**Suitability of the thawed algae for transmission electron microscopy study: Ultrastructural investigation on *Coccomyxa melkonianii* SCCA 048**

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## Abstract

Introduction. Morphological and ultrastructural investigations are crucial for the identification and characterization of species such as microalgae, microorganisms that greatly change their morphology and physiology during their life cycle. Transmission electron microscopy (TEM) is an excellent tool for the ultrastructural observation of cells and their components. To date, limited ultrastructural studies have been carried out on microalgae, due to the difficulties in sample preparation. The aim of this work is to establish an appropriate fixation method that allows to better preserve the algal ultrastructure and test the suitability of the thawed algae for TEM observation.

Methods. Fresh and thawed algae (*Coccomyxa melkonianii* SCCA 048) were fixed with different TEM fixation methods (a mix of glutaraldehyde and paraformaldehyde for several incubation times, sometimes preceded by a prefixation in cold methanol). The ultrastructural images obtained from fresh algae were compared to those obtained from frozen biomass.

Results and conclusion. The best morphological results were achieved by fixing fresh algae in 1% paraformaldehyde and 1.25% glutaraldehyde for 5 hours. Pretreating with frozen methyl alcohol reduced fixation time to 2 hours. Both fresh and frozen algae ultrastructure were rather well preserved also with 1% paraformaldehyde and 1.25% glutaraldehyde for 2 hours. Ultrastuctural morphological images of the thawed algae demonstrated that also frozen samples can be used in TEM research thus widening specimen suitability by means of this technique.

Key words: ultrastructure; Coccomyxa; frozen biomass; transmission electron microscopy (TEM)

## Introduction

Transmission electron microscopy (TEM) is one of the most informative methods for the documentation of the ultrastructure of cells and their components as emphasized by several authors (Stead et al. 1988; Griffiths 2006; Kimura et al. 2012; Dohnalkova and Cardon, 2018). Therefore, TEM is frequently used to determine the diversity of microorganisms in terms of morphology, ecology and physiological adaptations (Hernández Mariné et al. 2004). Microalgae are a diverse group of single-cell photosynthetic organisms, both prokaryotes and eukaryotes, which can rapidly grow in a wide range of habitats (Barsanti and Gualtieri, 2018). These microorganisms greatly change their morphology and physiology, and it is recognized that morphological, ultrastructural, and phylogenetic methods are crucial for the identification of species level. Despite the importance of TEM observations to study phytoplankton, studies conducted on microalgae by TEM remain quite limited (Sieburth et al. 1988; Kimura et al. 2012). This is partially due to methodological difficulties in sample preparation of phytoplankton (Kimura et al., 2012).

*Coccomyxa* is a genus of unicellular green algae of the class Trebouxiophyceae, well known for its cosmopolitan distribution and great ecological amplitude (Malavasi et al., 2016). It is a unicellular green alga, which is characterized by ellipsoid to elongated cell shape, a parietal chloroplast, and mucilage surrounding the cell (Schmidle 1901; Darienko et al., 2015; Malavasi et al., 2016; Gustavs et al, 2017). The type species of this genus is *Coccomyxa dispar* (Schmidle 1901), is a pyrenoid-lacking species.

The aim of this work is to find an appropriate fixation protocol that allows to better preserve the ultrastructure of *Coccomyxa melkonianii* SCCA 048. To this end algal samples were fixed with different fixation methods.

Moreover, we tested the suitability of exploiting thawed algae for TEM observation. To date, no satisfactory TEM protocols are available for thawed algal biomass. The cryopreservation is considered by most Biological Resource Centre (BRC) practitioners to be the optimal long-term conservation method for microbial cultures (Kirsop and Doyle 1991; Kapoore et al. 2019), although the access to ultra-low temperature refrigerators or liquid nitrogen storage is limited to few labs, while easy access to domestic freezers is widespread (Kapoore et al. 2019). We compared the results obtained in thawed and fresh pelleted biomass treated with the same fixation method.

## Materials and Methods

The protocols listed in Table 1 were tested on *C. melkonianii* cell pellets. Each protocol consists of the following steps: (i) cultivation, (ii) fixation, (iii) post-fixation and en bloc staining, (iv) dehydration and embedding, (v) ultrathin sectioning and staining, and (vi) visualization by TEM. Both fresh and thawed algal pellets were processed according to the following customized protocols:

### i. Cultivation of *Coccomyxa melkonianii*

*C. melkonianii*, SCCA 048 strain, was obtained from the Sardinian Culture Collection of Algae (SCCA) (Malavasi & Cao 2015). The cells were grown in BBM medium in 250 mL flasks under constant illumination (80-100 μm $m^{-2}$ $s^{-1}$) at 25°C. In each TEM sample preparation protocol cells in the exponential phase growth were used.

### ii. Fixation protocols, (iii) postfixation and en bloc staining, (iv) dehydration and embedding, (v) ultrathin sectioning and staining.

Fresh algae were harvested by centrifugation (2000g, 2 min, RT). The pellets were fixed for 2 and 5 hrs with a mixture of 1% paraformaldehyde and 1.25% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at room temperature (RT). Some pellets were fixed for 3 hrs in 3% glutaraldehyde at RT. Other pellets were pre-treated with methyl alcohol for 20' at -20°C and then with a mixture of 1% paraformaldehyde and 1.25% glutaraldehyde for 2 hrs at RT.

Algae were frozen in their culture medium at -20°C for several days. Then algae were thawed, pelleted and pellets were fixed for 2 hrs with a mixture of 1% paraformaldehyde and 1.25% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 2 hours a RT.

After fixation, all the pellets were rinsed in cacodylate buffer added with 3.5% sucrose, and post-fixed in 2% osmium tetroxide for 1 hour, and, after rinsing with bi-distilled water, samples were left overnight in 0, 25%, uranyl acetate.

Moreover, some fresh algae pellets were treated with a solution of 1,5% or 5% $KMnO_4$ for 20 minutes or 1 hour, respectively. These samples did not undergo to post-fixation and overnight staining with uranyl acetate while they were directly dehydrated and embedded. These samples were not counterstained after cutting as the others.

All pellets were dehydrated in an ascending acetone series (50–100%) and embedded in Epon Resin (Glycide Ether 100; Merck, Darmstadt, Germany). Randomly selected tissue blocks from each pellet were used to prepare sections for electron microscopic studies. Ultrathin sections (90nm) were cut with a diamond knife, collected on grids, and were contrasted with uranyl acetate and bismuth subnitrate. The investigation was performed according to the procedures shown in Table 1.

### vi. Visualization

Following the different fixation protocols described above, samples of *C. melkonianii*, were observed and randomly photographed in a JEOL 100S transmission electron microscope (TEM) and JEOL JEM 1400 Plus TEM (Tokyo, Japan) (the latter in the CeSAR facility).

## Results

***-Fixation in 3% glutaraldehyde***

Despite several papers indicate this fixation method as preferable, severe morphological damage of the cells was observed by fixation of fresh algae for 3 hours in 3% glutaraldehyde at room temperature (Fig. 1 A, B). All intracellular structures appeared rather darkly stained. Nucleus and mitochondria were often indistinguishable, the arrangement of chloroplasts was scarcely maintained, and the thylakoid membranes were almost completely disrupted.

***-Fixation in 5% $KMnO_4$***

This high $KMnO_4$ concentration was chosen to allow fixative penetration of the cell wall, since lower concentrations were ineffective (data not shown). Several alterations and artefacts were found in fresh *C. melkonianii* fixed for 20 minutes in 5% $KMnO_4$ at room temperature. Along the cell wall numerous interruptions were observed (Fig. 1 C, D). Although the chloroplast maintained a normal shape, the thylakoid membranes were swollen and very irregular. Moreover, the other ultrastructural features of the cell organelles were not clearly observable.

***-Fixation in 1% paraformaldehyde and 1.25% glutaraldehyde***

Exposure to the mixture of 1% paraformaldehyde and 1.25% glutaraldehyde for 5 h resulted in the best morphological appearance in fresh cells (Fig. 2). The overall cell structure retained the typical ultrastructural features of *Coccomyxa* sp. The peculiar external envelope, called mother cell wall, and the cell wall were easily discernible in Fig. 2A. The cup-shaped chloroplast, which occupied most of the inner cell volume, was well represented (Fig 2 A-C). The thylakoidal system, formed by small closed cisternae, seen as parallel membranes when they are visualized by cross section, was well distinguishable. Many plastoglobules, which are spherical electron-dense granules, different in size, were observed enclosed within cell wall, close to external face of chloroplasts and in the inter-thylacoidal spaces. (Fig. 2 A, B). Nuclei and mitochondria were always clearly observed.

This combination of fixatives has been used by our group for a long time (Isola et al, 2011; 2013; Lilliu et al. 2015; Riva et al., 2011; Isola et al., 2010), while exposure time to fixation was shorter since it was applied to mammalian cells which lack a cell wall and easily allow chemicals' penetration.

A good level of cell structure preservation was also achieved with 2 hours of fixation in 1% paraformaldehyde and 1.25% glutaraldehyde at room temperature in both fresh (Fig. 3) and thawed samples (Fig. 4). In fact, fresh and thawed algae displayed the mother cell wall, the cell wall and the chloroplasts as well as thylacoidal membranes. Nuclei were always observed.

***-Methyl alcohol for 20' at -20°C and fixation in 1% paraformaldehyde and 1.25% glutaraldehyde***

The ultrastructure of fresh algal pre-fixed in Methyl alcohol for 20' at -20°C and then exposed for 2 hours in 1% paraformaldehyde and 1.25% glutaraldehyde at room temperature (Fig. 5) appeared globally clearly observable, very similar to what observed after fixation with aldehyde mixture for 5 hours, albeit the fine structure of the chloroplast was sometimes altered with swollen tylakoidal membranes. In contrast, the nucleus and its nuclear envelope were clearly appreciated.

**Discussion**

We carried out this morphological investigation on *C. melkonianii* by TEM, using different fixation techniques, in order to evaluate which fixation method better preserves the structural morphology of these microalgae. In fact, in the current literature for microalgae few well-established TEM protocols are available for the ultrastructural studies of these cells. We then compare new morphological data with those found in published TEM protocols (Albertano et al., 1990; Honegger, 2009; Garbayo et al. 2012; Kimura et al., 2012; Malavasi et al., 2016; Rivasseau, 2016) on a member of Trebouxiophyceae family (green microalgae) by other researchers. As in the

published TEM protocols the essential steps, such as speed, duration of centrifugation and incubation temperatures, are not always well detailed, making reproducibility difficult, we filled this lack of information.

In addition, we tested whether thawed algae were suitable for TEM observation of phytoplankton species, which might have important implications in microbial biotechnology

The best morphological results were achieved by fixing fresh algae in 1% paraformaldehyde and 1.25% glutaraldehyde (for 5 hours). A rather good level of preservation of the cell morphology was reached also with fixation in 1% paraformaldehyde and 1.25% glutaraldehyde for 2 hours in both fresh and frozen algae as far as the cell wall, the chloroplasts' shape as well as the thylakoid membranes were concerned. Indeed, the global form of the parietal cupshaped chloroplast and thylakoid membranes were visible and well distinguishable As previous described in Malavasi et al. (2016), Trebouxiophycean genus *Coccomyxa* is characterized by tripartite cell walls with a thin, trilaminar outermost wall layer built up by algaenans, enzymatically non-degradable, sporopollenin-like biopolymers (Honegger 2009). This major organelle covered most of the inner cell. In Malavasi et al. (2016) was reported that *C. melkonianii* is characterized by high morphological variability, usually with a single parietal chloroplast without pyrenoid, sexual reproduction by cell division and a formation of 2–4 autospores released by rupture of the cell wall at the rounded apex of the cell. As the others Chlorophyta, chloroplasts are surrounded by a two-membrane envelope, with no chloroplast endoplasmatic reticulum (Lee 2008).

The spherical electron-dense inclusions always observed in our samples are in agreement with previous findings firstly reported Malavasi et al. (2016), that described several "plastoglobuli" filled with electron-dense material in the cytoplasm of this organism, which probably corresponded to lipid droplets previously observed by Rivasseau et al. (2016) in the cytosol of the strain *Coccomyxa actinabiotis*. Albertano et al. (1990), described similar structures as "electrodense vacuoles" in the cytoplasm of specimen of the same genus. Garbayo et al. (2012) reported the presence of "electron-dense deposits" whose appearance was probably induced by metal exposure, and "plastoglobules" located in the stroma of chloroplasts of *Coccomyxa onubensis*. The starch granules were deposited in the interthylacoidal spaces, as observed previously in this (Malavasi et al. 2016) and different species of the same genus (Albertano et al. 1990; Garbayo et al. 2012; Rivasseau et al. 2016;).

The peculiar external wall that we called as mother cell wall envelope was already reported by other authors. Peveling and Galun (1976) described an "additional homogenous envelope in the area between plasmalemma and cell wall, naming it "additional wall". Successively, Albertano et al. (1990) and Honegger (2009) reported a similar structure as "algal mother cell walls", whereas Tremouillaux-Guiller et al. (2002) defined this structure as "mother cell wall remnants". Indeed Garbayo et al. (2012) designated this structure as "distinct cell wall". Usually, this structure has been associated to cell division.

The post-thawing ultrastuctural morphology of these algae was comparable to that obtained from fresh biomass treated with the same protocol of fixation. Our results suggest that keeping the algal cultures at -20°C is a practical and low-cost technique to store this kind of cells and nevertheless allows obtaining high-resolution TEM images of microalgae. The authors believe that this freezing technique can be further improved and that the speed, ease, and low cost of the method makes it a

very attractive alternative to more conventional methods for freezing algal cells.The maintenance of cultures in domestic freezers without loss of viability might have important implications in microbial biotechnology, particularly for non-specialist algal labs that have no access to ultra-low temperature refrigerators or liquid nitrogen storage dewars, and are looking for suitable alternatives for cells storage and preservation.

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| **Pellet** | Fixation | Time | Post fixation | Dehydration | Embedding or Infiltration | Staining |
|---|---|---|---|---|---|---|
| **Fresh algae** | 1% paraformaldehyde and 1.25% glutaraldehyde | 2 hrs | 2% osmium tetroxide 1 h; 0, 25%, uranil acetate overnight. | acetone series (50–100%) | Epon Resin | uranyl acetate 10’ ; bismuth subnitrate 2’ |
| **Fresh algae** | 3% glutaraldehyde | 3 hrs | 2% osmium tetroxide 1 h; 0, 25%, uranil acetate overnight. | acetone series (50–100%) | Epon Resi n | uranyl acetate 10’ ; bismuth subnitrate 2’ |
| **Fresh algae** | 1% paraformaldehyde and 1.25% glutaraldehyde | 5 hrs | % osmium tetroxide 1 h; 0, 25%, uranil acetate overnight. | acetone series (50–100%) | Epon Resin | uranyl acetate 10’ ; bismuth subnitrate 2’ |
| **Fresh algae** | methyl alcohol + 1% paraformaldehyde and 1.25% glutaraldehyde | **20 ‘ 2 hrs** | % osmium tetroxide 1 h; 0, 25%, uranil acetate overnight. | acetone series (50–100%) | Epon Resin | uranyl acetate 10’ ; bismuth subnitrate 2’ |
| **Fresh algae** | 1,5% KMnO4 5% KMnO4 | **20 ‘ ; 1 hr 20 ‘ ; 1 hr** | no postfixation | acetone series (50–100%) | Epon Resin | no staining |
| **Thawed algae** | 1% paraformaldehyde and 1.25% glutaraldehyde | 2 hrs | % osmium tetroxide 1 h; 0, 25%, uranil acetate overnight. | acetone series (50–100%) | Epon Resin | uranyl acetate 10’ ; bismuth subnitrate 2’ |

Table 1 – List of procedures tested.

**Figure legends**

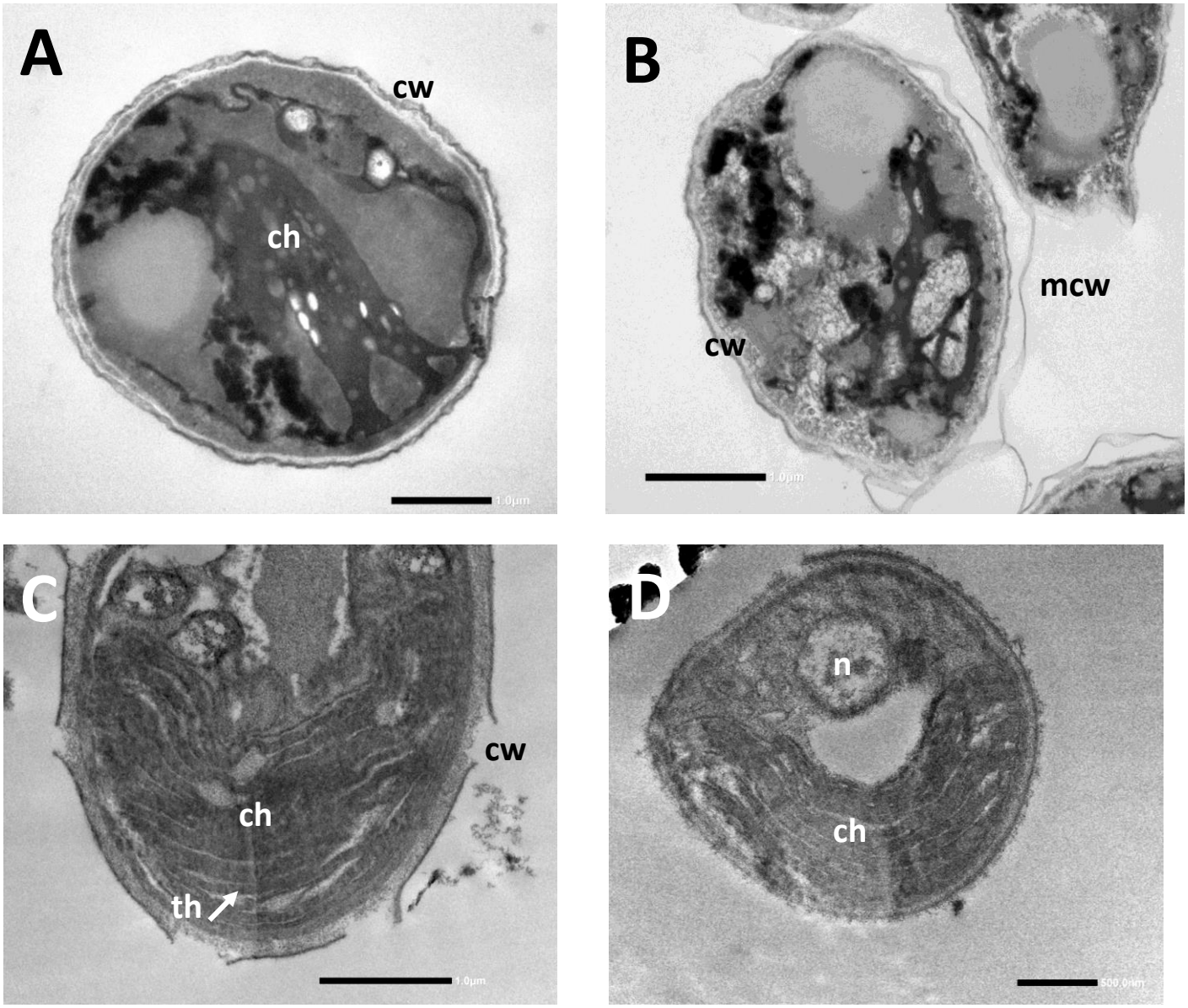
Fig. 1 - Transmission electron micrographs of *C. melkonianii* fresh cells fixed for 3h with 3% glutaraldehyde (A-B) or for 20 minutes with 5% $KMnO_4$. (C-D). Higher magnification of the chloroplast (C). Note the thylacoids and the disrupted cell wall (C). (ch) chloroplast, (cw) cell wall, (mcw) mother cell wall, (n) nucleus, (th) thylakoids. Scale bars = 1,0 μm (A-C); 500 nm (D).

Fig. 2 - TEM micrographs of fresh cells of *C. melkonianii* fixed for 5h with aldehyde mixture (see methods). The ultrastructure of the cells was well preserved: chloroplast ultrastructure was easy discernible (A-C), thylakoids were paired to form stacks (A-B); a number of electron-dense plastoglobuli were evident (arrowheads) (A-B). (C) Cell with cup-shaped chloroplast. Note the presence of distinct starch grain. Labels: (ch) chloroplast, (cw) cell wall, (ld) lipid droplets, (m) mitochondrion, (mcw) mother cell wall, (n) nucleus, (pg) plastoglobuli, (s) starch granules, (th) thylakoids. Scale bars = 1 μm.

Fig. 3 - Fresh cells of *C. melkonianii* were fixed with the aldehyde mixture (see methods) for 2h and then observed by TEM. (A) Vegetative cells with the presence of distinct starch grains. (B) Higher magnification of the chloroplast and the thylacoids (arrows). Note the presence of a number of electrondense plastoglobuli (arrowheads). Labels: (ch) chloroplast, (cw) cell wall, (pg) plastoglobuli, (s) starch granules, (th) thylakoids. Scale bars = 1 μm (A); 200nm (B).

Fig. 4 – TEM micrographs of thawed cells of *C. melkonianii* fixed with the aldehyde mixture for 2 h. (A) Cell with typical cup shaped chloroplast. Note a number of starch grains and the presence of the mother cell wall surrounding the cell. (B) Higher magnification of the cell wall and the thylacoids (arrows) in the chloroplast. (C) Higher magnification of newly formed autospores still enclosed within a mother cell wall. Note the electrondense plastoglobuli (arrowhaeds). Labels: (cf) cleavage furrow, (ch) chloroplast, (cw) cell wall, (mcw) mother cell wall, (pg) plastoglobuli, (s) starch granules, (th) thylakoids. Scale bars = 1 μm (A), 200nm (B), 500nm (C).

Fig. 5 - TEM electron micrographs of fresh cells *of C. melkonianii* fixed with the aldehyde mixture for 2 h after pretreatment with methyl alcohol for 20 minutes at -20°C. (A-B) Vegetative cell ultrastructure with typical cup shaped chloroplast. (A) Note the presence of a number of starch grains within the chloroplast. Labels: (ch) chloroplast, (cw) cell wall, (n) nucleus, (pg) plastoglobuli (arrowhead), (s) starch granules, (th) thylakoids (arrow). Scale bars = 1,0 μm.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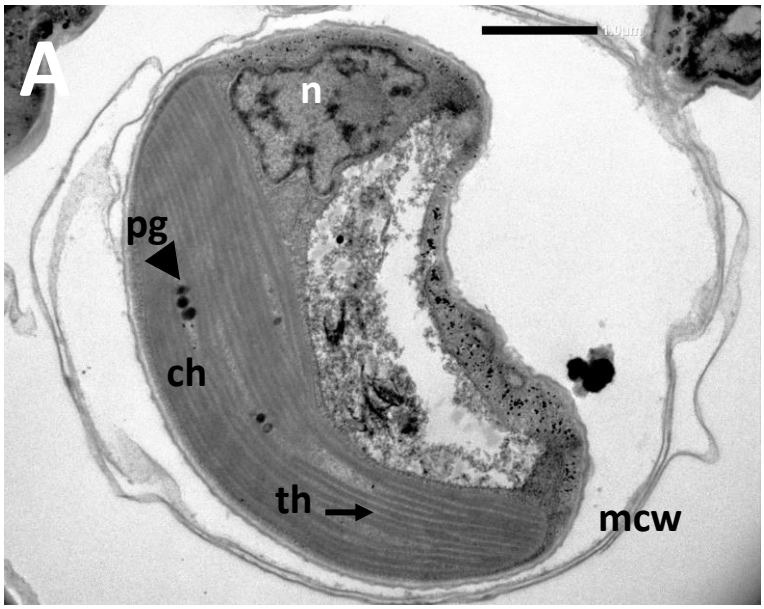
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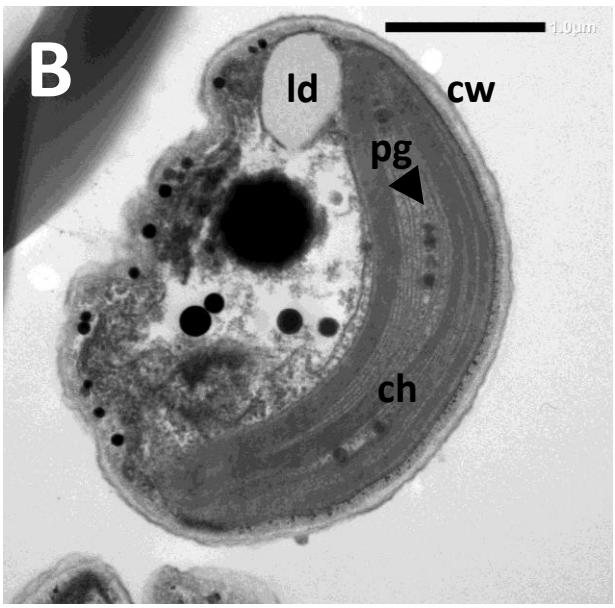
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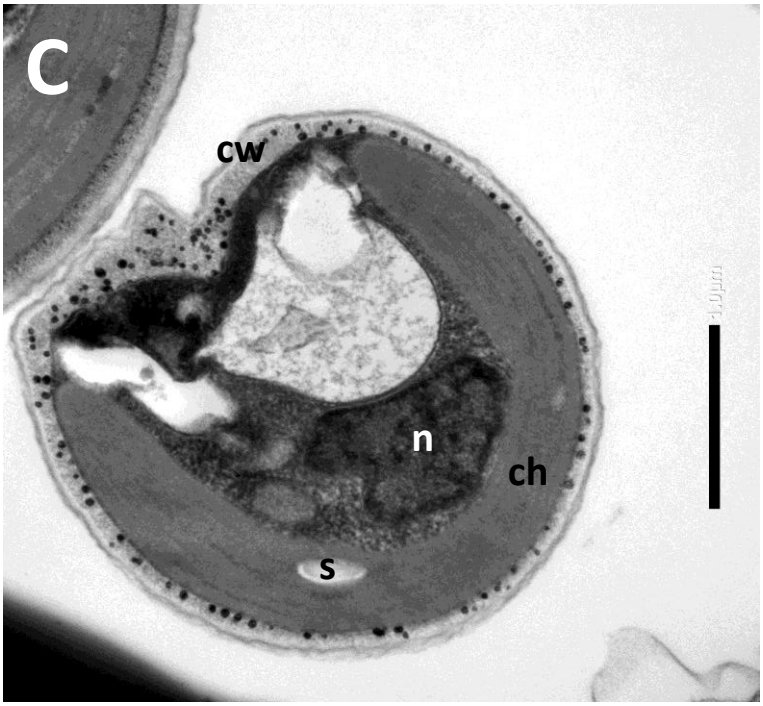
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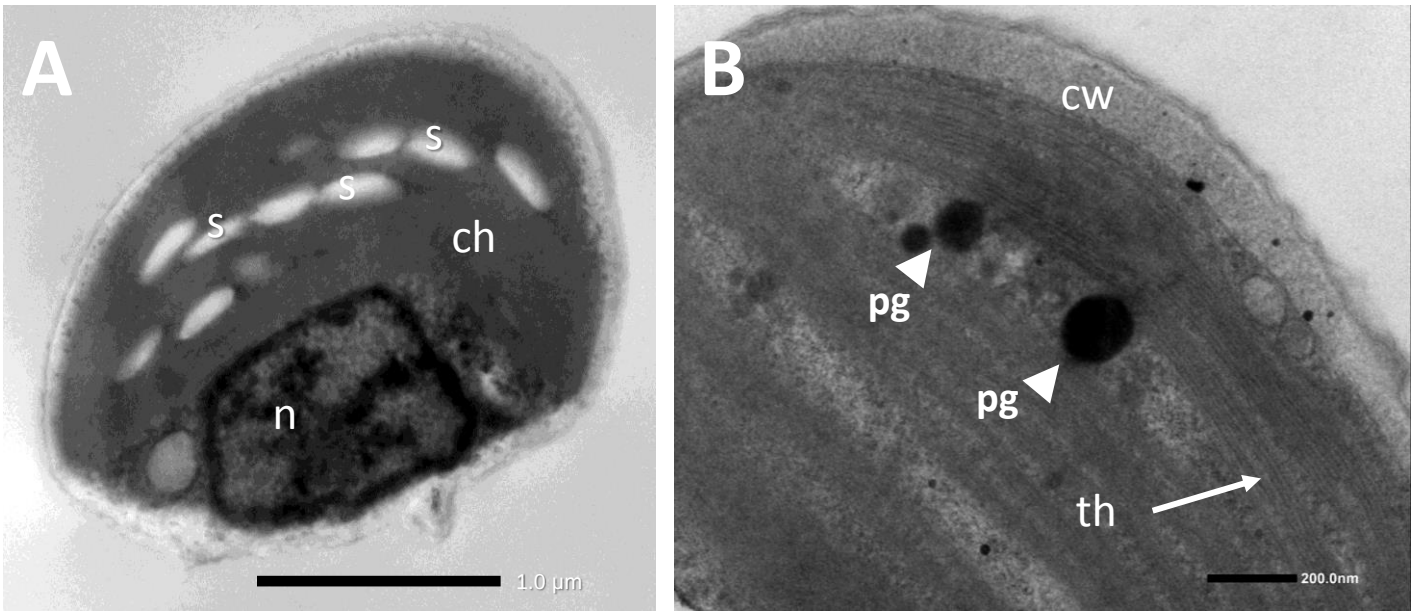
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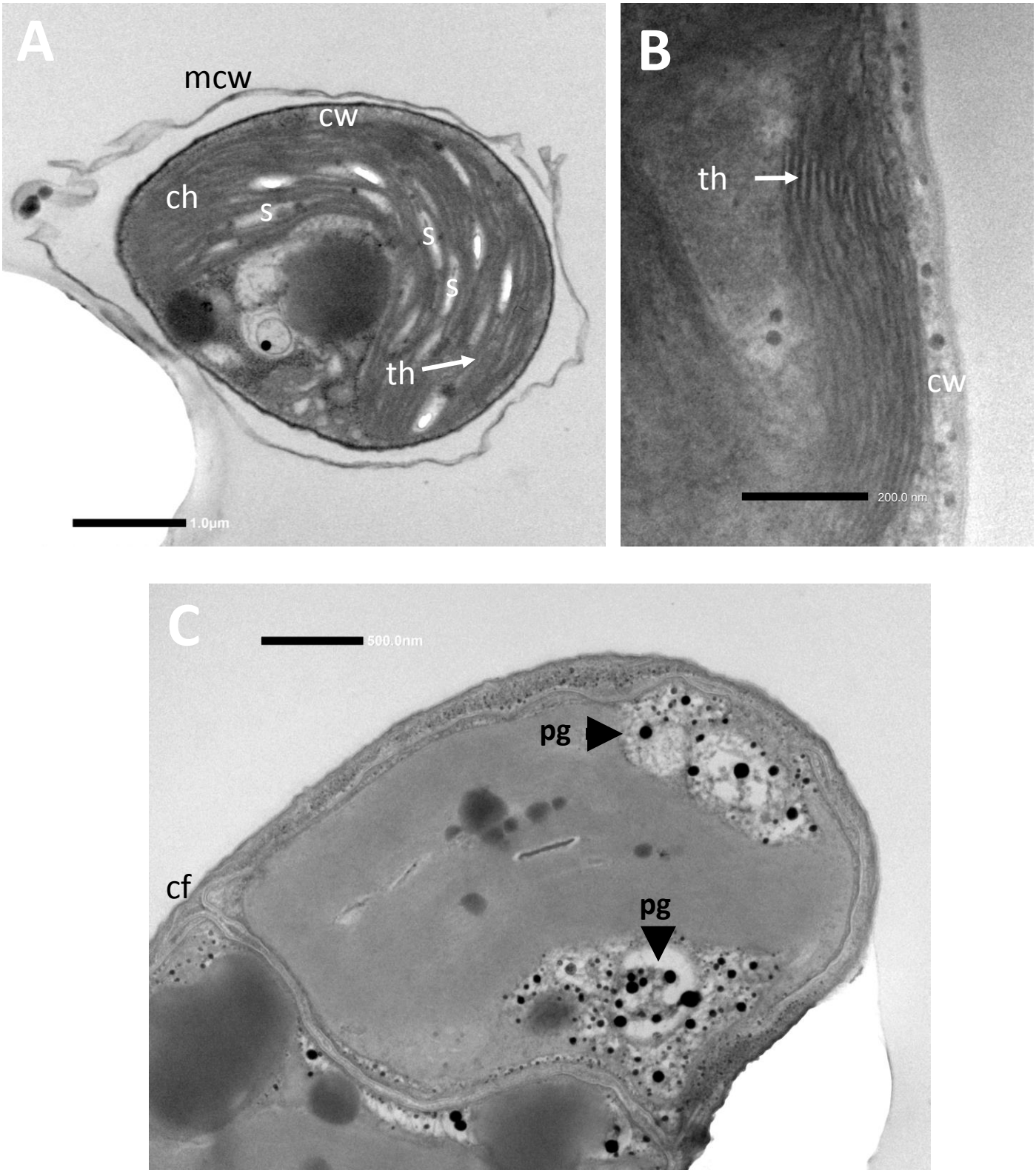
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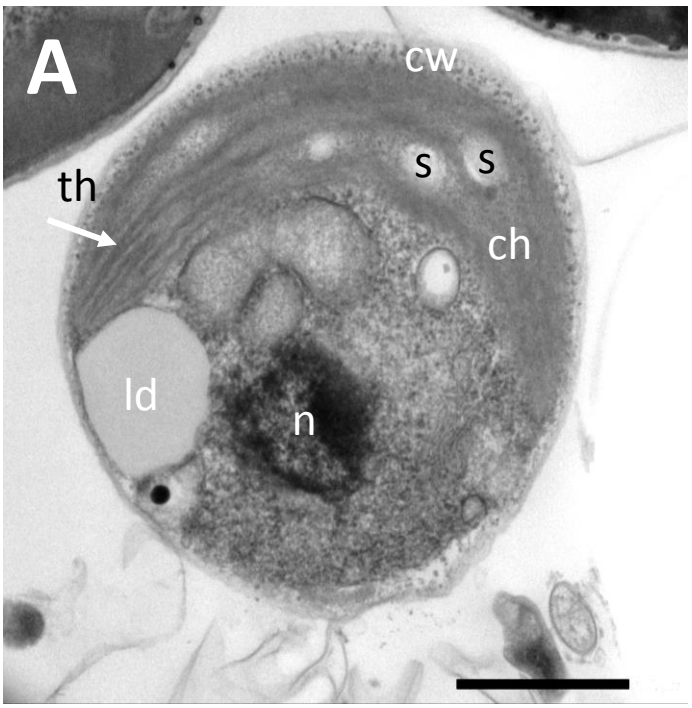
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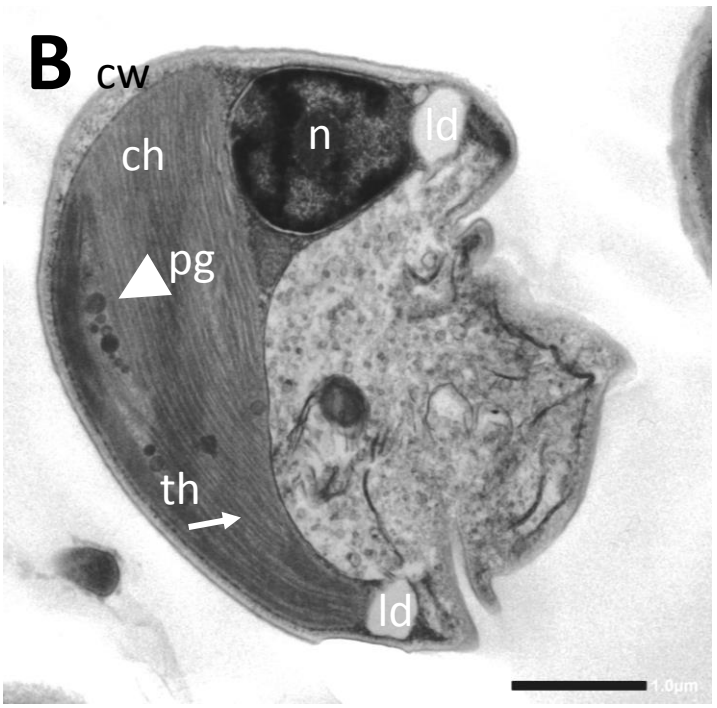
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