

Influence of darkness on pigments of *Tetraselmis indica* (Chlorodendrophyceae, Chlorophyta)

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Abstract

In the photic zone, phytoplankton experience diurnal variation in darkness. However, prolonged exposure to aphotic conditions influences their physiological state. Pigment composition is a useful biomarker to decipher cells physiological state and adaptive response to changing environmental conditions. Chlorophyll, a natural pigment is biosynthesised even in darkness and studies have shown such an ability is determined by the genetic characteristics of the organism. The purpose of this study was to examine the influence of darkness on pigments and chlorophyll autofluorescence of *Tetraselmis indica*. Dark exposure (up to 6 months) had no significant impact on chlorophyll *a* and *b* concentration, whereas carotenoids were enhanced. Upon re-illumination pigments gradually recovered to pre-dark phase concentrations. These adaptive survival strategies by altering pigment concentration in response to prolonged darkness is interesting. The absence of loroxanthin and loroxanthin esters in *T. indica* is reported in a first *Tetraselmis* species so far. The evaluation of autofluorescence and cellular chlorophyll concentration pointed out that they are not interdependent in this species. Hence, careful consideration of these two factors is needed when either of them is used as a proxy for other. The results obtained encourage a thorough study of pigment analysis, especially when subjected to darkness, to elucidate potential role in the evolution, chemotaxonomy, and survivability of species.

Keywords: *Tetraselmis indica*, green algae, survival, pigments, loroxanthin, chlorophyll autofluorescence

1. Introduction

Chlorodendrophyceae is an interesting deep-diverging lineage of core Chlorophyta (Turmel et al. 2016). It is a small class of green algae comprised of two genera *Tetraselmis* and *Scherffelia* (Guillou et al. 2004; Leliaert et al. 2012). These unicellular quadriflagellates, traditionally regarded as members of Prasinophyceae, have several ultrastructural features of core Chlorophyta and hence now belong to Chlorodendrophyceae (Leliaert et al. 2012). However, recent chloroplast genome study of a *Tetraselmis* species has revealed that they have still retained the ancestral quadripartite structure of prasinophytes and streptophytes (Turmel et al. 2016). Therefore, to elucidate the evolutionary history of the green algal lineage, *Tetraselmis* species are an important model organism.

The pigment composition of *Tetraselmis* genus has been extensively studied (Egeland et al., 1997, 1995; Garrido et al., 2009; Latasa et al., 2004). Based on carotenoid composition, a classification had been devised earlier (Egeland et al., 1997). Type 1 possesses green algal carotenoids such as neoxanthin, violaxanthin, antheraxanthin, lutein, zeaxanthin, and β,β -carotene. Type 2 includes siphonaxanthin, loroxanthin and their esters whereas Type 3 contains prasinoxanthin, uriolide, micromonol, micromonal, and dihydrolutein in addition to other green algal carotenoids (Egeland et al. 1997). Type 1 comprises of Chlorodendrales (e.g. genus *Tetraselmis*) whereas type 2 consist of Pyramimonadales and Pseudoscourfieldiales (Garrido et al., 2009; Latasa et al., 2004; Yoshii et al., 2002). Even though *Tetraselmis* species are grouped into Type 1, loroxanthin and loroxanthin esters (phylogenetically important pigments) have been detected in this genus (e.g. Garrido et al., 2009; Sansone et al., 2017). Hence, the study of loroxanthin distribution in algae provides a valuable tool to investigate phylogenetic questions in green algae (Fawley and Buchheim, 1995).

Light is a fundamental source of energy and its absence can trigger several biochemical or physiological responses in phytoplankton, including a change in pigment concentration (e.g. Luder et al., 2002; Montechiaro and Giordano, 2006; Peters, 1996; Peters and Thomas, 1996; Zhang et al., 2007). Pigment studies can decode cells physiological state and their adaptive response to environment (Brunet et al., 1996). Similar to pigments, flow cytometry (FC) obtained chlorophyll autofluorescence signatures are also an important tool to examine environmentally induced variation in phytoplankton physiology (Veldhuis and Kraay, 2000). These signatures are not only affected by choice of fixative but even storage temperature (Naik and Anil, 2017). Autofluorescence is often related to cellular pigmentation especially chlorophyll *a* (Zhang et al., 2007). Hence, the relationship between FC obtained fluorescence signals and cellular photosynthetic pigments need to be investigated thoroughly.

The goal of this paper is to evaluate the influence of darkness on pigments and chlorophyll autofluorescence. For this purpose, we used *Tetraselmis indica* as a model organism whose flexible physiology and survivability under prolonged darkness are known (Naik and Anil, 2018).

2. Materials and methods

2.1 Experimental setup

Tetraselmis indica was maintained in f/2 without silicate (Guillard and Ryther 1962) enriched natural seawater at $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$. The light intensity of $40 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ was provided by daylight white fluorescent tubes under a photoperiod of 12:12 light:dark cycle. The culture flasks were gently shaken every day to ensure homogenous mixing.

Details of the experimental setup are described in Naik and Anil (2018). Briefly, one set of flasks (3 flasks x 10 sampling days = 30; Set 1) was maintained as control (12:12 light:dark cycle) while the other set was subjected to complete darkness (3 flasks x 9 sampling days = 27; Set 2). To ensure complete darkness in Set 2, flasks were covered with black polythene and samples were collected in dark. Three flasks were randomly collected on day 0, 1, 4, 8, 15, 30, 45, 74, 147, and 182 from both sets. The growth potential of dark-adapted cells was tested by transferring 50 mL sub-sample to initial light condition.

2.2 FC analysis

The enumeration of cell abundance and red fluorescence (a proxy for chlorophyll autofluorescence) was performed on a FACS Verse FC (BD Bioscience- US) equipped with a blue laser (488 nm) as described earlier (Naik and Anil, 2017). To avoid the effect of fixative on red fluorescence fresh samples were used (Naik and Anil, 2017). The red fluorescence was recorded in list mode and processed using FACS Suite software. In order to compare red fluorescence of different samples, Fluoresbrite[®] YG microspheres (10.3 μm ; Polysciences Co., USA) were added as an internal standard.

2.3 Pigment analysis

Sample (3-5 mL) was filtered through glass fiber filters (GF/F Whatman, 25mm) at a low vacuum pressure and stored at -20°C until analysis. The pigment extraction was carried out using 100% acetone (HPLC grade from Merck Chemicals) in a centrifuge tube. After mixing and cold sonication (PRO250. Pro Scientific Inc. Oxford), tubes were stored at -20°C overnight. Next day, tubes were centrifuged in a fixed angle rotary centrifuge (Centrifuge 5804R, Eppendorf) at 3000 rcf for 15 min at 0°C . The supernatant was filtered using Acrodisc[®] syringe filters (Gelman laboratory). The entire procedure was carried out in dim light to avoid pigment degradation. High-performance

liquid chromatography (HPLC; Agilent Technologies 1200 series) with a photodiode detector was carried out following a Heukelem (2002) method as described earlier in Roy et al. (2015). To know the presence of linoxanthin and linoxanthin esters in this alga a modified Zapata method was also attempted (Garrido et al., 2009).

2.4 Statistical analyses

The normality of data was determined using Shapiro Wilk's W test to meet the assumptions of parametric tests. The variation in all parameters with days was carried using a one-way ANOVA and Tukey's post hoc test for both control and dark conditions. A two-way ANOVA for both the conditions was also carried out separately between conditions and days. All statistical analyses were performed using SPSS statistical package (Windows Ver. 22).

3. Results

HPLC technique allowed pigment identification and quantification in *T. indica*. Heukelem method could separate six pigments, namely neoxanthin (Neo), violaxanthin (Vio), lutein (Lut), chlorophyll *b* (Chl *b*), β -carotene (β -car), and chlorophyll *a* (Chl *a*) (Fig. 1A, Table 1). While linoxanthin and linoxanthin esters were not detected in *T. indica*, antheraxanthin (Anth) along with above six pigments were identified using a modified Zapata method (Fig 1B, Table 1).

Both control and complete darkness had a significant influence on pigment concentrations (ANOVA, $P < 0.001$; Fig. 2A-F). The pigment concentration in control condition was different compared to dark condition. In control condition, after initial fluctuations, pigment concentration decreased (Tukey's post hoc, $P < 0.01$). β -car initially remained unchanged (Tukey's post hoc, $P > 0.05$) but increased significantly on day 147 (Tukey's post hoc, $P < 0.05$). In dark condition, chlorophyll (both Chl *a* and *b*) varied insignificantly on day 0 and 182 (Tukey's post hoc, $P > 0.05$). The change in carotenoids (Neo, Vio, Lut, and β -car) was insignificant until day 30 (Tukey's post hoc, $P > 0.05$), but later increased (Tukey's post hoc, $P < 0.01$). Interestingly, pigment concentration was significantly higher in dark than the control condition.

Upon re-illumination of dark-adapted cells, pigment concentration gradually recovered and attained concentration similar to the pre-dark phase or more (Fig. 3A-H). The pigments immediately recovered when *T. indica* was exposed to the darkness of up to 8 days. However, with increasing darkness duration, pigments initially decreased and was affected by the lag phase. Beyond 45 days in darkness, initially, pigment concentration declined until it reached non-detectable levels but later concentration was either recovered to the original concentration or higher.

The experimental conditions also had a significant impact on chlorophyll autofluorescence (ANOVA, $P < 0.001$; Fig. 4). However, this influence was different compared to cellular pigments. In control condition, no change was observed on autofluorescence till the stationary phase (i.e., day 45; $P > 0.05$) but in dark it showed 71.81% decline by day 30 (ANOVA, $P < 0.001$).

4. Discussion

The pigment suites of *T. indica* were comparable with other *Tetraselmis* species (Borghini et al., 2009; Egeland et al., 1997, 1995; Sansone et al., 2017). Based on Egeland et al. (1997, 1995) classification, we infer that *T. indica* belong to type 1 pigment composition as expected for order Chlorodendrales. Heukelem method could separate Neo, Vio, Lut, Chl *b*, Chl *a*, and β -car whereas the modified Zapata method detected Anth in addition to other six pigments (Fig. 1). The aim of testing pigment composition using the latter method was to evaluate the presence of loroxanthin and loroxanthin esters in *T. indica*. Though loroxanthin has been detected in other species of *Tetraselmis* (Garrido et al., 2009; Sansone et al., 2017), it was absent in *T. indica*. This indicates that *T. indica* is the first *Tetraselmis* species studied so far not to have loroxanthin containing pigmentation. It is not impossible since 18S phylogeny studied by Arora et al. (2013) shows a separate branch for *T. indica* compared to other *Tetraselmis* species. It could also be due to the absence of favourable conditions necessary for the synthesis of loroxanthin and requires further study. Even though the type 1 pigment composition includes zeaxanthin (Zea), it was surprising that no Zea was detected by both the tested methods. Zea commonly coelutes with Lut, however, Heukelem method could efficiently separate these two pigments.

Pigments in the control condition decreased except for β -car (Fig. 2). The change in pigment concentration is generally to partially compensate for the variations in light intensity and nutrient concentration for cells to adapt under unfavourable conditions (Falkowski and Owens, 1980; Sosik et al., 1989). The pigments can also decrease when the light is not a limiting factor i.e., under high light intensity or in dilute cell suspensions (Beale and Appleman, 1971). This is because the light is essential to maintain chlorophyll stability but not synthesis (Beale and Appleman, 1971; Raymont, 1980). It has also been proven that under steady-state conditions, chlorophyll concentration is inversely related to light availability (Beale and Appleman, 1971). Although Neo, Vio, and Lut decreased with time, β -car increased at a later stage. A similar trend was observed in *Dunaliella bardawil*, where β -car increase was attributed to the nutritional imbalance, role of light intensity, and protective role of β -car against damaging radiation (Ben-Amotz and Avron, 1983).

Darkness can have varying influence on pigment concentration of algae (e.g. Deventer and Heckman 1996; Luder et al. 2002, 2001; Montecchiaro and Giordano 2006; Murphy and Cowles 1997). Different light intensities and spectral qualities can even result in the interconversion of pigments (or *ex novo* synthesis) in order to maintain photosynthetic performance (Garrido et al., 2016). In the present study, chlorophylls (both Chl *a* and *b*) were not much affected and remained almost stable whereas carotenoids were boosted (Fig. 2). Chlorophylls are abundant classes of natural pigments and are biosynthesized either in darkness or continuous light (Galova et al., 2008). The presence of *chlLNB* (*chlL*, *chlN*, and *chlB*) genes in chloroplast genome is accounted for as the ability of algae to synthesize chlorophyll in dark (Galova et al., 2008; Turmel et al., 2016). The absence of *chlLNB* genes reported in *Tetraselmis* species (Turmel et al., 2016) may be the reason for the inability of *T. indica* to synthesize chlorophyll in dark. The low impact on chlorophyll may also indicate good maintenance and preservation of photosynthetic capacity and photosynthetic apparatus (Dehning and Tilzer 1989; du Preez and Bate 1992). The maintenance of these cellular features can be shown by their growth and ability to synthesize pigments upon re-illumination (Fig. 3).

Carotenoids are known to dissipate excess energy via the xanthophyll cycle, have a structural role of stabilizing and photoprotecting chloroplast membrane, and protect photosynthetic reaction centers and antenna complexes against photochemical damage (Frank and Cogdell, 1993). They are also known as protective pigments due to their antioxidant properties (Pizarro and Stange 2009). This may have triggered the enhancement of carotenoids in *T. indica* under darkness. Another possible reason for carotenogenesis in dark is the presence of enzyme carotene isomerase (*CRTISO*) in *Tetraselmis* species. *CRTISO* is known to presumably enable carotenoid biosynthesis in dark from *cis*-form to *trans*-form; otherwise, this transformation occurs non-enzymatically in the presence of light by photoisomerization (Isaacson et al. 2002; Masamoto et al. 2001). The presence of this enzyme has been confirmed in halophilic microalga *Tetraselmis* sp. GSL018 isolated from Great Salt Lake, Utah (USA) (Jinkerson et al. unpublished GenBank data). *Tetraselmis* sp. GSL018 is also known to form a close phylogenetic relationship with *T. indica* as described earlier (Arora et al., 2013). Similar constancy in chlorophyll concentration and an increase in carotenoid (β -car) under dark condition has also been reported in a green alga *Scenedesmus quadricauda* (Deventer and Heckman 1996).

Red fluorescence is used as a proxy for chlorophyll autofluorescence in laser-based instruments (Jochem, 2000; Veldhuis and Kraay, 2000). In the present study, chlorophyll autofluorescence and cellular chlorophyll concentration showed a different trend in both the control (12:12 light:dark cycle) and dark conditions (Fig. 2E,F & 4). These variations in chlorophyll

autofluorescence can be due to the, i) differences in relative abundance of Chl *a* and accessory pigments, ii) change in efficiency of photon absorption, and iii) cell size which contributes to pigment packaging in cells (Olson et al., 1983; Sosik et al., 1989). These results demands careful consideration of FC obtained autofluorescence signatures as a proxy for chlorophyll.

5. Conclusion

Tetraselmis indica, when exposed to prolonged darkness, showed minimal alteration in chlorophyll concentration but carotenoids were enhanced. Our results highlight the role of carotenoids in survivability of *T. indica* in darkness. Unlike other *Tetraselmis* species, loroxanthin was absent in this species. This may contribute to understanding the evolutionary history of the green algal lineage, especially of *Tetraselmis* genus. The different trend of chlorophyll autofluorescence and chlorophyll concentration obtained also highlights the drawbacks of using chlorophyll autofluorescence as a proxy for chlorophyll concentration in this species.

Acknowledgement

This work was supported by Council of Scientific & Industrial Research (CSIR) funded project Ocean Finder (PSC0105). S.M.N. greatly acknowledges research fellowship awarded by University Grants Commission (UGC), India and support provided by Academy of Scientific and Innovative Research (AcSIR). S.M.N is also grateful to Suchandan Bernal, Dr. Dhiraj Narale, and Dr. Lalita Baragi for their immense encouragement and support. This is a CSIR-NIO contribution (#).

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Figure legends

Fig.1. HPLC chromatogram of pigments extracts of *Tetraselmis indica* using a) Heukelem method and b) Modified Zapata method. 1) Neoxanthin, 2) Violaxanthin, 3) Antheraxanthin, 4) Lutein, 5) Chlorophyll *b*, 6) Chlorophyll *a*, and 7) β -carotene

Fig. 2. Change in pigment concentrations (pg cell^{-1}) of *Tetraselmis indica* grown under control (12:12 light:dark cycle; white square) and dark (black square) conditions using the Heukelem method. (a) Neoxanthin (Neo), (b) Violaxanthin (Vio), (c) Lutein (Lut), (d) β -carotene (β -car), (e) Chlorophyll *a* (Chl *a*), and (f) Chlorophyll *b* (Chl *b*). Vertical lines indicate standard deviation from the mean.

Fig. 3. Change in pigment concentrations (pg cell^{-1}) of dark-adapted *Tetraselmis indica* upon re-illumination after 1 day, 4 days, 8 days, 15 days, 30 days, 45 days, 147 days, and 182 days of darkness using Heukelem method. (a) Neoxanthin (Neo), (b) Violaxanthin (Vio), (c) Lutein (Lut), (d) β -carotene (β -car), (e) Chlorophyll *a* (Chl *a*), and (f) Chlorophyll *b* (Chl *b*). Vertical lines indicate standard deviation from the mean.

Fig. 4. Change in red fluorescence intensity (a proxy for chlorophyll fluorescence) of *Tetraselmis indica* grown under control (12:12 light:dark cycle; white squares) and dark (black squares) conditions. Vertical lines indicate standard deviation from the mean.

Table legend

Table 1. Peak identification, abbreviation, retention time and online spectral characteristics

Figure 1

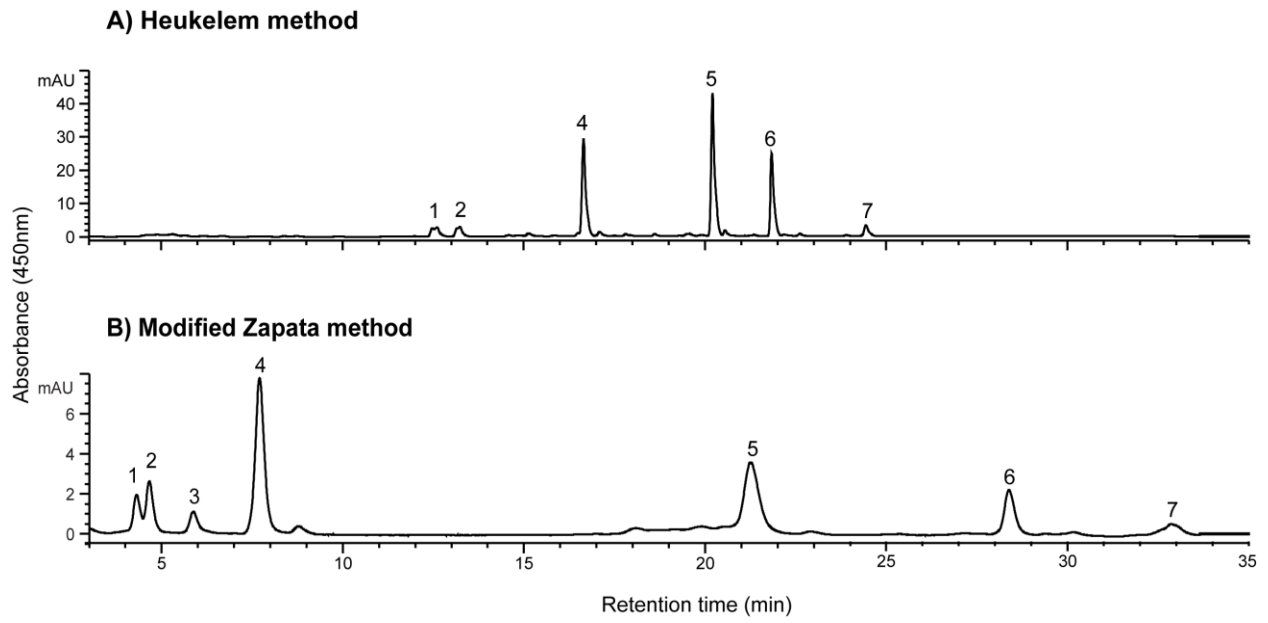


Figure 2

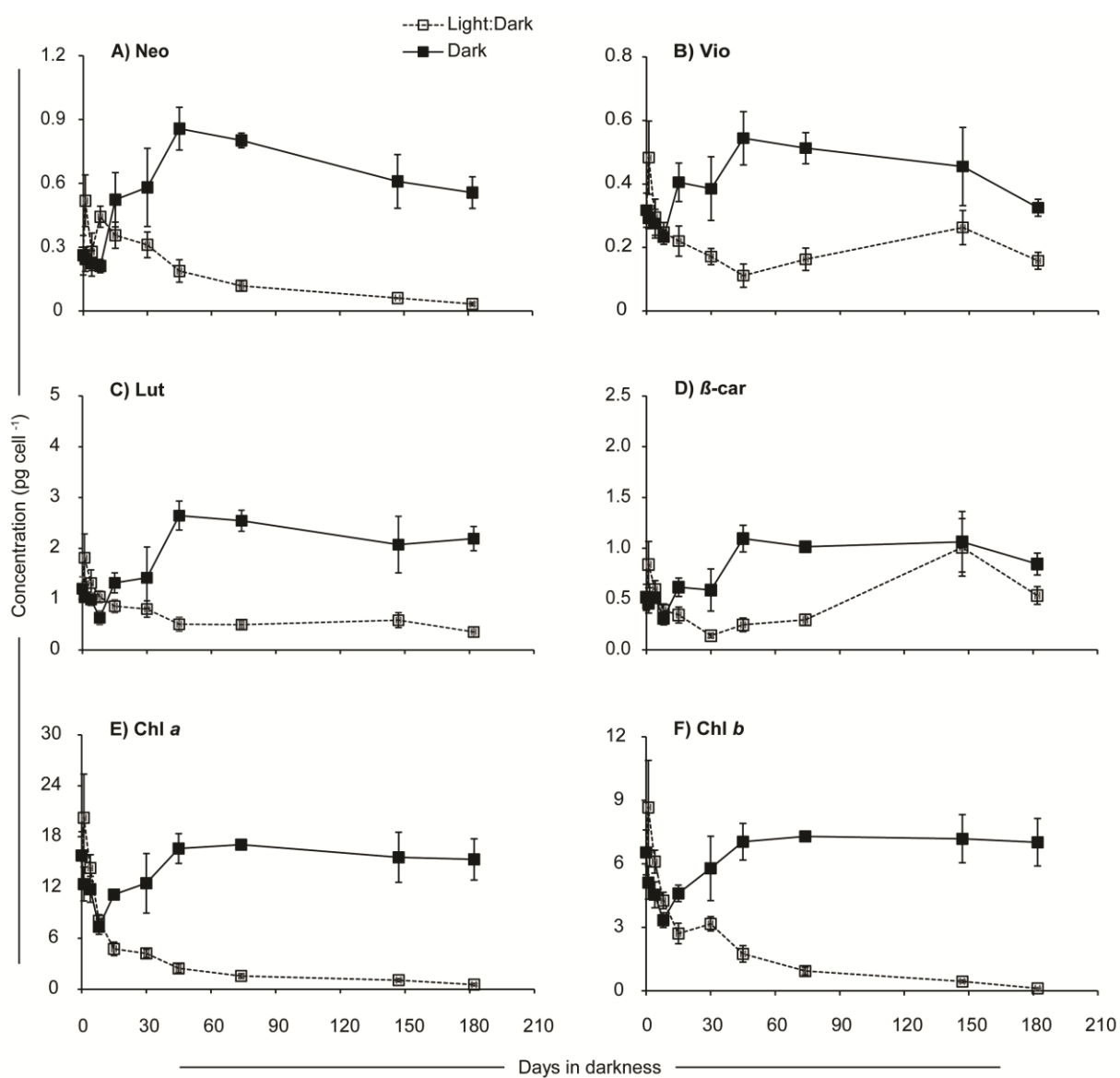


Figure 3

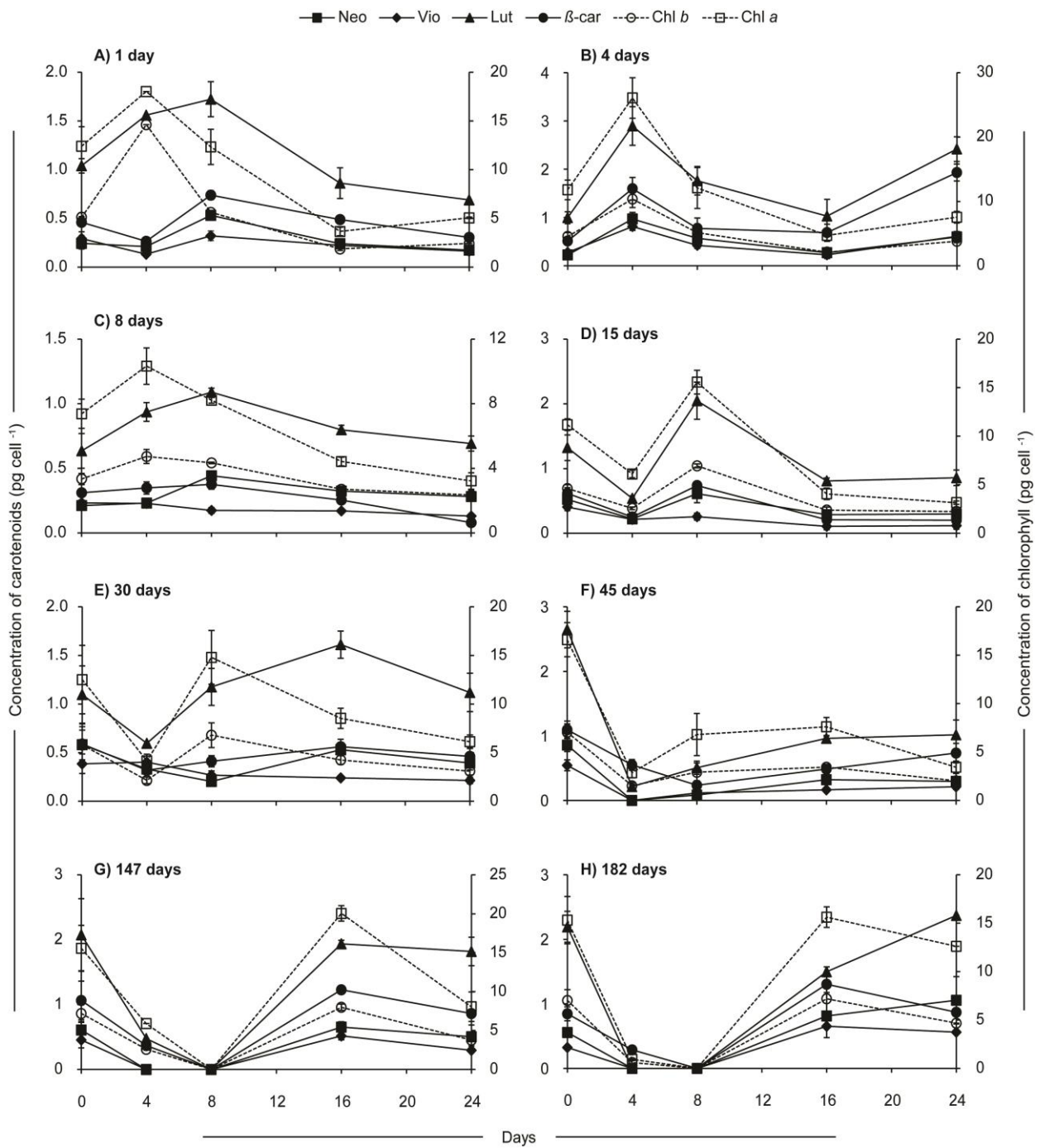


Figure 4

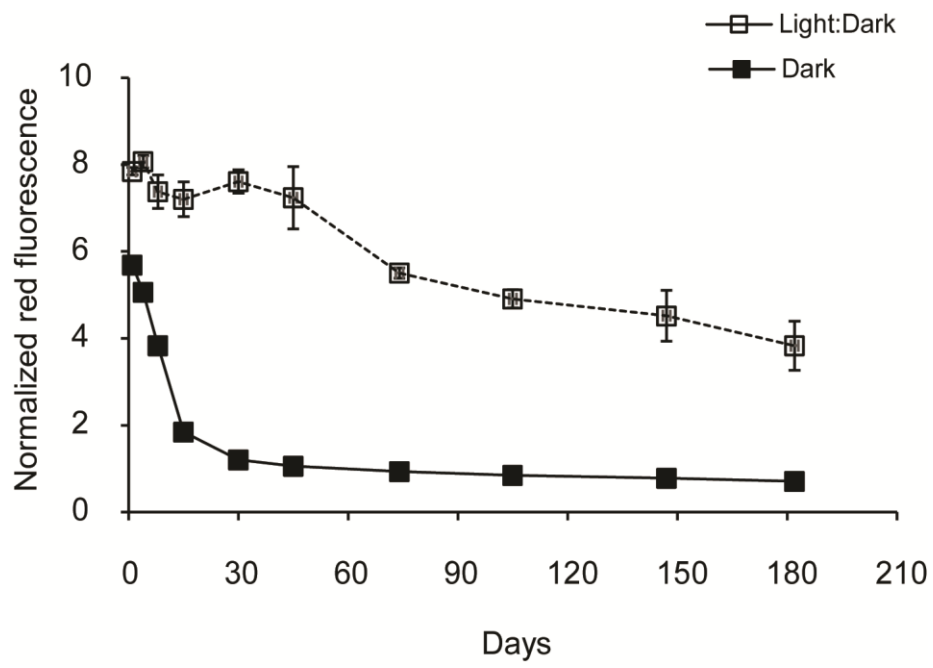


Table 1.

Peak	Pigment	Abbreviation	Retention Time (min)	Absorbance maxima (nm)		
Heukelem method						
1	Neoxanthin	Neo	12.48	438	466	416
2	Violaxanthin	Vio	13.24	438	470	416
4	Lutein	Lut	16.65	446	474	462
5	Chlorophyll <i>b</i>	Chl <i>b</i>	20.21	470	652	602
6	Chlorophyll <i>a</i>	Chl <i>a</i>	21.84	432	666	620
7	β -carotene	β -car	24.44	448	476	468
Modified Zapata method						
1	Neoxanthin	Neo	4.31	436	465	414
2	Violaxanthin	Vio	4.66	440	470	455
3	Antheraxanthin	Anth	5.87	446	477	472
4	Lutein	Lut	7.69	446	474	463
5	Chlorophyll <i>b</i>	Chl <i>b</i>	21.25	462	626	648
6	Chlorophyll <i>a</i>	Chl <i>a</i>	28.38	430	663	627
7	β -carotene	β -car	32.86	448	450	480