Appendix F

Spectrophotometric and fluorometric equations in common use in oceanography

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F.1 Introduction

A number of spectrophotometric and fluorometric equations have been published since 1949 for the simultaneous determination of extracted chlorophylls *a* and *b*, *a* and *c*, and *a*, *b* and *c*.

Their accuracy depends on:

- the accuracy of the chlorophyll extinction coefficients used to derive the equations,
- the spectral purity of these chlorophylls (see Chapter 7 and the Part IV Data sheets).
- the solvents used: some solvents, such as diethyl ether, dimethyl formamide and acetone, give sharp maxima and enhance absorbance bands; others, such as

methanol, flatten maxima and depress absorption bands. The former are the most accurate.

- the presence of water extracted from cells which can alter peak positions in pure solvents and affect accuracy (Porra *et al.* 1989). For this reason 80 and 90% aqueous acetone are frequently used, as cell water will not significantly alter the already high aqueous concentration.
- the relative proportions and concentration of chlorophylls *a*, *b* and *c* in the extracts being analysed.

When applying these equations, there are other sources of inaccuracy:

- the presence of chlorophyll degradation products of similar spectral and fluorescence properties to those of the parent chlorophylls, which causes significant errors (see Section F.5 and Chapter 14). Aqueous buffer is frequently added to acetone to minimize pheophytin formation, particularly with higher plant tissues (cf. Porra *et al.* 1989).
- the precision of the spectrophotometers and fluorometers used for analysis.

The following sections discuss the spectrophotometric and fluorometric techniques that are used in oceanography. The sections on spectrophotometry (F.1–F.3) were written by S. W. Jeffrey and those on spectrophotometry (for pheopigment corrections) and fluorometry (sections F.4–F.7) by N. A. Welschmeyer.

F.2 Extinction coefficients and spectrophotometric equations

The extinction coefficients that have been used to derive the most commonly used equations are given in Table F.1. The best extinction coefficients are usually the highest: *viz*, those of Zscheile and Comar (1941), Smith and Benitez (1955), Jeffrey and Humphrey (1975) and Strain *et al.* (1963), all of which are within ±3% of each other.

These extinction coefficients were determined by weighing out highly purified or crystalline preparations, dissolving them in a known volume of solvent (usually diethyl ether to give sharpest maxima and greatest intensity of the absorption bands), taking the visible absorption spectrum and calculating the extinction coefficient according to Beer's Law (see footnote to Table F.1 and Preamble to Part IV).

The extinction coefficients of Smith and Benitez (1955) in diethyl ether were used by Vernon (1960), Lichtenthaler and Wellburn (1983) and Porra *et al.* (1989) as the standard to calibrate their purified chlorophylls in other solvents. Porra *et al.* (1989) analysed the magnesium content by atomic absorption spectroscopy and showed that the Smith and Benitez (1955) coefficients had less than a 1% error. Thus the equations of the above authors are accurate for both chlorophylls *a* and *b* in mixtures of these two pigments. Extinction coefficients in other solvents (e.g., acetone, dimethyl formamide and methanol) were derived by evaporating an aliquot of a purified chlorophyll solution in diethyl ether, and replacing it with an equal volume of a different solvent. The extinction coefficients of Jeffrey and Humphrey (1975) were derived from crystalline preparations of chlorophylls *a*, *b*, c_1 and c_2, prepared by Jeffrey (see Jeffrey and Humphrey, 1975), weighed and measured as described above, and were not based on the extinction coefficients of Strain *et al.* (1963) as incorrectly stated by Porra *et al.* (1989) and Porra (1991).

References and comments on six sets of equations and the extinction coefficients used to derive them are listed in Table F.2. It is clear from this table, and care-

ful tests carried out by Lorenzen and Jeffrey (1980) on mixtures of pure pigments (see Table F.3), that all equations that used the chlorophyll *a* extinction coefficients of Zscheile and Comar (1941), Smith and Benitez (1955), Strain *et al.* (1963) or Jeffrey and Humphrey (1975) should be satisfactory, as they are within ±3% of the 'best' values.

Four sets of trichromatic equations for estimating chlorophylls *a*, *b* and *c* are detailed below: (Richards with Thompson, 1952; Parsons and Strickland, 1963; SCOR–UNESCO, 1966; Jeffrey and Humphrey, 1975). Equations for chlorophylls *a* and *c* follow (Jeffrey and Humphrey, 1975; Humphrey, 1979). Three sets of equations are then listed for chlorophylls *a* and *b* (Arnon, 1949; Jeffrey and Humphrey, 1975; Porra *et al.* 1989). The equations of Lichtenthaler and Wellburn (1983) for chlorophylls *a* and *b* are not given, as they are closely similar to those of Porra *et al.* (1989). E_x denotes the extinction (absorption) measured at wavelength x in a 1 cm cuvette.

A. Simultaneous equations for chlorophylls *a*, *b* and *c*

1. **Richards with Thompson (1952)**

Solvent 90% acetone; units for equations (1), (2) = mg/l; units for (3) = MSPU/l: Milli-Specified Pigment Units

Chlorophyll *a* = $15.6\ E_{665} - 2.0\ E_{645} - 0.8\ E_{630}$ (1)

Chlorophyll *b* = $25.4\ E_{645} - 4.4\ E_{665} - 10.3\ E_{630}$ (2)

Chlorophyll *c* = $109\ E_{630} - 12.5\ E_{665} - 28.7\ E_{645}$ (3)

Not recommended

2. **Parsons and Strickland (1963)**

Solvent 90% acetone; units, mg/l

Chlorophyll *a* = $11.6\ E_{665} - 0.14\ E_{630} - 1.31\ E_{645}$ (4)

Chlorophyll *b* = $20.7\ E_{645} - 4.34\ E_{665} - 4.42\ E_{630}$ (5)

Chlorophyll *c* = $55\ E_{630} - 16.3\ E_{645} - 4.64\ E_{665}$ (6)

Recommended for Chl a *only*

3. **SCOR–UNESCO (1966)**

Solvent 90% acetone; units, µg/ml

Chlorophyll *a* = $11.64\ E_{663} - 2.16\ E_{645} + 0.10\ E_{630}$ (7)

Chlorophyll *b* = $-3.94\ E_{663} + 20.97\ E_{645} - 3.66\ E_{630}$ (8)

Chlorophyll *c* = $-5.53\ E_{663} - 14.81\ E_{645} + 54.22\ E_{630}$ (9)

Recommended for Chl a *only*

4. **Jeffrey and Humphrey (1975)**

Units, µg/ml

For mixed phytoplankton populations (Chls *a*, *b*, c_1 and c_2; solvent 90% acetone)

Chlorophyll *a* = $11.85\ E_{664} - 1.54\ E_{647} - 0.08\ E_{630}$ (10)

Chlorophyll *b* = $-5.43\ E_{664} + 21.03\ E_{647} - 2.66\ E_{630}$ (11)

Chlorophylls $c_1 + c_2$ = $-1.67\ E_{664} - 7.60\ E_{647} + 24.52\ E_{630}$ (12)

Recommended for Chls a, b *and* c *(but see Appendix G for tests of accuracy of various chlorophyll mixtures)*

TABLE F.1 Specific extinction coefficients of the various chlorophylls at the major red bands used for the derivation of spectrophotometric equations. Numbers in parentheses indicate the wavelength in nm (λ_{max}) at which the extinction coefficient was determined.

	Extinction coefficients (α; l gm^{-1} cm^{-1}) and wavelengths (nm) at the red maximum									
Chlorophyll	Diethyl ether		100% acetone		90% acetone		80% acetone		methanol		Reference
Chlorophyll *a*	102.1	(660)									Zscheile and Comar (1941)
	90.1		84.0	(663)							Mackinney (1941)
	100.9	(662)									Smith and Benitez (1955)
	100.9[a]	(662)	92.6	(663)	91.1	(665)					Vernon (1960)
	96.6	(660.5)									Strain *et al.* (1963)
	98.07	(660.7)	88.15	(662.7)	87.67	(664.3)					Jeffrey and Humphrey (1975)
									79.81	(666.1)	Jeffrey (unpublished)
	101[a]	(662)	88.88	(662)			86.86	(663)	79.29	(666)	Lichtenthaler and Wellburn (1983)
	100.9[a, b]	(662)					85.95	(663.6)	79.95	(665.2)	Porra *et al.* (1989)
Chlorophyll *b*	56.8	(642.5)									Zscheile and Comar (1941)
			51.8	(648)							Mackinney (1941)
	62.0	(644)									Smith and Benitez (1955)
	62.0[a]	(644)	53.5	(647)	52.5	(648)					Vernon (1960)
	61.8	(642)									Strain *et al.* (1963)
	62.0	(643.3)			51.36	(646.8)					Jeffrey and Humphrey (1975)
	62[a]	(644)	56.11	(645)			57.70	(646)	45.88	(653)	Lichtenthaler and Wellburn (1983)
	62.0[a, b]	(644)					51.84	(646.6)	42.48	(652)	Porra *et al.* (1989)
Chlorophyll ($c_1 + c_2$)					19.44	(630)					Jeffrey (1963)
Chlorophyll c_1			39.2[c]	(629.1)	44.8[c]	(630.6)					Jeffrey (1972)
Chlorophyll c_2			37.2[c]	(629.6)	40.4[c]	(630.9)					Jeffrey (1972)

Extinction coefficients were determined in all cases, except Vernon (1960), Lichtenthaler and Wellburn (1983) and Porra *et al.* (1989), by weighing out highly purified or crystalline preparations, dissolving in a known volume of solvent, taking the visible absorption spectrum and calculating the extinction coefficient according to Beer's Law:

$$\alpha = \frac{1}{dC} \cdot \log \frac{I_0}{I} = \frac{A}{dC} \ ;$$

where d = inside length of the absorption cuvette in cm
C = concentration of chlorophyll in gm per litre
I_0, I = light intensities transmitted by pure solvent and the chlorophyll solution, respectively
A = Absorbance (or optical density)

a. Extinction coefficients of Smith and Benitez (1955) were used as the standard from which extinction coefficients in other solvents were experimentally determined (see text).
b. Porra *et al.* (1989) analysed the magnesium content of purified preparations of chlorophylls *a* and *b* by atomic absorption spectroscopy and showed that the Smith and Benitez (1955) extinction coefficients were accurate to within 1%.
c. Crystalline chlorophylls c_1 and c_2 are insoluble in common solvents (e.g., diethyl ether, acetone) and were dissolved in a small volume of pyridine before diluting with acetone or 90% acetone.

TABLE F.2 Evaluation of spectrophotometric equations based on the extinction coefficients used.

Author of equations	Source of extinction coefficients: Chl *a*	Chl *b*	Chl *c*	Solvent used for equations	Comments
Richards with Thompson (1952)	Zscheile (1934)	Zscheile (1934)	None available; MSPU[a] used	90% acetone	Extinction coefficients too low for Chl *a* and *b*; none available for Chl *c*. Equations not recommended.
Parsons and Strickland (1963)	Vernon (1960)[b]	Vernon (1960)[b]	Jeffrey (1963)[c]	90% acetone	Extinction coefficients acceptable for Chls *a* and *b*; those for Chl *c* are too low[c]. Equations recommended for Chl *a*.
SCOR–UNESCO (1966)	Mean of three extinction coefficients used: Zscheile and Comar (1941), Smith and Benitez (1955), and Strain *et al.* (1963). Chl *a*, 99.87 l gm^{-1} cm^{-1} in diethyl ether Chl *b*, 60.2 l gm^{-1} cm^{-1} in diethyl ether		Jeffrey (1963)[c]	90% acetone	Extinction coefficients within 5% of 'best value' for Chls *a* and *b*; those for Chl *c* are too low[c]. Equations recommended for Chl *a*.
Jeffrey and Humphrey (1975)	Jeffrey and Humphrey (1975)		Jeffrey (1972)	90% + 100% acetone	Extinction coefficients for Chls *a* and *b* within 2% of 'best' value (Smith and Benitez, 1955); those for Chl $c_1 + c_2$ are the 'best' available. Equations recommended.
Arnon (1949)	Mackinney (1941)	Mackinney (1941)	–	80% acetone	Extinction coefficients for chl *a* at λ_{max} chl *a* and λ_{max} chl *b* are 4,5% and 19,5% low respectively; for chl *b* at λ_{max} chl *b* and λ_{max} chl *a* are 12% and 14% low respectively (see Porra *et al.* 1989). Equations widely used for leaf pigments (Chls *a* and *b*) in plant physiology; not recommended.
Porra *et al.* (1989)	Porra *et al.* (1989)[b]	Porra *et al.* (1989)[b]	–	Various	Extinction coefficients of Smith and Benitez (1955) confirmed by Mg analysis. Equations recommended.

a. MSPU is a specific pigment unit of undetermined weight.
b. Based on Smith and Benitez (1955) extinction coefficients in diethyl ether (see Table F.1).
c. Low extinction coefficient due to unrecognized polymerization of purified chlorophyll *c*, which resulted in lowered intensities of absorption bands.

B. Simultaneous equations for chlorophylls *a* and *c*

5. **Jeffrey and Humphrey (1975) and Humphrey (1979)**
Units, μg/ml

(a) *For chromophyte algae* (Chls *a*, c_1 and c_2; solvent 90% acetone)

Chlorophyll *a* = $11.47\ E_{664} - 0.40\ E_{630}$ (13)

Chlorophylls $c_1 + c_2$ = $24.36\ E_{630} - 3.73\ E_{663}$ (14)

Recommended for Chls a *and* c

(b) *For dinoflagellates and cryptomonads* (Chls *a* and c_2; solvent 100% acetone)

Chlorophyll *a* = $11.43\ E_{663} - 0.64\ E_{630}$ (15)

Chlorophyll c_2 = $27.09\ E_{630} - 3.63\ E_{663}$ (16)

Recommended for Chls a *and* c

(c) *For dinoflagellates and cryptomonads* (Chls *a* and c_2; solvent 90% acetone)

Chlorophyll *a* = $11.43\ E_{664} - 0.40\ E_{630}$ (17)

Chlorophyll c_2 = $24.88\ E_{630} - 3.80\ E_{664}$ (18)

Recommended for Chls a *and* c

C. Simultaneous equations for chlorophylls *a* and *b*

6. **Arnon (1949)**
Units, μg/ml

For green algae and leaf pigments (Chls *a* and *b*; solvent 80% acetone)

Chlorophyll *a* = $12.7\ E_{663} - 2.69\ E_{645}$ (19)

Chlorophyll *b* = $22.9\ E_{645} - 4.68\ E_{663}$ (20)

Not recommended

7. **Jeffrey and Humphrey (1975)**
Units, μg/ml

For green algae and leaf pigments (Chls *a* and *b*; solvent 90% acetone)

Chlorophyll *a* = $11.93\ E_{664} - 1.93\ E_{647}$ (21)

Chlorophyll *b* = $20.36\ E_{647} - 5.50\ E_{664}$ (22)

Recommended for Chls a *and* b

8. **Porra *et al.* (1989)**
Units, μg/ml

For green algae and leaf pigments (Chls *a* and *b*; solvent, buffered 80% acetone)

Chlorophyll *a* = $12.25\ E_{663.6} - 2.55\ E_{646.6}$ (23)

Chlorophyll *b* = $20.31\ E_{646.6} - 4.91\ E_{663.6}$ (24)

Recommended for Chls a *and* b

For green algae and leaf pigments (Chls *a* and *b*; solvent methanol)

Chlorophyll *a* = $16.29\ E_{665.2} - 8.54\ E_{652}$ (25)

Chlorophyll *b* = $30.66\ E_{652} - 13.58\ E_{665.2}$ (26)

Recommended for Chls a *and* b

For green algae and leaf pigments (Chls *a* and *b*; solvent dimethyl formamide)

Chlorophyll *a* = $12.00\ E_{663.8} - 3.11\ E_{646.8}$ (27)

Chlorophyll *b* = $20.78\ E_{646.8} - 4.88\ E_{663.8}$ (28)

Recommended for Chls a *and* b

F.3 ACCURACY OF EQUATIONS TESTED WITH MIXTURES OF PURE PIGMENTS

Lorenzen and Jeffrey (1980) tested the accuracy of five spectrophotometric trichromatic equations, using 20 mixtures of pure pigments, to simulate a number of oceanographic situations.

Four sets of these equations have been used extensively in oceanography for measuring chlorophylls *a*, *b* and *c*: Richards with Thompson (1952), Parsons and Strickland (1963), SCOR–UNESCO (1966) and Jeffrey and Humphrey (1975). Humphrey (1966) modified the Richards–Thompson equations (fifth set) by correcting the chlorophyll *c* term (Jeffrey, 1963).

Table F.3 shows the accuracy of these equations, using one chlorophyll mixture from the many presented by Lorenzen and Jeffrey (1980). It contained chlorophylls *a*, *b* and *c* in the proportions 8:1.7:1. No chlorophyll degradation products were present. The results and percent errors of these analyses show that

a) Chlorophyll *a* is satisfactory when calculated by Jeffrey–Humphrey, SCOR–UNESCO or Parsons–Strickland (1–3 % error). Richards–Thompson methods are 30% high due to the use of the Zscheile (1934) extinction coefficient for chlorophyll *a* which is too low (see Table F.2).
b) Chlorophyll *b* is satisfactory only with Jeffrey–Humphrey or SCOR–UNESCO.
c) Chlorophyll *c* is satisfactory only with Jeffrey–Humphrey, but even this set of equations can give errors if chlorophyll *c* is much lower than chlorophyll *a* (see Appendix G). However, accuracy was high with other chlorophyll *c*-containing mixtures tested by Lorenzen and Jeffrey (1980) and Humphrey and Jeffrey (Appendix G).
d) Errors arising from using slightly different wavelengths for chlorophyll *a* are small (e.g., 663 nm (SCOR–UNESCO); 664 nm (Jeffrey–Humphrey); 665 nm (Parsons–Strickland and Richards–Thompson)). In 90% acetone, the chlorophyll *a* red band shows a plateau at 663–664 nm and drops 3% in optical density at 665 nm. The small errors in wavelength reading are insignificant for

TABLE F.3 Comparison of accuracy of five sets of trichromatic spectrophotometric equations using a mixture of spectrally pure chlorophylls *a*, *b* and *c* ($c_1 + c_2$) (from Lorenzen and Jeffrey, 1980).

Equations	Concentration of chlorophylls			% errors		
	Chl *a*	Chl *b* ($\mu g\ ml.^{-1}$)	Chl *c* ($c_1 + c_2$)	Chl *a*	Chl *b*	Chl *c*
Known chlorophyll mixture	1.84	0.39	0.296			
Jeffrey-Humphrey (1975)	1.85	0.39	0.225	<1%	<1%	24% low
Parsons-Strickland (1963)	1.78	0.26	0.672	3% low	33% low	208% high
SCOR-UNESCO (1966)	1.80	0.35	0.55	2% low	11% low	169% high
Richards-Thompson (1952)	2.38	0.21	1.00	30% high	54% low	300% high
Richards-Thompson modified Humphrey (1966)	2.38	0.21	0.43	30% high	54% low	45% high

chlorophyll *a*. For chlorophyll *b* the wavelengths used are 647 nm (Jeffrey–Humphrey; SCOR–UNESCO) and 645 nm (Parsons–Strickland and Richards–Thompson). In 90% acetone there is an 11% drop in optical density at 645 nm compared to that at 647 nm (λ_{max}), which results in the greater errors observed. The Jeffrey–Humphrey equations are the only ones available for the simultaneous measurement of chlorophylls *a*, *b*, c_1 and c_2. Tests of their accuracy with mixtures of pure chlorophylls in different proportions and concentrations are given in Appendix G.

F.4 Spectrophotometric equations that correct for pheopigments

The trichromatic equations for determining pigment concentrations in mixtures of chlorophylls *a*, *b* and c assume the absence of chlorophyll degradation products that can interfere with the assay. However, chlorophyll degradation products, such as pheophytin *a* and pheophorbide *a*, may be present in relatively high concentrations in some natural samples, such as in deep water from below the euphotic zone, detritus and sediments. The presence of pheopigments will result in an overestimate of chlorophyll *a*, since the absorption characteristics of pheopigments are similar to those of their parent chlorophylls (see Part IV Data sheets).

Methods to correct for pheopigment interference in spectrophotometric and fluorometric assays for chlorophyll *a* were developed by Vernon (1960), Yentsch and Menzel (1963), Holm-Hansen *et al.* (1965) and Lorenzen (1967). They all use spectrophotometric acidification techniques based on the following points:

- chlorophyll *a* treated with weak acid converts to pheophytin *a* with 100% molar stoichiometry
- the molar absorptivity of the red absorbance band of pheophytin *a* is lower than that of chlorophyll *a*
- the relative absorption ratio of the two pigments in the same solvent is a constant.

Lorenzen (1967) determined that the absorbance at 665 nm was reduced by a factor of 1.7 when pure chlorophyll *a* in 90% acetone was pheophytinized by weak acid, thus defining the 'acid factor' for pure chlorophyll *a* ($A\ 665_o/A\ 665_a$: for symbols, see below). The value of the acid factor for any unknown sample will range from 1.0 (pure pheophytin *a*) to 1.7 (pure chlorophyll *a*). For example, a sample consisting of 50% chlorophyll *a* and 50% pheophytin *a* on a molar basis will yield an acid factor of 1.35. The concentrations of chlorophyll *a* and pheophytin *a* in any unknown sample can be determined algebraically by the equations of Lorenzen (1967):

$$\text{Chl } a \text{ (mg m}^{-3}) = \frac{[AK(665_o - 665_a)v]}{V_f l}$$

$$\text{Pheo } a \text{ (mg m}^{-3}) = \frac{[AK((R665_a) - 665_o)v]}{V_f l}$$

where

A = inverse extinction coefficient in 90% acetone for chl *a* (x 1000); [1/(91.1 l g^{-1} cm^{-1})] x 1000 = 11.0 µg cm ml^{-1}

R = maximum absorbance ratio of $665_o/665_a$ in the absence of pheopigments, 1.7

K = R/(R–1.0); 1.7/0.7, or 2.43

665_o = absorbance at 665 nm before acidification

665_a = absorbance at 665 nm after acidification
v = volume of 90% acetone used for extraction (ml)
V_f = volume of water filtered (litres)
l = path length of cuvette (cm)

The basic principles of Lorenzen's (1967) spectrophotometric acidification technique outlined above are analogous to those used in previous fluorometric acidification techniques (e.g., Holm-Hansen *et al.* 1965). The original spectrophotometric method of Lorenzen (1967) used an extinction coefficient of 91.1 l g^{-1} cm^{-1} in 90% acetone (Vernon, 1960), which will give chlorophyll concentrations about 4% lower than those calculated with the recommended extinction coefficient of Jeffrey and Humphrey (1975) (see Table F.1). Lorenzen (1967) recommended reading absorbance values at 665 nm instead of 663 nm — the 'true' λ_{max} for chlorophyll *a* in acetone — to facilitate readings on the then-popular Beckman DU spectrophotometer, which used a non-linear grid on its monochromator settings. However, on other instruments any convenient wavelength setting near the red absorbance peak can be used as long as the acid-factor for pure chlorophyll *a* is measured at that wavelength and incorporated in the variables A and R above.

The units of concentration are calculated on a weight-basis (μg l^{-1}), which has unfortunately led to some confusion (Lorenzen and Newton-Downs, 1986). Acid pheophytinization results in stoichiometric pigment conversion on a molar basis (see above). Thus, the equations should be expressed on a molar basis to meet the assumptions of molar stoichiometry outlined earlier. Lorenzen cast the equations in terms of weight-specific concentrations to maintain dimensional consistency with extinction coefficients, which are most often cited in weight-specific units. There is only a 3% difference in the molecular weights of chlorophyll *a* and pheophytin *a* (893 and 869, respectively), and the error in converting from mass to moles was considered trivial. There is therefore only a small error in the chlorophyll *a* and pheopigment concentrations calculated as above, provided the unknown sample contains pheophytin *a* as the only degradation product of chlorophyll *a* (which is seldom the case). However, the calculated weight of pheopigment can be overestimated by a factor of 1.51 if the unknown sample contains pheophorbide *a* rather than pheophytin *a*, since the molecular weight of pheophorbide *a* (591) is 1.51-fold less than that of chlorophyll *a*. Any chlorophyll *b* present is also degraded by the acidification and is erroneously expressed as a 'pheopigment' component (see Section F.5).

The accuracy of pigment concentrations calculated from the spectrophotometric acidification technique is not uniform for all potential mixtures of chlorophyll *a* and pheopigments. The variable error results from the algebra of the acidification equations *per se*. Simple propagation of instrumental errors in reading unacidified and acidified absorbances will show that the greatest error in determining pheopigment concentration occurs when pheopigments constitute a small fraction of total pigment mass (chlorophyll + pheopigments), e.g., when the absorbance acid ratio is >1.35. Likewise, the greatest potential error in calculating chlorophyll *a* concentrations occurs when chlorophyll *a* constitutes a small fraction of the total pigment mass; when the acid ratio is <1.35.

For most samples from the euphotic zone, and especially for pure algal tissues, the mass of chlorophyll *a* greatly outweighs that of pheopigments. Calculated concentrations of chlorophyll *a* should be accurate for these samples, provided caution is exercised in judging the accuracy of the pheopigment (see above). Acidification

techniques were specifically designed to correct the chlorophyll *a* concentration for interference from pheopigments (Lorenzen and Newton-Downs, 1986), but the concentration of pheopigments calculated under low pheopigment/chlorophyll ratios should be viewed with caution. The same restrictions also apply directly to fluorescence acidification techniques (see Section F.5).

F.5 Fluorometric equations

Fluorometric equations for extracted chlorophyll a and pheopigments

Fluorescence assays for extracted chlorophyll *a* are two to three orders of magnitude more sensitive than spectrophotometric methods. For this reason, fluorometric chlorophyll techniques have been popular for studies of oligotrophic marine systems, where environmental chlorophyll concentrations can be very low (<0.1 μg l^{-1}). Fluorescence acidification techniques, which correct for the presence of pheopigments, have been routinely used for over 30 years (Holm-Hansen *et al.* 1965).

The assumptions of the fluorometric acidification technique are analogous to those used in spectrophotometric acidification methods (see Section F.4). It is assumed that

- when chlorophyll *a* is treated with weak acid it will be converted to pheophytin *a* with 100% molar stoichiometry
- the molar fluorescence response of pheophytin *a* is lower than that for chlorophyll *a*, yielding a constant relative ratio of fluorescence responses ($F_m=F_o/F_a$) for the two pigments.

In contrast to spectrophotometric methods, the relative fluorescence response (sensitivity coefficient, K) and the maximum fluorescence acid ratio (F_m) are highly dependent on the specific fluorometric optical configuration of each instrument and therefore must be empirically determined with a chlorophyll *a* standard that is free of both pheopigments and chlorophyll *b*. The equations for chlorophyll *a* and pheophytin *a* are listed below:

$$\text{Chlorophyll } a \text{ (mg m}^{-3}) = \frac{KF_m v(F_o - F_a)}{V_f(F_m - 1)}$$

$$\text{Pheophytin } a \text{ (mg m}^{-3}) = \frac{KF_m v(F_m F_a - F_o)}{V_f(F_m - 1)}$$

where

K = fluorescence sensitivity coefficient in extraction solvent
= [(μg Chl *a*/ml solvent)/instrument fluorescence unit]
F_m = maximum ratio F_o/F_a in the absence of pheopigments and chlorophyll *b*
F_o = fluorescence before acidification
F_a = fluorescence after acidification
v = volume of acetone used for extraction (ml)
V_f = volume of water filtered (litres)

The fluorometric calibration coefficients, K and F_m, can be determined empirically from a solvent solution of pure chlorophyll *a* of known concentration (determined spectrophotometrically, using an absorbance extinction coefficient of 87.67 l g^{-1} cm^{-1} at 664 nm in 90% acetone). The fluorometric calibration coefficients can also be determined with mixed extracts containing chlorophylls and carotenoids where the

chlorophyll *a* concentration is known, as long as the solution is free of pheopigments and chlorophyll *b*. It is well known that chlorophyll *b* undergoes wavelength shifts upon acidification which bias the fluorescence readings for chlorophyll *a*, resulting in significant overestimates of the true concentration of pheopigments (Gibbs, 1979; Trees *et al.* 1985). For this reason, the fluorescence acidification technique is not recommended for analysis of natural samples known to contain significant concentrations of chlorophyll *b*, e.g., freshwater or marine samples dominated by chlorophytes or prasinophytes (see Chapter 2).

Fluorometric equations for extracted chlorophylls a, b and c and their pheophytins

Several methods have been published which determine concentrations of extracted chlorophylls *a*, *b* and *c* and their pheophytins using multi-wavelength fluorometric readings (Loftus and Carpenter, 1971; Boto and Bunt, 1978; Bazzaz and Rebiez, 1979; Neveux and Panouse, 1987). These methods make use of the fact that each pigment is characterized by unique fluorescence excitation/emission spectra, and each pigment can be caused to fluoresce maximally through selective choice of excitation and emission wavelengths. The multi-wavelength fluorescence method is analogous to the trichromatic spectrophotometric technique, since it requires a number of readings equivalent to the individual pigments analyzed. The method of Neveux and Panouse (1987) determines concentrations of chlorophylls *a*, *b* and *c* and pheophytins *a*, *b* and *c* by making fluorescence readings at six pairs of excitation/emission settings, the calculation of which requires solution of six equations for six unknowns.

In general, the multi-wavelength fluorometric techniques have been less popular because of the tedious calibration procedures required. Each pigment to be analyzed must be available as a highly purified standard and its concentration known for the empirical determination of fluorescence response coefficients. The calibration coefficients are dependent on optical conditions, so any routine changes, such as adjusting the slit width, require re-determination of the calibration coefficients.

Fluorometric technique for determining chlorophyll a

A recent method uses a simple filter fluorometer to determine chlorophyll *a* in the presence of pheopigments and chlorophyll *b* (without acidification procedures) (Welschmeyer, 1994). It yields measurements of chlorophyll *a* only, and provides no estimate of pheopigments. Most of the advantages of fluorescence sensitivity are maintained and the method can be used on samples that are known to contain chlorophytes, e.g., freshwater oligotrophic systems.

The method calls for a change in the lamp and filters used in conventional filter fluorometers. The new lamp (Type 10–089 Turner Designs Inc.) contains little energy at the wavelength of maximum pheophytin *a* excitation (408 nm), and uses interference filters at 436 nm and 680 nm (10 nm bandwidth) at the excitation/emission positions. The optical configuration produces reasonable sensitivity to chlorophyll *a* while maintaining desensitized responses from both chlorophyll *b* and pheophytin *b*. Chlorophyll *a* is calculated from the following equation, using a single determination of fluorescence for each sample:

$$\text{Chlorophyll } a \text{ (mg m}^{-3}) = \frac{KF_o v}{V_f}$$

where

K = fluorescence sensitivity coefficient in extraction solvent
= [(μg Chl *a*/ml solvent)/instrument fluorescence unit]
F_o = fluorescence response (no acidification)
v = volume of acetone used for extraction (ml)
V_f = volume of water filtered (litres)

The method offers simple, low-cost determination of chlorophyll *a* and reduces the requirements for purity of chlorophyll *a* standards, since any solvent mixture containing chlorophyll *a* (e.g., leaf extracts) can be used for standardization.

In vivo fluorescence

Chlorophyll *a* determination from *in vivo* fluorescence (Lorenzen, 1966) is probably the most convenient but least accurate method of determining chlorophyll (see Section 17.1; Table 17.1). It is widely used in marine research for mapping broad geographic patterns of phytoplankton biomass. The fluorescence of individual phytoplankton cells is illustrated in Plate 4A, Chapter 6. The instruments used include ship-board flow-through fluorometers (Lorenzen, 1966) and *in situ* fluorometers (Mackey *et al.* 1995). Some problems with this method are discussed briefly by Lorenzen and Jeffrey (1980).

The calculation procedure reduces to:

$$\text{Chlorophyll } a \text{ (mg m}^{-3}) = KF$$

where

K = *in vivo* fluorescence sensitivity coefficient
= mg Chl *a* m^{-3} per fluorescence unit (obtained by prior calibration)
F = *in vivo* fluorescence response

To calibrate the instruments, extracted chlorophyll *a* concentrations and the associated *in vivo* fluorescence response must be determined. Instrument response is typically linear over natural concentration ranges, excluding turbid waters. Fluorescence response per unit chlorophyll is species-dependent and is also related to the photophysiological status of the algae. Variables such as cell-packaging, chloroplast shape, photosynthetic rate, presence of senescent cells, and temperatures at which the analyses are carried out, can result in changes in the apparent *in vivo* fluorescence sensitivity coefficient (see Falkowski and Kiefer, 1985). For these reasons the instrument must be calibrated frequently. These inaccuracies do not overshadow the important role that *in vivo* fluorometry has played in rapidly determining

- an index of chlorophyll (phytoplankton abundance) along the ship's track at sea
- vertical profiles of phytoplankton distributions
- concentrations of chlorophyll in discrete water samples, and
- long term continuous changes in phytoplankton biomass estimated from moored *in situ* fluorometers.

F.6 Problems with using spectrophotometric and fluorometric equations for phytoplankton field samples

Spectrophotometric assays

The most common problem in spectrophotometric assays of natural phytoplankton assemblages is inadequate sensitivity. Unless one can visually 'see colour' in the final solvent extract, the absorbance may be so low as to produce inaccuracies arising from unacceptably low signal-to-noise ratios. Ideally, enough seawater should be filtered to yield an absorbance (optical density) >0.1 at 664 nm when using the spectrophotometric acidification technique. Final absorbance should be even higher when using the trichromatic technique, since the absorbances at 647 and 630 nm will be more than two-fold lower than at 664 nm. The user should make adjustments to obtain the highest absorbance possible, such as filtering more sample, using the smallest solvent volume and using small-volume, long-path cuvettes. If sonication is used to disrupt cells, extractions with 1.2 ml 90% acetone in microcentrifuge tubes will yield at least 1 ml of usable extract, which should be adequate to fill most 1 cm spectrophotometric micro-cuvettes.

Fluorometric assays

Filter Fluorometers. The specific optical configurations of common filter fluorometers will result in wavelength-dependent response characteristics that are unique to each instrument. The user should be aware that changes in filters, lamps and/or photomultipliers, other than those specified in a given method, will often result in performance characteristics quite different from those described in the literature. It is necessary, for instance, to use red-sensitive photomultipliers (Hamamatsu R446, or equivalent) in all of the fluorometric chlorophyll analyses described above to gain full advantage of fluorometric sensitivity.

The colour spectra of common lamps used in filter fluorometers must also be considered with respect to specific excitation-emission spectra of pigments, since unwanted interferences may occur. For example, a shift of over 20 nm in the blue excitation band occurs when chlorophyll *b* is converted to pheophytin *b* by acidification (excitation/emission maxima of chlorophyll *b* and pheophytin *b* are 458/653 nm and 435/658 nm respectively, Fig. F.1). The resultant blue excitation shift causes pheophytin *b* to be detected with greater sensitivity than chlorophyll *b* in most filter fluorometers since the standard fluorometric lamp (Fig. F.2A and F.2B) has a strong energy band at 434 nm. This results in the well known 'chlorophyll *b* problem' which causes overestimates of pheopigments and underestimates of chlorophyll *a* in the conventional fluorometric acidification technique (see section F.5).

As can be seen in Fig. F.2, several of the common fluorometer lamps contain strong energy bands at 405 and 434 nm (Fig. F.2A–E) making them potentially useful in fluorometric acidification analyses of chlorophyll *a* and pheopigments. However, when combined with a blue excitation filter (Corning 5–60), the resultant acid ratio for pure chlorophyll *a* can vary greatly depending on the lamp used. Calibration coefficients should be determined empirically as described in section F.5 whenever making optical changes to filter fluorometers for chlorophyll analysis. Figs. F.1 and F.2 are provided to assist the user in selecting lamps for specific pigment analyses, which might also include fluorometric detection in HPLC analyses.

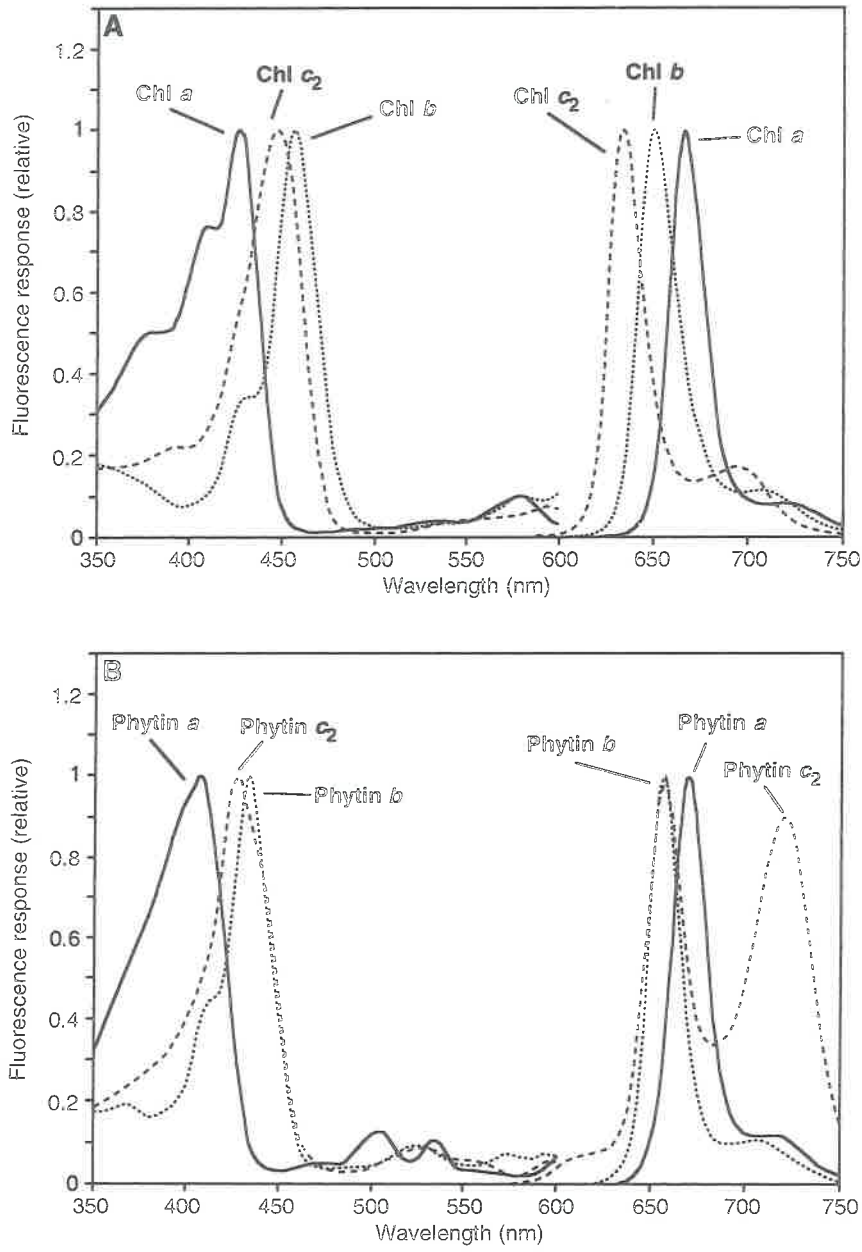

Figure E.1
Photon-corrected excitation-emission spectra of HPLC-purified 90% acetone solutions of A, chlorophylls *a*, *b* and c_2, and B, their acid-produced pheophytins. Fluorescence responses were measured at the maximum excitation-emission wavelength for each pigment. Results were normalized to unity, yielding concentration-independent spectra.

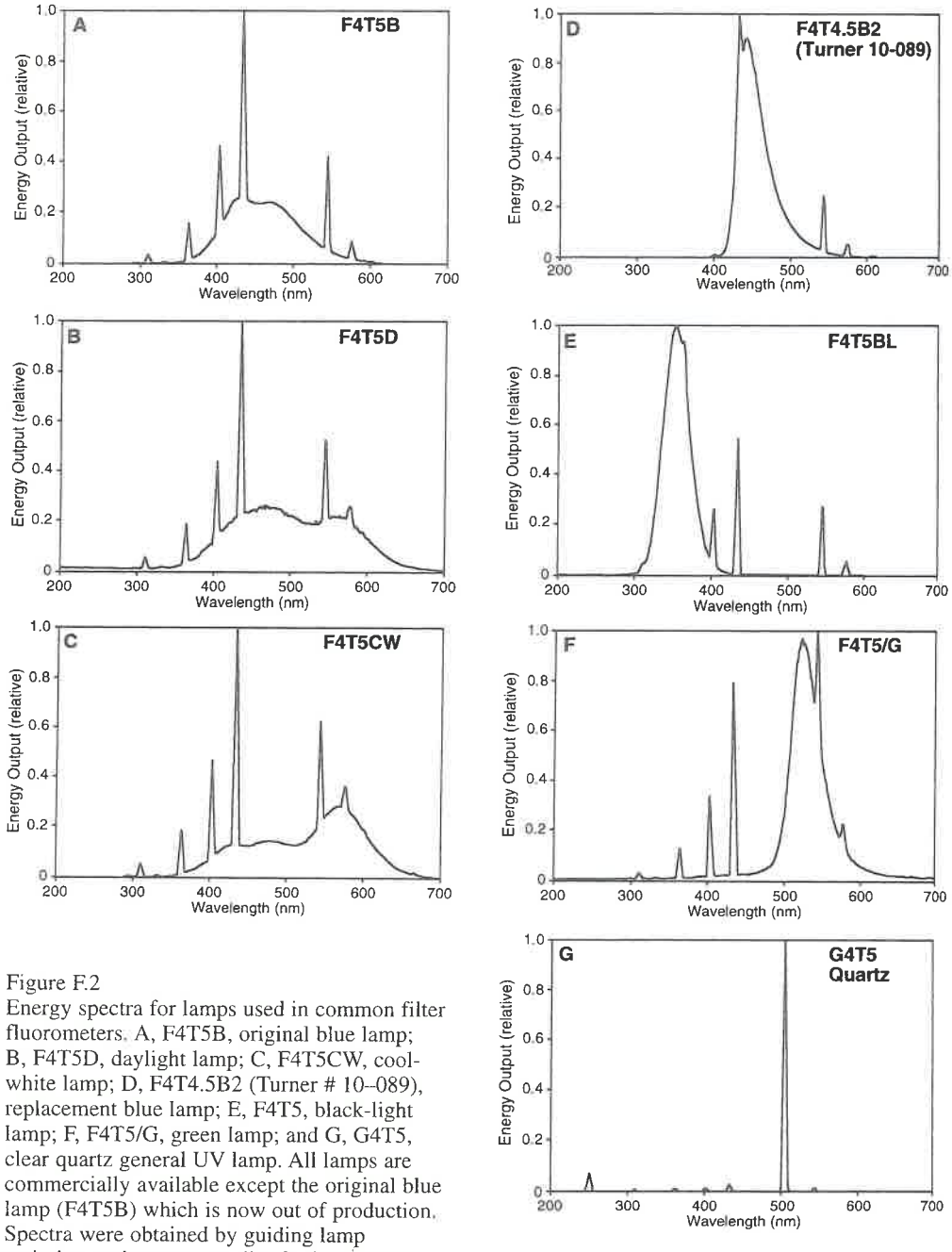

Figure F.2
Energy spectra for lamps used in common filter fluorometers. A, F4T5B, original blue lamp; B, F4T5D, daylight lamp; C, F4T5CW, cool-white lamp; D, F4T4.5B2 (Turner # 10–089), replacement blue lamp; E, F4T5, black-light lamp; F, F4T5/G, green lamp; and G, G4T5, clear quartz general UV lamp. All lamps are commercially available except the original blue lamp (F4T5B) which is now out of production. Spectra were obtained by guiding lamp emission to the entrance slit of a Spex model 1681 monochromator (Welschmeyer, 1994). All spectra were normalized to unity at the wavelength of greatest energy output. Energy correction in the UV range was not possible; the relative energy level of the quartz lamp line at 254 nm (G) is significantly greater than shown.

Spectrofluorometers. Quantitative analyses of chlorophylls by the spectrofluorometric technique is less common than simple filter-fluorometric techniques for reasons described in section F.5. In particular, the user should be aware of differences in excitation-emission spectra made with, and without, photon-correction. Uncorrected spectra contain wavelength-dependent fluctuations in both a) excitation lamp energy and b) photomultiplier sensitivity, thereby distorting the true fluorescence characteristics of pigments being analyzed. Corrected and uncorrected spectra of similar samples can appear quite different, making comparisons between techniques difficult. In general, each user is 'on their own' when attempting to adapt spectrofluorometry to quantitative pigment analyses, and it is highly recommended that photon-corrected instrumentation be used to yield optimum universality (cf. Bazzaz and Rebiez, 1979; Neveux and Panouse, 1987).

F.7 Summary

Most of our current knowledge of phytoplankton distributions in the ocean is based on chlorophyll analyses made by spectrophotometry and by popular filter-fluorometry. Both techniques will continue to be used whenever simple assays for chlorophyll *a* are required. It is clear, however, that fluorometry offers significant advantages in sensitivity which result in its overwhelming popularity in providing simple, low cost chlorophyll *a* analyses in most ocean environments. All pigment assays, including fluorometric and HPLC techniques, will ultimately be referenced to spectrophotometric absorbance measurements and, in this regard, spectrophotometric techniques are clearly indispensable.

Where concentrations of accessory chlorophylls are needed, the dichromatic and trichromatic spectrophotometric techniques can provide good accuracy for chlorophylls *a* and *b*, *a* and *c*, and *a*, *b*, and c_1+c_2 (see sections F.2, F.3 and Appendix G), providing that sample absorbances are high and degradation products are absent (e.g., algal cultures). However, if accurate analyses of accessory chlorophylls and degradation products are required, especially on natural field samples, then the isocratic HPLC system outlined in Chapter 11 is highly recommended. If the full suite of chlorophylls, carotenoids and chlorophyll degradation products in field samples is required, then an HPLC method, such to that described in Chapter 12, should be used.

Fluorometric analysis of chlorophyll *a* is accurate under conditions where chlorophyll *b* is absent. However, the accuracy of estimated pheopigment concentrations is questionable, especially under common conditions where the ratio chlorophyll/pheopigment is high. Since we now know that prochlorophytes rich in chlorophyll *b* and divinyl chlorophyll *b* are widely distributed, and even dominant, in the open ocean (Chisholm *et al.* 1988), absolute concentrations of pheopigments determined from the fluorometric acidification technique should be treated with caution.

In vivo fluorometry provides the most convenient, but least accurate method of determining chlorophyll *a*, and has become increasingly popular for studies involving long term monitoring and small-scale spatial distributions. There is currently no substitute for *in vivo* fluorescence for rapidly mapping real-time, vertical resolution in phytoplankton biomass over both temporal and spatial dimensions.

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