Studying the ultrastructure of the marine diatom *Seminavis robusta*.

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Abstract

Diatoms are a species-rich group of chromophytes which can be found both in marine and fresh-water environments. Diatoms contribute close to 20% of global primary production and close to 40% of marine primary production, and form the base of the trophic pyramid in many areas of the ocean. The most characteristic feature of diatoms is their ornate silica cell wall, called the frustule.

Seminavis robusta is a species of benthic, pennate diatom. *S. robusta* is especially well-suited for ultrastructural study because of its large size, up to 100µm, and ,being a benthic diatom adhering to surfaces, allowing the easy, non-intrusive observation of cellular behaviour and life cycle of living cells. They have two large vacuoles and phaeoplasts. The pyrenoids of *S. robusta* are elongated and run down the inside of the phaeoplasts. The large vacuoles store, among other things, neutral lipids. The nucleus is always located in the centre of the cell and is surrounded by Golgi apparatuses. ER can be found as lengths of double membrane in the cytosol.

In this thesis an attempt was made to design a method of preparation of *S. robusta* for TEM. During this method development the main focus was on fixatives for both primary and post fixation. There is still much research to be done in this field and future studies should focus on infiltration time during fixation, post fixation and embedding.

In addition to the work done with TEM two different fluorescent probes were tested on *S. robusta* in CLSM.

Preface

This thesis was written at the Department of Laboratory Medicine, Children's and Women's Health of the Norwegian University of Science and Technology (NTNU) and is the result of a 2-year master programme in molecular medicine.

The laboratory work for this thesis was done at the cellular and molecular biology lab at the Department of Biology as well as at the electron microscopy lab at the Department of Laboratory Medicine, Children's and Women's Health.

I would like to thank my supervisor, Atle Bones, IBI, for the opportunity to work on such an exciting thesis. I would also like to thank my assistant supervisor Per Winge, IBI, for showing great enthusiasm for and interest in my work, and for all the help he has given me the past year.

I would like to thank Linh Hoang, LBK, for teaching me to prepare samples for TEM and to use the ultramicrotome, and Nan Tostrup Skogaker, LBK, for teaching me to thin section and use the TEM. Thanks to Bjørnar Sporsheim, IBI, and Astrid Bjørkøy, IFY, for teaching me to use the CLSM. And finally another big thank you to Nan for providing me with all the good help, patience and advice this past year.

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1. Theory

1.1 Diatoms

Diatoms are a species-rich group of chromophytes which can be found both in marine and fresh-water environments, down to any depth to which photosynthetically available radiation can penetrate. Their most characteristic feature is their ornate silica cell wall, called the frustule (Falaciatore & Bowler, 2002). Diatoms contribute close to 20% of global primary production and close to 40% of marine primary production, and form the base of the trophic pyramid in many areas of the ocean. Diatoms are also important in the biogeochemical cycling of silica, owing to their requirement for this mineral for cell wall biogenesis (Chepurunov et al, 2008; Falaciatore & Bowler, 2002).

1.1.1 Characteristics

The frustule of diatoms consists of two almost equal halves, with the smaller fitting into the larger like a Petri dish. The larger of the halves is called the epitheca and the smaller is called the hypotheca. Each theca is generally composed of two parts; the valve, which forms the larger outer surface, and the girdle, which consists of circular bands of silica attached to the edge of the valve. The siliceous material of the frustule is deposited in highly regular patterns which are faithfully reproduced between the generations, implicating a strict genetic control of the process (Falaciatore & Bowler, 2002).

Diatoms are divided into two main groups based on the architecture of their frustules: “centric” diatoms and “pennate” diatoms. Centric diatoms are radially symmetrical with radially patterned valves, while pennate diatoms are elongated and bilaterally symmetrical with a feather-like valve structure (Chepurunov et al, 2008). Centric diatoms tend to be planktonic, while pennate diatoms are often benthic, living on sediments or other surfaces such as rocks or macroalgae (Falaciatore & Bowler, 2002).

The photosynthetic apparatus of diatoms are, similar to other photosynthetic eukaryotes, housed within plastids inside the cell. Diatoms are brown owing to the presence of the accessory pigment fuxoanthin, in addition to chlorophyll a and c, in their plastids. Therefore, the plastids of diatoms and other chromophytes are called phaeoplasts, in order to separate them from the rhodoplasts of red algae and chloroplasts of green algae. Centric diatoms generally have several small disc-shaped plastids, while pennate diatoms tend to have fewer, large, plastids (Falaciatore & Bowler, 2002).

1.1.2 Life Cycle

The Diatom life cycle has only been studied in detail in a minority of diatom species, but these have been found to represent many genera and almost all of the principal diatom

lineages. There are two principal stages in the diatom life cycle; a prolonged diploid vegetative stage, lasting months or years, and a sexual phase lasting hours or days, including the development of the zygote into a new vegetative cell (Chepurunov et al, 2008).

In the vegetative stage the diatoms multiply mitotically (Chepurunov et al, 2008). In each mitotic division each theca of the mother cell becomes the epitheca of one of the daughter cells. This leads to one of the daughter cells becoming smaller than the mother cell and, consequently, a decrease in the average size of the diatom population with repeated cell divisions (Chepurunov et al, 2008; Falaciatore & Bowler, 2002). Restoration of cell size occurs during sexual reproduction by the development of a specialized cell called an auxospore, which is larger than either parent. The induction of sex is size dependent; only cells of a particular, small, size become mating competent. During gametogenesis the old thecae are dropped and the zygote matures into an auxospore that is free to expand. After the auxospore has reached maximum size a new cell is formed inside the auxospore envelope. This new cell then begins a new round of vegetative multiplication (Chepurunov et al, 2008; Falaciatore & Bowler, 2002).

1.1.3 Movement

Planktonic diatoms are subjected to passive movements such as sinking and turbulence in the water. They do not have flagella and are therefore not able to move actively in their environment, though some species can control their buoyancy by making colonies that increase the surface to volume ratio and thus slows settling in the water column. The planktonic diatom *Ditylum brightwellii* can also move up and down in the water by changing the ionic composition of its large vacuole (Falaciatore & Bowler, 2002). However, some benthic diatoms are able to move much more freely on their growth surface (Falaciatore & Bowler, 2002). This movement is thought to be associated with the secretion of mucilage from the raphe, a long slit penetrating each valve of the frustule. This mucilage is believed to consist of acidic muco-polysaccharide (Edgar & Pickett-Heaps, 1982; Wang et al, 2013).

Conventional fixation and dehydration protocols used on diatoms known to have a substantial polysaccharide membrane enveloping the frustule, destroys the membrane (Edgar & Pickett-Heaps, 1982). Because of this, ruthenium red was used in the following experiment since this dye is known to stabilize and stain such polysaccharides (Fassel & Edmiston, 2000).

1.1.4 Frustule formation

The frustules of diatoms consist of both organic and siliceous components. In most diatoms the siliceous components are dominant, but in the first formed wall of the zygote and in some atypical diatoms, such as *Phaeodactylum tricornutum*, the inorganic components are dominant. In diatoms where the siliceous components are dominant it is believed that the organic components serve to maintain the integrity of the frustule while allowing cell

expansion through the sliding apart of the epitheca and the hypotheca (Mann & Marchant, 1989), as well as providing a scaffold for the construction of the siliceous components during frustule formation and maintenance of frustule function (Tesson & Hildebrand, 2013).

The silica components consists of two large plates, called the valves, and a number of smaller plates, called the girdle bands, which link the valves together (Mann & Marchant, 1989; Tesson & Hildebrand, 2013). The silica plates are formed inside the cells, in membrane-bound silica deposition vesicles (SDVs), which are associated with the plasma membrane throughout silification of the frustule. After formation of the silica structures in the SDV is complete the entire structure is exocytosed to contribute to the new wall (Mann & Marchant, 1989; Tesson & Hildebrand, 2013).

The SDVs of most of the siliceous components of the frustule expand as silification proceeds. The valve SDVs begin as small sacs and grow laterally beneath the plasma membrane. As they grow, systems of ribs form within them. The valve SDV and the rib system within expand until they reach the maximum size, limited by the parental thecae. At this point any further development of the valve happens at right angles to the original growth. This produces three-dimensional structures such as tubes, spines or ridges (Mann & Marchant, 1989).

The main source of silica to marine diatoms is silicic acid ($Si(OH)_4$), which is transported into the diatom via silica transporters located in the plasma membrane (Falaciatore & Bowler, 2002).

1.1.5 *Seminavis robusta*

Seminavis robusta is a species of diatoms especially well-suited for ultrastructural study because of its large size, up to 100µm, and ,being a benthic diatom adhering to surfaces, allowing the easy, non-intrusive observation of cellular behaviour and life cycle of living cells. Although the cells move and grow on surfaces they are only loosely attached and can easily be suspended if required. (Chepurunov et al, 2008; Gilard et al, 2008).

S. robusta is a benthic, pennate diatom which has only two large phaeoplasts (Vanstechelman et al, 2013). The movement of *S. robusta* is associated with mucilage secreted through the raphe (Wang et al, 2013).

The life cycle of *S. robusta.* has been proven to follow the typical patterns for diatoms: there is a size reduction and restitution cycle where restoration of size takes place via sexual auxosporulation, and the potential for sexual reproduction is size dependent. During auxosporulation two gametangia form two gametes each, which then fuse to produce two auxospores. The auxospores develop trough bipolar expansion, accompanied by the deposition of "auxospore-specific" siliceous elements, and are finally transformed into the enlarged cell of the next generation. The life cycle is short and can be completed in a few

weeks, if cells are kept growing in optimal conditions, and the cell cycle is correspondingly short, lasting about 0,5 days (Chepurunov et al, 2008).

1.2 Transmission Electron Microscopy

Transmission electron microscopy (TEM) is a microscopy technique where a beam of electrons is transmitted through an ultra-thin section and an image is formed from the electrons transmitted through the section. The image is magnified and focused by an objective lens and appears on an imaging screen, a fluorescent screen in most TEMs, and a monitor, or on a layer of photographic film, or to be detected by a sensor such as a CCD camera (Bozzola & Russell, 1999).

A TEM has magnification and resolution abilities that are over a thousand times better than that of a conventional light microscope. It is equipped with a set of electromagnetic lenses used to control a beam of imaging electrons in order to visualize extremely fine structural details in the ultrastructure of cells. The illuminating electrons pass through the specimens and the image is therefore referred to as a transmitted image. The modern TEM can achieve magnifications of one million with resolutions of 0,1 nm. Magnification is defined as a measure of the increase in diameter of a structure, while resolution is defined as the ability to discriminate two closely parted structures and to see more details in the objects viewed (Bozzola & Russell, 1999).

Tissue preparation for TEM can be divided into 4 steps; fixation, dehydration, infiltration and embedding. The process begins with living tissue and ends with water-free tissue preserved in a static state in a plastic resin (Bozzola & Russell, 1999).

1.2.1 Fixation

The purpose of fixation is to preserve the tissue with as little alteration as possible from the living state, as well as protecting the tissue from disruption during embedding and sectioning. No fixative can do this completely, so a fixation protocol should be selected for its ability to preserve the specific ultrastructural feature of interest. Most laboratories today use a fixation protocol where primary fixation with glutaraldehyde is followed by secondary exposure to osmium tetroxide. Most specialized fixation techniques are modifications of the glutaraldehyde-osmium protocol (Bozzola & Russell, 1999).

Glutaraldehyde is a five-carbon compound containing terminal aldehyde groups. Its attribute as a fixative is in its ability to cross-link protein through a reaction between glutaraldehyde's terminal aldehyde groups and ϵ-amino groups of lysine in adjacent proteins. Glutaraldehyde will also, to some degree, react to lipids, carbohydrates and nucleic acids. The rate of penetration of glutaraldehyde into tissue is very slow. This rate is dependent on the nature of the tissue; glutaraldehyde penetrates compact tissue with many membrane layers slower than it does tissue with large fluid spaces. A general rule of thumb to obtain rapid

immersion fixation in glutaraldehyde is that the immersed tissue should be in pieces under 1mm in thickness (Bozzola & Russell, 1999).

The use of formaldehyde with glutaraldehyde during fixation achieves a faster overall penetration of fixative than with only glutaraldehyde. Formaldehyde penetrates about five times faster than glutaraldehyde and it is theorized that formaldehyde temporarily stabilizes structures that are later fixated permanently by glutaraldehyde. Formaldehyde is a gas at room temperature, but when mixing fixatives one can use powdered, polymerized formaldehyde, or paraformaldehyde (Bozzola & Russell, 1999). Studies indicate that formaldehyde reacts with various functional groups of biological macromolecules, such as proteins, nucleic acids and polysaccharides by cross-linking them. The most reactive sites are primary amines, such as lysine, and thiols, such as cysteine, and then cross-linking them to less reactive groups such as primary amides, like glutamine and asparagine, guanidine groups, like arginine, and tyrosine ring carbons (Fox et al, 1985)

Osmium tetroxide (OsO_4) is a symmetrical molecule which contains four double-bonded oxygen molecules. It works as a secondary fixative by reacting primarily with lipid moieties. It is believed that when it does so it becomes reduced and this reduced version adds density and contrast to the tissue. The molecular weight of osmium tetroxide is high enough to be effective in scattering electrons, which makes it an important stain. It is also capable of combining with other stains or dyes at a later time, a trait which enhances the effect of lead staining for TEM. Tissues stained with osmium tetroxide look black when viewed with the naked eye. The penetration rate of osmium tetroxide is often slower than that of glutaraldehyde, and osmium tetroxide will generally not penetrate pieces of compact tissue thicker than 0,5mm in one hour. Too short exposure to osmium tetroxide results in under fixation, leaving specimen pieces with a core of unblackened tissue that is virtually useless for electron microscopy. Prolonged exposure results in loss of tissue components, especially later, during dehydration (Bozzola & Russell, 1999). Post-fixating samples with potassium ferrocyanide ($K_4Fe(CN)_6$) as well as OsO_4 enhances the contrast of membrane structures in the cell (Neiss, 1984).

Exopolysaccharide elements are generally too electron-translucent to be distinguished from the epoxy resin background in TEM images. The use of cationic reagents, such as ruthenium red, provides added stability to polysaccharide membranes, which are not well stabilized by the aldehydes or osmium tetroxide and are especially vulnerable to damage during the dehydration step. The addition of lysine to ruthenium red and glutaraldehyde increases the effectiveness of this fixative noticeably (Fassel & Edmiston, 2000).

Tannic acid is a polyphenolic compound which stains and prevents the extraction of biological membranes during the fixation and embedding procedures for TEM (Pietro & Merchan, 1986). The tannic acid in oolong tea enhances the contrast of various intra- and extracellular structures, including membranes. It has been suggested that the tannic acid enhances the effect of OsO_4, as well as the effect of lead citrate on osmicated tissues (Kalina & Pease, 1977).

1.2.2 Dehydration

Dehydration is the process of replacing the water in cells with a fluid that acts as a solvent between the environment of the cell and the embedding media, the cellular environment being highly aqueous and the embedding media being hydrophobic. Common agents used for dehydration are ethanol and acetone. The general protocol for dehydration is to gradually replace the water in the tissue by using a graded series of dehydration agents (Bozzola & Russell, 1999).

1.2.3 Infiltration and embedding

Infiltration is the process by which the dehydrating agents are gradually replaced by epoxy resin. It is usually necessary to first replace the dehydrating agents with another intermediary solvent which mixes easily with the plastic embedding medium. The standard solvent used is propylene oxide. Usually more than one change of propylene oxide is necessary to completely replace the dehydrating agent. Propylene oxide is then mixed with the epoxy and placed into vials with the tissue. The epoxy-solvent ratio is gradually increased until pure epoxy is used. The tissues infiltrated with pure epoxy resin are transferred into moulds or capsules containing resin and are finally placed into an oven where the epoxy components polymerize to form a solid block (Bozzola & Russell, 1999).

1.2.4 Ultamicrotomy

Ultramicrotomy is a procedure where the embedded tissue is cut into extremely thin sections for viewing in the TEM. The sections must be very thin because the electrons of the standard TEM cannot pass through biological material much thicker than 150nm. For the best resolution the sections should be between 30nm and 60nm thick. So called “thin” sections are 50-100nm thick, and are suitable for viewing in the TEM. “Semi”, “thick” or “blue” sections are 0,5-2 µm thick, and are viewed in the light microscope to find the right area for thin sectioning. For it to be possible to cut thin sections from biological tissue it is important that it is properly embedded (Bozzola & Russell, 1999).

An Ultramicrotome is a specialized instrument used for cutting sections. Ultramicrotomes advance the tissue blocks in precise, repeatable steps by using either a mechanical or thermal advancement mechanism. After the sections are cut and are floating on the surface of water contained in a trough attached to the knife they are picked up on a copper screen mesh, or grid, and stained using salts of heavy metals, such as lead and uranium, prior to viewing in the TEM (Bozzola & Russell, 1999).

It is necessary to shape the tissue block into a small cutting surface in order to minimize the stress on the edge of the knife during ultramicrotomy. This is usually done in two stages; the block is first roughly trimmed to remove excess plastic and to expose the surface of the tissue. After locating the area of tissue on the block face fine trimming is done

to remove unwanted areas next to the tissue. The final goal of the trimming is to produce a truncated pyramid with its sides sloped at about 45-60°. The pyramidal shape is used because it is a more stable than, for example, one with non-sloped sides (Bozzola & Russell, 1999).

Most microtomists use glass knives to make semi sections from the block, in order to get an overview of the embedded tissue, make the right orientation and find the right area for TEM. The sections are collected from the water-filled trough of the knife, using a hair-loop probe. The sections are then transferred to a droplet of distilled water on a microscope slide. The slide is then transferred to a hot plate to dry, and the sections are finally stained using a toluidine blue solution, before light microscopy is performed (Bozzola & Russell, 1999).

After the semi sections have been examined in the light microscope and the area of interest has been found, it is often necessary to trim the block more to exclude areas that are not of interest. After trimming the thin sections are cut in the same way as the semi sections, but for thin sections most microtomists use diamond knives, since these are generally sharper than glass knives and the knife edge does not dull as quickly. Another important difference is that after thin sectioning the sections are not collected with an hair loop probe and put onto microscope slides, but are collected directly on specimen grids, which are the electron microscope analogue of the microscope slide (Bozzola & Russell, 1999).

1.2.5 Staining

In the light microscope the light that illuminates the specimen consists of a spectrum of colours that become visible when the light strikes a specimen that has been stained with a dye. Colours do not exist in electron microscopy, however, since the illumination source is of a single wavelength determined by the accelerating voltage. Since the wavelength is outside the spectre visible to human eyes it is necessary to examine TEM images on viewing screens coated with phosphorescent materials. The phosphors convert the energy of the invisible short wavelength electrons into a visible longer wavelength. The images generated on such screens consist of various levels of brightness, ranging from very bright to completely dark. The darker areas are associated with areas of greater density in the tissue, while brighter areas of the tissue have less density. This is because denser areas of the tissue deflect electrons so that they are eliminated from the image. It is important to increase the image contrast of most biological specimens by reacting selective cellular components with heavy metals (Bozzola & Russell, 1999).

As mentioned earlier; osmium tetroxide works as a stain for TEM samples by reacting with many organic molecules of the tissue and becoming reduced to metallic osmium. Although there is an improvement in image contrast with osmium, only rarely will it be enough to get a good image. In most cases it is necessary to increase the contrast by using other heavy metals. Salts of heavy metals, such as uranyl acetate and lead citrate, increase the contrast of osmicated tissues further when the metal ions in the salt react with various cellular components (Bozzola & Russell, 1999).

One of the most common methods for staining thin sections is with solutions of uranyl acetate. Usually staining is done by floating the grids on droplets of uranyl acetate on a dental wax or Parafilm surface. After staining the grids are removed from the droplet using fine tipped tweezers and rinsed with distilled water. It is important to avoid carryover of the various staining reagents from one staining solution to another (Bozzola & Russell, 1999).

After the sections have been contrasted by uranyl acetate it is common to add a second stain, usually lead citrate. Unfortunately lead stains are easily precipitated upon contact with CO_2. Staining in lead citrate is done in much the same way as staining in uranyl acetate. The grid with the section is first floated on a droplet of distilled water, then it is removed using fine tipped tweezers and put to float on a drop of lead citrate solution. After staining the grid is again rinsed with distilled water. After the final rinse the grid is allowed to dry before examination in the transmission microscope (Bozzola & Russell, 1999).

1.3 Confocal laser microscopy

Confocal laser microscopy (CLSM) is a microscopy technique where a laser beam passes through a light source aperture and is focused by an objective lens into a small, preferably diffraction limited, focal point inside or on the surface of a specimen. Fractured and reflected light as well as fluorescent light from the illuminated point is then collected by the objective lens. A filter selectively passes through light with the fluorescence wavelength and blocks the original excitation wavelength. The light passes through a pinhole and is detected by a device which transforms the light signal into an electrical one that is recorded by a computer. The detected light from an illuminated point inside or on the specimen represents one pixel in the resulting image. As the laser scans over the plane of interest a whole image is obtained pixel by pixel (Claxton et al, 2006).

With a CLSM it is possible, among other things, to view thin sections out of thick specimens, view specimens in planes tilted to and running at right angles to the line of sight, penetrate deep into light scattering tissues and create three-dimensional images of a specimen (Inoué, 2006).

CLSM relies on fluorescence for imaging. Many fluorescent probes have been produced to bind to certain structures or molecules in order to monitor the localisation of these molecules inside the specimen, at specific times or conditions (Claxton et al, 2006).

BODIPY is the trade name for a group of photostable, neutrally charged, boron containing fluorescent probes that span much of the visible spectrum (Hardin, 2006). BODIPY probes are non-polar and relatively insoluble in water (Johnson, 2006). BODIPY 505/515 stains neutral lipids, such as fatty acids, phospholipids, cholesterol, cholesteryl, esters and ceramides, and has previously been used to monitor oil storage in living algal cells (Govender et al, 2012; Xu et al, 2013). BODIPY 505/515 has been found to penetrate all structures within a cell and the characteristic green fluorescence occurred when the probe is in

either non-polar or polar environments, primarily staining neutral storage lipid droplets in microalgal cells (Cooper et al, 2010).

Aniline blue is a water soluble probe that binds to the β 1,3-glucan callose (Scherp et al, 2001). Callose has been found to be associated with the frustule in diatoms (Waterkeyn & Bienfait, 1969).

Plant tissue generally contains a large number of light-absorbing pigments, many of which have autofluorescence. Since the plant tissue contains many different autofluorescent pigments, each with a different absorption and emission spectrum, the resulting fluorescence spectra will vary depending on the excitation wavelength used (Cheng, 2006).

2. Materials and Methods

2.1 Cultures

Seminavis robusta cultures were grown in culture flasks, 50mL or 125mL, in f/2 medium, at room temperature with days of 16h light and 8h darkness. The growth medium was changed every 3-4 days.

f/2 medium

In filtered and autoclaved sea water;

8.82×10^{-4} M $NaNO_3$

3.62×10^{-5} M NaH_2PO_4 H_2O

1.06×10^{-4} M Na_2SiO_3 $9H_2O$

Trace metals:

1.17×10^{-5} M $FeCl_3$ $6H_2O$

1.17×10^{-5} M Na_2EDTA $2H_2O$

3.93×10^{-8} M $CuSO_4$ $5H_2O$

2.60×10^{-8} M Na_2MoO_4 $2H_2O$

7.65×10^{-8} M ZnSO4 $7H_2O$

4.20×10^{-8} M $CoCl_2$ $6H_2O$

9.10×10^{-7} M $MnCl_2$ $4H_2O$

Vitamins:

2.96×10^{-7} M Thiamine HCl (vit. B_1)

2.05×10^{-9} M Biotin (vit. H)

3.69×10^{-10} M Cyanocobalamin (vit. B_{12})

2.2 TEM

All TEM images were taken using a JEOL JEM 1011 transmission electron microscope.

2.2.1 Method development

The methods tested in this section were based on the standard preparation methods for TEM from the electron microscopy lab of the Department of Laboratory Medicine, Children's and Women's Health at the Faculty of Medicine, NTNU (See Appendix B).

Samples

One 50mL culture flask of *Seminavis robusta* was used as sample for each of the following experiments. The samples were fixated in the flask before being scraped and transferred to a centrifuge tube.

Fixation

Method AAS1

The sample was pre-fixated in a solution of 1,37% lysine, 0,075% ruthenium red (RR) and 2,5% glutaraldehyde (GA) in HEPES buffer (0,1M), for 20min. After pre-fixation the sample was fixated for 2h in 0,075% RR and 2,5% GA in HEPES buffer (0,1M).

After fixation the sample rinsed 3x10min in HEPES buffer (0,1M) before being transferred to a centrifuge tube. The sample was then centrifuged for 10min at 1500rcf and the buffer removed. The remaining pellet was mixed out into a solution of 5% gelatine in phosphate buffer (0,1M) and the tube was put in an incubation oven at 40°C for 20min. After this time the sample was again centrifuged for 10min at 1500rcf and most of the supernatant removed. The cell pellet and the surrounding gelatine were then left to set at 4°C. After the gelatine had set, 2% GA in phosphate buffer (0,1M) was added to fixate the gelatine.

Once the gelatine was fixated the pellet was rinsed 2x10min with phosphate buffer (0,1M) and all excess gelatine was cut from around the cells. The cell pellet was then cut into smaller pieces and post-fixated in 1% osmium tetroxide (OsO_4) in phosphate buffer (0,1M) for 1 hour.

After post-fixation the pieces were rinsed 3x10min in phosphate buffer.

Method AAS2

The sample was pre-fixed in a solution of 1,37% lysine, 0,075% ruthenium red (RR) and 2,5% glutaraldehyde (GA) in f/2 medium, for 20min. After pre-fixation the cells were fixated for 2h in 0,075% RR and 2,5% GA in f/2.

After fixation the sample was rinsed 3x10min in f/2 before being transferred to a centrifuge tube. The sample was then centrifuged for 10min at 1500rcf and the supernatant removed. The remaining pellet was mixed out into a solution of 5% gelatine in f/2 and the tube was put in an incubation oven at 40°C for 20min. After this time the sample was again centrifuged for 10min at 1500rcf and most of the supernatant removed. The cell pellet and the surrounding gelatine were then left to set at 4°C. After the gelatine had set, 2% GA in f/2 was added to fixate the gelatine.

Once the gelatine was fixated the pellet was rinsed 2x10min with phosphate buffer (0,1M) and all excess gelatine was cut from around the cells. The cell pellet was then cut into smaller pieces and post fixated in 2% OsO_4 in phosphate buffer (0,1M) for 1 hour.

After post-fixation the pieces were rinsed 2x5min in phosphate buffer.

The sample was then divided into two separate containers marked AAS2S and AAS2H.

Method AAS3

The sample was fixated for 2 hours in 2,5% GA in f/2.

After fixation the sample was rinsed 3x10min in f/2 before being transferred to a centrifuge tube. The sample was then centrifuged for 10min at 1500rcf and the supernatant removed. The remaining pellet was mixed out into a solution of 5% gelatine in f/2 and the tube was put in an incubation oven at 40°C for 20min. After this time the sample was again centrifuged for 10min at 1500rcf and most of the supernatant removed. The cell pellet and the surrounding gelatine was then left to set at 4°C. After the gelatine had set, 2% GA in f/2 was added to fixate the gelatine.

The sample was rinsed and post-fixated in the same way as AAS2.

The sample was then divided into two separate containers marked AAS3S and AAS3H.

Method AAS4

The sample was pre-fixated in a solution of 1,37% lysine, 0,075% ruthenium red (RR) and 2,5% glutaraldehyde (GA) in f/2 medium, for 20min. After pre-fixation the cells were fixated for 2 hours in 0,075% RR and 2,5% GA in f/2.

After fixation the sample was rinsed 3x10min in f/2 before being transferred to a centrifuge tube. The sample was then left until the cells had precipitated to the bottom of the tube, then most of the f/2 was removed. The remaining sample was mixed with an equal amount of 10% gelatine in f/2 and the tube was put in an incubation oven at 40°C for 1h30min. After this time most of the gelatine was removed, and the cells that had gathered at the bottom of the tube

and the surrounding gelatine was then left to set at 4°C. After the gelatine had set, 2% GA in phosphate buffer (0,1M) was added to fixate the gelatine.

The sample was rinsed and post-fixated in the same way as AAS2.

Method AAS5

The sample was fixated for 2 hours in 0,075% RR, 2% GA and 4% paraformaldehyde (PAF) in f/2.

After fixation the sample was rinsed 3x10min in f/2 before being transferred to a centrifuge tube. The sample was then left until the cells had precipitated to the bottom of the tube, then most of the f/2 was removed. The remaining sample was mixed with an equal amount of 10% gelatine in f/2 and the tube was put in an incubation oven at 40°C for 1h30min. After this time most of the gelatine was removed, and the cells that had gathered at the bottom of the tube and the surrounding gelatine was then left to set at 4°C. After the gelatine had set, 2% GA in phosphate buffer (0,1M) was added to fixate the gelatine.

The sample was rinsed and post-fixated in the same way as AAS2.

Method AAS6

The sample was fixated for 2 hours in 1% GA and 1% PAF in f/2.

The same method of fixation, rinsing and post-fixation was used for this sample as in AAS5

Method AAS7

The sample was fixated for 2 hours in 2% GA and 4% PAF in f/2.

The same method of fixation, rinsing and post-fixation was used for this sample as in AAS5

Method AAS8

The sample was fixated for 2 hours in 0,075% RR , 2% GA and 4% PAF in f/2.

The same method of fixation and rinsing was used for this sample as in AAS2

Once the gelatine was fixated the pellet was rinsed 2x10min with phosphate buffer (0,1M) and all excess gelatine was cut from around the cells. The cell pellet was then cut into smaller pieces and post-fixated in 1,5% Potassium ferrocyanide and 2% OsO_4 in phosphate buffer (0,1M) for 2 hours.

After post-fixation the pieces were rinsed 2x5min in phosphate buffer.

Method AAS9

The sample was fixated for 2 hours in 0,075% RR , 2% GA and 4% PAF in f/2.

The same method of fixation and rinsing was used for this sample as in AAS2

Once the gelatine was fixated the pellet was rinsed 2x10min with phosphate buffer (0,1M) and all excess gelatine was cut from around the cells. The pieces were then post-fixated in a solution of 1,6% oolong tea in phosphate buffer (0,1M) for 21 hours. After this time the sample was rinsed 2x15min in imidazole buffer (0,2M) and post-fixated in 2% OsO_4 in imidazole buffer (0,1M) for 1 hour.

After post fixation the pieces were rinsed 2x5min in imidazole buffer and then transferred to phosphate buffer (0,1M) before dehydration.

Method AAS10

The sample was fixated in 0,075% RR , 2% GA and 4% PAF in f/2 for 2 hours at 5°C.

After fixation the sample was rinsed 3x10min in f/2 before being transferred to a centrifuge tube. The sample was then centrifuged for 10min at 1500rcf and the supernatant removed. The remaining pellet was mixed out into a solution of 5% gelatine in f/2 and the tube was put in an incubation oven at 40°C for 25min. After this time the sample was again centrifuged for 10min at 1500rcf and most of the supernatant removed. The cell pellet and the surrounding gelatine was then left to set at 4°C. After the gelatine had set, 2% GA in phosphate buffer (0,1M) was added to fixate the gelatine.

The same method of post-fixation and rinsing was used for this sample as in AAS8.

Dehydration.

After post-fixation all of the samples were dehydrated in a gradient of ethanol.

50%, 70%, and 90% for 15min followed by absolute ethanol: 1x15min, 2x30min + 2x15min from an unopened bottle.

Epoxy resin

The epoxy resins used in these experiments were based on Bozzola's epoxy resin (Bozzola & Russell, 1999)

"Soft" epoxy

LX112	153,4g
DDSA	81,1g
NMA	65,5g

"Hard" epoxy

LX112	53,33g
DDSA	20,0g
NMA	33,33g

0,15mL DMP-30 was added to every 10mL epoxy resin prior to use.

Infiltration and embedding

After dehydration the samples were immersed in propylenoxide: 4x15min. Followed by mixtures of propylenoxide and epoxy: 2:1, 1:1, 1:2 for 1h.

After that the samples were placed in pure epoxy overnight.

The nest day the samples were transferred to block moulds filled with new epoxy, and cured at 60°C.

AAS1 was embedded in "soft" epoxy resin and cured for 3 days.

AAS2S and AAS3S were embedded in "soft" epoxy resin and cured for 5 days.

AAS2H and AAS3H were embedded in "hard" epoxy resin and cured for 5 days.

AAS4, AAS5, AAS6, AAS7, AAS8, AAS9 and AAS10 were embedded in "hard" epoxy resin and cured for 6 days.

Sectioning

200 mesh copper grids with formvar film were used during thin sectioning of all the samples.

Staining

All grids, except those from AAS9, were stained with uranyl acetate for 25-30min and rinsed in RO-water before being stained for 5min with lead citrate.

The grids from AAS9 were only stained with lead citrate.

2.2.2 Silica starvation

Cell proliferation

Seminavis robusta was reseeded from one 50mL culture flask into two 125mL culture bottles, one control flask containing regular f/2, and one flask containing f/2 without added Na_2SiO_3. 1,5mL samples were taken from both bottles immediately after reseeding and again every day from day 3 after reseeding. The number of cells in these samples was counted and the result used to find the concentration of *S. robusta* in the flasks and find growth curves.

Based on the results from this experiment another two bottles were reseeded from another 50mL culture flask and the concentration of cells was calculated for every day from right after reseeding and until four days after reseeding.

TEM

Seminavis robusta was grown in a 125mL culture flask in f/2. At the start of this experiment the f/2 was exchanged for f/2 without added Na_2SiO_3. A 5mL sample was taken from the flask just after the change of the growth medium, three days, four days and five days after the change.

The samples were labelled AASi0, AASi3, AASi4 and AASi5, respectively, and prepared for TEM using method AAS8 from section 2.2.1.

One mistake was made during the post-fixation of AASi0, where the sample was left in the potassium ferrocyanide and OsO_4 solution for 2h50min instead of 2h.

2.2.3 *Thalassiosira pseudonana*

Samples of *Thalassiosira pseudonana* were provided by Matilde Skogen Chauton, department of Biotechnology, NTNU. Cells were prepared for TEM using method AAS8 from section 2.2.1 to check if the method would work on diatom species other than *Seminavis robusta*.

2.3CLSM

All CLSM images were taken with a Leica TCS SP5 confocal microscope.

2.3.1 BODIPY 505/515

A sample of *Seminavis robusta* was incubated in 3µM BODIPY 505/515 for 10 minutes and viewed in the CLSM. The excitation wavelength used was 488nm and the emission wavelengths scanned for were 500-550nm for the probe and 655-710nm for the auto fluorescence of the phaeoplasts.

2.3.2 Aniline Blue

A sample of *Seminavis robusta* in f/2 was mixed 1:1 with a solution of 0,1% aniline blue in distilled water and viewed in the CLSM. The excitation wavelength used was 514nm and the emission wavelengths scanned for were 530-610nm for the probe and 655-710nm for the auto fluorescence of the phaeoplasts.

3. Results

3.1 TEM

3.1.1 Method development

AAS1

The methods tested in this section were based on the standard preparation methods for TEM from the electron microscopy lab of the Department of Laboratory Medicine, Children's and Women's Health at the Faculty of Medicine. The dehydration and infiltration times were lengthened because it was known at the lab, based on earlier experiments, that infiltration takes longer into diatoms than it does into cells that do not have a silica cell wall. Ruthenium red was used to preserve the polysaccharide membrane enveloping the frustule.

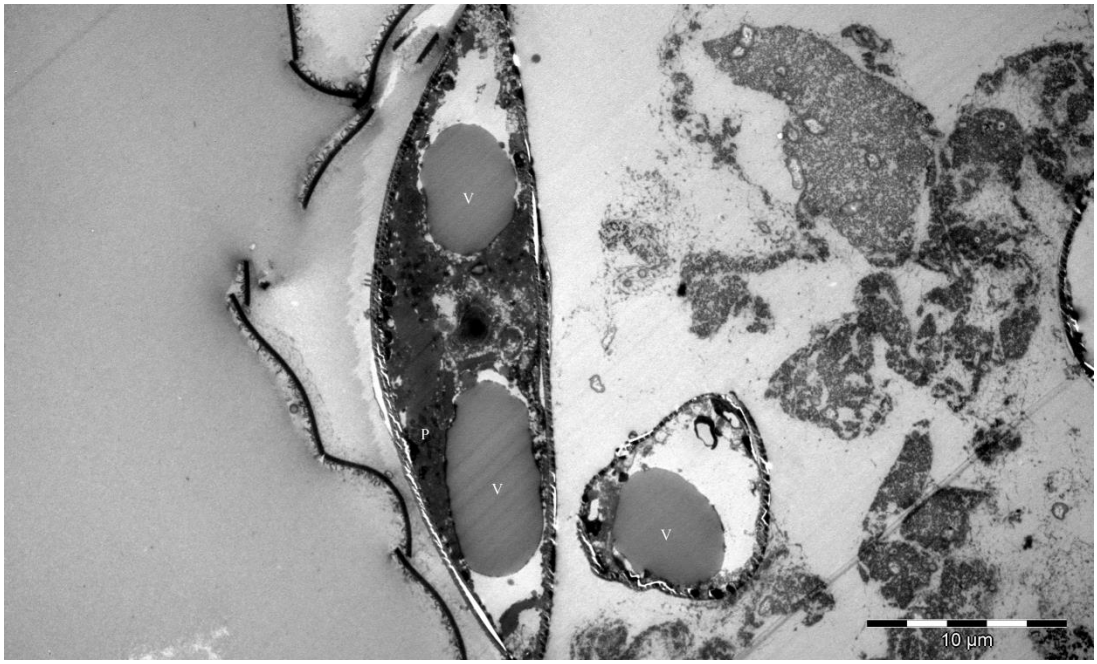


Figure 1: Longitudinal section and a cross section of *Seminavis robusta* cells prepared for TEM with the AAS1 method, with the vacuoles (V) and a phaeoplast (P) labelled.

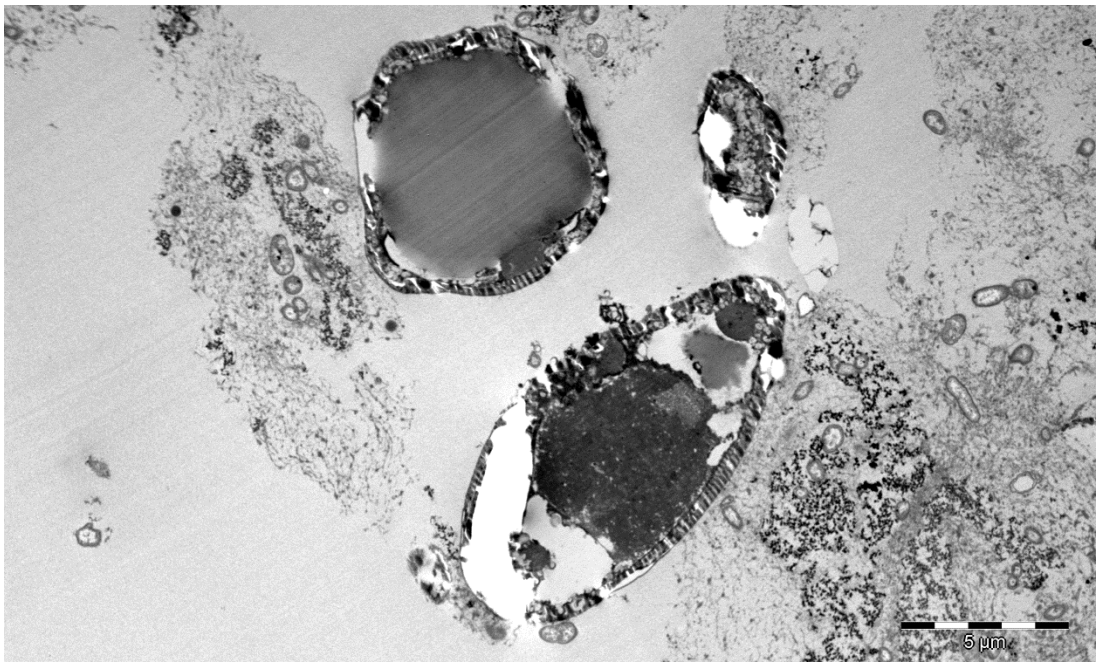


Figure 2: Cross section of two *Seminavis robusta* cells prepared for TEM with the AAS1 method.

Figure 1 and 2 show extensive damage to the intracellular structures of the *Seminavis robusta* cells. It is possible to identify the vacuoles, but there are empty spaces with no cytoplasm inside the cells. This suggests that the internal structures have shrunk or that parts of them have been washed out of the cells during preparation, due to poor fixation. In addition to the damaged cells shown in the figures, debris from shattered cells was also observed in the sample. HEPES buffer is not intended for use with marine organisms and it is likely that the cells ruptured as the result of osmotic shock.

AAS2

This fixation method was the same as the AAS1 method, but f/2 was used instead of HEPES buffer. This was done in order to lessen the osmotic shock on the cells.

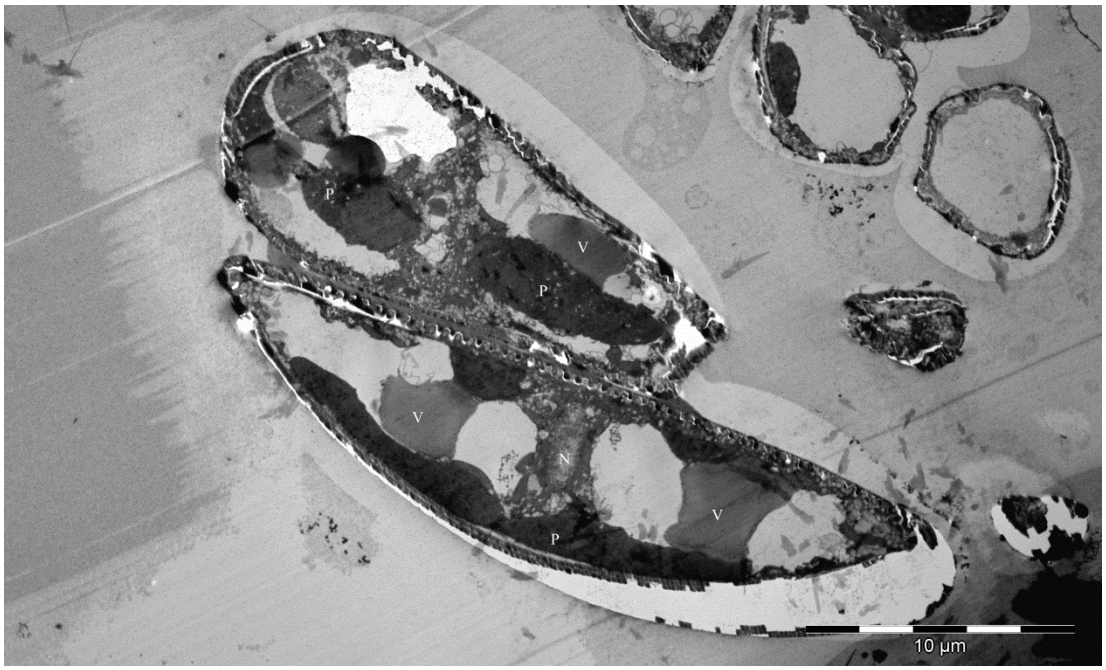


Figure 3: Longitudinal section and diagonal section of *Seminavis robusta* cells prepared for TEM with the AAS2S method. It is possible to see the remains of the vacuoles (V) and phaeoplasts (P) as well as the nucleus (N) of the cell in the bottom of the image. There are, however, large empty spaces inside the cells.

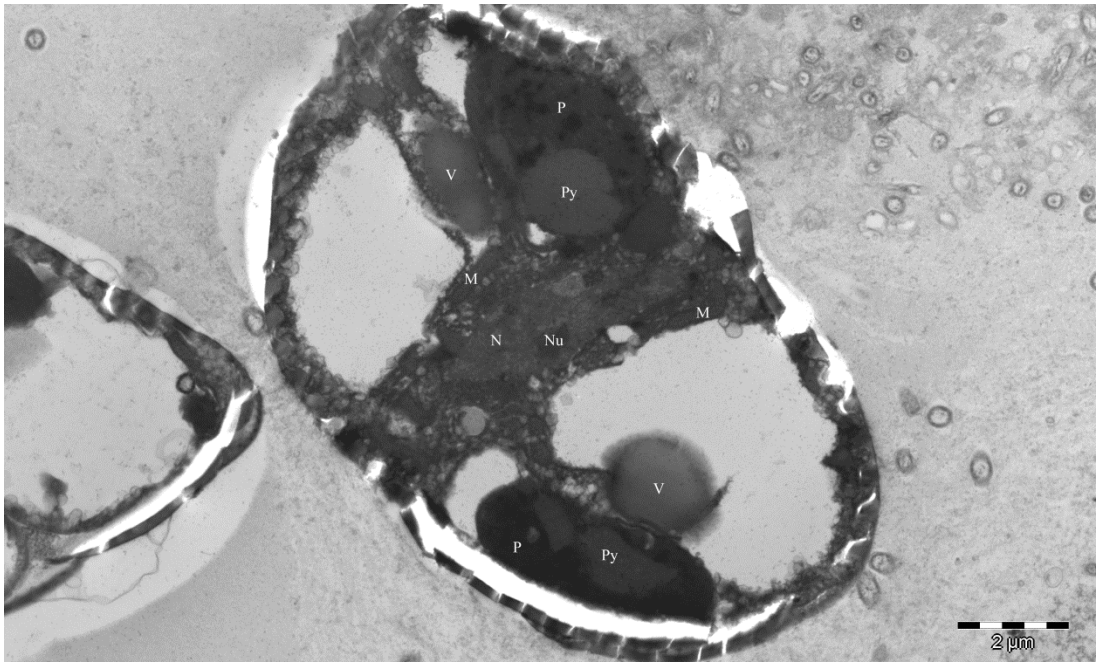


Figure 4: Central cross section of a *Seminavis robusta* cell prepared for TEM with the AAS2S method. There are large empty spaces inside the cell, it is, however, possible to see the remains of the vacuoles (V) and both phaeoplasts (P) as well as the pyrenoids (Py), nucleus (N), nucleolus (Nu) and some mitochondria (M) in this cell.

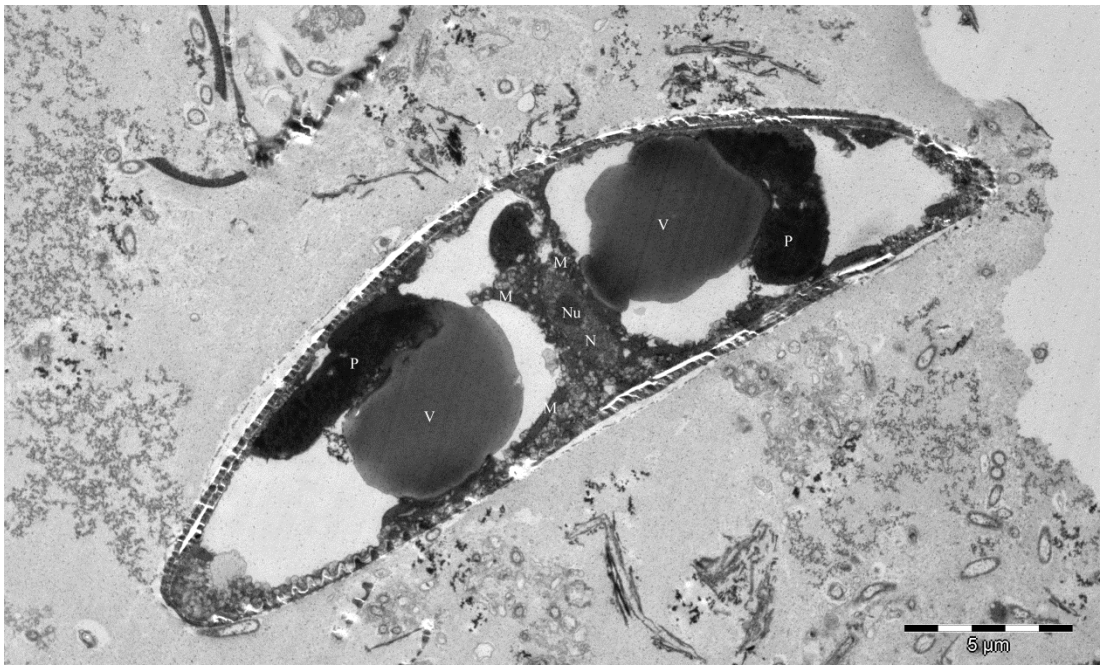


Figure 5: Longitudinal section of a *Seminavis robusta* cell prepared for TEM with the AAS2H method. There are large empty spaces inside the cell, but it is possible to see the remains of the vacuoles (V) and both phaeoplasts (P) as well as the nucleus (N), nucleolus (Nu) and some mitochondria (M) in this cell.

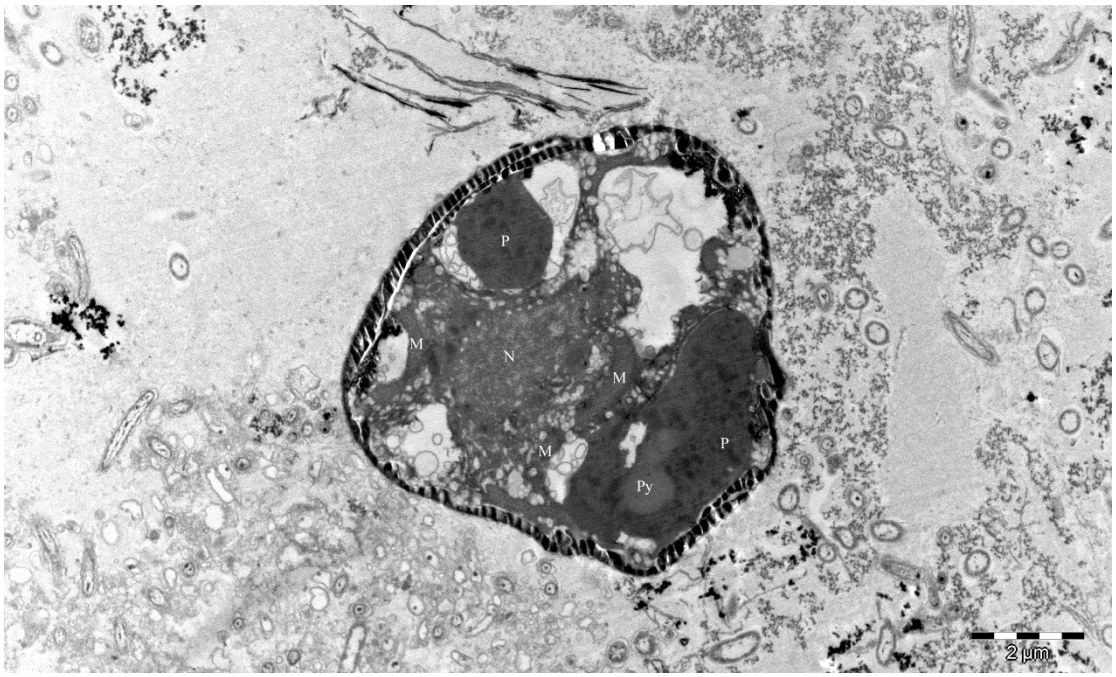


Figure 6: Central cross section of a *Seminavis robusta* cell prepared for TEM with the AAS2H method. It is possible to see the remains of both phaeoplasts (P) as well as a pyrenoid (Py), the nucleus (N), nucleolus (Nu) and some mitochondria (M) in this cell. There are, however, empty spaces inside the cell.

The cells in figures 3-6 show extensive damage to the intracellular structures of the diatoms. It is possible to identify the phaeoplasts, nucleus, vacuoles and mitochondria, but there is much space inside the cells without any structures or cytoplasm, which is a sign of poor fixation. There was observed less debris of ruptured cells in the AAS2 sample than in the AAS1 sample, which suggests that fixating the cells in f/2 does not give them the same osmotic shock as fixation in HEPES buffer. There was still, however, extensive damage to the intracellular structures of the diatoms in the AAS2 sample.

In addition to the change from HEPES buffer to f/2, half of the AAS2 sample was embedded in the same epoxy resin as the AAS1 sample, but cured for 5 days instead of 3, and the other half was embedded in the harder "H" epoxy resin. This was done because there were holes in the epoxy around most of the diatoms in the AAS1 sample. An examples of this can be seen in figure 2. These holes often appear if the resin is softer than the embedded cells, leading to the knife cutting faster through the resin and pushing the cells up, out of position, in the resin before it cuts through them. The resin gets harder the longer it is cured, but, as can be seen in figures 3 and 4, there were holes in the resin around the cells in the AAS2S sample. There were, however no holes around the cells in the AAS2H samples, which can be seen in figures 5 and 6. This means that the harder "H" epoxy, combined with the longer curing time stopped the cells from being pushed out of position during sectioning.

AAS3

This was the same fixation and embedding method as AAS2, but without the ruthenium red (RR). This was done to see if the RR had any special effect on the diatoms, since the standard fixation method only uses glutaraldehyde (GA).

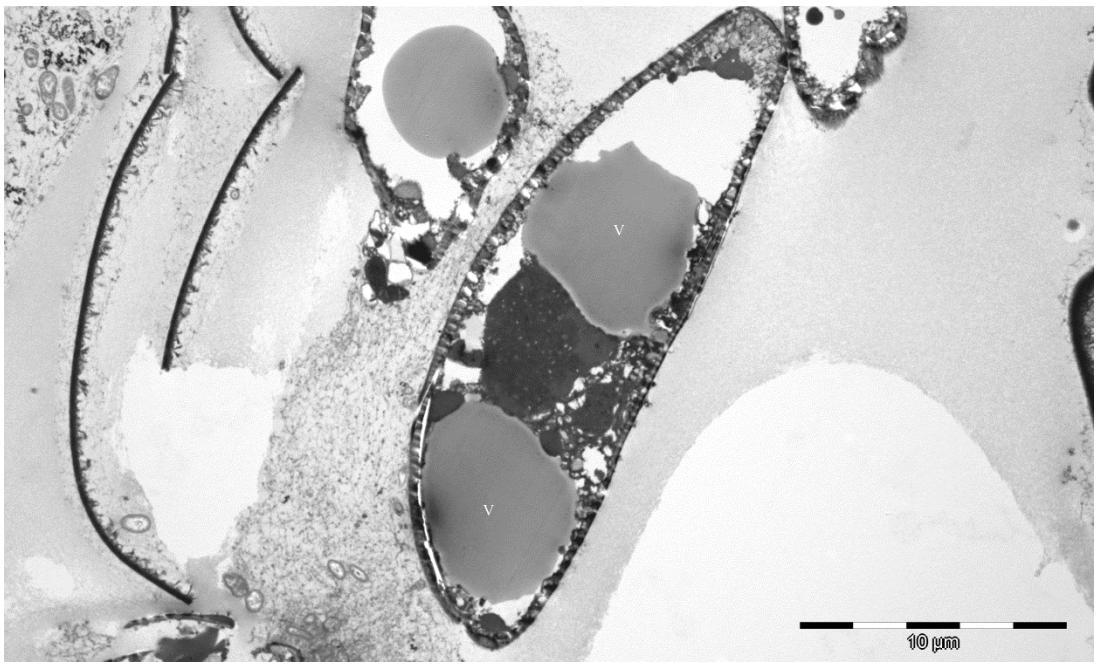


Figure 7: Longitudinal section of a *Seminavis robusta* cell prepared for TEM with the AAS3S method. There are large empty spaces inside the cell, but t is possible to identify the vacuoles (V).

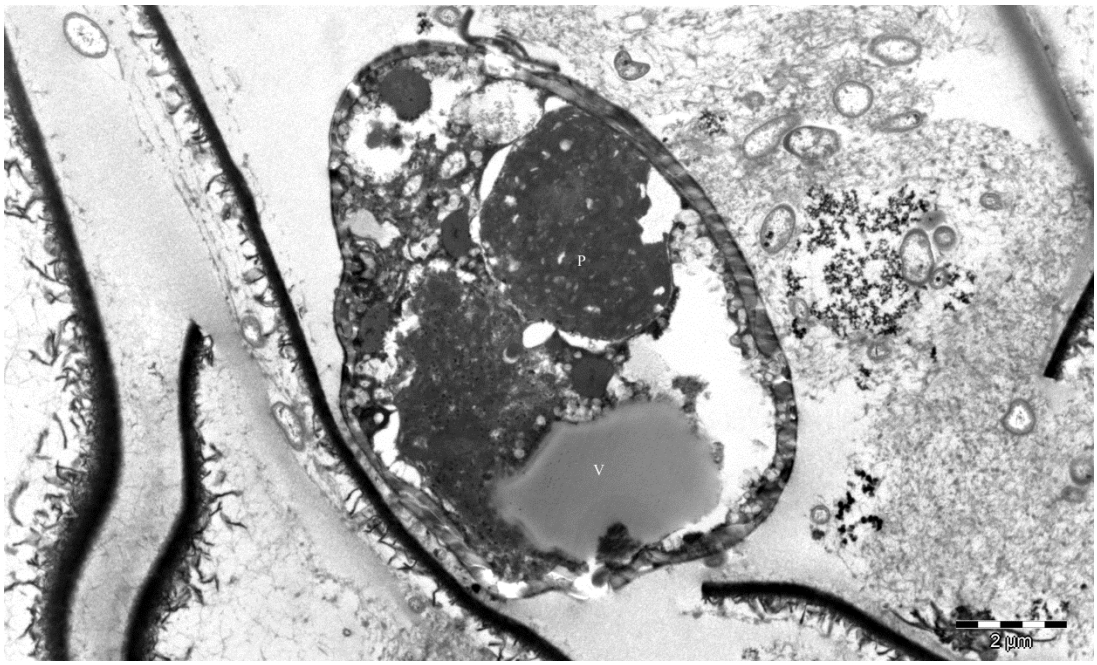


Figure 8: Cross section of a *Seminavis robusta* cell prepared for TEM with the AAS3S method, showing the remains of a vacuole (V) and phaeoplast (P).

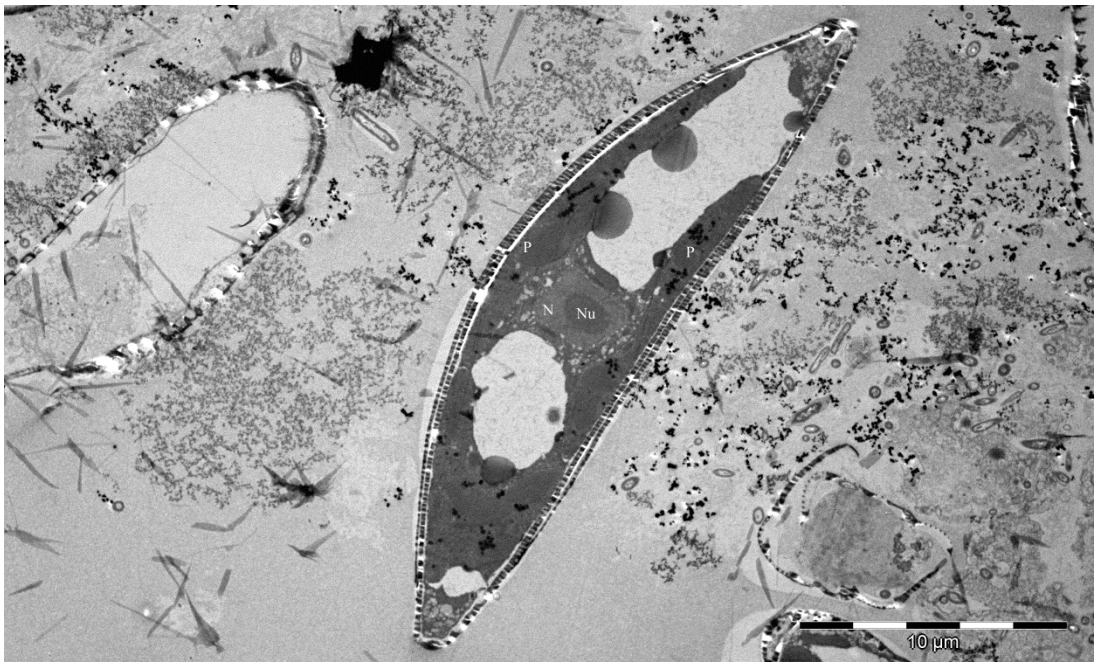


Figure 9: Longitudinal section of a *Seminavis robusta* cell prepared for TEM with the AAS3H method. It is possible to see both phaeoplasts (P) the nucleus (N) and nucleolus (Nu) in this cell. There are large empty spaces inside the cell.

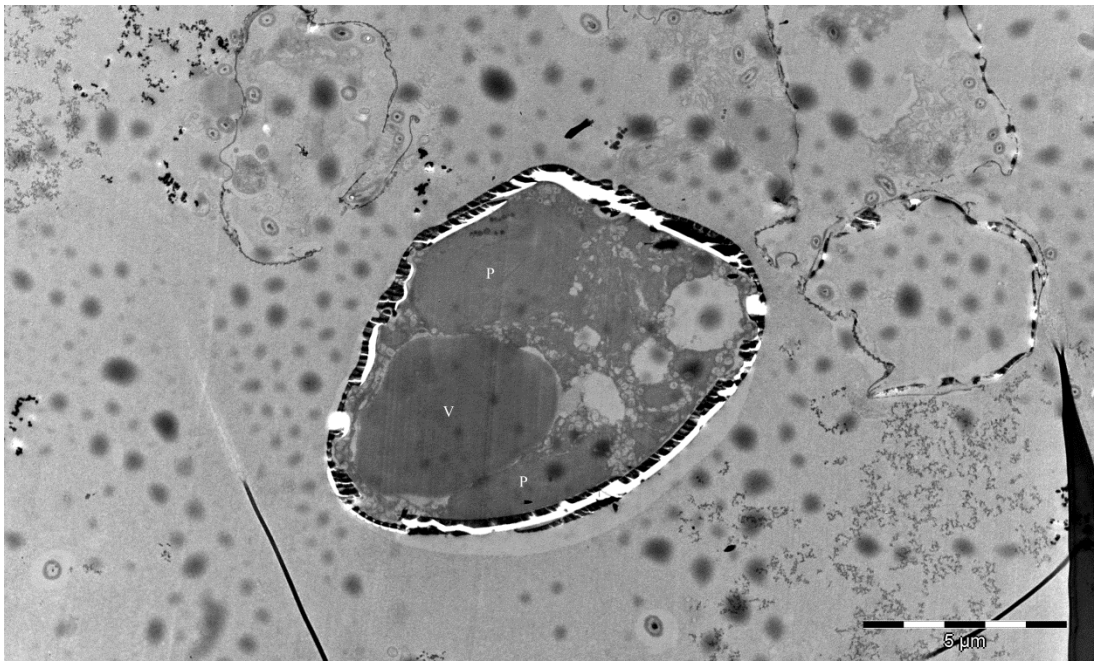


Figure 10: Distal cross section of a *Seminavis robusta* cell prepared for TEM with the AAS3H method. It is possible to see both phaeoplasts (P) and a vacuole (V) in this cell.

The cells in figures 7-10 show extensive damage to the intracellular structures of the diatoms. It is possible to identify the nucleus and nucleolus in figure 9 and the vacuoles in figure 7, but there are few other identifiable structures inside the cells. In the cells in figures 7-9 there are empty spaces without any structures or cytoplasm.

The intracellular structures of the diatoms were about as damaged as in the AAS2 sample. The phaeoplasts, however, were better preserved in the AAS2 sample as can be seen if figures 3-6 are compared with figures 7-10.

AAS4

This method was the AAS2 method without any centrifugation. The centrifugation was removed from the method in order to observe if this treatment damaged the cells.

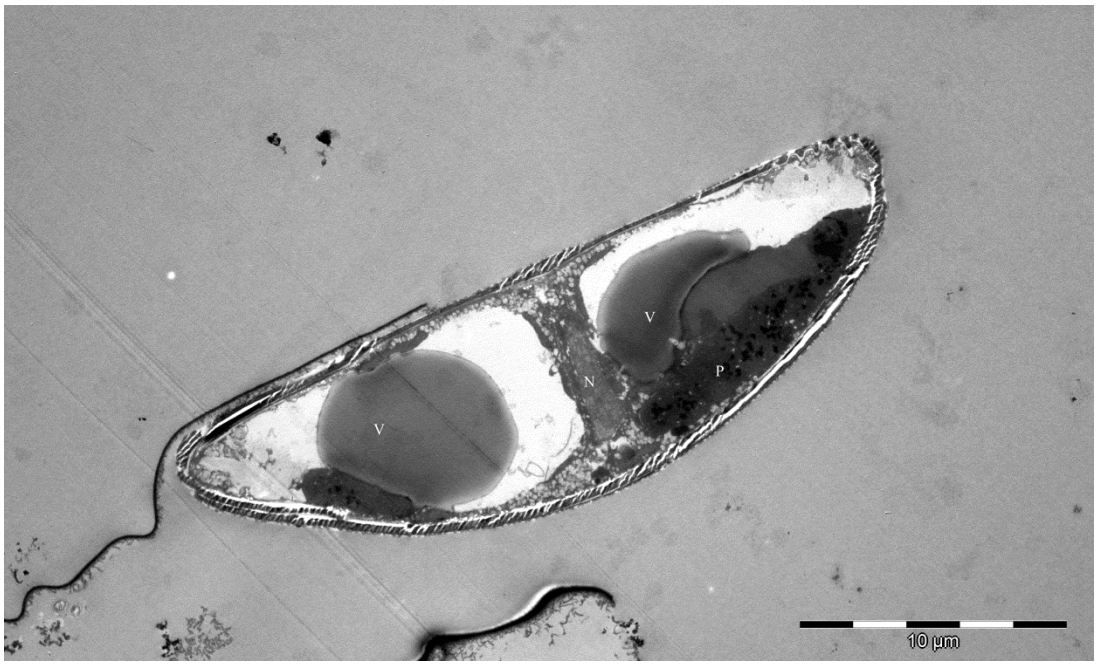


Figure 11: Longitudinal section of a *Seminavis robusta* cell prepared for TEM with the AAS4 method. The nucleus (N) and the remains of the vacuoles (V) and a phaeoplast (P) are labelled. There are large empty spaces on both sides of the nucleus.

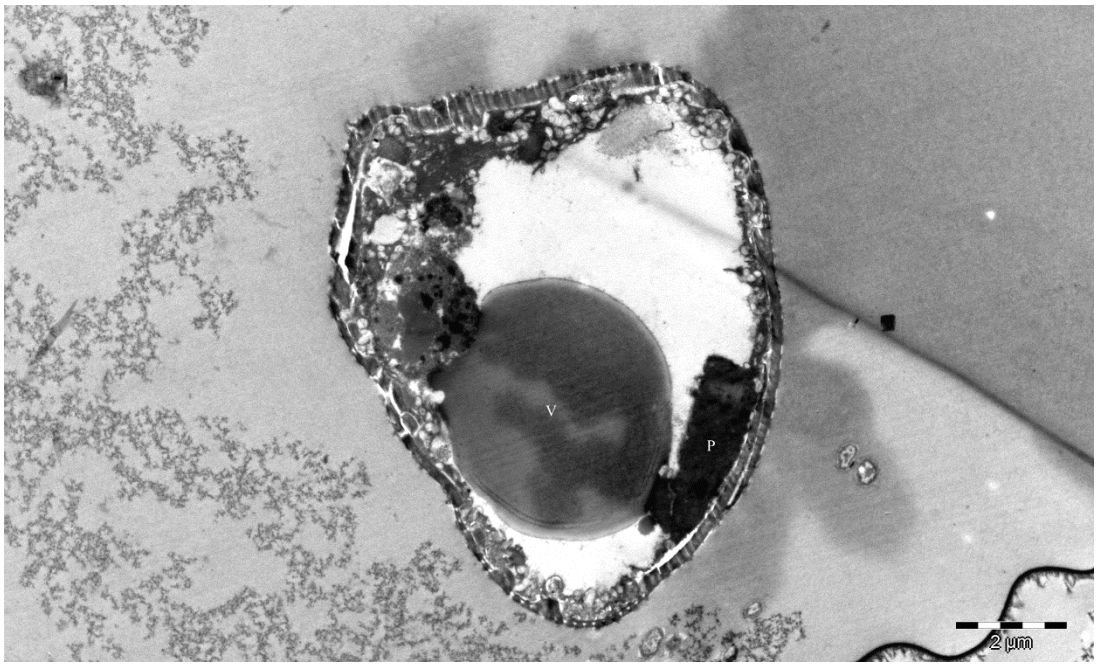


Figure 12: Distal cross section of a *Seminavis robusta* cell prepared for TEM with the AAS4 method. There is much empty space, without any cytoplasm inside the cell. It is, however, possible to identify the remains of a vacuole (V) and one phaeoplast (P).

A comparison between figures 5 and 11 and figures 4 and 12 shows that the diatoms in sample AAS2 and AAS4 are equally damaged, which suggests that the cells were not damaged by the centrifugation used in method AAS2. The cells in AAS4 were, however more difficult to find during sectioning and most sections viewed in the microscope only had one or two cells.

AAS5

In this method paraformaldehyde (PAF) was used in addition to glutaraldehyde (GA) and ruthenium red (RR). PAF fixates the same structures as GA, but at a faster rate, meaning the degradation of those structures should be halted faster when PAF is used.

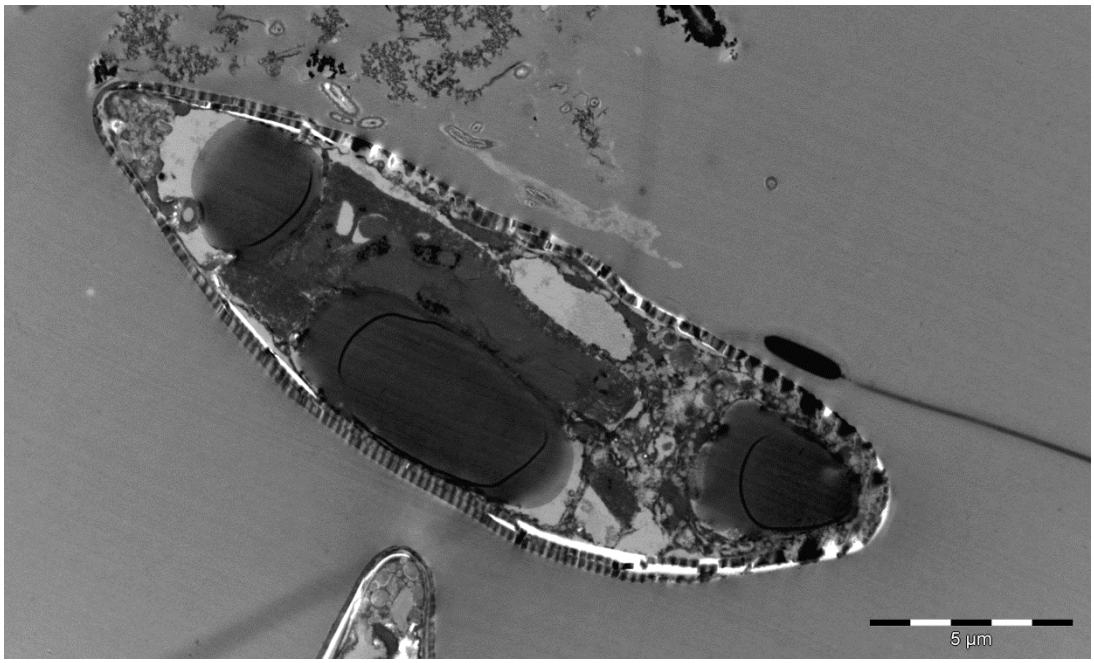


Figure 13: Longitudinal section of a *Seminavis robusta* cell prepared for TEM with the AAS5 method. There is less empty space inside this cell than in some of the earlier ones, it is however difficult to positively identify any of the internal structures.

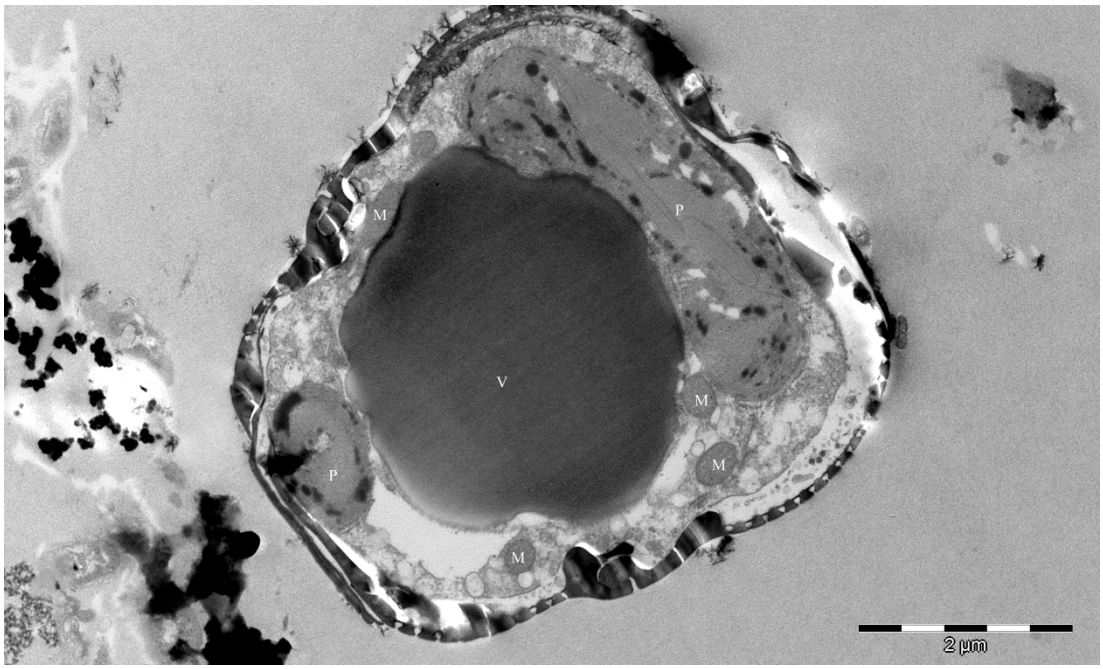


Figure 14: Distal cross section of a *Seminavis robusta* cell prepared for TEM with the AAS5 method. There is some empty space inside this cell, but it is possible to identify the vacuole (V), both phaeoplasts (P) and some mitochondria (M).

The phaeoplasts in figure 14 show more detail than the ones in the earlier figures. It is also possible to identify a few mitochondria and a vacuole in the cell in this figure. There are still empty spaces inside the cell in figure 14. In the cell in figure 13 there are also some empty spaces and the intracellular structures in this cell are difficult to recognize.

AAS6

Fixatives of 1% GA and 1% PAF have been successfully used to preserve marine algae (Lazinsky & Sicko-Goad, 1979). In this method this fixative was tested on *Seminavis robusta*.

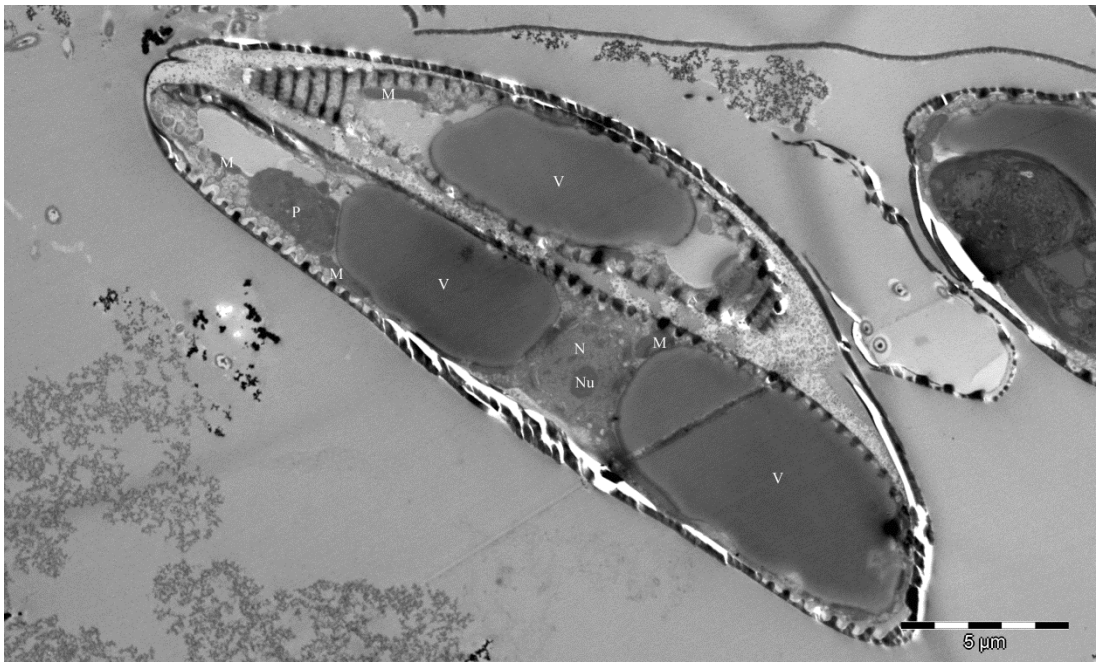


Figure 15: Longitudinal section of a *Seminavis robusta* cell prepared for TEM with the AAS6 method. This cell was caught during division. It is possible to see a mitochondria (M) and one of the vacuoles (V) of one of the daughter cells, as well as both vacuoles (V), the nucleus (N), nucleolus (N), one of the phaeoplasts (P) and some mitochondria (M) of the other daughter cell.

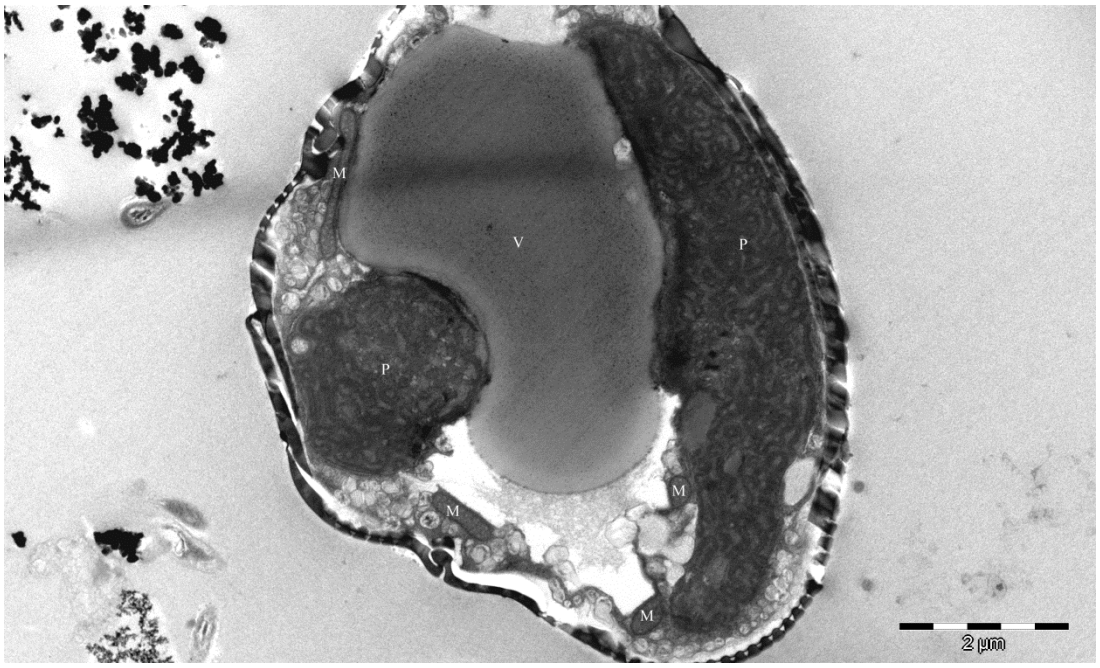


Figure 16: Distal cross section of a *Seminavis robusta* cell prepared for TEM with the AAS6 method. It is possible to see the stacks of the thyllacoid membranes clearly in both the phaeoplasts (P) of the cell. It is also possible to identify a vacuole (V) and some mitochondria (M).

In the cell in figure 15 the nucleus, nucleolus, vacuoles, and several mitochondria and the remains of a phaeoplast can easily be identified. In the cell in figure 16 the phaeoplasts, vacuole and four mitochondria are easily identified, and the stacks of thylakoid membranes inside the phaeoplasts are much clearer than in earlier figures. There are still some empty spaces inside the cells in figures 15 and 16.

AAS7

It was suggested that the concentration of fixatives in AAS6 was not high enough to be effective, so in this method the concentration was increased to 2% GA and 4% PAF.

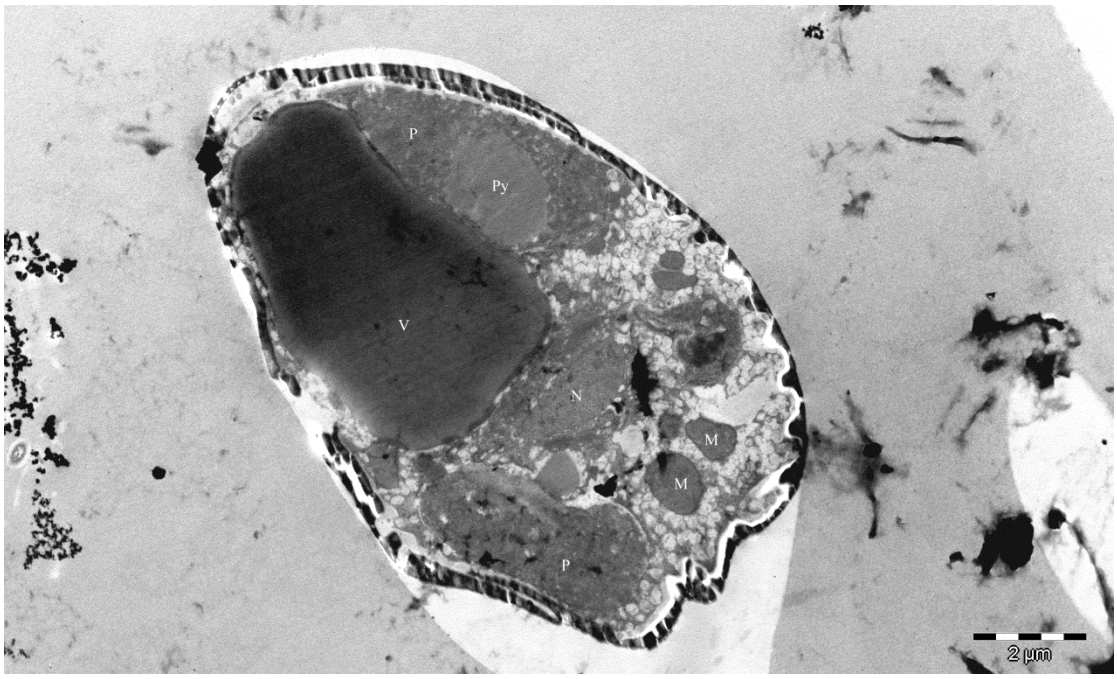


Figure 17: Central cross section of a *Seminavis robusta* cell prepared for TEM with the AAS7 method, with the nucleus (N), phaeoplast (P), a pyrenoid (Py) and some mitochondria labelled

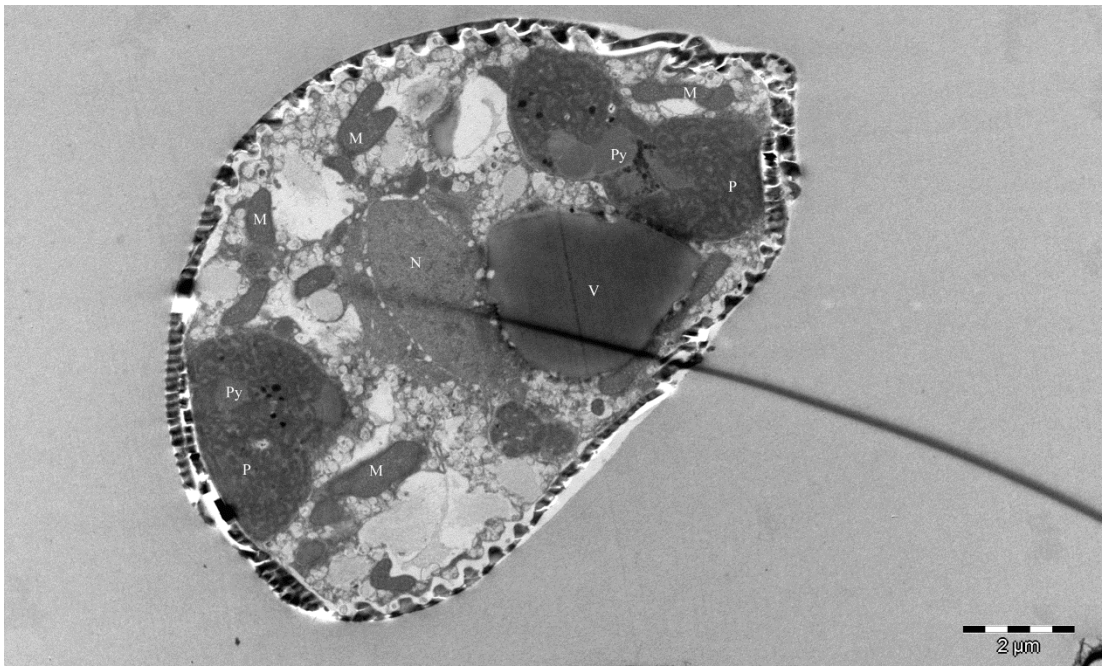


Figure 18: Central cross section of a *Seminavis robusta* cell prepared for TEM with the AAS7 method. It is possible to see both phaeoplasts (P), pyrenoids (Py), the nucleus (N) and several mitochondria in this cell. There are, however, empty spaces inside the cell.

In the cells in figures 17 and 18 the nucleus, vacuole, both phaeoplasts and several mitochondria can be identified. In figure 18 the stacks of thylakoid membranes inside the phaeoplasts are about as clear as in figure 16. There are still some empty spaces inside the cells in figures 17 and 18.

AAS8

In this method potassium ferrocyanide was used during post fixation in an attempt to better preserve the intracellular membrane structures of the diatoms.

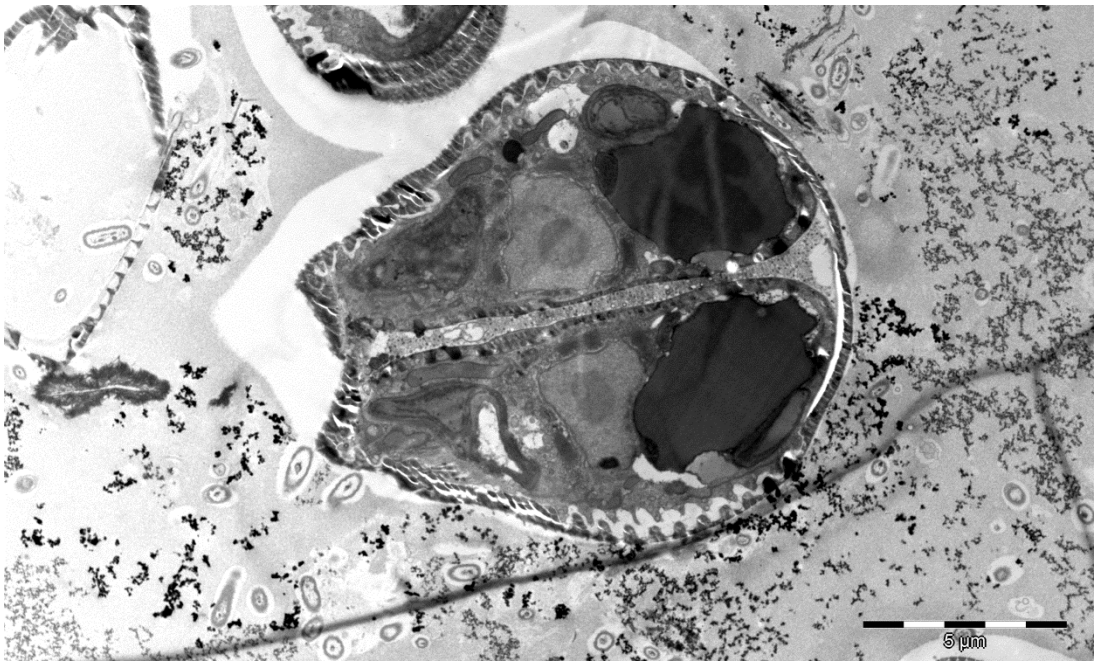


Figure 19: Central cross section of a dividing *Seminavis robusta* cell prepared for TEM with the AAS8 method. It is possible to see the nucleus, nucleolus, vacuoles, phaeoplasts an mitochondria of both daughter cells.

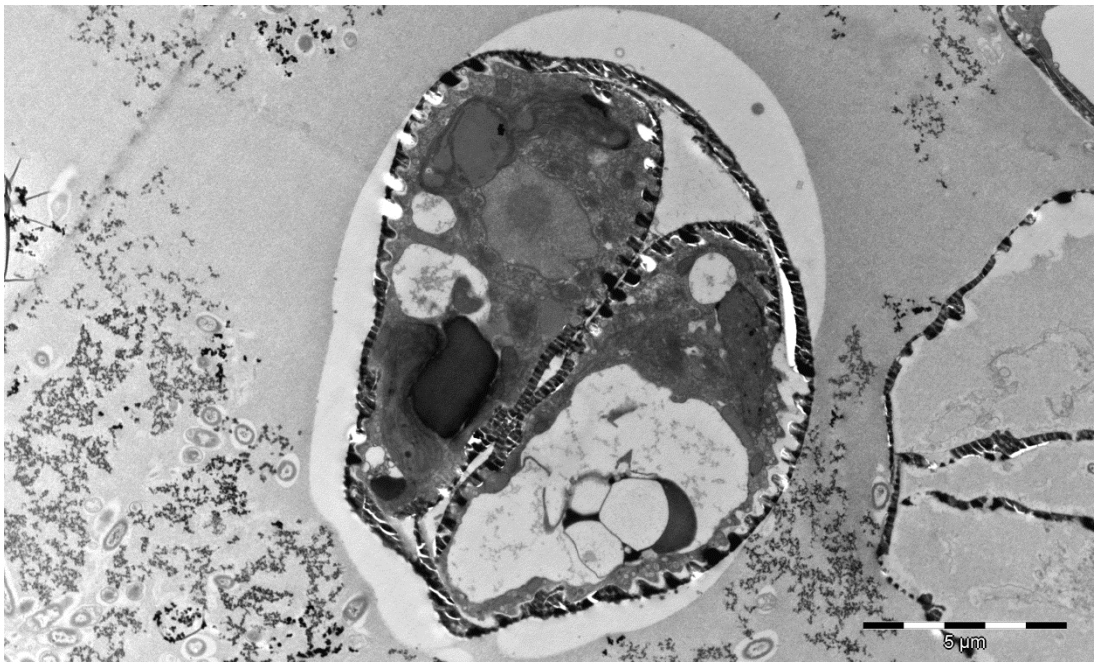


Figure 20: Central ross section of a dividing *Seminavis robusta* cell prepared for TEM with the AAS8 method. It is possible to see the nucleus, nucleolus, vacuole, phaeoplasts an mitochondria of the daughter cell on the left. Most of the cytoplasm in the other daughter cell has been washed out of the cell. It is, however possible to identify one of the phaeoplasts of this cell as well as some mitochondria.

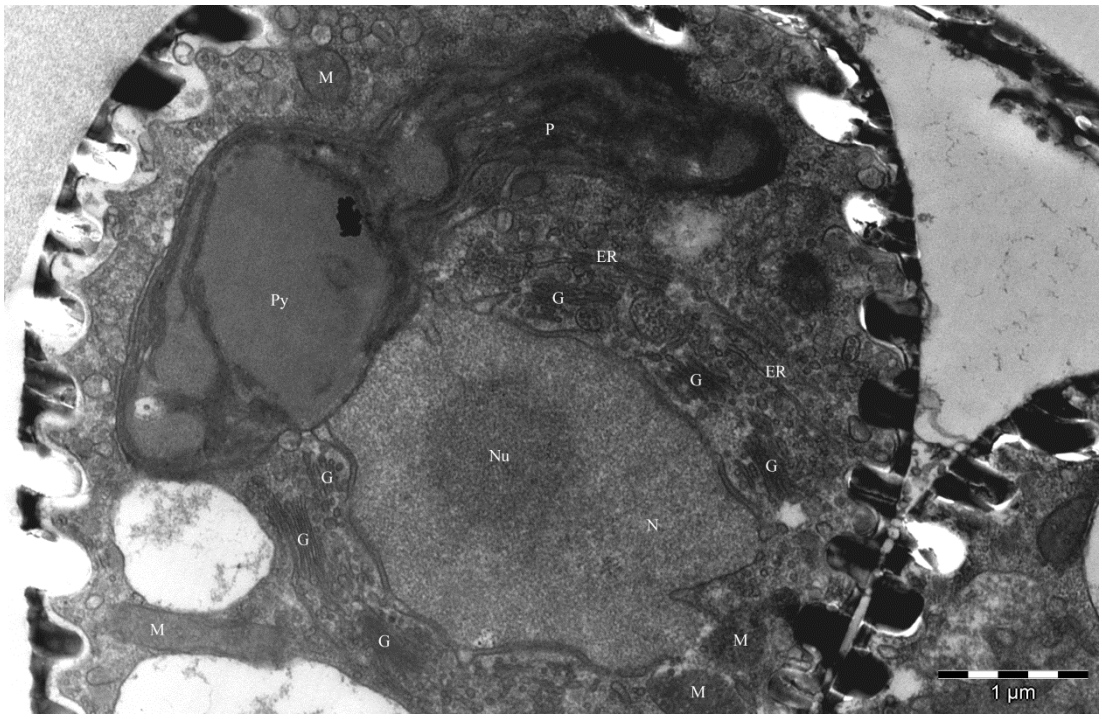


Figure 21: Close-up of the cell in figure 20, with the nucleus (N), nucleolus (Nu), phaeoplast (P), pyrenoid (Py), mitochondria, Golgi apparatuses (G) and some endoplasmic reticulum (ER) labelled.

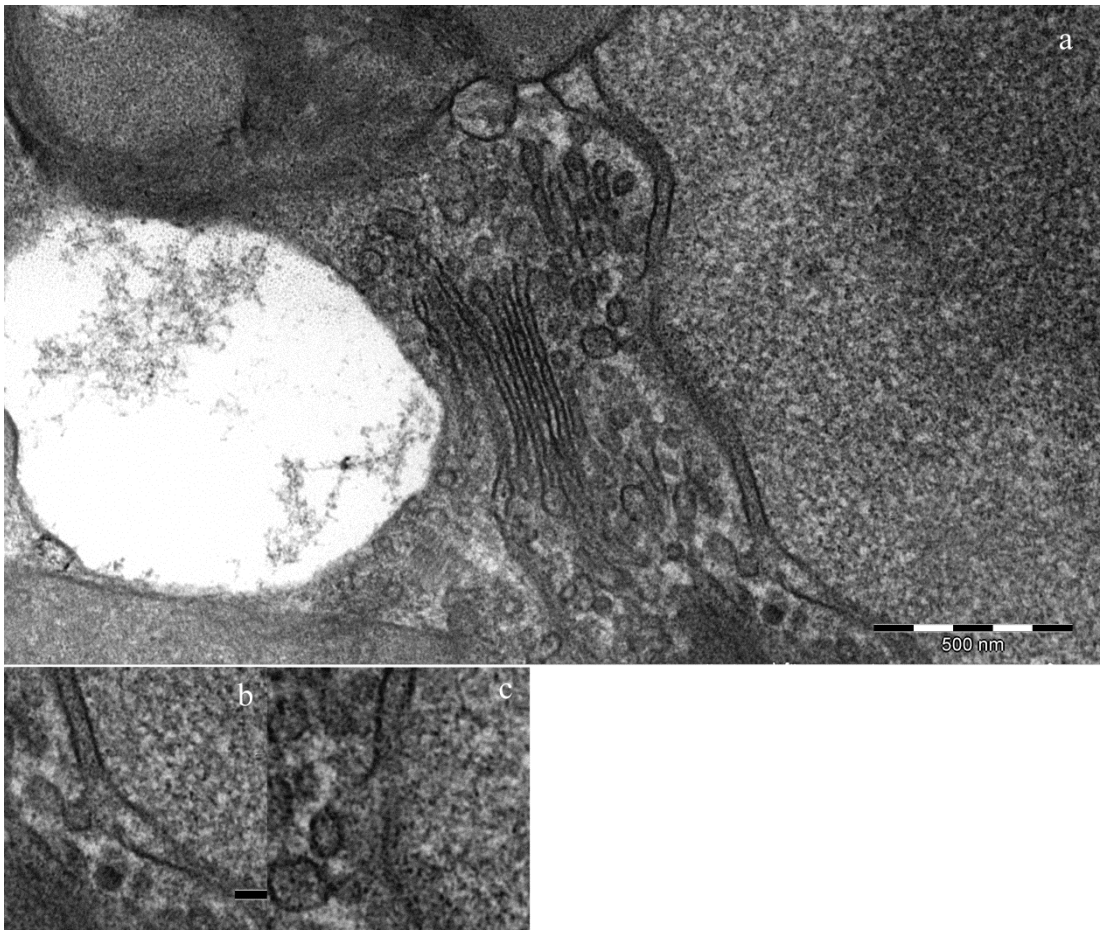


Figure 22: Close-up of the cell in figures 20 and 21, showing Golgi apparatuses and nuclear envelope (a). It is possible to see two nuclear pores in this section of the nuclear envelope (b, c).

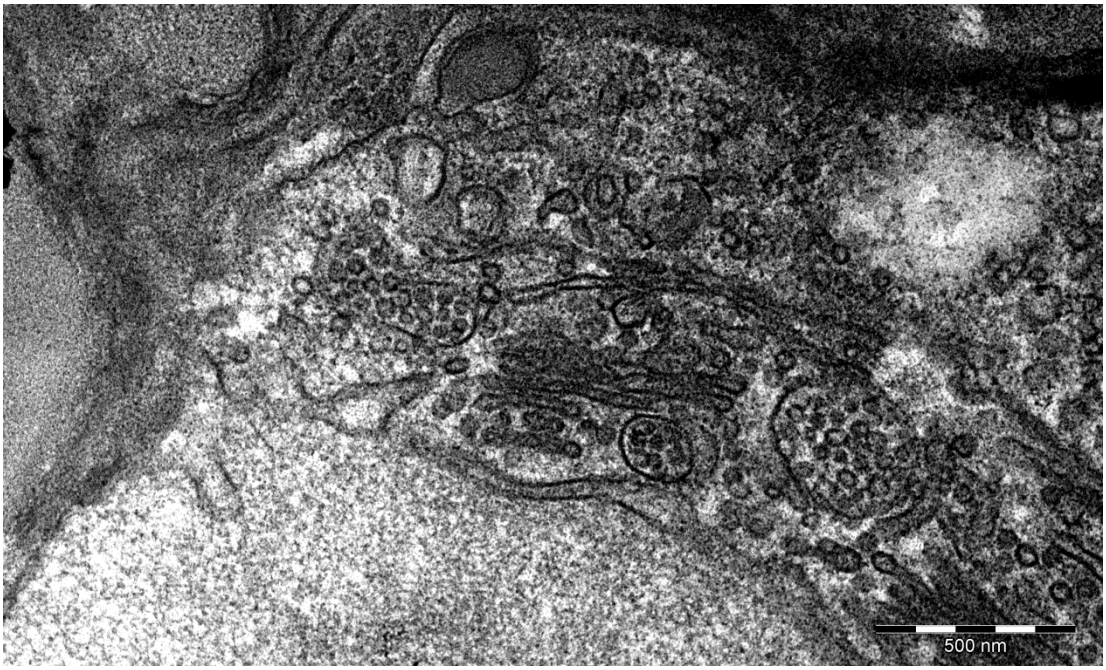


Figure 23: Close-up of the cell in figures 20 and 21, showing vesicles, Golgi apparatuses, some ER and a section of the nuclear envelope.

In the cells in figures 19-23 the nucleus, vacuoles, phaeoplasts, mitochondria, Golgi apparatuses and several vesicles in the cytoplasm can be identified. That these structures can be observed in the cells of this sample so clearly supports the hypothesis that potassium ferrocyanide helps preserve membrane structures during TEM preparation. There are, however, still some empty spaces inside the cells in the AAS8 sample, meaning this fixation method does not completely preserve the cells during TEM preparation .

AAS9

In this method the cells were post fixated in tannic acid prior to osmium tetroxide. Uranyl acetate is toxic and radioactive, while tannic acid can be found in high concentrations in oolong tea, which is why it would be beneficial if staining with oolong tea could replace uranyl acetate staining.

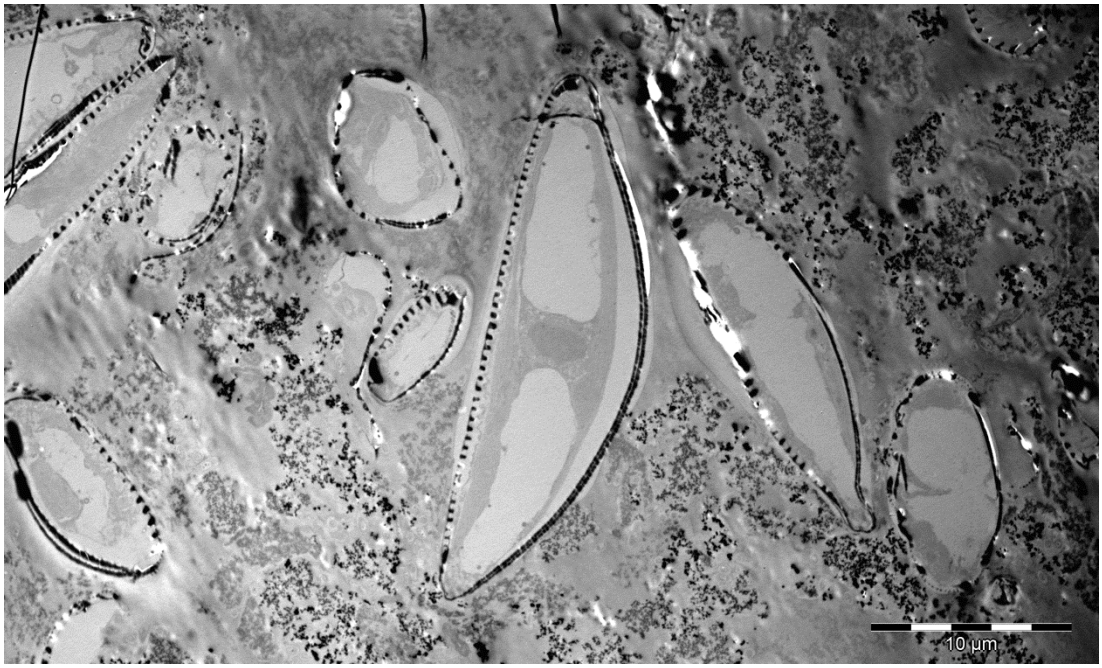


Figure 24: Longitudinal and cross sections of *Seminavis robusta* cells prepared for TEM with the AAS9 method. The internal structures are faint and difficult to distinguish, though hit is possible to identify the nucleus of the cell in the center of the image.

After an initial inspection of the sample on the fluorescent screen of the microscope, the inside of the frustules appeared to be empty. However, figure 24 shows that some intracellular structures were captured by the camera that took this micrograph, though they are still faint.

AAS10

This fixation method is the same as the AAS8 method, except that the primary fixation, with GA, PAF and RR, was done at 5ºC instead of at room temperature (ca. 20ºC) like in the other

methods. This was done because the intracellular structures of the diatom cells decay faster at room temperature than at 5ºC.

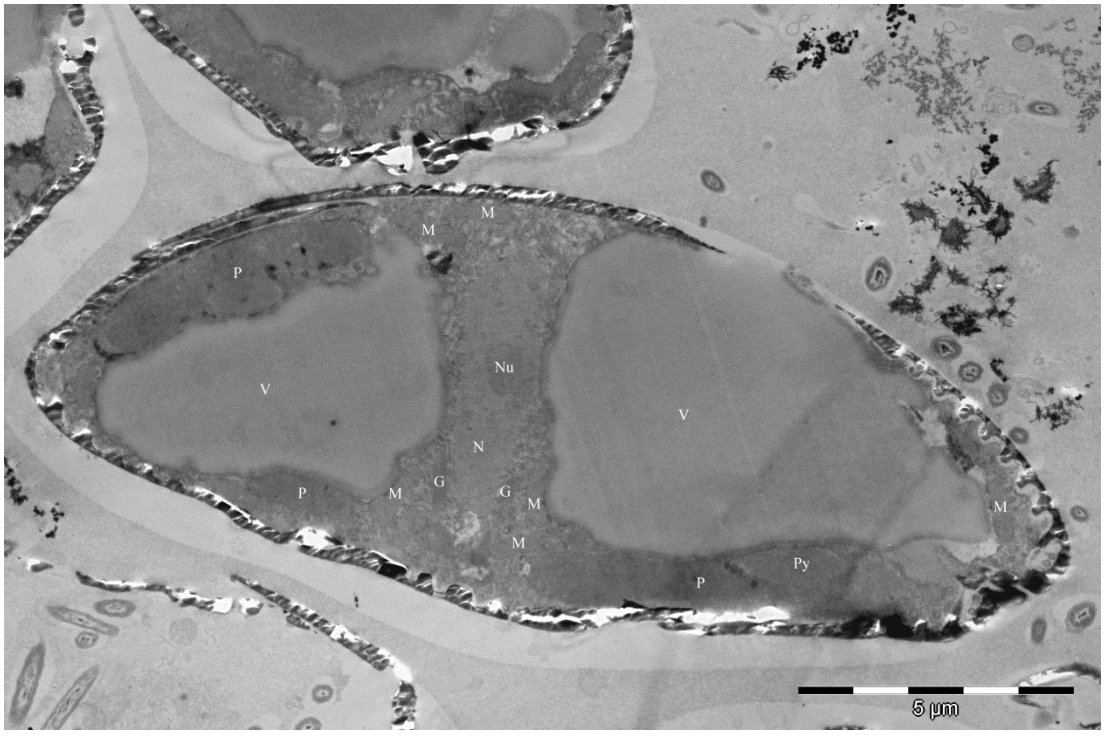


Figure 25: Longitudinal section of a *Seminavis robusta* cell prepared for TEM with the AAS10 method. It is possible to see the nucleus (N), nucleolus (Nu), both vacuoles (V), phaeoplast (P), a pyrenoid (Py), some mitochondria (M) and Golgi apparatuses (G) in this cell.

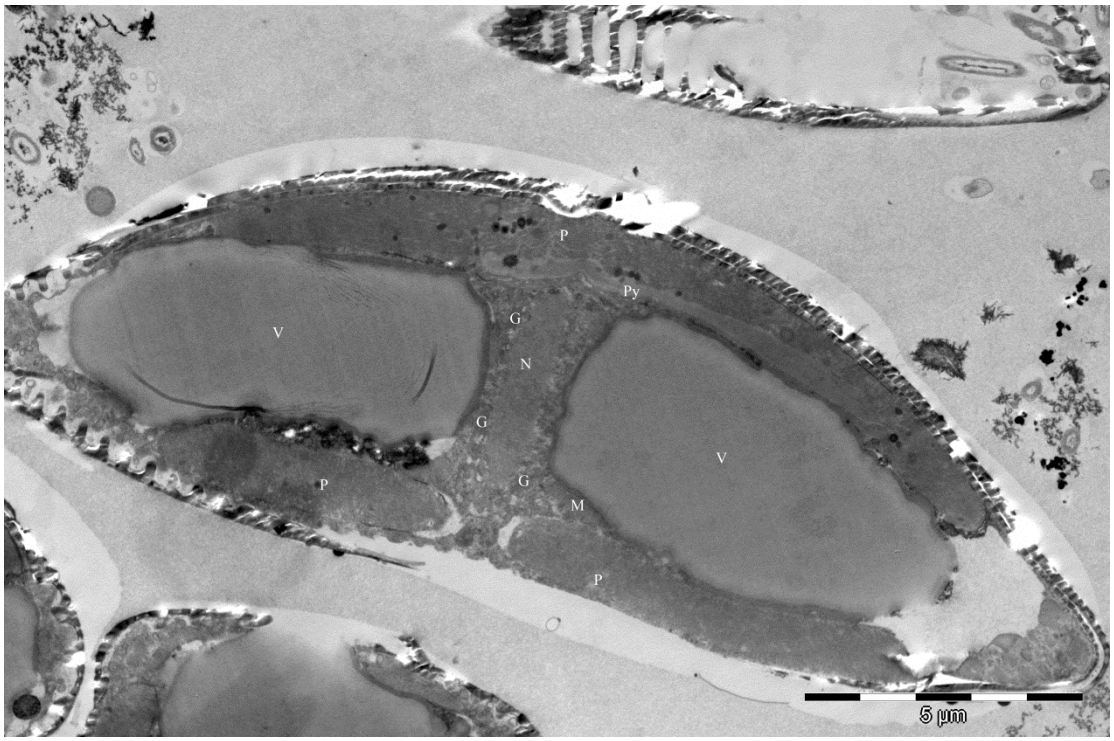


Figure 26: Longitudinal section of a *Seminavis robusta* cell prepared for TEM with the AAS10 method. It is possible to see the nucleus (N), both vacuoles (V), phaeoplasts (P), a pyrenoid (Py), some mitochondria (M) and Golgi apparatuses (G) in this cell.

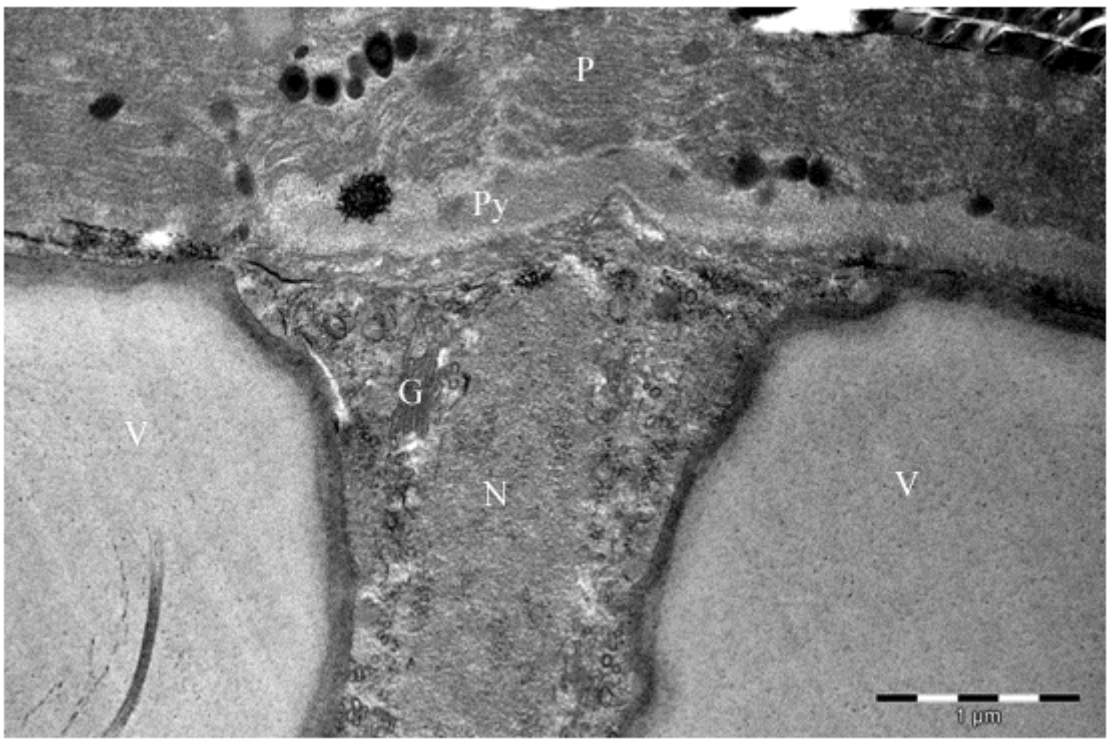


Figure 27: Close-up of the cell in figure 26, with the nucleus (N), vacuoles (V), phaeoplast (P), pyrenoid (Py) and Golgi apparatus (G) labelled.

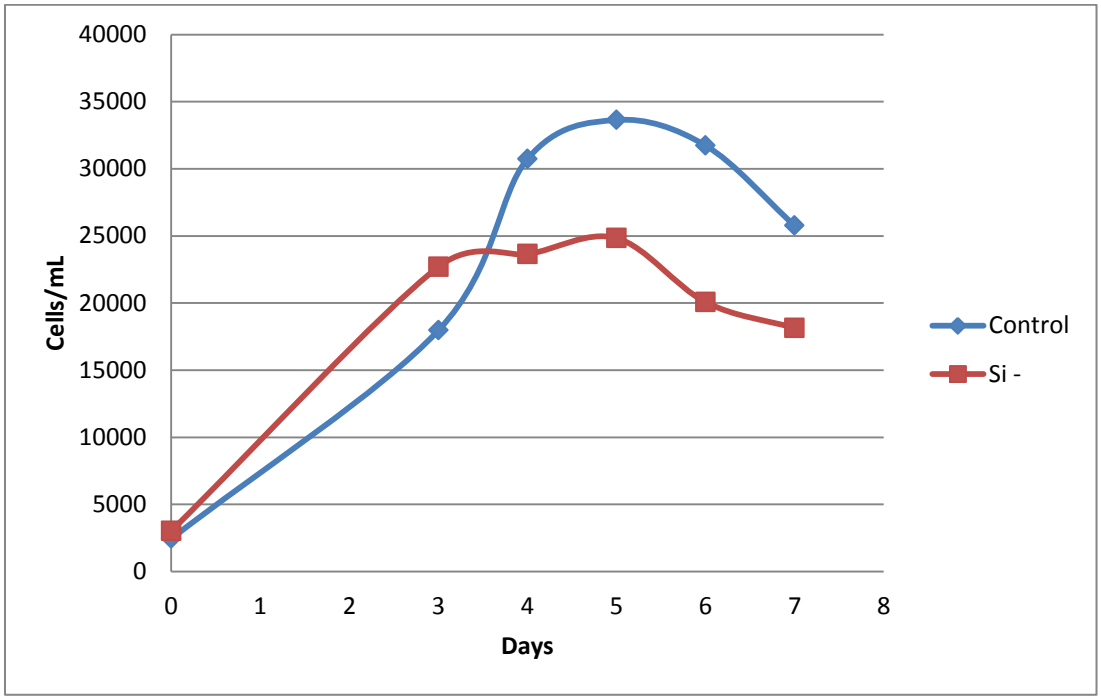
In the cells in figures 25 and 26 the nucleus, vacuoles, phaeoplasts, mitochondria, Golgi apparatuses and several vesicles in the cytoplasm can be identified. There are still some empty spaces inside the cells in this sample, which means this fixation technique is not completely successful.

The intracellular structures of the diatom cells decay faster at room temperature than at 5ºC, but the fixative will infiltrate the cells slower at the lower temperature. If figures 19-21 are compared with 25-27 it is possible to see that the AAS10 and AAS8 fixation methods preserve the intracellular structures for TEM equally well.

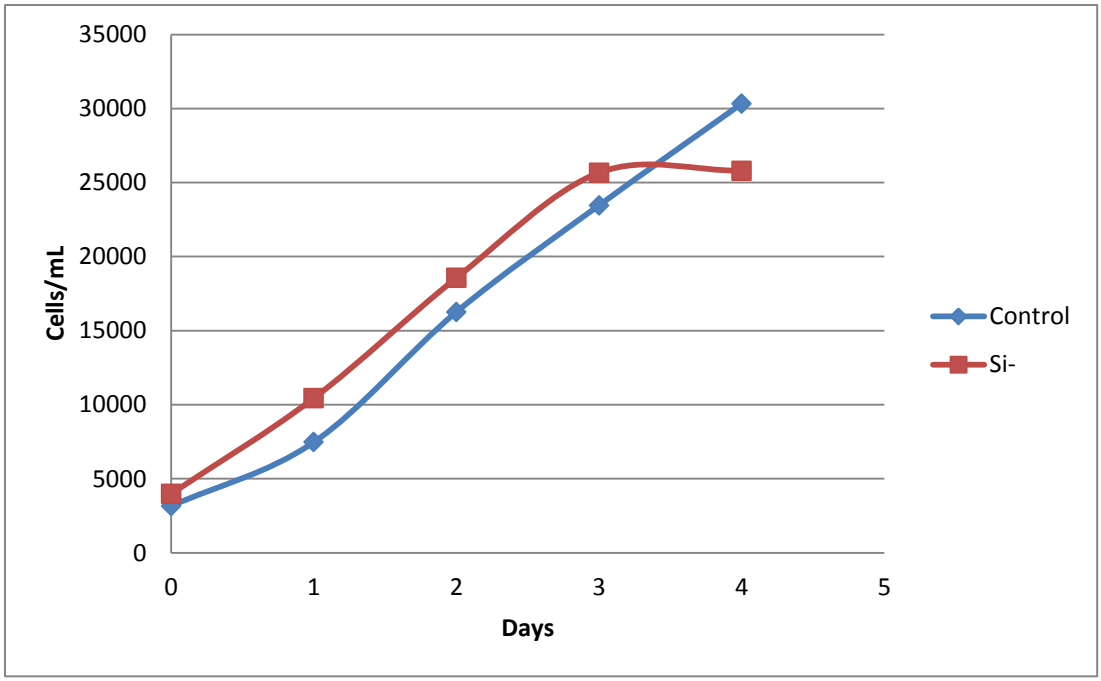
3.1.2 Silica starvation

This experiment was done to study the effect of silica deprivation on the frustules of *Seminavis robusta*. Niu et al (2012) discovered that thinning the frustule of *Phaeodactylum triconutum* through silica deprivation allowed transformation of the diatom by electroporation. The following experiment was done in order to discover whether the frustule of *S. robusta* could be thinned enough to allow transformation of this diatom by electroporation.

Cell proliferation



Graph 1: Cell proliferation over time in regular f/2 (control) and f/2 without added Na_2SiO_3 (Si-), based on the numbers in table 1 in Appendix A.



Graph 2: Cell proliferation over time in regular f/2 (control) and f/2 without added Na_2SiO_3 (Si-), based on the numbers in table 2 in Appendix A.

Graph 1 shows that the concentration of cells in regular f/2 increases until the cells have been in the medium for five days, at which point the concentration of cells in the flask starts decreasing. In the same graph it is shown that the growth in concentration of cells in f/2 without added Na_2SiO_3 (Si- f/2) flattens out after 3 days and the concentration decreases after five days. This graph does not show what happens between day 0 and 3, so it is not possible to see based on this experiment alone when the cells grown in Si- f/2 stop dividing. That is why the second cell proliferation experiment was performed.

Graph 2 shows that the cells continue to divide for three days after being introduced to the Si- f/2. That the cells grown in Si- f/2 stopped dividing before the control cells proves that silica is important for cell proliferation. The reason why the cells grown in Si- f/2 are able to divide for three days after being seeded into the medium is that there are other sources of silica in the filtered seawater the medium is made of, than the Na_2SiO_3 that is added to regular f/2. That both the control and the Si- cells started dying after five days suggest that the reason of death was lack some other nutrient than silica, or the Si- cells would have started dying before the control cells.

TEM

For TEM studies the AAS8 fixation method from section 2.2.1 was used, since this method most effectively preserved the internal structures of the diatoms.

AASi0

Seminavis robusta before silica deprivation.

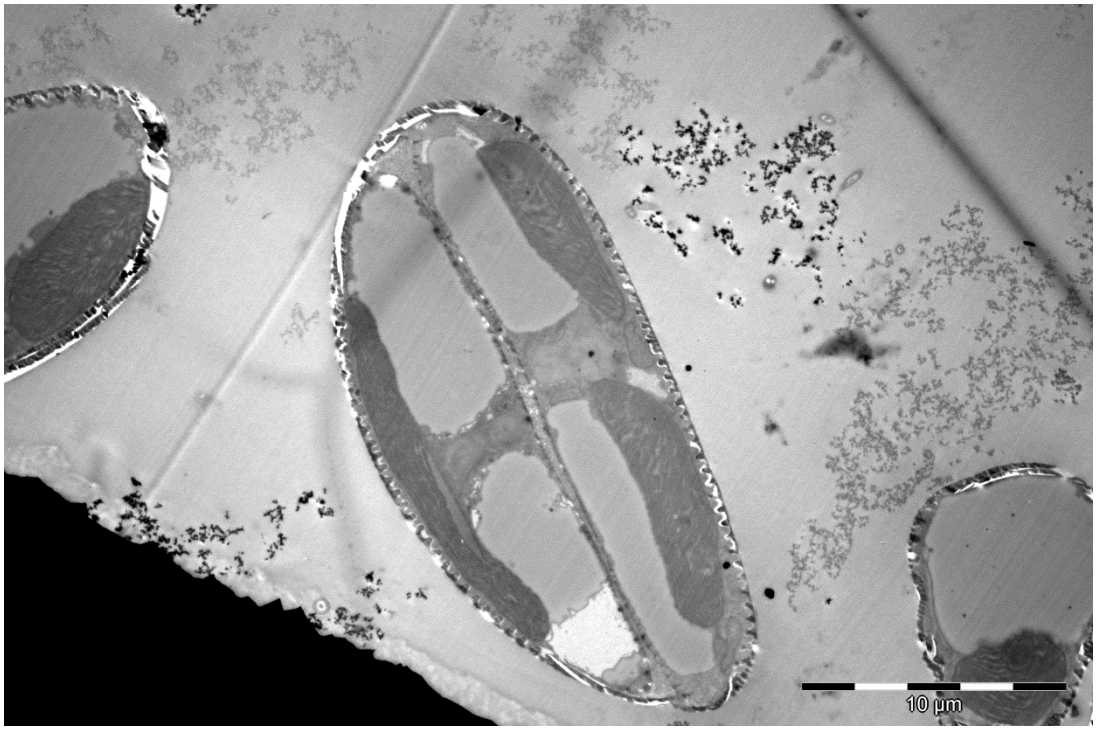


Figure 28: Longitudinal section of a dividing *Seminavis robusta* cell that has not been exposed to silica starvation.

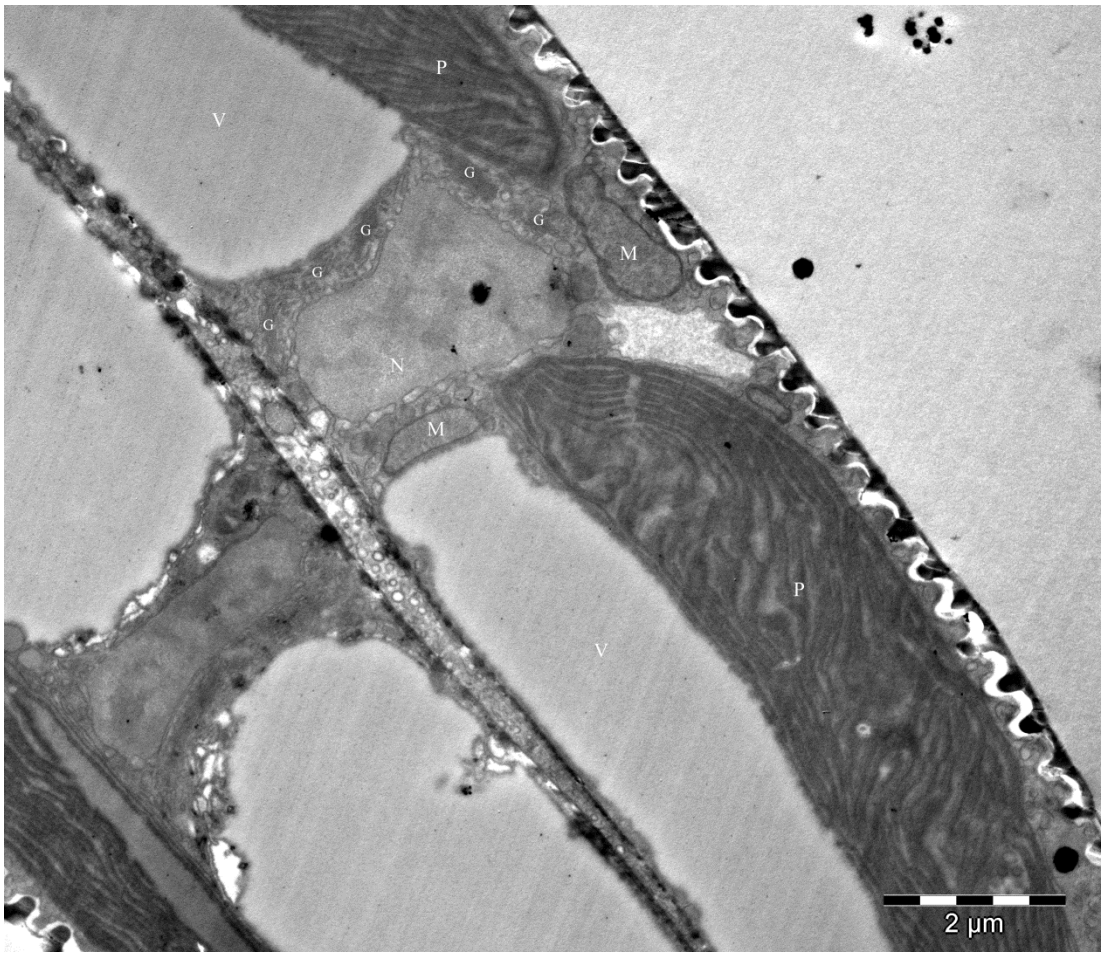


Figure 29: Close-up of the cell in figure 28, with the nucleus (N), vacuoles (V), phaeoplasts (P), mitochondria (M) and Golgi apparatuses (G) labelled.

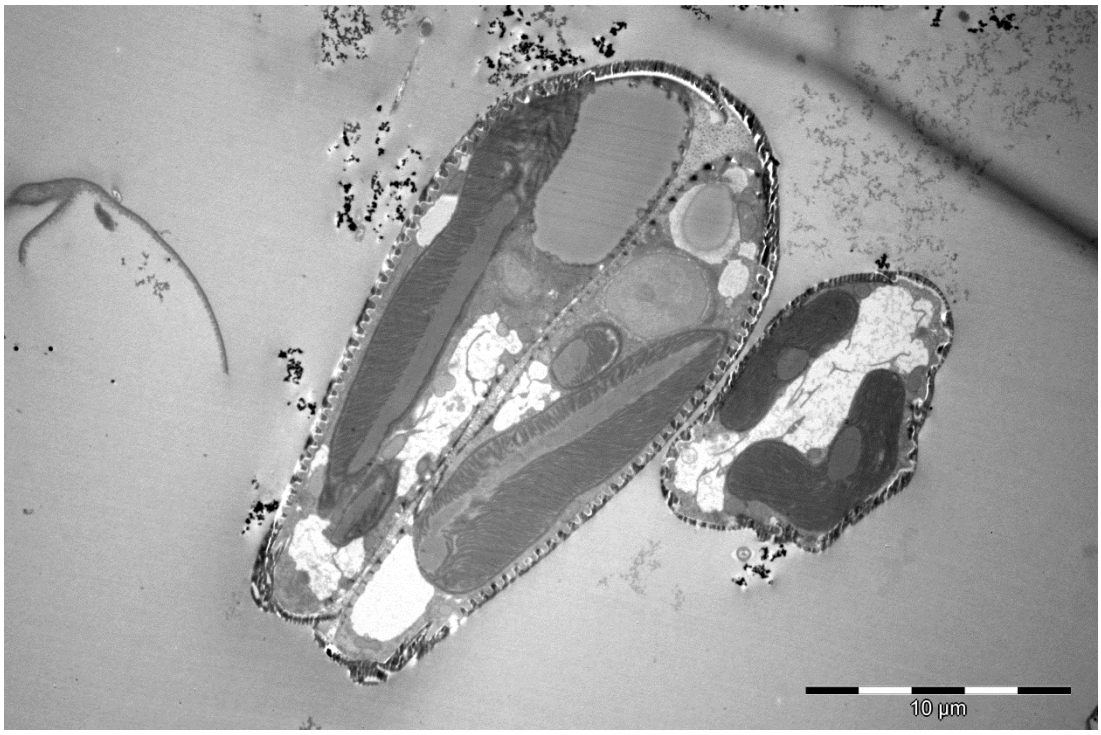


Figure 30: Diagonal and cross sections of *Seminavis robusta* cells that have not been exposed to silica starvation. The cell on the left has been caught during division. There are empty spaces inside the cells, it is, however possible to identify the phaeoplasts and pyrenoids of both daughter cells as well as the nucleus, nucleolus and remains of the vacuole of the daughter cell on the right, and the vacuole and edge of the nucleus of the daughter cell on the left.

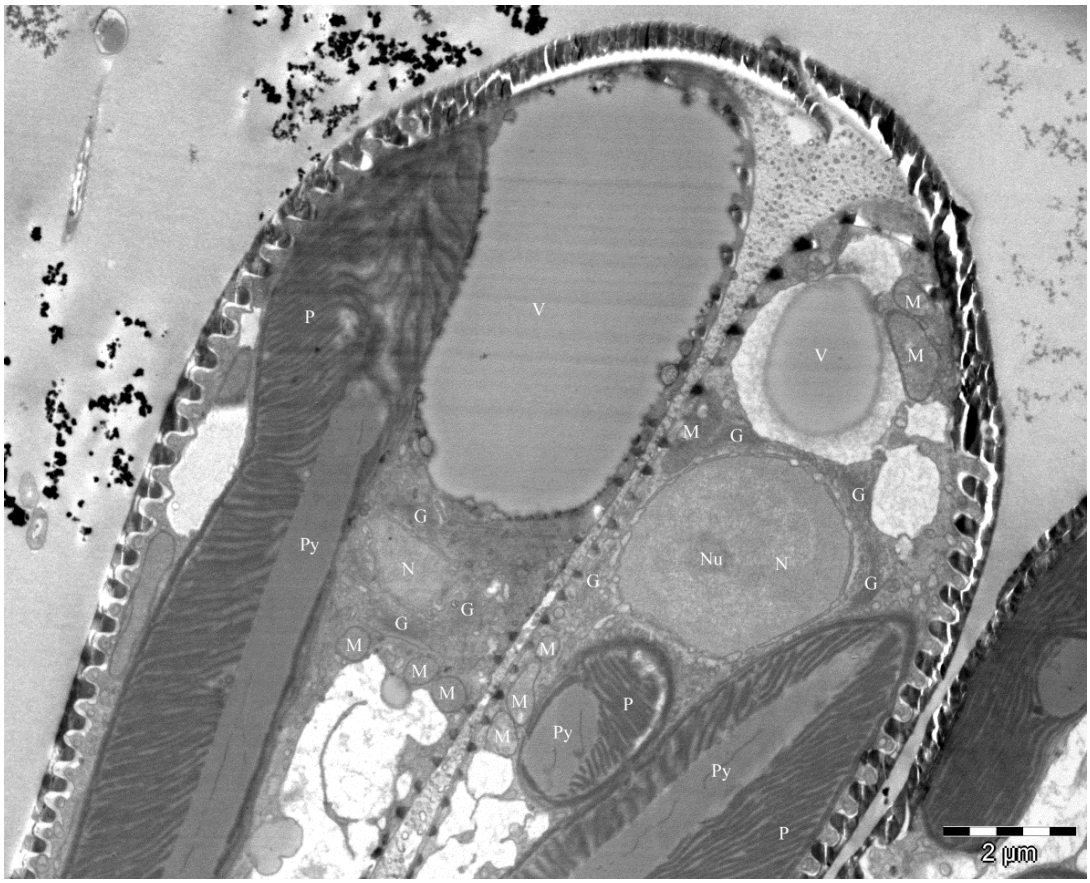


Figure 31: Close-up of one of the cells in figure 30, with the nucleus (N), nucleolus (Nu) vacuoles (V), phaeoplasts (P), pyrenoids (Py), mitochondria (M) and Golgi apparatuses (G) labelled. The area between the two developing thecae is rich in vesicles. These might be SDVs carrying silica towards the new thecae.

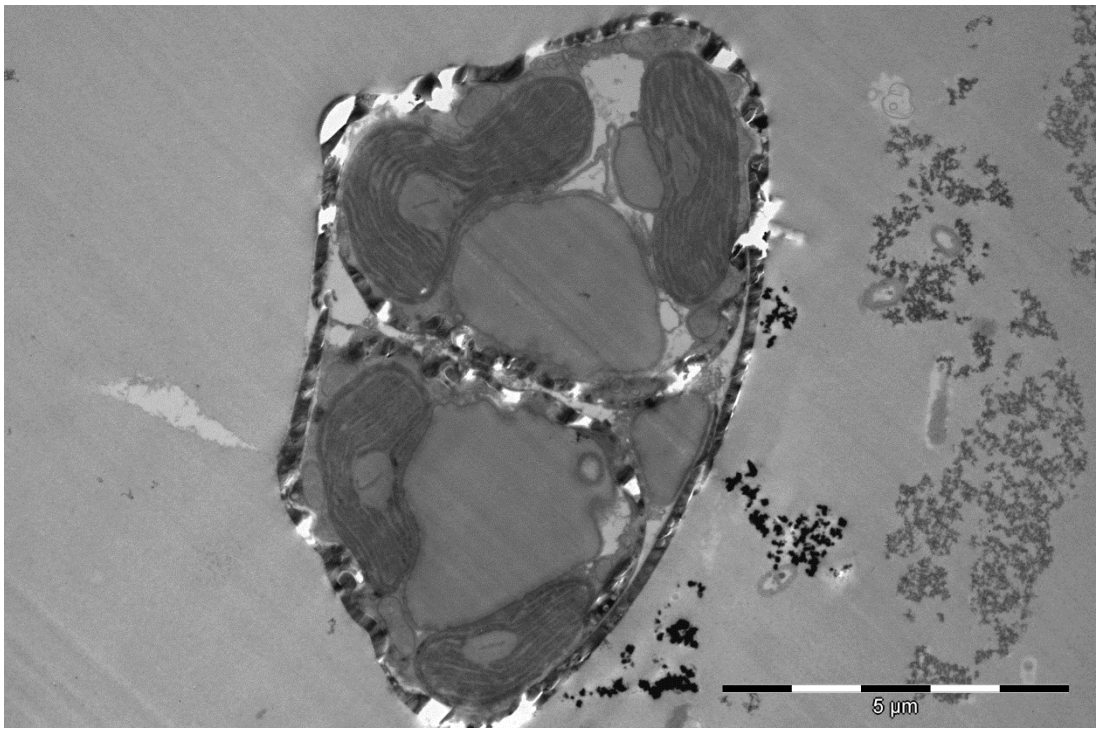


Figure 32: Distal cross section of a dividing *Seminavis robusta* cell that has not been exposed to silica starvation. There are empty spaces inside the cells, it is, however possible to identify the phaeoplasts and pyrenoids as well as the vacuoles and some mitochondria in both daughter cells.

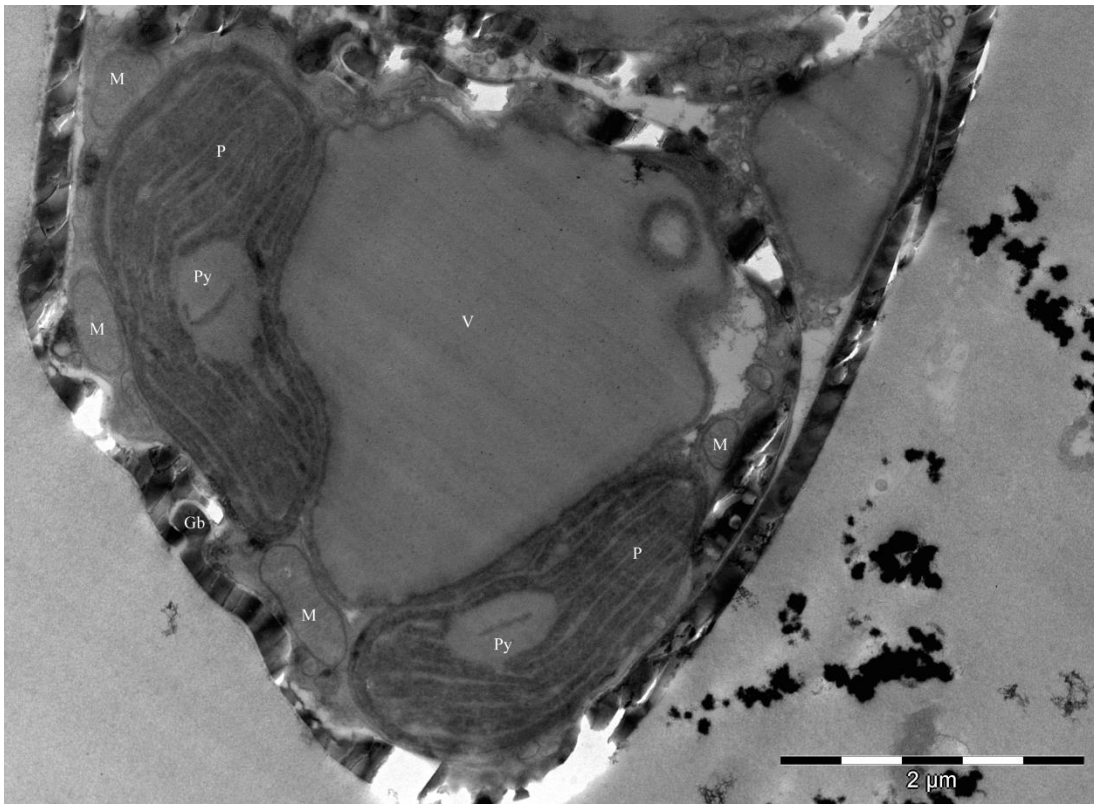


Figure 33: Close-up of the cell in figure 32, with the vacuole (V), phaeoplasts (P), pyrenoids (Py), mitochondria (M) and girdle bands (Gb) labelled. Notice the central lamellar structure in the pyrenoid. Pyrenoids are frequently traversed by single thylakoids, as can be seen in this image. The area between the two developing thecae is rich in vesicles. Some of these might be SDVs carrying silica towards the new thecae.

AASi3

Seminavis robusta three days after silica deprivation.

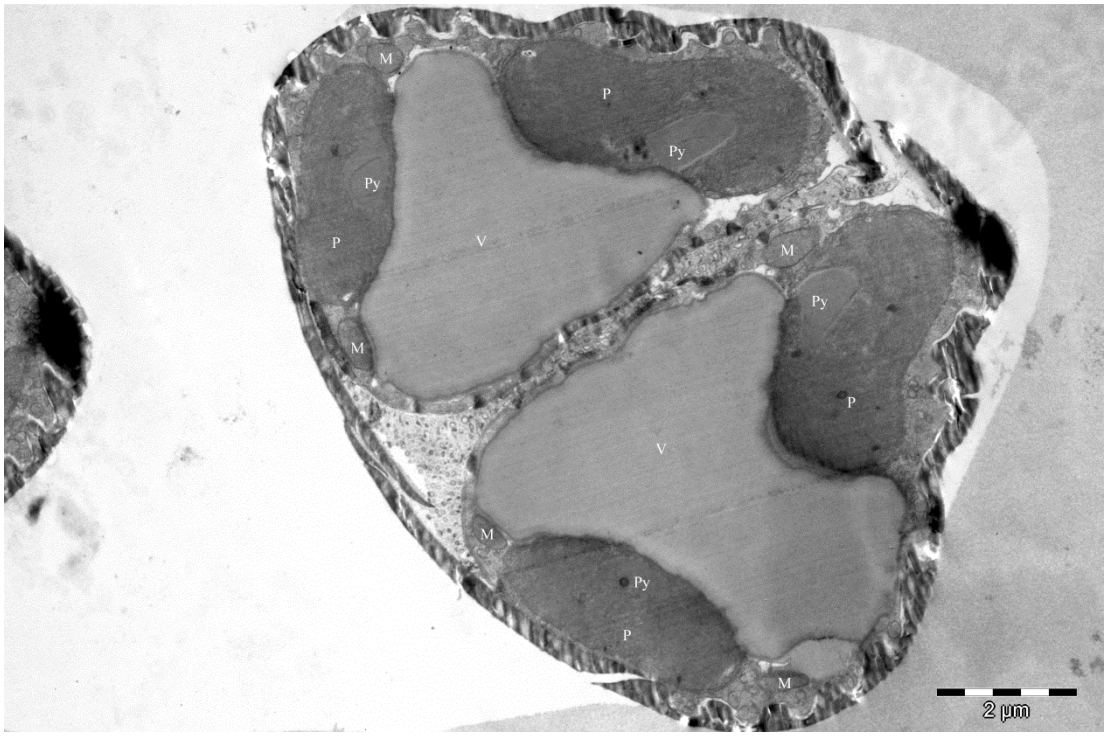


Figure 34: Distal cross section of a dividing *Seminavis robusta* cell after three days in Si- f/2. It is possible to identify the phaeoplasts (P) and pyrenoids (Py), vacuoles (V) and some mitochondria (M) in both daughter cells. The area between the two developing thecae is rich in vesicles. Some of these might be SDVs carrying silica towards the new thecae. There is still no difference in the frustules of the cells in this sample compared to the ones in AASi0.

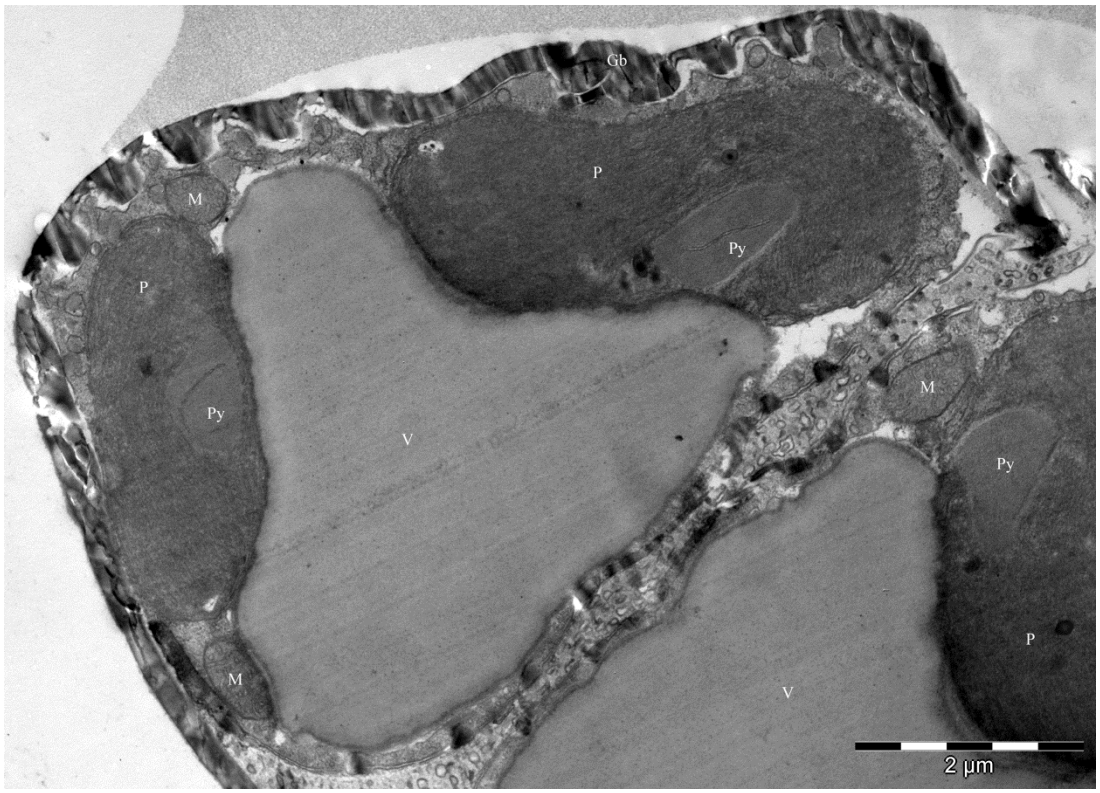


Figure 35: Close-up of the cell in figure 34, with the vacuoles (V), phaeoplasts (P), pyrenoids (Py), mitochondria (M), and girdle bands (Gb) labelled. The area between the two developing thecae is rich in vesicles. Some of these might be SDVs carrying silica towards the new thecae.

Figure 36: Distal cross section of a dividing *Seminavis robusta* cell after three days in silica-free f/2. It is possible to identify the phaeoplasts (P) pyrenoids (Py), vacuoles (V) and mitochondria (M) of both daughter cells. The area between the two developing thecae is rich in vesicles. Some of these might be SDVs carrying silica towards the new thecae. There is still no difference in the frustules of the cells in this sample compared to the ones in AASi0.

Figure 37: Close-up of the cell in figure 36, with the vacuole (V), phaeoplasts (P), pyrenoids (Py), mitochondria (M) and the girdle bands (Gb) labelled. The area between the two developing thecae is rich in vesicles. Some of these might be SDVs carrying silica towards the new thecae.

AASi4

Seminavis robusta after four days of silica deprivation.

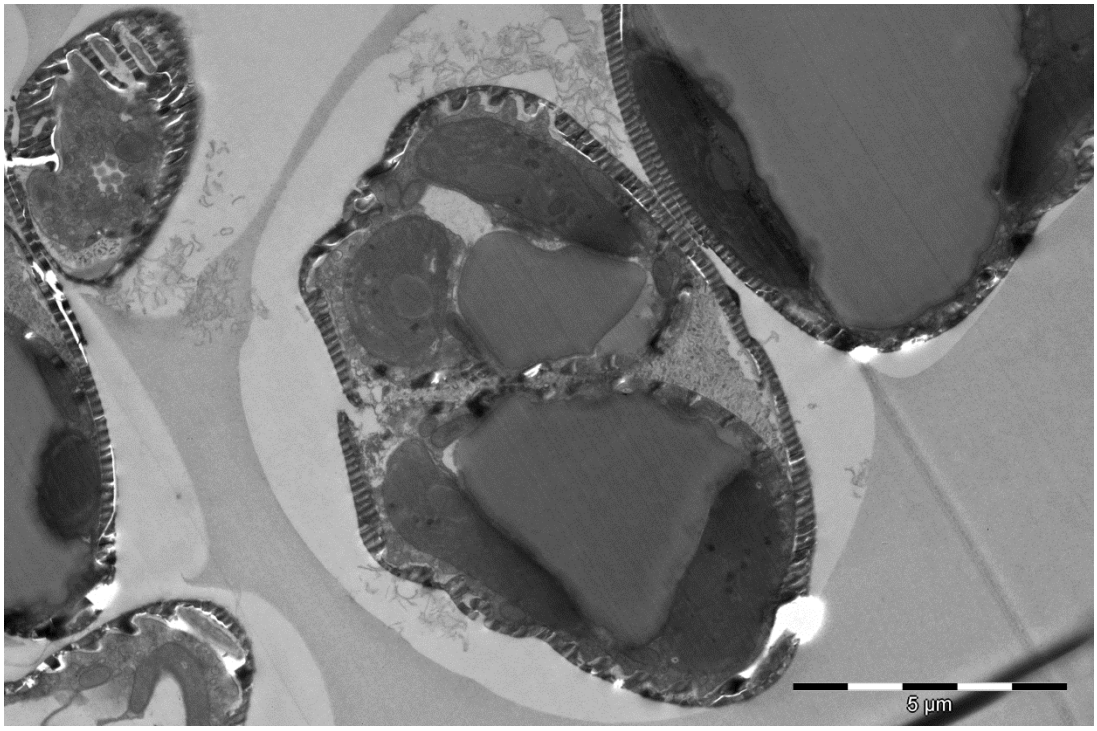


Figure 38: Distal cross section of a dividing *Seminavis robusta* cell after four days in Si- f/2.

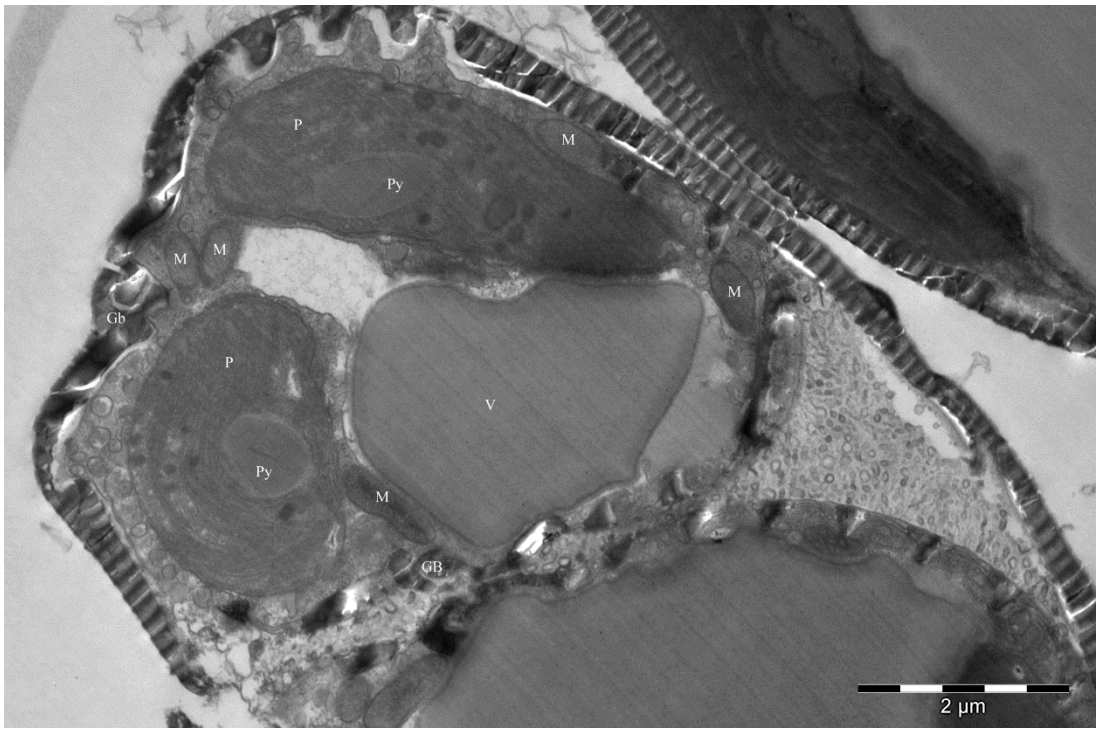


Figure 39: Close-up of the cell in figure 38, with the phaeoplasts (P), pyrenoids (Py), a vacuole (V), mitochondria (M), the girdle bands of the parental theca (Gb) and the girdle bands of the new theca (GB) labelled. The area between the two developing thecae is rich in vesicles. Some of these might be SDVs carrying silica towards the new thecae.

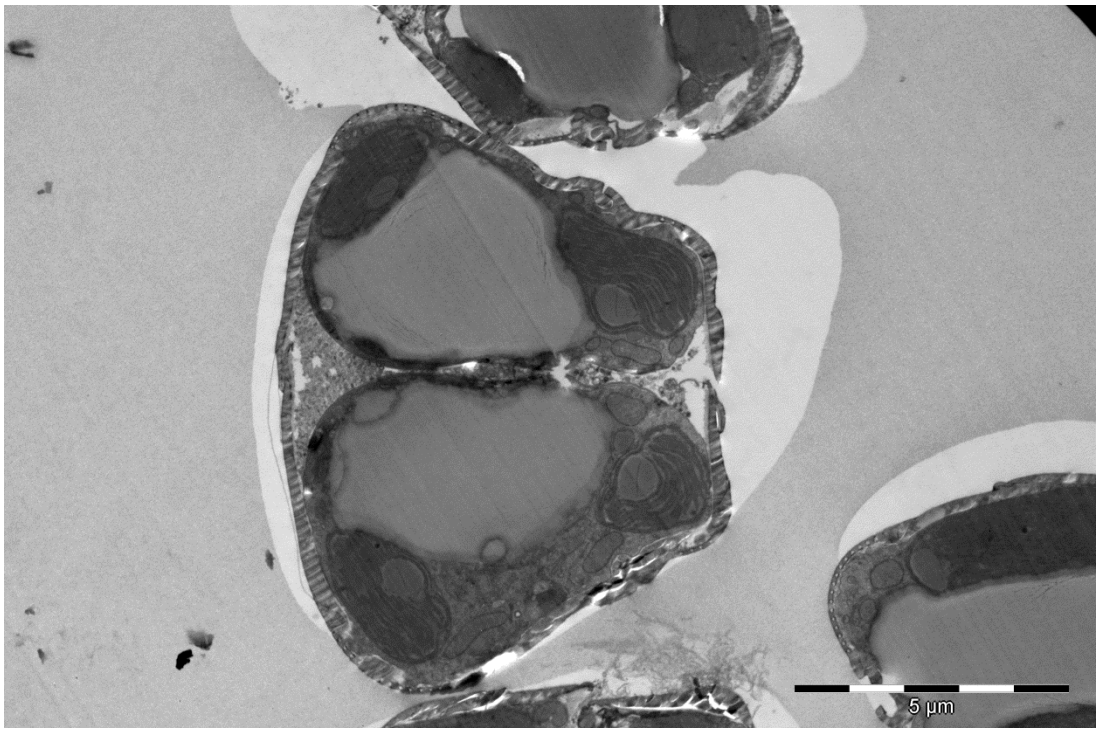


Figure 40: Distal cross section of a dividing *Seminavis robusta* cell after four days in Si- f/2.

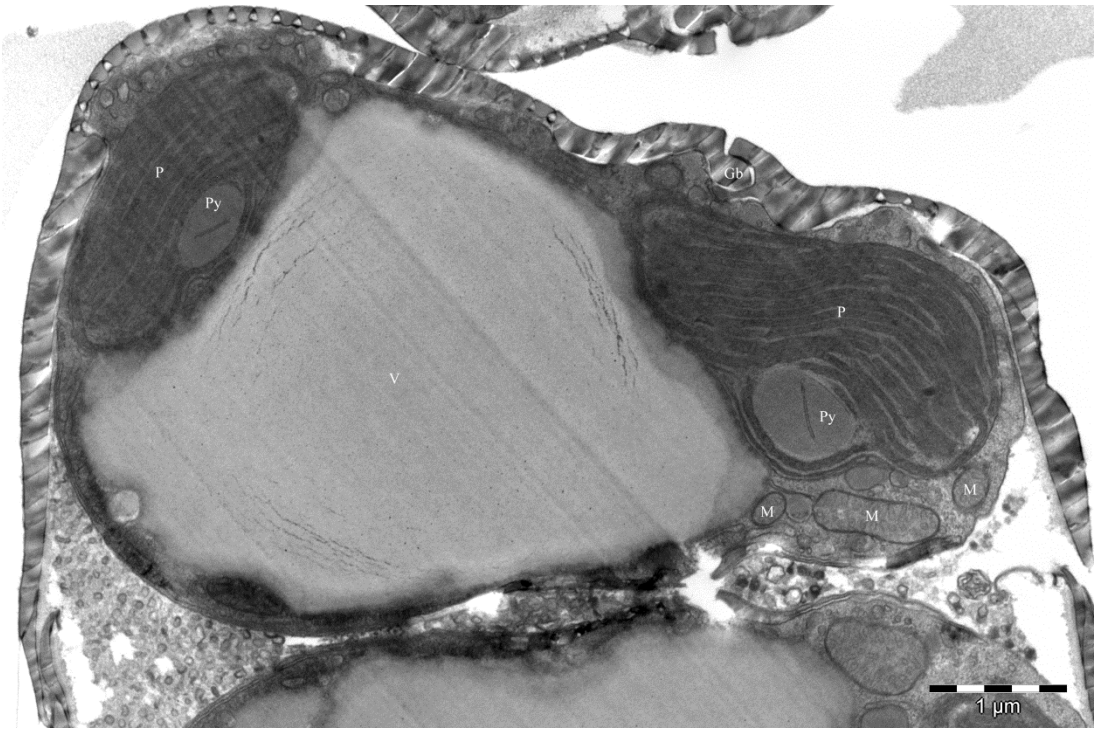


Figure 41: Close-up of the cell in figure 40, with the phaeoplasts (P), pyrenoids (Py), vacuole (V), mitochondria (M) and girdle bands (Gb) labelled. The area between the two developing thecae is rich in vesicles. Some of these might be SDVs carrying silica towards the new thecae.

AASi5

Seminavis robusta five days after silica deprivation.

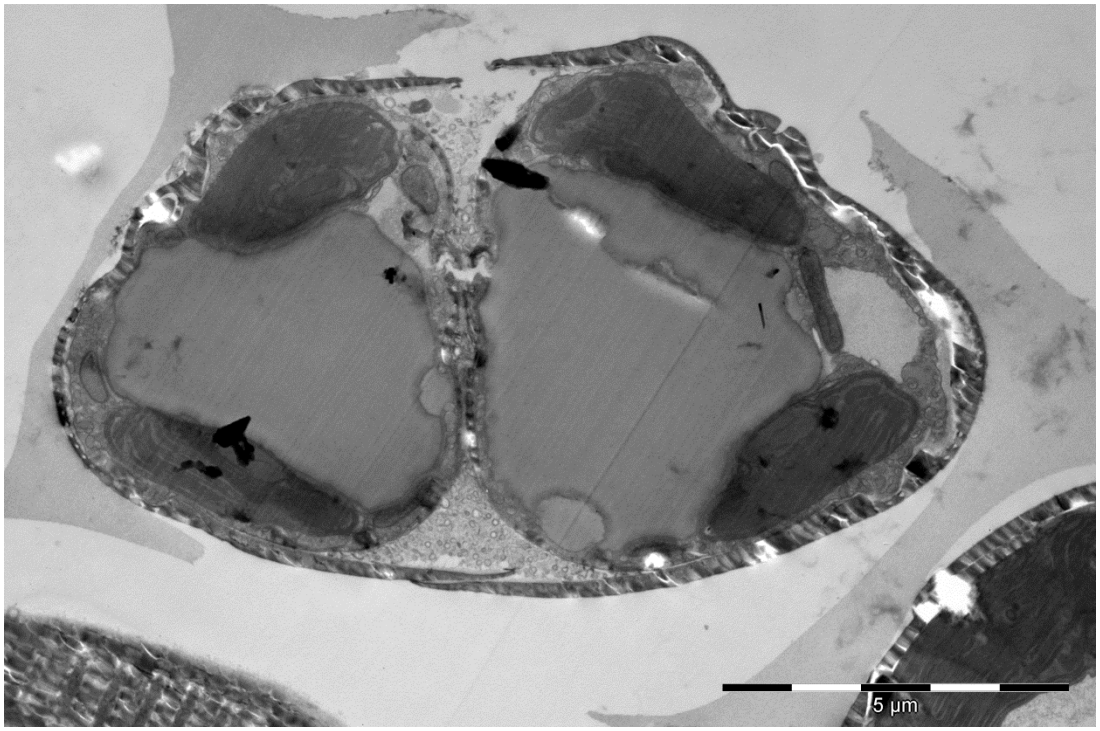


Figure 42: Cross section of a dividing *Seminavis robusta* cell after five days in Si- f/2. The frustule of this cell is of uneven thickness and generally thinner than the frustules of *S. robusta* that have not been exposed to silica deprivation. This is an example of barely enough silica having been deposited by the SDVs during cell division.

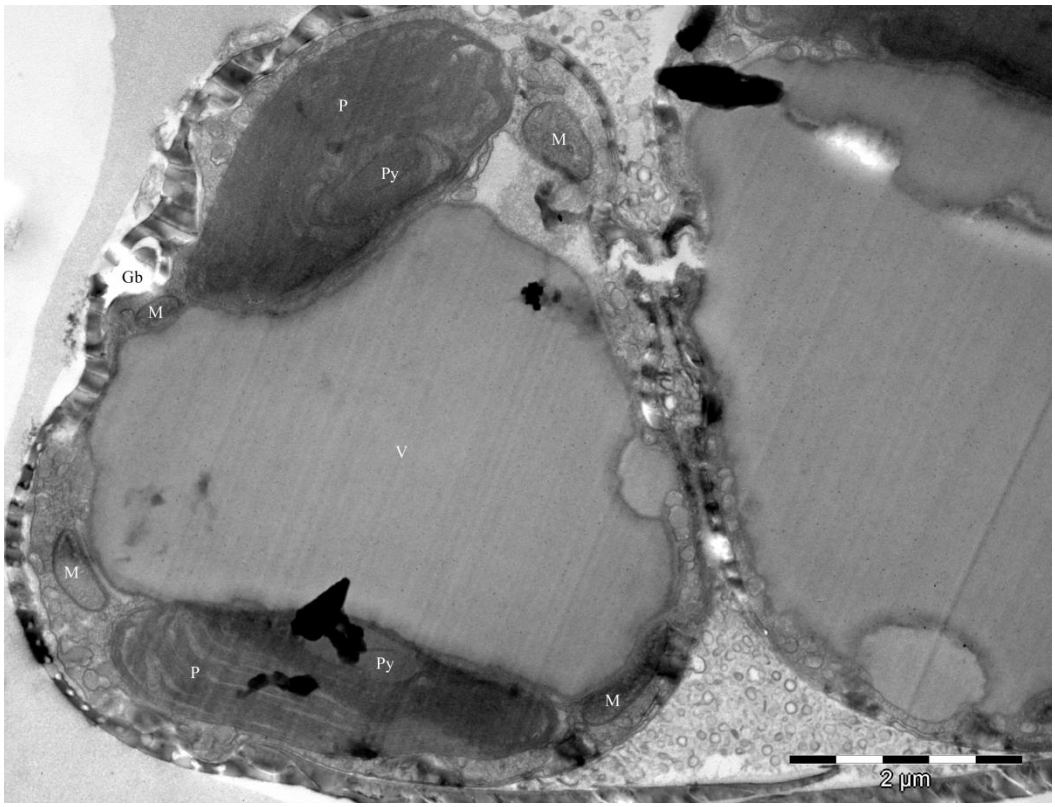


Figure 43: Close-up of the cell in figure 42, with the phaeoplasts (P), pyrenoids (Py), vacuole (V), mitochondria (M) and girdle bands (Gb) labelled.

It was observed that the percentage of cells in division, compared to cells not in division, increased with each day. This, along with the numbers showing that after three days the number of cells in the flask stops increasing, suggests that the lack of silica during frustule formation means the cell will not be able to complete the division and is arrested in telophase, this being the phase all the cells in division were found in. After four and five days in Si- f/2 cells with deformed frustules or frustules of varying thickness, were observed. This might indicate that when their parental cells divided there was just barely enough silica to make incomplete new thecae.

More cellular debris and empty frustules were found after five days than after three and four days. The appearance of cellular debris in the samples after four and five days suggests that the cells became increasingly more fragile and less able to endure the preparation protocol without shattering. The appearance of completely empty frustules in the sample taken after five days suggests the presence of many dead cells in the culture flask the sample was taken from.

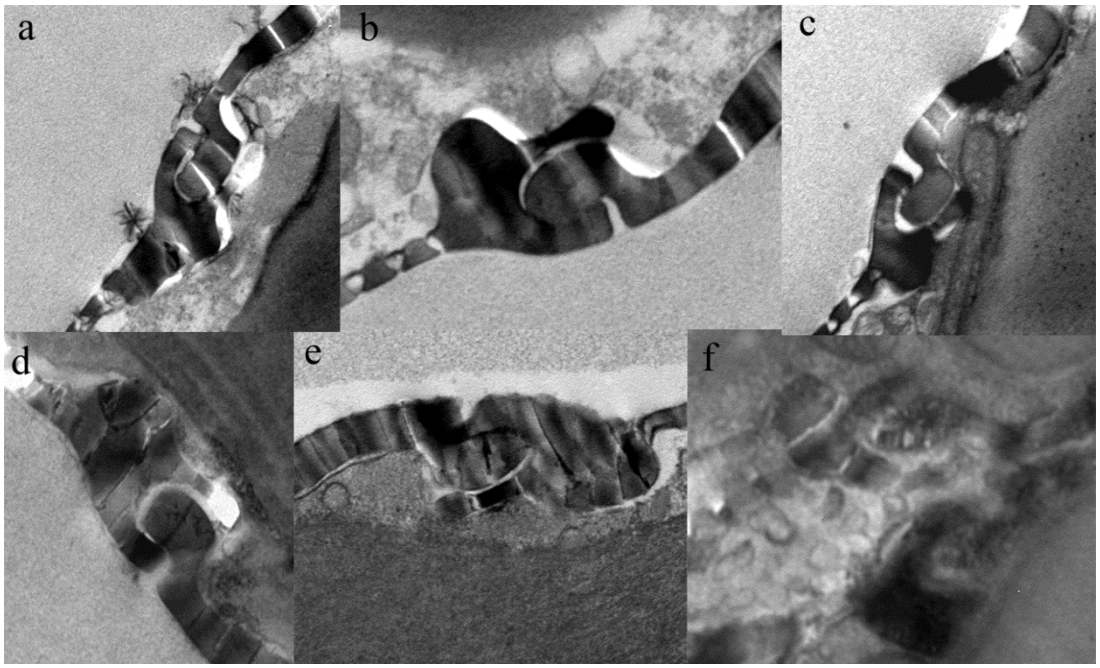


Figure 44: Cross sections of girdle bands. The girdle bands are circular bands of silica attached to the edge of the valve, which link the valves together. In cross section these bands appear as hinge like structures in the frustule (a-e). This figure also shows the girdle bands of two thecae being formed during mitosis of the cell in figure 38 and 39 (f).

Frustule formation during mitosis.

The silica starvation experiment resulted in many images of cells in different stages of telophase. Figure 45 shows a model of frustule formation during cell division, put together based on those images.

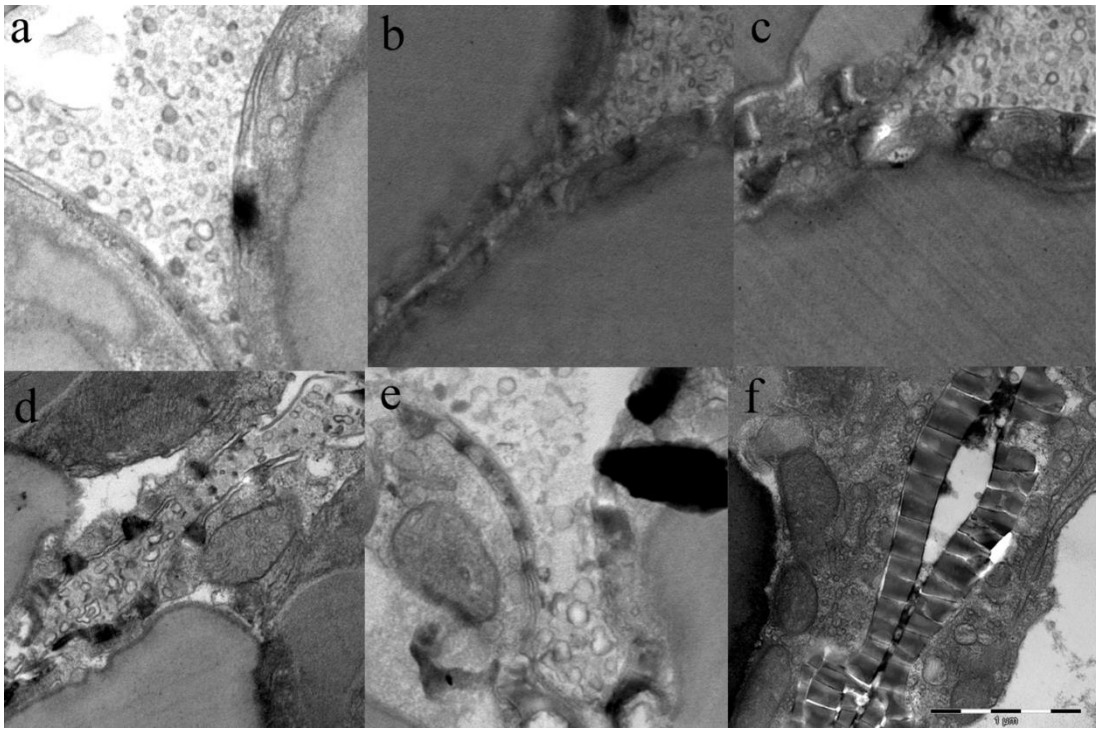


Figure 45: Different stages of frustule formation during mitosis. A new membrane appears under the plasma membrane on the side away from the parental theca (a). Silica forms into rods at even intervals between the membranes (b). These then expand, first into cones with the point towards the intracellular space (c, d) and then into squares (e) until they fuse and complete the formation of the frustule (f). During the formation of the new frustule there can be found a large number of vesicles between the two new cell walls (a-e). Most of these are most likely SDVs, this suggests that there is still much traffic over the plasma membrane by e.g. silica transporters.

3.1.3 Thalassiosira pseudonana

For TEM studies of *Thalassiosira pseudonana* the AAS8 fixation method from section 2.2.1 was used since this method most effectively preserved the internal structures of *Seminavis robusta*.

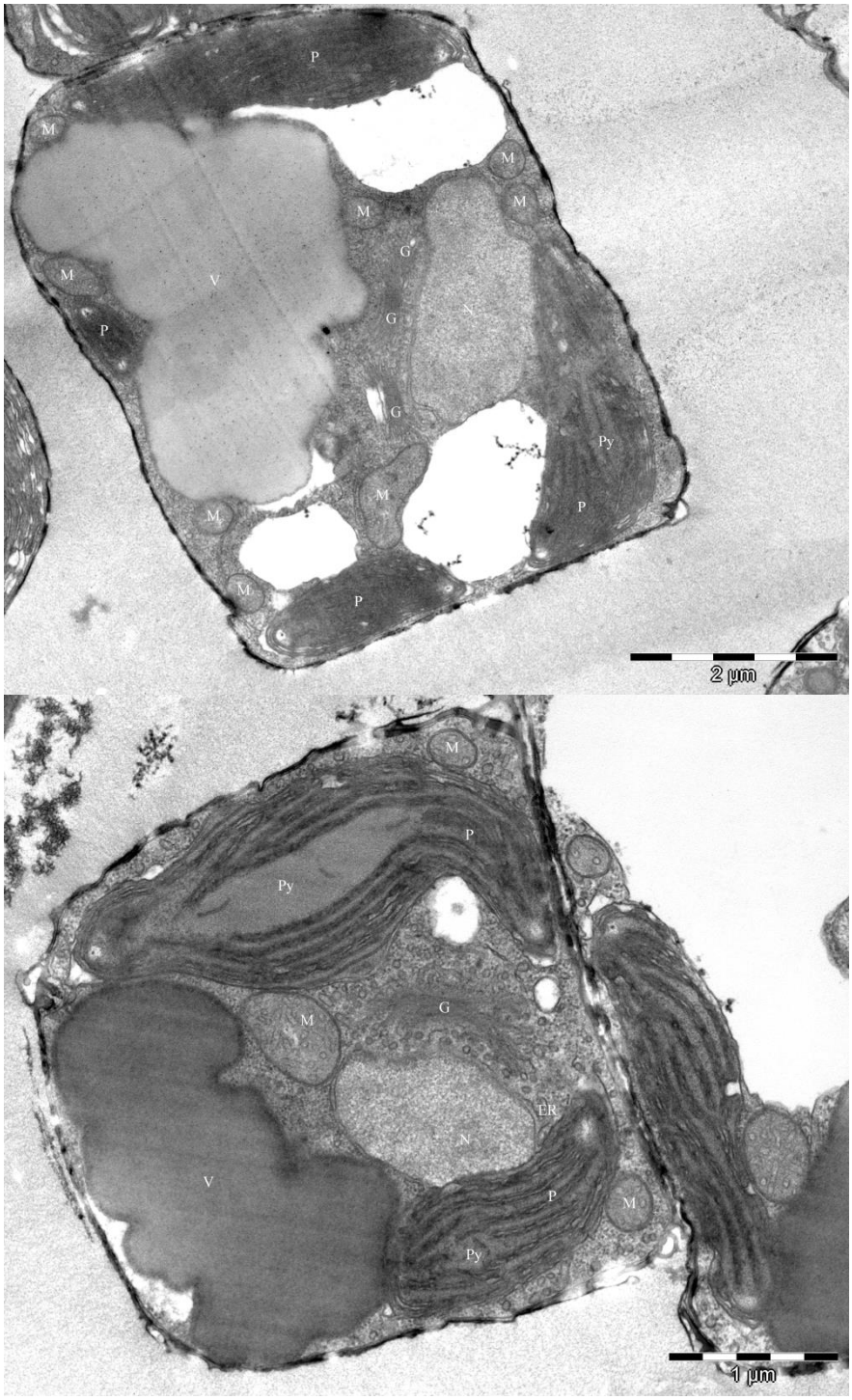


Figure 46: Longitudinal sections of *Thalassiosira pseudonana* cells. It is possible to see the nuclei (N), vacuoles (V), phaeoplasts (P), pyrenoids (Py), some mitochondria (M), endoplasmic reticulum (ER) and Golgi apparatuses (G). There are some empty spaces inside these cells.

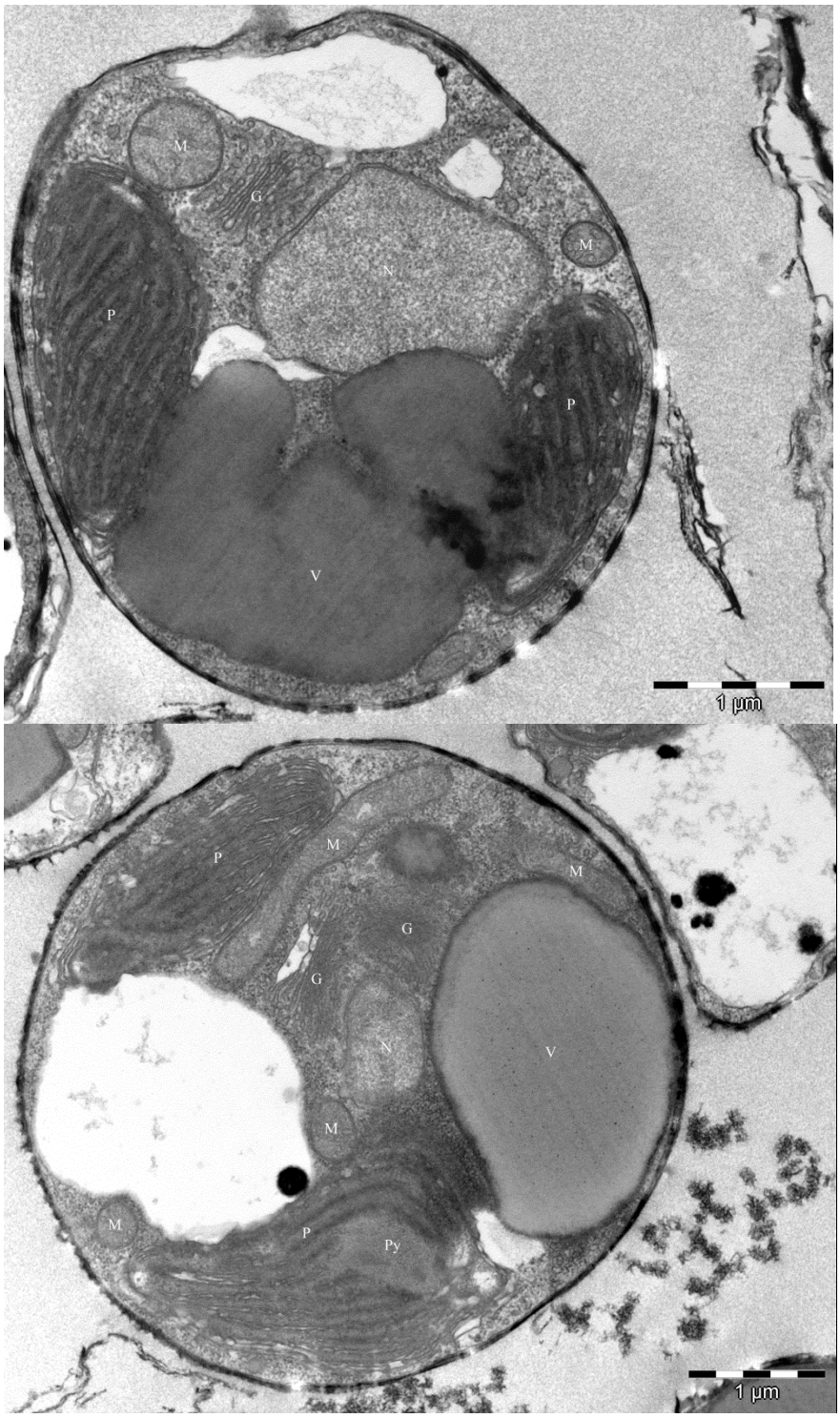


Figure 47: Cross sections of *Thalassiosira pseudonana* cells. It is possible to see the nuclei (N), vacuoles (V), phaeoplasts (P), pyrenoids (Py), some mitochondria (M) and Golgi apparatuses (G). There are some empty spaces inside these cells.

In the sections shown in figures 46 and 47 many intracellular structures of *Thalassiosira pseudonana* can be seen, such as the nucleus, vacuole, mitochondria, phaeoplasts and Golgi apparatuses. There are holes in all four cells, where no intracellular structures or cytoplasm can be seen.

Fixatives infiltrate smaller cells faster than larger ones. This should mean that *T. pseudonana* would be better fixated than *Seminavis robusta* when using the same fixatives and infiltration time. The cells in the *T. pseudonana* experiment done for this thesis were, however, not better preserved than any of the *S. robusta* cells fixated with the same method. As can be seen if figures 46 and 47 are compared with e.g. figures 19, 26 and 31. The reason for this might be that the pores in the frustule of *T. pseudonana* are smaller than those of *S. robusta*, making the fixative infiltrate the *T. pseudonana* cells slower.

3.2 CLSM

3.2.1 BODIPY 505/515

In this experiment living *Seminavis robusta* cells were treated with BODIPY 505/515 in order to test the hypothesis that the large vacuoles of the cells store lipids.

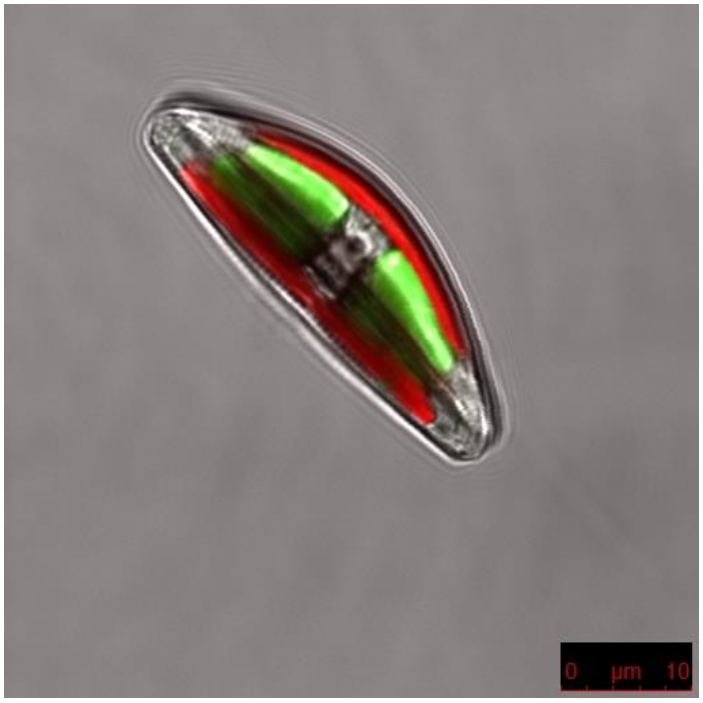


Figure 48: *Seminavis robusta* treated with BODIPY 505/515. The green colour represents the fluorescence of the BODIPY probe. The red colour represents the autofluorescence of the phaeoplasts.

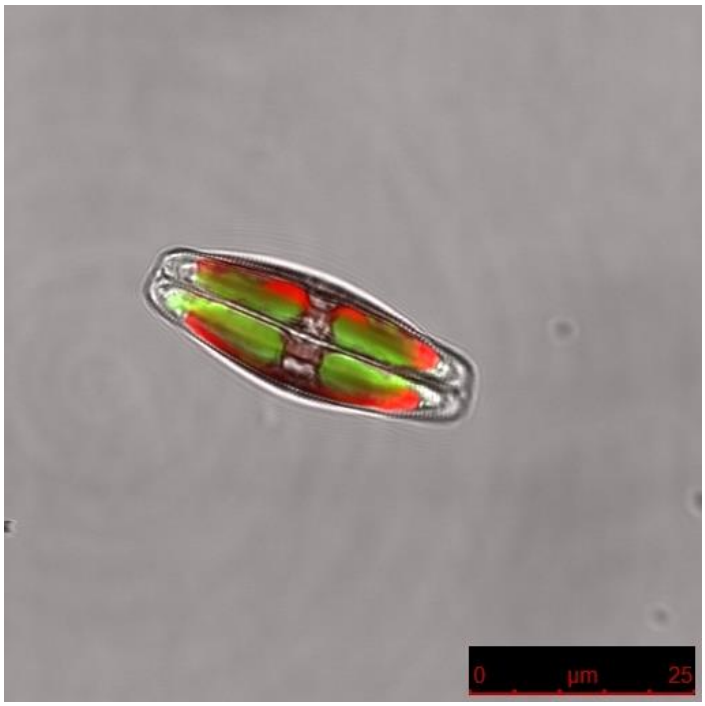


Figure 49: A dividing *Seminavis robusta* treated with BODIPY 505/515. The green colour represents the fluorescence of the BODIPY probe. The red colour represents the autofluorescence of the phaeoplasts.

Figures 48 and 49 show that the green colour of the BODIPY 505/515 is located inside the two large vacuoles of the *Seminavis robusta* cells. This suggests that neutral lipids are stored inside these vacuoles.

3.2.2 Aniline blue

In this experiment *Seminavis robusta* cells were imaged in a timeline after the addition of aniline blue to the medium, in order to see how the cells absorb the probe and how it is distributed throughout the cells.

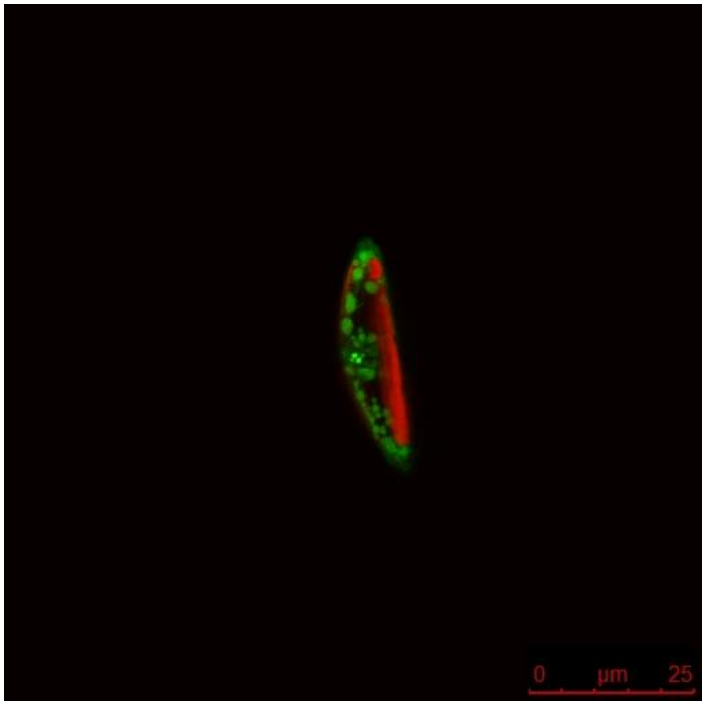


Figure 50: *Seminavis robusta* cell one minute after addition of aniline blue. The green colour represents the fluorescence of the probe. The red colour represents the autofluorescence of the phaeoplasts. The aniline blue has been absorbed by the cell and is now contained in vesicles throughout the cytoplasm.

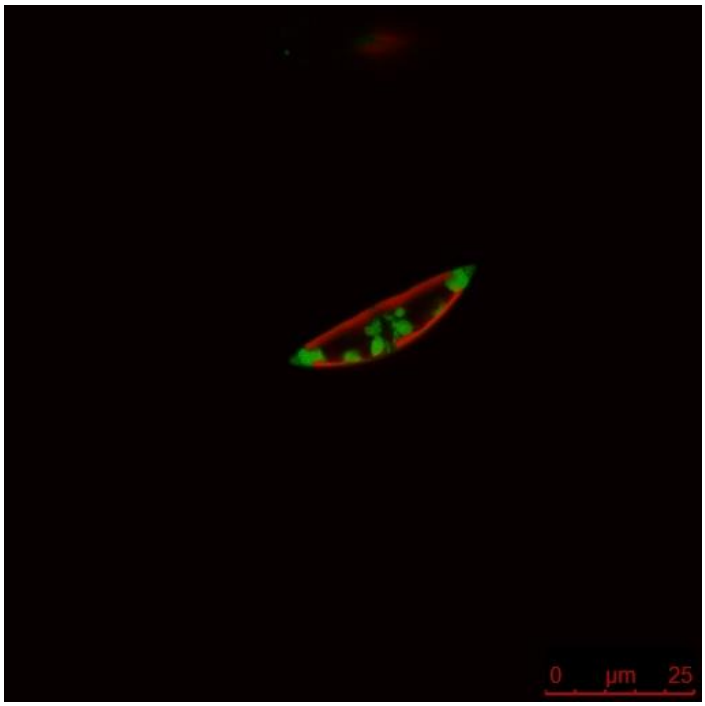


Figure 51: *Seminavis robusta* cell nine minutes after addition of aniline blue. The green colour represents the fluorescence of the probe. The red colour represents the autofluorescence of the phaeoplasts. The vesicles have now merged into larger vesicles and are mostly located in the cytoplasm around the nucleus and at the distal ends of the cell.

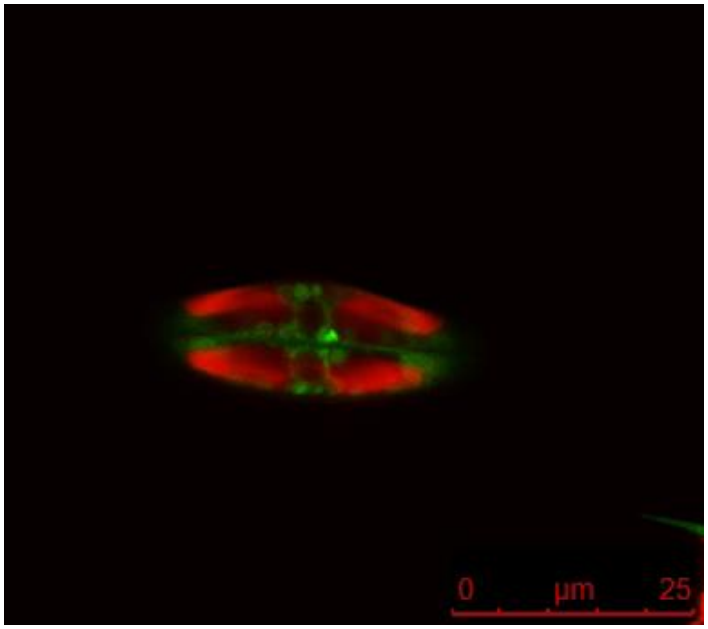


Figure 52: Dividing *Seminavis robusta* cell twelve minutes after addition of aniline blue. The green colour represents the fluorescence of the probe. The red colour represents the autofluorescence of the phaeoplasts. The aniline blue has begun to be distributed in the cytoplasm, the vesicles are getting smaller.

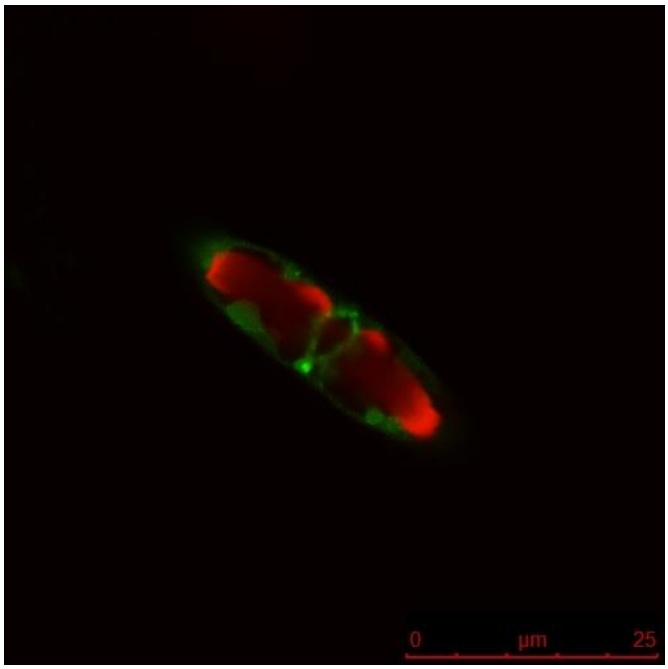


Figure 53: *Seminavis robusta* cell seventeen minutes after addition of aniline blue. The green colour represents the fluorescence of the probe. The red colour represents the autofluorescence of the phaeoplasts. The aniline blue has begun to be distributed in the cytoplasm, the vesicles are getting smaller. There is a bright green ring forming around the nucleus. As we can see in figures 21 and 26 it is in this area that the Golgi apparatuses are located.

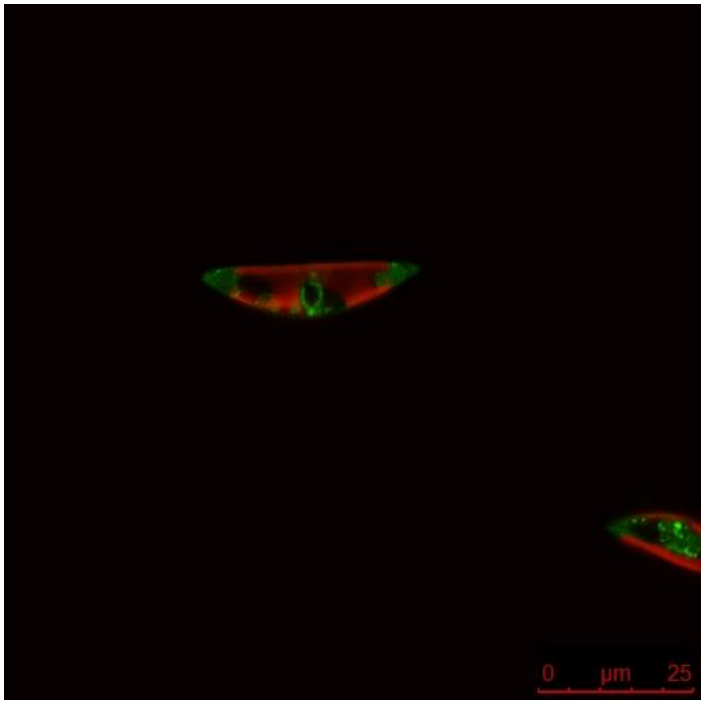


Figure 54: *Seminavis robusta* cell 30 minutes after addition of aniline blue. The green colour represents the fluorescence of the probe. The red colour represents the autofluorescence of the phaeoplasts. The probe is mostly distributed in the cytoplasm. There is no aniline blue in the vacuoles, but there is a bright green ring around the nucleus.

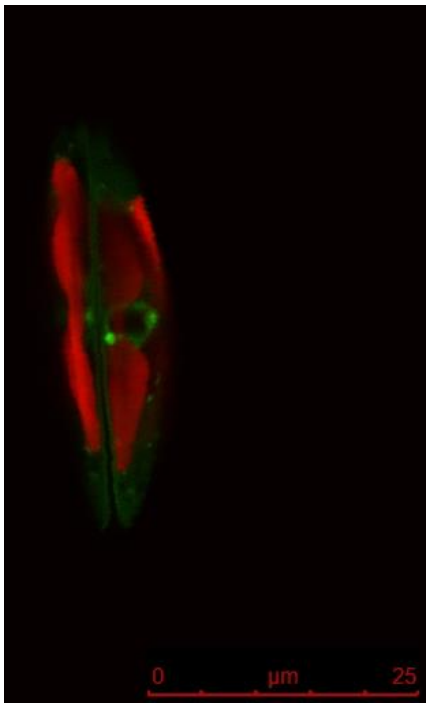


Figure 55: *Seminavis robusta* cell 42 minutes after addition of aniline blue. The green colour represents the fluorescence of the probe. The red colour represents the autofluorescence of the phaeoplasts. At this time the probe is nearly completely distributed throughout the cytoplasm. The bright ring around the nucleus remains, however.

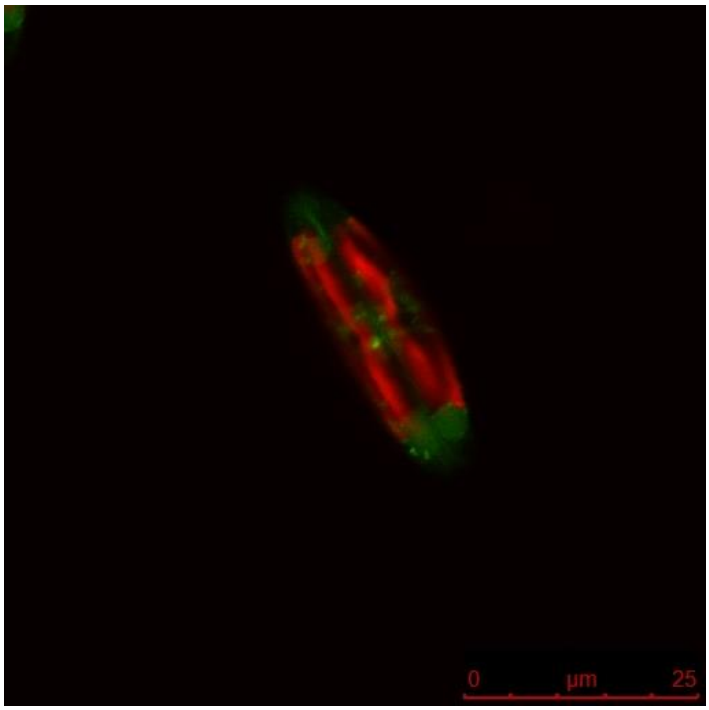


Figure 56: *Seminavis robusta* cell 56 minutes after addition of aniline blue. The green colour represents the fluorescence of the probe. The red colour represents the autofluorescence of the phaeoplasts.

Figures 50-56 show a timeline view of the intake and distribution of aniline blue inside *Seminavis robusta* cells. In figure 50 the probe is located inside vesicles in the cells, which suggests that the probe is taken into the cells by endocytosis. In figures 51-53 the vesicles grow larger. In figures 54-56 the probe is distributed throughout the cytosol, but does not enter the vacuoles. This indicates that there are β 1,3-glucans in the cytosol, or vesicles too small to distinguish from the surrounding cytosol, of *S. robusta*. There is a bright green ring forming around the nucleus. As we can see in figures 21 and 26 it is in this area that the Golgi apparatuses are located.

4. Discussion

Method development.

The methods tested in this section were based on the standard preparation method for TEM from the electron microscopy lab of the Department of Laboratory Medicine, Children's and Women's Health at the Faculty of Medicine. The dehydration and infiltration times were lengthened because it was known from previous experiments that infiltration requires longer incubation time in diatoms than it does in cells that do not have a silica cell wall. Ruthenium red was used to preserve the polysaccharide membrane enveloping the frustule.

The density of the frustule protects the diatoms from outside influences that might be harmful to the cell by limiting the permeability of the cell's surface (Armburst, 2009). The density and thickness of the frustule varies between the different species of diatoms (Armburst, 2009) and any TEM preparation methods need to be optimised for the specific diatom that is to be studied.

Figure 1 and 2 show *Seminavis robusta* prepared with the AAS1 method from section 2.2.1. The Intracellular structures of the diatoms are extensively damaged and there are large empty spaces inside the cells. This suggests that the internal structures have shrunk or that parts of them have been washed out of the cells during preparation, due to poor fixation. In addition to the damaged cells shown in the figures, debris from ruptured cells was also observed in the sample. HEPES buffer is not intended for use with marine organisms and it is likely that the cells ruptured as the result of osmotic shock.

To save time, the AAS2 and AAS3 method were tested at the same time. The AAS2 fixation method was the same as the AAS1 method, but f/2 was used instead of HEPES buffer. There was observed less debris of ruptured cells in the AAS2 sample than in sample AAS1, which suggests that fixating the cells in f/2 does not give them the same osmotic shock as fixation in HEPES buffer. There was still, however, extensive damage to the intracellular structures of the diatoms in the AAS2 sample.

In addition to the change from HEPES buffer to f/2, half of the AAS2 sample was embedded in the same epoxy resin as the AAS1 sample, but cured for 5 days instead of 3, and the other half was embedded in the harder "H" epoxy resin. This was done because there were

holes in the epoxy around most of the diatoms in the AAS1 sample. An example of this can be seen in figure 2. These holes often appear if the resin is softer than the embedded cells, leading to the knife cutting faster through the resin and pushing the cells up, out of position, in the resin before it cuts through them (Bozzola & Russell, 1999). Any holes in the resin will cause problems during microscopy due to the thermic effects of the electron beam. The energy of the beam melts the resin around the edge of a hole, enlarging it very quickly.

The resin gets harder the longer it is cured, but, as can be seen in figures 3 and 4, there were holes in the resin around the cells in the AAS2S sample. There were, however no holes around the cells in the AAS2H samples, which can be seen in figures 5 and 6. This means that the harder "H" epoxy, combined with the longer curing time stopped the cells from being pushed out of position during sectioning.

AAS3 was the same fixation and embedding method as AAS2, but without the ruthenium red (RR). This was done to see if the RR had any special effect on the diatoms, since the standard fixation method only uses glutaraldehyde (GA). RR was used in AAS1 and AAS2 in order to preserve the polysaccharide membrane believed to envelop the frustule of *S. robusta*. However, as seen in figures 1and 2, no evidence of this membrane was found in the AAS1 experiment. Figures 7-9 show some of the cells from the AAS3 sample. The intracellular structures of the diatoms were about as damaged as in the AAS2 sample. The phaeoplasts, however, were better preserved in the AAS2 sample as can be seen if figures 3-6 are compared with figures 7-9.

To save time the AAS4, AAS5, AAS6 and AAS7 methods were tested at the same time. The AAS4 method was the AAS2 method without any centrifugation. This was done to eliminate any damage to the cells caused by mechanical stress from the centrifugation process. A comparison between figures 5 and 11 and figures 4 and 12, however, shows that the diatoms in sample AAS2 and AAS4 are equally damaged, which suggests that the cells were not damaged by the centrifugation used in method AAS2. The cells in AAS4 were, however, more difficult to find during sectioning, due to being scattered in the gelatine instead of collected in a pellet, and most sections viewed in the microscope only showed one or two cells.

Figures 13 and 14 show *S. robusta* prepared with the AAS5 method, where paraformaldehyde (PAF) was used in addition to GA and RR. The intracellular structures of the diatom in figure 13 are extensively damaged, and there are empty spaces inside the cells in both figures. The phaeoplasts were, however better preserved in this sample than in any of the earlier ones, as can be seen in figure 14.

Fixatives containing 1% GA and 1% PAF have been used successfully to fixate single-celled, marine algae (Lazinsky & Sicko-Goad, 1979; Lazinsky & Sicko-Goad, 1983). In AAS6 this fixative was tested on *S. robusta*. It was suggested that this concentration was not high enough to be effective, so in method AAS7 it was increased to 2% GA and 4% PAF. Figures 15 and 16 show cells fixated with the AAS6 method, and figures 17 and 18 show cells fixated with the AAS7 method. In the cell in figure 15 the nucleus, nucleolus, vacuoles, and several mitochondria and the remains of a phaeoplast can easily be identified. In the cell in

figure 16 the phaeoplasts, vacuole and four mitochondria are easily identified, and the stacks of thylakoid membranes inside the phaeoplasts are clearer than in earlier samples. This might suggest that PAF helps fixate these structures better than GA and RR alone, but there are still some empty spaces inside the cells in this sample. In the cells in figures 17 and 18 the nucleus, vacuole, both phaeoplasts and several mitochondria can be identified. In figure 18 the stacks of thylakoid membranes inside the phaeoplasts are about as clear as in figure 16, but here are still some empty spaces inside the cells in this sample as well.

To save time the AAS8 and AAS9 methods were tested at the same time. In the AAS8 method potassium ferrocyanide was used during post fixation in an attempt to better preserve the intracellular membrane structures of the diatoms. Apart from that it was the same method as AAS5, except with the same centrifugation steps as AAS2. In figure 22 some Golgi apparatuses and a section of the nuclear envelope of a diatom can be seen. Figure 23 shows some vesicles inside the cytoplasm as well as some ER, Golgi apparatuses and a section of the nuclear envelope. That these structures can be observed in the cells of this sample so clearly supports the hypothesis that potassium ferrocyanide helps preserve membrane structures during TEM preparation. There are, however, still some empty spaces inside the cells in the AAS8 sample, meaning this fixation method does not completely preserve the internal structures of the cells.

Uranyl acetate is toxic and radioactive, while tannic acid can be found in high concentrations in oolong tea, which is why it would be beneficial if staining with tannic acid could replace uranyl acetate staining. The contrast of the cells of sample AAS9, however, was so poor that no intracellular structures could be observed on the fluorescent screen of the microscope. Figure 24 shows that some intracellular structures were captured by the camera that took this micrograph, though these structures are very faint. As staining with oolong tea has been known to give good contrast to tissues fixated in GA (Sato et al, 2003) it is possible that the RR in the fixative blocked the effect of the tannic acid in the oolong tea post-fixative.

AAS10 is the AAS8 method where the primary fixation, with GA, PAF and RR, was done at 5ºC instead of at room temperature (ca. 20ºC) like in the other methods. The intracellular structures of the diatom cells decay faster at room temperature than at 5ºC, but the fixative will infiltrate the cells slower at the lower temperature. If figures 20 and 21 are compared with 25-27 it is possible to see that the AAS10 and AAS8 fixation methods preserve the intracellular structures for TEM equally well.

The AASi0 sample was accidentally left in the osmium tetroxide and potassium ferrocyanide post fixative for 2h 50min, instead of the intended 2h. Figures 31 and 33 show that the stacks of thylakoid membranes in the phaeoplasts of the cells in this sample are better preserved and easier to study than in any of the AAS samples. This might be an indication that in any future experiment on method development further study of the post fixation time should be done.

During method development for this thesis the main focus was on fixatives for both primary and post fixation. There is still much research to be done in this field and future studies should also focus on incubation time during fixation, post fixation and infiltration.

Characterizing the ultrastructure of *Seminavis robusta*

Seminavis robusta is a pennate diatom, which means it is elongated and bilaterally symmetrical. Figure 26 shows that they have two large vacuoles, one on each side of the nucleus, and that the two phaeoplasts are situated close to the frustule at the top and bottom of the cell. Figures 25 and 26 show that the cytoplasm is pressed up around the inside of the frustule by the large vacuoles and the nucleus is squeezed in the middle. Figures 21- 23 show that the nucleus is surrounded by Golgi apparatuses. In most cells ER can be found as elongated, folded membrane structures closely associated with the nuclear envelope. In *S. robusta*, however, ER can be found as short lengths of double membrane in the cytosol around the nucleus, as can be seen in figures 21 and 23. Figures 30 and 33 show that the pyrenoids in *S. robusta* are elongated and run down the inside of the phaeoplasts.

Frustule formation

Figure 45 shows the different stages of frustule formation during mitosis. A new membrane appears under the plasma membrane on the side away from the parental theca. Silica forms into rods at even intervals between the membranes. These then expand until they fuse and complete the formation of the frustule. During the formation of the new frustule there can be found a large number of vesicles between the two cell new walls. Most of these are most likely SDVs, which suggests that there is still much traffic over the plasma membrane by e.g. silica transporters.

Due to the large distance between the second membrane and the plasma membrane, seen clearly in figure 45 a, it is possible that the second membrane is not an actual membrane, but a protein-based structure associated with the plasma membrane. This structure might work as a scaffolding structure which helps control the formation of the new theca. The silica rods which form the first stage of the silica deposition into the new theca are very evenly spaced, as can be seen in figure 45 b-d. This suggests strict control of this stage of the frustule formation.

Silica starvation

Niu et al (2012) discovered that silica deprivation lead to the frustule of *Phaeodactylum tricornutum* becoming thin enough to allow transformation of the diatom by electroporation. In the silica starvation experiment done for this thesis *Seminavis robusta* was grown in f/2 medium without added Na_2SiO_3 in order to see if silica deprivation would affect cell proliferation and whether the frustules would become thinner due to the silica deprivation, like that of *P. tricornutum*.

The growth curve in graph 1 shows that the concentration of cells in regular f/2 increases until the cells have been in the medium for five days, at which point the concentration of cells in the flask starts decreasing. In the same graph it is shown that the growth in concentration of cells in f/2 without added Na_2SiO_3 (Si- f/2) flattens out after 3 days and the concentration decreases after five days. This graph does not show what happens

between day 0 and 3, so it is not possible to see based on this experiment alone when the cells grown in Si- f/2 stop dividing. That is why the second cell proliferation experiment was performed. In graph 2 we see that the cells continue to divide for three days after being introduced to the Si- f/2. That the cells grown in Si- f/2 stopped dividing before the control cells proves that silica is important for cell proliferation. The reason why the cells grown in Si- f/2 are able to divide for three days after being seeded into the medium is that there are other sources of silica in the filtered seawater the medium is made of, than the Na_2SiO_3 that is added to regular f/2. That both the control and the Si- cells started dying after five days suggest that the reason of death was lack some other nutrient than silica, or the Si- cells would have started dying before the control cells.

In the TEM experiment it was observed that the percentage of cells in division, or arrested in cell division, compared to cells not in division, increased with each day. This, along with the numbers showing that after three days the number of cells in the flask stops increasing, suggests that the lack of silica during frustule formation means the cell will not be able to complete the division, which is arrested in telophase. After four and five days in Si- f/2 cells with deformed frustules or frustules of varying thickness, were observed. This might indicate that when their parental cells divided there was just barely enough silica to make incomplete new thecae.

More cellular debris and empty frustules were found in the samples taken after five days than after three and four days. The appearance of cellular debris in the samples after four and five days suggests that the cells became increasingly more fragile and less able to endure the preparation protocol without shattering. The appearance of completely empty frustules in the sample taken after five days suggests the presence of many dead cells in the culture flask the sample was taken from.

The availability of silica in the environment is important for the formation of the frustule of most diatoms. *P. tricornutum*, however, has the unusual property of being pleiomorphic, meaning they have the ability to alter their shape or size in response to environmental conditions. This plasticity is related to the atypical nature of the cell wall, which is only poorly silicified compared with other diatoms, and as a result of this *P. tricornutum* does not have an obligate requirement for silica (Martino et al, 2007). Based on the silica starvation experiment it is possible to deduce that if there is not enough silica in the environment *S. robusta* will stop dividing during telophase. If only barely enough silica can be found in the environment *S. robusta* will make incomplete thecae for the daughter cells in order to exit telophase. It is also possible that the cells could perform asymmetric distribution of silica, sacrificing one daughter cell to ensure the survival of the other.

Thalassiosira pseudonana

While *Seminavis robusta* is a relatively large species of pennate, benthic diatoms, which can grow to be up to 100µm long (Chepurunov et al, 2008), *Thalassiosira pseudonana* is a species of centric, planktonic diatoms. They have a cylindrical shape with a diameter of about 4-6µm and a length of about 4-5µm (Armbrust et al, 2004).

Samples of *T. pseudonana* were provided by Matilde Skogen Chauton, Department of Biotechnology, NTNU, and prepared for TEM using the same method as AAS8 in section 2.2.1 to see if this method would work on other diatom species than *S. robusta*.

Fixatives infiltrate smaller cells faster than larger ones. This should mean that *T. pseudonana* would be better fixated than *S. robusta* when using the same fixatives and incubation times. If figures 46 and 47 are compared with e.g. figures 19, 26 and 30 it is possible to see that the *T. pseudonana* cells were, however, not better preserved than the *S. robusta* cells. Many intracellular structures are preserved in both species of diatoms, such as vacuoles, phaeoplasts, nuclei, mitochondria and Golgi apparatuses, but there are also large, empty spaces inside both species, where the intracellular structures have been washed out of the cells due to poor fixation. The reason why *T. pseudonana* are not better preserved than the *S. robusta* cells fixated with the same method might be that the frustule of *T. pseudonana* is denser and the pores smaller than those of *S. robusta*, making the fixative infiltrate the *T. pseudonana* cells slower. These findings confirm that TEM preparation methods need to be optimised for the specific diatom that is to be studied.

BODIPY 505/515

In this experiment living *Seminavis robusta* cells were incubated in 3µM BODIPY 505/515 in f/2 for 10 minutes in order to test the hypothesis that the large vacuoles of the cells are used to store lipids. BODIPY 505/515 stains neutral lipids and has previously been used to monitor oil storage in living algal cells (Cooper et al, 2010; Xu et al, 2013).

In figures 48 and 49 the green colour of the BODIPY 505/515 is located inside the two large vacuoles of the *S. robusta* cells. This suggests that neutral lipids are stored inside these vacuoles.

Aniline blue

Aniline blue is a water soluble dye that binds to the β 1,3-glucan callose in plant cells. Callose has been found to be associated with the frustule in diatoms. In this experiment *Seminavis robusta* cells were imaged in a timeline after the addition of aniline blue to the medium, in order to observe how the cells absorb the probe and how it is distributed throughout the cells.

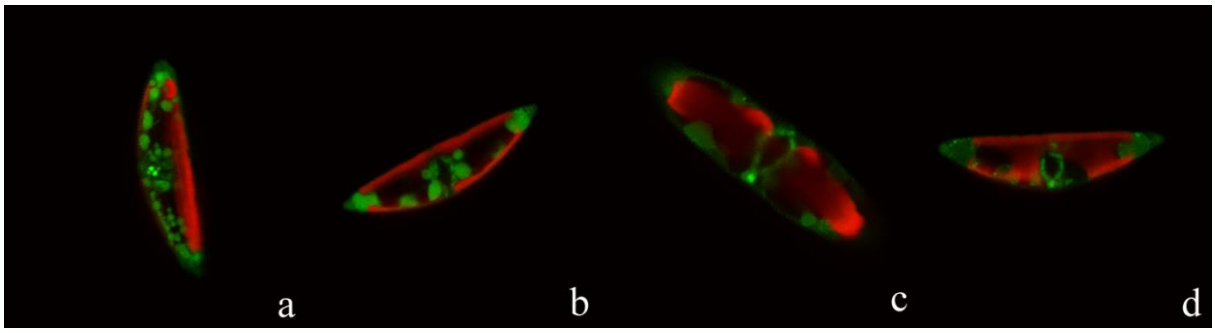


Figure 57: Timeline view of the intake and distribution of aniline blue inside *Seminavis robusta* cells. The green colour represents the fluorescence of the probe. The red colour represents the autofluorescence of the phaeoplasts. The probe is absorbed by the cells through endocytosis and contained in many small vesicles inside the cells (a). These vesicles fuse together into larger vesicles which are located in the cytosol around the nucleus and at the lateral ends of the cells (b). The aniline blue is then distributed in the cytoplasm, the vesicles begin shrinking and the green fluorescence from them becomes fainter. A bright green ring forms around the nucleus (c). The probe is mostly distributed in the cytosol or vesicles too small to distinguish from the surrounding cytosol, suggesting there is callose at these locations. There is no aniline blue in the vacuoles, but there is a bright green ring around the nucleus (d), suggesting there is a high concentration of callose being packaged and transported by the Golgi apparatuses.

5. Conclusion

The method of TEM preparation for *Seminavis robusta* that worked best of the ones that were tested in this thesis was method AAS8, which worked about equally well on *Thalassiosira pseudonana*.

During method development for this thesis the main focus was on fixatives for both primary and post-fixation. There is, however, still much research to be done in this field and future studies could with benefit, also focus on incubation time during fixation, post fixation and infiltration.

The availability of silica in the environment is important for the formation of the frustule of most diatoms. If there is not enough silica in the environment *S. robusta* will stop dividing during telophase. If only barely enough silica can be found in the environment *S. robusta* will make incomplete thecae for the daughter cells in order to exit telophase. It is also possible that the cells could perform asymmetric distribution of silica, sacrificing one daughter cell to ensure the survival of the other.

External nutrients are key regulators of diatom growth. In the marine environment nutrients, such as nitrate, silica and phosphate are important, and evidence also indicates dissolved iron as being a limiting resource for phytoplankton growth (Falaciatore & Bowler, 2002). In the natural environment of diatoms, silica starvation is rare, and it is more likely that the proliferation of *S. robusta* would be inhibited by some other factor before silica deprivation becomes a problem for the cells. Because of this, *S. robusta* may not have mechanisms to detect when the concentration of silica in the environment is too low for it to be able to complete a cell division.

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Appendix

Appendix A

Silica starvation

Cell proliferation

Days	Control (cells/mL)	Si - (cells/mL)
0	2440	3026
3	17966	22700
4	30733	23633
5	33633	24833
6	31733	20067
7	25766	18133

Table 1: Cell proliferation over time in regular f/2 (control) and f/2 without added Na_2SiO_3 (Si-).

Days	Control (cells/mL)	Si - (cells/mL)
0	3160	3987
1	7473	10447
2	16267	18567
3	23467	25667
4	30333	25800

Table 2: Cell proliferation over time in regular f/2 (control) and f/2 without added Na_2SiO_3 (Si-).

Appendix B

STANDARD PROCEDURE

DEHYDRATION AND EMBBEDING

FOR TEM

Chemicals and buffer

0,1 M Sørensens phosphate buffer (pH 7,2)

2% osmium tetrokside (OsO4) i 0,1 M Sørensens phosphate buffer (frozen in 2 mL 4% OsO4, diluted 1+1 with distilled water)

Ethanol (without additives)

Propylene oxide

Epoxy resin LX-112

DMP-30

Equipments

Glass pipettes, pasteur pipettes, cellulose wadding, 1 mL disposable syringe, dram glass, embedding molds, preparation neddle, labels, tweezer

Fume hood

Rotator

Heating cabinet connected to the exhaust, 60°C

Gloves (EN374)

Procedure

The entire procedure takes about 1 week.

- The tissues are pre-fixed in 2 % glutaraldehyde, a minimum of 4 hours.
- 2X5 minutes rinse in 0,1 M Sørensens phosphate buffer
- 1 hour post fixation in osmium tetroxide (protected from light)
- 2X5 minutes rinse in 0,1 M Sørensens phosphate buffer
- 1X10 minutes **50 %** alcohol
- 1X10 minutes **70 %** alcohol
- 1X10 minutes **90 %** alcohol
- 4X15 minutes **absolute** alcohol (The last 2 bottles from full bottle)
- 2X15 minutes **propylene oxide**

All epoxy resin must be added 0,15 ml DMP-30 in 10 mL epoxy and stir well before using.

- 30 minutes 2+1 **propylene oxide+epoxy resin**
- 30 minutes 1+2 **propylene oxide+epoxy resin**
- **Pure and freshly prepared** epoxy esin overnight (2 mL each glass)
- The glasses are placed on rotator overnight (without lid!)

- Use **freshly prepared** epoxy resin for embedding

- Add labels to the molds using a tweezer

- Transfer the tissue to the molds using a preparation neddle. Wait until the tissue have sunk to the bottom. Fill to the top with epoxy resin!

- Polymerize at 56-60°C for 3 days in a heating cabinet

- Once polymerized, put the molds in the fume hood for cooling

- Remove the blocks from the molds with a tong and store them in a macro-cassette

The molds can be reused!