Pigment composition and photoacclimation as keys to the ecological success of Gonyostomum semen (Raphidophyceae, Stramenopiles)

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Aquatic habitats are usually structured by light attenuation with depth resulting in different microalgal communities, each one adapted to a certain light regime by their specific pigment composition. Several taxa contain pigments restricted to one phylogenetic group, making them useful as marker pigments in phytoplankton community studies. The nuisance and invasive freshwater microalga *Gonyostomum semen* (Raphidophyceae) is mainly found in brown water lakes with sharp vertical gradients in light intensity and color. However, its pigment composition and potential photoadaptations have not been comprehensively studied. We analyzed the photopigment composition of 12 genetically different strains of *G. semen* by high performance liquid chromatography after acclimation to different light conditions. We confirmed the pigments chl *a*, chl *a*1146, diadinoxanthin, trans-neoxanthin, cis-neoxanthin, α and β carotene, which have already been reported for *G. semen*. In addition, we identified, for the first time, the pigments violaxanthin, zeaxanthin, and alloxyanthin in this species. Alloxanthin has never been observed in raphidophytes before, suggesting differences in evolutionary plastid acquisition between freshwater lineages and the well-described marine species. The amount of total chl *a* per cell generally decreased with increasing light intensity. In contrast, the increasing ratios of the prominent pigments diadinoxanthin and alloxanthin per chl *a* with light intensity suggest photoprotective functions. In addition, we found significant variation in cell-specific pigment concentration among strains, grouped by lake of origin, which might correspond to genetic differences between strains and populations.

**Key index words:** alloxanthin; *Gonyostomum semen*; HPLC; photopigment composition; pigments; raphidophyte

**Abbreviations:** allo, alloxanthin; chl, chlorophyll; diadino, diadinoxanthin; DOM, dissolved organic matter; HPLC, high performance liquid chromatography; neox, neoxanthin; violax, violaxanthin; zeax, zeaxanthin

Microalgae are usually highly adapted to different light conditions, populating habitats suited for their specific pigment composition and absorption maxima. This is especially evident in marine ecosystems, as stratified water columns and significant changes in light intensity and color often cause selection and niche partitioning of phytoplankton taxa according to the prevailing light environment (Bidigare et al. 1990a, Stomp et al. 2004, Hickman et al. 2009). For example, Bidigare et al. (1990a) observed a vertically structured distribution of different phytoplankton taxa (cyanobacteria, diatoms, and prymnesiophytes) in the Sargasso Sea suggesting chromatic adaptation. Picoplanktonic cyanobacteria like *Synechococcus* sp. and *Prochlorococcus* sp. are well-adapted to high irradiance in blue tropical waters by generating high concentrations of the photoprotective pigment zeaxanthin (zeax) (Lutz et al. 2003, Hickman et al. 2009).

In environments with varying light conditions, photoacclimation is frequently manifested as alterations in the photosynthetic pigment content in response to changing irradiance (MacIntyre et al. 2002). Haptophytes, for example, adjust to heterogeneous light climates through highly plastic concentrations of fucoxanthin-related carotenoids. The concentrations of these pigments per chl *a* can differ among haptophyte species and strains (Buma et al. 1991, Stolte et al. 2000). Thus, acclimation does not involve a change in the genetic structure of the population under investigation whereas adaptation does.

The nuisance freshwater microalga *Gonyostomum semen* (Ehrenberg) has recently spread to several new habitats and currently dominates the phytoplankton community in many Scandinavian lakes (Lepistö et al. 1994, Rengefors et al. 2012, Lebret et al. 2013). Originally, it was encountered in dark, brown water lakes (Sörens 1954) with very sharp gradients of
light intensity because of rapid attenuation of light with depth. In these lakes, it may form extensive blooms during the summer and its cell number can represent up to 97% of the phytoplankton community (Cronberg et al. 1988, Findlay et al. 2005, Lebret et al. 2012b). *Gonyostomum semen* is considered a nuisance species, as its mucous threads and trichocysts may cause skin irritation in swimmers (Sørensen 1954). Its rapid expansion and high competitiveness has recently aroused much scientific interest. There is evidence that a lack of grazers, the ability to feed heterotrophically on dissolved organic matter (DOM), preferences for low pH and higher water temperatures enable this species to outcompete other algae (Findlay et al. 2005, Rengefors et al. 2008, Lefebvre et al. 2013). Furthermore, *G. semen* is able to migrate vertically through the water column (Cronberg et al. 1988, Salonen and Rosenberg 2000), presumably as a way of choosing an optimal light intensity for photosynthesis during the day, and at night it migrates down in anoxic zones below the thermocline to access higher nutrient concentrations (Salonen and Rosenberg 2000). This is an important advantage in the competition with many other microalgae, which are dependent on buoyancy or suspension for access to light near to the surface (Olli 1999, Richardson et al. 2001). Despite the importance of light as a structuring environmental resource, there is very little known about the photophysiology of *G. semen*, particularly with respect to their pigment composition, photoadaptation to brown water and photoacclimation responses to variation in light environments.

Pigments in *G. semen* were first analyzed by Chapman and Haxo (1966), who identified chl a and four different but unidentified carotenoids in natural bloom samples. Fiksdahl et al. (1984) later identified the carotenoids diadinoxanthin (diadino), dinoxanthin, β,β-carotene, heteroxanthin, neoxanthin (neo), chl c1 and c2 by mass spectrometry and chemical derivatizations. Yamaguchi et al. (2010) analyzed the closely related species *Gonyostomum latum* by microspectrophotometry and found diadino, chl a, chl c1, and c2.

The purpose of this study was to characterize the pigment composition in *G. semen* by high performance liquid chromatography (HPLC) and compare it with other raphidophytes. We acclimated and grew 12 genetically distinct strains of this species at six different light intensities to study intra-specific differences and changes in pigment concentration because of photoacclimation. We hypothesized that *G. semen* is equipped with a very powerful light harvesting system to effectively photosynthesize under low light intensity, but negligible photoprotective capabilities since they can migrate downwards to avoid supersaturating irradiance. As the strains originated from habitats with different water color and light conditions, we expected to find significant differences in pigment ratios among strains because of genetic adaptation to local environmental conditions.

### MATERIALS AND METHODS

**Culturing conditions and microscopy.** Twelve genetically distinct strains of the microalgae *G. semen*, which were distinguished earlier by amplified fragment length polymorphism (Lebret et al. 2013), were used for this experiment. These strains originated from five lakes with varying absorbance (Table 1), resulting in different light climates. Cultures were incubated for 6 weeks at six different light conditions (5, 10, 25, 150, 200 μmol photons·m⁻²·s⁻¹ of white light and 25 μmol photons·m⁻²·s⁻¹ of red light, AURA Luminette 36W 840, Aura Light International AB, Karlskrona, Sweden) in artificial medium (modified Woods Hole medium, pH 7.0; Guillard and Lorenzen 1972). The red light climate was achieved by filtering out all other wavelength (<600 nm) with a Rosulcux filter (#19, Port Chester, NY, USA). The light intensities and spectra were measured with an Ocean Optics Inc. USB 2000 spectrometer, Dunedin, FL, USA (software OOIBase32) and a LI-COR light meter (Model LI-189; Lincoln, NE, USA). All cultures were grown in four replicates on a 14:10 light:dark cycle at 20°C on benches that were isolated by black foil or cardboard from the surrounding light climate in a walk-in incubator. The algae were cultured in 50 mL sterile, vented, polystyrene tissue culture flasks (VWR, Radnor, PA, USA) with a starting concentration of 500 cells·mL⁻¹.

At the end of the incubation 60 living cells per treatment were photographed with a Nikon Digital Sight-F1 camera (Nikon Instruments Inc., Melville, NY, USA) to measure cell width and length to calculate the cell area (A = width·length / 2 + (π·width radius) / 2; NIS Element Imaging Software, Nikon Instruments Inc., Melville, NY, USA). Subsamples of each culture were fixed with Lugol’s solution and manually counted in a Sedgewick-Rafter chamber using an inverted microscope (Nikon Eclipse TS100; Melville, NY, USA) at 40× magnification.

**HPLC and spectrophotometry.** For analysis of the pigment composition of *G. semen*, aliquots of 25–115 mL were filtered onto 25 mm GF/F glass fiber filters using a vacuum pump (<20 kPa) followed by immediate freezing at −80°C. Filters

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**TABLE 1. Location and water color of lakes of origin for all *Gonyostomum semen* strains.** Letters in name of strain correspond to lake. LI, Liasjön; DM, Dammen; MJ, Mjörträsket; TO, Torsjön; KY, Kylänalainen.

<table>
<thead>
<tr>
<th>Lake</th>
<th>Location</th>
<th>Longitude</th>
<th>Latitude</th>
<th>Absorbance, 420 nm·cm⁻¹</th>
<th>Water color</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liasjön</td>
<td>Southern Sweden</td>
<td>56.762604</td>
<td>13.990871</td>
<td>0.256</td>
<td>883</td>
</tr>
<tr>
<td>Dammen</td>
<td>Southern Sweden</td>
<td>56.551346</td>
<td>14.320550</td>
<td>0.058</td>
<td>200</td>
</tr>
<tr>
<td>Mjörträsket</td>
<td>Northern Sweden</td>
<td>66.035358</td>
<td>22.100891</td>
<td>0.052</td>
<td>179</td>
</tr>
<tr>
<td>Torsjön</td>
<td>Southern Sweden</td>
<td>56.762604</td>
<td>14.395361</td>
<td>0.041</td>
<td>141</td>
</tr>
<tr>
<td>Kylänalainen</td>
<td>Southern Finland</td>
<td>60.410038</td>
<td>23.754930</td>
<td>0.029</td>
<td>100</td>
</tr>
</tbody>
</table>
were subsequently hophilized for 12 h at −50°C, placed in 0.75 mL of 90% acetone, and extracted at −20°C in the dark for 20 h. Extracts were filtered through a 0.45-mm Teflon filter (Gelman Acrodisc, Port Washington, NY, USA), dispensed into amber glass vials and placed in a refrigerated autosampler (4°C). Filtered extracts (200 mL) were injected into a Shimadzu-HPLC equipped with a monomeric (Rainin Microsorb-MV, 0.46 × 10 cm, 3 mm) and two polymeric (Vydac 201TP54, 0.46 × 25 cm, 5 mm) reverse-phase C18 column in series. A nonlinear binary gradient consisting of the solvents 80% methanol:20% 0.50 M ammonium acetate and 80% methanol:20% acetone was used for pigment separations (Pinckney et al. 1996). Absorption spectra and chromatograms (440 ± 4 nm) were acquired using a Shimadzu SPD-M10av photodiode array detector. Pigment peaks were identified by comparison of retention times and absorption spectra with pure standards (DHI, Aarhus, Denmark). The synthetic carotenoid β-apo-8′-carotenal was used as an internal standard. Two samples of a culture of *Vacuolaria virescens* (1195-1, SAG Culture Collection of Algae, Göttingen, Germany), a closely related freshwater raphidophyte, were also analyzed for the first time by this HPLC method for comparison of pigment composition.

Eight replicates of peaks thought to be from the pigment alloxanthin (allo) were collected from the HPLC effluent for determination of pigment identity by spectroscopy. The methanol-acetone solvent was first evaporated under nitrogen and the precipitated pigment dissolved in 1.5 mL of 100% acetone. The spectrum (350–700 nm) was collected with a Shimadzu UV-2450 dual beam spectrophotometer (Kyoto, Japan) with 100% acetone as the blank. The spectrum was compared to a reference spectrum for allo from Jeffrey et al. (1997).

**Statistical analyses.** A non-parametric Spearman Rho correlation analysis between light treatment, total chl *a*, total carotenoids, and accessory pigment/chl *a* ratios was performed. A Kruskal–Wallis Test was used to analyze differences in pigment composition among strains, grouped by lake of origin, and compared to a reference spectrum for allo from Jeffrey et al. (1997).

RESULTS

**Pigment composition.** We acquired the first complete HPLC pigment spectrum showing photoacclimation to different light conditions for several strains of *G. semen*. All 12 strains contained the pigments chl *c1/c2*, trans-neox, cis-neox, violaxanthin (violax), diadino, allo, zeax, chl *a* and β carotene (Fig. 1). The other freshwater raphidophyte *Vacuolaria virescens* contained exactly the same pigments plus diatoxanthin, although the pigment ratios differed between the two species. The most evident differences with *G. semen* included extremely high amounts of chl *c1/c2* and trans-neox, reduced concentrations of diadino and small amounts of allo (Fig. 2).

**Photoacclimation.** Total chl *a* per cell decreased in all *G. semen* strains with increasing light intensity (Spearman’s Rho coefficient −0.669, *P* < 0.001; Fig. 3). The randomized block design ANOVA (Chl *a* by light, *F*<sub>5,66</sub> = 26.685, *P* < 0.001) and the post-hoc test showed that chl *a* per cell in the red light treatment (25 μmol photons · m<sup>−2</sup> · s<sup>−1</sup>) was significantly higher than cellular concentration at the equivalent white light intensity (25 μmol photons · m<sup>−2</sup> · s<sup>−1</sup>; Mean difference = 40.5%, Tukey HSD, *P* < 0.001; Fig. 3). Instead it was more similar to the chl *a* concentrations at 5 μmol photons · m<sup>−2</sup> · s<sup>−1</sup> (white light; Tukey HSD, *P* = 0.137).

The total amount of chl *c1/c2* per cell and most carotenoids also decreased with increasing light intensity, but several carotenoids decreased at a slower rate than chl *a*. Cellular concentrations of allo were not significantly correlated to light intensity (Spearman-Rho correlation: *P* = 0.774). However,
the ratios of the accessory pigments chl c1c2, violax, zeax, diadino and allo per chl a increased with increasing light intensity (Spearman’s Rho coefficient: 0.239, 0.434, 0.364, 0.818, 0.724, respectively; \( P \leq 0.007 \)).

We found significant differences between cell areas depending on light intensity (ANOVA \( F_{5,354} = 30.022, P < 0.001 \)). Cells grown under red light were the largest (average 1,276 \( \mu \text{m}^2 \); Tukey HSD; \( P \leq 0.005 \)) while cells grown under 200 \( \mu \text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \) white light and additionally 25 \( \mu \text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \) red light. Means with identical letters are not significantly different. Error bars represent SE.

**Alloxanthin.** The pigment peak with a retention time of \( \sim 19.7 \text{ min} \) was identified as allo by comparison with the absorption spectra of a pure standard and of extracted pigments from the cryptophyte *Rhodomonas salina* and by direct spectrophotometry. The UV-Vis spectrum clearly showed the two characteristic peaks of allo at 454 nm (2) and 484 nm (1) (Fig. 4).

There was a significant correlation between the two major accessory pigments allo and diadino \(( R = 0.59; R^2 = 0.687 \)). Both pigments per chl a were correlated to increasing light intensity (Spearman Rho correlation: \( P < 0.000 \)) and reached maximum concentrations at 200 \( \mu \text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \) (Fig. 5). With increasing light...
intensity the Diadino/Chl a ratio increased first, but at higher light intensities the concentrations of diadino and allo per chl a were similar. This correlation is very different from other allo containing algae like Rhodomonas salina, as this species has little diadino in comparison to allo. The other freshwater raphidophyte Vacuolaria virescens contains little allo in comparison to G. semen, resulting in a different ratio of allo to diadino as well (Fig. 5).

Differences among strains. The amount of total chl a per cell differed significantly among strains at both low (5, 10, 25 μmol photons · m⁻² · s⁻¹) and high (150, 200 μmol photons · m⁻² · s⁻¹) light intensities (Kruskal–Wallis Test: \( P = 0.02, \ P < 0.001 \), respectively). However, the ratio of the main light harvesting accessory pigment chl c1c2 to chl a did not differ among strains in low light conditions (Kruskal-Wallis test; \( P = 0.31 \)). In high light conditions the ratio of the main photoprotective pigment diadino to chl a was similar in all cultures (Kruskal-Wallis test; \( P = 0.156 \)). The ratios of all other accessory pigments to chl a differed significantly in both high and low light conditions among the strains.

Similar results could be observed for differences in pigment ratios among strains grouped by lake of origin. At pooled low light intensities the lake groups differed in all pigment concentrations significantly (Kruskal-Wallis test; \( P \leq 0.024 \)) except zeax and the main light harvesting pigments chl c1c2 and α carotene per chl a (Kruskal–Wallis Test: \( P = 0.498, \ P = 0.061, \ P = 0.15 \), respectively). However, focusing on the 5 μmol photons · m⁻² · s⁻¹ treatment, the amounts of α and β carotene per chl a were approximately two times higher in strains from lake Kylišanlainen than in all other strains (Kruskal–Wallis Test: \( P = 0.034, \ P = 0.013 \), respectively). At pooled high light intensities total chl a, cis-neox, diadino, and allo per chl a occurred in similar concentrations (Kruskal-Wallis test; \( P = 0.162, \ P = 0.733, \ P = 0.936, \ P = 0.076; \) respectively). All other pigments differed significantly among groups (Kruskal-Wallis test; \( P \leq 0.002; \) Fig. 6).

DISCUSSION

In this study, we characterized the pigment composition in 12 strains of the freshwater alga G. semen that were grown under a range of light conditions. Our results showed major differences to marine
Pigment Composition and Photoacclimation in Gonyostomum

The potentially photoprotective pigments allo and diadino might enable *G. semen* to establish successfully in many new habitats, which do not feature a sharp light gradient like brown water swamp lakes. In lakes with little attenuation of irradiance, algae are probably exposed to higher light levels for a longer time and need photoprotective mechanisms like the xanthophyll cycle to avoid photoinhibition (Ragni et al. 2008, Harrison and Smith 2011). Photosystem II is constantly damaged by photons and therefore, the rate of photoinhibition is directly proportional to the excitation energy, which is transferred to the reaction center (Melis 1999, Ross et al. 2008, Kirk 2011). There is an especially strong photoinhibitory effect of blue light, which is filtered out in the red light treatment in this study. This might explain the high chl *a* content per cell, which we observed in this treatment. However, the maximal light intensity in this experiment (200 μmol photons · m⁻² · s⁻¹) does not reflect the maximal possible light intensity in lakes in Sweden (up to 1,000 μmol photons · m⁻² · s⁻¹ near the surface; Hellström 1991). Therefore, *G. semen* might not have experienced severe photoinhibition as shown by the low concentrations of photoprotective pigments per cell.

The origin of the atypical pigment allo in *G. semen* remains unclear. All chromista (haptophytes, cryptomonads, heterokonts, dinoflagellates, and apicomplexas) obtained their plastids originally by secondary endosymbiosis of red algal cells (Bhattacharya and Medlin 1998, Cavalier-Smith 2000, 2002, Keeling 2010). Therefore, chloroplasts of most raphidophytes are surrounded by four
membranes originating from the engulfed endosymbiont (Ishida et al. 2000, Chaal and Green 2005). However, there are several examples for microalgae, especially in dinoflagellates, which have reduced their original plastids again or replaced them by tertiary endosymbiosis (Keeling 2010, Stiller 2014). It has been assumed that the pigment allo evolved after secondary endosymbiosis exclusively in cryptophytes. Carpenter et al. (1995) and Hackett et al. (2003) suggested that the plastids of the dinoflagellate *Dinophysis norvegica* originate from a tertiary endosymbiosis of an engulfed cryptophyte based on monophyletic plastid sequences and the ultrastructure of their chloroplasts and thylakoids.

There are also several reports of acquired phototrophy in the ciliate genus *Mesodinium* and the dinoflagellate genus *Dinophysis* (Stoecker et al. 2009, Hansen et al. 2013). The ciliates ingest cryptophytes and keep the cells as “reduced symbionts” (Esteban et al. 2010, Johnson 2011). The dinoflagellates graze on *Mesodinium* species, which contain cryptophyte symbionts, and integrate the “prey chloroplasts” at least partly into their metabolism (Stoecker et al. 2009, Hansen 2011, Hansen et al. 2013). These microalgal genera are dependent on a continuous supply of new chloroplasts, as they are not able to replicate the plastids (Hansen et al. 2013).

Symbionts, parasites, or food vacuoles containing other algae have never been observed in *G. semen* (Rengefors et al. 2008) and this study was conducted on unialgal strains, which have been cultivated in the laboratory for at least 1.5 years. This suggests direct synthesis of allo by chloroplasts owned by *G. semen*. This species might have acquired the pigment allo by tertiary endosymbiosis of a cryptophyte, which would represent different evolutionary plastid acquisition in marine taxa, like *Chattonella*, *Fibrocapsa* and *Heterosigma*, than in freshwater raphidophytes. This hypothesis is supported by differences in fatty acid and sterol composition between brown-pigmented, marine raphidophytes and green-pigmented species (*Gonyostomum* and *Vau-ularia*; Roche and Leblond 2011, Leblond et al. 2013). However, further ultrastructural studies are needed to confirm tertiary endosymbiosis in the freshwater lineages.

Our data suggest a high plasticity in pigment concentrations in all *G. semen* strains based on the significant differences between light treatments. In addition to its ability to optimize light harvesting conditions by vertical migration, we found that this species is able to decrease its chl *a* content with increasing light intensity (from 25 μmol photons · m⁻² · s⁻¹ red light to 200 μmol photons · m⁻² · s⁻¹ white light) by 76.4%. Flexible concentrations of the potentially photoprotective pigments allo and diadino per chl *a* might enable this species to photosynthesize under many different light intensities and adjust quickly to changing environmental conditions. The increase in chl *a* concentrations under low light appears to be related to an increase in the cell surface area, presumably to magnify the area of absorbance and decrease the “package effect” (Duysens 1956). This is caused by the patchy and stacked distribution of pigment molecules in the cells, leading to a lower specific absorption per unit pigment in vivo than expected from the absorption of extracted pigments (Bidigare et al. 1990b, Kirk 2011).

The significant differences among strains when grouped by lake of origin regarding the ratios of the carotenoids violax, trans-neox, and β carotene may be based on genetic differences between the isolates. These differences are especially pronounced in the carotene to chl *a* ratios under very low light intensity (5 μmol photons · m⁻² · s⁻¹). Strains from the Finnish lake Kylänalainen, which is the clearest of all lakes (Table 1), contained significantly more of these pigments than all other strains. Large geographic distances to the other Swedish lakes and less absorption of light in the water column of lake Kylänalainen might have promoted genetic differentiation into different phenotypes. It is difficult to relate variations in other pigment ratios to the lake of origin, as the exact functions of trans-neox and violax in *G. semen* are unknown. All strains contain comparable amounts of chl *c1*c2 per chl *a* under low light intensities. This might be caused by the dependency of microalgae on efficient light harvesting under these conditions, resulting in an equally high concentration of the accessory pigment chl *c1*c2 among all strains. Under high light intensities dissipation of excessive light energy by photoprotective pigments is very important, which might explain the occurrence of similar concentrations of diadino and allo in all strains.

This study provides the first complete pigment analysis of *G. semen* by HPLC, which gives insight into photoacclimation processes in this species. These consist of major variation in chl *a* concentration depending on light intensity and regulation of accessory pigments like diadino and allo. The high plasticity in pigment concentrations and the variability among genetically different strains might have facilitated the recent spreading of *G. semen*. In addition, the significant differences in pigment composition to closely related marine taxa give further evidence for a different evolutionary acquisition of plastids within groups of raphidophytes.

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Gonyostomum semen of the flagellate Gonyostomum semen (Raphidophyta) - a new picoplanktonic green alga. J. Phycol. 36:1135–44.


