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Pigment Markers for Phytoplankton Production

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Abstract Chlorophylls and carotenoids are commonly used as quantitative biomarkers for the composition and biomass of marine phytoplankton. This chapter provides an overview of the molecular diversity of pigment markers, their distribution across algal taxa (based on theories of plastid diversity through endosymbiosis), and their environmental variability. Three new methods for analysis of pigments by HPLC are compared with the original SCOR method. Guidelines for interpreting HPLC pigment

chromatograms from field samples are given to determine the likely algal types present, thus enabling optimal computational analysis. Mathematical techniques for analysis of complex pigment data sets (multiple linear regression, inverse simultaneous equations and matrix factorization methods, using CHEMTAX software) are discussed. Methods for converting pigment data to carbon biomass are considered, with suggested strategies for improving biomass estimates.

Keywords Carotenoid · Chemotaxonomy · Chlorophyll · Computational methods · Phytoplankton

Abbreviations

But-fuco	19′-butanoyloxyfucoxanthin
Chl	Chlorophyll
Chl c_2-MGDG	chlorophyll c_2-monogalactosyl diacylglyceride ester. A suffix, e.g. [18 : 4/14 : 0], denotes the chain lengths (18, 14) and the number of double bonds (4, 0) of the two esterified fatty acids, respectively
Chlide	chlorophyllide
DMF	dimethyl formamide
DV	divinyl
Hex-fuco	19′-hexanoyloxyfucoxanthin
HPLC	high performance liquid chromatography
IS	internal standard
4-k Hex-fuco	4-keto-19′-hexanoyloxyfucoxanthin
MgDVP	magnesium divinyl pheoporphyrin a_5 monomethyl ester
MV	monovinyl
Neox	neoxanthin
Np	non-polar
SCOR	Scientific Council for Oceanic Research
TBAA	Tetrabutylammonium acetate
tr	trace
Unk	unknown

1 Introduction

Chromatographic analysis of algal pigments is a powerful tool for characterization of phytoplankton in field populations. Chlorophylls, carotenoids and phycobiliproteins have many favourable characteristics as chemotaxonomic markers. They are present in all photosynthetic algae, but not in most bacteria, protozoa or detritus, allowing phytoplankton to be distinguished from other components of the microbial community. Many pigments are limited to particular classes or even genera, allowing the taxonomic composition of the phytoplankton to be determined to class level or better. They are strongly coloured, and in the case of chlorophylls and phycobiliproteins, fluorescent at

visible wavelengths, allowing them to be sensitively detected. Finally, they are labile and are rapidly degraded after the death of the cell, thus distinguishing living from senescent cells.

Pigments also suffer several disadvantages as markers. Their lability means that special conditions must be employed to preserve them, as they are sensitive to light, heat, oxygen, acids and alkalis, as well as spontaneously forming families of isomers in solution. Their distribution is complex, with few unambiguous markers. Their expression is variable, even within a particular class, and their content per cell varies with environmental factors such as irradiance and nutrients. Due to the fact that some pigments span many algal classes [1], interpretation of pigment data is difficult. It is important that other techniques (e.g. microscopy or genetic analysis) are used on representative samples to identify the algal types present.

Pigment analysis, however, offers the best technique for mapping phytoplankton populations and monitoring their abundance and composition. Modern automated HPLC analysis of pigments makes it feasible to analyse several hundred phytoplankton samples from a single oceanographic cruise, a task that would be completely impractical by microscopy. Pigment analysis is a powerful means of recognizing nano- and pico-planktonic organisms, which are normally unrecognizable by light microscopy (unless they possess special features such as fluorescent phycobilins), and are often difficult to preserve. Flow cytometry can be used to rapidly count cells but it is poor at identification. DNA analysis is powerful for identifying the composition of phytoplankton, but is still slow and complex for analyzing mixed populations.

A detailed account of the theory and practice of pigment chromatography is given in the 1997 UNESCO monograph *Phytoplankton Pigments in Oceanography*: *Guidelines to Modern Methods* [2], a widely used publication that gives a comprehensive account of the field to 1996 (history, applications, specific recommendations for techniques, and data for identifying and quantifying pigments). Subsequent reviews [3, 4] highlight new developments in pigment chromatography. This chapter updates the current understanding of pigment analytical methods and data interpretation.

2 Pigment Markers

Phytoplankton contain three types of pigments involved in light harvesting and photoprotection: chlorophylls, carotenoids and biliproteins. Their chemical structures and properties have been extensively reviewed [5–8], as have their metabolism [9] and applications in oceanography [10]. Comprehensive data and graphics sheets were compiled for 47 of the most important chlorophylls and carotenoids found in marine algae [11]. Marker pigments discovered since 1996 are described in a recent review [1].

2.1 Chlorophylls

All photosynthetic phytoplankton contain one or more types of chlorophylls as part of the light-harvesting complexes in their chloroplasts. Chlorophylls are magnesium coordination complexes of conjugated cyclic tetrapyrroles with a fifth isocyclic ring and often an esterified long-chain alcohol [7, 12].

Figure 1 shows the structure and numbering system for chlorophyll *a* (Chl *a*), with four rings (A–D) of the tetrapyrrole macrocycle, a cyclopentanone ring (E) conjoint with ring C, and a propionic acid side-chain at C-17, esterified to the C_{20} alcohol, phytol. The central magnesium atom is bound to the nitrogen atoms of the pyrrole rings, but can also bind to electron donors on either side of the plane of the macrocycle: water, proteins, or the 13-keto group of another chlorophyll molecule.

Other chlorophylls differ according to the oxidation state of the macrocycle, the type of side-chains, and the type of esterifying alcohol, if present. The macrocycle may be a porphyrin, with rings A–D all fully unsaturated (Chl *c* family); a chlorin (17,18-dihydroporphyrin), with ring D reduced (Chls *a*, *b*, and *d*); or a bacteriochlorin (7,8,17,18-tetrahydroporphyrin), with rings B and D reduced (bacteriochlorophylls *a*, *b*, *g* [7]). Chl *b* differs from Chl *a*

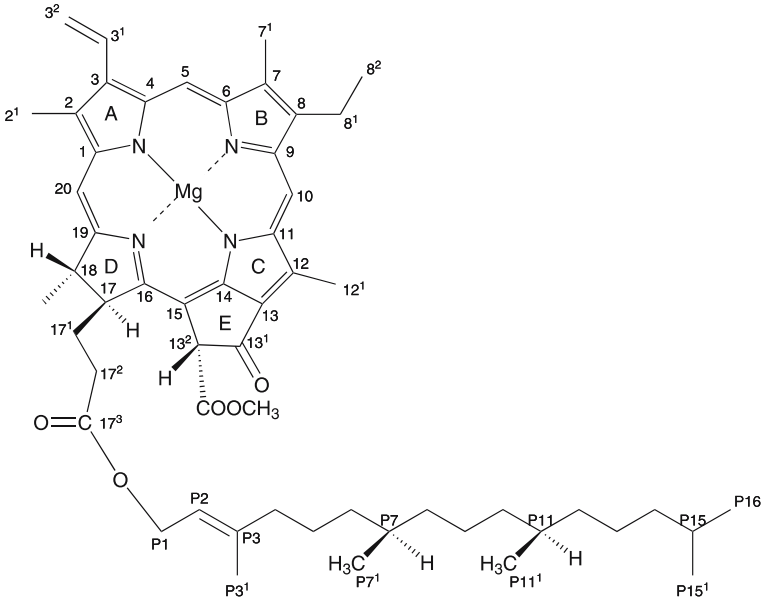

Fig. 1 Structure of Chl *a*, with numbering scheme, showing four rings (A–D) of the tetrapyrrole macrocycle, a cyclopentanone ring (E), a propionic acid side chain at C-17, esterified to phytol, from Data Sheets in [11]

by having an aldehyde rather than a methyl group at position C-7 of ring B, which alters its spectral properties and increases both its polarity and stability to photooxidation. Divinyl (DV) forms of both Chl *a* and Chl *b* are found in prochlorophytes, in which the C-8-ethyl group is replaced by a second vinyl group (in addition to that at C-3).

Nine members of the Chl *c* family have been identified in phytoplankton, mostly from the algal division Haptophyta [13]. In addition to being porphyrins, they differ from Chl *a* by having an acrylic, rather than a propionic acid side chain at C-17, in ring D (except MgDVP and Chl $c_{cs\text{-}170}$, which retain the propionic acid). In Chls *a* and *b*, this very acidic carboxyl group is esterified to phytol, but in the Chl *c* family, it is usually unesterified and can significantly affect the chromatographic properties of the molecule depending on pH and the presence of counter-ions. Chls c_1 and c_2 differ in having an ethyl group (c_1) or a vinyl group (c_2) at C-8 of ring B of the macrocycle [14]. Chl c_3 has a carbomethoxy group at position C-7 on ring B, as well as a vinyl group at C-8 [15, 16]. Monovinyl (MV) Chl c_3 has recently been identified in haptophytes [17]. Chl $c_{cs\text{-}170}$ [18] is thought to be the propionate derivative of Chl c_3 [11]. In several non-polar Chl *c* pigments the C-17 acrylic acid is esterified to a massive galactolipid side chain (Chl c_2-MGDG [19]).

Many chlorophyll derivatives are found both naturally and as artefacts of extraction. These may have lost the Mg atom (pheophytins), the phytol chain (chlorophyllides), both Mg and phytol (pheophorbides), and/or the C-13^2 carbomethoxy group (pyro-derivatives), and they may also spontaneously rearrange (epimers) or oxidize (allomers) [9]. Analytical techniques should be optimized to prevent the formation of these artefacts.

2.2 Carotenoids

Carotenoids are a diverse family of yellow, orange or red isoprenoid, polyene pigments—the carotenes (hydrocarbons) and xanthophylls (oxygenated carotenoid derivatives). Many are involved in light-harvesting, with the ability to absorb light of blue and green wavelengths (420–550 nm), bridging the gap between Chl *a* and *b* absorption bands. Certain carotenoids are involved in photoprotection, notably diadinoxanthin and diatoxanthin in chromophytes and violaxanthin, antheraxanthin, and zeaxanthin in green algae. These pigments are taxonomically useful but quantitatively variable since their abundance can change dramatically in response to irradiance. Carotenoids may also help to stabilize the photosynthetic apparatus [8]. Most have a C_{40} skeleton with alternating single and double bonds that form the chromophore, responsible for the spectral characteristics of the molecule [20]. The IUPAC nomenclature of carotenoids [21] (see http://www.chem.qmw.ac.uk/iupac/carot/) differs from general IUPAC rules [22]. The base name for a carotene depends on the end groups, four

types of which are found in phytoplankton (Fig. 2). IUPAC names specify both end groups, in order of their appearance in the Greek alphabet. For instance, Fig. 3a shows β,ε-carotene, along with the numbering system for carotenes and their derivatives. Trivial names were used for carotenes in older literature and are still sometimes encountered.

Common carotenoid modifications include the degree of unsaturation of the isoprenoid skeleton (with rearrangements including acetylenic and allenic units), oxygen functional groups (e.g. hydroxyl, ketones, and epoxides), and esterification of hydroxyl derivatives with acyl or large (705 Daltons) glycosidic groups. Many of these structural groups are found in 19′-hexanoyloxyfucoxanthin (Hex-fuco, Fig. 3b), formally (3*S*,5*R*,6*S*,3′*S*,5′*R*,6′*S*)-5,6-epoxy-3,3′,5′,19′-tetrahydroxy-6′,7′-didehydro-5,6,7,8,5′,6′-hexahydro-β,β-caroten-8-one 3′-acetate19′-hexanoate. Loss of in-chain carbons may result in shortened skeletons, e.g. the C_{37} skeleton of the abundant light-harvesting dinoflagellate carotenoid, peridinin.

Over 600 carotenoids are known in nature [23–25]. Many of the enzymatic pathways required for their synthesis are taxonomically restricted (particu-

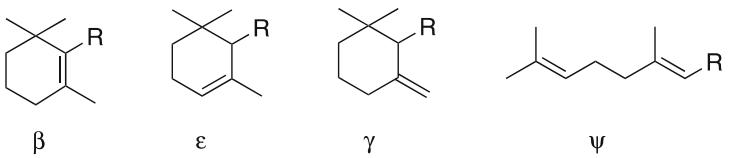

Fig. 2 Structure of the four types of end groups commonly found in carotenoids from phytoplankton, modified from Bjørnland [22]

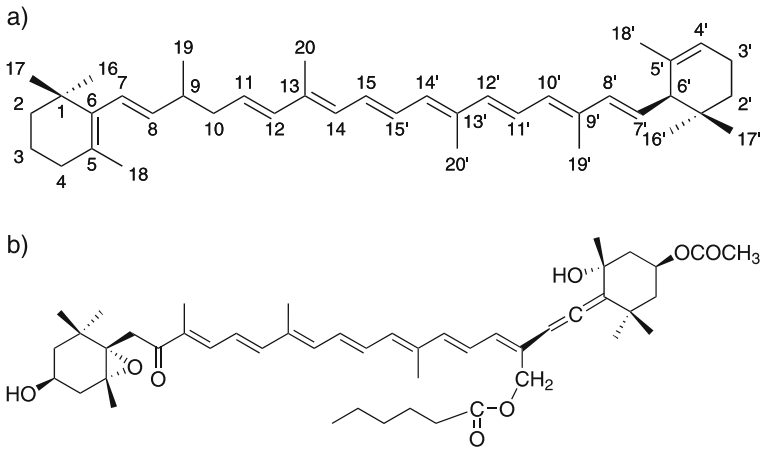

Fig. 3 Structures of carotenoids: **a** β,ε-carotene, with the numbering system for carotenes and their derivatives, **b** 19′-hexanoyloxyfucoxanthin, modified from Data Sheets in [11]

larly ε-cyclization, 5,6 epoxidation, allenic and acetylenic bond formation, 19-hydroxylation, 8-keto formation, acetylation, allelic 3-hydroxylation [26]).

Carotenoids are particularly labile molecules. Pigments with 5,6-epoxides, like diadinoxanthin and violaxanthin, readily form 5,8 furanoxides, especially under acidic conditions. Esters may be hydrolyzed. Carotenoids also spontaneously rearrange in solution. Nearly all carotenoids found in phytoplankton (with the notable exception of 9′-*cis*-neoxanthin), have the carbon atoms arranged around the skeletal double bonds in the *trans* form, producing linear molecules such as those shown in Fig. 3a. Once extracted into an organic solvent, carotenoids will form a stereoisomeric equilibrium mixture of all-*trans* and *cis* carotenoids. The *cis* forms are bent, exposing their (generally) hydrophobic mid-chains and usually retarding their elution in reverse-phase HPLC systems, producing additional peaks that complicate analyses.

Carotenoid absorption spectra generally have three peaks (depending on the solvent used), labelled I, II and III from short to long wavelengths. Often peaks I and III are reduced to shoulders or hidden altogether by overlapping of the peaks due to interference of functional groups with the carotenoid chromophore. The shape and position of the peaks vary in different solvents, and are useful in identification [11, 27]. The %III/II ratio is also useful, being the relative heights of the III and II peaks over the valley between them (not the baseline, see Data Sheets [11]). Identification of carotenoids is facilitated by descriptions of UV/visible spectra [20, 27], mass spectra [28], other data compilations [11], and analytical approaches [29].

2.3 Phycobiliproteins

Phycobiliproteins are the third type of light harvesting pigment found in cyanobacteria, rhodophytes and cryptophytes. Three main subtypes are found—the phycoerythrobilins, phycocyanobilins and the phycourobilins. The chromophore consists of an open-chain tetrapyrrole, which does not contain a central metal ligand like the chlorophylls, but is bound covalently to an *apo*protein [9].

Although phycobiliproteins are taxonomically restricted, they are generally not used as chemical markers, probably because the algal classes that contain them are so easily recognized and counted by techniques that detect biliprotein fluorescence directly in situ. These include epifluorescence microscopy [30, 31], flow cytometry [32–34] and delayed fluorescence excitation spectroscopy [35]. Since biliproteins are water soluble, they are not extracted by organic solvents used in analysis of chlorophylls and carotenoids. Their chemical analysis will not be considered further in this chapter.

2.4 Pigment Diversity and Chemotaxonomy

A recent review of pigment characteristics of microalgal classes [1] considered patterns of 56 pigments across 32 algal groups. The complexity of these pigment patterns is better understood through recent developments in endosymbiotic theories of the origins of plastid diversity [36–39].

These theories suggest that plastids originally arose by ingestion of a previously free-living cyanobacterium by a non-photosynthetic protist of unknown origin. Permanent symbioses that developed during subsequent evolution produced three major primary lineages, each monophyletic: the Glaucocystophyta, the Chlorophyta and the Rhodophyta radiations [40]. Modern Cyanophyta evolved from the ancestral cyanobacterium without further endosymbioses. Cyanophyta (e.g. *Richelia intracellularis*) may be found residing in modern-day algal taxa (e.g. the diatom *Rhizosolenia clevei*) in an association, in which the cyanophyte is not reduced to an organelle [41].

Further diversity was introduced by secondary and tertiary endosymbioses derived from engulfment of members of primary lineages by other protists. Thus plastids from the diatoms, brown algae, chrysophytes, haptophytes, cryptophytes and most dinoflagellates were derived by ingestion of rhodophytes by non-photosynthetic protists [1, 37]. Similarly euglenophytes, chlorarachniophytes and green dinoflagellates acquired their plastids from the chlorophytes. In some groups, multiple plastid losses and replacements have occurred—particularly in the dinoflagellates, which are now known to possess five different pigment suites, reflecting the various origins of their plastids.

In spite of the great diversity of pigments across algal taxa [1], relatively few are unambiguous markers. Many pigments are shared across taxa, making it necessary to consider suites of pigments when interpreting field data.

The most complete list of pigment distributions currently available (Table 20 in Jeffrey and Wright [1]) distinguished multiple major pigment suites (defined as Types 1 to *n*) within various algal divisions: Cyanophyta (two Types), Prochlorophyta (three), Prasinophyta (three), Chrysophyta (three), Dinophyta (five), and Haptophyta (eight). The remaining divisions (Chlorophyta, Euglenophyta, Rhodophyta, Cryptophyta, Bacillariophyta, Bolidophyta, Raphidophyta, and Eustigmatophyta) contained only one major pigment type. A key problem is that many taxa share the same pigment patterns, and thus they often cannot be distinguished on the basis of pigments alone. Microscopy or other methods should be used to assist identification of phytoplankton types in field samples.

Table 1 summarizes the pigment distribution of commonly encountered phytoplankton groups, using the type definitions of Jeffrey and Wright described above.

Environmental factors strongly influence pigment composition of microalgae. These include irradiance [42–45], spectral distribution of light [46–48],

Table 1 Definitive pigments in selected algal taxa are listed using the Type definitions from Table 20 in Jeffrey and Wright [1]. Thirteen chemotaxonomically useful pigment suites are presented in *bold*, while eight single pigments unique to specific algal Types are marked with an *asterisk*

Algal type	Definitive pigments
Cyanophyta (e.g. *Synechococcus* sp.)	**Chl *a*, zeaxanthin**
Prochlorophyta (e.g. *Prochlorococcus marinus*)	**DV Chl *a**, DV Chl *b****, β,ε-carotene, zeaxanthin
Chlorophyta (e.g. *Chlorella* sp.) Prasinophyta Type 1 (e.g. *Tetraselmis* sp.)	**Chl *a*, Chl *b*, lutein**, neoxanthin, violaxanthin, zeaxanthin (tr)
Prasinophyta Type 2 (e.g. *Pyramimonas amylifera*)	**Chl *a*, Chl *b*, MgDVP, siphonaxanthin ester***, neoxanthin, violaxanthin (minor)
Prasinophyta Type 3 (e.g. *Micromonas pusilla*) Dinophyta Type 5 (e.g. *Gymnodinium chlorophorum*)	**Chl *a*, Chl *b*, MgDVP, prasinoxanthin***, **uriolide, micromonal**
Cryptophyta (e.g. *Rhodomonas*) Dinophyta Type 4 (e.g. *Dinophysis norvegica*)	**Chl *a***, Chl c_2, **alloxanthin***, crocoxanthin, monadoxanthin
Bacillariophyta Type 1 (e.g. *Phaeodactylum tricornutum*) Haptophyta Type 1 (e.g. *Pavlova lutheri*) Dinophyta Type 3 (e.g. *Kryptoperidinium foliaceum*)	**Chl *a***, **Chl c_1**, Chl c_2, **Fucoxanthin**, diadinoxanthin
Haptophyta Type 6 (e.g. *Emiliania huxleyi*)	**Chl *a***, Chl c_2, **Chl c_3**, MV-Chl c_3 (tr)*, **Hex-fuco**, fucoxanthin, diadinoxanthin, 4-keto-Hex-fuco
Haptophyta Type 7 (e.g. *Chrysochromulina polylepis*)	**Chl *a***, Chl c_2, **Chl c_3**, **Chl c_2-MGDG[14 : 0/14 : 0]***, **Hex-fuco**, fucoxanthin, diadinoxanthin, 4-keto-Hex-fuco
Haptophyta Type 8 (e.g. *Phaeocystis antarctica*)	**Chl *a***, Chl c_2, **Chl c_3**, Chl c_2-MGDG[18 : 4/14 : 0], **Hex-fuco**, **But-fuco**, fucoxanthin, diadinoxanthin

Table 1 (continued)

Algal type	Definitive pigments
Chrysophyta Type 3 (Pelagophytes) (e.g. *Pelagococcus subviridis*)	**Chl *a***, Chl c_2, **Chl c_3**, **But-fuco**, fucoxanthin, diadinoxanthin
Dinophyta Type 1 (e.g. *Amphidinium carterae*)	**Chl *a***, Chl c_2, **peridinin***, diadinoxanthin
Dinophyta Type 2 (e.g. *Gymnodinium galatheanum*)	**Chl *a***, Chl c_2, **Chl c_3**, **Hex-fuco**, **gyroxanthin diester***, fucoxanthin, diadinoxanthin

daylength [49], diurnal cycle [50], nutrient status [43, 45], iron concentration [51, 52], growth phase [44, 53], and strain differences [13, 54]. This variability is usually limited to changes in the total pigment quantity per cell rather than the type of pigments present, although in senescent or nutrient-limited populations secondary pigments may be produced.

Pigment concentrations within taxa may also vary regionally due to strain variations. For instance, most strains of *Phaeocystis* spp. contain significant quantities of Hex-fuco [13, 44, 55], but many northern European strains lack this pigment [56–58]. It is therefore advantageous to have cultures of local strains for reference.

3 Methods of Analysis

Many methods of varying accuracy are available for chlorophyll analysis, with HPLC the most powerful. If an estimate of total phytoplankton biomass is all that is required, then spectrophotometric or fluorometric analysis of Chl *a* may be appropriate. The accuracy of spectrophotometry, fluorometry and HPLC were compared in the SCOR/UNESCO volume [59]. Briefly, it was found that the "Jeffrey and Humphrey (1975)" spectrophotometric method [60] and the "Holm-Hansen et al. (1965) extracted" fluorometric method [61] were reasonably accurate if chlorophyll degradation products were absent. The acid-spectrophotometric and fluorometric methods reduced interference from pheophytins and pheophorbides, but chromatography was required to accurately assess chlorophylls in the presence of degradation products. If analysis of marker pigments is required, then HPLC is the best method.

In situ and in vivo fluorometry are useful aids to HPLC pigment sampling, giving continuous fine scale resolution of phytoplankton populations

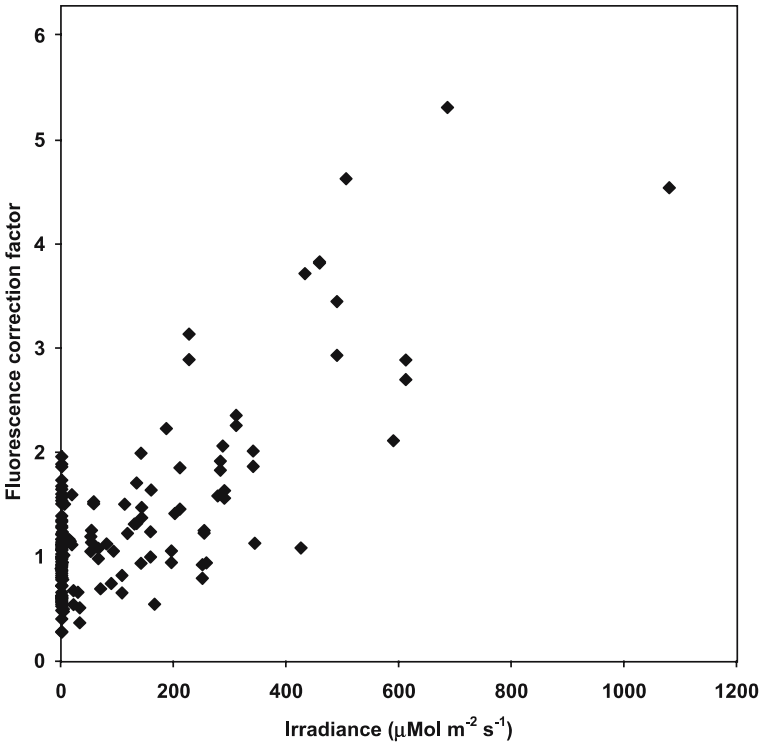

Fig. 4 Change in HPLC Chl *a*: in vivo fluorescence correction factor correlated with ambient irradiance. The correction factor equals unity for night time samples, and increases at high irradiances due to fluorescence quenching

that cannot be matched by discrete sampling regimes. In situ profiles by fluorometers attached to CTDs allow directed sampling of stratified populations. Surface fluorometry also provides immediate (but approximate) underway data.

Fluorometry, being non-discriminate, suffers from interferences from compounds other than Chl *a*. In estuarine samples, fluorometry sometimes overestimates Chl *a* by nearly two orders of magnitude compared to HPLC, probably due to fluorescent compounds abundant in dissolved organic matter [62]. The in vivo fluorescence response per unit Chl *a* is altered by irradiance, and the response curve changes markedly during the day. A five-fold variation was recently found in chlorophyll fluorescence (in vivo) in Southern Ocean phytoplankton due to diurnal changes in irradiance (Wright, unpublished; Fig. 4). It is, therefore, desirable to collect at least five HPLC samples per day, throughout the 24-hr cycle, to calibrate underway Chl *a* fluorescence.

3.1
Collection and Storage of Field Samples

Water samples may be collected from surface samplers, Niskin bottles or a clean seawater line. From the moment of collection, the pigment ratios will begin to change due to the altered light environment. In multidisciplinary cruises, water samples may not be available from Niskin bottles for 40 minutes or more, while gas samples are taken, during which time pigments of the violaxanthin or diadinoxanthin cycles can change markedly.

Suitable filtration equipment and recommended procedures are described elsewhere [63]. If the volume of seawater sample is limited, overall method sensitivity can be increased by using small diameter glass fibre filters that can subsequently be extracted in a small volume of solvent. The filters should be folded, blotted and stored immediately in liquid nitrogen. Alternatively, they may be stored for several days at – 20 °C or several weeks at – 90 °C [64]. Samples should not be freeze-dried since this causes degradation and reduces extractability of the pigments [64]. It is important to blot remaining seawater reproducibly and as completely as possible from the folded filter, since any remaining water will dilute the extraction solvent, altering its effectiveness and potentially altering HPLC retention times. Representative samples should also be preserved for light and electron microscopy.

3.2
Extraction of Pigments

Accurate analysis of phytoplankton pigments depends on the effectiveness of the extraction technique yet, despite the importance of this step, several extraction techniques of varying effectiveness remain in current use. There is disagreement over the best physical techniques of sample disruption (grinding, bath sonication, high-powered probe sonication, or soaking) and the most suitable solvents [acetone, methanol, dimethyl formamide (DMF)]. Three criteria are important—the ability to completely extract all pigments from field samples irrespective of the phytoplankton species composition, compatibility with the chromatographic technique (the ability to produce sharp peaks), and stability of pigments in the extraction solvent (since samples must often wait many hours in an autosampler before injection).

Common methods and solvents for extraction are reviewed in the UNESCO monograph [65]. The relative performance of different solvents varies according to the extraction methods used and the type of alga being extracted. DMF was the best solvent for pigment extraction when used with a high-powered probe sonicator, but the toxicity and ease of skin absorption of DMF for the operators make it too hazardous for safe use at sea. Sonication in 100% methanol was recommended as the second best method. The JGOFS protocol instead recommended extraction in acetone using bath soni-

cation and soaking overnight in a freezer (– 18 °C), and this technique is used by many groups.

Methanol has some advantages for extraction. It has lower volatility than acetone, and produces sharper HPLC peaks than acetone extracts, particularly for polar pigments [66]. A disadvantage is that methanol promotes allomerization of Chl *a* [67], and recent data [68] suggests that holding methanol-extracted samples in an autosampler at 4 °C produces significant pigment degradation over 24 hours, more than occurs in samples which are extracted in acetone.

Experiments comparing extraction protocols in our laboratory (Wright and van den Enden, unpublished) using local estuarine seawater, confirmed the superiority of methanol for extracting Chl *b* [65]. Pigment degradation was not regularly observed in either methanol or acetone. Occasional losses of pigments were seen in both solvents, suggesting that variations in sample composition, perhaps lipid content, may have affected the losses of the pigments, through coprecipitation and/or adherence to surfaces.

Clearly more work is required to better characterize different protocols and to determine factors that affect pigment stability in the various solvents. The type of microalgae being extracted is also important.

3.3 Choice of HPLC Methods

HPLC analysis of algal pigments presents a major challenge due to the diversity of large molecules spanning a wide range of polarities, but also including many that have closely similar chemical structures, some differing only by the position of a double bond.

Routine pigment analysis of field samples became feasible with the development of HPLC methods in the 1980s [69–74] with automated analysis and quantitation of pigments, and the possibility of on-line identification using diode-array detection. All current methods use reverse-phase chemistry, in which compounds are resolved primarily on their polarity, with C_8–C_{30} stationary phases and gradient elution. Resolution of acidic chlorophylls, for which buffering and ion-pairing or ion-suppression reagents are required, has until recently been achieved using ammonium acetate, sometimes coupled with tetrabutylammonium acetate (TBAA) [75, 76].

The Wright et al. (1991) technique recommended in the UNESCO monograph [77] used a reverse-phase C_{18} monomeric column and ammonium acetate modifier that provided good resolution of 40 algal carotenoids and 12 chlorophylls and their derivatives, but lacked resolution of monovinyl and divinyl chlorophyll pairs, in particular Chl c_1/c_2, and the important MV/DV forms of Chls *a*, *b*, and c_3. This method remains in common use but it has been superseded by methods that exploit the subtle polarity differences between MV/DV Chl pairs on monomeric C_8 columns or use molecular shape

selectivity of pigments on polymeric stationary phases. Such approaches have been recently evaluated and reviewed [3, 4].

Two new methods using monomeric C_8 columns have improved resolution by replacing ammonium acetate with either pyridine or TBAA modifier:

- The Zapata et al. (2000) method [78] uses a gradient from aqueous methanol/acetonitrile to methanol/acetonitrile/acetone, with pyridine modifier (as the acetate, pH 5.0), achieving resolution of seven polar Chl *c* derivatives, the Chl *a*/DV Chl *a* pair and partial resolution of Chl *b*/DV Chl *b*.
- The Van Heukelem and Thomas (2001) method [76] uses an aqueous methanol to methanol gradient at 60 °C with TBAA modifier (pH 6.5).

Both of these C_8 methods have greater resolution of the Chl *c* family than the C_{18} Wright et al. (1991) method [77], and are recommended for routine analysis, but neither is perfect. Relative differences in their resolution of significant pigment pairs are summarized in Table 2, and two representative chromatograms are presented in Fig. 5a,b.

Two other methods offer particular advantages:

- The Garrido and Zapata (1997) method [75] employs molecular shape selectivity on a polymeric C_{18} column with an aqueous methanol/acetonitrile to acetone gradient with pyridine modifier at 15 °C. It achieves excellent resolution of MV/DV pairs, but poorer resolution of carotenoids than the other methods due to the slower mass transfer characteristics of polymeric coatings. The retention order of several pigments differs from other methods, offering a useful means of confirming pigment identities [79].
- The Airs et al. (2001) method [80] achieves excellent resolution of bacteriochlorophylls and should be used whenever photosynthetic bacteria are present.

Pigment retention times of these three methods are compared with those of the Wright et al. (1991) method in Table 3. Whichever HPLC method is em-

Table 2 Relative differences in the resolution of significant pigment pairs in the Zapata et al. (2000) and Van Heukelem and Thomas (2001) methods. Resolution factors (*R*) are the difference in retention times divided by the average of peak widths [134]

Pigment pair	Resolution factors (*R*)	
	Zapata et al. (2000)	Van Heukelem and Thomas (2001)
MgDVP/Chl c_2	Good ($R > 1$)	Poor ($R < 0.5$)
Chl *b*/DV-Chl *b*	Poor ($R < 0.5$)	Partial ($R = 0.8$)
4-k Hex/9-*cis* Neox	Complete ($R > 1.25$)	Not resolved
Lutein / Zeaxanthin	Partial ($R = 0.8$)	Good ($R = 1$)

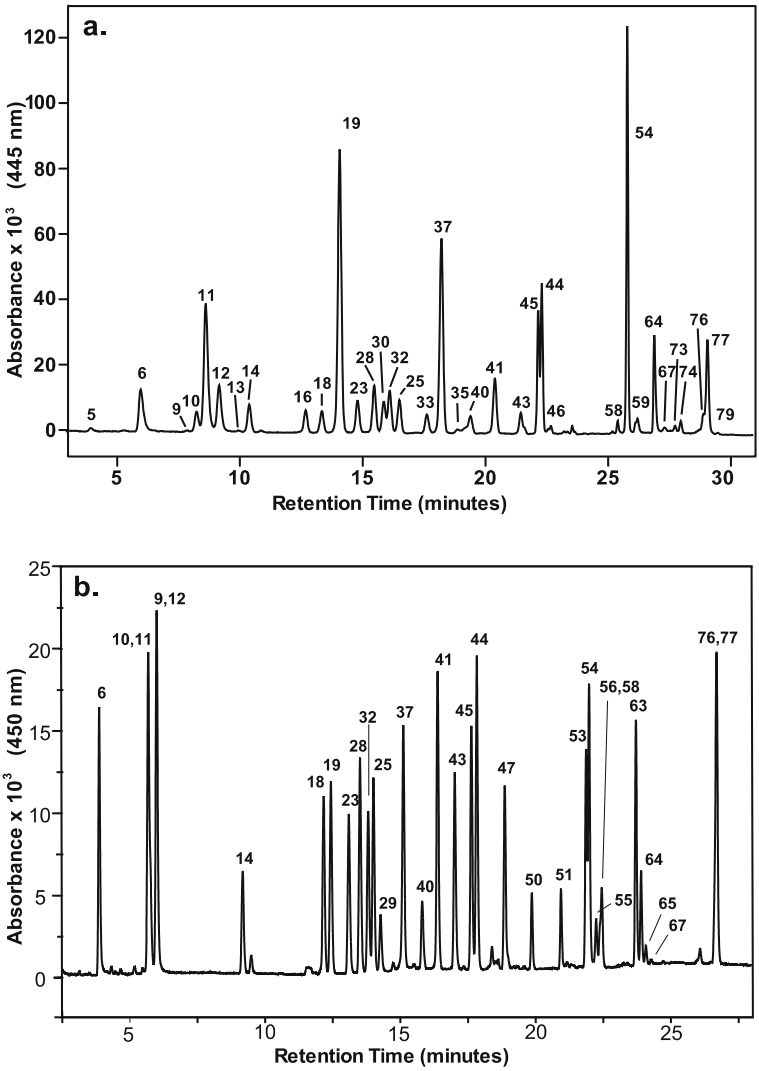

Fig. 5 Example chromatograms from HPLC methods: **a** Zapata et al. (2000) method [78]: from mixed cultures (see Sect. 3.3, S. Wright, unpublished); **b** Van Heukelem and Thomas (2001) method [76]: mixed pigments. For peak identities, see Table 3

ployed, it is important that the HPLC system is correctly optimized to produce the best results (see guidelines [81]).

Table 3 Comparisons of pigment retention times (RT) between four chromatographic methods. Pigment peak numbers correspond to those in Fig. 5a,b. Species names as suffixes refer to the original sources of the pigments: *Pavlova gyrans*, *Prymnesium parvum* and *Micromonas pusilla*

Peak No.	Pigment	RT (min) in various Methods			
		Wright et al. (1991) [77]	Zapata et al. (2000) [78]	Van Heukelem & Thomas (2001) [76]	Garrido & Zapata (1997) [75]
0	(solvent front)	2.56	1.93		
1	Chlide *b*	4.48	5.43		
2	Carotenoid P468	4.50			
3	Peridininol		6.06		
4	Methyl Chlide *b*		7.19		
5	Carotenoid P457	5.11			
6	Chl c_3	5.38	7.94	3.88	13.85
7	Chl *c P. gyrans*		8.27		8.42
8	MV Chl c_3	5.38	8.66	4.14	13.31
9	Chlide *a*	5.01	10.46	6.06	4.89
10	MgDVP	6.40	11.01	5.81	10.59
11	Chl c_2	6.40	11.44	5.70	14.94
12	Chl c_1	6.40	12.14	6.05	13.04
13	Methyl Chlide *a*	6.40	13.13		
14	Peridinin	7.42	14.20	9.32	7.88
15	Siphonaxanthin	8.11	14.76		
16	Uriolide		17.03		9.51
17	4-Keto-fucoxanthin		17.60		
18	But-fuco	8.11	17.94	12.31	8.15
19	Fucoxanthin	8.70	18.87	12.63	9.23
20	*trans*-Neoxanthin	9.11			
21	*cis*-But-fuco	9.12			
22	Neochrome	9.21			
23	9′ *cis*-Neoxanthin	9.31	19.62	13.29	11.14
24	4-Keto-Hex-fuco	9.31	20.92	13.31	8.96
25	Hex-fuco	9.31	21.75	14.16	10.05
26	*cis*-Fucoxanthin	9.68			
27	*cis*-Hex-fuco	9.97			
28	Prasinoxanthin	10.20	20.46	13.74	12.77
29	Micromonol		20.99		13.58
30	Phaeophorbide *a*	10.39			
31	Violaxanthin	10.59	21.32	13.99	14.12
32	Micromonal		23.24		15.75
33	Phaeophorbide *a*-like	10.62			
34	Dinoxanthin	10.76	25.22	15.49	14.67
35	*cis*-Prasinoxanthin	11.11			
36	Diadinoxanthin	11.61	24.11	15.23	
37	Diadinochrome I	11.79	23.27	15.02	

Table 3 (continued)

Peak No.	Pigment	RT (min) in various Methods			
		Wright et al. (1991) [77]	Zapata et al. (2000) [78]	Van Heukelem & Thomas (2001) [76]	Garrido & Zapata (1997) [75]
38	Diadinochrome II	11.96			
39	Antheraxanthin	12.24	25.38	15.99	17.93
40	Alloxanthin	12.51	26.25	16.53	19.83
41	Monadoxanthin	12.78	27.07	17.22	25.53
42	Diatoxanthin	13.08	26.90	17.12	
43	Lutein	13.36	27.65	17.98	20.37
44	Zeaxanthin	13.59	27.49	17.79	21.46
45	Dihydrolutein	13.83	28.00		
46	Canthaxanthin	14.00		19.07	
47	*trans*-β-*apo*-8′-Carotenal	14.00			
48	Siphonein	14.36	29.37		
49	Gyroxanthin diester			19.94	
50	Gyroxanthin diester			21.00	
51	Chl *b* allomer	14.85	31.28		
52	DV Chl *b*	15.15	31.58	21.92	
53	Chl *b*	15.15	31.62	22.03	23.9
54	DV Chl *b* epimer			22.29	
55	Chl *b* epimer		31.87	22.50	
56	Ethyl 8′-β-apocarotenoate	15.43			
57	Crocoxanthin	15.87	31.11	22.42	
58	Chl c_2-MGDG[18 : 4/14 : 0]		32.18		31.51
59	Np-Chl (c_1 like) *P. parvum*		32.44		28.79
60	Chl *a* allomer	15.87	32.63	23.30	
61	Np-Chl c_2			23.53	
62	DV Chl *a*	16.15	32.83	23.76	
63	Chl *a*	16.15	33.15	23.96	27.16
64	DV Chl *a* epimer	16.53		24.13	
65	Chl c_2-MGDG[14 : 0/14 : 0]		33.50		34.49
66	Chl *a* epimer	16.53	33.48	24.33	28.47
67	Echinenone	16.74			
68	Lycopene	17.59			
69	Phaeophytin *b*	17.68			
70	*cis*-Lycopene	17.84			
71	Phaeophytin *a*	18.56	35.41		
72	β,y-Carotene	18.26	34.25		
73	Unk carotenoid *M. pusilla*		34.95		30.42
74	ε,ε-Carotene	18.40	35.52		
75	β,ε-Carotene	18.64	35.74	26.65	32.59
76	β,β-Carotene	18.76	35.95	26.71	32.86
77	*cis*-β,ε-Carotene	18.83			
78	*cis*-β,β-Carotene	18.94	36.26		

3.4
Peak Detection and Integration

For samples containing a variety of pigments, a diode-array detector is essential to allow identification of peaks from their spectra collected during elution.

The following wavelengths are useful for routine detection and integration

- 435 nm—detects all common pigments except phaeophytin *a*, phaeophorbide *a* and their derivatives;
- 470 nm—detects carotenoids, Chl *b*, and Chls *c*, without interference from Chl *a* derivatives;
- 665 nm—detects Chl *a*, phaeophytin *a*, phaeophorbide *a* and their derivatives.

Sensitivity can be increased in most systems by including a channel that sums a range of wavelengths (e.g. 427–464 nm). Such "wavelength bunching" improves the signal to noise ratio, but reduces the selectivity of the detection.

Fluorescence detection is a valuable addition to diode-array detection, due to its sensitivity and selectivity. Broad excitation and emission bandwidths are better than narrow bandwidths since sensitivity and detection of all chlorophyll derivatives are increased [81].

3.5
Peak Identification and Quantitation

Peaks are identified by comparison of their retention times and spectra with those of standard pigments. Many are commercially available, but expensive, and not always pure. It is far more efficient (and cheaper) to employ pigments from well-characterized reference phytoplankton cultures [82].

A standard mixture of pigments is injected (after a blank column conditioning cycle) before each batch of samples. This is prepared by mixing extracts of (typically) *Pavlova lutheri*, *Pelagococcus subviridis*, *Micromonas pusilla*, *Dunaliella tertiolecta*, *Amphidinium carterae*, and *Chroomonas salina*. Individual algae are analyzed first, then mixed so that the peak heights of major pigments are approximately equal. Aliquots (0.5 ml) of the mixture are dispensed into cryotubes that are immediately frozen in liquid nitrogen. A freshly thawed sample is injected each day, providing an invaluable monitor of system performance as well as the basis of a retention time table.

Peaks can be quantified using either the internal standard (IS) or external standard methods [83]. Using an IS gives increased accuracy and precision, since it accounts for any volume changes due to evaporation or dilution, and it also provides a check on the injection status. It is thus recommended for routine oceanographic samples, but for unfamiliar samples it is prudent to run a sample without an IS in case there are pigments that co-chromatograph

with it. The most commonly used commercially available internal standards are ethyl 8′-apo-β-carotenoate, 8′-apo-β-carotenal and canthaxanthin, the first of which is most suitable due to its stability and non-occurrence in natural systems.

3.6 Data Quality

Diagnoses of general HPLC instrumental problems are beyond the scope of this chapter, but several problems may be encountered during pigment analysis that can reduce data quality.

Significant peaks of chlorophyllide *a* (Chlide *a*) are often seen in chromatograms of some diatoms because chlorophyllase enzymes are activated when the cell is damaged, e.g. during filtration, storage or extraction [63, 84, 85]. Significant degradation of chlorophyll may occur if the cells are left too long on the filter, frozen too slowly or not cold enough, or extracted in a solvent that does not inactivate the chlorophyllase. Chlorophyllases are inactivated more rapidly by 100% acetone than 90% acetone [84, 86]. Sonication in methanol can produce some Chlide *a* (although the resultant methyl-Chlide *a* is clearly recognizable as an extraction artefact [87]). Chlide *a* concentration is generally included in the total Chl *a* fraction for biomass estimation [88].

Chromatography problems may be caused by animal lipids in a sample, e.g. from a copepod on the filter. These are extracted along with the pigments and may cause smeared chromatographic peaks (eluting later, with lower peak heights and severe tailing). Any animals visible on the filter should be removed before freezing the sample [63]. In extracting symbiotic microalgal pigments from animal tissue, the microalgae should first be isolated before pigment extraction to prevent such artefacts [89].

Peak integration errors may be introduced by reproducible baseline changes that occur during gradient elution due to refractive index changes in the solvents. These are often most pronounced in the region of Chl *a* elution [at least in the Wright et al. (1991) and Zapata et al. (2000) methods] and may interfere with the integration of Chl *a* in samples with low concentrations. It is thus preferable to use a fluorescence detection channel for such samples as this is unaffected by refractive index.

For large peaks, an optimized HPLC system can achieve a precision of about 1% [90], but the uncertainty increases as the peak size is reduced and detector noise becomes more significant. A recent intercalibration exercise [91] found average percentage differences of 7.0% for total Chl *a* between laboratories, which was reduced to 5.5% after excluding very low values, standardizing quantitation and accurately accounting for DV-Chl *a*. The authors suggested that all pigment data submitted to databases should include information on the limits of detection for each pigment.

4
Taxonomic Interpretation and Quantitative Analysis of Pigment Data

This section describes a systematic approach for determining the likely taxonomic components of field samples from HPLC pigment chromatograms, and the various mathematical techniques available for estimating the relative abundance of algal types. The merits of these mathematical techniques are then considered in a summary of some recently published quantitative studies.

4.1
Hierarchical Guide to Interpreting Pigment Data

Due to the large number of pigments involved, a formal approach should be used to interpret chromatograms, based on knowledge of the most recent pigment distributions [1]. After considering Chl *a*, an index of total phytoplankton biomass (excluding prochlorophytes), one works through a pigment hierarchy, starting with unambiguous markers for algal types, then deducing the algal composition from the content of other pigments. Table 4 suggests a possible approach. Having derived the likely taxonomic composition of field samples from the HPLC chromatograms, quantitative analysis of pigment data can be undertaken. Due to the environmental variability of pigment composition, one cannot simply use pigment ratios from cultures and apply them to field populations. Pigment : Chl *a* ratios must be determined from analysis of the field data [3], using the analytical tools below.

4.2
Mathematical Tools for Interpretation of Pigment Data Sets

Until recently, most applications of pigment methods to oceanography [10] were semi-quantitative and based on the use of single marker pigments for particular taxa. Qualitative analysis was introduced through the application of multiple regression, inverse simultaneous equations, and matrix factorization through CHEMTAX software.

4.2.1
Multiple Regression

Multiple regression allows a statistically sound analysis of the relationship between various marker pigments and total Chl *a* [70]. It does not allow for shared pigments between algal taxa, so that while the contribution of "fucoxanthin-containing" algae, for example, may be accurately determined, it is not known whether the fucoxanthin came from diatoms, haptophytes, chrysophytes or mixed populations. The contribution of minor groups may be ignored altogether if they are swamped by noise in the data [92]. As an ex-

Table 4 Hierarchical guide for interpreting pigment field data

Pigment	Significance
Chl *a*	An index of total algal biomass, excluding prochlorophytes
Unambiguous markers for algal types	
DV-Chl *a*	An index of prochlorophyte biomass
DV-Chl *b*	Unambiguous marker for prochlorophytes
Siphonaxanthin esters	Unambiguous marker for Type 2 prasinophytes [135]
Prasinoxanthin	Unambiguous marker for Type 3 prasinophytes
Peridinin	Type 1 dinoflagellates
Alloxanthin	Cryptophytes
Gyroxanthin diester	Dinoflagellates Type 2
Chl c_2 MGDG [14 : 0/14 : 0]	*Chrysochromulina* spp. (Haptophyte Type 7) [13]
Chl *b*	Distinguishes "green algae" (chlorophytes, prasinophytes, euglenophytes and green dinoflagellates) from all other algal types. The relative proportion of these groups can be deduced [111] from the proportions of the following major carotenoids [44, 45, 90, 105]. Types 2 and 3 prasinophytes are distinguished by the presence of **siphonaxanthin esters** and **prasinoxanthin**, respectively (see above). Chlorophytes and Type 1 prasinophytes can be identified by their relative ratios of **Lutein** to **Chl *b*** (Lut : Chl *b* = 0.30–1.77, 0–0.18, respectively). Euglenophytes are difficult to distinguish since their major carotenoid, **diadinoxanthin**, is a major component of the chromophytes. Green dinoflagellates have no known distinguishing pigments and must be identified microscopically before fixation.
Chl *c* series	Distinguishes chromophyta from all other algal types
Chl c_1	Widely distributed. A useful marker for diatoms in populations dominated by Type 6 and Type 8 haptophytes [108]
Chl c_2	The major Chl *c* component in chromophyte algae
Chl c_3	A significant component of Haptophytes Types 4–8 [13] including coccolithophorids (Type 6), *Chrysochromulina* sp. (Type 7), and *Phaeocystis* sp. (Type 8). Chl c_3 is also present in Chrysophytes Type 3 (pelagophytes), bolidophytes, and some diatoms [136], notably the harmful bloom-forming genus, *Pseudonitzschia* [137].
Chl c_2 MGDG	Haptophytes Type 3–8 contain Chl c_2 MGDG [18 : 4/14 : 0] [13]. See also Chl c_2 MGDG [14 : 0/14 : 0], above.
MgDVP	A marker for prasinophyte Types 2 and 3, but it occurs in trace amounts in most algae
Chl c_2 *P. gyrans* type	(Minor pigment) chrysophytes Type 1 and haptophytes Type 2

Table 4 (continued)

Pigment	Significance
Np-Chl c_1 like	(Minor pigment) haptophytes Type 4 [13]
MV-Chl c_3	(Minor pigment) haptophytes Type 6 (coccolithophorids)
Fucoxanthin and derivatives	
Fucoxanthin	Erroneously regarded as a unique marker for diatoms. Also present in haptophytes, chrysophytes, raphidophytes, bolidophytes and some dinoflagellates [1].
Hex-fuco	Restricted to haptophytes Types 6–8 (but see Sect. 2.4) and dinoflagellates Type 2 (which also have **gyroxanthin diester**, see above).
But-fuco	Restricted to haptophytes Type 8 (with traces in haptophytes Types 6 and 7), where it always co-occurs with Hex-fuco. Pelagophytes contain But-fuco, but no Hex-fuco.
Other pigments	
Zeaxanthin	A useful marker for cyanobacteria when they are a major component of the population; widespread in low concentrations in prochlorophytes, chlorophytes, prasinophytes, euglenophytes, chrysophytes, raphidophytes and eustigmatophytes.
Vaucheriaxanthin esters	Markers for eustigmatophytes [1] and chrysophytes Type 1 (Jeffrey et al., unpublished data).
Loroxanthin	An occasional component of chlorophytes [138]; a useful marker for chlorophytes in Antarctic waters [108].
Bacteriochlorophyll *a*	A marker for photosynthetic proteobacteria [139, 140].
Diadinoxanthin and Diatoxanthin	Major pigments of the chromophyte algae are found in most oceanic HPLC chromatograms. While they are not definitive taxonomically, their role in the light-regulated epoxide cycle [9] allows their ratio to Chl *a* to be used as an index of the light history [141] of the algae in the water column. This can be used to compute vertical mixing velocities [142]. The two pigments are interconverted on a timescale of minutes [143–145], too fast for conventional oceanographic sampling methods.

ploratory tool, multiple regression is excellent for pigment data since it does not require assumptions about the composition of the phytoplankton population, and it remains in common use [92–94].

Even if more advanced mathematical techniques are to be employed, it is helpful to first plot or run multiple regressions of the concentrations of major pigments against Chl *a* to determine the likely main components. Similarly, running multiple regressions of green algal pigments (lutein, prasinoxanthin, siphonaxanthin esters) against Chl *b*, and fucoxanthin and its derivatives

against Chl c_2 may be useful. An important check for interpreting such relationships is to test the correlation between two pigments that are not found in the same organism, e.g. Hex-fuco and Chl *b*. This will show to what extent there is a correlation between unrelated taxa, such as might occur following a nutrient incursion. Where such correlation exists, other correlations must be treated with caution.

4.2.2 Inverse Simultaneous Equations

This approach [95–98] uses a series of simultaneous equations in which the contribution to total Chl *a* by each algal group is calculated from the concentration of each marker pigment, choosing the appropriate marker pigment : Chl *a* ratios (see Sect. 4.2.4) and including proportionate subtraction of markers shared between groups. Each equation includes the chosen values for each marker pigment : Chl *a* ratio and the proportion of shared markers. These ratios are modified by inverse methods to find the least squares best solution to the total concentration of Chl *a*. This approach is less flexible than matrix factorization (see the following section).

4.2.3 Matrix Factorization (CHEMTAX Software)

CHEMTAX analysis [90, 99] is fundamentally similar to the simultaneous equations method, except that instead of building a series of simultaneous equations, the operator constructs a matrix of the algal types and their pigment content (from microscopy and examination of chromatograms in the data set, see Sect. 4.1). A second matrix constrains how far each pigment ratio can change. Each element of the pigment ratio matrix is iteratively modified to optimize the agreement between observed and computed pigment abundance, to estimate the marker pigment : Chl *a* ratios in the field samples.

Entering pigment data as a matrix is more flexible for adjusting the taxonomic makeup and more suited to handling shared markers than building sets of equations. It readily accommodates hypothesis testing whereby several different models of a field population can be compared. The study by Havskum et al. [100] provides an excellent approach to using CHEMTAX on field samples.

4.2.4 Characteristics of Computational Methods

Simultaneous equations and CHEMTAX are both able to distinguish broad algal groups within a phytoplankton population, making it possible to map the distribution geographically or in relation to oceanographic features. Both ap-

proaches require the pigment ratios to be stable within the data set. Thus any known factors affecting pigment ratios must be eliminated as far as possible, e.g. by breaking the data set into subsets by water masses (controlling nutrient status) and sample depth (controlling irradiance [101, 102]). The range of pigment : Chl *a* ratios calculated by CHEMTAX in field studies is smaller than observed in culture (Higgins and Descy, in preparation), probably because phytoplankton in the mixed layer never get the chance to adapt their pigmentation to a constant irradiance, and develop a composition closer to the median.

Unlike multiple regression, the computational methods do not ignore algal groups with low abundance, but in general pigment ratios for such groups are not optimized and the groups are quantified on the basis of the chosen initial ratio, upon which the accuracy of the final determination depends.

CHEMTAX was originally tested using synthetic data sets based on culture analyses [90], with which it performed well, and then with field data from a surface transect across the Southern Ocean [99]. In the latter case, CHEMTAX could distinguish two populations of haptophytes with identical pigment compositions; the northern population, considered to be mainly coccolithophorids, showed a distribution consistent with a previous microscopic study. The southern populations were considered to be *Phaeocystis sp*. In a recent study of the Rio de La Plata [103], CHEMTAX was able to distinguish four categories of haptophytes as well as pelagophytes and diatoms.

Choosing starting ratios for CHEMTAX (or inverse simultaneous equations) remains the biggest problem due to insufficient knowledge of algal pigment ratios in the field. If local data are not available, then ratios recommended in the CHEMTAX manual [90] supplemented by recent surveys [13, 104, 105] will serve as a guide. However, these ratios are known to vary according to region, and it is far better to use pigment data from cultures of local isolates, grown under an appropriate range of conditions.

It is worthwhile performing multiple runs of CHEMTAX using a range of initial marker pigment : Chl *a* ratio matrices. CHEMTAX works by iteratively minimizing the pigment residual (i.e. the difference between observed and calculated concentrations of the pigment to be optimized, usually Chl *a*). It is apt to find a local minimum rather than the overall minimum for the data set, particularly if there is a poor signal-to-noise ratio in the data. During testing of CHEMTAX Version 2, Wright (unpublished) performed multiple CHEMTAX runs on pigment data from Antarctic picoplankton using 28 pigment ratio tables that had been multiplied by a scaled random number to adjust each ratio up to $\pm 50\%$. Each calculation produced a slightly different result. When the results were sorted in order of decreasing pigment residual, the taxonomic estimates were found to converge towards stable values. So although CHEMTAX was encountering local minima, it was tending towards the overall minimum in the data. Performing such multiple estimates from randomized starting points gives the analyst an indication of the stability of the results and its confidence limits.

4.3 Comparison of Pigment and Microscopic Analysis

Several recent studies have compared pigment chemotaxonomy and microscopic analysis of phytoplankton groups, using individual pigments [106], linear regression [58, 92], simultaneous equations [107], or CHEMTAX [44, 45, 100, 103, 108–112]. The microscopic studies attempted cell identifications and in many of them carbon cell biomass was calculated from cell volume measurements of each species group in the sample. Most studies compared patterns and proportions of algal groups by the two methods—cell volume/carbon equivalent (microscopy) and μg Chl *a* (HPLC analysis)—rather than comparing biomass in the same units. Major threads from these studies include:

- Microscopic and pigment analyses are difficult to compare quantitatively due to the poor precision of cell counts. Counting precision was not considered explicitly in most studies, but (where given) cell numbers normally ranged from 100 total to 100 per category, implying counting precision ranging from ±50% to ±20%, respectively for five categories [113]. Pigment analysis can achieve 1% precision of concentration estimates, but estimating taxonomic proportions from such data is less precise.
- Microscopic identification of cells and estimation of cell biomass was time-consuming and difficult [44, 92, 100, 107, 112], particularly when hampered by poor cell preservation, because the best fixatives were not used in all cases.
- Correlation of pigments with cell biomass was improved if very large diatoms were removed from the analysis [100, 111].
- Correlation was improved if the pigment data set was split into similar regions [92] or depths [107], in order to have stable pigment ratios within the data set (see Sect. 4.2).
- Pigments gave good estimates of groups with well-defined markers [92, 100, 103], but multiple regression worked well only for groups that had a well-defined pigment marker that was present in reasonable concentration [92]. Pigments sometimes detected taxa having unambiguous markers that were missed by microscopy [70, 100, 112].
- Dinoflagellates presented particular problems when non-pigmented species [112] or those with major pigments other than peridinin [108] were present.
- Minor pigments, in particular Chls *c*, were very useful in discriminating algal types [103, 108].
- Groups with similar pigment patterns were often confused, e.g. *Phaeocystis pouchetti* was erroneously attributed to diatom Chl *a*, suggesting that it contained unexpectedly high fucoxanthin concentrations [112]; confusion between *Synechococcus* sp. and *Trichodesmium* sp. was also noted [114].

- In a mesocosm study [100], cyanobacteria biomass was negatively correlated with estimates from pigment data in treatments with added nutrients (but not the control, which was positively correlated). This was ascribed to variations in the zeaxanthin : Chl *a* ratio, perhaps due to different light environments between treatments.
- CHEMTAX was sometimes found to be sensitive to erroneous initial pigment ratios [45] and insensitive in other cases [44]. A thorough knowledge of the phytoplankton community is necessary for trustworthy results [45], as shown in the excellent study of the Urdaibai Estuary [110].
- No clear relationship was found between CHEMTAX community analysis and estimates of carbon derived from microscopic cell counts from two years of data from the English Channel [112]. Much of the variability appeared to be due to problems with microscopic analysis as well as the changing pigment ratios due to changes in irradiance, nutrient concentrations and community composition within the data set. Nevertheless, computed pigment : Chl *a* ratios were similar to those of other studies [44, 45, 115], giving some confidence in the results. Other authors [92, 103, 107] found that computed field pigment ratios were within the range of literature values.

4.4
Selected Bibliography of Recent Field Studies

Most studies of community composition still employ pigments as independent chemotaxonomic markers, using them as indicators of particular algal taxa. This can be very useful in distinguishing patterns of distribution without determining contributions to Total Chl *a* [116, 117]. Where formal computational methods are applied for quantitative analysis, CHEMTAX is a popular choice. A summary of recent publications employing multiple linear regression, simultaneous equations or CHEMTAX analysis of quantitative pigment HPLC data of phytoplankton populations is given in Table 5, along with the focus of the study and the geographical region in which it was conducted.

The objective in most cases was to determine the community composition of phytoplankton. Excellent examples include a cross-Atlantic survey [118], and a South Pacific transect [119] that provided impressive large scale synopses of phytoplankton distributions.

5
Estimating Biomass from Pigment Content

Chl *a* concentration is a useful index of phytoplankton biomass when modeling primary production, but carbon content is preferable for studies of

Table 5 Examples of recent studies employing HPLC pigment analysis for quantitative phytoplankton biomass estimation, with the method of data analysis—multiple linear regression (MLR), simultaneous equations (SE), or CHEMTAX—as well as the focus of each study and geographic region in which it was performed

Authors	Data analysis method	Focus	Region	Ref.
Abrahamsson et al. 2004	CHEMTAX	Halocarbons	Southern Ocean	[146]
Andersson et al. 2003	MLR	Grazing	Baltic	[147]
Ansotegui et al. 2003	CHEMTAX	Size distribution	Urdaibai estuary, France	[110]
Bergmann et al. 2002	CHEMTAX	Photosynthetic efficiency	Neuse Estuary, USA	[148]
Breton et al. 2000	MLR	Community composition	English Channel	[58]
Carreto et al. 2003	CHEMTAX	Springtime communities	Argentina	[103]
Di Tullio et al. 2003	CHEMTAX	Community composition	South Pacific	[119]
Ediger et al. 2001	MLR	Community composition	Galway Bay, Ireland	[149]
Furuya et al. 2003	CHEMTAX	Community dynamics	East China Sea	[150]
Garibotti et al. 2003	MLR	Community composition	Southern Ocean	[92]
Gibb et al. 2001	CHEMTAX+MLR	Community composition	NE Atlantic	[151]
Gin et al. 2003	SE	Community composition	Singapore	[152]
Havskum et al. 2004	CHEMTAX	Method comparison	Mesocosms, Denmark	[100]
Henriksen et al. 2002	CHEMTAX	Light and nutrients	Danish waters	[45]
Li et al. 2002	MLR	Community composition	East China Sea	[153]
Llewellyn et al. 2005	CHEMTAX	Community composition	English Channel	[112]
Mackey et al. 2002	CHEMTAX	Community composition	Equatorial Pacific	[154]
Riegman & Kraay 2001	CHEMTAX	Community composition	Faroe-Shetland Channel	[155]
Rodriguez et al. 2002	CHEMTAX	Community composition	Southern Ocean	[108]
Rodriguez et al. 2003	CHEMTAX	Community composition	Bay of Biscay	[109]
Schlüter et al. 2000	CHEMTAX	Light and nutrients	Danish waters	[44]
Steinberg et al. 2001	SE	Time series	Bermuda	[156]
Suzuki et al. 2005	CHEMTAX	Iron fertilization	Subarctic Pacific	[157]
Veldhuis & Kraay 2004	CHEMTAX	Community composition	Subtropical Atlantic	[114]
Vidussi et al. 2000	SE	Community composition	Mediterranean	[98]

ecosystem dynamics or carbon flux. Conversion is not straightforward. The carbon-to-Chl *a* ratio (C : Chl *a*) of phytoplankton varies widely, from $< 10–200\ g\ C\ g^{-1}$ Chl *a* in culture [120–123]. The range observed in the field is similar [92, 112, 124] but varies seasonally [45]. A wider range has been observed for prochlorophytes, from $450\ g\ C\ g^{-1}$ DV-Chl *a* at the surface to $15\ g\ C\ g^{-1}$ DV-Chl *a* at 150 metres depth [114].

A model of C : Chl *a* versus irradiance, daylength, temperature, and nutrients [125] predicted values of C : Chl *a* that matched field studies. Application of this model to a one-dimensional model of phytoplankton production dynamics [123] predicted a range of $20–160\ g\ C\ g^{-1}$ Chl *a* with lowest values predicted at the top of the nutricline within the seasonal thermocline and highest values in the nutrient-depleted surface mixed layer in mid-summer. The model produced excellent correlations with observed data for a variety of cultures ($r^2 = 0.75–0.89$), but is not immediately applicable to field studies since it includes a term K_I, the saturation parameter for the growth irradiance curve, that depends on α, the initial slope of the photosynthesis irradiance curve. This is species-dependent and varies with environmental conditions. Nevertheless, the model predicts a linear relationship between C : Chl *a* and irradiance, suggesting that the vertical change in C : Chl *a* can be interpolated between representative samples in field studies.

The carbon content per cell for species observed in field samples is generally determined by microscopic measurements of individual cells [126] and application of a cellular carbon-to-volume ratio ($C_c : V_c$) that may range from $0.04–0.4\ pg\ C\ \mu m^{-3}$ for typical phytoplankton [127]. Recent examples of this approach [100, 112] summarize previous literature values. A new $C_c : V_c$ relationship has recently been developed for dinoflagellates, diatoms, and other protists [128]. C : Chl *a* can also be measured by incorporation of ^{14}C [114, 120, 129–131]. A promising flow cytometric method [114] uses fluorescent labeling of DNA, which has a strong correlation with cellular carbon.

Estimates of biovolume may have standard deviations of 15 to 50% [53], and $C_c : V_c$ may vary 5-fold depending on the choice of regression model [132]. $C_c : V_c$ also varies markedly according to size [58, 128].

Possible avenues for reducing uncertainties in estimates of chlorophyll or carbon biomass include:

- Size-fractionating representative field samples to get more accurate estimates of Pigment : Chl *a* and C : Chl *a* for different size classes.
- Improved knowledge of the pigment and carbon content of major taxa in field samples through further isolation and culture studies under different environmental conditions.
- Exploring the use of particular species to indicate the history of the light environment, using the following rationale. Certain taxa, notably cyanobacteria, cryptophytes and prochlorophytes, have distinctive pigment markers (Table 1) and can also be reliably distinguished and accu-

rately counted in field samples using flow cytometry. For these species it should be possible to determine the content of each pigment per cell in field samples, which varies according to light environment, as well as other factors (see Sect. 2.4). The light history of these cells could then be deduced by comparison with culture data and used to constrain possible values of marker pigments : Chl *a* and C : Chl *a*, in CHEMTAX and in biomass calculations, respectively.

Finally, it may be preferable to calculate the biovolume of the algal class directly from the concentrations of suites of marker pigments rather than indirectly through computed Chl *a* concentrations [133].

6 Conclusions

HPLC pigment analysis is currently the best means of mapping phytoplankton populations in the oceans and gaining an in-depth view of the dynamics of such populations. Much more work should be done to get basic parameters of key species by culturing them and determining their pigment content, particularly under simulated field conditions, such as rapidly varying irradiance and nutrient stress.

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