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Integrating pigment and fatty acid profiles for enhanced estimation of seston community composition

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Abstract

Climate change, nutrition pollution, and land use alterations influence the primary production of lakes. While light-microscopy counting remains the standard for estimating phytoplankton community composition, its expense and time-consuming nature necessitate cost-effective alternatives for seston analysis. Furthermore, estimating the contribution of seston constituents other than primary producers, or non-algal particles, is not possible with light-microscopy counting. Biotracer approach using computational methods and chemotaxonomic biomarkers such as carotenoid pigments and fatty acids have been used as an alternative in seston analysis when species-level taxonomy is not required. However, a comprehensive testing of how well carotenoid and fatty acids can be used in estimating a wide range of seston phytoplankton communities using different estimation methods is lacking. To assess the accuracy of a suite of state-of-the-art biotracer-based computational methods, namely CHEMTAX, FASTAR (Fatty Acid Source-Tracking Algorithm in R), MixSIAR, and QFASA (Quantitative Fatty Acid Signature Analysis), lake water samples were collected in 2016, 2018, 2019, 2020, and 2021 for seston composition analysis in a boreal eutrophic lake with light-microscopy counting serving as the reference for seston composition. Absolute errors between the biotracer-based estimates were calculated to evaluate method performance. A small laboratory experiment to assess the reliability of estimating the contribution of non-algal particles using the computational methods with fatty acids was also conducted. The closest alignment to light-microscopy counting in terms of absolute error was achieved when both carotenoids and fatty acids were used together in the QFASA method. For CHEMTAX, FASTAR, and MixSIAR, using carotenoids alone produced the closest results. Additionally, the estimation methods accurately assessed the proportion of non-algal particles in the seston when using fatty acid profiles, a capability not possible with light-microscopy counting. Our findings demonstrate that the biotracer approach provides a viable and cost-effective alternative to light-microscopy counting when group-level

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information of phytoplankton community composition suffices. Furthermore, we show that non-algal particles can be effectively estimated together with phytoplankton when using fatty acids.

INTRODUCTION

Primary production by autotrophic microbes in seston forms the basis for the functioning of aquatic food webs (Underwood & Kromkamp, 1999). Seston consists of phytoplankton that encompasses prokaryotic cyanobacteria and various groups of eukaryotic phytoplankton. In addition to phytoplankton, seston contains heterotrophic protozoa and bacteria, as well as detritus from aquatic and terrestrial origins. The assessment of phytoplankton community and, more widely, seston composition is important in understanding the changes happening in aquatic ecosystems in response to environmental changes (Domis et al., 2014; Reynolds, 2006; Senar et al., 2021; Taipale, Vuorio, et al., 2016).

The predominant method for estimating phytoplankton communities involves using a light microscope to identify different species based on their morphological characteristics (Utermöhl, 1958). While species-level accuracy is achieved with morphology-based counting, estimates can vary significantly between analysts (Abad et al., 2016; Vuorio et al., 2007), and moreover, the method is time consuming (Culverhouse et al., 2003). Furthermore, there is evidence suggesting that the preservation method and duration can have significant effects on estimates obtained through light-microscopy counting as species-specific cell swelling, shrinking, or breakage has been observed (Bergkemper & Weisse, 2017; Menden-Deuer et al., 2001; Zarauz & Irigoien, 2008). Additionally, seston contains nano- and picoplankton (Callieri, 2008), microorganisms such as bacteria (Seymour et al., 2017), and organic detritus (Pace et al., 2004), all contributing to the basal aquatic food web. These constituents are not usually included in the monitoring efforts as their morphological identification and quantification are cumbersome (Havskum et al., 2004; McManus & Katz, 2009). Due to the time-intensiveness and shortcomings of light-microscopy counting, alternative methods have been developed for phytoplankton community estimation with biomarker and molecular approaches (Hering et al., 2018; Mackey et al., 1996; Strandberg et al., 2015; Vuorio et al., 2020).

Biomolecules used as chemotaxonomic biomarkers (hereafter biotracers) offer a cost-effective approach for the high-throughput estimation of phytoplankton community composition of water samples with the help of computational methods (Mackey et al., 1996; Strandberg et al., 2015). The approach is based on biotracer profiles that are unique to phytoplankton phyla or class (i.e., phytoplankton groups). Photosynthetic pigments (i.e., chlorophylls and carotenoids) that are produced by autotrophic phytoplankton can be analyzed from seston samples with high-performance liquid chromatography (HPLC) and can be used as biotracers due to many pigments being phytoplankton group-specific (Mackey et al., 1996). Fatty acids (FAs) of seston samples analyzed with gas chromatogram–mass spectrometry (GC–MS) can also serve as biotracers for estimating the proportions of phytoplankton groups. Similar to pigments, FA profiles are unique to phytoplankton groups (Taipale et al., 2013). Importantly, FAs also enable the estimation of additional seston constituents such as bacteria (Carvalho et al., 2014; Dijkman et al., 2010; Dijkman & Kromkamp, 2006; Strandberg et al., 2015). Iso- and anteiso-branched FAs are effective biomarkers for bacteria (Dijkman & Kromkamp, 2006; Taipale et al., 2015), whereas heterotrophic bacteria do not contain pigments. Furthermore, allochthonous particulate organic matter such as reed and deciduous tree leaf litter can be characterized with long-chain saturated fatty acids (LSAFA) (Taipale et al., 2015). The viability of FAs as biotracers in the estimation phytoplankton community composition of seston samples has been shown to align with both pigment-based estimation and light-microscopy counting estimates (Dijkman & Kromkamp, 2006; Strandberg et al., 2015). Peltomaa et al. (2023) found that simultaneous use of FAs, carotenoids, and sterols produced the highest possible chemotaxonomic resolution achievable with biotracers, and suggested that phytoplankton composition estimates could be improved with including all three biomolecules compared with only carotenoids or FAs. Using multiple classes of biotracers simultaneously helped to avoid overlapping biotracer profiles, as only few biotracers are group-specific. While sestonic sterol concentration is usually relatively low (Taipale, Vuorio, et al., 2019), carotenoids and FAs are present at higher

concentrations, and are thus practical for seston phytoplankton community estimation.

A common computational biotracer approach to estimate phytoplankton community composition is using pigment data in CHEMTAX, a program for estimating class abundances from chemical markers (Mackey et al., 1996). This method is based on pigment-to-chlorophyll *a* ratios that are unique to phytoplankton groups, and it gives quantitative estimates of biomass at phytoplankton group level, also accounting for nano- and picoplankton (Wright et al., 2009). While CHEMTAX is commonly employed in marine contexts, its applicability in freshwater settings has been demonstrated, producing estimates aligning with estimates derived from light-microscopy counting (e.g., Descy et al., 2000; Lauridsen et al., 2011; Sarmento & Descy, 2008). CHEMTAX has also been used as an estimation method with FAs (e.g., Cañavate et al., 2019; Carvalho et al., 2014; Dijkman et al., 2010; Dijkman & Kromkamp, 2006). Recently, computational methods created specifically for FA-based estimation have been employed for estimating proportional phytoplankton community compositions. Fatty Acid Source-Tracking Algorithm in R (FASTAR) (Galloway et al., 2015), a Bayesian estimation method, has been effectively used to estimate the phytoplankton community composition in freshwater (Strandberg et al., 2015) and estuary (Cañavate et al., 2019) systems. Likewise, a numerical optimization method Quantitative Fatty Acid Signature Analysis (QFASA) (Iverson et al., 2004) has been used to estimate freshwater seston community composition (Keva et al., 2023). R package MixSIAR (Stock et al., 2018) is the current state-of-the-art framework of Bayesian estimation methods for stable isotope-based estimation and claims an improvement over the algorithm used in FASTAR. MixSIAR has been previously employed using FAs as biotracers (e.g., Guerrero & Rogers, 2020; Litmanen et al., 2020) but it has not yet been applied in seston community composition analysis.

Although the biotracer approach lacks the taxonomic resolution of light-microscopy counting, group level information of phytoplankton community composition can be sufficient in many studies. For example, considering the effects of eutrophication or browning in lakes from the perspective of nutritional quality of phytoplankton to consumers it is often enough to distinguishing the prevalence of the phytoplankton groups that synthesize important biomolecules such as long-chain polyunsaturated fatty acids (LC-PUFA) (e.g., cryptophytes, diatoms, dinoflagellates, and golden algae) opposed to other phytoplankton groups that can be deficient in them (e.g., cyanobacteria and green algae) (Taipale et al., 2013). Generally, eutrophication increases the total biomass of phytoplankton, but cyanobacteria often dominate under eutrophic conditions (Ptacnik et al., 2008), at the expense of high-quality phytoplankton groups (Taipale, Vuorio, et al., 2019). Conversely, browning leads to changes in the light and chemical conditions that may reduce primary production and nutritional quality of lake seston by favoring heterotrophic production (Blomqvist et al., 2001; Forsström et al., 2013; Karlsson et al., 2015; Taipale, Vuorio, et al., 2016).

Currently, systematic testing of how well carotenoids and FAs can estimate a wide range of seston phytoplankton communities using different estimation methods is lacking. Thus, we set out to evaluate the effectiveness of the biotracer approach in estimating the phytoplankton composition of seston on a group level. To achieve this, we monitored the seston composition of a boreal eutrophic lake with occasional cyanobacterial blooms (Lake Vesijärvi, southern Finland), and conducted light-microscopy counting and biomolecule analyses from the same lake water samples. We used two types of biotracers, namely carotenoids and FAs, to estimate the phytoplankton community composition of seston using four different contemporary computational estimation methods (CHEMTAX, FASTAR, MixSIAR, and QFASA) and compared the results with light-microscopy counting estimates. To our knowledge, the present study is the first to simultaneously employ these two biotracers, aiming to enhance phytoplankton composition estimates. Our distinct focus was on detecting cyanobacteria blooms and identifying nutritionally high-quality phytoplankton taxa in the lake water samples. Additionally, we investigated how FAs could be used as biotracers to estimate the proportion of terrestrial and aquatic detritus as well as bacteria in seston, a task not achievable with light-microscopy counting.

MATERIALS AND METHODS

Field sampling

We collected lake water and seston samples in 2016, 2018, 2019, 2020, and 2021 from eutrophic Lake Vesijärvi, Lahti, southern Finland, between June and October. Lake Vesijärvi has been extensively studied, and was also a subject of many different restoration efforts in recent decades (Salonen et al., 2020, 2023). Integrated samples were collected with a 1-m-long water sampler (Limnos, 7 L) from the surface down to a depth of 10 m (0–10 m). The water was collected into an 80-L plastic bucket. The phytoplankton samples for light microscopy analyses were placed in 100 mL clear polyethylene terephthalate bottles or 100 mL dark brown glass bottles and preserved immediately with acid Lugol's iodine solution (0.5 mL per 100 mL), and then stored in a

refrigerator. The water samples for FA and carotenoid analysis were pre-sieved through a 250-μm mesh to remove larger zooplankton and stored in ice in 1 or 3 L covered polyethene bottles before further processing. FA samples were collected in all five sampling years with a total of 21 sampling dates matching light-microscopy counting, whereas carotenoids were collected only in 2018 and 2021 with a total of 9 sampling dates.

Lipid extraction

For FA analysis, from each sample 100–250 mL of lake water was filtered onto a water filter (Whatman Cellulose Nitrate Membrane, 0.2 μm nominal pore size, 47 mm diameter) and for carotenoid analysis, 500–1500 mL of lake water was filtered onto a glass fiber filter (Whatman GF/C, 1 μm nominal pore size, 47 mm diameter) in the dark. Visible zooplankton was removed from the filters with tweezers under a stereo microscope. The filters were kept at −80°C before freeze-drying and stored in Kimax-tubes in 2:1 chloroform:methanol solution at −20°C before extraction. Lipids from the freeze-dried seston (~1 mg) were extracted with chloroform:methanol:water in a 8:4:3 ratio (Folch et al., 1957). The samples were sonicated for 10 min and centrifuged, after which the lower phase was transferred into a new tube and evaporated to dryness under nitrogen flow. The FAs were redissolved into 1 mL of toluene, while the carotenoids were redissolved in 200 μL of acetone.

FA analysis

FAs were methylated using mild sulfuric acid in methanol (Taipale, Hiltunen, et al., 2016). Methyl esterified samples were analyzed on a Shimadzu GC–MS-QP2010 Ultra (Shimadzu, Kyoto, Japan) with helium as a carrier gas. The samples from 2016 were run with the instrument equipped with an Agilent DB-23 (Santa Clara, CA, USA) column (30 m × 0.25 mm × 0.25 μm) using the same temperature program as Taipale, Hiltunen, et al. (2016). The samples from 2018 and 2019 were run with the instrument equipped with a Zebron ZB-FAME (Torrance, CA, USA) column (30 m × 0.25 mm × 0.20 μm) using the same temperature program as Peltomaa et al. (2019). The samples from 2020 and 2021 were run with the instrument equipped with an Agilent DB-23 (Santa Clara, CA, USA) column (60 m × 0.25 mm × 0.25 μm) using the same temperature program as Taipale et al. (2021). Each column had a 5-m guard column attached. FAs from all different runs were identified by the retention times, and specific ions, which were also used for

quantification (Taipale, Hiltunen, et al., 2016). FA concentrations were calculated using calibration curves based on known standard solutions (15, 50, 100, and 250 ng) of a fatty acid methyl ester (FAME) standard mixture (GLC standard mixture 566c, Nu-Chek Prep, Elysian, MI, USA). The Pearson correlation coefficient was >0.99 for each FA calibration curve. The FA concentrations were then normalized to create FA profiles for each sample.

Carotenoid analysis

Carotenoids were analyzed by reversed-phase liquid chromatography using Shimadzu 30-series HPLC system (Shimadzu, Kyoto, Japan) consisting of an LC-30 AD pump, an autosampler (SIL-30 AC), an online degasser, a column oven (CTO-20 AC), and a photodiode array detector (PDA; SPD-M20A). A final volume of 10 μL for 2018 samples and 20 μL for 2021 samples was used for injection into HPLC. Carotenoids were separated on a YMC Carotenoid column (250 × 4.6 mm C30, 5 μm) coupled to a 10 × 4 mm C30 guard column (YMC Co., Kyoto, Japan) using mobile phases of MeOH and MTBE. The samples were run using the same chromatographic method as Peltomaa et al. (2023). Carotenoids were identified by the retention times and absorption spectra, and quantified by internal standard (Trans-β-Apo-8′-carotenal [Sigma Aldrich]) calibration based on response factors as described by Peltomaa et al. (2023). The carotenoid concentrations were then normalized to create carotenoid profiles for each sample.

Light-microscopy counting

Phytoplankton light microscopy analyses were performed according to Utermöhl technique (Utermöhl, 1958) using an inverted light microscope following the EN 15204 standard (2006) Phytoplankton were identified to species level by morphology where possible. Phytoplankton abundances were converted to biomass by applying geometric formulas, assuming a phytoplankton density equivalent to water (1 g cm^{-3}). Carbon content was determined using the Menden-Deuer et al. (2001) method and from that data the relative contribution of each phytoplankton group was calculated. It is important to note that the determination of non-algal particle (NAP) proportions is not possible through light-microscopy counting. For the purpose of our study, light-microscopy counting estimates serve as the reference against which the other computational methods are evaluated. For some sampling days we used light-microscopy counting estimates from the same week derived from an open-source

phytoplankton database of the Finnish Environment Institute. Notably, the aforementioned samples were collected at depths of 0–4 m or 0–6 m on the basis of secchi disk visibility, in contrast to the sampling depth of 0–10 m in the samples that we processed.

Biotracer-based estimation methods

All four biotracer-based estimation methods that we used in this study follow the principle of a mixing model, where the composition of seston in a sample (mixture) is computationally estimated based on all possible constituents in the seston (sources). The seston sample is presented as a biotracer profile. The source library is formed by grouping (to phylum or class level) the seston constituents that are represented by species-specific biotracer profiles derived from laboratory analysis of multiple samples, embracing the inherent variability within each phytoplankton group. All biotracer-based estimations were conducted using the R statistical software (R Core Team, 2023; version 4.3.1).

We constructed five different versions of the seston source library to study the performance of different biotracers in phytoplankton and seston composition estimation. Three versions were created on the basis of a previous study (Peltomaa et al., 2023): one using only carotenoids (CAR), another using only FAs, and a third incorporating both simultaneously (CAR + FA). All versions of the source library consisted of six phytoplankton groups, namely, cryptophytes (crypto), cyanobacteria (cyano), diatoms (diatom), dinoflagellates (dino), golden algae (golden), and green algae (green). As seston consists of more than just phytoplankton, we created two more versions of the source library that included three NAP groups in it, namely, reed (reed powder), tPOM (terrestrial particulate organic matter, including deciduous tree litter), and tPOMb (terrestrial particulate organic matter through bacterial loop, i.e., leaf litter incubated with aquatic bacteria). These groups were assumed to contain negligible amounts of carotenoids compared with FAs, thus creating the last two versions where NAPs were included when either FAs or carotenoids and FAs were used (FA/NAP and CAR + FA/NAP). FAs 14:0 and 18:0 were excluded from the source library due to contamination in many of the samples. Overall, a total of 38 FAs and 18 carotenoids were included in the source library (Appendix S1: Table S1). Raphidophytes were omitted from our source library due to their infrequency in eutrophic lakes such as Lake Vesijärvi (Strandberg et al., 2020). This decision is supported by light-microscopy counting, which revealed only minor amounts of raphidophytes in five out of our 21 samples.

To visually illustrate the distinct characteristics of each constituent in the source library, we conducted a principal components analysis (PCA) using the prcomp function from the stats package in R.

We employed CHEMTAX (Mackey et al., 1996), a program for estimating class abundances from chemical markers, implemented in R as part of the limSolve package (Soetaert et al., 2009). Specifically, we used the xsample function (Van den Meersche et al., 2009), employing Markov chain Monte Carlo (MCMC) algorithms to uniformly sample the feasible region of constrained linear problems. The use of CHEMTAX is typically associated with carotenoids and other pigments, and we included it in our study due to its widespread use in the scientific community. However, it is important to note that CHEMTAX does not take the variability in the source library into account. While it uses mean values for each constituent group based on the available data, it does not consider the range or diversity within each group. Although CHEMTAX has the capability to provide quantitative estimates with the help of chlorophyll a concentrations, we standardized all chlorophyll a: biotracer ratios to 1 to ensure compatibility with other methods. In our estimation process, a random sample from the posterior probability distribution of the model parameters, with a sample size of 3000, was saved for subsequent analysis.

The Fatty Acid Source Tracing Algorithm for R FASTAR (Galloway et al., 2015) has been used in phytoplankton community composition estimation before (Strandberg et al., 2015). Given its established utility, we incorporated FASTAR into our study. This method uses Bayesian inference, and for approximation of the posterior probability distribution of the model parameters, we used the Stable Isotope Analysis in R (SIAR) algorithm developed by Parnell et al. (2010), producing a posterior sample of sample size 3000.

MixSIAR (Stock et al., 2018) is the current pinnacle of Bayesian estimation methods for stable isotope-based estimation and claims an improvement over the SIAR algorithm used in FASTAR. We used the MixSIAR framework to run the model with both residual error and process error ("model 4"; Stock & Semmens, 2016) to generate a posterior sample of sample size 3000.

QFASA is a numerical optimization-based method designed for estimating the diets of marine mammals (Iverson et al., 2004). QFASA has been found to excel in zooplankton diet estimation (Litmanen et al., 2020) and it has been used in seston composition estimation in a previous study (Keva et al., 2023), so we included it in the study. We used an R implementation, QFASAR (Bromaghin, 2017), where the chi-squared metric (Stewart, 2017) was used as the objective function. It is

important to note that QFASA, in contrast to Bayesian methods, provides a point estimate minimizing the objective function. To facilitate comparability with the Bayesian methods, we modified the QFASAR code. Specifically, we generated a bootstrapping sample of size 3000, treating it as a posterior-like probability distribution. This approach allows us to simulate a form of uncertainty estimate for the QFASA solution and enhances the comparability between the methods, despite the inherent differences in their output structures.

Comparison and statistics

In our comparative analysis of various estimation methods (CHEMTAX, FASTAR, MixSIAR, and QFASA) and different combinations of biotracers and seston constituents included (FA, FA/NAP, CAR, CAR + FA, and CAR + FA/NAP), the primary evaluation metric was the absolute error, representing the absolute difference between the reference proportions and the median of each posterior sample generated by the various estimation methods for each seston constituent. For comparability between the reference proportions obtained through light-microscopy counting and estimated proportions from the estimation methods, the three NAP groups were not included in the comparisons. This exclusion was necessary because light-microscopy counting cannot determine the amount of NAP, which are a part of the seston composition. Instead, the NAP proportions were removed from the estimates and the proportions of phytoplankton groups were normalized to facilitate a meaningful comparison with the reference proportions.

We calculated mean absolute errors (MAEs) for each phytoplankton group individually, but also for all phytoplankton groups collectively, providing an assessment of overall estimation accuracy. Additionally, we examined MAEs for high-quality phytoplankton groups, including cryptophytes, diatoms, dinoflagellates, and golden algae, to evaluate the effectiveness of the methods in assessing nutritionally high-quality phytoplankton. A threshold of 0.1 was established as an acceptable level of MAE. We also compared the performance of the estimation methods in terms of MAE with the versions of the source library with or without NAP (FA compared with FA/NAP, and CAR + FA compared with CAR + FA/NAP).

We assessed whether the estimation methods consistently identified the dominant phytoplankton group, which we refer to as the dominant constituent identification frequency (DCIF). In essence, we aimed to determine whether the estimation methods reliably detected the most abundant phytoplankton group in the reference proportions, irrespective of the specific proportion, by calculating the frequency of highest proportion attributed to the same phytoplankton group as in the reference proportions. These tests were specifically conducted for cyanobacteria, and the high-quality algae group treated as a single entity in our analysis.

In our analysis, we also assessed the consistency of the estimation methods by calculating the frequency with which the reference proportions fell within the estimated range at both the 68% (median $\pm 1\sigma$) and 95% (median $\pm 2\sigma$) credible intervals of the estimation method. Furthermore, we calculated the frequency of under- or overestimation for both credible intervals. This evaluation of consistency is paramount because it provides insights into the reliability and robustness of the estimation methods in quantifying uncertainty. It serves as a critical aspect of the assessment, ensuring that the methods consistently provide results that not only estimate proportions but also convey the associated level of confidence or uncertainty. This comprehensive approach allows for a more informed understanding of the methods' performance, addressing the essential aspect of quantifying and correctly characterizing uncertainty in the estimation process.

All statistical analyses and figure generation were executed using the R statistical software (R Core Team, 2023; version 4.3.1).

Assessing NAP estimation performance

In addition to evaluating the performance of different computational methods against the reference proportions obtained by light-microscopy counting, we conducted a small laboratory study to test how accurately the estimation methods could estimate the proportion of NAPs when utilizing the version of the source library with only FAs and including NAP (FA/NAP). A gradient was created with 10%, 30%, 50%, 70%, and 90% of alder leaf extract (incubated in natural lake water) of sample carbon, representing tPOM and tPOMb, with the remaining part being green algae *Chlamydomonas reinhardtii* P.A. Dangeard. The correct carbon contribution of components was determined with a turbidity meter. Subsequently, FA analysis was conducted similar to section 0 and the contributions were estimated with each estimation method utilizing FA/NAP. The estimate medians were then compared to the known proportions to calculate absolute errors.

RESULTS

The estimation accuracy of the proportions of the different constituents in the seston samples varied greatly with

the MAE averaged across all groups ranging from 0.065 to 0.180 depending on the used method and biotracers, and if NAP were included (Figure 1a; Appendix S1: Table S2). The estimation was most accurate when using QFASA which utilized carotenoids alone (CAR) or both carotenoids and FAs and included CAR + FA/NAP in the estimation (MAE of 0.065). Overall, QFASA performed most accurately with all of the five different biotracer combinations and NAP inclusion in terms of MAE (FA: MAE = 0.127, FA/NAP: MAE = 0.132, CAR: MAE = 0.065, CAR + FA: MAE = 0.071, and CAR + FA/NAP: MAE = 0.065). Thus, we chose to visually differentiate between the different biotracer and constituent combinations using QFASA illustrated in Figure 2 while the other methods can be found in the supplementary materials (Appendix S1: Figure S1) Carotenoids produced the most accurate results for CHEMTAX (MAE = 0.079), FASTAR (MAE = 0.076), and MixSIAR (MAE = 0.070). The estimation of phytoplankton proportions was found to be particularly challenging when relying solely on FAs and FA/NAP, with the average MAE ranging from 0.127 to 0.180, staying above the set threshold of 0.1 (Figure 1a). The highest errors with FAs alone (FA and FA/NAP) ranged from 0.623 to 0.941 while with carotenoids (CAR, CAR + FA, and CAR + FA/NAP) the highest errors ranged from 0.258 to 0.648 (Figure 3; Appendix S1: Table S3).

Despite QFASA producing the most accurate results in terms of MAE, it was found to have the least estimate consistency with only 0.09–0.30 of proportions obtained from light-microscopy counting falling within the estimated 68% CI and 0.22–0.57 within the 95% CI (Figure 4). On the other hand, FASTAR using FAs and including NAP (FA/NAP) produced the most consistent
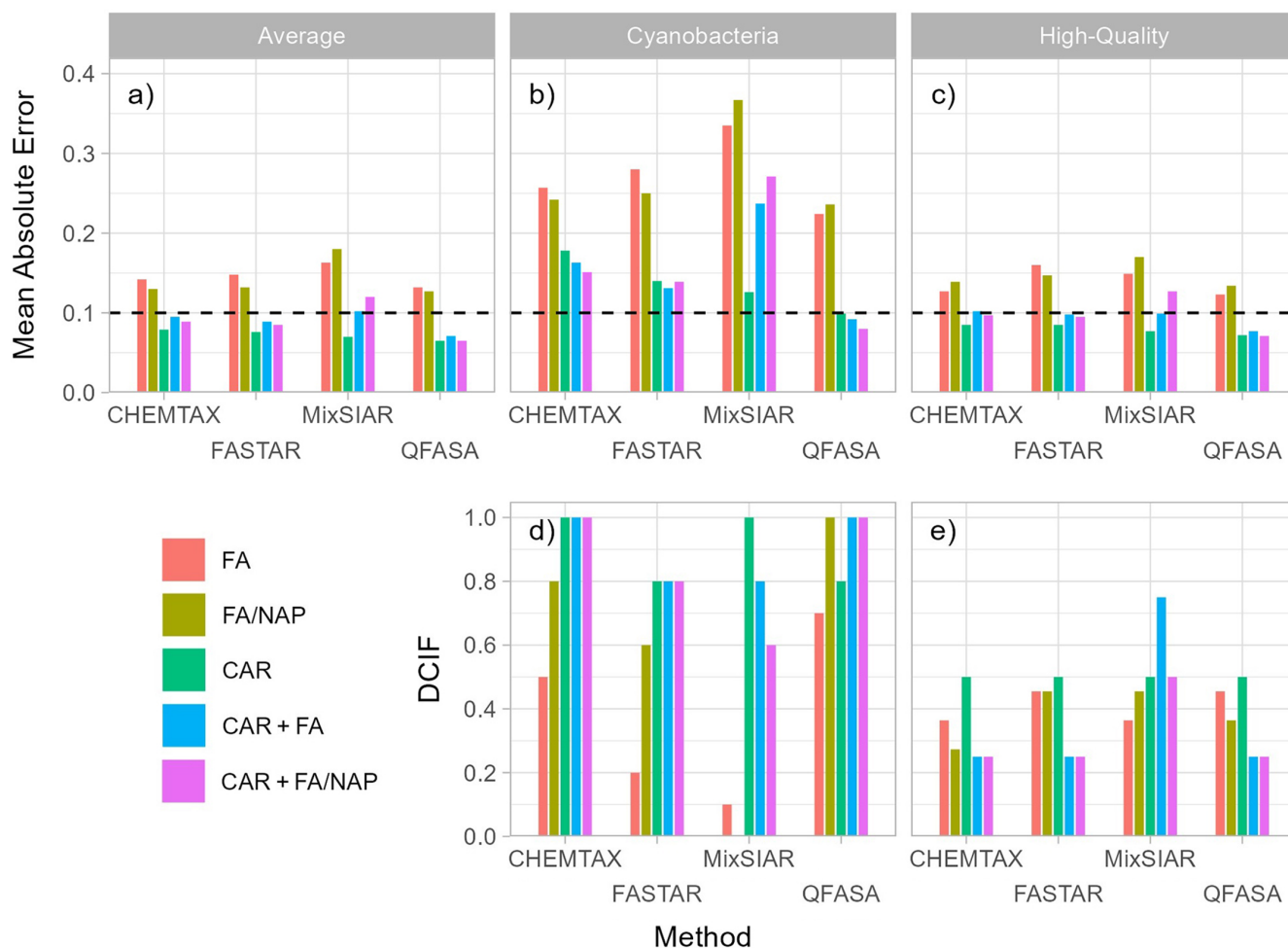
FIGURE 1 Mean absolute errors (MAEs) for (a) all algae groups, (b) cyanobacteria, and (c) high-quality algae, and dominant constituent identification frequency (DCIF) for (d) cyanobacteria, and (e) high-quality algae, for each biotracer and non-algal particles (NAP) combination (represented by color) and estimation method. The dotted line indicates the threshold for good performance set at 0.1 in MAE figures (a–c). High-quality algae refers to the nutritionally high-quality phytoplankton groups (cryptophytes, diatoms, dinoflagellates, and golden algae) for herbivorous consumers. Numeric values can be found in Appendix S1: Table S2. CAR, carotenoids; FA, fatty acid; FASTAR, Fatty Acid Source-Tracking Algorithm in R; QFASA, Quantitative Fatty Acid Signature Analysis.
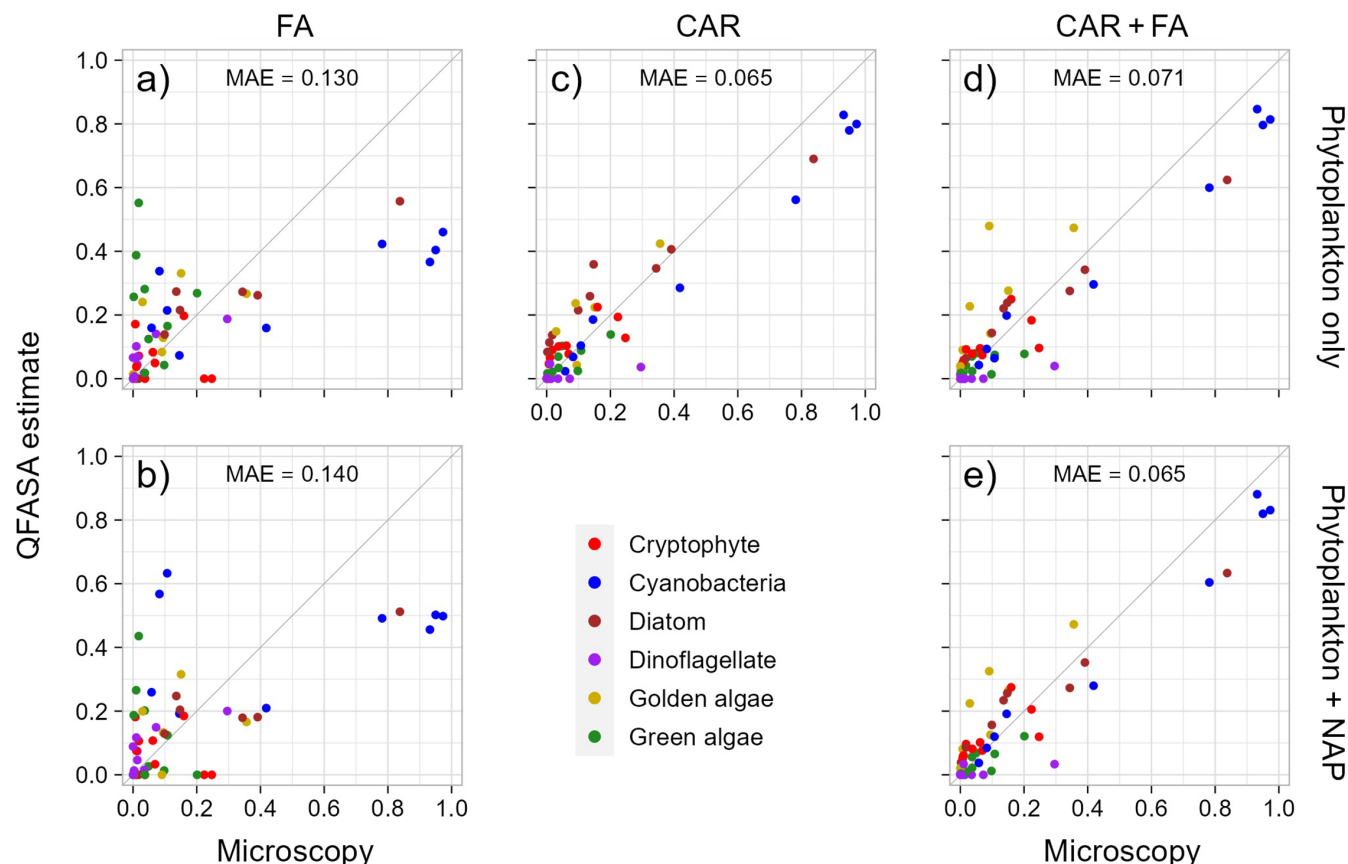
FIGURE 2 Relationship between Quantitative Fatty Acid Signature Analysis (QFASA) estimates and reference proportions obtained through light-microscopy counting for all phytoplankton groups using (a, b) the fatty acids (FAs), (c) carotenoids (CAR), and (d, e) CAR + FA biotracers, nine field samples (a, c, d) with phytoplankton alone in the estimation and (b, e) with the inclusion of non-algal particles (NAPs) in the estimation. Each phytoplankton group is represented by a distinct color. The mean absolute error (MAE) for each combination of biotracer and NAP is indicated at the top of each graph. The diagonal light gray line represents the ideal one-to-one mapping between estimates and reference proportions. See Appendix S1: Figure S1 for results obtained using other estimation methods.

estimates, with 0.41 of light-microscopy-counting proportions found within the 68% CI and 0.79 found within the 95% CI. The most consistent estimates were achieved for both CI ranges with FAs and NAP included (FA/NAP) with FASTAR (0.41 and 0.79), and QFASA (0.30 and 0.57), but without NAP (FA) for MixSIAR (0.29 and 0.57). For CHEMTAX using both carotenoids and CAR + FAs lead to most consistent estimates (0.33 and 0.69). FA-based estimation resulted in least consistency for CHEMTAX (0.29 and 0.56), and utilizing carotenoids and FAs together (CAR + FA) resulted in least consistency for FASTAR (0.26 and 0.54), and the 95% CI for QFASA (0.22). Carotenoid-based estimation (CAR) provided the least consistency for both CI's for MixSIAR (0.13 and 0.35), and for the 68% CI for QFASA (0.09). When the light-microscopy counting estimate did not fall within the CI's, the estimate was an overestimation rather than underestimation with the exception of FASTAR using FAs and NAP included (FA/NAP).

Cyanobacteria and green algae

The estimation of the cyanobacteria proportion in the seston samples proved difficult for most methods with the MAE ranging between 0.080 and 0.367 (Figure 1b; Appendix S1: Table S2). The most accurate estimates of cyanobacteria proportion were achieved with QFASA when both carotenoids and FAs were used, and NAP was included (CAR + FA/NAP; MAE = 0.080). The performance was more dependent on the biotracers than the method, as MAE ranged for CHEMTAX between 0.151 and 0.257, for FASTAR between 0.131 and 0.280, for MixSIAR between 0.126 and 0.367, and for QFASA between 0.080 and 0.236. Using FAs alone in the estimation of the cyanobacteria proportion the estimates were always less accurate (MAE for all methods ranging from 0.224 to 0.367) compared with the same method's estimate that included carotenoids (MAE for all methods ranging from 0.080 to 0.271). Only QFASA using
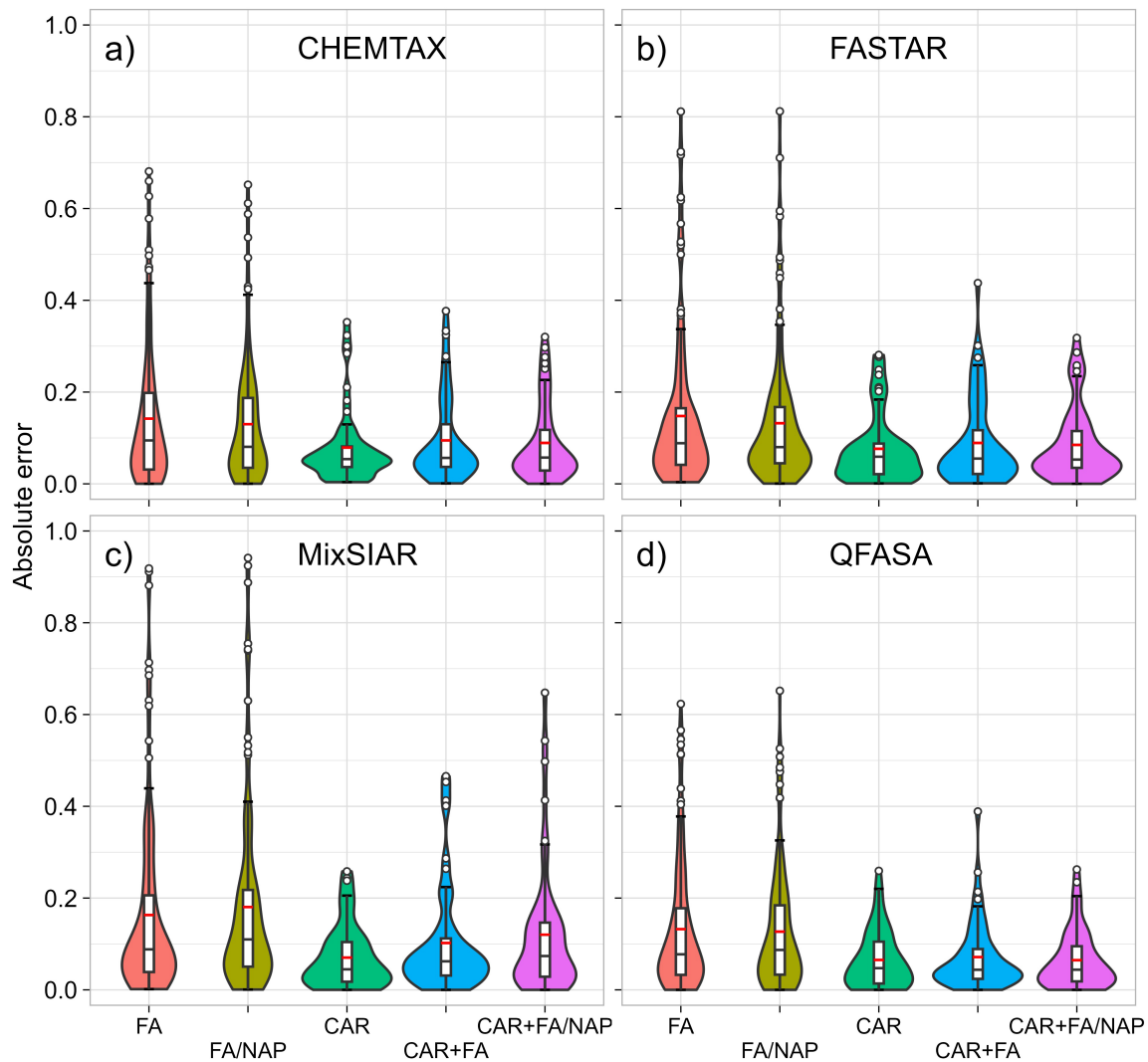
FIGURE 3 The absolute errors compared with reference proportions obtained through light-microscopy counting visualized using violins plots for different methods using fatty acids (FAs), fatty acids with the inclusion of non-algal particles (NAPs) in the estimation (FA/NAP), carotenoids (CAR), carotenoids and fatty acids simultaneously (CAR + FA) and carotenoids and fatty acids simultaneously with the inclusion of NAP in the estimation (CAR + FA/NAP). The vertical axis shows the absolute errors of the estimated source proportions in seston. The black and red horizontal lines, the box, and the whiskers inside the violin represents the median, the mean, the interquartile range (IQR), and the median $\pm 1.5 \times$ IQR, respectively. The white dots represent outliers. Distribution statistics are presented in Appendix S1: Table S3.

carotenoids with or without FAs achieved the desired performance with a level below the 0.1 threshold (Figure 1b). The most accurate estimates of green algae proportion were achieved for all methods when carotenoids were used (CAR; MAE ranging between 0.030 and 0.049, CAR + FA; MAE ranging from 0.041 to 0.117, and CAR + FA/NAP; MAE ranging from 0.031 to 0.087) compared with using FAs alone (FA; MAE ranging from 0.086 to 0.231, and FA/NAP; MAE ranging from 0.060 to 0.233).

The estimated consistencies for cyanobacteria proportion ranged between 0.095 and 0.444 for the 68% CI, and between 0.222 and 0.571 for the 95% CI (Appendix S1:

Table S4). In the 68% CI over half of the estimates were underestimated with the exception of CHEMTAX using FAs with NAP included (FA/NAP) (0.476). Contrarily, the proportion of green algae was mostly overestimated when NAP is included in the estimation but much less overestimated when NAP was not included. For example, the estimated consistency of 68% CI QFASA using FAs overestimated 71.4% of green algae proportion when NAP was included (FA/NAP) but overestimated only 23.8% when NAP was not included.

When "cyanobacteria" was the primary component, CHEMTAX, FASTAR, and QFASA could identify the primary component regularly with the dominant

FIGURE 4 Estimate consistencies for 68% and 95% CI. The figure shows the frequencies of finding the reference proportions in the indicated CI ("within range"), and for the reference proportion falling under the CI thus being overestimated by the estimation method ("overestimated"), or the reference proportion falling above the CI thus being underestimated by the estimation method ("underestimated"). CAR, carotenoids; FA, fatty acid; FASTAR, Fatty Acid Source-Tracking Algorithm in R; NAP, non-algal particle; QFASA, Quantitative Fatty Acid Signature Analysis.

component identification frequency (DCIF) ranging from 0.20 to 1.00 (Figure 1d). MixSIAR could not estimate the primary component correctly when relying solely on FAs (FA/NAP; DCIF = 0.00, and FA; DCIF = 0.10), but when carotenoids were included in the estimation DCIF ranged between 0.60 and 1.00 for all methods. The inclusion of NAP in the FA-based estimation (FA compared with FA/NAP) improved all the other methods (from DCIF = 0.50 to DCIF = 0.80 for CHEMTAX, from DCIF = 0.20 to DCIF = 0.60 for FASTAR, and from 0.70 to 1.00 for QFASA) except MixSIAR. The inclusion of NAP to the carotenoid and FA-based estimation (CAR + FA compared with CAR + FA/NAP) did not affect the method's performance (DCIF = 1.00 for CHEMTAX and QFASA, DCIF = 0.80

for FASTAR) other than decreasing MixSIAR's performance (DCIF = 0.80 to DCIF = 0.60).

High-quality phytoplankton

Estimation of the proportions of "high-quality phytoplankton" group (cryptophytes, dinoflagellates, diatoms, and golden algae) was generally easier for the methods than cyanobacteria proportion estimation evidenced with the MAEs ranging between 0.071 and 0.170 (Figure 1c; Appendix S1: Table S2). The best results for estimating high-quality phytoplankton group proportions were achieved using QFASA using carotenoids and FAs with NAP included in the estimation (CAR + FA/NAP;

MAE = 0.071). The most accurate estimates with other methods were produced with carotenoids alone (CAR; CHEMTAX: MAE = 0.085, FASTAR: MAE = 0.085, and MixSIAR: MAE = 0.077). Contrarily, the least accurate estimates were produced with only FAs (FA; CHEMTAX: MAE = 0.139, MixSIAR: MAE = 0.170, QFASA: MAE = 0.134, FA/NAP; FASTAR: MAE = 0.160). When using carotenoids and FAs the estimates were more accurate with NAP included (CAR + FA/NAP) for CHEMTAX (MAE = 0.097), FASTAR (MAE = 0.095), and QFASA (MAE = 0.071) but without NAP (CAR + FA) for MixSIAR (MAE = 0.099). The high-quality phytoplankton estimates met the 0.1 threshold only when carotenoids were used in the estimation (Figure 1c).

Cryptophyte proportion estimates generally had an error less than 0.1 with some exceptions, as MAE ranged from 0.055 to 0.119. The best estimates of cryptophyte proportions were produced with CHEMTAX, MixSIAR, and QFASA using carotenoids alone (CAR; MAE = 0.063, 0.062, 0.055, respectively) and FASTAR using the carotenoids and FAs (CAR + FA; MAE = 0.064). Relying solely on FAs in the estimation of the cryptophyte proportions the estimates were always less accurate (MAE for all methods ranging from 0.084 to 0.119) compared with the same method's estimate that included carotenoids (MAE for all methods ranging from 0.055 to 0.107). When the estimation included carotenoids, the proportion of cryptophytes was often overestimated in contrast to much less overestimation when using FAs alone (Appendix S1: Table S4).

The least errors across the estimation of the high-quality phytoplankton groups were in dinoflagellate proportions with MAEs ranging from 0.037 to 0.138. The poor performance of MixSIAR using FAs alone and including NAP (FA/NAP; MAE = 0.138) was an outlier and all other combinations had MAE of 0.087 or less. The proportion of dinoflagellates was often estimated within the 95% CI of CHEMTAX and FASTAR regardless of biotracers used, whereas with MixSIAR and QFASA the proportion was often overestimated with FAs alone (FA and FA/NAP) but underestimated when carotenoids were used.

Contrarily, diatom proportion estimates had the most MAE out of the high-quality phytoplankton groups (MAEs ranging from 0.045 to 0.183). Specifically, the estimation using FAs alone posed challenges as evidenced by MAEs ranging from 0.139 to 0.183. When carotenoids were included in the estimation the MAEs were considerably smaller (MAE ranging from 0.045 to 0.127). The proportion of diatoms was often estimated within the 95% CI of CHEMTAX, FASTAR, and QFASA, whereas with MixSIAR the proportion was often underestimated with FAs alone but overestimated when carotenoids were used.

The most accurate golden algae proportion estimates were achieved using carotenoids alone (CAR; MAE ranging from 0.056 to 0.087) with the exception of MixSIAR using the carotenoids and FAs (CAR + FA; MAE = 0.040). Contrarily to most phytoplankton groups, golden algae proportion estimates were more accurate when estimated using FAs alone compared with carotenoid and FA-based estimations with CHEMTAX and QFASA. The proportion of golden algae in the seston sample was often overestimated.

When the dominant component was among the "high-quality algae" group, most methods could not identify that regularly. The best performer was MixSIAR when carotenoids and FAs were used (CAR + FA; DCIF = 0.75; Figure 1e). For CHEMTAX, FASTAR, and QFASA the best performance was obtained using carotenoids alone (CAR; DCIF = 0.50). The poorest performance for CHEMTAX, FASTAR, and QFASA was obtained when using both carotenoids and FAs (CAR + FA and CAR + FA/NAP; DCIF = 0.25). For MixSIAR the poorest performance was obtained with FAs alone (FA; DCIF = 0.36). When carotenoids and FAs were used, there was no difference in performance whether NAP was included or not for CHEMTAX, FASTAR and QFASA (DCIF = 0.25), but the performance of MixSIAR decreased with NAP (CAR + FA; DCIF = 0.75, CAR + FA/NAP; DCIF = 0.50).

Assessing NAP estimation performance

The methods had differing success in estimating NAP and tPOM of the alder leaf extract test, with MAEs ranging from 0.107 to 0.385 for NAP. FASTAR performed best in terms of MAE (NAP MAE = 0.107). CHEMTAX NAP median estimates were overestimated for true value of 0.1, 0.3, and 0.5 and underestimated of true proportions 0.9 (MAE = 0.118) with absolute error ranging from 0.059 to 0.258 (Figure 5; Appendix S1: Table S5). CHEMTAX estimated that 46%–60% of NAP would be tPOM. FASTAR overestimated NAP for all true proportions but 0.9 with absolute error ranging from 0.010 to 0.166 (MAE = 0.107). FASTAR estimated that 83%–88% of NAP would be tPOM. MixSIAR consistently overestimated the proportion of NAP, resulting in the highest MAE (MAE = 0.385) with a range from 0.097 to 0.628. For example, the lowest median estimate for true proportion of 0.1 was 0.728 for NAP resulting in absolute error of over 0.6. MixSIAR estimated consistently that about 92% of NAP would be tPOM. QFASA overestimated the proportion of all true NAP proportions with absolute errors ranging between 0.026 and 0.201 (MAE = 0.128). QFASA estimated that, for the true proportion of 0.1, 86% of NAP
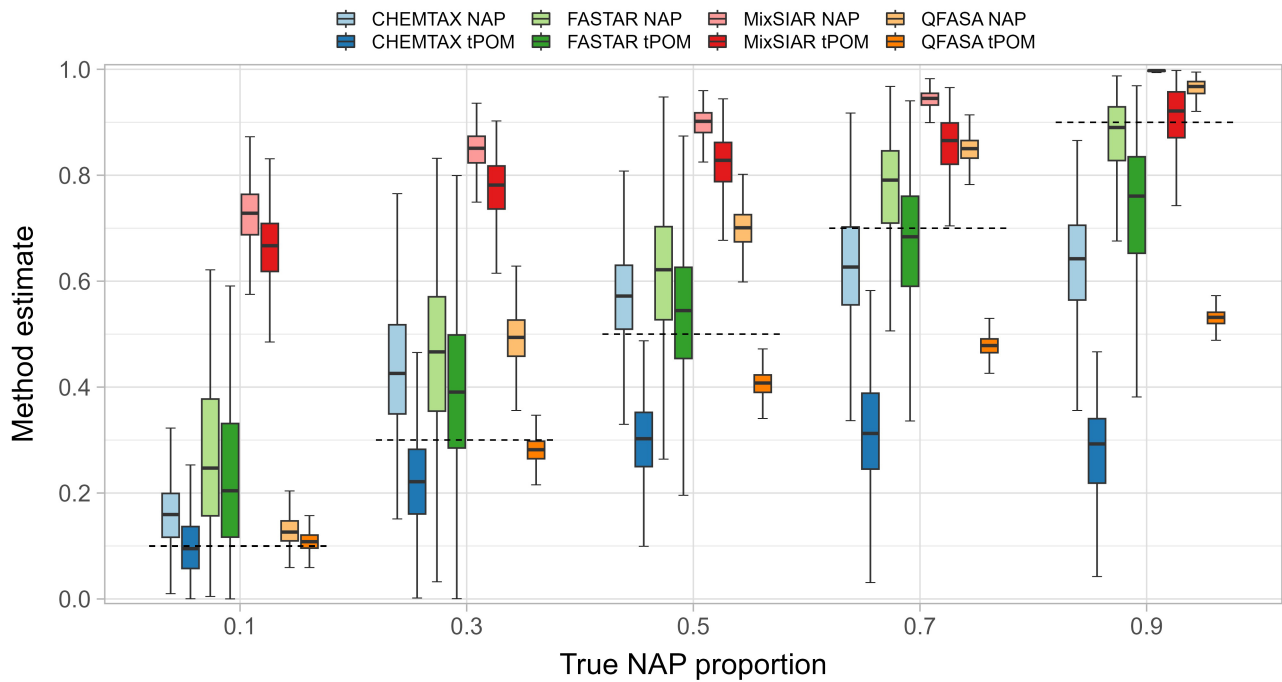
FIGURE 5 The performance of each estimation method to estimate the correct proportion of non-algal particle (NAP) and terrestrial particulate organic matter (tPOM). Notably tPOM is a fraction of NAP. Side by side are the estimated distributions of NAP and tPOM with different shades of the same color for each estimation method. The vertical axis shows the estimated source proportions in seston. The dotted horizontal lines represent the true proportion of NAP that the estimates are compared against. For each boxplot the horizontal line, the box, and the whiskers represents the median, the interquartile range (IQR), and the median $\pm\,1.5\times$ IQR, respectively. Outliers are not shown for clarity. Distribution statistics are presented in Appendix S1: Table S5. FASTAR, Fatty Acid Source-Tracking Algorithm in R; QFASA, Quantitative Fatty Acid Signature Analysis.

would be tPOM, while for the remaining true proportions, it estimated that approximately 57% of NAP would be tPOM.

The proportion of NAP was also estimated in the seston samples. See supplementary material for analysis (Appendix S1: Section S1).

PCA of source libraries

When solely utilizing FAs, some overlap among phytoplankton groups was observed (Figure 6). Notably, improved separation, especially between cyanobacteria and cryptophytes, was achieved when carotenoids (CAR) were used. The most distinct separation between different phytoplankton groups was achieved when both carotenoids and FAs were used (CAR + FA). Furthermore, the inclusion of NAP in the source library (FA/NAP and CAR + FA/NAP) resulted in a clear separation between detritus (tPOM and reed) and tPOM of bacterial origin from both phytoplankton and each other. However, tPOM and reed exhibited some overlap. Detailed loadings for the PCA can be found in the supplementary materials (Appendix S1: Figure S2, Table S6).

DISCUSSION

The combination of FAs and carotenoids allows for phytoplankton community composition estimates on group level that are close to light-microscopy counting estimates with computational estimation methods while simultaneously estimating the proportion of NAPin seston. In terms of MAE compared with light-microscopy counting, QFASA was the most accurate estimation method for estimating the composition of phytoplankton community when carotenoids and FAs were USED. When using carotenoids alone, CHEMTAX, FASTAR, and MixSIAR gave the most accurate estimates for each method with MAEs close to QFASAs.

Using solely FAs, contrary to a previous study (Strandberg et al., 2015), proved to be suboptimal for predicting phytoplankton composition in our samples due to considerable issues in correctly estimating high proportions of cyanobacteria and diatoms. This difference in performance could be due to the fact that cyanobacteria or diatom blooms were not observed in the study samples of Strandberg et al. (2015) while many of our samples contained more than 90% of cyanobacteria and up to 84% of diatoms. Our results
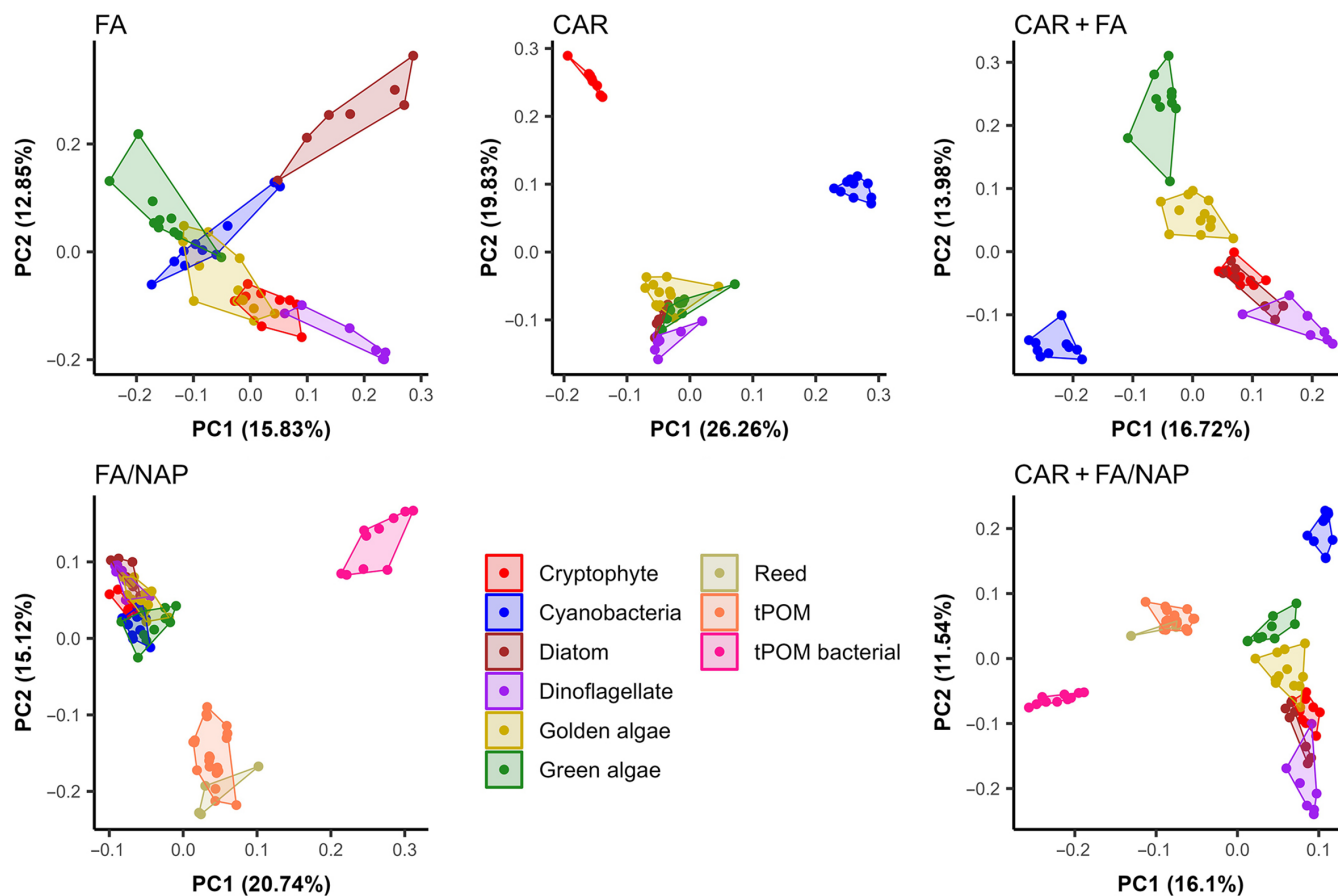
FIGURE 6 Principal components analysis (PCA) depicting the separation of phytoplankton groups and other constituents in the source library for each biotracer source library version: Fatty acids (FAs), carotenoids (CAR) or both (CAR + FA), along with versions including non-algal particles (FA/NAP and CAR + FA/NAP). The percentage of the variability explained by each principal component (PC) is indicated next to the respective PC. Loading arrows and detailed loading values for the PCA can be found in Appendix S1: Figure S2, Table S6.

suggest that accurately estimating these phytoplankton blooms is not estimation method dependent, but instead the challenge lies in the overlapping of certain FA biotracer profiles, hindering the clear separation between cyanobacteria, diatoms, and green algae. Los and Mironov (2015) have noted that cyanobacteria can be grouped to four different FA groups, one which lacks any PUFAs, and three other groups that differ in their 16 carbon monounsaturated FAs and 18 carbon PUFAs resulting in overlapping FA profiles with green algae and diatoms (Peltomaa et al., 2023). A possible explanation for the issue with diatoms lies in the dynamic nature of diatom FA profiles during blooms. As diatoms multiply rapidly, their FA content is different compared with the stationary phase of growth, thus leading to changes in the FA profiles (Leu et al., 2006; Taipale et al., 2020). Furthermore, there is evidence of changes in the FA profiles of diatoms caused by nutrient or light deficiency (Mekhalfi et al., 2014; Wacker et al., 2016).

Our results show that carotenoids separate the problematic groups much more clearly, and thus, the estimation methods produced more accurate estimates. This can be explained by many carotenoids being unique to phytoplankton groups. For example, echinenone, and myxo and aphanizophyll are dominant carotenoids in cyanobacteria while also being unique to cyanobacteria. However, not all phytoplankton groups have as distinct carotenoid profiles as cyanobacteria and, therefore, using FAs at the same time can improve estimates. Interestingly, during cyanobacteria blooms the estimates were most accurate when carotenoids and FAs were usedd simultaneously. Furthermore, using carotenoids and FAs with NAP included in the estimation allows the estimation of proportions of NAP in seston.

All of the studied biotracer-based estimation methods produced their results as distributions, therefore allowing us to investigate whether the reference proportions obtained through light-microscopy counting would be found within the range of the estimated distributions,

i.e., their estimate consistency. None of the methods would consistently align with the reference proportions in their estimated distributions when the 68% credible interval (median $\pm$ 1σ) was considered. However, when the credible interval was extended to 95% (median $\pm$ 2σ), CHEMTAX and FASTAR consistently aligned with the light-microscopy counting estimates regardless of the biotracers used, while for MixSIAR and QFASA this was true only when FAs were used. While the absolute errors with MixSIAR were high, explaining the poor estimate consistency, QFASA did not excel in estimate consistency despite the high point-estimate accuracy. The estimation distributions produced by QFASA are derived from bootstrapping by contrast with Markov Chain Monte Carlo algorithms employed by the other methods, resulting in narrower distributions. Whether the narrow distributions are a problem as opposed to accurate estimates is something that should be considered when choosing the estimation method for a study.

When high point estimate accuracy is a primary concern, the results presented in this study further confirms the advantage of QFASA over other methods observed in our previous work with herbivorous zooplankton diet composition estimation (Litmanen et al., 2020). CHEMTAX and FASTAR seemed to perform quite evenly on phytoplankton composition estimation in terms of MAEs with FASTAR being usually slightly more accurate. FASTAR performed best if estimate consistency, that is, finding the light-microscopy counting result in the estimate distribution, is the primary concern. MixSIAR seemed reliable only when using carotenoids in terms of point estimate accuracy, but simultaneously, estimated consistency was the lowest for the method. Contrary to other methods tested here, the MAEs of MixSIAR estimates were higher if NAP were included in the estimation. Furthermore, significant issues arose due to the computational demands of MixSIAR when using carotenoids and FAs and including NAP in the estimations; the computation of these estimates required over a week to complete, in stark contrast to estimates produced with carotenoids alone, which were processed typically within just a few hours. Notably, MixSIAR estimated the proportion of NAP in seston to be exceedingly high compared to the other methods.

Light-microscopy counting is often perceived as a reliable method for determining the proportions of various phytoplankton groups, such as cyanobacteria, in the seston sample. However, it is essential to recognize that light microscopy-based estimates rely on size-based criteria and biomass calculations involving volumetric coefficients (Hillebrand et al., 1999) and species-specific cell swelling, shrinking, or breakage has been observed as a result of the preservation (Bergkemper & Weisse, 2017; Menden-Deuer et al., 2001; Zarauz & Irigoien, 2008). Thus, light-microscopy counting might under- or overestimate the concentrations of the different phytoplankton groups (Cermeño et al., 2014). Consequently, uncertainties arise regarding whether either light-microscopy counting or the biotracer approach aligns more closely with an accurate representation of the true phytoplankton proportions. Hence, a comprehensive assessment considering the uncertainties associated with both light-microscopy counting, and biotracer-based estimation is essential for meaningful comparisons.

Eutrophication, the estimation of cyanobacteria abundance

Generally, the biotracer-based estimation methods could detect cyanobacteria blooms. Solely using FAs in estimation for the intensity of the bloom was underestimated compared with the reference proportions obtained through light-microscopy counting. When the reference proportion of cyanobacteria exceeded 75% the methods often underestimated the proportion by tens of percentage points. We assessed that this challenge primarily stemmed from the limitations of the estimation methods in differentiating between cyanobacteria and green algae, but also to a lesser extent dinoflagellates and golden algae. With all methods except MixSIAR including NAP in the estimation with FAs improved the detection of cyanobacteria blooms and decreased the overestimation of cyanobacteria proportions in lower concentrations while not having a large effect on underestimation of high abundances. Notably, the overestimation of green algae proportions was also greatly reduced when NAP was included. This highlights that seston samples are partly composed of FA-containing material other than phytoplankton and thus, the estimates are improved when the source library contains all feasible components in the field sample (Iverson et al., 2004).

The biotracer-based estimation method estimates aligned much closer to the light-microscopy counting estimates when carotenoids were used, although the contribution of cyanobacteria was still underestimated to a slighter degree. The most accurate estimates for the contribution of cyanobacteria were achieved with QFASA when both carotenoids and FAs were used, and NAP were included in the estimation. Based on our systematic testing, almost equally good results could be achieved when using carotenoids alone with any of the estimation methods, although only QFASA with both carotenoids and FAs could pass under the 0.1 threshold when estimating the proportion of cyanobacteria.

The estimation of nutritionally high-quality algae abundance

All employed estimation methods estimated the overall proportion of high nutritional-quality phytoplankton relatively well, yet they encountered notable challenges in differentiating between the various high-quality phytoplankton groups, namely cryptophytes, diatoms, dinoflagellates, and golden algae. When a high-quality phytoplankton group was observed as the most abundant phytoplankton taxa by light-microscopy counting, the methods faced challenges accurately identifying the dominant group, although the estimates as a whole usually indicated that high-quality phytoplankton groups dominated the sample.

Much like cyanobacteria, estimating large diatom proportions resulted in significant discrepancies with the FA-based estimations. For example, when the reference proportion was around 78%, the closest FA-based estimate was less than 21%. In instances of high diatom reference proportions, the methods allocated the difference between the estimated and reference proportions of diatoms to the three other high-quality phytoplankton groups, and surprisingly, to cyanobacteria. This could pose a potential issue since in boreal setting, as diatoms are characteristic of oligotrophic lakes, whereas cryptophytes and dinoflagellates are characteristic of dystrophic lakes, and cyanobacteria and diatoms of eutrophic lakes (Lepistö & Rosenström, 1998; Taipale, Vuorio, et al., 2019). Unfortunately, we did not acquire carotenoids samples in the summer of 2019 when Lake Vesijärvi was mostly dominated by diatoms. However, based on the samples from summer of 2021, another time diatoms were dominant, when carotenoids were used the estimation methods were able to account for the substantial diatom presence in the samples aligning much more closely with the light-microscopy counting estimates although the FA-based estimates were also more closely aligned compared with 2019.

Cryptophytes, dinoflagellates, and golden algae were not dominant in many samples unlike diatoms. Estimation of these three high-quality phytoplankton groups resulted in overestimation of small proportions and underestimation of large proportions with all estimation methods. Notably, the estimates of golden algae were the only ones that aligned more closely with the light-microscopy counting results when using FAs alone compared with simultaneous usage of carotenoids and FAs. We cannot explain this since according to the PCA, golden algae is one of the most clearly separated phytoplankton groups when both biotracers are used as opposed to slight overlapping of groups when relying solely on FAs.

While the carotenoid and FA profiles of freshwater phytoplankton have been shown to follow more phylogenetical groups than physical–chemical parameters (Galloway & Winder, 2015; Tamm et al., 2015), slight variations in FA or carotenoid profiles may be caused by changes in the physical and chemical parameters, such as temperature and phosphorus content, and growth stage (Calderini et al., 2023; Juneja et al., 2013; Latasa, 1995; Taipale et al., 2020). Furthermore, we did not have comprehensive estimates of nano- or picoplankton abundances in the seston samples while they contribute to the biomolecule content of seston samples (Wright et al., 2009). Many of the high-quality phytoplankton taxa are mixotrophic and there is evidence that the FA profile of a mixotrophic green algae might change based on the energy acquiring mode (Wacker & Weithoff, 2009). However, recent studies have shown that mixotrophy has only a minor impact on the FA profiles of high-quality phytoplankton (Calderini et al., 2022; Liu et al., 2011; Peltomaa & Taipale, 2020).

Another possible cause for difficulties with especially FA-based estimation could be ciliates and other heterotrophic protozoans that are not included in the seston source library but are present in the field samples and can have an effect on the seston FA profiles (Galloway & Winder, 2015). The contribution of heterotrophic protozoans to the carbon pool in boreal lakes can be up to 20% (Strandberg et al., 2015, 2020) and they can significantly contribute to the highly unsaturated fatty acid (HUFA) contents of seston (Bec, Perga, et al., 2010) also being capable of producing HUFA de novo (Bec, Martin-Creuzburg, et al., 2010).

The estimation of non-algal particles in seston

Using FAs in the estimation allows for estimation of other components of seston NAP, namely macrophyte detritus (reed), tPOM, and microbes on tPOM (tPOMb). We investigated the methods' ability to accurately estimate the proportion and composition of NAP within our samples through a small-scale laboratory experiment. This assessment aimed to evaluate the methods' performance in this specific context. Notably, at low proportions of NAP in the samples, all methods, except MixSIAR, demonstrated a high level of accuracy. Although the majority of the samples were composed of tPOM, tPOMb was also present. Unfortunately, we cannot confirm the proportional contribution of tPOMb in the samples. However, our results indicate that the methods successfully distinguished between tPOM and tPOMb. This aligns with our previous study, where we

demonstrated that QFASA and FASTAR can effectively differentiate between phytoplankton, tPOM, and tPOMb when assimilated to *Daphnia* (Litmanen et al., 2020).

When estimating the proportions of phytoplankton and different NAP components in the seston samples the estimates of seston composition varied widely between the four estimation methods. This raises doubts about the reliability of the estimates in terms of total proportion of NAP and composition of NAP. With CHEMTAX and QFASA, the proportion of NAP in seston were estimated to be under 20% with a few exceptions, and most of NAP was determined to consist of tPOM. Contrarily, FASTAR estimated NAP to account for 20% to 60% in most samples and the bulk of NAP consisted of tPOMb. MixSIAR estimated the NAP to account for mostly over 50%, and the bulk of NAP consisted of tPOM. In a previous study on the same lake (Lake Vesijärvi) the proportion of NAP was determined to be over 50% (<3% of bacteria and 54% of detritus and terrestrial particles) based on mass calculations (Taipale, Aalto, et al., 2019).

Outlook

We strive to have a robust and cost-effective method for estimating seston composition. In future studies, adding more phytoplankton species to the source library to represent the phytoplankton community more fully could enhance the accuracy of estimations. While it requires prior knowledge of phytoplankton community composition obtained by light-microscopy counting or molecular methods, customizing the source library to reflect the specific taxa of phytoplankton within each studied system can help achieve a more precise representation of the community and thus lead to more accurate estimates. Furthermore, the difference between laboratory-derived biotracer profiles and natural samples in different systems should be more thoroughly studied. The addition of a third biotracer type, namely sterols, could also improve the estimates (Peltomaa et al., 2023) provided the sterol content of seston samples are sufficiently high. Also, the introduction of compound-specific isotope data to these estimation methods could help improve accuracy especially with the differentiation of phytoplankton and NAP (Taipale et al., 2015), which would, however, require the construction of a completely new source library. Another step further would also be the quantification of the estimates, that is, estimating the biomass of the phytoplankton groups. Chlorophyll *a*, a pigment whose concentration can be determined simultaneously with carotenoid analysis, could be used to quantify the carotenoid results and, thus, the estimates of this study's approach (Mackey et al., 1996; Tamm et al., 2015). With the aforementioned development, the biotracer approach could prove to be efficient in fulfilling the three of the most important European Union Water Framework Directive (WFD; European Parliament, 2000) metrics for phytoplankton monitoring; chlorophyll *a* concentration, phytoplankton trophic index, and cyanobacterial biovolume (Carvalho et al., 2013). Furthermore, in the realm of lake biochemical state assessment, where prior studies have primarily focused on FAs and sterols (e.g., Peltomaa et al., 2017; Taipale, Hiltunen, et al., 2016), the incorporation of carotenoid analysis not only enriches the scope of evaluation but also offers an additional benefit in the form of robust community composition estimation. Moreover, species-level composition estimates could be achieved by having a simultaneous molecular primer-free measurement of seston composition (Vuorio et al., 2020).

Conclusions

In conclusion, our study highlights the successful application of the biotracer approach for accurately estimating freshwater phytoplankton community composition proportions at the group level. Using carotenoid and FA data through computational estimation methods, we can effectively identify specific phytoplankton groups serving as indicators for eutrophication or other ecological processes that affect the composition of phytoplankton community. The approach can be helpful in long-term studies to assess the response to environmental change, particularly when taxonomic expertise is lacking, or group-level information suffices. Moreover, the approach allows for the assessment of lake restoration efforts by evaluating seston quality. Additionally, we demonstrate the methods' utility in estimating the proportion and composition of NAPs in seston. Careful selection of the estimation method is crucial to ensure precise biotracer-based assessments of phytoplankton composition and avoid misleading conclusions. The biotracer approach emerges as a valuable alternative to light-microscopy counting, particularly when adequate information on phytoplankton group levels is available in community composition studies.

(supporting), investigation (supporting), formal analysis (supporting), and conceptualization.

CONFLICT OF INTEREST STATEMENT
The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT
Data (Litmanen et al., 2024) are available from Dryad: 10.5061/dryad.t1g1jwt9v.

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