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# Morphological Transition in Kleptochloroplasts after Ingestion in the Dinoflagellates *Amphidinium poecilochroum* and *Gymnodinium aeruginosum* (Dinophyceae)

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Running title: Transition of kleptochloroplast

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The unarmoured marine dinoflagellate *Amphidinium poecilochroum* and the unarmoured freshwater *Gymnodinium aeruginosum* both belonging to the same clade, are known to possess cryptomonad-derived kleptochloroplasts. Previous studies revealed that *G. aeruginosum* can synchronise the division of the chloroplast with its own cell division while no simultaneous division takes place in *A. poecilochroum*, which is interpreted to mean that state of kleptochloroplastidy in *G. aeruginosum* is closer to that of the initial acquisition of the 'true chloroplast' within the lineage. Although the general ultrastructure of these two species has been reported, the changes in the kleptochloroplast with time have never been followed. We observed morphological changes in kleptochloroplasts of *A. poecilochroum* and *G. aeruginosum* following the ingestion of cryptomonad cells, using light and transmission electron microscopes. In *A. poecilochroum*, the cryptomonad ejectosomes, mitochondria and cytoplasm were all actively transferred into digestive vacuoles within 1 h of ingestion. The chloroplasts were deformed and the cryptomonad nucleus was digested after 3 h. By contrast, in *G. aeruginosum*, the cryptomonad cytoplasm and nucleus were retained for 24 h following ingestion, and the chloroplast was substantially enlarged. These differences imply that the retention of the cryptomonad nucleus is important for the maintenance of the chloroplast.

Key words: *Amphidinium poecilochroum*; cryptomonad organelle; *Gymnodinium aeruginosum*; kleptochloroplast; morphological change; ultrastructure

## Introduction

Dinoflagellates are considered to have followed highly diverse evolutionary strategies, especially with regard to their chloroplast origins. The ancestor of all dinoflagellates is considered to be photosynthetic and to have acquired a chloroplast derived from a red alga via secondary endosymbiosis, although the timing of acquisition is unclear (Horiguchi 2006; Keeling 2010). Typical photosynthetic dinoflagellates resulting from such an endosymbiotic event possess the peridinin chloroplasts. However, a number of dinoflagellates have lost their chloroplasts and have become heterotrophic (Saldarriaga et al. 2001). Moreover, some dinoflagellates are considered to have replaced their original peridinin chloroplasts with those of diatom or haptophyte origin via tertiary endosymbiotic events, or with that of chlorophyte origin via serial secondary endosymbiosis (Hackett et al. 2004; Horiguchi 2006; Saldarriaga et al. 2001; Stoebe and Maier 2002). Thus, dinoflagellates have a very complex history with regard to the evolution of their chloroplasts.

In addition to these permanent chloroplasts mentioned above, some dinoflagellates possess a unique form of 'chloroplast'. These dinoflagellates, which have lost the original peridinin chloroplast ingest chloroplasts (often together with other organelles) of other photosynthetic algae, and utilize them for photosynthesis. The ingested chloroplasts are temporarily retained in the dinoflagellate cell, but are eventually lost during cell division or digestion and the dinoflagellates need to feed on other photosynthetic algal cells to regain its temporary 'chloroplast'. This type of chloroplast is called a "stolen chloroplast" or "kleptochloroplast" (Schnepf and Elbrächter 1992).

The kleptochloroplast phenomenon is widely spread in dinoflagellates, from the armoured species of *Dinophysis* (Schnepf and Elbrächter 1988) and *Amylax* spp. (Koike and Takishita 2008) to unarmoured species, i.e. *Amphidinium latum* (Horiguchi and Pienaar 1992), *A. poecilochroum* (Larsen 1988), *Gymnodinium aeruginosum* (Schnepf et al. 1989), *G. acidotum* (Wilcox and Wedemayer 1984), *G. myriopyrenoides* (Yamaguchi et al. 2011), *G. eucyaneum* (Xia et al. 2013), *G.* '*gracilentum*' (Skovgaard 1998), a novel dinoflagellate (RS24 and W5-1 strains) (Gast et al. 2007). *Dinophysis* spp. acquire kleptochloroplasts not by engulfing cryptomonad cells directly, but by ingesting the ciliate *Mesodinium rubrum*, the chloroplasts of which are acquired from the cryptophyte *Teleaulax* (Nagai et al. 2008; Park et al. 2006). *Dinophysis* spp. keep only the chloroplasts in the dinoflagellate cytoplasm, and the chloroplasts are surrounded by two membranes (Schnepf and Elbrächter 1988). *Amylax* spp. possess cryptomonad chloroplasts drerived from *Teleaulax* sp. (Koike and Takishita 2008). Recent phylogenetic studies indicated that the unarmoured kleptochloroplastidic dinoflagellates *A. poecilochroum, G. acidotum*, *G. eucyaneum* and *G. myriopyrenoides* are monophyletic (Xia et al. 2013; Yamaguchi et al. 2011). Moreover, it was reported that *A. latum*, *G. acidotum* and *G. aeruginosum* also form a monophyletic group (Takano and Horiguchi 2007). Taking these results into consideration, all the unarmoured kleptochloroplastidic dinoflagellates are

monophyletic and a single origin of kleptochloroplastidy within this linage is highly likely. Interestingly, among the unarmoured kleptochloroplastidic dinoflagellate clade, several differences between marine and freshwater representatives have been noted with regard to the specificity of cryptomonads ingested and the dynamics of the kleptochloroplast within the host cell as mentioned below.

The marine dinoflagellate *Amphidinium poecilochroum* is sand-dwelling and usually possesses 4-8 blue-green or yellow-green cryptomonad chloroplasts in a single cell, the colour depending on the species of cryptophyte engulfed (Larsen 1988). Another marine species, *A. latum*, is also sand-dwelling and can have several cryptomonad cells, often of different colour or structure (Horiguchi and Pienaar 1992). Therefore, these marine kleptochloroplastidic species are capable of ingesting more than one species belonging to the class Cryptophyceae. These kleptochloroplasts are surrounded by four membranes; two chloroplast membranes, two chloroplast endoplasmic reticulum (ER) membranes, which is the same membrane composition as that of free-living cryptomonads. In addition to the chloroplast, the dinoflagellate engulfs cryptomonad cytoplasm and a single membrane (referred as 'phagotrophic' vacuole in Larsen (1988)) separates the cryptomonad cytoplasm from the dinoflagellate cytoplasm. The cryptomonad cytoplasm contains the cryptomonad nucleus, mitochondria and the periplastidal compartment (PPC) inside of which is the nucleomorph (Horiguchi and Pienaar 1992; Larsen 1988). When the dinoflagellates divide, the cryptomonad kleptochloroplasts are randomly distributed between the daughter cells, and no synchronization of divisions of the kleptochloroplasts and host cell has been observed.

By contrast, the freshwater dinoflagellates *Gymnodinium acidotum* and *G. aeruginosum* possess only blue-green kleptochloroplasts (Schnepf et al. 1989; Wilcox and Wedemayer 1984). In fact, the cryptomonads that *G. acidotum* can ingest are members of the genus *Chroomonas* (which are blue-green) only, and no other cryptophytes, such as *Cryptomonas*, can be ingested (Fields and Rhodes 1991). It appears that the specificity of the dinoflagellate for its cryptomonad prey is stricter in freshwater dinoflagellates than it is in marine species. Usually only one kleptochloroplast is retained at a time in field-sampled freshwater dinoflagellates and it is usually so enlarged that it pervades most of the host cell. The kleptochloroplast membranes and the cryptomonad organelles are intact, as found in marine species (Farmer and Roberts 1990; Schnepf et al. 1989; Wilcox and Wedemayer 1984). Moreover, the division of the kleptochloroplast of the freshwater *Gymnodinium* is synchronised with the host cell division and each half of the kleptochloroplast is inherited by each daughter cell (Fields and Rhodes 1991; Schnepf et al. 1989). Considering all above, the kleptochloroplasts in *G. acidotum* and *G. aeruginosum* represent a much more advanced stage of development of a true chloroplast from an endosymbiont than that seen in *Amphidinium poecilochroum* and *A. latum* (Fields and Rhodes 1991; Yamaguchi et al. 2011).

It is well known that there are significant differences between the structure of a free-living cryptomonad cell and that of the kleptochloroplast in the dinoflagellate cell. An ultrastructural study of *Amphidinium poecilochroum* revealed that the cryptomonad ejectosomes and mitochondria are sequestered in an 'accumulation body' (Larsen 1988 Fig. 14, referred as 'digestive vacuole' in this study). Cryptomonad ejectosomes, periplasts and basal bodies have never been observed in the cells of *Gymnodinium aeruginosum* and *G. acidotum* (Farmer and Roberts 1989; Schnepf et al. 1989; Wilcox and Wedemayer 1984). Moreover, all kleptochloroplastidic species possess chloroplasts that are considerably enlarged relative to the original cryptomonad chloroplast, especially in *G. acidotum*, where it is additionally highly-lobed and ramifies throughout the dinoflagellate cytoplasm (Farmer and Roberts 1989; Horiguchi and Pienaar 1992; Larsen 1988; Schnepf et al. 1989; Wilcox and Wedemayer 1984). However, it is unknown how and when organelles such as the ejectosomes are eliminated, and how the kleptochloroplasts are modified to become highly-lobed. Although previous studies revealed the general ultrastructure of the host and the kleptochloroplast (Fields and Rhodes 1991; Horiguchi and Pienaar 1992; Larsen 1988), few studies examine structural changes in the kleptochloroplasts over time, from ingestion to their disappearance.

Recently, morphological changes of kleptochloroplasts in *Dinophysis caudata* were investigated over time from ingestion of prey (Kim et al. 2012). The study of the kleptochloroplasts in *D. caudata* revealed that the structural differences between the chloroplasts within the host and those of the original cryptomonad cell were caused by a rearrangement in the host cell (Kim et al. 2012). On the other hand, morphological changes in kleptochloroplasts with time have never been observed in unarmoured kleptochloroplastidic dinoflagellates, especially using the TEM. Of particular interest is how the dinoflagellates develop their kleptochloroplast from the original organization of the chloroplasts of free-living cryptomonads.

This study focuses on *Amphidinium poecilochroum* and *Gymnodinium aeruginosum,* two dinoflagellates exhibiting different possible evolutionary stages. The morphological changes in the prey of these two organisms at different times after ingestion were compared using the LM and TEM.

## Results

We have observed more than one cell of *Amphidinium poecilochroum* and *Gymnodinium aeruginosum* at each stage and ultrastructure of both species were investigated using serial sections. The number of the cells used for each observation was listed in Supplemental Table 1.

### Morphological changes in the ingested cryptomonad cells of *Amphidinium poecilochroum*

### LM observations

The ingested cryptomonad cells (= the entire ingested cryptomonad cytoplasm including the cryptomonad chloroplast) were located in the posterior of the dinoflagellate cell, and their original shape was retained until the 10 min stage. During this period, a pyrenoid was detectable in the chloroplast at the LM level, but other structures or newly formed structures (like the digestive vacuole) in the dinoflagellate cell could not be observed (Fig. 1A-D). At 20 min from ingestion, most of cryptomonad cells were elliptic but some were slightly deformed (Fig 1E). By this stage, the host cell had formed several spherical digestive vacuoles that were visible under the LM. These digestive vacuoles were transparent, and occasionally small granules were observed in them (Fig. 1E), but no autofluorescence was detectable in them (Fig 1F). The ingested chloroplasts deformed gradually beyond 20 mins of ingestion and by 2 h they had lost their original cup-shape seen in Fig. 1B (Fig. 1E-L). At the 3 h and 4 h stage, the cryptomonad cells became elongated, developed lobes and were restricted to the periphery of the cell (Fig. 1M-P). Although the dinoflagellate cells at these stages seem larger than those of other stages, this is simply reflecting the fact that these particular cells engulfed more prey cell than other cells and this enlargement of cell size is not specific phenomenon for this timing. At the 6 h and 12 h stage, the cryptomonad cells were still deformed, but less so than at the 3 h and 4 h stage (Fig. 1Q-T). Digestive vacuoles could be observed from the 20 min stage to 12 h stage. At the 12 h stage, relatively large cells underwent cell division (data not shown), but the morphological change of the cryptomonad cell after host cell division was not monitored in this study.

## TEM observations

**Membranes of the cryptomonad cell:** Right after ingestion, the ingested cryptomonad chloroplast was surrounded by two chloroplast membranes (Fig. 2A white arrowhead) and two chloroplast ER membranes (Fig. 2A arrowhead), just like in the free-living condition. The cryptomonad cytoplasm, which had less electron-dense than the dinoflagellate cytoplasm and contained a chloroplast and additional organelles, was separated from the dinoflagellate cytoplasm by a single membrane (Fig. 2A arrow). Twelve hours after ingestion, the cryptomonad cytoplasm had been removed around the chloroplast and the dinoflagellate cytoplasm was detected in close proximity with the chloroplast (Fig 2B). Several membranes were observed around the chloroplast, probably the result of digestion of membranes or an artifact of fixation. This made it impossible to determine how many membranes were retained of the original chloroplast (the two chloroplast membranes and the two chloroplast ER membranes) or of the membrane between the cryptomonad cytoplasm and the dinoflagellate cytoplasm (Fig 2B).

**The chloroplast and additional organelles:** Immediately following ingestion, the chloroplast was cup-shaped, like that of the free-living cryptomonad. It was confirmed that the cryptomonad organelles and their arrangement were well-retained. The cryptomonad cytoplasm contained a

chloroplast, a cryptomonad nucleus, a nucleomorph, ejectosomes (gullet-surrounding ejectosomes only) and mitochondria with flat cristae (Fig. 3A). No cryptomonad periplast, flagella or basal bodies were observed in 7 cells used for TEM observation. The peripheral ejectosomes of the cryptomonad (= smaller ejectosome; see Supplementary Fig. S1B) were accumulated (see below for more detail). At the 20 min stage, the chloroplast became slightly irregular in shape, and the cryptomonad nucleus and the nucleomorph were still observed (Fig.3B, the nucleomorph is not shown). In contrast, the cryptomonad mitochondria and most of its cytoplasm were removed from the vicinity of the chloroplast and the cryptomonad nucleus. As a result, the dinoflagellate cytoplasm approached a direct connection with the chloroplast (Fig. 3B). At the 30 min stage, the gullet-surrounding ejectosomes (= larger ejectosomes; see Supplementary Fig. S1B) were still retained in a small pocket of cryptomonad cytoplasm rather than within a digestive vacuole (Fig. 3C). At the 1 h stage, the cryptomonad mitochondria and ejectosomes were removed, and no cryptomonad cytoplasm could be discerned around the chloroplast (Fig. 3D). At the 3 h stage, the chloroplast was located at the periphery of the host cell and the cryptomonad cell had lost its original organelles (Fig. 3E). At the 12 h stage, the chloroplast was significantly modified and large starch granules had accumulated in the periplastidal compartment of the chloroplast (Fig. 3F). The nucleomorph was still detected at this stage, although the cryptomonad nucleus could no longer be observed in the host cell (Fig. 3F).

**Cryptomonad ejectosomes and the digestive vacuole:** The peripheral ejectosomes accumulated in the space between the cryptomonad cytoplasm and dinoflagellate cytoplasm right after ingestion. The membranous material, which encircles the accumulated ejectosomes could be observed (Fig. 4A arrow). At the 20 min stage, the accumulated peripheral ejectosomes together with cryptomonad mitochondria became surrounded by a distinct membrane, which is thought to be a digestive vacuole membrane (Fig. 4B). At this stage, the gullet-surrounding ejectosomes were not contained in a digestive vacuole but remained in the cryptomonad cytoplasm (not shown). The gullet-surrounding ejectosomes were transferred into the digestive vacuole at the 1 h stage together with the peripheral ejectosomes and the cryptomonad mitochondria (Fig. 4C). At the 4 h stage, the peripheral ejectosomes were difficult to recognize due to digestion (Fig. 4D). At the 6 h stage, all ejectosomes were digested or had lost their original shape, but the mitochondrial membranes were not digested (Fig.4E). By the 12 h stage, the cryptomonad organelles in the digestive vacuole had become unrecognisable and the contents of digestive vacuole were homogenous (Fig. 4F).

**The cryptomonad nucleus and nucleomorph:** TEM observations indicated that the cryptomonad nucleus was intact at ingestion (0 min stage) (Fig. 5A). It was still intact at the 2 h stage (Fig. 5B), but at the 3 h stage, its surface was undulated and the nucleoplasm was more electron dense than during the previous stages (Fig. 5C). At the 4 h stage, two different states of modification in nuclei were observed; either the majority of the cryptomonad

nucleus was removed from the vicinity of the chloroplast, or it remained in close proximity with the chloroplast. Where the cryptomonad nucleus was kept near the chloroplast, the nucleoplasm and the surface of nuclear membranes were as described at the 3 h stage (Fig. 5D). The removed cryptomonad nucleus should have been transferred to digestive vacuoles, but we could not detect it in the cells at 4 h stage. At the 6 h stage, an intact cryptomonad nucleus was never observed but the homogenous content of the digestive vacuole was reminiscent of cryptomonad nucleus. This is because it contained the same spots of low electron-density typical of the cryptomonad nucleus, suggesting that the damaged nucleus have transferred to the digestive vacuole (Fig. 5E). At the 12 h stage, trace of the cryptomonad nucleus was no longer detected because the content of digestive vacuole was completely digested (Fig. 5F).

By contrast, the nucleomorph was highly preserved throughout the experimental period. It was intact at the time of ingestion (Fig. 6A) and later, even up to the 12 h stage, its membranes were obvious, indicating that the nucleomorph was not modified by the dinoflagellate (Fig. 6B-F). Division of the nucleomorph was not observed in this study.

## The morphological change of the kleptochloroplast in *Gymnodinium aeruginosum*

### LM observations

Newly-ingested cryptomonad cells retained their original shape in the hypocone of the dinoflagellate cell and kept their eyespots and pyrenoids (Fig. 7A, B). The cryptomonad cells were oval or elliptic and were not deformed or modified up to the 2 h stage (Fig. 7C-L). At the 3 h stage, they started to deform slightly (Fig. 7M, N) and, at the 4 h and 6 h stages, they gradually modified (Fig. 7O-Q). At the 12 h and 24 h stages, the cryptomonad cells became highly lobed and took on complex shapes, making their original shape unrecognisable (Fig. 7S-V, see Supplementary Fig. S2C). No digestive vacuole was detected under LM in *Gymnodinium aeruginosum*. By the 24 h stage, the newly-ingested cryptomonad cells had enlarged gradually, but they were restricted to the periphery of the hypocone of the dinoflagellate cells rather than being situated throughout the host cell. Thus, the chloroplasts, by the 24 h stage, were not of the same dimensions as those of kleptochloroplasts assimilated in the field, which enlarge into every part of the host cell.

### TEM observations

**Membranes of the cryptomonad cell:** Right after ingestion, there were four surrounding membranes to the chloroplast: two chloroplast membranes (Fig. 8A white arrowhead) and two chloroplast ER membranes (Fig. 8A arrowhead), and the cryptomonad cytoplasm was separated from the dinoflagellate cytoplasm by a single membrane (Fig. 8A arrow). All these membranes were well-preserved at the 24 h phase (Fig. 8B). The

cryptomonad cytoplasm was kept between the outer membrane of the chloroplast ER and the membrane separating the cryptomonad cytoplasm from the dinoflagellate cytoplasm (Fig. 8B arrow).

**The chloroplast and additional organelles:** The ingested chloroplast was nearly cup-shaped and the cryptomonad cell kept its original arrangement of organelles (Fig. 9A, see Supplemental Fig. S2C). It was confirmed that almost all cryptomonad organelles were preserved at this stage; i.e. a cryptomonad nucleus, a nucleomorph, ejectosomes, cryptomonad mitochondria and basal bodies (Fig. 9A, 10A, B). Flagella and periplasts were not observed. The digestive vacuole was absent. At the 20 min stage, the shape of the chloroplast had not changed significantly and the cryptomonad organelles were preserved in their original positions. The cryptomonad nucleus was located in the posterior position, surrounded by the cup-shaped chloroplast. The nucleomorph was near the eyespot. At this stage, the cryptomonad cytoplasm was still present around the chloroplast (Fig.9B). At the 3 h stage, the chloroplast had slightly changed by losing its original cup shape and the cryptomonad cytoplasm was still present (Fig. 9C). By the 6h stage, although the cryptomonad nucleus and mitochondria were in the cytoplasm of the cryptomonad cell and the nucleomorph was still observable, the chloroplast have obviously deformed from the original cup-shape and the relative position of the nucleus, the pyrenoid and the nucleomorph was changed, indicating the original dorsiventrality in the arrangement of the cryptomonad organelles was lost (Fig. 9D). By the 12 h stage, the chloroplast was much more deformed and irregularly elongated than it was at the 6 h stage (Fig. 9E). Even this late, the cryptomonad cytoplasm was retained and contained the cryptomonad nucleus and mitochondria and a nucleomorph was also observed (Fig. 9E). At the 24 h stage, the chloroplast continued to be deformed in a more irregular fashion than witnessed at the 12 h stage. The cryptomonad cytoplasm was still present around the chloroplast and a cryptomonad nucleus and a nucleomorph could still be discerned (Fig. 9F).

**Cryptomonad ejectosomes and the digestive vacuoles:** The peripheral ejectosomes of the cryptomonad cell did not accumulated like they were in *Amphidinium poecilochroum* right after ingestion (Fig. 10A, B). At the 1 h stage, the gullet-surrounding ejectosomes were still present in the cryptomonad cytoplasm (Fig. 10C) and no digestive vacuole had formed in the dinoflagellate cytoplasm. Both sets of ejectosomes were not actively removed from the cryptomonad cytoplasm as they were in *A. poecilochroum*. Digestive vacuoles appeared at the 6 h stage (1 μm in diameter), but ejectosomes were not transferred into them (Fig. 10D, E). Rather they were retained in the cryptomonad cytoplasm at this stage (Fig. 10D, E). At the 12 h stage, the ejectosomes disappeared from the cryptomonad cytoplasm. Although the digestive vacuole was discernible at this stage too, neither type of ejectosomes was found in the digestive vacuole (Fig. 10F).

**The cryptomonad nucleus and nucleomorph:** The cryptomonad nucleus immediately following ingestion was intact and held in the cryptomonad

cytoplasm (Fig. 11A). It was retained without any modification throughout all investigated times and its membranes were obvious, even at the 24 h stage (Fig. 11B).

The nucleomorph was also intact right after ingestion and located in the periplastidal compartment (Fig. 11C). The structure and position of nucleomorph were maintained unaltered up to the 24 h stage, and no sign of digestion was evident using the TEM (Fig. 11D). No divisions of the cryptomonad nucleus and the nucleomorph were observed.

## The estimates of kleptochloroplast volumes with confocal laser scanning microscope

We obtained the serial sectioned images of chloroplast autofluorescence using confocal laser scanning microscope, and estimated the volume of ingested chloroplast by 3D reconstruction at 0 min, 1 h, 4 h, 6 h, 12 h, 24 h and 72 h after ingestion of cryptomonad in both *Amphidinium poecilochroum* and *Gymnodinium aeruginosum* (see Supplementary Movies S1-4 showing *A. poecilochroum* at 0 min and 24 h, and *G. aeruginosum* at 0 min and 72 h). The number of samples we measured the volume is shown in supplemental table 1. In *A. poecilochroum*, the volume of chloroplast at 0 min stage was estimated as 117.72 (± 37.83 SD) $\mu m^3$. Despite modification of the shape of the chloroplast seen in LM and TEM observation, the volume of chloroplast remained almost constant, i.e. around 100 $\mu m^3$ during the incubation time, and enlargement of kleptochloroplast was not observed based on this volume estimate (Fig. 12). In *G. aeruginosum* at 0 min stage, the volume of the chloroplast was 70.49 (± 25.09 SD) $\mu m^3$. The volume was not changed up to 12 h (Fig. 12). The chloroplast, however, started to grow up significantly from 12 h and reached the volume of 1091.17 (± 378.35 SD) $\mu m^3$ at 72 h, more than ten-fold compared with that at 0 min (Fig. 12). The chloroplasts at 72 h were pervaded throughout the host cell, forming ramified shape (Supplemental Movie S4). Unfortunately, we failed to measure volume at 72 h in *A. poecilochroum*, because the cells were disappeared after the incubation time. In our preliminary observation, it was revealed that the individual kleptochloroplast was retained only about for 3 days in *A. poecilochroum*, while *G. aeruginosum* could retain it more than a month.

## Discussion

In this study, using the single-cell LM and TEM methods, detailed structural changes of ingested cryptomonad cells were followed at 0 min, 10 min, 20 min, 30 min, 1 h, 2 h, 3 h, 4 h, 6 h, 12 h and 24 h after ingestion of cryptomonad prey both in *Amphidinium poecilochroum* and *Gymnodinium aeruginosum* respectively (in case of the former, only up to 12 h). These two species process their prey after ingestion in a very different fashion and might provide clues about the evolutionary steps required between simple ingestion as prey to the establishment of a true chloroplast.

**Formation of the digestive vacuole and isolation of the kleptochloroplast**

Directly after ingestion, the ingested cryptomonad cells lack periplast and flagella in the cells of *Amphidinium poecilochroum* and *Gymnodinium aeruginosum*. In *A. poecilochroum*, basal bodies were also not found in spite of TEM observation using serial sections of 7 cells. The ingested cells were encircled by a single membrane. These conditions of the ingested cryptomonad cells are same as those observed by Larsen (1988). He observed *A. poecilochroum* just ingesting cryptomonad cell under TEM and suggested that the periplast may be digested in feeding process, and that the membrane encircling the ingested cell (referred as 'phagotrophic vacuole') was likely to be formed by the dinoflagellate (Larsen 1988). This study also suggests that outer structures of the cryptomonad were removed in the feeding process. The membrane separating the cryptomonad and the dinoflagellate cytoplasm should not be derived from the cytoplasmic membrane of the cryptomonad because the cytoplasmic membrane lies just outside the inner periplast component (Brett et al. 1994).

*Dinophysis* spp. are known to obtain kleptochloroplasts by myzocytosis from *Mesodinium rubrum* that in turn possesses kleptochloroplasts derived from the cryptomonad genus *Teleaulax* (Nagai et al. 2008; Nishitani et al. 2008; Park et al. 2006). *Dinophysis fortii*, heavily fed on *M. rubrum*, possesses solitary kleptochloroplasts (= restricted to chloroplasts only) and several food vacuoles that contain membrane-like and mitochondria-like structures. The kleptochloroplasts lie directly in the dinoflagellate cytoplasm, and not in the food vacuole (Nagai et al. 2008). Recently, the process of kleptochloroplast isolation in *D. caudata* was revealed ultrastructurally (Kim et al. 2012). The study showed that *D. caudata* takes up kleptochloroplasts together with other organelles of *M. rubrum* into a food vacuole, and then isolated the kleptochloroplasts from the food vacuole into the dinoflagellate cytoplasm (Kim et al. 2012). In *A. poecilochroum* and *G. aeruginosum*, the chloroplast and cryptomonad organelles were ingested into the dinoflagellate cytoplasm directly, not via a digestive vacuole, making the mechanism of kleptochloroplast incorporation into the dinoflagellate cytoplasm clearly different from that employed by *D. caudata*.

This study revealed that digestive vacuole formation in *Amphidinium poecilochroum* was quite rapid, occurring within 20 minutes of ingestion. The digestive vacuoles initially (20 min) contained cryptomonad mitochondria, peripheral ejectosomes and later (1 h) added the gullet-surrounding ejectosomes. The digestive vacuoles increased in number and volume with time, indicating that the digestive vacuole formation involves modification of the prey cell after ingestion. Larsen (1988) showed that the membrane between the cryptomonad cytoplasm and the dinoflagellate cytoplasm was very close to the chloroplast ER because of a highly reduced cryptomonad cytoplasm. He showed that *A. poecilochroum* possessed digestive vacuoles containing cryptomonad ejectosomes and mitochondria, but failed to demonstrate the process of formation of the

digestive vacuoles and the resultant decrease of the cryptomonad cytoplasm. In this study, the volume of the cryptomonad cytoplasm was demonstrated to be decreased and the cryptomonad mitochondria and ejectosomes were not observed by TEM after 1 h. This supports the interpretation that *A. poecilochroum* removes the cryptomonad cytoplasm together with its organelles, such as mitochondria and ejectosomes, by actively transferring them into a digestive vacuole and as a result, the cryptomonad cytoplasm is removed and the membrane between the host and the prey approaches to the chloroplast ER.

In contrast to *Amphidinium poecilochroum*, *Gymnodinium aeruginosum* did not form a digestive vacuole rapidly, and if digestive vacuoles were formed, cryptomonad mitochondria and ejectosomes were not transferred into them until later stages; the ejectosomes being removed from the cytoplasm and disappearing at the 12 h and 24 h stages. These results suggest that *G. aeruginosum* can eliminate and digest ejectosomes between 6 h and 12 h of ingestion, transferring them into the digestive vacuole. Unlike *A. poecilochroum* which removed the prey's cytoplasm together with the non-plastidial organelles, including the nucleus, *G. aeruginosum* seemed to select and remove only the unnecessary cryptomonad organelles, because the prey's cytoplasm was retained around the chloroplast until much later. Wild material of *G. aeruginosum* has never been seen to possess cryptomonad ejectosomes, although this could be because digestive vacuoles were not encountered (Schnepf et al. 1989). Such a condition probably represents a stage after the selective elimination of ejectosomes, as observed in this study. The underlying mechanism behind the selective removal of particular organelles, however, remains unknown.

**Enlargement of the kleptochloroplast**

The host organisms that undergo kleptochloroplastidy tend to enlarge or deform the ingested chloroplast. The katablepharid, *Hatena arenicola*, is known to possess a single kleptochloroplast derived from a *Nephroselmis* sp. (Prasinophyceae, Viridiplantae) (Okamoto and Inouye 2006). The symbiont in *H. arenicola* is enlarged after engulfment, occupying most of the host cytoplasm. The volume of the symbiont is more than ten-fold relative to the free-living *Nephroselmis*. The eyespot of the symbiont is invariably positioned at anterior of the host cell and the pyrenoid is duplicated from the single pyrenoid in the free-living *Nephroselmis* (Okamoto and Inouye 2006). In the case of *Dinophysis caudata*, kleptochloroplasts make their way into the dinoflagellate cytoplasm via the food vacuoles, and then elongate toward the periphery of the cell. At the same time, the kleptochloroplasts are highly modified not only with regard to their shape but also with regard to the arrangement of the thylakoids relative to the pyrenoid (Kim et al. 2012). As in *H. arenicola* and *D. caudata*, the kleptochloroplast of *Amphidinium poecilochroum* and *Gymnodinium aeruginosum* also become highly modified under experimental conditions. The modification of the kleptochloroplast witnessed in *H. arenicola* and the unarmoured kleptochloroplastidic

dinoflagellates represents a significant shift from the response of the prey chloroplasts during ordinary phagotrophy, where it is immediately digested. Therefore, the modification of the prey seems to be one of the characteristic features of kleptochloroplastidy and distinguishes it from the phagotrophy.

The kleptochloroplasts of currently-observed cells of *Amphidinium poecilochroum* (LM at 3 h and 4 h stages) were more elongated and lobed than those formerly observed (Larsen 1988), but they did not become more ramified after this stage. This implies that kleptochloroplast modification reaches its maximum state at around the 4 h stage. However, the volume of the kleptochloroplast is not increased up to 24 h, indicating *A. poecilochroum* can modify the kleptochloroplast shape but cannot enlarge it.

In case of *Gymnodinium aeruginosum*, highly-elongated chloroplasts were observed at 12 h and 24 h stages, but the shape and the number of chloroplasts was significantly different from those found in field samples. Previously, *G. aeruginosum* was shown to possess only a single kleptochloroplast, enlarged throughout the cell (Schnepf et al. 1989). In *G. acidotum*, the kleptochloroplast is also single and enlarged like in *G. aeruginosum* (Wilcox and Wedemayer 1984). By contrast, experimentally-fed *G. aeruginosum* in this study possessed several chloroplasts and the chloroplasts were not enlarged and were not even as large as those found in natural populations up to 24 h. Nevertheless, the passage of time sees the chloroplasts of cultured cells becoming larger and in fact, the chloroplast at the 24 h stage is larger than that of 12 h stage, indicating that growth is occurring. However, by 24 h, not all chloroplasts were enlarged and they were restricted in distribution to the hypocone of the cell. In the cells at 72 h after ingestion, the chloroplasts were even larger, almost comparable in size to those found in natural populations. Indeed, confocal laser scanning microscopic observation indicated that the kleptochloroplast at 72 h pervaded throughout the host cell and increased the volume more than ten-fold relative to the chloroplast right after ingestion. Therefore, the size of field-sampled chloroplasts might be equally variable and just happened to be the size encountered because the cells had fed on the prey at least three days ago. At the moment, no clues exist to allow one to postulate the possible mechanisms involved in the enlargement of the chloroplast. More detailed studies in this regard should be undertaken to facilitate this.

Another difference between cultured and field-sampled cells is the number of chloroplasts in a cell. In culture, *G. aeruginosum* ingested multiple *Chroomonas* cells as they encountered them and therefore has the potential to possess several chloroplasts if the prey is sufficiently available. However, in the field, *G. aeruginosum* possesses only one chloroplast probably because of the low prey (*Chroomonas*) density. Once the dinoflagellate obtains its prey, it enlarges the chloroplast slowly, taking more than three days to reach the maximum photosynthetic efficiency. Colourless individuals are sometimes encountered in field samples. This food-deprived dinoflagellate must ingest a *Chroomonas* cell to survive.

## Retention and digestion of cryptomonad organelles

Unarmoured kleptochloroplastidic dinoflagellates are known to possess additional organelles from the cryptomonad other than the chloroplast; these include the nucleus, nucleomorph and often mitochondria (Horiguchi and Pienaar 1992; Larsen 1988; Wilcox and Wedemayer 1984). However, previous studies on these dinoflagellates have never focused on morphological changes to the ingested cells with time.

This study revealed that the surface of the cryptomonad nucleus wrinkles shortly after ingestion by *Amphidinium poecilochroum* and that the nuclear membranes become indistinct as early as the 3 h stage. Moreover, it was completely absent in the ingested cryptomonad cell after the 6 h stage. These results strongly suggest that the cryptomonad nucleus is selectively and rapidly digested by *A. poecilochroum*. By contrast, the nucleomorph was highly preserved at least up to the 12 h stage. Cryptomonads that belong to *Rhodomonas*, position their nucleomorph within the pyrenoid matrix (Hill and Wetherbee 1989). The *Rhodomonas* sp. used in this study also has the nucleomorph embedded in the pyrenoid matrix. The embedded nature of this nucleomorph might contribute to its retention.

In *Gymnodinium aeruginosum*, the cryptomonad cell is retained relatively intact. The host cell always contains a chloroplast, a cryptomonad nucleus, a nucleomorph, and cryptomonad mitochondria within the cryptomonad cytoplasm that is separated by a single membrane from its own cytoplasm. This single membrane, the double chloroplast ER membranes and the double chloroplast envelope membranes were highly retained at least until the 24 h stage.

Thus, this represents the first report of two highly divergent strategies in dinoflagellate kleptochloroplastidy: in *Amphidinium poecilochroum* the chloroplast and the accompanying cryptomonad organelles are modified drastically, while in *Gymnodinium aeruginosum* the cryptomonad cell is retained more or less intact. As will be shown later, *G. aeruginosum* possesses a more advanced type of kleptochloroplast than that of *A. poecilochroum*, and it tends to preserve the accompanying cryptomonad organelles, implying that the retention of the cryptomonad organelles, especially the cryptomonad nucleus, might be critical as a first step in the evolution of a permanent chloroplast.

In *Amphidinium latum*, it has been reported that a single kleptochloroplast contained up to three nucleomorphs (Horiguchi and Pienaar 1992), indicating that the nucleomorph has undergone multiplication in the host cell after ingestion, because free-living cryptomonad cells usually possess only one nucleomorph. Although no evidence of nucleomorph multiplication was encountered in this investigation in both species, a cultured cell of *Gymnodinium aeruginosum* in a preliminary study was noted to have multiple nucleomorphs. In the field-collected cells of *G. aeruginosum* and *G. acidotum*, the nucleomorph tends to be retained despite loss of cryptomonad nucleus, implying that the nucleomorph was important for maintenance of the kleptochloroplast

(Farmer and Roberts 1990; Schnepf et al. 1989; Wilcox and Wedemayer 1984). Because the role of the nucleomorph is still remained unknown, future work will focus on following the fate of the nucleomorph and the nucleus in ingested cryptomonads.

**Evolution of kleptochloroplastidy**

This study confirmed that the organelles of ingested cryptomonads were less modified in *Gymnodinium aeruginosum* than in *Amphidinium poecilochroum*. *G. aeruginosum* retained the cryptomonad cytoplasm together with the nucleus and selectively digested the ejectosomes, while *A. poecilochroum* tended to digest the cryptomonad cytoplasm altogether. Interestingly, *G. aeruginosum* is considered to exhibit a more advanced stage of kleptochloroplastidy leading to the acquisition of a true chloroplast and this condition goes hand-in-hand with a retardation in the digestion of non-plastidial components of the cryptomonad. An important difference between *G. aeruginosum* and *A. poecilochroum* is whether the dinoflagellate retains the cryptomonad nucleus for an extended period or not. This implies that the cryptomonad nucleus plays a certain function in maintaining the kleptochloroplast and, certainly, the presence of functional cryptomonad nucleus is known to be important in the kleptochloroplastidic ciliate, *Mesodinium rubrum*.

*Mesodinium rubrum* possesses several kleptochloroplasts derived from the cryptomonad, *Teleaulax* (Johnson et al. 2006). This ciliate forms a "chloroplast-mitochondria complex" (CMC), composed of a kleptochloroplast, a nucleomorph and cryptomonad mitochondria. The cryptomonad cytoplasm is separated by a single membrane from the ciliate cytoplasm, which contains a highly-enlarged cryptomonad nucleus outside of the CMCs (Hansen and Fenchel 2006; Hibberd 1977; Lindholm 1985). The CMC is retained for at least 99 days and the cryptomonad nucleus for up to 30 days. Interestingly, the loss of the cryptomonad nucleus causes a significant decline in the kleptochloroplast number and the photosynthetic quantum efficiency, suggesting that cryptomonad nucleus is transcriptionally active. Thus, it has been suggested that both the performance and replication of the kleptochloroplast are dependent on the cryptomonad nucleus. This phenomenon is termed 'karyoklepty' (Johnson et al. 2007). In the case of the unarmoured kleptochloroplastidic dinoflagellates, the longest duration time ever reported for the maintenance of a kleptochloroplast is 14 days (Fields and Rhodes 1991), which is considerably shorter than that of the kleptochloroplasts of *M. rubrum*. In addition, karyoklepty is not reported in unarmoured kleptochloroplastidic dinoflagellates.

*Durinskia baltica* and *Kryptoperidinium foliaceum* are known to possess permanent chloroplasts derived from a diatom via tertiary endosymbiosis (Chesnick et al. 1996; Horiguchi 2006; Tomas and Cox 1973). These species possess an endosymbiotic cytoplasm, which contain a nucleus, chloroplasts and mitochondria, and which is separated from the host cytoplasm by a single membrane (Schnepf and Elbrächter 1999). When the cell of

dinoflagellate divides, the simultaneous division of the endosymbiotic nucleus takes place and the divided nucleus is inherited to each daughter cell in addition to chloroplasts (Figueroa et al. 2009; Tippit and Pickett-Heaps 1976). In this way, these dinoflagellates retain the relationship with the endosymbiont permanently.

By contrast, in the unarmoured kleptochloroplastidic dinoflagellates, the cryptomonad organelles are not stable as shown by the presence or the absence of cryptomonad nucleus and nucleomorph (Farmer and Roberts 1990; Schnepf et al. 1989). As a result, the relationship between the host and the prey is not as advanced as in the diatom-harbouring dinoflagellates. Restricting considerations to these impermanent relationships, it seems that the retention of the cryptomonad nucleus is key to extending the duration (i.e. reducing the instability) of the functional relationship between the two compartments. It thus seems to be an important step in the evolution towards the acquisition of a true chloroplast in unarmoured (initially kleptochloroplastidic) dinoflagellates. Thus *A. poecilochroum*, which loses the cryptomonad nucleus and cytoplasm early after prey ingestion, is interpreted as representing a relatively primitive condition in the relationship between the host and the endosymbiont. In *A. poecilochroum*, the cryptomonad nucleus is unlikely to be functional because it is digested very early on and the relationship between the two organisms is thus destined to be very short-lived. On the other hand, *G. aeruginosum* is considered to be an advanced stage in the evolution of the kleptochloroplastidic dinoflagellate, one which possesses a highly-modified kleptochloroplast that occupies most of the host cell (Schnepf et al. 1989). The current study showed that *G. aeruginosum* gradually enlarges the kleptochloroplast and retains the cryptomonad nucleus. Like karyoklepty in *M. rubrum*, it is possible that the cryptomonad nucleus in *G. aeruginosum* is transcriptionally active and modifies the kleptochloroplast to extend its period of retention. However, the cryptomonad nucleus in *G. aeruginosum* is eventually lost (Schnepf et al. 1989). The role of the cryptomonad nucleus needs to be investigated at the genetic level to unravel what is happening in the kleptochloroplast of *A. poecilochroum* and *G. aeruginosum*.

## Methods

**Sampling and Establishment of culture strains:** *Amphidinium poecilochroum* was collected at Shibagaki Beach, Hakui City, Ishikawa Prefecture (36°57'03" N: 136°45'34" E) on 29 March 2010. Sand samples were collected at the edge of the surf. The sand sample was then placed in a plastic cup and enriched with Daigo IMK medium (Wako, Osaka, Japan). This was cultured at 20 °C in a culture cabinet using fluorescent light with a photon flux density of 30-50 μmol photon $m^{-2}s^{-1}$ and a 16 h L/8 h D cycle. Individual cells of *A. poecilochroum* observed in the enrichment culture using an inverted microscope (CK X41, Olympus, Tokyo) were picked up and placed into a compartment of a 48-well microplate containing IMK medium. *Rhodomonas*

sp. (strain Mr06; collected at Denshin-Beach, Muroran-City, Hokkaido Prefecture in June 2010) was added to each well of microplate as prey. After the number of cells of *A. poecilochroum* increased sufficiently to allow their successful transfer, the cells were placed in a plastic petri dish together with *Rhodomonas* sp. and maintained as a culture strain under the conditions described above.

A sample containing *Gymnodinium aeruginosum* was collected at the South pond of the Municipal building (Docho), Sapporo-City, Hokkaido Prefecture (43°03'48" N: 141°20'56"E) on 24 September 2010, using a plankton net with a pore size of 25 µm. Cells of *G. aeruginosum* were isolated as described for *A. poecilochroum*, but were placed in individual wells of a microplate filled with AF-6 medium (Kato 1982). As prey, *Chroomonas* sp. (strain Dc01; isolated from the same sample as *G. aeruginosum*) cells were added to each well. *G. aeruginosum* was cultured under the same conditions as those of *A. poecilochroum*. After successful growth, the cells were transferred to a 24-well microplate with *Chroomonas* sp. and maintained as culture strains.

**Feeding of cryptomonad cells for experiments:** In order to compare the morphological changes of prey at different times after feeding, the following methods were used. For *Amphidinium poecilochroum*, colourless cells (= without kleptochloroplasts) were picked up from the culture strain and placed in a drop of fresh medium on a depression glass slide, and *Rhodomonas* sp. cells were added to the drop. After *A. poecilochroum* ingested the *Rhodomonas* sp., they were again isolated into a drop of fresh culture medium placed in a plastic petri dish (35mm in diameter) and incubated under the same conditions for different durations (0, 20, 30 minutes, 1, 2, 3, 4, 6 and 12 hours, respectively). Directly after ingestion (0 min), *A. poecilochroum* was observed or fixed without isolation. After the incubation period, the sample was observed using the LM and fixed for TEM observation (see below for detail).

For *Gymnodinium aeruginosum*, cells were placed in a well of a microplate without adding *Chroomonas* sp. and allowed to starve. When the cells became colourless, cells of *Chroomonas* sp. were added into the microplate well and *G. aeruginosum* was isolated after confirming the ingestion of *Chroomonas* sp. Incubation for different durations was performed by the same way as that described for cells of *A. poecilochroum*.

The number of cells used for each observation is listed in supplement table S1.

**Light microscopical (LM) observations:** For LM observations, the cells of *A. poecilochroum* or *G. aeruginosum* were observed using the ZEISS Axioskop2 Plus (Carl Zeiss Japan, Tokyo) and photographs were taken with a CCD camera DS-Fi1 (Nikon, Tokyo, Japan). To observe the autofluorescence of kleptochloroplast, a Fs 15 filter of the same microscope was used.

**Transmission electron microscopical (TEM) observations:** In this study, we used the single-cell TEM method for all TEM samples. The medium containing the incubated cells mentioned above was mixed with an equal

volume of Karnovsky fixative (5% glutaraldehyde and 4% paraformaldehyde) in 0.1M cacodylate buffer at pH 7.0. For the fixation of cells of *A. poecilochroum*, 0.3M sucrose was added in the same fixative. The samples were pre-fixed at room temperature for 2 h. After fixation, the cell was transferred by micropipette onto a poly-L-lysine coated Thermanox plastic coverslip (Thermo Scientific, Kanagawa, Japan), pre-cut to a size that can be accommodated in a 1.5 ml microtube, and the cell was allowed to settle and attach to the coverslip. The coverslip with attached cell was placed in a 1.5 ml microtube filled with 0.1M cacodylate buffer, and rinsed 3 times by exchanging the buffer on ice. The cell on the coverslip was then post-fixed for 1 h with 1% $OsO_4$ in the buffer. After fixation, the cell was dehydrated through a graded acetone series (25, 50, 80, 90, 95 and 100%). Post-fixation and dehydration were performed on ice. The dehydrated cell was infiltrated with Agar LV resin (EM Japan, Tokyo, Japan) and polymerized at 65°C for 16 h. The polymerized block that contained the cell was removed from the coverslip and sectioned on an ME-Ultracut S ultramicrotome (Leica, Wetzlar, Germany). Serial sections were placed onto formvar-coated copper one-slot grids, and then observed under a transmission electron microscope Hitachi H-7650 (Tokyo, Japan) without staining.

**Measurements of kleptochloroplast volumes with confocal laser scanning microscope:** To measure precise chloroplast volumes, we fed colourless cells of *A. poecilochroum* and *G. aeruginosum* with a single cryptomonad cell with the same method as feeding experiment shown above. For observation of the cell right after ingestion, cryptomonad-ingesting cells were quickly isolated to 3 µl of IMK or AF-6 medium on cover glass, and 3 µl of 5% glutaraldehyde was added to the medium. For observation of the cells at 1 h, 4 h, 6 h, 12 h, 24 h, 72 h after ingestion, the cells isolated on cover glass were cultured respectively in a humidified petri dish to prevent drying up, and fixed at each period. The fixed samples were observed using confocal laser scanning microscope Zeiss LSM-DUO (Carl Zeiss Japan, Tokyo) and obtained serial sectioning images (0.5 µm interval) of chloroplast autofluorescence. The images were stored as TIFF files using software IMARIS (Carl Zeiss Japan, Tokyo), and TIFF files are put into ImageJ (http://rsb.info.nih.gov/ij/). According to manufactures' instruction, the volumes of chloroplast were estimated using ImageJ plugin Sync Measure 3D downloaded from ImageJ website (http://rsbweb.nih.gov/ij/plugins/sync-windows.html).

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## Figure legends

**Figure 1.** Bright field and fluorescence micrographs of the morphological change in the cryptomonad cells ingested by *Amphidinium poecilochroum*. Times shown on bright field micrographs indicate the elapsed times after the ingestion of *Rhodomona*s sp. Each fluorescence micrograph corresponds to the bright field micrograph directly above it. Note that the cryptomonad cells have deformed gradually after the 20 min stage. Digestive vacuoles (arrowheads) are formed after 20 min, and remain visible until the 12 h stage. Arrows indicate pyrenoids. Bar = 10 µm.

**Figure 2.** TEM micrographs of membranes surrounding the kleptochloroplast in *Amphidinium poecilochroum*. **A.** Membranes directly following ingestion. Ingested chloroplast is enclosed by four membranes; two chloroplast membranes (white arrowhead) and two chloroplast ER membranes (arrowhead). The cryptomonad cytoplasm is separated from the dinoflagellate cytoplasm by a single membrane (arrow). **B.** Membranes 12 h after ingestion. Membranes of the chloroplast and the single membrane between the cryptomonad cytoplasm and the dinoflagellate cytoplasm cannot be resolved because they are very close to each other. The dinoflagellate cytoplasm is adjacent to the chloroplast, and the cryptomonad cytoplasm is not visible even under the TEM. Abbreviations: Chl, chloroplast; cS, cryptomonad starch; cCy, cryptomonad cytoplasm; dCy, dinoflagellate cytoplasm; dM, dinoflagellate mitochondria. Bar = 200 nm.

**Figure 3.** TEM micrographs of the morphological change in the cryptomonad cell at various times following ingestion by *Amphidinium poecilochroum*. **A.** Ingested cryptomonad cell directly after ingestion. The cell of *A. poecilochroum* contains a chloroplast, a cryptomonad nucleus, a nucleomorph, cryptomonad mitochondria and cytoplasm. The peripheral ejectosomes of the cryptomonad are accumulated. (ae; and see Fig. 4A). **B.** Cryptomonad cell 20 min after ingestion. Most of cryptomonad cytoplasm is removed from cryptomonad cell. The dinoflagellate nucleus is close to the chloroplast. **C.** Cryptomonad cell 30 min after ingestion. The gullet-surrounding ejectosomes of the cryptomonad are retained in the cryptomonad cytoplasm. **D.** Cryptomonad cell 1 h after ingestion. Almost all the cryptomonad's mitochondria, ejectosomes and cytoplasm are removed from the vicinity of the chloroplast. **E.** Cryptomonad cell 4 h after ingestion. Note that the chloroplast is elongated and on the periphery of the dinoflagellate cell and that the cryptomonad nucleus has become more electron dense than during the previous stage (see D). **F.** Chloroplast 12 h after ingestion. The chloroplast completely loses its original shape. Large

cryptomonad starch granules and digestive vacuoles are observed. No cryptomonad nucleus is found around the chloroplast. Arrows indicate that the boundary between the cryptomonad and the dinoflagellate cytoplasm. Abbreviations: Chl, chloroplast; cN, cryptomonad nucleus; Nm, nucleomorph; cM, cryptomonad mitochondria; Py, pyrenoid; cE, gullet-surrounding ejectosome; cS, cryptomonad starch; cCy, cryptomonad cytoplasm; dN, dinoflagellate nucleus; DV, digestive vacuole. Bar = 2 µm.

**Figure 4.** TEM micrographs of digestive vacuole formation in *Amphidinium poecilochroum* cell. **A.** Cryptomonad peripheral ejectosomes directly following ingestion. Note that the ejectosomes are accumulated between the cryptomonad and dinoflagellate. Membranous material can be seen (arrow). **B.** Digestive vacuole at the 20 min stage containing accumulated ejectosomes and cryptomonad mitochondria. White arrows indicate the digestive vacuole membrane. **C.** Digestive vacuole at the 1 h stage containing the peripheral ejectosomes and the gullet-surrounding ejectosomes. **D.** The digestive vacuole at the 4 h stage with digested contents. Peripheral ejectosomes cannot be discerned due to digestion. **E.** The digestive vacuole at the 6 h stage showing the digested cryptomonad mitochondria and gullet-surrounding ejectosomes. **F.** The digestive vacuole at the 12 h stage containing homogeneous material. The cryptomonad organelles cannot be recognised. Arrowhead indicates an individual peripheral ejectosome. Abbreviations: cM, cryptomonad mitochondria; cE, gullet-surrounding ejectosomes; dM, dinoflagellate mitochondria. Bar = 500 nm.

**Figure 5.** TEM micrographs of the cryptomonad nucleus (cN) in *Amphidinium poecilochroum*. **A.** Cryptomonad nucleus showing intact structure at the time of ingestion. **B.** Cryptomonad nucleus at the 2 h stage showing intact structure. **C-D.** The surface of cryptomonad nucleus becomes undulated at the 3 h and 4 h stages. Note that increase in electron density of the cryptomonad nucleus relative to that in the previous stages (see B). **E.** The digestive vacuole at the 6 h stage containing a cryptomonad nucleus-like structure. **F.** The content of digestive vacuole at the 12 h stage becoming homogeneous. No cryptomonad nucleus can be found in the digestive vacuole. Bar = 500 nm.

**Figure 6.** TEM micrographs of the nucleomorph in *Amphidinium poecilochroum*. **A**. Ingested nucleomorph showing intact structure within pyrenoid matrix. **B-F.** Nucleomorphs at each stage investigated showing that its structure remains intact and with no visible signs of digestion. Abbreviations: Chl, chloroplast; Nm, nucleomorph; Py, pyrenoid. Bar = 500 nm.

**Figure 7.** Bright field and fluorescent micrographs following the morphological change of cryptomonad cells in *Gymnodinium aeruginosum* with time after ingestion. Times shown on the bright field micrographs

indicate that elapsed times after ingestion of *Chroomonas* sp. Each fluorescent micrograph corresponds to the bright field micrograph shown above it. Note that cryptomonad cells are deformed significantly by the 6 h stage. White arrows and arrows indicate an eyespot and a pyrenoid of cryptomonad respectively. A digestive vacuole is not observed in *G. aeruginosum*. Bar = 10 μm.

**Figure 8.** TEM micrographs of membranes of the chloroplast in *Gymnodinium aeruginosum*. **A.** The ingested chloroplast is surrounded by four membranes; two chloroplast membranes and two chloroplast ER membranes. The cryptomonad cytoplasm is separated from that of the dinoflagellate by a single membrane. **B.** Intact membranes around the chloroplast. Four membranes and a cytoplasmic boundary membrane are still retained after 24 h. White arrowheads, arrowheads and arrows indicate chloroplast membranes, chloroplast ER membranes and the membrane between the cryptomonad and the dinoflagellate cytoplasm. Abbreviations: Chl, chloroplast; cCy, cryptomonad cytoplasm; cM, cryptomonad mitochondria; cS, cryptomonad starch; dCy, dinoflagellate cytoplasm; dS, dinoflagellate starch. Bar = 200 nm.

**Figure 9.** TEM micrographs following the morphological change of cryptomonad cells in *Gymnodinium aeruginosum* with time after ingestion. **A.** Ingested cryptomonad cell directly after ingestion. The cell of *G. aeruginosum* possesses a chloroplast, a cryptomonad nucleus, a nucleomorph, cryptomonad mitochondria and cytoplasm. **B.** Cryptomonad cell 20 min after ingestion. The chloroplast is slightly deformed. Cryptomonad organelles are not removed by this stage. **C.** Cryptomonad cell 3 h after ingestion. The chloroplast is enlarged and has lost the original elliptic shape. **D.** Cryptomonad cell 6 h after ingestion. Note that the complete loss of the original dorsiventrality of the cryptomonad. Cryptomonad organelles are well retained. **E.** Cryptomonad cell 12 h after ingestion. Cryptomonad organelles within cryptomonad cytoplasm can be observed. **F.** Cryptomonad cell 24 h after ingestion. Note that the cryptomonad nucleus, the nucleomorph and the cryptomonad cytoplasm are retained although the chloroplast has been modified. Arrows indicate the boundary between the cryptomonad and the dinoflagellate cytoplasm. Abbreviations: Chl, chloroplast; cN, cryptomonad nucleus; Nm, nucleomorph; cM, cryptomonad mitochondria; Py, pyrenoid; E, eyespot; cCy, cryptomonad cytoplasm; dM, dinoflagellate mitochondria. Bar = 2 μm.

**Figure 10.** TEM micrographs of cryptomonad ejectosomes and digestive vacuoles in *Gymnodinium aeruginosum* cell. **A, B.** Cryptomonad cytoplasm containing peripheral ejectosomes, a basal bodies and cryptomonad mitochondria directly following ingestion. Note that peripheral ejectosomes are not accumulated as they are in *A. poecilochroum*. Bar = 500 nm. **C.** Cryptomonad cytoplasm containing gullet-surrounding ejectosomes at the 1

h stage. Note that gullet-surrounding ejectosomes are retained in the cryptomonad cytoplasm. Bar = 500 nm. **D.** Digestive vacuoles containing unknown material at the 6 h stage. Note that gullet-surrounding ejectosomes remain in the cryptomonad cytoplasm. Bar = 1 µm. **E**. Cryptomonad cytoplasm containing peripheral ejectosomes at the 6 h stage. Bar = 500 nm. **F.** Digestive vacuole at the 12 h stage showing an increase in size (2.5 µm in diameter) relative to that at the 6 h stage (1 µm in diameter). The contents of the digestive vacuole are unknown. Bar = 500 nm. Arrowhead indicates an individual peripheral ejectosome. Abbreviations: Chl, chloroplast; cN, cryptomonad nucleus; cM, cryptomonad mitochondria; cE, gullet-surrounding ejectosome; B, basal body; cCy, cryptomonad cytoplasm; DV, digestive vacuole; dM, dinoflagellate mitochondria.

**Figure 11.** TEM micrographs of the cryptomonad nucleus and nucleomorph in *Gymnodinium aeruginosum*. **A.** Cryptomonad nucleus showing its intact structure at the time of ingestion. Bar = 500 nm. **B.** Cryptomonad nucleus retaining its intact structure without any sign of digestion 24 h after ingestion. Bar = 1 µm. **C.** Nucleomorph showing its intact structure directly after ingestion. Bar = 500 nm. **D.** Nucleomorph at the 24 h stage, showing no effect of digestion. Bar = 500 nm. Abbreviations: Chl, chloroplast; cN, cryptomonad nucleus; Nm, nucleomorph; Py, pyrenoid.

Figure 12. Volume ($\mu m^3$) of ingested chloroplast in each stage from ingestion of cryptomonad in *Amphidinium poecilochroum* (diamond) and *Gymnodinium aeruginosum* (square). Error bars mean SD.

Supplemental Table 1. The number of cells of *Amphidinium poecilochroum* and *Gymnodinium aeruginosum* used for light microscopy (LM), transmission electron microscopy (TEM) and confocal laser scanning microscopy (CLSM).

Supplemental Figure 1. **A.** light micrograph of *Rhodomonas* sp. used as prey for *Amphidinium poecilochroum*. A pyrenoid (Py) is visible. Bar = 5 µm. **B.** TEM micrograph of gullet-surrounding (arrow) and peripheral (arrowhead) ejectosomes. Chloroplast (Chl) and starch granule (cS) are shown. Bar = 1 µm. **C.** Longitudinal section of *Rhodomonas* sp. showing a chloroplast (Chl), a nucleus (cN), a nucleomorph (Nm), mitochondria (cM), a Golgi body (G), a pyrenoid (Py) and starch granules (cS). Bar = 2 µm.

Supplemental Figure 2. **A.** light micrograph of *Chroomonas* sp. used as prey for *Gymnodinium aeruginosum*. A pyrenoid (Py) is visible. Bar = 5 µm. **B.** TEM micrograph of gullet-surrounding (arrow) and peripheral (arrowhead) ejectosomes. Chloroplast (Chl), mitochondria (cM) and gullet (Gu) are shown. Bar = 1 µm. **C.** Longitudinal section of *Chroomonas* sp. showing a chloroplast (Chl), a nucleus (cN), a nucleomorph (Nm), mitochondria (cM), a gullet (G), a pyrenoid (Py), starch granules (cS), peripheral ejectosomes(arrowhead). Bar

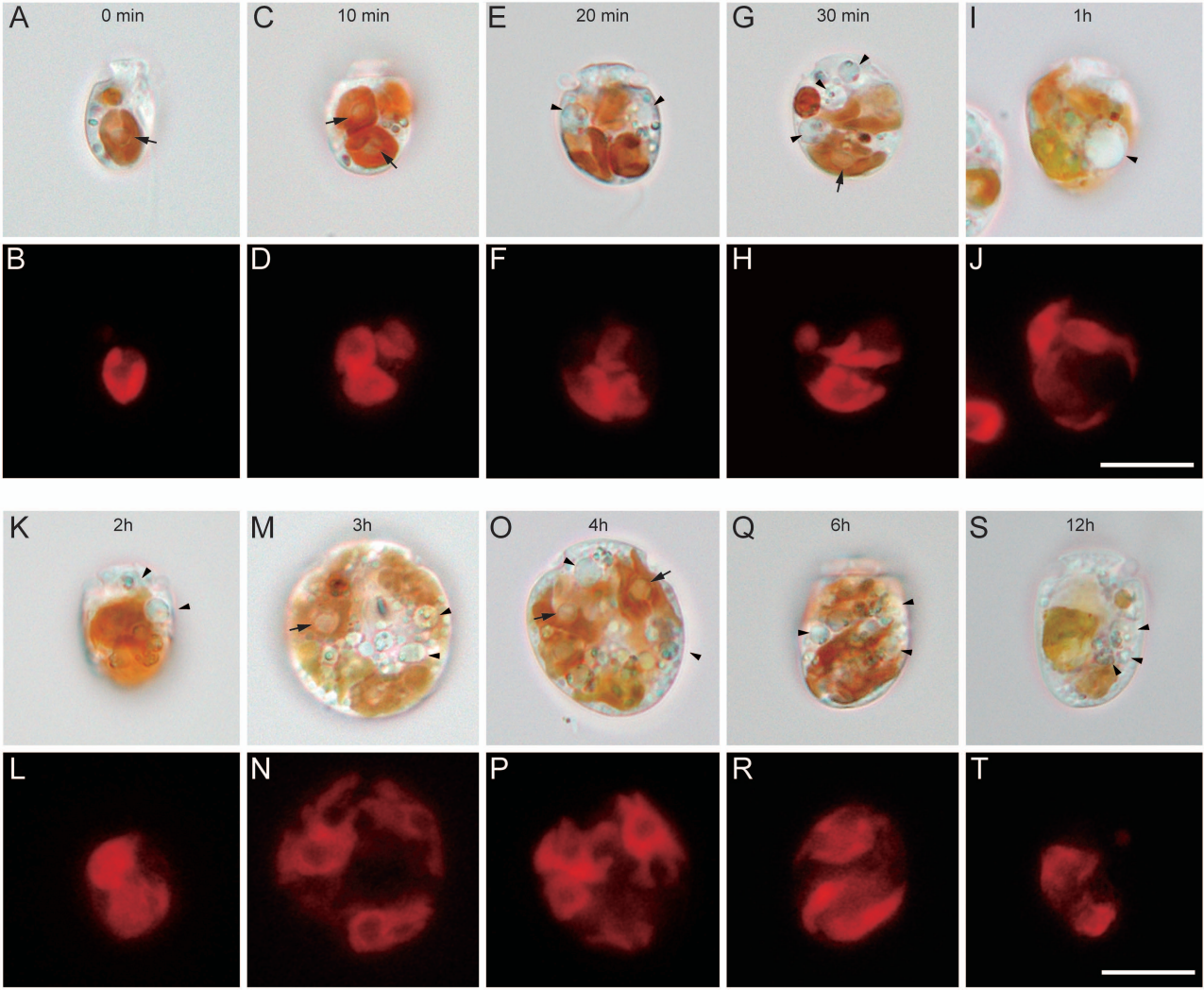
= 2 µm.

Supplemental Movie 1. Animation of serial sectioning images of chloroplast autofluorescence in *Amphidinium poecilochroum* at 0 min stage. Bar = 10 µm.

Supplemental Movie 2. Animation of serial sectioning images of chloroplast autofluorescence in *Amphidinium poecilochroum* at 24 h stage. Bar = 10 µm.

Supplemental Movie 3. Animation of serial sectioning images of chloroplast autofluorescence in *Gymnodinium aeruginosum* at 0 min stage. Bar = 10 µm.

Supplemental Movie 4. Animation of serial sectioning images of chloroplast autofluorescence in *Gymnodinium aeruginosum* at 72 h stage. Bar = 10 µm.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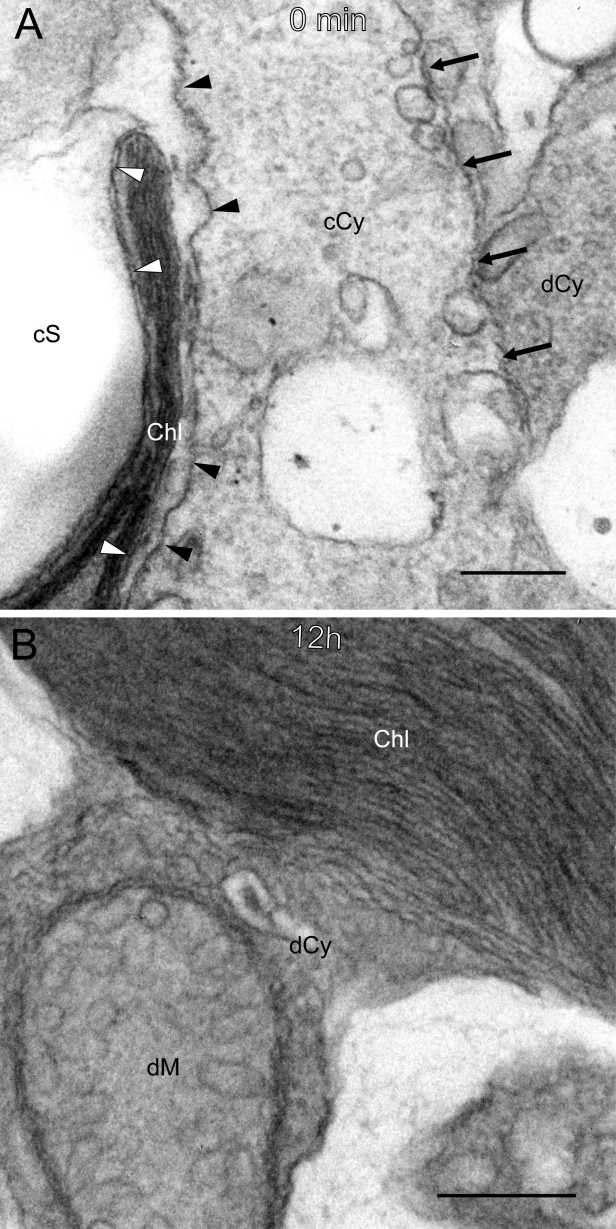
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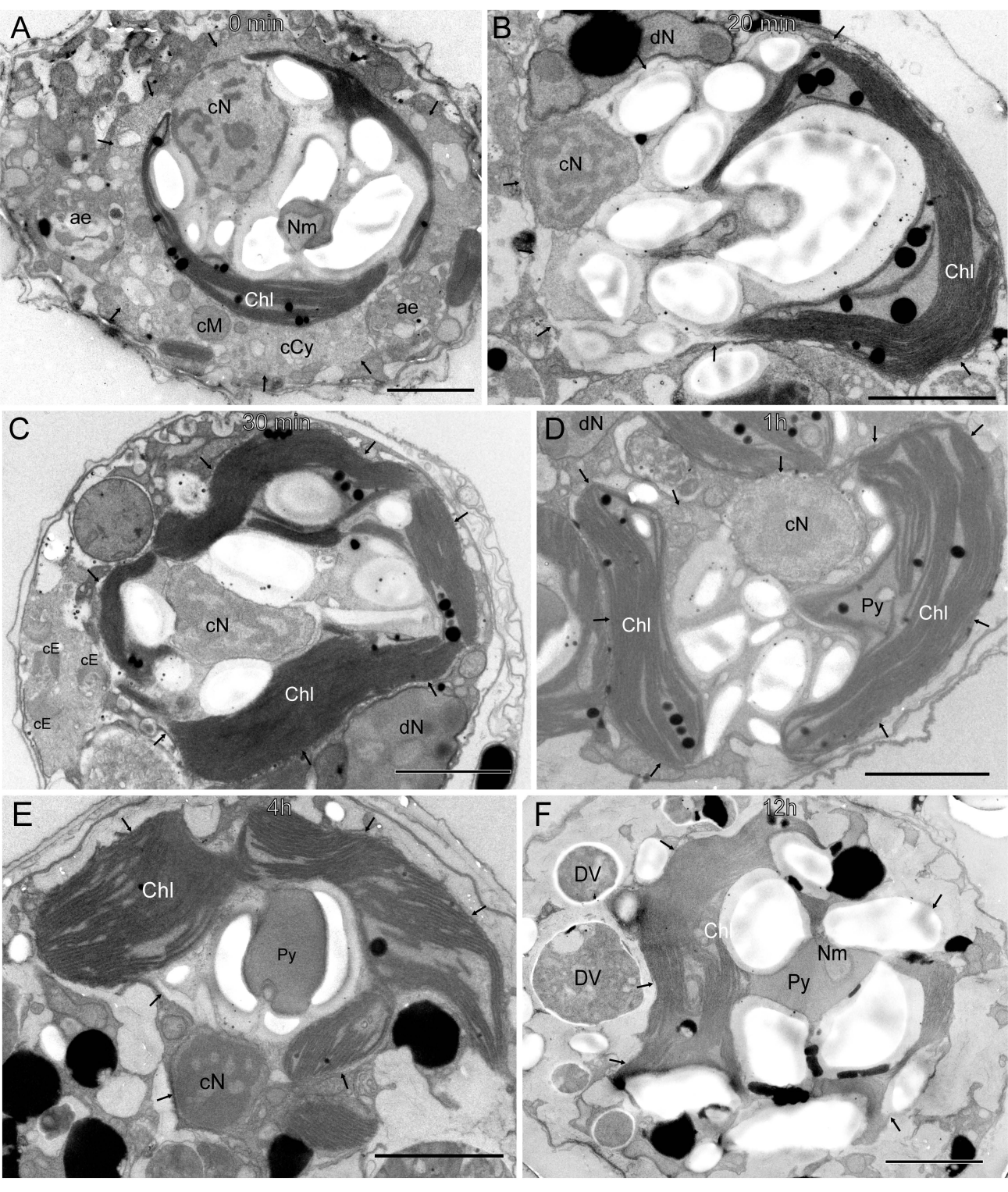
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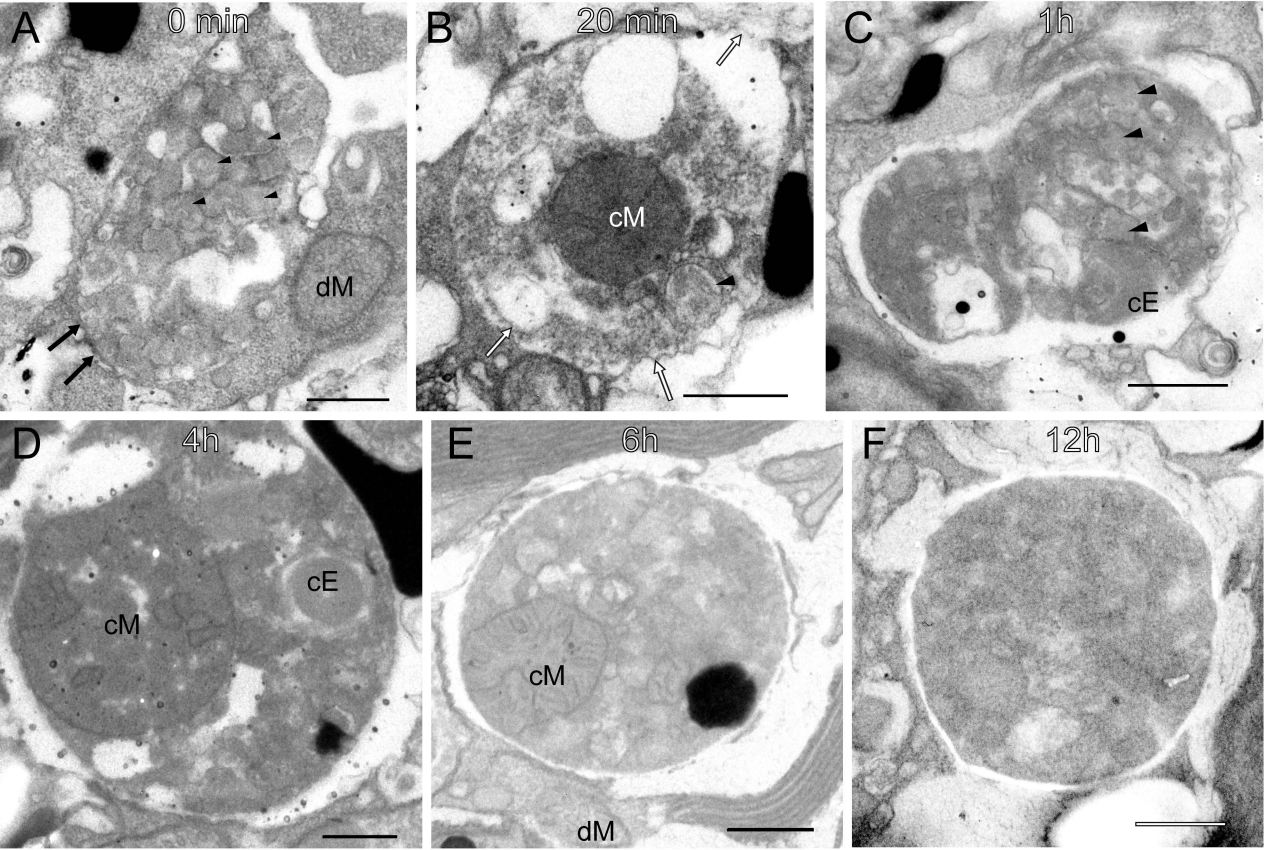
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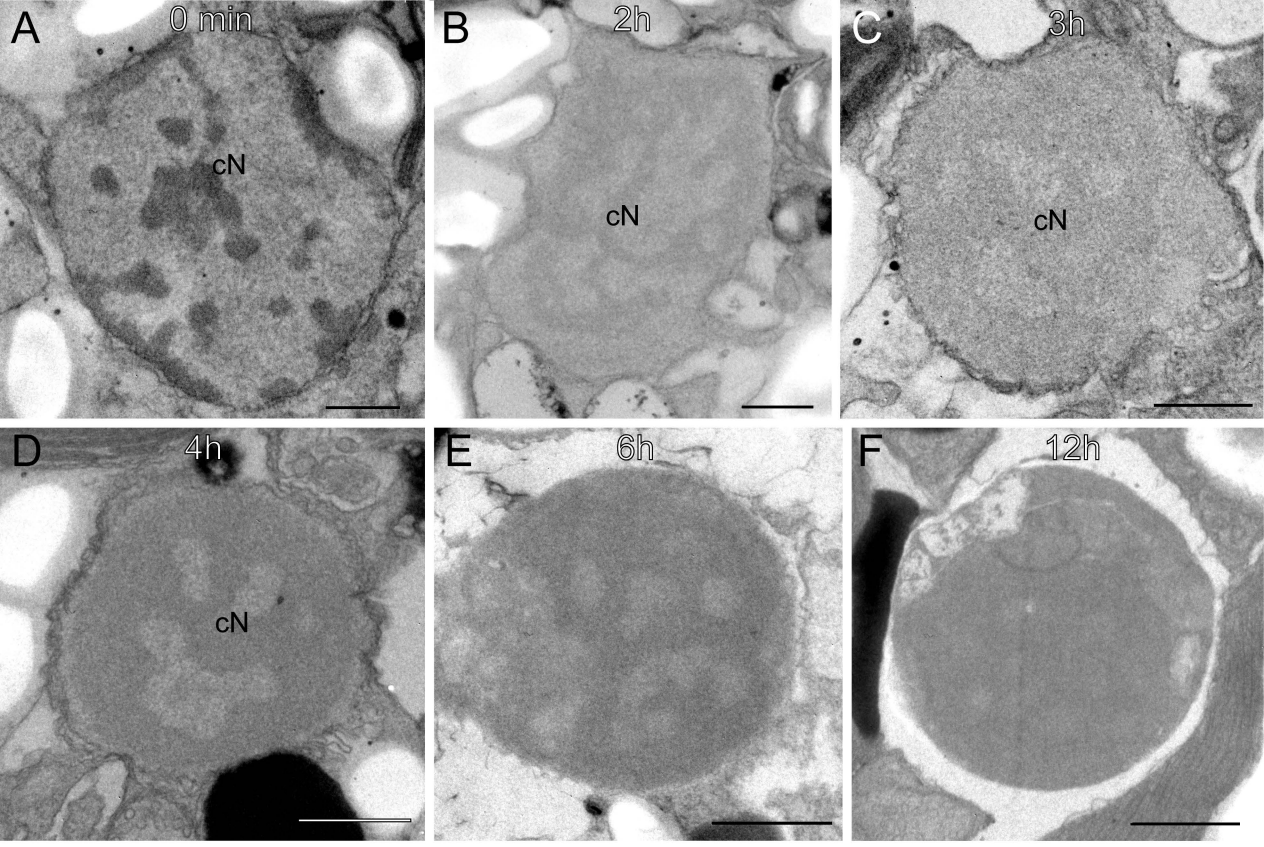
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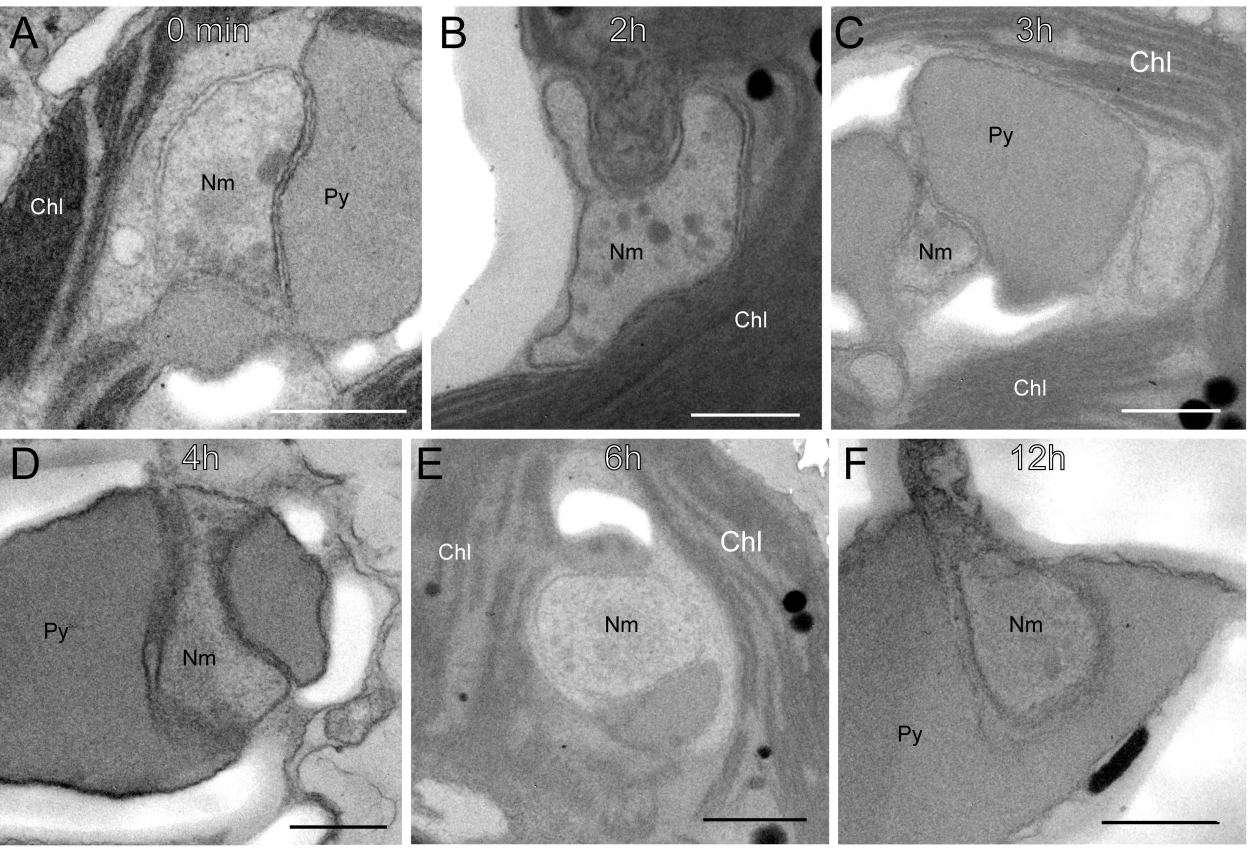
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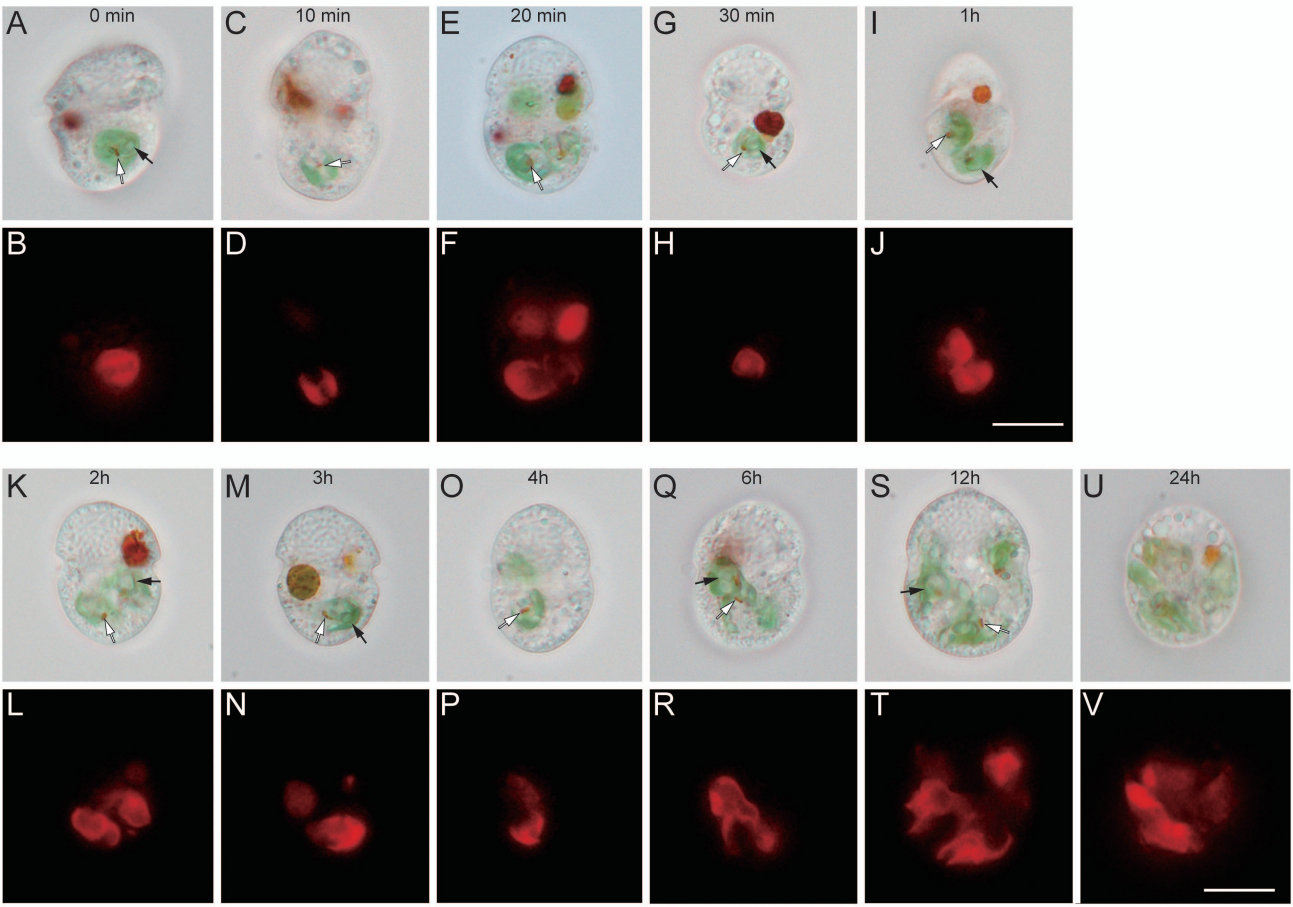
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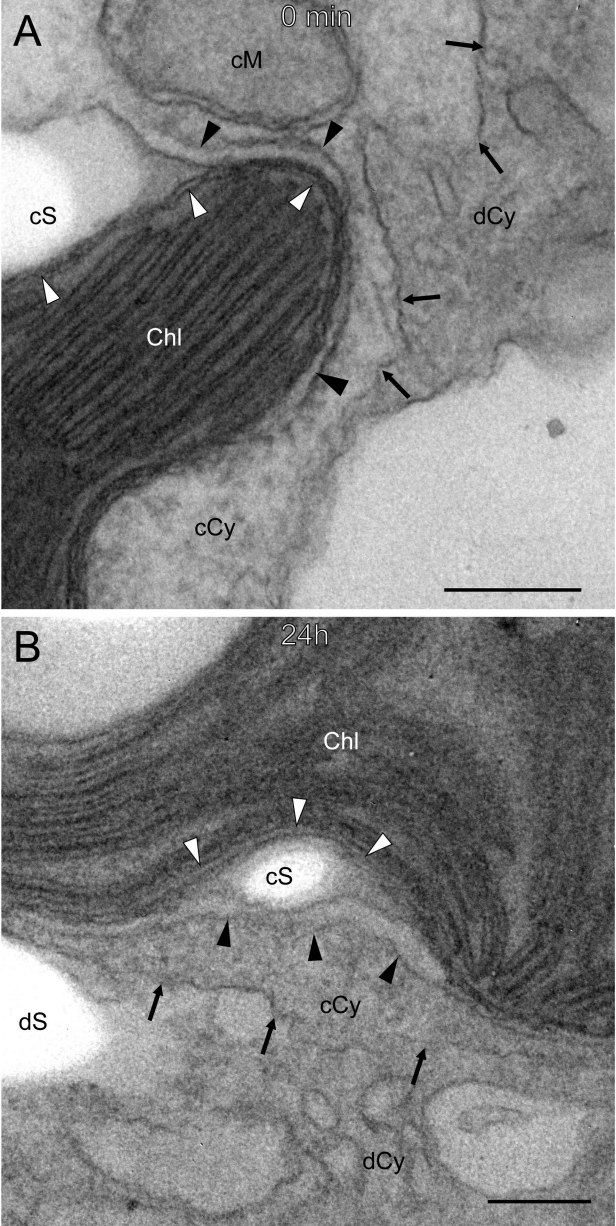
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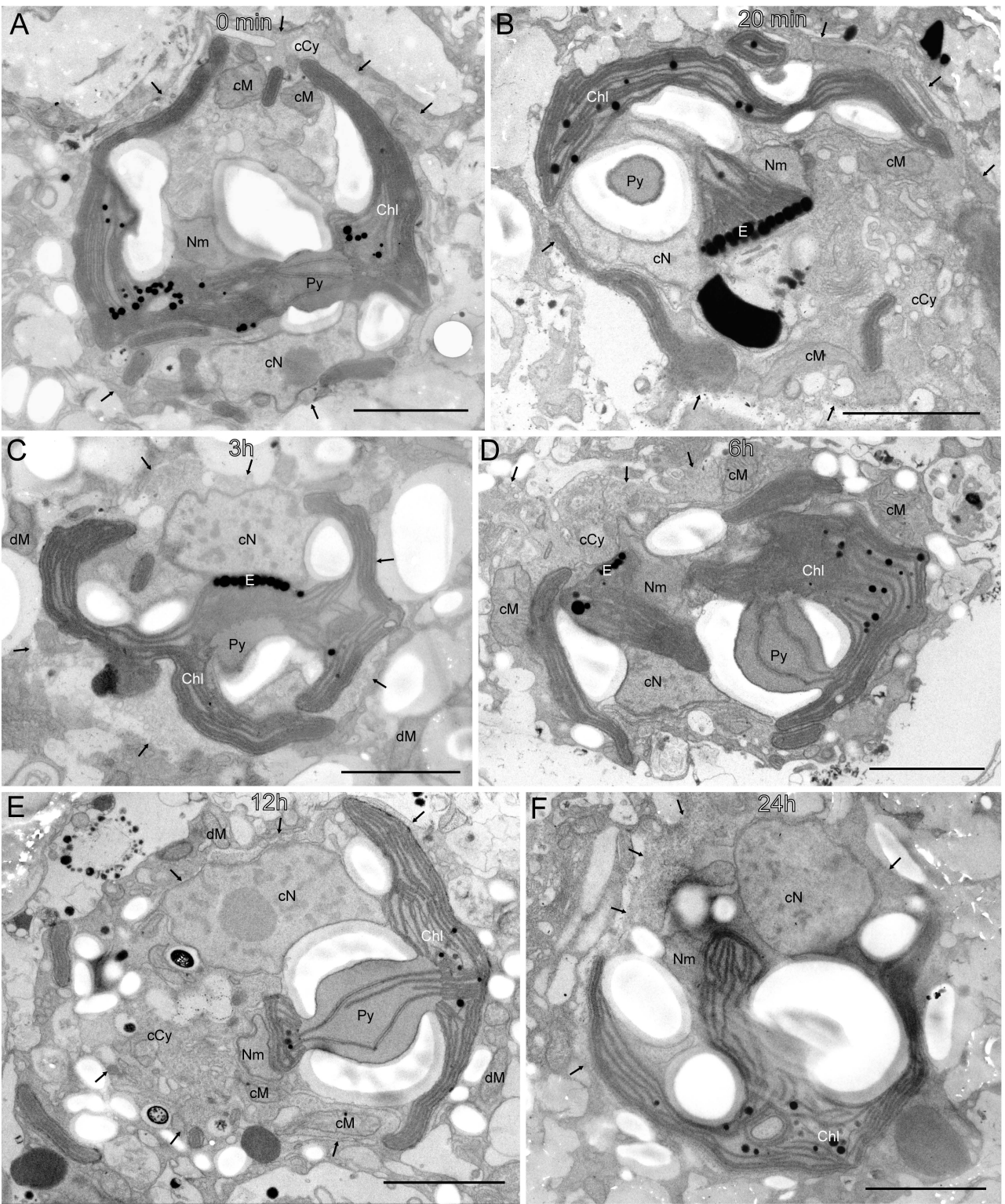
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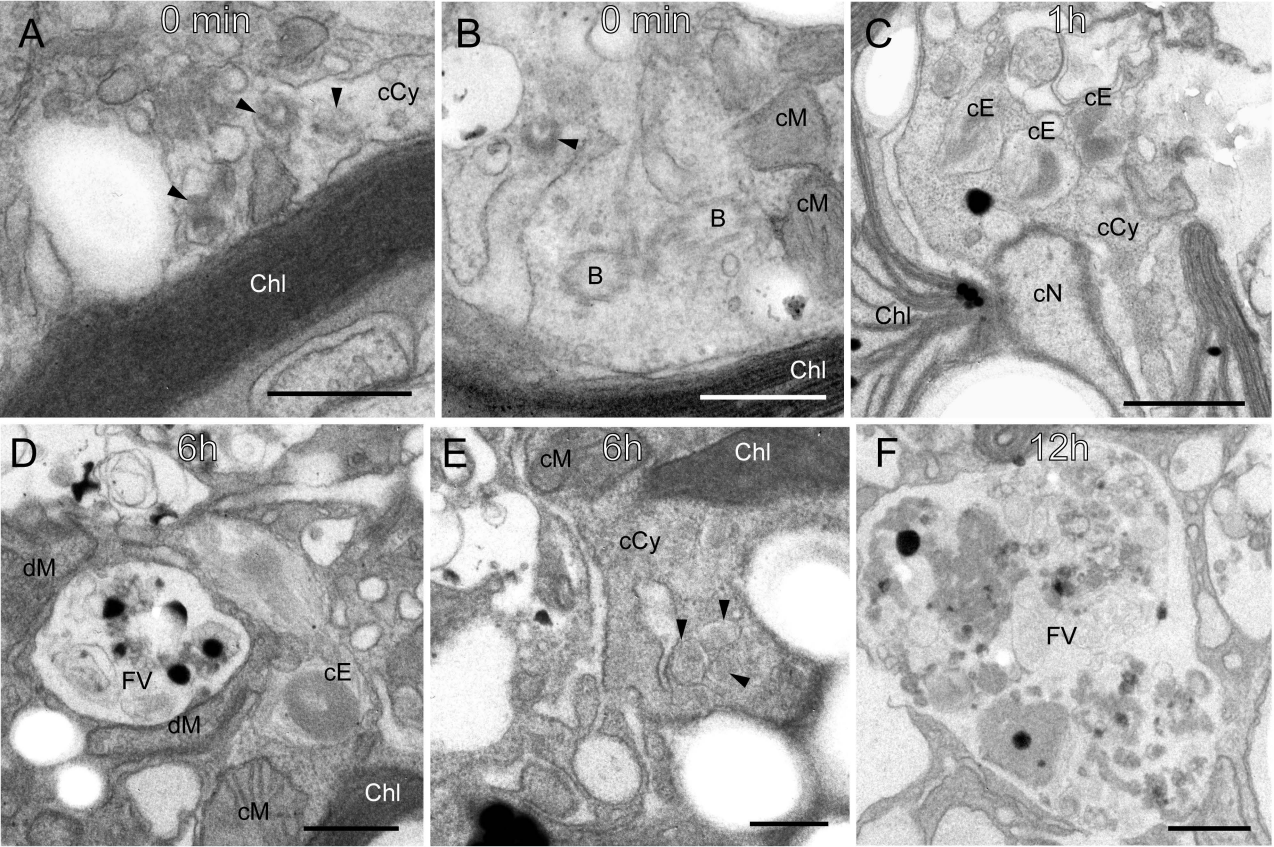
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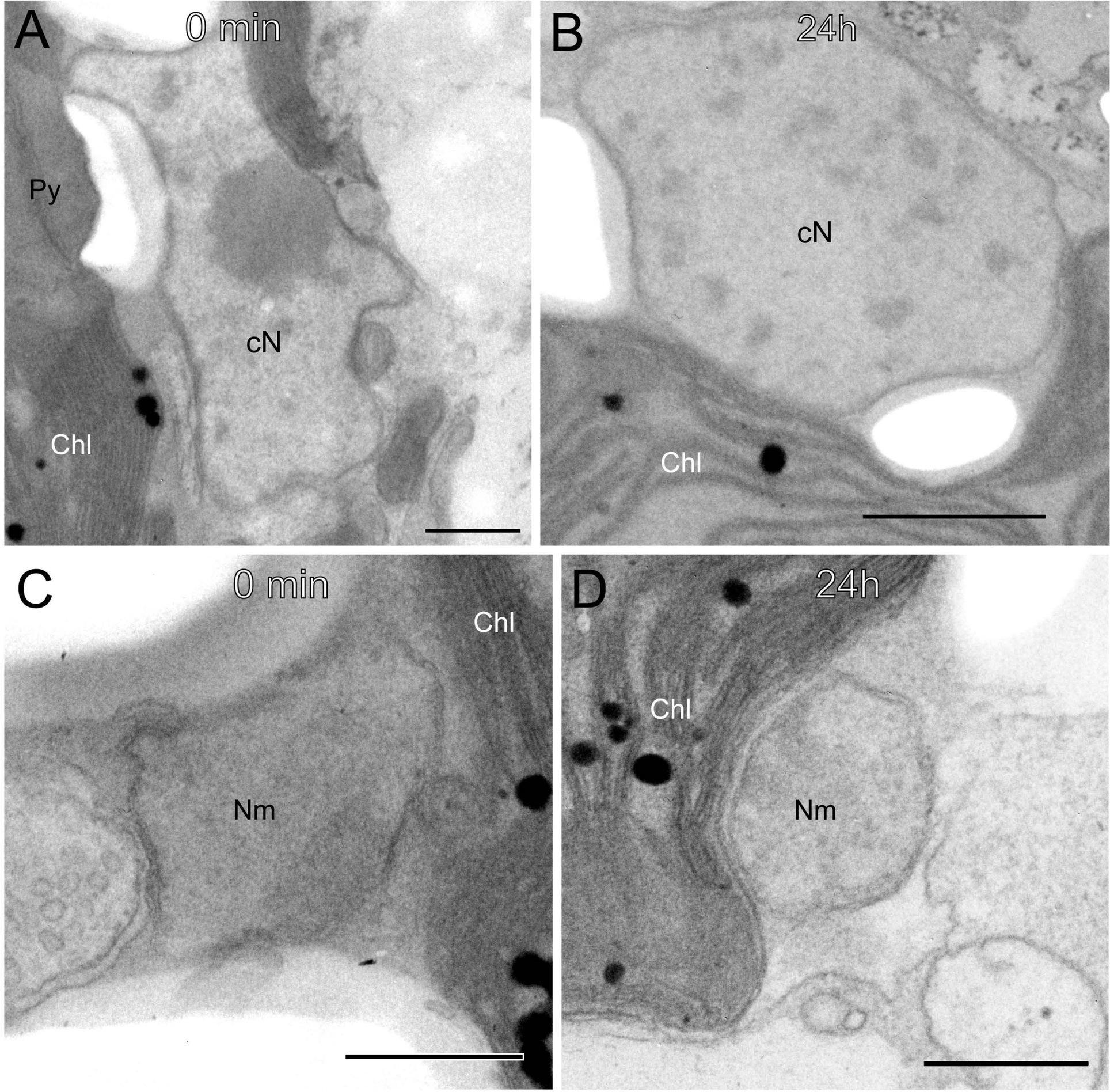
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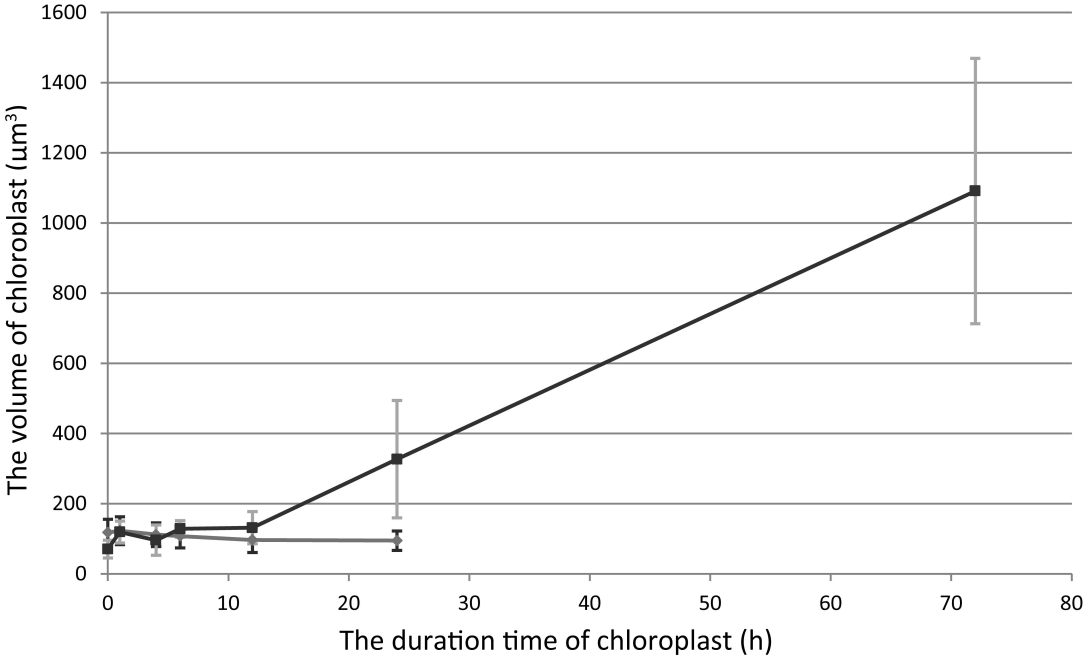
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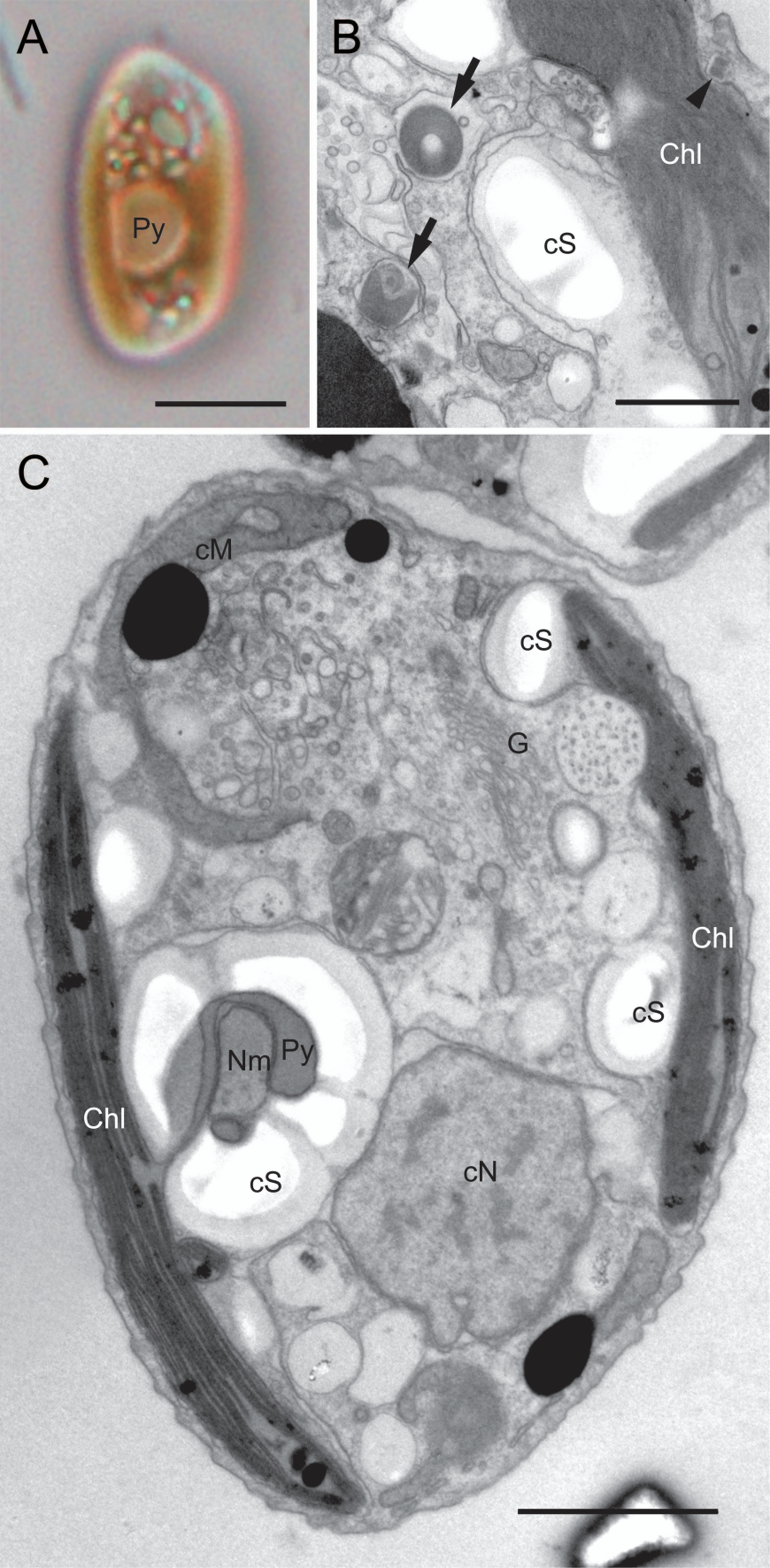
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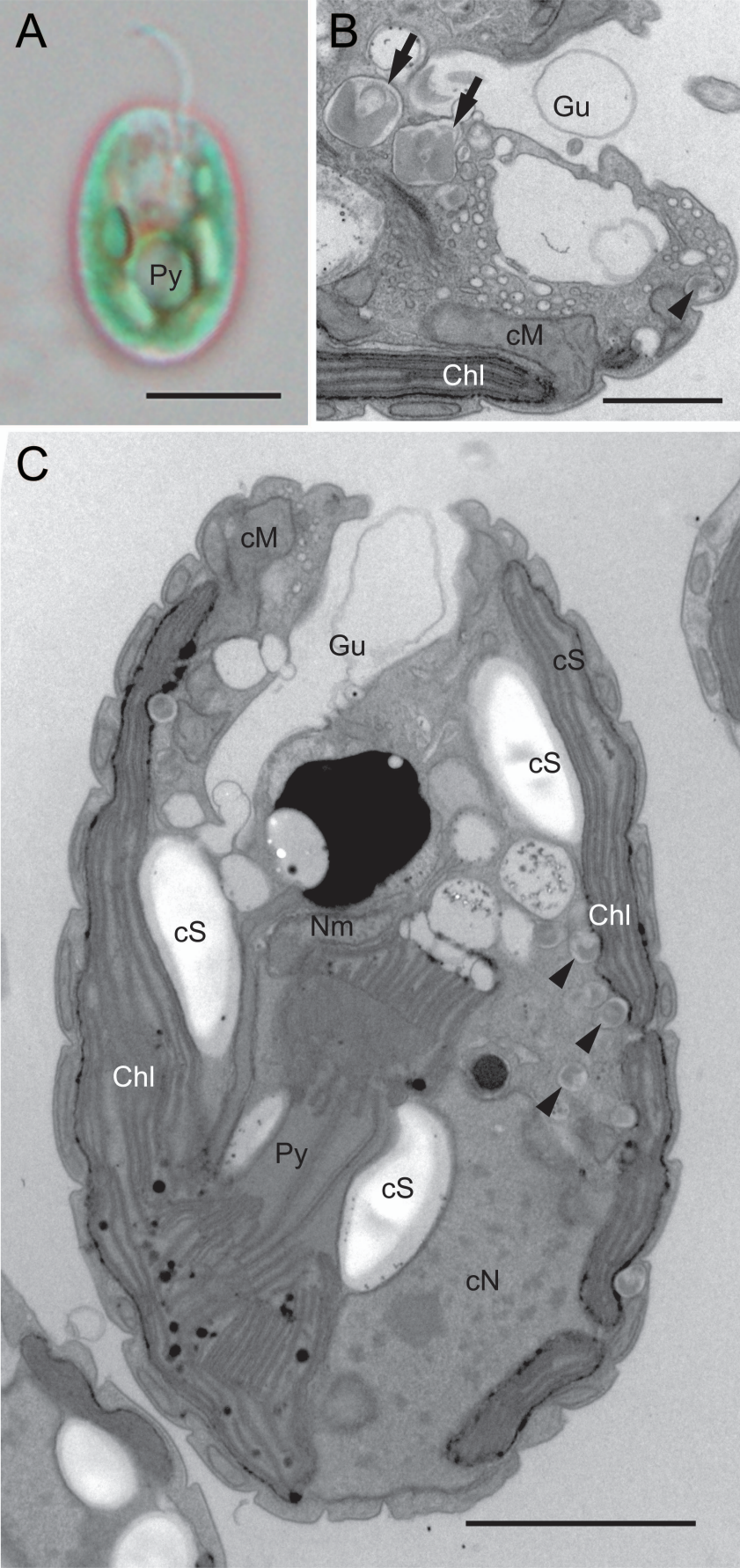
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| 20 min | 3 | 2 | - | 3 | 2 | - |
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| 1 h | 9 | 3 | 22 | 2 | 2 | 16 |
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| 24 h | - | | 24 | 5 | 2 | 16 |
| 72 h | - | | - | - | - | 10 |