Fottea, Olomouc, 23(2): 223–234, 2023
DOI: 10.5507/fot.2023.001

Differences in pigment composition and concentration between phototrophic, mixotrophic, and heterotrophic Chrysophyceae

Jens Boenigk[1,2*], Daniela Beisser[1,2], Luise Franke[1], Lea Klar[1], Maja Ilić [3,4] & Patrick Fink[3,5]

[1] *University of Duisburg–Essen, Biodiversity, Universitätsstr. 5, 45141 Essen, Germany; *Corresponding author e–mail: jens.boenigk@uni–due.de*
[2] *University of Duisburg–Essen, Centre for Water and Environmental Research, Universitätsstr. 5, 45141 Essen, Germany*
[3] *University of Cologne, Institute for Zoology, Zuelpicher Strasse 47b, 50674 Koeln, Germany*
[4] *present address: Swiss Federal Institute for Forest, Snow and Landscape Research WSL, Forest Health and Biotic Interactions, Zuercherstrasse 111, 8903 Birmensdorf, Switzerland*
[5] *present address: Helmholtz–Centre for Environmental Research – UFZ, Department River Ecology and Department Aquatic Ecosystem Analysis and Management, Brueckstrasse 3a, 39114 Magdeburg, Germany*

Abstract: The presence of distinct pigments is characteristic of different algal groups while the relative concentration of these pigments may vary with light conditions. Here we investigate the pigment pattern of 35 strains of Chrysophyceae by means of high–performance liquid chromatography. We further investigated shifts in pigment concentrations between light and dark conditions and the conservation of these pigment patterns in heterotrophic, mixotrophic, and phototrophic taxa. We did not find chlorophyll in heterotrophic strains but carotenoids were present in all taxa. In phototrophs and mixotrophs, we confirm the presence of the chlorophylls–a, c_1, and c_2 as well as of fucoxanthin. We provide evidence for the violaxanthin cycle as the dominant xanthophyll cycle. Moreover, we found pigments of the diadinoxanthin cycle in low concentrations. While pigment concentrations were regulated depending on light availability in photosynthetic taxa, they were unaffected by light in heterotrophic taxa.

Key words: carotenoids, chlorophylls, Chrysophyceae, light adaptation, pigments, trophic mode

Introduction

Chrysophyceae are widespread in marine and freshwater habitats and constitute an important fraction of eukaryotic organisms in aquatic ecosystems (Boenigk & Arndt 2002; Andersen 2007). They are important primary producers (Bock et al. 2014) and are among the dominant bacterivores in most habitats channelling bacterial secondary production to higher trophic levels (Finlay & Esteban 1998). Chrysophyceae comprise a broad range of nutritional modes. Phototrophic chrysophytes, such as the unicellular *Mallomonas* Perty spp. or the colonial *Synura* Ehrenberg spp. vary between 5 µm and 100 µm in size while heterotrophic (colourless) taxa are usually small unicellular flagellates hardly exceeding a cell size of 5 µm (Cavalier–Smith & Chao 2006; Grossmann et al. 2016a,b; Kristiansen & Škaloud 2017). Chrysophytes evolved from obligate phototrophic ancestors but comprise many lineages which secondarily lost photosynthesis (Graupner et al. 2018). Mixotrophy, i.e., complementing photoautotrophy with chemoheterotrophy (bacterivory), probably appeared early in their evolution and obligate chemoorganoheterotrophy evolved several times independently (Olefeld et al. 2018). Within the extant chrysophytes, the Synurales are the only predominantly phototrophic lineage, while most other lineages comprise heterotrophic and mixotrophic taxa. In the context of our study, we use the term mixotrophic for strains which have the ability for photosynthetic carbon fixation and are known to regularly ingest bacteria. In chrysophytes, mixotrophy spans from a predominantly phototrophic to a predominantly heterotrophic nutrition (Holen & Boraas 1995).

Photopigments

Chlorophylls and photosynthetically active carotenoid derivatives, such as fucoxanthin, are classified as light–harvesting pigments (Brunet et al. 2011).

Chlorophyll–a as the main pigment of photosynthesis can be found in all photosynthetically active organisms in the reaction center of the photosystems (Schopfer 2010). Stramenopiles further possess chlorophyll–c in their light–harvesting complex (Dorrell & Bowler 2017). The variants of chlorophyll–c differ slightly in their chemical structure and absorption maxima (Fookes & Jeffrey 1989).

Carotenoids are divided into oxygen–free hydrocarbons (α–carotene, β–carotene) and their oxygen–containing derivates, the xanthophylls (e.g., fucoxanthin, zeaxanthin) (Latowski et al. 2014; Lohr et al. 2005). Carotenoids play an important role in photosynthesis as they function as both light–harvesting (Lichtenthaler & Buschmann 2001) and photoprotection pigments (Demmig–Adams & Adams III 1992; Horton & Ruban 2005). They can be divided into primarily light–harvesting pigments and primarily photo–protective pigments based on their function (Brunet et al. 2011). The group of photo–protective pigments includes β–carotene and xanthophylls (e.g., violaxanthin, zeaxanthin) (Rodríguez et al. 2006; Brunet et al. 2011). They are important for avoiding photo–damage in the photosynthetic apparatus by dissipating excess of energy to heat (Falkowski & Raven 1998). The most widespread dissipation process is the xanthophyll cycle: under high–light conditions, violaxanthin is first converted to the intermediate state antheraxanthin and finally to zeaxanthin. Under low–light conditions, the reaction is reversed and zeaxanthin is transformed into violaxanthin (Demming–Adams & Adams 1996; Young et al. 1997). Some microalgal groups also possess another carotenoid–based energy dissipation process, the diadinoxanthin–cycle which interconverts between diadinoxanthin and diatoxanthin depending on the light conditions (Lohr & Wilhelm 1999).

The actual pigment concentrations (i.e., not the presence of distinct pigments but their relative and absolute concentration) may be dynamic within strains. Microalgae have evolved a diversity of adaptive and acclimation mechanisms for optimizing the rate of photosynthesis (Garrido et al. 2016). Adaptive differentiation of pigment patterns may eventually lead to speciation while pigment patterns may physiologically vary to a certain extent depending on light intensity (Anning et al. 2000; Garrido et al. 2016). Algae experience high fluctuations in their light environment, e.g., due to diurnal changes and decreasing light intensity in the water column. Photosynthetic organisms can physiologically adjust to changes in their light environment by photo–acclimation based on changes in pigment composition that helps to adjust to changes in light regime (Brunet et al. 2011). The relative concentration of light–harvesting pigments, such as chlorophylls and fucoxanthin decrease under high light conditions, while photoprotective pigments, such as β–carotene and the elements of the xanthophyll–cycle are enhanced under high light conditions (Dubinsky & Stambler 2009).

Chrysophyte plastids and pigmentation

All chrysophytes taxa contain plastids but the plastids are strongly reduced in heterotrophic lineages (Smith & Chao 2006; Bock et al. 2014; Cavalier– Kristiansen & Škaloud 2017). So far, there is no evidence of a complete loss of plastids in any heterotrophic lineage (Graupner et al. 2018; Grossmann et al. 2016a) even though the presumable complete loss of the plastid genome has been shown in at least one species (Dorrell et al. 2019). However, the loss of photosynthesis and plastid reduction in the evolution of heterotrophy was accompanied by a reduction or loss of pigmentation (Beisser et al. 2017; Graupner et al. 2018; Dorrell et al. 2019).

The pigments chlorophyll–a and c, β–carotene, and carotenoids derived from β–carotene, i.e., antheraxanthin, diadinoxanthin, diatoxanthin, fucoxanthin, neoxanthin, violaxanthin, and zeaxanthin were described to be present in Chrysophyceae with β–carotene as the main carotene and fucoxanthin as the main xanthophyll (Dales 1960; Withers et al. 1979; Withers et al. 1981; Falkowski & Raven 1998; Fig. 1). The high fucoxanthin concentrations are responsible for the yellow–gold to yellow–brown colour of chrysophytes (Evert & Eichhorn 2013; Kristiansen & Škaloud 2017).

So far, pigment patterns have not been investigated in the context of the evolution of mixotrophy and heterotrophy in chrysophytes. It is unclear whether heterotrophic strains still possess photopigments and to what extent pigmentation was reduced in mixotrophic and heterotrophic taxa. As heterotrophy evolved at least five times independently, pigment patterns in heterotrophic lineages may or may not show similar patterns in different taxa, i.e., to what extent such shifts in pigment concentrations are conserved during the evolution of heterotrophy remains unknown.

Further, it is unclear which role adaptation and acclimation play in the expression of distinct pigment patterns in chrysophytes and whether their importance differs between trophic modes. The actual (relative and absolute) pigment concentrations may not be genetically fixed but may vary to a certain extent depending on light intensity as shown for other algae (Anning et al. 2000; Garrido et al. 2016). It remains unclear to what extent pigment patterns in chrysophytes are modified by light intensity. In order to judge the specificity of pigment patterns, it is therefore important to consider intraspecific variation.

Thus, here we address the pigmentation pattern of 35 chrysophytes (table S1) including phototrophic, mixotrophic, and different heterotrophic lineages by means of high–performance liquid chromatography (HPLC). We further investigated the extent of physiological regulation of pigment concentrations due to light and dark conditions in phototrophic, mixotrophic, and heterotrophic taxa. For this latter aspect, we particularly focused on pigment patterns for taxa that are at the edge between mixotrophy and heterotrophy and compare the extent of regulation in these taxa to that of an obligate phototrophic species.

Materials and Methods

Culture media and origin of strains. A total of 35 chrysophyte strains (Table S1) including 12 heterotrophic, 14 mixotrophic, and nine phototrophic strains were investigated. All chrysophyte strains are in culture in the Department of Biodiversity at the University of Duisburg–Essen in 50 ml cell culture flasks in either WC medium (Guillard & Lorenzen 1972), modified DY–V medium (Andersen 2007), or in inorganic basal medium with a wheat grain (Hahn et al. 2003) (Table S1). All heterotrophic and predominantly heterotroph mixotrophic organisms were supplemented with sterile wheat grains and fed with the bacterium *Limnohabitans planktonicus* Kasalický, Jezbera, Šimek et Hahn as a food source.

Prior to the experiments the wheat grain was removed, and the strains were grown with the bacterial strain *Limnohabitans planktonicus* II–D5 as food source (1–2×10^7 bacteria.ml^{-1}). *Limnohabitans planktonicus* strain II–D5 was grown in an inorganic basal (IB) medium supplemented with 3 $g.l^{-1}$ NSY (Hahn et al. 2003), called NSY medium hereafter, at room temperature on a shaker. The bacteria were centrifuged for 15 min at 5000 rpm (Eppendorf 5804) in 50 ml tubes. The supernatant was discarded, and the bacteria were resuspended in 50 ml IB medium. This washing step was repeated for a second time and subsequently the bacteria were suspended in 25 ml IB medium.

Cultivation and determination of cell abundance and biovolume. For the experiments the strains were transferred to a) 500 ml cell culture flasks or b) 5 l Erlenmeyer glass flasks. All strains were grown in culture cabinets under a 14:10 h light:dark regime (100 $\mu mol.m^{-2}.s^{-1}$) at 16 °C. The heterotrophic strains as well as *Poterioochromonas* spp. were fed with bacteria every second day (adjusted to 1–2×10^7 bacteria.ml^{-1}) and supplemented with sterile wheat grains as a food source for *Limnohabitans planktonicus*.

The strains transferred to 500 ml cell culture flasks were grown for four to six weeks depending on the growth rates of the different strains before samples were taken to measure pigment concentrations, cell abundances, and biovolumes.

The strains transferred to 5 l Erlenmeyer glass flasks were used in the experiments aiming at photo–acclimation (see description of experiment below). Three strains, *Synura sphagnicola* (Korshikov) Korshikov (LO234K–E), *Cornospumella fuschlensis* Boenigk et Grossmann (A–R4–D6), and *Poterioochromonas malhamensis* (Pringsheim) Péterfi (DS) were cultivated in triplicates under different light conditions (light: 100 $\mu E \cdot m^{-2}.s^{-1}$ and dark: 0 $\mu E.m^{-2}.s^{-1}$) for six days. Additionally, *Poterioochromonas malhamensis* (DS) was cultured osmotrophically under axenic conditions in NSY medium and phagotrophically in WC medium supplemented with wheat grain and *Limnohabitans planktonicus* as food bacteria. Temperature and day/night rhythm were the same as described above.

For all samples, 35 ml of the culture for phototrophic and mixotrophic taxa and 150 ml of the culture for heterotrophic taxa were filtered onto three 25 mm glass fiber filters (mean 0.7 µm pore size, grade GF/F; VWR) for pigment analysis. They were stored at –20 °C until further processing. Negative controls without flagellates were also processed in order to check for potential contaminations by food organisms.

A 2.5 ml subsample of each sample was fixed with 100 µl Lugol's iodine solution for the determination of the flagellate abundance. Flagellates were subsequently counted in Sedgewick–Rafter counting chambers under the light microscope (Nikon EclipseTS100) at 400× total magnification. Further, 35 cells for each culture (exponential growth phase) were measured in order to determine the biovolume assuming an ellipsoid cell form (Hillebrandt et al. 1999).

Extraction and analysis of pigments. The pigment extraction was performed following Ilić et al. (2023). Briefly, the frozen filters were transferred into separate 10 ml glass test tubes and 4 ml acetone (100%, HiPerSolv Chromanorm, HPLC Grade) were added. Depending on the estimated biomass on the filters a defined volume (25–200 ml) of an internal standard (ISTD; trans–β–Apo–8'–carotinal, 5 $\mu g.ml^{-1}$) was also added.

The tubes were mixed with a vortex–shaker and placed into an ultrasonic bath for 10 min (to prevent overheating the samples were removed every two minutes from the ultrasonic bath and cooled for one minute on ice). The samples were stored overnight at 4 °C and after removal of the filter the tubes were centrifuged for 15 min at 5,000 rpm (Eppendorf Centrifuge 5804). One ml of the clear supernatant was transferred into a 1.5 ml glass vial and stored at –20 °C. For all samples including all biological replicates, three filters were prepared (technical replicates) and were analysed with HPLC within 72 hours. In the case that only very low peaks (close to the limit of detection) were detected in the chromatogram, indicating a potentially low concentration of the pigments in the analysed sample, the samples were transferred to vials with 200 µl glass insert and were concentrated by evaporation (under gentle N_2 stream), resuspended in 200 µl acetone and subsequently measured again with HPLC.

We used a modified high–performance liquid chromatography (HPLC) protocol based on the protocol by Garrido & Zapata (1993), see Ilić et al. (2023) for details on the methodology. We applied reversed–phase HPLC using the Shimadzu Prominence series system (DGU–20A3 degasser, LC–20AB binary pump, CTO–10AC column oven set to 40 °C, SPDM20A diode array detector). The separation of different pigments was performed using a Spherisorb Octadecyl Silica 2 (ODS2) 5 µm column (250 mm × 4.6 mm) equipped with a tungsten lamp at 350–700 nm. The pigments were eluted by solvent A (methanol:acetonitrile:1 M ammonium acetate (50:30:20, v/v/v)) and solvent B (acetonitrile:ethyl–acetate (50:50, v/v)) with a flow rate of 1 $ml.min^{-1}$ and the following gradient: 0 min: A: 90%, B: 10%; 2 min: A: 90%, B: 10%; 26 min: A: 40%, B: 60%; 28 min: A: 10%, B: 90%; 30 min: A: 10%, B: 90%. The chemicals used were purchased from VWR–Chemicals (methanol, acetonitrile, both HPLC grade) and Merck Millipore (ammonium acetate, ethyl acetate, both ACS, ISO, Reag. Ph Eur).

Pigments were identified based on the comparison with pigment absorption spectra and retention times from previous measurements of pure pigment standards with the same HPLC system and separation method. All pigment standards were obtained from DHI Laboratory (Hørsholm, Denmark). For the calibration, previously available and new data were used. Calibration curves were established based on dilution series (at least five dilution steps in triplicates) of chlorophyll–c_2 and c_3 and diadinoxanthin, neoxanthin, and violaxanthin, while for all other pigments, calibration curves from previous analyses were used (Ilić et al. 2023). All pigment peak areas were integrated at 436 nm and corrected for the internal standard, and the pigment concentration was calculated based on established calibration curves.

Because the peaks of diadinoxanthin and the internal standard were not entirely separated on the baseline of the chromatogram, diadinoxanthin could not be clearly identified

(diadinoxanthin could be identified for a non–chrysophyte strain, i.e., *Tetrachrysis* A. J. Dop sp. (PR30K–A), data not shown). Due to overlapping retention times and thus peak areas, the peaks areas of chlorophyll–a and pheophytin–a were combined and shown as chlorophyll–a.

Data analysis. The peaks of each chromatogram were integrated using LabSolution, peaks with a height lower than 75 mAU (milliabsorption units) were not processed further (limit of detection as established from previous measurements (ILIĆ et al. 2023). We analysed the data using R (version 3.1.1, R Core Team 2014; ggplot2 version 2.2.1, WICKHAM 2009; reshape2 version 1.4.2, WICKHAM 2007) and RStudio (version 1.0.153, RStudio Team 2020). For the different treatments (light intensity: light and dark) separate linear models were fitted to the time series and the coefficient for the slope including the p–value were extracted from the model. To test for differences between treatments a model with and without interaction term (time × light intensity) was fitted. The fits of the complete (time + time × light intensity) versus the reduced model (time) were tested using an ANOVA and the p–value of the interaction term was extracted from the model.

Photo–acclimation experiment. The phototrophic *Synura sphagnicola* strain LO234K–E, the mixotrophic *Poterioochromonas malhamensis* strain DS, and *Cornospumella fuschlensis* strain A–R4–D6, which is at the edge of exclusive heterotrophy, were used for photo–acclimation experiments. *Poterioochromonas malhamensis* was tested when grown phagotrophically with bacteria and when grown osmotrophically (axenic) in organic medium. The phagotrophic treatment was supplemented with *Limnohabitans planktonicus* strain II–D5 as food bacteria.

Before the experiments the strains were split into triplicates (biological replicates) and incubated as described above, i.e., at 16 °C and with a 14:10 h light:dark cycle. 24 hours before the start of the experiment, each replicate was first mixed and then divided into two 5 l Erlenmeyer flasks each and diluted with fresh medium. The final volume was between 1.5 and 3.5 l depending on the growth rate and density of the strain. The strains were either exposed to continuous light (100 µmol photons light.m^{-2}.s^{-1}) or grown in the dark.

At the start of the experiment the first subsample for analysis was taken and the experimental vessels were placed in light or dark conditions immediately afterward. Samples were taken on days 0, 2, 4, and 6 for the analysis of cell abundance, cell volume, and pigment composition. All experiments were run in triplicates.

Subsamples were filtered and analysed for pigment concentrations as described above.

RESULTS

Pigments of phototrophic, mixotrophic, and heterotrophic chrysophytes

We detected pigments in all investigated taxa but pigment concentrations and in part also pigment composition differed considerably. β–carotene and zeaxanthin were present in phototrophic, mixotrophic, and heterotrophic taxa, while the other pigments were largely restricted to the phototrophic and mixotrophic taxa (Figs 1, 2, S1, and S2). In particular, chlorophylls were only present in phototrophic and mixotrophic strains. Chlorophyll–a and fucoxanthin were the dominant pigments, followed by β–carotene and neoxanthin. We did not detect the pigments alloxanthin, peridinin, and echinenone in any of the samples. However, we found low concentrations of either diadinoxanthin or diatoxanthin in *Mallomonas kalinae* Rezákova, *Cornospumella fuschlensis*, and *Pedospumella encystans* Boenigk et Findenig strain 1006. In *Poterioochromonas malhamensis* strain DS, an unknown pigment was detected.

While the pigment content per cell correlated with the mode of nutrition, the pigment concentration per biomass did only weakly reflect this relation. The ratio of chlorophylls to carotenoids was surprisingly similar between strains (Fig. 3A), even though absolute pigment concentrations varied strongly between taxa and were related to the mode of nutrition (Figs S1 and S2). For phototrophic taxa pigment concentration was inversely related to cell volume while we did not find such a relation in mixotrophic and heterotrophic taxa (Fig. 3B).

Differential pigment profiles in light and dark treatments

During the course of the experiment, cell densities of the phototrophic *Synura sphagnicola* remained constant in the dark, but their cell volumes decreased by roughly 40%, even though this was not statistically significant ($p = 0.0543$). In the light, the cell volume increased slightly but also not significantly ($p = 0.3864$), but cell densities increased ($p = 0.006$). Pigment concentrations (both per cell and per biovolume) remained rather constant in the light, but increased significantly in the dark ($p = 0.0003$ and $p = 0.003$, respectively). This was significant for all pigments (Figs S3 and S4) except for chlorophyll c_1, which showed a slight but insignificant decrease or no change over time (per cell: $p = 0.1955$ and per biovolume: $p = 0.9175$).

Different patterns were observed in *Poterioochromonas malhamensis*, as a taxon capable of photosynthesis, but at the edge between mixotrophy and heterotrophy. Due to the low pigment concentrations and the superimposition of pigment peaks by the unknown pigment, only the chlorophylls–a and c_2, and the carotenoids β–carotene and fucoxanthin were analysed for the mixotrophic *P. malhamensis* from both light and dark treatments.

When grown osmotrophically, the cell volumes declined in the light ($p = 0.0031$), but cell numbers increased ($p = 0.0282$). The same pattern was visible in the dark, but not significant (abundance: $p = 0.482$; volume: $p = 0.0693$). Even though pigment sums increased in the dark (per cell: $p = 0.044$; per biovolume: $p = 0.01$), the changes were marginal. Chlorophyll–a and fucoxanthin show significant differences between light and dark treatment (chlorophyll–a (per cell: $p = 0.0062$; per biovolume: $p = 0.0029$) and fucoxanthin (per cell: $p = 0088$; per biovolume: $p = 0.0037$)).

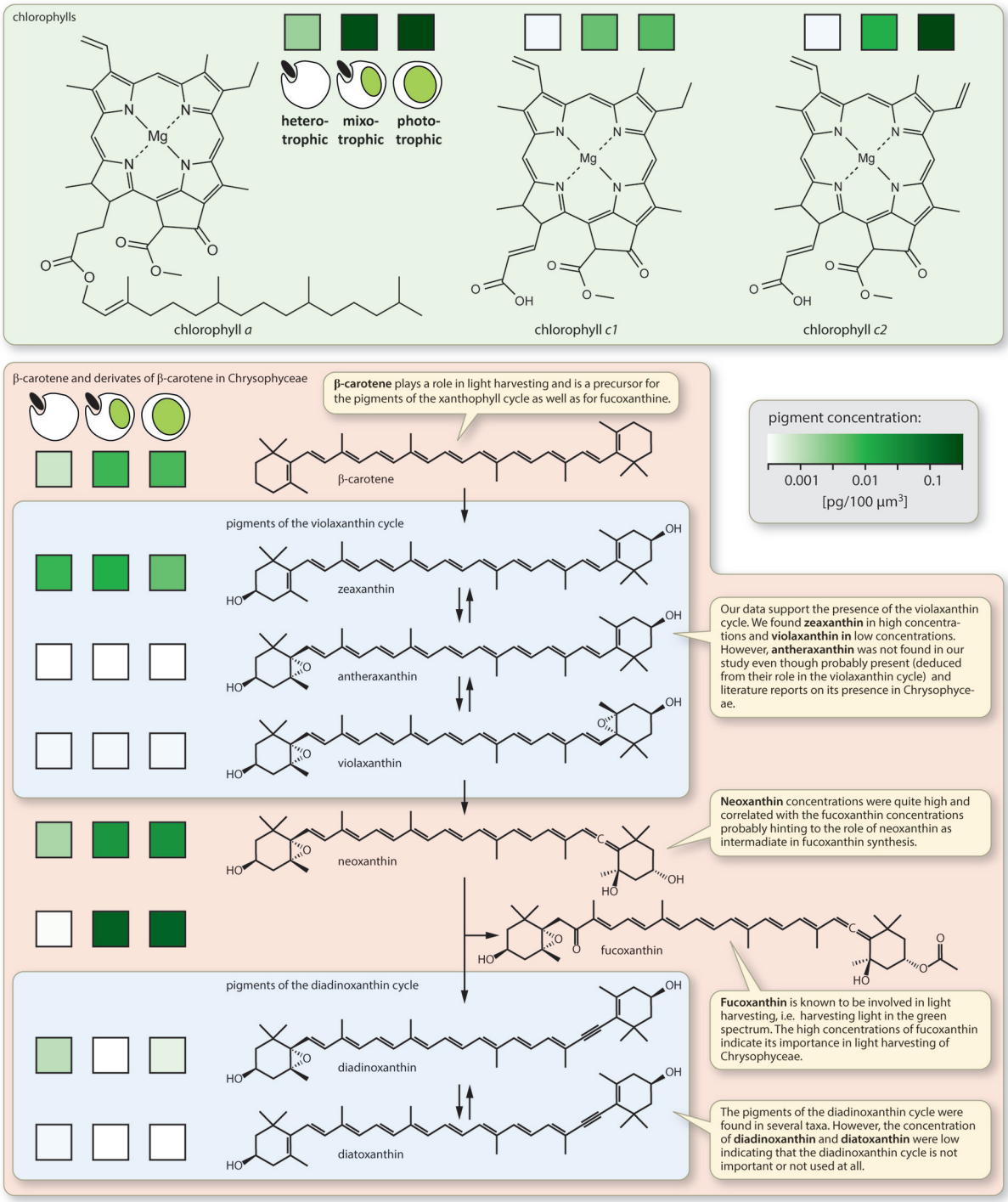

Fig. 1. Pigments in Chrysophyceae based on literature (Dales 1960; Withers et al. 1979; Withers et al. 1981; Falkowski & Raven 1998) and this study. Mean pigment concentrations for heterotrophic, mixotrophic, and phototrophic taxa are indicated by color.

When grown phagotrophically, cell volumes did not change significantly, while cell abundances decreased in the dark ($p = 0.0084$) and increased in the light ($p = 0.0009$). Pigment sums did not change significantly in the phagotrophic treatments (per cell dark: $p = 0.238$ and light: $p = 0.1871$; per biovolume dark: $p = 0.4264$ and light: $p = 0.3616$). The pigments diatoxanthin and zeaxanthin were only detected when *P. malhamensis* was fed with bacteria in the dark treatment, while the concentrations of these pigments, as well as those of violaxanthin and diadinoxanthin, were below the detection limit in all other treatments (Fig. S3). In contrast to the expectation from their function in light protection, diatoxanthin and zeaxanthin could only be detected in the dark treatments and the concentration of diatoxanthin increased over time ($p = 0.0015$).

Cornospumella fuschlensis was chosen as a strain at the extreme edge towards the evolution

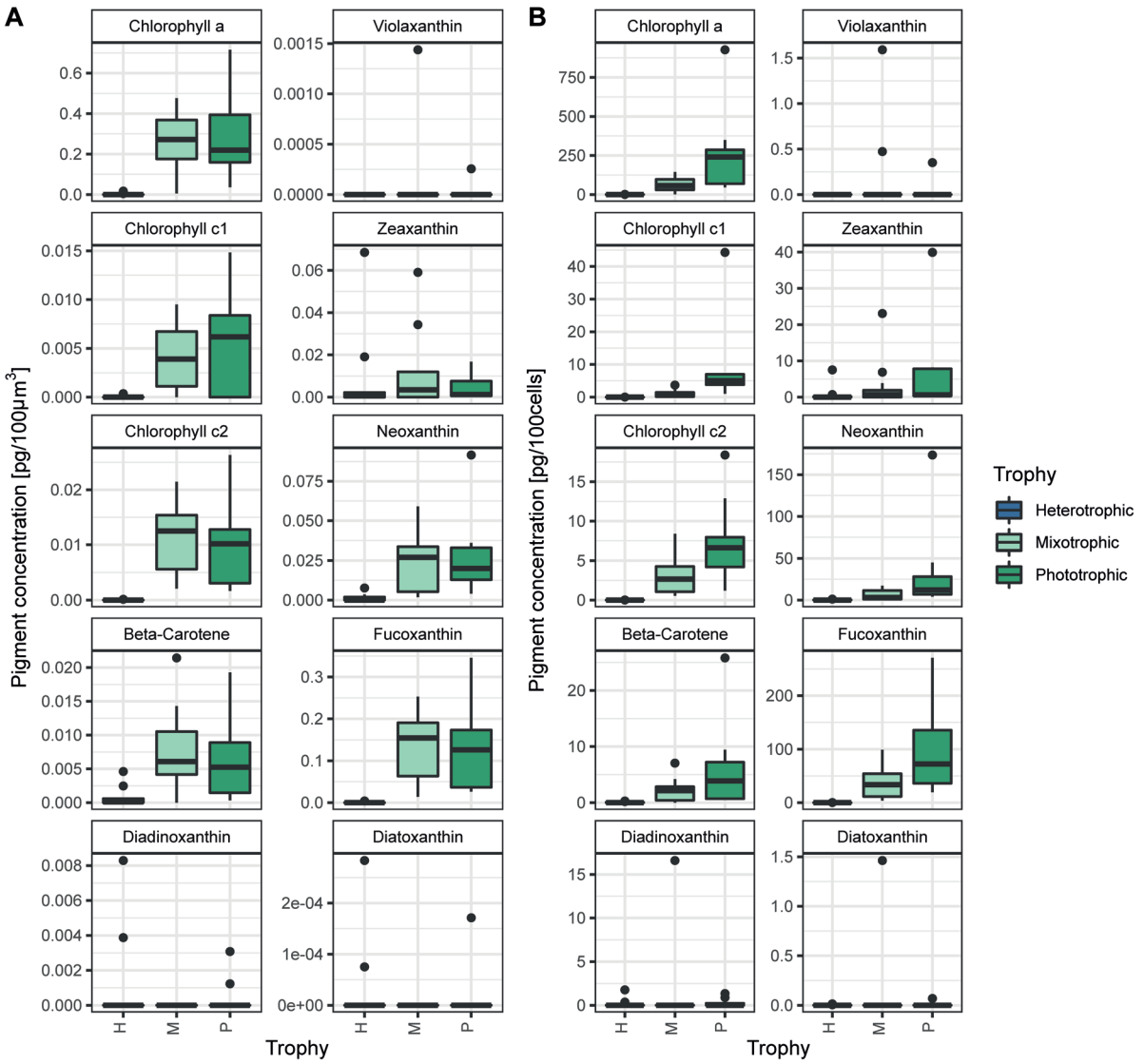

Fig. 2. Pigment concentrations in chrysophyte taxa. (A) Boxplots of pigment concentrations per biovolume of all strains divided for the different trophic modes (x–axis): heterotrophic (H, blue), mixotrophic (M, light green), and phototrophic (P, green); (B) boxplots of pigment concentrations per cell of all strains divided for the different trophic modes; each subplot depicts one of the measured pigments; the boxplots' whiskers indicate the minimum and maximum values (within 1.5 × interquartile range), outliers are shown as points; the median is indicated by the line in the box, which itself shows the interquartile range; individual data per strain is available in Figs S1 and S2.

of heterotrophy as it is heterotrophic but contains minimal amounts of pigments including chlorophylls. Cell volume ($p = 0.9266$) and abundance ($p = 0.7246$) developed similarly, i.e., not significantly different, in light and dark conditions. Overall pigment concentration did not change significantly both in the light and in the dark (Fig. 4). On the level of individual pigments per cell, some pigments increased in the light and in the dark (i.e., diadinoxanthin: $p = 0.0406$ and $p = 0.0174$; zeaxanthin: $p = 0.0218$ and $p = 0.0274$) and some only in the dark (i.e., chlorophyll–c_2: $p = 0.027$; β–carotene: $p = 0.046$). As the delimitation of the diadinoxanthin and the diatoxanthin peaks was uncertain these pigments were not included in the further analyses even though both pigments were present at low concentrations.

Discussion

Even though the photosynthetic apparatus is evolutionarily well conserved, different organisms show great variability in the presence and abundance of carotenoids (ESTEBAN et al. 2015). Here we show that pigment composition and concentrations differ between chrysophyte taxa and are related to their mode of nutrition.

So far, different pigments have been described for Chrysophyceae (Fig. 1; DALES 1960; WITHERS et al. 1979). In our study, we could confirm the described pigments even though several of the pigments were found only in a few of the investigated strains. We found the chlorophylls–a, c_1, and c_2, β–carotene, xanthophylls of the violaxanthin cycle (i.e., violaxanthin and zeaxanthin), xanthophylls of the diadinoxanthin cycle (i.e., diadinoxanthin and

diatoxanthin), as well as fucoxanthin and neoxanthin.

Pigment content and nutritional mode

The total pigment concentrations in the phototrophic and most mixotrophic taxa were considerably higher than in the heterotrophic taxa. However, pigment concentrations of the phototrophic taxa varied strongly. Interestingly, pigment concentration was inversely related to cell size in the phototrophic taxa, indicating that plastid size and number do not correlate with cell size (Fig. 3B). Therefore, pigment concentration alone appears not to be a good indicator for the nutritional mode of the analysed chrysophyte strains. Several mixotrophic taxa had a much higher pigment concentration than most phototrophic taxa. Pigment concentration per cell correlated much better with the nutritional mode, even though again a clear distinction between phototrophic and mixotrophic taxa was not possible based on pigment concentrations alone.

Phototrophic and mixotrophic taxa. Pigment patterns were generally similar for taxa containing functional chloroplasts capable of photosynthesis. In these taxa chlorophyll–a and fucoxanthin were the dominant pigments, which is consistent with the literature (Evert & Eichhorn 2013; Kristiansen & Škaloud 2017).

Aside from chlorophyll–a, we also found variants of chlorophyll–c. Only a few taxa have been analysed for pigment content so far and even fewer differentiated chlorophyll–c variants. We found chlorophyll–c_1 as well as chlorophyll–c_2 but not chlorophyll–c_3. This corresponds to the expectations (Kristiansen & Škaloud 2017). In general, the chlorophylls–c_1 and c_2 are the more common forms, while chlorophyll–c_3 has first been reported for the microalga *Emiliania huxleyi* (Lohmann) W.W. Hay et H. Mohler (Fookes & Jeffrey 1989) and is not known for Chrysophyceae. Both chlorophyll–c_1 and c_2 are presumably protein–bound in the light–harvesting antenna (Wilhelm & Wiedemann 1991; Scheer 1991).

We further detected numerous carotenoids. We detected β–carotene and fucoxanthin in all phototrophic taxa. Peaks of β–carotene were clearly visible in all taxa except for *Synura sphagnicola*, where the presumable β–carotene peak was masked by high concentrations of phaeophytin–a. However, the experiments aiming at light/dark regimes provided evidence for the presence of β–carotene also in *S. sphagnicola* (Figs S1 and S2). In *Poterioochromonas* we detected one unknown pigment which is most likely either a degradation product of chlorophylls or a pigment of the food bacteria. *Poterioochromonas* is known to be cannibalistic (Caron et al. 1990) which is observed particularly at high cell densities (own observation). A certain fraction of cells is therefore predated and digested which may be reflected by degradation products of pigments. However, as we can exclude phaeophytin due to our calibration curves the unknown pigment may represent a bacterial pigment as the prey bacterium was a photosynthetic bacterium possessing pigments, i.e. bacteriochlorophyll–a and the carotenoids hydroxyspheroidenone and spheroidenone (cf. Kasalický et al. 2017). As we did not include reference standards for bacterial pigments in our study the exact chemical nature of this pigments remains, however, speculative.

Our results also confirm the presence of pigments involved in xanthophyll cycling. All photosynthetic organisms using xanthophyll cycling for photoprotection employ either the violaxanthin cycle or the diadinoxanthin cycle (Lohr & Wilhelm 1999). However, algae possessing the diadinoxanthin cycle have been shown

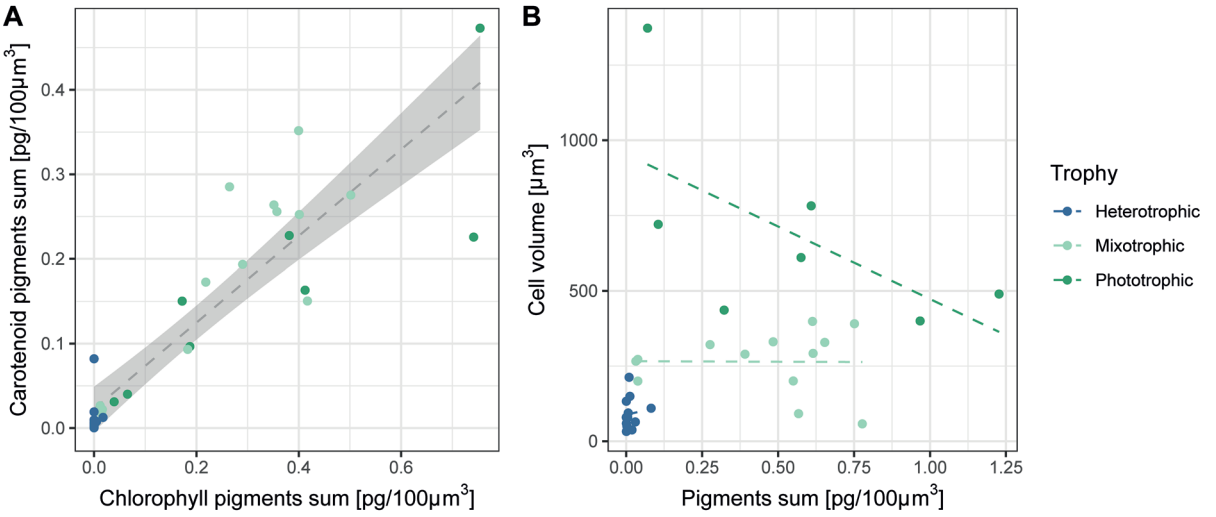

Fig. 3. Summed pigment concentrations. (A) The ratio of chlorophyll to carotenoid pigments; the concentration per biovolume of chlorophyll pigments (sum over all pigments) versus carotenoid pigments (sum) per 100 µm³ in the analysed chrysophyte taxa; the point color depicts the tropic mode of the chrysophyte: heterotrophic (blue), mixotrophic (light green), and phototrophic (green); a regression line was fitted to the data and shown as gray dashed line in the plot. (B) The pigments sum per 100 µm³ versus cell volume; for each trophic mode a regression line was fitted; colors are according to subplot (A).

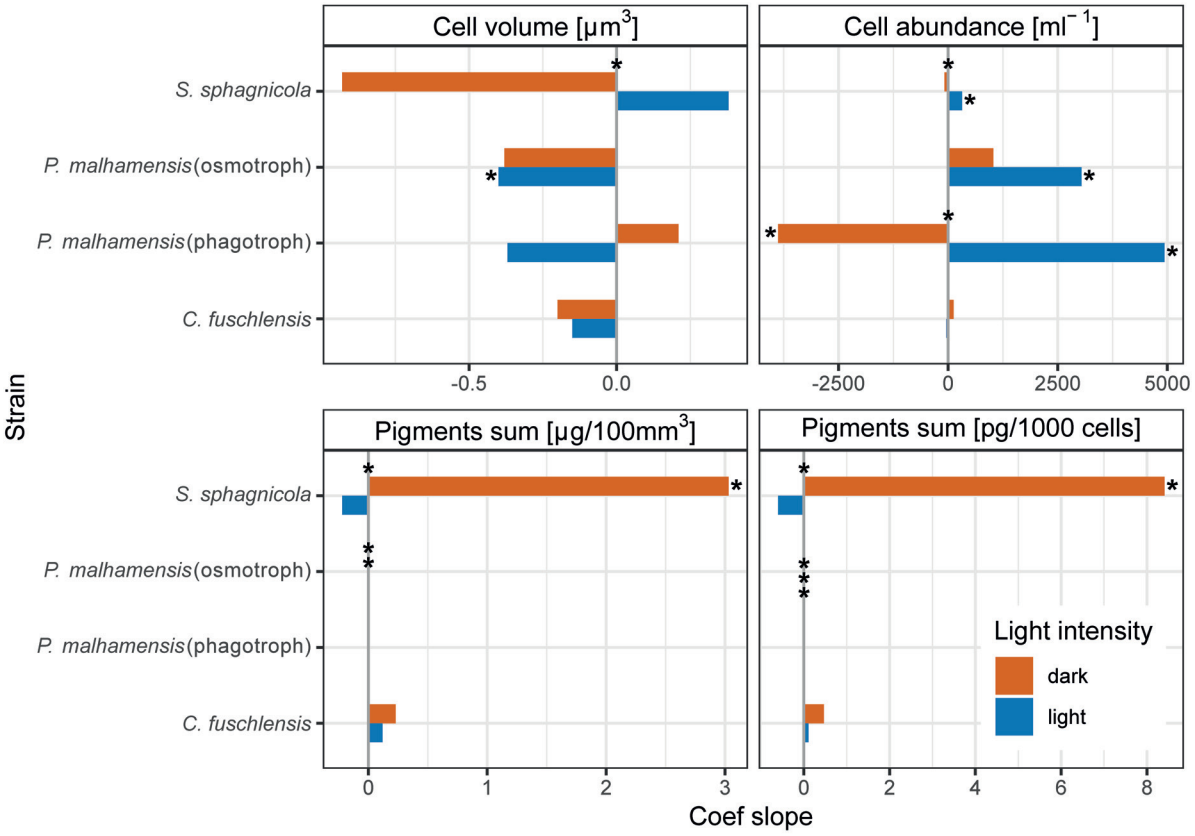

Fig. 4. Development of cell abundance, cell volume, and pigment concentration in light and dark treatments; the subplots depict the cell volume, cell abundance, pigments sum per biovolume, and pigments sum per cell for four chrysophyte taxa; each subplot depicts the second coefficient (i.e., the slope) of a linear regression model fitted to the time series data for the dark and light treatment; for individual data see Figs S3 and S4; significant coefficients are marked with an asterisk next to the bar; a significant difference between treatments is marked with an asterisk above the bars for the respective strain at the zero line; the bar colors depict the light intensity: dark (orange) and light (blue).

to synthesize also pigments of the violaxanthin cycle (LOHR & WILHELM 1999). In fact, we found evidence for both xanthophyll cycles in Chrysophyceae.

Xanthophylls of the violaxanthin cycle were found in nearly all investigated strains, i.e., zeaxanthin was detected in most cultures, while violaxanthin was only found in few taxa (Figs S1 and S2). As all cultures were grown in the light, a relative dominance of zeaxanthin was expected (DEMMIG–ADAMS & ADAMS III 1992, 1996). Our results suggest that the violaxanthin cycle is used by chrysophytes for photoprotection. In organisms using the zeaxanthin cycle, components of the diatoxanthin cycle (diatoxanthin, diadinoxanthin) are not expected (DEMMIG–ADAMS & ADAMS III 1992).

Former studies investigating these latter pigments for Chrysophyceae reported these pigments primarily for taxa nowadays excluded from the Chrysophyceae: the study by DALES (1960) on chrysophyte pigments focused mainly on *Isochrysis* Parke, i.e., a taxon which is now known to be a haptophyte, not a chrysophyte. Similarly, WITHERS et al. (1981) reported in their study on carotenoids of Chrysophyceae the presence of diadinoxanthin and diatoxanthin only for *Sarcinochrysis marina* Geitler, i.e., a Pelagophyceae. However, our study confirmed the presence of diadinoxanthin and diatoxanthin for few chrysophycean strains affiliated with, e.g., Synurales and Ochromonadales. Diadinoxanthin may also serve as a precursor of fucoxanthin (LOHR & WILHELM 1999) but the presence of diatoxanthin in some strains may hint to the presence of the diadinoxanthin cycle. As the detection of these pigments did neither correspond to a phylogenetic pattern nor to the mode of nutrition, it is likely that these pigments are generally present, albeit at low concentrations, and thus escaped detection in several of the investigated strains. However, the concentration of these pigments was always lower as in the Phaeothamniophyceae *Tetrachrysis* A.J. Dop (data not shown) and suggests that the diadinoxanthin cycle is not used or at least not important in Chrysophyceae.

Heterotrophic taxa. Heterotrophic strains in general showed a low overall pigment content and concentration. This corresponds to the reduction of photosynthesis and plastids in the course of evolution of heterotrophy in chrysophytes (GROSSMANN et al. 2016a; GRAUPNER et al. 2018).

However, we detected two different patterns of pigment reduction in the heterotrophic strains: in one group comprising the genera *Pedospumella* Boenigk et Findenig, *Acrispumella* Boenigk et Grossmann and some

species affiliated with *Spumella* (Cienkowsky) Boenigk et Findenig, we detected β–carotene and zeaxanthin, while all other pigments were absent or below the detection limit. In the second group comprising the heterotrophic *Poteriospumella* as well as *Spumella bureschii* (as well as the mixotrophic *Poterioochromonas*), we did not detect these two pigments, but several other pigments, such as neoxanthin and diadinoxanthin. According to the transcriptome analysis of Graupner et al. (2018), some genes essential for β–carotene biosynthesis are downregulated (or missing) in *Poterioochromonas* and *Poteriospumella*. Assuming that genes for the subsequent synthesis of β–carotene derived xanthophylls are not down–regulated, the final product in the synthesis pathway, i.e., neoxanthin, may become the dominant pigment. In contrast, β–carotene and xanthophylls at intermediate positions of the pathway would become limiting – a pattern observed in a number of mixotrophic and heterotrophic taxa. This different pattern is therefore to some extent presumably caused by the low overall pigment concentrations (near the limit of quantitation) in these strains. However, the systematic difference in pigment patterns between these two groups indicates a true biological pattern. We assume that several pigments are present in all strains even though below the detection limit – most probably at least β–carotene, zeaxanthin, and neoxanthin, but the relative importance of these different pigments differ between taxa.

The differences in the pigment pattern at first sight follow a rough phylogenetic pattern with most strains affiliated with the C3 clade (Grossmann et al. 2016a) belonging to the first group while most strains affiliated with the C2 and C1 clade (Grossmann et al. 2016a) belong to the second group. However, in both clades, distinct taxa, in particular *Acrispumella msimbasiensis* Boenigk et Grossmann affiliated with the C3 clade and *Spumella bureschii* (Valkanov) Boenigk et Grossmann affiliated with the C2 clade, deviate from the phylogenetic pattern.

In particular, taxa affiliated with the C3 clade of the Ochromonadales are at the edge between mixotrophic and heterotrophic nutrition, i.e., photosynthesis may play a certain even though probably minor role at least in some taxa. While *Poterioochromonas malhamensis* and *Chlorochromonas danica* (E.G. Pringsheim) R.A. Andersen, L. Graf, Y. Malakhov et H.S. Yoon possess functional plastids, other genera affiliated with this clade are considered to be obligate heterotrophs (Findenig et al. 2010a): *Cornospumella fuschlensis* possess a complete set of pigments as do the mixotrophic and phototrophic strains indicating that this strain is presumably still mixotrophic even though at the far heterotrophic end of the spectrum. *Poteriospumella lacustris* Boenigk et Findenig contains chlorophyll–a however only in low concentration. As chlorophyll–a indicates photosynthetic activity the pigment pattern of *P. lacustris* presumably indicates a potentially remaining functional plastid similar to the case of *Cornospumella fuschlensis*. This was not expected, since genes for chlorophyll synthesis were not detected in a transcriptome study of *P. lacustris* (Graupner et al. 2018). However, the latter study already indicated that *P. lacustris* may serve as a model for an early stage of plastid reduction. Based on our results, some of the genes that have not been found based on transcriptomics (Graupner et al. 2018) and were considered absent, might be expressed under different conditions. This is supported by genome analyses as some genes of the chlorophyll synthesis pathway are present (Majda et al. 2021). Overall, our pigment analyses suggest that the genera *Poterioochromonas*, *Cornospumella*, and *Poteriospumella* cover the boundary between mixotrophic and heterotrophic nutrition. These taxa are all affiliated with the extended C3 clade (Grossmann et al. 2016a) of Ochromonadales. The closer relation of *Cornospumella* to *Ochromonas danica* and of *Poteriospumella* to *Poterioochromonas* hint to an independent reduction of pigments and of photosynthesis in these two lineages of the C3–clade.

Light acclimation of pigment concentration

Phototrophic taxa. In the light treatment, the population of *Synura sphagnicola* grew, i.e., cell number increased, while cell volume and pigment concentration remained constant. In contrast, in the dark treatment, the cell numbers did neither increase nor decrease but the cell volume decreased by 40%. This indicates that individual cells can survive several days in the absence of light and cell metabolism is maintained presumably through the use of storage products. Total pigment concentration strongly increased in the dark both per cell and per biovolume. Assuming that pigments are not degraded during dark incubation, this relative increase is largely due to the decreasing cell size. Further, starvation due to light limitation may stimulate metabolic pathways related to light harvesting and photosynthesis which matches expectations for this phototrophic strain (cf. Su 2018).

Heterotrophic taxa. The heterotrophic *Cornospumella fuschlensis* did not show shifts in cell volume and pigment concentration – neither in the dark nor in the light treatment. Further, cell numbers did not significantly differ between both treatments. This pattern indicates that the growth of this strain is rather independent of light, which matches the expectations for heterotrophic chrysophytes. This observation, however, leaves the question of why pigments are synthesized in this taxon unanswered. As pigment concentrations are low and pigment synthesis was independent on light availability, the synthesis could be a relic of the phototrophic ancestry. Possibly some pathways related to photosynthesis may become important in the absence of food, i.e., in starved populations, but this was not the focus of the present study.

Similarly, for taxa at the edge between mixotrophy and heterotrophy, such as *Poterioochromonas malhamensis* (cf. Holen 1999) we did not observe

considerable differences between the light and dark treatment. In contrast to the expectation from their function in light protection, diatoxanthin and zeaxanthin could only be detected in the dark treatments with an increase in concentration over time. This may possibly be related to other functions of these pigments, such as stress response as discussed in Fernández–Marin et al. (2021).

While mixotrophic but predominantly phototrophic algae cannot survive with permanent light deprivation (Caron et al. 1993), *Poterioochromonas* may keep its maintenance metabolism in the dark, allowing for survival but not growth (Rottberger et al. 2013). The axenic mixotroph cultures showed a similar development in the light and in the dark, i.e., a slight increase in cell abundance was counterbalanced by a slight decrease in cell volume. In the axenic cultures, pigment concentrations increased in the dark while pigment concentrations decreased in the light. When fed with bacteria, their cell abundance decreased in the dark, but increased in the light, whereas the cell volume did hardly change, i.e., biomass changed inversely in the light and dark treatment. As *Poterioochromonas* is far on the heterotrophic side of the spectrum, growth was expected under the high food concentrations in our experiment. The decline in abundance in the dark treatment indicated that an essential nutrient or growth factor was missing and thus, the population did not grow despite high food concentrations. When grown osmotrophically, the growth rates in the light were lower as compared to the phagotrophically grown culture, indicating that phagotrophic food uptake is more efficient, and thus allows for higher growth rates (cf. Holen 2010; Rottberger et al. 2013). In contrast, the osmotrophically grown cultures performed better in the dark than the phagotrophic cultures. This may indicate that the unknown growth–limiting substance is missing in the phagotrophic treatment, but present in the complex organic medium. The different growth response in the light indicates that this limiting substance presumably can be synthesized by *Poterioochromonas* in a light–dependent reaction. Light–dependent reactions in plants comprise, for instance, the synthesis of the rate–limiting enzyme for carotenoid biosynthesis, phytoene synthase (PSY), as well as determine its activity in the condensation of two geranylgeranyl pyrophosphate molecules (Cazzonelli 2011).

In summary, the detected pigment patterns confirm that the whole range between photosynthetic and heterotrophic nutrition is realized. For phototrophic and mixotrophic strains the ratio of chlorophylls to carotenoids is rather constant. This may indicate a physiologically similar adaptation level for both, photosynthesis and photoprotection, in phototrophs and mixotrophs cultivated under similar light conditions. Chlorophyll is not present in heterotrophic lineages (except for taxa at the edge between mixotrophy and heterotrophy) while different carotenoids are found in heterotrophic lineages. Accordingly, pigment concentrations are regulated depending on light availability only in photosynthetic taxa while pigment concentrations are unaffected by light in heterotrophic taxa. The presence of carotenoids in heterotrophic taxa is therefore most likely a relic of the phototrophic ancestry.

References

Andersen R.A. (2007): Molecular systematics of the Chrysophyceae and Synurophyceae. – In: Brodie J. & Lewis J.S. (eds): Unravelling the algae – The past, present, and future of algal systematics. – pp. 285–313, CRC Press, Boca Raton.

Anning, T.; MacIntyre, H.L.; Pratt, S.M.; Sammes, P.J.; Gibb, S. & Geider, R.J. (2000) Photoacclimation in the marine diatom *Skeletonema costatum*. – Limnol. Oceanogr. 45: 1807–1817.

Beisser, D.; Graupner, N.; Bock, C.; Wodniok, S.; Grossmann, L.; Vos, M. & Boenigk, J. (2017): Comprehensive transcriptome analysis provides new insights into nutritional strategies and phylogenetic relationships of chrysophytes. – PeerJ. 5: e2832.

Bock, C.; Medinger, R.; Jost, S.; Psenner, R. & Boenigk, J. (2014): Seasonal variation of planktonic chrysophytes with special focus on *Dinobryon*. – Fottea 14: 179–190.

Boenigk, J. & Arndt, H. (2002): Bacterivory by heterotrophic flagellates: community structure and feeding strategies. – Antonie van Leeuwenhoek 81: 465–480.

Brunet, C.; Johnsen, G.; Lavau, J. & Roy, S. (2011): Pigments and photoacclimation processes. – In: Roy, S.; Llewellyn, C.; Egeland, E. & Johnsen, G. (eds): Phytoplankton pigments: characterization, chemotaxonomy and applications in oceanography. – pp. 445–471, Cambridge University Press, New York.

Caron, D. A.; Porter, K. G.; Sanders, R. W. (1990) Carbon, nitrogen, and phosphorus budgets for the mixotrophic phytoflagellate *Poterioochromonas malhamensis* (Chrysophyceae) during bacterial ingestion. – Limnol. Oceanogr. 35: 433–443.

Caron, D.A.; Sanders, R.W.; Lira, E.L.; Marras, C.; Amaral, L.A.; Whitney, S. & Porter, K.G. (1993): Light–dependent phagotrophy in the freshwater mixotrophic chrysophyte *Dinobryon cylindricum*. – Microb. Ecol. 25: 93–111.

Cavalier–Smith, T. & Chao, E.E.–Y. (2006): Phylogeny and megasystematics of phagotrophic heterokonts (kingdom Chromista). – J. Mol. Evol. 62: 388–420.

Cazzonelli, C.I. (2011): Carotenoids in nature: insights from plants and beyond. – Functional Plant Biology 38: 833–847.

Dales, R.P. (1960): On the pigments of the Chrysophyceae. – J. Mar. Biol. Ass. U.K. 39: 693–699.

Demmig–Adams, B. & Adams III, W.W. (1992): Photoprotection and other responses of plants to high light stress. – Annual review of plant physiology and plant molecular biology 43: 599–626.

Demmig–Adams, B. & Adams III, W.W. (1996): The role of xanthophyll cycle carotenoids in the protection of photosynthesis. – Trends in plant science 1: 21–26.

Dorrell, R.G.; Azuma, T.; Nomura, M. & Kamikawa, R.

(2019): Principles of plastid reductive evolution illuminated by nonphotosynthetic chrysophytes. – PNAS 116: 6914–6923.

Dorrell, R.G. & Bowler, C. (2017): Secondary plastids of Stramenopiles. – Advances Bot. Res. 84: 57–103.

Esteban, R.; Moran, J.F.; Becerril, J.M. & Garcia–Plazaola, J.I. (2015): Versatility of carotenoids: an integrated vire on diversity, evolution, functional roles and environmental interactions. – Environmental and Experimental Botany 119: 63–65.

Evert, R.F. & Eichhorn, S.E. (2013): Protists: algae and heterotrophic protists: photosynthetic Stramenopiles. – In: Raven, J.A. (ed.): Biology of plants. – pp. 330–340, W. H. Freeman and Company Publishers, New York.

Falkowski, P.G. & Raven, J.A. (1998): Aquatic photosynthesis. Blackwell Science, Malden, Mass.

Fernández–Marín, B.; Roach, T.; Verhoeven, A. & García–Plazaola, J.I. (2021): Shedding light on the dark side of xanthophyll cycles. – New Phytol. 230: 1336–1344.

Finlay, B.J. & Esteban, G.F. (1998): Freshwater protozoa: biodiversity and ecological function. – Biodiversity and Conservation 7: 1163–1186.

Findenig, B.M; Chatzinotas, A. & Boenigk, J. (2010): Taxonomic and ecological characterization of stomatocysts of *Spumella*–like flagellates (Chrysophyceae). – Journal of Phycology 46: 868–881.

Fookes, C.J.R. & Jeffrey, S.W. (1989): The structure of chlorophyll c3, a novel marine photosynthetic pigment. – Journal of the Chemical Society: 1827–1828.

Garrido, J.L.; Brunet, C. & Rodríguez, F. (2016): Pigment variations in *Emiliania huxleyi* (CCMP370) as a response to changes in light intensity or quality. – Environ. Microbiol. 18: 4412–4425.

Garrido, J.L. & Zapata, M. (1993): High performance liquid chromatographic separation of polar and non–polar chlorophyll pigments from algae using a wide pore polymeric octadecylsilica column. – Hrc–journal of High Resolution Chromatography 16: 229–233.

Graupner, N.; Jensen, M.; Bock, C.; Marks, S.; Rahmann, S.; Beisser, D. & Boenigk J. (2018): Evolution of heterotrophy in chrysophytes as reflected by comparative transcriptomics. – FEMS Microbiol. Ecol. 94: fiy039.

Grossmann, L.; Bock, C.; Schweikert, M. & Boenigk, J. (2016a): Small but manifold – hidden diversity in '*Spumella*–like flagellates'. – J. Euk. Mic. 63: 419–39.

Grossmann, L.; Jensen, M.; Pandey, R.V.; Jost, S; Bass, D.; Psenner, R. & Boenigk, J. (2016b): Molecular investigation of protistan diversity along an elevation transect of alpine lakes. – Aquat. Microb. Ecol. 78: 25–37.

Guillard, R.R.L. & Lorenzen, C.J. (1972): Yellow–green algae with chlorophyllide c. – J. Phycol. 8: 10–14.

Hahn, M.W.; Lünsdorf, H.; Wu, Q.; Schauer, M.; Höfle, M.G. & Boenigk, J. (2003): Isolation of novel ultramicrobacteria classified as Actinobacteria from five freshwater habitats in Europe and Asia. – Appl. Environ. Microbiol 69: 1442–1451.

Hillebrand, H.; Dürselen, C.–D.; Kirschtel, D.; Pollingher, U. & Zohary, T. (1999): Biovolume calculation for pelagic and benthic microalgae. – J. Phycol. 35: 403–424.

Holen, D.A. (1999): Effects of prey abundance and light intensity on the mixotrophic chrysophyte *Poterioochromonas malhamensis* from a mesotrophic lake. – Freshwater Biology 42: 445–455.

Holen, D.A. (2010) Mixotrophy in two species of *Ochromonas*. – Nova Hedwigia Beiheft 136: 153–165.

Holen, D.A. & Boraas, M.E. (1995): Mixotrophy in chrysophytes. – In: Sandgren, C.D.; Smol, J.P. & Kristiansen, J. (eds): Chrysophyte algae ecology, phylogeny and development. – pp. 119–140, Cambridge University Press, Cambridge.

Horton, P. & Ruban, A. (2005): Molecular design of the photosystem II light harvesting antenna: photosynthesis and photoprotection. – Journal of Experimental Botany 56: 365–373.

Ilić, M.; Walden, S.; Hammerstein, S. K.; Stockenreiter, M.; Stibor, H. & Fink, P. (2023): Pigment and fluorescence proxies to estimate functional diversity of phytoplankton communities. – Fundamental and Applied Limnology 196: 229–249.

Kasalický, V.; Zeng, Y.; Piwosz, K.; Šimek, K.; Kratochvilová, H. & Koblížek, M. (2017): Aerobic anoxygenic photosynthesis is commonly present within the genus *Limnohabitans*. – Applied and Environmental Microbiology 84: e02116–17.

Kristiansen, J. & Škaloud, P. (2017): Chrysophyta. – In: Archibald, J.; Simpson, A. & Slamovits, C. (eds): Handbook of the protists. – pp. 331–366, Springer, Cham.

Latowski, D.; Szymanaska, R.; & Strzałka, K. (2014). Carotenoids involved in antioxidant system of chlorophyll. – In: Ahmad, P. (ed): Oxidative damage to plants: antioxidant networks and signaling. – pp. 289–319, Elsevier, San Diego, USA.

Lichtenthaler, H.K. & Buschmann, C. (2001): Chlorophylls and Carotenoids: Measurement and Characterization by UV–VIS Spectroscopy. – Current Protocols in Food Analytical Chemistry 1: F4.3.1–F4.3.8.

Lohr, M.; Im, C.–S. & Grossmann, A.R. (2005): Genome–based examination of chlorophyll and carotenoid biosynthesis in *Chlamydomonas reinhardtii*. – Plant Physiology 138: 490–515.

Lohr, M. & Wilhelm, C. (1999): Algae displaying the diadinoxanthin cycle also posess the violaxanthin cycle. – Proceedings of the National Academy of Sciences 96: 8784–8789.

Majda, S.; Beisser, D. & Boenigk, J. (2021): Nutrient–driven genome evolution revealed by comparative genomics of chrysomonad flagellates. – Commun. Biol. 4: 328.

Olefeld, J.L.; Majda, S.; Albach, D.C.; Marks, S. & Boenigk, J. (2018): Genome size of chrysophytes varies with cell size and nutritional mode. – Organisms Div. Evol. 18: 163–173.

R Core Team (2014): R: A language and environment for statistical computing. R Foundation for Statistical Computing, URL http://www.R–project.org/. Vienna, Austria.

Rodríguez, F.; Chauton, M.; Johnsen, G.; Andresen, K.; Olsen, L.M. & Zapata, M. (2006): Photoacclimation in phytoplankton: implications for biomass estimates, pigment functionality and chemotaxonomy. – Marine Biology 148: 963–971.

Rottberger, J.; Gruber, A.; Boenigk, J. & Kroth, P.G.

(2013): Influence of nutrients and light on autotrophic, mixotrophic and heterotrophic freshwater chrysophytes. – Aquat Microb Ecol 71: 179–191.

RStudio Team (2020): RStudio: Integrated Development for R. RStudio, PBC, URL http://www.rstudio.com/. Boston, MA.

Scheer, H. (1991): Chlorophylls. CRC Press: Boca Raton.

Schopfer, P. (2010): Pflanzenphysiologie. Springer Spektrum, Berlin.

Su, Y. (2018): The effect of different light regimes on pigments in *Coscinodiscus granii*. – Photosynthesis Research 140: 301–310.

Wickham, H. (2009): ggplot2: elegant graphics for data analysis. Springer, New York.

Wickham, H. (2007): Reshaping data with the reshape package. – J. Stat. Software 21: 1–20.

Wilhelm, C. & Wiedemann, I. (1991): Evidence of protein–bound chlorophyll c3 in a light–harvesting protein from the flagellate alga *Prymnesium parvum* (Prymnesiophyceae). – Photosynthetica 25: 249–255.

Withers, N.W.; Fiksdahl, A.; Tuttle, R.C. & Liaaen–Jensen, S. (1979): Carotenoids of the Chysophyceae. – Comp. Biochem. Physiol. 68 B: 345–349.

Withers, N.W.; Fiksdahl, A.; Tuttle, C. & Liaan–Jensen, S. (1981): Carotenoids of the Chrysophyceae. – Comp. Biochem. Physiol. 68: 345–349.

Young, A.J.; Phillip, D.; Ruban, A.V.; Horton, P. & Frank, H.A. (1997): The xantophyll cycle and carotenoid–mediated dissipation of excess escitation energy in photosynthesis. – Pure and Applied Chemistry 69: 2125–2130.

Supplementary material

The following supplementary material is available for this article:

Table S1. Origin of strains. All except for *Mallomonas kalinae* (CAUP, Charles University in Prague) and *Bitrichia* sp. (CCAC, Central Collection of Algal Cultures at the University of Essen) were taken from the culture collection of Jens Boenigk. Strains were grown in WC medium (Guillard 1972), modified DY-V medium (Andersen 2007), or in inorganic basal medium (Hahn et al. 2003).

Fig. S1. Pigment concentrations in different chrysophyte taxa per cell number. The subplots depict the different pigments and their concentration with standard deviation (n = 3) per 100 cells in the analysed chrysophyte taxa (x–axis). The x–axes are ordered by trophic mode: heterotrophic (blue), mixotrophic (light green), and phototrophic (green) and within each group alphabetically.

Fig. S2. Pigment concentrations in different chrysophyte taxa per biovolume. The subplots depict the different pigments and their concentrations with standard deviation (n = 3) per 100 μm^3 in the analysed chrysophyte taxa (x–axis). The x–axes are ordered by trophic mode: heterotrophic (blue), mixotrophic (light green), and phototrophic (green) and within each group alphabetically.

Fig. S3. Development of single pigment concentrations in light and dark treatments per cell number. The subplots depict the development of pigment concentrations with standard deviation (n = 3) per 1000 cells for (A) *Synura sphagnicola*, (B) *Poterioochromonas malhamensis* (grown osmotrophically), (C) *Poterioochromonas malhamensis* (grown phagotrophically), and (D) *Cornospumella fuschlensis*. All values are shown with standard deviation and for light (blue) and dark treatment (orange).

Fig. S4. Development of single pigment concentrations in light and dark treatments per biovolume; the subplots depict the development of pigment concentrations with standard deviation (n = 3) per 100 mm^3 for (A) *Synura sphagnicola*, (B) *Poterioochromonas malhamensis* (grown osmotrophically), (C) *Poterioochromonas malhamensis* (grown phagotrophically), and (D) *Cornospumella fuschlensis*; all values are shown with standard deviation and for light (blue) and dark treatment (orange).

Fig. S5. Development of cell abundance, cell volume, and pigment concentrations in light and dark treatments; the subplots depict the cell volume (1st row), cell abundance (2nd row), pigments sum per biovolume (3rd row), and pigments sum per cell (4th row) with standard deviations (n = 3) for four chrysophyte taxa.; the first column shows these measures for *Synura sphagnicola*, the second for *Poterioochromonas malhamensis* (grown osmotrophically), the third *Poterioochromonas malhamensis* (grown phagotrophically), and the last column *Cornospumella fuschlensis*; all values are shown with standard deviation and for light (blue) and dark treatment (orange).

Fig. S6. Calibration curves based on dilution series (at least five dilution steps in triplicates) of chlorophyll–c_2 and c_3 and diadinoxanthin, neoxanthin, and violaxanthin, while for all other pigments, calibration curves from previous analyses were used (Ilić et al., in press); all pigment peak areas were integrated at 436 nm and corrected for the internal standard, and the pigment concentration was calculated based on established calibration curves.

Fig. S7. Chromatograms of the pigments retention times. Chromatograms are shown for different channels (wavelengths of the channels are provided in the figure subheadings).

This material is available as part of the online article (http://fottea.czechphycology.cz/contents)

Received October 7, 2022
Accepted January 15, 2023