Fundam. Appl. Limnol.
published online January 2023

EarlyAcces-Article

Pigment and fluorescence proxies to estimate functional diversity of phytoplankton communities

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With 8 figures and 2 tables

Abstract: Given the global loss of biodiversity, trait-based studies are needed to assess the associated consequences for ecosystem functions and services. Many studies focus on the assessment of functional diversity of natural communities as a mechanistic link between biodiversity and ecosystem functioning. In freshwater ecosystems, diversity of primary producers is crucial for resource use efficiency and trophic transfer of energy. Furthermore, one indicator of the ecological status of surface waters is the composition of natural phytoplankton communities. The number of available techniques for the quantification and discrimination of different phytoplankton groups have increased in recent years. For example, phytoplankton community composition can indirectly be assessed via CHEMTAX, a matrix factorization program, which calculates the contribution of different phytoplankton taxa to the total chlorophyll-*a* using concentrations of pigments analysed via liquid chromatography. A more direct, *in vivo* assessment can be achieved with instruments based on spectral fluorometry, such as the Algae Lab Analyser, which allows for a differentiation of four phytoplankton groups depending on spectral fluorescence signatures. In this study, we compared both methods by analyses of phytoplankton biomass and functional diversity from phytoplankton communities of three lakes of different trophic states, while a subset of biomass and diversity estimates derived from microscopic counts served as a reference. We found marked differences in biomass estimates of all assessed phytoplankton groups, with cyanobacteria being significantly underestimated by the Algae Lab Analyser. Furthermore, we show that the level of agreement between the methods somewhat depends on the trophic state of the lake. We conclude that both methods are suitable to estimate phytoplankton functional diversity with specific advantages and disadvantages. Here we provide users with a flow chart to help them find the most suitable method for their respective purposes.

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DOI: 10.1127/fal/2023/1466

www.schweizerbart.de
1863-9135/23/1466 $ 5.25

20230414-122309
A2239/32525/C186CAF5

Keywords: algae; biodiversity; cyanobacteria; functional traits; phytoplankton composition; pigments; spectral fluorescence; Algae Lab Analyser; HPLC; CHEMTAX; cyanobacteria; water quality monitoring

Introduction

The global loss of freshwater biodiversity, which is mainly driven by human-induced climate change and eutrophication, is predicted to affect the stability of freshwater ecosystems and challenge ecosystem functioning and services (Dudgeon 2010; Janse et al. 2015). Phytoplankton communities, which are primary producers at the base of pelagic food webs, show fast generation times and thereby respond rapidly to environmental changes (Winder & Sommer 2012; Pomati et al. 2013). Phytoplankton pigment composition plays a crucial role for the ecology and competitive interactions in phytoplankton via traits linked to light use efficiency and light use niches (Litchman & Klausmeier 2008; Striebel et al. 2009; Behl et al. 2011; Lewandowska et al. 2015). On the other hand, phytoplankton groups differ in food quality for higher trophic levels with far reaching trophic consequences (Ahlgren et al. 1990; Marzetz et al. 2017; Trommer et al. 2019; Titocci & Fink 2022). Well known examples are cyanobacteria that can even reduce the transfer efficiency of energy from primary production to herbivores (Von Elert et al. 2003; Martin-Creuzburg et al. 2008). Therefore, light use and food quality help identify functionally distinct groups in phytoplankton communities.

However, with increasing interest in the role of biodiversity *per se*, alpha diversity measures such as taxonomic richness and evenness also have received closer scrutiny (Hillebrand et al. 2008; Filstrup et al. 2014). Natural as well as anthropogenic drivers have been identified to shift dominance among phytoplankton groups (Sommer et al. 1986; Sommer et al. 2012). For example, the PEG model (Sommer et al. 1986) identifies abiotic drivers responsible for seasonal shifts in phytoplankton, depending also on lake trophic state. The assessment of phytoplankton community shifts with corresponding alterations in functional trait diversity and consequences for ecosystem functioning have become key challenges of modern aquatic ecology (Kremer et al. 2017; Martini et al. 2021).

In parallel, to ensure the sustainable use of aquatic ecosystems, European Community legislation has introduced the EU Water Framework Directive (WFD, Directive 2000/60/EC). The WFD defines the composition of the phytoplankton community as one of the most important biological parameters that determines the quality and ecological status of surface water bodies (Sarmento & Descy 2008; Izydorczyk et al. 2009; Catherine et al. 2012; Escoffier et al. 2015). Hence, both for basic and applied research aspects, the frequent monitoring of the taxonomic and trait diversity of the natural phytoplankton communities is essential.

Frequent monitoring of natural phytoplankton communities leads to a high number of samples that have to be processed, which results in very high labour costs when traditional methods such as microscopic counting are used. Microscopy is extremely time consuming, and the result largely depends on the taxonomic knowledge of the respective person. With the reduction in taxonomic instruction at many universities, the lack of taxonomic expertise increases, which further highlights the need for alternative methods (Drew 2011). Also, pico-phytoplankton ($< 2\,\mu m$) cannot be differentiated accurately via the traditional Utermöhl (1958) microscopic counting techniques (Booth 1993). These challenges, however, can be partly overcome by assessing phytoplankton community composition via epifluorescence (Callieri & Stockner 2002; Crosbie et al. 2003; Salmi et al. 2021) or DNA metabarcoding (Groendahl et al. 2017; MacKeigan et al. 2022). Advantages and disadvantages of these methods are further described in the Supplementary Material.

To overcome challenges associated with these aforementioned approaches, chemotaxonomic alternatives have been proposed, such as *in vivo* pigment-based spectrofluorometry and *in vitro* high pressure liquid chromatography (HPLC) of pigments, in combination with the matrix factorization programme CHEMTAX (Mackey et al. 1996). Both approaches are based on the differences in pigment composition of the main phytoplankton groups. For example, dinoflagellates contain the pigment peridinin, which is specific for dinoflagellates (Prézelin & Haxo 1976; Norris & Miller 1994; Schulte et al. 2010), while alloxanthin and lutein are group-specific pigments for cryptophytes and chlorophytes, respectively (Gieskes & Kraay 1983; Jeffrey et al. 2011). The CHEMTAX approach uses input ratio matrices containing ratios of group-specific pigments to chlorophyll-*a*, which can be found in the literature. For example, such ratio matrices were developed using data from 46 German lakes by Schlüter et al. (2016). We further introduce and discuss both approaches in Supplementary Mate-

rial. The advantage of both chemotaxonomic methods is that they are less time consuming than conventional methods. However, they only allow for a relatively low taxonomic resolution (class level), compared to microscopy and DNA metabarcoding, which both usually allow for genus or even species level, given there is enough morphological and genetic differences, as well as a comprehensive DNA barcode library.

Some previous studies compared the suitability of spectrofluorometry and HPLC/CHEMTAX for the assessment of the phytoplankton community composition, e.g., usage of the bbe Moldaenke FluoroProbe (See et al. 2005) and the bbe Moldaenke Algae Online Analyser (Richardson et al. 2010) compared to CHEMTAX-derived community composition data (for more details on findings from these studies, please see Supplementary Material).

In this study, we for the first time compare phytoplankton community composition assessed via Algae Lab Analyzer and HPLC/CHEMTAX in lakes across a gradient of trophic status. This is important, as trophic status not only can lead to strong differences in phytoplankton composition and dynamics (Sommer et al. 1986), but also change important traits of biodiversity *per se*. For example, the dominance of cyanobacteria generally increases with trophic state, resulting in biodiversity loss and decreased species evenness within phytoplankton communities (Watson et al. 1997; Kosten et al. 2012; Paerl & Paul 2012; Rigosi et al. 2014). Low nutrient supply accompanied by low growth rates often result in more even and diverse communities, whereas high nutrient supply often results in the dominance of a few fast growing taxa or groups (Huston & DeAngelis 1994; Huston 2014) or inedible cyanobacteria, which may become the dominant group in the community as a consequence of selected grazing by the zooplankton on eukaryotic algae (Leitão et al. 2018; Ger et al. 2019). Hence, the accuracy of phytoplankton diversity estimations by proxies should be robust and not be affected by the trophic status of lakes or an interaction between trophic status and major algal groups. Otherwise, such bias could result in misinterpretations of observed, but methodologically-generated, patterns as trophic effects on biodiversity – ecosystem functioning relationships.

We addressed the following hypotheses: i) both methods allow for a rapid assessment of phytoplankton community composition, albeit at lower taxonomic resolution compared to microscopic counting; ii) the congruence of the methods does not depend on the trophic state of the lakes and does not differ between different phytoplankton groups; iii) pigment diversity derived from HPLC can be used as a proxy for phytoplankton diversity.

Methods

Field experiment and sampling

During summer 2014, we sampled pelagic mesocosms deployed simultaneously in three lakes of different trophic state situated in Upper Bavaria (Germany): Brunnsee (47° 59′ 01″ N, 12° 26′ 12″ E, area: 5.8 ha, maximum depth: 20 m), Klostersee (47° 58′ 26″ N, 12° 27′ 10″ E, area: 47.0 ha, maximum depth: 16 m) and Thaler See (47° 54′ 16″ N, 12° 20′ 17″ E, area: 3.8 ha, maximum depth: 7 m). Depending on the average epilimnetic total phosphorus concentration (TP, mean ± standard deviation) determined in summer 2014, Brunnsee can be classified as an oligotrophic lake (TP = 5.62 ± 1.09 µg L^{-1}), Klostersee as an oligo-mesotrophic lake (TP = 9.88 ± 2.47 µg L^{-1}) and Thaler See as a mesotrophic lake (16.80 ± 5.18 µg L^{-1}), based on classifications given in Nürnberg (1996).

The mesocosms were made of transparent plastic foil, forming cylindrical enclosures closed at the bottom and open at the top to allow exchange with the atmosphere. They had a diameter of 0.95 m and a length of 5 m (Thaler See) and 6 m (Brunnsee and Klostersee), resulting in a total volume of approx. 3.5–4.2 m^3, respectively. Twenty mesocosms were installed (per lake) and filled with surrounding water from the respective lake, which was pre-filtered over a 250 µm gaze, to exclude mesozooplankton and thus prevent major grazing effects. The mesocosms were part of another study, but we made use of their availability to obtain samples of differing phytoplankton communities for the present investigation. We took weekly water samples from the mesocosms and from the lake itself, using a 2 L integrated water sampler (KC Denmark), from depths ranging from 0.5–2.5 m. The water samples were transported to the laboratory and were kept cool and dark until further analyses (within a few hours). In total, we analysed 562 samples (186 from Brunnsee, 187 from Klostersee and 189 from Thaler See).

Assessment of the phytoplankton community composition via microscopic counts

For microscopic identification of phytoplankton, we followed the Utermöhl technique (Utermöhl 1958). For this purpose, 100 mL subsamples from each me-

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socosm were fixed with Lugol's solution. Based on phytoplankton biomass (data from Algae Lab Analyser) corresponding amounts of fixed sample were filled in plankton sedimentation chambers (Hydrobios Kiel, Kiel, Germany) to ensure sufficient density of phytoplankton. Phytoplankton composition was then analysed by using an inverted microscope (Wild M40, Heerbrugg, Switzerland), at a magnification of 400×. Phytoplankton were identified to species level, if possible, otherwise to genus level. The samples were analysed in transects and at least 100 individuals of each species/genus were counted per sample (Lund et al. 1958). This procedure was valid for most of the taxa in the samples. To ensure rare (fewer than 100 individuals) and larger species, mainly dinoflagellates, were determined, the bottom of the whole sedimentation chamber was screened at a magnification of 200×. For the final phytoplankton biovolume, counts were calculated by species/genus-cell-specific biovolume. Specific biovolume data used were from (Kremer et al. 2014). We analysed all 63 samples from the first sampling event (8^{th}–10^{th} July 2014; $n = 21$ per lake, including samples from all 20 enclosures and the sample from the lake itself).

In vivo fluorometric analysis (Algae Lab Analyser)

The fluorometric measurement of the chlorophyll-*a* concentration was done using the Algae Lab Analyser (bbe Moldaenke, Germany). The Algae Lab Analyser contains five different light-emitting diodes (LEDs) with λ 450 nm, 525 nm, 570 nm, 590 nm, and 610 nm, respectively. Based on the group-specific excitation spectra, also called norm spectra, the Algae Lab Analyser allows for differentiation of four spectral groups: the green group (chlorophytes), the blue-green group (cyanobacteria), the brown group (chromophytes, which includes diatoms, chrysophytes and dinoflagellates) and mixed-group (cryptophytes and phycoerythrin-containing algae (Beutler et al. 2002). Calculation of the contribution of each phytoplankton group to the total chlorophyll-*a* is based on linear unmixing (i.e., solving linear equations). See the supplemental materials for further details.

In vitro chromatographic analysis (HPLC)

For the HPLC analyses, up to 1000 ml of the water samples from the lakes were filtered onto precombusted glass fibre filters (VWR GF/F, Ø 25 mm). The filters were wrapped in aluminium foil and stored at –20 °C until analysis. See the supplemental materials for further details.

Based on our previous knowledge on phytoplankton groups that are usually present in the examined lakes (data from long-term monitoring), we chose 10 pigment standards, of which 9 were obtained from DHI Water (Hoersholm, Denmark): alloxanthin (marker pigment for cryptophytes), β-carotene, chlorophyll-*a*, chlorophyll-*b* (marker pigment for chlorophytes), diatoxanthin, echinenone (marker pigment for cyanobacteria), fucoxanthin (marker pigment for diatoms and chrysophytes), lutein (another marker pigment for chlorophytes) and zeaxanthin [usually used as the only marker pigment for cyanobacteria (Havskum et al. 2004; Llewellyn 2004; Lewitus et al. 2005), but also shared with other groups like chlorophytes]. Peridinin (marker pigment for dinoflagellates, extracted from *Symbiodinium* spp. following the protocol from Rogers & Marcovich 2007) was kindly provided by D. Langenbach, University of Cologne. With the applied solvent gradient (given in the supplementary materials), we were able to separate all pigment peaks to the baseline except for lutein and zeaxanthin (Supplementary Material Fig. S1). Although well separated, diatoxanthin was excluded from the subsequent CHEMTAX analysis as it was detected in very low amounts and only in a few samples. Also, we excluded β-carotene as it did not have any effect on the output data (previous CHEMTAX runs, data not shown).

CHEMTAX

Pigment : chlorophyll-*a* ratio matrices established for oligo- and for meso- and eutrophic lakes (including our three study lakes) were taken from Schlüter et al. (2016). These ratio matrices should thus be highly suitable for our study and were used in the present study to calculate the contribution of six phytoplankton groups (diatoms, chlorophytes, chrysophytes, cryptophytes, cyanobacteria, and dinoflagellates) to the total chlorophyll-*a* via CHEMTAX (Mackey et al. 1996; version 1.95 provided by S. Wright). See the supplemental materials for further details.

Data analysis

Estimates of phytoplankton biomass, given as total chlorophyll-*a* (TChl-*a*, in μg L^{-1}) derived from Algae Lab Analyser or via HPLC, were compared between these two methods for each lake separately, by performing paired Wilcoxon-Mann-Whitney tests, to account for the fact that the data originated from the same samples (20 enclosures per lake and the lake itself). These tests were based on all available data from

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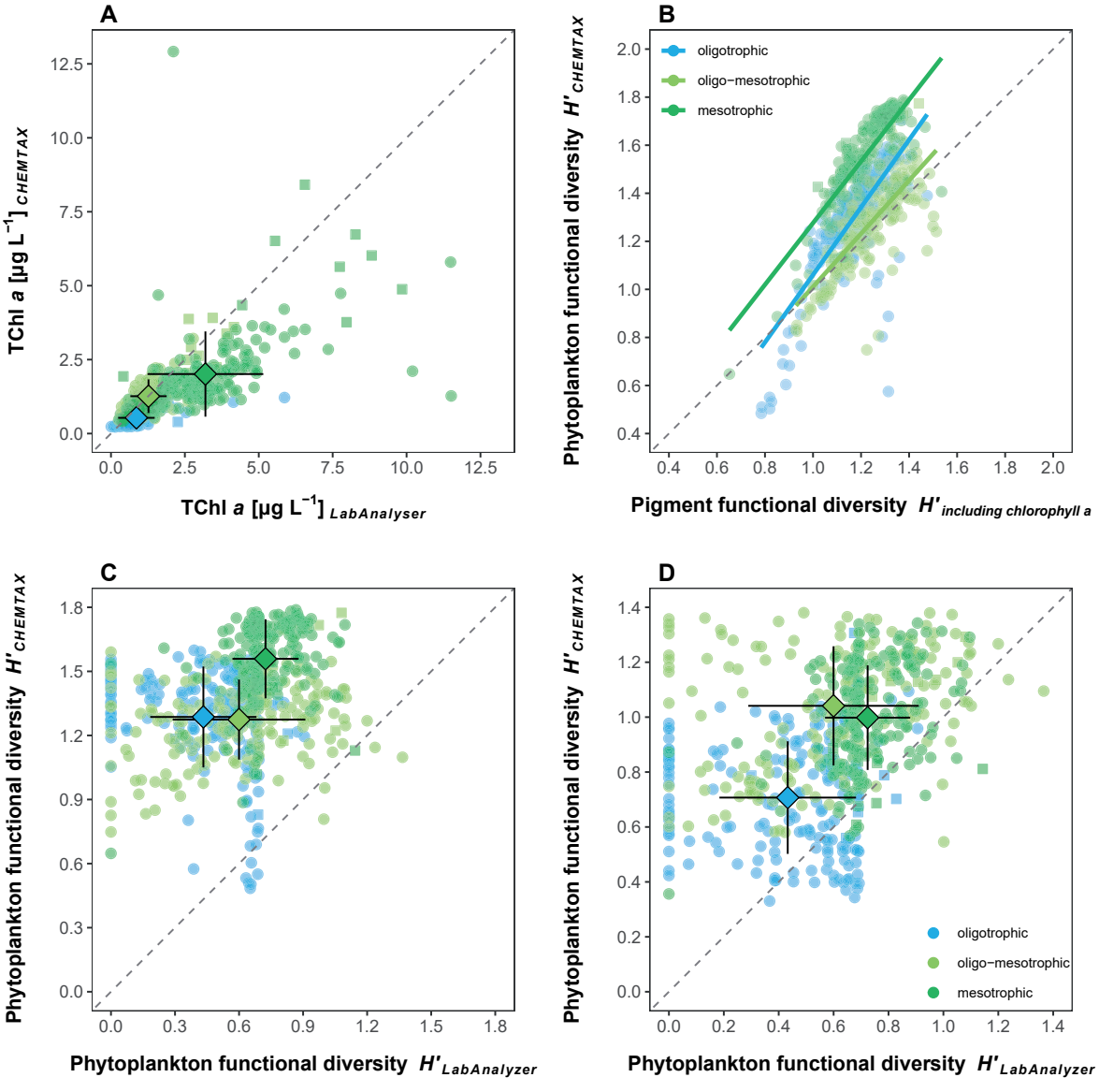

Fig. 1. **(A)** Total chlorophyll-*a* concentration (µg L^{-1}) and **(C, D)** phytoplankton functional diversity *H'* determined spectrofluorometrically *in vivo* with Algae Lab Analyser (y-axis) and chromatographically *in vitro* via HPLC and CHEMTAX (x-axis). **(B)** Pigment functional diversity $H'_{including\ chlorophyll\text{-}a}$ (x-axis) and phytoplankton functional diversity $H'_{CHEMTAX}$ (y-axis), determined via HPLC and CHEMTAX. Data from all 9 sampling events (July to September 2014) is shown. Coloured lines in (B) represent the linear regression for each lake. Colour of the symbols represents the trophic state of the lakes, blue: oligotrophic (lake Brunnsee, $n = 186$); light green: oligo-mesotrophic (lake Klostersee, $n = 187$); dark green: mesotrophic (lake Thaler See, $n = 189$); n in parentheses indicates the number of water samples per lake analysed within this study. Data originating from enclosures are depicted as circles, while data originating directly from the lakes are depicted as squares. Diamonds in panels (A, C, D) represent the mean values, while horizontal and vertical error bars represent the standard deviation (based on all data points per lake).

all 9 sampling events. Additionally, we calculated Spearman's correlation coefficient r_S between both phytoplankton biomass estimates for each lake separately, as well as across all three lakes.

Based on pigment concentrations and phytoplankton community composition, derived from microscopy, CHEMTAX and Algae Lab Analyser, we calculated Shannon Diversity Indices as estimates of pigment and phytoplankton functional diversity (Shannon & Weaver 1949). For this, we used the equation:

$$H' = -\sum_{i=1}^{n} p_i \times ln(p_i)$$

where p_i is the proportion of the pigment or phytoplankton class relative to the total amount of the pig-

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ments or the total biovolume or biomass of the phytoplankton community, respectively. Pigment functional diversity was calculated based on all 10 pigments (including chlorophyll-*a*, β-carotene and diatoxanthin). Phytoplankton functional diversity derived from microscopic counts and CHEMTAX was calculated based on biovolume or biomass of six phytoplankton classes: diatoms, chlorophytes (incl. euglenophytes), chrysophytes, cryptophytes, cyanobacteria, and dinoflagellates. In order to compare the performance of CHEMTAX and Algae Lab Analyser irrespectively of their taxonomic resolution, we additionally calculated the functional diversity derived from CHEMTAX based on four classes only, by treating diatoms, chrysophytes and dinoflagellates as chromophytes, to match the phytoplankton classes derived from Algae Lab Analyser.

As the data from microscopic counts were available only for the first sampling event, the statistical data analysis was split in two parts. In the first part, we compared the functional diversity derived from microscopy, CHEMTAX (based on all six classes) and Algae Lab Analyser both between the methods for each lake separately, as well as across all three lakes, considering one method at a time. With this approach, we determined if the differences in phytoplankton functional diversity between lakes of different trophic state could be equally revealed with any of the three methods. Similarly, to test the ability of CHEMTAX and Algae Lab Analyser to correctly estimate the contribution of the different phytoplankton classes to the total biomass, relative abundances of the phytoplankton classes based on the biomass estimates derived from CHEMTAX and Algae Lab Analyser were compared to the relative abundances of these classes based on the biovolume derived from the microscopic counts (treated as a reference).

The second part of the analysis was based on all available data from all 9 sampling events (July to September 2014, $n = 562$ analysed samples). We compared the functional diversity derived from CHEMTAX (based on six or four classes) and Algae Lab Analyser, both across all lakes for each method separately (to test for differences related to the trophic state of each lake) and across all methods, considering one lake at a time. Additionally, we compared the pigment functional diversity between all three lakes and calculated the Spearman's correlation coefficient r_S between pigment functional diversity and phytoplankton functional diversity based on CHEMTAX biomass estimates, separately for each lake, as well as across all three lakes.

Table 1. Biomass estimates (given as contribution to total chlorophyll-*a* in µg L^{-1}) of the four main phytoplankton groups as determined with Algae Lab Analyser or via HPLC/CHEMTAX. Given are mean, standard deviation, median, minimum, and maximum value for each phytoplankton group and each lake, based on samples from all 9 sampling events (July to September 2014; oligotrophic: $n = 186$; oligo-mesotrophic: $n = 187$; mesotrophic: $n = 189$). Total biomass (TChl-*a*) and associated summary statistics are given in bold.

Trophic state	Group	Biomass (µg TChl-*a* L^{-1})									
		Algae Lab Analyser					CHEMTAX				
		Mean	SD	Median	Min	Max	Mean	SD	Median	Min	Max
oligotrophic	Chlorophytes	0.21	0.50	0.08	0.00	5.18	0.07	0.13	0.03	0.01	0.96
	Chromophytes	0.60	0.27	0.59	0.00	1.74	0.37	0.18	0.34	0.08	1.33
	Cryptophytes	0.05	0.17	0.00	0.00	1.41	0.01	0.04	0.00	0.00	0.48
	Cyanobacteria	0.00	0.01	0.00	0.00	0.07	0.09	0.07	0.07	0.01	0.47
	TChl-*a*	**0.86**	**0.62**	**0.70**	**0.01**	**5.87**	**0.53**	**0.25**	**0.50**	**0.22**	**1.79**
oligo-mesotrophic	Chlorophytes	0.18	0.37	0.00	0.00	2.20	0.28	0.16	0.23	0.07	1.02
	Chromophytes	0.72	0.47	0.64	0.00	2.09	0.64	0.42	0.57	0.00	2.28
	Cryptophytes	0.35	0.33	0.26	0.00	1.78	0.14	0.21	0.04	0.00	1.51
	Cyanobacteria	0.02	0.06	0.00	0.00	0.43	0.20	0.14	0.15	0.03	0.83
	TChl-*a*	**1.27**	**0.61**	**1.20**	**0.26**	**4.16**	**1.26**	**0.57**	**1.15**	**0.42**	**3.91**
mesotrophic	Chlorophytes	0.70	1.05	0.41	0.00	8.05	0.24	0.30	0.18	0.04	2.97
	Chromophytes	1.89	1.15	1.78	0.00	5.51	1.32	1.16	1.00	0.18	10.58
	Cryptophytes	0.60	0.76	0.40	0.00	6.75	0.22	0.15	0.20	0.00	1.02
	Cyanobacteria	0.01	0.08	0.00	0.00	0.86	0.23	0.13	0.22	0.01	0.98
	TChl-*a*	**3.19**	**1.96**	**2.90**	**0.28**	**11.51**	**2.01**	**1.44**	**1.69**	**0.42**	**12.92**

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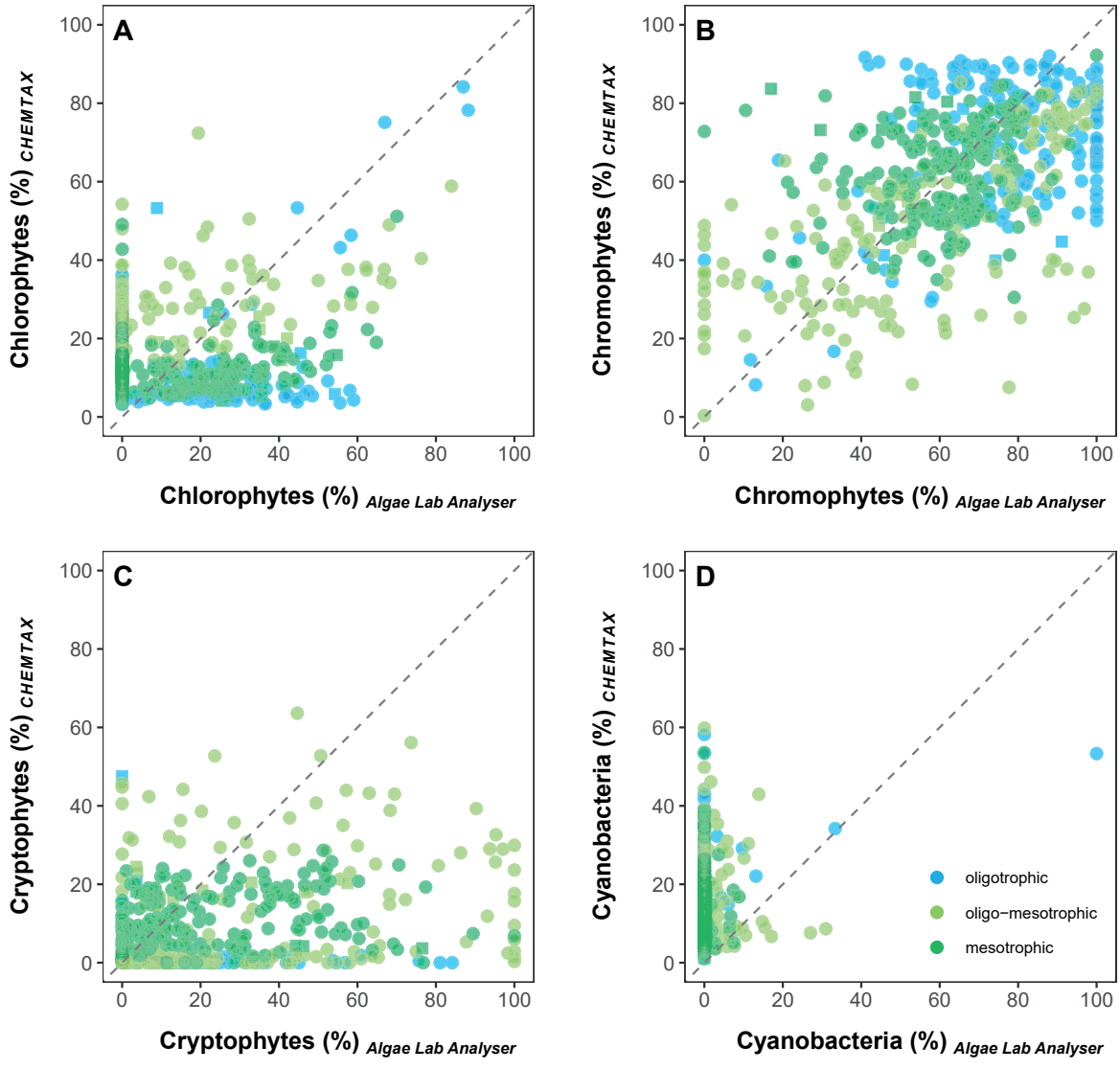

Fig. 2. Relative abundance (%) of **(A)** chlorophytes, **(B)** chromophytes, **(C)** cryptophytes and **(D)** cyanobacteria determined spectrofluorometrically *in vivo* with Algae Lab Analyser (x-axis) and chromatographically *in vitro* via HPLC and CHEMTAX (y-axis). Data from all 9 sampling events (July to September 2014) are shown. For legend, see Fig. 1.

Finally, the relative abundances of the four phytoplankton classes were compared for each lake and method separately (to reveal dominant or rare classes in each lake), as well as between the two methods considering only one class at a time, to test for potential identification mismatches between Algae Lab Analyser and CHEMTAX.

When estimates from only two methods were compared (e.g., relative abundance of Chlorophytes derived from CHEMTAX vs Algae Lab Analyser), we applied paired Wilcoxon-Mann-Whitney tests. In all other cases, when comparing estimates from all three methods or across all three lakes, we performed Kruskal-Wallis tests, followed by paired Wilcoxon-Mann-Whitney tests with Holm correction of the *p*-values. Paired tests were used to account for the fact that the data originated from the same samples (20 enclosures per lake and the lake itself).

For all calculations, statistics, and figures, we used the statistical packages R (version 4.4.1, R Core Team 2021), *dplyr* (version 1.0.7, Wickham et al. 2021), *ggplot2* (version 3.3.5, Wickham 2016), *tidyr* (version 1.1.4, Wickham 2021), and *vegan* (version 2.5-7, Oksanen et al. 2020).

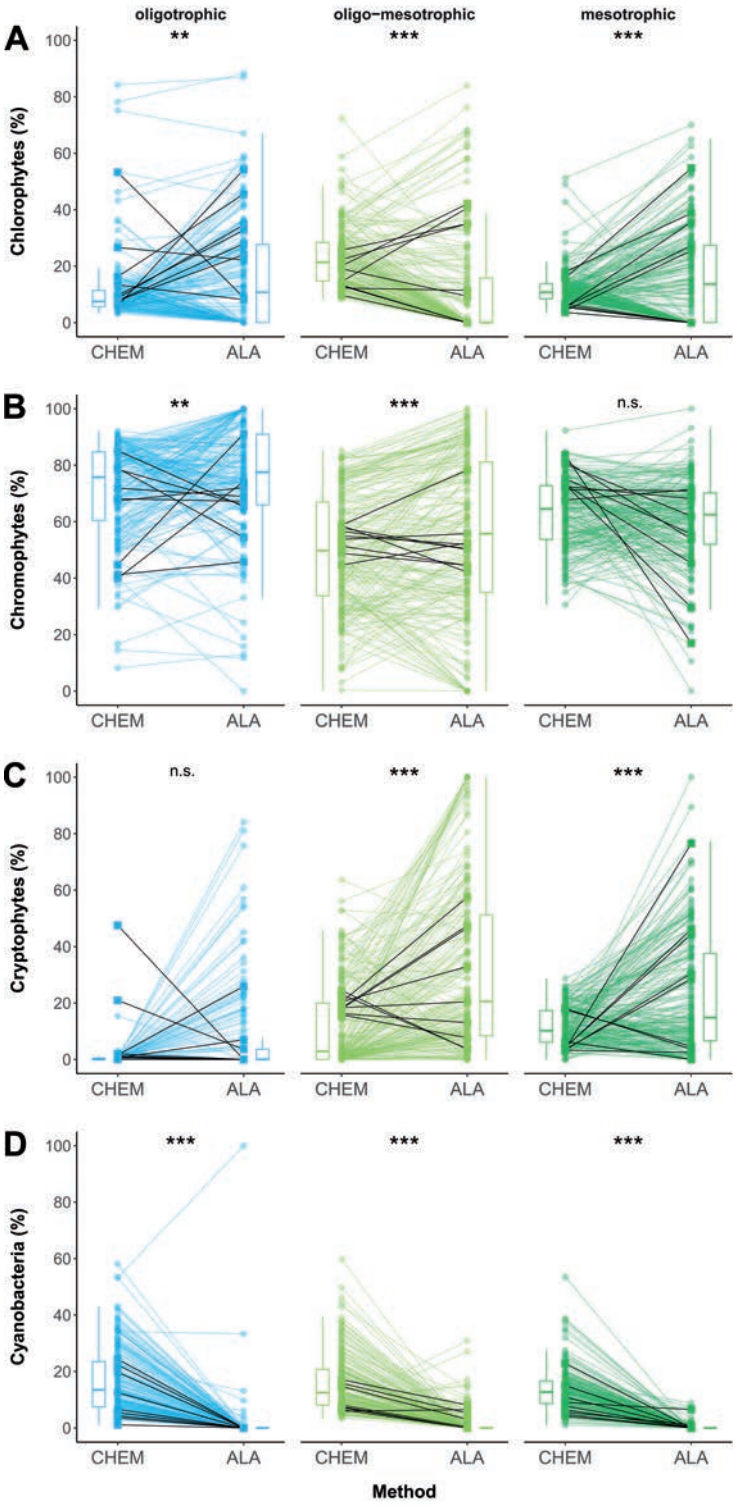

Fig. 3. Relative abundance (%) of **(A)** chlorophytes, **(B)** chromophytes, **(C)** cryptophytes and **(D)** cyanobacteria determined chromatographically *in vitro* via HPLC and CHEMTAX (CHEM) and spectrofluorometrically *in vivo* with Algae Lab Analyser (ALA). Data from all 9 sampling events (July to September 2014) are shown (blue: oligotrophic, $n = 186$; light green: oligo-mesotrophic, $n = 187$; dark green: mesotrophic, $n = 189$). Data originating from enclosures are depicted as circles and connected with coloured lines, while data originating directly from the lakes are depicted as squares and connected with black lines. Boxplots on each side show the median (thick line), interquartile range between 25th percentile 75th percentile (IQR, box) and smallest and largest value within the 1.5× IQR below 25th percentile and above 75th percentile, respectively (whiskers), while outliers were omitted from plotting. Significant differences based on paired Wilcoxon-Mann-Whitney tests are depicted as follows: ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$, n.s.: $p \geq 0.05$ (not significant).

Results

Total biomass

The biomasses per sample, given as TChl-*a*, ranged between 0.01 and 11.51 µg TChl-*a* L^{-1}, as determined with Algae Lab Analyser, and between 0.22 and 12.92 µg TChl-*a* L^{-1}, as determined via HPLC (across all lakes and the entire experimental period, Fig. 1A, Table 1). The average TChl-*a* per lake was significantly higher when determined with Algae Lab

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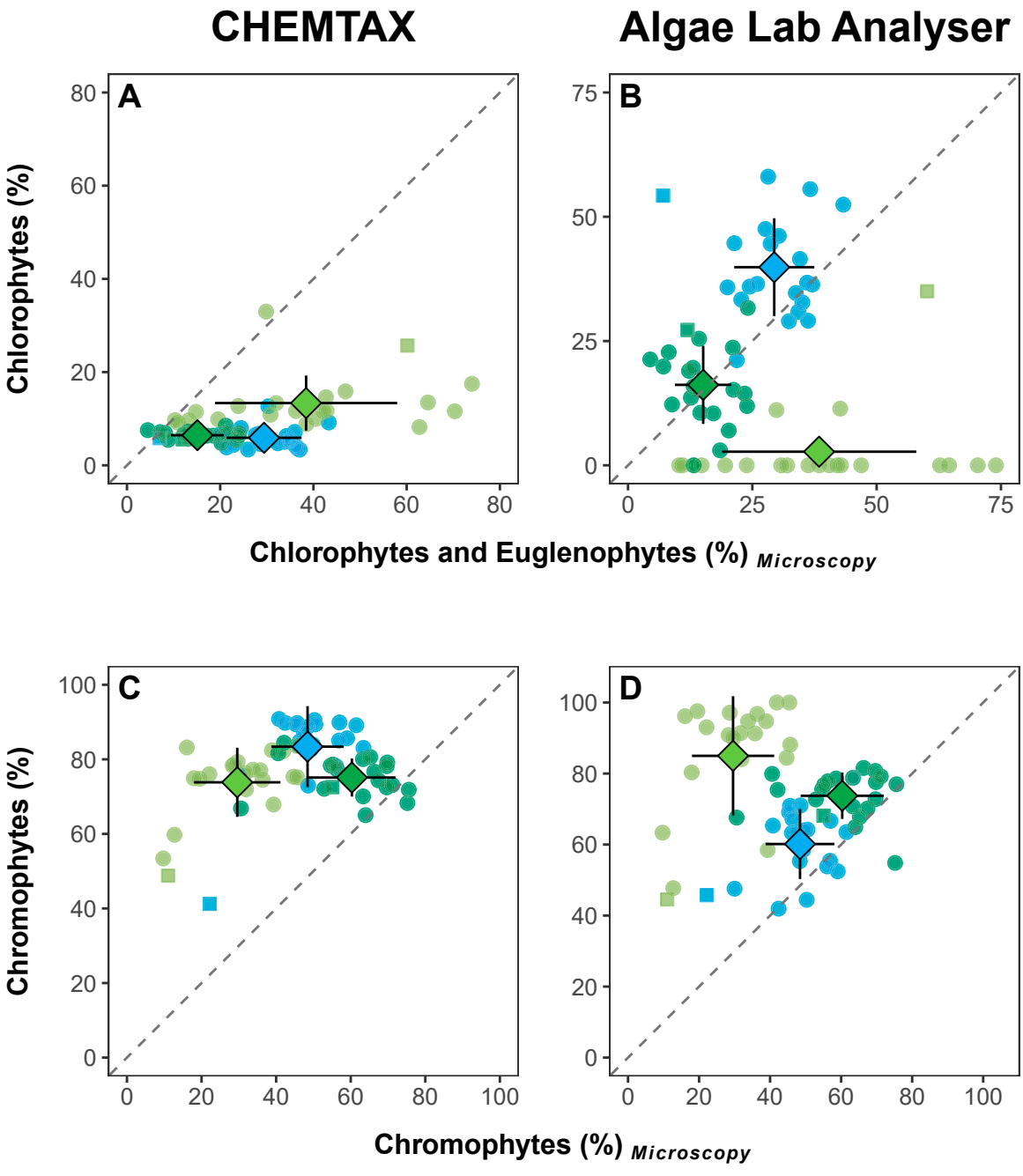

Fig. 4. Relative abundance (%) of **(A, B)** chlorophytes (and euglenophytes in the case of microscopy) and **(C, D)** chromophytes (including diatoms, chrysophytes and dinoflagellates) determined via microscopic counts (x-axis in all panels), chromatographically *in vitro* via HPLC and CHEMTAX (panels A and C, y-axis) and spectrofluorometrically *in vivo* with Algae Lab Analyser (panels B and D, y-axis). For legend, see Fig. 1. Only data from the first sampling event (8th –10th July 2014) are shown ($n = 21$ for each lake).

Analyser (0.86 ± 0.62 µg L^{-1} in the oligotrophic lake and 3.19 ± 1.96 µg L^{-1} in the mesotrophic lake; mean ± standard deviation) compared to the values determined via HPLC (0.53 ± 0.25 µg L^{-1} and 2.01 ± 1.44 µg L^{-1}, respectively; Wilcoxon-Mann-Whitney test, $p < 0.001$ for both lakes), while the total biomass estimates for the oligo-mesotrophic lake did not differ between these two methods (1.27 ± 0.61 µg L^{-1} as determined with Algae Lab Analyser and 1.26 ± 0.57 µg L^{-1} as determined via HPLC; Wilcoxon-Mann-Whitney test, $p = 0.72$). Nevertheless, we found a high positive correlation for the estimated TChl-*a* between the two

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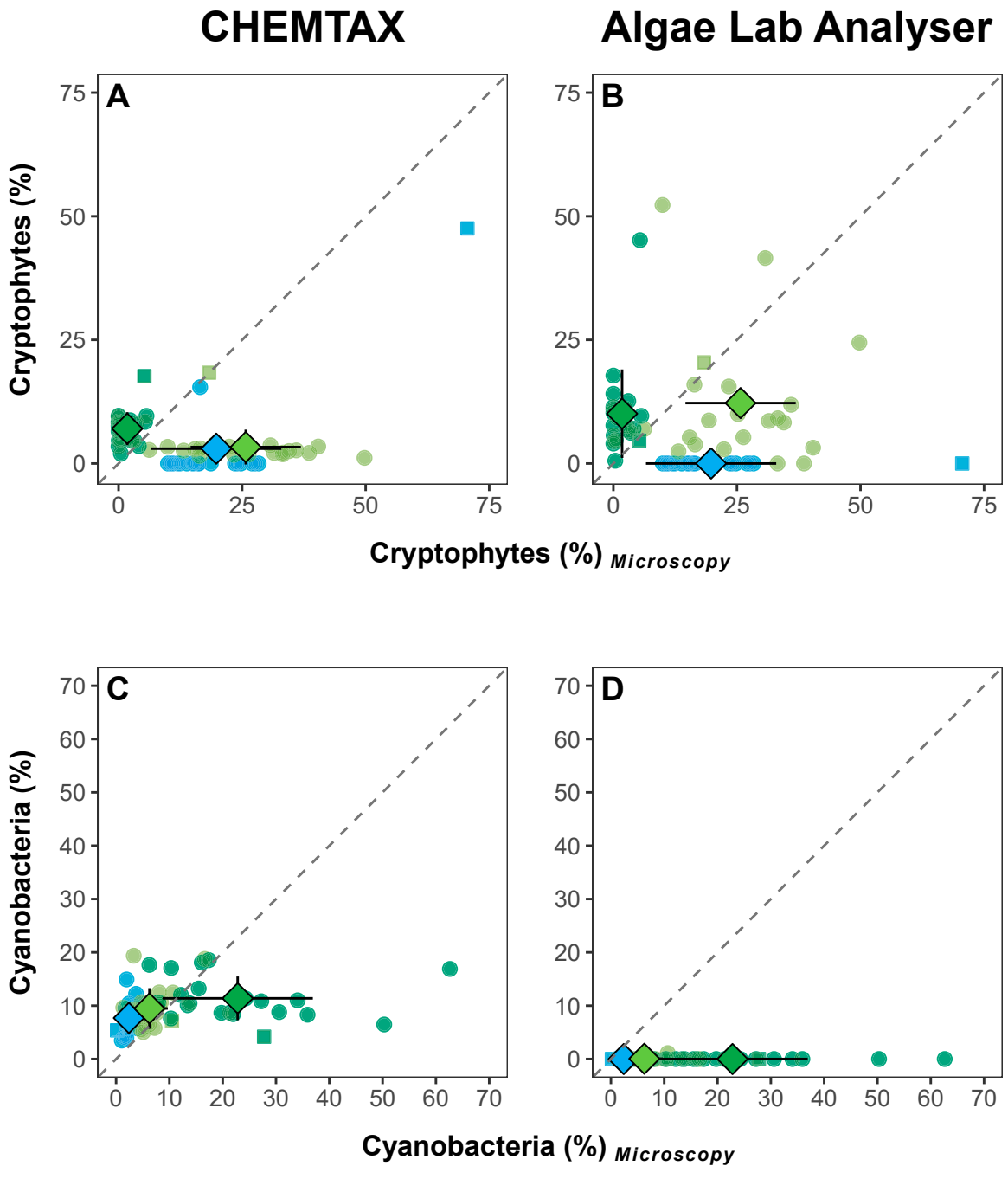

Fig. 5. Relative abundance (%) of **(A, B)** cryptophytes and **(C, D)** cyanobacteria determined via microscopic counts (x-axis in all panels), chromatographically *in vitro* via HPLC and CHEMTAX (panels A and C, y-axis) and spectrofluorometrically *in vivo* with Algae Lab Analyser (panels B and D, y-axis. For legend, see Fig. 1. Only data from the first sampling event (8^{th} – 10^{th} July 2014) are shown ($n = 21$ for each lake).

methods ($r_s = 0.82$, $p < 0.001$) across all three lakes (all sampling events considered). This correlation, albeit significant, was less strong when lakes were considered individually (oligotrophic: $r_s = 0.56$, $p < 0.001$; oligo-mesotrophic: $r_s = 0.59$, $p < 0.001$; mesotrophic: $r_s = 0.72$, $p < 0.001$).

Phytoplankton composition

The phytoplankton communities of all three lakes were strongly dominated by chromophytes (Supplementary Material Table S6). Based on biomass estimates from Algae Lab Analyser, the mean relative abundance of

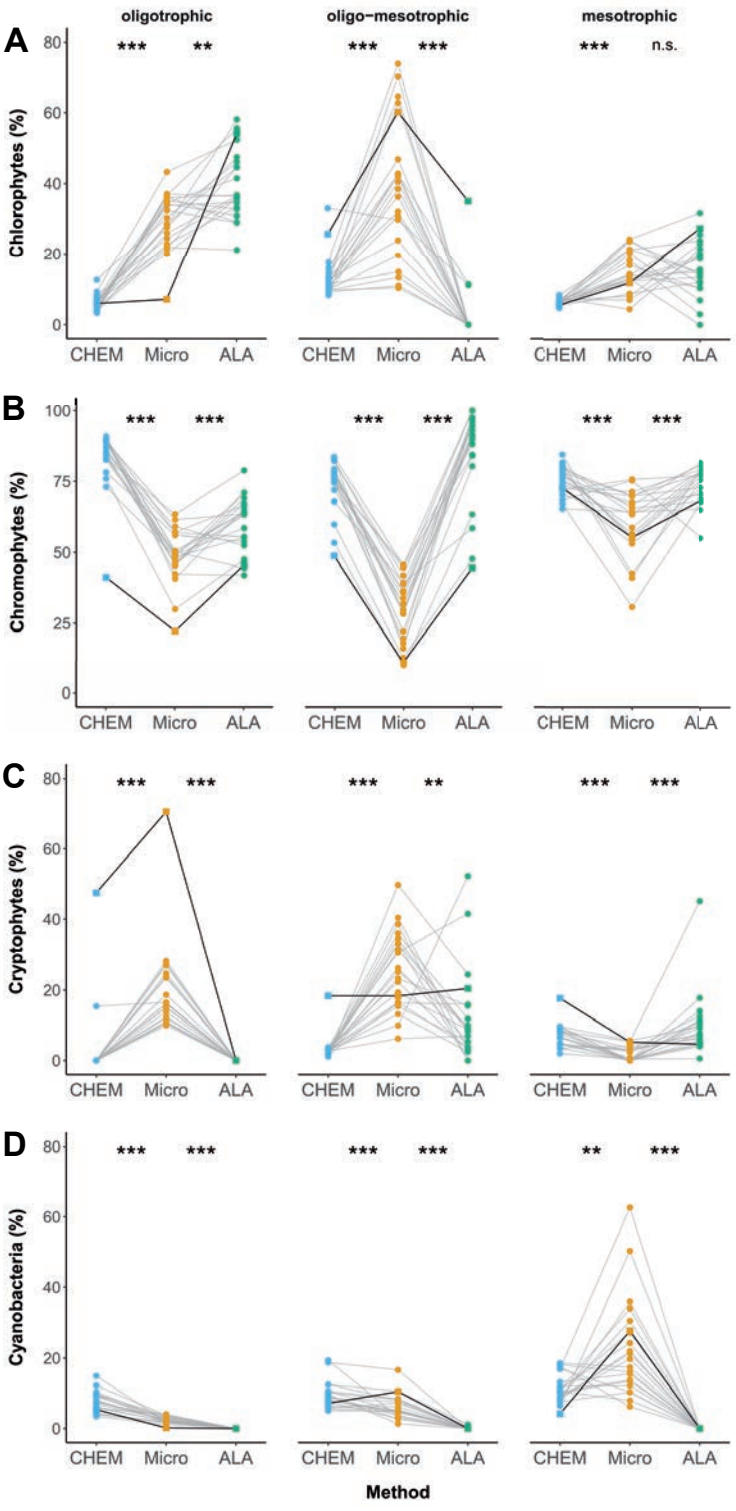

Fig. 6. Relative abundance (%) of **(A)** chlorophytes, **(B)** chromophytes, **(C)** cryptophytes and **(D)** cyanobacteria determined via microscopic counts (Micro, orange symbols), chromatographically *in vitro* via HPLC and CHEMTAX (CHEM, blue symbols) and spectrofluorometrically *in vivo* with Algae Lab Analyser (ALA, green symbols). Data originating from enclosures are depicted as circles and connected with grey lines, while data originating directly from the lakes are depicted as squares and connected with black lines. Only data from the first sampling event (8^{th} –10^{th} July 2014) are shown ($n = 21$ for each lake). Significant differences based on paired Wilcoxon-Mann-Whitney tests are depicted as follows: ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$, n.s.: $p \geq 0.05$ (not significant).

chromophytes ranged from 55 ± 29 % in the oligo-mesotrophic lake (all available data considered, Supplementary Material Fig. S3) to 76 ± 20 % as found in the oligotrophic lake. The second most abundant group in the oligo-mesotrophic and the mesotrophic lake were cryptophytes (32 ± 30 % and 23 ± 21 %, respectively), while the chlorophytes were the second most abundant group in the oligotrophic lake (16 ± 19 %). Cyanobacteria were found only in very low abundances being even below 2 %. When the lakes were considered indi-

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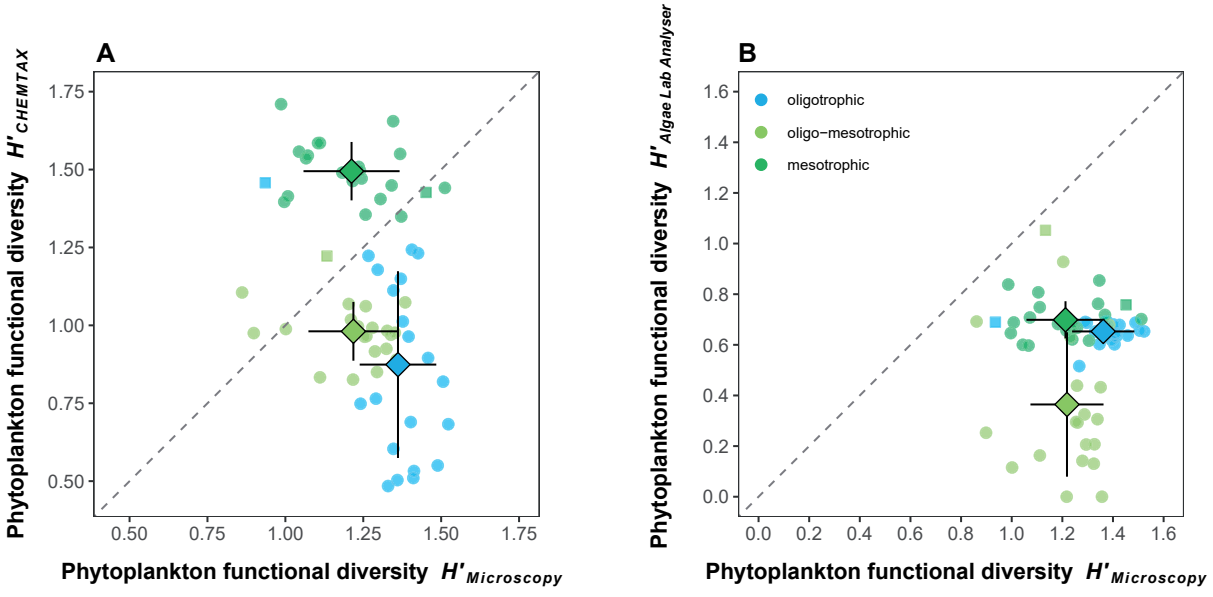

Fig. 7. Phytoplankton functional diversity *H'* determined via microscopic counts (x-axis in both panels), chromatographically *in vitro* via HPLC and CHEMTAX (based on all 6 phytoplankton classes; panel A, y-axis) and spectrofluorometrically *in vivo* with Algae Lab Analyser (panel B, y-axis). The dashed lines in all four panels represent the 1:1 relationship. Colour of the symbols represents the trophic state of the lakes, blue: oligotrophic; light green: oligo-mesotrophic; dark green: mesotrophic. Data originating from enclosures are depicted as circles, while data originating directly from the lakes are depicted as squares. Diamonds represent the mean values, while horizontal and vertical error bars represent the standard deviation (based on all data points per lake). Only data from the first sampling event (8^{th} –10^{th} July 2014) are shown ($n = 21$ for each lake).

vidually, we found significant differences between all four phytoplankton groups (Kruskal-Wallis test, oligotrophic: $X^2_{3,182} = 514.5$, $p < 0.001$; oligo-mesotrophic: $X^2_{3,183} = 381.8$, $p < 0.001$; mesotrophic: $X^2_{3,185} = 511.4$, $p < 0.001$).

Similarly, based on CHEMTAX biomass estimates, we found significant differences between all four phytoplankton groups, except in the mesotrophic lake, where we found no difference between the relative abundance of cyanobacteria (13 ± 7 %) and chlorophytes (12 ± 6 %, Wilcoxon-Mann-Whitney test, $p = 0.071$) or cryptophytes (12 ± 7 %, Wilcoxon-Mann-Whitney test, $p = 0.315$). In all three lakes, chromophytes were the most abundant group (oligotrophic: 71 ± 17 %; oligo-mesotrophic: 49 ± 21 %; mesotrophic: 63 ± 12 %).

The congruence of CHEMTAX and Algae Lab Analyser was in general very low, and partly differed across the lakes and the phytoplankton groups,but did not follow any patterns. The relative abundance of chlorophytes was lower in the oligotrophic and mesotrophic lake, and higher in the oligo-mesotrophic lake when determined via CHEMTAX compared to the estimates derived from Algae Lab Analyser (Fig. 2A and Fig. 3A). While the relative abundances of chromophytes in the mesotrophic lake was equally estimated by both CHEMTAX and Algae Lab Analyser, CHEMTAX found less chromophytes in both the oligotrophic and oligo-mesotrophic lakes compared to Algae Lab Analyser (Fig. 2B and 3B). The relative abundance of cryptophytes was significantly lower in the oligo-mesotrophic and mesotrophic lake when determined via CHEMTAX compared to the estimates derived from Algae Lab Analyser, while both methods equally estimated the relative abundance of cryptophytes in the oligotrophic lake (Fig. 2C and 3C). In contrast, the relative abundance of cyanobacteria was consistently higher when determined via CHEMTAX compared to the estimates derived from Algae Lab Analyser, independent of the lakes' trophic state (Fig. 2D and 3D).

We found differences in the ability of CHEMTAX and Algae Lab Analyser to correctly differentiate between the four main phytoplankton classes: compared to the phytoplankton community composition derived from microscopic counts, CHEMTAX significantly underestimated the relative abundance of chlorophytes (in all three lakes, Fig. 4A and 6A) and cryptophytes (in the oligotrophic and oligo-mesotrophic lake, Fig. 5A and 6C), and overestimated the relative abundance of chromophytes (in all three lakes, Fig. 4C and 6B) and cyanobacteria (in the oligotrophic and ol-

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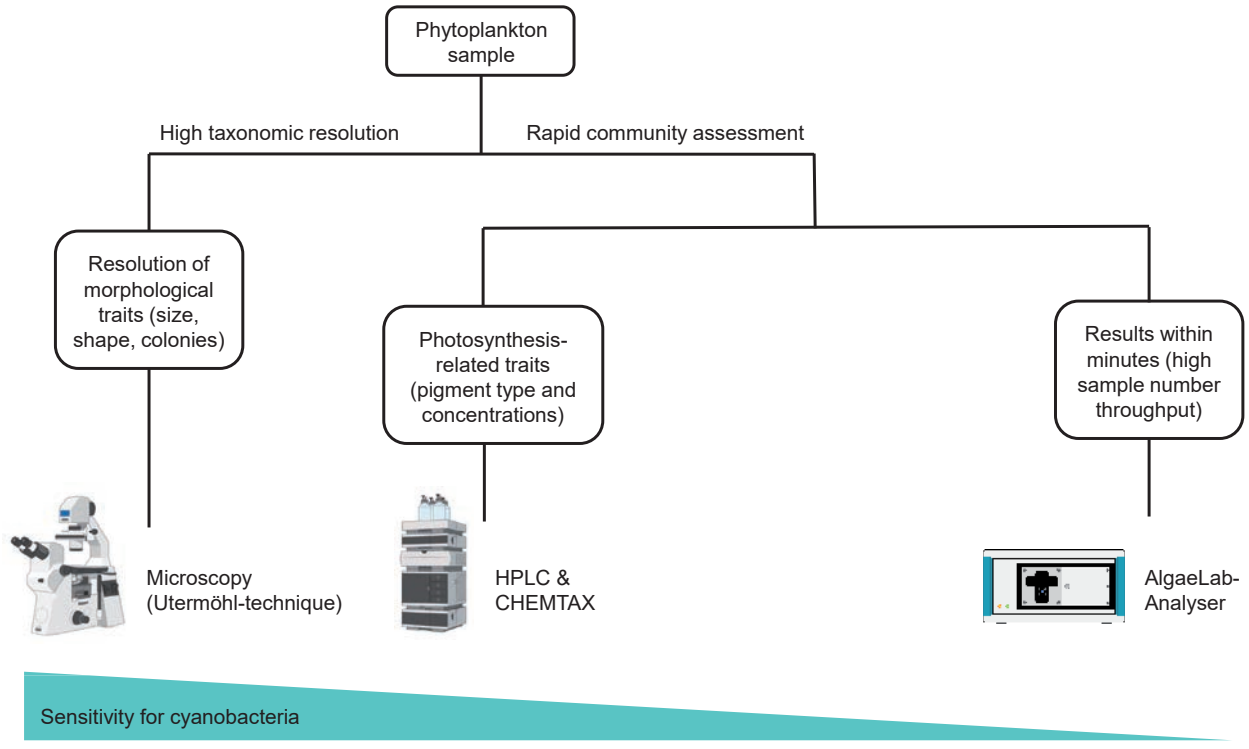

Fig. 8. Flow chart to support decision-making for the most suitable method, depending on time effort, taxonomic resolution and traits of interest. We focused only on the approaches presented in the study: microscopic counting following the Utermöhl-technique (Utermöhl 1958), HPLC in combination with CHEMTAX, and Algae Lab Analyser. Inverted microscope and HPLC system originate from BioRender (https://biorender.com/).

igo-mesotrophic lake, Fig. 5C and 6D). In contrast, in the mesotrophic lake, CHEMTAX significantly overestimated the relative abundance of cryptophytes, and underestimated the relative abundance of cyanobacteria. In all three lakes, Algae Lab Analyser significantly overestimated the relative abundance of chromophytes (Fig. 4D and 6B) while it underestimated the relative abundance of cyanobacteria (Fig. 5D and 6D). The ability of Algae Lab Analyser to accurately estimate the relative abundance of chlorophytes and cryptophytes differed between the three lakes: while it equally estimated the relative abundance of the chlorophytes in the mesotrophic lake, Algae Lab Analyser significantly overestimated their relative abundance in the oligotrophic lake and underestimated their relative abundance in the oligo-mesotrophic lake (Fig. 4B and 6A). The relative abundance of cryptophytes was significantly underestimated in the oligotrophic and oligo-mesotrophic lake and overestimated in the mesotrophic lake (Fig. 5B and 6C). All significant differences are based on paired Wilcoxon-Mann-Whitney tests ($p < 0.001$ or $p < 0.01$ for all comparisons, Fig. 6).

With CHEMTAX, we were able to differentiate between the subgroups of chromophytes (diatoms, chrysophytes and dinoflagellates) and thus achieved a higher taxonomic resolution of the phytoplankton community composition compared to the Algae Lab Analyser (Fig. 7). According to the CHEMTAX calculations, in the oligotrophic lake (Supplementary Material Fig. S4), the diatoms were the most abundant phytoplankton group (44 ± 19 %, all available data considered), followed by dinoflagellates (17 ± 11 %), cyanobacteria (17 ± 11 %), chlorophytes (11 ± 12 %) and chrysophytes (10 ± 5 %), while the average relative abundance of the cryptophytes was below 1 %, as indicated by the very low amount of alloxanthin (Supplementary Material Fig. S2). The phytoplankton community in both the oligo-mesotrophic and the mesotrophic lakes was dominated by chrysophytes (37 ± 20 % and 33 ± 16 %, respectively). As indicated by high amounts of zeaxanthin, chlorophyll-*b* and lutein in the oligo-mesotrophic lake (Supplementary Material Fig. S2), the chlorophytes (23 ± 11 %) were the second most abundant phytoplankton group in this lake (Supplementary Material Fig. S4), followed by cyanobacteria (16 ± 10 %), while dinoflagellates were the least abundant group (1 ± 5 %). In the mesotrophic lake, diatoms were the second most abundant phyto-

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Table 2. Functional diversity of the natural phytoplankton communities, given as Shannon Diversity Index *H'*, based on biomass estimates derived from Algae Lab Analyser (four groups) or via HPLC/CHEMTAX (six or four groups). Additionally, pigment functional diversity is given, based on pigment concentration derived via HPLC (all 10 pigments considered). Given are mean, standard deviation, median, minimum, and maximum value for each lake, based on samples from all 9 sampling events (July to September 2014; oligotrophic: $n = 186$; oligo-mesotrophic: $n = 187$; mesotrophic: $n = 189$). Highest average functional diversity (highest mean *H'*) for each method is given in bold, while the lowest average functional diversity (lowest mean *H'*) is given in italics.

Trophic state	Functional diversity				
	Mean	SD	Median	Min	Max
$H'_{Algae\ Lab\ Analyser}$					
oligotrophic	*0.43*	0.25	0.51	0	0.91
oligo-mesotrophic	0.6	0.31	0.65	0	1.37
mesotrophic	**0.72**	0.15	0.69	0	1.14
$H'_{CHEMTAX\ (6\ groups)}$					
oligotrophic	1.29	0.24	1.35	0.48	1.7
oligo-mesotrophic	*1.27*	0.19	1.29	0.75	1.77
mesotrophic	**1.56**	0.19	1.59	0.65	1.79
$H'_{CHEMTAX\ (4\ groups)}$					
oligotrophic	*0.71*	0.21	0.7	0.33	1.31
oligo-mesotrophic	**1.04**	0.22	1.08	0.55	1.38
mesotrophic	1	0.19	1	0.36	1.34
$H'_{Pigments\ (incl.\ Chl\text{-}a)}$					
oligotrophic	*1.16*	0.13	1.17	0.78	1.48
oligo-mesotrophic	**1.24**	0.13	1.24	0.93	1.51
mesotrophic	**1.22**	0.12	1.23	0.65	1.54

plankton group (16 ± 8 %), while the other four phytoplankton groups were all present in relatively similar abundances, ranging from 12 ± 7 % (cryptophytes) to 13 ± 8 % (cyanobacteria and dinoflagellates, respectively, Supplementary Material Fig. S4).

We found that CHEMTAX significantly underestimated the contribution of the diatoms to the total biomass in the oligo-mesotrophic and mesotrophic lake, and overestimated the biomass estimates of chrysophytes, compared to the phytoplankton community composition derived from microscopic counts (Wilcoxon-Mann-Whitney tests, $p < 0.001$ for all comparisons; Supplementary Material Fig. S8A and S8B). In contrast, CHEMTAX overestimated the relative abundance of diatoms and underestimated the relative abundance of chrysophytes in the oligotrophic lake (Wilcoxon-Mann-Whitney tests, $p < 0.001$ for all comparisons, Supplementary Material Fig. S8A and S8B). As for dinoflagellates, the agreement between microscopic counts and CHEMTAX differed across the three lakes (Supplementary Material Fig. S8C): while CHEMTAX significantly overestimated the abundance of dinoflagellates in the oligotrophic lake (Wilcoxon-Mann-Whitney tests, $p < 0.01$), it underestimated their abundance in the oligo-mesotrophic lake (Wilcoxon-Mann-Whitney tests, $p < 0.05$). The relative abundance of dinoflagellates in the mesotrophic lake was equally estimated by both microscopic counts and via CHEMTAX (Wilcoxon-Mann-Whitney tests, $p = 0.66$).

Interestingly, in more than 63 % of the samples, we found only 1 or 2 functional groups when using Algae Lab Analyser (46 and 310 samples, respectively), while 3 or 4 groups were found in 186 and 20 samples, respectively. With CHEMTAX, we found all four phytoplankton groups in 482 out of 562 samples (86 %), while 78 samples had a functional richness of 3 (Supplementary Material Fig. S9).

Functional diversity

We found a strong correlation between the functional diversity based on pigment concentrations and CHEMTAX biomass estimates of all six phytoplankton groups, especially when each lake was considered separately (oligotrophic: $r_S = 0.77$; oligo-mesotrophic: $r_S = 0.77$; mesotrophic: $r_S = 0.78$, Fig. 1B). The correlation was less strong when all three lakes were considered together ($r_S = 0.62$). Based on pigment concentrations, the functional diversity in the oligo-mesotrophic and mesotrophic lakes was higher than

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in the oligotrophic lake (Kruskal-Wallis test, $X^2_{2,559} = 33.96$, $p < 0.001$, Table 2).

The phytoplankton functional diversity based on the biomass estimates of four phytoplankton groups (chlorophytes, chromophytes, cryptophytes and cyanobacteria) determined with Algae Lab Analyser was the highest in the mesotrophic lake, while the oligotrophic lake was the least diverse (Kruskal-Wallis test, $X^2_{2,559} = 131.5$, $p < 0.001$; Fig. 1C, Table 2).

Similar to Algae Lab Analyser, CHEMTAX revealed the highest average phytoplankton functional diversity in the mesotrophic lake, but the oligo-mesotrophic lake was the least diverse (Kruskal-Wallis test, $X^2_{2,559} = 200.4$, $p < 0.001$; Fig. 1C, Table 2).

However, when only four groups were considered, the CHEMTAX based phytoplankton functional diversity was the highest in the oligo-mesotrophic lake and the lowest in the oligotrophic lake (Kruskal-Wallis test, $X^2_{2,559} = 183.3$, $p < 0.001$; Fig. 1D, Table 2).

We found significant differences between lakes and methods when comparing the phytoplankton functional diversity derived from microscopic counts, CHEMTAX (based on all six groups) and Algae Lab Analyser based on the first set of samples (8^{th} –10^{th} July 2014). The highest average diversity, derived from microscopic counts, was found in the oligotrophic lake ($H' = 1.36 \pm 0.12$; Kruskal-Wallis test, $X^2_{2,60} = 17.40$, $p < 0.001$), while no differences were found between the oligo-mesotrophic ($H' = 1.22 \pm 0.14$) and mesotrophic lake ($H' = 1.21 \pm 0.15$; Wilcoxon-Mann-Whitney test, $p = 0.69$). In contrast, CHEMTAX revealed the highest average diversity in the mesotrophic lake ($H' = 1.49 \pm 0.09$; Kruskal-Wallis test, $X^2_{2,60} = 40.47$, $p < 0.001$), while no differences were found between the oligotrophic ($H' = 0.87 \pm 0.30$) and the oligo-mesotrophic lake ($H' = 0.98 \pm 0.09$; Wilcoxon-Mann-Whitney test, $p = 0.21$). Finally, based on biomass estimates derived from Algae Lab Analyser, the oligo-mesotrophic lake was less diverse ($H' = 0.36 \pm 0.29$; Kruskal-Wallis test, $X^2_{2,60} = 20.04$, $p < 0.001$) compared to the oligotrophic ($H' = 0.65 \pm 0.04$) and the mesotrophic lake ($H' = 0.70 \pm 0.07$). None of the methods revealed similar estimates of phytoplankton functional diversity when lakes were considered separately.

Discussion

Both, the Algae Lab Analyser and HPLC/CHEMTAX, allowed for a rapid assessment of natural phytoplankton communities and their functional diversity, albeit at a somewhat limited taxonomic resolution. Phytoplankton biomass estimates (determined as total chlorophyll-*a*) were very similar with both methods. This demonstrates the general utility of both approaches and corroborates our first hypothesis, as both methods require relatively little time in comparison to e.g., microscopic counting, which makes them highly suited for monitoring and routine phytoplankton analyses.

Despite their general comparability in estimating the total phytoplankton biomass, both methods differed markedly in some important aspects. This applies in particular, but not exclusively, to the determination of cyanobacterial abundances, which are a major focus of phytoplankton community assessment in the context of water quality management (Izydorczyk et al. 2009; UBA 2012; Carmichael & Boyer 2016; Huisman et al. 2018). In general, the congruence of Algae Lab Analyser and CHEMTAX was low, irrespective of phytoplankton group and the trophic state of the lakes. Despite a few exceptions, these findings to a large part support our second hypothesis, which postulates that the congruence of the methods does not depend on the trophic state of the lakes and does not differ between different phytoplankton groups. Finally, we found a strong correlation between pigment diversity derived from HPLC and functional diversity based on CHEMTAX biomass estimates, confirming our third hypothesis, and suggesting that pigment diversity can be used as a proxy for phytoplankton functional diversity.

Comparative assessment of methods

In our study, the Algae Lab Analyser was frequently unable to detect any cyanobacteria in the lakes' phytoplankton, even though the detection of echinenone in the HPLC gave clear indications of cyanobacterial presence. This finding is also supported by microscopic observations of a subset of the samples that showed a presence of cyanobacteria in the majority of analysed samples, especially in those from the mesotrophic lake. It should be noted that the manufacturer suggests calibrating the Algae Lab Analyser with phytoplankton species isolated from the water bodies of interest to get a more accurate assessment of the phytoplankton community composition. However, this may not be realistic in practice, in particular, for routine laboratories and water authorities that monitor numerous surface water bodies in parallel.

An important aspect that might explain the observed differences between the two methods is the possibility to adjust the sensitivity of the HPLC/CHEMTAX method via the filtered volume of samples. While

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only 25 ml of the water samples were measured in the Algae Lab Analyser, we filtered 500–1000 ml of water for each sample for the pigment-analyses via HPLC. Thus, the concentration of the pigments extracted from the filters and detected via HPLC was higher compared to the pigment concentrations in the water sample measured *in vivo* with Algae Lab Analyser. Furthermore, HPLC has the advantage of measuring the total concentration of pigments within the cells (as pigments are extracted from the phytoplankton cells prior to HPLC analysis), while Algae Lab Analyser is applied *in vivo* without cell extraction, and therefore only allows for detection of pigments on the surface of the cells. This probably allowed for the higher sensitivity of the HPLC/CHEMTAX method and its accuracy in the estimation of low cyanobacterial abundances. The sensitivity of HPLC could potentially be further increased by applying a lower flow rate of the solvents within the HPLC system and using microbore HPLC columns (Zweigenbaum et al. 2000; Barco et al. 2002).

Three specific aspects in the comparative evaluation of the HPLC-based and the *in vivo* method merit particular attention. The first applies to the distinction between cryptophytes and cyanobacteria, which is of particular relevance for water quality assessment and monitoring (Catherine et al. 2012; Gregor et al. 2005; Izydorczyk et al. 2009): the detection of cryptophytes by the Algae Lab Analyser depends not only on the main cryptophyte marker pigment alloxanthin, but further on the specific absorption of phycoerythrin (Beutler et al. 2002; Beutler et al. 2004), which is also an important pigment for many "red" and "blue" cyanobacteria (Bryant 1982; Gregor et al. 2005; Haverkamp et al. 2009). As the lipophilic extraction commonly applied prior to the HPLC separation of pigments does not capture the hydrophilic pigment groups of phycoerythrins and phycocyanins, these pigments cannot be evaluated by the CHEMTAX approach. This led us to the assumption that CHEMTAX may underestimate the abundance of cyanobacteria in lake phytoplankton. Interestingly, our data indicated quite the opposite, i.e., a much higher relative abundance of cyanobacteria in the phytoplankton community assessment via CHEMTAX as compared to the Algae Lab Analyser. Catherine et al. (2012) also reported a "potentially strong misattribution towards cryptophytes of "red" cyanobacteria" when they compared the biomass estimates of cryptophytes and cyanobacteria from FluoroProbe to the microscopic counts. When examining cyanobacterial blooms in reservoirs, in some samples dominated by cyanobacteria, Gregor et al. (2005) detected certain amounts of cryptophytes (approx. 1–20 % of TChl-*a*) via FluoroProbe, although microscopic counts revealed no cryptophyte abundances. This may be explained by the inclusion of phycoerythrins into the detection of cryptophytes by the Algae Lab Analyser (and FluoroProbe). Admittedly, there have been attempts to account for this potential problem by the manufacturers of the Algae Lab Analyser (Beutler et al. 2003; Beutler et al. 2004). Nevertheless, our data indicate that under certain conditions, the CHEMTAX approach may be more sensitive for the detection of low cyanobacterial abundances in comparison to the *in vivo* approach of the Algae Lab Analyser.

Beyond the distinction between cryptophytes and cyanobacteria, it may also be challenging to distinguish chlorophytes from cyanobacteria under certain conditions. Most published HPLC gradients have difficulties in separating the peaks of lutein and zeaxanthin (Latasa et al. 1996; Van Heukelem & Thomas 2001; Ston-Egiert & Kosakowska 2005). This was also the case for our HPLC gradient. Therefore, lutein may be frequently underestimated, which would lead to an underestimation of chlorophytes relative to cyanobacteria. In our HPLC data, no lutein peak could be identified in some samples, although microscopic counts indicated the presence of chlorophytes. Such an underestimation of chlorophyte abundances due to an insufficient separation of lutein and zeaxanthin and consequently the misattribution of chlorophytes towards cyanobacteria may explain the disagreement between CHEMTAX and Algae Lab Analyser and differences in their ability to accurately identify chlorophytes and cyanobacteria. CHEMTAX estimates the relative abundance of chlorophytes mainly based on the occurrence of lutein and chlorophyll-*b*. If chlorophyll-*b*, but no lutein is detected, this is probably a consequence of the aforementioned weak separation of the lutein and zeaxanthin peaks in the HPLC. An alternative explanation could be the occurrence of euglenophytes that are characterised by the possession of chlorophyll-*b* without a concomitant abundance in lutein (Fietz & Nicklisch 2004; Schlüter et al. 2006; Sarmento & Descy 2008). However, microscopic observations of our samples gave little indications of common occurrences of euglenophytes in our study lakes.

The third important difference of the two methods is related to the distinction of diatoms and chrysophytes. As both groups share the characteristic pigment fucoxanthin, the Algae Lab Analyser does not allow for a distinction between them. This is somewhat unfortunate, as these two algal groups often domi-

nate phytoplankton communities in oligo- and mesotrophic lakes (Buchaca et al. 2005; Ptacnik et al. 2008; Järvinen et al. 2013; Poxleitner et al. 2016; Schlüter et al. 2016). CHEMTAX provides the distinct advantage of separating chrysophytes from diatoms based on their specific fucoxanthin : chlorophyll-*a* ratios. As mentioned before, the final output ratio of fucoxanthin : chlorophyll-*a* for diatoms and chrysophytes differed between the oligotrophic lake and the oligo-mesotrophic and the mesotrophic lake, resulting in a different ratio of diatoms to chrysophytes depending on the trophic state (oligotrophic lake: diatoms more abundant than chrysophytes, while the opposite was the case in the oligo-mesotrophic and the mesotrophic lake). However, microscopic counts indicated an overall lower biovolume (common proxy for phytoplankton biomass) of chrysophytes compared to diatoms across all three lakes. One possible explanation might be the use of different pigment ratio matrices for initial CHEMTAX calculations, which were chosen according to the trophic state of each lake. Specifically, in the case of the oligo-mesotrophic lake, we used a ratio matrix with average pigment : chlorophyll-*a* ratios based on the two matrices established in Schlüter et al. (2016). However, CHEMTAX calculations for the oligo-mesotrophic lake with the ratio matrix established for oligotrophic lakes (Schlüter et al. 2016) yielded unaltered results (data not shown). This suggests that the choice of the initial pigment ratio matrix is less important for an accurate assessment of the phytoplankton community, and that the final output is strongly driven by the pigment concentrations measured via HPLC. However, it also indicates that a differentiation between diatoms and chrysophytes based on their specific fucoxanthin : chlorophyll-*a* ratios is not sufficient to accurately discriminate these two phytoplankton groups.

Similar results were found by Simmons et al. (2016), who compared the phytoplankton community composition via HPLC/CHEMTAX estimates to biovolume estimates derived from microscopic counts for oligotrophic Lake Michigan. There, CHEMTAX overestimated chrysophytes versus diatoms. Interestingly, the output fucoxanthin : chlorophyll-*a* ratios for both groups of Simmons et al. (2016) were similar to the final output fucoxanthin : chlorophyll-*a* ratios for the oligo-mesotrophic and the mesotrophic lakes from our study, which leads to a consistent favouring of chrysophytes over diatoms. To overcome the observed mismatch between diatoms and chrysophytes, Simmons et al. (2016) suggested including chlorophyll-c_1 and -c_2 into CHEMTAX analyses. This is because (freshwater) diatoms contain both chlorophyll-c_1 and -c_2, while most chrysophytes contain only chlorophyll-c_2 (Jeffrey et al. 2011). We additionally suggest including other pigments into the CHEMTAX approach for a more accurate differentiation of diatoms and chrysophytes, e.g., violaxanthin, which is a commonly used marker pigment for chrysophytes (Descy et al. 2000; Buchaca et al. 2005; Lauridsen et al. 2011; Schlüter et al. 2016).

Phytoplankton functional diversity

Although both, Algae Lab Analyser and HPLC/CHEMTAX, allow for a lower taxonomic resolution compared to microscopy, this may not be a major constraint, as multiple studies have shown functional phytoplankton diversity to be a better predictor of ecosystem functioning than species richness (Striebel et al. 2009; Behl et al. 2011; Stockenreiter et al. 2013). While reducing data complexity (e.g., by aggregating taxa into functional groups based on traits such as pigment composition) might result in loss of ecological information, this might not necessary be the case if functional diversity highly correlates with taxonomic diversity, thereby highlighting complementarity. In fact, the use of functional approaches is crucial to improve our understanding of how community composition can be linked to ecosystem functioning (Abonyi et al. 2018, and references therein). This means that Algae Lab Analyser and HPLC/CHEMTAX approach might be an alternative and/or complementary tools to assess the phytoplankton community composition and address research questions related to the biodiversity – ecosystem functioning relationship.

In general, the functional diversity of the natural phytoplankton communities based on CHEMTAX biomass estimates was overall higher than the functional diversity derived from Algae Lab Analyser, which may be related to the observation that in more than 63 % of the samples, the Algae Lab Analyser identified only one or two phytoplankton groups. This seems highly unlikely for samples from natural phytoplankton communities. Thus, for studies on functional diversity of phytoplankton communities, CHEMTAX appears to be more suitable, as it in general allows for a higher functional resolution of natural phytoplankton communities. Furthermore, the high positive correlation between the pigment-based and the phytoplankton-based functional diversity derived via HPLC and CHEMTAX indicates that the pigments can be used as a proxy for functional groups. This provides estimates of functional diversity within natural

phytoplankton communities without the necessity to perform CHEMTAX calculations. Moreover, assessing the pigment diversity of the phytoplankton may be crucial to predict compositional shifts and potential consequences of biodiversity changes for functions provided by phytoplankton, such as biomass production, as pigments are a functionally relevant trait linked to light use efficiency.

Interestingly, we found the lowest average functional diversity in the oligotrophic Lake Brunnsee with both Algae Lab Analyser and CHEMTAX (when only the four main groups were considered). This was surprising, as former studies claim that oligotrophic lakes usually harbour more diverse phytoplankton communities (in terms of species richness) compared to mesotrophic or eutrophic lakes (Leibold 1999; Dodson et al. 2000). This is probably due to a strong dominance of chromophytes and in particular diatoms in Lake Brunnsee. Nevertheless, we cannot exclude that despite the low functional diversity observed in Brunnsee, there may be an underlying high species richness within one functional group. It needs to be noted that the diversity of the phytoplankton communities does not depend on the trophic state alone, but is also determined by other variables, such as physical environment or stratification (layering) conditions in the lake (Borics et al. 2021; Stockenreiter et al. 2021), which however have not been assessed here.

Role of trophic state

In most cases, the agreement between both methods was low, irrespective of the lake trophic status. Compared to CHEMTAX, the Algae Lab Analyser consistently underestimated the cyanobacterial abundances across all three lakes. However, with the exception of cyanobacteria, we did not find a clear pattern. For example, the relative abundance of chromophytes in the mesotrophic lake was equally estimated by the two methods, but differed significantly in the oligotrophic and the oligo-mesotrophic lakes. The best agreement for cryptophytes was found in the oligotrophic lake, while the relative abundance of cryptophytes in the two other lakes significantly differed between the two methods. Furthermore, the estimates of total biomass (given as TChl-*a*) were similar between these two methods in the case of the oligo-mesotrophic lake, while TChl-*a* derived from Algae Lab Analyser was significantly higher in the oligotrophic and mesotrophic lakes compared to TChl-*a* determined via HPLC. This indicates that the agreement between the two methods might depend on the overall biomass found in the lakes: too low or too high chlorophyll-*a* concentrations might be difficult to estimate accurately via HPLC. Based on our results, we present an overview of the advantages and disadvantages of both approaches. This allows us to provide a flow chart to support decision-making for the most suitable method (Fig. 8).

Conclusions

Both the Algae Lab Analyser and HPLC/CHEMTAX can be fast and useful tools for the assessment of phytoplankton community composition. However, the agreement between the methods was not always satisfactory, which may be due to different marker pigments utilised by the two methods. In general, more pigments should be included in the HPLC analysis, especially to be able to distinguish between diatoms and chrysophytes, e.g., violaxanthin and chlorophylls-c_1 and -c_2. As both methods have advantages and disadvantages, the method of choice depends on the aim of the study or the field of use. While the Algae Lab Analyser is more suitable for rapid monitoring, CHEMTAX provides a higher resolution of the functional diversity in the community and better estimates of cyanobacterial abundances.

Authors' contribution

MI, SKH, MS, HS and PF designed the study; SKH and MS conducted field samplings, microscopic counting, and measurements with the Algae Lab Analyser; MI and SW developed the HPLC method for the pigment analysis, extracted and analysed pigments via HPLC; MI parametrized and ran CHEMTAX calculations; MI and PF analysed the data and MI wrote the manuscript with input from all co–authors. All authors gave their final approval for publication.

Acknowledgments

We would like to thank Christian Werner and Achim Weigert for laboratory assistance. We are grateful to Simon Wright (Australian Antarctic Division, CSIRO) for providing the CHEMTAX software v. 1.95 and his useful advice and to Dorothee Langenbach, Institute for Botany, University of Cologne, for providing the peridinin standard. We thank András Abonyi and an anonymous reviewer for their thoughtful feedback and valuable comments on the manuscript. This study was supported by the Deutsche Forschungsgemeinschaft in the project DYNATLOSS (grant DFG FI-1548/6-1, STI-180/5-1, STO-1075/1-1) as part of DFG Priority Programme 1704 "DynaTrait".

This manuscript is dedicated to Winfried Lampert; he was always exploring new techniques and method developments in biology and chemistry for their applicability in limnoecology. He was using innovative and modern methods and instrumentation to explore conceptual aspects of ecophysiology and ecology during his entire career. Hence, he also was interested in

instruments helping to ease experimental protocols; for example, such as by using automated cell counter systems to measure the concentration and size distribution of phytoplankton serving as food in *Daphnia* experiments. We are sure that he would have been interested in modern developments of phytoplankton quantification by chemical and fluorescence methods and their integration in experimental and observational studies.

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Manuscript received: 16 February 2022
Revisions requested: 11 August 2022
Revised version received: 20 November 2022
Manuscript accepted: 04 December 2022

The pdf version of this paper includes an electronic supplement
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20230414-122309
A2239/32525/C186C-AF5