Spectra of light absorption by phytoplankton pigments in the Baltic; conclusions to be drawn from a Gaussian analysis of empirical data*

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Abstract

Analysed by differential spectroscopy, 1208 empirical spectra of light absorption $a_{pl}(\lambda)$ by Baltic phytoplankton were spectrally decomposed into 26 elementary Gaussian component bands. At the same time the composition and concentrations of each of the 5 main groups of pigments (chlorophylls *a*, chlorophylls *b*, chlorophylls *c*, photosynthetic carotenoids and photoprotecting carotenoids) were analysed in 782 samples by HPLC. Inspection of the correlations between the intensities of the 26 elementary absorption bands and the concentrations of the

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Fig. 2 and eq. (6) contain errors in the printed original and are corrected in this electronic version.

pigment groups resulted in given elementary bands being attributed to particular pigment groups and the spectra of the mass-specific absorption coefficients established for these pigment groups. Moreover, balancing the absorption effects due to these 5 pigment groups against the overall absorption spectra of phytoplankton suggested the presence of a sixth group of pigments, as yet unidentified (UP), undetected by HPLC. A preliminary mathematical description of the spectral absorption properties of these UP was established. Like some forms of phycobilins, these pigments are strong absorbers in the 450–650 nm spectral region.

The packaging effect of pigments in Baltic phytoplankton was analysed statistically, then correlated with the concentration of chlorophyll *a* in Baltic water. As a result, a Baltic version of the algorithm of light absorption by phytoplankton could be developed. This algorithm can be applied to estimate overall phytoplankton absorption spectra and their components due to the various groups of pigments from a knowledge of their concentrations in Baltic water.

1. Introduction

The decision to undertake a thorough investigation of the spectra of light absorption by phytoplankton pigments in the Baltic was inspired by research aiming to continue the development of satellite remote-sensing methods for monitoring the Baltic ecosystem. This work is being carried out at IO PAS, in conjunction with the Institute of Oceanography at the University of Gdańsk, the Institute of Physics at the Pomeranian Pedagogical Academy in Słupsk, and the Sea Fisheries Institute in Gdynia (Woźniak et al. 2004). The theory of these remote-sensing methods is based on the bio-optical multi-component marine photosynthesis model (MCM), which we developed some time ago (Woźniak et al. 2003). MCM enables various inherent (IOPS) and apparent (AOPS) optical properties of the sea, along with a number of chemical and biological characteristics of the ecosystem (phytoplankton pigment content, primary production etc.), to be estimated from three remotely sensed parameters: the downward irradiance PAR[1] at the sea surface, the sea surface temperature SST, and the chlorophyll *a* concentration in the surface layer of the sea. Analysis and empirical validation of the functions of MCM have confirmed its practical utility, as well as the considerable accuracy of its estimates with respect to oceanic waters, in particular case 1 waters (see Ficek et al. 2003). However, similar attempts to apply MCM in remote-sensing algorithms with respect to case 2 waters, in particular to Baltic Sea waters, have yielded much less accurate data with regard to the desired bio-optical and biological characteristics. This is because the IOPS, AOPS and the other parameters

[1]PAR – Photosynthetically Available Radiation – radiation of wavelengths in the spectral range c. 400–700 nm.

of case 2 waters are very much more complex in such waters (Morel & Prieur 1977, Dera 1995, 2003). The efficient application of MCM to remote sensing algorithms for the Baltic ecosystem thus demands that the complexity of these factors be taken into consideration. Hence, several of the partial sub-algorithms of the model require modification.

One of these sub-algorithms in MCM which need to be modified to cater for conditions in the Baltic provides a quantitative and spectral description of light absorption by phytoplankton in the sea (see Annex 1, Section B, Block 7, eqs. A1.15–A1.23 in Woźniak et al. 2003). With this sub-algorithm, the individual absorption properties of particular groups of phytoplankton pigments and the overall absorption properties of marine algae in vivo can be determined from known concentrations of pigments. For this purpose, we use the system of eqs. (1) to (7) (see later), as well as the characteristics of the component bands making up the absorption spectrum, described in the model by Gaussian functions, for the five principal groups of phytoplankton pigments – chlorophylls *a*, chlorophylls *b*, chlorophylls *c*, photosynthetic carotenoids (PSC) and photoprotecting carotenoids (PPC) (see Table 2(A), this paper, p. 543). These characteristics were described earlier (Woźniak et al. 1999, 2000, Majchrowski et al. 2000, Majchrowski 2001) on the basis of analyses of empirical data gathered in various regions of the World Ocean as well as the Baltic and Black Seas. These data included the spectra of the total absorption of light by algae and the concentrations of their various pigments, with the exception of phycobilins. Despite the omission of the effects of phycobilins from the total pigment absorption, this sub-algorithm of MCM in practice (Woźniak et al. 2003) reproduced the coefficients of absorption for oceanic phytoplankton with satisfactory accuracy. Henceforth, then, this sub-algorithm will be referred to as the 'oceanic version' of the algorithm.

Nevertheless, application of this 'oceanic version' to Baltic phytoplankton yields much poorer results. This can be seen in Figs 1a and 2a. In Fig. 1a the empirical mean spectra of light absorption by phytoplankton *in vivo* a_{pl} are compared with the spectra computed from the model for selected trophic types of Baltic Sea water. Similarly, Fig. 2a compares the average specific absorption spectra by all pigments in Baltic phytoplankton in the so-called *in solvent* state $a^*_{pl,\,s}$, calculated according to the oceanic version of the algorithm, to the average value for all trophic types of water. *In solvent* means that the empirical spectra of the specific absorption coefficients *in vivo* a^*_{pl} have been converted to coefficients *in solvent* $a^*_{pl,\,s}$, in accordance with the guidelines set out in the 'Theoretical background' section. These comparisons (Figs 1a and 2a) show that the

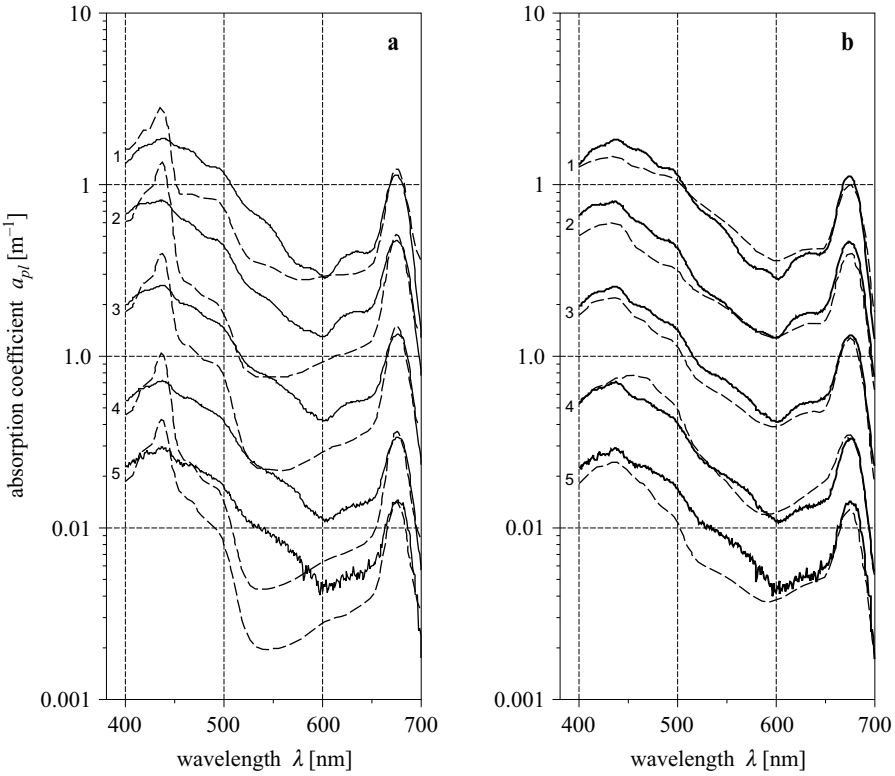

Fig. 1. Comparison of absorption spectra of Baltic phytoplankton $a_{pl}(\lambda)$ – empirical means for selected trophic types of Baltic Sea water (solid lines) with those calculated with the algorithm (dashed lines): oceanic version of the algorithm (a), Baltic version of the algorithm (b). The curve numbers correspond to the following mean values (and variability intervals) of the chlorophyll a concentration C_a [mg tot. chl a m^{-3}] taken into account in these analyses: 1 – 64.2 (52–86), 2 – 22.6 (15–31), 3 – 6.79 (5.05–9.50), 4 – 1.43 (1.00–1.93), 5 – 0.55 (0.25–0.95)

modelled absorption spectra provide a fairly satisfactory picture of the real (measured) absorption of light by Baltic phytoplankton only in the case of red light (wavelengths $\lambda =$ c. 650–700 nm). In contrast, there is a substantial discrepancy between the empirical and modelled values of a_{pl} and $a^*_{pl,\,s}$ with respect to the other spectral ranges of visible light. It will be noticed that these discrepancies recur on an almost regular basis. They are positive in the so-called Soret band ($\lambda =$ c. 440 nm), i.e. the modelled coefficients a_{pl} and $a^*_{pl,\,s}$ are higher in value than the empirical ones, whereas in the middle part of the visible light spectrum ($\lambda =$ c. 460–650 nm) they are negative, i.e. a_{pl} and $a^*_{pl,\,s}$ modelled using the oceanic version of the algorithm are usually smaller than the empirical values. A whole series of hypothetical causes

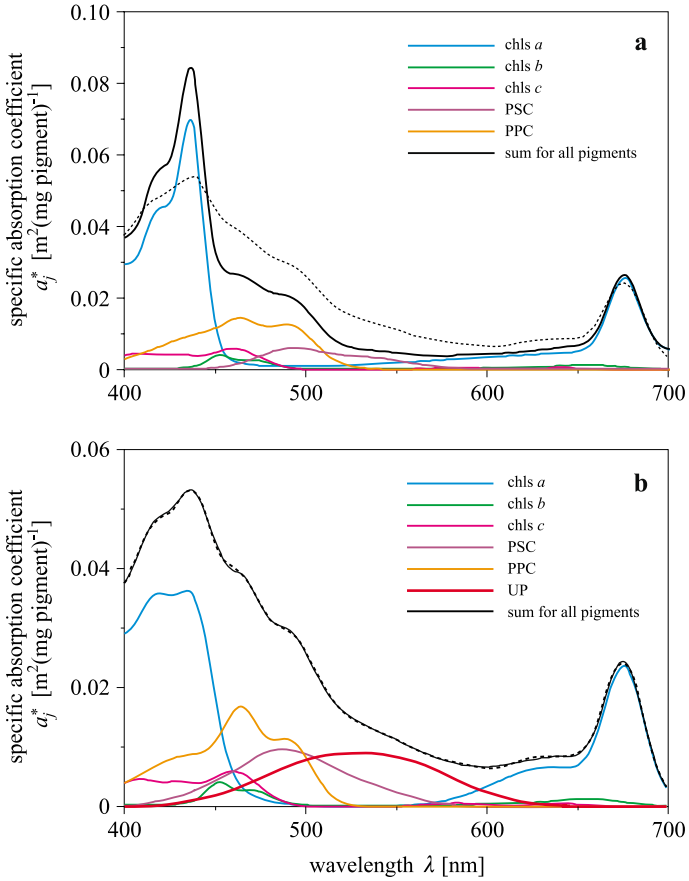

Fig. 2. Specific absorption spectra of phytoplankton pigments *in solvent*: the mean for groups determined (after correction for the packaging effect) on the basis of measured data sets (dashed lines) and modelled from the sum of the spectra of the major component pigments (solid lines): computed from oceanic version of the model (a), computed from the Baltic version of the model (b)

can be put forward to explain these discrepancies. They could be due on the one hand to inaccuracies in the empirical data arising out of less-than-perfect methods of measuring a_{pl} and the pigment concentration C_j, on the other hand to formal inaccuracies in the mathematical description of the relationships used in the algorithm. We have undertaken comprehensive investigations in an attempt to eliminate the methodological and formal causes of these discrepancies. One thing we established was that even though a range of such causes may occur, they must not be allowed to give rise to such considerable and systematic discrepancies as we find in Figs 1a and 2a. Hence the oceanic version of the algorithm, as applied to Baltic phytoplankton, probably leads to these discrepancies for other

reasons, mainly natural ones, which can be summarised by the two research hypotheses discussed below.

(1) We can postulate a diversity of native 'optical' forms and also different chemical forms of phytoplankton pigments in different seas. The compositions of the concentrations of these optical and chemical forms in the five main pigment groups in the case of Baltic phytoplankton may differ from the 'average' of such compositions in oceanic phytoplankton. As a result we have a diversity of spectra of mean coefficients of light absorption by the various groups of pigments in 'Baltic' and 'oceanic' phytoplankton. Thus, in each of these cases the component Gaussian bands of the total phytoplankton absorption spectra differ in intensity, peak position and width. The overestimation in the calculated values of a_{pl} with respect to values in the Soret band measured in the Baltic could therefore be due to a chlorophyll a pigment composition, which displays strong absorption in the Soret region, that is different from the average oceanic value. Hence the component Gaussian bands of the summary spectrum will also be different. To a lesser extent, this overestimation of a_{pl} and $a^*_{pl,\,s}$ may be due to the composition of pigments from the other four groups. This composition, especially that of the PSC group, may also be responsible (but only partially!) for the underestimation in the Baltic of the modelled coefficients a_{pl} and $a^*_{pl,\,s}$ in the extensive middle region of the spectrum (460–650 nm).

(2) We can also postulate the occurrence, besides the five main groups of pigments (chlorophylls a, chlorophylls b, chlorophylls c, photosynthetic carotenoids (PSC) and photoprotecting carotenoids (PPC)), of hitherto unidentified or unrecorded pigments that can affect the overall absorption of light by Baltic phytoplankton. The effect of these unidentified pigments on the total absorption by algae is not taken into consideration by the oceanic version of the algorithm. Such unidentified pigments could be strong absorbers of light from the mid-regions of the spectrum ($\lambda \sim$ 460–650 nm) – possibly phycobilins, which are not determined by the techniques usually applied in oceanology. They could also be certain carotenoids, which remain undetected by the HPLC apparatus currently used by the authors (see the section later on the empirical material used in the analyses). Omitting these 'unidentified pigments' (UP) from the oceanic version of the algorithm could be an important reason for the systematic underestimation of the calculated values of a_{pl} and $a^*_{pl,\,s}$ in the 460–650 nm band.

In view of the above arguments, we undertook the work described in this report, the aim of which was to attempt a confirmation of these two hypotheses. The research question we have tried to respond to was formulated thus: Can the diversity of native optical forms and chemical

forms of pigments and also the possible existence of unidentified pigments in Baltic phytoplankton explain the discrepancies between the coefficients of light absorption by phytoplankton as computed with the oceanic version of the algorithm (after Woźniak et al. 1999, 2003), and the empirically determined values of these coefficients? An additional, practical aim of this work was to establish a novel, modified mathematical description of the spectra of light absorption by phytoplankton pigments that could be applied to the construction of a Baltic version of the algorithm – one which could serve to determine the spectra of light absorption by Baltic phytoplankton from known concentrations of its pigments.

To achieve these aims the authors gathered a suitably large set of empirical material regarding the spectra of light absorption by phytoplankton and the concentrations of its pigments in the Baltic Sea. This material was then subjected to comprehensive theoretical and statistical analyses, which involved, among other things, the so-called spectral decomposition of the absorption spectra and their Gaussian analysis, in accordance with the rules discussed below.

2. Theoretical background

The model equations suitable in practice for describing the spectra of the light absorption coefficient by a monodispersive, homogeneous suspension of phytoplankton in the sea $a_{pl}(\lambda)$ [m^{-1}] can be simply written as follows (Bricaud et al. 1995, Woźniak et al. 1999):

$$a_{pl}(\lambda) = a^*_{pl}(\lambda)\, C_a, \tag{1}$$

$$a^*_{pl}(\lambda) = Q^*(\lambda) a^*_{pl,\,s}(\lambda), \tag{2}$$

where the index s stands for *in solvent*, and the other symbols denote

$a^*_{pl}(\lambda)$ [m^2 (mg tot. chl a)$^{-1}$] – the specific absorption coefficient of phytoplankton,

$\mathrm{a}^*_{pl,\,s}(\lambda)$ [m^2 (mg tot. chl a)$^{-1}$] – the specific absorption coefficient of phytoplankton pigments *in solvent*,

C_a [mg tot. chl a m^{-3}] – the total concentration of chlorophylls (chl a + divinyl chl a) in sea water,

$Q^*(\lambda)$ [dimensionless] – the spectral function of the pigment packaging effect.

The spectral function of the pigment packaging effect in phytoplankton cells with a spherical symmetry, assuming that phytoplankton cells are optically soft particles, is equal to (according to van de Hulst 1981, Morel & Bricaud 1981):

$$Q^*(\lambda) = \frac{3}{2\rho'(\lambda)}\left[1 + \frac{2e^{-\rho'(\lambda)}}{\rho'(\lambda)} + 2\frac{e^{-\rho'(\lambda)} - 1}{\rho'^2(\lambda)}\right], \tag{3}$$

$$\rho' = a^*_{pl,\,s}\ C_I d, \tag{4}$$

where

C_I [mg tot. chl a m^{-3}] – intracellular chlorophyll a concentration,

d [m] – cell diameter.

Notice that the above set of eqs. (1)–(4) refers to a monodispersive, homogeneous set of spherical phytoplankton cells. As applied to natural populations of marine algae (of various shapes and sizes and of complex, non-homogeneous internal structure), these equations are a far-reaching simplification. Nevertheless, practice has shown that they are acceptable so long as the product $C_I d$ in eq. (4) is treated as an equivalent, approximate magnitude, averaged for a given natural population of phytoplankton. One should bear in mind, however, that this assumption will always give rise to certain discrepancies between the modelled and real values of absorption.

The set of eqs. (1)–(4) describes the relationships between the spectra of absorption coefficients a_{pl} and a^*_{pl} of algae *in vivo* and the spectrum of the specific absorption coefficient $a^*_{pl,\,s}$ of the sum of all phytoplankton pigments in the *in solvent* state. So in order to complete the algorithm for determining the absorption properties of phytoplankton from known concentrations of all its pigments, the dependence of the coefficient $a^*_{pl,\,s}$ on these concentrations has to be added to it, and some dependence or method of working out the value of $C_I d$ for the algal population in question has to be found.

In the algorithms we have already developed – both the oceanic version and the Baltic version proposed here – the first of these relationships (between $a^*_{pl,\,s}$ and the pigment concentrations), described with the aid of the decomposition of the absorption spectra of the separate pigment groups into elementary Gaussian bands, is as follows:

$$a^*_{pl,\,s}(\lambda) = \frac{1}{C_a} \sum \left[a^*_j(\lambda)\, C_j \right], \tag{5}$$

$$a^*_j(\lambda) = \sum a^*_{\max,\,i}\ e^{-\frac{1}{2}\left(\frac{\lambda-\lambda_{\max}}{\sigma_i}\right)^2}, \tag{6}$$

where

j – denotes the pigment group index (i.e. $j = a$ for chlorophylls a; $j = b$ for chlorophylls b; $j = c$ for chlorophylls c; $j =$ PSC for photosynthetic carotenoids; $j =$ PPC for photoprotecting carotenoids; $j = phyc$ for phycobilins; and $j =$ UP for other, unidentified pigments);

$a^*_j(\lambda)$ [m^2 (mg pigment)$^{-1}$] – spectral mass-specific absorption coefficient for the j-th group of unpackaged pigments (i.e. in the solvent);

C_j [mg pigment m^{-3}] – concentration of the j-pigment group (i.e. a, b, c, PSC, PPC, $phyc$);

$a^*_{\max,i}$ [m^2 (mg pigment)$^{-1}$] – mass-specific absorption coefficient for the spectral peak of the Gaussian band;
$\lambda_{\max,i}$ [nm] – centre of the spectral band;
σ_i [nm] – dispersion of the band.

The values of these last three parameters ($a^*_{\max,i}$, $\lambda_{\max,i}$ and σ_i) are given in Table 2. They characterise the individual, elementary Gaussian absorption bands of the separate pigments that we defined previously (Woźniak et al. 1999) for the oceanic version of the algorithm (Table 2(A), p. 543), and established in the present paper for the Baltic version of the algorithm (Table 2(B), p. 543).

The final component relationship in the algorithms for determining the absorption properties of phytoplankton from known concentrations of its pigments is the statistical relationship that we developed earlier for the oceanic version of the algorithm between the values of $C_I d$ (the product of the intracellular concentration of chlorophyll a and the cell diameter d) and the concentration of chlorophyll a in the sea C_a, in the form (after Woźniak et al. 1999):

$$C_I d = 24.65\, C_a^{0.75015}. \tag{7}$$

A similar relationship established in this paper for the Baltic version of the algorithm is also described by eq. (11).

3. Empirical material and the methods used in the analyses

The empirical data for the Baltic utilised in this work were gathered during numerous research cruises of r/v 'Oceania' in various parts of the Baltic, mainly its southern basins, from 1994 to 2004. Among the many bio-optical and chemical parameters of sea water obtained during this time, the following were used for the purposes of the present analyses:

- 1208 spectra of coefficients of light absorption by phytoplankton $a_{pl}(\lambda)$ from different depths in the sea;
- 782 sets of concentrations of the main groups of phytoplankton pigments, determined in the same samples for which the absorption $a_{pl}(\lambda)$ was measured.

Table 1 shows the specification of these data sets, split in accordance with the trophic types of water present in the Baltic.

The water samples for the bio-optical measurements, that is, of the spectra of light absorption by suspended matter a_p and the concentration of photosynthetic pigments by HPLC, were taken with a bathometer, and from the surface waters with a pail. All the water was filtered immediately after sampling.

Table 1. Specification of the input empirical data collection

Trophic type of water*	Surface chlorophyll $C_a(0)$ concentration range [mg m^{-3}]	Number of a_{pl} spectra data	Number of pigments (HPLC) data
M	0.2–0.5	19	19
I	0.5–1	107	107
E–1	1–2	251	166
E–2	2–5	507	265
E–3	5–10	137	88
E–4	10–20	111	73
E–5	20–50	64	53
E–6	>50	12	11
	total	1208	782

* The symbols denote the trophic types of sea waters (M – mesotrophic, I – intermediate, E – eutrophic), where the trophicity index is the concentration of chlorophyll a in the surface layer of the sea $C_a(0)$, in accordance with the convention that we suggested in the paper by Woźniak et al. (1992).

The spectra of light absorption by suspended matter in sea water were measured by means of a spectrophotometer equipped with an integrating sphere. The methodology of this technique is described in Tassan & Ferrari (1995, 2002), and in Ferrari & Tassan (1999).

The sea water samples were passed through Whatman GF/F filters (ϕ = 22 mm). The volumes of filtered water ranged from 100 ml to 2 l and were selected such that the layer of suspended matter collected by the filter had an optical density (OD) no greater than 0.5 after subtraction of the filter's own OD. In these measurements it was of great importance that the layer of sediment should be homogeneous; if this was not the case, the filtering was repeated.

After filtration the samples were immediately frozen in liquid nitrogen. They were defrosted immediately prior to the spectrophotometric measurements. These were carried out on a UNICAM UV4-100 spectrophotometer equipped with a LABSPHERE RSA-UC-40 integrating sphere with an internal diameter of 63.5 mm. The measurements were carried out over a range of wavelengths from 350 to 750 nm in accordance with the procedure given by Tassan & Ferrari (1995). Thus, to obtain the absorption spectrum of the suspended matter, a reading was taken with the filter placed just in front of the transmittance port of the integrating sphere, and then a second reading was taken with the filter placed in the sphere's reflectance port. The optical density OD_f of the suspended particles on the filter was calculated from these two readings (see Tassan & Ferrari 1995). In order to determine the light absorption by suspended particles that were not pigments,

Table 2. Model characteristics of the specific absorption components of Gaussian bands: oceanic version (A), Baltic version (B)

chlorophylls *a*

	Characteristic	Gaussian band number					
		A 1	A 2	A 3	A 4	A 5	A 6
A	$\lambda_{\max,\,i}$	381	420	437	630	675	700
	σ_i	33.8	8.25	6.50	89.8	8.55	101
	$a^*_{\max,\,i}$	0.0333	0.0268	0.0580	0.0005	0.0204	0.005
B	$\lambda_{\max,\,i}$	381	418	439	635	676	708
	σ_i	37.7	10.0	9.72	29.9	10.7	14.4
	$a^*_{\max,\,i}$	0.0296	0.0151	0.0238	0.0067	0.0210	0.0008

chlorophylls *b*

	Characteristic	Gaussian band number					
		B 1	B 2	B 3	B 4	B 5	B 6
A, B	$\lambda_{\max,\,i}$	380	442	452	470	609	655
	σ_i	194	7.45	5.6	10.5	16.0	18.5
	$a^*_{\max,\,i}$	0.0059	0.0145	0.0631	0.0514	0.0083	0.0257

chlorophylls *c*

	Characteristic	Gaussian band number				
		C 1	C 2	C 3	C 4	C 5
A	$\lambda_{\max,\,i}$	408	432	460	583	
	σ_i	16.1	7.93	14.2	32.2	
	$a^*_{\max,\,i}$	0.0561	0.0234	0.0072	0.0133	
B	$\lambda_{\max\ i}$	408	432	460	583	640
	σ_i	16.1	7.93	14.2	16.0	16.0
	$a^*_{\max,\,i}$	0.0561	0.0234	0.0720	0.0073	0.0060

photosynthetic carotenoids

	Characteristic	Gaussian band number			
		PSC 1	PSC 2	PSC 3	PSC 4
A	$\lambda_{\max,\,i}$	490	532		
	σ_i	17.1	22.8		
	$a^*_{\max,\,i}$	0.0313	0.0194		
B	$\lambda_{\max\ i}$	468	490	515	532
	σ_i	26.7	17.1	13.1	22.8
	$a^*_{\max,\,i}$	0.0311	0.0313	0.0096	0.0194

Table 2. (*continued*)

photoprotecting carotenoids

	Characteristic	Gaussian band number		
		PSC 1	PSC 2	PSC 3
A	$\lambda_{\max,\,i}$	451	464	493
	σ_i	32.0	8.60	12.0
	$a^*_{\max,\,i}$	0.0632	0.0253	0.0464
B	$\lambda_{\max\,i}$	438	465	492
	σ_i	29.7	9.24	11.7
	$a^*_{\max,\,i}$	0.0516	0.0622	0.0560

unidentified pigments

	Characteristic	Gaussian band number	
		UP 1	UP 2
B	$\lambda_{\max\,i}$	502	557
	σ_i	33.2	31.2
	$a^*_{\max,\,i}$	0.0015	0.0013

where
$\lambda_{\max,\,i}$ – centre of band [nm],
σ_i – dispersion of band [nm],
$a^*_{\max,\,i}$ – specific absorption coefficient at the maximum [m^2 (mg pigment)$^{-1}$].

the sample was bleached (the pigments were broken down) and the above two measurements were repeated. The samples were bleached by saturating them with a 2% solution of NaClO. The bleaching time ranged from 1 to 15 minutes or so, depending on the species composition of the phytoplankton.

A problem that crops up with this type of measurement is the amplification of the optical path of the light in the sediment samples on the filter. To eliminate this effect, the optical path length amplification factor β, defined as the ratio of the optical path to the geometrical path in the sample, is introduced into the calculation of the real absorption coefficients of the suspended matter (Butler 1962). In practice, the application of this factor involved determining the real optical density of the suspension in water (OD_{sus}) from the optical density obtained from measurements made on the filter (OD_f). Experiments carried out by many authors have shown that the dependence of OD_{sus} on OD_f is non-linear and can be described approximately by the equation

$$OD_{sus}(\lambda) = a\ OD_f^2 + b\ OD_f. \tag{8}$$

The values of the coefficients in this equation were determined empirically: $a = 0.592$, $b = 0.4$.

Once the optical densities of all the suspensions $OD_{sus,\,ses}(\lambda)$ and of all suspended matter that is not pigment $OD_{sus,\,det}(\lambda)$ were calculated, the values of the corresponding coefficients of absorption $a_{ses}(\lambda)$ and $a_{det}(\lambda)$ could be derived from them.

The spectra of the coefficient of absorption by phytoplankton pigments a_{pl} was calculated as the difference

$$a_{pl}(\lambda) = a_{ses}(\lambda) - a_{det}(\lambda). \tag{9}$$

Since phytoplankton pigments do not absorb radiation in the near-IR region, the non-zero value of $a_{pl}(750)$ should be regarded as a measurement error. This error is assumed to be independent of wavelength: whenever a spectrum $a_{pl}(\lambda)$ was obtained with a non-zero value of $a_{pl}(750)$, the result was corrected by subtracting this non-zero value from all values of $a_{pl}(\lambda)$.

The overall concentrations of the main groups of pigments referred to diverse forms of chlorophylls a (C_a), chlorophylls b (C_b), chlorophylls c (C_c), photosynthetic carotenoids (C_{PSC}) (e.g. fucoxanthin, 19′but-fucoxanthin, 19′hex-fucoxanthin, peridinin, prasinoxanthin, α-carotene) and photoprotecting carotenoids (C_{PPC}) (e.g. antheraxanthin, diadinoxanthin, alloxanthin, diatoxanthin, lutein, violaxanthin, neoxanthin, zeaxanthin and β-carotene). They were defined as the sum of different individual pigments from these groups as determined by HPLC techniques.

Pigments were extracted by grinding and sonication (5 min, 20 kHz, Cole Palmer, 4710 Series) in 3 cm^3 of 90% acetone as extraction solvent at 4°C in the dark for 2 hours, after which the extracts were centrifuged (20 min, 5°C, 2150 g, Beckman, GS-6R), clarified and then subjected to chromatographic analysis.

Pigments were isolated using the RP-HPLC technique. The chromatographic system was equipped with an HP 1050 pump, diode array detector (model HP 1100), an HP 1046 fluorescence detector and Rheodyne injector with a 100 μl sample loop. Two types of C_{18} analytical columns: LichroCART™ Hypersil ODS (dimensions: 250×4 mm, particle size: 5 μm, Merck) and LichroCART™ LiChrospher™ 100 RP18e (dimensions: 250×4 mm, particle size: 5 μm, Merck) were used for pigment separation and identification. The diode array absorbance detector ('dad') was set at $\lambda = 440$ nm. The fluorescence detector with an excitation wavelength $\lambda_{ex} = 431$ nm and emission $\lambda_{em} = 660$ nm was used only to confirm the presence of chloropigments in the extract.

The solvents used for chromatography were filtered and degassed with helium before use. The mobile phases used in the gradient elution were made up of a primary eluant (A) consisting of methanol and 1 M ammonium

acetate (80 : 20 v/v), and a secondary eluant (B) prepared from methanol and acetone (60 : 40 v/v). Separation was achieved by changing the solvent mixture composition from 100% of solvent A to 100% of solvent B over 10 minutes after injection, after which the mixture was maintained isocratically at a constant flow rate of 0.8 ml min^{-1} until the end of the analysis (Mantoura & Llewellyn 1983, Barlow et al. 1993, Stoń & Kosakowska 2002). Equilibrium was attained after 10 min, when the solvent composition returned to the initial conditions.

The qualitative and quantitative analysis of the pigment content in natural samples was performed using commercially available pigment standards from the International Agency for C^{14} Determination in Denmark. Qualitative analysis was based on a comparison of the retention times and the absorbance spectra of eluting peaks with those of the standards. Identification was confirmed by co-injection and on-line diode array spectra.

The quantitative characteristics of the pigments occurring in natural samples were based on the external standardisation equation (Mantoura & Repeta 1997):

$$C_p = \frac{A_p f_p \nu_{ext} 10^3}{\nu_{inj} \nu_{filt} B}. \tag{10}$$

This allows for the precise designation of the concentration of a particular pigment (C_p; ng dm^{-3}) with respect to the peak area (A_p; mAU s) of the eluted pigment, the slope of the calibration curve (f_p; ng $(\mathrm{mAU\ s})^{-1}$), the volume of filtered seawater (ν_{filt}; dm^3), the solvent used for the extraction (ν_{ext}; cm^3), the solvent injected into the chromatographic system (ν_{inj}; μl), and the buffer dilution factor B.

4. Statistical analyses – description and results

The empirical data described above were meticulously analysed with a number of statistical methods that we developed and described in earlier papers (Woźniak et al. 1999, Woźniak 2000). Therefore, without entering into the fine detail of the various stages of these analyses, we will now outline the main points:

- (*stage 1*) All 1208 spectra $a_{pl}(\lambda)$ of Baltic phytoplankton were decomposed by differential spectroscopy. Generally speaking, this procedure showed that the spectra can be decomposed with considerable precision into 26 elementary absorption bands described by Gaussian functions. These are identical for most of the spectra as far as the position of the spectral maximum (peak) and the half-width are concerned.

• (*stage 2*) The correlations between the intensities of the 26 elementary Gaussian bands and the concentrations of all 5 main groups of pigments i.e. C_a, C_b, C_c, C_{PSP}, C_{PPC} were examined in a set of 782 spectra, i.e. those for which the concentrations of the separate pigment groups were defined. As a result, 24 of the 26 elementary bands could be ascribed to particular groups of pigments, albeit under the assumption that a given band is due to the pigment group whose coefficient of correlation between band intensities and pigment concentrations takes the largest value and is very much greater than the other correlations. However, there was no significant correlation between band intensity and the concentrations of the five pigment groups for the two remaining bands, which lie in the middle region of the visible light spectrum (with peaks at 492 nm and 547 nm – see Fig. 3a). This therefore suggests that there are pigments in Baltic phytoplankton which are strong absorbers of light from this part of the spectrum, whose concentrations are not detectable with the methods of pigment identification that we used. The second of the hypotheses postulated in the introduction therefore seems more likely to reflect reality, constituting as it does the 'optical' proof of existence of this putative group of unidentified pigments (UP).

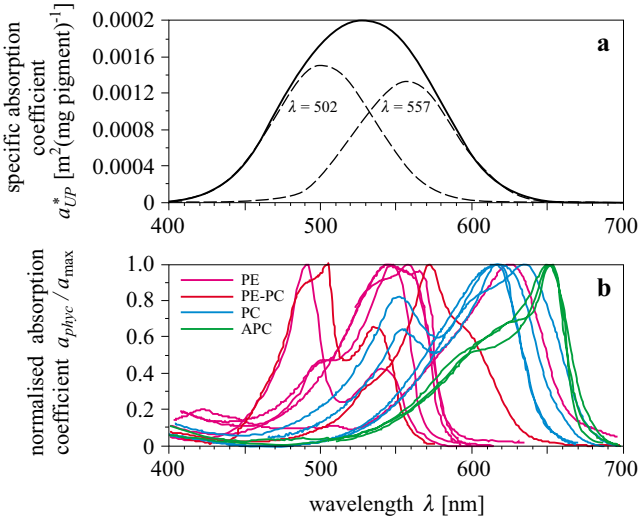

Fig. 3. Decomposition of 'unpackaged' specific absorption spectra of the unidentified pigments (UP) into elementary Gaussian bands (a). Normalised distributions of phycobilin absorption bands a_{phyc} found in the literature (Tarchevsky 1977, Grabowski 1984, Bidigare et al. 1990, Hall & Rao 1999); PE – phycoerythrin, PE-PC – phycoerythrin-phycocyanin complexes, PC – phycocyanin, APC – allophycocyanin (b)

• (*stage 3*) Application of the method of successive approximations in accordance with the algorithm described in our earlier papers (Woźniak et al. 1999, Woźniak 2000) yielded, among other things, approximate values of the product $C_I d$. The values of this product for all 782 spectra are given in Fig. 5a with respect to measured concentrations of chlorophyll *a* in the sea C_a; they will be discussed later. Knowing the values of $C_I d$ for all the spectra $a_{pl}(\lambda)$, we were able to reduce the overall *in vivo* absorption spectra (i.e. in the packaged state) and the component spectra due to the various pigments to the *in solvent* state. In other words, we converted the empirical coefficients *in vivo* a^*_{pl} to *in solvent* coefficients $a^*_{pl,\,s}$, in line with the principles discussed earlier in the 'Theoretical background' section. Then, taking into account the dependence of these components of absorption on the pigment concentrations, and appropriate averaging, the various parameters ($\lambda_{\max}$, σ_i, $a^*_{\max,\,i}$) of the elementary absorption bands could be defined once they had been reduced to the *in solvent* state. The values of these parameters determined for the various groups of pigments, including the group of unidentified pigments (UP), are given in Table 2(B). Since the UP concentrations were not known, the values of mass-specific absorption at the spectral peak ($a^*_{\max,\,i}$) given in this table for the UP group were determined indirectly and approximately in units equivalent to the mass-specific absorption of phycobilins. In this case it was assumed that such a mass-specific integral absorption (i.e. the surface area of the entire spectral absorption band – see the plot in the bottom right-hand corner of Fig. 4) for UP is equal to the similar integral absorption for phycobilins. In these calculations we took this integral absorption to be the mean value we had determined from the analysis of a dozen or so specific absorption spectra of different phycobilins, including the two forms of phycoerythrin postulated by Bidigare et al. 1990 (see curves 8103PE and DC2PE in Fig. 4). By taking into consideration at the current stage of our analyses this new 'Baltic' mathematical description of elementary bands of light absorption by the various pigment groups (including UP – see Table 2(B)) in our estimation of the summary absorption spectra $a^*_{pl,\,s}$ of Baltic phytoplankton in the *in solvent* state (see Fig. 2b), we obtain a distinct improvement in the accuracy of this estimation in comparison with the estimation using the 'oceanic' description (see Fig. 2a).

• (*stage 4*) The final stage of these analyses was to establish the connection, characteristic of the Baltic, between the calculated magnitudes of the product $C_I d$ (see *stage 3*) and the concentration of chlorophyll *a* in the sea, C_a. This relationship is a statistical one, displaying a characteristically

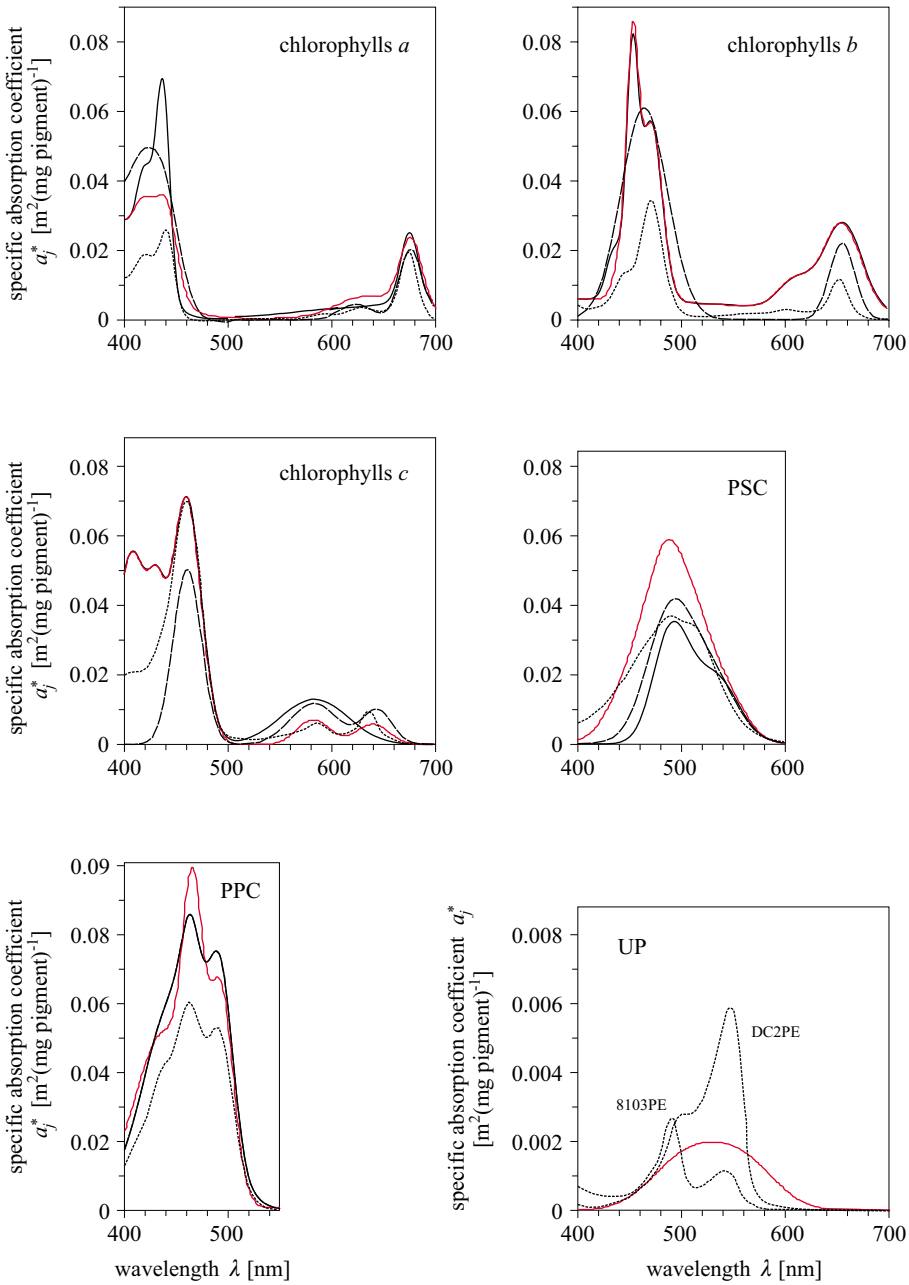

Fig. 4. 'Unpackaged' specific absorption spectra modelled in this paper for the major pigment groups (solid red lines – Baltic version of the algorithm) and postulated by other authors: Woźniak et al. 1999 (solid black lines – oceanic version of the algorithm), Hoepffner & Sathyendranath 1991 (dashed lines), Bidigare et al. 1990 (dotted lines)

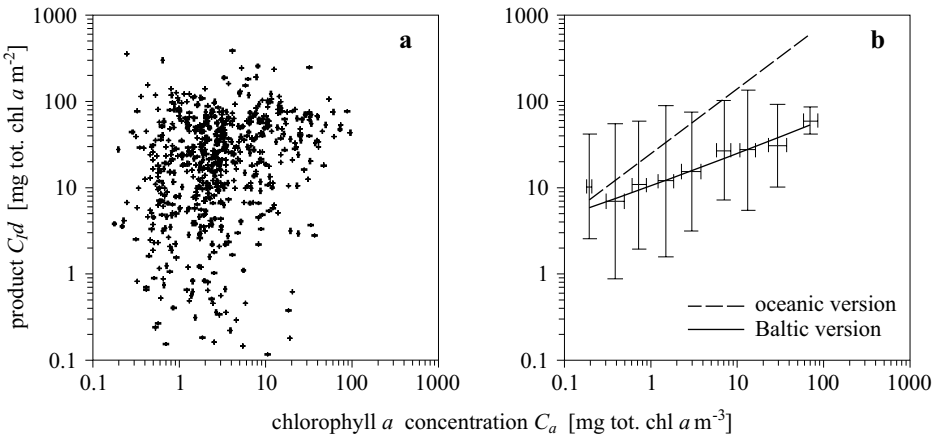

Fig. 5. Empirical relationships of the product of $C_I d$ and the chlorophyll a concentration C_a in the Baltic: individual readings (a), averaged readings – standard deviations given (b). The solid regression line for the Baltic in Fig. (b) was calculated from eq. (11), and the dashed line (for comparison) for the ocean from eq. (7)

large scatter (see Fig. 5), and is described by the approximate regression equation:

$$C_I d = 10.77\, C_a^{0.3767}. \tag{11}$$

The plot of this relationship is illustrated in Fig. 5b. If this relationship is allowed for in the complete Baltic version of the algorithm for determining the coefficients of light absorption by Baltic phytoplankton, we obtain very much better results than with the oceanic version of the algorithm, despite the broad scatter of its empirical data (see Fig. 1).

5. Discussion and summary

These results of the Gaussian analysis of the empirical spectra of light absorption by phytoplankton in the Baltic and the relationships between the spectra and the concentrations of the various groups of pigments are to a certain extent confirmation of the two hypotheses that we formulated and discussed in the Introduction. We can summarise the results as follows:

(a) We assigned the spectra of the coefficients of light absorption by the main groups of pigments in Baltic phytoplankton (see *stage* of the statistical analyses above). In general outline these spectra resemble the averaged spectra for oceanic algae, but certain significant differences between them are also noticeable, especially if one compares the relevant data for the elementary Gaussian absorption bands of pigments calculated according to the oceanic (Table 2(A)) and Baltic (Table 2(B)) versions of the algorithm,

and the absorption spectra of these pigments defined on this basis (Fig. 4). So, for example, the coefficients of light absorption by chlorophylls *a* in the Soret band are, in the case of Baltic algae, less than the 'average' values obtained for phytoplankton from other seas and oceans. At the same time, the corresponding resultant absorption bands for these pigments in the Baltic are broader than the average for oceanic algae. As far as the PSC absorption spectra of Baltic algae are concerned, their structure is more intricate than that of oceanic algae, because they consist of 4 elementary absorption bands, which our analyses identified (Table 2(B)). Only 2 such bands have been identified for oceanic phytoplankton (Table 2(A)). As can be seen from the remaining data in Table 2 and Fig. 4, differences of a similar nature apply to all the groups of pigments analysed here, although they are of greatest significance in the case of the chlorophylls *a* and PSC. It is highly likely that all these differences stem from the diversity of native optical forms and also the chemical forms of compounds belonging to the same groups of phytoplankton pigments in different seas. The decomposition of the overall spectra of light absorption by phytoplankton into elementary Gaussian bands appears to confirm the first of the two hypotheses formulated at the outset.

(b) The present work has also established important facts that appear to endorse the second of the two hypotheses. Analysis of the correlations (see *stage 2*) between the intensities of the 26 elementary absorption bands in the Baltic phytoplankton absorption spectra and the concentrations of the 5 pigment groups (C_a, C_b, C_c, C_{PSP}, C_{PPC}) showed that the intensities of two of these bands (at wavelengths 492 nm and 547 nm) are not significantly correlated with these concentrations. One may infer from this that significant quantities of the suggested group of unidentified pigments (UP) occur in Baltic algae, which have not been reported in 'normal' oceanic phytoplankton (or are present in very much smaller amounts) and which absorb light from this middle region of the visible light spectrum. At the present time we are unable to define the chemical nature of these UP, because so far we have neither isolated them nor directly defined their concentration in marine phytoplankton. It is nevertheless very probable that UP are compounds from the PSC group, whose concentrations are not detected with the HPLC apparatus that we have been using, or that they are phycobilins. We think that the latter possibility is the more likely. Evidence for this is to be found in Fig. 3, which we obtained indirectly by comparing the component elementary absorption bands and the overall absorption of these UP with the absorption spectra of more than a dozen natural phycobilins isolated from different plants. This figure shows that the UP absorption bands bear a striking resemblance to the absorption

bands of the phycobilins, especially the various forms of phycoerythrin and phycoerythrin-phycocyanin complexes. The exact classification of these UP, however, remains an open question. How it is to be answered is a matter for the future. In the first instance it will require fresh, comprehensive bio-optical studies to be performed in conjunction with the determination of the concentrations of all the known groups of plant pigments that phytoplankton may contain, including the phycobilins.

These arguments have served as evidence to justify our two hypotheses. At the same time, however, they give a positive answer to the question posed at the start of this paper: Can the diversity of native optical forms and chemical varieties of pigments and also the possible existence of unidentified pigments in Baltic phytoplankton explain the discrepancies between the coefficients of light absorption by phytoplankton as computed with the oceanic version of the algorithm and the empirically determined values of these coefficients? In particular:

(c) The coefficients a_{pl} (and also $a^*_{pl,\,s}$) calculated using the oceanic version of the algorithm are overestimated as against the values measured in the Baltic in the Soret band (see Figs 1a and 2a), which is due to the composition of chlorophyll *a* pigments, strong absorbers of light in the Soret band, being different in the Baltic from that in the oceans. Hence the component Gaussian bands of the overall absorption spectrum of chlorophyll *a* for both these types of phytoplankton are also different, lower in the Baltic than in the oceans (see Fig. 4). To a lesser extent these overestimated values may be due to the pigment composition in the other pigment groups. This composition, especially of the PSC group, may also be responsible, even if only slightly, for the underestimation of these absorption coefficients in the extensive middle region of the light spectrum (460–650 nm) calculated for the Baltic using the oceanic version (Figs 1a and 2a). The specific coefficients of absorption of these pigments are lower for 'normal' oceanic phytoplankton than for Baltic algae (see Fig. 4).

(d) The underestimation of a_{pl} (and also $a^*_{pl,\,s}$) calculated using the oceanic version vis-à-vis the values measured in the Baltic for the middle part of the spectrum (460–650 nm, see Figs 1a and 2a) is due largely to the occurrence of the postulated groups of UP in Baltic algae. They it is that absorb light from that very spectral region which the oceanic version of the algorithm did not take into account.

(e) Apart from enabling important cognitive objectives to be achieved, the results of our work have also had a practical effect: this is the establishment of a new, modified mathematical description of the spectra of light absorption by Baltic phytoplankton pigments, in other words, a Baltic version of the algorithm. The intention is for this version of the algorithm to

be used to determine the spectra of light absorption by Baltic phytoplankton both *in vivo* and *in solvent*, and also such spectra referring to the various groups of pigments in these algae on the basis of known concentrations of these pigments. The algorithm consists of eqs. (1)–(6) and (11), supplemented by the input data given in Table 2(B). This Baltic version takes in all the modifications discussed earlier. The 'Baltic' description of the mass-specific absorption coefficients of the various groups of pigments clearly differs from the 'oceanic' version; the effect of a new group of hitherto unidentified pigments (UP) is taken into account; and the algorithm is based on the relation, modified for the Baltic, between the product $C_I d$ and the chlorophyll *a* concentration. As a result of these modifications, the Baltic version of the algorithm we have developed in this work describes the empirical spectra of light absorption by Baltic phytoplankton (see e.g. Figs 1b and 2b) far more exactly than the algorithm in its oceanic version (Figs 1a and 2a).

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