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Phytoplankton group identification with chemotaxonomic biomarkers: In combination they do better

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ABSTRACT

Chemotaxonomic biomarkers are needed to monitor and evaluate the nutritional quality of phytoplankton communities. The biomolecules produced by different phytoplankton species do not always follow genetic phylogeny. Therefore, we analyzed fatty acids, sterols, and carotenoids from 57 freshwater phytoplankton strains to evaluate the usability of these biomolecules as chemotaxonomic biomarkers. We found 29 fatty acids, 34 sterols, and 26 carotenoids in our samples. The strains were grouped into cryptomonads, cyanobacteria, diatoms, dinoflagellates, golden algae, green algae, and raphidophytes, and the phytoplankton group explained 61%, 54%, and 89% of the variability of fatty acids, sterols, and carotenoids, respectively. Fatty acid and carotenoid profiles distinguished most phytoplankton groups, but not flawlessly. For example, fatty acids could not distinguish golden algae and cryptomonads, whereas carotenoids did not separate diatoms and golden algae. The sterol composition was heterogeneous but seemed to be useful for distinguishing different genera within a phytoplankton group. The chemotaxonomy biomarkers yielded optimal genetic phylogeny when the fatty acids, sterols, and carotenoids were used together in multivariate statistical analysis. Our results suggest that the accuracy of phytoplankton composition modeling could be enhanced by combining these three biomolecule groups.

1. Introduction

Phytoplankton have a central role in aquatic ecosystems by supplying essential biochemicals to organisms of a higher trophic level and by participating the biogeochemical cycles (Falkowski, 1994; Ward et al., 2012). Phytoplankton communities are phylogenetically highly diverse, as they have representatives in most supergroups of the eukaryotic tree of life (Burki et al., 2020) as well as in cyanobacteria (Elliott, 2012). Due to their phylogenetic diversity, phytoplankton vary in their environmental preferences, e.g. for temperature, light, and nutrient availability (Isles et al., 2021; Litchman et al., 2010; Reynolds, 1998; Tanioka and Matsumoto, 2020). Moreover, their chemical composition is genetically determined, but the biomolecule concentrations are affected by the environment (Galloway and Winder, 2015). The composition of the phytoplankton community is important when defining aquatic production since phytoplankton synthesize many taxon-specific biomolecules that consumers cannot synthesize *de novo*. Therefore, phytoplankton partially determine the production of the upper trophic levels and the whole food web (Danielsdottir et al., 2007; Dickman et al., 2008; Elliott, 2012; Peltomaa et al., 2017; Taipale et al., 2019). Phytoplankton are thus often used for estimating ecological status and stability of aquatic ecosystems.

Microscopy has been an important tool for phytoplankton identification, abundance, and diversity determinations for decades. However, even though microscopy may allow very precise determinations, it has some severe limitations; microscopy is very time-consuming, and the reliability of the identification is dependent on the skills of the identifier (Abad et al., 2016). Species identification relies entirely on morphological characteristics, and thus, morphologically similar species cannot be separated from each other (Nair et al., 2008). Nowadays, molecular

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methods, such as amplicon and shotgun sequencing and metabarcoding, have increasingly been used to phytoplankton identification. However, the intragenomic variation with many copies of individual genes causes overestimation of cell numbers and diversity, leading to discrepancies when combining morphological and molecular methods (McManus and Katz, 2009). Moreover, there is insufficient coverage of freshwater phytoplankton in the currently available databases (e.g. SILVA, Greengenes), and due to a lack of reference material, molecular methods in freshwaters are largely limited to specific phytoplankton groups (Huo et al., 2020; Malashenkov et al., 2021). In addition to the morphological and molecular taxonomy, phytoplankton have been grouped by their functional, ecometabolomic, and chemotaxonomic differences (Descy et al., 2009; Litchman et al., 2010; Peñuelas and Sardan, 2009), which, besides giving taxonomic information, provide important information for ecological studies.

Chemotaxonomic biomarkers can be used for monitoring phytoplankton communities, and they are valuable for providing additional information for precise taxonomic ranking different from morphological and genetic analyses. Simultaneously, they provide information on the nutritional quality of phytoplankton for higher trophic levels. Thus, they are valuable in food web studies and can be used when modeling phytoplankton community composition (Strandberg et al., 2015), predator-prey interactions, and even ecosystems (Pethybridge et al., 2018; Petrišič and Ogrinc, 2013). Fatty acids and carotenoids have already been used for class-level identification (Cañavate et al., 2019; Kramer et al., 2020; Paliwal et al., 2016; Sahu et al., 2013; Stamenković et al., 2020; Taipale et al., 2013), but both of them have some limitations due to overlapping profiles. Fatty acids have been reported to be unable

Table 1
Investigated freshwater algal and cyanobacterial strains and their culture collection numbers. The taxonomy is based on AlgaeBase (July 25, 2022).

Number	Group	Class	Species	Strain number
1	Cryptomonads	Cryptophyceae	*Cryptomonas curvata* Ehrenberg	CCAP 979/63
2	Cryptomonads	Cryptophyceae	*Cryptomonas erosa* Ehrenberg	CPCC 466
3	Cryptomonads	Cryptophyceae	*Cryptomonas lundii* Hoef-Emden & Melkonian	CCAP 979/69
4	Cryptomonads	Cryptophyceae	*Cryptomonas marssonii* Skuja	CCAP 979/70
5	Cryptomonads	Cryptophyceae	*Cryptomonas ovata* Ehrenberg	CCAC 0064
6	Cryptomonads	Cryptophyceae	*Cryptomonas pyrenoidifera* Skuja	NIVA 2/81
7	Cryptomonads	Cryptophyceae	*Cryptomonas* sp.	CPCC 336
8	Cryptomonads	Cryptophyceae	*Rhodomonas lacustris* Pascher & Ruttner	NIVA 8/82
9	Cryptomonads	Cryptophyceae	*Rhodomonas* sp.	CCAC 0194
10	Cyanobacteria	Cyanophyceae	*Anabaena flos-aquae* Elenkin	NIVA-CYA 138
11	Cyanobacteria	Cyanophyceae	*Aphanothece* cf. *Clathrata*	NIVA-CYA 369
12	Cyanobacteria	Cyanophyceae	*Chroococcus* cf. *Dispersus*	NIVA-CYA 370
13	Cyanobacteria	Cyanophyceae	*Limnothrix planktonica* (Wołoszyńska) Meffert	NIVA-CYA 107
14	Cyanobacteria	Cyanophyceae	*Microcystis* sp.	NIVA-CYA 642
15	Cyanobacteria	Cyanophyceae	*Planktothrix rubescens* (De Candolle ex Gomont) Anagnostidis & Komárek	NIVA-CYA 624
16	Cyanobacteria	Cyanophyceae	*Pseudanabaena limnetica* (Lemmermann) Komárek	NIVA-CYA 276/11
17	Cyanobacteria	Cyanophyceae	*Pseudanabaena* sp.	CPCC 678
18	Cyanobacteria	Cyanophyceae	*Snowella lacustris (Chodat)* Komárek & Hindák	NIVA-CYA-339
19	Cyanobacteria	Cyanophyceae	*Synechococcus elongatus* (Nägeli) Nägeli	UTEX LB 563
20	Diatoms	Bacillariophyceae	*Fragilaria capucina* Desmazières	CCAC 1767 B
21	Diatoms	Bacillariophyceae	*Nitzschia communis* Rabenhorst	CCAC 1762 B
22	Diatoms	Bacillariophyceae	*Surirella* sp.	CCAC 3416 B
23	Diatoms	Bacillariophyceae	*Tabellaria* sp.	CCAC 3717
24	Diatoms	Bacillariophyceae	*Asterionella formosa* Hassall	CCAC 3890 B
25	Diatoms	Coscinodiscophyceae	*Melosira* sp.	CCAC 1935 B
26	Diatoms	Mediophyceae	*Cyclotella* sp.	CCAC 3539 B
27	Dinoflagellates	Dinophyceae	*Ceratium* sp.	Own isolation, Lake Köyhälampi, Finland
28	Dinoflagellates	Dinophyceae	*Gymnodinium impatiens* Skuja	CCAC 0025
29	Dinoflagellates	Dinophyceae	*Peridinium centenniale* (Playfair) Er.Lindem.	CCAC 0002
30	Dinoflagellates	Dinophyceae	*Peridinium cinctum* (O.F.Müller) Ehrenberg	CCAC 0102 B
31	Dinoflagellates	Dinophyceae	*Peridinium cinctum* (O.F.Müller) Ehrenberg	SCCAP K-1721
32	Dinoflagellates	Dinophyceae	*Peridinium volzii* Lemmermann	SCCAP K-1611
33	Dinoflagellates	Dinophyceae	*Peridinium bipes* F.Stein	CCAC 1426 B
34	Golden algae	Chrysophyceae	*Dinobryon* sp.	CCAC 2950 B
35	Golden algae	Chrysophyceae	*Ochromonas perlata* Doflein	K-1208
36	Golden algae	Chrysophyceae	*Poterioochromonas malhamensis* (Pringsheim) Péterfi	CCAC 3498
37	Golden algae	Chrysophyceae	*Spumella* sp.	CCAP 955/2
38	Golden algae	Chrysophyceae	*Uroglena* sp.	CCAC 2977 B
39	Golden algae	Chrysophyceae	*Uroglena* sp.	CPCC 276
40	Golden algae	Coccolithophyceae	*Chrysochromulina parva* Lackey	CCAC 1889B
41	Golden algae	Synurophyceae	*Mallomonas caudata* Iwanoff [Ivanov]	CCAP 929/8
42	Golden algae	Synurophyceae	*Mallomonas insignis* Penard	CCAC 2924 B
43	Golden algae	Synurophyceae	*Mallomonas kalinae* Rezácova	SCCAP K-1759
44	Golden algae	Synurophyceae	*Synura petersenii* Korshikov	K-1875
45	Golden algae	Synurophyceae	*Synura* sp.	NIVA-5/09
46	Golden algae	Synurophyceae	*Synura uvella* Ehrenberg	CCAC 1159 B
47	Green algae	Chlorophyceae	*Acutodesmus* sp.	Basel University
48	Green algae	Chlorophyceae	*Chlamydomonas multitaeniata* Korschikov	CCAC 0008
49	Green algae	Chlorophyceae	*Chlamydomonas reinhardtii* P.A.Dangeard	UWCC
50	Green algae	Chlorophyceae	*Desmodesmus maximus* (West & G.S.West) Hegewald	CCAC 3524 B
51	Green algae	Chlorophyceae	*Haematococcus pluvialis* Flotow	K-0084
52	Green algae	Chlorophyceae	*Monoraphidium griffithii* (Berkeley) Komárková-Legnerová	NIVA-CHL8
53	Green algae	Chlorophyceae	*Selenastrum* sp.	SCCAP K-1877
54	Green algae	Trebouxiophyceae	*Botryococcus braunii* Kützing	CCAC 0121
55	Green algae	Trebouxiophyceae	*Choricystis* sp.	NIVA-CHL 88
56	Green algae	Trebouxiophyceae	*Stichococcus bacillaris* Nägeli	NIVA-CHL 136
57	Raphidophytes	Raphidophyceae	*Gonyostomum semen* (Ehrenberg) Diesing	CCAC 2816 B

to separate cryptomonads and golden algae (Taipale et al., 2013). Carotenoids do not always distinguish between cyanobacteria and green algae. Many cyanobacteria include myxoxanthophyll (Srivastava et al., 2022), while some cyanobacteria species lack that (Takaichi, 2011), however, it has concluded that myxoxanthophyll contribute significantly to the vigor of cyanobacteria, as it stabilizes thylakoid membranes and is critical for S-layer formation (Mohamed et al., 2005). Sterols have not been successfully established as biomarkers due to their low class-level taxonomic specificity, however, some sterols are very specific to certain genera (Taipale et al., 2016; Volkman et al., 1998). All three, fatty acids, sterols, and carotenoids have been widely studied in marine environments (Cañavate, 2018; Ding et al., 2019; Huang et al., 2017; Jónasdóttir, 2019; Véron et al., 1998), and especially as sedimentary biomarkers (Summons et al., 2022; Wakeham et al., 1997). However, the phytoplankton community composition differs in response to salinity (Obolewski et al., 2018). Thus, the findings from marine environments may not be applicable to freshwaters.

Here, we analyzed the fatty acid, sterol, and carotenoid profiles of seven phytoplankton groups (see Table 1) to determine how specific their production is within taxa. We also evaluated how well the chemotaxonomic phylogeny matches the molecular identification. This is important because misinterpretations in the classification can have serious consequences when modeling ecological processes. We focused on freshwater phytoplankton and assessed the suitability of fatty acids, sterols, and carotenoids as well as their combination in identification using multivariate statistics. The analysis included 57 phytoplankton strains from cryptomonads, cyanobacteria, diatoms, dinoflagellates, golden algae, green algae, and raphidophytes, i.e. the most abundant phytoplankton groups in freshwaters (Hedlund and Hagman, 2020; Lepistö and Rosenström, 1998). We hypothesized that the chemotaxonomic descriptive grouping best corresponds to molecular taxonomy when all three groups of biomolecules are used together. Additionally, we expected to find chemotaxonomic differences within morphologically and/or genetically related phytoplankton groups.

2. Results and discussion

2.1. Biochemical profiles

We were able to determine 29 fatty acids, 34 sterols, and 26 carotenoids from seven phytoplankton groups (cryptomonads, cyanobacteria, diatoms, dinoflagellates, golden algae, green algae, and raphidophytes; see Table 1) consisting of 57 freshwater strains (Figs. 1–3, Supplemental Table 1). All studied strains contained various kinds of fatty acids, sterols, and carotenoids, excluding cyanobacteria, which lack sterols as also previously shown (Peltomaa et al., 2017; Volkman, 2016). We aimed to evaluate how well the chemotaxonomic phylogeny matches the plastid 23 S rRNA gene identification (Supplement 2) and if using all biomarkers together improves the accuracy of the identification. We also aimed to evaluate the fatty acid, sterol, and carotenoid profiles of the phytoplankton groups and to determine how specific their production is within taxa. Our results show that the phytoplankton groups differ from each other based on their biochemical composition; however, there were great differences in the similarity, dispersion, and clustering within phytoplankton groups (Figs. 4 and 5, Tables 2 and 3). Cyanobacteria generally had the lowest similarity (Table 3) and the highest dispersion (Fig. 5) in all measured biomarkers, which means higher within-group diversity in cyanobacteria than in the other studied groups of phytoplankton. The detailed results on the performance of each biomarker group are found below, but, to summarize, carotenoids explained most of the variation in phytoplankton groups among all biomolecules, resulting in good separation of dinoflagellates, cyanobacteria, cryptomonads, and raphidophytes (Fig. 4C, Table 2). However, all three biomarker groups (fatty acids, sterols, and carotenoids) were required to separate the seven phytoplankton groups from each other at 60% similarity level (Fig. 4D).

2.2. Fatty acids

The within-group similarities of the fatty acid profiles were high (Fig. 1, Table 2). According to our results, fatty acids accounted for 61% of variation in phytoplankton, which is similar to the results of Galloway and Winder (2015), who showed that phylogeny explains the greatest proportion of total variation in phytoplankton fatty acid profiles. This has permitted the use of fatty acids in seston composition studies and modeling (Strandberg et al., 2015; Taube et al., 2019). However, it should be noted that light and nutrients can influence the fatty acid content of algal cells, affecting the detection if the concentration of a specific fatty acid is low (Guschina and Harwood, 2006). There has been a lack of data on the fatty acids of freshwater golden algae in previous biomarker studies (Ahlgren et al., 1992; Galloway and Winder, 2015; Taipale et al., 2013, 2016). Here, we investigated the fatty acid composition of 13 golden algae, including all main genera common in the boreal lakes (Lepistö and Rosenström, 1998). Our analysis shows that the fatty acid profiles of golden algae, especially Synurophyceae, overlap with those of cryptomonads (Fig. 4A). In contrast, the fatty acid

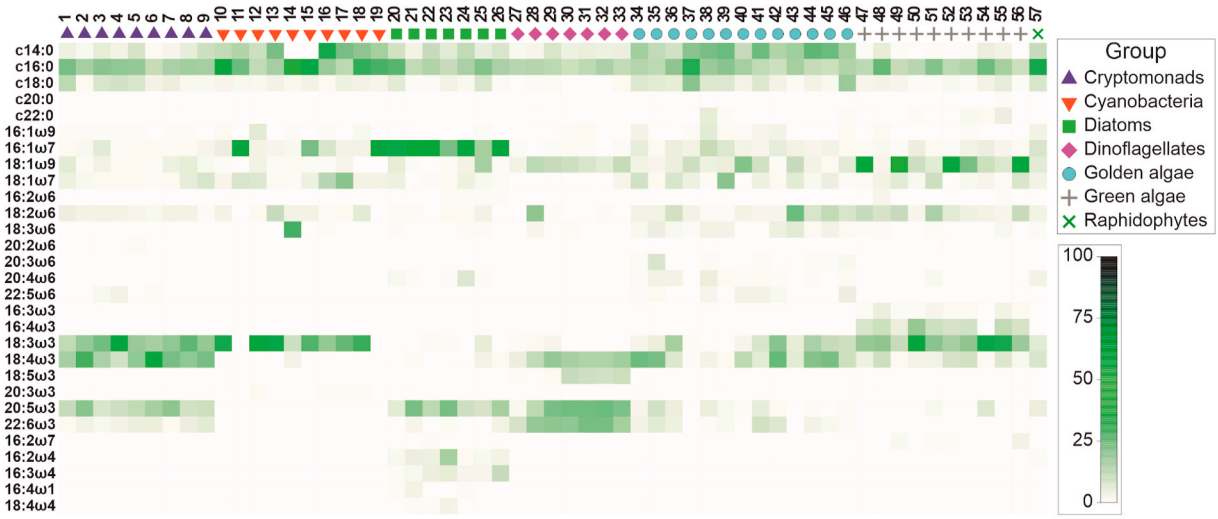

Fig. 1. Fatty acid profiles of cultured freshwater strains. Abundance refers to the percentage of all fatty acids. Numbers refer to the number of strains in Table 1. The algal groups have been confirmed with plastid 23 S rRNA gene sequence data of the studied samples (Supplement 2).

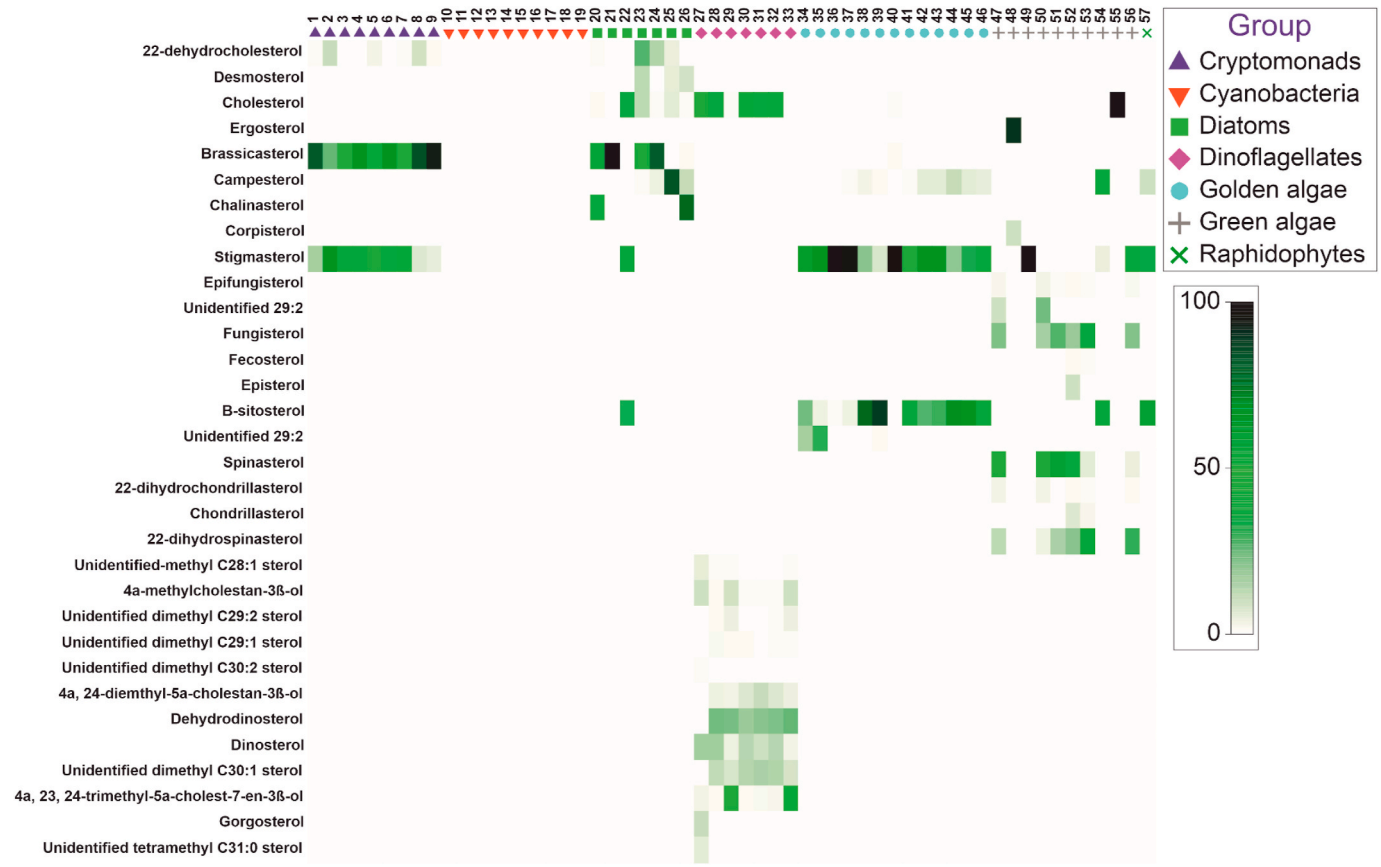

Fig. 2. Sterol profiles of cultured freshwater strains. Abundance refers to the percentage of all sterols. Numbers refer to the number of strains in Table 1. The algal groups have been confirmed with plastid 23 S rRNA gene sequence data of the studied samples (Supplement 2).

profiles of *Spumella* sp. (Ochromonadaceae) and *Uroglena* sp. (Ochromonadaceae) differ from those of cryptomonads and are closer to the diatoms in the nMDS (Fig. 4A). Thus, fatty acids can be used to separate golden algae from cryptomonads and diatoms only partially.

The group of diatoms was recently split into several different classes. Interestingly, *Melosira* sp. (Melosiraceae), which belongs to the Coscinodiscophyceae, differed from the other diatom classes (Bacillariophyceae and Mediophyceae) in the nMDS, and was actually more similar to golden algae (Fig. 4A). Likewise, one of the dinoflagellates, *Ceratium* sp. (Ceratiaceae), differed from the other studied dinoflagellates; *Ceratium* was not within the 60% similarity circle in the nMDS plot even though the dinoflagellates in general had high similarity and low dispersion within taxa (Fig. 4A). The examples given by *Melosira* and *Ceratium* show that the fatty acid-based separation between diatoms and dinoflagellates must be done with caution.

The fatty acid profiles of cyanobacteria have previously been classified into four different categories based on their omega-3 (ω-3) or omega-6 (ω-6) polyunsaturated fatty acids (PUFAs), i.e. linoleic acid (LIN), α-linolenic acid (ALA), 18:3ω6, or stearidonic acid (SDA) content (Los and Mironov, 2015). However, some cyanobacteria, e.g. *Synechococcus elongatus* (Nägeli) Nägeli (Synechococcaceae) and *Aphanothece* cf. *Clathrata*, do not contain any ω-3 or ω-6 PUFAs, and our nMDS analysis categorized these cyanobacteria strains together with diatoms (Fig. 4A). This is because diatoms are also low in LIN, ALA, and SDA but can contain high amounts of 16:1ω7. Diatoms have eicosapentaenoic acid (EPA), which is a long-chain ω-3 PUFA and separates diatoms from cyanobacteria, however, the contribution of EPA varied greatly (from 6% to 23%) among the studied diatoms. Moreover, our previous studies have shown that EPA content in diatoms may differ by habitat and growth stage (Peltomaa et al., 2019; Taipale et al., 2020), which challenges its usability in identification.

Overall, our results show differences in fatty acid profiles within phytoplankton groups, indicating that fatty acids cannot be unequivocally used to separate the groups from each other. Thus, the variability between phytoplankton groups should be taken into account, for example, in modeling with modern approaches such as Bayesian modeling (Strandberg et al., 2015).

2.3. Sterols

Sterols had systematically lower similarity percentages and higher dispersion in every phytoplankton group (Table 3, Fig. 5). The lowest within-group similarity was observed in diatoms and green algae, both of which differed greatly in their sterol profiles (Table 3). However, our results confirm that sterols could be used to separate some specific genera, e.g. *Chlamydomonas* (Chlamydomonadaceae), which produce ergosterol (IUPAC name: (22 E)-ergosta-5,7,22-trien-3β-ol) and corbisterol ((22 E)-stigmasta-5,7,22-trien-3β-ol), thus differing from other green algae (Martin-Creuzburg and Merkel, 2016; Taipale et al., 2016). *Chlamydomonas* belongs to the order of Chlamydomonadales, whereas the other studied green algae belonged to Sphaeropleales and Trebouxiophyceae. The sterols produced by Trebouxiophyceae differed from Sphaeropleales, which mainly contained Δ7 -sterols (spinasterol ((22 E)-5α-stigmasta-7,22-dien-3β-ol), fungisterol (5α-ergo-st-7-en-3β-ol); Table 3, Supplemental Table 1). However, the sterol profiles of the three Trebouxiophyceae (*Botryococcus braunii* Kützing (Botryococcaceae), *Stichococcus bacillaris* Nägeli (Stichococcaceae), and *Choricystis* sp. (Coccomyxaceae)) also differed from each other; *B. braunii* contained only campesterol/22-dihydrobrassicasterol (campest/ergost-5-en-3β-ol) and β-sitosterol/clionasterol (stigmast/poriferast-5-en-3β-ol), having sterol profiles similar to golden algae (Fig. 4B). *S. bacillarialis* contained also stigmasterol/poriferasterol ((22

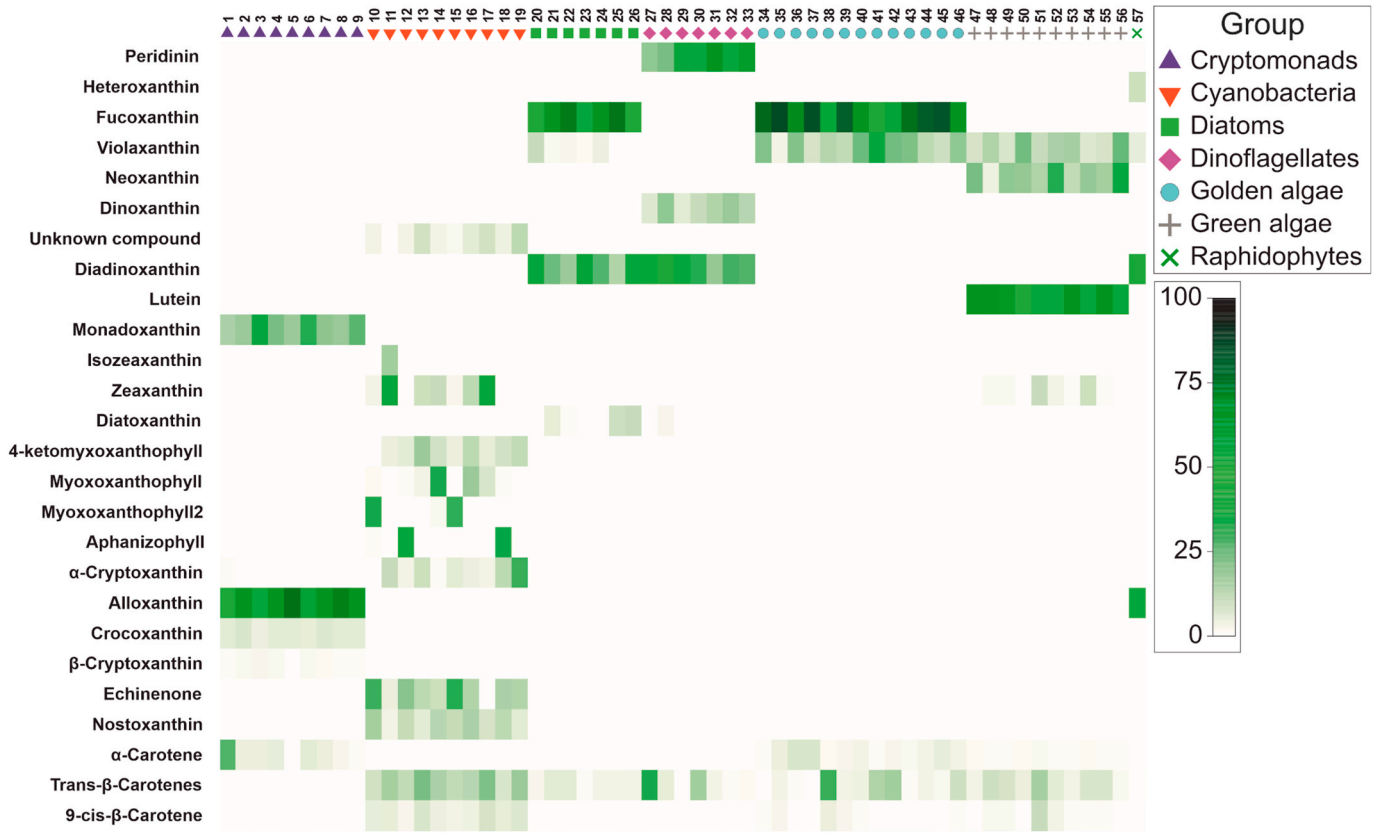

Fig. 3. Carotenoid profiles of cultured freshwater strains. Abundance refers to the percentage of all carotenoids. Numbers refer to the number of strains in Table 1. The algal groups have been confirmed with plastid 23 S rRNA gene sequence data of the studied samples (Supplement 2).

E)-stigmasta/poriferasta-5,22-dien-3β-ol) but clustered closely with golden algae, whereas *Choricystis* sp. Contained only cholesterol (cholest-5-en-3β-ol) and clustered together with dinoflagellates in the nMDS (Fig. 4B).

In diatoms, only three strains, *Nitzschia communis* Rabenhorst (Bacillariaceae), *Fragilaria capucina* Desmazières (Fragilariaceae), and *Asterionella formosa* Hassall (Tabellariaceae), clustered together, having brassicasterol/epibrassicasterol ((22 E)-ergosta/campesta-5,22-dien-3β-ol) as their main sterol (Fig. 2). However, since brassicasterol/epibrassicasterol is the main sterol also in some cryptomonads, the nMDS clustered some diatoms and cryptomonads together (Fig. 4B). The sterol profiles of cryptomonads and golden algae differed from each other since cryptomonads have primarily brassicasterol/epibrassicasterol, whereas stigmasterol/poriferasterol is the primary sterol in golden algae. Therefore, it is not surprising that in our previous study fatty acids and sterols together gave better separation than fatty acids alone (Taipale et al., 2016).

Dinoflagellates were the most diverse phytoplankton group in sterols as previously found in marine dinoflagellates (LeBlond and Chapman 2002). We were able to identify 15 different sterols even though many sterols were close to detection limit. In our previous study we concluded that gorgosterol was typical for *Ceratium* sp. (Ceratiaceae) that differed from the other dinoflagellates (Taipale et al., 2016, Fig. 4B). Here, we also found tetrametnyl C31:0 from *Ceratium*. Furthermore, we cultured five different *Peridinium* -dinofalgellates, of which *Peridinium centenniale* (Playfair) Er. Lindem and *Peridinium bipes* (Peridiniaceae) have lower contribution of cholesterol but higher contribution of 4α-methylcholestan-3β-ol and unidentified diemethyl C29:2 sterol than the other *Peridinium* strains (Fig. 2, Supplemental Table 1). This, together with the above-mentioned results, shows that the sterol composition of phytoplankton can differ significantly at genus level, confirming that sterols alone are not suitable biomarkers at class level.

2.4. Carotenoids

Among the three studied biomarkers, the carotenoid profiles of phytoplankton resulted in the highest similarity and lowest dispersion within each phytoplankton group, excluding cyanobacteria (Table 3, Figs. 3 and 5). However, the nMDS did not separate diatoms and golden algae when using 60% similarity as criteria for group separation (Fig. 4C). This was because fucoxanthin was the characteristic carotenoid for both of these phytoplankton groups (Table 3). Additionally, as shown before (Casper-Lindley and Björkman, 1998; Withers et al., 1981), both diatoms and golden algae contained violaxanthin and β-carotene (Fig. 3, Supplemental Table 1). These two phytoplankton groups differed only in the diadinoxanthin, which the studied diatoms contained at some levels, but the golden algae did not. However, diadinoxanthin was the characteristic carotenoid together with peridinin for all dinoflagellates, including *Ceratium* sp. (Fig. 4C). Additionally, diadinoxanthin was also found in the raphidophyte *Gonyostomum semen* (Ehrenberg) Diesing (Vacuolariaceae), i.e. this carotenoid was found in three phytoplankton groups, which complicates its usability as a chemotaxonomic biomarker (Supplemental Table 1).

Alloxanthin was found in both *G. semen* and cryptomonads (Fig. 4C). *G. semen* contained also heteroxanthin, which is reported to be a suitable pigment biomarker for this species (Hagman et al., 2019), and separated *G. semen* from the cryptomonads in our nMDS ordination (Fig. 4C). Cryptomonads also contained monadoxanthin, crocoxanthin, and β-cryptoxanthin, as previously reported for marine cryptomonads (Margulis and Chapman, 2009; Pennington et al., 1985), however, α-cryptoxanthin was found only at a trace level. Moreover, we found a higher contribution of α-cryptoxanthin from some cyanobacteria than from any strains of cryptomonads (Supplemental Table 1).

Cyanobacteria as a group differed greatly in their carotenoids (Fig. 3), and thus, carotenoids can be poorly utilized as biomarkers at the

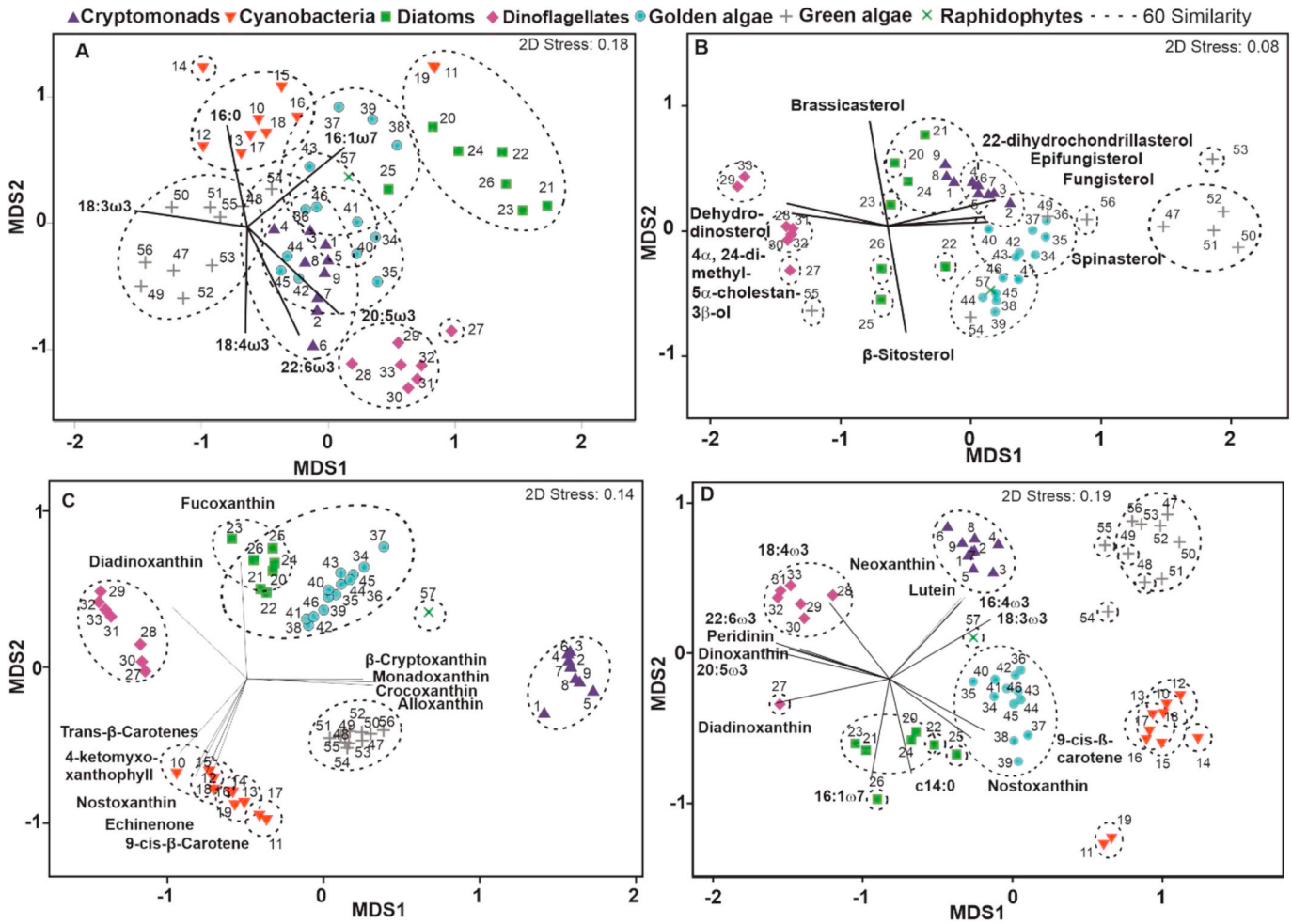

Fig. 4. Non-metric multidimensional scaling (nMDS) of fatty acids (A), sterols (B), carotenoids (C), and all biomolecules together (D). For the figures, 60% similarity was used. Vectors stand for Pearson correlation (>0.6) between biomolecules and nMDS axes 1 and 2. Numbers refer to the number of strains in Table 1. Cyanobacteria and green algae strain no 48 (*Chlamydomonas multitaeniata*) are not included in sterol panel (B). The algal groups have been confirmed with plastid 23 S rRNA gene sequence data of the studied samples (Supplement 2). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

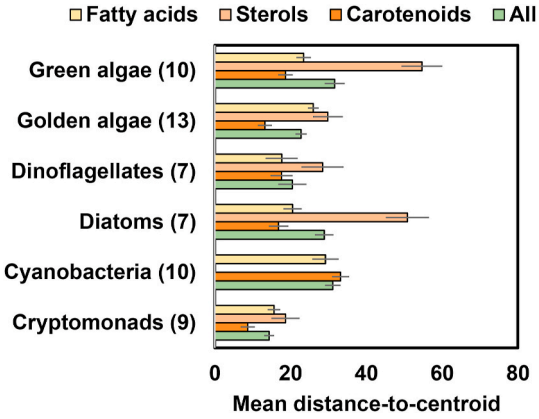

Fig. 5. PERMDISP was used to evaluate dispersion within each phytoplankton group. The highest mean distance-to-centroid, i.e. highest dissimilarity, was measured when sterols were used alone, and the lowest with carotenoids, excluding Cyanobacteria. Algal groups have been confirmed with plastid 23 S rRNA gene sequence data of the studied samples (Supplement 2).

group level for cyanobacteria. However, this offers a great opportunity to separate different cyanobacteria strains by their pigments and especially to use spectral imaging by determining blooms of different cyanobacterial species (Salmi et al., 2022). Mostly, cyanobacteria differed in their contribution to different myxoxanthophylls. Myxoxanthophylls have the same carotenoid (glycoside), but they can vary by the attached sugar, resulting in a similar UV-VIS absorption spectra (Britton et al., 2004). In addition to the standard myxoxanthophyll, we found a high contribution of myxoxanthophyll with a similar UV-VIS spectra with a later retention time from *Anabaena flos-aquae* Elenkin (Nostocaceae) and *Planktothrix rubescens* (De Candolle ex Gomont) Anagnostidis & Komárek (Microcoleaceae). Moreover, we noted a high contribution of aphanizophyll only from *Snowella lacustris* (Chodat) Komárek & Hindák (Coelosphaeriaceae). The β-carotene and an unidentified derivative (UID) of β-carotene were unifying pigments in cyanobacteria (Fig. 3). Additionally, we detected echinenone in all cyanobacterial strains, excluding *Pseudanabaena* sp. (Pseudanabaenaceae).

2.5. Comparison of chemotaxonomic analysis with phylogeny

The chemotaxonomic trees (Fig. 6) reconstructed from the biomolecule data were compared with the phylogenetic tree of the phytoplankton (Supplemental Figure 2.1). The chemotaxonomic information from the fatty acids and carotenoids allowed good reconstruction of

Table 2
Main PERMANOVA results and pairwise tests for comparing phytoplankton groups using fatty acids (FA), sterols (STE), carotenoids (CAR), and all biomolecules together (ALL). For the main test, numbers stand for Pseudo-F values and for pairwise comparison t-values. An asterisk indicates Monte Carlo (MC) permutation (*p < 0.05, **p = 0.001). The percentage in parentheses indicates how much of the variability of biomolecules is explained by the phytoplankton group.

Comparison	FA	STE	CAR	ALL
Main test	13.2** (61%)	9.7** (54%)	65.5** (89%)	25.4 (75%)**
Cryptomonads vs. Cyanobacteria	3.5**	–	7.5**	6.1**
Cryptomonads vs. Diatoms	5.2**	1.9*	14.1**	5.6**
Cryptomonads vs. Dinoflagellates	4.1**	6.5**	12.3**	7.9**
Cryptomonads vs. Golden algae	3.3**	4.8**	16.1**	7.2*
Cryptomonads vs. Green algae	4.0**	3.4**	14.9**	6.0**
Cyanobacteria vs. Diatoms	3.4**	–	6.1**	4.4**
Cyanobacteria vs. Dinoflagellates	4.7**	–	5.7**	5.4**
Cyanobacteria vs. Golden algae	2.8**	–	7.9**	5.7**
Cyanobacteria vs. Green algae	3.2**	–	6.6**	4.4**
Diatoms vs. Dinoflagellates	4.5**	2.9*	6.8**	4.5**
Diatoms vs. Golden algae	4.1**	3.4**	4.5**	4.1**
Diatoms vs. Green algae	5.3**	2.0*	11.1**	4.8**
Dinoflagellates vs. Golden algae	3.9**	5.6**	11.7**	7.0**
Dinoflagellates vs. Green algae	4.9**	2.9**	10.4**	5.3**
Golden algae vs. Green algae	3.9**	3.1**	12.1**	5.5**

phylogenetic relationships, whereas the sterols did not. The best results were again obtained with the combined analysis, which clustered all strains, except the diatom *Surirella* sp., according to their group-level genetic similarity (Fig. 6D; Supplemental Figure 2.1). However, the precise locations of some specific phytoplankton strains differed between the chemotaxonomic and phylogenetic trees. This might be partially because the phylogenetic tree was supplemented with GenBank data in order to confirm the branching of the tree but also because there were class-level differences in the biochemical profiles within the phytoplankton groups. The largest discrepancy was in golden algae, in which the strains belonging to Chrysophyceae and Synurophyceae could not be separated by the chemotaxonomic biomarkers applied (Fig. 6). Andersen (2007) had earlier questioned whether these two closely related classes should be separated. They differ in many morphological features, which speaks in favor of separating them despite their chemotaxonomic similarities (Andersen, 2007). Another unresolved group was green algae, in which Chlorophyceae and Trebouxiophyceae were partly mixed, and a wider analysis of Trebouxiophyceae is required to determine whether they differ chemotaxonomically from Chlorophyceae (Fig. 6). These two classes are closely related, which explains the similar results from fatty acids and carotenoids (Marin, 2012). However, these two groups seem to diverge in their sterol profiles. The major diatom classes Bacillariophyceae, Coscinodiscophyceae and Mediophyceae were separated from each other in the combined tree (Fig. 6D). There has been lively discussion on whether or not these three classes are paraphyletic (Theriot et al., 2009, and references therein). However, wider screening of Coscinodiscophyceae and Mediophyceae is needed before any final conclusions can be drawn.

The other groups, i.e. cryptomonads, dinoflagellates, and cyanobacteria, consisted only of one class, and thus, within-group differences at a class level cannot be discussed. However, we emphasize the additional value of chemotaxonomic clustering when trying to construct precise taxonomic rankings. In our data, the two non-ω-3 PUFA-producing cyanobacteria *A.* cf. *Clathrata* and *S. elongatus* were separated clearly in the fatty acid tree and formed their own branch of cyanobacteria also in the combined tree (Fig. 6). Otherwise, the cyanobacteria were separated into several clusters, confirming the already acknowledged diversity of this group (Palinska and Surosz 2014; Willis and Woodhouse, 2020). In dinoflagellates, the *Peridinium* strains were separated from *Ceratium* (52% similarity between *Peridinium* and *Ceratium*) in the combined analysis (Fig. 6D), which is in accordance with the molecular and morphological differences of these genera. Lastly, the cryptophytes were separated into two clusters, one of which comprised six *Cryptomonas* strains and the other two *Rhodomonas* and one *Cryptomonas* strain (*C. curvata*) (Fig. 6D). *Cryptomonas* and *Rhodomonas* have earlier been reported to belong to different clades of Cryptophyta based on SSU rRNA-sequencing (Marin et al., 1998). The discrepancy in our results was mainly due to the similarity in the sterol profiles of *C. curvata* and *R. lacustris* (Fig. 6), and therefore, more data are needed to draw accurate conclusions about the exceptionality of *C. curvata* or other *Cryptomonas* strains.

3. Concluding remarks

In this study, we were able to show that chemotaxonomic identification can offer a great tool for the assessment of the phytoplankton community composition. Additionally, biomolecule analysis can provide new insights when assessing the taxonomic rankings of

Table 3
Results of similarity percentage (SIMPER) analysis of freshwater microalgae fatty acid (FA), sterol, carotenoid signatures, and all biomolecules together in six classes. The result shows the main component(s) together contributing to >50% of similarity within taxa. The analysis was run on untransformed data. No results are reported for Raphidophyceae because only one strain was sampled within this group.

Phytoplankton taxa	Fatty acids Avg. Similarity/	Compounds	Sterols Avg. Similarity/	Compounds	Carotenoids Avg. Similarity/	Compounds	All Avg. Similarity/	Compounds
Cryptomonads	76.5	ALA, SDA, 16:0	72.6	Brassicasterol	86.8	Alloxanthin, Monadoxanthin	78.7	Alloxanthin, Brassicasterol, Stigmasterol, Monadoxanthin
Cyanobacteria	56.5	C16:0, ALA	–	–	51.4	Trans-β-carotenes, Echinenone, Nostoxanthin, 4-ketomyxoxanthophyll	53.4	16:0, ALA, Trans-β-carotenes, Echinenone, 14:0
Diatoms	68.8	16:1ω7, 16:0	23.5	Brassicasterol	78.0	Fucoxanthin	56.7	Fucoxanthin, 16:1ω7, Diadinoxanthin, Brassicasterol
Dinoflagellates	73.4	DHA, EPA, 16:0	58.7	Cholesterol, Dehydrodinosterol,	73.8	Peridinin, Diadinoxanthin	68.7	Peridinin, Diadinoxanthin, Cholesterol, DHA, Dehydrodinosterol
Golden algae	62.0	C16:0, C14:0, SDA	57.9	Stigmasterol	81.1	Fucoxanthin	67.1	Fucoxanthin, Stigmasterol, β-Sitosterol
Green algae	66.0	ALA, 16:0, 18:1ω9	19.9	Spinasterol, Fungisterol	78.3	Lutein, Neoxanthin	54.5	Lutein, ALA, Neoxanthin,16:0

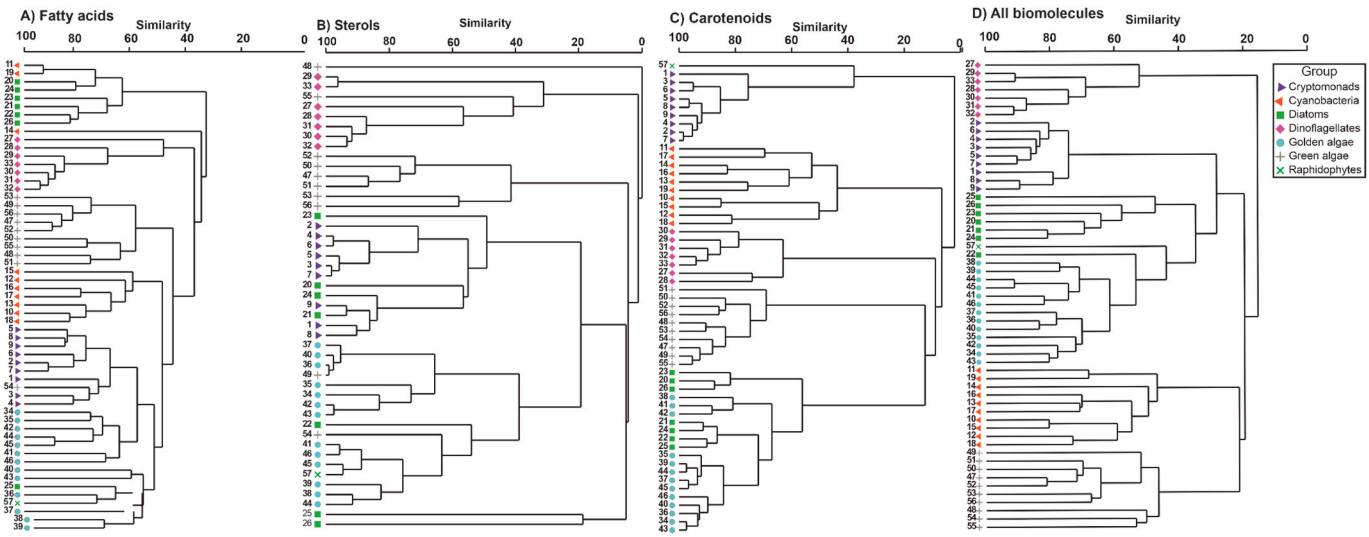

Fig. 6. Chemotaxonomic clustering of the phytoplankton strains. The chemotaxonomic trees (cluster analysis) resulting from the hierarchical clustering of the phytoplankton with (A) fatty acid, (B) sterol, and (C) carotenoid profiles as well as with (D) their combination.

phytoplankton. As expected, all studied biomarkers differed to some extent at a class level, but the fatty acid, sterol, and carotenoid profiles of the phytoplankton groups overlapped, resulting in insufficient separation of freshwater phytoplankton groups. The highest within-group similarities and the lowest dispersions among all measured biomolecules were in carotenoids for all but cyanobacteria, which were most similar in their fatty acid profiles. However, we did not sum up the different myxoxanthophylls, which could have increased the similarity in carotenoids in cyanobacteria. Additionally, in dinoflagellates the carotenoids and fatty acids had equal similarity. When the usability of these compounds in identification was examined, carotenoids were found to be the most reliable since they distinguished all but diatoms and golden algae from each other. The fatty acids performed best in separating diatoms from cryptomonads and green algae, whereas the sterols performed best in separating cryptomonads and dinoflagellates from each other. The most reliable identification was obtained with the combined analysis of all three biomarker groups. Thus, we conclude that the accuracy of phytoplankton group-level description is significantly improved when using fatty acids, carotenoids, and sterols in tandem.

4. Experimental

4.1. Phytoplankton strains and culturing

Forty-seven strains of planktonic freshwater eukaryotic algae and ten stains of planktonic freshwater cyanobacteria (Table 1) were grown in MWC medium (Guillard and Lorenzen, 1972) in 800 mL plastic tissue culture flasks. Each strain had two to three replicates that were grown at 18 °C and under a light:dark cycle of 16:8 h. The intensity of the illumination was between 10 and 80 μmol quanta s^{-1} m^{-2} and selected based on known preferences such that strains belonging to the same class were exposed to similar light levels to ensure possible differences in biomolecule profiles were due to genetics and not growth conditions. The biomasses were collected by filtering 100–1000 mL of cultures onto sterile cellulose nitrate membrane filters (pore size 3 μm, Whatman, Maidstone, Kent, UK). Samples for identification were frozen (−20 °C), and the DNA was isolated within a week, whereas the FA, sterol, and carotenoid samples were frozen (−80 °C), freeze-dried, and stored at −80 °C until analysis. For the fatty acids and sterol profiles of *Ceratium* sp. We used our previously published data (Taipale et al., 2016).

4.2. Lipid extraction

Lipids from the freeze-dried phytoplankton (3–10 mg) were extracted with chloroform:methanol:water in a 8:4:3 ratio (Folch et al., 1957). The samples were sonicated for 20 min and centrifuged, after which the lower phase was transferred into a new tube and evaporated to dryness under nitrogen. Samples were diluted in 400 μL of toluene and split for sterol, fatty acid, and carotenoid analyses in a ratio of 2:1:1.

4.3. Fatty acid analysis

Fatty acids were methylated using mild sulfuric acid (Taipale 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 temperature of the injector was 260 °C, and we used a splitless injection mode (for 1 min). Temperatures of the interface and ion source were 250 °C and 220 °C, respectively. Agilent DP-23 (60 m × 0.25 mm x 0.25 μm) with 5 m Guardian (Agilent, Santa Clara, CA, USA) was used with the following temperature program: 60 °C was maintained for 1 min, then the temperature was increased at a rate of 30 °C min^{-1} to 130 °C, followed by 7 °C min^{-1} to 180 °C and 1.5 °C min^{-1} to 200 °C. This temperature was held for 10 min. The total program time was 47.14 min, and the solvent cut time was 9 min. Fatty acids were identified by the retention times (RT) and specific ions, which were also used for quantification (Taipale et al., 2016). Fatty acid concentrations were calculated using calibration curves based on known standard solutions (15 ng, 50 ng, 100 ng, and 250 ng) of a FAME standard mixture (GLC standard mixture 566c, Nu-Chek Prep, Elysian, MI, USA) and using recovery percentage of internal standards. The Pearson correlation coefficient was >0.99 for each fatty acid calibration curve. Additionally, we used the free fatty acid of C23:0 (Larodan, Malmö, Sweden) as an internal standard and for the calculation of recovery percentages.

4.4. Sterol analysis

Toluene was evaporated from the sterol samples, after which 100 μL of pyridine was added to dissolve the sterols. Sterols were silylated using 70 μL of N,O-bis [trimethylsilyltrifluoroacetamide] (BSTFA) at 70 °C. Trimethylsilyl (TMSi) derivatives of sterols were analyzed with Shimadzu GC-MS-QP2010 Ultra with helium as a carrier gas. The temperature of the injector was 280 °C, and we used a splitless injection mode (for 1 min). Temperatures of the interface and ion source were 250 °C and 250 °C, respectively. Sterol TMSi was analyzed with a Phenomenex

(Torrance, CA, USA) ZB-1701 column (30 m × 0.25 mm × 0.25 μm) according to Laakso et al. (2014). We followed the temperature program of Dutta and Normén (1998), in which 80 °C was maintained for 1 min, and then the temperature was increased by 20 °C min^{-1} to 260 °C, after which 260 °C was held for 1 min. The temperature was finally increased to 275 °C at a rate of 1 °C min^{-1}, and this temperature was held for 25 min. The total program time was 51.0 min, and the solvent cut time was 15 min.

Sterols were identified using external standards, retention indices, and MS spectra in a NIST 2.3 Library. Since we had two identical MS spectra for 24 epimers of Spinasterol/Chondrillasterol and 22-dihydrospinasterol/22-dihydrochondrillasterol, we were able to separate these epimers using commercially available standards (Table 4, Fig. 7). However, we did not find two epimers (MS spectra) for all potential pairs (epibrassicasterol/brassicasterol, sitosterol/clionasterol, stigmasterol/poriferasterol, campesterol/dihydrobrassicasterol) and thus it is possible that both epimers elutes at the same retention time (Table 4, Fig. 7). Therefore in the text we use both names.

Sterols were quantified using characteristic ions (Taipale et al., 2016) and using authentic standard solutions of plant sterol mixture from Larodan (Malmö, Sweden). Identification of dinoflagellates is based on previously published MS spectra (Amo et al., 2010; Atwood et al., 2014; Leblond and Chapman 2002; Piretti et al., 1997). Phytosterol mixture included 53% β-sitosterol (stigmast-5-en-3β-ol), 7% stigmasterol ((24 E)-stigmasta-5,22-dien-3β-ol), 26% of campesterol (campest-5-en-3β-ol), and 13% of brassicasterol ((22 E)-ergosta-5, 22-dien-3β-ol). Cholesterol (cholest-5-en-3β-ol), desmosterol (cholest-5, 24-dien-3β-ol), ergosterol, fucosterol ((24 E)-stigmasta-5,24 (24^1)-dien-3β-ol), and spinasterol ((24 E)-stigmasta-7,22-dien-3β-ol) were purschased from Merck KGaA (Darmstadt, Germany). The 22, 23-dihydrospinasterol (schottenol, 5α-stigmast-7-en-3β-ol), fungisterol (5α-Ergost-7-en-3β-ol), episterol (5α-Ergosta-7,24 (24^1)-dien-3β-ol), corbisterol (3β-stigmasta-5,7,22-trien-3-ol), and 24-methylcholestanol (4α-methyl-5α-cholestan-3β-ol) were purschased from Cymit Quimica SL (Barcelona, Spain). For those compounds for which no external standards were available, we used the closest similar compound. The recovery percentage of sterol samples was calculated using 5α-cholestane (Merck KGaA) as an internal standard.

4.5. Carotenoid analysis

Toluene was evaporated from the samples and redissolved in 200 μL of acetone. Carotenoids were analyzed by reversed-phase liquid chromatography using Shimadzu 30-series ultra high performance LC

Table 4
Retention time (RT) for analyzed sterols from phytoplankton samples. The chemical structures and the trivial names of sterols are based on Goad and Akihisa (1997). Identification was based on standards and MS spectra. The order of stereoisomers was based on standards and MS spectra. Star in the trivial name indicates uncertainty in the order of stereoisomers. Other M+ ions refer to major ions of the MS spectra (the first ion is the base peak). Data for 24-methylcholestanol, 4-methylcholestanol, and gorgosterol are from Taipale et al. (2016), thus retention times are not given here.

RT (min)	Chemical structure	IUPAC name	Trivial name	Identification	MW TMSi	Other M+ ions
24.7	$C_{27}H_{44}O$	(22 E)-Cholesta-5,22-dien-3β-ol	22-Dehydrocholesterol	MS spectra	456	69, 111, 129, 215, 327, 366
25.7	$C_{27}H_{46}O$	Cholest-5-en-3β-ol	Cholesterol	Standard	458	129,329, 443
27	$C_{28}H_{46}O$	(22 E)-Ergosta-5,22-dien-3β-ol	Brassicasterol	Standard	470	129, 145, 255, 343, 367, 382, 457, 472
27.17	$C_{27}H_{44}O$	Cholesta-5,24-dien-3β-ol	Desmosterol	Standard	456	129, 253, 327, 366, 441
28.2	$C_{28}H_{44}O$	(22 E)-Ergosta-5,7,22-trien-3β-ol	Ergosterol	Standard	468	363, 211, 237, 251, 253, 337, 362, 363, 376, 378
28.1	$C_{28}H_{48}O$		Unidentified methyl-C28:1 sterol	Ms spectra	472	73, 147, 207, 227, 269, 367, 457
28.3	$C_{28}H_{50}O$	4α-Methyl-5α-cholestan-3β-ol	4-Methylcholestanol	MS spectra	474	75, 345, 369, 384, 459
28.9	$C_{28}H_{46}O$	Campest-5-en-3β-ol	Campesterol	Standard	472	129, 145, 255, 343, 367, 382, 457
29.1	$C_{28}H_{48}O$	5α-Campest-7-en-3β-ol	24-Epifungisterol*	MS spectra	472	107, 147, 182, 213, 255, 343, 367
29.2	$C_{28}H_{46}O$	Ergosta-5,24 (24^1)-dien-3β-ol	24-Methylenecholesterol (Chalinasterol)	MS spectra	470	129, 73, 296, 386, 455
29.2	$C_{29}H_{48}O$		Unidentified dimethyl-C29:2	MS spectra	484	125, 69, 269, 354, 394
29.3	$C_{29}H_{50}O$		Unidentified dimethyl-C29:1	MS spectra	486	69, 95, 125, 271, 298, 388
30	$C_{29}H_{48}O$	(24 E)-Stigmasta-5,22-dien-3β-ol	Stigmasterol	Standard	484	83, 129, 255, 355, 379, 394, 469, 484
30.2	$C_{29}H_{48}O$		Unidentified C29:2 sterol	MS spectra	484	83, 107, 147, 213, 229, 255, 318, 343, 379, 427, 469
30.6	$C_{28}H_{46}O$	5α-Ergosta-8,24 (28^1)-dien-3β-ol	Fecosterol	MS spectra	470	343, 75, 107, 145, 213, 255, 318, 343, 365, 413, 455
30.8	$C_{28}H_{48}O$	5α-Ergost-7-en-3β-ol	Fungisterol*	Standard	472	255, 213, 229, 351, 367, 377, 457
31.2	$C_{28}H_{46}O$	5α-Ergosta-7,24 (24^1)-dien-3β-ol	Episterol	Standard	472	343, 75, 107, 145, 213, 253, 281, 365, 386, 455
31.5	$C_{29}H_{46}O$	3β-Stigmasta-5,7,22-trien-3-ol	Corbisterol	Standard	482	377, 211, 251, 253, 392, 467
31.6	$C_{30}H_{50}O$		Unidentified trimethyl-C30:2	MS spectra	498	393, 69, 109, 187, 241, 351, 483
32	$C_{29}H_{50}O$	Stigmast-5-en-3β-ol	β-Sitosterol	Standard	486	129, 255, 357, 381, 396, 471, 486
32.1	$C_{29}H_{48}O$	(24 E)-Stigmasta-7,22-dien-3β-ol	Spinasterol	Standard	484	343, 255, 318, 372, 379, 441, 469
32.3	$C_{29}H_{52}O$	4α, 24-dimethyl-5α-cholest-3β-ol		MS spectra	498	69, 83, 139, 368, 408
32.5	$C_{29}H_{50}O$	5α-Poriferasta-7-en-3β-ol	22-Dihydrochondrillasterol	MS spectra	486	147, 107, 213, 229, 255, 318, 345, 381, 429, 471
33.0	$C_{30}H_{48}O$	4α,23,24-Trimethyl-5α-cholest-5, 22 E-dien-3β-ol	Dehydrodinosterol	MS spectra	498	69, 83, 129, 139, 269, 368, 408
33.2	$C_{30}H_{52}O$	4α,23,24-Trimethyl-5α-cholest-22 E-en-3β-ol	Dinosterol	MS spectra	500	69, 271, 359, 388
34	$C_{29}H_{48}O$	(24 E)-Poriferasta-7,22-dien-3β-ol	Chondrillasterol	MS spectra	484	343, 255, 318, 371, 379, 441, 469
34.4	$C_{29}H_{50}O$	5α-Stigmast-7-en-3β-ol	Schottenol/22-dihydrospinasterol	Standard	486	255, 147, 107, 213, 229, 318, 345, 381, 429, 471
34.4	$C_{30}H_{50}O$		Unidentified trimethyl-C30:1		500	73, 156, 207, 269, 281, 429, 471
35	$C_{30}H_{50}O$	4α,23,24-Trimethyl-5α-cholest-7-en-3β-ol	–	MS spectra	500	95, 121, 229, 283, 297, 359, 387, 485
36.1	$C_{30}H_{52}O$	4α,23,24-trimethyl-5α-cholestan-3β-ol	–	MS spectra	502	75, 130, 397, 412
38.4	$C_{30}H_{50}O$	Gorgost-5-en-3β-ol	Gorgosterol	MS spectra	498	129, 253, 337, 343, 386, 400, 408, 483
43	$C_{31}H_{54}O$		Unidentified tetramethyl-C31:0	MS spectra	514	129, 204, 229, 271, 383, 422, 497

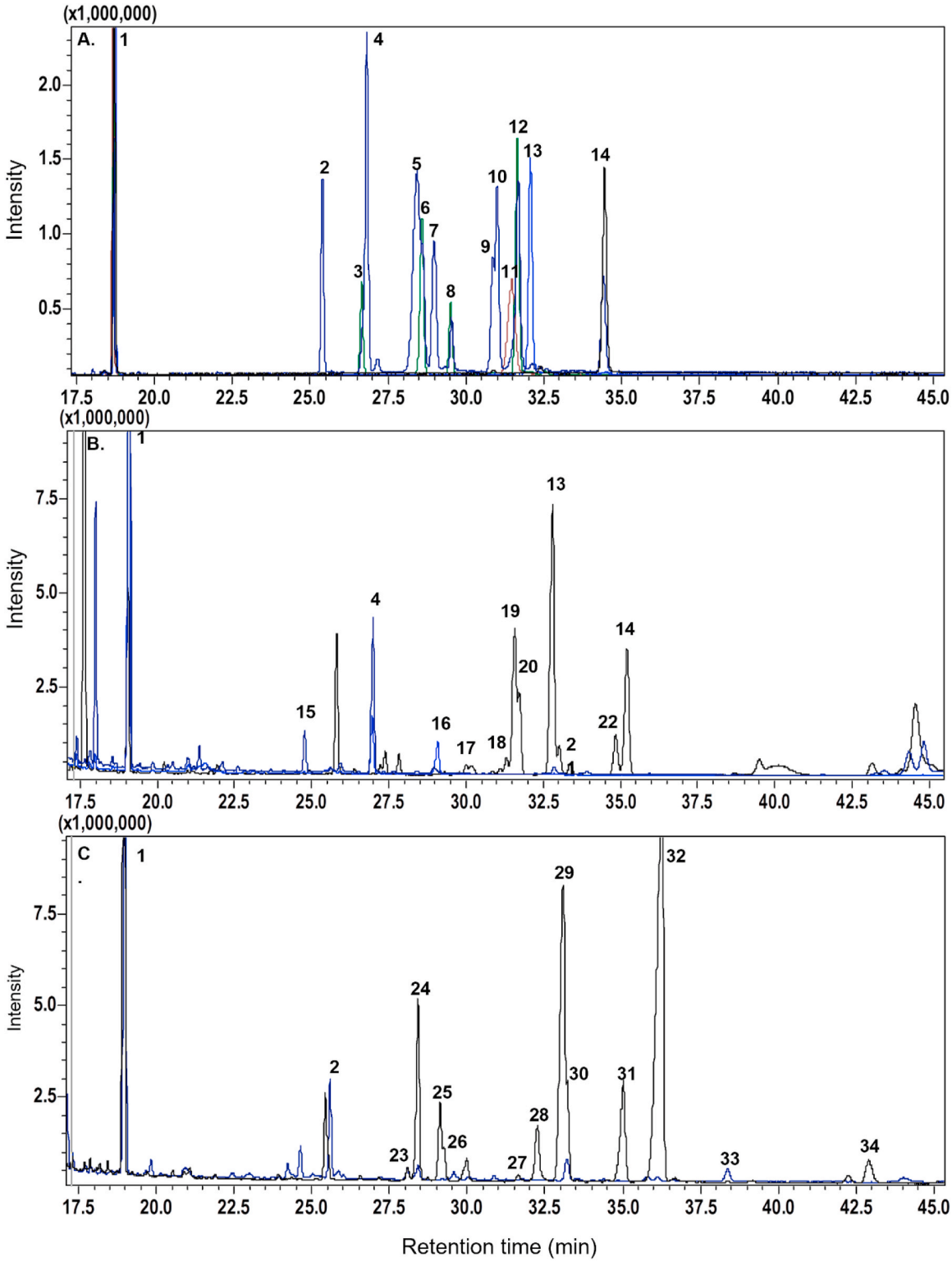

Fig. 7. Chromatograms of sterols found from freshwater phytoplankton strains: (A) sterols idendified with external sterol standards, and (B) chromatograms of sterols identified from diatoms (*Fragilaria capucina*, *Asterionella formosa*) and green algae (*Monoraphidium griffithii*), C) sterols identidied from dinoflagellates (*Peridinium centeniale* and *Ceratium* sp.). The IUPAC names of the sterols are found in Table 4. Chromatographic conditions are described in the text, the peaks are as follows: (1) α-Cholestane (ISTD), (2) Cholesterol, (3) Brassicasterol, (4) Desmosterol, (5) Ergosterol, (6) Campesterol, (7) 24-methylcholestanol, (8) Stigmasterol, (9) Fungisterol, (10) Episterol, (11) Corbisterol, (12) β-Sitosterol, (13) Spinasterol, (14) 22-dihydrospinasterol (schottenol), (15) 22-Dehydrocholesterol, (16) Chalinasterol, (17) Epifungisterol, (18) Fecosterol, (19) Fungisterol, (20) Episterol, (21) 22-dihydrochondrillasterol (22) Chondrillasterol, (23) Unidentified methyl C28:2 sterol, (24) 4α-methyl-5α-cholestan-3β-ol, (25) Unidentified dimethyl C29:2 sterol, (26) Unidentified dimethyl C29:1 sterol, (27) Unidentified trimethyl C29:2 sterol (28) 4α, 24-Dimethyl-5α-cholestan-3β-ol, (29) Dehydrodinosterol, (30) Dinosterol, (31) Unidentified trimethyl C30:1 sterol, (32) 4α,23,24-Trimethyl-5α-cholest-(7)-en-3β-ol, (33) Gorgosterol, (34) Unidentified tetramethyl-C31:0. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(Shimadzu, Kyoto, Japan) consisting of an LC-30AD pump, an autosampler (SIL-30 A C), an online degasser, a column oven (CTO-20 A C), and a photodiode array detector (PDA; SPD-M20A). A final volume of 10 or 50 μL 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 (A) MeOH and (B) MTBE. The flow rate was 1.0 mL min^{-1} and the gradient used was as follows: initial flow ratio was 100:0 MeOH: MTBE

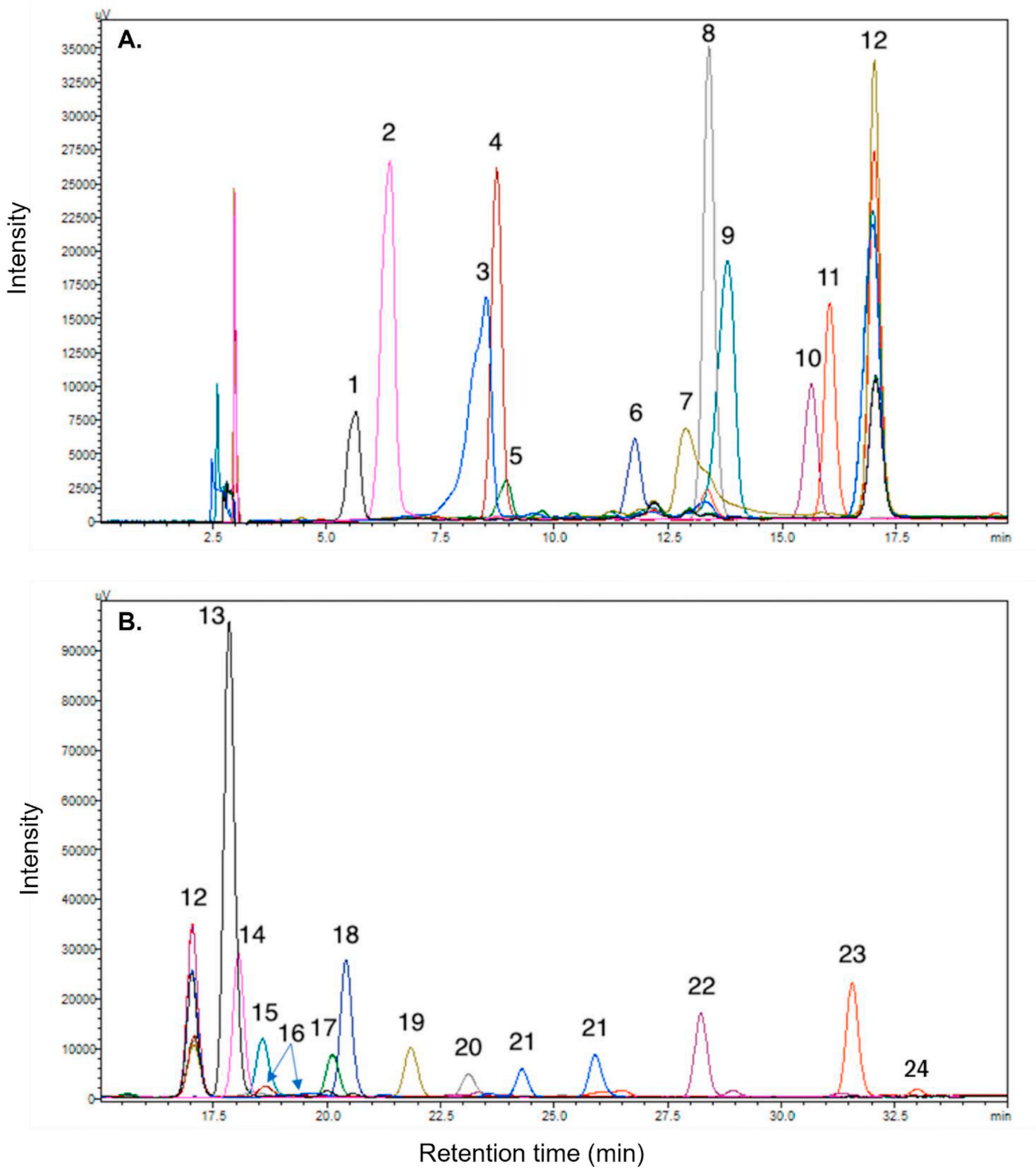

Fig. 8. Chromatograms of carotenoid standards. Chromatographic conditions are described in the text, and peaks are as follows: (1) Peridinin, (2) Fucoxanthin, (3) Violaxanthin, (4) Neoxanthin, (5) Dinoxanthin, (6) Antheraxanthin, (7) Astaxanthin, (8) Diadinoxanthin, (9) Lutein, (10) Monadoxanthin, (11) Zeaxanthin, (12) *Trans*-β-Apo-8′-carotenal (ISTD), (13) Canthaxanthin, (14) Diatoxanthin, (15) Myxoxanthophyll, (16) Aphanizophyll, (17) α-Cryptoxanthin, (18) Alloxanthin, (19) Crocoxanthin, (20) β-Cryptoxanthin, (21) Echinenone, (22) α-Carotene, (23) β-Carotene, and (24) unidentified derivative (UID) of β-Carotene.

(v/v) ramped evenly from time 1 min–35 min to 65:35 MeOH: MTBE (v/v), then ramped from 35 min to 40 min to 50:50 MeOH: MTBE (v/v) following a cleaning ramp from 40 min to 42 min to 30:70 MeOH: MTBE (v/v) for 6 min and then returned to initial eluent composition over 2 min. Finally, a re-equilibration (10 min) was carried out at initial concentrations of 100:0 MeOH: MTBE (v/v). The column temperature was maintained at 30 °C. The eluting peaks were monitored at a range of 250–600 nm (slit 1.2 nm) using PDA. Quantification was performed using wavelength 450 ± 4 nm (for the zeaxanthin 480 ± 4 nm due to overlapping with chlorophyll *a*) and Shimadzu LabSolutions software (version 5.93) comparing peak area with standard reference curves.

Peaks were identified by comparing the retention times and UV–Vis spectral data with those of the corresponding standards and literature (Britton et al., 2004; Roy et al., 2011). Combined chromatograms of the standards are presented in Fig. 8. Response factors of carotenes and xanthophylls (Table 5) were calculated using Equation 1.

$$F = \left(\frac{A_x}{c_x}\right) \cdot \left(\frac{c_{ISTD}}{A_{ISTD}}\right) \quad (1)$$

where F is the response factor, A_x is the peak area of the analyte, c_x is the concentration of the analyte, c_{ISTD} is the concentration of the internal standard, and A_{ISTD} is the peak area of the internal standard. The concentration of each analyte was calculated based on the response factors.

Standards for peridinin, fucoxanthin, violaxanthin, neoxanthin, dinoxanthin, antheraxanthin, astaxanthin, diadinoxanthin, lutein, monadoxanthin, zeaxanthin, canthaxanthin, diatoxanthin, myxoxanthophyll, aphanizophyll, α-cryptoxanthin, alloxanthin, crocoxanthin, β-cryptoxanthin, echinenone, α-carotene, and β-carotene were purchased from DHI Laboratory Products (Hoersholm, Denmark). Trans-β-Apo-8′-carotenal was used as an internal standard and was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The concentrations of standard carotenoids were assessed by DHI Laboratory Products using spectrophotometry and the absorption coefficients of the carotenoids. To a 200 μL sample of each standard solution of carotenes and xanthophylls (c = 0.5–1.5 μg mL^{-1}), 10 μL of internal standard in acetone (c = 20.04–1.00 ng μL^{-1}) was added. After that, each standard sample together with an internal standard was used for HPLC analysis.

4.6. Statistical analysis

We used Similarity percentages (SIMPER; Clarke and Warwick, 1994) to identify the characteristic fatty acids, sterols, and carotenoids of each phytoplankton group. SIMPER assesses the average percentual contribution of individual variables to the dissimilarity in a Bray-Curtis dissimilarity matrix. The similarity of fatty acid, sterol, and carotenoid profiles was studied with similarity profile routine (SIMPROF; Clarke et al., 2008).

PERMDISP (distance-based test for homogeneity of multivariate dispersions; Anderson, 2014) was used to investigate the within-group variation in fatty acid and/or sterol and/or carotenoid composition. PERMDISP compares among-group differences based on the distance each observation is from its group centroid. In PERMDISP, a high mean distance-to-centroid denotes high dissimilarity. Additionally, permutational multivariate analysis of variance (PERMANOVA; Anderson, 2014) was used to test the differences in fatty acid, sterol, and carotenoid contents between phytoplankton groups. The pairwise comparisons determined whether the groups were significantly different from one another. Because the within-group sample sizes were limited for certain groups, Monte Carlo p-values were used to assess the significance of the PERMANOVA test statistic by random sampling of the asymptotic permutation distribution (Anderson, 2014).

Non-metric multidimensional scaling nMDS (Primer 7) (Clarke and Gorley, 2015) was used to separate the phytoplankton groups by their fatty acid, sterol, and carotenoid composition in the hierarchical cluster analysis, i.e. for creating similarity groups in nMDS ordination.

Table 5
Retention time (RT) and response factor (F) for analyzed carotenoids from phytoplankton samples.

Carotenoid	P_n	RT (min)	F
Peridinin	1	5.6	0.657
Fucoxanthin	2	6.4	0.721
Violaxanthin	3	8.5	0.889
Neoxanthin	4	8.7	0.547
Dinoxanthin	5	8.9	0.509
Antheraxanthin	6	11.7	0.903
Astaxanthin	7	12.9	0.508
Diadinoxanthin	8	13.4	1.060
Lutein	9	13.8	0.864
Monadoxanthin	10	15.6	1.197
Zeaxanthin	11	16.0	0.752
Trans-β-Apo-8′-carotenal[a]	12	16.9	
Cantaxanthin	13	17.9	2.358
Diatoxanthin	14	18.0	0.952
Myxoxanthophyll	15	18.6	0.466
Aphanizophyll	16	19.4	0.037
α-Cryptoxanthin	17	20.1	0.702
Alloxanthin	18	20.4	1.116
Crocoxanthin	19	21.8	1.101
β-Cryptoxanthin	20	23.1	0.361
Echinenone	21	25.9	0.419
α-Carotene	22	28.2	0.725
β-Carotene	23	31.3	0.703
Unidentified derivative of β-Carotene	24	32.7	nd

[a] Internal standard; nd = not determined.

Additionally, we applied nMDS using all biomolecules together. We used 60% similarity for the limit of good separation of phytoplankton groups. The interactions between MDS1 and MDS2 and variables were analyzed with Spearman correlation analysis ($r > 0.6$ shown in outputs).

Author contributions

E.P. and S.J.T. conceptualized the study and wrote the first draft of the paper. C.R. carried out the molecular analyses. J.B. carried out the phylogenetic data analyses. H.P. guided and assisted in the methodology of the chemical analyses. H.A. participated in acquiring the carotenoid data. P.S. helped in carotenoid data acquisition and analysis. All authors reviewed the manuscript.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.phytochem.2023.113624.

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