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Phycocyanin-specific absorption coefficient: Eliminating the effect of chlorophylls absorption

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Abstract

The applicability of algorithms for estimation of phycocyanin (PC) concentration based on light spectral reflectance heavily depends on the specific absorption of the pigment. But the determination of PC-specific absorption coefficient is not a straightforward task, as PC optical activity is overlapped by absorption of chlorophylls. The aim of our study was to determine $a^*_{PC}(625)$—the specific absorption coefficient of PC at 625 nm, in samples with PC concentrations ranging from 0.5 mg m^{-3} to 126.4 mg m^{-3} and varying proportions of chlorophylls *a*, *b*, and *c* in the samples. The effect of chlorophylls was subtracted from total absorption at 625 nm using Chl *a* absorption at 675 nm as a reference; the contribution of the chlorophylls was computed on the basis of their relative absorption at 625 nm and concentrations. The major effect on the precision of $a^*_{PC}(625)$ determination was imposed by Chl *a* absorption, but the effect of the accessory chlorophylls ought to be accounted for in order to arrive at reliable PC-specific absorption values. $a^*_{PC}(625)$ varied widely, from 0.002 mg m^{-2} to 0.027 mg m^{-2}, and decreased with increase of PC concentration. As PC concentration exceeded 10 mg m^{-3}, $a^*_{PC}(625)$ was almost invariable, slightly oscillating around 0.007 mg m^{-2} and similar to PC specific absorption coefficient estimated by alternative methods. It is clear that a universal value is unfeasible, but $a^*_{PC}(625)$ of 0.007–0.008 mg m^{-2} seems an appropriate value for use in algorithms destined for estimation of phycocyanin in typical mesotrophic and eutrophic inland waters dominated by cyanobacteria.

A useful way to determine the potential of vegetation to capture light is by measuring mass-specific light absorption. Practically, the value of specific light absorption is vital for the development of analytical models for determination of pigment concentration (Gons 1999), and for algorithms destined to estimate phytoplankton primary productivity (Marra et al. 2007). In the case of phytoplankton, it usually defined as chlorophyll *a* (Chl *a*)-specific absorption (Kiefer and Soo-Hoo 1982). Synthetic datasets of reflectance spectra showed that the relationship between optical information at the red-NIR domain, essential for development of algorithms for Chl *a* estimation in productive waters, heavily depends on Chl *a*-specific absorption value at 665 nm (Gilerson et al. 2010).

The measurement of phytoplankton absorption is accomplished using cell suspension or particles captured on a glass fiber filter. The specific absorption of a pigment in vivo is calculated by division of the absorption value at a given wavelength by the concentration of the pigment in question, and in the case of Chl *a* is a straight forward task, as the absorption peak is located beyond the optical activity of other pigments and is only slightly influenced by detritus and colored dissolved organic matter. The variation of the specific absorption coefficient of Chl *a* was extensively studied and the inverse relationship between pigment concentration and its specific absorption is well documented (Bricaud et al. 1995; Allali et al. 1997; Dall'Olmo and Gitelson 2005; Gurlin et al. 2011).

Determination of specific absorption coefficient of other pigment than Chl *a* is complicated due to overlapping absorption of the pigments themselves, as well as by the effects of the noncellular water constituents. Of particulate importance is to establish real values for the water soluble phycobilins, signature pigments for cyanobacteria (Rowan 1989); some representatives of that phylum are toxin-producers and thus confer a potential environmental risk (Carmichael 1997). Cyanophytes often dominate phytoplankton in marine and freshwater ecosystems, and current knowledge leads to the conclusion that they will increase in

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importance in the foreseen future (Paerl and Huisman 2008). With the increasing importance of these organisms attempts are made to improve methods for monitoring, among others by means of remotely sensed information, mostly by acquisition of spectral information in the visible and near-infrared domains (Simis et al. 2005, 2007; Hunter et al. 2009; Mishra et al. 2013, 2014; Ogashawara et al. 2013). In cyanobacteria dominating phytoplankton in inland waters, the major pigment is mostly phycocyanin (PC). PC absorption peaks around 610–620 nm and impairs a blue-green color in combination with Chl *a*. Phycobilins are located in protein-based structures, phycobilisomes, and the procedures applied for extraction of phycobilins are those used for most proteins, i.e., in aqueous solutions (Colyer et al. 2005). Those procedures are laborious and require specialized equipment, needed for cell disruption for the liberation of protein cellular components. The existing laboratory methods for phycobilins extraction do not suit large-scale work, as mostly required for field samples, and therefore, may introduce large margin of experimental error (Millie et al. 1992). Moreover, the bond between the phycobilins and the protein is not broken following cell disruption, and the spectrum showed by the released phycobilin is species specific, and dependent on the interaction between the protein and the chromophore.

So far, a major effort was directed to develop an optical technique for remote estimation of PC, a water-soluble pigment common in freshwater cyanobacteria. The absorption signal of PC is detectable from reflectance spectra in eutrophic waters near 625 nm (Dekker 1993; Jupp et al. 1994; Gitelson et al. 1999; Schalles and Yacobi 2000). Following the same logic as for Chl *a* retrieval (Gitelson et al. 1985), a semianalytical algorithm for PC estimation has been proposed (Simis et al. 2005). The algorithm attributed the absorption signal in the 625-nm band to both PC and Chl *a* and to the absorption of Chl *a* around 670 nm. The initial tests have shown that the algorithm generally provides significant overestimations of the PC, except when extremely high PC concentrations associated with massive blue-green algae blooms were observed in the lakes (Simis et al. 2007). These authors conclude that correction for the red absorption by chlorophyll *c* (Chl *c*) and chlorophyll *b* (Chl *b*) is needed to yield more realistic PC assessments in inland waters. Thus, the state-of-the-art for PC retrieval can be summarized as follows: (1) PC prediction best matches observed values only during periods of high relative abundance of cyanobacteria in the plankton community, and the algorithm in its current form (Simis et al. 2005) is considered suitable for detection of the PC concentration above 50 mg m^{-3} in cyanobacteria-dominated waters. (2) Applying a fixed value of the specific absorption coefficient for PC, $a^*_{PC}(625)$, caused an overestimation of the PC concentration that increases drastically at lower concentrations of that pigment. (3) The results of Simis et al. (2005) algorithm calibration suggest strong (up to 15-fold) seasonal variation in $a^*_{PC}(625)$. This difference is probably the result of package effect as well as various physiological states of algae (nutrient/light effects). (4) The presence of pigments other than PC and Chl *a*, and the variable influence of Chl *a* on retrieved absorption at 625 nm, are potential causes of error in PC retrieval. (5) Due to significant and systematic overestimation of Chl *a* by the algorithm developed by Gons et al. (2000) for Chl $a < 20$ mg m^{-3}, there are significant errors in subtraction of Chl *a* contribution at 625 nm in the Simis et al. (2005) algorithm. It is likely that non-phytoplankton absorption as well as absorption by Chl *c* and Chl *b* may explain this tendency to overestimate Chl *a* (Gitelson et al. 2008).

Absorption of all chlorophylls partly overlaps and also overlaps the range of the optical activity of PC. It is therefore necessary to take into account the effect of Chl *a* and the accessory Chl *b* and Chl *c* absorption in the spectral range of PC absorption in order to achieve a reliable estimation of PC-specific absorption. The goals of this study were: (1) to determine the net effect of PC absorption at the wavelength around 625 nm (where a peak is observed in absorption spectra and a small but discernible trough in reflectance spectra); (2) to establish the quantitative relationship between PC concentration and specific absorption coefficient of PC; (3) to define the minimum concentration of PC or relative proportion of it to Chl *a* where the optical activity of PC is detectable in natural samples.

Materials and Procedures

Sampling sites

Water samples were collected in Lake Kinneret (Israel) in eight different campaigns (June, July, August, September, and November 2012 and June and July 2013) and in three campaigns in Berlin lakes (Germany) in August 2013.

Experimental work

Collected water samples were kept in a dark box, shipped to the laboratory, and processed within four hours from the first collection. Qualitative microscopic examination was undertaken to determine the dominant components of phytoplankton, identification limited to the level of genus. Subsamples of 200–250 mL in Lake Kinneret, and 40–200 mL in Berlin lakes were filtered onto glass fiber filter (Whatman GF/F) using vacuum of < 15 kPa and the wavelength-dependent absorption of the collected particles was determined spectrophotometrically in the range from 400 nm to 750 nm. To elucidate the optical impact of phytoplankton pigments, the sample (origin) was bleached for 20 min by 1% sodium hypochlorite solution, following Ferrari and Tassan (1999). The depigmented tripton (bleached) displayed an exponential decline of absorption from 400 nm toward longer wavelengths. The absorption of the depigmented material was subtracted from the absorption of the origin, resulting in a wavelength-dependent description of phytoplankton pigments absorption. The pigments absorption wavelength-dependent

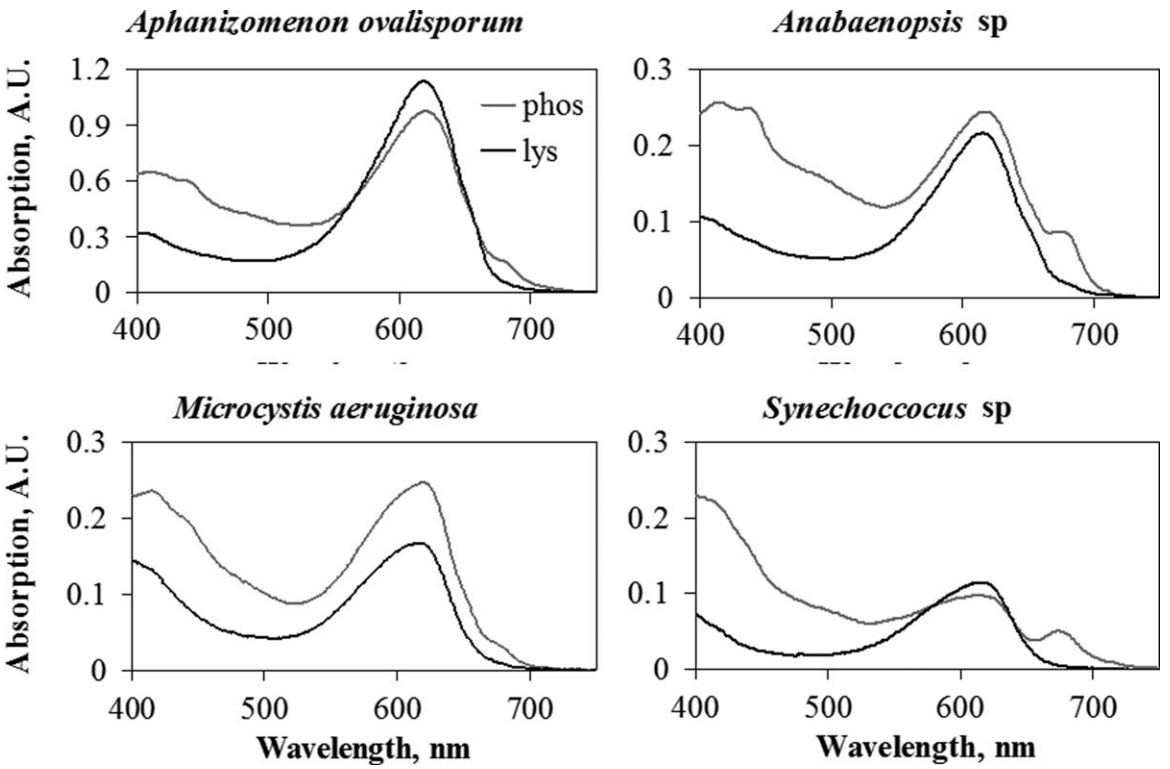

Fig. 1. Comparison of absorption spectra of aqueous extracts of cyanobacteria. Spectra were measured following clearing of the suspensions by centrifugation. The extraction was into phosphate buffer medium (phos) following Sarada et al. (1999) or into lysozyme medium (lys) following the protocol suggested by Stewart and Farmer (1984).

coefficient, $a_{ph}(\lambda)$, was calculated correcting for the path length amplification effect, filtered volume, and the filter collecting area, following Allali et al. (1997).

For the determination of chlorophyll concentrations, samples of particulate matter were collected by filtration of water samples onto glass-fiber filters (Whatman GF/F), frozen at -20°C and subsequently ground in 90% acetone and left overnight at 4°C in the dark. We did not maintain frozen filters for period that exceeded one week. Following clarification of the extract by centrifugation of three minutes at $1100 \times g$, Chl *a*, *b*, and *c* concentrations were determined by spectrophotometrical measurement and subsequent calculation using the algorithms developed by Ritchie (2008).

For determination of PC concentration, cells were collected by filtration onto glass-fiber filters (GF/F, Whatman) and frozen at -20°C. Grinding was facilitated in an all-glass mortar and pestle set (Kontess Glass Co.).

We ran a preliminary study to choose a method for the extraction of the water soluble pigments using dense cyanobacteria cultures. Cultures were grown in BG-11 medium, at 17°C, at continuous light of 35 μmol m^{-2} s^{-1}. Two extraction media were tested: (1) 50 mM phosphate buffer following Sarada et al. (1999), and (2) lysozyme-based extraction medium (lysozyme solution), following Stewart and Farmer (1984). Extraction lasted 24 h, samples were clarified by one of the following means: (1) centrifugation of three minutes at $1100 \times g$, (2) filtration onto glass-fiber filters (Whatman GF/F), with an average pore size of 0.7 μm, (3) filtration onto Durapore® five-micrometer filters (Millipore). Concentrated aqueous extracts were scanned in the range from 400 nm to 750 nm, using a double-beam spectrophotometer (Uvikon 930, Kontron) and the concentration of PC as mg L^{-1} was determined using a slightly modified formula devised by Bennett and Bogorad (1973):

$$PC = (A_{617} - 0.474A_{652})/5.34 \quad (1)$$

where, $A6_{17}$ and A_{652} is the absorbance at 617 nm and 652 nm, respectively.

Comparison of means for clarification of the aqueous extracts showed that filtration onto GF/F filter consistently reduces the concentration of PC, regardless of the extraction medium and the cyanobacteria extracted. The concentration of PC in extracts clarified by centrifugation was slightly higher than in extracts clarified by filtration onto five-micrometer membrane filters, but the difference between these two means was not consistent, and we used the centrifugation as a routine mean for extract clarification.

The spectra of the pigment(s) released into extraction media peaked at a range from 615 nm to 620 nm in all

examined cultures and natural samples. The difference in the location of the peak was not consistent between media or species extracted. A consistent and stark difference showed, however, between the spectra of extract into phosphate buffer and spectra resulting from the application of the lysozyme solution (Fig. 1). In the latter, light absorption curve showed almost linear decline from 400 nm to about 450 nm, subsequently a wide trough up to approximately 530 nm, and next a linear increase toward the peak at 615–620 nm; the decline from the peak also showed a linear pattern down to 670 nm, and continued in an apparently exponential decline to 750 nm. Phosphate buffer extracts showed in all cases absorption properties that necessarily are contributed by thylakoid-bound pigments, i.e., carotenoids and chlorophylls. The most prominent and consistent are Chl *a* (and its degradation products) signature peaks, visible near 415 nm, 433 nm, and 675 nm. A peak near 482 nm is with a high probability a signature of carotenoids, optically active in the violet, blue, and blue-green range of the spectrum. Chl *a* was detected in most samples; however, its concentration in phosphate buffer extracts was on average eight times higher than in the lysozyme solution extracts. Calculation of PC concentration (Eq. 1) showed mostly higher values in the phosphate buffer, but that result was not consistent and cases where PC concentration in the lysozyme extract was higher than in the phosphate buffer extract were also recorded. Considering these findings, we used the lysozyme protocol for routine PC extraction.

In extracts of field samples, PC concentration was mostly too low to be measured spectrophotometrically. In field samples, we therefore measured the concentration of PC in Lake Kinneret by a Turner Designs fluorometer equipped with a cool white lamp (10-047) and an excitation filter 580-620 nm and emission 640 nm. Calibration of the fluorometer was done with concentrated PC extract prepared as mentioned above, and measured spectrophotometrically. In Berlin, PC concentrations were measured using a spectrofluorometer (Perkin Elmer, LS 50B), with the excitation wavelength set to 580 nm and emission from 600 nm to 660 nm, and calibrated as in Lake Kinneret.

Correction of absorption coefficients

To estimate PC net absorption at 625 nm (the wavelength where we assume the peak of that pigment was located in absorption spectra of pad-collected particles), the contribution of absorption by chlorophylls at that wavelength was subtracted. The contribution of Chl *a*, Chl *b*, and Chl *c* at relevant wavelengths was based on specific absorption coefficients spectra presented in Bidigare et al. (1990) and are summarized in Table 1. We assumed that the measured absorption of particles at 675 nm, $a_{ph}(675)$, represents exclusively the optical activity of Chl *a*, neglecting the slight effect imposed by the accessory chlorophylls, detritus, and CDOM. We therefore assumed that the effect of Chl *a* on

Table 1. Specific absorption values of chlorophylls (multiplied by 1000) at chosen wavelengths based on "unpackaged" specific absorption coefficients (Bidigare et al. 1990).

	Pigment		
Wavelength (nm)	Chl *a*	Chl *b*	Chl *c*
625	40.5	23	55
636	38	36	98
652	32	118	27
675	201	10.5	2

absorption at 625 nm is 0.201 of that at 675 nm. The putative absorption coefficient of other pigments at 625 nm, $a_{Chl\ a}(625)$, was therefore calculated as:

$$a_{Chla}(625) = a_{ph}(675) \times 0.201 \quad (2)$$

Whenever accessory chlorophylls are present, they also contribute to absorption at 625 nm. To estimate the contribution of the accessory chlorophylls at 625 nm, we undertook the following steps: (1) calculated the difference between the absorption of phytoplankton at 625 nm ($a_{ph}(625)$ and the absorption attributed to Chl *a* at 625($a_{Chl\ a}(625)$; (2) multiplied the result of step 1 by the ratio of the concentrations of Chl *b* and Chl *c* to Chl *a*, and (3) by the ratio between the specific absorption of the Chl *b* and Chl *c* to the absorption of Chl *a* at 625 nm (derived from Table 1):

$$a_{Chlb}(625) = [a_{ph}(625) - a_{Chla}(625)] \times [Chlb/Chla] \times 0.57 \quad (3)$$

$$a_{Chlc}(625) = [a_{ph}(625) - a_{Chla}(625)] \times [Chlc/Chla] \times 1.36 \quad (4)$$

PC net absorption at 625 nm was therefore corrected by subtraction of the effect of all chlorophylls:

$$a_{PC}(625) = a_{ph}(625) - [a_{Chla}(625) + a_{Chlb}(625) + a_{Chl\ c}(625)] \quad (5)$$

Chl *a* specific absorption coefficient, $a^*_{ph}(675)$, was calculated as a ratio of phytoplankton absorption at 675 nm and Chl *a* concentration, while the specific absorption coefficients of PC were calculated by the division of calculated pigment absorption at the peak location by the correspondent pigment concentration and marked $a^*_{PC}(625)$.

As an alternative approach for the estimation of a^*_{PC}, the presumptive absorption of Chl *a* was simulated and subtracted from the phytoplankton absorption function measured for each sample. The presumptive Chl *a* absorption was established as follows:

$$a_{Chl\,a}(\lambda) = a^*_{Chl\,a}(\lambda) \times [Chl\,a] \quad (6)$$

$a^*_{Chl\,a}(\lambda)$ was derived from a previous study (Gilerson et al. 2010) and [Chl *a*] was Chl *a* concentration determined in samples. Assuming that the contribution of CDOM and detritus is negligible in wavelengths higher than 550 nm, we

Table 2. Descriptive statistics of particular water constituents: total suspended solids (TSS) and phytoplankton pigments (Chl = chlorophyll, PC = phycocyanin) and specific absorption coefficients in Lake Kinneret (n = 88). a^*_{ph} (675) and a^*_{ph} (625)—phytoplankton-specific absorption at 675 nm and 625 nm, respectively, and a^*_{PC} (625)—PC-specific absorption at 625 nm.

Variable	Min	Max	Median	Average	Std	c.v.
TSS (g m^{-3})	1.3	4.3	3.1	3.0	0.7	22
Pigment concentration						
Chl *a* (mg m^{-3})	6.5	17.4	11.6	11.5	3.0	26
Chl *b* (mg m^{-3})	0.3	2.8	0.9	1.2	0.7	56
Chl *c* (mg m^{-3})	0.5	2.9	1.0	1.1	0.5	45
Phycocyanin (mg m^{-3})	1.4	9.6	4.0	4.0	1.9	48
Pigment ratio						
Chl *a*/Chl *b*	5.5	37.5	10.0	11.9	5.4	46
Chl *a*/Chl *c*	3.4	25.6	11.2	11.7	5.4	46
Chl *a*/PC	0.9	6.7	3.3	3.4	1.2	36
Specific absorption (m^2 mg^{-1})						
a^*_{ph} (675)	0.0124	0.0244	0.0153	0.0156	0.0021	13
a^*_{ph} (625)	0.0073	0.0524	0.0222	0.0228	0.0087	38
a^*_{PC} (625)	0.0028	0.0287	0.0088	0.0099	0.0060	60

assumed that absorption is the result of optical activity of the accessory pigments, and if Chl *b* and *c* are only a minor component, the major contributor to absorption in wavelengths from 550 nm to 710 nm are Chl *a* and PC. Finally, the difference between the measured wavelength-dependent absorption function and the calculated function of Chl *a* absorption was divided by PC concentration to yield $a^*_{PC}(\lambda)$.

Assessment

Water constituents characteristics

The sampling sites differed conspicuously in concentration of total suspended solids (TSS), concentration of phytoplankton—as estimated by the concentration of Chl *a*, and phytoplankton composition, indicated by the ratio of Chl *a* to accessory chlorophylls and PC (Tables 2, 3). In Lake Kinneret, particulate water constituents varied within limited ranges in the samples presented in the current report (Table 2). Chl *a* concentrations ranged from 6.5 mg m^{-3} to 17.4 mg m^{-3}, with an overall average of 11.5 mg m^{-3}, which is lower than the multiannual average in the lake (16.9 mg m^{-3}) and with low spatial and temporal variation. The coefficient of variation of the latter was only 26% in the current database, while the multiannual average surpasses 130%. The ratios of Chl *a* to the accessory Chl *b* and Chl *c* and to PC indicate that seldom one of the major phytoplankton groups (chlorophytes, diatoms and/or dinoflagellates, and cyanobacteria) was decisively dominant. The (weight/weight) ratio between Chl *a* and PC was 3.4 in average, with relatively little variation indicated by a median of 3.3 and a coefficient of variation of 36% (Table 2).

The average concentration of TSS in Berlin lakes was almost three times higher than the average in Lake Kinneret and Chl *a* concentration was more than four times higher than the average in Lake Kinneret (Table 3). With few exceptions, the concentration of accessory chlorophylls in Berlin lakes was low, relatively to the concentration of Chl *a* and PC (Table 3). Chl *c*-containing algae (mostly diatoms) comprised a relatively high proportion in few locations, as indicated by the low Chl *a*/Chl *c* ratio, but most of the samples were clearly dominated by cyanobacteria. The (weight/weight) ratio between Chl *a* and PC was 4.5 in average, but highly variable as indicated by a median of 1.9 and a coefficient of variation of 162% (Table 3).

Absorption spectra of collected particles

Several clear features were identified in the spectra of material collected onto a pad and examined spectrophotometrically. The nonpigmented component of TSS in Lake Kinneret exerted only a minor effect on light absorption in wavelengths longer than 550 nm (Fig. 2a), while in Berlin lakes the effect of nonpigmented particles was prominent almost all along the scanned range (Fig. 2b). The most prominent peak of the pigmented fraction was located at 675 nm, visible in all pad spectra of particles collected in our samples and reflecting Chl *a* absorption. Chl *a* absorption at 436 nm was prominent in all Lake Kinneret samples (Fig. 2a), and also detectable in Berlin samples (Fig. 2b), but in the latter, another peak at the shorter wavelengths range, around 410

Table 3. Descriptive statistics of particular water constituents: total suspended solids (TSS) and phytoplankton pigments (Chl = chlorophyll, PC = phycocyanin) and specific absorption coefficients in Berlin lakes (n = 52). a^*_{ph} (675) and a^*_{ph} (625)—phytoplankton-specific absorption at 675 nm and 625 nm, respectively, and a^*_{PC} (625)—PC-specific absorption at 625 nm.

Variable	Min	Max	Median	Average	Std	c.v.
TSS (g m^{-3})	2.6	28.8	7.4	8.7	5.5	64
Pigment concentration						
Chl *a* (mg m^{-3})	7.6	160.7	42.4	49.7	32.5	65
Chl *b* (mg m^{-3})	0.3	3.0	1.1	1.3	0.9	69
Chl *c* (mg m^{-3})	0.6	7.3	3.0	3.3	1.7	50
Phycocyanin (mg m^{-3})	0.5	126.4	26.1	36.6	37.5	102
Pigment ratio						
Chl *a*/Chl *b*	10.4	346.7	22.4	80.8	113.1	140
Chl *a*/Chl *c*	4.2	30.6	16.8	15.8	7.2	45
Chl *a*/PC	0.6	35.9	1.9	4.5	7.2	162
Specific absorption (m^2 mg^{-1})						
a^*_{ph} (675)	0.0121	0.0342	0.0204	0.0203	0.0043	21
a^*_{ph} (625)	0.0068	0.1934	0.0137	0.0317	0.0401	127
a^*_{PC} (625)	0.0034	0.0760	0.0069	0.0137	0.0155	113

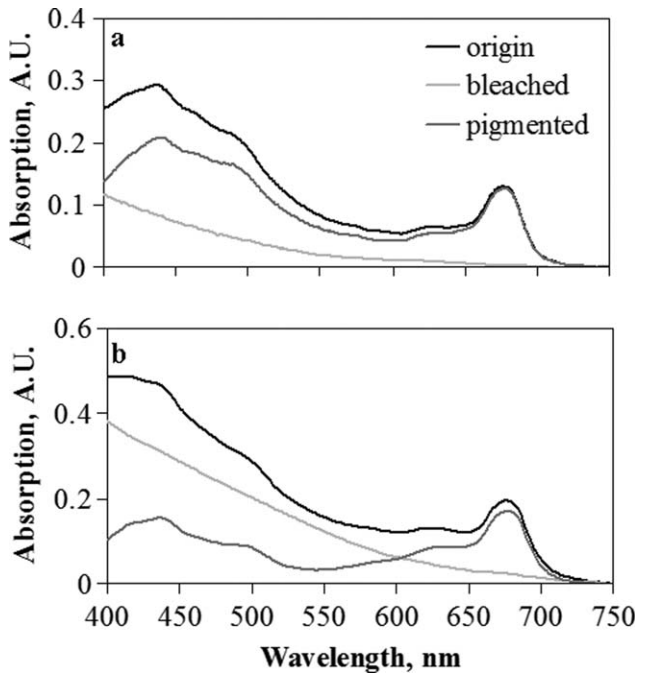

Fig. 2. Absorption spectra of particles collected onto GF/F filter. "Origin" is nontreated particles; "bleached" is sample treated by 1% sodium hypochlorite (Ferrari and Tassan 1999); "pigmented" is the difference between "origin" and "bleached". (a) Lake Kinneret: phytoplankton biomass dominated by dinoflagellates, accompanied by chlorophytes, diatoms, and cyanobacteria, with Chl a = 9.2 mg m^{-3}, PC = 2.2 mg m^{-3}; (b) Berlin lakes: phytoplankton composed exclusively by cyanobacteria, with Chl a = 50 mg m^{-3}, PC = 41.9 mg m^{-3}.

nm, was mostly higher. A broad peak, centered mostly at 625 nm was also seen in all spectra, but its prominence, i.e., the ratio of heights of the peaks at 625 nm and 675 nm assumed different relationships in samples with relatively low PC concentrations and samples where PC/Chl a ratio was high. The 625 nm peak was positively correlated with the ratio of PC/Chl a/ in samples where the concentration of PC was at least half the concentration of Chl a or higher, and the ratio between absorption peak at 625 nm to the absorption at 675 nm exceeded the value of 0.4 (Fig. 3); in samples with lower PC/Chl a ratio, and lower 625/675 nm absorption ratio no defined pattern of relationship was apparent (Fig. 3).

The relationship between phytoplankton absorption at 625 nm, $a_{ph}(625)$, and PC concentration varied between sampling locations. In the samples collected in Berlin lakes, the relationship between PC concentration and $a_{ph}(625)$ was close (r^2 = 0.89, n = 52, $p < 0.001$), but in Lake Kinneret, the relationship between PC concentration and $a_{ph}(625)$ was effectively null. In samples of Berlin lakes where PC concentrations were below 10 mg m^{-3}, $a_{ph}(625)$ was also not related to PC concentration, and essentially was not different from that of Lake Kinneret.

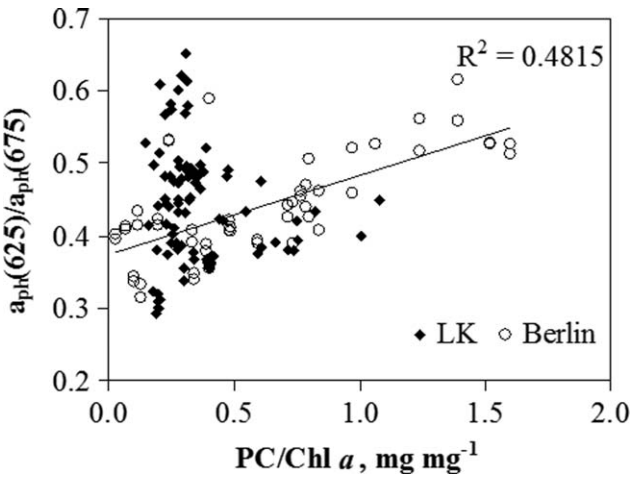

Fig. 3. The plot of the ratio between concentration of PC to Chl a vs. the ratio of pigment absorption at 625 nm to the absorption at 675 nm. The regression line pertains to Berlin lakes results.

The $a_{PC}(625)$ vs. PC relationship for Berlin lakes was very close with r^2 of 0.92 (Fig. 4a). However, the deduction of the effect of chlorophylls on absorption at 625 nm did not enhance correlation between PC concentration and absorption at 625 nm when the concentrations of PC was below 10 mg m^{-3}, both in Berlin lakes and in Lake Kinneret (Fig. 4b).

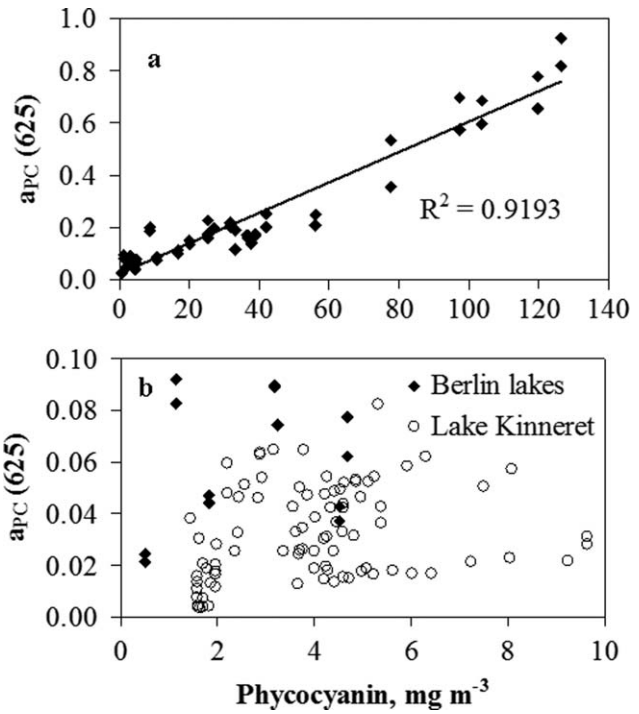

Fig. 4. Relationship between PC concentration and PC absorption at 625 nm in (a) Berlin lakes (all data), and (b) in Lake Kinneret and Berlin lakes as PC concentration in latter was below 10 mg m^{-3}. PC net absorption was calculated by Eq. 5.

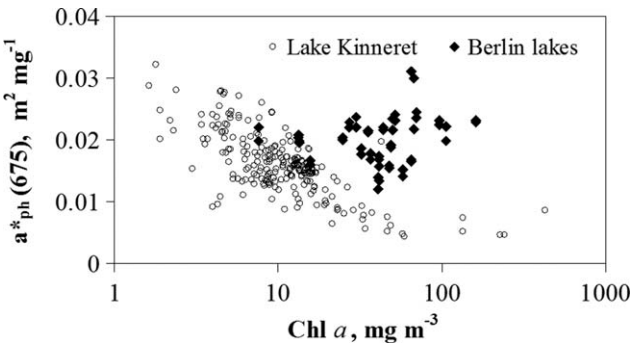

Fig. 5. The relationship between Chl *a* concentration and Chl *a* specific absorption coefficient at 675 nm, $a^*_{ph}(675)$, in Lake Kinneret and Berlin lakes. The relationship in Lake Kinneret was $a^* = 0.342 \times [\text{Chl } a]^{-0.3463}$, with $r^2 = 0.564$, $n = 348$, $p < 0.001$. Kinneret results include data collected in the current study and another 260 sample collected in previous years, to increase the range of Chl *a* concentration in the analysis.

Pigment-specific absorption coefficients

In Lake Kinneret samples, the specific absorption coefficient at 675 nm, $a^*_{ph}(675)$, was in average 0.0156 m^2 mg^{-1}, ranged from 0.0124 m^2 mg^{-1} to 0.0244 m^2 mg^{-1} and was negatively correlated with Chl *a* concentration (Fig. 5). The variability of $a^*_{ph}(675)$ at each one of the cruises was low, with coefficient of variation ranged from 2% to 25%, whereas the coefficient of variation for Chl *a* concentration ranged from 4% to 73%. The average $a^*_{ph}(675)$ in Berlin lakes was higher—0.0203 m^2 mg^{-1}, and, in contrast to the Kinneret samples, did not show any specific trend of change with Chl *a* concentration variation (Fig. 5). It is hardly surprising that $a^*_{ph}(675)$ values for low (Lake Kinneret data) and moderate (in most Berlin lakes) Chl *a* concentrations are different and showed a different pattern of relationship with Chl *a*, as $a^*_{ph}(675)$ depends on photophysiological acclimation and taxonomic changes (Fujiki and Taguchi 2002). The negative nonlinear relationship between $a^*_{ph}(675)$ and Chl *a* is typical for low Chl a concentrations (e.g., Allali et al. 1997; Suzuki et al. 1998). Conversely, the $a^*_{ph}(675)$ values in Berlin lakes as well as the relationship $a^*_{ph}(675)$ vs. Chl *a* in this study resemble findings from water bodies with diverse Chl *a* concentrations and diverse complexity in term of water constituents. In such waters, $a^*_{ph}(675)$ for Chl *a* concentration of tens of mg m^{-3} may assume values differing by a factor of two and three for the same concentration of the pigment (Gurlin et al. 2011; Gilerson et al. 2010; Shi et al. 2013).

In Lake Kinneret samples, the value of PC specific absorption coefficient, when the effect of absorption by all chlorophylls was subtracted, $a^*_{PC}(625)$, was in average 0.0099 m^2 mg^{-1}. In Berlin samples following subtraction of the chlorophylls effect, the average $a^*_{PC}(625)$ was 0.0137 m^2 mg^{-1}. Comparison of $a^*_{PC}(625)$ to the not corrected values of $a^*_{ph}(625$ nm) is shown in Tables 2, 3 and underline the caveat of calculation of the specific activity of PC without accounting for the optical activity of chlorophylls at 625 nm. When the absorption at 625 nm was corrected for the contribution of Chl *a* only, the average $a^*_{PC}(625)$ in Lake Kinneret was 0.0119 m^2 $mg^{-1}$and in Berlin lakes it was 0.0156 m^2 mg^{-1}, indicating that without the subtraction of the accessory chlorophylls $a^*_{PC}(625)$ was overestimated by 20% and 14%, respectively. For samples with PC concentration higher than 10 mg m^{-3} (all in Berlin lakes), the overestimation caused by not accounting for the absorption at 625 nm by accessory chlorophylls was 6%.

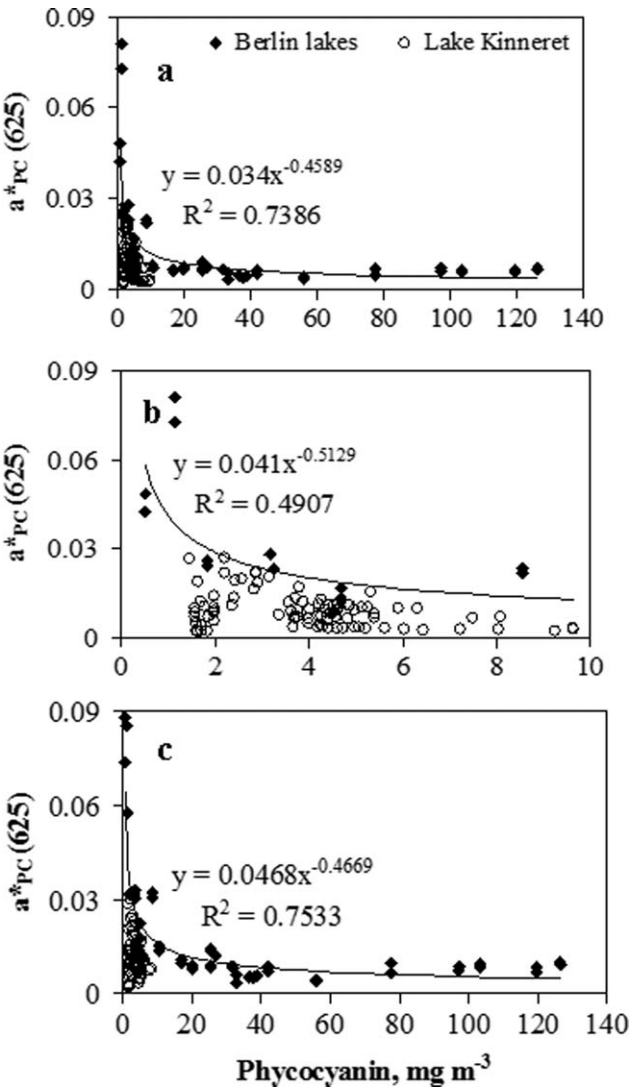

Fig. 6. The relationship between PC concentration and specific absorption coefficient of PC at 625 nm, $a^*_{PC}(625)$, in Lake Kinneret and Berlin lakes (a) all results; (b) $(a^*_{625})_{PC}$ for samples where PC concentration was below 10 mg m^{-3}; (c) $(a^*_{625})_{PC}$ in Lake Kinneret and Berlin lakes calculated on the basis of the difference between phytoplankton absorption and the simulated absorption of Chl *a* using Eq. 6. In all panes, the trend line stands for Berlin data only.

$a^*_{PC}(625$ nm) was negatively, nonlinearly correlated with PC concentration and in Berlin lakes showed a high

correlation (Fig. 6a). When PC concentrations were lower than 10 mg m^{-3}, i.e., the database that encompassed all Lake Kinneret and some results from Berlin lakes, $a^*_{PC}(625)$ was also negatively correlated with the PC concentration, but showed a much smaller correlation (Fig. 6b); in Berlin lakes, it was $r^2 = 0.49$ and in Lake Kinneret, $r^2 = 0.13$. However, when PC concentrations exceeded 10 mg m^{-3}, $a^*_{PC}(625)$ oscillated around 0.007 m^2 mg PC^{-1} in a wide range of PC concentration (Fig. 6c).

The alternative calculation of $a^*_{PC}(625$ nm), based on the difference between phytoplankton absorption and the simulated absorption of Chl *a* (Eq. 6) yielded a similar result in Berlin lakes, $a^*_{PC}(625)$ oscillated around 0.008 m^2 mg PC^{-1} where PC concentrations were > 10 mg m^{-3}.

Discussion

Determination of the net effect of PC absorption at 625 nm

The published information on the specific absorption coefficient of PC is rather scarce, and often does not take into account the accumulating effect of several pigments at a given point along the absorption spectrum. It is, therefore, not surprising that the published values of $a^*_{PC}(625)$ vary by a factor of almost 60 (Table 4), far larger than the variation of specific absorption coefficient of Chl *a* (Bricaud et al. 1995). Comparison within each set of results also shows a large variability, which spans from a ratio of approximately 1 : 3 up to 1 : 22 between the minimum and maximum $a^*_{PC}(625)$ in each one of the sets (Table 4). Schemes for correction of $a^*_{ph}(625)$ due to Chl *a* absorption were already suggested in previous studies (Dekker 1993; Simis et al. 2005) and are apparently correct in instances where cyanobacteria are by-and-large the dominant phytoplankton component. However, the suggested methods of correction (Dekker 1993; Simis et al. 2005) that account for the absorption effect exercised by Chl *a* disregard the effect of the accessory chlorophylls. An indirect evidence for the necessity of including correction for the accessory chlorophylls was provided by Simis et al. (2007), that showed that a model for the prediction of PC concentration overestimates the actual PC concentrations in water where Chl *b*- and Chl *c*-containing algae. The lack of correction for the accessory chlorophylls may be the reason for the exceedingly high $a^*_{ph}(620)$ values in Lake IJsselmeer (The Netherlands), whenever low PC concentrations prevailed in that lake (Table 4). The correction procedure we suggested herein reduced $a^*_{ph}(625)$ values by approximately 52% in Berlin lakes (Table 3) and lowered the noncorrected value by approximately 59% in Lake Kinneret (Table 2). Our correction factors for $a^*_{ph}(625)$ were higher than the correction value of 24% suggested by Simis et al. 2007, and achieved by a different methodology for cyanobacteria dominated water bodies. We applied the correction scheme for "net" absorption by PC at 620 nm as suggested by Simis et al. (2005):

$$a_{PC}(620) = a_{ph}(620) - [\varepsilon \times a_{ph}(665)] \quad (7)$$

where, $a_{ph}(620)$ and $a_{ph}(665)$ are phytoplankton absorption at 620 nm and 665 nm, respectively, and ε is "the value where the slope of the linear least-squares fit of modeled against observed PC concentration was ~ 1"(Simis et al. 2005). We used $\varepsilon = 0.24$ and found that $a^*_{ph}(620)$ were in average 0.0118 mg PC m^{-2} and 0.0174 mg PC m^{-2} for Lake Kinneret and Berlin lakes, respectively. These averages are higher than the averages computed by our method, but closely correlated. The two sets showed in Lake Kinneret a slightly lower coefficient of fit (r^2) and a relatively higher values for $a^*_{PC}(620)$ (Fig. 7a) than in Berlin lakes (Fig. 7b). That difference is probably the outcome of the relatively higher proportion of accessory chlorophylls in Lake Kinneret samples, which are not accounted for when Eq. 7 was applied. In both locations, part of the difference in the calculated specific coefficients should be attributed to the fact

Table 4. Specific absorption of PC, a^*_{PC} or a^*_{ph}, determined for wavelengths varying between studies from 620 nm to 625 nm (n.d. = data not available)

Origin	Dominant organism	Specific absorption (m^2 mg^{-1})	Phycocyanin (mg m^{-3})	Reference
Vecht lake area	*Oscillatoria* sp.	0.0032	7–130	Dekker (1993)
Lake Loosdrecht	*Limnothrix/Pseudanabaena* group	0.0059–0.0174	21.7–79.8	Simis et al. (2005)
Lake IJsselmeer	*Microcystis* sp. and *Aphanizomenon* sp.	0.0088–0.1868	0.8–64.8	Simis et al. (2005)
cultures	Several cultured species	0.0071	n.d.	Simis and Kauko (2012)
Branched Oak lakes	Not indicated	0.0193	0.7–159	Gurlin (2012), p. 96
Lake Kinneret	*Microcystis* sp., *Aphanizomenon* sp., *Cylindrospermopsis* sp.	0.0028–0.0287	1.4–9.6	Current work
Berlin lakes	*Microcystis* sp., *Anabaena* sp.	0.0034–0.076	0.5–126.4	Current work

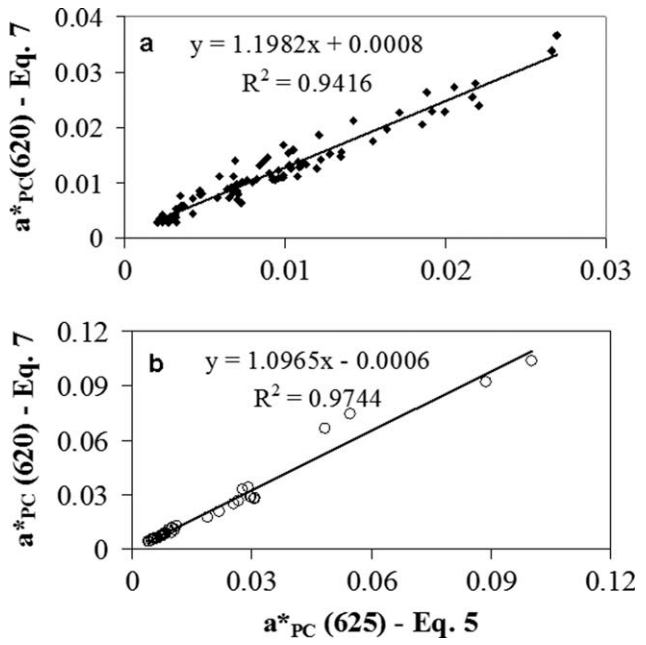

Fig. 7. Comparison of a^*_{PC}—PC-specific absorption calculated by accounting for all chlorophylls absorption at 625 nm (based on Eq. 5) vs. a^*_{PC} calculated by subtraction of the effect of Chl *a* at 620 nm, based on Simis et al. (2005) (Eq. 7). (a) Lake Kinneret and (b) Berlin lakes.

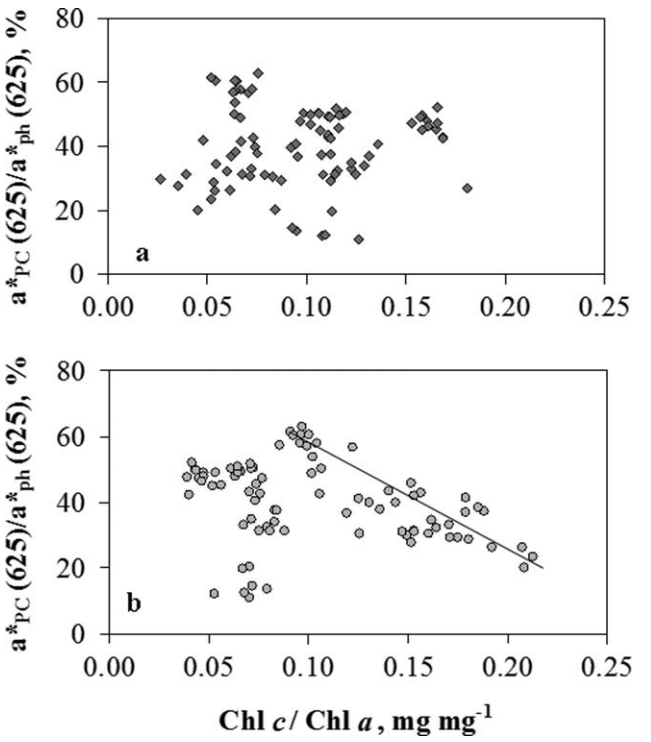

Fig. 8. The ratio of the calculated PC-specific absorption coefficient at 625 nm, $a^*_{PC}(625)$, to the phytoplankton specific absorption coefficient at 625 nm, $a^*_{ph}(625)$ vs. the ratio of accessory pigments Chl *b* (a), and Chl *c* (b) to Chl *a*. Relationships are based on data collected in Lake Kinneret. Note the apparent decrease of $a^*_{PC}(625)/a^*_{ph}(625)$ as Chl *c*/Chl *a* exceeded 0.1.

that we used the published value of ϵ computed from a different data base (Simis et al. 2005) and also to the difference in chosen wavelengths in the formulae used for the computation of $a^*_{ph}(\lambda)$. Thus, when $a_{ph}(665)$ was substituted by $a_{ph}(675)$ in Eq. 7 $a^*_{ph}(620)$ was reduced to 0.0097 mg PC m^{-2} in Lake Kinneret, and in Berlin lakes decreased to 0.0166 mg PC m^{-2}. Substitution of absorption at 620 nm by substitution at 625 nm hardly changed $a^*_{ph}(620)$ values. Using Eq. 7, for samples with PC concentration $>$ 10 mg m^{-3} the average $a^*_{PC}(625)$ was 0.007, similar to the result achieved by the use of Eq. 5 (divided by PC concentration) and Eq. 6.

We are aware of the fact that one of our correction methodology is based on pigment absorption functions of "unpackaged" pigments (Bidigare et al. 1990), and absorption of phytoplankton is apparently not synonymous with the combination of computed absorption of isolated pigments. Full deduction of the effect of chlorophylls on absorption at 625 nm probably was not achieved in this study, but the fact that three different approaches yielded quite similar $a^*_{ph}(625)$ values indicate that our estimation of $a^*_{ph}(625)$ is close to the real value.

When Chl *b* and Chl *c* containing phytoplankton is not negligible, like the situation we had in Lake Kinneret, the effect of the accessory chlorophylls must also be taken into account for reliable $a^*_{PC}(625)$ estimation. In our sets, $a^*_{PC}(625)/a^*_{ph}(625)$ oscillated widely around 40% when Chl *b*/Chl *a* varied by factor 8 (Fig. 8a). However, when Chl *c*/Chl *a* was above 0.1, the magnitude of $a^*_{PC}(625)/a^*_{ph}(625)$ oscillation became smaller with tendency to decrease (Fig. 8b). It implies that when Chl *c*-containing phytoplankton comprises 20–30% of the algal assemblage, contribution of Chl *c* to phytoplankton absorption at 625 nm became significant and may affect the quantification of $a^*_{PC}(625)$. We assume that the effect of Chl *c* is more pronounced as a result of the relatively high absorption of that pigment at 625 nm (Table 1).To our knowledge, this effect was not observed before and requires further thoughtful investigation.

$a^*_{PC}(625)$ and $a^*_{ph}(675)$ were virtually not correlated (Fig. 9), indicating that thylakoid-bound pigments (chlorophylls) and pigments packed in the phycobilisomes (phycocyanin) are probably affected differently by changing environmental enforcing conditions. In the case of thylakoid-bound pigments, light climate has a conspicuous effect on cellular pigmentation and on the way pigmented membranes are packed. It was shown that $a^*_{ph}(675)$ may vary by a factor of two in a given algal species, both as an effect of increased pigment synthesis and increased density of thylakoid membranes with decrease of light (Berner et al. 1989), but that relationship is not consistent (Dubinsky et al. 1986). Phycobilin cellular concentrations do respond to the light regime

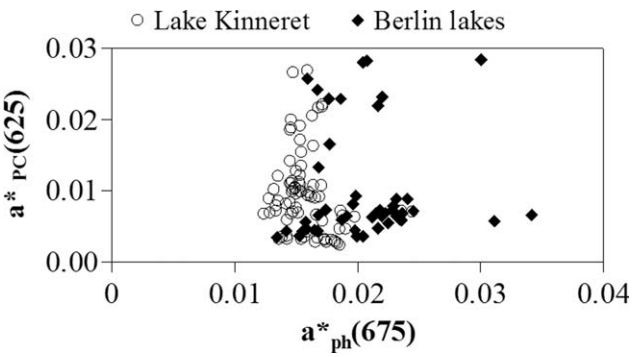

Fig. 9. Relationship between the specific absorption coefficient of Chl *a*—$a^*_{ph}(675)$, and the specific absorption coefficient of PC $a^*_{PC}(625)$. Note that the specific absorption coefficients of Chl *a* and PC are not related, implying that the packaging of these pigments is governed by different factors.

the cells are exposed to, but phycobilins are also potential nitrogen storage compounds and as such may accumulate in cells of cyanobacteria regardless of the light climate (Allen and Smith 1969; Boussiba and Richmond 1980).

Chlorophyllous pigments absorb in the spectral region around 625 nm. Thus, the challenge of quantifying the contribution of PC absorption around 625 nm is how to subtract accurately the absorption of all pigments but PC from total absorption. As we showed, it could be done providing that PC concentration is above 10 mg m^{-3} when the effect of PC becomes the governing factor in total absorption around 625 nm. For smaller PC concentration, our approach failed due to comparable contribution of all pigments to total absorption at 625 nm.

The relationship between PC-specific absorption coefficient and concentration

The sets of measurements included in this study represented different ranges of PC concentrations, but wherever overlapping of concentrations exists, $a^*_{PC}(625)$ also overlapped to a large extent. The plot of $a^*_{ph}(625)$ or $a^*_{PC}(625$ nm) (Fig. 6) against PC concentration showed a negative nonlinear relationship which is also typical for $a^*_{ph}(675)$ and Chl *a* concentration (e.g., Bricaud et al. 1995; Le et al. 2009), and is also characterized by high scatter around the average. Mishra et al. (2013) plotted $a^*_{PC}(620)$ against PC concentrations of up to approximately 3000 mg m^{-3} and showed that from approximately 200 mg m^{-3} and on $a^*_{PC}(620)$ oscillated around 0.004 m^2 mg^{-1}. An important point of our study is that with PC concentrations above 10 mg m^{-3}, $a^*_{PC}(625)$ was almost invariant with an average of approximately 0.007 m^2 mg PC^{-1}, up to PC concentration of 130 mg m^{-3} (Fig. 6a,c), and showed limited variation, with a coefficient of variation of 24%. This $a^*_{PC}(625)$ value is quite close to $a^*_{PC}(625)$ calculated directly from pigment absorption function following chemical stripping of chlorophylls, and representing pure PC in vivo (Simis and Kauko 2012). We therefore assume that $a^*_{PC}(625) = 0.007$ m^2 mg^{-1} is a useful parameter for use in analytical model linking optical activity to PC concentration in mesotrophic-eutrophic water bodies.

Comments and Recommendations

Reflectance spectra measured in cyanobacteria-dominated waters show a bump at 650 nm (e.g., Gitelson et al. 1999) and that feature also appears in modeled spectra based on absorption in waters dominated by cyanobacteria (Kutser et al 2006). The peak of reflectance around 650 nm may be the consequence of decline in absorption and/or increase in backscattering at that range of the electromagnetic spectrum; in our view, the feature in the vicinity of 650 nm reflects a combined decline of PC and Chl *a* absorptions, as there is no physical background on which one expects backscattering to be featured as peak around 650 nm. The mechanism of formation of the reflectance peak around 650 nm is equivalent to that of the peak around 700 nm, which is the result of a combination of minimum absorptions by Chl *a* and water (Gitelson 1992). The peak around 650 nm in cyanobacteria dominated waters contains, therefore, information on both PC and Chl *a* optical activity, and maybe used for qualitative characterization of cyanobacteria dominance (Metsamaa et al. 2006) but it is not a metric of PC. Mishra et al. (2009) have discussed the dynamics and utility of the 650 nm peak in PC prediction models, and the results show that the magnitude of the peak is highly sensitive to the changes in Chl *a* concentration, therefore, algorithms using the peak at 650 nm offer poor PC predictive ability. We therefore think that absorption by PC is main feature that should be used for PC remote estimation, and that the specific absorption coefficient we calculated in this study is an expedient entry for such modeling efforts.

Inferring from the comparison between our sets acquired in different aquatic environments, we conclude that: (1) if PC concentration is below 10 mg m^{-3}, and/or PC/Chl *a* is below 0.5, the optical activity of PC can not be detected in reflectance spectra as it is masked by the optical activity of chlorophyllous pigment. Thus, PC can not be quantified by remote sensing technique wherever the mentioned conditions prevail. (2) Stability of the specific pigment absorption coefficient was suggested a key factor in the predicted robustness of reflectance-based algorithms for estimation of Chl *a* concentration (Gilerson et al. 2010), and by high probability it applies as well to PC. $a^*_{PC}(625)$ is reasonably stable as PC concentration varied from 10 mg m^{-3} to 140 mg m^{-3}. However, if PC concentrations are higher, the suggested algorithm for PC estimation (Simis et al. 2005, 2007) should be calibrated for each ecosystem separately. Considering the limited database we present herein, it appears that a universal, reflectance-based algorithm for determination of PC is

not feasible. $a^*_{PC}(625)$ value changes with PC concentration, and probably with other environmental factors, however, 0.007–0.008 mg m^{-2} seems as an appropriate value for use in algorithms destined for estimation of PC concentration in typical mesotrophic and eutrophic inland waters dominated by cyanophytes.

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