Consistent Sets of Spectrophotometric Chlorophyll Equations for Acetone, Methanol and Ethanol Solvents

Article *in* Photosynthesis Research · August 2006

DOI: 10.1007/s11120-006-9065-9 · Source: PubMed

1 author:

Raymond J Ritchie
Prince of Songkla University - Phuket
139 PUBLICATIONS **2,794** CITATIONS

SEE PROFILE

Photosynth Res (2006) 89:27–41
DOI 10.1007/s11120-006-9065-9

REGULAR PAPER

Consistent sets of spectrophotometric chlorophyll equations for acetone, methanol and ethanol solvents

Raymond J. Ritchie

Received: 1 August 2005 / Accepted: 8 December 2005 / Published online: 9 June 2006

Abstract A set of equations for determining chlorophyll *a* (Chl *a*) and accessory chlorophylls *b*, c_2, $c_1 + c_2$ and the special case of *Acaryochloris marina*, which uses Chl *d* as its primary photosynthetic pigment and also has Chl *a*, have been developed for 90% acetone, methanol and ethanol solvents. These equations for different solvents give chlorophyll assays that are consistent with each other. No algorithms for Chl **c** compounds (c_2, $c_1 + c_2$) in the presence of Chl *a* have previously been published for methanol or ethanol. The limits of detection (and inherent error, ± 95% confidence limit), for chlorophylls in all organisms tested, was generally less than 0.1 μg/ml. The Chl *a* and *b* algorithms for green algae and land plants have very small inherent errors (< 0.01 μg/ml). Chl *a and d* algorithms for *Acaryochloris marina* are consistent with each other, giving estimates of Chl *d/a* ratios which are consistent with previously published estimates using HPLC and a rarely used algorithm originally published for diethyl ether in 1955. The statistical error structure of chlorophyll algorithms is discussed. The relative error of measurements of chlorophylls increases hyperbolically in diluted chlorophyll extracts because the inherent errors of the chlorophyll algorithms are constants independent of the magnitude of absorbance readings. For safety reasons, efficient extraction of chlorophylls and the convenience of being able to use polystyrene cuvettes, the algorithms for ethanol are recommended for routine assays of chlorophylls. The methanol algorithms would be convenient for assays associated with HPLC work.

R. J. Ritchie (✉)
School of Biological Sciences, The University of Sydney, Sydney, NSW 2006, Australia
e-mail: rrit3143@usyd.edu.au

Keywords Chlorophyll · Acetone · Methanol · Ethanol · Algorithms · Chlorophyll *a* · Chlorophyll *b* · Chlorophyll c_2 · Chlorophyll $c_1 + c_2$ · Chlorophyll *d* · Error structure

Introduction

Measurements of chlorophyll content of oxygenic photosynthetic organisms are a fundamental measurement in many branches of plant biology and ecology. The amount of chlorophyll in photosynthetic organisms is an important measurement in itself, particularly for chlorophyll *a* (Chl *a*), which is the primary photosynthetic pigment in nearly all known oxygenic photosynthetic organisms. The presence or absence of other chlorophylls (*b*, c_1, c_2, c_3 and *d*) is of taxonomic importance. In addition, the relative amounts of secondary chlorophylls, such as Chl *b* in chlorophyte algae and vascular plants compared to Chl *a* varies with both light intensity and the spectral quality of light (Atwell et al. 1999). Chlorophyll *a* (Chl *a*) or sometimes total chlorophyll is used as the standard basis on which to calculate photosynthetic and respiratory rates (mol O_2. mg Chl a^{-1}.hr^{-1}), the metabolically active biomass and the productivity of terrestrial and aquatic ecosystems (gC. mg Chl a^{-1}. d^{-1}). The amount of chlorophyll per unit of protein is crucial in studies of chlorophyll-protein complexes because knowing the number and type of chlorophylls in each type of chlorophyll complex is important in working out their structure and function (Porra et al. 1989; Porra 1991, 2002). For example, Hu et al. (1998) showed in the novel organism *Acaryochloris marina* that the photosynthetic reaction centre of PS I used Chl *d* rather than Chl *a* as in other oxygenic photosynthetic organisms.

This paper presents a consistent set of algorithms for the routine assays of chlorophylls *a, b,* c_1 ($c_1 + c_2$) and *d* in acetone, methanol and ethanol solvents which give closely similar estimates of the chlorophylls. Chlorophyll estimates can now be made using either acetone, methanol or ethanol solvent systems appropriate to the particular experimental situation. Previously published algorithms for estimation of chlorophylls using different solvents such as acetone and methanol were only available for Chl $a + b$ and in any case were not always consistent with each other (Lichtenthaler 1987).

Although acetone solvent gives very sharp chlorophyll absorption peaks and so is the solvent of choice for chlorophyll assays (see Arnon 19490; Jeffrey and Humphrey 1975; Jeffrey et al. 1997; Humphrey and Jeffrey 1997; Porra et al. 1989, Porra 1991, 2002; Wright et al. 1997), acetone is sometimes a poor extractant of chlorophyll from many vascular plants and some algae, particularly green algae such as *Scenedesmus*, *Chlorella* and *Nannochloris* (Sartory and Grobbelaar 1984; Porra et al. 1989; Porra 1991, 2002; Jeffrey et al. 1997; Wright et al. 1997). Methanol and ethanol are often more efficient extractants (Lichtenthaler and Wellburn 1983; Sartory and Grobbelaar 1984; Wright et al. 1997; Lichtenthaler 1987). Neutralised methanol and ethanol were used in the present study to avoid formation of allomerisation products of chlorophylls, which are spectrally different to chlorophylls. Porra (1990) found that 1.5 mM sodium dithionite improved extraction of chlorophylls from recalcitrant algae and discouraged the formation of breakdown products in methanol solvent (use of sodium dithionite would not affect the equations developed in the present study). The equations developed for spectrophotometric assay of Chl *a* and *b* in these solvents (methanol: Porra et al. 1989; Porra 1990, 1991, 2002; ethanol: Lichtenthaler 1987; Rowan 1989) are not widely used. The chlorophyll red peaks (Q_y) for Chl $c_1 + c_2$ and Chl c_2 are much lower and broader in methanol and ethanol and there do not appear to be any published algorithms for determinations of Chl *a* and Chl $c_1 + c_2$ or c_2 in methanol or ethanol.

There is a real need for a consistent set of algorithms for routinely calculating chlorophyll contents of environmental samples, cultured algae and for biochemical studies of chlorophyll–protein complexes with some choice of solvents to use. There is also a trade-off between choosing the best solvent for efficient quantitative extraction of chlorophylls and use of a solvent best suited for spectrophotometric assay. Acetone is not the ideal solvent for extraction, although it has great merit as the solvent for assay of chlorophylls. In many practical situations, any gains in accuracy from using acetone as the assay solvent are lost because of its inefficient extraction of chlorophylls from cells. Replacing one solvent with another by driving off the extracting solvent using a stream of N_2 and then replacing it with another solvent is inefficient when large numbers of samples need to be assayed and leads to inevitable losses and oxidation.

There are two other reasons why acetone is not a desirable solvent for chlorophyll assays. The first is safety. Acetone is very volatile, highly flammable, causes headache, is narcotic in high concentrations and is a skin irritant (erythema). Plastic or latex gloves provide little protection or actually worsen the situation by being attacked by the acetone. It is not a desirable solvent to use in a teaching laboratory and its flammability, security concerns and volatility make it problematic to transport by air for fieldwork. The widespread use of plastic laboratory-ware also leads to difficulties because acetone attacks polystyrene and polymethylacrylates (PMMA) and, therefore, plastic spectrophotometer cuvettes cannot be used for acetone based chlorophyll assays.

Methanol is a very good extractant for chlorophylls, particularly from recalcitrant vascular plants and algae (Porra et al. 1989; Porra 1990, 1991, 2002). It is less volatile and flammable than acetone but is notoriously toxic. It is an insidious poison because it is readily adsorbed by inhalation and through the skin and so should not be used in a teaching laboratory if it can be replaced by ethanol. Methanol slowly fogs polystyrene spectrophotometer cuvettes leading to false readings and cannot be used at all with PMMA cuvettes. Methanol is the usual solvent for HPLC systems to assay chlorophylls: a set of spectrophotometric equations for a methanol solvent system would therefore be a very useful adjunct to HPLC work on chlorophylls (Wright and Jeffrey 1997; Jeffrey and Wright 1997; Mantoura et al. 1997).

Ethanol is a much safer solvent than either acetone or methanol (Wright et al. 1997) but is not used very often for the assay of chlorophylls although equations for *Chl a* and *b* are available (Lichtenthaler 1987; Rowan 1989). Although flammable it is not very toxic and is suitable for use in a teaching laboratory. Ethanol does not attack polystyrene and so polystyrene plastic spectrophotometer cuvettes can be used. There are considerable practical, safety and economic advantages in using ethanol as the solvent for chlorophyll extract and assay. Algorithms developed for ethanol in the present study now allow routine assays of chlorophylls *a,* $a + b$, $c_1 + c_2$, c_2 and *d* in a very safe and convenient solvent.

Diethyl ether is a very popular solvent for chlorophylls for research purposes, particularly for preparing pure pigments (see Porra et al. 1989, Porra 1991; Scheer 1991; Jeffrey et al. 1997). Many of the diagnostic spectra of chlorophyll pigments are for diethyl ether as solvent (Jeffrey et al. 1997). Except for freeze-dried material, it cannot be directly used as a chlorophyll extractant because it is not miscible in water. It is not a solvent of choice for routine and class laboratory work because it is extremely volatile, flammable, explosive and narcotic. The explosion hazard in particular restricts it use. Ether also attacks plastic cuvettes and most plastic laboratory ware. Diethyl ether had to be used in part of the present study because the only published formulae for determining Chl *a* and *d* in mixtures of these chlorophylls were for diethyl ether solvent (Smith and Benitez 1955; French 1960).

Porra et al. (1989) and Wright et al. (1997) discuss the merits of other solvents used for chlorophyll extraction and assay such as chloroform, dimethyl sulphoxide (DMSO) and dimethyl formamide (DMF). These solvents are more dangerous and even less desirable than acetone and methanol for routine research purposes and in the teaching laboratory but chlorophyll algorithms for a DMSO solvent system are currently under development in our laboratory.

The present study compares the use of the solvents acetone, methanol and ethanol in determining concentrations of chlorophylls *a, b,* $c_1 + c_2$, c_2 and *d*. New algorithms for determining concentrations of chlorophyll mixtures in the three solvents are presented along with error estimates.

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 0.35–0.25, consistent with being grown in bright light. Where shade adapted plants were required, spinach plants were kept in a south-facing shaded sunroom for several days and the new shade-adapted leaves were used as a source of Chl *a* and *b*. The marine diatom, *Phaeodactylum* sp. (Sydney University Teaching Collection) was used as a source of Chl *a* and $c_1 + c_2$. *Heterocapsa pygmae* (a small marine dinoflagellate, Sydney University Teaching Collection), and zooxanthellae (*Symbiodinium* sp., Dinophyta) from a zoanthid (*Zoanthus robustus* (Carlgren, Coelenterata)) and *Rhodomonas spN23* (Chroomonas spN23, Cryptophyta, Sydney University Teaching Collection) were tried out as sources of Chl *a* and c_2. *Rhodomonas spN23* could be grown easily in large amounts and was used as the standard source of Chl *a* and c_2 preparations. *Acaryochloris marina* was a kind gift from Dr Min Chen (Sydney University). *Acaryochloris marina* 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). A free-living oxyphotobacterium with Chl *d* as its dominant chlorophyll was recently isolated from the Salton Sea in the USA (CCMEE 5410, Miller et al. 2005) and another as epiphytes on rhodophyte algae (Murakami et al. 2004). *Phaeodactylum* sp., *Heterocapsa pygmae*, *Rhodomonas spN23* and *Acaryochloris marina* were all grown in enriched f-2 seawater as described by McLachlan (1973) but using Fe-citrate rather than Fe-EDTA as the iron source.

The algae were grown on an orbital shaker (≈80 rpm) fitted with overhead fluorescent lights (Sylvania Gro-Lux). The light intensity was approximately 80 μE m^{-2} s^{-1} (PAR, using a Li-Cor photon flux meter Model LI-189). However, *Acaryochloris marina* consistently grew better on the edge of the shaker where the light intensity was lower (≈ 40 μE m^{-2} s^{-1}). *Acaryochloris marina* consistently grows better at low light intensities.

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 μE m^{-2} s^{-1} (400–700 nm PAR) (Li-Cor Quantum photometer Model LI-189). Exposure of chlorophyll extracts to light was avoided.

Analytical grade acetone, methanol and ethanol were from Mallinckrodt Baker BV, Deventer, Holland. Denatured dry alcohol (Ethyl alcohol 99.5%, Chem-Supply Ltd, Gillman, SA, Australia), denatured with denatonium benzoate 0.00066%, fluorescein 0.0001% and methyl isobutyl ketone 0.25%, was found to be free of spectroscopic contaminants in the visible range and so could also be successfully used. Analytical diethyl ether was from Merck Pty Ltd, Kilsyth, Victoria, Australia.

Commercial acetone and methanol are often highly acidic. A 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. Excessively alkaline extractants should

also be avoided because of allomerisation of chlorophylls and in particular the formation of rhodochlorins in alkaline solvent (Porra et al. 1989; Porra 1990). Aqueous preparations of methanol and ethanol were not used in the present study. Papista et al. (2002) reported that chlorophylls form Chl-monosolvate and Chl-disolvate mixtures in methanol/water and ethanol/water mixtures lead to misleading results. Solvents were kept at 4°C.

Microalgae were collected by first centrifuging them at 3000 × *g* for 10 min, then resuspending in deionised water and pelleted a second time. After decanting and resuspension of the hard pellet, the pigments were extracted in a 1:1:1 mixture of 90% acetone, 100% methanol and 100 % (99.5%) ethanol, all neutralised with magnesium carbonate. The crude extract was allowed to stand in a refrigerator in the dark at 4 °C for about 30 min before being cleared by centrifugation and the pellet discarded. To extract Chl *a* and *b* from spinach, the leaves were cut up into small pieces and ground in a glass-glass tissue grinder in the mixed acetone/methanol/ethanol extractant. The extract was then pelleted by centrifugation and the pellet discarded. All concentrated extracts were made up to about 6 ml and stored in the dark in a freezer at –20°C for no more than 7 days.

Extraction of chlorophyll by soaking algae or vascular plants in solvents overnight was not employed because it provides an opportunity for chlorophyllase to convert chlorophylls to chlorophyllides.

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. Quartz cuvettes were used unless otherwise stated. Polystyrene cuvettes were from Sarstedt International (Numbrecht, Germany). Concentrated pigment extracts were used to make up diluted samples for the spectrophotometric study. A 50 μl of pigment extract was diluted with assay solvent to make up to 1.0 ml of assay mixture. Where mixtures of chlorophyll extracts were being assayed it was ensured that the diluted sample was not contaminated with more than 6.7% of foreign solvents. Thus a 1.0 ml mixture of Chl *a* from *Synechococcus* and Chl *a* and *b* from spinach, made up in 90% acetone would contain no more than 3.3% methanol and 3.3% ethanol. All chlorophyll assays on the concentrated extracts were run in acetone, methanol and ethanol so that direct cross-comparisons of chlorophyll assays using the three solvent systems could be made.

In the present study, all error-bars are ±95% confidence limits (CL) with the number of replicates in brackets. All chlorophyll 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 mg l^{-1} cm A^{-1}.

Spectrophotometry theory

French (1960), Porra (1991, 2002) and Jeffrey and Welschmeyer (1997) give general outlines of the simultaneous equation approach to estimating separately the chlorophylls in mixtures of chlorophylls. This method of estimating the component chlorophylls in plants was popularised by Arnon (1949). In spectrophotometry of chlorophylls, it is customary to zero spectrophotometers at 750 nm to correct for turbidity and contaminating coloured compounds. Thus, the simplest chlorophyll algorithms capable of resolving two chlorophylls (1 and 2) in a mixture have the general form,

$$\begin{aligned} \mathrm{Chl}(1)\,(\mu\mathrm{g/ml}) &\approx E_{\lambda 1,1}A_{\lambda 1} + E_{\lambda 2,1}A_{\lambda 2} \\ \mathrm{Chl}(2)\,(\mu\mathrm{g/ml}) &\approx E_{\lambda 1,2}A_{\lambda 1} + E_{\lambda 2,2}A_{\lambda 2} \end{aligned} \quad (1a, b)$$

where, $E_{\lambda 1,1}$ is the absorbance coefficient (mg l^{-1} cm A^{-1}) for the red peak ($\lambda 1$) of Chlorophyll (1), $E_{\lambda 1,2}$ is the absorbance coefficient (mg l^{-1} cm A^{-1}) for the red peak ($\lambda 1$) of Chlorophyll (2), $A_{\lambda 1}$ is the absorbance of the pigment extract at wavelength ($\lambda 1$) nm.

More complex algorithms using measurements at three or more wavelengths can be developed, however, the more complex the algorithm, the more difficult it is to fit to a data set and the larger the inherent error (Appendix 1). Hence, the least complex algorithm, consistent with a good fit to the data, should be adopted.

For organisms containing two types of chlorophyll, readings at two wavelengths (the red absorption peaks Q_y of the two chlorophylls present) are usually employed and so chlorophyll equations are usually of the form $z = ax + by$. Jeffrey and Humphrey (1975) also published a trichroic formula to determine Chl *a, b* and $c_1 + c_2$ in acetone extracts from mixed phytoplankton populations.

In most oxygenic photosynthetic organisms (*Acaryochloris marina* is an exception), Chl *a* is the predominant chlorophyll. Absorption by Chl *a* over most of the red part of the spectrum strongly interferes with determinations of Chl *b,* c_2 and $(c_1 + c_2)$. Chlorophyll algorithms require measurements of absorbances at two wavelengths: one is at the red absorption peak for Chl *a* and the other is at the red peak of the other chlorophyll. The Chl *a* equation is usually very accurate but the equation for the minor pigment will be less

reliable. For example, one of the most accepted Chl *a* and Chl *b* formulae for 90% acetone (Humphrey and Jeffrey 1975) are,

$$\text{Chl } a \ (\mu\text{g/ml}) \approx -1.93 \times A_{647} + 11.93 \times A_{664}$$
$$\text{Chl } b \ (\mu\text{g/ml}) \approx 20.36 \times A_{647} - 5.50 \times A_{664} \qquad (2a,b)$$

Unfortunately, estimates of the errors of these equations or of the absorbance coefficients (mg l^{-1} cm A^{-1}), $E_{647,\ \text{Chl } a}$ = 1.93, $E_{664,\ \text{Chl } a}$ = 11.93, $E_{647,\ \text{Chl } b}$ = 20.36 and $E_{664,\ \text{Chl } b}$ = –5.50 were not published.

The error structure of equations of the form illustrated by Eqs. (1a, b) and (2a, b) is outlined in Appendix 1. The error is a constant, independent of the magnitude of the absorbance readings. Consequently, the errors of the extinction coefficients on which such simultaneous equations are based have to be very small (< 1%), otherwise chlorophyll algorithms derived from them will have unacceptable total errors.

Appendix 3 presents the published chlorophyll formulae from Smith and Benitez (1955), Humphrey and Jeffrey (1975), Rowan (1989), Porra et al. (1989) and Porra (1991, 2002) that were used in the present study. For comparative purposes it has been assumed that the absorption coefficients quoted by Smith and Benitez (1955), Humphrey and Jeffrey (1975) and Rowan (1989) had an error of ±1.0%. This is consistent with Porra et al. (1989) who found the standard deviations of the extinction coefficients for *Chl a* and *b* in 80% acetone and 100% methanol to be about 0.3–1.0% of the mean values. The inherent error of each equation has been calculated using the error formulae in Appendix 1.

The red peak (Q_y) for *Chl c* (c_1 and c_2 and related chlorophylls) is much lower than for equimolar amounts of *Chl a* (Jeffrey et al. 1997). Chlorophyll **c** compounds normally represent less than 20% of the total chlorophyll of algal cells containing chlorophyll *c* compounds and hence they are difficult to assay in chlorophyll mixtures. Absorption by Chl *a* at the red peak for Chl *c* compounds tends to drown out the signal from Chl *c*. Inherently, formulae to calculate Chl c_2 or Chl $c_1 + c_2$ are not very accurate, particularly if only small amounts of Chl c_2 or $c_1 + c_2$ are present (Humphrey and Jeffrey 1997).

Chlorophyll *d* was originally described as an accessory chlorophyll in extracts from rhodophyte algae (Manning and Strain 1943). A Chl *a* and *d* algorithm has been published for diethyl ether (Smith and Benitez 1955) but not for acetone, methanol or ethanol. The availability of this algorithm seems to have been neglected in the current resurgence of interest in Chl *d*. Currently the usual method for determining Chl *a* and *d* in *Acaryochloris marina* and related cyanobacteria is HPLC (Miyashita et al. 1997; Akiyama et al. 2001; Murakami et al. 2004; Miller et al. 2005).

Acaryochloris marina contains very little Chl *a*; about 95% or more of its total chlorophyll is Chl *d* (Miyashita et al. 1996, 1997). In the case of *Acaryochloris marina* it is difficult to estimate Chl *a* in the organism spectrophotometrically because the absorption of Chl *d* obscures the contribution of Chl *a*. The absorption peak and extinction coefficients for Chl *d* have been published for diethyl ether (Smith and Benitez 1955, p 169–171; French 1960, p 254). The molecular weight of Chl *d* is 894.5 (not 893.5 assumed by Smith and Benitez 1955) and so its molar extinction coefficient in ether is $\varepsilon = 98.6 \times 10^3$ l mol^{-1} A cm^{-1}. It has a specific extinction coefficient of $\alpha = 110.23$ l g^{-1} A cm^{-1} and thus an absorbance coefficient of $E_{688} = 0.0720$ mg l^{-1} A^{-1} cm. The red absorption peaks (Q_y) had to be determined experimentally in acetone and ethanol in the present study.

Spectroscopically pure Chl *d* was a kind gift from Dr Min Chen. Care was taken to avoid formation of breakdown products (Manning and Strain 1943; Nieuwenburg et al. 2003). The red absorption peak in methanol is at 696 nm (Manning and Strain 1943) and at 688 nm in diethyl ether (Smith and Benitez 1955). Using a matched set of 48 samples of pure Chl *d* the absorbance ratio of the red peak (Q_y) in methanol (696 nm) to that in diethyl ether (688 nm) was found to be 0.704 ± 0.00327 leading to an estimated specific extinction coefficient of 77.62 ± 0.360 l g^{-1} A cm^{-1} for methanol and thus an absorbance coefficient of $E_{696} = 12.883$ mg l^{-1} A^{-1} cm.

Scans of pure Chl *d* were also used to estimate the absorbance ratios (relative to the Q_y Chl *d* peak) for 630, 647 and 663 nm for the acetone solvent, 632, 652 and 665 nm for the methanol solvent and 629, 449 and 665 nm for the ethanol solvent. The A_{665}/A_{696} ratio ($R_{d,\ 665/696}$) for methanol was 0.2383 ± 0.01182 (four samples, n = 42 determinations). The $R_{d,\ 665/696}$ ratio for methanol agrees with that found by Manning and Strain (1943). The $R_{d,\ 665/696}$ ratios for pure Chl *d* in methanol was used as a method of estimating the contribution of Chl *d* to absorbance at 665 nm (Porra 2002). The contribution of Chl *a* to absorbance at 665 nm in *Acaryochloris marina* pigment extracts could then be estimated and were used in fitting the algorithm for determining the Chl *a* and *d* absorption coefficients shown in Table 2.

$$\text{Chl a } (\mu\text{g/ml}) \approx E_{665,\ \text{chl } a} \cdot (A_{665} - R_{d,665/696} \cdot A_{696}) \qquad (3)$$

where, $E_{665,\text{Chl }a}$ is the absorption coefficient ($1/\alpha$) for pure Chl a in methanol (Table 2b), A_{665} is the absorbance of an *Acaryochloris* pigment extract at 665 nm, $R_{d,665/696}$ is the absorbance ratio of A_{665} to A_{696} of pure Chl *d*, 0_{696} is the absorbance of the *Acaryochloris* extract at 696 nm.

Algorithms for determination of chlorophylls in mixtures of Chl *a* and *b* (Chlorophyte algae and higher plants), Chl *a* and c_2 (Dinophytes and Cryptophytes) and Chl *a* and ($c_1 + c_2$) (Phaeophytes and diatoms) 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. Estimates of the chlorophylls in 90% acetone were determined using the equations of Jeffrey and Humphrey (1975) and used to fit algorithms (2 or 3 wavelength) of the form shown in Eqs. (1a, b) by least squares. Mean square residuals (MSR) were then calculated for the fits. Sums of the squares (SS) of the absorbance readings were used to set up 2×2, or 3×3 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 coefficients, $E_{\lambda 1}$, $E_{\lambda 2}$, $E_{\lambda 3}$, $E_{\lambda 4}$ (see Appendix 2). The ± 95% confidence limits of the coefficients could then be calculated. Using the calculation of the error of coefficient $E_{\lambda 1}$ as a worked example,

$$\pm E_{\lambda 1} \approx t_{0.05,\mathrm{df},2} \cdot \sqrt{\frac{\mathrm{MSR.varE}_{\lambda 1}}{n}} \quad (4)$$

where $\pm E_{\lambda 1}$ is the asymptotic error of the absorbance coefficient (*E*) for absorbance readings at wavelength ($\lambda 1$), *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 (1,2,3 or 4), two-tailed, MSR is the mean square residual of the fit (SS/df), var$E_{\lambda 1}$ is the variance estimate from the inverse matrix for the absorbance coefficient $E_{\lambda 1}$.

The estimates of chlorophylls using the equations of Jeffrey and Humphrey (1975) were transferred from the acetone spreadsheet to the methanol and ethanol EXCEL spreadsheets and used to fit chlorophyll equations for methanol and ethanol solvents. A set of chlorophyll equations for acetone, methanol and ethanol determined in this way should yield estimates of chlorophyll content of a concentrated extract that are similar whether the acetone, methanol or ethanol-diluted sample is used for spectrophotometry.

The algorithm for determination of Chl *a* and *d* in extracts from *Acaryochloris marina* were also determined by least squares fitting. Estimates of the Chl *d* in methanol were determined using the calculated extinction coefficient for Chl *d* (see above). No corrections for absorbance by Chl *a* were necessary because *Acaryochloris marina* contains only small amounts of Chl *a* (Miyashita et al. 1996, 1997; Akiyama et al. 2001). The estimated $R_{a,\ 696/665}$ value for Chl *a* was determined experimentally to be only 0.02333 ± 0.0008921 (144) for pure Chl *a* from *Synechococcus*. Thus, even if 20% of the total chlorophyll of *Acaryochloris marina* was Chl *a*, the contribution by Chl ***a*** to absorbance at 665 nm would be less than 0.5% of the total absorbance at 696 nm in methanol. Chlorophyll **a** was estimated using Eq. (3). Least squares fitting procedures, calculation of MSR, setting up matrices and matrix inversion routines to obtain estimates of the errors of the coefficients, were carried out as described above for the other chlorophyll mixtures.

In the case of *Acaryochloris marina*, the determination of Chl *a* and *d* in methanol was transferred to the acetone and ethanol EXCEL spreadsheets and used to determine the equations for the acetone and ethanol systems. For comparison, some Chl *a* and *d* determinations were made in diethyl ether using the equations published by Smith and Benitez (1955) and French (1960) and compared to those made in acetone, methanol and ethanol. No more than 50 μl of acetone/methanol/ethanol based chlorophyll extract was added to 950 μl (nominal volume) of ether. Nevertheless, assay samples in the diethyl ether solvent system had to be cleared by centrifugation because of precipitation of some of the Magnesium salts in the acetone/methanol/ethanol extraction mixture.

Initial comparisons of Chl *a* and *d* determinations showed that the estimates of Chl *d* were consistent in acetone, methanol, ethanol and ether but the assays in ether using the equations of Smith and Benitez (1955) overestimated Chl ***a*** by over 50%. It was found that they had underestimated $C_{\text{Chl }a,\ 688/663}$ for Chl ***a*** and $C_{\text{Chl }d,\ 663/688}$ for Chl *d* and hence underestimated the extinction coefficients of Chl ***a*** at 688 nm ($\varepsilon_{\text{Chl }a,\ 688} = 1.5\ \text{l g}^{-1}\ \text{A cm}^{-1}$) and Chl d at 663 ($\varepsilon_{\text{Chl }d,\ 663} = 11\ \text{l g}^{-1}\ \text{A cm}^{-1}$). Using my own spectrophotometric data these extinction coefficients were: for Chl ***a*** at 688 nm, $\varepsilon_{\text{Chl }a,688} = 1.848\ \text{l g}^{-1}\ \text{A cm}^{-1}$ and for Chl *d* at 663 nm, $\varepsilon_{\text{Chl }d,663} = 12.83\ \text{l g}^{-1}\ \text{A cm}^{-1}$. Modified equations were calculated as described by French (1960) (Appendix 3). It was assumed that the extinction coefficients at the Qy peaks of Chl *a* and *d* were correct.

Results

Table 1 shows the red band (Soret) absorption peaks for Chl *a, b* and *d*, and for Chl c_2 and (c_1 + c_2). Figure 1 shows the spectra of Chl *a* (from *Synechococcus R-2*) compared to Chl *a* and *b* from spinach, in acetone, methanol and ethanol. In general, chlorophyll peaks are displaced to the red end of the spectrum in polar solvents such as methanol and ethanol and the peaks are notably flatter and wider than in acetone. The Chl c_2 peak was small but noticeable in the *Rhodomonas spN23* extracts but the chlorophyll $c_1 + c_2$ peak in extracts from *Phaeodactylum* sp. was not readily identifiable but could be detected on a difference spectrum against an extract from *Synechococcus R-2* which contained only Chl *a* but had a similar amount of Chl *a* as an extract of *Phaeodactylum* sp.

The methanol and ethanol peaks for Chl $c_1 + c_2$ were identified using difference spectra of Chl *a* and (c_1 + c_2) from *Phaeodactylum* sp. standardised to an absorption of 1.0 at 665 nm, compared to a similarly standardised preparation of Chl *a* from *Synechococcus R-2*. The chlorophyll peaks for Chl *a* and c_2 in extracts from *Rhodomonas spN23* were readily apparent and could be located using the peak-finding software of the dual beam spectrophotometer. These were found to be the same as for Chl *a* and (c_1 + c_2) in *Phaeodactylum* sp. using the difference spectra method: the baseline of the spectrophotometer was set on a Chl *a* extract with a red absorbance peak near 1.0, then a scan was made of a Chl *a* and (c_1 + c_2) extract with a similar Chl *a* absorbance peak. The Chl *d* peaks in acetone and ethanol in *Acaryochloris marina* extracts were identified using the peak-finding software.

Table 2 shows the algorithms for assay of Chl *a, a* and *b, a* and c_2, *a* and $c_1 + c_2$ and *a* and *d* in acetone, methanol and ethanol. The ± 95% confidence limits for each absorbance coefficient were calculated as described in the Theory and Appendix 2. The inherent error of each spectrophotometric equation was then calculated as described in Appendix 1. This error is independent of the magnitude of the absorbances measured using the spectrophotometer and so the relative error of chlorophyll estimates increases hyperbolically if the extract is diluted.

The Chl *a* and *b* equations are based on 328 sets of spectrophotometric readings at the red peak absorbances of Chl c_2 and Chl (c_1 + c_2), Chl *b,* Chl *a* and Chl *d* in acetone, methanol and ethanol solvent systems. Measurements were usually made in sets of 12: measurements on four replicate diluted samples of an acetone/methanol/ethanol extract from *Synechococcus R-2* (zero Chl *b*), measurements on four replicate diluted samples of an acetone/methanol/ethanol extract from spinach and measurements on four replicate samples with equal volumes of *Synechococcus R-2* and spinach extracts (see Fig. 1). The chlorophyll assays of the *Synechococcus R-2* and spinach samples could be used to calculate the amounts of Chl *a* and *b* to be found in the combined samples. Spinach grown in bright light and dim light was used and the sample preparation routine ensured that data sets covered samples with a wide range of Chl *b/a* ratios. Seven sets of 12 determinations on high light-grown spinach and five sets of 12 determinations from shade grown spinach were included in the data set.

Least squares fitting showed that algorithms using only two terms (at the absorption peaks for Chl *a* and *b*) gave excellent estimates of Chl *a* and *b* (Tables 2A, B, C). Expanding the algorithm to 3 or 4 terms only served to increase the error. The coefficients determined by least squares fitting in acetone are very similar to those of Jeffrey and Humphrey (1975) (see Eqs.2a, b). Taking the values from Table 2A, the equations for Chl *a* and *b* in 90% acetone would be,

$$\begin{aligned} \text{Chl } a\ (\mu\text{g/ml}) &\approx -1.7858 \times A_{647} + 11.8668 \\ &\quad \times A_{664}\ (\pm 0.000669\ \mu\text{g/ml}) \\ \text{Chl } b\ (\mu\text{g/ml}) &\approx 18.9775 \times A_{647} - 4.8950 \\ &\quad \times A_{664} (\pm 0.006935\ \mu\text{g/ml}) \end{aligned} \qquad (5a, b)$$

The inherent errors of the spectrophotometric equations for Chl *a* and *b* are both less than 0.01 μg/ml. These errors can be taken as the lower detection limits for both chlorophylls in plants containing Chl *a* and *b*. Equations for all the chlorophylls in the different solvents can be written out from the data in Table 2A, B and C as for Eqs. (5a, b).

Table 3A and B are comparisons of Chl *a* and *b* determinations made using the published chlorophyll algorithms with the algorithms determined in the present study. The values in Table 3A were based on sets of 12 replicate extracts diluted with acetone,

Table 1 Red absorption peaks (Q_y) for chlorophylls in acetone, methanol, ethanol and diethyl ether

	Chl *a*	Chl *b*	Chl *c* (c_2, $c_1 + c_2$)	Chl *d*
90% acetone	664[a]	647[a]	630[a]	691
100% methanol	665[b]	652[b]	632	696[d,e,f]
100% ethanol	665[c]	649[c]	629	696
100% Diethyl ether	663[e]	643[e]	628[e]	688[e]

Sources: [a]Jeffrey and Humphrey (1975); [b]Porra et al. (1989), Porra (1991, 2002); [c]Rowan (1989); [d]Manning and Strain (1943), [e]French (1960) and [f]Miyashita et al. (1997)

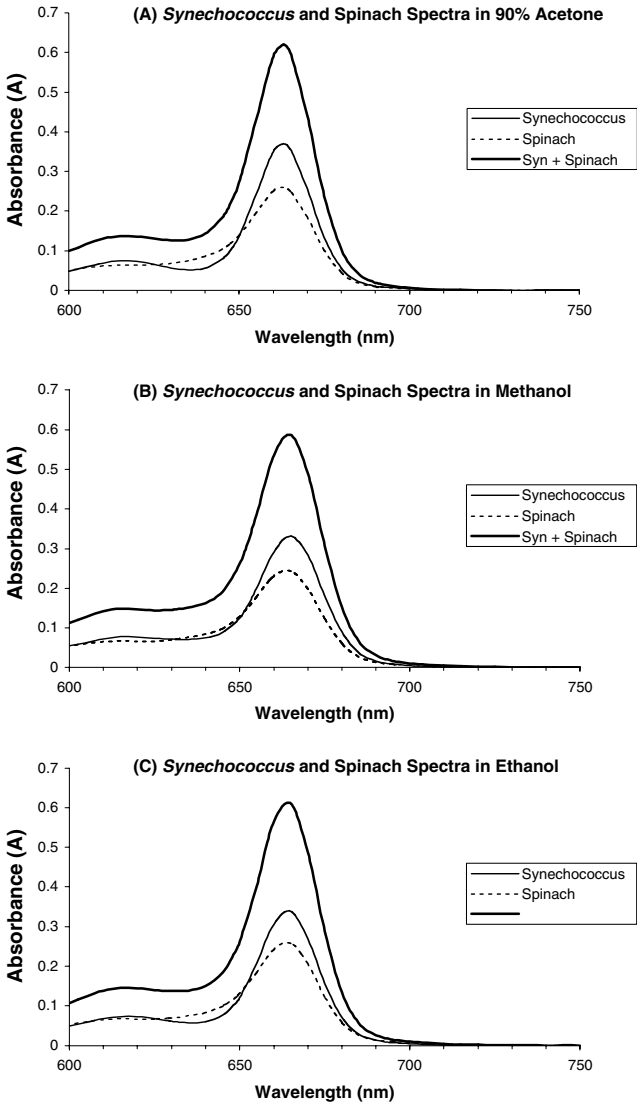

Fig. 1 Spectra of solvent extracts from *Synechococcus* and spinach and a spectrum of the two extracts combined. All samples were made up from two concentrated extracts of pigments in a 1:1:1 mixture of 90% acetone, 100% methanol and 100%(99.5%) ethanol. 50 μl of pigment extract was added to make up to 1.0 ml of solvent: no sample was contaminated with more than 6% of foreign solvents

methanol and ethanol prepared from a concentrated extract from spinach plants adapted to dim light. Table 3B were based on 12 replicate extracts prepared from spinach grown in sunlight. The algorithms determined in the present study yield similar estimates of Chl *a*, *b* and *b/a* ratios (errors calculated as described in Appendix 1) in acetone, methanol and ethanol. These estimates are not significantly different to those calculated from the Jeffrey–Humphrey formulae for acetone solvent. The methanol formulae from Porra et al. (1989), Porra (1991, 2002) and the ethanol formulae from Rowan (1989) give very similar estimates of Chl *a* as the other formulae but both their formulae seem to overestimate Chl *b* and hence give significantly higher Chl *b/a* ratios.

The Chl *a* and c_2 equations are based on 336 sets of spectrophotometric readings including some on the dinoflagellates *Heterocapsa pygmae* (20) and *Symbiodinium* sp. (32) but most are based on extracts from *Synechococcus R-2*, *Rhodomonas spN23* or combinations of the two. Measurements were usually made in sets of 12 as described above for the development of the Chl *a* and *b* equations. Ten sets of 12 determinations on *Rhodomonas spN23* in each solvent were included in the analysis.

Algorithms with two terms (at the absorption peaks for Chl *a* and c_2) gave acceptable estimates of Chl *a* and c_2 (Tables 2A, B, C). The coefficients determined by least squares fitting in acetone are very similar to those of Jeffrey and Humphrey (1975). All the experimentally determined inherent errors (and hence the detection limits) of the spectrophotometric equations for Chl *a* and c_2 are less than 0.1 μg/ml. The original algorithm of Humphrey and Jeffrey (1975) is likely to have a similar inherent error in its Chl c_2 algorithm (Appendix 3).

Table 2 Coefficients (E_λ) for spectrophotometric equations for organisms with chlorophyll *a*, *a* and *b*, *a* and c_2, *a* and $c_1 + c_2$, *a* and *d*

Organism	Chlorophyll	630 nm	647 nm	664 nm	691 nm	±95% CL, Detection limit (μg/ml)
A: 90% Acetone						
Cyanobacteria[a]	*a*			11.4062		0.0005343
Chlorophytes, bryophytes	*a*		–1.7858	11.8668		0.0006687
and vascular plants	*b*		18.9775	–4.8950		0.006935
Cryptophytes, dinophytes	*a*	–0.4574		11.4754		0.03879
	c_2	23.3900		–3.5322		0.0270
Diatoms and phaeophytes	*a*	–0.4504		11.4902		0.01154
	$c_1 + c_2$	22.6792		–3.4041		0.02434
Acaryochloris marina	*a*			11.4711	–1.6841	0.02787
	d			–0.1372	10.9660	0.05077
B: Methanol						
Organism	Chlorophyll	632 nm	652 nm	665 nm	696 nm	±95% CL, Detection limit (μg/ml)
Cyanobacteria[a]	*a*			12.9447		0.03234
Chlorophytes, bryophytes	*a*		–8.0962	16.5169		0.04696
and vascular plants	*b*		27.4405	–12.1688		0.05776
Cryptophytes, dinophytes	*a*	–3.4551		13.6849		0.05646
	c_2	32.9371		–7.0140		0.04146
Diatoms and phaeophytes	*a*	–2.6839		13.2654		0.1097
	$c_1 + c_2$	28.8191		–6.0138		0.02500
Acaryochloris marina	*a*			13.0161	-3.1022	0.005536
	d			–0.3270	12.9367	0.007669
C: Ethanol						
Organism	Chlorophyll	629 nm	649 nm	665 nm	696 nm	±95% CL, Detection limit (μg/ml)
Cyanobacteria[a]	*a*			11.9035		0.01888
Chlorophytes, bryophytes	*a*		–5.2007	13.5275		0.03125
and vascular plants	*b*		22.4327	–7.0741		0.02623
Cryptophytes, dinophytes	*a*	–2.6094		12.4380		0.05180
	c_2	29.8208		–5.6461		0.07523
Diatoms and phaeophytes	*a*	–1.4014		12.1551		0.08072
	$c_1 + c_2$	27.2641		–5.0212		0.07074
Acaryochloris marina	*a*			11.9780	–2.3238	0.02934
	d			–0.2006	12.0995	0.1148

[a]Chlorophyll *a* formula calculated from the *Chl a* and *b* algorithms assuming *Chl b* = 0. The absorbance coefficients have the dimensions mg l^{-1} cm A^{-1} because the absorbance readings have units of A cm^{-1}

Table 3 Comparison of chlorophyll *a* and *b* assays on spinach using published chlorophyte and vascular plant chlorophyll equations

	Published algorithms (μg ml^{-1})			Algorithms from the present study (μg ml^{-1})		
	Chl *a*	Chl *b*	Chl *b/a*	Chl *a*	Chl *b*	Chl *b/a*
A: Chlorophyll a and b assays on shade-adapted spinach						
90% acetone[a]	5.7696 ± 0.1547	2.9086 ± 0.2249	0.5039 ± 0.0413	5.7774 ± 0.0969	2.8337 ± 0.0755	0.4903 ± 0.0154
100% methanol[b]	5.4193 ± 0.6154	3.3192 ± 1.147	0.6124 ± 0.2227	5.6821 ± 0.1150	2.9632 ± 0.0720	0.5214 ± 0.0198
100% ethanol[c]	5.6560 ± 0.1545	3.6753 ± 0.2719	0.6498 ± 0.0512	5.7320 ± 0.0533	2.9627 ± 0.0420	0.5169 ± 0.00876
B: Comparison of chlorophyll a and b assays on sun-adapted spinach						
90% acetone[a]	9.3982 ± 0.1520	2.9827 ± 0.2700	0.3174 ± 0.0307	9.3987 ± 0.0538	2.9766 ± 0.0142	0.3167 ± 0.00236
100% methanol[b]	8.8522 ± 0.6095	3.5386 ± 1.144	0.3998 ± 0.1322	9.2365 ± 0.0856	3.1554 ± 0.0641	0.3417 ± 0.00763
100% ethanol[c]	9.0328 ± 0.1520	3.8577 ± 0.2700	0.4271 ± 0.0307	9.1107 ± 0.0448	2.9984 ± 0.0328	0.3291 ± 0.00395

Sources: [a]Jeffrey and Humphrey (1975); [b]Porra et al. (1989) and Porra (1991, 2002); [c]Rowan (1989). The error-bars quoted in Table 3 include the error calculated between replicate samples (n = 12) and the inherent errors of the chlorophyll algorithms (see Appendix 3)

Using a data set of 12 scans on matched Chl $a + c_2$ extracts in acetone, methanol and ethanol, Table 4 shows that the algorithms determined in the present study yield similar estimates of Chl *a*, c_2 and c_2/a ratios (errors calculated as described in Appendix 1). These estimates are not significantly different to those cal-

Table 4 Comparison of chlorophyll *a* and c_2 assays on the Cryptophyte, *Rhodomonas spN23*, using published chlorophyll equations and from the present study

	Published algorithms (μg ml^{-1})			Algorithms from the present study (μg ml^{-1})		
	Chl *a*	Chl c_2	Chl c_2/a	Chl *a*	Chl c_2	Chl c_2/a
90% acetone[a]	4.9577 ± 0.1347	1.0737 ± 0.2555	0.2164 ± 0.05183	4.9712 ± 0.08116	1.0270 ± 0.04989	0.2064 ± 0.0106
100% methanol	NA	NA	NA	4.9761 ± 0.09081	1.2129 ± 0.0728	0.2437 ± 0.0153
100% ethanol	NA	NA	NA	5.0755 ± 0.05630	1.1310 ± 0.07679	0.2234 ± 0.0153

Sources: [a]Jeffrey and Humphrey (1975). The error-bars quoted in this table include the error calculated between replicate samples (n = 12) and the inherent errors of the chlorophyll algorithms (see Appendix 3). No methanol or ethanol formulae for Chl *a* and c_2 appear to have been published

culated from the Jeffrey-Humphrey formulae for the acetone solvent system.

The Chl *a* and (c_1 + c_2) equations are based on 412 sets of spectrophotometric readings on extracts from *Synechococcus R-2*, *Phaeodactylum* sp. or combinations of the two. Measurements were usually made in sets of 12 as described above for the development of the Chl $a + b$ equations. In total of 15 sets of 12 determinations on *Phaeodactylum* sp. in each solvent were included in the analysis.

The algorithms for Chl *a* and (c_1 + c_2) were the most difficult to determine because only small amounts of Chl (c_1 + c_2) were present in *Phaeodactylum* sp. Initially an algorithm with three absorbance terms was needed to consistently estimate Chl *a* and Chl c_1 + c_2. After more than 200 measurements had been made, a simpler algorithm with only two terms, at the absorption peaks for Chl *a* and c_1 + c_2, gave estimates of Chl *a* and c_1 + c_2 that were consistent between the three different solvents (Table 2A, B, C). The inherent errors (and hence the detection limits) of the spectrophotometric equations for Chl *a* and (c_1 + c_2) are less than 0.11 μg/ml.

A matched data set of 12 scans on chlorophyll extracts in acetone, methanol and ethanol was used to test the Chl *a* and c_1 + c_2 algorithms. Table 5 shows that the two-factor algorithms determined in the present study yield similar estimates of Chl *a*, (c_1 + c_2) and Chl (c_1 + c_2)/*a* ratios (errors calculated as described in Appendix 1) in acetone, methanol and ethanol. These estimates are not significantly different to those calculated from the Jeffrey-Humphrey formulae for the acetone solvent system.

The Chl *a* and *d* equations are based on 436 sets of spectrophotometric readings based on extracts from *Synechococcus* sp., *Acaryochloris marina* or combinations of the two. A set of 12 readings on a preparation of pure Chl *d* in acetone, methanol and ethanol solvents is included in the data set. Measurements were usually made in sets of 12 as described above for the development of the Chl $a + b$ equations. In total 13 sets of 12 determinations on *Acaryochloris marina* in each solvent were included in the analysis.

Algorithms with two terms, at the absorption peaks for Chl *a*, *d*, were necessary to give estimates of Chl *a* and *d* (Table 2A, B, C). Estimates of Chl *a* and *d* in extracts from *Acaryochloris marina* were calculated as described in Theory. All the inherent errors (which can also serve as the detection limits) of the spectrophotometric equations for Chl *a* and *d* are both less than 0.1 μg/ml.

The diethyl ether equations for Chl *a* and *d* modified from Smith and Benitez (1955) and French (1960) were used to obtain estimates of Chl *a* and *d* in extracts from *Acaryochloris marina*. These were compared to estimates based on the equations calculated in the present study for acetone, methanol and ethanol solvents (Table 2A, B, C). A concentrated extract of Chl *a* and *d* was used to make up a matched set of 12 replicate

Table 5 Comparison of chlorophyll *a* and c_1 + c_2 assays on the diatom, *Phaeodactylum* sp., using published chlorophyll equations and from the present study

	Published algorithms (μg ml^{-1})			Algorithms from the present study (μg ml^{-1})		
	Chl *a*	Chl c_1 + c_2	Chl (c_1 + c_2)/*a*	Chl *a*	Chl c_1 + c_2	Chl (c_1 + c_2)/*a*
90% acetone[a]	12.3414 ± 0.1424	1.2831 ± 0.2469	0.1040 ± 0.02005	12.3522 ± 0.08519	1.2688 ± 0.02855	0.1027 ± 0.00242
100% methanol	NA	NA	NA	12.2621 ± 0.1176	1.2289 ± 0.02931	0.1002 ± 0.00258
100% ethanol	NA	NA	NA	12.3949 ± 0.09383	1.1350 ± 0.07336	0.0916 ± 0.00596

Sources: [a]Jeffrey and Humphrey (1975). The error-bars quoted in this table include the error calculated between replicate samples (n = 12) and the inherent errors of the chlorophyll algorithms (see Appendix 3). No methanol or ethanol algorithms for Chl *a* and c_1 + c_2 appear to have been published

diethyl ether, acetone, methanol and ethanol samples. It is difficult to do quantitative spectrophotometry using diethyl ether because its volatility makes it difficult to dispense in accurate volumes. In this study, the actual volumes of ether were calculated from measurements of the weight of added ether converted to volume. The acetone, methanol and ethanol data were used to calculate Chl *a* and *d* using the absorbance coefficients shown in Table 2. Table 6 shows that the algorithms determined in the present study, using the non-linear least squares fitting methods, yield similar estimates of Chl *a*, *d* and *d/a* ratios (errors calculated as described in Appendix 1) in acetone, methanol and ethanol with acceptable levels of error. The estimate of Chl *a* for this extract of pigments from this particular batch of *Acaryochloris marina* is slightly lower in acetone than in the other solvents. The Chl *a*, *d* and *d/a* ratios in methanol and ethanol are very similar to estimates based on the equations for diethyl ether in Appendix 3.

Discussion

This study has shown that it is possible to determine algorithms for the spectrophotometric determination of chlorophylls in acetone, methanol and ethanol that give estimates of Chl *a, b,* c_2, $c_1 + c_2$ and *d* that are consistent with each other and where inherent errors can be calculated and taken into account. The error analysis in Appendix 1 shows that multiple linear equations for chlorophylls have the inherent limitation that the inherent errors of chlorophylls are constant, independent of the magnitude of the absorbance readings. Thus, the more dilute the chlorophyll solution the greater the relative error: this effect is most pronounced for the accessory chlorophylls. The relative error of measurements of chlorophylls, particularly minor chlorophylls, increases hyperbolically in diluted chlorophyll extracts. The statistical limitations of chlorophyll determinations are generally ignored. This is unfortunate, because important information about the limits of precision and accuracy of chlorophyll determinations and chlorophyll ratios is lost.

Chlorophyll **c** compounds (c_1, c_2, c_3 and related chlorophyll *c*-like pigments such as *MgDVP*) (Jeffrey et al. 1997) are minor pigments, usually constituting less than 20% of total chlorophyll in algae where they occur. They are well known to be difficult to assay in chlorophyll extracts because their absorption peaks in the red part of the spectrum are low compared to other chlorophylls. Evidence for their presence in mixed chlorophyll extracts tends to be drowned out by the absorbance of Chl *a*. Jeffrey and Humphrey (1975) pointed out in their original paper that their equations for Chl c_2 and $c_1 + c_2$ had increasing error as the abundance of the Chl ***c*** compounds decreased with reference to Chl *a*. The chlorophyll **c** compounds became harder to detect. Humphrey and Jeffrey (1997) tested their chlorophyll equations, originally published in Jeffrey and Humphrey (1975), on a wide range of mixtures of chromatographically pure chlorophylls. They confirmed that the Chl *a* and *b* equations were highly accurate for both Chl *a* and *b* but the Chl *a* and c_2 and the Chl *a* and $(c_1 + c_2)$ overestimated the chlorophyll **c** compounds when their abundance was 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 Mantoura et al. (1997). This problem has been partially corrected for in the present study by including chlorophyll extracts containing only Chl *a* and samples of chlorophyll mixtures diluted with a known amount of Chl *a* in the derivation of the algorithms.

Comparison of the accuracy of the algorithms has shown that the methanol formulae of Porra et al. (1989) and Porra (1991, 2002) and the ethanol formulae of Rowan (1989) for Chl $a + b$ do not give estimates of Chl *a* and *b* that are consistent with those obtained using the algorithms determined in the present study or with the acetone formulae of Jeffrey and Humphrey (1975) (Table 3A, B). The inherent errors

Table 6 Chlorophyll *a* and *d* assays on the unusual Cyanophyte, *Acaryochloris marina*

	Chlorophyll assay (μg ml^{-1})		
	Chl *a*	Chl *d*	Chl *d*/*a*
90% acetone	0.5199 ± 0.0290	16.4188 ± 0.1606	31.5942 ± 1.787
100% methanol[a]	0.5838 ± 0.00781	16.7616 ± 0.1158	28.7138 ± 0.4322
100% ethanol	0.6037 ± 0.0297	16.8392 ± 0.1519	27.8928 ± 1.394
100% diethyl ether[b]	0.6116 ± 0.1002	16.8320 ± 0.1174	27.527 ± 4.512

Sources: [a]Algorithm used a Chl *a* extinction coefficient calculated from Table 2B, [b]Chlorophyll *a* and *d* estimated using equations published by Smith and Benitez (1955) and later by French (1960), refer to Appendix 3. The error-bars quoted in this table include the error calculated between replicate samples ($n = 12$) and the inherent errors of the chlorophyll algorithms (see Appendix 3)

in the methanol equations of Porra are large and so his equations would not give satisfactory estimates of the chlorophylls in diluted samples (see Table 3A, B and Appendix 3). Mantoura et al. (1997) showed that the methanol equations of Porra et al. (1989) and Porra (1991, 2002) gave spurious values for Chl *b* in algae where it is known that Chl *b* does not occur (for example, *Phaeodactylum* sp. and *Amphidinium* sp.). Table 14.4 in Mantoura et al. (1997) lists a number of organisms known to contain Chl *a* and *b* but their chlorophylls were assayed using acetone or methanol algorithms by different researchers. The Chl *b/a* ratios obtained were considerably different.

Hu et al. (1998) found using HPLC that the Chl *d/a* ratio of *Acaryochloris marina* grown in bulk cultures in dim light (25 μE m^{-2} s^{-1}) (PAR) was 30 ± 4. This value is similar to that found in the present study (about 18–30) where higher light intensities were used for growing the alga. Miyashita et al. (1996, 1997) found Chl *d/a* ratios of about 10.9–37, again consistent with those found in the present study.

Acaryochloris marina in nature lives commensally (Kuhl et al. 2005), along with the obligate symbiont *Prochloron didemni* (Lewin) (Larkum et al. 1994), in didemnid ascidians (Miyashita et al. 1997, 2003). Kuhl et al. (2005) proposed that the use of Chl *d* as a primary photosynthetic pigment in *Acaryochloris marina* was an adaptation to growth in dim light deficient in wavelengths shorter than 680 nm due to the growth of colonies of *Prochloron didemni* situated above where *Acaryochloris marina* grows inside the basal tissues of the ascidian. Experience with growing *Acaryochloris marina* in culture has shown that the relative amounts of Chl *a* and *d* varies considerably according to the age of the culture and the lighting conditions. However, this needs to be systematically investigated. Free-living cyanobacteria containing Chl *d* have also been found recently in an artificial hypersaline lake (Salton Sea, USA, Miller et al. 2005) and as epiphytes (species specific commensals or symbionts?) on red algae (Murakami et al. (2004). It appears likely that the Salton Sea population is an accidental introduction resulting from marine fish stocking experiments conducted in the Salton Sea when it was first created in the early 20th century.

Chlorophyll *d* was originally found in extracts from certain red algae (Manning and Strain 1943). Later studies found that its occurrence was inconsistent which lead to the suggestion that Chl *d* was only an artefact of the chlorophyll extraction process. Reports of Chl *d* in organisms tended to be dismissed until Miyashita et al. (1996) found that in *Acaryochloris marina* Chl *d* was the dominant photosynthetic pigment and could no longer be dismissed as an artefact of extraction. The existence Chl *d* in vivo was questioned as late as 1991 (Scheer 1991).

There are a number of problems in accurately determining Chl *d* that require further work. Chlorophyll *d* is considerably less stable than Chl *a*. Manning and Strain (1943), confirmed by Nieuwenburg et al. (2003), point out that Chl *d* in methanol slowly isomerises to form isochlorophyll *d*, which has an absorption peak at 661 nm (near the red peak for Chl *a*). Manning and Strain (1943) reported an approximately 25% increase in absorbance at 661 nm after storage of samples in methanol for 9 days. They reported that exclusion of oxygen had no effect on this isomerisation process. Chlorophyll *d* appeared to be stable in the acetone/methanol/ethanol extraction cocktail used in the present study if kept in the dark at –20°C. If substantial isomerisation had occurred during storage it would have been apparent as a spurious increase in the apparent amount of Chl *a* present in extracts from *Acaryochloris marina* after storage for several days.

Another potential problem with the Chl *a* and *d* algorithms developed in the present study is that they are ultimately based on the extinction coefficient for pure Chl *d* in diethyl ether determined by Smith and Benitez (1955). More modern determinations may be needed. Nevertheless, the extinction coefficients used by Smith and Benitez (1955) to develop their Chl *a* and *d* algorithms for diethyl ether were carefully chosen: the extinction coefficient at 663 nm they used for *Chl a* is within ± 1% of modern values (Porra 2002). Unfortunately the spectroscopic scans they used do not appear to be accurate by modern standards (see Theory).

Murakami et al. (2004) suggested that the irregular detection of Chl *d* in extracts from red algae was due to the presence of epiphytic chlorophyll *d*-containing oxyphotobacteria. It appears that chlorophyll *d*-containing oxygenic photobacteria are likely to be much more widespread in nature than the three examples currently known, particularly under light regimes depleted in red light in the visible part of the spectrum. Exploratory searches for the presence of Chl *d* in such habitats are likely to discover some interesting oxygenic phototrophs.

One of the reasons why there is some hesitancy in using alternative solvents to acetone is the problem of chlorophyllase degradation of chlorophylls. Chlorophyllase (EC 3.1.1.14) hydrolyses the phytol chain off phytollated chlorophylls (Chl *a, b* and *d*) to produce the corresponding chlorophyllides (Matile et al. 1999). It is a very unusual class of enzyme because they are activated by the presence of organic solvents such as

acetone, methanol and ethanol, their pH optima vary considerably from 6 to 8.5 and often retain 30% or more of maximum activity at 0°C (BRENDA database 2005). Chlorophyllases are inhibited in acetone if the organic solvent is maintained above 40% v/v (BRENDA database 2005) but substantial activity can occur in even 95% ethanol or methanol forming methyl and ethyl chlorophyllides (Hynninen 1991). Like other proteins, chlorophyllases are not soluble in organic solvents. Chlorophyllase can easily be removed by filtration or centrifugation. Chlorophyllase is also easily denatured by heating above 60°C. Soaking of plants in solvents for long periods as an extraction technique should be avoided unless some treatment to inactivate chlorophyllases has been used. In the present study, there was little or no significant degradation of Chl *a* stored in acetone/methanol/ethanol at –20°C (after clearing by centrifugation) for 7 days.

A set of chlorophyll equations that give consistent estimates of chlorophylls in acetone, methanol and ethanol has long been needed to allow for comparable estimates of chlorophylls to be possible in a variety of solvents. Acetone is an inconvenient solvent for routine work and often proves to be an inefficient extractant of chlorophylls. Ethanol based chlorophyll extraction and assay is much more suited to the teaching laboratory and for routine assays and poses no significant solvent disposal problems. The use of ethanol also offers the convenience of being able to use polystyrene cuvettes. The methanol algorithms would be convenient for assays associated with HPLC work.

Acknowledgements The author wishes to thank Dr Min Chen for her kind gift of preparations of pure Chlorophyll *d* and for providing advice on growing *Acaryochloris marina* in culture. The author wishes to thank Dr George F Humphrey for his critical reading of the manuscript. Access to facilities in the laboratory of Prof AWD Larkum and Dr RG Quinnell is also gratefully acknowledged.

Appendices

Appendix 1: 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 $\pm A$, $\pm B$, $\pm C$ and $\pm D$, the asymptotic error ($\pm Z$) is,

$$\pm Z^2 \approx \left(\frac{\mathrm{d}Z}{\mathrm{d}v}\right)^2 \pm A^2 + \left(\frac{\mathrm{d}Z}{\mathrm{d}w}\right)^2 \pm B^2 + \left(\frac{\mathrm{d}Z}{\mathrm{d}x}\right)^2 \pm C^2 + \left(\frac{\mathrm{d}Z}{\mathrm{d}y}\right)^2 \pm D^2$$

since $\frac{\mathrm{d}z}{\mathrm{d}_v}, \frac{\mathrm{d}z}{\mathrm{d}_w}, \frac{\mathrm{d}z}{\mathrm{d}_x}$ & $\frac{\mathrm{d}z}{\mathrm{d}_y} = 1$,
$\pm Z \approx \sqrt{\pm A^2 + \pm B^2 + \pm C^2 + \pm D^2}$ (Note that the error is independent of absorbance readings v,w,x or y).

For example, for a spectrophotometric equation for Chl *a* using absorbancesat two wavelengths, A_{630} and A_{664} and calculated absorbance coefficients E_{630} and E_{664},

$$\text{Chl } a\ (\mu\text{g/ml}) = A_{630}.E_{630} + A_{664}.E_{664},$$
$$\pm \text{Chl } a\ (\mu\text{g.ml}) \approx \sqrt{\pm E_{630}^2 + \pm E_{664}^2}.$$

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 ± B and ± A, the error is approximately, $\pm Z \approx \sqrt{\left(\frac{\mathrm{d}Z}{\mathrm{d}A}\right)^2(\pm A)^2 + \left(\frac{\mathrm{d}Z}{\mathrm{d}B}\right)^2(\pm B)^2}$, which simplifies to $\pm Z \approx Z\sqrt{\left(\frac{\pm A}{A}\right)^2 + \left(\frac{\pm B}{B}\right)^2}$.

A Chl *b/a* ratio can therefore be expressed as,

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

Appendix 2: Matrix algebra

The example shown are for sets of spectrophotometric readings in acetone solvent where absorbances are measured at 630, 647, 664 and 691 nm which are the Q_y values are for Chl c_2 and Chl $c_1 + c_2$, b, a and d respectively. It can be shown that all the chlorophyll equations have the same solution matrix. For a chlorophyll equation based on absorbance readings at two wavelengths, Chl = $E_{647}.A_{647} + E_{663}.A_{664}$,

$$\begin{bmatrix} \sum_{i=1}^{N}\frac{\mathrm{dChl}^2}{\mathrm{d}A_{647}} & \sum_{i=1}^{N}\frac{\mathrm{dChl}}{\mathrm{d}A_{647}}\cdot\frac{\mathrm{dChl}}{\mathrm{d}A_{664}} \\ \sum_{i=1}^{N}\frac{\mathrm{dChl}}{\mathrm{d}A_{647}}\cdot\frac{\mathrm{dChl}}{\mathrm{d}A_{664}} & \sum_{i=1}^{N}\frac{\mathrm{dChl}^2}{\mathrm{d}A_{664}} \end{bmatrix}^{-1} = \begin{bmatrix} \mathrm{varE}_{647} & \mathrm{covE}_{647}.\mathrm{E}_{664} \\ \mathrm{covE}_{647}.\mathrm{E}_{664} & \mathrm{varE}_{664} \end{bmatrix}$$

for a chlorophyll equation requiring measurements at three wavelengths, $\mathrm{Chl} = E_{630}.A_{630} + E_{647}.A_{647} + E_{664}.A_{664}$,

$$\begin{bmatrix} \sum_{i=1}^{N} \frac{\mathrm{dChl}^2}{\mathrm{d}A_{630}} & \sum_{i=1}^{N} \frac{\mathrm{dChl}}{\mathrm{d}A_{630}} \cdot \frac{\mathrm{dChl}}{\mathrm{d}A_{647}} & \sum_{i=1}^{N} \frac{\mathrm{dChl}}{\mathrm{d}A_{630}} \cdot \frac{\mathrm{dChl}}{\mathrm{d}A_{664}} \\ \sum_{i=1}^{N} \frac{\mathrm{dChl}}{\mathrm{d}A_{647}} \cdot \frac{\mathrm{dChl}}{\mathrm{d}A_{630}} & \sum_{i=1}^{N} \frac{\mathrm{dChl}^2}{\mathrm{d}A_{647}} & \sum_{i=1}^{N} \frac{\mathrm{dChl}}{\mathrm{d}A_{647}} \cdot \frac{\mathrm{dChl}}{\mathrm{d}A_{664}} \\ \sum_{i=1}^{N} \frac{\mathrm{dChl}}{\mathrm{d}A_{664}} \cdot \frac{\mathrm{dChl}}{\mathrm{d}A_{630}} & \sum_{i=1}^{N} \frac{\mathrm{dChl}}{\mathrm{d}A_{664}} \cdot \frac{\mathrm{dChl}}{\mathrm{d}A_{647}} & \sum_{i=1}^{N} \frac{\mathrm{dChl}^2}{\mathrm{d}A_{664}} \end{bmatrix}^{-1} = \begin{bmatrix} \mathrm{varE}_{630} & \mathrm{covE}_{630}.\mathrm{E}_{647} & \mathrm{covE}_{630}.\mathrm{E}_{664} \\ \mathrm{covE}_{630}.\mathrm{E}_{647} & \mathrm{varE}_{647} & \mathrm{covE}_{647}.\mathrm{E}_{664} \\ \mathrm{covE}_{630}.\mathrm{E}_{664} & \mathrm{covE}_{647}.\mathrm{E}_{664} & \mathrm{varE}_{664} \end{bmatrix}$$

Appendix 3: Published chlorophyll formulae used in the present study

Authority	Solvent	Chlorophyll	Formulae (μg ml^{-1})	Inherent error* (μg ml^{-1})
Humphrey and Jeffrey (1975)*	90% acetone	*a*	$11.93 \times A_{664} - 1.93 \times A_{647}$	0.1209
		b	$-5.5 \times A_{664} + 20.36 \times A_{647}$	0.2108
		a	$11.43 \times A_{664} - 0.40 \times A_{630}$	0.1144
		c_2	$-3.80 \times \mathrm{A}_{664} + 24.88 \times A_{630}$	0.2516
		a	$11.47 \times A_{664} - 0.40 \times A_{630}$	0.1148
		$c_1 + c_2$	$-3.73 \times A_{664} + 24.36 \times A_{630}$	0.2464
Porra et al. (1989), Porra (1991, 2002)**	100% methanol	*a*	$16.29 \times \mathrm{A}_{665} - 8.54 \times \mathrm{A}_{652}$	0.6056
		b	$-13.58 \times A_{665} + 30.66 \times A_{652}$	1.1438
Rowan (1989)*	100% ethanol	*a*	$13.70 \times \mathrm{A}_{665} - 5.76 \times \mathrm{A}_{649}$	0.1486
		b	$-7.60 \times A_{665} + 25.8 \times A_{649}$	0.2690
Modified from Smith and Benitez (1955)*	100% diethyl ether	*a*	$9.92 \times A_{663} - 1.15 \times A_{688}$	0.0999
		d	$-0.166 \times A_{663} + 9.09 \times A_{688}$	0.0909

The equations of Smith and Benitez (1955) have been recalculated using the following extinction values: $\varepsilon_{\mathrm{Chl\ a},663} = 101$ l g^{-1} A cm^{-1}, $\varepsilon_{\mathrm{Chl}\ a,688} = 1.848$ l g^{-1} A cm^{-1}, $\varepsilon_{\mathrm{Chl}\ d,663} = 12.83$ l g^{-1} A cm^{-1}, $\varepsilon_{\mathrm{Chl}\ d,663} = 110.23$ l g^{-1} A cm^{-1}). *Inherent errors calculated on the assumption that the relative errors of the absorbance coefficients were ± 1.0%. **Inherent errors quoted here are ± 2 × standard deviations of the extinction values found by Porra et al. (1989). The acetone equations for *Chl a* and *b* by Porra et al. (1989) are for 80% acetone and so have not been included in the present study.

References

Akiyama M, Miyashita H, Kise H, Watanabe T, Miyachi S, Yobayashi M (2001) Detection of chlorophyll *d′* and pheophytin a in a chlorophyll *d* – dominating oxygenic photosynthetic prokaryote, *Acaryochloris marina*. Anal Sci 17:205–208

Allen MM (1973) Methods for cyanophyceae. In: Stein JR (ed) Handbook of phycological methods: culture methods and growth measurements. Cambridge University Press, Cambridge, pp 127–138

Arnon DI (1949) Copper enzymes in isolated chloroplasts: polyphenoloxidase in *Beta vulgaris*. Plant Physiol 24:1–15

Atwell BJ, Kreidermann PE, Turnbull CGN (1999) Plants in action. MacMillan Education Australia, South Yarra, Australia

BRENDA (2005) Brenda: the comprehensive enzyme information system. Retrieved from [http://www.brenda.uni-koeln.de/index.php4 on 13 July 2005

French CS (1960) The chlorophyll *in vivo* and *in vitro*. In: Ruhland W (ed) Encyclopedia of plant physiology, vol 5/1. Springer-Verlag Publ., Berlin, pp 252–297

Hu Q, Miyashita H, Iwasaki I, Kurano N, Miyachi S, Iwaki M, Itoh S (1998) A photosystem I reaction center driven by chlorophyll *d* in oxygenic photosynthesis. Proc Nat Acad Sci USA 95:13319–13323

Humphrey GF, Jeffrey SW (1997) Test of accuracy of spectrophotometric equations for the simultaneous determination of chlorophylls *a*, *b*, c_1 and c_2. In: Jeffrey SW, Mantoura RFC, Wright SW (eds) Phytoplankton pigments in oceanography: guidelines to modern methods. UNESCO Publ., Paris, pp 616–621

Hynninen PH (1991) 1.7 Chemistry of chlorophylls: modifications. In: Scheer H (ed) Chlorophylls. CRC Press, Boca Raton-Ann Arbor-Boston-Landon pp 145–209

Jeffrey SW, Humphrey GF (1975) New spectrophotometric equations for determining chlorophyll *a, b,* c_1 and c_2 in higher plants and natural phytoplankton. Bioch Physiol Pflanz (BPP) 165:191–194

Jeffrey SW, Mantoura RFC, Bjornland T (1997) Data for the identification of 47 key phytoplankton pigments. In: Jeffrey SW, Mantoura RFC, Wright SW (eds)Phytoplankton pigments in oceanography: guidelines to modern methods. UNESCO Publ., Paris, pp 449–559

Jeffrey SW, Mantoura RFC, Wright SW (eds) (1997) Phytoplankton pigments in oceanography: guidelines to modern methods. UNESCO Monographs on Oceanographic Methodology, vol 10. UNESCO Publishing, Paris

Jeffrey SW, Welschmeyer NA (1997) Spectrophotometric and fluorometric equations in common use in oceanography. In: Jeffrey SW, Mantoura RFC, Wright SW (eds) Phytoplankton pigments in oceanography: guidelines to modern methods. UNESCO Publ., Paris, pp 597–621

Jeffrey SW, Wright SW (1997) Qualitative and quantitative HPLC analysis of SCOR reference culture collections. In: Jeffrey SW, Mantoura RFC, Wright SW (eds) Phytoplankton pigments in oceanography: guidelines to modern methods. UNESCO Publ., Paris, pp 343–360

Johnson ML, Faunt LM (1992) Parameter estimation by least squares methods. Methods Enzymol 210:1–37

Kuhl M, Min Chen, Ralph PJ, Schreiber U, Larkum AWD (2005) A niche for cyanobacteria containing chlorophyll *d*. Nature 433:820

Larkum AWD, Scaramuzzi C, Cox GC, Hiller RG, Turner AG (1994) Light harvesting chlorophyll *c*-like pigment in *Prochloron*. Proc Nat Acad Sci USA 91:679–683

Lichtenthaler HK (1987) Chlorophylls and carotenoids: pigments of photosynthetic membranes. Method Enzymol 148:350–382

Lichtenthaler HK, Wellburn AR (1983) Determinations of total carotenoids and chlorophylls *a* and *b* of leaf extracts in different solvents. Biochem Soc Trans 11:591–592

Manning WM, Strain HH (1943) Chlorophyll *d*, a green pigment of red algae. J Biol Chem 151:1–19

Mantoura RFC, Jeffrey SW, Llewellyn CA, Claustre H, Morales CE (1997) Comparison between spectrophotometric, fluorometric HPLC methods for chlorophyll analysis. In: Jeffrey SW, Mantoura RFC, Wright SW (eds) Phytoplankton pigments in oceanography: guidelines to modern methods. UNESCO Publ., Paris, pp 361–380

Matile P, Hortensteiner S, Thomas H (1999) Chlorophyll degradation. Ann Rev Plant Physiol Plant Mol Biol 50:67–95

McLachlan J (1973) Growth media – marine. In: Stein JR (ed) Handbook of phycological methods: culture methods and growth measurements. Cambridge University Press, Cambridge, pp 25–51

Miller SR, Augustine S, Olson TL, Blankenship RE, Selker J, Wood AM (2005) Discovery of a free-living chlorophyll *d*-producing cyanobacterium with a hybrid proteobacterial/cyanobacterial small-subunit rRNA gene. Proc Nat Acad Sci USA 102:850–855

Miyashita H, Ikemoto H, Kurano N, Adachi K, Chihara M, Miyachi S (1996) Chlorophyll *d* as a major pigment. Nature 383:402

Miyashita H, Adachi K, Kurano N, Ikemoto H, Chihara M, Miyachi S (1997) Pigment composition of a novel oxygenic photosynthetic prokaryote containing chlorophyll d as the major chlorophyll. Plant Cell Physiol 38:274–281

Miyashita H, Ikemoto H, Kurano N, Miyachi S, Chihara M (2003) *Acaryochloris marina* gen. et. sp. nov. (Cyanobacteria), an oxygenic photosynthetic prokaryote containing *Chl d* as a major pigment. J Phycol 39:1247–1253

Murakami A, Miyashita H, Iseki M, Adachi K, Mimuro M (2004) Chlorophyll *d* in an epiphytic cyanobacterium of red algae. Science 303: 1633

Nieuwenburg P, Clarke RJ, Cai Z-L, Min Chen, Larkum AWD, Cabral NM, Ghiggino KP, Reimers JR (2003) Examination of the photophysical processes of Chlorophyll *d* leading to a clarification of proposed uphill energy transfer processes in cells of *Acaryochloris marina*. Photochem Photobiol 77:638–637

Papista E, Acs E, Boddi B (2002) Chlorophyll-*a* determination with ethanol – a critical test. Hydrobiologia 485:191–198

Porra RJ (1990) A simple method for extracting chlorophylls from the recalcitrant alga, *Nannochloris atomus*, without formation of spectroscopically-different magnesium-rhodochlorin derivatives. Biochim Biophys Acta 1019: 137–141

Porra RJ (1991) 1.2 recent advances and re-assessments in chlorophyll extraction and assay procedures for terrestrial, aquatic and marine organisms, including recalcitrant algae. In: Scheer H, (ed) Chlorophylls. CRC Press Boca Raton, Ann Arbor, Boston, Landon, pp 31–57

Porra RJ (2002) The chequered history of the development and use of simultaneous equations for the accurate determination of chlorophylls *a* and *b*. Photosynth Res 73:149–156

Porra RJ, Thompson WA, Kreidemann PE (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls *a* and *b* extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectrometry. Biochim Biophys Acta 975:384–394

Rowan KS (1989) Photosynthetic pigments of algae. Cambridge University Press, Cambridge

Sartory DP, Grobbelaar JU (1984) Extraction of chlorophyll *a* from freshwater phytoplankton for spectrophotometric analysis. Hydrobiologia 114:177–187

Scheer H (ed) (1991) Chlorophylls. CRC Press, Boca Raton, Ann Arbor, Boston, Landon

Smith JHC, Benitez A (1955) Chlorophylls: analysis of plant materials. In: Paech K, Tracey MV (eds) Modern methods of plant analysis, vol 4. Springer Publ., Berlin, pp 142–196

Straume H, Johnson ML (1992) Analysis of residuals: criteria for determining goodness-of-fit. Method Enzymol 210:87–105

Wright SW, Jeffrey SW (1997) High resolution HPLC system for chlorophylls and carotenoids of marine plankton. In: Jeffrey SW, Mantoura RFC, Wright SW (eds) Phytoplankton pigments in oceanography: guidelines to modern methods. UNESCO Publ., Paris, pp 327–341

Wright SW, Jeffrey SW, Mantoura FRC (1997) Evaluation of methods and solvents for pigment analysis. In: Jeffrey SW, Mantoura RFC, Wright SW (eds) Phytoplankton pigments in oceanography: guidelines to modern methods. UNESCO Publ., Paris, pp 261–282