\frac{dChl}{dA_{647}} & \sum_{i=1}^{N} \frac{dChl}{dA_{664}}^2 & \sum_{i=1}^{N} \frac{dChl}{dA_{664}}\frac{dChl}{dA_{691}} \\ \sum_{i=1}^{N} \frac{dChl}{dA_{691}}\frac{dChl}{dA_{630}} & \sum_{i=1}^{N} \frac{dChl}{dA_{691}}\frac{dChl}{dA_{647}} & \sum_{i=1}^{N} \frac{dChl}{dA_{691}}\frac{dChl}{dA_{664}} & \sum_{i=1}^{N} \frac{dChl}{dA_{691}}^2 \end{bmatrix}^{-1} = \begin{bmatrix} \text{var}\,E_{630} & \text{cov}\,E_{630}\,E_{647} & \text{cov}\,E_{630}\,E_{664} & \text{cov}\,E_{630}\,E_{691} \\ \text{cov}\,E_{630}\,E_{647} & \text{var}\,E_{647} & \text{cov}\,E_{647}\,E_{664} & \text{cov}\,E_{647}\,E_{691} \\ \text{cov}\,E_{630}\,E_{664} & \text{cov}\,E_{647}\,E_{664} & \text{var}\,E_{664} & \text{cov}\,E_{664}\,E_{691} \\ \text{cov}\,E_{630}\,E_{691} & \text{cov}\,E_{647}\,E_{691} & \text{cov}\,E_{664}\,E_{691} & \text{var}\,E_{691} \end{bmatrix}$$