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Effect of light on photosynthetic efficiency of sequestered chloroplasts in intertidal benthic foraminifera (*Haynesina germanica* and *Ammonia tepida*)

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Abstract. Some benthic foraminifera have the ability to incorporate functional chloroplasts from diatoms (kleptoplasty). Our objective was to investigate chloroplast functionality of two benthic foraminifera (*Haynesina germanica* and *Ammonia tepida*) exposed to different irradiance levels (0, 25, 70 µmol photon m^{-2} s^{-1}) using spectral reflectance, epifluorescence observations, oxygen evolution and pulse amplitude modulated (PAM) fluorometry (maximum photosystem II quantum efficiency (Fv/Fm) and rapid light curves (RLC)). Our results clearly showed that *H. germanica* was capable of using its kleptoplasts for more than 1 week while *A. tepida* showed very limited kleptoplastic ability with maximum photosystem II quantum efficiency (Fv/Fm = 0.4), much lower than *H. germanica* and decreasing to zero in only 1 day. Only *H. germanica* showed net oxygen production with a compensation point at 24 µmol photon m^{-2} s^{-1} and a production up to 1000 pmol O_2 $cell^{-1}$ day^{-1} at 300 µmol photon m^{-2} s^{-1}. *Haynesina germanica* Fv/Fm slowly decreased from 0.65 to 0.55 in 7 days when kept in darkness; however, it quickly decreased to 0.2 under high light. Kleptoplast functional time was thus estimated between 11 and 21 days in darkness and between 7 and 8 days at high light. These results emphasize that studies about foraminifera kleptoplasty must take into account light history. Additionally, this study showed that the kleptoplasts are unlikely to be completely functional, thus requiring continuous chloroplast resupply from foraminifera food source. The advantages of keeping functional chloroplasts are discussed but more information is needed to better understand foraminifera feeding strategies.

1 Introduction

Benthic foraminifera colonize a wide variety of sediments from brackish waters to deep-sea environments and can be the dominant meiofauna in these ecosystems (Gooday, 1986; Pascal et al., 2009). They may play a relevant role in the carbon cycle in sediments from deep sea (Moodley et al., 2002) to brackish environments (Thibault de Chanvalon et al., 2015). Their minor role in organic carbon cycling in aerobic sediments, compared to bacteria, contrasts with their strong contribution to anaerobic organic matter mineralisation (Geslin et al., 2011) and they can be responsible for up to 80 % of benthic denitrification (Pina-Ochoa et al., 2010; Risgaard-Petersen et al., 2006).

Some benthic foraminiferal species are known to sequester chloroplasts from their food source and store them in their

cytoplasm (Lopez, 1979; Bernhard and Bowser, 1999) in a process known as kleptoplasty (Clark et al., 1990). A kleptoplast is thus a chloroplast, functional or not, that was "stolen" and integrated by an organism. Kleptoplastic foraminifera are found in intertidal sediments (e.g. *Haynesina*, *Elphidium* and *Xiphophaga*) (Lopez, 1979; Correia and Lee, 2000, 2002a, b; Goldstein et al., 2010; Pillet et al., 2011), low oxygenated aphotic environments (*Nonionella*, *Nonionellina*, *Stainforthia*) (Bernhard and Bowser, 1999; Grzymski et al., 2002) and shallow-water sediments (*Bulimina elegantissima*) (Bernhard and Bowser, 1999). The role of chloroplasts sequestered by benthic foraminifera is poorly known and photosynthetic functions have only been studied in a few mudflat species (*Elphidium williamsoni*, *Elphidium excavatum* and *Haynesina germanica*) (Lopez, 1979; Correia and Lee, 2000, 2002a, b; F. Cesbron, personal communication, 2015). Amongst the deep-sea benthic foraminifer living in the aphotic zone, only *Nonionella stella* has been studied (Grzymski et al., 2002). The authors suggest that the sequestered chloroplasts in this species may play a role in the assimilation of inorganic nitrogen, even when light is absent. It has also been hypothesised that chloroplast retention may play a major role in foraminiferal survival when facing starvation periods or in anoxic environments (F. Cesbron, personal communication, 2015). Under these conditions, kleptoplasts could potentially be used as a carbohydrate source, and participate in inorganic nitrogen assimilation (Falkowski and Raven, 2007) or, when exposed to light, to produce oxygen needed in foraminiferal aerobic respiration (Lopez, 1979).

Foraminifera pigment and plastid ultrastructure studies have shown that the chloroplasts are sequestered from their food source, i.e. mainly from diatoms (Lopez, 1979; Knight and Mantoura, 1985; Grzymski et al., 2002; Goldstein, 2004). This was confirmed by experimental feeding studies (Correia and Lee, 2002a; Austin et al., 2005) and by molecular analysis of kleptoplastic foraminifera from different environments (Pillet et al., 2011; Tsuchiya et al., 2015). Foraminifera from intertidal mudflat environments (e.g. *H. germanica*, *A. tepida*) feed mostly on pennate diatoms (Pillet et al., 2011) which are the dominant microalgae in intertidal mudflat sediments (MacIntyre et al., 1996; Jesus et al., 2009). Furthermore, in these transitional coastal environments (e.g. estuaries, bays, lagoons) *A. tepida* and *H. germanica* are usually the dominant meiofauna species in West Atlantic French coast mudflats (Debenay et al., 2000, 2006; Morvan et al., 2006; Bouchet et al., 2009; Pascal et al., 2009; Thibault de Chanvalon et al., 2015). Their vertical distribution in the sediment is characterised by a clear maximum density at the surface (Alve and Murray, 2001; Bouchet et al., 2009; Thibault de Chanvalon et al., 2015) with access to light, followed by a sharp decrease in the next two centimetres (Thibault de Chanvalon et al., 2015).

Foraminiferal kleptoplast retention times can vary from days to months (Lopez, 1979; Lee et al., 1988; Correia and Lee, 2002b; Grzymski et al., 2002). The source of this variation is poorly known but longer kleptoplast retention times were found in dark treatments (Lopez, 1979; Correia and Lee, 2002b), thus suggesting an effect of light exposure, similar to what is observed in kleptoplastic sacoglossans (Trench et al., 1972; Clark et al., 1990; Evertsen et al., 2007; Vieira et al., 2009), possibly related to the absence of some components of the kleptoplast photosynthetic protein complexes in the host (Eberhard et al., 2008).

Most recent studies on kleptoplastic foraminifera focused on feeding, genetics and microscopic observation related to chloroplast acquisition (e.g., Austin et al., 2005; Pillet et al., 2011; Pillet and Pawlowski, 2013). To our knowledge little is known about the effects of abiotic factors on photosynthetic efficiency of sequestered chloroplasts in benthic foraminifera, particularly on the effect of light intensity on kleptoplast functionality. Non-invasive techniques are ideal to follow photosynthesis and some have already been used to study foraminifera respiration and photosynthesis, e.g. oxygen evolution by microelectrodes (Rink et al., 1998; Geslin et al., 2011) or ^{14}C radiotracer (Lopez, 1979). Recently, pulse amplitude modulated (PAM) fluorometry has been used extensively in the study of kleptoplastic sacoglossans (Vieira et al., 2009; Costa et al., 2012; Jesus et al., 2010; Serodio et al., 2010; Curtis et al., 2013; Ventura et al., 2013). This non-invasive technique has the advantage of estimating relative electron transport rates (rETR) using rapid light curves (RLC) and photosystem II (PSII) maximum quantum efficiencies (Fv/Fm) very quickly and without incubation periods. The latter parameter has been shown to be a good parameter to estimate PSII functionality (e.g. Vieira et al., 2009; Jesus et al., 2010; Serodio et al., 2010; Costa et al., 2012; Curtis et al., 2013; Ventura et al., 2013).

The objective of the current work was to investigate the effect of irradiance levels on photosynthetic efficiency and chloroplast functional times of two benthic foraminifera feeding in the same brackish areas, *H. germanica*, which is known to sequester chloroplasts and *A. tepida*, not known to sequester chloroplasts. These two species were exposed to different irradiance levels during 1 week and chloroplast efficiency was measured using epifluorescence, oxygen microsensors and PAM fluorometry.

2 Materials and methods

2.1 Sampling

Haynesina germanica and *A. tepida* were sampled in January 2015 in Bourgneuf Bay (47.013° N, 2.019° W), a coastal bay with a large mudflat situated south of the Loire estuary on the French west coast. In this area, all specimens of *A. tepida* belong to genotype T6 of Hayward et al. (2004) (M. Schweizer, personal communication, 2015). In the field, a large amount ($\sim$ 20 kg) of the upper sediment layer (roughly first 5 mm) was sampled and sieved over 300 and 150 µm meshes us-

ing in situ sea water. The 150 µm fraction was collected in dark flasks and maintained overnight in the dark at 18 °C in the laboratory. No additional food was added. In the following day, sediment with foraminifera was diluted with filtered (GF/C, 1.2 µm, Whatman) autoclaved sea-water (temperature: 18 °C and salinity: 32) and *H. germanica* and *A. tepida* in healthy conditions (i.e. with cytoplasm inside the test) were collected with a brush using a stereomicroscope (Leica MZ 12.5). The selected specimens were rinsed several times using Bourgneuf bay filtered-autoclaved seawater to minimize bacterial and microalgal contamination.

2.2 Size and biovolume determination

Foraminifera test mean maximal elongation (µm, the length of the axes going from the last chamber to the other side of the test and passing by the umbilicus) was measured using a micrometre mounted on a Leica stereomicroscope (MZ 12.5). Mean foraminiferal volume was approximated with the equation of a half sphere, which is the best resembling geometric shape for *H. germanica* and *A. tepida* (Geslin et al., 2011). The cytoplasmic volume (or biovolume) was then estimated by assuming that the internal test volume corresponds to 75 % of the total foraminiferal test volume (Hannah et al., 1994).

2.3 Spectral reflectance

Pigment spectral reflectance was measured non-invasively to determine and compare the relative pigment composition on 50 fresh specimens of *H. germanica*, on 50 fresh specimens of *A. tepida* and on a benthic diatom as explained in Jesus et al. (2008). Concisely, a USB2000 (Ocean Optics, Dunedin, FL, USA) spectroradiometer with a VIS-NIR optical configuration controlled by OObase32 software (Ocean Optics B.V., Duiven, the Netherlands) was used. The spectroradiometer sensor was positioned so that the surface was always viewed from the nadir position. Foraminiferal reflectance spectra were calculated by dividing the upwelling spectral radiance from the foraminifera (Lu) by the reflectance of a clean polystyrene plate (Ld) for both of which the machine dark noise (Dn) was subtracted (Eq. 1).

$$\rho = \frac{(\mathrm{Lu} - \mathrm{Dn})}{(\mathrm{Ld} - \mathrm{Dn})} \quad (1)$$

2.4 Image analysis

Foraminifera kleptoplast fluorescence was measured using epifluorescence microscopy (× 200, Olympus Ax70 with Olympus U-RFL-T, excitation wave length 485 nm). Two Tif images (1232 × 964 px) of each foraminifer were taken (one bright field photography and one epifluorescence photography) using LUCIA G™ software. The bright field photography was used to trace the contours of the foraminifer and an ImageJ macro was used to extract the mean pixel values of the corresponding epifluorescence photography. Higher mean pixel values corresponded to foraminifera emitting more fluorescence and thus, as a proxy, contain more chlorophyll. In an RGB image each channel contains pixels between 0 and 255 values. The majority of the information regarding chlorophyll fluorescence is encoded in the red channel, therefore the green and blue channel were discarded and only the red channel was kept. The images from the different treatments were directly comparable as all images were taken using the same acquisition settings. Thus, the mean red pixel values were used as a proxy for chlorophyll fluorescence.

2.5 Oxygen measurements

Oxygen was measured using advanced Clark type oxygen microelectrodes of 50 µm in diameter (Revsbech, 1989) (OXI50 – Unisense, Denmark). Electrodes were calibrated with a solution of sodium ascorbate at 0.1 M (0 %) and with seawater saturated with oxygen by bubbling air (100 %). Foraminiferal photosynthesis and oxygen respiration rates were measured following Høgslund et al. (2008) and Geslin et al. (2011). Measurements were carried out in a micro-tube made from glass Pasteur pipette tips with an inner diameter of 1 mm. The micro-tube was fixed to a small vial, filled with filtered autoclaved seawater from Bourgneuf Bay. The vial was placed in an aquarium with water kept at room temperature (18 °C). A small brush was used to position a pool of 7 to 10 foraminifera in the glass micro-tube after removing air bubbles. Oxygen micro-profiles started at a distance of 200 µm above the foraminifers to avoid oxygen turbulences often observed around the foraminifers. Measurements were registered when the oxygen micro-profiles were stable; they were then repeated five time in the centre of the micro-tube, using 50 µm steps until 1000 µm away from the foraminifers (Geslin et al., 2011). The oxygen flux (J) was calculated using the first law of Fick:

$$J = -D \times \frac{\mathrm{d}C}{\mathrm{d}x}, \quad (2)$$

where D is the oxygen diffusion coefficient ($\mathrm{cm^2\,s^{-1}}$) at experimental temperature (18 °C) and salinity (32) (Li and Gregory, 1974), and $\mathrm{d}C/\mathrm{d}x$ is the oxygen concentration gradient ($\mathrm{pmol\,O_2\,cm^{-1}}$). The O_2 concentration gradients were calculated with the oxygen profiles and using the R^2 of the regression line to determine the best gradient. Total O_2 consumption and production rates were calculated as the product of O_2 fluxes by the surface area of the micro-tube and subsequently divided by the foraminifera number to finally obtain the cell specific rate ($\mathrm{pmol\,O_2\,cell^{-1}\,d^{-1}}$) (Geslin et al., 2011).

2.6 Fluorescence

All pulse amplitude modulated fluorescence measurements were carried out with a Water PAM fluorometer (Walz, Ger-

many) using a blue measuring light. Chloroplast functionality was estimated by monitoring PSII maximum quantum efficiency (Fv/Fm) and by using *P-I* rapid light curve (RLC, e.g., Perkins et al., 2006) parameters (α, initial slope of the RLC at limiting irradiance; rETRmax, maximum relative electron transport rate; Ek, light saturation coefficient; and Eopt, optimum light) (Platt et al., 1980). Rapid light curves were constructed using eight incremental light steps (0, 4, 15, 20, 36, 48, 64, 90 and 128 µmol photons m^{-2} s^{-1}), each lasting 30 s. The PAM probe was set up on a stand holder at a 2 mm distance from a group of 10 foraminifera.

2.7 Experimental design

Haynesina germanica, a species known to sequester chloroplasts, were placed in plastic Petri dishes and starved for 7 days under three different light conditions: dark (D and Dark-RLC), low light (LL, 25 µmol photons m^{-2} s^{-1}) and high light (HL, 70 µmol photons m^{-2} s^{-1}); whereas for comparison, *A. tepida*, a foraminifer not known to sequester chloroplasts was starved but only exposed to the dark condition. A short-term experiment was thus carried out (7 days) to study the effect of light on healthy specimens rather than the effect of starvation. For each condition, 10 specimens were used per replicate and three replicates per light treatment; furthermore all plastic Petri dishes were filled with Bourgneuf bay filtered-autoclaved seawater. This experiment was carried out in a thermo-regulated culture room at 18 °C, equipped with cool light fluorescent lamp (Lumix day light, L30W/865, Osram) and using a 14:10 h (Light : Dark) photoperiod. The distances between the light and the experimental conditions were assessed using a light-metre and a quantum sensor (ULM-500 and MQS-B of Walz) to obtain the desirable light intensities. Concerning the dark condition, the Petri dishes were placed in a box covered with aluminium foil.

Haynesina germanica kleptoplast fluorescence was measured using epifluorescence microscopy, as explained above, before and after the different light treatments. At the beginning of the experiment it was done on 30 independent specimens to assess the natural and initial variation of *Haynesina germanica* kleptoplast fluorescence. At the end of the experiment, the measurements were done on all foraminifera exposed to the different light conditions (a total of 30 specimens per condition). This was also measured on *A. tepida*, but results are not presented because no chlorophyll fluorescence was observed at the end of the experiment.

Haynesina germanica and *A. tepida* oxygen production and consumption were measured at the beginning of the experiment on three independent replicates with seven specimens in each replicate. Six different light steps were used to measure O_2 production (0, 25, 50, 100, 200 and 300 µmol photons m^{-2} s^{-1}) for *H. germanica* and only two light steps (0 and 300 µmol photons m^{-2} s^{-1}) for *A. tepida*. Photosynthetic activity (P) data of *H. germanica* were fitted with a Haldane model, as modified by Papacek et al. (2010) and Marchetti et al. (2013) but without photoinhibition (Eq. 3).

$$P(I) = \frac{\mathrm{Pm} \times I}{I + \mathrm{Ek}} - \mathrm{Rd}, \tag{3}$$

where Pm is the maximum photosynthetic capacity (pmol O_2 $cell^{-1}$ d^{-1}), I the photon flux density (µmol photons m^{-2} s^{-1}), Ek the half-saturation constant (µmol photons m^{-2} s^{-1}) and Rd the dark respiration, expressed as an oxygen consumption (pmol O_2 $cell^{-1}$ d^{-1}). The initial slope of the *P-I* (Photosynthesis–Irradiance) curve at limiting irradiance α (pmol O_2 $cell^{-1}$ day^{-1} (µmol photons m^{-2} s^{-1})$^{-1}$) and the compensation irradiance Ic were calculated according to Eqs. (4) and (5).

$$\mathrm{Ic} = \frac{\mathrm{Ek} \times \mathrm{Rd}}{\mathrm{Pm} - \mathrm{Rd}} \tag{4}$$

$$\alpha = \frac{\mathrm{Rd}}{\mathrm{Ic}} \tag{5}$$

Oxygen measurements were repeated at 300 µmol photons m^{-2} s^{-1} and in the dark at the end of the experiment (7 days of incubation) for all different light treatments (D, LL, HL) using 10 specimens, to assess their production or consumption of oxygen at these two light levels (300 µmol photons m^{-2} s^{-1} and in the dark) in all treatments.

For all conditions (D, LL, HL and Dark-RLC) Fv/Fm was measured daily at early afternoon, after a 1-hour dark adaptation period and measurements were done in triplicate for each Petri dish.

Rapid light curves were also carried out in all light treatments at the beginning and end of the experiment, after 1-hour dark adaptation for the two tested species. Additionally, RLC were carried out daily in an extra triplicate kept in the dark (Dark-RLC) throughout the duration of the experiment.

2.8 Statistical analysis

Data are expressed as mean ± standard deviation (SD) when $n = 3$ or standard error (SE) when $n = 30$. Statistical analyses consisted of a t test to compare the foraminifera test mean maximal elongation, a non parametric test (Kruskal Wallis) to compare the mean chlorophyll fluorescence of the foraminifera exposed to the different experimental conditions and a multifactor (experimental conditions (D, LL, HL), irradiance (0–300 µmol photons m^{-2} s^{-1})) analysis of variance (ANOVA) with a Fisher's LSD test to compare the respiration rates at the end of the experiment. Differences were considered significant at $p < 0.05$. Statistical analyses were carried out using the Statgraphics Centurion XV.I (StatPoint Technologies, Inc.) software.

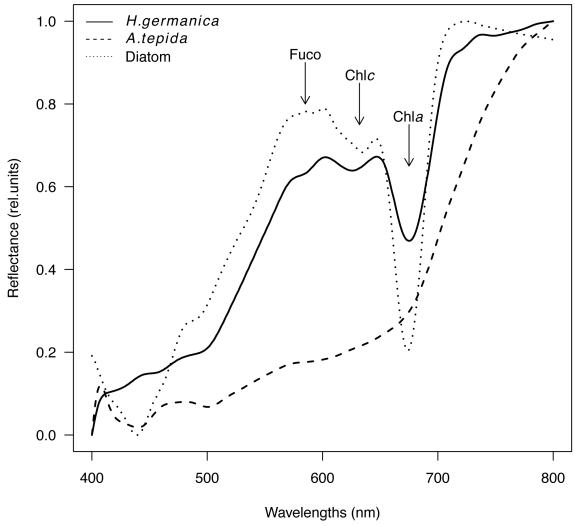

Figure 1. Spectral reflectance signatures of *Haynesina germanica*, *Ammonia tepida* and of a benthic diatom in relative units (x axis legend: Wavelength (nm)).

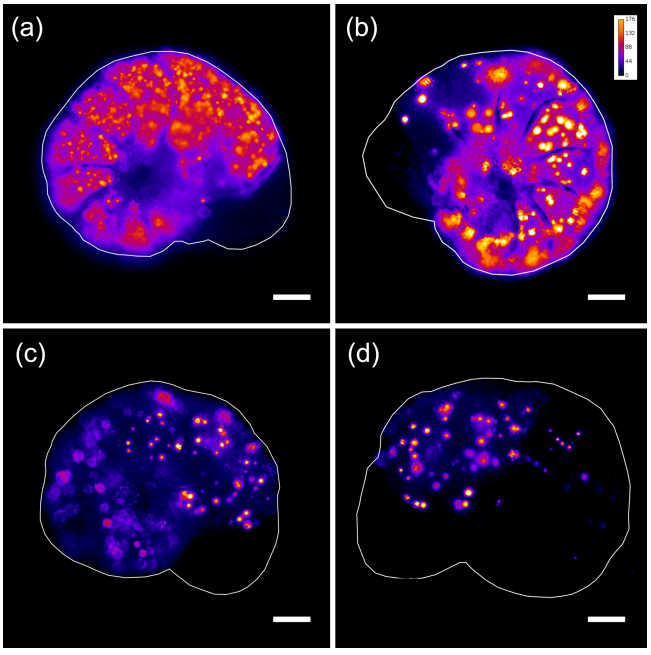

Figure 2. Illustration of *Haynesina germanica* chloroplast content at the beginning **(a)** and at the end of the experiment for the three experimental conditions, Dark **(b)**, Low Light **(c)** and High Light **(d)**. Higher colour scale values correspond to foraminifera emitting more fluorescence and likely containing more chlorophyll *a*; fluorescence in pixel values between 0 and 255, (scale bar = 50 µm).

3 Results

3.1 Size and biovolume

Ammonia tepida specimens were larger than *H. germanica* with a mean maximal elongation of 390 ± 42 µm (SD, $n = 34$) and 366 ± 45 µm (SD, $n = 122$), respectively ($p < 0.01$, $F_{121,33} = 1.15$). This resulted in cytoplasmic biovolumes equal to $1.20 \times 10^7 \pm 3.9 \times 10^6$ µm^3 (SD) and $1.01 \times 10^7 \pm 3.65 \times 10^6$ µm^3 (SD), respectively.

3.2 Chloroplast functionality

Fresh *Haynesina germanica* and *A. tepida* showed very different spectral reflectance signatures (Fig. 1). *Haynesina germanica* showed a typical diatom spectral signature with high reflectance in the infrared region (> 740 nm) and clear absorption features around 585, 630 and 675 nm; the absorption feature around 675 nm corresponds to the presence of chlorophyll *a*; the 585 nm feature is the result of fucoxanthin and the 630 nm absorption feature is the result of chlorophyll *c* (arrows, Fig. 1). *Ammonia tepida* showed no obvious pigment absorption features apart from 430 nm (Fig. 1).

Epifluorescence images showed a clear effect of the different light treatments (Dark, Low Light, Hight Light) on *H. germanica* chlorophyll fluorescence (Fig. 2). Visual observations showed a clear decrease in chlorophyll fluorescence for the LL and HL treatments from the beginning of the experiment (Fig. 2a) to the end of a 7-day period of light exposure (Fig. 2c and d, respectively). Samples kept in the dark did not show an obvious decrease but showed a more patchy distribution compared to the beginning of the experiment (Fig. 2b). This was confirmed by a non-parametric test (Kruskal Wallis) showing that the differences in chlorophyll *a* fluorescence were significant ($p < 0.01$, Df = 3, Fig. 3). It is also noteworthy to mention that there was a large individual variability within each treatment leading to large standard errors in spite of the number of replicates ($n = 30$).

Oxygen measurements carried out at the beginning of the experiment (T0) differed considerably between the two species. *Ammonia tepida* did not show any net oxygen production although respiration rates measured at 300 µmol photons m^{-2} s^{-1} were lower (2485 ± 245 pmol O_2 cell^{-1} d^{-1}) than the ones measured in the dark (3531 ± 128 pmol O_2 cell^{-1} d^{-1}) ($F_{2,2} = 3.7$, $p = 0.02$). *Haynesina germanica* showed lower dark respiration rates (1654 ± 785 pmol O_2 cell^{-1} d^{-1}) and oxygen production quickly increased with irradiance, showing no evidence of photoinhibition within the light range used (Fig. 4). Compensation irradiance (Ic) was reached very quickly, as low as 24 µmol photons m^{-2} s^{-1} (95 % coefficient bound: 17–30 µmol photons m^{-2} s^{-1}, values calculated from the fitted model Eq. 4) and the half-saturation constant (Ek) was also reached at very low light levels, i.e. at 17 µmol photons m^{-2} s^{-1}. No photoinhibition was observed under the experimental light conditions (0 to 300 µmol photons m^{-2} s^{-1}), which resulted in an estimation of ~ 2800 pmol O_2 cell^{-1} d^{-1} for maximum photosynthetic capacity. The *P-I* curve initial slope at limiting

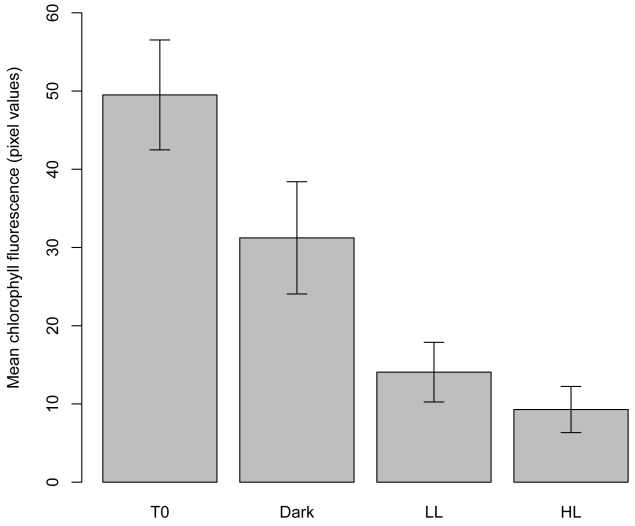

Figure 3. Mean chlorophyll *a* fluorescence (±SE, $n = 30$) at the end for the three experimental conditions (Dark, Low Light and High Light) and the beginning (T0) of the experiment using *Haynesina germanica*. Higher mean values likely corresponded to foraminifera containing more chlorophyll.

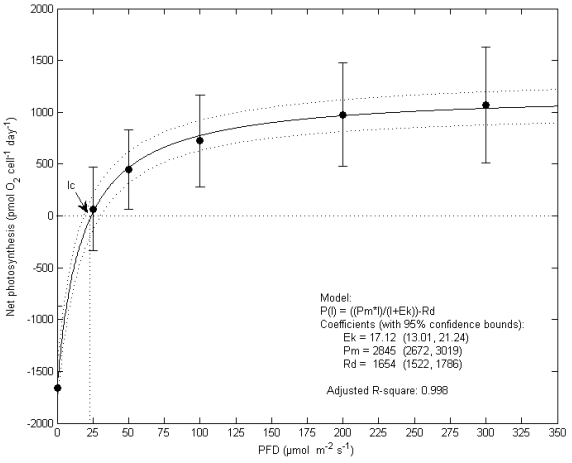

Figure 4. Net photosynthesis of *Haynesina germanica* (pmol O_2 cell^{-1} d^{-1}) as a function of the photon flux density (PFD, µmol photons m^{-2} s^{-1}). The half-saturation constant, Ek, was found at 17 (13–21), the dark respiration, Rd, at 1654 (1522–1786) pmol O_2 cell^{-1} d^{-1} and the maximum photosynthetic capacity, Pm, at 2845 (2672–3019) pmol O_2 cell^{-1} d^{-1}. The Ic, calculated compensation irradiance (24 (17–30) µmol photons m^{-2} s^{-1}). The adjusted R^2 of the model was equal to 0.998, $n = 3$.

irradiance (α) was estimated at 70 pmol O_2 cell^{-1} d^{-1} (µmol photons m^{-2} s^{-1})$^{-1}$ (95 % coefficient bound: 58–88).

Oxygen measurements carried out at the end of the experiment (T7) showed significant different dark and light respiration rates, with light respiration being lower than dark respiration but not reaching net oxygen production rates (D, LL, HL) (Table 1). Moreover, respiration rates were different between conditions ($p < 0.001$), with significantly lower respiration rates of specimens incubated under High Light conditions than those under Dark and Low Light conditions ($p < 0.05$, Fisher's LSD test).

Table 1. Light and dark respiration rates (pmol O_2 cell^{-1} d^{-1}) ± SD of *Haynesina germanica* in the three experimental conditions (Dark, Low Light and High Light) at the end of the experiment (Df, degree of freedom, PFD photon flux density).

Condition	PFD	Respiration rate (pmol O_2 cell^{-1} d^{-1})		
D	300	2452 ± 537		
	0	3542 ± 765		
LL	300	3468 ± 305		
	0	4015 ± 110		
HL	300	1179 ± 261		
	0	1905 ± 235		
Anova		Df	F test	p
Condition	p ($\alpha = 0.05$)	2	13.1	< 0.001
PFD	p ($\alpha = 0.05$)	1	5.4	0.026
Interaction	p ($\alpha = 0.05$)	2	0.3	0.78

PAM fluorescence rapid light curve (RLC) parameters (α, rETRmax, Ek and Eopt) showed significant differences between foraminiferal species and over the duration of the experiment (Figs. 5 and 6). Highest rETRmax, α and Eopt were always observed in *H. germanica*. After only one starvation day *A. tepida* RLC parameters dropped to zero or close to zero. In contrast, *H. germanica* RLC parameters showed a slow decrease throughout the experiment (Figs. 5 and 6) with rETRmax and α decreasing from 6 to 4 and 0.22 to 0.15, respectively (Figs. 6a and b). The parameters Ek and Eopt stayed constant over the 7 days of the experiment, with values oscillating around 30 and 90, respectively (Fig. 6c and d).

PSII maximum quantum yields (Fv/Fm) were clearly affected by light and time (Fig. 7). Both species showed high initial Fv/Fm values, i.e. > 0.6 and 0.4 for *H. germanica* and *A. tepida*, respectively (Fig. 7). However, while *A. tepida* Fv/Fm values quickly decreased to zero after only one starvation day, *H. germanica* exhibited a large variability between light conditions (D, LL, HL) throughout the duration of the experiment (Fig. 7); decreasing from 0.65 to 0.55 in darkness (D), from 0.65 to 0.35 under low light (LL) conditions and from 0.65 to 0.20 under high light (HL). Using these Fv/Fm decreases, *H. germanica* kleptoplast functional times were estimated between 11 and 21 days in the dark (D), 9–12 days in low light (LL) and 7–8 days in high light (HL), depending on whether or not an exponential or linear model was applied. *Ammonia tepida* chloroplast functional times were estimated between 1 and 2 days (exponential and linear model, respectively) and light exposure reduced the functional time to less than 1 day (data not shown).

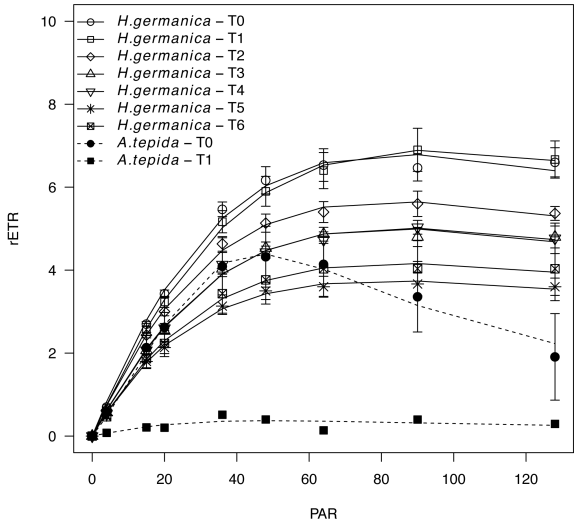

Figure 5. Rapid light curves (RLC, $n = 3$) expressed as the relative electron transport rate (rETR) as a function of the photosynthetic active radiation (PAR in µmol photons m^{-2} s^{-1}) of *Haynesina germanica* (black lines) and *Ammonia tepida* (black dashed lines) during the 7 days of the experiment.

4 Discussion

4.1 Chloroplast functionality

Our results clearly show that only *H. germanica* was capable of carrying out net photosynthesis. *Haynesina germanica* had typical diatom reflectance spectra (Fig. 1), showing the three major diatom pigment absorption features: chlorophyll *a*, chlorophyll *c*, and fucoxanthin (Meleder et al., 2003, 2013; Jesus et al., 2008; Kazemipour et al., 2012). Conversely, in *A. tepida* these absorption features were not detected, suggesting that diatom pigments ingested by this species were quickly digested and degraded to a degree where they were no longer detected by spectral reflectance measurements. These non-destructive reflectance measurements are thus in accordance with other studies on benthic foraminifera pigments by HPLC showing that *H. germanica* feed on benthic diatoms (Knight and Mantoura, 1985). Similarly, Knight and Mantoura (1985) also detected higher concentrations and less degraded diatom pigments in *H. germanica* than in *A. tepida*.

Furthermore, *H. germanica* has the ability to produce oxygen from low to relatively high irradiance, as shown by the low compensation point (Ic) of 24 µmol photons m^{-2} s^{-1} and the high onset of light saturation (> 300 µmol photons m^{-2} s^{-1}) (Fig. 4). Thus, *H. germanica* seems to be well adapted to cope with the high light variability observed in intertidal sediments that can range from very high irradiance levels (> 1000 µmol photons m^{-2} s^{-1}) at the surface of the sediment during low tide to very low levels within the sediment matrix or during high tide in turbid mudflat waters. *Ammonia tepida* was found to carry out aerobic respiration, but respiration rates measured at 300 µmol photons m^{-2} s^{-1} were lower than those measured in the dark. We thus suppose that in *A. tepida* oxygen production by ingested diatom or chloroplasts might be possible, provided that this species is constantly supplied with fresh diatoms. However, another possibility to explain this reduction in oxygen consumption could be a decrease of its metabolism or activity under light exposure. The light and dark oxygen production or consumption values measured for both species are in accordance with previous studies (Geslin et al., 2011).

According to Lopez (1979), measured oxygen data can be used to estimate *H. germanica* carbon fixation rates. Thus, using 1000 pmol O_2 $cell^{-1}$ d^{-1} at 300 µmol photons m^{-2} s^{-1}, ∼ 200 to 4000 cells per 50 cm^3 in the top 0.5 cm (Morvan et al., 2006; Bouchet et al., 2007) and assuming that photosynthesis produced one mol O_2 per mol of C fixed, *H. germanica* primary production would be between 1.8×10^{-5} and 4.0×10^{-4} mol C m^{-2} d^{-1}. This is a very low value compared to microphytobenthos primary production in Atlantic mudflat ecosystems, which usually range from 1.5 to 5.9 mol C m^{-2} d^{-1} (e.g. Brotas and Catarino, 1995, reviewed in MacIntyre et al., 1996). The estimated values represent thus less than 0.1 % of microphytobenthos fixated carbon and are in the same range of values than what has been described by Lopez (1979) using ^{14}C radioactive tracers. These results should be interpreted with caution because a wide variety of factors probably affect *H. germanica* in situ primary production, e.g. diatom availability, kleptoplast densities, nutrient supply, light exposure, sea water turbidity, local biogeochemical processes and migration capability are all factors that can potentially affect *H. germanica* kleptoplast functionality. Nevertheless, although carbon fixation seems not to be relevant at a global scale, the oxygen production could be important at a microscale and relevant in local mineralization processes in/on mudflat sediments (e.g. iron, ammonium, manganese).

At sampling time (T0) *H. germanica* rETR and Fv/Fm values were similar to microphytobenthic species (i.e. Fv/Fm > 0.65) (Perkins et al., 2001), suggesting that the kleptoplast PSII and electron transport chain were not much affected after incorporation in the foraminifers' cytoplasm. In contrast, *A. tepida* Fv/Fm and RLC parameters were already much lower on the sampling day and quickly decreased to almost zero within 24 h, suggesting that plastids were not stable inside the *A. tepida* cytoplasm. Complete diatoms inside *A. tepida* were already observed in feeding studies (Le Kieffre, pers. com), this low Fv/Fm value might thus come from recently ingested diatoms by *A. tepida*. Fv/Fm has previously been used to determine kleptoplast functional times and to follow decrease in kleptoplast efficiency in other kleptoplastic organisms, e.g. the sea slug *Elysia viridis* (Vieira et al., 2009). Fv/Fm measurements carried out on *H. germanica* at different light conditions showed that light had a significant effect on the estimation of kleptoplast functional

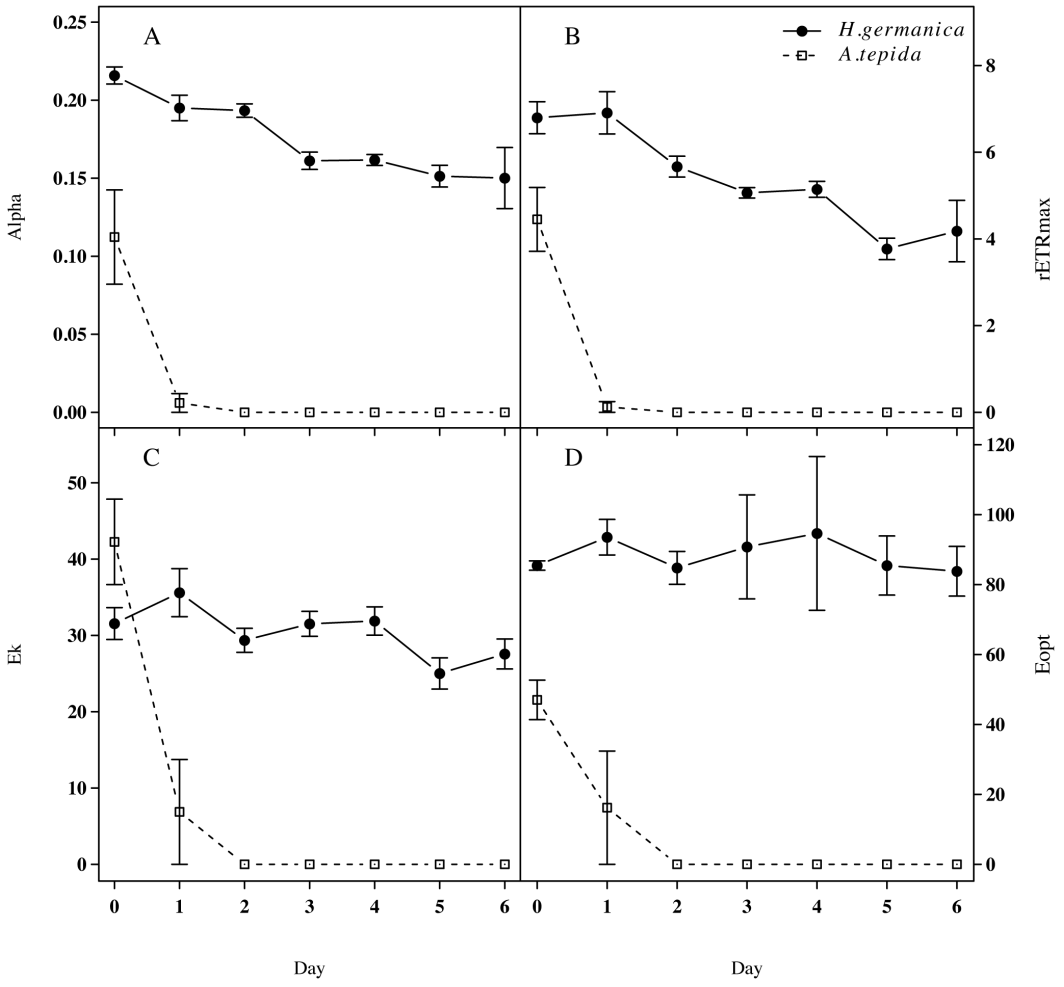

Figure 6. Rapid light curve (RLC, $n = 3$) parameters for *Haynesina germanica* (Dark-RLC) and *Ammonia tepida* maintained in the dark during the experiment, Alpha is the initial slope of the RLC at limiting irradiance, rETRmax is the maximum relative electron transport rate, Ek is the light saturation coefficient and Eopt is the optimum light, all of them were estimated by adjusting the experimental data to fit the model of Platt et al. (1980).

time, with the longest functional time estimated at 21 days for dark conditions. This time frame would qualify *H. germanica* as a long-term kleptoplast retention species (Clark et al., 1990); however, our 7 days estimation for the high light treatment would place *H. germanica* in the medium-term retention group. This clearly shows that light exposure has an important effect on this species kleptoplast functionality. Concerning *A. tepida*, the short dark diatom or chloroplast functional time (< 2 days) places this species directly in the short or medium-term retention group.

Additionally, *H. germanica* kept in darkness showed a slow decrease of the RLC parameters, α and rETRmax, throughout the 7 experimental days; this decrease is likely related to overall degradation of the light-harvesting complexes and of other components of the photosynthetic apparatus, which gradually induced a reduction of light harvesting efficiency and of carbon metabolism. This decrease was amplified in low and high irradiance and it should be pointed out that the actual light level of the HL treatment (i.e. 70 µmol photons m^{-2} s^{-1}) is very low compared to irradiances in their natural environment, which are easily going above 1000 µmol photons m^{-2} s^{-1}, showing that the foraminifera kleptoplasts lack the high photoregulation capacity exhibited by the benthic diatoms that they feed upon (Cartaxana et al., 2013). This is consistent with the observation at the end of the experiment that no net oxygen production was occurring under the different light conditions. Nevertheless, a small difference was still found between dark and light respiration (Table 1), suggesting that some oxygen production was still occurring but it was not sufficient to compensate for the respiration oxygen consumption. We also noticed that the respiration was higher in the foraminifera maintained in low light and dark conditions in comparison to the high light foraminifera. In the line of the lower Fv/Fm values

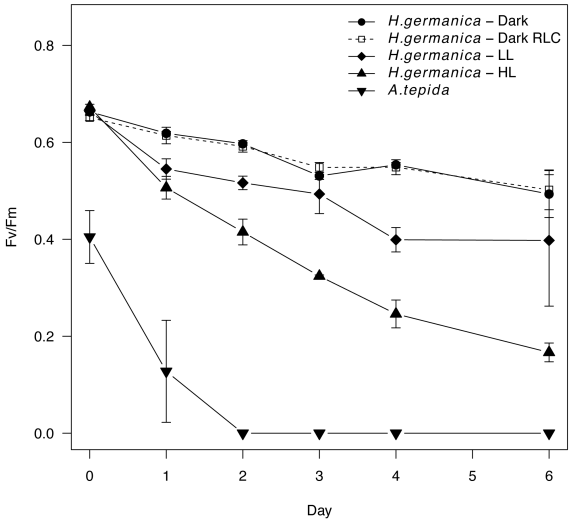

Figure 7. Maximum quantum efficiency of the photosystem II (Fv/Fm, $n = 3$) during the experiment for the different applied conditions (Dark, Low Light and High Light) and species (*Haynesina germanica* and *Ammonia tepida*).

observed, this suggests that kleptoplasts and possibly other metabolic pathways might have been damaged by the excess light. Clearly, in *H. germanica* light exposure had a strong effect on PSII maximum quantum efficiency and on the retention of functional kleptoplasts (Fig. 7), which can explain the absence of net oxygen production after the 7 days of the experiments. Comparable results for *H. germanica* were also obtained by counting the number of chloroplasts over time with cells exposed or not to light (Lopez, 1979). One of the most probable explanations for the observed Fv/Fm decrease is the gradual inactivation of the protein D1 in PSII reaction centres. This protein is an essential component in the electron transport chain and its turnover rate is frequently the limiting factor in PSII repair rates (reviewed in Campbell and Tyystjärvi, 2012). Normally, protein D1 is encoded in the chloroplast and is rapidly degraded and resynthesized under light exposure with a turnover correlated to irradiance (Tyystjärvi and Aro, 1996). However, although D1 is encoded by the chloroplast genome, its synthesis and concomitant PSII recovery require further proteins that are encoded by the algal nuclear genome (Yamaguchi et al., 2005). Thus, when D1 turnover is impaired it will induce an Fv/Fm decrease correlated to irradiance (Tyystjärvi and Aro, 1996) consistent to what was observed in the present study. In another deep sea benthic species (*Nonionella stella*) the D1 and other plastid proteins (RuBisCO and FCP complex) were still present in the foraminifer 1 year after sampling (Grzymski et al., 2002). This shows that some foraminifera can retain both nuclear (FCP) and chloroplast (D1 and RuBisCO) encoded proteins. However, contrary to *H. germanica*, *N. stella* lives in deeper environments never exposed to light and thus is unlikely to carry out oxygenic photosynthesis (Grzymski et al., 2002). This fundamental difference could explain why kleptoplast functional times are much longer in *N. stella*, reaching up to 1 year in specimens kept in darkness (Grzymski et al., 2002). On the other hand, it has been shown that isolated chloroplasts are able to function for several months in Sacoglossan sea slugs provided with air and light in aquaria (Green et al., 2001; Rumpho et al., 2001), which demonstrates the existence of interactions between the kleptoplast and the host genomes, and/or of mechanisms facilitating and supporting such long-lasting associations. In *H. germanica* exposed to high light it is also possible that reactive oxygen species (ROS) production rates of the sequestered chloroplasts might exceed the foraminifera capacity to eliminate those ROS, thus inducing permanent damage to the foraminifera. This ROS production could also eventually damage the kleptoplasts resulting in higher kleptoplast degradation rates.

4.2 Possible advantages of kleptoplasty for intertidal benthic foraminifera

Much is still unknown about the relationship between kleptoplastic benthic foraminifera and their sequestered chloroplasts. The relevance of the photosynthetic metabolism compared to predation or organic matter assimilation is unknown; however, it would be of great interest to understand the kleptoplast role in the foraminiferal total energy budget. Oxygenic photosynthesis comprises multiple reactions leading to the transformation of inorganic carbon to carbohydrates. However, to produce these carbohydrates all the light-driven reactions have to be carried out, as well as the Calvin cycle reactions. With fresh kleptoplasts this hypothesis seems possible (e.g. Lopez, 1979), especially if the plastid proteins are still present and functional. However, we showed that the maximum quantum efficiency of the PSII decreased quickly under light exposure, suggesting that substantial direct carbohydrate production is unlikely without constant chloroplast replacement. Conversely, the production of intermediate photosynthetate products such as adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH) could be possible and would be of metabolic value for the foraminifera. It is also possible that in situ the foraminifera have better photoregulation capacities. Not only will they have easy access to fresh diatom chloroplasts, as *H. germanica* is mainly living in the first few millimetres of the superficial sediment (Alve and Murray, 2001; Thibault de Chanvalon et al., 2015), but they will also have the possibility of migrating within the sediment (Gross, 2000) using this behavioural feature to enhance their photoregulation capacity, similar to what is observed in benthic diatoms from microphytobenthic biofilms (e.g. Jesus et al., 2006; Mouget et al., 2008; Perkins et al., 2010). However, below the photic limit (max 2 to 3 mm in estuarine sediments reviewed in MacIntyre et al., 1996; Cartaxana et al., 2011) it is unlikely that oxygenic photosynthesis will occur, even if live *H. german-*

ica are also found below this limit (Thibault de Chanvalon et al., 2015; Cesbron et al., 2016).

5 Conclusion

Comparing *H. germanica* with *A. tepida* showed that the former species potentially has the capacity of retaining functional kleptoplasts up to 21 days, much longer than *A. tepida* that showed almost no PSII activity after 24 h. Nevertheless, the capacity of *H. germanica* to keep functional kleptoplasts was significantly decreased by exposing it even to low irradiance levels, which resulted in low Fv/Fm values and decreased oxygen production. This shows clearly that in our experimental conditions, *H. germanica* had reduced photoregulation capacities. These results emphasize that studies on kleptoplast photophysiology of benthic foraminifera must be interpreted with care, as results are strongly influenced by the foraminiferal light history before incubation. Additionally, this study shows that the cellular machinery necessary for chloroplast maintenance is unlikely to be completely functional, suggesting that *H. germanica* has to continuously renew its chloroplasts to keep them functional. We hypothesize that kleptoplasts might have an added value by providing extra carbon, mainly under light exposure, but also as energy stock to be digested during food impoverished periods, in dark or light conditions.

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