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RESEARCH ARTICLE

REVISED Carbon and nitrogen uptake through photosynthesis and feeding by photosymbiotic Acantharia

[version 2; peer review: 2 approved with reservations, 1 not approved]

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Abstract

Background: Mixotrophy, combining phagotrophy and photoautotrophy in order to acquire nutrients and energy, is a widespread trophic mode in marine protist plankton. Acantharia (Radiolaria) are ubiquitous, but still uncultured oceanic protists. Many of them are mixoplanktic by endosymbiotic relations with microalgae. Here we aimed at quantitatively assessing phototrophy (inorganic nutrients) and phagotrophy (organic nutrients) of photosymbiotic Acantharia, to understand their physiology, and thereby improve integrations of mixotrophy into ecological models of oceanic ecosystems.

Methods: Freshly collected Acantharia were incubated with stable isotopes of inorganic carbon and nitrogen to determine photosynthetic uptake rates. Grazing experiments (prey disappearance) were done with different algal cultures as potential food organisms to measure the contribution of prey ingestion to the acantharian metabolism. Fluorescently (and isotopically) labelled prey was used to verify prey uptake, qualitatively.

Results/Conclusions: Carbon uptake rates were unaffected by the nitrogen source (*i.e.*, nitrate or ammonium). Total carbon inorganic uptake rate was 1112±82 pgC h^{-1} $Acantharia^{-1}$, 22.3±1.6 pgC h^{-1} symbiont $cell^{-1}$assuming 50 symbionts per Acantharia, at ~155-µmol photons m^{-2} s^{-1} irradiance. The Acantharia studied could use both inorganic ammonium and nitrate, but ammonium was taken up at a ~5 times higher rate. Prey ingestion of the haptophyte, *Isochrysis galbana*, was detected using labelled algae. Significant grazing by Acantharia could only be established on the dinoflagellate *Effrenium voratum*, with a grazing rate of 728 prey $Acantharia^{-1}$ $hour^{-1}$ (*i.e.*, ~56.3 ngC h^{-1}, 46% of total holobiont carbon content) at a ratio of 1.06x10^4 prey $predator^{-1}$. Daily photosynthetic carbon uptake rates made up ~14.5% of the total holobiont carbon content (0.9% hourly). The extent to which photosynthates are used and assimilated by the acantharian

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cell and/or if it is used for catabolic processes to obtain energy is still to be studied. Isotopic ratios further suggests seasonal differences in the usage of each trophic mode.

Keywords
Radiolaria, photosymbiosis, carbon fixation, mixotrophy, plankton, stable isotopes, primary production, grazing



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REVISED **Amendments from Version 1**

Typos have been corrected, including the absence of minus signs and incorrect units.

In the discussion, the photosynthetic C uptake rate of Acantharia has now been compared to previous research by Caron *et al.*, 1995. The confusing sentence/argument about δ ^{13}C of heterotrophic food sources has been deleted. We have elaborated an argument on the use of recycled CO_2 from the host for photosynthesis by the symbionts.

Additional images of the experiments have been added to the extended data.

Any further responses from the reviewers can be found at the end of the article

Plain language summary

Background: Many marine plankton can feed on prey like animals and also perform photosynthesis like plants, we call these mixoplankton. Among them, the group of single cell organisms Acantharia (Radiolaria) are widespread but can't be maintained in culture. Many of them are mixoplankton *via* a symbiotic partnership with microalgal cells that they host in their own cell. Here we aimed to quantitatively assess the respective contribution of phototrophy and feeding for such photosymbiotic Acantharia to understand their functioning, and thereby improve integrations of mixoplankton into ecological models of oceanic ecosystems.

Methods: We performed experiments using stable isotopes of inorganic carbon and nitrogen to determine photosynthetic uptake rates, as well as prey disappearance to measure the uptake of food.

Results/Conclusions: We observed that carbon uptake rates didn't depend on the nitrogen source and that the Acantharia studied could use both inorganic ammonium and nitrate. Though ammonium was taken up at a ~5 times higher rate. Significant grazing by Acantharia could only be established on the dinoflagellate *Effrenium voratum*, with a grazing rate of 728 prey Acantharia^{-1} hour^{-1} (*i.e.*, ~56.3 ngC h^{-1}, 46% of total organism's carbon content). Daily photosynthetic carbon uptake rates made up ~14.5% of the total organism's carbon content (that is 0.9% hourly). The extent to which photosynthates are used and assimilated by the acantharian cell and/or if it is used for catabolic processes to obtain energy is still to be studied. Isotopic ratios further suggest seasonal differences in the usage of photosynthesis *versus* feeding. Figure 1 shows the graphical summary of the study.

Introduction

Mixotrophy is a widespread trophic mode that, in marine plankton, is defined as a nutritional strategy combining phagotrophy and phototrophy autotrophy in order to acquire nutrients and energy (Flynn *et al.*, 2019; Selosse *et al.*, 2017). The perceived relevance of mixoplankton to the marine food web and nutrient cycles has been increasing in the last years. It is now recognised that mixoplankton are ubiquitous worldwide (Faure *et al.*, 2019; Leles *et al.*, 2017; Schneider *et al.*, 2020). Ecologically, mixoplankton can form an important (alternative) path for nutrient transfer at the base of the food web (Caron, 2016). To improve predictive models, attempts have been made to explicitly incorporate mixoplankton into aquatic ecosystem models (Ghyoot *et al.*, 2017a; Ghyoot *et al.*, 2017b; Schenone *et al.*, 2022; Ward & Follows, 2016).

Among mixoplankton, some harbour entire photosynthetic algae as endosymbionts to account for their photosynthetic ability. Their symbiotic partner exploits light and carbon dioxide to produce photosynthates, and these photosynthates might be transferred and used by the host, as is seen in many photosymbiotic systems (Trench, 1979; Yellowlees *et al.*, 2008).

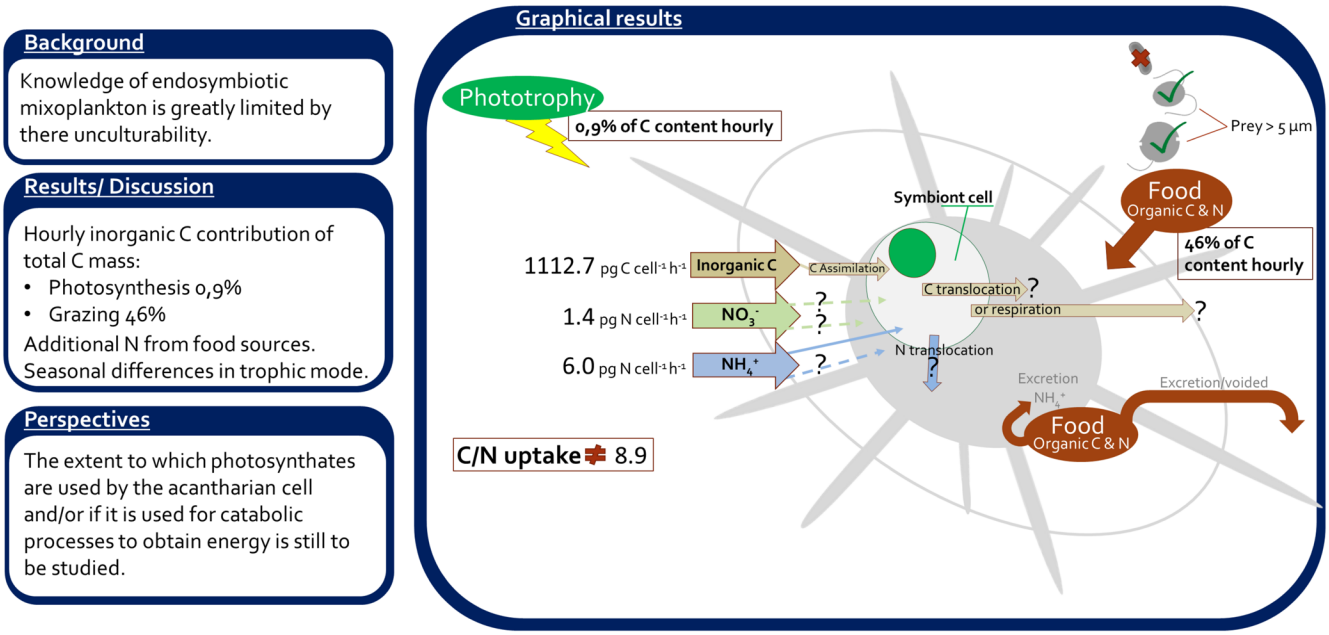

Figure 1. Graphical summary of the study. C, carbon; N, nitrogen.

The acquired ability to photosynthesise can give mixoplankton a competitive advantage over pure phototrophs or heterotrophs since they can supplement their metabolic needs with either nutritional mode. This will be of particular relevance when, for example, prey concentrations are low (Schoener & McManus, 2017). The increase in gross growth efficiency gained from photosynthesis by mixoplankton will be transferred to higher trophic levels. Models have suggested enhanced biomass transfer to higher trophic levels and the biological carbon pump by up to 35% (Mitra *et al.*, 2014; Ward & Follows, 2016). Understanding their physiology, and knowledge of rates of activity (*e.g.*, primary production, grazing, nutrient uptake) is therefore crucial in the parametrisation of carbon budgets in planktic ecosystem models.

Endosymbiotic Acantharia (Radiolaria), predominantly hosting the haptophyte alga, *Phaeocystis* (Decelle *et al.*, 2012a), are prominent planktic members of the epipelagic surface community (Biard & Ohman, 2020; Michaels, 1988). In the upper 20 m of the water column (where Acantharia are most abundant) in the central North Pacific Ocean, Acantharia have been estimated to account for up to 4% of the total primary production, or even 20% of the surface production by virtue of their endosymbionts (Michaels, 1988; Michaels, 1991). However, like for most mixoplankton, very little data on the contribution of photosynthesis to the carbon budgets of endosymbiotic Acantharia is available in the literature and virtually none related to prey uptake. Likewise, little is known about how the availability of dissolved inorganic nutrients influences acantharian physiology. A recent gene expression study of a solitary Collodaria (Radiolaria) suggests that they cannot take up nitrate but can use ammonium (Liu *et al.*, 2019).

Some mixoplanktic colonial Radiolaria (Collodaria) have previously been hypothesized to use photosynthesis merely for subsistence, whereas additional nutrients for growth would be obtained through phagotrophy; net hourly photosynthesis has been measured to constitute at a maximum only 0.4% of the radiolarian's carbon content (Swanberg, 1983). In stark contrast, the spumellarian Radiolaria, *Physematium muelleri*, was estimated to obtain more than half of its carbon by photosynthesis (Swanberg *et al.*, 1986)

A study by Swanberg and Caron (1991) microscopically investigated the prey content of Acantharia and other Radiolaria. Although most (60%) of collected Acantharia contained no detectable prey; the identifiable presumed prey consisted (numerically) mostly of tintinnids, other (*i.e.*, non-oligotrich) ciliates, and other protozoa, including smaller Radiolaria. It was also estimated that copepods contributed to 40% of the acantharian consumption in terms of carbon. Further qualitative accounts of radiolarian feeding indicate a general preference for metazooplankton over algal food (Anderson *et al.*, 1984 and references therein). *Spongodrymus* sp., a large 800 µm Spumellaria (Radiolaria), was shown to be able to consume 791 cells of *Isochrysis* galbana (haptophyte), 466 cells of *Amphidinium carterae* (dinoflagellate), or 0.5 *Artemia* spp., nauplius larvae (crustacean) h^{-1} in the laboratory (Anderson *et al.*, 1984). A recent study reported that Acantharia used pseudopodial extensions with drop-shaped terminal structures, which could function as a fishing apparatus similar to those observed in other radiolarians (Mars Brisbin *et al.*, 2020; and Extended Data Figure 2 in the *Extended data* section [Mansour *et al.*, 2022]). Despite this, prey ingestion by Acantharia is still an unresolved process, and quantitative estimates of acantharian grazing rate are not available. One of the main reasons for this lack of quantitative data is the inability in keeping laboratory cultures.

To gain insights into the reliance of Acantharia on prey ingestion *versus* symbiotic photosynthesis we measured photosynthetic carbon uptake using stable isotopes under different conditions of nitrogen availability (nitrate (NO_3^-) or ammonium (NH_4^+)). Additionally, photosynthetic carbon uptake of free-living symbionts was also measured, to compare the change in physiological rates between *in-hospite* and *ex-hospite* symbionts. Coupled with this, we estimated feeding rates on potential prey using calculations based on prey disappearance and tested this with (stable isotope and fluorescent) labelled prey to qualitatively explore acantharian feeding.

Methods

Samples

Acantharia. Acantharia (molecular clade F, see Extended Data Text in the *Extended data* section [Mansour *et al.*, 2022]) with endosymbiotic *Phaeocystis* were collected daily around 9 AM, by gentle plankton net tows along the sub-surface in the bay of Villefranche (Mediterranean Sea, Villefranche-sur-Mer, France 43°41′10″ N, 7°18′50″ E) as described previously (Mansour *et al.*, 2021). Acantharia specimens were collected during April and May 2020 and prepared as described in (Mansour & Not, 2021: https://dx.doi.org/10.17504/protocols.io.bqvrmw56). Briefly, following collection, individual cells were promptly isolated under a microscope and deposited in 0.22 µm-filtered seawater (FSW). Isolated specimens were incubated for an hour and transferred again to fresh filtered seawater; this was repeated three times before experimental incubations. This procedure allows for self-cleaning of particles attached to the cells and dilution to achieve extinction of any organisms accidentally taken with during isolation.

Algal cultures. Cultures of the microalgae *Isochrysis galbana* (RCC178), *Phaeocystis cordata* (RCC1383), *Synechococcus* sp. (RCC307), and *Effrenium voratum* (formerly *Symbiodinium voratum*, RCC1521) were obtained from the Roscoff Culture Collection, France (Extended Data Table 1 in the *Extended data* section [Mansour *et al.*, 2022]). *Synechococcus* was maintained in 'Red Sea medium' as described in https://dx.doi.org/10.17504/protocols.io.sz3ef8n. All other species were grown in K/5 medium consisting of 0.22 µm filter-sterilised and pasteurised aged natural seawater with a salinity of 35%, and a dissolved inorganic carbon (DIC) concentration of 24.65 µg C mL^{-1}. Seawater was obtained offshore near Roscoff, France (48°46'18'' N, 3°58'6'' W). Added macronutrients consisted of 288 µM NO_3; 5 µM NH_4 and 18 µM PO_4 (K-medium preparation is described in https://dx.doi.org/10.17504/protocols.io.7bxhipn; full K medium composition in Extended Data

Table 2 in the *Extended data* section [Mansour *et al.*, 2022]). Stock cultures were maintained in light of 125 µmol photons m^{-2} s^{-1} with a light-dark cycle of 16:8 h and grown at 20°C. Aliquots of dense algal stock culture were diluted in FSW, with no addition of nutrients, before being used as prey for Acantharia.

Experimental designs

Acantharian photosynthesis. Experiments were designed to determine rates of photosynthetic uptake of inorganic carbon and nitrogen in photosymbiotic Acantharia (see a conceptual drawing of the experiment in Extended Data Figure 2 in the *Extended data* section [Mansour *et al.*, 2022]). The used specimens all harbour intact photosynthetic symbionts that provide the hosts with photosynthates (Anderson, 1983; Decelle *et al.*, 2012b; Probert *et al.*, 2014; Yellowlees *et al.*, 2008). Seawater was collected from the bay of Villefranche-sur-Mer and filtered (0.22 µm) for experimental incubations. The concentration and isotopic ratio of dissolved inorganic carbon in the seawater was measured using Delta V Plus mass spectrometer and Gas Bench II at the IRMS platform in Brest, France.

Acantharia were transferred to 6-well plates containing 10 mL FSW enriched with $NaH^{13}CO_3$ (99% ^{13}C, Eurisotop) and either $^{15}NH_4Cl$ (99% ^{15}N, Eurisotop) or $Na^{15}NO_3$ (98% ^{15}N, Eurisotop). Each treatment was accompanied by a control incubation containing non-enriched nutrients in the same concentrations (natural medium incubations). The experimental treatments were as follows:

- Treatment 1: 10 µM $NaH^{13}CO_3$;
- Treatment 2: 10 µM $NaH^{13}CO_3$ and 0.3 µM $^{15}NH_4Cl$;
- Treatment 3: 10 µM $NaH^{13}CO_3$ and 0.5 µM $Na^{15}NO_3$;
- Treatment 4: 10 µM $NaH^{13}CO_3$ and 0.3 µM $^{15}NH_4Cl$ in complete darkness. This treatment was not accompanied by a similar incubation using non-enriched nutrients.

Specimens were incubated for 4 h in an incubator set at seawater temperature (16.5°C, April 2021), with a 16:8 h light:dark cycle at 150–160 µmol photons m^{-2} s^{-1}. After the incubation 60 to 100 cells were manually transferred through three series of clean filtered seawater and finally deposited one by one on a pre-combusted (450°C for 4 h) GF/F filter (25 mm, Whatman). The volumes of medium used to transfer all Acantharia were kept track of, and analytical blanks were made by wetting a separate GF/F filter with an equal amount of seawater leftover from the incubation. All filters were dried at 60°C for a minimum of 24 h and subsequently kept in sealed containers in the dark before isotopic analysis. Experiments were always initiated at the same time of day (*i.e.*, between 15:00 and 16:00) to avoid any confounding effects owing to circadian rhythms.

Additionally, several Acantharia were reserved for measurements of photochemical efficiency, using a MICROSCOPY-PAM (Walz), before and after incubation. This provided us an indication of the symbionts', and thereby presumably the holobiont's, general health, and allowed insights into the treatments where photosynthetic efficiency was determined.

Symbiont (Phaeocystis) photosynthesis. Photosynthetic rate of the free-living state (*i.e.*, in algal culture, not in symbiosis) of *Phaeocystis cordata* (RCC1383) was measured from cultures. Prior to the experiments, cultures were grown in 50 mL flasks, partially submerged in an aquarium with a water temperature of 20°C, a light:dark cycle 14:10 h at a photon irradiance of 150–170 µmol photons m^{-2} s^{-1} as measured in the water. The aquarium with culture flasks was placed on a glass table with light provided from below. Light was provided by cool white fluorescent tubes (OSRAM 58W, 840) and photon irradiance was measured using a light meter equipped with a spherical quantum sensor (ULM & US-SQS/L, Walz GmbH, Germany).

Cultures in exponential growth phase (as estimated by daily flowcytometric cell counts) were used for photosynthesis measurements employing the ^{14}C methodology. Different incubations were performed for a duration of 0, 3, 6, 8 and 24 h. For each incubation-time, one set of triplicates was incubated in the light, while the other was kept in complete darkness to compensate for passive incorporation of the radioisotope.

Simultaneous with the ^{14}C experimental procedure aliquots of the culture were taken for cell enumeration and algal chlorophyll *a* (Chl*a*) content measurements.

Acantharian grazing. Grazing experiments were done with different potential prey algal cultures to measure the contribution of prey ingestion to the acantharian metabolism (see a conceptual drawing of the experiment in Extended Data Figure 3 in the *Extended data* section [Mansour *et al.*, 2022]). In triplicate, Acantharia (15 to 31 cells) were transferred to 6-well plates prefilled with 1 mL FSW after which 10 mL of algal culture (from the same culture flask) was added. Control monocultures of each algal culture were also set up in triplicates allowing for the calculation of prey ingestion rate as the reduction in prey concentration in grazing treatments compared to control treatments with the prey algae alone. Incubations were done under the same conditions as for Acantharian photosynthesis measurements. At 4 h and 24 h the samples were gently mixed by pipetting and triplicate 1 mL samples were taken and fixed in 0.25% (final concentration) glutaraldehyde, and frozen at -20°C for flow-cytometry cell counts. These samples excluded the Acantharia, thus only the potential prey alga were taken. Following the results, we note that possibly the ratios between prey and Acantharia were too high at the chosen prey densities to effectively measure the number of prey eaten from a much larger prey pool.

Additionally, to qualitatively observe ingested prey, a similar incubation was performed, though with beforehand staining of *I. galbana* (RCC178) cells using the fluorochrome CellTracker Blue CMAC (7 amino-4-chloromethylcoumarin, ThermoFisher), a vital cytoplasmic stain, as described by

Martínez *et al.* (2014). *Isochrysis galbana* is used here as a species that has been shown to be ingested by Spumellaria (Radiolaria) (Anderson *et al.*, 1984). Cells were stained for 4–6 h with the CellTracker at a final concentration of 10 µM. To reduce the carryover of stain that could enter Acantharia, excess stain was removed from the medium after the staining period by washing three times. This was performed by centrifugation of the live fluorescently labelled algae (LFLA) at 2,000 x g for 5 min; the supernatant was discarded and the cells were re-suspended in FSW. Acantharia were incubated with the labelled prey as before, triplicate Acantharia were individually isolated after 0, 30, 60, and 240 min; transferred to 0.5 mL tubes; fixed in a final concentration of 0.25% glutaraldehyde (Sigma, ref. G5882-100ML) and 1% paraformaldehyde (Electron Microscopy Sciences, ref. 15714, diluted in phosphate-buffered saline); and stored at 4°C for fluorescence microscopy.

Analyses

Stable isotope analysis. The C and N isotopic composition and content of the particulate organic matter was determined as CO_2 and N_2 released by flash combustion using an elemental analyser (EA Isolink CN/OH, Thermo Fisher) coupled to an isotope ratio mass spectrometer (IRMS, Delta V Advantage, Thermo Fisher) *via* an open split and dilution-controlled interface (ConFlow IV, Thermo Fisher) with a continuous flow of helium at 180 mL min^{-1}. In addition to the samples, each run contained international isotopic standards from International Atomic Energy Agency (IAEA) (these were IAEA-600 (caffeine), and CH6 (sucrose) for C; N2 (Ammonium Sulfate) and USGS34 (Potassium Nitrate) for N). IAEA standards were used for manual normalization of the δ-unit to the Vienna Pee Dee Belemnite-limestone (VPDP) or atmospheric N_2 scale, for carbon and nitrogen respectively, using a linear calibration curve (Coplen *et al.*, 2006; Paul *et al.*, 2007). An isotopic laboratory working standard (casein, certified Elemental Microanalysis) was run every 15 samples for verification of system stability. Though it was not needed in our case, a correction for linearity could be performed using the employed casein standard. A laboratory weight standard (acetanilide, certified Elemental Microanalysis) of different masses were used to calibrate the analyser and determine C and N content of the samples. Standard deviations were 0.11 and 0.06 µg for C and N content respectively, and 0.1 ‰, and 0.05 ‰ for $\delta^{13}C$ and $\delta^{15}N$, respectively. Additionally, a quality check (urea, certified Elemental Microanalysis) for isotopic ratios and weight (urea, certified Elemental Microanalysis) was run randomly to validate the quality of analysis. Isotopic Ratio Mass Spectrometry data were processed in the manufacturer's ISODAT 3.0 software and Microsoft Excel (RRID:SCR_016137) for the output of values expressed in delta notation (δ) in units of per mil (‰).

Carbon and nitrogen signal of the samples was corrected by accounting for the related analytical blank. The blank correction was performed for the normalized δ-values using the following equation:

$$\delta_{blkcorr} = \frac{(\delta_{meas} \times Area_{meas}) - (\delta_{blk} \times Area_{blk})}{Area_{meas} - Area_{blk}}$$

$\delta_{blkcorr}$ = blank corrected δ-value of the sample

δ_{meas} = δ-value of the sample

δ_{blk} = δ-value of the blank (*i.e.,* the medium the specimen was kept in)

$Area_{mea}$ = area of the sample peak

$Area_{blk}$ = area of the blank peak

For qualitative measurements of isotope uptake, or the calculation of excess, ^{13}C and ^{15}N of each sample relative to natural abundance $\delta^{13}C$ and $\delta^{15}N$ were converted to fractional abundances (F) using the following equations (Fry, 2006; Moodley *et al.*, 2005):

$$R = \left(\frac{\delta X}{1000} + 1\right) \times R_{VPDP}$$

$$F = \frac{R}{R+1}$$

Where R is the ratio of either $^{13}C/^{12}C$ or $^{15}N/^{14}N$; δX is the normalised-blank-corrected-δ-value of the sample, either $\delta^{13}C$ or $\delta^{15}N$; R_{vpdp} = 0.0112372, for nitrogen this is substituted with R_{air} = 0.0036765, and F is the fractional abundance. Finally, total uptake (I) of the heavier isotope in µg C $cell^{-1}$ h^{-1} or µg N $cell^{-1}$ h^{-1} was calculated as:

$$I = (F_{sample} - F_{control}) \times m_{sample} / t / n$$

Here F_{sample} is the fractional abundance of the sample after incubation and $F_{control}$ the non-enriched samples. m_{sample} is the total C or N mass of the sample in µg and t is the incubation time in hours. The ^{13}C uptake was further normalised per acantharian cell by division with the total number of Acantharia in the sample (n).

The total uptake of carbon (^{12}C + ^{13}C) was calculated by division of the total ^{13}C uptake (*I*) by the fractional abundance of total ^{13}C of dissolved inorganic carbon in the incubation. The dissolved inorganic carbon concentration of the seawater was measured to average 5,900 µM, with a natural abundance of ^{13}C of 1.08%. The total fraction of ^{13}C in the experimental incubations was thus 1.9%. For total uptake of nitrogen, the concentrations of nitrite, nitrate, and ammonium at the sampling location were considered The natural ^{15}N fraction was assumed as 0.368% (data from https://www.somlit.fr/visualisation-des-donnees, Villefranche point B; surface), we estimated the total fraction of ^{15}N as 43.95 and 32.53% for the nitrate and ammonium treatments, respectively.

Microscopy chlorophyll fluorescence measurements. Chlorophyll fluorescence parameters were measured using a Pulse Amplitude Modulated (PAM) fluorometer coupled to a microscope (MICROSCOPY-PAM; Walz, Effeltrich, Germany), equipped with a 10X objective lens. After a minimum of 5 min dark incubation, the basal level of fluorescence (F_0) was measured under modulated light (9 µmol photons m^{-2} s^{-1}, frequency: 8 Hz at 625 nm), after which the maximum fluorescence level (F_m) was determined with a saturating light pulse (1719 µmol photons m^{-2} s^{-1}, during 8 × 60 ms at 625 nm).

Dark-adapted maximal quantum yield (F_v/F_m) of photosystem II (PSII) was calculated as follows: $F_v/F_m = (F_m - F_0)/F_m$.

ImagingWin (v2.46i) software (WALZ) was used to determine areas of interest (AOI) that encompass the entire Acantharia cell. Due to the patchiness of the photosynthetic activity, only pixels for which $F_v/F_m > 0$ were used to calculate fluorescence parameters, hence non-photosynthetically active regions (such as those of the host cell) are not taken into consideration.

Growth and ingestion rate calculations. Algal culture growth rates (μ, d^{-1}) were measured as change in cell concentrations over time and calculated assuming exponential growth.

$$\mu = ln(N_x / N_0) / (t_x - t_0)$$

Where N_x and N_0 are the cell concentrations in cells mL^{-1} at time point t_x and time t_0 respectively.

The ingestion rates (U = prey predator^{-1} h^{-1}) were calculated by Frost (1972) equations as modified by Heinbokel (1978):

$$U = C\,x\,F$$

Where C is the average prey concentration in the experimental incubations with prey and grazer, calculated as:

$$C = (Xt_x - Xt_0) / (Ln(Xt_x) - Ln(Xt_0))$$

With Xt_x being the prey concentration at the end of the incubation and Xt_0 at the start.

F is the clearance rate (mL grazer^{-1} h^{-1}) calculated as:

$$F = (k - g) / P$$

k is the intrinsic growth rate of prey in monoculture incubations, calculated as:

$$k = (Ln(Xt_2) - Ln(Xt_1)) / (t_2 - t_1)$$

g is the grazing coefficient, calculated as the growth rate of prey in the experimental incubations with prey and grazer:

$$g = (Ln(Xt_2) - Ln(Xt_1)) / (t_2 - t_1)$$

Xt_2 and Xt_1 being the prey concentrations at t_2 and t_1, respectively.

P is the average grazer concentration in the experimental incubations with prey and grazer. Considering that Acantharia do not proliferate during the experiments (only death might be observed), P is simply:

$$P = A / V$$

Where A is the Acantharia count at the end of the incubation and V the volume of the incubation.

Fluorescence microscopy. Fluorescence microscopy observations of ingested prey were conducted using an inverted Leica SP8 confocal laser scanning microscope (Leica Microsystems, Germany) equipped with a compact supply unit that integrates a LIAchroic scan head and several laser lines (405 nm, 488 nm, 552 nm, 638 nm), and either an HC PL APO CS2 63 × 1.40 OIL objective or an HC PL APO CS2 40 × 1.1 WATER objective, according to specimen size. Single cells were isolated from the fixative solution and placed on LabTek II chambered coverglass (Nunc 155382; Thermo Fisher Scientific, MA, USA). To visualise the membrane and skeletal structures, the cells were additionally stained with 50 μM Poly-L-lysine conjugated with Alexa Fluor 546 for 15 min (Colin *et al.*, 2017), and then washed by means of cell transfer through FSW. This stain binds among others strontium sulphate, protein, and polysaccharide materials (Colin *et al.*, 2017).

Imaging was performed with excitation using the 405 nm, and 552 nm lasers, and signal collection at emission wavelengths 460–472 nm (histogram adjustment: minimum 5; maximum 105), 603–635 nm (histogram adjustments: minimum 0; maximum 210), and 693–782 nm (histogram adjustment: minimum 0; maximum 160) to capture fluorescence of the Celltracker blue stain, carotenoids/Alexa Fluor 546 stain, and chlorophyll, respectively. Image processing and 3D rendering from z-stacks was performed using the software Imaris (RRID:SCR_007370) (Bitplane AG, Zurich, Switzerland) (free alternative, FIJI ImageJ), following the guidelines of Schmied and Jambor (2020).

Algal cell enumeration. Fixed algal culture cells (glutaraldehyde 0.25% final concentration) of the grazing experiments were enumerated on an Agilent NovoCyto Advanteon flowcytometer. Except for cells < 1.5 μm (*Synechococcus* sp.), cells were enumerated on a Guava Easycyte Ht flowcytometer. Free-living stages of the algal symbionts (*Phaeocystis*) for the symbiont photosynthesis experiments, were enumerated on a Cytoflex flowcytometer (Beckman Coulter, USA). Thresholds were on red fluorescence, based on fluorescence patterns and cell size from side scatter (Olson *et al.*, 1991).

Chlorophyll a content of Phaeocystis cultures. Samples for Chl*a* measurements from algal suspensions were filtered or deposited on a GF/F filter and Chl*a* was extracted by adding an equal amount of 96% ethanol (*i.e.*, 2 mL sample with 2 mL 96% ethanol). Samples were stored at 4°C for 24 h in darkness. Fluorescence and Chl*a* concentrations were measured using a bench-top fluorometer (Trilogy, Turner designs, CA) equipped with a manufacturer's Chl*a* non-acidification insert for Chl*a* determination. The fluorometer was calibrated against a pure Chl*a* standard (2.13 mg Chl*a* L^{-1}) of cyanobacterial origin (DHI, Hørsholm, Denmark).

Photosynthetic rates measurements of Phaeocystis cultures. Photosynthetic rates were measured using the ^{14}C technique by Rivkin and Seliger (1981) and previously described by Skovgaard *et al.* (2000) and Hansen *et al.* (2016). Sample aliquots of 2 mL from algae suspensions were taken in two sets

of triplicates. To each sample 20 µL of $NaH^{14}CO^3$ stock solution (specific activity = 100 µCi mL^{-1}; Carbon-14 Centralen, Denmark) was added for a final concentration 1 µCi mL^{-1}. For each experimental incubation, one set of triplicates was incubated in the light, while the other was kept in complete darkness to compensate for passive incorporation of the isotope. After incubation, a 100 µL sub-sample was transferred to a new vial containing 200 µL phenylethylamine for determination of specific activity. The remaining 1.9 mL were acidified with 2 mL 10% glacial acetic acid in methanol and evaporated overnight at 60°C to remove inorganic carbon. The residue was then re-dissolved in 1.5 mL Milli-Q water. All vials were treated with 10 mL Ultima Gold XR scintillation cocktail, and disintegrations per minute were measured using a Perkin Elmer, Tri-Carb 2910TR liquid scintillation analyzer with 10-minute acquisition time. As per Skovgaard *et al.* (2000), new caps (Packard poly screw caps) were mounted after adding the scintillation cocktail.

Photosynthetic activity (PA in pg C $cells^{-1}$ h^{-1}) was calculated from the disintegration per minute using the following equation.

$$PA = \frac{(lightDPM - darkDPM) \times [\mathrm{DIC}]}{DPM specific\,activity \times \mathrm{h} \times \mathrm{N}}$$

Where DPM is disintegrations min^{-1} mL^{-1}, either in the 'light', 'dark' or 'specific activity' vial; h is the incubation time; N is cells mL^{-1} and [DIC] is the concentration of dissolved inorganic carbon (pg C mL^{-1}) in the medium. Total dissolved inorganic carbon concentrations [DIC] were measured on 25 mL of the medium used for the experiments using a TOC-L analyser, Shimadzu.

Statistical analyses. Mean $\delta^{13}C$ (or $\delta^{15}N$) differences between treatments were tested by comparing means with an ANOVA and Tukey-Kramer HSD post-hoc test. Effects of Acantharia grazing were tested by comparing the grazing coefficient with the intrinsic growth rate using a Student's t-test. Tests were done at significance level 0.01. The software SAS JMP Pro (RRID:SCR_022199) 15.0 (SAS Institute, Inc) was used for statistical analyses pertaining isotopic ratios (free alternative, the R statistical environment (R Core Team 2013, RRID:SCR_001905)). Microsoft Excel (RRID:SCR_016137) was used for statistical analyses pertaining grazing. Results are presented as mean ± standard deviation.

Results

Acantharia used in these experiments all belong to clade F3b and are phylogenetically assigned to *Acanthostaurus* sp. or *Acanthometra* sp. (see Extended Data Text in the *Extended data* section [Mansour *et al.*, 2022]).

Maximum quantum yield (F_v/F_m) measurements averaged 0.7 ± 0.08 (n = 86) for acantharian cells and did not significantly change as a function of time after isolation or cleaning during the 4 h incubation, (Extended Data Figure 4 in the *Extended data* section [Mansour *et al.*, 2022]). Morphological observations, *i.e.*, extended cytoplasm and pseudopodal extension, confirmed that the Acantharia cells were in a healthy state during the experimental incubations (Extended Data Figure 1 in the *Extended data* section [Mansour *et al.*, 2022])

Elemental content of acantharian cells averaged cellular carbon and nitrogen contents of 122.8 ± 25.1 ng C $cell^{-1}$ and 14.9 ± 5.0 ng N $cell^{-1}$, and a C:N ratio of 9.0 ± 1.7. The C:N ratio did not significantly change with nutrient addition (ANOVA $F_{2,19}$ = 0.323, p = 0.8), and the previously given average values are thus for all samples combined (n = 27).

Inorganic nutrient uptake rates—photosynthesis

Carbon uptake rates by Acantharia holobiont. Acantharia in control incubations with natural medium averaged a $\delta^{13}C$ value of -22.5 ± 2.3‰ (n = 11). After 4 h incubation with ^{13}C-bicarbonate, all treatments showed significant ^{13}C enrichment compared to the relevant control incubation (ANOVA $F_{2,19}$ = 23.55, p < 0.01; Figure 2A [Mansour *et al.*, 2022]) (Extended Data Table 3 in the *Extended data* section [Mansour *et al.*, 2022]).

Incubations in complete darkness do not differ significantly in $\delta^{13}C$ values from control incubations, implying that ^{13}C uptake requires light (photosynthesis), and thus excludes passive ^{13}C uptake. Addition of ammonium or nitrate did not significantly affect acantharian ^{13}C uptake, as compared to without their addition. However, there seems to be a significantly higher carbon enrichment with the nitrate treatment than with the ammonium treatment (Extended Data Table 3 in the *Extended data* section [Mansour *et al.*, 2022]).

To determine quantitative uptake rates of total C per Acantharia cell, the relative abundance of naturally occurring ^{13}C (1.1%) was combined with the relative abundance of added ^{13}C tracer. Hence, the total ^{13}C (1.9%) in our incubations was used to calculate total carbon uptake. Table 1 shows the C uptake rates for each treatment. Considering an average of 50 symbionts per Acantharia cell, the total carbon uptake rate per symbiont was 22.3 ± 1.6 pg C h^{-1} symbiont $cell^{-1}$.

Nitrogen uptake rates by Acantharia holobiont. Isotopic ratios of nitrogen in Acantharia were only measurable for a fraction of the samples due to insufficient sample biomass, and the ratios were best measured when isotopic enrichment was high. Thus, most measurements came from the labelled ammonium treatment with highest ^{15}N enrichment (Figure 2B). There is indication that nitrate is also taken up, as there is an increase of $\delta^{15}N$ by 31.8‰ (Table 2). No nitrogen enrichment by ammonium can be observed in the dark, though it is unclear whether this is due to biological or technical effects. An estimate of total nitrogen uptake is made by accounting for the fractional abundance of ^{15}N in the respective treatments (Table 2).

Photosynthesis by free-living Phaeocystis. *Phaeocystis cordata* is the dominant symbiont in the acantharian photosymbiosis in the Mediterranean Sea. We measured the C uptake of free-living *P. cordata* cultures to average 0.38 ± 0.043 pg C $cell^{-1}$ h^{-1} (n = 21) or 2.2 ± 0.2 pg C Chl*a*$^{-1}$ h^{-1} (n = 12,) with Chl*a* ranging between 0.094 and 0.18 pg Chl*a* $cell^{-1}$ (n = 3). With an estimated cell diameter of 4 µm the carbon content of a *Phaeocytis* cell was calculated as 5.8 pg C $cell^{-1}$, according to the protist volume to carbon content conversion by Menden-Deuer and Lessard (2000). Thereby carbon-specific photosynthesis rate for *Phaeocystis* is 0.065 pg C [pg C]$^{-1}$ h^{-1}.

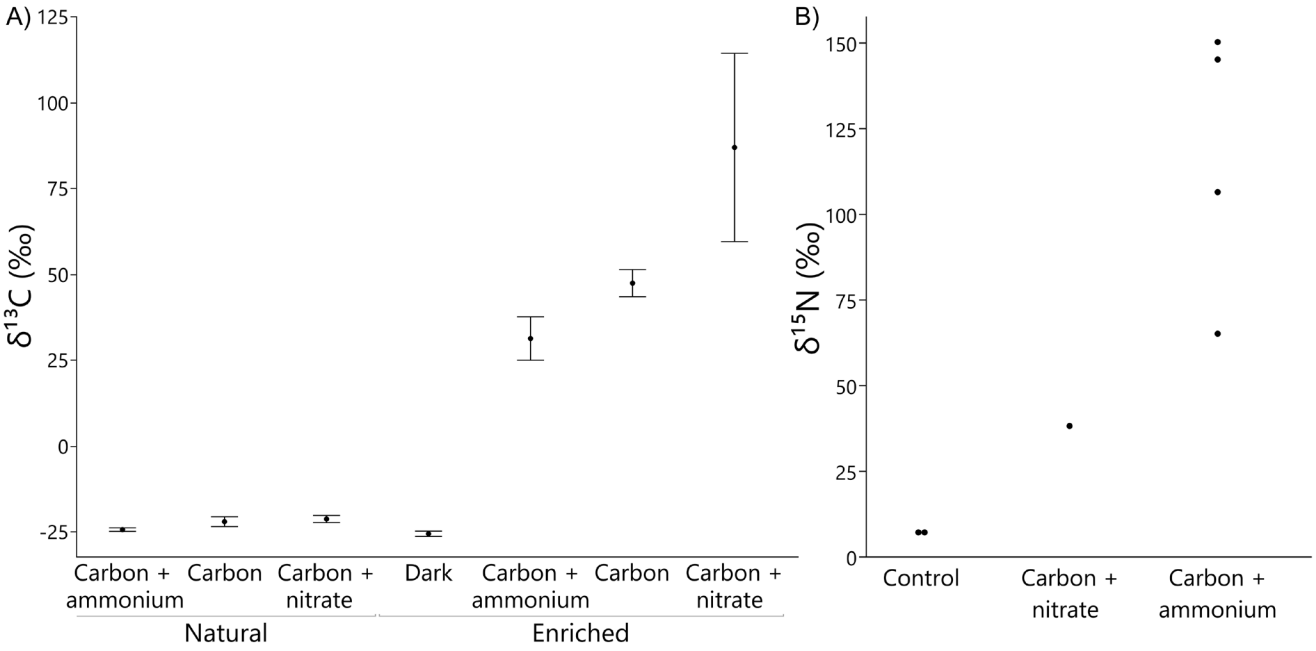

Figure 2. Carbon and Nitrogen stable isotope ratios. Carbon (**A**) and nitrogen (**B**) isotopic composition of Acantharia in delta (‰) notation for each treatment and control treatment. The dark treatment involved incubation with labelled carbon and ammonium. For $\delta^{15}N$, control is both values from the natural nitrate and ammonium treatments. Values are means and standard deviations for $\delta^{13}C$, and individual sample data for $\delta^{15}N$; n for each treatment is indicated in Table 1 for carbon and in Table 2 for nitrogen.

Table 1. Carbon isotopic ratio and uptake data for Acantharia. Data shown are the $\delta^{13}C$ (‰) for each treatment, and the uptake rates of labelled and total carbon for enriched samples. Data are presented as means and standard deviations. NA, not applicable.

Treatment		$\delta^{13}C$ (‰)	pg ^{13}C h^{-1} $cell^{-1}$	pg C h^{-1} $cell^{-1}$
Controls	Carbon (n = 4)	-21.9 ± 2.8	NA	NA
	Carbon + ammonium (n = 4)	-24.2 ± 1.0	NA	NA
	Carbon + nitrate (n = 3)	-21.1 ± 1.8	NA	NA
Enriched	Carbon (n = 4)	47.5 ± 7.9	21.2 ± 1.6	1,112.7 ± 82.1
	Carbon + ammonium, Dark (n = 3)	-25.4 ± 1.3	-1.1 ± 0.3	-59.3 ± 15.0
	Carbon + ammonium (n = 5)	31.4 ± 14.1	20.5 ± 3.9	1,075.6 ± 203.2
	Carbon + nitrate (n = 3)	87.0 ± 27.4	32.5 ± 12.8	1,706.5 ± 671.1

Table 2. Nitrogen isotopic ratio and uptake data for Acantharia. Data shown are the $\delta^{15}C$ (‰) for each nitrogen treatment, and the uptake rates of labelled and total nitrogen for enriched samples. Data are presented as means and standard deviations. NA, not applicable.

Treatment		$\delta^{15}N$ (‰)	pg ^{15}N h^{-1} $cell^{-1}$	pg N h^{-1} $cell^{-1}$
Controls	Carbon + ammonium (n = 1)	7.8	NA	NA
	Carbon + nitrate (n = 1)	6.4	NA	NA
Enriched	Carbon + ammonium (n = 4)	116.9 ± 39.6	2.0 ± 1.3	6.0 ± 4.1
	Carbon + nitrate (n = 1)	38.1	0.6	1.4

Organic nutrient uptake—grazing

Calculated grazing rates of Acantharia on different prey were in most cases not different from zero when measured using the prey disappearance approach (Table 3). Incubations with the cyanobacteria *Synechococcus* sp. (RCC307), and the haptophyte *Isochrysis galbana* (RCC178) were done with different initial concentrations, but that did not change the significance of the ingestion rates. Incubation of 4 h with the dinoflagellate *Effrenium voratum* (RCC1521) led to a significant prey ingestion rate of 728 ± 89 cells Acantharia^{-1} h^{-1} (*i.e.*, 56.3 ng C h^{-1}, assuming a C content of 77.3 pg C prey based on 10 µm estimated spherical diameter, which is an hourly uptake of 46% of total acantharian C content). Incubation for 24 h, however, did not show significant grazing for all three algae tested (Table 3).

Qualitative observation showed that incubation of Acantharia with LFLA *I. galbana* does indicate prey ingestion (Figure 3

Table 3. Feeding rate data of Acantharia. Ingestion rates for each incubation of Acantharia with algal culture, as well as the incubation time point, and the average Acantharia to algal culture ratio over the incubation time. The 24 h incubations are the same incubations following the respective 4 h incubations. A negative ingestion rate implies a higher growth rate of algal prey with Acantharia than in monoculture. Shown is the p-value for the Student's t-test testing the hypothesis that incubations with and without Acantharia do not differ in algal growth rate, rejection of the hypothesis is indicated in bold. Data are presented as mean ± standard deviation, n = 3.

Algal species	Time point (h)	Prey to Acantharia ratio	Ingestion rate (prey Acantharia^{-1} hour^{-1})	p-value
*Isochrysis galbana**	4	7.23E+04	3,681 ± 4,468	0.198
Isochrysis galbana	4	3.50E+04	2 ± 112	0.994
Isochrysis galbana	24	4.03E+04	319 ± 189	0.157
Isochrysis galbana	4	1.33E+04	51 ± 100	0.350
Isochrysis galbana	24	1.64E+04	9 ± 25	0.583
Synechococcus sp.	4	7.16E+06	246,577 ± 156,953	0.068
Synechococcus sp.	24	4.49E+06	-14,105 ± 37,361	0.454
Synechococcus sp.	4	8.24E+05	-8,837 ± 9,905	0.165
Synechococcus sp.	24	9.52E+05	490 ± 2,255	0.865
Synechococcus sp.	4	3.69E+05	-4,276 ± 5,215	0.618
Synechococcus sp.	24	2.01E+05	2,088 ± 2,476	0.167
Synechococcus sp.	4	3.90E+03	36 ± 0.01	0.318
Synechococcus sp.	24	3.52E+03	-0.59 ± 5.9	0.973
Effrenium voratum	**4**	**1.06E+04**	**728 ± 89**	**0.000**
Effrenium voratum	24	1.17E+04	-137 ± 109	0.089

* incubation performed using live fluorescent labelled algae, samples only taken at 4 h

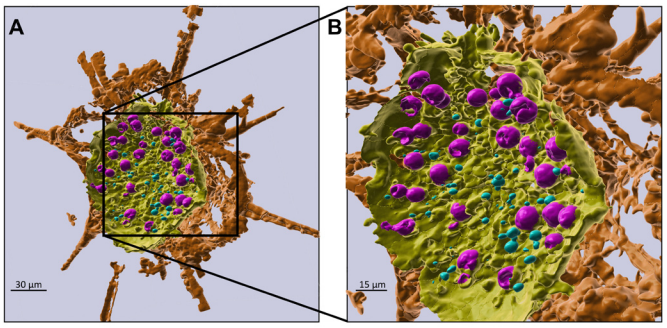

Figure 3. 3-D reconstruction of (half) an Acantharia after 60-minute incubation with LFLA *I. galbana* imaged using confocal microscopy. Membranes and skeletons have been stained using Poly-L-lysine Alexa Fluor 546. **A**) Cyan indicates captured emission wavelengths at 460–472 nm (Celltracker Blue fluorescence), yellow and maroon at 603–635 nm (Alexa Fluor 546, masking the cell's autofluorescence), and magenta at 693 782 nm (chlorophyll autofluorescence). The signal of the Alexa Fluor has been coloured maroon to indicate the outer membrane and spicules whereas the central capsule membrane is shown in yellow. **B**) Zoomed and angled frame of the same cell. LFLA and symbiont chlorophyll signal is found inside the central capsule membrane. Z-stack images were three-dimensionally reconstructed with Imaris software. A 3D pan around video of this image is available at https://doi.org/10.5281/zenodo.6868821. LFLA, live fluorescently labelled algae.

and Figure 4) (Extended Data Figure 7 in the *Extended data* section [Mansour *et al.*, 2022]). Control Acantharia experiments that were not subjected to LFLA gave a baseline (auto)fluorescence for the cells. Acantharia show inherent autofluorescence independent of symbionts as is seen when observing fixed cells, as well as live cells (not shown), and is likewise the case for non-symbiotic species (Extended Data Figure 5 in the *Extended data* section [Mansour *et al.*, 2022]).

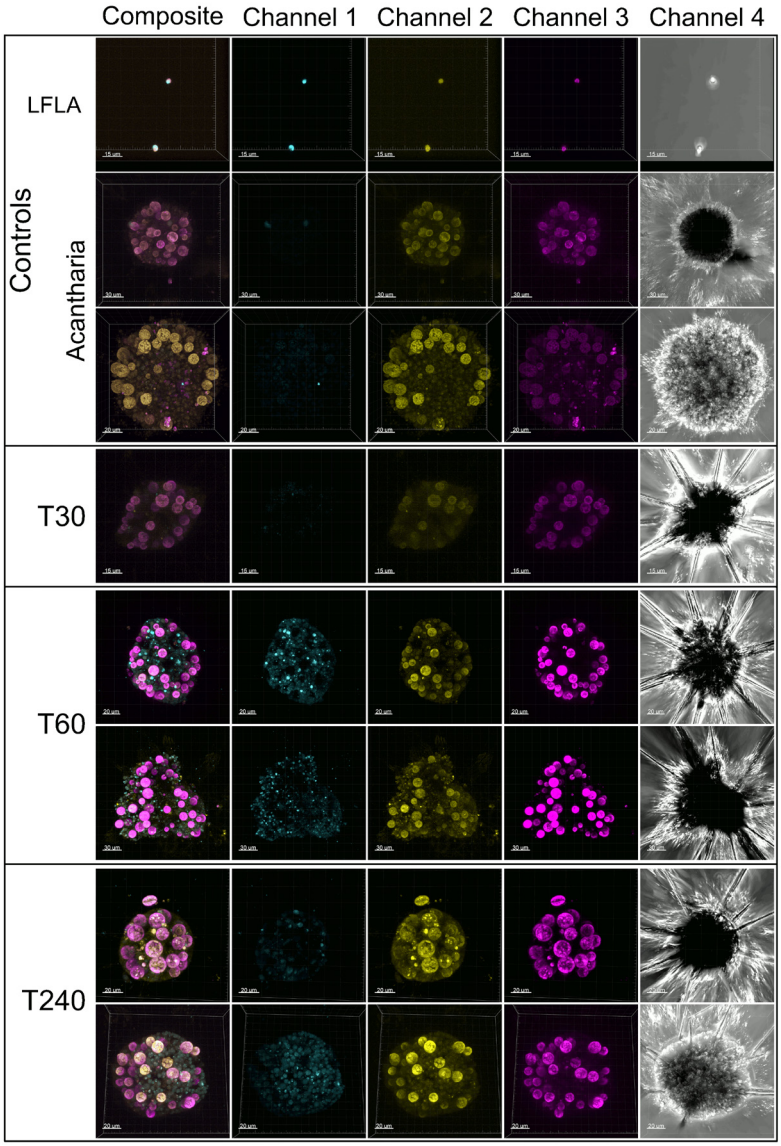

Figure 4. Acantharia after incubation with LFLA *I. galbana* at different time points imaged using confocal microscopy. Panels show the snapshots of different time points (30, 60, and 240 min) with 3-D projections of separate channels and a composite of channels 1 through 3. Channel 1 (cyan) is for emission wavelengths 460–472 nm (Celltracker Blue fluorescence), and channel 2 (yellow) for 603–635 nm (unknown autofluorescence, likely carotenoids), and channel 3 (magenta) for 693–782 nm (chlorophyll autofluorescence). Channel 4 represents transmission light images. The first row represents stained *I. galbana* (LFLA)**.** The Acantharia controls show a lack of spicules, as seen in the channel 4 panels, due to it being cells that have been stored longer in fixative and spicules appear to be dissolved. Lower fluorescence intensity is observed with control and T30 panel images, due to those having been imaged using an oil immersion objective, whereas the other images were made using a water immersion objective. Z-stack images were three-dimensionally reconstructed with Imaris software. All image data files can be found at https://doi.org/10.5281/zenodo.6868821. The fluorescence signal assumed to be of prey (Channel 1) is clearly observed in Acantharia after 60 minutes. Subsequent hours show a loss of this signal indicating a higher rate of digestion than uptake. LFLA, live fluorescently labelled algae.

The ingestion of LFLA in these non-symbiotic cells appears initially faster, *i.e.*, after already 30 min. Signals of LFLA in symbiotic Acantharia were best observed after 60 min, and apparently declined after 4 h of incubation (Figure 4). Fluorescence of LFLA cells was observed inside of the central capsule membrane (Figure 3 and Figure 4).

Discussion

Photosynthetic carbon uptake by Acantharia

Results obtained in this study showed that carbon uptake rates do not significantly vary according to nitrogen source (ammonium/nitrate). We measured a total carbon uptake rate of 1112.7 ± 82.1 pg C h^{-1} Acantharia^{-1}, or 14.5% of total holobiont C content assuming 16h of light. This is within range of previous average rates measured in the central North Pacific Ocean (816.7 ± 875 pg C Acantharia^{-1} h^{-1}, Michaels, 1991). As well as, in the Sargasso Sea, 9% of total carbon content (12h light period) (Caron *et al.*, 1995). However, these studies found a huge variation in their experiments. They used ^{14}C while we used ^{13}C as a tracer, but the two techniques should indeed give comparable results (López-Sandoval *et al.*, 2018). The data of Michaels (1991) was most likely highly variable due to a small number of Acantharia cells per sample, specimen size variation, symbiont number differences, and uncontrolled irradiance levels, making direct comparisons difficult. Here we used stable laboratory light and temperature conditions, as well as more specimens per sample that were isolated for specific species. Hence, the variation in our data is much less.

Free-living versus in-hospite symbiont carbon uptake. Acantharia typically harbour 30 to 50 photosymbiotic algae, though symbiont numbers can vary from 10 to > 65 among different species and sizes of Acantharia (Michaels, 1991), see also Figure 3. The primary productivity of one acantharian symbiont, as measured here, averaged 22.3 ± 1.6 pg C symbiont^{-1} h^{-1}, assuming an average of 50 *Phaeocystis* symbionts per Acantharia. For comparison, the free-living alga (*Phaeocystis*) has a primary productivity rate ~55 times lower (*i.e.*, 0.38 ± 0.04 pg C cell^{-1} h^{-1}). Consistent with this increase of *in hospite* carbon uptake and fixation, *Phaeocystis* has been shown to be heavily modified *in hospite* (Decelle *et al.*, 2019; Uwizeye *et al.*, 2021), by increasing the number of chloroplasts as well as several-fold increase in thylakoid density, thereby increasing the photosynthetic efficiency (Decelle *et al.*, 2019). Concurrently, carbon fixation genes, like genes for Calvin Benson cycle enzymes, are found to be upregulated in symbiotic *Phaeocystis* as compared to free-living (Uwizeye *et al.*, 2021).

However, accounting for an increase in the size of *Phaeocystis in hospite*, one should compare the carbon-specific uptake rates. Doing so, a cell diameter of 10 µm can be assumed for enlarged symbiotic cells (Decelle *et al.*, 2019, see also Figure 3 and Figure 4), which would, by estimation, account for a carbon content of 77.3 pg C per symbiont cell. The carbon-specific primary productivity per acantharian symbiont cell would then be 0.29 ± 0.02 pg C [pg C]$^{-1}$ h^{-1}. This rate is less than 5 times more than the free-living symbiont's carbon-specific uptake rate (0.065 pg C [pg C]$^{-1}$ h^{-1}). The symbiont's cellular composition and structural changes, like the increased number of plastids (up to 60, Uwizeye *et al.*, 2021), are not accounted for in this estimation. The carbon-specific photosynthetic rates are thus not so different between *in hospite* and *ex hospite Phaeocystis* (only a ~5 times higher rate *in hospite*), nonetheless the algae does not achieve the same carbon uptake rates in its free-living state.

Influence of nitrogen on inorganic carbon uptake. Ammonium has been suggested to increase the rate at which carbon is fixed by dinoflagellate coral symbionts in darkness (Cook *et al.*, 1992; Ezzat *et al.*, 2015), although this was not seen in photosymbiotic *Cassiopea* sp. (Lyndby *et al.*, 2020). Possibly this can proceed through the synergy of both host and symbiont pathways in a photosynthesis-independent manner in the urea cycle where ammonium is combined with bicarbonate (*e.g.*, Allen *et al.*, 2011; Burns *et al.*, 2020). Under the conditions described here, we do not see statistically significant differences in C uptake with nitrogen additions. Nevertheless, we saw indications that C uptake might be lower in the treatment with ammonium addition and higher with nitrate addition, as compared to without the addition of these N-sources. Internal recycling of N is expected in endo-photosymbioses, and thus acantharian symbionts may not be N-limited. However, it has previously been shown that with increasing ammonium assimilation, catabolic process and stored carbon (*e.g.*, starch, sucrose) may be used to fulfil the extra carbon demands (Elrifi & Turpin, 1986; Huppe & Turpin, 1994; Turpin, 1991). If stored carbon is used, this might explain the lower $\delta^{13}C$ ratio observed. Whereas nitrate would demand more photosynthetic activity to satisfy the energetic demands for its reduction, it was however still taken up at a lower rate than ammonium.

In our experiments, no photosynthesis independent carbon uptake was measured, even though carbon assimilation in the dark may be significant in other photosymbiotic associations (Röthig *et al.*, 2021). In a preliminary photosynthesis experiment we have measured photosynthetic carbon uptake at low irradiances, see Extended Data Figure 6 in the *Extended data* section (Mansour *et al.*, 2022). In that low irradiance experiment uptake of carbon was only seen in the treatments with ammonium addition, though at a low rate, suggesting there might be a role for ammonium in affecting carbon uptake under certain conditions.

Inorganic nitrogen uptake by Acantharia

Both ammonium and nitrate are significantly taken up by the Acantharia. Though ammonium is taken up at a higher rate, as might be expected due to its lower energetic demands (von Wirén *et al.*, 2000), and is similar in planktic photosymbiotic Foraminifera (LeKieffre *et al.*, 2020). Lower nitrate uptake could also be attributed to the sufficient presence of ammonium available to the microalgal photosymbiont through the use of waste products of the acantharian host, as in photosymbiotic corals (Ezzat *et al.*, 2015; Grover *et al.*, 2002).

The nitrogen uptake rate from ammonium is, however, rather low (6.0 ± 4.0 pg N h^{-1} $cell^{-1}$). For the ammonium treatment, the ratio of carbon to nitrogen uptake is 178 (mass basis); other sources of nitrogen (inorganic or organic) must thus be used to constitute a cellular C/N ratio of 8.9 (mass basis) for Acantharia.

We were not able to measure ammonium uptake in the dark. This could be due to technical limitations, seeing as no isotopic ratio for nitrogen could be established in the dark. However, considering that it was possible to measure ammonium uptake in the light, this seems less likely. Photosynthesis-independent processes for ammonium assimilation might still be possible. The fact that we did not see a significant uptake of ammonium in complete darkness could mean that the energy needed for its assimilation might need to come from symbiont processes (photosynthesis); assimilation of ammonium in complete darkness might thus not be possible or efficient. For radiolarian Collodaria, we did see ammonium uptake in the dark (Extended Data Figure 8 in the *Extended data* section [Mansour *et al.*, 2022]). An important difference here between the Acantharia and Collodaria is that Acantharia host haptophyte symbionts, whereas Collodaria host dinoflagellates. Some dinoflagellates have been reported to use ammonium in the dark (Dagenais-Bellefeuille & Morse, 2013). In dinoflagellate bearing photosymbioses ammonium uptake in the dark has been demonstrated in several studies. For example, corals efficiently take up ammonium in the dark (Grover *et al.*, 2002). Photosymbiotic planktic Foraminifera and the photosymbiotic jellyfish *Cassiopea* are likewise shown to take up ammonium in the dark (LeKieffre *et al.*, 2020; Lyndby *et al.*, 2020). Additionally, nitrate uptake in the dark has also been shown in *Cassiopea,* as well as in corals (Freeman *et al.*, 2016; Kopp *et al.*, 2013).

Grazing—organic nutrient uptake—by Acantharia

Observations and quantitative measurements of grazing remain a difficult task on uncultured planktic species. Out of the three microalgal species tested, statistically significant grazing could only be established on *E. voratum,* with a grazing rate of 729 prey $Acantharia^{-1}$ $hour^{-1}$ (56.3 ng C h^{-1}). Such an ingestion rate could potentially provide 46% of total acantharian C content in an hour. The method used for measurements of grazing rates depends on the initial ratios of prey to predator concentrations. If the prey to predator concentration is too high, grazing rates cannot be measured. The cell densities of the algae used in these experiments were quite high, higher than they typically will be in natural systems.

Despite being unable to establish significant Acantharia ingestion rates on *I. galbana*, indication of prey ingestion has been observed using LFLA (Figure 4) and stable isotope labelled algae (Extended Data Figure 7 in the *Extended data* section [Mansour *et al.*, 2022]). The LFLA fluorescent signal did not coincide with a chlorophyll signal of the algae, possibly due to rapid digestion of algal pigments upon ingestion and internalisation (Moore & Gelder, 1983; Mayzaud & Razouls, 1992). Staining signal visible in the Acantharia host could correspond to labelled food vacuoles, as the size is already smaller than that of the original LFLA, and/or alternatively stain that has dissipated into the Acantharia. Dispersal of stain to the predator has been observed previously, but it is more commonly an issue with other feeding mechanisms such as peduncle feeding (Ferreira *et al.*, 2021). Continuous fluorescent monitoring of algal cells during grazing could better elucidate the digestion process and would be possible using this method, considering the stability of the Celltracker Blue stain that experiences minimal bleaching (Ferreira *et al.*, 2021).

Our grazing rates on *E. voratum* were obtained from 4 h incubation experiments. No significant grazing rates by Acantharia could be obtained from experiments lasting 24 h. Visual inspections revealed more death of Acantharia, and dinoflagellates accumulated around and circled near-dead Acantharia cells. It is unclear if this was a form of predation by the dinoflagellates, nor whether this would have been sapotrophy by the dinoflagellates, or if they were cause of acantharian mortality. Indeed, *ex hospite Symbiodinium* clade E cultures (*E. voratum* was formerly *Symbiodinium* clade E) have been shown to be both prey and predator capable of grazing to supplement their nutrients (Jeong *et al.*, 2012; Jeong *et al.*, 2014).

Natural isotopic ratios to investigate trophic modes of Radiolaria

Isotopic ratios are widely used to analyse food webs and define trophic levels of organisms. The $\delta^{13}C$ trophic fractionation over trophic levels is close to zero *i.e.*, 0.4‰ (SD = 1.3) (Post, 2002), thus $\delta^{13}C$ change with trophic level is not large, though slightly more negative values are expected with more heterotrophy. The $\delta^{13}C$ can, however, shift more substantially based on photoautotrophy. For plants, generally, those with a CO_2 concentrating mechanism (like C4, CAM) have higher $\delta^{13}C$ values, ~-7‰, while those without (C3 plants) have $\delta^{13}C$ values of ~-22‰ (Beardall, 1989). In contrast to the $\delta^{13}C$ ratio, the $\delta^{15}N$ ratio increase due to predation, typically ~3 to 4.5% with each trophic level. $\delta^{15}N$ ratios can thus be used to help define the trophic level of an organism (Fry, 2006; Post, 2002), whereas $\delta^{13}C$ ratios would rather give clues on the source of the nutrients (Fry & Sherr, 1989).

In mixotrophic organisms both the photosynthetic and phagotrophic metabolisms influence nutrient assimilation, and thus isotopic ratios will reflect a mix between the organic and inorganic food sources, making it difficult to assess the original nutrient source (Ferrier-Pagès & Leal, 2018). Both the $\delta^{13}C$ (-22.5‰) and $\delta^{15}N$ (7.1‰) ratios of Acantharia might imply a trophic level similar to Mediterranean zooplankton (Bănaru *et al.*, 2014; Vizzini & Mazzola, 2006). The natural isotopic ratios of carbon and nitrogen of Acantharia might suggest a higher contribution of organic (grazing) than inorganic (photosynthesis) nutrition.

This is, however, better compared locally, as nutritional status and nutrient sources can differ seasonally and geographically, affecting C/N and delta ratios (Bănaru *et al.*, 2014; Dobberfuhl & Elser, 2000).

Taking Collodaria as an example, we measured an average $\delta^{13}C$ ratio of -14.9 ± 4.1‰ (n = 18) in autumn (September) 2018, and -20.9 ± 1.5‰ (n = 4) during spring (April) 2019 (Extended Data in the *Extended data* section [Mansour *et al.*, 2022]). This could imply seasonal differences in nutrient sources, for example, towards spring collodarian trophic mode could be more heterotrophic than autotrophic, and *vice versa* towards autumn. In spring, a phytoplankton bloom would subsequently allow heterotrophic organisms to increase due to the abundance of biomass to graze on (Hansen *et al.*, 2019). This results in more heterotrophy and a lower $\delta^{13}C$, as we observed for the Collodaria during spring. During the following summer period of high light and low nutrients, the Collodaria could resort to a more autotrophic lifestyle, which would explain the higher $\delta^{13}C$ values we measured nearing autumn. Such a trend in changing isotopic ratios throughout the seasons has also been observed in corals (Ferrier-Pagés *et al.*, 2011), and seasonal dependency on differing trophic modes has also been predicted by model simulations (Gonçalves Leles *et al.*, 2021; Mitra *et al.*, 2014). The seasonal difference in $\delta^{13}C$ for Acantharia was not as pronounced as for Collodaria, with for Acantharia a $\delta^{13}C$ of -19.7± 6.6‰ (n = 10) in autumn (September) 2018 (Extended Data in the *Extended data* section [Mansour *et al.*, 2022]) and -22.5 ± 2.3‰ (n = 11) during spring (May) 2021. Overall, it might suggest that these mixotrophic Radiolaria rely more on heterotrophy and external (organic) food sources around spring, whereas summer and autumn would be a period where phototrophy is exploited.

Additionally, we measured a lower $\delta^{13}C$ ratio for Acantharia than for Collodaria (Extended Data in the *Extended data* section [Mansour *et al.*, 2022]). As noted, $\delta^{13}C$ tends to increase with photoautotrophy and decrease with heterotrophy, which might suggest a more heterotrophic role in the food web for Acantharia than for Collodaria. Symbiont differences likely play a major role in the isotopic difference between these Radiolaria. The relevant β-carboxylase for dinoflagellates and haptophytes (major symbionts for Collodaria and Acantharia respectively) are different (Raven, 1997). This is not only relevant for possible carbon isotopic discrimination differences, but also for the fact that in the marine environment two inorganic carbon sources are available for photosynthesis: CO_2 and HCO_3^-. Dissolved CO_2 in seawater has a $\delta^{13}C$ value of around -7‰ whilst that of HCO_3^- is more enriched in ^{13}C and close to 0‰. When the activity of the photosynthetic carbon reduction cycle decreases fixation of HCO_3^- by β carboxylase becomes more important. Additionally, compared to haptophytes, dinoflagellates generally have higher dark respiration (Geider & Osborne, 1989), where the utilisation of HCO_3^- might be of more importance. Rost *et al.* (2006) estimated that HCO_3^- uptake accounted for > 80% of photosynthetic carbon fixation for three species of marine dinoflagellates. Hence using HCO_3^- (*i.e.*, the product with the higher $\delta^{13}C$ value) could increase the overall $\delta^{13}C$ of the collodarian holobiont. Using natural isotopic ratios of these Radiolaria in comparison to other organisms in the local food web might further help identify their reliance on either phototrophy or phagotrophy.

Inorganic and organic nutrient uptake balance of Acantharia

Photosynthesis has been shown to be a requirement for survival of other Radiolaria belonging to the Spumellaria (Swanberg & Anderson, 1985). Photosynthesis is expected to be a major part of the acantharian metabolism since modifications are made on the photosynthetic system in symbiont cells. Though we do not have data on daily respiration, photosynthesis could contribute 17.8 ng C on a 16 h light period per day. Thereby constituting daily 14.5% (0.9% hourly) of total Acantharia carbon content (122.8 ng C), 3.86 ng C (or 3.1% of the holobiont C content) is estimated to be attributed to symbionts, assuming 50 symbionts of 10 µm ESD with 77.3 pg C per symbiont. The photosynthetic C uptake would thus be expected to fulfil the symbionts nutritional requirements. The net daily photosynthetic carbon uptake is likely much lower due to respiration, which we cannot currently account for. In contrast, the possible overestimation might be counteracted by the fact the symbionts which live in the central capsule of host can use 'recycled' CO_2 from the host's respiration (as also suggested previously by Caron *et al.*, 1995). Thus possibly being a factor for underestimating photosynthetic rates, particularly when the respired CO_2 would be more easily accessible than the seawater carbon source that has to pass several host membranes.

Grazing could potentially provide 46% of total acantharian holobiont carbon content in as little as an hour. The variety of food items utilised by these Radiolaria is wide (Swanberg & Caron, 1991). Still, food in absence of light could not extend the survival of Spumellaria, thus light is required for growth and survival Spumellaria (Swanberg & Anderson, 1985). Likewise, it has been suggested that photosynthesis needs to be supplemented with food uptake for spumellarian and collodarian Radiolaria (Swanberg, 1983; Swanberg *et al.*, 1986). Photosynthesis is hypothesised to predominately provide energy in some mixotrophic organisms (Terrado *et al.*, 2017; Wilken *et al.*, 2014). This might also be expected in Acantharia considering the inorganic and organic carbon uptake potentials. It will be important to understand acantharian respiration rates to be able to conclude if photosynthetic rates can provide the Acantharia with the carbon for growth (assimilation) and/or survival (energy).

Results obtained in this study suggest that the uptake of inorganic nutrients by Acantharia depends on the symbiont, as light is needed for uptake of dissolved inorganics. Though considering the high inorganic C/N uptake ratio a supplemental uptake of N from organic food sources is expected. Hence food might constitute the majority of N uptake. How symbiont and host benefit from phototrophic or phagotrophic nutrient sources might be further investigated by comparison

of natural isotopic ratios of the symbiont and host (symbiont would need to be separated from the host). The uptake of inorganic nitrogen has not been entirely resolved with our results. The current bulk sample methodology is limited by biomass requirements. An increased amount of cell material would be needed to consistently quantify inorganic nitrogen uptake for these unculturable protists. The feasibility of such work is low because of the need for sufficient replicates. The next step of this work will be to verify and assess how the assimilated nitrogen (and carbon) is partitioned between algae and host. We suggest that by elucidating the primary location of nitrogen assimilation (*e.g.*, by single-cell nanoSIMS), and the genetical/transcriptional capability of host and symbiont for processes involved in nitrogen metabolism (*e.g.*, urea cycle), the role of the symbiont and host in nitrogen uptake and assimilation could be addressed. Thereby it might also resolve if the photosynthates are used and assimilated by the acantharian cell and/or if it is used for catabolic processes to obtain energy.

Data availability

Underlying data

NCBI Nucleotide: Acantharia cells and swarmers 18S. Accession numbers OK157864 to OK157871.

Zenodo: Data for: Carbon and nitrogen uptake through photosynthesis and feeding by photosymbiotic Acantharia (Radiolaria). https://doi.org/10.5281/zenodo.6868821 (Mansour *et al.*, 2022).

This project contains the following underlying data:

- EAIRMS_data_for_Acantharia-Table1_Table2_Figure1_ETable3.xlsx (*pertains isotopic data of the* Acantharia *photosynthetic C and N uptake experiments)*
- Flowcytometry_data-Grazing_rates-Table3.xlsx (pertains cell count data of prey items used for acantharian grazing experiments)
- Photosynthetic_rates_and_chla_summary_of_algal_culture_data.xlsx (pertains photosynthetic rates of Phaeocystis cultures as measured using ^{14}C techniques, and chla concentrations of relevant cultures)
- Imaging_data-Acantharia_grazing.zip (This is data of the fluorescence confocal microscopy and is organised by experimental timepoint. It includes the .tif files used in the manuscript, and the original .lif files of the Leica microscope)

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

Extended data

Zenodo: Data for: Carbon and nitrogen uptake through photosynthesis and feeding by photosymbiotic Acantharia (Radiolaria). https://doi.org/10.5281/zenodo.6868821 (Mansour *et al.*, 2022).

This project contains the following extended data:

- Extended_data_Text_Figures_Tables.pdf (Supplementary information to "Carbon and nitrogen uptake through photosynthesis and feeding by photosymbiotic Acantharia". Contains all figures, table and legends, figures are also separately available in this dataset)
- E Fig 1 cells.tiff
- E fig 2 photo exp design 2022.png
- E fig 3 feeding exp design.png
- E fig 4 PAM supp image.png
- E fig 5- nonsymbiotic Acantharia.tif
- E fig 6 Acantharia prelimn d13C Graph exl outlier.png
- E Fig 7 - Acantharia SI-prey feeding graph
- E fig 8 - Graph collodaria d15N.png
- E table 1 - Culture information.xlsx
- E Table 2 - medium specifics.xlsx
- Extended_data-PAM raw data_EFig4.xlsx (Data underlying E Fig 4)
- Extended_data-isotopic values september 2018 Collodaria and Acantharia.xlsx (pertains isotopic data of Collodaria and Acantharia samples from September 2018; data underlying E Fig 6)
- Extended_data-EAIRMS data Collodaria -april2019-reprocessed25-9-2019_E fig 8.xls (pertains underlying data for E Fig 8)
- Extended_data-Isotopically labeled prey plus Acantharia_EAIRMS_and_Flowcyto_prey_count_data_E Fig7.xls
- Fig 2- 3D reconstruction video simulation-Acantharia T60_cell_1_stained.avi
- Image_data-Acantharia_grazing.zip (Photographs of the grazing experiments)

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

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Open Peer Review

Current Peer Review Status: ? ✗ ?

Version 2

Reviewer Report 05 July 2023

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Ramon Massana
Ecology of Marine Microbes, Institut de Ciènces del Mar (ICM-CSIC), Barcelona, Spain

This is an interesting and complete study that compares the role of photosynthesis and phagocytosis for energy and food acquisition in acantharean cells, a group of large phagotrophic protists that act as mixotrophs by virtue of their endosymbiotic algae. The authors used stable isotope probing with inorganic carbon and nitrogen to measure the incorporation of inorganic carbon by photosynthesis and grazing experiments (by prey disappearance) to measure the prey capture. The results are interesting, but I guess could be better explained in several parts of the manuscript. I only have a few comments and suggestions for further improvement of this manuscript.

A big issue when working with acantharean species is that no culture exists for them. So, experiments and measures taken here have been done with freshly collected cells from the Mediterranean Sea. Which is perfectly fine, but the way that the data is presented (photosynthesis or grazing rates) is scaled to "acantharean cells", giving little attention that different species can be involved, and that each species may have different physiological performances. Later at the beginning of the results there is the mention that most cells belong to 2 genera, but this should be central in the manuscript (already at the first page). It does not make sense to refer to "acantharean cells", giving the expected large phylogenetic and physiological diversity within this group. A bit related to this issue, what is the cell size of the cells manipulated here? Are all manipulated cells within a comparable size range?

The section Results/Conclusions in page 1 could be much more focused. "Carbon uptake rates" could refer as well to organic carbon. You can also mention the three preys used for phagotrophy and the results for each prey. Also, according to the data, 46% of total halobiont content was replaced per hour by phagotrophy (in a way, I found this way too high, is this realistic?). In contrast, photosynthesis represents only 0.9% hourly. Based in these two values, one would make the strong conclusion that phagotrophy is much more important than photosynthesis, the later contributing very little to the needs of the acantharean cells. If this is right, I miss this conclusion in a very explicit way.

There is not mention to prey abundance in the grazing experiments, which is critical for the measured rates. Were the experiments done at saturating concentrations?

Page 5. I didn't get how the acantharean cells were manipulated in the 6-wells plates. For photosynthesis, one cell in a well or many cells together? Later for phagotrophy, I do not follow the set-up, which included "triplicates", "15-31 cells", and 6-wells plates.

I am puzzled by the difference in photosynthetic rates between free-living and symbiont Phaeocystis. Initially rates were 55 larger in symbionts, which for me was a bit counterintuitive. Then, after some calculations, it remains only 5 times larger, to end up saying that they were similar....

Fig. 2A. It is not clear the border between "Natural" and "Enriched". In Fig 2B, all treatments (x-axis) are enriched, right? Perhaps you should add it here, for consistency.

Table 3. For grazing rates, what is important is the "prey concentration", not the prey/predator ratio, as grazing rates may change with prey abundance (this is the functional response). Also, it is not clear to me why showing so many numbers, if they are not credible, does it make sense? It adds more confusion than real value...

Figure 3. In the legend, it would help to be much more specific: blue dots for the prey, purple dots for symbionts.

Page 13. Is there anything that can be said about the dynamics of the food vacuoles? Is each prey in a separate position of the cytoplasm, so potentially one food vacuole for prey? Or there are some prey cells compacted in the same vacuole?

Page 13. The section on the discussion about isotopic rates of freshly collected cells, to infer the heterotrophic-phototrophic status, appears here but has been not presented in the results section. Shouldn't the primary data be shown in the Results section?

Page 14. The two values reported of delta 13C for acantharean cells in different seasons, -19.7 and -22.5, together with their deviations, can justify to infer seasonal differences in the trophic status?

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and does the work have academic merit?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Microbial ecology, protist ecology, genomics

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 23 June 2023

https://doi.org/10.21956/openreseurope.16748.r32157

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Joaquim Goes
Columbia University, New York, New York, USA

This research on Acantharia is clearly an important study, that seeks to quantify the role and importance of prey ingestion and symbiont photosynthesis in meeting the metabolic requirements of this mixoplanktonic entity. The study is well designed and the methods and approach are state-of-art and adequate to address the central goals of this study. Unfortunately ambiguities in the results and discussion left me disappointed and questioning whether other than getting an estimate of the inorganic carbon uptake rates, and its preference for ammonium over nitrate, the results and discussion left me with more questions than answers. I have made several of my comments in the pdf version of this article with the hope that the authors will make edits and strengthen their conclusions. For instance I found difficulty in understanding what kind of data the grazing experiments yielded if the host cells were dead at the end of the experiment? Obviously they were physiologically unhealthy and super-stressed if they died during the experiments.

I have made several comments throughout the text and I will urge the authors to pay close attention to language as many a times I had to read a paragraph several times to understand its content.

Throughout the discussion, I found it difficult how earlier work being cited was related to the results of this study. If this study was focused on Acantharia why bring in Collodaria?

At this moment I would recommend going carefully through the issues and questions that I have in the pdf version.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and does the work have academic merit?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Mixotrophy

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Version 1

Reviewer Report 22 December 2022

https://doi.org/10.21956/openreseurope.16196.r30327

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Haruka Takagi
[1] Department of Earth Sciences, Faculty of Science, Chiba University, Chiba, Japan
[2] Department of Earth Sciences, Faculty of Science, Chiba University, Chiba, Japan

General comments

The manuscript entitled “Carbon and nitrogen uptake through photosynthesis and feeding by photosymbiotic Acantharia” by Mansour et al. examined the trophic ecology of unculturable protist Acantharia that harbor photosymbionts. They conducted multiple experiments to

investigate phototrophy (symbiont photosynthetic activity) and heterotrophy (grazing of various prey), both quantitatively and qualitatively. I am impressed by their devoted time and efforts to conduct all the laborious work. Their experiments were overall well designed and the analyses were sound in most part. However, I have several concerns, especially about the grazing experiment (see below). I hope my comments outlined below will be helpful.

Major points

1. Grazing rate
The authors concluded that 46% of total host C content could be provided by predation of dinoflagellate *E. voratum* (728 prey Acantharia^{-1} h^{-1}). I somehow feel weird that a small host can ingest relatively large phytoplankton in such a large amount. According to the text, the dinoflagellate prey size is 10 μm in diameter. The size of Acantharia is not provided, but it seems ca. 80 μm in endoplasm diameter. Is it physically possible to consume such a large amount of large prey?

I tried to find photo images of the grazing experiment of *E. voratum* in Extended data files, but I couldn't find any. In the text, the authors say, "Visual inspections revealed more death of Acantharia, and dinoflagellates accumulated around and circled near-dead Acantharia cells" for 24h experiment (p13-right column L9–21). If so, similar phenomena could have happened at 4 h time point; it may be possible that the decrease in cell concentration of *E. voratum* was attributable for their accumulation around Acantharia due to predation (*E. voratum* predation on Acantaria) or just by attaching to Acantharia, not by grazing. If such possibility can be denied by visual inspection, please include such statements, and describe how the prey and predator were interacting (e.g., *E. voratum* cells could be observed within the endoplasm, pseudopods seemed to capture *E. voratum* cells, etc...). If the authors insist on arguing that their interpretation on the grazing experiment results simply reflect grazing by Acantharia, I strongly request images to indicate that.

This is an option, but I would suggest to use the other type of control to conduct grazing experiment. The same number of dead Acantharia individuals can be added to the control wells (C1–C3). In this experimental design, you can cancel out the effect of predation by the algae or the other effect causing accumulation of the algae due to the presence of Acantharia.

2. Calculation of grazing/ingestion rate

The authors applied Frost (1972)'s and Heinbokel (1978)'s calculations. I checked the papers, and found that the calculations were designed for copepods (Frost, 1972) and tintinnids ciliate (Heinbokel, 1978). The clearance rate *F* is indeed applicable to copepods, of course, and ciliate as well because they have cilia that enable them to swim around. However, Acantharia is immotile, as far as I know, so the "clearance rate" sounds strange for this taxon. Alternatively, prey can swim; dinoflagellates can actively swim around using their flagella. So I can imagine that the encounter rate of *E. voratum* is much higher than the other algae, resulting in a higher value of ingestion rate of the dinoflagellate prey. I would recommend using different calculation methods to estimate the ingestion rate.

In addition, the point I feel the gap is that the contribution of grazing is consistently discussed

based on the *E. voratum*data, whereas the evidence of grazing is all based on *I. galbana* data (the quantitative part is derived from *E. voratum*data, and the qualitative part is derived only from *I. galbana* data). It is no doubt that *I. galbana* is incorporated and ingested by Acantharia, as shown in Figure 4, but this phenomenon was not detected by quantitative experiments (probably due to the too-high concentration of prey and different time point investigated). So, from this study, quantitative data and qualitative data were not coupled. I think you need to be very careful to say about the rate. At least, the evidence that *E. voratum* is eaten by Acantharia is strongly needed (as I mentioned above).

3. C assimilation rates of Caron et al. 1995[1]
Caron et al. (1995) also measured host-symbiont primary production of rhizaria, though their method is ^{14}C experiment. Comparing the results of this study to theirs would be very useful. Actually, in their paper, Acantharian primary production is reported as 0.0076 ng C [ng C]$^{-1}$ h^{-1} in average (daily production is 9% of host-symbiont C), which is comparable to the results of this study. This literature should be cited, and the data needs to be compared in the discussion. It would also be helpful to include a comparison table showing data of previous studies and this study.

4. Possible effect of host respired CO_2
Based on the confocal images, the symbionts are all inside the host's central capsule membrane. It is presumed that access to the seawater carbon source (HCO^{3-}) is harder than the carbon source from the host respired CO_2, if any. In other words, the calculated carbon assimilation rate was more or less underestimated if the symbiont used the respired CO_2. Such a phenomenon (use of host's respired CO_2 for symbiont photosynthesis) has been discussed, at least in planktonic foraminiferal symbiosis (see Caron et al. 1995). I suggest including such a possibility in the discussion section because it directly affects the estimation of the contribution of phototrophy. I understand that without information on respiration rate or photosynthetic rate measured by different methods (e.g., O_2 production), it is difficult to quantitatively discuss the contribution of respired CO_2. However, considering the location of symbionts and the close relationship between the host and symbionts, it is very likely that this is the case and is worthy of inclusion in the text.

Minor points

Unit of carbon-specific photosynthesis rate: throughout the text, the unit is written as "pg C C^{-1} h^{-1} ", but it must be "pg C [pg C]$^{-1}$ h^{-1}". (e.g., for free-living *Phaeocystis* value, 0.38 pg C cell^{-1} h^{-1} ÷ 5.8 pg C cell^{-1} = 0.065 pg C [pg C]$^{-1}$ h^{-1})

p4-right column L42: galbana. Please italicize.

p5-left column L26: "In triplicate, Acantharia (15 to 25 cells) were …" According to the Extended data table, I found the smallest number of Acantharia of 13 and the largest number of 31. Please check and correct the numbers in the text.

p8-left column L16–19: "Total dissolved inorganic…TOC-L analyzer, Shimadzu." The same sentence

has already appeared in the method section (p5-right column L17–20). Please delete either of the two.

p8-left column L47–48: 122.8 ± 25.1 µg C cell^{-1} and 14.9 ± 5.0 µg C cell^{-1} Is the unit correct? µg should be ng?

p10 caption of Table 3: "rejection of the hypothesis is indicated in bold. There seem to be no bold letters in the table. Please correct.

p12-left column L10: *Cassiopea* sp. Later on, it is explained that *Cassiopea* is a photosymbiotic jellyfish, but should be noted at this point since here is the first appearance of this taxon name for easier understanding for readers.

p14-left column L3: 20.9 ± 1.5 ‰ Maybe –20.9 ± 1.5 ‰?

p14-left column L25: 22.5 ± 2.29 ‰ Maybe –22.5 ± 2.29 ‰?

p14-right column L1–4: "A lower $\delta^{13}C$ could also imply a heterotrophic food source; big Collodaria colonies are more likely to consume larger zooplankton. These can all result in a higher $\delta^{13}C$ value for Collodaria compared to Acantharia." This part confuses me. "A lower $\delta^{13}C$" of what? Collodaria? In my understanding, Collodaria's $\delta^{13}C$ is higher than Acantharian's (as written in the text), and it is attributable to the more photoautotrophic nature of Collodaria. In that sense, what does the sentence "big Collodaria colonies consume larger zooplankton" explain?

p14-right column L16: "4.6 ng C (or 3.1% of the holobiont C content) is estimated to be attributed to symbionts". I could not understand what this value is based on. Please explain it in more detail. 122.8 ng C is the C content of holobiont (Acantharia+symbionts), in my understanding. If so, 3.1% of holobiont C content is 3.8 ng.

References

1. Caron D, Michaels A, Swanberg N, Howse F: Primary productivity by symbiont-bearing planktonic sarcodines (Acantharia, Radiolaria, Foraminifera) in surface waters near Bermuda. *Journal of Plankton Research*. 1995; **17** (1): 103-129 Publisher Full Text

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and does the work have academic merit?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: photosymbiosis in planktonic foraminifera

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 27 Feb 2023

Joost MANSOUR

Thank you kindly for taking the time to review our manuscript in detail and bring very valuable comments. We address each comment below.

Major points

1. Grazing rate
The authors concluded that 46% of total host C content could be provided by predation of dinoflagellate *E. voratum* (728 prey Acantharia^{-1} h^{-1}). I somehow feel weird that a small host can ingest relatively large phytoplankton in such a large amount. According to the text, the dinoflagellate prey size is 10 µm in diameter. The size of Acantharia is not provided, but it seems ca. 80 µm in endoplasm diameter. Is it physically possible to consume such a large amount of large prey?

The central capsule of our Acantharian samples we estimate to be ~100µm (ESD) (E fig 1); the outer cytoplasm can add another 150+ um not including the many pseudopodal extensions. So overall, the Acantharian cells are easily 250+ µm. For comparison, ciliates (ESD of (~38µm) can have ingestion rates up to 58 prey predator^{-1} h^{-1} (Hughes et al., 2021, Maselli et al., 2020). Indeed relative to size Acantharian uptake rate would be twice that of ciliates. Or, with 77.3 ng C prey^{-1}, the C uptake rate of 728 prey/predator/h would be 56.3 ng C h $^{-1}$. When assuming 122.8 ng C per Acantharia the C specific ingestion is then 11 ngC [ng C]$^{-1}$ d^{-1} **, assuming 24 hrs of activity, less activity is more likely, so this is an overestimation. This is thus indeed a large number of prey ingested per unit time, though not more than their own C content.**

Indeed, prey concentrations used in this experiment are unlike natural encounters, thereby probably overestimating actual grazing rates. Also, the uptake of larger organisms such as copepods has been reported (Swanberg and Caron 1991), which would be a faster means to acquire nutrients. A single copepod would provide many times the C (Traboni et al.,2021) of these other prey.

I tried to find photo images of the grazing experiment of *E. voratum* in Extended data files,

but I couldn't find any. In the text, the authors say, "Visual inspections revealed more death of Acantharia, and dinoflagellates accumulated around and circled near-dead Acantharia cells" for 24h experiment (p13-right column L9–21). If so, similar phenomena could have happened at 4 h time point; it may be possible that the decrease in cell concentration of *E. voratum* was attributable for their accumulation around Acantharia due to predation (*E. voratum* predation on Acantaria) or just by attaching to Acantharia, not by grazing. If such possibility can be denied by visual inspection, please include such statements, and describe how the prey and predator were interacting (e.g., *E. voratum* cells could be observed within the endoplasm, pseudopods seemed to capture *E. voratum* cells, etc...). If the authors insist on arguing that their interpretation on the grazing experiment results simply reflect grazing by Acantharia, I strongly request images to indicate that.

All experiments were visually inspected during sampling at each time point, and some photos were taken. Samples were of course gently mixed before sampling to minimize the accumulation/sticking confounding factor. This has now been emphasized in the methods. Images were taken initially only at T0 to minimize time spent under the bright microscope light and only with the goal to assess the Acantharia and their physiological state. Thus giving a snapshot of the Acantharia used for the experimental incubations. Indeed, for *E. voratum,* we also made images after 4 hrs. Yes, some Acantharia death was observed after 4hrs, but this was very much intensified after 24hrs. Although, these images were not made with the intention of verifying feeding behavior we have added these to the Extended data for completeness. The dinoflagellates that might be sticking to the Acantharia were not present in such numbers that are likely to affect the statistical significance testing. Even if assuming 100 dinoflagellates stick to one Acantharia, thus putting all those back in the free algal pool for the calculations (effectively increasing the cells ml^{-1} by approx. 2000), the p value would change to 0.00685 (from 0.00040). Finally, as mentioned, we cannot actually account for apparent loss of prey in flow cytometry counts which would be due to sticking to the Acantharia cell surface without actual ingestion, therefore one would consider these preys to eventually be consumed.

This is an option, but I would suggest to use the other type of control to conduct grazing experiment. The same number of dead Acantharia individuals can be added to the control wells (C1–C3). In this experimental design, you can cancel out the effect of predation by the algae or the other effect causing accumulation of the algae due to the presence of Acantharia.

The experimental/control wells were of course gently mixed (by pipetting) before sampling to minimize the accumulation/sticking confounding factor. Adding fixed Acantharia to the control might be a good option for future experiments. But the number of non-surviving Acantharia to the experimental bottles cannot be predicted. Nevertheless, the number of dead Acantharia in the experiment was accounted for in the calculations.

2. Calculation of grazing/ingestion rate

The authors applied Frost (1972)'s and Heinbokel (1978)'s calculations. I checked the papers,

and found that the calculations were designed for copepods (Frost, 1972) and tintinnids ciliate (Heinbokel, 1978). The clearance rate *F* is indeed applicable to copepods, of course, and ciliate as well because they have cilia that enable them to swim around. However, Acantharia is immotile, as far as I know, so the "clearance rate" sounds strange for this taxon. Alternatively, prey can swim; dinoflagellates can actively swim around using their flagella. So I can imagine that the encounter rate of *E. voratum* is much higher than the other algae, resulting in a higher value of ingestion rate of the dinoflagellate prey. I would recommend using different calculation methods to estimate the ingestion rate.

Indeed, we generally assumed no mobility of the Acantharia. However, Acantharia do have some surprising mobility (videos of this can be found online, e.g., https://twitter.com/JoostMansour/status/1571088356955820032 ; https://brisbinplankton.wordpress.com/a-very-active-acantharian/). Nonetheless, the calculations do not take mobility into account, they are solely based on a growth rate or cell density, which is how such rates are commonly calculated for all kinds of planktonic organisms. The rates are in essence only based on the concentration of prey and predator. We are not aware of any more appropriate calculations, but would be happy to be made aware if those exists.

In addition, the point I feel the gap is that the contribution of grazing is consistently discussed based on the *E. voratum* data, whereas the evidence of grazing is all based on *I. galbana* data (the quantitative part is derived from *E. voratum*data, and the qualitative part is derived only from *I. galbana* data). It is no doubt that *I. galbana* is incorporated and ingested by Acantharia, as shown in Figure 4, but this phenomenon was not detected by quantitative experiments (probably due to the too-high concentration of prey and different time point investigated). So, from this study, quantitative data and qualitative data were not coupled. I think you need to be very careful to say about the rate. At least, the evidence that *E. voratum* is eaten by Acantharia is strongly needed (as I mentioned above).

Yes, we totally agree. Unfortunately, due to the unculturablility of Acantharia, we have yet been unable to do similar labelling and microscopy experiments for other potential prey species other than I. galbana. This would be a great place to start follow up research.

3. C assimilation rates of Caron et al. 1995[1]
Caron et al. (1995) also measured host-symbiont primary production of rhizaria, though their method is ^{14}C experiment. Comparing the results of this study to theirs would be very useful. Actually, in their paper, Acantharian primary production is reported as 0.0076 ng C [ng C]$^{-1}$ h^{-1} in average (daily production is 9% of host-symbiont C), which is comparable to the results of this study. This literature should be cited, and the data needs to be compared in the discussion. It would also be helpful to include a comparison table showing data of previous studies and this study.

Indeed, we should have compared the rates to Caron et al., 1995. For different Acantharia the measured primary production rates are between 10 and 100000 pg C organism^{-1} h^{-1} with the overall average at 2200 pg C organism^{-1} h^{-1}. This is a huge range, as is also noted in that original manuscript. We added a sentence comparing

our values to the studies of Michaels et al., 1991 & Caron et al., 1995. We have decided to not include a table due to the limited number of measurements on these organisms.

4. Possible effect of host respired CO_2
Based on the confocal images, the symbionts are all inside the host's central capsule membrane. It is presumed that access to the seawater carbon source (HCO^{3-}) is harder than the carbon source from the host respired CO_2, if any. In other words, the calculated carbon assimilation rate was more or less underestimated if the symbiont used the respired CO_2. Such a phenomenon (use of host's respired CO_2 for symbiont photosynthesis) has been discussed, at least in planktonic foraminiferal symbiosis (see Caron et al. 1995). I suggest including such a possibility in the discussion section because it directly affects the estimation of the contribution of phototrophy. I understand that without information on respiration rate or photosynthetic rate measured by different methods (e.g., O_2 production), it is difficult to quantitatively discuss the contribution of respired CO_2. However, considering the location of symbionts and the close relationship between the host and symbionts, it is very likely that this is the case and is worthy of inclusion in the text.

We agree with the reviewer. We elaborated a bit more on this potential photosynthetic rate underestimation, in addition to the single sentence we had previously had on this subject, in the paragraph 'Inorganic and organic nutrient uptake balance of Acantharia'

Minor points
All minor comments have been corrected and integrated.
Unit of carbon-specific photosynthesis rate: throughout the text, the unit is written as "pg C C^{-1} h^{-1}", but it must be "pg C [pg C]$^{-1}$ h^{-1}". (e.g., for free-living *Phaeocystis* value, 0.38 pg C cell $^{-1}$ h^{-1} ÷ 5.8 pg C cell^{-1} = 0.065 pg C [pg C]$^{-1}$ h^{-1})

Thank you for the correction, it has been rectified.

p4-right column L42: galbana. Please italicize.

Corrected

p5-left column L26: "In triplicate, Acantharia (15 to 25 cells) were …" According to the Extended data table, I found the smallest number of Acantharia of 13 and the largest number of 31. Please check and correct the numbers in the text.

Corrected. 13 was after 24 hour incubation the incubation started with more. Hence, the range was adjusted to 15 to 31

p8-left column L16–19: "Total dissolved inorganic…TOC-L analyzer, Shimadzu." The same sentence has already appeared in the method section (p5-right column L17–20). Please delete either of the two.

Corrected

p8-left column L47–48: 122.8 ± 25.1 µg C cell^{-1} and 14.9 ± 5.0 µg C cell^{-1} Is the unit correct? µg should be ng?

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p14-right column L1–4: "A lower $\delta^{13}C$ could also imply a heterotrophic food source; big Collodaria colonies are more likely to consume larger zooplankton. These can all result in a higher $\delta^{13}C$ value for Collodaria compared to Acantharia." This part confuses me. "A lower $\delta^{13}C$" of what? Collodaria? In my understanding, Collodaria's $\delta^{13}C$ is higher than Acantharian's (as written in the text), and it is attributable to the more photoautotrophic nature of Collodaria. In that sense, what does the sentence "big Collodaria colonies consume larger zooplankton" explain?

This has been rectified and omitted for simplicity

p14-right column L16: "4.6 ng C (or 3.1% of the holobiont C content) is estimated to be attributed to symbionts". I could not understand what this value is based on. Please explain it in more detail. 122.8 ng C is the C content of holobiont (Acantharia+symbionts), in my understanding. If so, 3.1% of holobiont C content is 3.8 ng.

Indeed this seems to be a typo, and the value has been further explained as well.

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