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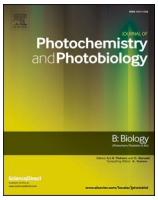

Spectroscopic analysis of sequestered chloroplasts in *Elphidium williamsoni* (Foraminifera)

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ABSTRACT

Foraminifera are unicellular, marine organisms that occur worldwide. A very common species in the German Wadden Sea is *Elphidium williamsoni*. Some foraminifera (such as elphidia) are able to use kleptoplastidy, which allows them to incorporate chloroplasts from their algal food source into their own cell body. The experiments reported here are based on the fact that chlorophyll (a and c) can be detected in the intact cells with spectroscopic methods in the visible spectral range, which allows an indirect investigation of the presence of sequestered chloroplasts. Starving experiments of *E. williamsoni* in the light (24 h continuous) showed that the greatest decrease in chlorophyll content was recorded within the first 20–30 days. From day 60 on, chlorophyll was hardly detectable. Through subsequent feeding on a renewed algal food source a significant increase in the chlorophyll content in foraminifera was noticed. The degradation of chlorophyll in the dark (24 h continuous darkness) during the starving period was much more complex. Chlorophyll was still detected in the cells after 113 days of starving time. Therefore, we hypotheses that the effect of photoinhibition applies to chloroplasts in foraminifera under continuous illumination.

1. Introduction

Foraminifera are marine unicellular protists. Their cytoplasm is surrounded by a single to multi-chambered test, which is mainly composed of calcium carbonate, but also biopolymers such as chitin and proteins, and silica. Foraminifera live in different habitats and generally feed on phytoplankton and phytodetritus, which they absorb using pseudopods (extension of the cytoplasm outside the test) and transport into the cell [10]. The foraminifera *Elphidium williamsoni*, which was studied here, is one of the most abundant species in the mudflats and benthic ecosystems of the German Wadden Sea. Mudflats are important habitats for a large number of microorganisms [27]. Due to the tides, in combination with strong variations in temperature and salinity, this region provides a special and extreme habitat for its residents. Studies by Tillmann et al. [36] showed that the growth of phytoplankton is high in spring to fall but very low or almost zero in the winter months, causing nutritional stress for foraminifera during winter time when little fresh phytodetritus is available.

Moreover, experimental studies have shown that the food uptake of foraminifera is highly dependent on environmental parameters. Not only the size of the food or its freshness [23], but also the temperature [40, 42], the salinity [25] and heavy metal concentrations (Lintner et al., submitted) have a significant influence on their food uptake.

An important nutritional aspect, particularly for elphidia (organisms belonging to the genus Elphidium), is kleptoplastidy – the process of isolation of chloroplasts from the algal food source and their subsequent incorporation into the own metabolic cycle (e.g. [24]). The chloroplasts are not digested and retain their photosynthetic activity [15]. Kleptoplasts may also be used as a carbon source during longer starving periods [8,24] and can be preserved in foraminifera from several days to several months [5,21,28]. A study by Lopez [28] showed that foraminifera have to sequester a certain number of chloroplasts per hour from fresh food in order to counteract a natural decrease in chloroplasts numbers per cell. Experiments with *Haynesina germanica* showed that chloroplasts remain stable and active in the cell for more than a week [31]. Further observations showed that not only foraminifera from the shallow marine environment have kleptoplasts, but also those living below the photic zone [1]. Kleptoplastidy has been found in different foraminiferal

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genera, such as *Bulimina*, *Haynesina*, *Nonionella*, *Nonionellina*, *Reophax*, and *Stainforthia*. Generally, not all algae are equally good chloroplast donors for foraminifera [22]. A study by Correia and Lee [6] showed that elphidia absorb up to 5 times more chloroplasts from diatoms compared to a diet with green algae. Although food uptake of *Elphidium excavatum* is strongly dependent on light exposure [26], the light / dark rhythm plays no role in the uptake of chloroplasts from the food [5].

However, currently no study has been published on the effects of light regime on the lifetime of kleptoplasts in foraminifera. In addition, this will be the first study that deals with kleptoplasts in foraminifera over such a long period of time (>100 days). To shed more light on this aspect, elphidia were incubated in complete darkness or in continuous light for several months and the chloroplasts were analyzed with transmission spectroscopy in the visible (VIS) to near infrared (NIR) spectral ranges. In this study we examined the effect of permanent light and of permanent darkness (24-h). It is well known that both conditions represent a stress factor for the foraminifera and should maximize the light-driven oxidative stress. Past studies have shown a good correlation between the spectral signature (light absorption) of foraminifera and their food source [15]. This correlation and the fact that chlorophyll (a and c) only occurs in chloroplasts [4,20] allows the investigation of the presence of intact chloroplasts (or kleptoplasts) in foraminifera through spectroscopic methods. The application of VIS spectroscopy can be used to detect chloroplasts in cells and was never applied on foraminifera for that long time.

2. Methods

2.1. Sampling and Cultivation

For the experiments, elphidia were collected from the mudflats near Dorum-Neufeld in northern Germany (53° 44′ N, 8° 31′ E, 0 m a.s.l.). Immediately after collecting the surface sediment (0.5–1 cm) sample in the mudflats in September 2019, they were wet-sieved through a 125 μm sieve with sea water from the location. Then the fraction >125 μm was placed in a cool box, filled with water from the sampling point and kept at ambient air temperature until departure in Vienna. A permanent culture (aquarium with a 2 cm sampled sediment layer covered with 15 cm natural seawater from the sampling site) was created back in the laboratory, which was operated at room temperature (20 °C) and salinity of 20. For all the spectroscopic examination, elphidia specimen were taken with a brush directly from the permanent culture (sediment), cleaned from any adhering material and placed in separate aquaria with natural, sterile filtered seawater without food and sediment. For the taxonomic identification of the individuals, a stereomicroscope (Nikon SMZ18) was used. Three yellowish (see Fig. 1 – individual in the middle, which was most common in the culture and lives, epifaunal on top of the sediment) elphidia were cultivated in the incubator (Cooled incubator ST2/Thermoschrank PolEko ST2/3+) at 20 °C and with a salinity of 20, once under continuous (24 h) light at 30 μmol photons m^{-2} s^{-1} using LED as a light source and additionally under continuous (24 h) darkness. Each individual was measured spectroscopically over a period of 113 days. The cultivation of foraminifera for this long period of time is very complex and the spectroscopic measurements require a large amount of time. For this reason, only three individuals per setup were examined. After 60 days of starvation the specimens were fed by adding a pulse of algal food source (5 ml sediment from the sampling site fraction <32 μm, which contains the natural food source). If the amount of chlorophyll inside the foraminifera increases after this algal pulse, it can be concluded, that the foraminifera is still alive and started again with food uptake in incorporation of kleptoplasts. The algae pulse was produced once from the sediment and used for all aquariums to ensure that each individual (foraminifera) was offered the same algae as a source of food.

2.2. Spectroscopic Analysis

For the Fourier Transform VIS/NIR spectroscopic measurements in transmission geometry, foraminifera had to be transferred intermittently to a small quartz glass container, which was filled with 2 ml sea water. In order to estimate the content of chlorophyll *a*, chlorophyll *c* and fucoxanthin over time, VIS/NIR absorption spectra of *E. williamsoni* individuals were measured at room temperature. The spectra were collected in the wavenumber range 22,000–8500 cm^{-1} (equivalent to wavelength range of 454–1176 nm) on a mirror-optics microscope IR-ScopeII, attached to a Bruker IFS66v/S FTIR spectrometer. A tungsten lamp was used as light source, with a Si detector and a quartz beam splitter. The measuring spot was 120–250 μm in diameter, depending on the individual sample (best fit for foraminiferal test diameter). Background spectra were measured through the quartz glass container filled with plain sea water. The spectral resolution was 40 cm^{-1} (wavenumber, equivalent to a wavelength of 250,000 nm or 250 μm) and the spectra were averaged from 512 scans. Integration of the peak areas of

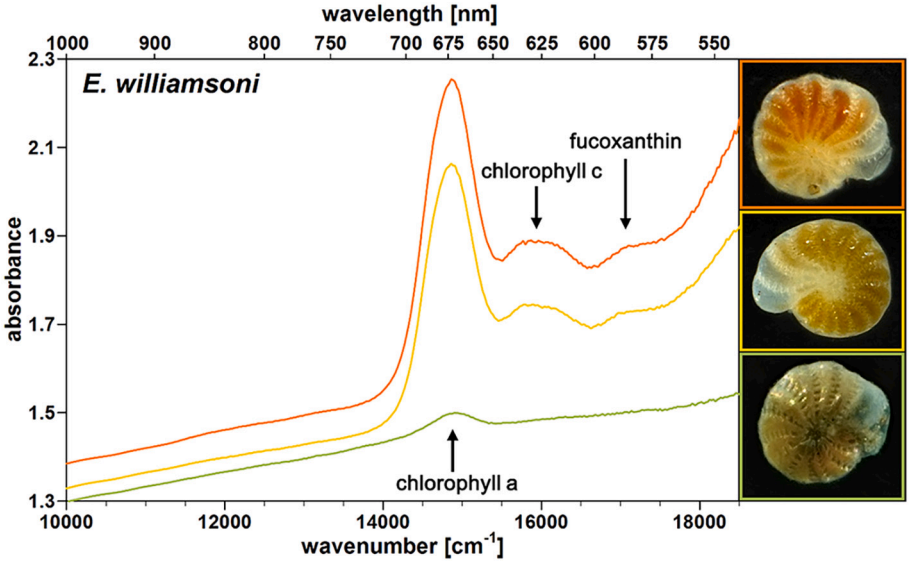

Fig. 1. VIS/NIR absorption spectra of *E. williamsoni* from different sediment layers (right insert, top and middle – individuals from the sediment surface, bottom – infaunal specimen).

chlorophyll *a*, chlorophyll *c* and fucoxanthin was performed – after a background correction accounting for the increasing absorption towards the (ultra)violet spectral region – with the program Fityk 1.3.1 [39]. The statistical evaluation (ANOVA, confidence interval = 95.0%) of changes in chlorophyll *a*nd fucoxanthin content was carried out using StatGraphics Centurion XVI.

3. Results

3.1. VIS/NIR Absorption Spectra of Foraminifera

The spectral analysis of foraminifera showed clear bands, with absorbance peaks for chlorophyll a at ~14,800 cm^{-1} (~675 nm), chlorophyll *c* at ~15,850 cm^{-1} (~630 nm) and fucoxanthin at ~17,000 cm^{-1} (~590 nm; Fig. 1). Several differently colored foraminifera of the same species (*E. williamsoni*) could be found in the untreated sediment in the aquarium in the lab. There, the sediment was not affected by bioturbation or tidal effects and the foraminifera can settle down in their preferred habitat. Those living on the sediment surface were yellow or red-orange in color and had a higher chlorophyll *a* content than infaunal (foraminifera living 1–2 cm inside the sediment of the aquarium) individuals (Fig. 1), where those with a yellow-green to brownish color containing hardly any chlorophyll *c* or fucoxanthin.

3.2. Changes in Chlorophyll Content over Time

Elphidia incubated under continuous light were designated L1-L3, elphidia that were incubated under continuous darkness were referred to as D1-D3. After each measurement, the foraminifera were placed in the middle of the aquaria. At the next measurement, the individuals were always found at a different location. Therefore, it can be assumed that the foraminifera moved during the incubation and were not in a resting phase. However, the individuals incubated in the dark showed less activity (motility). The pigment levels (chlorophyll *a*, c and fucoxanthin) changed highly significant ($p < 0.0001$) with time (all time points) which signaling, a starving process. Further, the amount of all pigments (chlorophyll *a*, c and fucoxanthin) during the experiments (all time points) where highly significant ($p < 0.0001$) different depending on light exposure (light or dark incubated individual). Multiple range tests (Fisher's LSD) show different homogeneous groups for D and L elphidia for all tested pigments. The chlorophyll *a* content of elphidia specimens L1-L3 decreased over time (Fig. 2), with the sharpest decline

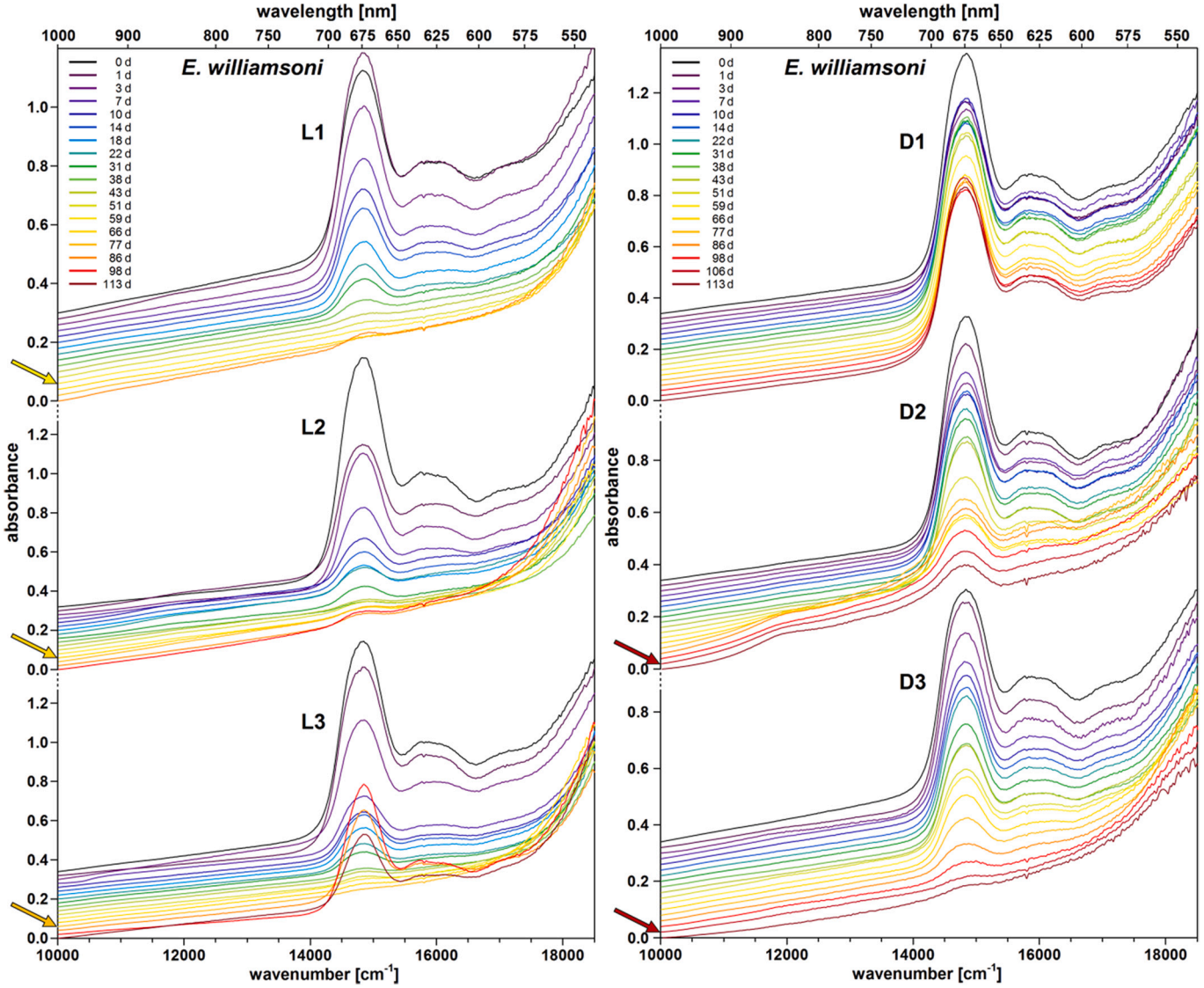

Fig. 2. VIS/NIR absorption spectra of *E. williamsoni* at different times, up to 113 days after start of the experiment. Left: L1-L3 elphidia were incubated under continuous light. Right: D1-D3 elphidia were incubated under continuous darkness. Arrows mark measurements just before an algal food source was supplemented again after the starvation period.

occurring within the first 20–30 days (Fig. 3), roughly following an exponential decay trend (results are summarized in supplementary Table S1). Between the 30th and 60th day the concentration of chlorophyll *a* was very low, but almost constant. From day 60 onwards (or day 77 for L3) the individuals were continuously fed (one algae pulse, followed by natural growth of the algae during the remaining experiment) and the content of chlorophyll increased again in two of three elphidia specimens, remarkably strong in L3 (Figs. 2 and 3). L1 and L2 have reached their "steady state" earlier than L3. This means that the chlorophyll content remained constant (based on the calculated function – red line in Fig. 3) after around 40 days (up to 60 days). At L3, this constant phase began later (around day 57). Therefore, a constant period of time (20 days) was produced in which the chlorophyll content is approximately stable. The increase of Chlorophyll a also shows that the elphidia were still alive, even after 60 days of starvation. Chlorophyll c showed a temporal trend highly similar to that of chlorophyll *a*. In contrast, fucoxanthin contents were low, decreased to negligible levels during starvation, and did not, or only marginally, increase after food alimentation. It seems that the foraminifera (L1−L3) have a different response to the algae pulse. All of the investigated individuals were alive and showed movement during the whole experiment. No foraminifera were damaged during the experiment, so the test always remained intact. It appears that the response of foraminifera to permanent light is highly individual and depends on several factors that were not examined in this study. In more detail, the response to starving during darkness seems much more individual than the response under continuous illumination.

Elphidia incubated under continuous darkness (D1-D3) showed a different trend from those under continuous light. Specimen D1 showed only very slight negative changes in chlorophyll concentrations and none in fucoxanthin over the entire duration of the experiment (113 days) (Fig. 4). Specimens D2 and D3, on the other hand, showed a curvilinear yet slower decrease in the photosynthetic pigments compared to L1-L3, and fucoxanthin decreased to reach negligible levels at the end of the incubation period. The pattern of decreases in chlorophyll *a* and chlorophyll *c* were again in parallel, and also follow those of fucoxanthin. However, these differences between D1 and the other foraminifera (D2, D3) are not obviously recognizable. All three individuals appeared healthy throughout the experiment and were moving about within the aquaria. After 105 days, the aquariums of individuals D2 and D3 were provided with an algae pulse in order to offer the foraminifera new food. This was not done with D1 because the concentration of chlorophyll was still very high. After feeding, the decrease of chlorophyll stopped by D2 and by D3 the chlorophyll content even increased again, which means that these organisms were still alive.

4. Discussion

4.1. Differences in Coloration of Foraminifera

In our study, foraminifera of the same species and collected at the same time, showed different color variations of the cytoplasm (Fig. 1). Similar examples have already been recorded in the foraminifer *Nonionella labradorica,* which also have kleptoplasts [3]. Cedhagen [3] examined the seasonal changes in coloration on *N. labradorica* and recognized that foraminifera collected in February were yellow and those from March or April were greenish. He hypotheses that this was due to different food sources. However, our foraminifera were all sampled at the same time and still showed a color difference. As mentioned in the method part, the used foraminifera for the time series had all the same color to avoid differences at the beginning of the observation. A possible explanation for this observation could be differences in the choice of the food source in elphidia collected from the sediment surface and in infaunal specimens. Past studies have shown that the chlorophyll content in foraminifera can vary between 27 and 0.6 ng cell^{-1}, depending on the size of the individual and the food uptake activity shortly before the examination [19]. In order to minimize this effect in our experiments, only foraminifera that were

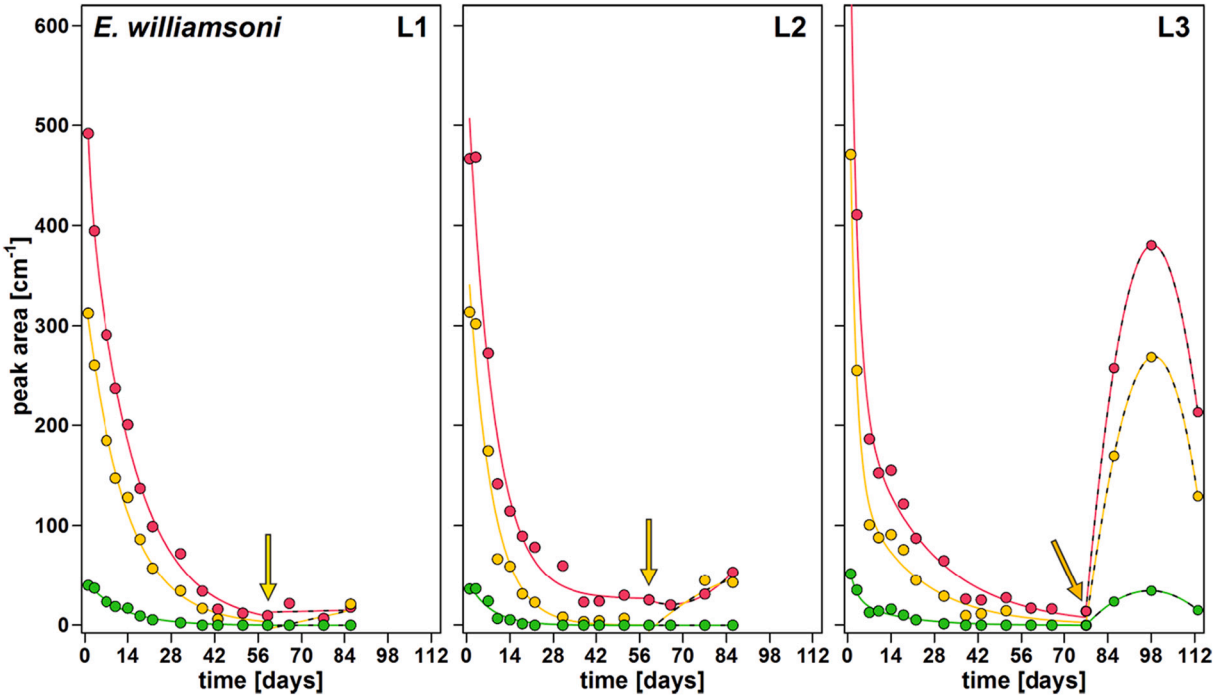

Fig. 3. Changes in the content (absorption peak area) of chlorophyll *a*, chlorophyll *c* and fucoxanthin in *E. williamsoni* individuals under continuous light (specimens L1-L3) over 86 days (113 in L3) of time. Day 0–60 (77 in L3) was the starvation period; an algal food source was supplemented thereafter. Time kinetics during starvation were fitted to exponential decay regressions. Red symbols and lines indicate peak areas of chlorophyll *a*, orange one's chlorophyll *c*, and green ones fucoxanthin. Arrows (color-coded as in Fig. 2) mark measurements just before an algal food source was supplemented; respective dashed lines serve as a guide to the eye for trends after re-feeding. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

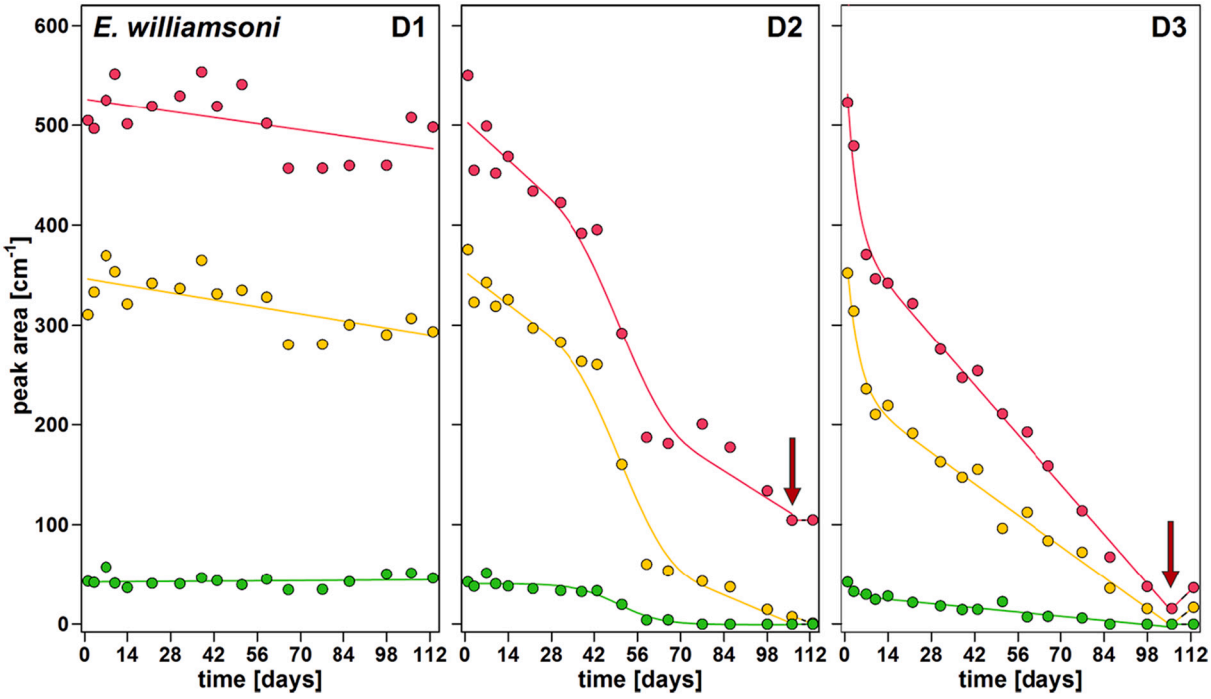

Fig. 4. Changes in the content (absorption peak area) of chlorophyll *a*, chlorophyll *c* and fucoxanthin in *E. williamsoni* individuals under continuous darkness (specimens D1-D3) over 113 days of time. Day 0–106 was the starvation period in D2 and D3; an algal food source was supplemented thereafter. Time kinetics were fitted to linear (D1), combined linear+sigmoidal (D2), or combined linear+exponential decay (D3) regressions. Red symbols and lines indicate peak areas of chlorophyll *a*, orange one's chlorophyll *c*, and green ones fucoxanthin. Arrows (color-coded as in Fig. 2) mark measurements just before an algal food source was supplemented; respective dashed lines serve as a guide to the eye for trends after re-feeding. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

approximately the same size order (150 μm) were used. Our results showed a variation in the chlorophyll content of the foraminifera. This can be attributed to the lifestyle of the foraminifera (epifaunal or infaunal) or the degradation of the food source (on or in the sediment, see Fig. 1). One possible explanation is that foraminifera in their different habitats feed on phytodetritus of varying decomposition stage, which will lead to different concentrations of chlorophyll in the foraminifera. Knight and Mantoura [19] also observed that *E. oceanens* tends to accumulate carotenoids from the food source in their cytoplasm. The different levels of carotenoids in the cytoplasm of *E. williamsoni* could also explain the color variations in the foraminifera as examined here.

4.2. Chlorophyll Degradation in E. williamsoni

Foraminifera in this study were examined using Fourier Transform VIS/NIR absorption spectroscopy to detect changes in the relative amount of chlorophyll in the foraminifera upon starvation, food amendment, and investigate the effect of light regime on plastid integrity. Measuring of the spectral absorption is a useful tool for monitoring changes in the chlorophyll *a* content of invertebrate / algal symbiotic associations [33] and based on our study it seems to be also very successful in protists. Chlorophyll a is a molecule that can only be found in chloroplasts, i.e., kleptoplasts, in foraminifera [4,20]. This means that the number or content of kleptoplasts in foraminiferal cells can be assumed based on the chlorophyll *a* content in the cells. It should be noted, however, that foraminifera get a greenish color in laboratory feeding experiments, when they are fed with fresh green algae [14]. After a few days they lose this color, due to the normal metabolic degradation of their food source [14]. To make a link between the degradation of kleptoplasts and chlorophyll with time, the experiments should take place under starving conditions, like in our experiments, which means that there is no new input of chloroplasts.

Our results thus showed that the chlorophyll content decreased faster and more rapidly over time when foraminifera were permanently exposed to light (see Fig. 2). Jauffrais et al. [15] used pulse amplitude modulated (PAM) fluorometry to investigate the activity of kleptoplasts in *Haynesina germanica* at different light intensities. On the basis of their PAM observations over seven days, they estimated that the kleptoplasts remained functional under high light (70 μmol photons m^{-2} s^{-1}) for about 7–8 days and in the dark, this extended to 11–21 days [15]. The results of our study showed that the chloroplasts were still present in *E. williamsoni* after 113 days under dark conditions (even in one of three replicates). However, in individuals under continuous light (30 μmol photons m^{-2} s^{-1}) the chlorophyll content and therefore chloroplast numbers rapidly decreased during the first 30 days and then more slowly. We hypothesize that under normal daylight conditions in summer (light: 16:8 h day:night) the chloroplasts remain intact for longer time periods in the foraminifera, based on the assumption that continuous illumination (exposed time) and not the irradiance supply (amount of photons) increased chlorophyll degradation.

The degradation of chlorophyll by light is a well-known phenomenon in botany and is also linked to photoinhibition (e.g., [41]). In general, a distinction can be made between irreversible photobleaching and reversible photoinhibition. A reverse photoinhibition means a decrease of photosynthetic activity due to a reversible energy-dissipating mechanisms, without involving real damage to PSII [2]. Biochemically, this photoinhibitory effect is triggered by the degradation of a PS II reaction center protein (D1 protein) and thus the electron transport chain becomes interrupted [12]. In our experiments, photoinhibition may leads to a permanent degradation of chloroplasts (kleptoplasts), due to the fact, that the chloroplasts are separated from their nucleus. Usually repair proteins are enclosed in the nucleus and then imported to organelles (e.g., [35]). Vieira et al. [37] reported, that the kleptoplasts in the mollusk *Elysia viridis* react different during starving under low light (30 μmol photons m^{-2} s^{-1}) and high light (140 μmol photons m^{-2} s^{-1}) conditions. They reported a rapid, exponential decrease of the

functionality of their kleptoplasts during high light and a lower decrease during low light. This exponential decay of the photoactive compounds can be explained by the absence of D1 protein repair capacity [37]. During our experiments we observed most probably the same correlation between decrease of kleptoplasts functionality and absence of D1 protein, which indicates that this mechanism could be present in all kleptoplasts bearing organisms.

A very high light intensity is necessary for photobleaching. Light intensities between 390 and 2100 μmol photons m^{-2} s^{-1} can be measured in the surface Baltic Sea [38]. Measured as maximum oxygen production rate, diatoms, naturally occurring in the Baltic Sea and therefore suited as food source and chloroplast donor for *Elphidium*, show light saturation of photosynthesis at 150 μmol photons m^{-2} s^{-1} [38]. Considering the rather low light intensity in our experiments (30 μmol photons m^{-2} s^{-1}), it is likely that the kleptoplasts can handle higher light intensities and therefore photobleaching is not the case. One aspect worth considering, however, is that our foraminifera were incubated without any sediment, which could dim the light. A further decrease of light in the natural environment comes with increasing water depth. At the sampling site, the water depth was 14 m. Previous investigations showed that only about 0.05% of the light intensity that can be measured on the water surface arrives at a depth of 15 m [29]. As a result, the light factor plays an important role in the degradation of chlorophyll in foraminifera, when adapted to low light environments, even with photobleaching requiring higher irradiance values. The second effect that could provide a possible explanation for the degradation of the chloroplasts is reversible photoinhibition. However, this reversible photoinhibition is associated with the operation of the xanthophyll cycle, which is still not investigated in kloroplasts of foraminifera. A study by Jauffrais et al. [17] showed that the photosynthetic activity of *E. williamsoni* is not inhibited by an increase in light intensity from 100 to 600 μmol photons m^{-2} s^{-1}, but decreases with the duration of the starving time. This observation corresponds to our results. Another observation made with *E. williamsoni* is the color change of cytoplasm when changing from light exposed to dark conditions. This process is reproducible and can be observed for at least several days. However, in order to discuss this aspect in more detail, further investigations using PAM, spectroscopy or transmission electron microscopy (TEM) will be needed.

Based on our study, the effect of light on kleptoplasts in combination with starving of foraminifera has different impacts on their metabolism. A study by Jauffrais et al. [17] described an increase in lipid droplets in *E. williamsoni* with longer starvation periods. Combining this with results of our study, where the decrease in chloroplast numbers also correlates with time, two different mechanisms can be deduced. First, the kleptoplasts may have a time-limited 'expiry date' that is reached sooner under excessive light than in the dark. The increased or continuous light exposure can damage the kleptoplasts, which are thus broken down and digested by the foraminifera. Although this is only an assumption, since in our study only the degradation of chloroplasts was observed and not their uptake by the foraminifera themselves. Further studies on this topic are necessary to clarify this aspect in detail. Concerning the study by Lopez [28], which showed that foraminifera have to ingest a certain number of chloroplasts per hour in order to keep the number of chloroplasts constant, our study showed once again that foraminifera degrade their damaged and/or aged chloroplasts over time. The second aspect could be related to the photosynthetic products. During the exposure to light, the chloroplasts produce photosynthates and other assimilates that foraminifera could possibly use as a source of food. If the chloroplasts become saturated with its products, the excessive assimilates may be transported outwards into the cytoplasm of the foraminifera across the symbiosome membrane. TEM observations, where increased lipid droplets were found in chloroplasts or in their surroundings during light exposure, confirm this theory [24]. As soon as the chloroplast becomes saturated with these lipid droplets it may be broken down and digested by the foraminifera. Since the saturation of lipids in chloroplasts takes place faster under light exposed conditions than in the dark this could possibly explain the more rapid degradation of chloroplasts in the light exposed environments (Fig. 2), whereas in the experiments in the dark the chloroplasts are more likely to be consumed after natural aging via digestion processes [28]. The exact function of kleproplasts in foraminifera is not exactly clarified at the moment. Currently it can be assumed, that kleptoplasts bring an advantage for the foraminifera, especially in anoxic sediments [11], but can also be used as an additional carbon source like discussed in this study.

5. Conclusion

In our study, kleptoplasts were observed in foraminifera over a long period of 113 days for the first time. It turned out that VIS spectroscopy is an efficient tool to check the activity of foraminifera regarding on the presence of their kleptoplasts. Based on this study, additional environmental parameters can now be tested to check how the kleptoplasts in the foraminifera react. Further, our results show that the degradation of chlorophyll in *E. williamsoni* clearly reacts to the presence of light. Under permanent light, after a relatively short time (20–30 days), the chlorophyll *a*nd thus the chloroplasts in the cells are degraded. In total darkness this effect occurs more slowly. However, chlorophyll can still be found in *E. williamsoni* cells after 113 days. In the natural environment, several color variants of a single species can be found, which is probably a consequence of differences in food source uptake or of the uptake of food in different degradation states. Our measurements showed that the chlorophyll content in the foraminiferal cells can *v*ary with the color. The color of foraminifera my reflect several factors like algal food source, degradation stage of incorporated chloroplasts or the amount of accumulated carotenoids in foraminiferal cytoplasm.

Declaration of Competing Interest

None

Data availability

The data that has been used is confidential.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jphotobiol.2022.112623.

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