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# Hijacking and Integration of Algal Plastids and Mitochondria in a Polar Planktonic Host

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## 24 **SUMMARY**

25 In oceanic plankton, various host organisms are capable of engulfing and temporarily integrating  
26 microalgae (photosymbiosis) or just their photosynthetic plastids (kleptoplastidy) as a solar-  
27 powered energy source. These cellular interactions can be considered to be representative of  
28 evolutionary steps in plastid acquisition in eukaryotes, but the underlying cellular mechanisms  
29 and dynamics are not fully understood. Here, we studied a kleptoplastidic dinoflagellate (RSD:  
30 Ross Sea Dinoflagellate), which is known to steal plastids of the microalga *Phaeocystis antarctica*.  
31 We tracked the morphology and activity of stolen plastids over several months by combining  
32 multimodal subcellular imaging and photophysiology. Upon integration inside a host vacuole, the  
33 volume of plastids and pyrenoids significantly increased and photosynthetic activity was boosted  
34 along with carbon fixation and transfer to the host. This may be supported by the retention of a  
35 50-fold larger algal nucleus for ~1 week. Once the algal nucleus was lost, there was a decrease  
36 in plastid volume and photosynthesis, but plastids were still beneficial for the host after > 2  
37 months. Unlike other kleptoplastidic interactions, we showed that the algal mitochondrion was  
38 also stolen and retained for several months, transforming into an extensive network in close  
39 proximity with plastids. This highlights a new strategy in plankton along the continuum of plastid  
40 symbioses where both the energy-producing plastid and mitochondrion of a microalga are  
41 hijacked for several months by a host. This symbiosis that we found to be widely-distributed in  
42 polar regions suggests that plastid-mitochondrion interaction may have played a role in the  
43 evolution of plastid acquisition.

44

45

## 46 **INTRODUCTION**

47 Marine plankton include a wide diversity of single-celled eukaryotes that can form complex cell-  
48 cell interactions during their life cycle [1,2]. Among the most intriguing examples are interactions  
49 between heterotrophic hosts that can engulf and temporarily integrate microalgae  
50 (photosymbiosis) or just their photosynthetic plastids (kleptoplastidy) as solar-powered carbon  
51 factories [2]. These plastid symbioses are widespread in the ocean and contribute significantly to  
52 biogeochemical cycles [56]. Photosymbiosis and kleptoplastidy are widely considered to  
53 represent fundamental evolutionary steps in the origin and distribution of plastids in eukaryotes  
54 [3,4]. Despite the ecological and evolutionary importance of photosymbiosis and kleptoplastidy,  
55 the structural and metabolic integration of a photosynthetic machinery into a host remains poorly  
56 understood. This may be explained by the fact that plastid integration in hosts is highly dynamic  
57 and can change over time. Therefore, in order to identify the nature and functioning of complex

58 cellular interactions, it is crucial to acquire quantitative data at relevant spatial and temporal  
59 resolutions by integrating advanced high-resolution imaging.

60 Kleptoplastidy has been observed in multicellular organisms (sea slugs [5], acoels [6]),  
61 and protists (ciliates [7], dinoflagellates [8,9], Euglenozoa [10], Foraminifera [11], centrohelids  
62 [12]) in coastal and oceanic waters. A wide diversity of micro- and macro-algae species can be  
63 the source of plastids, but hosts typically rely on specific algae as prey. For instance, dinoflagellate  
64 hosts can acquire plastids from specific cryptophytes [14], diatoms [19] or haptophytes [9]. The  
65 most intriguing aspect of kleptoplastidy is how stolen plastids function in a host without their  
66 original nucleus for periods lasting from days to weeks [13]. In several kleptoplastidic interactions,  
67 stolen plastids have been reported to benefit to the host, providing photosynthetically-derived  
68 carbon energy (sugars and lipids), as well as nitrogen and sulfur compounds, and vitamins [10,  
69 16]. In some cases, dinoflagellate and ciliate hosts can also steal and sequester the nucleus  
70 (called a kleptokaryon) and mitochondrion of the algal prey for several days [17-19]. When the  
71 algal nucleus is retained, stolen plastids can increase in size, divide and photoacclimate, but  
72 degrade after the nucleus is lost [18, 42 20,60-61]. The mechanisms by which stolen plastids  
73 morphologically and physiologically adapt within a foreign host over time and the underlying  
74 molecular processes regulating functional stolen plastids remain unresolved. Exploring the  
75 cellular mechanisms of kleptoplastidy in protists can provide critical insights into the evolutionary  
76 acquisition of plastids in eukaryotes.

77 A kleptoplastidic dinoflagellate species found in Antarctic waters (Ross Sea Dinoflagellate,  
78 RSD) steals plastids from the bloom-forming haptophyte microalga *Phaeocystis antarctica* [9, 21,  
79 22]. This dinoflagellate is part of the Kareniaceae family, most members of which harbor  
80 permanent plastids derived (*via* tertiary endosymbiosis acquisition) from haptophytes [15]. The  
81 RSD host strictly depends on kleptoplastidy for its growth, most likely by benefiting from  
82 photosynthetic products. In laboratory culture, stolen plastids have been reported to remain for  
83 over two years inside hosts [21]. This capacity to retain plastids for extended periods could be  
84 explained by the genetic toolkit of the host, composed of nucleus-encoded photosynthesis genes  
85 acquired from haptophyte and other microalgal sources (Horizontal Gene Transfer, HGT) [22].  
86 This includes certain genes from Photosystem I and the cytochrome b6/f complex with specific  
87 sequences to target proteins to the stolen plastid. However, some essential nucleus-encoded  
88 genes, such as photosystem II (PsbO), are missing, which should dramatically limit photosynthetic  
89 activity of stolen plastids over time. A potential dependence of photosynthesis on PSI through  
90 Cyclic Electron Transport has therefore been proposed [22, 25]. Fundamental information on this

91 kleptoplastidy is lacking, particularly concerning the dynamics of the interaction and the fate of  
92 stolen plastids over time and whether other algal organelles are retained in the host.

93 In this study, we used the Ross Sea dinoflagellate to investigate how stolen plastids are  
94 integrated and maintained within a host at relevant temporal and spatial scales. We employed an  
95 innovative combination of multimodal imaging and molecular tools, including volume electron  
96 microscopy for three-dimensional (3D) reconstruction of plastid morphology, Nano-scale  
97 Secondary Ion Mass Spectrometry (NanoSIMS) to track carbon fluxes, and photophysiology to  
98 monitor the photosynthetic activity of stolen plastids over several months. This multiscale study  
99 revealed a new strategy in protists whereby the host hijacks not only the plastids, but also the  
100 mitochondria of microalgae, both of which remain functional for several months without their  
101 original nucleus. These closely interacting stolen organelles were morphologically and  
102 metabolically remodeled in the host, with plastids notably exhibiting increased volume and  
103 boosted photosynthetic activity during the first two weeks. This study improves our knowledge on  
104 plastid symbioses in marine ecosystems and provides insights into the potentially fundamental  
105 role of the mitochondrion during the evolutionary acquisition of plastids in eukaryotes.

106

## 107 **RESULTS AND DISCUSSION**

### 108 **Enlargement and enhanced photosynthetic activity of newly integrated stolen plastids**

109 The subcellular organization of the kleptoplastidic association between the microalga *Phaeocystis*  
110 *antarctica* and the dinoflagellate host (RSD), with a focus on the morphology of stolen plastids,  
111 was first investigated by multiscale imaging in the presence of algal prey (fed conditions, Figure  
112 1A). Confocal fluorescence microscopy revealed that host cells contained numerous stolen  
113 plastids ( $n \approx 40$  per cell), which appeared to be larger in size compared to native plastids of the  
114 prey (Figures 1B and 1C). 3D reconstruction and volumetric analyses based on FIB-SEM  
115 (Focused-Ion Beam Scanning Electron Microscopy) images, conducted from three host cells and  
116 eight prey cells, revealed that the volume of stolen plastids was 6 times greater than that of native  
117 plastids of the algal prey ( $11.11 \mu\text{m}^3 \pm 3.30$ ,  $n=14$  vs  $1.91 \mu\text{m}^3 \pm 0.43$ ,  $n=12$ , respectively), and 3  
118 times greater than that of plastids from dividing algal prey ( $3.50 \mu\text{m}^3 \pm 0.62$ ,  $n=9$ ) (Figures 1F and  
119 2A-C; Table S1). Stolen plastids were not only larger but also contained 12 times more chlorophyll  
120 ( $1.24 \text{ ng} \pm 0.17$  per stolen plastid vs  $0.11 \text{ ng} \pm 0.014$  per native plastid, Table S1) and contained  
121 more thylakoid membranes, which sustain the light reactions of photosynthesis (Figures 1D and  
122 1E). More specifically, the ratio of thylakoids to the total surface area was higher in stolen plastids  
123 ( $0.71 \pm 0.05$ ,  $n = 9$ ) than in the prey ( $0.57 \pm 0.09$ ,  $n = 18$ ) (Table S1). Some of the stolen plastids  
124 that were observed were seemingly undergoing division based on their large volumes and

125 possible constriction sites with a continuous pyrenoid (Figure 2B). FIB-SEM images also revealed  
126 that stolen plastids were enclosed within a single large vacuole (hereafter called symbiosome)  
127 (Figure 1E).

128 In order to evaluate how morphological changes of stolen plastids influence their  
129 physiology, photosynthetic activity was compared with native plastids of *P. antarctica*. The relative  
130 electron transfer rate through PSII (rETR II) was significantly higher ( $P < 0.001$  (Table S2)) in  
131 stolen plastids in a range of light conditions from 15 to 219  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  (Figure 2E). The  
132 alpha value from the ETR curve tended to be higher in stolen plastids, which could indicate a  
133 higher effective antenna size (Figure S5; Table S2). In addition, oxygen measurements showed  
134 that gross production per plastid was 2.5 times higher in stolen plastids compared to native ones  
135 in *P. antarctica* ( $1.01 \text{ fmol O}_2 \text{ plastid}^{-1} \text{ min}^{-1} \pm 0.16$ ,  $n = 3$  and  $0.41 \text{ fmol O}_2 \text{ plastid}^{-1} \text{ min}^{-1} \pm 0.01$ ,  $n$   
136  $= 3$ ) (Figure 2F). Our results indicate that photosynthetic production of newly acquired plastids  
137 was enhanced in the new host, correlating with increased volume, chlorophyll content and  
138 thylakoid stacking. These fluorescence-based measurements on PSII suggest that the host RSD  
139 retains functioning plastids, conflicting with previous studies reporting that photosystem II (PSII)  
140 was absent or diminished in its function ([22] [25]). We further verified PSII activity with DCMU (3-  
141 (3,4-dichlorophenyl)-1,1-dimethylurea), an inhibitor of PSII, which caused a drop in Fv/FM (from  
142 0.64 to 0.35) and no detectable ETR. Combining Western blot analyses and Ultrastructure  
143 Expansion Microscopy (U-ExM; [63]), recently applied on various planktonic eukaryotes [64] [65],  
144 we validated the presence and localization of PsbA (plastid-encoded photosystem II subunit) in  
145 the thylakoids of stolen plastids (Figure 1C; Figures S1 and S2).

146

### 147 **Carbon fixation by stolen plastids and carbon transfer to the host cell**

148 In order to further understand the function and potential benefits of the newly integrated plastids  
149 in host cells (fed condition), their capability for carbon fixation was also assessed. We first  
150 examined the morphology of the pyrenoid, the  $\text{CO}_2$ -fixing compartment within the plastids,  
151 containing the enzyme Rubisco [28]. Pyrenoids in stolen plastids tended to change from a round  
152 to an elongated rod-like shape (Figures 2A and 2B) and their volume was 5-fold higher on average  
153 than pyrenoids in prey plastids ( $1.19 \mu\text{m}^3 \pm 0.45$ ,  $n = 14$  and  $0.23 \mu\text{m}^3 \pm 0.07$ ,  $n = 12$ ; respectively)  
154 (Figure 2C; Table S1). Pyrenoids occupied a similar volume (11-12%) within stolen and native  
155 plastids, demonstrating that pyrenoid to plastid volume ratio is maintained in the enlarged stolen  
156 plastids (Figure 2D). UexM and Western blots indicated that the plastid-encoded enzyme Rubisco  
157 (RbcL, large subunit) was present and localized in pyrenoids of stolen plastids as expected  
158 (Figure 1C; Figures S1 and S2).

159 To assess whether stolen plastids can fix inorganic carbon and provide photosynthates to  
160 their new host,  $^{13}\text{C}$  isotope ratio images were obtained using NanoSIMS for several host cells  
161 incubated for 24 hours with labeled  $^{13}\text{C}$ -bicarbonate. NanoSIMS images revealed significant  $^{13}\text{C}$   
162 enrichment in stolen plastids, especially in pyrenoids (6000-8000‰) and the thylakoid area  
163 (4000‰) (Figures 2G-I and S3). Significant  $^{13}\text{C}$  enrichment was also detected in the host cell,  
164 especially in trichocysts and several unidentified structures (4000-8000‰), as well as in the host  
165 nucleus (2100-4000‰), demonstrating that stolen plastids export carbon to the host. Thus, once  
166 engulfed in their new host, stolen plastids undergo significant volume expansion with higher  
167 photosynthetic activity, which is clearly beneficial to the host as a source of carbon. This raises  
168 the question about the underlying mechanisms of plastid remodeling and photosynthetic  
169 enhancement in this association.

170

### 171 **Retention and enlargement of the algal mitochondrion and nucleus in the host**

172 Electron microscopy revealed the presence of intact engulfed algal cells within fed hosts,  
173 indicating that plastid uptake takes place through phagotrophy of the entire algal cell (Figure S4).  
174 Upon engulfment, observations with expansion and electron microscopy revealed that the host  
175 not only retained several plastids but also the nucleus, mitochondria and possibly other organelles  
176 from the algae within a single vacuole (Figures 1 and 3, Figure S4). Of note,  $^{13}\text{C}$  enrichment was  
177 observed in these stolen organelles (2100-4000‰) by NanoSIMS, indicating that they utilize  
178 photosynthetically-derived carbon (Figures 2G-I; Figure S3). FIB-SEM imaging revealed that the  
179 algal mitochondrion dramatically expanded in volume (more than 20-fold increase, Table S1), and  
180 transformed into a highly reticulated network, in close association with plastids (Figures 1F and  
181 3D). Mitochondrial protrusions into a “pocket-like” structure were observed within the plastid  
182 (Figures 3D and S4). A similar phenotypic transformation of the mitochondrion and close  
183 interaction with plastids has been reported in the microalga *Phaeocystis* in symbiosis within  
184 acantharian hosts [29]. This significant mitochondrial development in photosymbiosis and  
185 kleptoplastidy models indicates an integral role of the algal mitochondrion in photosynthesis and  
186 bioenergetics, as reported in diatoms and plants [31, 32].

187 In addition to the algal mitochondrion, one or several microalgal nuclei was/were observed  
188 in continuously-fed host cells (Figures 1C and 1E; Figures S1 and S4). Compared to the original  
189 nucleus of the microalga, the volume of stolen nucleus increased 50-fold, with a ~90 and ~15 fold  
190 increase in the volume of the nucleolus (ribosome factory; [33]) and heterochromatin, respectively  
191 (Figures 3A-C; Table S1). There was also a remodeling of the nuclear compartments, whereby  
192 heterochromatin occupancy (within total nucleus volume) decreased by 25% while nucleolus

193 occupancy increased by 8% (Figure 3C), suggesting higher transcriptional activity in the algal  
194 nucleus. At this stage of the kleptoplastidy when prey is available, the presence of the algal  
195 nucleus and mitochondrion may support the growth and activity of stolen plastids by providing  
196 proteins and metabolites [18, 42].

197

### 198 **Tracking the morphology and activity of stolen plastids during several months of** 199 **starvation**

200 Whereas preceding observations were based on newly acquired plastids, in culture conditions  
201 where algal prey was continuously present, in the natural environment hosts may be temporarily  
202 starved when prey availability decreases (e.g. austral winter). In this kleptoplastidic association,  
203 a previous study reported that stolen plastids could be maintained for over two years in the  
204 absence of algal prey [9]. However, there is no information on the activity of stolen plastids (except  
205 for the absence of host nucleus-encoded PSII genes [22, 25]) and the fate of the algal  
206 mitochondrion and nucleus during starvation. In order to address this knowledge gap, a time-  
207 course experiment was conducted to monitor starved host cells in the absence of algal prey for  
208 up to 30 weeks (Figure 4). Physiological measurements and microscopy observations were  
209 carried out at different time points (weeks 1, 3, 9 and 30). After 1 week of starvation, electron  
210 microscopy observations showed that ~2% of host cells still contained at least one algal nucleus  
211 (n= 53 cells), whereas none were present at week 3 and beyond. It is likely that algal nuclei rapidly  
212 disappear in most host cells by dilution over multiple host divisions. Because the nucleus of  
213 photosynthetic organisms is fundamental for the maintenance and physiology of plastids [34], its  
214 absence raises questions around how stolen plastids function and provide benefits for the host.

215 During the first week of starvation, dinoflagellate host cells divided (0.6 division/day on  
216 average), but plastid number per host remained relatively stable (~30 on average; Figures 4A-C).  
217 This indicates that stolen plastids were able to divide during the first week, which was supported  
218 by our FIB-SEM observations (Figure 2B). Between week 1 and week 30, host cells decreased in  
219 size and gradually lost plastids (down to ~7 on average; Figures 4A-C), indicating that plastids do  
220 not divide in the host once the algal nucleus is lost. On rare occasions, host cells with ~40 plastids  
221 were still observed after 30 weeks of starvation, but the morphology of these cells suggests that  
222 they were resting stages.

223 Physiological activity and morphology of stolen plastids were then investigated during the  
224 starvation period. Fv/Fm decreased from 0.64 in “continuously-fed” condition to 0.46 at week 9  
225 (Figure 4D). Photosynthetic oxygen production per plastid was relatively stable during the first two  
226 weeks of starvation (around 1 fmol O<sub>2</sub> plastid<sup>-1</sup> min<sup>-1</sup>, n = 3 culture replicates) and similar to that



227 of freshly acquired plastids (Figure 4E). Subsequently, a continuous decrease in oxygen  
228 production per plastid was observed at week 3 ( $0.5 \pm 0.09$  fmol O<sub>2</sub> plastid<sup>-1</sup> min<sup>-1</sup>) and week 9  
229 ( $0.12 \pm 0.08$  fmol O<sub>2</sub> plastid<sup>-1</sup> min<sup>-1</sup>). Corroborating these results, ETR measurements, including  
230 the alpha parameter, also indicated that PSII activity decreased during starvation (Figure S5).  
231 Yet, these measurements indicate that PSII was present and functional in stolen plastids for more  
232 than 9 weeks. Western blot analyses confirmed that subunits of PSII (PsbA) and PSI (PsaC) were  
233 still present during this starvation period (potentially still encoded by the stolen plastids or stable  
234 over time) (Figure S2). The nucleus-encoded PSII subunit PsbO was detected at week 3, but not  
235 at week 9 (likely below the detection limit due to the very low number of plastids/cell). We  
236 hypothesize that PSII starts to degrade at later stages of the kleptoplastidy, impairing the electron  
237 transport chain within stolen plastids as observed here.

238 The diminished photosynthetic activity of stolen plastids was also accompanied by drastic  
239 morphological changes (Figures 5A-B): their volume significantly decreased by 35% at week 9  
240 ( $7.18 \mu\text{m}^3 \pm 2.72$ ,  $n = 14$ ) compared to freshly engulfed plastids, with much fewer thylakoid  
241 membranes and lower chlorophyll content (Figures 5 and S6; Table S1). Note that these plastids  
242 at week 9 were still larger than the native ones in *P. antarctica*. Old plastids also exhibited internal  
243 vacuoles/vesicles that seem to have been expelled into the symbiosome (Figures 5A and S6). This  
244 could be a sign of senescence in aging plastids (or gerontoplasts) where autophagic degradation  
245 can take place [35, 58-59].

246

### 247 **Carbon fixation by stolen plastids during starvation**

248 The gradual decrease of photosynthetic activity of stolen plastids during starvation raises the  
249 question about whether they are still able to fix and translocate carbon to the host. Contrary to  
250 the thylakoids and stroma, the pyrenoid volume was comparable between week 9 ( $1.20 \mu\text{m}^3 \pm$   
251  $0.44$ ,  $n= 14$ ) and freshly engulfed plastids ( $1.19 \mu\text{m}^3 \pm 0.45$ ,  $n= 14$ ). As a consequence, pyrenoid  
252 occupancy in plastids was higher (17% vs 11% in fed hosts) (Table S1). Western blot analyses  
253 showed that RbcL was still present in stolen plastids at weeks 3 and 9, suggesting the ability to  
254 maintain carbon fixation (Figure S2). NanoSIMS <sup>13</sup>C mapping confirmed that carbon fixation in  
255 plastids (low enrichment levels;  $\sim 1500\text{‰}$ ), as well as carbon transfer to the host (including nucleus  
256 and trichocysts) still took place after 9 weeks of starvation (Figure 5D). Of note, high <sup>13</sup>C  
257 enrichment ( $\sim 5000\text{-}9000\text{‰}$ ) was observed in some vacuoles, localized in the symbiosome,  
258 suggesting carbon storage that may accumulate over time (Figures 5D and S6). Very low content  
259 of <sup>32</sup>S, <sup>31</sup>P and <sup>12</sup>C<sup>14</sup>N as unveiled by NanoSIMS elemental images was found in these vacuoles,  
260 supporting this hypothesis of carbon storage (Figure S7). Thus, despite decreased photosynthetic

261 activity and volume during starvation, stolen plastids were still able to fix and transfer carbon to  
262 the host, likely providing nutritional benefits after several months.

263 Except for the two plastid-encoded Rubisco subunits, all Calvin–Benson–Bassham (CBB) genes  
264 are encoded in the nucleus, raising the question of how carbon fixation still takes place.  
265 Transcriptomic analyses identified plastidial isoforms of several CBB transcripts (Table S3), all of  
266 dinoflagellate origin. The N-terminal extensions of most of these CBB transcripts contain the  
267 hallmarks of peridinin-plastid targeting peptides [36], suggesting targeting to the remnant/cryptic  
268 peridinin plastid, although such a relict plastid has not been identified in RSD. However, as the  
269 NanoSIMS analysis confirmed continued carbon fixation, we consider here the possibility of dual  
270 targeting to the peridinin as well as stolen plastids, which presumably co-exist in the same host  
271 cell.

272

### 273 **Retention of the algal mitochondrion for several months**

274 Remarkably, the algal mitochondrion was still observed inside host cells after several months of  
275 starvation. At week 9, the mitochondrion formed a thread-like network connecting different stolen  
276 plastids (Figure 5E) or was closely associated with a single remaining plastid (Figure 5F). At week  
277 30, the mitochondria were still associated with stolen plastids with normal cristae (Figure S6). We  
278 hypothesize that the retention of the algal mitochondrion and its intimate interaction with the  
279 plastid may contribute to the longevity and functioning of plastids (e.g., ATP and NADPH supply,  
280 metabolite exchange, ROS balance, CO<sub>2</sub> delivery) [37, 38]. To our knowledge, this observation  
281 revealing long-term retention of an algal mitochondrion without its original nucleus is  
282 unprecedented and unveils a new strategy in kleptoplastidy [39]. Like for the plastid, the  
283 transcriptome of the fed host was analyzed to assess the genetic basis of mitochondrion  
284 maintenance. We identified all but one protein of the nuclear-encoded Tricarboxylic acid cycle  
285 (Krebs cycle; TCA), but all were of dinoflagellate origin. This suggests that, like for the CBB cycle,  
286 no gene transfers from the current kleptoplast or former associations have occurred in this  
287 pathway. However, with the exception of two candidates, more than one isoform of each TCA  
288 protein was identified, which allows for the possibility of dual targeting: "alga-mitochondria  
289 targeted" and "host-mitochondria targeted" isoforms (Table 4).

290

### 291 **Worldwide geography of the dinoflagellate host in polar regions**

292 The results of our multimodal study clearly demonstrate the important impact of kleptoplastidy on  
293 carbon fluxes at the cellular scale in this association. In order to determine the potential ecological  
294 significance of this strategy, originally found in the Ross Sea (Antarctica), we searched for the

295 18S rRNA V4 sequence marker of RSD in the EukBank database [41] that contains  
296 metabarcoding samples from worldwide oceanographic expeditions. This analysis showed that  
297 the RSD dinoflagellate is not only found in different regions of the Southern Ocean, but is also  
298 widespread in the Arctic (Figure 6). We carried out similar analyses for *Phaeocystis antarctica*  
299 and *Phaeocystis pouchetii* (the endemic *Phaeocystis* species in the Antarctic and Arctic regions  
300 respectively), which revealed their presence in most samples where RSD sequences were  
301 detected during the polar spring and summer. We hypothesize that the RSD dinoflagellate is  
302 present in polar regions, including the Arctic, relying on the abundant polar *Phaeocystis* species  
303 as prey. These results further highlight the significance of this strategy in the ocean plankton. We  
304 hypothesize that this (and potentially other) kleptoplastic association(s) play an important  
305 ecosystem-level role in primary productivity and carbon cycling in polar regions.

306

## 307 **CONCLUSIONS**

308 The combination of photophysiology, 3D electron microscopy and nanoscale carbon flux imaging  
309 in this study illuminates, at pertinent temporal and subcellular scales, a new interaction strategy  
310 in the plankton whereby the host captures entire algal cells and hijacks expanded nucleus,  
311 mitochondrion and plastids. The 6-fold expansion of plastid and C-fixing pyrenoid volumes and  
312 boosted photosynthesis, likely supported by the stolen algal nucleus and mitochondrion, is clearly  
313 beneficial for the host as we showed that they provide carbon energy. Increase in the volume of  
314 the photosynthetic machinery in a foreign host was also found in kleptoplastidic (*Mesodinium*  
315 *rubrum* [17] and *Nusuttodinium aeruginosum* [18]) and photosymbiotic [29] interactions,  
316 suggesting the existence of unknown cellular/signaling mechanisms for plastid remodeling. The  
317 algal mitochondria also developed into an extensive network in close interaction with plastids and  
318 likely contributed to the control and activity of photosynthesis, as shown in diatoms and plants  
319 [31-32]. Thus, when algal prey is available, this interaction is more cytolept (cell stealing) than  
320 kleptoplastidy, whereby a host steals and manipulates most of the organelles of an ecologically  
321 successful microalgal cell, such as *Phaeocystis*.

322 When algal prey is no longer available, however, our study showed that host cells lose the  
323 algal nucleus after ~1 week. Despite the absence of essential algal nucleus-encoded proteins,  
324 the stolen plastids and mitochondria remain active for the next few months. This may be explained  
325 by the complex genomic toolkit of the host, composed of photosynthesis-related genes inherited  
326 i) from the vestigial permanent plastids (peridinin and haptophyte origins), by endosymbiotic gene  
327 transfer, and ii) from various algal lineages (including haptophytes) by horizontal gene transfer.  
328 Although they still fix and transfer carbon to the host, plastids decrease in volume with diminished

329 photosynthetic activity over several weeks in the absence of the algal nucleus. It is possible that  
330 stolen plastids switch to electron cyclic flow mode when PSII is no longer functional.

331 The host must therefore eventually acquire new plastids from the environment in order to  
332 resume vigorous growth. It is clearly advantageous for hosts to rely on an abundant bloom-  
333 forming phytoplankton taxon as a source of new organelles, such as *Phaeocystis* (up to 20 million  
334 of cells l<sup>-1</sup>) that are often present across seasons [43] [44]. *Phaeocystis* can be present during the  
335 austral spring, but reaches extremely high abundances in austral summer (December and  
336 January), providing unlimited resources for the host dinoflagellate. During the polar night, the  
337 retention time of functional plastids may be extended since degradation could be slower due to  
338 the absence of light. In early spring, retained plastids could enable the host to persist until levels  
339 of prey are high enough to be encountered and incorporated.

340 The use of a combination of multiscale approaches with relevant temporal resolution is  
341 fundamental for deciphering highly complex cellular interactions in protists and unveiling  
342 remarkable mechanisms along the spectrum of plastid endosymbiosis. From an ecological  
343 perspective, this study shows the capacity of hosts to hijack plastids and temporarily boost  
344 photosynthesis, a process which likely impacts the oceanic carbon cycle. From an evolutionary  
345 perspective, the interaction between RSD and *Phaeocystis* provides key insights into cellular and  
346 metabolic processes that potentially led to the permanent incorporation of photosynthetic  
347 machinery and thus improves our understanding of how eukaryotes can acquire new organelles.  
348 This unprecedented example of extended retention of the algal mitochondrion suggests that this  
349 organelle may have played a key role in the early stages of plastid acquisition in addition to  
350 fundamental endosymbiotic steps (e.g. gene transfer, protein targeting).

351

352

## 353 MATERIALS AND METHODS

### 354 Culture conditions of the microalga *Phaeocystis antarctica* and the kleptoplastidy system

355 A co-culture of *Phaeocystis antarctica* and Ross Sea Dinoflagellates (RSD) isolated from the Ross  
356 Sea, Antarctica was maintained in L1 medium [50] (36 ppt salinity) at 4°C with a 12-12 hour  
357 light/dark cycle at 45-50  $\mu\text{mol}/\text{m}^2/\text{s}$  photon flux intensity. In parallel, the microalga *Phaeocystis*  
358 *antarctica* (called here the prey) was isolated from this co-culture and grown separately in the  
359 same conditions. All microscopy images and physiological measurements were performed on  
360 solitary cells for accurate counting of cells. Prey *P. antarctica* cells in the exponential phase (~  
361 day 5) were used to feed RSD cultures. For measurements on “Fed Hosts”, co-cultures were  
362 filtered with a 70  $\mu\text{m}$  Corning® Cell strainer, followed by sequential filtrations on a 20  $\mu\text{m}$  mesh  
363 fabric to remove prey, RSD clumps and solitary prey cells. Measurements on algal prey were  
364 performed on cells filtered with a 40  $\mu\text{m}$  strainer to remove colonies.

365 For the starvation experiment, the prey-host co-culture was first gently filtered using a 70  $\mu\text{m}$  cell  
366 strainer to remove colonies of *P. antarctica*, followed by filtrations on a 20  $\mu\text{m}$  mesh fabric to  
367 remove solitary *Phaeocystis* cells and recover RSD dinoflagellate cells (larger than 20  $\mu\text{m}$  in size).  
368 These RSD cells were washed three times with cold filtered seawater and once with L1 medium,  
369 and transferred to a culture flask. After these filtrations, the presence of *Phaeocystis* (both solitary  
370 and colonial stages) was visually checked through an inverted light microscope using both bright  
371 field and chlorophyll fluorescence. RSD cells were then placed at 4°C and the same conditions  
372 described above (45-50  $\mu\text{mol}/\text{m}^2/\text{s}$  with a 12h-12h light-dark cycle). The culture medium was  
373 refreshed every 4 weeks during starvation by filtering the host cells on a clean (sonicated) 10  $\mu\text{m}$   
374 mesh fabric followed by washing with cold fresh L1 medium. All subsequent measurements on  
375 “Starved Hosts” were performed with no further filtering.

376

### 377 Photosynthesis measurements and oxygen production

378 The rapid light curves to measure Electron Transport Rate through PSII (rETR II) were performed  
379 using Pulse-Amplitude Modulation (PAM) fluorometry on the microalgae culture *P. antarctica* and  
380 the host RSD (fed and starved conditions) with 300 milliseconds saturating pulse followed by 20  
381 seconds between 8 increasing actinic light exposures (15 to 219  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ). 500 ml of  
382 each sample (with biological triplicates) was pipetted directly from the growth light condition into  
383 a WALZ KS-2500 water-jacketed chamber equipped with a stir bar maintained at 4°C by a Mini-  
384 PAM-II fluorometer. The instrument is operated with WinControl-3 software (Heinz Walz GmbH).  
385 Curve-fitting for ETR values and extraction of the parameters alpha, beta, ETRmax, Ek and ps  
386 from the fitted curves was performed using the R package phytotools [51] and to extract the

387 coefficients/parameters alpha, beta, ETR<sub>max</sub>, E<sub>k</sub> and p<sub>s</sub> from the fitted curve. A modified Chi-  
388 square test was performed on the fitted-curved to compare the curves using the Excel sheet (N>1,  
389 mean and SE) from [27]. The first Y(II) value from the rapid light curve was considered for  
390 maximum potential quantum efficiency of Photosystem II (F<sub>v</sub>/F<sub>m</sub>). The samples were dark-  
391 adapted for one minute before starting the light curve. Dark-adapting the cells separately for 40  
392 minutes before F<sub>v</sub>/F<sub>m</sub> measurement did not significantly change the value of F<sub>v</sub>/F<sub>m</sub> (±0.01  
393 compared to 1-minute dark prior to rapid light curve). This is possibly due to the light level (45-50  
394 μmol/m<sup>2</sup>/s photon flux intensity). For the oxygen measurements, 500 ml of samples was pipetted  
395 in a WALZ KS-2500 water-jacketed chamber (Heinz Walz GmbH) paired with a FSO<sub>2</sub>-1 oxygen  
396 meter and optical microsensor - FireStingO<sub>2</sub> (PyroScience GmbH) controlled by the WinControl-  
397 3 software. Net Oxygen production was measured by exposing cultures to actinic light set at 90  
398 μmol/m<sup>2</sup>/s for 9 minutes followed by 2 minutes in the dark to measure respiration. Gross maximum  
399 production was calculated by: O<sub>2</sub> Gross = O<sub>2</sub> Net – (Respiration (negative slope)).

400

#### 401 **Sample Preparation for electron microscopy (EM)**

402 The microalga *Phaeocystis antarctica* and host RSD dinoflagellate cultures in exponential phases  
403 were centrifuged at 2000 g for two minutes and cryofixed using High Pressure Freezing (HPM100,  
404 Leica Microsystems, Austria) at a pressure of 210 MPa at -196°C as in [29]. This was followed by  
405 freeze-substitution (EM ASF2, Leica Microsystems, Austria), where vitrified ice was replaced with  
406 dried acetone and 2% osmium tetroxide for TEM and nanoSIMS, with 0.5% uranyl acetate added  
407 for FIB-SEM. Samples are embedded in EPON-substitute (for TEM) or Araldite (for SEM). More  
408 details of the sample preparation protocols are provided in protocol.io [52]. The resin blocks were  
409 then stored in dry conditions prior to imaging.

410

#### 411 **Transmission and Scanning EM observations**

412 For transmission electron microscopy (TEM), 70 nm thick sections obtained using a diamond knife  
413 and a UC7 ultramicrotome (Leica Microsystems, Austria) were collected on 200 mesh formvar-  
414 carbon coated grids. Sections were stained with 4% uranyl acetate (10 min) and lead citrate  
415 (5 min). Micrographs were obtained using a Tecnai G2 Spirit BioTwin microscope (FEI) operating  
416 at 120 kV with an Orius SC1000 CCD camera (Gatan). For scanning electron microscopy (SEM),  
417 200 nm thick sections were transferred to a silicon wafer and observed at low voltage (3 kV, 800  
418 pA) with a Sense BSD backscattered electron detector in a Zeiss GeminiSEM 460.

419

#### 420 **FIB-SEM acquisition, segmentation, and morphometric analyses**

421 Focused Ion beam Scanning Electron Microscopy (FIB-SEM) tomography was performed with a  
422 Zeiss CrossBeam 550 microscope (Zeiss, Germany) as [66]. The resin block containing the cells  
423 was fixed on a stub with silver paste and surface-abraded with a diamond knife in a microtome to  
424 obtain a flat and clean surface. Samples were then metallized with 4-8 nm of platinum to avoid  
425 charging during observations. Inside the FIB-SEM, a second platinum layer (1–2  $\mu\text{m}$ ) was  
426 deposited locally on the analyzed area to mitigate possible curtaining artifacts. The sample was  
427 then abraded slice by slice with the  $\text{Ga}^+$  ion beam (typically with a current of 700 pA at 30 kV).  
428 Each exposed surface was imaged by SEM at 1.5 kV and with a current of  $\sim 1$  nA using the in-  
429 column EsB backscatter detector. Similar milling and imaging modes were used for all samples.  
430 Automatic focus and astigmatism correction were performed during image acquisition, typically at  
431 approximately hourly intervals. For each slice, a thickness of 6 or 8 nm was removed and SEM  
432 images were recorded with a corresponding pixel size of 6 to 8 nm in order to obtain an isotropic  
433 voxel size. Entire volumes were imaged with 800–3000 frames, depending on the cell type and  
434 volume. The first steps of image processing were performed using Fiji software for registration  
435 (adapted Stackreg plugin), and noise reduction (3D mean function of the 3D suite plugin). Raw  
436 electron microscopy data have been deposited in the Electron Microscopy Public Image Archive  
437 (EMPIAR), accession code EMPIAR-XXX.

438 Two segmentation strategies (automatic with a new algorithm and semi-automatic) were used to  
439 reconstruct and generate models of cells and organelles in three dimensions. The overview of  
440 plastid and mitochondria morphologies was obtained using the FeatureForest, a napari plugin  
441 that uses the Segment Anything Model (SAM) [62] to extract features from images and train a  
442 random forest classifier based on these features and inputs from the user. Once trained on a  
443 small subset of the data, the random forest model, together with SAM, allows segmenting the  
444 entire stack. In order to obtain smoother object segmentation, FeatureForest applies post-  
445 processing steps by filling holes in the segmentation, removing connected components smaller  
446 than a certain threshold and using the remaining connected components to generate bounding  
447 boxes used as input prompts to SAM, yielding well-defined object boundaries. The plugin was  
448 created in collaboration with the Jug lab and the Bioimage Analysis Unit of the National Facility  
449 for Data Handling and Analysis at the Human Technopole, Milan, Italy through the AI4Life project  
450 ([https://github.com/ai4life-opencalls/oc\\_1\\_project\\_52](https://github.com/ai4life-opencalls/oc_1_project_52)). The plugin repository is publicly available  
451 at <https://github.com/juglab/featureforest/blob/main/docs/index.md>.

452

453 Semi-automatic and manual segmentation using 3D slicer [48] was used to obtain volumes of  
454 organelles. Plastids, pyrenoids, mitochondria and nuclei of the microalga (native and stolen

455 states) and RSD dinoflagellate host cells were segmented to reconstruct the cellular organization  
456 of kleptoplastidy and quantify volumes ( $\mu\text{m}^3$ ). Each segment (e.g. pyrenoid, plastid, mitochondria)  
457 was colored using paint tools, adjusting the threshold range of the pixel values (intensity) of the  
458 images. Different views and slices were generated for the model using the EasyClip module.  
459 Volume measurements were then calculated using the Segmentation Statistics module. Paraview  
460 was also used to edit the 3D models and generate images and videos [49].

461

#### 462 **NanoSIMS experiment with labeled $^{13}\text{C}$ -bicarbonate: correlative imaging and analysis**

463 NanoSIMS experiments were conducted on two culture conditions: fed and starved RSD  
464 dinoflagellate host cells. For the fed conditions, around 1 ml of prey microalgae (*P. antarctica* in  
465 exponential phase) was added one week before cryofixation to culture flasks containing 90 ml  
466 RSD culture. For the starved conditions, RSD dinoflagellates were washed several times to  
467 remove the *Phaeocystis* prey as explained above and maintained for 9 weeks without prey before  
468 cryofixation for nanoSIMS. For both conditions, cells were incubated with stable isotopes for 24  
469 hours. More specifically, 10 ml of an isotopic solution with 10X concentration containing  $\text{H}^{13}\text{CO}_3$   
470 was added to the culture flasks. A control culture without isotope labeling was also maintained in  
471 the same culture conditions. After 24h incubation with  $\text{H}^{13}\text{CO}_3$ , cells were harvested by  
472 centrifugation for two minutes at 2000 g and cryofixed with High Pressure Freezing (see above).  
473 Thin sections (100 nm - 200 nm) were first imaged using TEM or SEM in order to observe the  
474 ultrastructure of the RSD dinoflagellates containing stolen plastids and identify the cells of interest  
475 for isotopic analysis. The TEM grids or SEM silicon wafers were then introduced into a NanoSIMS  
476 ion microprobe (Cameca, Gennevilliers, France) at either EPFL, Lausanne (Switzerland) or at  
477 Institut Curie, Orsay (France) and analyses were performed on the preselected cells. The  
478 instrument delivers a primary beam of Cesium ( $\text{Cs}^+$ ) ions to the sample surface, focused to a  
479  $\sim 50\text{-}100\text{nm}$  spot. The secondary ions are extracted from the sample surface and transferred to a  
480 high-resolution mass-spectrometer.

481 For NanoSIMS on fed host cells, the secondary  $^{12}\text{C}^{14}\text{N}^-$  and  $^{13}\text{C}^{14}\text{N}^-$  ions were collected in  
482 electron multipliers at the exit of the mass spectrometer with a mass resolution ( $M/\Delta M$ ) of  $\sim 9000$ .  
483 Each capture consisted of six sequential images. For 9-week starved cells, five ion species ( $^{12}\text{C}_2^-$   
484 ,  $^{12}\text{C}^{13}\text{C}^-$ ,  $^{12}\text{C}^{14}\text{N}^-$ ,  $^{31}\text{P}^-$  and  $^{32}\text{S}^-$ ) were collected in parallel, also captured in multiframe acquisition  
485 mode. Individual frames were recorded at  $512 \times 512$  pixel definition, with a dwell time of 0.5 ms  
486 per pixel, with up to 100 frames accumulated for each acquisition. The collection of  $\text{C}_2^-$  was  
487 preferred to  $\text{C}^-$  for enhanced ion emission yield thus higher count rate and improved counting  
488 statistics for  $^{13}\text{C}$  quantification. However, appropriate mass resolving power has to be applied to



489 ensure specific detection of  $^{12}\text{C}^{13}\text{C}^-$  ions against the interference  $^{12}\text{C}_2^1\text{H}^-$  ions (although at a  
490 moderate relative mass separation,  $M/\Delta M$ , of 5600, but with an intensity of 10 times that of  $^{12}\text{C}^{13}\text{C}^-$   
491 ). Sequential images acquired from the NanoSIMS instruments were aligned frame-to-frame with  
492 the L'IMAGE software (developed by Larry Nittler; marketed by the Carnegie Institution of  
493 Washington, USA) for drift compensation. The ratio images for the carbon isotope were then  
494 obtained by pixel-to-pixel division of accumulated  $^{12}\text{C}^{15}\text{N}^-$  and  $^{12}\text{C}^{14}\text{N}^-$ , or  $^{12}\text{C}^{13}\text{C}^-$  and  $^{12}\text{C}_2^-$  images.  
495 The resulting isotopic images were then superimposed on the TEM/SEM images to precisely  
496 measure the isotopic ratios in specific subcellular locations thus allowing us to study carbon  
497 allocation within the RSD [17].

498

### 499 **Ultrastructural expansion microscopy (U-ExM) and Confocal Microscopy**

500 Dinoflagellate RSD cells were gently pelleted at 4°C by centrifugation at 1000 g (3170 rpm) for 3  
501 minutes. The pellets were fixed in a final concentration of 4% formaldehyde for 20 minutes at  
502 room temperature. Pellets were then washed twice with 1 ml 1x PBS followed by centrifugation  
503 at 1000 g for 3 minutes at 20°C. Finally, pelleted cells were resuspended in 1 ml of 1x PBS and  
504 stored at 4°C until expansion. Expansion was performed as previously described [63-65]. Fixed  
505 cells were either attached to a poly-D-Lysine coverslip or sedimented in a microtube before being  
506 cross-linked in AA/FA solution (1% acrylamide (AA)/0.7% formaldehyde (FA)) for 12 hours at  
507 37°C. The cells in microtubes were then allowed to sediment to the bottom of a 12-well plate, and  
508 excess liquid was carefully removed with minimal disturbance. Gelation was performed using a  
509 monomer solution consisting of 19% (wt/wt) sodium acrylate (Combi-Blocks, ref: QC-1489), 10%  
510 (wt/wt) acrylamide (Sigma-Aldrich; ref: A4058), and 0.1% (wt/wt) N, N'-  
511 methylenebisacrylamide (Sigma-Aldrich; ref: M1533) in PBS. The process was carried out at 37°C  
512 for 1 hour in a moist chamber. Gel denaturation was performed in denaturation buffer (50 mM Tris  
513 pH 9.0, 200 mM NaCl, 200 mM SDS, pH to 9.0) for 15 min at RT. The gel was then incubated at  
514 95°C for 1.5 hours. Following denaturation, expansion was performed with several water rinses  
515 as previously described [63-65]. Post expansion, gel diameter was measured and used to  
516 determine the expansion factor (4.1875X). Scale bars in the images are corrected to indicate  
517 actual size. Sequential antibody staining was performed for imaging thylakoids with pyrenoids  
518 and protein localization (Figure 1C). Antibodies were prepared in PBS supplemented with 3%  
519 BSA and 0.1% Tween20. For thylakoid staining, a rabbit primary antibody targeting Photosystem  
520 II PsbA (Ref: AS05 084, Agrisera) was used at a 1/250 dilution. The primary antibody was tagged  
521 with an Invitrogen Donkey-anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody,  
522 Alexa Fluor™ 488 (Ref: A-21206, Thermo Fisher) at a 1/250 dilution. The secondary antibody

523 was washed 5 times with PBS supplemented with 3% BSA and 0.1% Tween20. Subsequently,  
524 pyrenoid staining was performed using a rabbit primary antibody targeting RuBisCO (Ref: AS03  
525 037, Agrisera) at a 1/250 dilution. This primary antibody was tagged with a different Invitrogen  
526 Donkey-anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 568  
527 (Ref: A10042, Thermo Fisher) at 1/500 dilution. For other protein localization (Figure S1), primary  
528 antibodies (beta-tubulin antibody, Ref: AA344-R, Geneva Antibody Facility. Alpha-tubulin  
529 antibody, Ref: AA345-R, Geneva Antibody Facility. Centrin2 (20H5) antibody, Ref: 04-1624,  
530 Merck. RuBisCO and Photosystem II PsbA antibodies, see above) were incubated overnight at  
531 37°C at dilutions of 1:250 to 1:500. After three washes in 0.1% PBST, secondary antibodies  
532 (Invitrogen Donkey or Goat-anti-Rabbit and anti-Mouse IgG (H+L) Highly Cross-Adsorbed  
533 Secondary Antibody, Alexa Fluor™ 488 or 647 Plus Ref: A21206, A28175, A32795, A32728,  
534 Thermo Fisher) were added at 1:500 to 1:1000 and incubated for 4hrs at 37°C. For protein pan-  
535 labelling, gels were incubated with Alexa Fluor™ 405 or 594 NHS Ester (Ref: A30000 and  
536 A20004, Thermofisher) at 1/500 dilution in NaHCO<sub>3</sub> (pH=8.28) for 1.5 hrs. For staining of nuclei  
537 of the dinoflagellate and algal prey, BioTracker NIR694 Nuclear Dye (Ref: SCT118, Merck  
538 Millipore) or Hoechst 33342 (Ref: 62249, Thermo Fisher) was incubated at a dilution of 1/500 and  
539 1/1000, respectively, in PBS for 30 minutes before re-expansion in water.

540 For gel mounting, gels were cut to appropriate sizes and attached to pre-coated Poly-D-  
541 lysine coverslips and sealed using i-Spacers (Ref: #IS013, Sunjin Lab). For imaging of expanded  
542 samples (Figures 1C and Figure S1), an upright Leica SP8 confocal microscope with an HC PL  
543 APO 40X/1.25 Glycerol objective was used. For confocal microscopy of non-expanded samples,  
544 cells were allowed to adhere to a 35 mm Poly-D-lysine-coated glass- bottom Petri dish for 15  
545 minutes at 4°C. Z-stack images of cells were imaged with a Zeiss LSM880 inverted confocal  
546 microscope equipped with a Zeiss Plan-Apochromat 963 (1.4) Oil DIC M27 objective, and Zeiss  
547 Airyscan super-resolution detector.

548

### 549 **Transcriptomic analysis and prediction of signal peptide origin**

550 Known CBB cycle proteins from several eukaryotic model organisms were selected from NCBI,  
551 while myzozoan representatives from the TCA cycle were obtained from  
552 (<https://bmcbiol.biomedcentral.com/articles/10.1186/s12915-021-01007-2>). Candidates were  
553 used as BLASTP queries against a comprehensive custom protein database containing  
554 representatives from most major eukaryotic groups, with a focus on plastid-containing lineages  
555 plus selected taxa from non-plastidial lineages and RefSeq data from all bacterial phyla at NCBI  
556 (last accessed December 2017). The database was subjected to CD-HIT with a similarity

557 threshold of 85% to reduce redundant sequences and paralogs, except for the two RSD data sets  
558 created in [22] that were clustered at 98%. Of note, as described in [22], the RSD transcriptome  
559 is substantially contaminated by the prey *Phaeocystis antarctica*. A cleaned dataset, 'RSD  
560 allclean noPhaeo' was therefore generated and used in conjunction with the uncleaned dataset  
561 'RSD Temp01'. Search results of the BLASTP step were parsed for hits with an e-value threshold  
562  $\leq 1e-25$  and a query coverage of  $\geq 50\%$  to reduce the possibility of paralogs and short sequences.  
563 The number of bacterial hits was restrained to 20 hits per phylum (for FCB group, most classes  
564 of Proteobacteria, PVC group, Spirochaetes, Actinobacteria, Cyanobacteria (unranked) and  
565 Firmicutes) or 10 per phylum (remaining bacterial phyla) as defined by NCBI taxonomy.  
566 Parsed hits of queries corresponding to the same protein were combined, deduplicated and  
567 aligned with MAFFT, using the --auto and the --reorder option  
568 (<https://academic.oup.com/mbe/article/30/4/772/1073398>), phylogenetically informative sites  
569 were identified with ClipKIT, using default options (10.1371/journal.pbio.3001007) and initial  
570 Maximum likelihood tree reconstructions were performed with FastTree v. 2.1.7, using default  
571 options (<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2835736/>). Due to the complexity of CBB  
572 cycle protein phylogenies, the BLASTP step against the custom database was repeated with the  
573 initial query protein set plus all RSD orthologs identified in the initial phylogeny, to ensure a  
574 comprehensive number of hits for sequences highly similar to RSD. Resulting phylogenies and  
575 underlying alignments were inspected manually to remove contaminations and poor-quality  
576 sequences in several iterations, repeating the tree reconstruction as described above. Cleaned  
577 alignments were then aligned with MAFFT L-INS-i, using the --reorder option, before trimming  
578 again with ClipKIT, using default options. Final trees were calculated with IQ-TREE v. 1.6.12  
579 (<https://doi.org/10.1093/molbev/msu300>), using the LG model, with branch support assessed with  
580 1000 ultrafast bootstrap replicates (<https://doi.org/10.1093/molbev/msx281>). Bootstraps for the  
581 TPI phylogeny did not converge even after increasing to 10,000 iterations. All phylogenies have  
582 been deposited to a repository as pdf figures and in newick format (preliminary links:  
583 <https://figshare.com/s/91c1a6c484d7e03fa61e> for the TCA cycle phylogenies and  
584 <https://figshare.com/s/7d005e093646410a6fb9> for the CBB cycle phylogenies).  
585 To investigate N-terminal extensions and thus intracellular location of RSD sequences,  
586 dinoflagellate and haptophyte clades containing RSD sequences were isolated from the  
587 corresponding alignments, realigned using MAFFT L-INS-I and then manually inspected for  
588 completeness of the sequences as well as for similarity in the N-terminal region to known plastid-  
589 targeted sequences of peridinin and haptophyte plastid-harboring dinoflagellates. Prediction of  
590 signal peptides as part of N-terminal bipartite leader sequences was performed with SignalP-6.0

591 selecting “Eukarya” as organism and “Slow” as the model mode  
592 (<https://pubmed.ncbi.nlm.nih.gov/34980915/>). Mitochondrial transit peptides and haptophyte  
593 plastid transit peptides were predicted with TargetP-2.0 (doi:10.26508/lsa.201900429). To predict  
594 N-terminal transmembrane domains, typical for peridinin-plastid transit peptides, DeepTMHMM  
595 (<https://www.biorxiv.org/content/10.1101/2022.04.08.487609v1>) and Phobius, as implemented in  
596 InterProScan (<https://europepmc.org/article/MED/24451626>), were used. Curated alignments  
597 underlying the plastidial clades of CBB cycle proteins containing RSD representatives have been  
598 deposited to a repository (preliminary link: <https://figshare.com/s/3bce42bce704e90ec5a6>).

599

### 600 **Protein extraction and Western Blot**

601 Total protein extracts were obtained from prey *Phaeocystis antarctica*, and fed and starved RSD  
602 dinoflagellate hosts at weeks 1, 3 and 9 in 50 mM Tris buffer pH 8.0 supplemented with protein  
603 inhibitor cocktail (539131, Calbiochem). A Precellys device (Bertin Technologies) and micro glass  
604 beads (500 µm) were used to disrupt cells with two 30 second cycles at 5000 rpm. After  
605 centrifugation, proteins in the supernatant were precipitated overnight at -20°C in 100 % acetone.  
606 After a second centrifugation, the pellet was solubilized for 5 min (RT) in 50 mM Tris (pH 6.8), 2%  
607 sodium dodecyl sulphate, 10 mM EDTA, and protein inhibitor cocktail. After a second  
608 centrifugation, supernatant was retained and protein quantified with the DC Protein assay kit II  
609 (Biorad). Protein samples (3µg - 5µg) were loaded on 10% SDS-PAGE gels (Mini-PROTEAN  
610 TGX Precast Protein Gels, Biorad) and blotted onto nitrocellulose membranes. Membranes were  
611 blocked for 1h with 5% low fat milk powder in TBS-T Tween 0.1% and probed with antiRBCL  
612 antibody (AS03 037), antiPsbA (PSII - chloroplast encoded subunit, AS05 084), antiPsaC (PSI -  
613 chloroplast encoded, AS10 939) (for Gels 1 and 2), antiPsbO (PSII - nuclear encoded subunit,  
614 AS21 4689) (dilution for all mentioned above: 1:10000 TBS-T, overnight) and antiH3 antibody  
615 (Histone 3, internal control AS10 710, dilution 1/5000 in TBS-T). For the secondary antibody, HRP  
616 conjugated anti rabbit antibody (111-035-003) (Interchim, 1:10000, 1h) in TBS-T was used.  
617 Antibody incubations were followed by washing in TBS-T. All steps were performed at room  
618 temperature with agitation. Blots were developed for 1min with the ECL Prime detection kit  
619 (RPN2232, Amersham) according to the manufacturer’s instructions (GE Healthcare). Images of  
620 the blot were obtained using a CCD imager (Chemidoc MP system, Biorad) and ImageJ software.

621

### 622 **18S rDNA sequencing and global biogeographic analyses using metabarcoding datasets**

623 Cultures of the RSD without *Phaeocystis* prey were centrifuged at 7500 rpm for 5 minutes and  
624 the supernatant was gently removed with a pipette and filtered tips. Then, 20µl of the buffer Phire

625 of the Plant Direct PCR Master Mix (Thermofisher) was added and the DNA sample was  
626 incubated at 95°C for 5 minutes. PCR amplification and sequencing of the SSU region was  
627 performed using primers as described in [14]. To obtain the entire 18S rDNA sequence of the  
628 RSD, three different primer sets were used with the Phire Plant Direct PCR Master Mix  
629 (Thermofisher) in the following conditions: 30s at 98°C pre-denaturation, followed by 35 cycles of  
630 10s at 98°C, 30s at 55°C and 30s at 72°C, with a final extension of 10 min at 72°C. PCR products  
631 were then purified using the QIAquick gel extraction kit (QIAGEN) after electrophoresis on 1%  
632 agarose gel. Sequences were manually aligned and edited using SnapGene software and were  
633 deposited in GenBank as a single contig of 1784 bp (GenBank accession no. = XXXX).  
634 Prior to metabarcoding analyses, we verified that the 18S rDNA sequence of RSD dinoflagellate  
635 has a unique V4 region sequence among the family Kareniaceae, including its closest relative  
636 *Shimiella gracilenta* (a kleptoplastidic dinoflagellate with a cryptophyte as prey [14]). Global  
637 biogeographic analysis of the dinoflagellate host RSD was carried out using the EukBank  
638 metabarcoding dataset of the V4 hypervariable region of the 18S rRNA accessed at doi:  
639 10.5281/zenodo.7804946. The V4 sequence of the RSD host was used to search reads with  
640 100% match in the EukBank dataset [41] and relative abundance of reads (number of reads from  
641 RSD divided by the total number of reads in the sample) was assessed. The same approach was  
642 conducted with the V4 region of the 18S rDNA sequences of *Phaeocystis antarctica* and  
643 *Phaeocystis pouchetii* retrieved on GenBank (KF925339 for *P. antarctica* and AF182114 for *P.*  
644 *pouchetii*) to investigate their global biogeography in the EukBank dataset.

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646

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## 672 **AUTHOR CONTRIBUTIONS**

673 A.K.R. and J.D. designed research and write the manuscript. B.G., J.D. and A.K.R. jointly  
674 performed the sample preparation for electron microscopy. C.L. and T.W. conducted nanoSIMS  
675 experiments, and T.W. and A.K.R. processed and interpreted data. A.K.R. and D.Y. carried out  
676 and interpreted the photophysiology measurements. M.P. conducted rDNA sequencing. M.O. and  
677 O.D. performed the expansion microscopy and E.H. the transcriptomic analyses. PH.J acquired  
678 and pre-processed the FIB-SEM imaging data. M.S., A.K.R. and J.Deschamps. contributed to the  
679 image analysis from FIB-SEM data. A.K.R. and F.C. performed the protein extraction and western  
680 blots analyses. R.G. helped to draft the manuscript.

681

## 682 **DECLARATION OF INTERESTS**

683 The authors declare no competing interests

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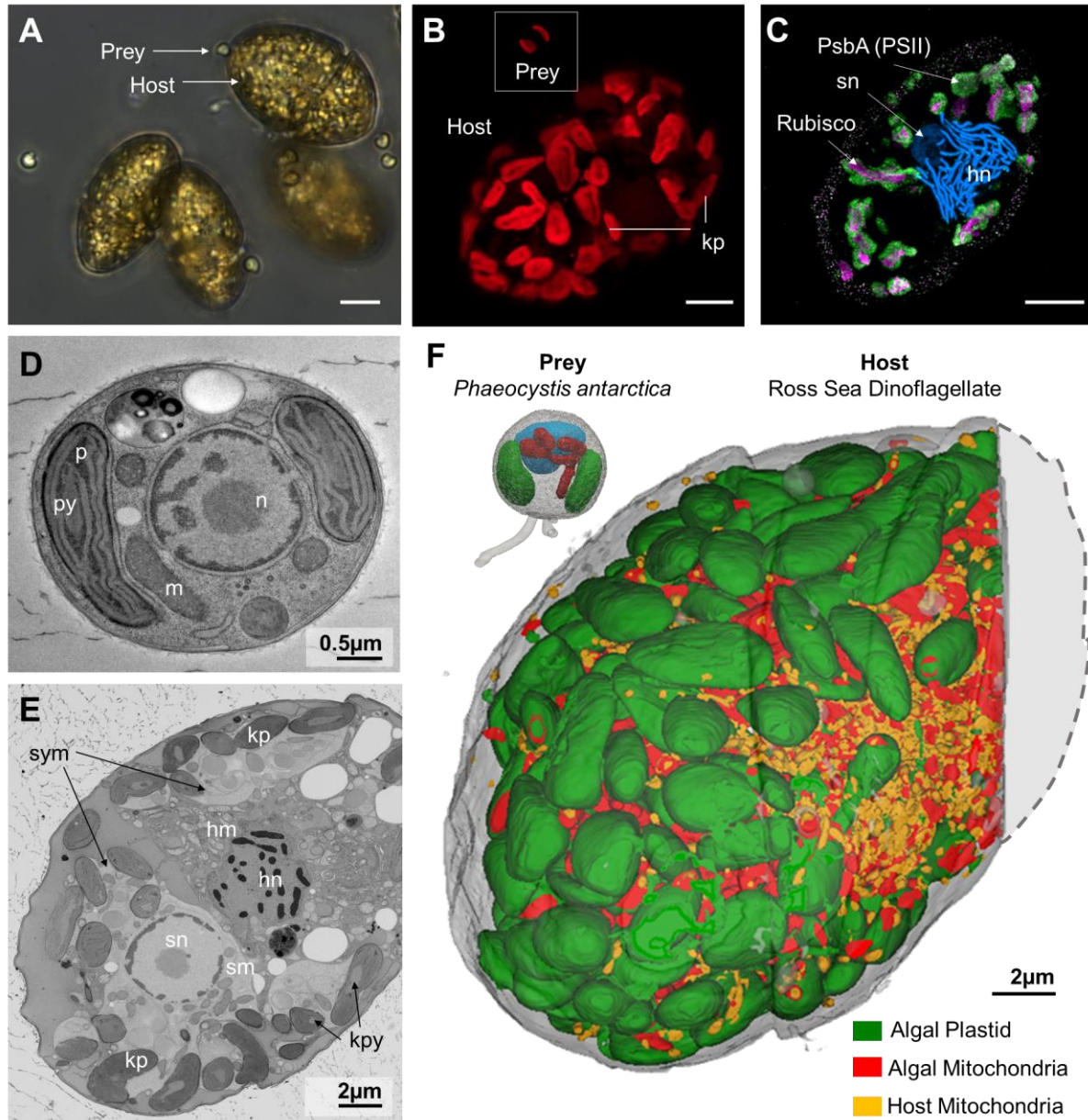
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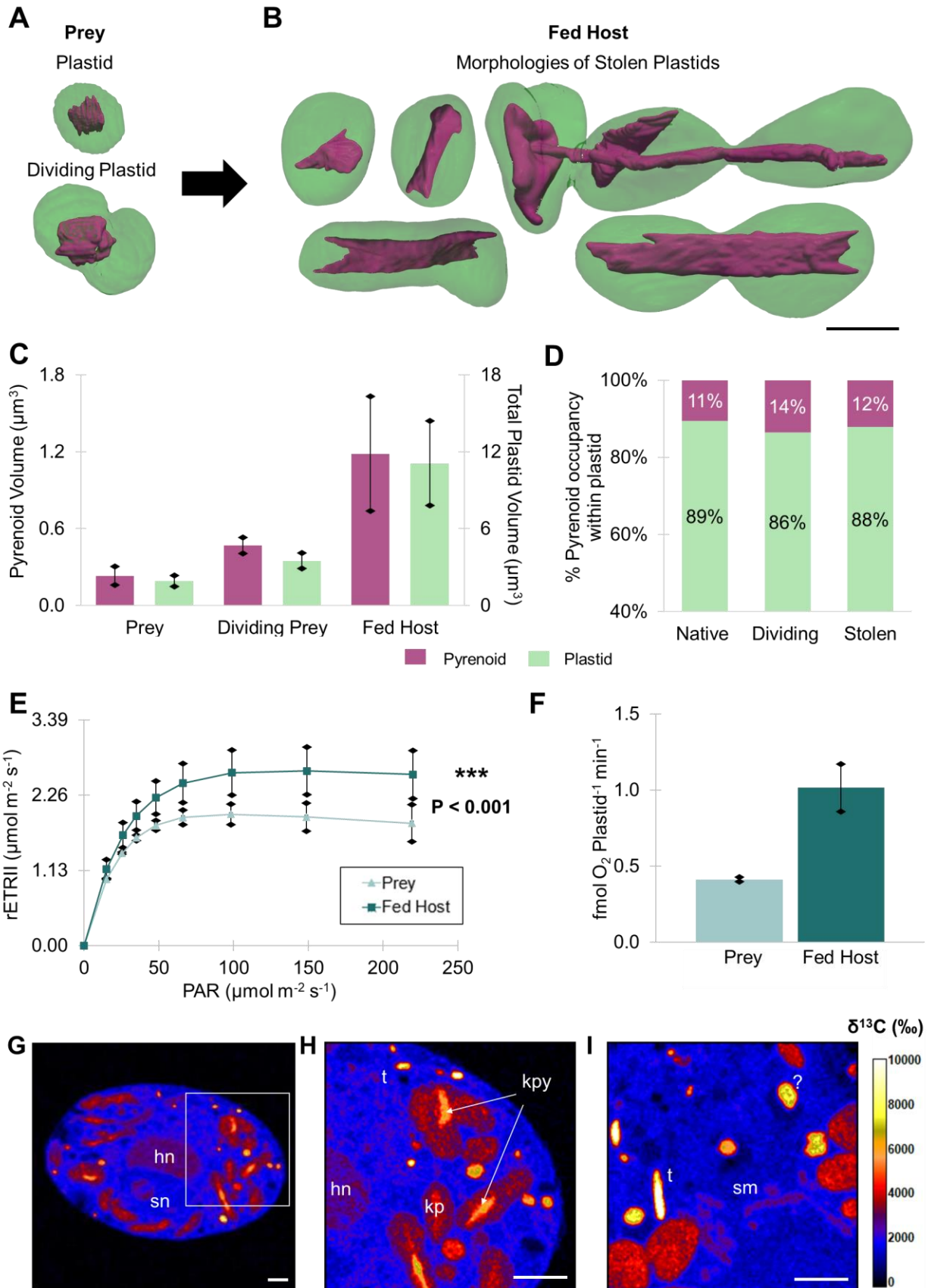
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## FIGURES

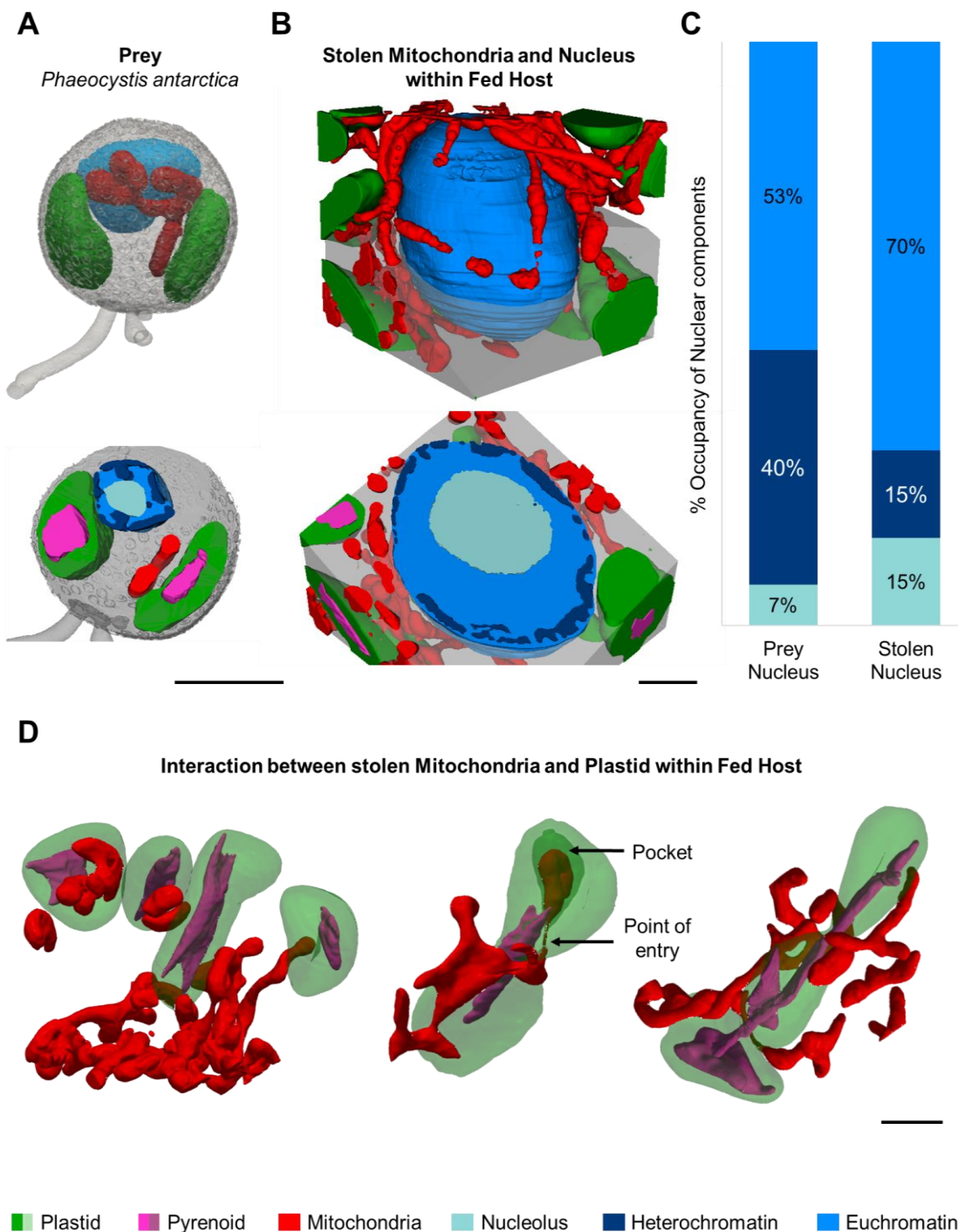


**Figure 1. Subcellular organization of the kleptoplastidy between the host dinoflagellate (Ross Sea Dinoflagellate; fed condition) and its microalga prey *Phaeocystis antarctica* unveiled by multimodal imaging.** (A) Light microscopy image of the co-culture of the Ross Sea Dinoflagellate (RSD, Kareniaceae; 20-40  $\mu\text{m}$ ) and its microalgal prey *Phaeocystis antarctica* (haptophyte; 2-5  $\mu\text{m}$ ). (B) Confocal fluorescence images showing the morphology of plastids unveiled by the red chlorophyll fluorescence in the microalgal *Phaeocystis* (prey, in the box) and in the host dinoflagellate (kleptoplast, kp). Up to 40 stolen plastids could be observed in the host and tended to be larger than native plastids in the algal prey. (C) Confocal fluorescence image of an expanded host cell after Ultrastructural Expansion Microscopy (UExM), stained using PSII PsbA antibody (thylakoids; green), RuBisCO antibody (pyrenoid; magenta), and NIR694 Nuclear

Dye (host nucleus (hn), stolen nucleus (sn); blue). This shows the presence of the algal nucleus at this stage (fed conditions). **(A-C)** Scale = 5  $\mu\text{m}$ . **(D)** Transmission Electron Microscopy (TEM) image of the algal prey *P. antarctica* with its plastids (p), pyrenoid (py), mitochondria (m) and nucleus (n); Scale = 0.5  $\mu\text{m}$ . **(E)** Micrograph from a Focused-Ion Beam Scanning EM (FIB-SEM) stack of a dinoflagellate host with its nucleus (hn) and mitochondria (hm), as well as the stolen organelles of the microalga *P. antarctica*: plastids (kp) and their pyrenoids (kpy), mitochondria (sm) and nucleus (sn). These stolen algal organelles were enclosed in a large compartment, (symbiosome; sym); Scale = 2  $\mu\text{m}$ . **(F)** Three-dimensional reconstruction based on FIB-SEM of the microalga *P. antarctica* (top left; prey) with its plastids (green), mitochondria (red) and nucleus (blue), and the subcellular organization of the RSD host in fed condition with stolen plastids (green) and mitochondria (red), and host mitochondria (orange) (based on a stack of ~2800 images, 6 nm resolution). 3D reconstruction confirmed the significant enlargement of stolen plastids compared to their native state. The outline of the host cell was completed with a dashed line; Scale = 2  $\mu\text{m}$ .



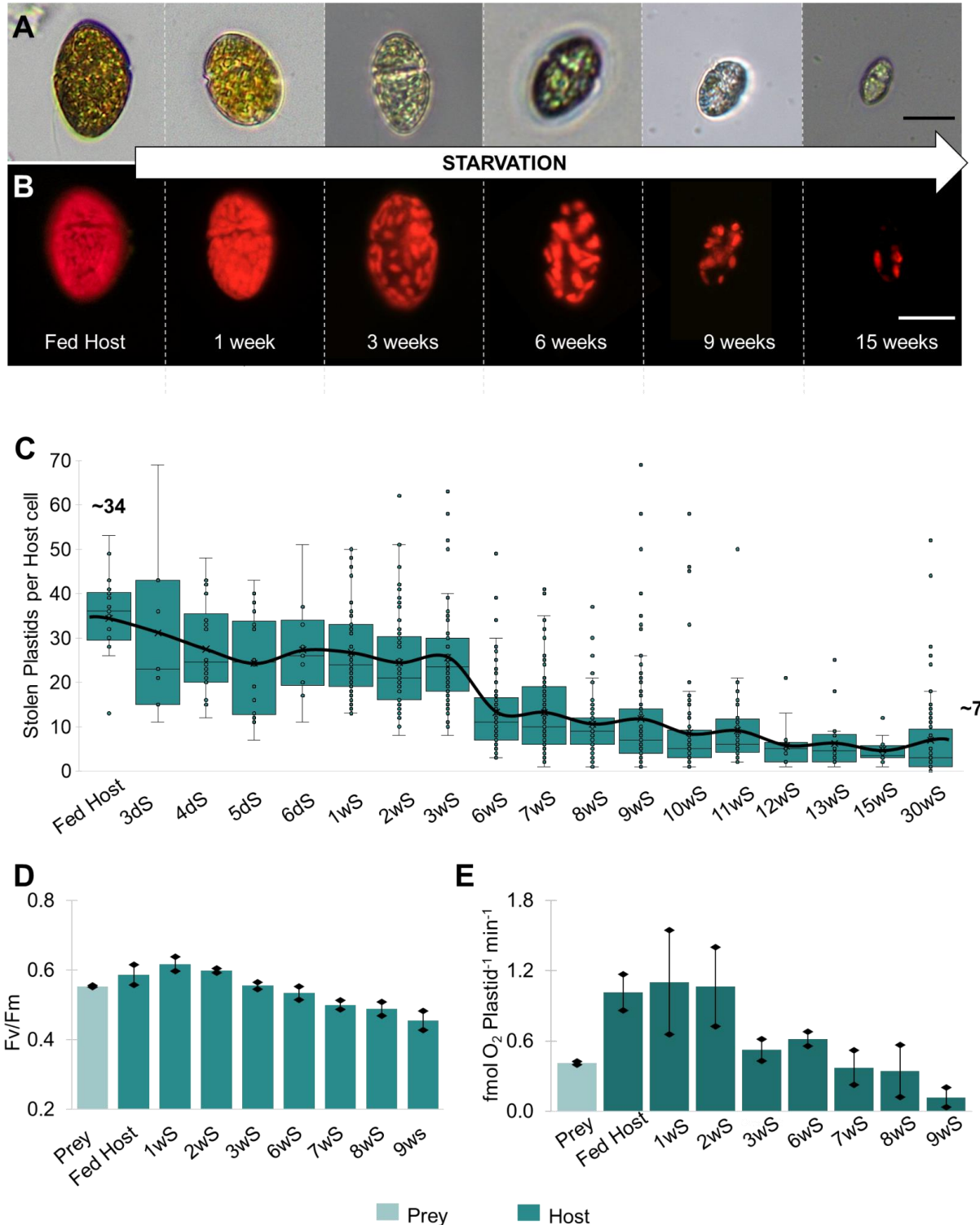
**Figure 2: Morphometrics and photophysiology of stolen plastids within the dinoflagellate host (fed condition).** **(A-B)** FIB-SEM based 3D reconstructions of native plastids (green) from non-dividing and dividing algal prey *P. antarctica* (A), and stolen plastids (green) in the dinoflagellate host (RSD) (B), including their carbon-fixing pyrenoid (magenta). Stolen plastids displayed different morphologies and some of them seemed to divide as shown by the constriction point and continuous pyrenoid (observations done on three different host cells); Scale = 2  $\mu\text{m}$ . **(C)** Volume of individual plastids (green; right axis) and their pyrenoids (magenta; left axis) in native (non-dividing microalga,  $n = 12$  and dividing microalga,  $n = 9$ ) and stolen states (fed host;  $n = 14$ ) assessed from 3D models ( $\mu\text{m}^3$ ). On average, volumes of stolen plastids and pyrenoids increased six and five times, respectively, compared to native plastids. Data available in Table S1. **(D)** Occupancy (%) of the pyrenoid (magenta) within the native and stolen plastids (green) assessed from FIB-SEM reconstructions. **(E)** Photosynthetic efficiency was measured by the relative Electron Transport Rate through Photosystem II (rETR II,  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) from a culture of *P. antarctica* (triangle) and a culture of fed dinoflagellate hosts (square) over a range of eight light intensities from 15 to 219  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  ( $n = 3$ ; each measure was obtained from three culture replicates). ETR curves showed that photosynthetic efficiency was higher in stolen plastids compared to native plastids ( $P$  value  $< 0.001$ ). **(F)** Gross photosynthetic oxygen per plastid ( $\text{fmol O}_2 \text{ plastid}^{-1} \text{ min}^{-1}$ ) in fed hosts was 2.5 times higher compared to the microalga ( $n = 3$ ; each measure was obtained from three culture replicates). **(G-I)** NanoSIMS  $^{13}\text{C}/^{12}\text{C}$  isotope ratio images of fed hosts from resin sections showing  $^{13}\text{C}$  enrichment in stolen algal organelles (plastids (kp) and their pyrenoids (kpy), mitochondria (sm), nucleus (sn)), as well as in the host nucleus (hn), trichocysts (t) and other unknown structures. **(H)** Zoom in of G. Overlays available in Figure S3. **(G-I)** Scale = 2  $\mu\text{m}$ . See also Tables S1 and S2.



**Figure 3: Three-dimensional reconstruction of newly stolen mitochondria and nucleus of the algal prey within the dinoflagellate host. (A)** 3D reconstruction based on FIB-SEM of a *Phaeocystis antarctica* cell (prey) and a coronal section (below), with nucleus (blue), two plastids (green) and mitochondria (red). **(B)** 3D reconstruction of a host region focusing on the stolen nucleus and mitochondria of *P. antarctica*, and an axial section (below) showing the significant increase in volume of these algal organelles. Particularly, the algal mitochondria formed an extensive network around the plastids and algal nucleus. **(C)** Volume occupancy (%) of different

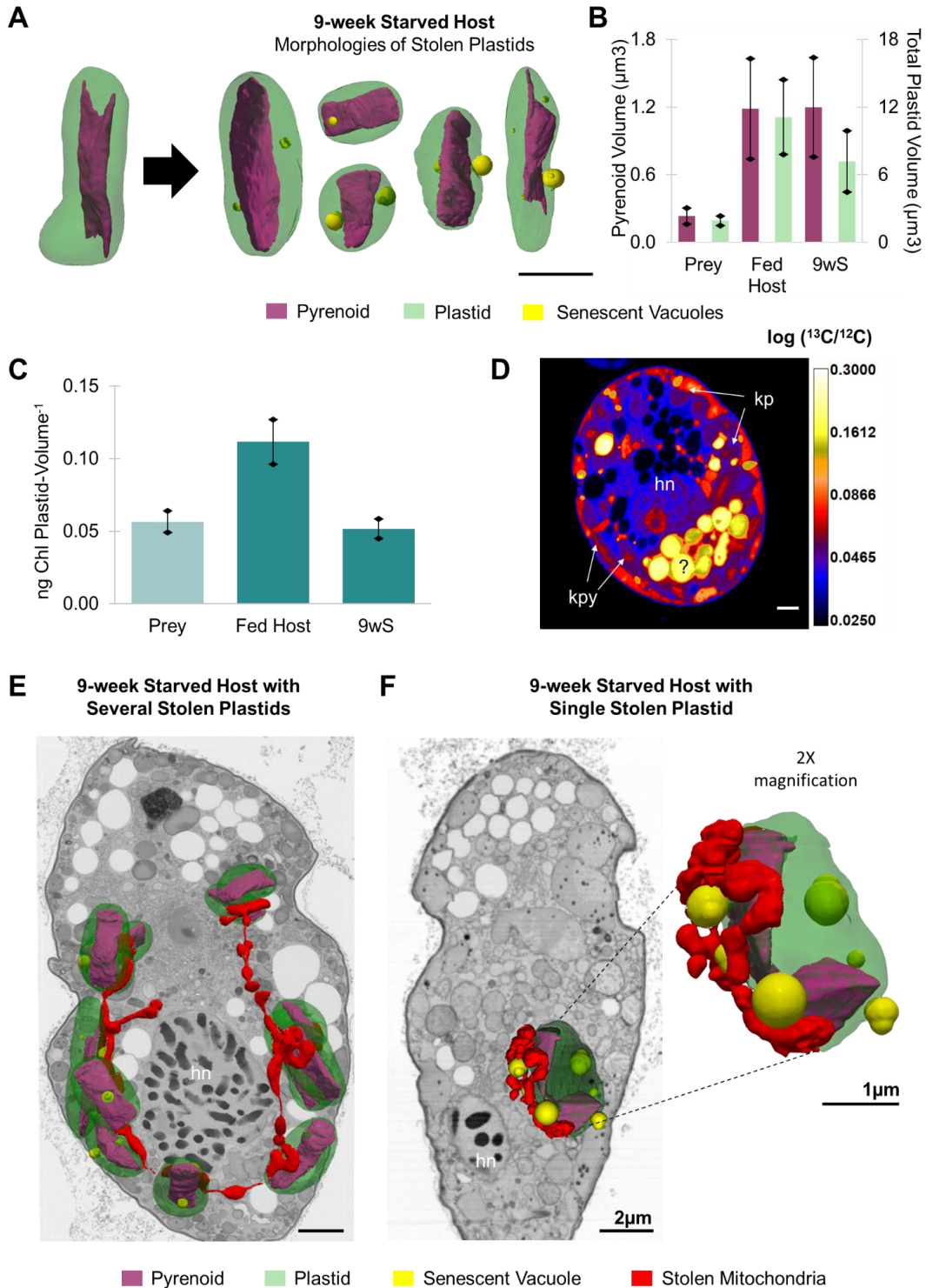


nuclear compartments - euchromatin (blue), heterochromatin (dark blue, electron dense nuclear structures), and nucleolus (light blue) - within the volume of native and stolen prey nucleus (prey nucleus,  $n = 3$ ; stolen nucleus,  $n = 1$ ). This shows that heterochromatin volume occupancy decreased and the nucleolus volume occupancy increased in stolen nucleus. **(D)** 3D reconstructions of different interactions between the stolen mitochondria (red) and plastids (green) within the host. Black arrows indicate the site of entry of the mitochondrion into the plastid and a pocket-like structure showing close interaction between both organelles. **(A,B,D)** Scale = 2  $\mu\text{m}$ . Plastid (green), pyrenoid (magenta), mitochondria (red), nucleolus (light blue), heterochromatin (dark blue), euchromatin (blue), rest of the cell/symbiosome (grey). See also Table S1.



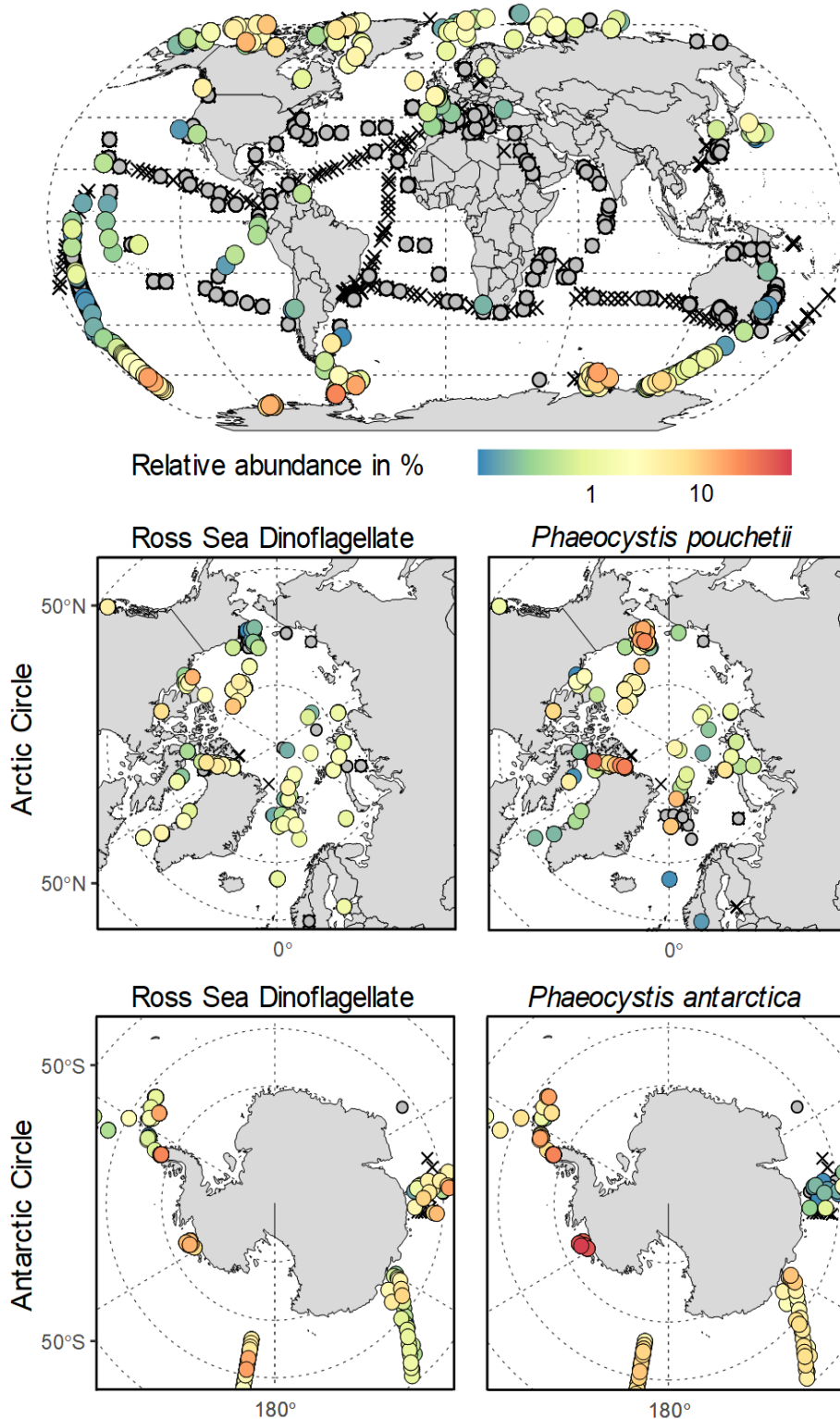
**Figure 4: Retention time and photophysiology of stolen plastids in host cells during several weeks of starvation (in the absence of algal prey).** (A-B) Light microscopy images of host cells (RSD dinoflagellate) over 15 weeks of starvation in bright field (A) and fluorescence (B, red chlorophyll fluorescence); Scale = 20  $\mu\text{m}$ . (C) Box plot showing the number of stolen plastids per host cell over 30 weeks of starvation (abbreviated as “wS”). Plastid counts were

performed from fluorescence images taken at different time points from 18 to 88 host cells. At the beginning of starvation, the number of stolen plastids per host cell was around 34 and decreased to ~ 7 at week 30. Plastid numbers were also assessed every day during the first week (3dS, 4dS, 5dS and 6dS). The black line connects the average plastid number of each week. Increased number of outliers were observed in the later weeks (> 3 weeks) of starvation and corresponded to very large host cells in a resting-like stage. **(D)** Bar plot of Maximum Quantum Yield (Fv/Fm) of Photosynthesis from a culture of microalga *Phaeocystis antarctica* (prey) and culture of fed and starved host cells over 9 weeks. Fv/Fm tended to slightly increase at week 1 (1wS, Fv/Fm = 0.618) compared to the fed host (Fv/Fm = 0.587), followed by a continuous decrease (Fv/Fm = 0.455 at week 9; 9wS) (n = 3; each measure was obtained from three culture replicates). **(E)** Gross oxygen production per plastid (fmol O<sub>2</sub> Plastid<sup>-1</sup> min<sup>-1</sup>) measured from the microalga *P. antarctica* (prey) and starved host cells over 9-weeks. Compared to newly stolen plastids (fed conditions), oxygen production per plastid was similar at week 1 (1.10 fmol O<sub>2</sub> Plastid<sup>-1</sup>min<sup>-1</sup> compared to 1.01 fmol O<sub>2</sub> Plastid<sup>-1</sup>min<sup>-1</sup>), and then gradually decreased over the next few weeks (0.12 fmol O<sub>2</sub> Plastid<sup>-1</sup>min<sup>-1</sup> at week-9; 9wS) (n = each measure was obtained from three culture replicates). See also Table S2.



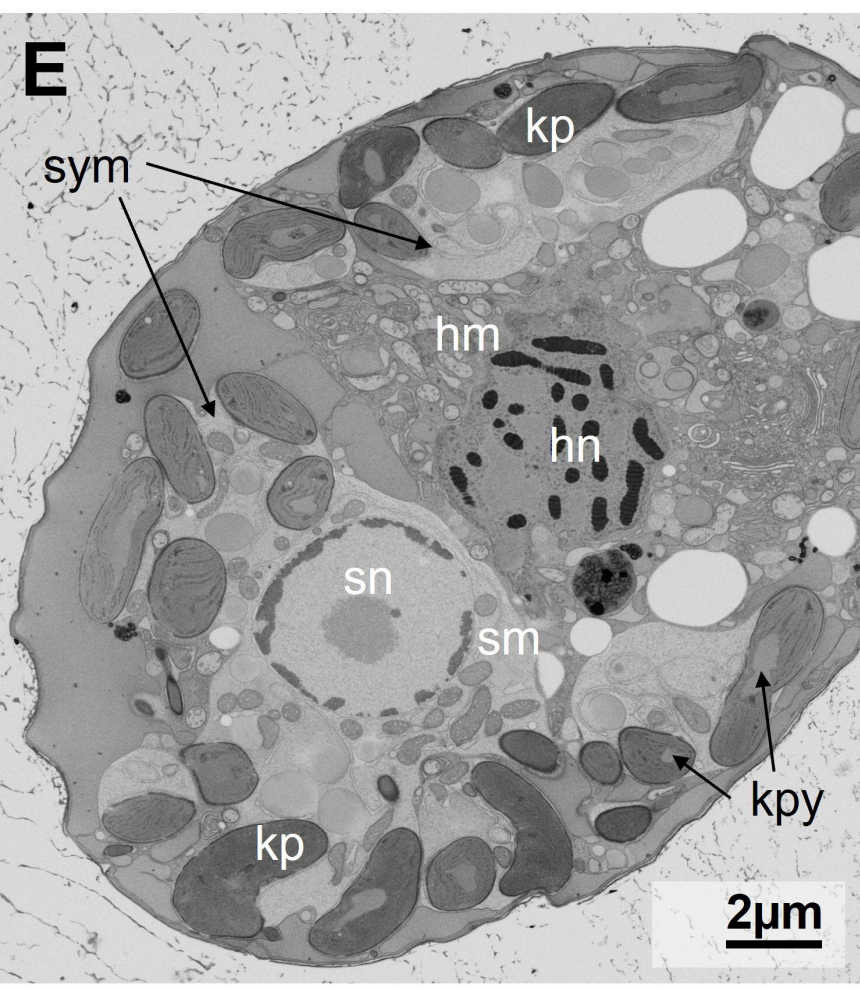
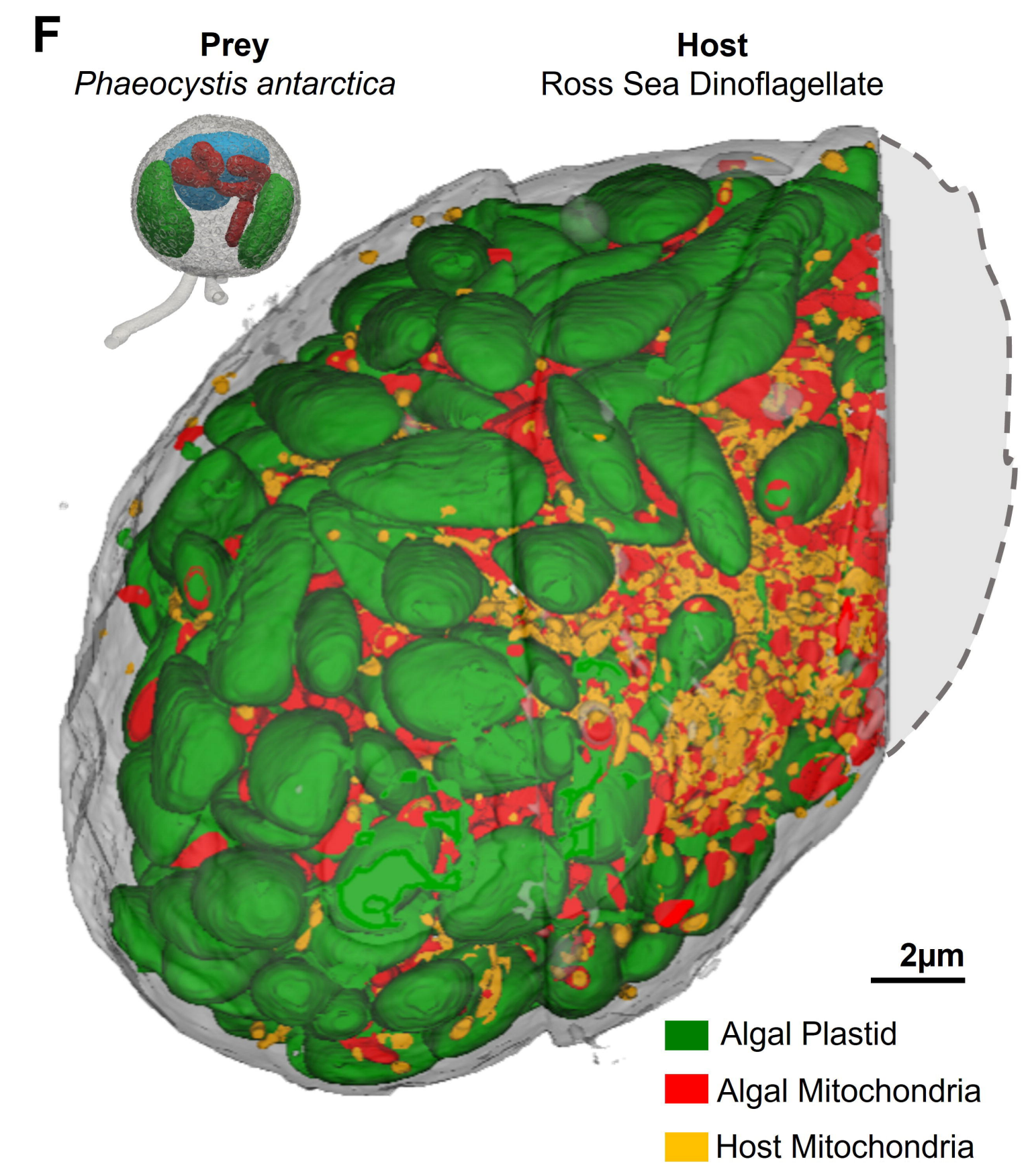
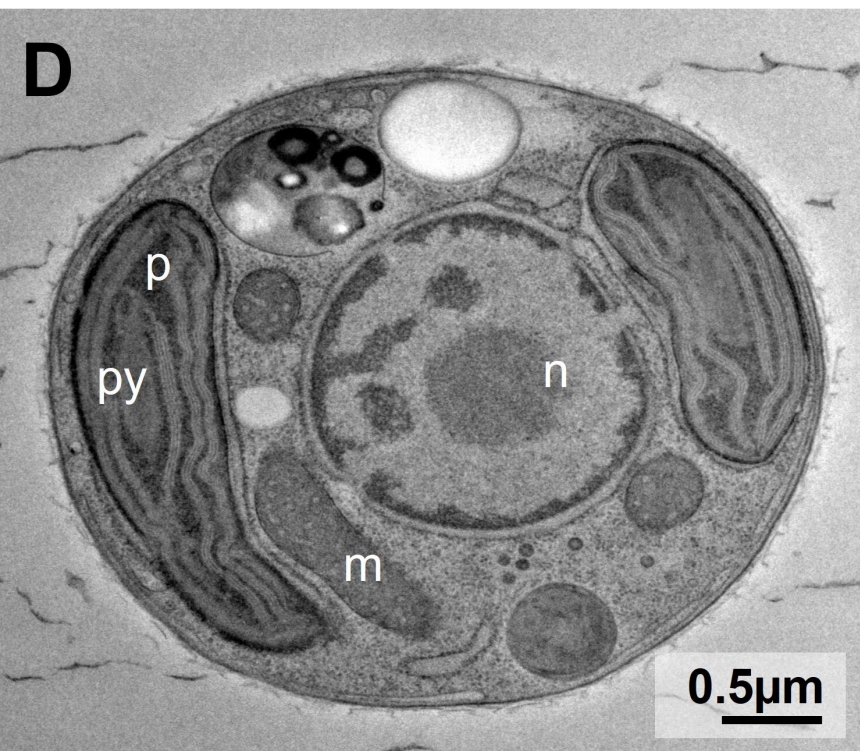
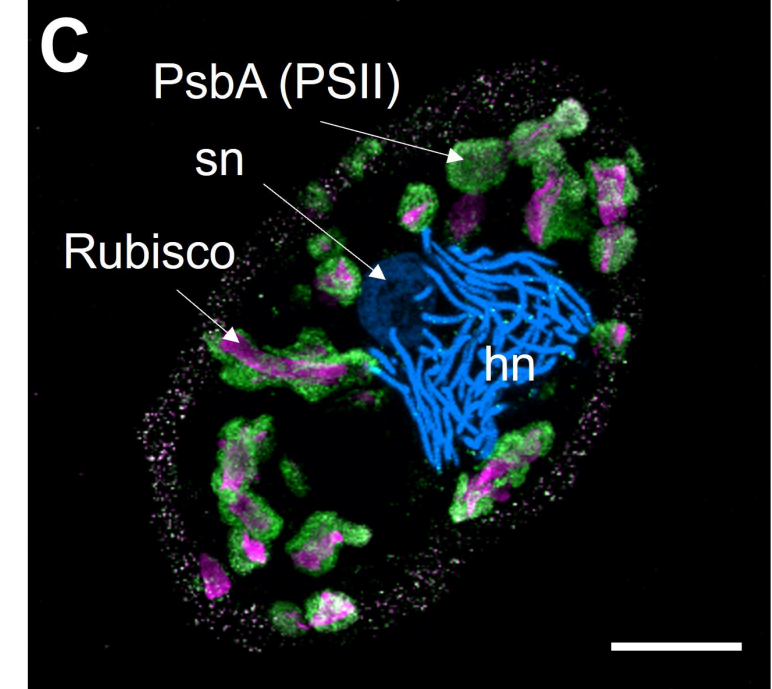
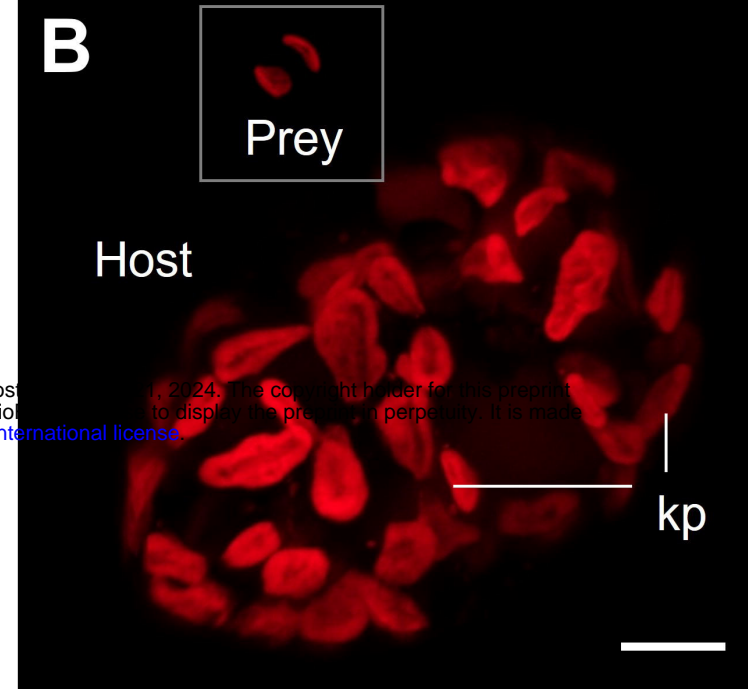
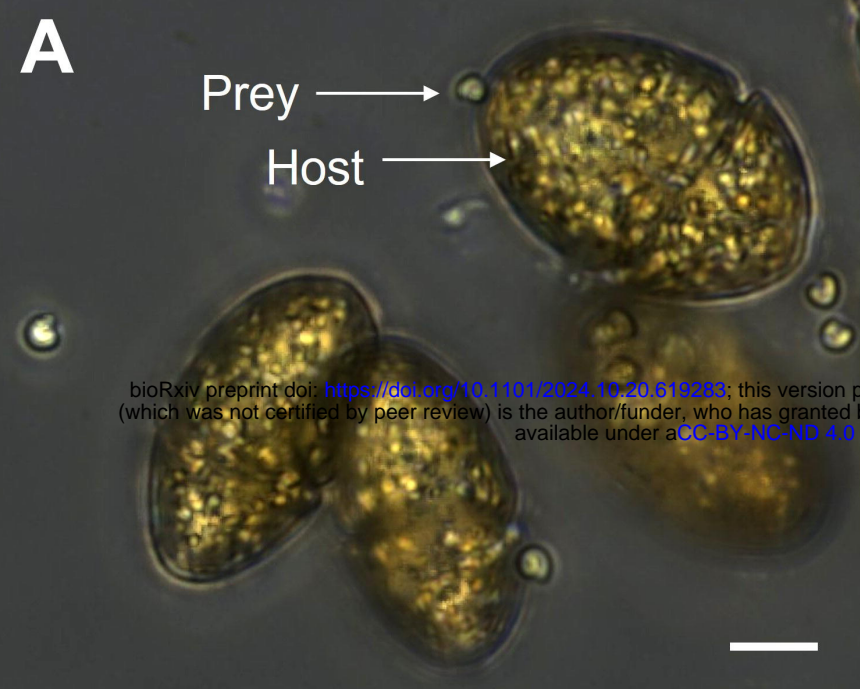
**Figure 5. Morphology and physiology of “old” stolen organelles (the algal plastids and mitochondria) at week 9 of starvation. (A) 3D-reconstruction of stolen plastids (green) and their internal pyrenoid (magenta) from fed and starved (9 weeks) hosts. Several unknown spherical structures (yellow) were observed at week 9, possibly vacuoles that could be a sign of senescence**

[58]; Scale = 2  $\mu\text{m}$ . **(B)** Volumes of individual plastids (green, right axis) and their pyrenoid (magenta, left axis) within algal prey ( $n = 12$  plastids), fed host ( $n = 14$  plastids from three hosts) and a 9-week starved host ( $n = 14$  plastids from two hosts). This shows a significant decrease of the plastid volume over time but the pyrenoid volume tended to remain relatively stable. Note that these 9-week-old plastids were still larger than the ones in the algal prey. **(C)** Chlorophyll per plastid volume ( $\text{ng Chl Plastid}^{-1}$ ) was assessed from *P. antarctica* (prey), stolen states in fed and starved (9 weeks) hosts (Chlorophyll measured on triplicate cultures). **(D)** NanoSIMS  $^{13}\text{C}/^{12}\text{C}$  isotope ratio image of starved host cell (week 9), showing  $^{13}\text{C}$  enrichment (log) in several host structures, so photosynthetically-fixed carbon was transferred to the host cell. Stolen plastids (kp) and their pyrenoids (kpy) exhibited lower  $^{13}\text{C}$  enrichment similar to the host nucleus (hn). Highly  $^{13}\text{C}$  labeled unknown vacuoles in the symbiosome were observed. The very low content of sulfur and phosphorus in these vacuoles suggests that it could be storage of carbohydrates that are produced by photosynthesis and accumulate over time (Figure S7). Scale = 2  $\mu\text{m}$ . **(E)** EM slice of a 9-week starved host overlaid with 3D reconstruction of stolen plastids (green), pyrenoids (magenta) and thread-like network of stolen mitochondria (red); host nucleus (hn); Scale = 2  $\mu\text{m}$ . **(F)** Starved host cell (week 9) with one stolen plastid surrounded by stolen algal mitochondria (scale = 2  $\mu\text{m}$ ) and a zoomed-in view with spherical vacuoles as in (A) (yellow) (Scale = 1  $\mu\text{m}$ ). See also Tables S1 and S2.

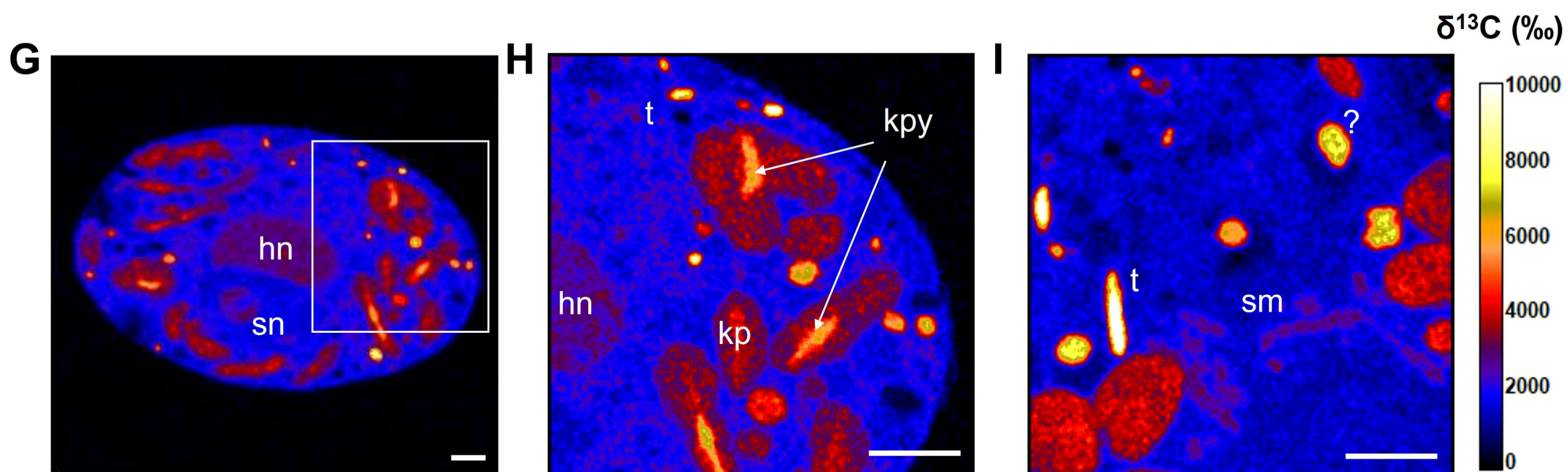
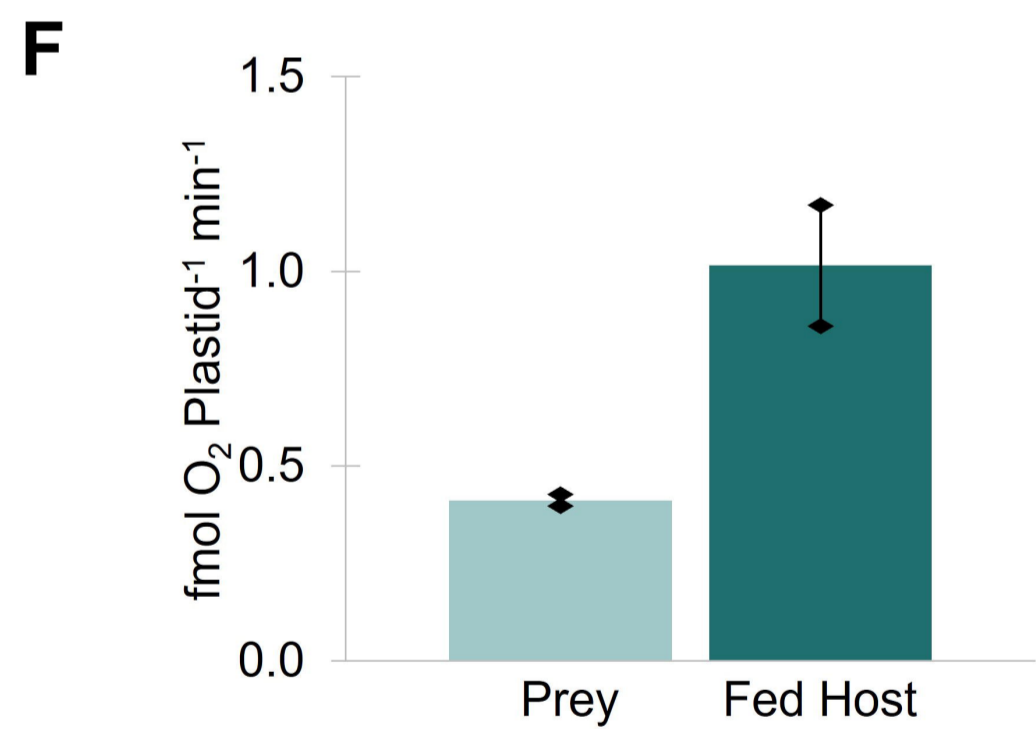
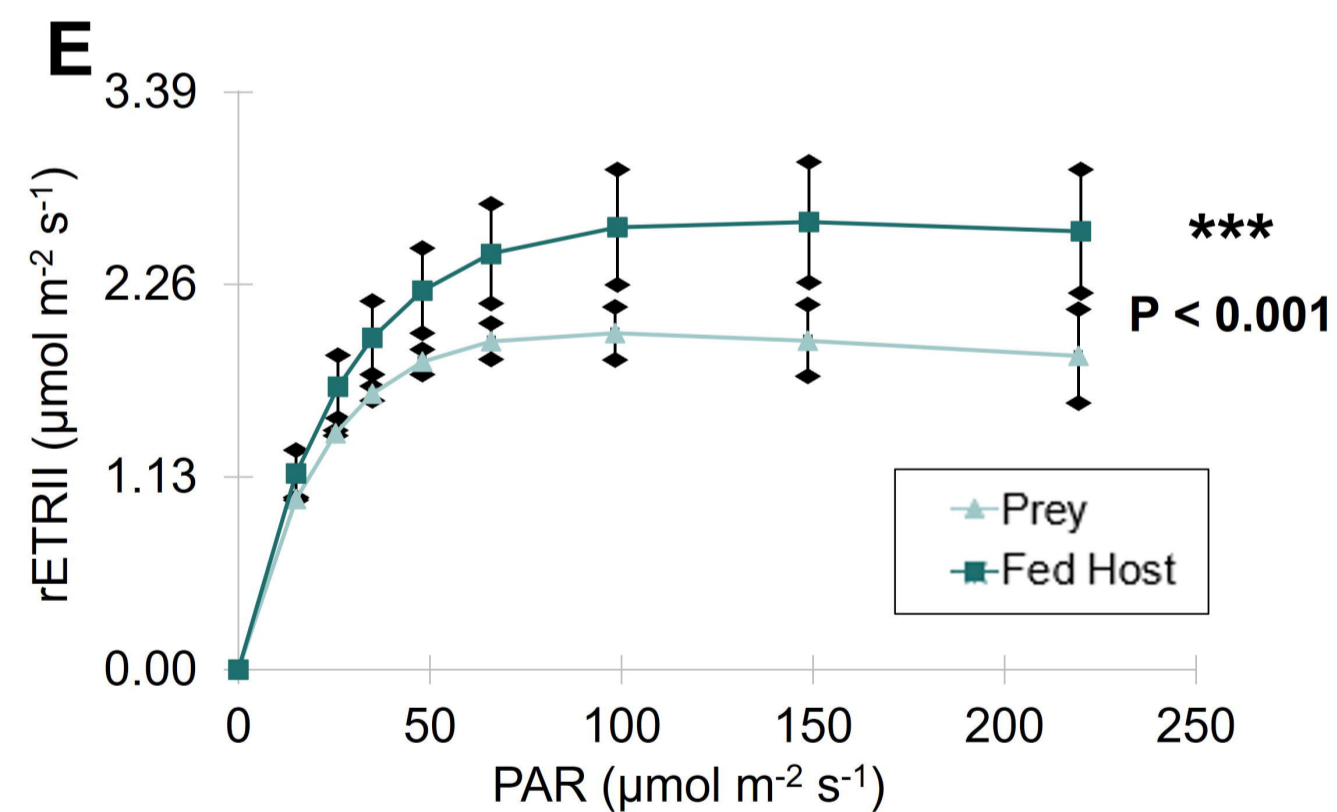
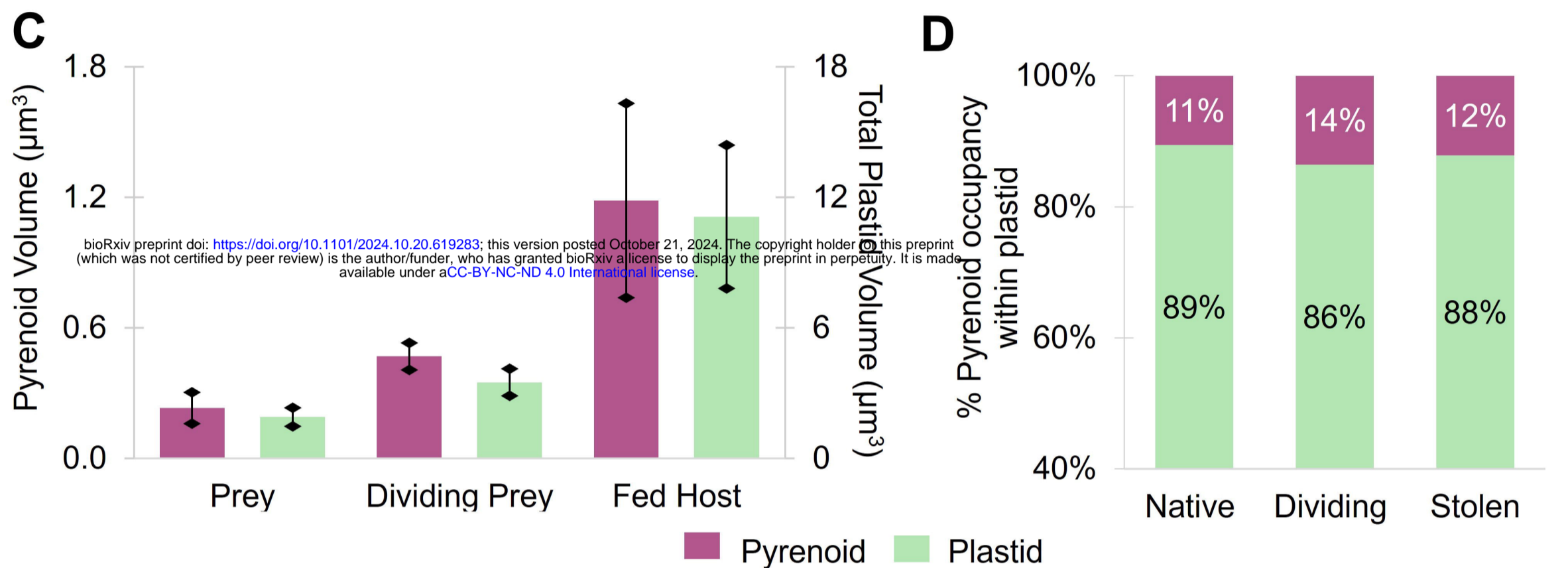
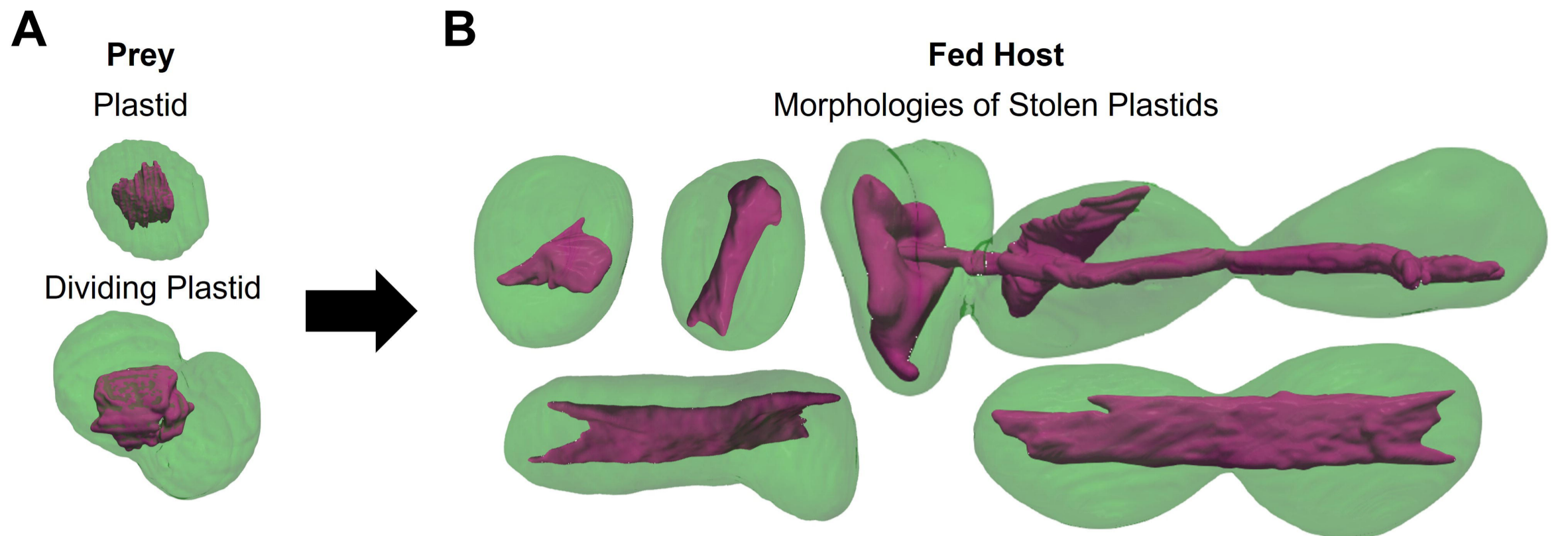


**Figure 6: Distribution of kleptoplastidic dinoflagellate RSD as unveiled by environmental metabarcoding datasets.** World map showing the relative abundance of metabarcoding V4 rDNA reads that matched 100% to query sequences of the RSD dinoflagellate, *Phaeocystis pouchetii*

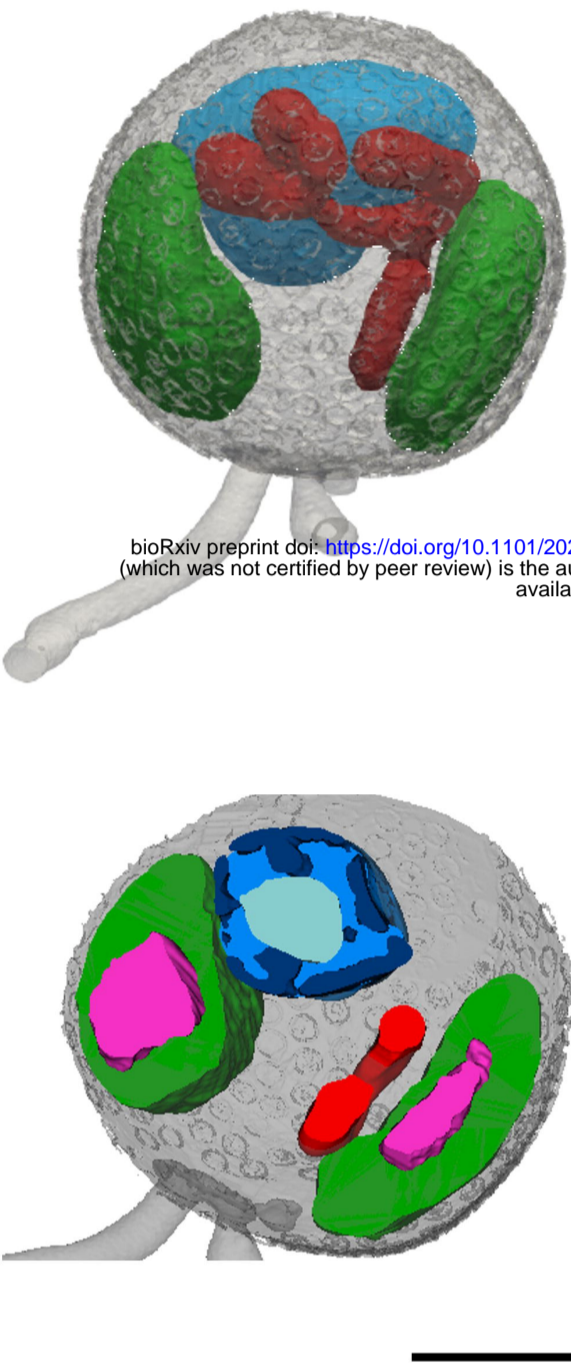
and *Phaeocystis antarctica* (% RSD reads among total reads of the community including protists and metazoans in a given sample) in different stations of the ocean. Higher relative abundance of RSD and polar *Phaeocystis* species reads was observed in the Arctic and Antarctic regions. Note that RSD dinoflagellate was not only found in different regions of Antarctic, but also widely distributed in the Arctic. Grey crosses on the map indicate when RSD or *Phaeocystis* reads were not detected.





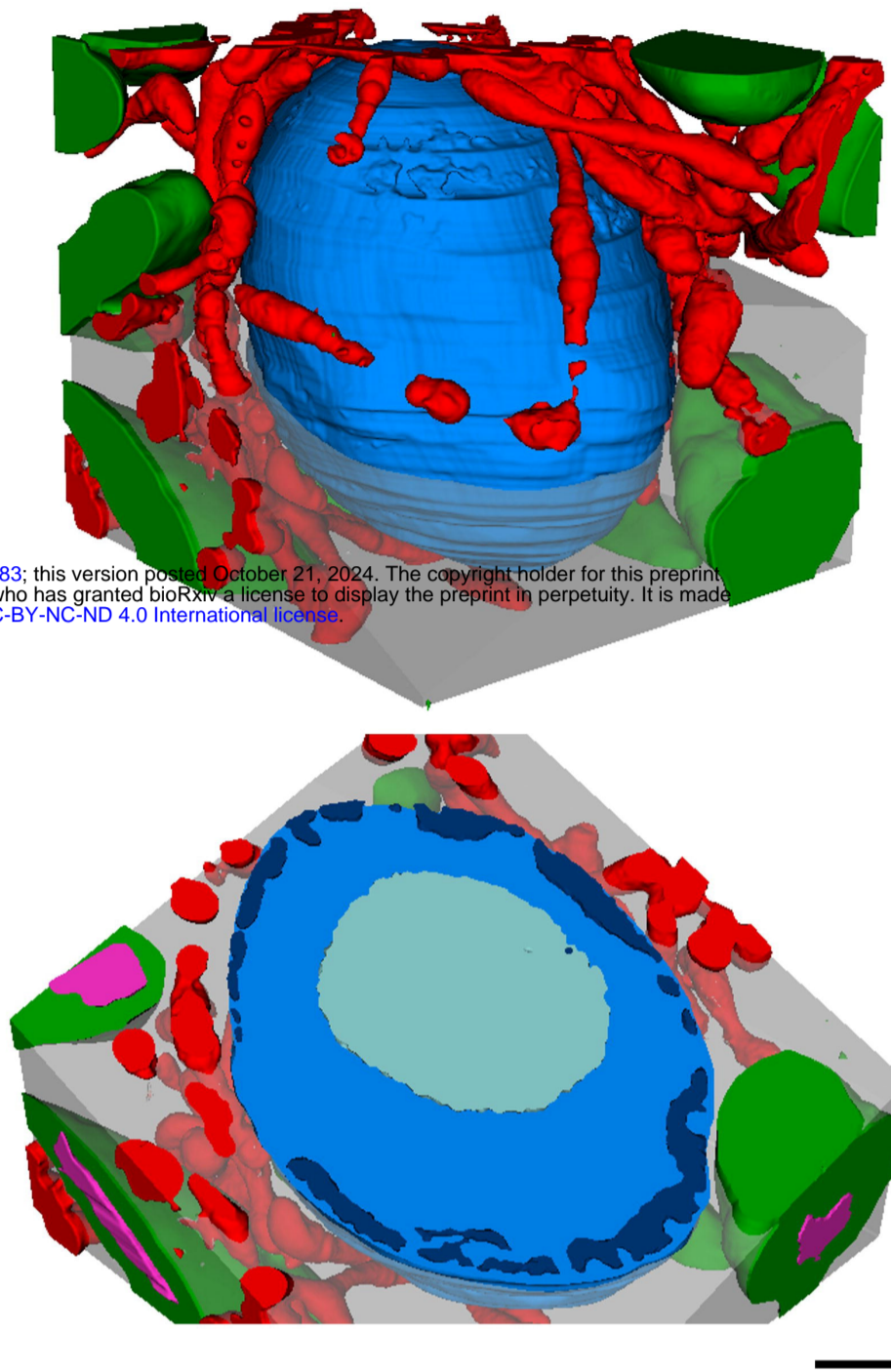


**A** Prey  
*Phaeocystis antarctica*



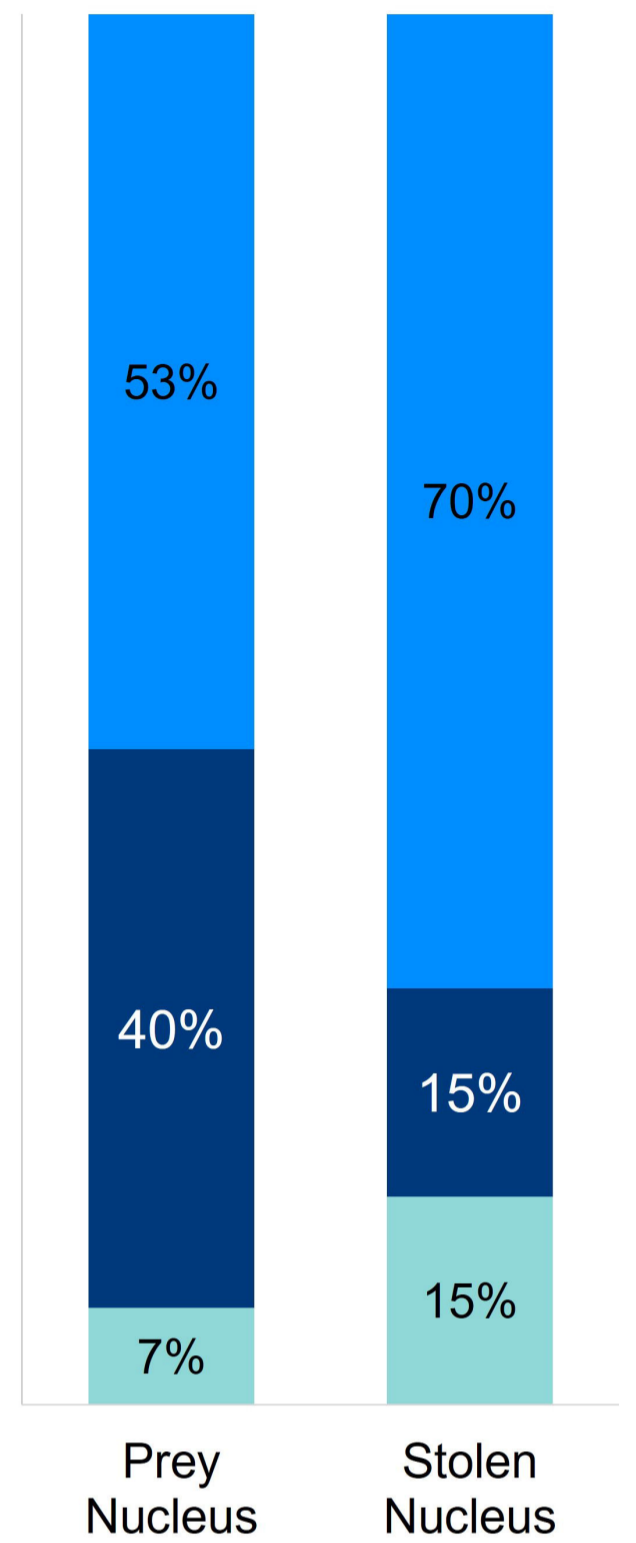
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**B** Stolen Mitochondria and Nucleus within Fed Host

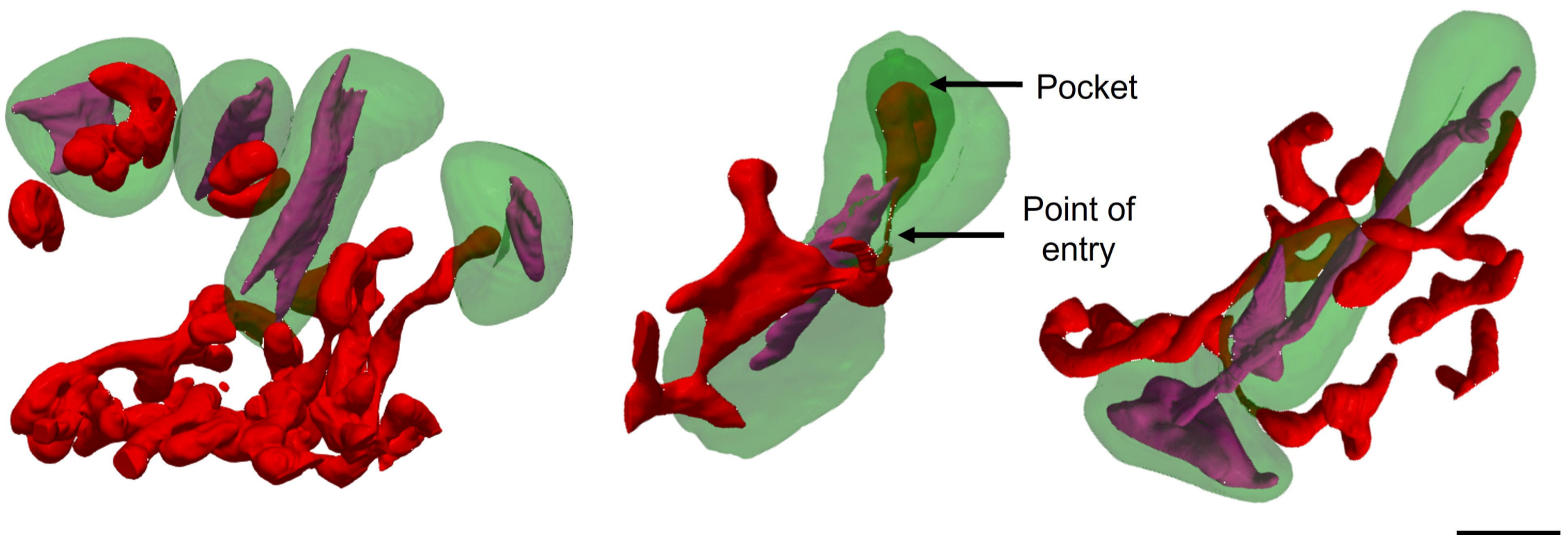


**C**

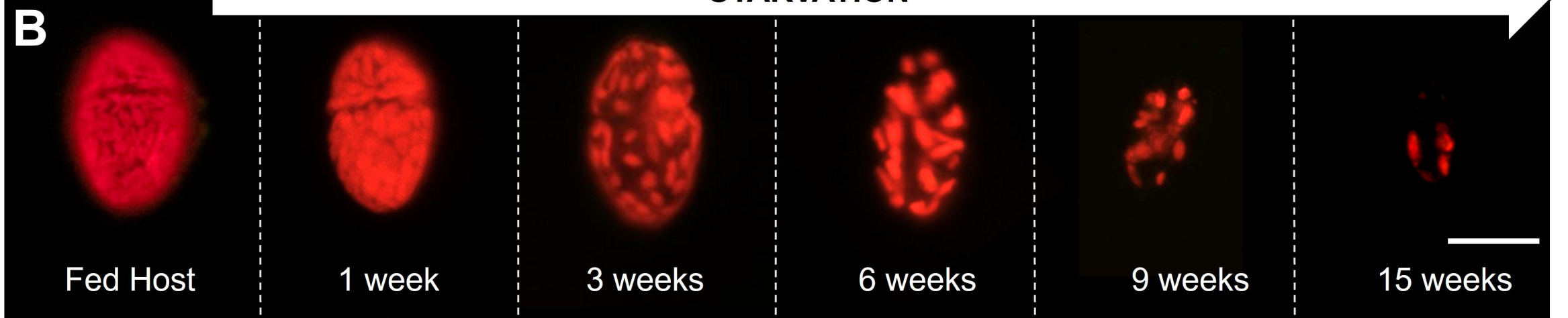
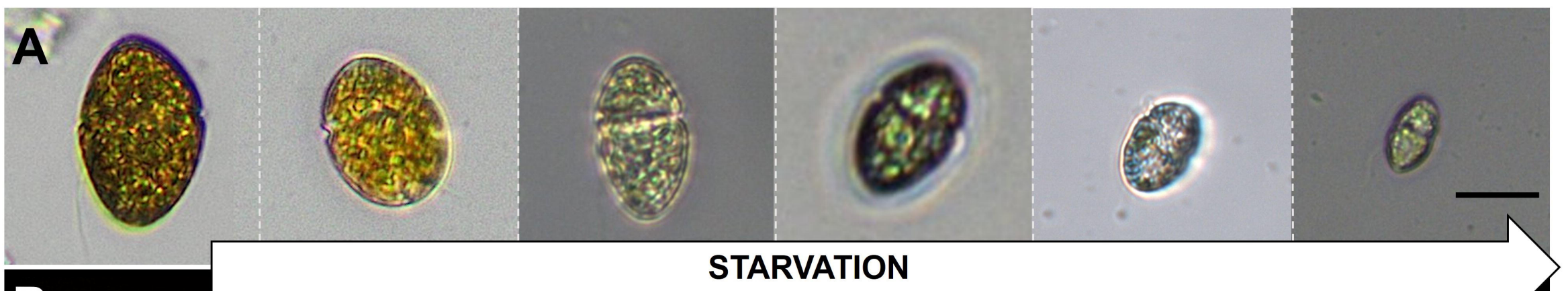
% Occupancy of Nuclear components



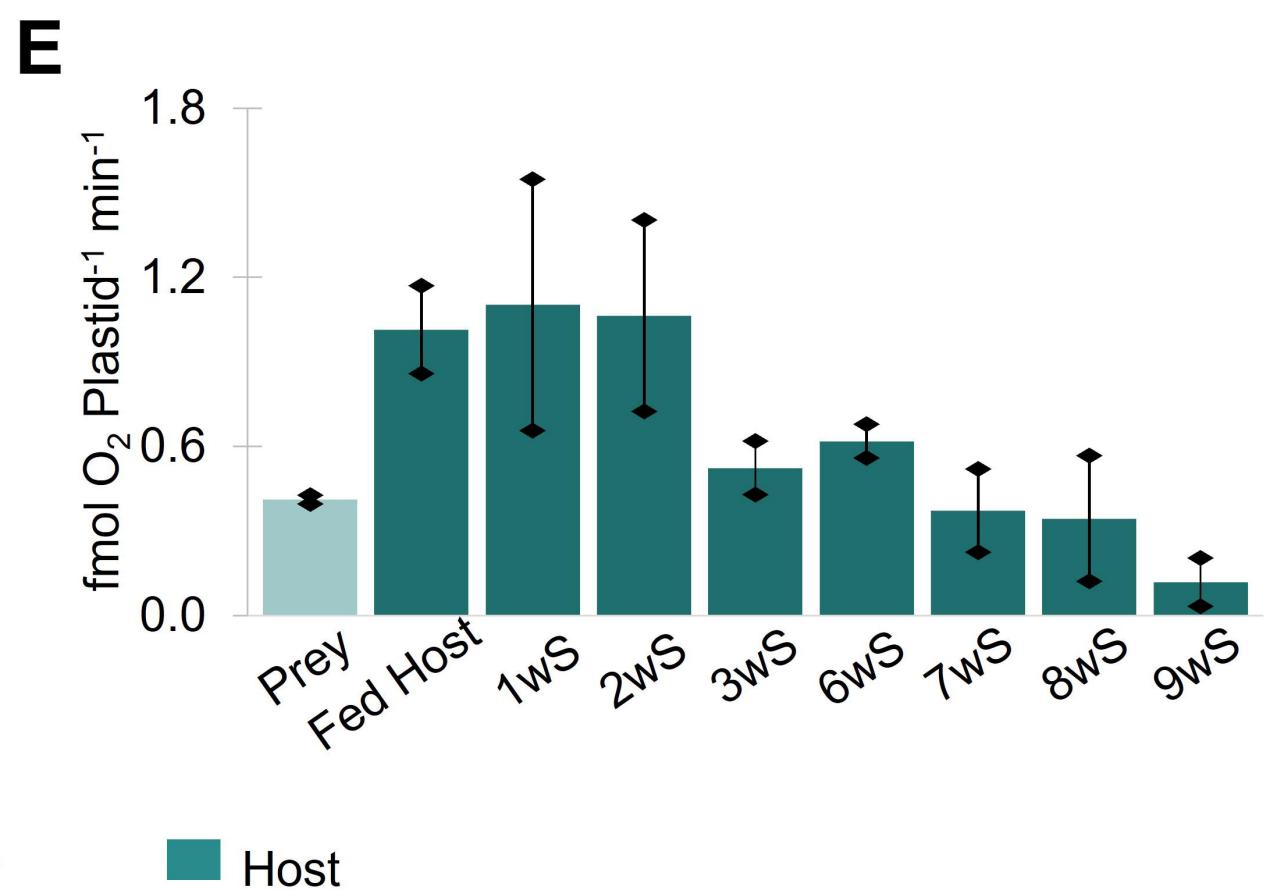
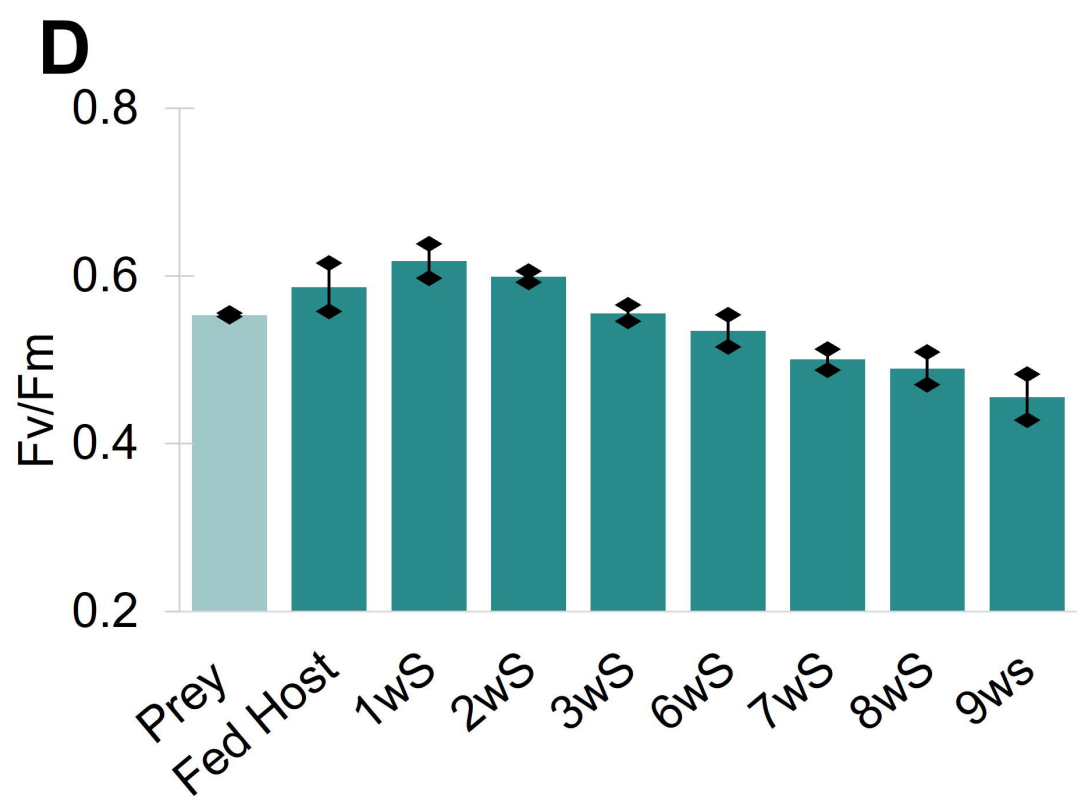
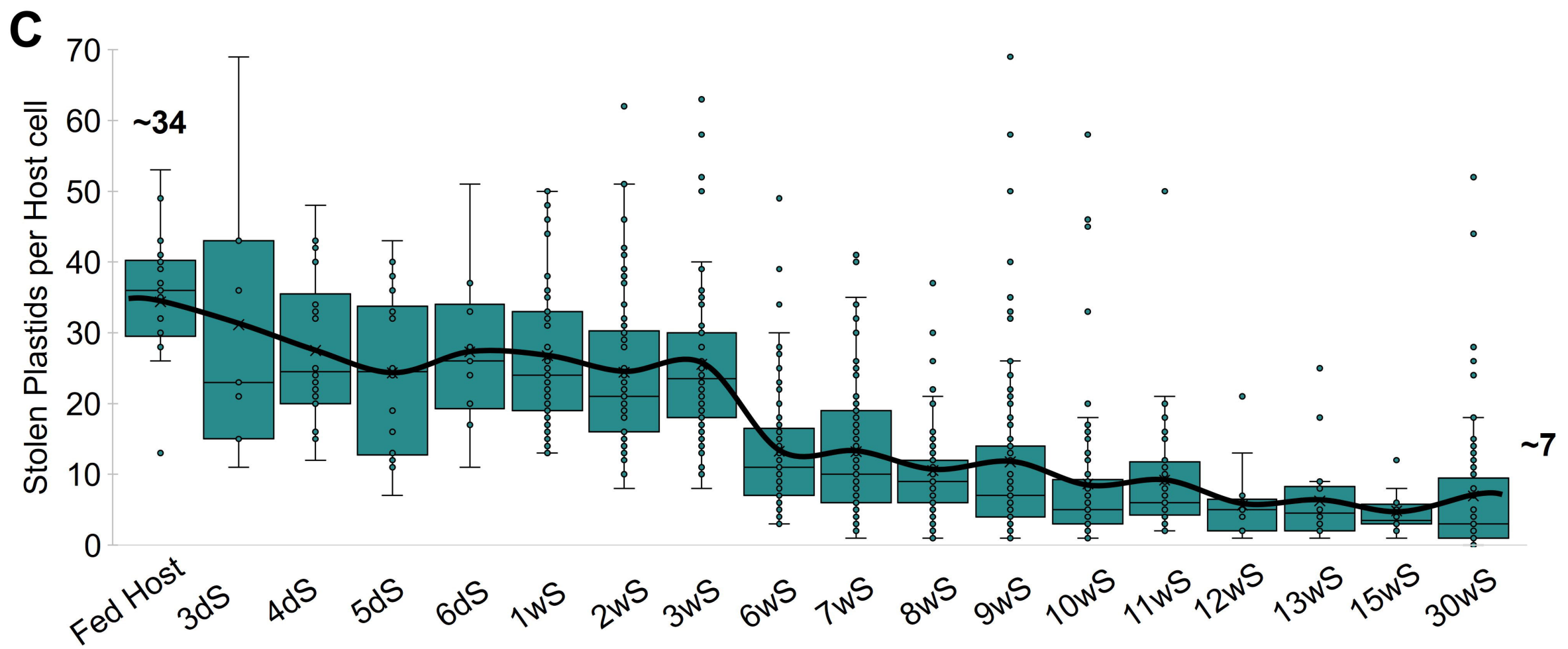
**D** Interaction between stolen Mitochondria and Plastid within Fed Host

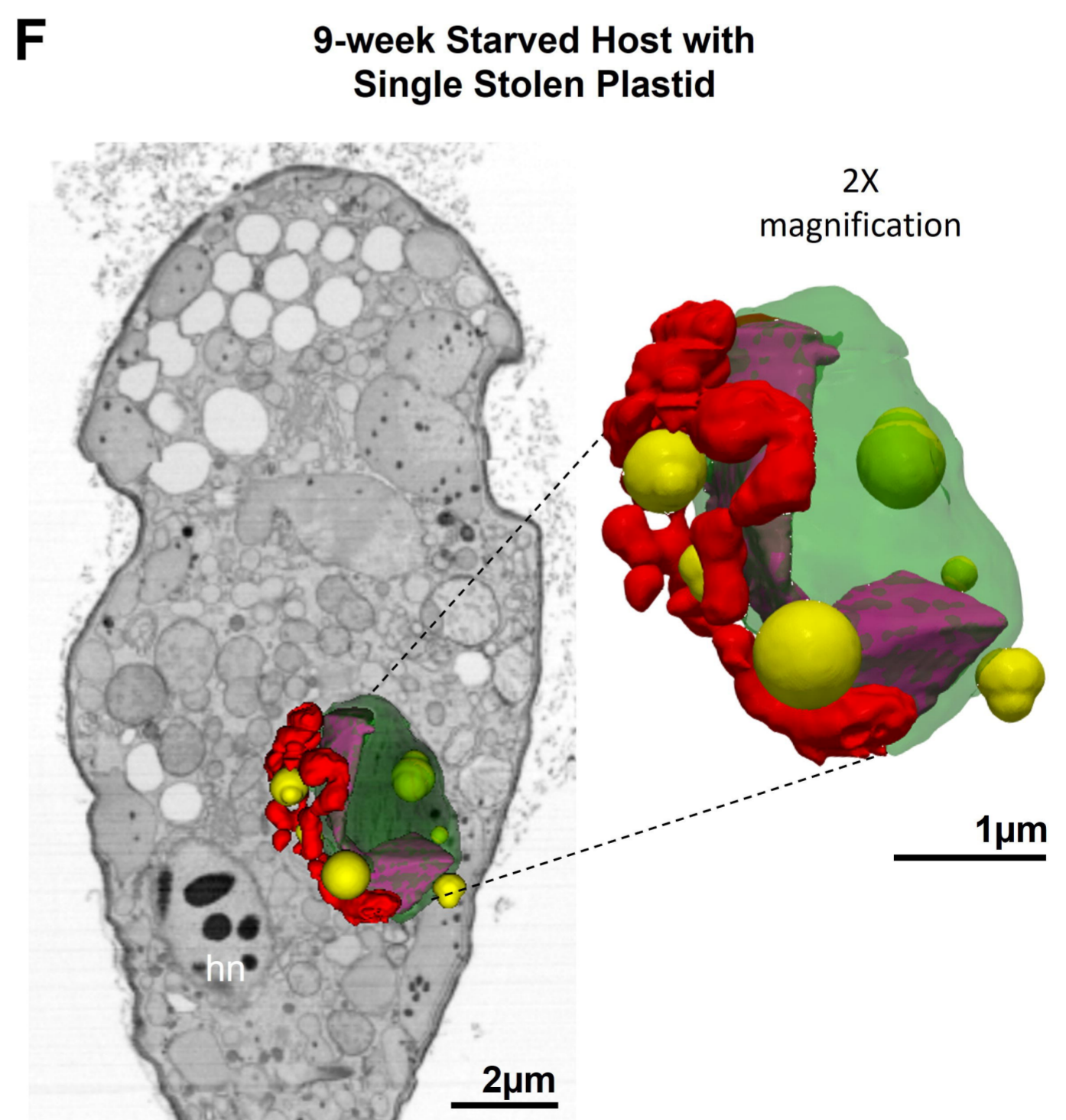
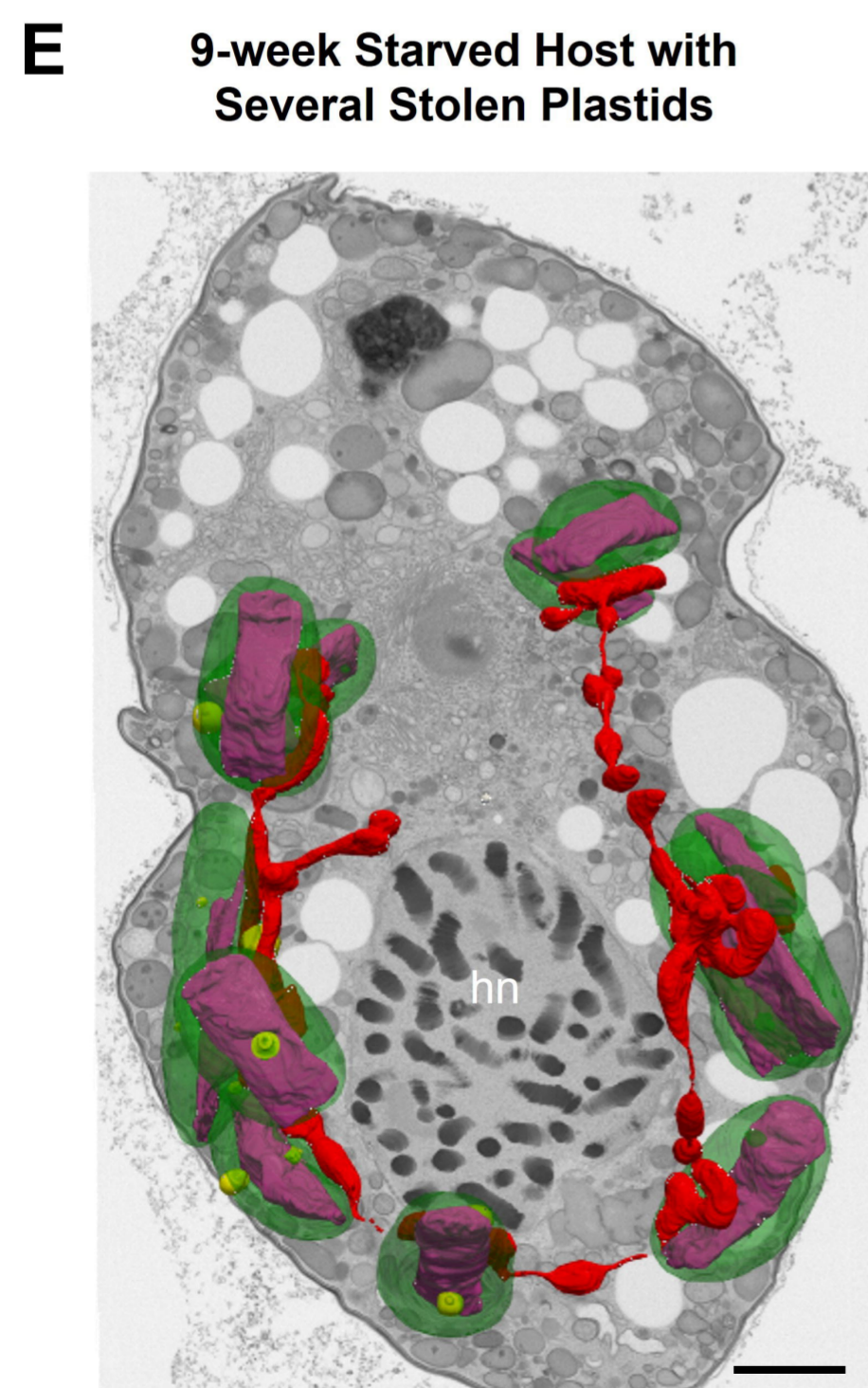
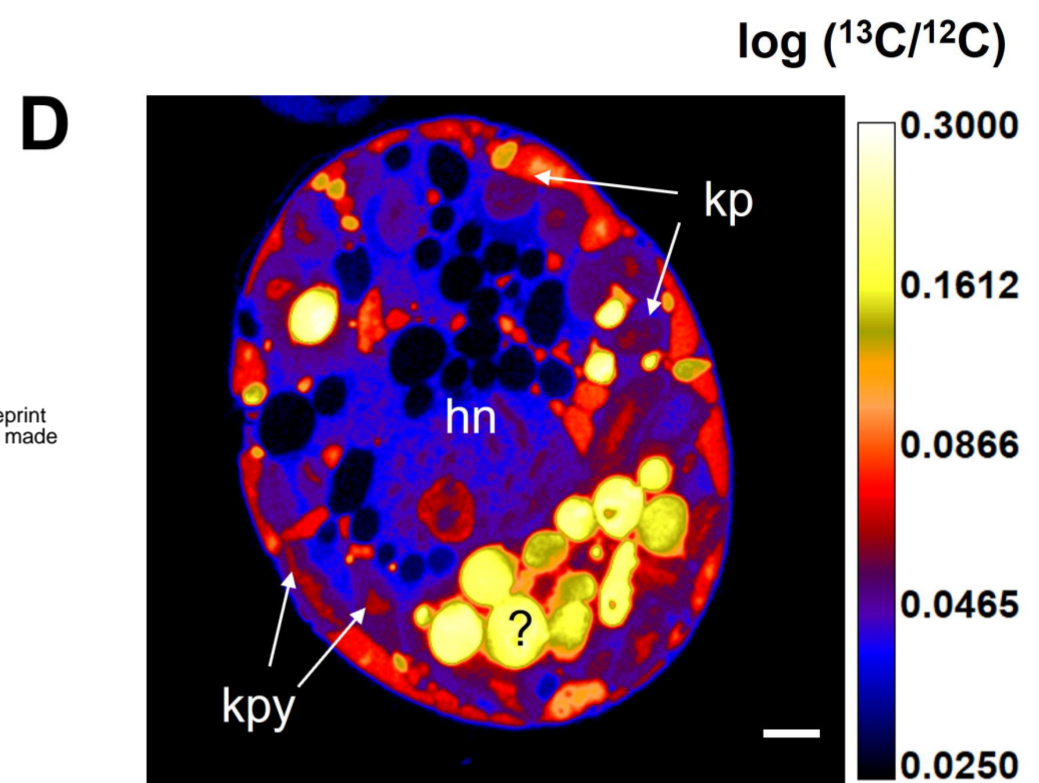
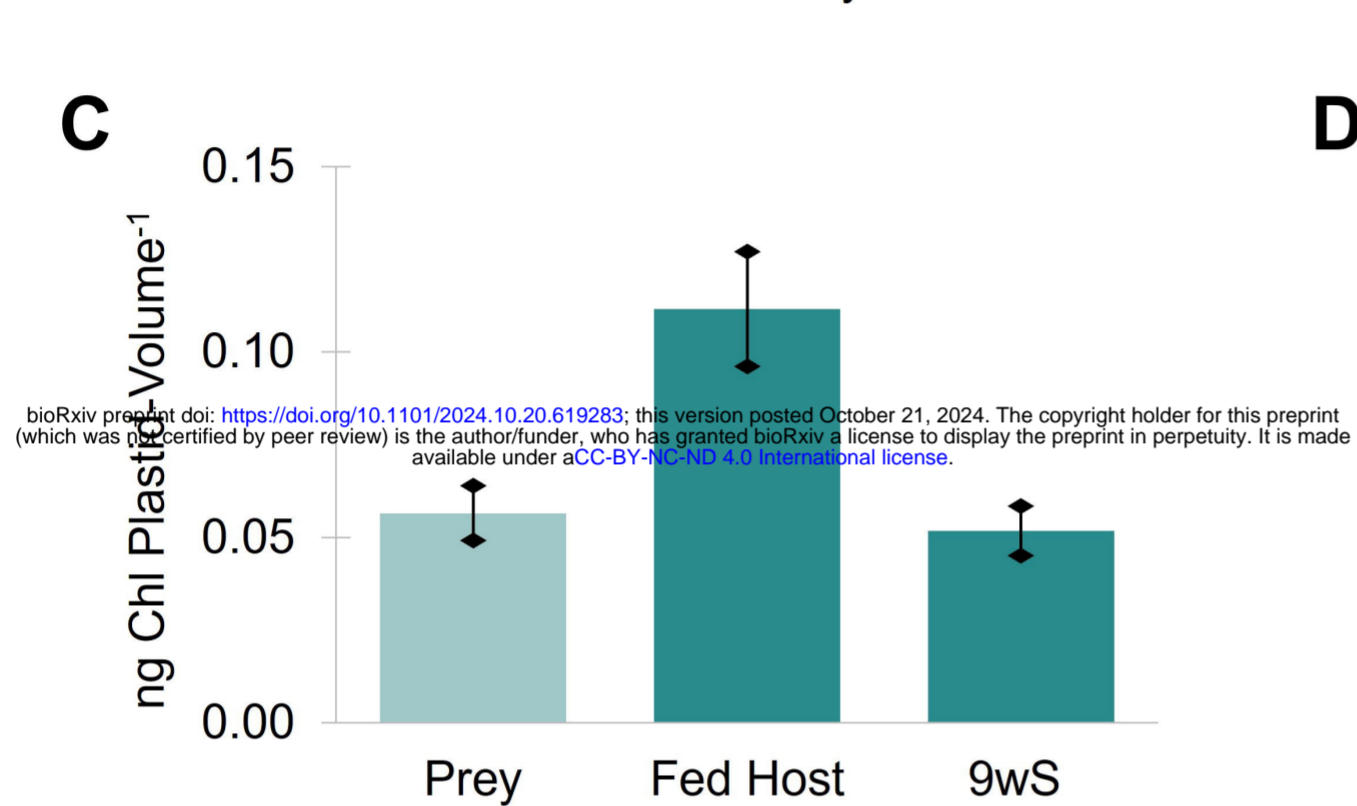
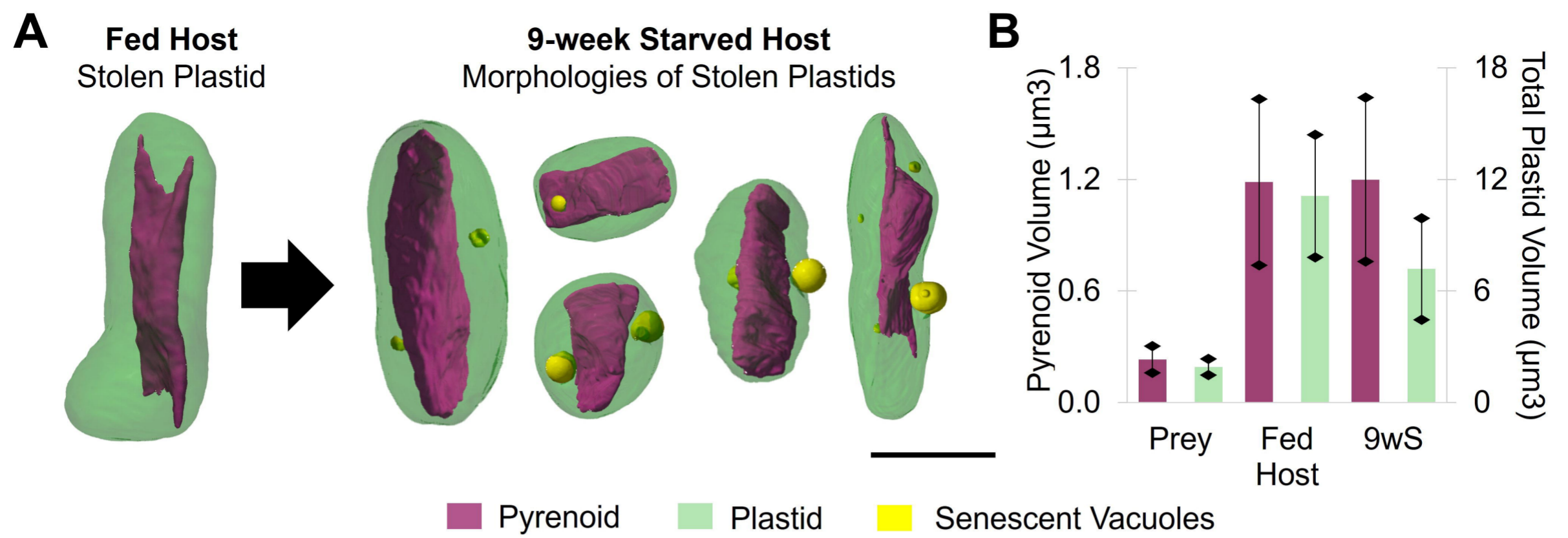


Plastid Pyrenoid Mitochondria Nucleolus Heterochromatin Euchromatin

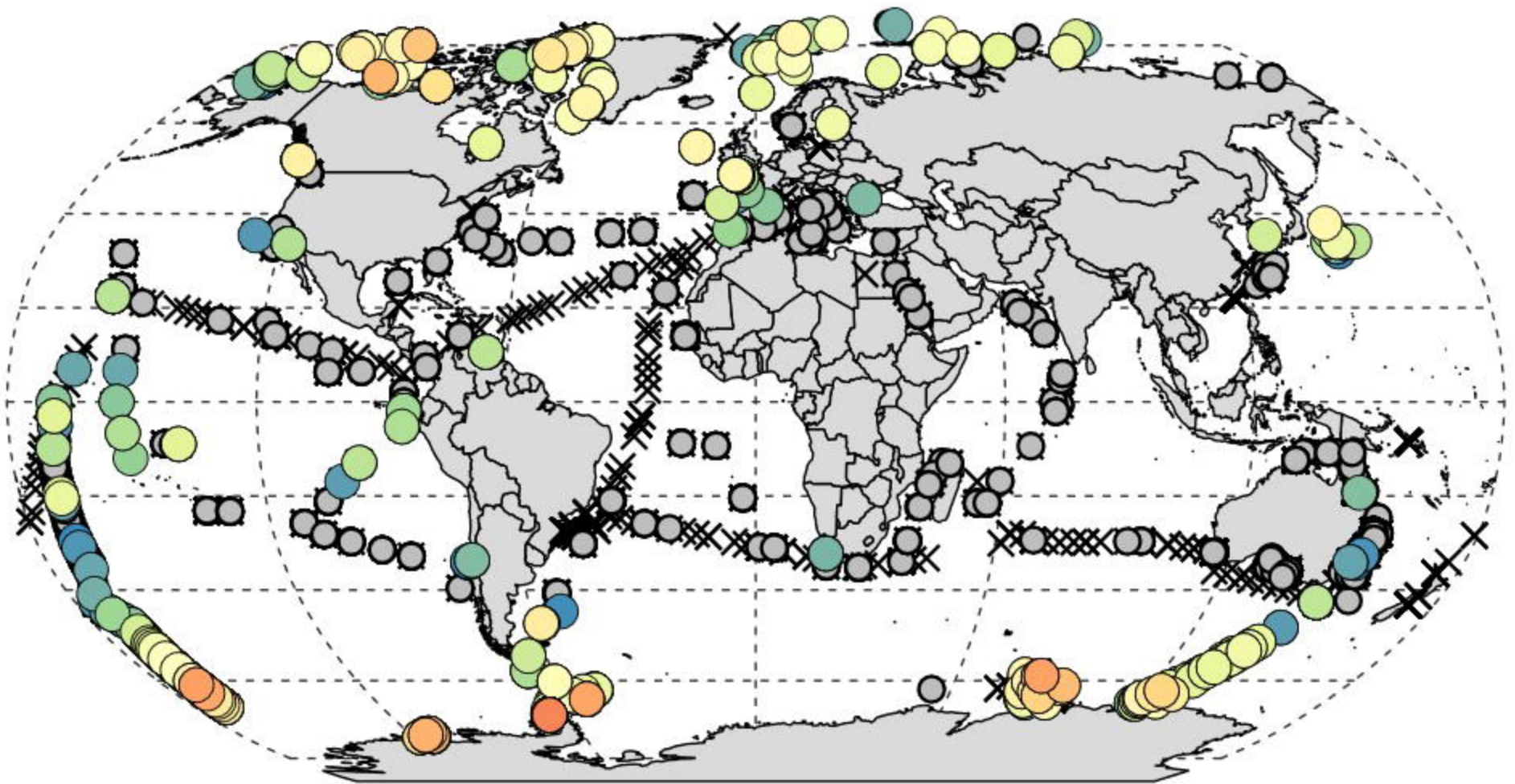


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Pyrenoid      Plastid      Senescent Vacuole      Stolen Mitochondria



Relative abundance in %



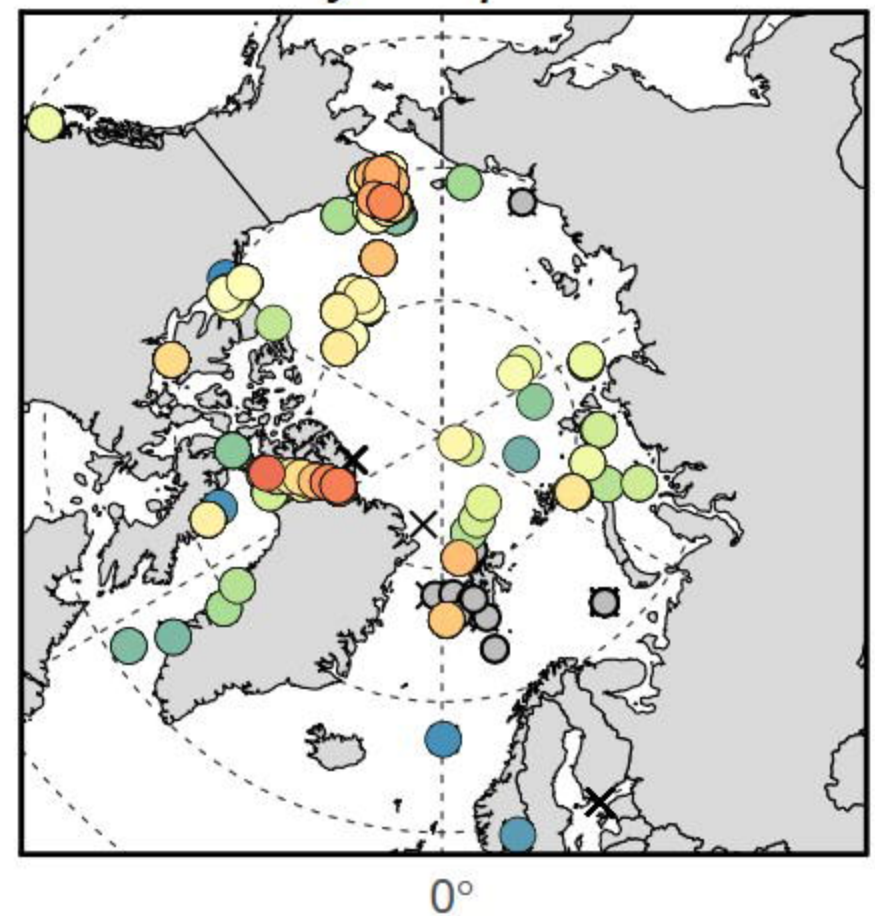
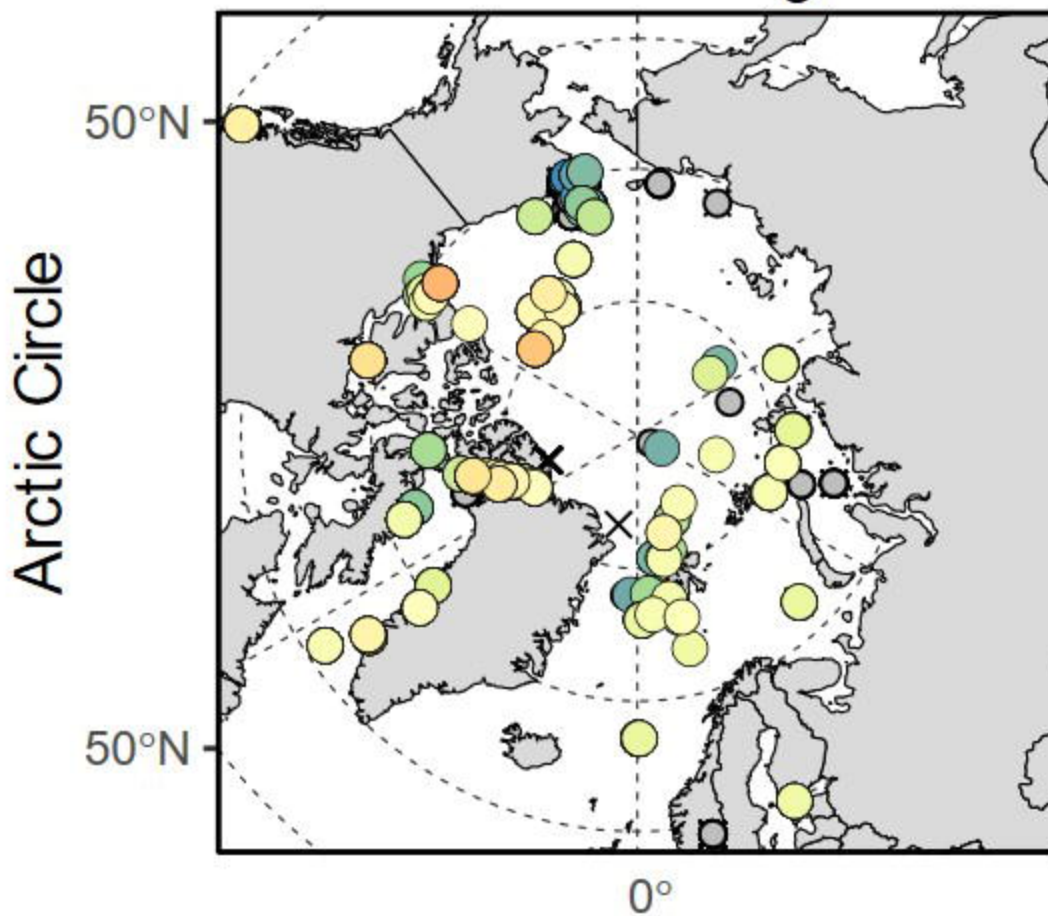
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Ross Sea Dinoflagellate

*Phaeocystis pouchetii*



Ross Sea Dinoflagellate

*Phaeocystis antarctica*

