A rapid cleaning method for diatoms

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Abstract

We describe here a protocol for cleaning diatoms when time is short and the amount of sample is very limited. Essentially, the method consists of drying material onto coverslips and cleaning it directly *in situ* using nitric acid (or hydrogen peroxide), which is evaporated to dryness. After washing twice or a few times with deionized water, the coverslips are ready for mounting in resin for light microscopy as usual, or attachment to stubs for scanning electron microscopy. Besides speed, the method has the advantage that it often preserves some frustules intact or leaves their different elements (and stages of valve formation) closely associated with each other. Examples where the method is especially advantageous are to clean small aliquots of cultures for identification or to act as vouchers, or to explore diversity of the most abundant species in natural material (e.g. periphyton). It is less suitable for counts in ecological or palaeoecological studies. We tabulate the many other cleaning methods to provide context for the new method described here.

Keywords: *cleaning procedure, diatoms, digestion, nitric acid cleaning, peroxide cleaning, preparation methods*.

Introduction

Perception of the importance of diatoms has grown for various reasons, including the realization that they are major players in the global carbon cycle, their almost unique capacity to transform dissolved silicate into patterned silica structures, and the insights their remains give about present and past environments. Increasingly, species are being brought into culture for genomic and transcriptomic studies, to investigate phylogenetic relationships and population genetics, to study cellular and life history processes, and to provide material for experimentation and surveys of secondary metabolite production. For all of these, accurate identification and vouchering are necessary for results to be interpreted in relation to other findings and made repeatable.

To identify diatoms it is necessary to clean material so that the details of the valve and frustules are clearly visible during study under either the light microscope (LM) or the scanning electron microscope (SEM). Diatom cleaning methods vary (Table 1) but most work by removing organic material through chemical oxidation, often by H_2O_2 or concentrated acid (e.g. HNO_3 or H_2SO_4). These methods have different pros and cons, but in our experience almost all of them require substantial amounts of material (sample and reagents) and take many person-hours to obtain clean material ready for mounting in LM preparations or on SEM stubs, because of the oxidization itself and then the successive rinsings with deionized water. These two aspects – time and sample quantity – are often a problem when dealing with cultures because i) we need to know what is isolated as soon as possible in order to decide whether the culture is worth keeping or studying; and, ii) some studies, e.g. to construct metabarcoding reference databases (Kelly et al. 2018, Rimet et al. 2018, and unpublished) and population genetic studies (e.g. Vanormelingen et al. 2015), require very large numbers of clones to be grown and vouchered.

We describe here a fast cleaning method suitable for very small amounts of material, which uses very small volumes of oxidizing reagents (ca 1–2 ml per preparation) and requires a minimum of expensive equipment and consumables (e.g. no centrifuge or filtration devices). The method works best when the material contains a high proportion of diatoms and does not contain much sediment; it can be applied to both freshwater and marine material, though for the latter there needs to be a pre-wash to remove salt. The oxidizing agents tested here are HNO_3 and H_2O_2 but any other agent may be suitable provided it does not contain dissolved salts. We have been using this method for about 10 years (e.g. Mann et al. 2011, Kahlert et al. 2019). The inspiration for it came from Prof. Dr. H.A. von Stosch (e.g. von Stosch 1982), who

heated material with perchloric acid on coverslips to clean and disrupt perizonia, and from Prof. Dr. Aloisie Poulíčková, who was already cleaning material on coverslips with H_2O_2 during the early 2000s.

Material and Methods

Equipment and consumables

- Coverslips, which should preferably be circular, since this makes it easier to add sufficient acid or water without spillage. We use Ø 18 mm coverslips for LM preparations and Ø 13 mm coverslips for SEM.
- Two glass Pasteur pipettes and bulbs (one for acid, one for deionized water).
- Two 100–150 ml beakers, one for nitric acid (the rapid method uses very little acid; probably only 10–20 ml will be needed to produce 10 LM preparations), one for deionized water.
- Ceramic hotplate in a fume cabinet (the ceramic nature of the hotplate is important, because of steps 4–6).
- Paper towel folded x3 to give thickness (for absorbing water drained off coverslips as below); numbers 1 to *x* written on it depending on the number of samples (i.e. coverslips) to be prepared (see step 10 for the reason).
- Extra paper towel (if marine samples need to be pre-treated to remove marine salts)
- Fine forceps, preferably with curved ends.
- Oxidizing agent: nitric acid (60–70% HNO_3) or hydrogen peroxide (ca 35% H_2O_2).
- Deionized water.

Protocol

1. Using a pipette, take a small volume of the sample or culture and place it on a clean, dry cover slip. The volume of sample to be taken depends on the size of the coverslip. For example, for a circular Ø 18 mm coverslip, 0.5 ml is an appropriate volume to work with, and c. 0.3 ml for Ø 13 mm coverslips. The amount of material in the sample should correspond to the final density required in the LM or SEM preparation since all the preparation and processing will occur on the coverslip itself; the amount should not be so high that large clumps or swathes of cells are present.
2. Leave the coverslips to dry undisturbed (e.g. overnight) and avoid dust contamination.

3. Once the coverslips are fully dry, place them on a ceramic hotplate in a fume cabinet with the diatoms uppermost, using fine forceps (NB. To minimize cross-contamination, clean the forceps between different coverslips). Leave enough room between coverslips to avoid the possibility of splashing material from one to another (e.g. space between the coverslips on the hot plate about 3 or 4 times the diameter of the coverslips) and to make manipulation easier. Record which coverslips (i.e. which samples) are placed where on the hotplate.
4. Heat to approximately 90°C (the hotplate can be preheated to reduce processing time).
5. Add the oxidizing agent (65–70% HNO_3 or 35% H_2O_2, previously placed in a beaker) from a Pasteur pipette to give a domed meniscus, taking care not to overflow onto the hotplate (though if it does, it's not a disaster: a ceramic hotplate will not be damaged by the concentrated hot oxidizing agents, which will simply evaporate away. The problem with overflow is that the coverslip may stick to the hotplate and then need to be gently prised off, and also that a little of the material will probably be lost).
6. Allow the acid to evaporate (it should take 10–15 min; if shorter, the temperature of the plate should be reduced), but just before the coverslip dries, add more nitric acid to give a domed meniscus, as before.
7. Repeat steps 5 and 6 as necessary: there should be no coloured residue – any visible residue (the diatoms) should be white or grey.
8. After the second or final acid treatment, allow all the acid to evaporate.
9. If the treatment has been successful, the coverslip should now bear cleaned diatoms , with virtually all organic material oxidized away and only small amounts of salts left from the original water and from decomposition of the diatoms (or other material); these will be removed in the next steps by washing with deionized water.
10. Transfer the coverslips to the paper towel with forceps, taking care to place them with the diatoms uppermost in known positions and record the positions; space them at intervals of at least c. 3–4 coverslip diameters.
11. Add deionized water carefully from a Pasteur pipette to give a fully domed meniscus covering the whole of the coverslip. Try to avoid spillage, but if it happens (it frequently does happen, even when taking lots of care), move the coverslip a little and try again: it is important that the coverslip is exposed to a reasonably high volume of water, to dissolve the residual salts.
12. After 10 min, drain the coverslip by tilting it to c. 80° to the horizontal, then make it horizontal again and repeat step 11. Depending on the sample, further washes may be given, though each one may dislodge a few more frustules.

13. Drain and fully air-dry the coverslip. Drying can be accelerated either by placing the coverslips back on a hotplate, or placing under a heat lamp.
14. Mount the coverslip in Naphrax diatom mountant for LM or attach to stub for SEM examination. Before this is done, it might be good to look at the coverslip under a microscope. With experience, one can estimate from this whether the cleaning has been effective or whether another nitric acid treatment might be worthwhile. To inspect the coverslip with a microscope, transfer it carefully to a microscope slide, with the diatoms facing up, and examine it with dry lenses. This needs care, since the coverslip will easily slide off the slide if not carried very carefully.

If desired, material can be stored dry after step 2, e.g. in a box or slide tray, analogous to the storage of material on mica by nineteenth century diatomists (e.g. Wetzel & Williams 2018).

Pre-treatment for saline samples

Marine, brackish and hypersaline samples need to be pre-washed to remove most of the salts because these will otherwise prevent adhesion of the cells to the coverslips in step 2, so that all cells would eventually be washed away.

a) Put the sample or aliquot of culture into a vial and fix the diatoms with ethanol (final concentration circa 30%). This will fix cells and promote their sedimentation and also begin to dilute the salts.
b) After allowing the cells to sediment (e.g. overnight) gently remove the supernatant without disturbing the suspension at the bottom, and add deionized water with a Pasteur pipette.
c) Allow to sediment and repeat step b, and then add deionized water if the sample contains lots of diatoms.
d) Sample is now ready to enter the main protocol at step 1.

This preliminary washing should also be done with freshwater samples if they have been fixed with a reagent containing dissolved solids (e.g. Lugol's iodine).

Testing **(Figs 1-14)**

To demonstrate the method for this paper, we used four samples: three marine clones (IRTA-CC-1, IRTA-CC-2, and IRTA-CC-3: Figs 1, 7–10) and a natural sample from riu Algars, Catalonia,

Spain (freshwater) (Figs 11–14). Previously, the method has been used to prepare vouchers of freshwater clones or to study vegetative cell and auxospore structure (Mann et al. 2011; Vanormelingen et al. 2013, 2015; Kelly et al. 2018; Mann & Poulíčková 2019) but without including a full explanation of the protocol. Material related to some of these studies is also included here (Figs 2–6).

We asked two staff with different levels of experience in traditional diatom cleaning methods (none and some) to execute the protocol in order to evaluate its description and performance.

The adequacy of cleaning was assessed by LM and SEM. LM observations of 18-mm diameter cover-slip preparations (mounted in Naphrax: Brunel Micoscopes, Chippenham, UK) were made using a Zeiss Axio Imager M2 with a Plan-Apochromat ×100 objective (nominal numerical aperture: 1.4) and differential interference contrast optics; photographs were taken using an Axiocam HRc digital camera and processed using Adobe Photoshop. For SEM, 13-mm cover-slip preparations were fixed to aluminium stubs and sputtered with platinum for 70–80 s at 5 nm min^{-1} (at 25 mA) using an Emitech K575X coater. They were observed using a LEO Supra 55 SEM at 5 kV and 4–5 mm working distance.

Results

Samples prepared by the rapid cleaning method are illustrated in Figs 1–7 (LM) and 8–14 (SEM). HNO_3 produced cleaner valves and frustules than H_2O_2, usually without destroying fine structures (e.g. the hymenes: Fig. 12 shows hymenes with pores less than 10 nm in diameter in *Brachysira* Kützing), though some evidence of erosion was seen in a few specimens (Figs 8, 9). It is not known whether these were frustules that had begun to dissolve after death of the cell. Preparations made following the protocol and using HNO_3 were just as good for identifying species as the more traditional methods listed in Table 1. The results with H_2O_2 were more disappointing and inconsistent: some organic and or inorganic matter was removed but not all (Figs 3, 9), making it difficult to identify all the valves and frustules in a sample. This was not a major problem when dealing with monocultures, except that it took longer to locate well-cleaned specimens, but it was a disadvantage when studying natural samples.

In the natural sample from riu Algars many frustules remained intact (Figs 13, 14) and allowed determination of girdle structure. In the most successful preparations (predominantly HNO_3-treated) of clones or natural material, forming valves were often observed alongside

fully formed valves and girdle bands (Fig. 10). The raphe and pseudo-raphe valves of monoraphid diatoms were often side-by-side in preparations (e.g. Fig. 5), as a result of single frustules being split and spread flat by the oxidative treatment but not scattered far apart.

The first trials of marine material (IRTA clones 1–3) revealed to us that without the pre-wash most of the frustules were lost at steps 10–12, probably because salt prevented firm attachment of the cells to the coverslips.

Discussion

We found that HNO_3 produces cleaner frustules than H_2O_2. This advantage of HNO_3 has already been pointed out by F.E. Round (see personal communication to Nagy 2011 p. 8), although the reasons for any such advantage are unclear and our survey of more than 150 papers published in *Phytotaxa* between 2010 and 2018 (available from the authors on request) showed that H_2O_2-cleaning can often be highly effective, without loss of fine structure (e.g. Tudesque et al. 2016). Unfortunately, there seem to have been no systematic investigations of the effectiveness of different preparation techniques. In some cases where fine structures have been lost, this may be because of the preservative used before or after cleaning, rather than the cleaning method itself.

HNO_3 and H_2O_2 were the oxidizing agents tested here but the method will probably work with other oxidizing agents (e.g. H_2SO_4, $HClO_4$) that do not crystallize at steps 2 and 5 (salts like $K_2Cr_2O_7$, $KMnO_4$, or $KClO_3$ will not evaporate when the coverslips are dried). HNO_3 is not a particularly strong oxidizing agent, compared to several of the chemicals and mixtures listed in Table 1 [see for example https://en.wikipedia.org/wiki/Standard_electrode_potential_(data_page)], but it has the advantage that it is also effective in solubilizing inorganic components, because all common nitrates are soluble.

The advantages of the method described here are speed and the small amounts of sample, chemicals and equipment necessary. In our experience, once samples have been dried onto coverslips, the whole process can be completed and slides made within c. 2–3 h, half of which is unsupervised while the cleaned material is air-drying. It is a practical method when large numbers of clones need to be processed to provide vouchers and for sampling and characterizing the same clones many times during their life cycle. It is also very useful when the identity of a clone needs to be established as soon as possible after isolation, as well as

when the sample is sparse and/or precious, such as when working with type material or the gut contents of small grazers. The method is also particularly suitable for living motile diatoms, which will colonize and attach themselves to coverslips, obviating the need for steps 1 and 2 of the protocol.

Our method is not meant as a replacement for all the methods used previously (Table 1). For example, it is not suitable when many slides are needed from a particular sample, and it is not very good for counting diatoms in ecological or palaeoecological studies. This is because very small subsamples may not be representative of the whole sample and because it is likely that diatoms will not be homogeneously distributed on the coverslip, e.g. as a result of extracellular polysaccharides or unequal drying during the oxidation steps. On the other hand, the fact the cells are not redistributed evenly during specimen preparation can be an advantage if one is interested in studying e.g. auxosporulation, since mother cells and auxospores often remain associated and perizonia often remain partially or fully intact (e.g. Mann et al. 2011). Very few of the traditional methods give this possibility (see Table 1).

Furthermore, even though many frustules are dissociated during cleaning, the valves and girdle bands often remain closely associated, so that there is opportunity to determine the morphology and arrangement of the bands. In the case of monoraphid or other heterovalvar diatoms, the fact that the two valves of a frustule are often located close together after cleaning could be a major advantage for identifying species, especially in natural samples.

With use of this rapid cleaning method, it is common to find delicate early stages in morphogenesis (cf. Fig. 10), which can easily be lost with methods that depend on filtration or sedimentation during washing of bulk-cleaned samples to remove excess oxidant and breakdown products. As might be expected, forming valves are more common in material from actively growing populations.

Several of the advantages of our method are shared with the two ‘dry’ methods listed in Table 1. We have used the muffle-furnace ourselves (e.g. Mann 1988) and, apart from the capital cost of a good quality muffle furnace (one with accurate control over ramping and the final temperature), it is certainly easy and fairly fast. The dry methods also keep cells in the same positions they occupied when dried onto the coverslip, so that, for example, auxospores and gametangia remain together. In our experience, the disadvantages of the dry methods are that all frustules remain complete, inorganic components of cells (e.g. polyphosphate deposits) are not extracted (this must also be true of the low temperature plasma method of Watanabe et al. 2010), distortion and breakage are more frequent, and LM mountant (Naphrax) seems to

penetrate less easily into frustules, many of which therefore contain air bubbles. In addition, coverslips sometimes warp at c. 560°C, unless they are in contact with a flat support over their whole area. These properties can reduce the quality and usefulness of preparations. Photographs of cells prepared by the furnace method were given by Riznyk (1973) and can be compared with our figures.

Finally, we would note that no cleaning method is perfect. The need for pre-treatment of marine samples before the oxidation and washing is probably the weakest aspect of our approach, since it adds to the time before material can be examined. One way around this for cultures of marine or brackish diatoms that adhere to solid substrata (because of raphe-associated movement or through production of stalks or pads) is to place sterile coverslips in the culture vessel to become colonized. These can then be removed with cells attached and drained of almost all saline medium before drying and further treatment. The approach mentioned above for living motile diatoms also permits marine (or brackish) material to be processed without pre-treatment, again because coverslips can be drained of seawater without loss of many cells before entering the cleaning procedure at step 2 (or 3).

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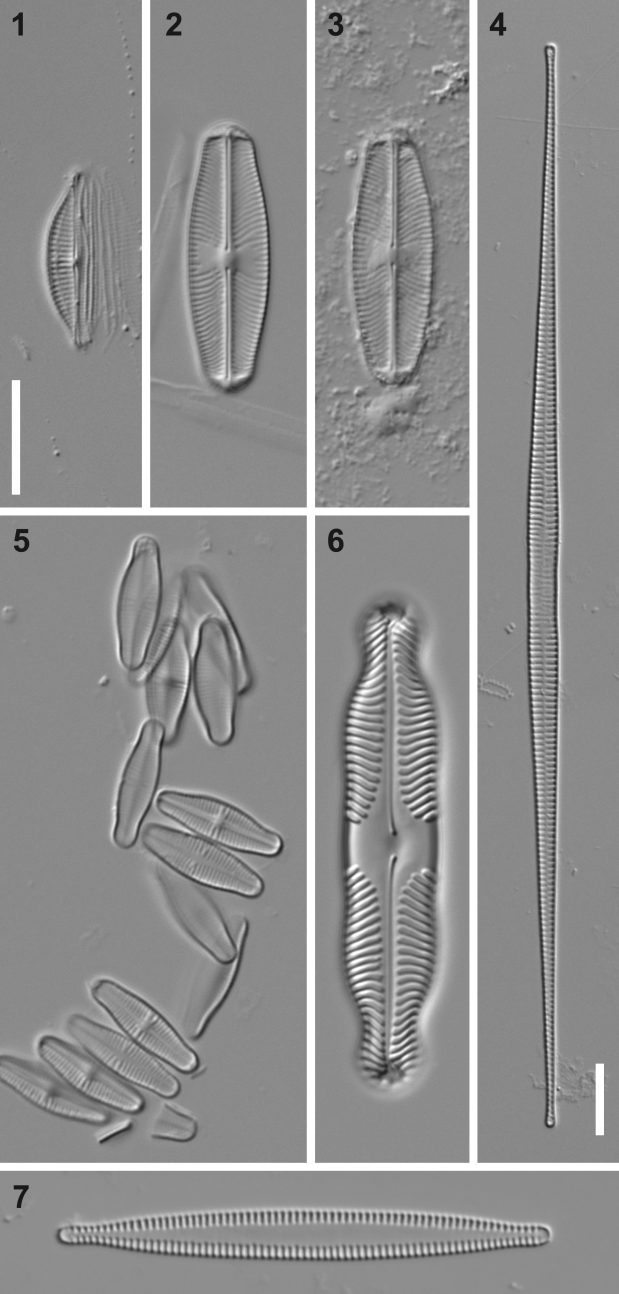
Figure captions

Figures 1–7. Valves prepared for light microscopy by the rapid cleaning method (all except Fig. 3 were prepared using nitric acid), differential interference contrast optics. The voucher slides are held in the Royal Botanic Garden Edinburgh (E). Fig. 1. *Halamphora* (Cleve) Levkov sp. valve with associated girdle bands, clone IRTA CC-2 (slide E6097). Fig. 2. *Sellaphora pausariae* Mann & Poulíčková, clone SEL703B (slide E4201). Fig. 3. *Sellaphora pausariae*, clone Sel515B (slide E5208), prepared using hydrogen peroxide; note the extraneous undigested material, which does not in this case prevent identification but is unattractive. Fig. 4. *Ulnaria acus* (Kützing) Aboal, UK barcode clone 0024 (slide BC0024). Fig. 5. *Achnanthidium minutissimum* (Kützing) Czarnecki species complex, UK barcode clone 0208 (slide BC0208). Fig. 6. *Pinnularia grunowii* Krammer, UK barcode clone 0057 (slide BC0057). Fig. 7. *Tabularia* (Kützing) D.M. Williams & Round sp., clone IRTA CC-3 (slide E6098). Scale bars = 10 µm (for all except Fig. 4, see the bar in Fig. 1).

Figures 8–14. Frustules and valves prepared for SEM by the rapid cleaning method. All except Fig. 9 were prepared using nitric acid; *Halamphora* (Figs 8, 9) was washed beforehand to remove salt. Figs 8, 9. *Halamphora* sp. frustules, clone IRTA-CC-2. Note the poorer cleaning with hydrogen peroxide (Fig. 9; cf. Fig. 3). Fig. 10. *Nitzschia inconspicua* Grunow species complex, clone IRTA-CC-1. Here, as often occurs with the rapid cleaning method, disassembled frustule elements have remained in close association, in this case including an early stage in valve formation (arrow) and separated girdle bands. Figs 11–14. Frustules and valves in a natural periphyton sample from riu Algars, Catalonia. Fig. 11. Interphase frustule of *Cyclotella* Kützing sp. showing the inequality of the epitheca (top, with four girdle bands) and hypotheca (bottom, two bands). Figs 12–14. *Brachysira* sp: hymenate pore occlusions with c. 7-nm diameter pores (Fig. 12), complete frustule in valve view (Fig. 13), and frustule end in girdle view (Fig. 14), showing the structure of the epicingulum, shown to consist of six open bands (bands 1–4 are numbered, and the open end of band 5 is indicated by an arrow). Scale bars = 2 µm (Figs 8–11, 13), 500 nm (Fig. 14), and 100 nm (Fig. 12).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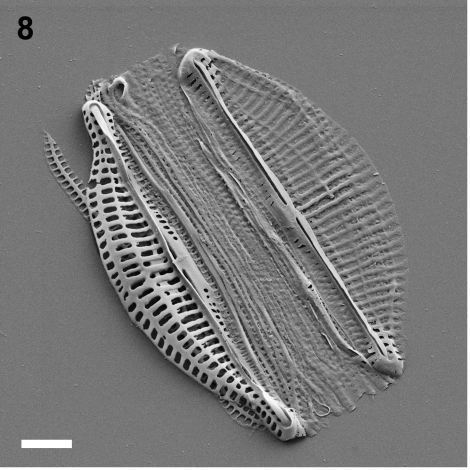
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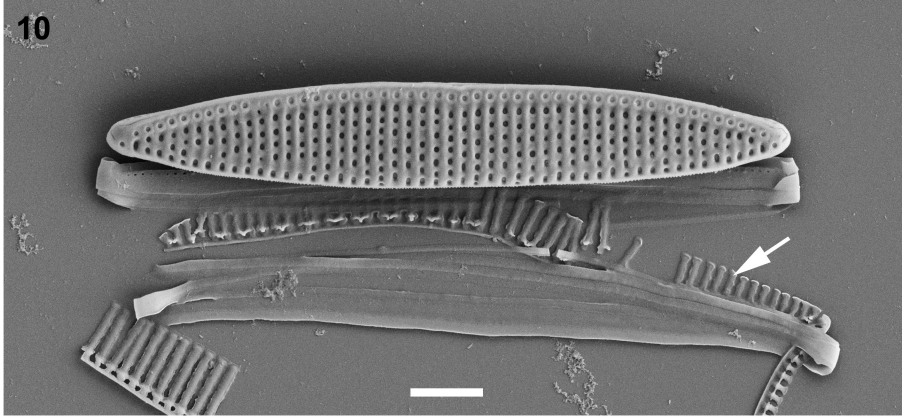
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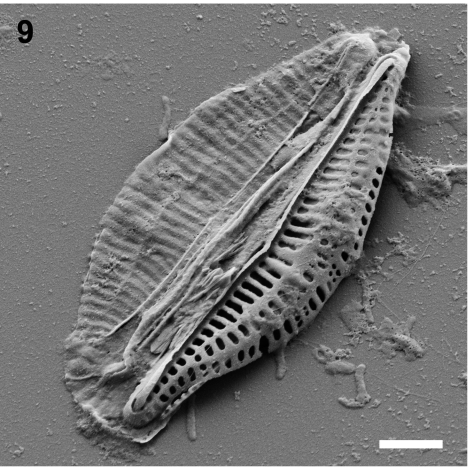
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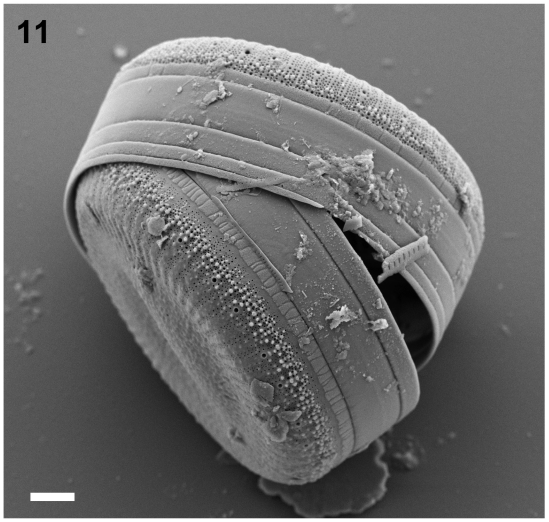
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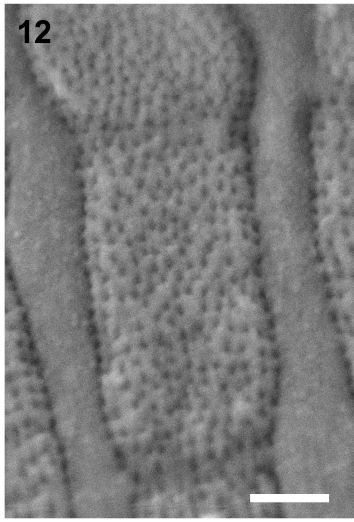
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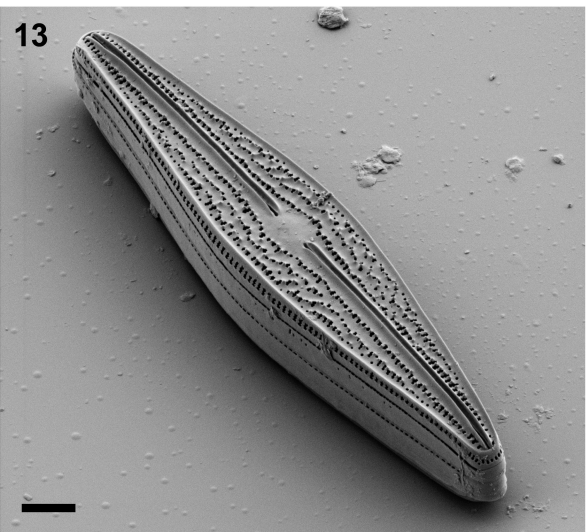
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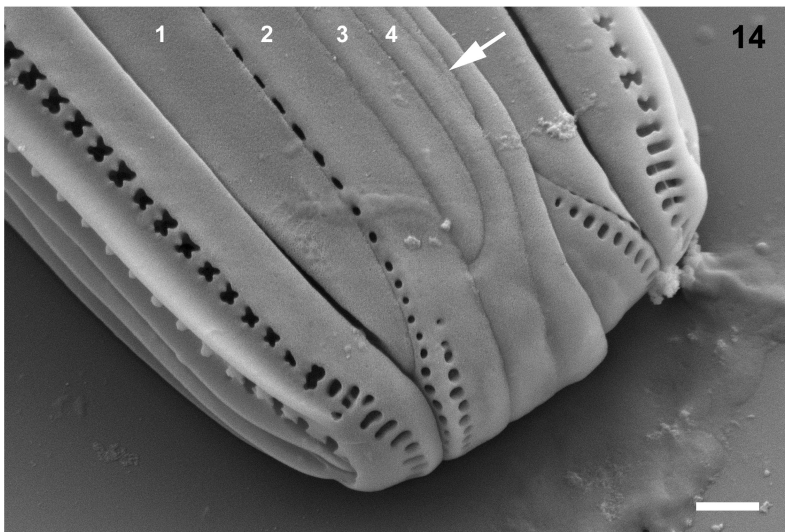
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