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DOES PIGMENT COMPOSITION REFLECT PHYTOPLANKTON COMMUNITY STRUCTURE IN DIFFERING TEMPERATURE AND LIGHT CONDITIONS IN A DEEP ALPINE LAKE? AN APPROACH USING HPLC AND DELAYED FLUORESCENCE TECHNIQUES[1]

Sonja Greisberger[2] *and Katrin Teubner*[3]

Department of Freshwater Ecology, Faculty of Life Sciences, University Vienna, Althanstrasse 14, A-1090 Vienna, Austria

In vivo delayed fluorescence (DF) and HPLC/CHEMTAX pigment analyses were used to investigate seasonal and depth distributions of phytoplankton in a deep alpine mesotrophic lake, Mondsee (Austria). Using chl *a* equivalents, we determined significant relationships with both approaches. Community structure derived from pigment ratios of homogenous samples was compared with microscopic estimations using biovolume conversion factors. An advantage of the HPLC/CHEMTAX method was that it gave good discrimination among phytoplankton groups when based on a pigment ratio matrix derived from multiple regression analysis. When a single algal group was dominant, such as epilimnetic diatoms or hypolimnetic cyanobacteria in the deep chl maxima, HPLC/CHEMTAX results were significantly correlated with microscopic estimations (diatoms: $r = 0.93$; cyanobacteria: $r = 0.94$). Changes in the composition of photosynthetically active pigments were investigated with DF and benefited from excitation spectra that considered all light-harvesting pigments, which made it possible to assess the enhancement of accessory photosynthetically active pigments relative to active chl *a* (chl a_{DF672}). Changes in similarity index, based on normalized DF spectra, confirmed compositional shifts observed by microscopy. At chosen wavelengths of DF spectra, 534 and 586 nm, we generally observed a significantly inverse relationship between normalized DF intensities and temperature and light along both seasonal and depth gradients. The relative increase in photosynthetically active pigments other than chl a_{DF672} under low light and temperature was caused by an increasing dominance of diatoms and/or phycobilin-rich cyanobacteria and Cryptophyta. DF spectra provided a more accurate picture of community pigments acclimated to light and temperature conditions than the β-carotene:chl *a* ratio derived from HPLC.

Key index words: **cryptophytes; cyanobacteria; diatoms; marker pigment ratios; metalimnion; oscillaxanthin**

Abbreviations: **Chl*a*Equivalent$_{biov}$, chl *a* equivalents of phytoplankton related to biovolume; Chl*a*Equivalent$_{DF}$, chl *a* equivalents of phytoplankton in relation to DF; Chl*a*Equivalent$_{HPLCCH}$, chl *a* equivalents of phytoplankton in reference to HPLC pigment-based CHEMTAX estimates; DF, delayed fluorescence**

In the last decade, the use of pigment-related methods to identify different taxonomic groups of phytoplankton has increased, mainly due to improvements in modern analytical techniques such as HPLC, which yields, depending on the procedure, quantitative data on lipophilic (chlorophylls and carotenoids) or water-soluble (phycobiliproteins) pigments (Wright et al. 1991, Jeffrey 1997, Descy et al. 2000, Teubner et al. 2003).

Quantification of pigments is a necessary first step in determining the contribution of individual taxonomic groups, with most attempts concentrating mainly on using multiple linear regression analysis between marker pigments and chl *a* (Gieskes et al. 1988, Woitke et al. 1996, Descy et al. 2000). These studies have shown that the individual contribution to total chl *a* by a given algal class can be established, if a unique marker pigment is present. This determination is not possible for algal classes where a pigment is shared (e.g., diatoms and chrysophytes sharing fucoxanthin). Furthermore, many approaches assume that marker pigment to chl *a* ratios are constant within a taxonomic group, independent of the species composition or physiological condition, which is not always the case. Changing environmental conditions, such as light and nutrients, have an important effect on marker pigment to chl *a* ratios (Descy et al. 2000, Schlüter et al. 2000), and, therefore, multiple regression analyses using constant ratios provide less accurate estimations of algal biomass (Woitke et al. 1996).

The application of the CHEMTAX procedure for calculating algal class abundances overcomes these limitations by taking into account variations in the marker pigment to chl *a* ratios (Mackey et al. 1996, 1998). The CHEMTAX program uses a factor analysis and steepest descent algorithm to find the best fit to the data based on an initial pigment ratio matrix for the classes to be determined. In addition, several in vivo methods, mainly based on fluorescence properties (prompt or delayed fluorescence, DF),

[1]Received 9 June 2006. Accepted 22 May 2007.
[2]Author for correspondence: e-mail sonja.greisberger2@sbg.ac.at.
[3]Author for correspondence: e-mail Katrin.Teubner@univie.ac.at.

have been developed recently for assessing biomass (Friedrich et al. 1998, Gerhardt and Bodemer 2000, Bodemer 2004, Istvánovics et al. 2005). The measurement of DF has the advantage of being rapid and nondestructive and offers the possibility of continuous monitoring due to the immediate reading of the results. DF is emitted between 670 and 750 nm from live, dark-adapted algal cells after excitation by monochromatic light. DF excitation spectroscopy can be used to determine chl *a* concentrations and phytoplankton composition, because algal groups with differing pigment composition have different excitation spectra over wavelengths from 400 to 730 nm. It is a measure of photosynthetic activity that takes into account the photosynthetic pigments, occurring only in photosynthetically active material. A summary of the main features of the two techniques used to infer algal class abundances is shown in Table 1.

The major objectives of the field study were to answer the following questions: (i) How effective are the pigment-based techniques DF and CHEMTAX in quantifying the abundances of different algal groups in a mixed community? (ii) How are changes in pigment composition of phytoplankton assemblages related to environmental parameters such as light climate and temperature?

MATERIALS AND METHODS

The results are based on two data sets from Mondsee in Austria (47°48′ N, 13°24′ E; $n = 158$). The seasonal data set was obtained by means of biweekly to monthly depth-integrated sampling (0–20 m) from February to December 2000. The second data set is a diel study of depth distribution of phytoplankton at 3 h sampling intervals over 2 d at the stable thermal stratification period in mid-July 2002 (22.7.: 4, 7, 10, 13, 16, 19, 21 h; 23.7.: 1, 4, 7, 10, 13, 16, 19 h).

Profiles of photosynthetically available radiation (PAR) were measured with a 4π quantum sensor (Li-Cor Biosciences, Lincoln, NE, USA), and temperature was measured with a multiparameter profiler (YSI 6920). Seasonal underwater light measurements were integrated over the depth of the epilimnion and over the 1 m layer of the respective sampling depth for profiles of the diel cycle.

Chl *a* was measured spectrophotometrically after extraction with hot ethanol following ISO 10260 (1992) and additionally by HPLC and DF. Lipophilic pigments were analyzed by HPLC in accordance with Wright et al. (1991). After filtration of 2 L of lake water (Whatman GF/F, Maidstone, UK), filters were frozen, and pigments extracted in 90% acetone. Pigments were identified by their retention time and spectra. Calibration was achieved using a mixed pigment standard, which contained carotenoids in quantitative proportion to chl *a*, as measured in algal cultures (Wilhelm et al. 1991). In addition, the biovolume of phytoplankton was estimated from counted abundance and size measurements by light microscopy (Zeiss LSM510 inverted microscope, Zeiss, Jena, Germany) using the sedimentation technique (Rott 1981).

Depth samples for DF spectra were measured in the laboratory by five replicates at 22°C after 30 min dark acclimation. The DF excitation spectra were analyzed using several deconvolution programs described in Bodemer (2002). These programs were based on calibration spectra of cultures shown in Figure 6A, which were then used to determine the concentrations of the algal classes, using cross-correlation between measured and calculated spectra until the cross-correlation reached a maximum (Gerhardt and Bodemer 2000).

Homogeneous data sets were required for the CHEMTAX procedure (Mackey et al. 1996), so we selected three subsets of similar species composition based on microscopic analysis. The seasonal cycle includes the depth-integrated samples from the annual data set ($n = 18$). The other two data sets from diel-cycle measurements during summer stratification were from the euphotic epilimnion, including the top 9.5 m (depths at 0, 0.5, 1, 2, 3.5, 5.5, 7.5, and 9.5 m; 14 time intervals; $n = 112$) and from the dim-light (meta-) hypolimnion, including deeper water layers below the euphotic zone at 12.5 and 14.5 m ($n = 28$). The mixing depth, defined by maximum values of the relative thermal resistance versus mixing, was 10.1 m; the euphotic depth, defined by 1% light intensity, was 12.1 m. To aid comparison of HPLC and DF methods, results for DF were also displayed separately for the three subsets of data.

The CHEMTAX procedure (Mackey et al. 1996) was based on three initial pigment ratio matrices (S, E, and H in Table 2), each constructed from phytoplankton samples (Descy et al. 2000). Therefore, instead of algal cultures, 6–12 phytoplankton samples with significant biovolumes of cyanobacteria; diatoms; dinoflagellates; and crypto-, chryso-, and chlorophytes were selected to calculate the initial ratio for the respective phytoplankton group for each of the three subsets of data. In the case of the last four taxonomic groups, which rarely reached >20%–30% of total biovolume in Mondsee, a few integrated, seasonal epilimnetic and hypolimnetic samples were collected from sporadic measurements in Mondsee in 2001 and considered in addition to the data set presented here. Consequently, only ~45% of the 158 plankton samples were involved in creating the initial ratio matrix. The initial pigment ratios are based on multiple regression analysis (using SPSS; SPSS Inc., Chicago, IL,

TABLE 1. Main features of the two methods used for phytoplankton assessment, delayed fluorescence (DF) and HPLC.

	DF	HPLC
Method	In vivo (no sample preparation needed) No size fractionation possible	Extraction method, adjustment to algal cell density by sample volume filtered Size fractionation possible
Measured pigments	Quantification of pigments by physico-physiological properties: photosynthetically active pigments (i.e., pigments contributing to charge separation at PSII, e.g., chlorophylls, fucoxanthin, peridinin, phycobiliproteins)	Separation of pigments due to physicochemical properties (solubility in certain solvents): lipophilic pigments were analyzed in this study (chlorophylls and all carotenoids)
Calculation of algal class abundances	Calibration spectra used	CHEMTAX program, considering multiple linear regressions among various marker pigments

TABLE 2. CHEMTAX processing. Initial pigment ratios for cyanobacteria (Cyano), diatoms (Bacill), dinoflagellates (Dino), cryptophytes (Crypto), chrysophytes (Chryso), and chlorophytes (Chloro) of three homogenous subsets of data: seasonal cycle (S), diel cycle epilimnion (E), and hypolimnion (H).

	Echi	Myx	Osci	Diad	Mon	Zeax	Fuco	Peri	Allo	Viol	Lute	Chlb
Cyano												
S	0.075	0.787	0.161	–	–	0.405	–	–	–	–	–	–
E	0.038	0.390	0.066	–	–	0.303	–	–	–	–	–	–
H	0.048	–	–	–	–	0.112	–	–	–	–	–	–
Bacill												
S	–	–	–	0.094	–	–	0.455	–	–	–	–	–
E	–	–	–	0.094	–	–	0.227	–	–	–	–	–
H	–	–	–	0.053	–	–	0.227	–	–	–	–	–
Dino												
S	–	–	–	0.063	–	–	–	0.210	–	–	–	–
E	–	–	–	0.036	–	–	–	0.115	–	–	–	–
H		–	–	0.019	–	–	–	0.115	–	–	–	–
Crypto												
S	–	–	–	–	0.143	–	–	–	0.532	–	–	–
E	–	–	–	–	0.143	–	–	–	0.532	–	–	–
H	–	–	–	–	0.035	–	–	–	0.532	–	–	–
Chryso												
S	–	–	–	–	–	–	0.275	–	–	0.174	–	–
E	–	–	–	0.021	–	–	0.182	–	–	0.174	–	–
H	–	–	–	–	–	–	0.182	–	–	0.174	–	–
Chloro												
S	–	–	–	–	–	–	–	–	–	0.055	0.654	0.472
E	–	–	–	–	–	–	–	–	–	0.043	0.654	0.472
H	–	–	–	–	–	–	–	–	–	0.043	0.654	0.472

Pigments abbreviated from left to right: echinenone, myxoxanthophyll, oscillaxanthin, diadinoxanthin, monadoxanthin, zeaxanthin, fucoxanthin, peridinin, alloxanthin, violaxanthin, lutein, chl *b*.

–, pigment not used as marker for a respective group.

USA), where chl *a* is used as the dependent variable, and the specific marker pigments are used as independent variables. For all calculations and results presented in this paper, pigment ratios were normalized to chl *a*. The pigments used for fitting the algal class abundances were echinenone, oscillaxanthin, myxoxanthophyll, and zeaxanthin for the cyanobacteria; diadinoxanthin and peridinin for the dinoflagellates; fucoxanthin and diadinoxanthin for the diatoms; monadoxanthin and alloxanthin for the cryptophytes; diadinoxanthin, fucoxanthin, and violaxanthin for the chrysophytes; and violaxanthin, lutein, and chl *b* for the chlorophytes. The final pigment ratio matrix created by CHEMTAX varied less from the initial matrix than allowed from setting the ratio limit to 500 (the variation from 6× ratio to ratio/6 was allowed). The final ratios deviated from the original ratios by only a factor of 1.1 for diatoms to 2.7 for chlorophytes (final matrix not shown).

The results of both CHEMTAX and DF estimates are given as chl *a* equivalents. To allow direct comparison of the microscopically derived biovolume with these results, biovolume was converted to chl *a* equivalents, assuming that a given equivalent of the biomass contributes to chl *a*. As this percentages varies in the different algal classes, the following conversion factors were used: cyanobacteria, 0.44; diatoms, 0.53; dinoflagellates, 0.99; cryptophytes, 1; chrysophytes, 0.54; and 1.2 for converting the chlorophyte biomass to chl *a* equivalents (Donabaum 1992). We define chl *a* equivalents of phytoplankton related to the biovolume as Chl*a*Equivalent$_{biov}$, in relation to DF as Chl*a*Equivalent$_{DF}$, and in reference to HPLC pigment-based CHEMTAX estimates as Chl*a*Equivalent$_{HPLCCH}$.

To test the success of the three techniques for assessing algal class abundances (biovolume, DF, and HPLC-CHEMTAX), parametric tests were used (Pearson product-moment correlation). The comparison of biovolume equivalents was displayed in notched box-whisker plots using SYSTAT 10 (SPSS Inc.). Boxes were notched at the median; the length of the notches indicated 95% confidence intervals. The persistence in the pattern of photosynthetically active pigments was measured as the Bray–Curtis similarity index between each pair of successive time and depth samples using PRIMER 5 (PRIMER-E Ltd., Plymouth, UK). The similarity index was based on continuous data of DF excitation spectra over the wavelength range from 400 to 730 nm normalized to the chl *a* peak at 672 nm (chl a_{DF672}). The variability of photosynthetically active pigments within season and depth gradients was estimated by the coefficient of variation (CV), equal to SD/mean.

RESULTS

The seasonal and vertical variations of chl *a* concentrations are shown in Figure 1 (spectrophotometrically measured ethanol extraction). The spring maximum of chl *a* reached 8 $\mu g \cdot L^{-1}$ during a period mainly dominated by diatoms (Fig. 1A). The profile of the water column shows a hypolimnetic chl *a* maximum mainly due to *Planktothrix rubescens* (D. C. ex Gomont) Anagn. et Komárek, which occurred during summer below or at the euphotic depth of 12.1 m (Fig. 1B). The median chl *a* concentrations in the hypolimnetic layer were $\sim$6.5 $\mu g \cdot L^{-1}$, while those measured in the summer epilimnion and for the seasonal cycle were much lower, ranging between 1.8 and 3 $\mu g \cdot L^{-1}$. In addition to spectrophotometrically analyzed ethanol extraction, chl *a* was measured using HPLC and DF. In contrast to the HPLC and ethanol technique, the absolute chl *a* concentrations measured by DF were

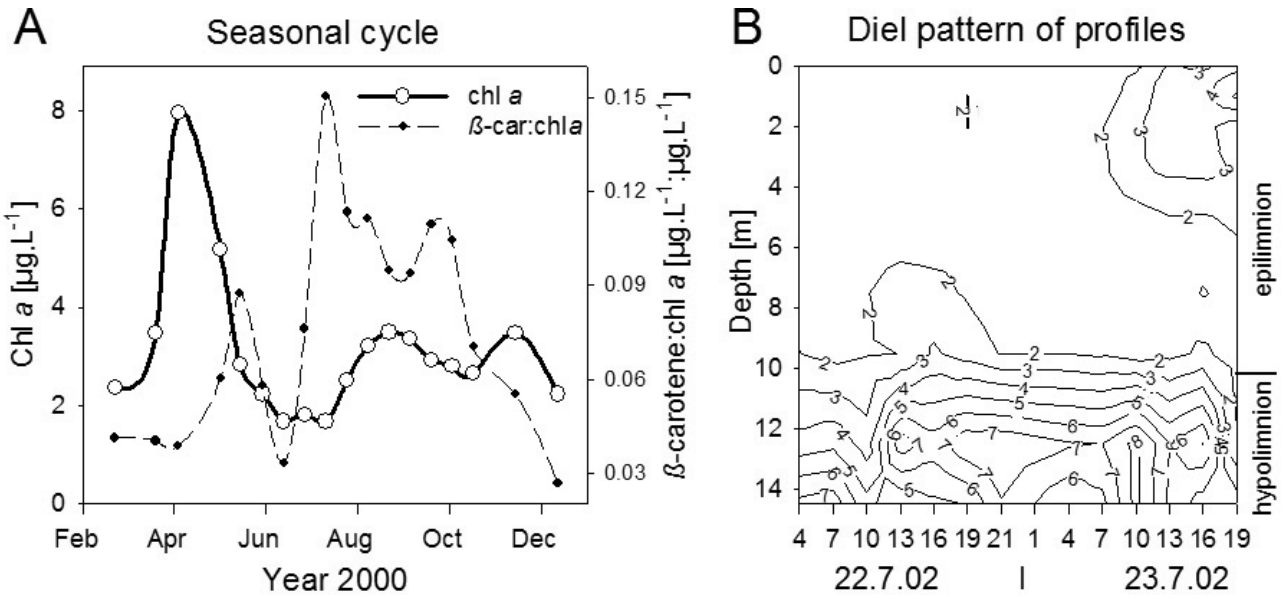

FIG. 1. Chl *a* spectrophotometrically measured by ethanol extraction (ISO 10260 1992) for seasons (A, February–December 2000) and diel depth cycle (B, stratified conditions in mid-July 2002) in Mondsee. The ratio of β-carotene:chl *a* (β-car:chl *a*, HPLC) is shown for seasons only (see text).

significantly lower, especially in the hypolimnion (data not shown). This finding was also evident from the percentage of DF chl *a* to ethanol-extracted chl *a*. The DF percentage varied between 40% and 70%, with the lowest values in the hypolimnion, whereas the percentage obtained by HPLC remained fairly constant at 70% to 80% over the three data subsets.

HPLC/CHEMTAX analysis. The CHEMTAX processing of the three subsets of data provided a detailed description of the phytoplankton composition in Mondsee. Chl*a*Equivalent$_{biov}$ values of all major plankton groups were statistically in the same range as Chl*a*Equivalent$_{HPLCCH}$ values, as shown by the overlapping confidence intervals in the box-whisker plots in Figure 2. The best agreement between the two techniques was observed when single algal groups dominated, as when cyanobacteria contributed up to 86% to biovolume in the hypolimnion (Fig. 3H), and diatoms up to 85% biovolume in the seasonal-cycle data set (seasonal cycle: $r = 0.93$; epilimnetic depth layer: $r = 0.82$; $P < 0.001$). This trend was also the case for subdominant cryptophytes in the seasonal and hypolimnetic depth-layer data set (Fig. 4, S and H) when they made up 33% of total biovolume. Even most CHEMTAX estimates, and marker pigments used for calculation of the remaining algal groups, were in reasonable agreement with the Chl*a*Equivalent$_{biov}$ (e.g., for epilimnetic dinoflagellates, $r = 0.63$, $P < 0.001$; peridinin: $r = 0.62$, $P < 0.001$; diadinoxanthin: $r = 0.57$, $P < 0.001$; chrysophytes (seasonal cycle): $r = 0.59$, $P < 0.05$; violaxanthin: $r = 0.75$, $P < 0.001$).

Correlations between single marker pigments and the Chl*a*Equivalent$_{biov}$ of cyanobacteria and cryptophytes were mostly significant as well (e.g., Figs. 3 and 4; the correlation between the cyanobacterium *P. rubescens* and oscillaxanthin was $r = 0.73$, $P < 0.001$). It is worth mentioning, however, that stronger correlations to Chl*a*Equivalent$_{biov}$ were determined with CHEMTAX analysis, because it included a combination of several marker pigments rather than single marker pigment inference (see Figs. 3 and 4; further, the correlations for seasonal diatoms inferred by single pigments were $r = 0.88$ for diadinoxanthin and $r = 0.89$ for fucoxanthin, whereas the combination of both pigments used for CHEMTAX was $r = 0.93$, $P < 0.001$). Hence, the application of a combination of marker pigments via CHEMTAX improved the discrimination between algal groups even if some groups shared pigments (chrysophytes and diatoms shared fucoxanthin).

Delayed fluorescence. DF of eukaryotic groups, such as diatoms (seasonal cycle: $r = 0.91$; epilimnetic depth layer: $r = 0.80$) and cryptophytes (hypolimnetic depth layer, Fig. 4H), showed the best agreement between Chl*a*Equivalent$_{DF}$ and Chl*a*Equivalent$_{biov}$. In the case of hypolimnetic cyanobacteria, estimates of chl *a* equivalents by both CHEMTAX and microscopy were $\sim 5\ \mu g \cdot L^{-1}$, whereas DF results were lower at only $3\ \mu g \cdot L^{-1}$ (Fig. 2). Nevertheless, in the hypolimnetic layers, a strong positive correlation between Chl*a*Equivalent$_{DF}$ and Chl*a*Equivalent$_{biov}$ was obtained (Fig. 3H). With the exception of chlorophytes, Chl*a*Equivalent$_{DF}$ values of all other groups were significantly lower in the hypolimnion

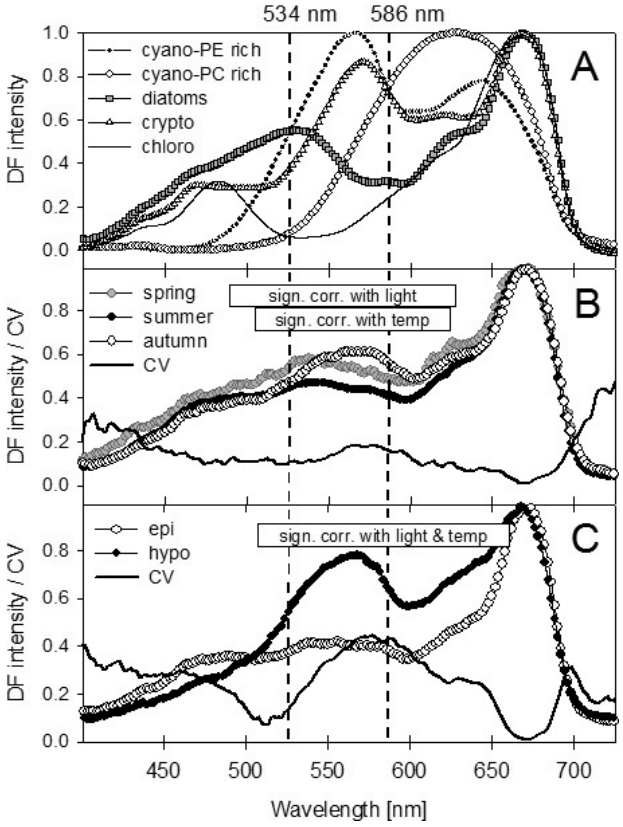

FIG. 6. Normalized delayed fluorescence (DF) excitation spectra of cultures (A) and phytoplankton of seasons (B) and depths (C) over the wavelength range from 400 to 730 nm. (A) Spectra from phycoerythrin-rich and phycocyanin-rich cyanobacteria (cyano-PE rich and cyano-PC rich, respectively), diatoms, cryptophytes (crypto), and chlorophytes (chloro). Chosen wavelengths (dashed lines) are associated with pigments of particular taxa described in the text. (B) Seasonal cycle: mean values of DF excitation spectra are shown for three seasons due to the persistent pattern of photosynthetically active pigments shown in Figure 5; coefficient of variation (CV) illustrates the intra-annual variability of DF excitation spectra. (C) As in (B), but for epi- and hypolimnetic layers from depth gradients; CV illustrates the variation of the spectra among depths. Horizontal bars in (B) and (C) indicate the wavelength range of significant inverse correlations ($P < 0.05$) between normalized DF intensity and light and temperature, respectively ([B] for light, 480–623 nm, and temperature, 521–640 nm; [C] for both light and temperature, 508–665 nm). Correlations within this range for selected wavelengths at 534 and 586 nm are shown in Figure 7. Normalization of spectra as in Figure 5.

caution. The use of chl *a* to quantify the phytoplankton community becomes even more complicated if the phytoplankton is dominated by cyanobacteria. This was evident in the deep, hypolimnetic layer of Mondsee dominated by *P. rubescens* (86% to total biovolume), by the particularly low chl *a* content measured by DF spectroscopy. Cyanobacteria generally exhibit a lower cellular chl *a* content than other algal groups (Donabaum 1992, Feuillade and Davies 1994). Furthermore, the content of active chl *a* decreases under low-light conditions relative to the light-harvesting phycobiliproteins as shown, for example, by Rücker et al. (1995), details discussed below).

Assessment of phytoplankton composition by marker pigments. The application of the CHEMTAX procedure on our phytoplankton data sets yielded satisfactory results for all major algal groups studied. An appropriate pigment ratio matrix was obtained by multiple regression analysis of the three subsets of data and, therefore, included variations due to the dominance of algal species as well as acclimation to the environment. The analyzed marker pigment to chl *a* ratios were in the range of values commonly observed for both pelagic algae in situ and cultures (Wilhelm et al. 1991, Woitke et al. 1996, Descy et al. 2000, Schlüter et al. 2000, 2006, Schagerl and Donabaum 2003, Fietz and Nicklisch 2004). Moreover, CHEMTAX distinguished between fucoxanthin-sharing diatoms and chrysophytes, due to the inclusion of violaxanthin into the initial pigment ratio matrix. Violaxanthin occurs in some freshwater chrysophytes, but it reached only low concentrations due to low cell abundances in Mondsee. Investigation of individual marker pigments also made it possible to quantify specific taxonomic groups—for example, the close relationship between *P. rubescens* and oscillaxanthin in Mondsee, similar to findings reported from the deep chl maximum in prealpine Ammersee (Bavaria, Germany; Teubner et al. 2003). Reasonable success in quantifying algal class abundances was also obtained with DF, especially when a single phytoplankton group was dominant, even when the chl *a* equivalents derived by DF were generally lower than those obtained by HPLC-CHEMTAX and microscopy, as discussed above (with exception for the chlorophytes discussed below).

A drawback of the DF spectroscopy was that this in vivo method allowed no adjustment of the sample volume and so gave noisy DF spectra if chl *a* concentrations were close to the detection limit at low phytoplankton abundances. In contrast, all techniques based on prior extraction of the pigments, as well as microscopic methods, overcome this limitation by analyzing an appropriate sample volume, depending on the prevailing algal density. DF particularly complicated the evaluation of subdominant algal groups—such as diatoms in the hypolimnetic layer dominated by cyanobacteria—when their spectral response was masked by the presence of more abundant algae. In the case of chrysophytes, the main reason for their underestimation lay in the similarity of their pigment composition to diatoms. Although Bodemer (2002) produced evidence that DF intensity of chrysophytes is apparently higher at wavelengths where chl *c* absorbs light (at 460–470 nm) and lower where fucoxanthin absorbs light (at 520–540 nm), the spectra are highly variable, and so separation from diatoms becomes more difficult at low abundances. The high pigment variability of chrysophytes can be attributed to their facultative photoautotrophy under natural conditions