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Capsules of the diatom *Achnanthidium minutissimum* arise from fibrillar precursors and foster attachment of bacteria

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Achnanthidium minutissimum is a benthic diatom that may form biofilms on submerged, aquatic surfaces. Within these biofilms, *A. minutissimum* cells produce extracellular structures which facilitate substrate adhesion, such as stalks and capsules. Both consist of extracellular polymeric substance (EPS), but the microstructure and development stages of the capsules are so far unknown, despite a number of hypotheses about their function, inlcuding attachment and protection. We coupled scanning electron microscopy (SEM) to bright-field microscopy (BFM) and found that *A. minutissimum* capsules mostly possess an unstructured surface. However, capsule material that was mechanically stressed by being stretched between or around cells displayed fibrillar substructures. Fibrils were also found on the frustules of non-encapsulated cells, implicating that *A. minutissimum* capsules may develop from fibrillar precursors. Energy-dispersive X-ray (EDX) spectroscopy revealed that the capsule material contains little to no silicon, suggesting that the capsule does not arise from the cell wall. We furthermore show that bacteria attach preferentially to capsules, instead of non-encapsulated *A. minutissimum* cells, which supports the idea that capsules mediate diatom-bacteria interactions.

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Introduction

Diatoms (Bacillariophyceae) are among the most productive photoautotrophic, aquatic micro-organisms. They contribute an estimated 40-45% to the net primary production (NPP) of the oceans (Mann, 1999), which themselves contribute approx. 45-50% to the global NPP (Field et al., 1998). Additionally, diatoms are important for the biogeochemical cycling of silicon, due to their ornate cell walls (called "frustules") composed of biomineralised silica (Bradbury, 2004). Cell division includes the separation of the two frustule parts ("thecae") along a "girdle" region. Each daughter cell then complements its inherited epitheca with a newly synthesised, smaller hypotheca. Within these thecae, slits (called "raphes") and pores may be present, facilitating the secretion of extracellular polymeric substances (EPS) (Wetherbee et al., 1998; Wang et al., 2000). This in turn conveys substrate attachment and motility to benthic diatoms, which often form biofilms with other photoautotrophic algae, as well as heterotrophic bacteria (Buhmann, Kroth & Schleheck, 2012).

The diatom *Achnanthidium minutissimum* (renamed from *Achnanthes minutissima* by (Czarnecki & Edlund, 1995)) colonises the littoral zone of Lake Constance. It represents a dominant species complex of early colonisers (Johnson, Tuchman & Peterson, 1997), forming epilithic biofilms in association with a variety of satellite bacteria (Bahulikar, 2006). These bacteria may influence the production of extracellular polymeric substances (EPS) by the diatom (Bruckner et al., 2008, 2011). Similarly to the rhizosphere of terrestrial environments (composed largely of fungi and bacteria associated with plant roots), a "phycosphere" has been defined as the space surrounding algal cells including the multitude of inter-kingdom interactions between bacteria and algae (Bell, Lang & Mitchell, 1974). EPS secretion is of ecological and biogeographical relevance contributing for instance to the stabilisation of sediments (Cyr & Morton, 2006; Lubarsky et al., 2010). Studying biofilm formation can therefore assist in the understanding of shore- and coast-line erosion as a result of climate-related changes (see section 3.2.1 of (Widdows & Brinsley, 2002) plus references therein). At the same time diatom settlement is one of the major causes of biofouling of man-made machinery in aquatic applications (Molino & Wetherbee, 2008).

A. minutissimum is an excellent model for studying diatom biofilms, because this alga is abundant *in natura* (Patrick & Reimer, 1966; Krammer K & Lange-Bertalot H, 1991) and can be cultivated in the laboratory both as "xenic" biofilms (Myklestad et al., 1989) and "axenic"

suspension cultures (Windler et al., 2014b). Xenic cultures contain bacteria from the diatom's natural habitat. Removal of these bacteria is possible (for example by antibiotic treatment) and yields viable axenic cultures (Bruckner & Kroth, 2009; Windler, Gruber & Kroth, 2012). Bacteria-free cultures allow the establishment of bioassays in order to study the interactions between diatoms and bacteria, although potentially unwelcome long-term effects have to be taken into account. For example, axenic growth can lead to a reduction of average cell size and to frustule deformations (MacDonald, 1869; Pfitzer, 1871; Geitler, 1932; Windler et al., 2014a)

Our model species *A. minutissimum* forms biofilms and extracellular structures like and capsules. Stalks have been investigated previously by transmission electron microscopy and biochemical techniques to elucidate structural morphology and chemical composition (Daniel, Chamberlain & Jones, 1987). Additionally, a phase model of diatom adhesion involving stalks has been developed (Wang et al., 1997). Stalks may protrude from so called "basal pads" of aggregated EPS at the apical valve faces within hours to a few days, thus elevating the cells above the substrate. Capsule formation in *A. minutissimum* biofilms occurs later in the stationary phase, is possibly triggered by bacterial influences, and may cement diatom attachment (Windler et al., 2014b). That study also found that axenic *A. minutissimum* cultures mostly secrete soluble carbohydrates while the presence of insoluble carbohydrates in xenic cultures coincided with the appearance of capsules. This strengthened the argument that *A. minutissimum* capsules consist of carbohydrates and are important for the inter-kingdom interactions of diatoms and bacteria.

Diatom capsules have puzzled phycologists for a long time and their potential physiological and ecological function have elicited a variety of hypotheses (Lewin, 1955; Geitler, 1977). For example, capsules have been proposed participate in locomotion, flotation, attachment, waste removal, catchment of inorganic nutrients, storage of polysaccharides, sexual reproduction, as well as protection against grazing and dehydration. More recently, it was demonstrated that capsule formation is dependent on at least "a certain minimum light intensity", sparking the idea that capsules might serve as an additional polysaccharide storage pool, once intracellular capacities are saturated (Staats et al., 2000).

While diatom capsules have mostly been characterised biochemically, electron microscopical analyses was so far focussed on the morphology of diatom frustules (Toyoda et al., 2005, 2006; Morin, Coste & Hamilton, 2008) and the development of its morphological variations as environmental markers (Potapova & Hamilton, 2007; Hlúbiková, Ector & Hoffmann, 2011; Cantonati et al., 2014). In the present study, scanning electron microscopy (SEM), as well as

energy-dispersive X-ray (EDX) spectroscopy were employed to analyse the microstructure and development stages of *Achnanthidium minutissimum* capsules in order to further develop this species as a model system for diatom-bacteria interactions, and to elucidate one aspect of the complex interactions of diatoms and other micro-organisms.

Materials & Methods

Cultivation conditions

Achnanthidium minutissimum (Kützing) Czarnecki (previously called *Achnanthes minutissima*) strains previously isolated from benthic biofilms of Lake Constance were cultivated as described (Windler et al., 2014b). In order to grow diatom biofilms directly on sample carriers for scanning electron microscopy, 6- (instead of 48)-well plates (Sarstedt, USA, order number 83.1839.500) were used. Sample carrier disks of ca. 1 cm in diameter were punched from Thermanox tissue culture cover slips (Miles Laboratories Inc., USA). Because the Thermanox material has two different sides, care was taken to always store and handle the disks right-side-up. Because autoclavation proved to melt them, sterilisation was instead conducted by immersion in 70% isopropanol (v/v in H_2O) over night and subsequent irradiation with UV light for 2 h in a laminar flow cabinet. Sterile disks were placed into the wells and covered with 3 to 5 mL of modified Bacillariophycean Medium (BM; (Schlösser, 1994; Windler, Gruber & Kroth, 2012). Well areas may allow for the parallel cultivation of several disks, but due to the risk of them sliding on top of one another, placing only a single disk into each well is recommended. Culture wells were inoculated with 5×10^5 to 1×10^6 *A. minutissimum* cells in 3 to 5 ml BM. Cells were confirmed to be axenic or xenic by SYBR Green staining. Well plates were sealed with Parafilm and incubated at 16°C under an illumination regime of 12 h dark and 12 h light at 20-50 µmol photons$\times m^{-2}\times s^{-1}$ for 11 to 31 days.

Crystal violet staining and bright-field microscopy

Thermanox disks were removed from stationary cultures after 11, 20 and 31 days with inverted ("soldering" or "cover glass") forceps (Hammacher, Germany) and rinsed with 1 mL sterile-filtered tap water. A Gram-staining protocol adapted from Kaplan & Fine (2002) was applied to visualise adherent cells and their extracellular polymeric structures as follows: A droplet of 200 µL solution of 0.02% crystal violet (CV) in sterile filtered tap water was applied onto the disk for 1-2 min, which was held suspended by forceps. Disks were rinsed with 1 to

3 mL water, until the run-off no longer contained visible CV. In order to find the same cell clusters in both microscopic approaches, pointing or encircling scratches were made into the biofilm-covered disk surfaces.

Disks were placed on moistened glass slides and moistened additionally with 20 µL sterile-filtered tap water. Cover slips were applied carefully and marked regions were observed under a BX51 (Olympus, USA) microscope with bright-field using chlorophyll fluorescence filters. Images of these areas at various magnifications were taken with AxioCams MRm and MRc using AxioVision software (Zeiss, Germany). See figures 1 and 2 for results. Disks were recovered from between glass slides and cover slips by separating the glass pieces with 1 mL of water.

Scanning electron microscopy (SEM) and energy-dispersive X-ray (EDX) spectroscopy

Diatom cells on thermanox disks were fixed by incubation in a mixture of 2% glutaraldehyde, 10 mM $CaCl_2$ and 10 mM $MgCl_2$ in 0.1 M sodium cacodylate buffer at pH 7 and room temperature for 2 h. Dehydration was conducted first with 30% and 50% EtOH, at room temperature for 2 h each, followed by 70% EtOH at 4°C over night, 90% EtOH at room temp. for 2 h and finally with 96% and 100% EtOH twice for 1 h each. Critical point drying in CO_2 followed (Balzers CPD030, Liechtenstein) and samples were finally sputtered with gold and palladium to a thickness of 5 nm (Balzers SCD030, Liechtenstein).

After fixation, dehydration and Au/Pd-sputtering, the biofilm-covered Thermanox disks were imaged with a Zeiss "AURIGA" scanning electron microscope, controlled with the "SmartSEM" software v05.04.05.00. See figures 2 to 6 for results. Energy-dispersive X-ray (EDX) spectra were recorded with an Oxford Instruments "X-Max 20 mm^2" device, controlled with the "INCA" software v4.15. See figure 7 for results.

Bacterial counting and statistical analyses

Bacteria (rod-shaped particles) on fully visible *A. minutissimum* valve faces were counted in scanning electron micrographs. Valve faces were classified into frustules and capsules, depending on whether pores were visible or completely disappeared under layer of capsule material. Diatom cells with partial encapsulation were not included in the counting, and neither were bacteria cells which attached to the girdle bands of diatom cells.

ImageJ v1.46r with the Cell Counter plug-in v2010/12/07 was used to count diatoms and bacteria cells. This data was evaluated in the R environment for statistical computing v3.1.1 and RStudio v0.98. See figure 8 for results.

Results and Discussion

Xenic *Achnanthidium minutissimum* were grown on disks of ca. 1 cm diameter punched from Thermanox tissue culture cover slips. These disks were used both for bright-field light and scanning electron microscopy to correlate the appearance of hydrated capsules with their microstructure after dehydration.

After an incubation period of 11 days, thermanox disk surfaces were densely covered by a mono-layer of xenic *A. minutissimum* cells (Fig. 1). The biofilm was visible as light greenish-brown coloration on the substrate disks. Staining with the dye crystal-violet (CV) and subsequent bright-field microscopy showed that large portions of the diatom cells were surrounded by capsules, which absorbed the chlorophyll fluorescence (chl).

In contrast, axenic *A. minutissimum* cells did not form biofilms on the substrate disks, so that careful rinsing already removed most of the cells. This observation is in agreement with studies that utilised other growth substrates to compare biofilm formation by axenic and xenic diatom cultures. By measuring chlorophyll (chl) contents, the possibility that axenic cells might simply be less proliferate was excluded (Windler et al., 2014b). Xenic *A. minutissimum* cultures on the other hand have also been found to develop biofilms on glass beads as well as in plastic multi-well plates (Lubarsky et al., 2010; Windler et al., 2014b). Our results therefore demonstrate, that *A. minutissimum* biofilms can easily be grown on thermanox disks, enabling direct preparation for electron microscopy of native biofilm samples.

Identification of *A. minutissimum* capsule microstructures

In order to correlate the hydrated *A. minutissimum* capsules visible in light microscopy to their dehydrated appearance in SEM, areas were marked by scratches on the crystal violet (CV) stained disks and cells of interest were identified by bright-field microscopy (BFM). Subsequently, the same cells were observed by scanning electron microscopy (SEM; Fig. 2). In BFM, the CV stained capsules were visible as balloon-like structures around most of the cells. As extracellular polymeric structures in the genus *Achnanthidium* are composed mostly of carbohydrates (Wustman et al., 1998), strong hydration in the native biofilm is likely the source

of this appearance of capsules. In SEM, however, we were able to distinguish two types of *A. minutissimum* cells in xenic biofilms already at low magnifications: cells with pores in their frustules still visible, and cells covered by an apparently unstructured material masking the pores.

The frustules of non-encapsulated xenic, as well as axenic *A. minutissimum* cells appeared identical to those from scanning electron micrographs shown in previous studies (Mayama & Kobayasi, 1989; Potapova & Hamilton, 2007; Hlúbiková, Ector & Hoffmann, 2011). The low prevalence of raphes in our images is most likely due to the orientation of raphes towards the substrate for mucilage secretion (Gordon & Drum, 1970, p. 197; Wetherbee et al., 1998). Natural attachment and orientation of cells on our biofilm disks was retained because we did not employ harsh preparation techniques, such as boiling diatom cells in sulphuric acid (Mayama & Kobayasi, 1989). Such harsh treatments are designed to prepare only frustules and would in our case have resulted in cell detachment from the growth substrate and random orientation on the SEM sample carrier. We however, applied the final SEM sample carrier disks directly as growth substrates for the biofilms.

The SEM images in figure 3 show that the capsule material appears to be unstructured, resembling the "adhering film and tube" of *Cymbella microcephala* and *Cymbella prostrata* reported in figures 31 & 32 of Hoagland et al. (1993).

Closer inspection provided many examples that the frustules of non-encapsulated xenic *A. minutissimum* cells were not be completely free of extracellular polymeric substances (EPS). Instead, they were covered by a mesh of fibrils (Fig. 3B), arranged mostly around the frustule pores, sometimes crossing them and sometimes sticking out. The average diameter of these fibrils was about 45 ± 9 nm. The fibrils were rarely observed to be secreted through the pores, although these were found to be large enough (from 60 to140 nm in diameter), showing round to elongated shapes. Fibrils were generally longer than pores where in diameter, but quantification was not performed because branching and crossing points made it infeasible to determine the respective beginnings or ends. Similarly structured, thinner fibrils were reported previously only for marine diatoms (Bosak et al., 2012). To the best of our knowledge, this is the first report of frustule-attached fibril structures in fresh water diatoms.

The dehydrated capsule material displayed a slight granularity in scanning electron micrographs (Fig. 3C), but unlike on frustules (with or without fibrils) few distinct features were apparent. The capsule material appears to be similar to the shaft ultra-structure of the marine diatom *Achnanthes longipes* displayed in figure 8 of Wang et al. (2000). Material secretion was

observed to appear in apical pore fields; the site of pad and stalk formation (Wang et al., 2000). Although originally reported for that marine diatom by (Wustman et al., 1998), we observed this also on the rapheless epitheca of the fresh water diatom *A. minutissimum*.

In addition to covering the cells, the capsule material also had sheet-like structures (arrows in figure 3A) in portions stretched between *A. minutissimum* cells and the anchoring points on the substrate. This pattern is most likely due to dehydration during SEM sample preparation (Hoagland et al., 1993). Due to fixation of the samples prior to drying, the hydrated capsules most likely shrank in their entirety to the dehydrated, envelope-like structures.

Fibrillar precursors may give rise to *A. minutissimum* capsules

In order to elucidate the process of capsule formation, we analysed the observed cell surface morphologies more closely. We found intermediate stages between the fibrillar meshes that only partially covered the frustule surface and the complete encapsulation with apparently unstructured material (Fig. 4).

Fibrillar meshes of varying densities were detected in both axenic and xenic cultures during the stationary phase. Axenic cultures additionally contained cells with even fewer and shorter fibrils than displayed in figure 4A. This indicates that there is a bias introduced by washing the substrate disks prior to SEM preparation. Probably only those axenic cells possessing at least a minimum amount of EPS on their surface were able to adhere to the disks. In xenic cultures, bacteria likely induced the secretion of EPS (Bruckner et al., 2011) and thus substrate adherence by the majority of cells. This is in line with the observation that capsules were only found in xenic cultures, particularly later in the stationary phase.

Based on the visual impression, we think that the dense fibrillar meshes represent precursors of the mature capsules. For example, the disordered arrangement of fibrils shown in figure 4B is also a feature of the unstructured capsule material. In it, no particular order of the slightly granular substructures is discernible either (Fig. 4C). An alternative explanation for the capsule structure may be the polymerisation of a secondary type of fibrils upon the primary mesh (Fig. 4A), relegating the latter to a scaffolding function.

In order to elucidate whether or not fibrils and capsules might be related, we observed mechanically stressed capsule areas (Fig. 5). Here, tension yielded an alignment of capsule micro-structures, as well as fraying on the edges. Fibrillar structures resulting from these processes were similar in diameter to the fibrils covering the frustules.

There are two possible sources for the mechanical force. Motility of the cells relative to each other in the native biofilm could induce force. However, it has been reported for a related species in the order *Achnanthales*, that a loss of motility following the production of EPS structures occurs (Wang et al., 1997). It is therefore also possible that mechanical force could be caused by the dehydration during SEM sample preparation. Both explanations lead to the question why mechanical force highlighted the fibrillar microstructure, while relaxed capsule areas (see previous figures) appeared unstructured.

Inter-fibril cross-linkage by for example hydrogen bonds was apparently weaker than intra-fibril connections, which are likely of covalent nature. Also, the integrity of fibrillar building blocks was apparently preserved during capsule formation. Otherwise, mechanical force would likely have favoured shearing of fibrillar microstructures over re-emergence from the capsule material.

We therefore suggest fibrils as a precursor candidate for capsules, into which they may condense as depicted in figure 4, for example by by enzymatic cross-linking or transglycosylase activity. Fibrils may be disguised in relaxed capsule material because they are arranged in a disorderly fashion, but mechanical stress may yield a visible alignment. Micromanipulation experiments that artificially apply mechanical force to encapsulated cells in the native biofilm should be developed to investigate this possibility further.

Capsule material does not contain silica

In order to exclude the possibility that the capsule structures we observed might be frustule deformations or extensions, two further experiments were conducted. First, because stress-induced frustule deformations (Cantonati et al., 2014; Windler et al., 2014a) might also result in the disappearance of pores, we imaged frustules at a higher excitation voltage (10 instead of 5 keV; Fig. 6), resulting in translucency of some capsule regions. This way, frustule pores beyond thicker capsule material became visible, demonstrating that capsules are an additional layer of material around the frustule.

Secondly, capsule material was screened for the presence of silicon (Si). This chemical element is a major component of diatom frustules, in which it is present as hydrated silicon dioxide. Capsules on the other hand may consist mostly of extracellular polymeric carbohydrates (Windler et al., 2014b). Energy-dispersive X-ray (EDX) spectra were therefore recorded from capsule areas with and without a frustule below them (Fig. 7).

As expected, Si signals around 1.75 keV were obtained from control areas with frustule material below the capsules (Guerra et al., 2013; Chandrasekaran et al., 2014). Si signals were 2.5 to 12 times stronger from such control areas then from capsule material only. We can therefore exclude the possibility that *A. minutissimum* capsule material is some kind of frustule extension or deformation.

Low Si counts (black line in grey highlighted area in figure 7) are likely contributed by the frustule edges in close proximity due to the "pear effect" (Arnould & Hild, 2007). It explains, how excited electrons diffuse into the sample, so that a pear-shaped volume of ca. 0.5-1 µm diameter below the measurement point or area also emits detectable X-rays. The cell bodies in figure 7 are separated by approximately that distance. The stronger gold (Au) signal around 2.15 keV most likely resulted from the larger sputtered surface area within the measurement volume.

Because no notable nitrogen signals (N; 0.39 keV) were recorded from the capsule material, we tentatively exclude chitin as a major capsule building block candidate. Chitin fibrils have been found to be secreted by diatoms into the surrounding water body (Gardner & Blackwell, 1971; Herth, 1979). In contrast, the fibrillar meshes we describe here, tightly covered the frustule surfaces of individual *A. minutissimum* cells and therefore likely represent different EPS structures.

Bacteria preferentially attach to encapsulated diatom cells

It has been proposed that *A. minutissimum* capsules might be an asset in the mutualistic relationship of the diatom with its satellite bacteria (Windler et al., 2014b). Previous findings suggest a pattern of bacterial attachment to xenic *A. minutissimum* cells that would support this hypothesis (Windler, Gruber & Kroth, 2012) as diatom cells seemingly are surrounded by a bacteria-free space, followed by a layer of densely aggregated bacteria cells. Although no CV stains were conducted in that study, the bacteria free regions resemble the EPS structures reported as capsules.

It became apparent during the analyses of SEM images, that diatom-attached bacteria cells occurred more often on capsules than on frustules. To substantiate this observation, bacteria cells were counted on both diatom cell surface types (Fig. 8).

Notably higher numbers of bacteria (ca. 25 times more on average) were attached to capsules as compared to frustules throughout the stationary phase (means: 11.41±8.23 and 0.46±0.82

respectively). As figure 8 shows, the variance in the numbers of bacteria per diatom (black dots) was larger (ca. 100 times) on encapsulated cells than on frustules, indicating that not all encapsulated *A. minutissimum* cells were equally strongly colonised by bacteria.

Bacterial cells as well as non-encapsulated diatom cells were able to individually retain attachment to the substrate during SEM sample preparation. The lower prevalence of bacteria attached to frustules is therefore probably not due to lower ability of the bacteria to remain attached to frustules during SEM sample preparation. Instead, the bacteria preferred capsules over frustules.

It is possible that diatom capsules serve as a common nutrient pool to some of the diatom's satellite bacteria in a mutualistic relationship (Bruckner et al., 2008). In bacterial biofilms, nutrient distribution is predominantly determined by diffusion, sometimes along strong gradients within a biofilm (Stewart, 2003). Similarly, variations of cellular nutrient distributions within fresh water diatom biofilms exist (Murdock et al., 2010). Therefore, competition between individual diatom cells for re-mineralising bacteria could occur. Nutrient-limited, but still photosynthetically active diatom cells may produce predominantly insoluble carbohydrates to foster close attachment of heterotrophic bacteria that re-mineralise EPS or secrete vitamins. Axenic *A. minutissimum* cultures have been shown to secrete carbohydrates predominantly in soluble form (Windler et al., 2014b). The same may be true for individual *A. minutissimum* cells under non-limiting nutrient conditions, despite being embedded in a xenic biofilm.

Our finding that bacteria attach preferentially to capsules strengthens the argument that capsules play a role in the inter-kingdom relationship of satellite bacteria and benthic diatoms. Whether this relationship is antagonistic, mutualistic or commensal in nature remains to be elucidated. Labelling experiments with isotopes or fluorophores may assist in the determination of carbohydrate fluxes from the diatom's EPS structures to bacteria feeding on those.

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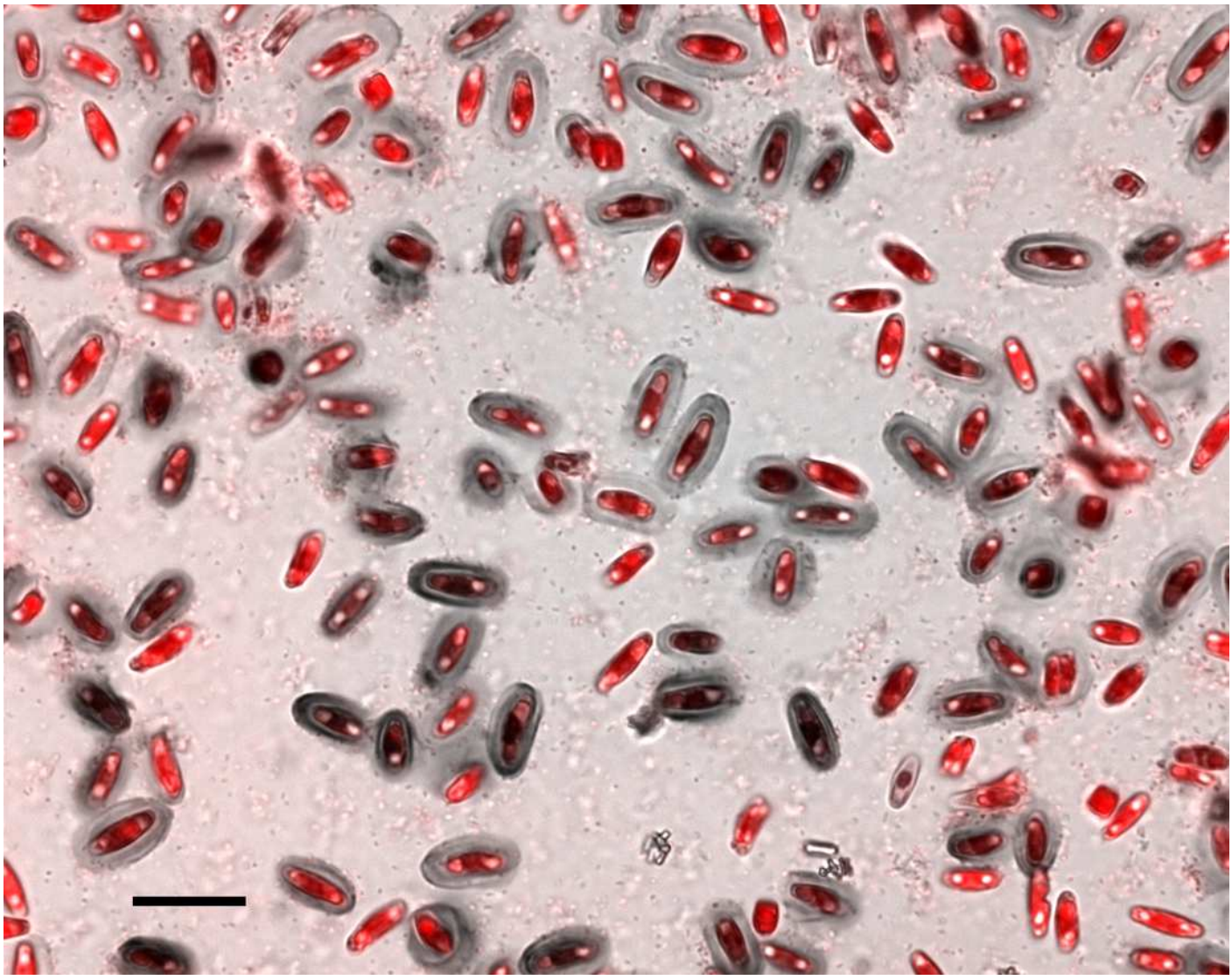
1

Chlorophyll fluorescence image (red) merged with the bright-field image of crystal violet (CV) stain (grey) of a xenic *A. minutissimum* biofilm after 11 days of incubation (scale: 20 µm).

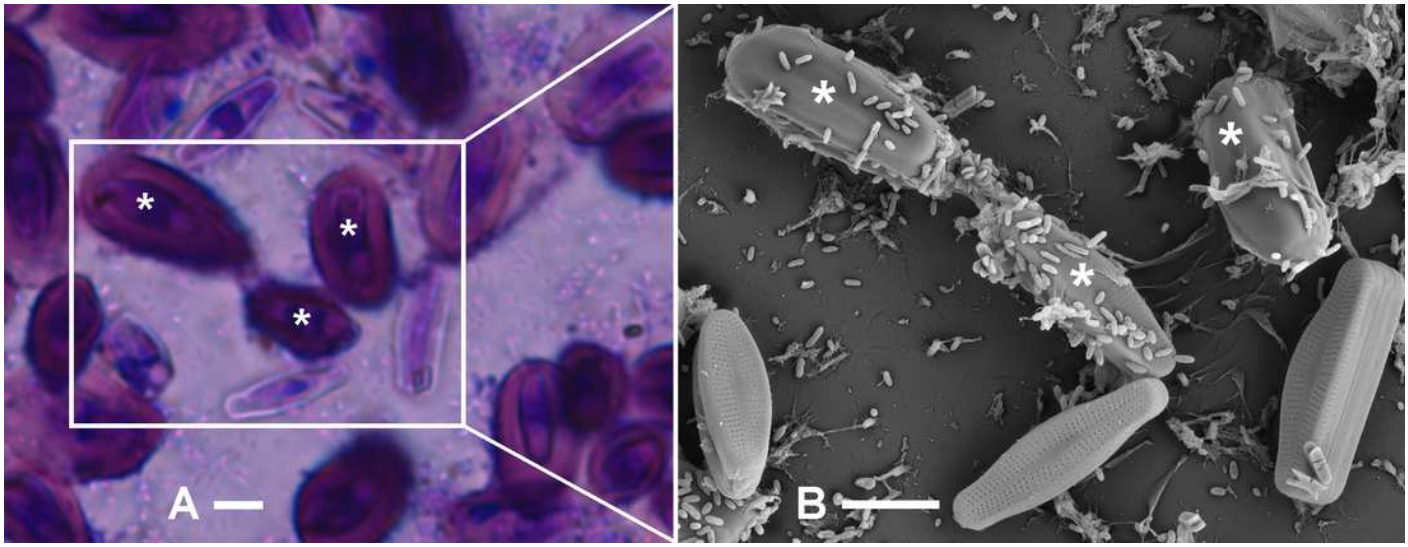
Bacteria are visible as dark speckles between the diatom cells. The dark grey, balloon-like structures surrounding the diatom cells and absorbing chlorophyll fluorescence are capsules, as visualised in real colours in figure 2A.



2

Identification of *A. minutissimum* capsules (asterisks) by subsequent observation of identical cell clusters in bright-field (A) and scanning electron (B) micrograph (scales: 5 µm).

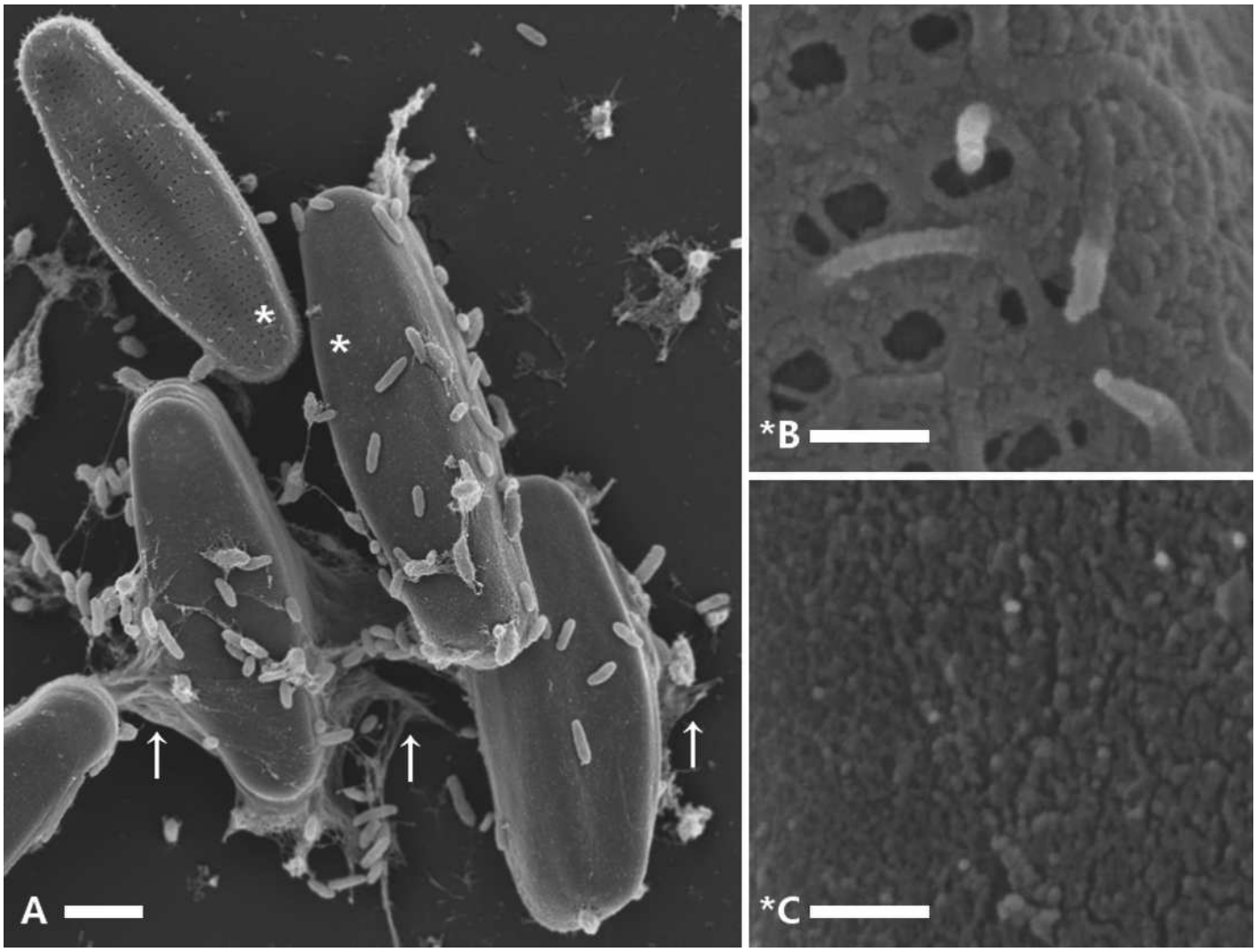
A: 30 days old xenic A. minutissimum biofilm stained with crystal violet (CV). Encapsulated cells are strongly stained, while weak staining indicates a lack of extracellular polymeric substances (EPS) on the frustule surfaces. **B:** In the same cell cluster, encapsulated cells (asterisks) are surrounded by an opaque material. Frustule pores and raphe typical for *A. minutissimum* are only visible on cells that did not possess a capsule in the hydrated biofilm. Note also the unequal distribution of bacteria cells onto capsule material versus non-encapsulated frustules.



3

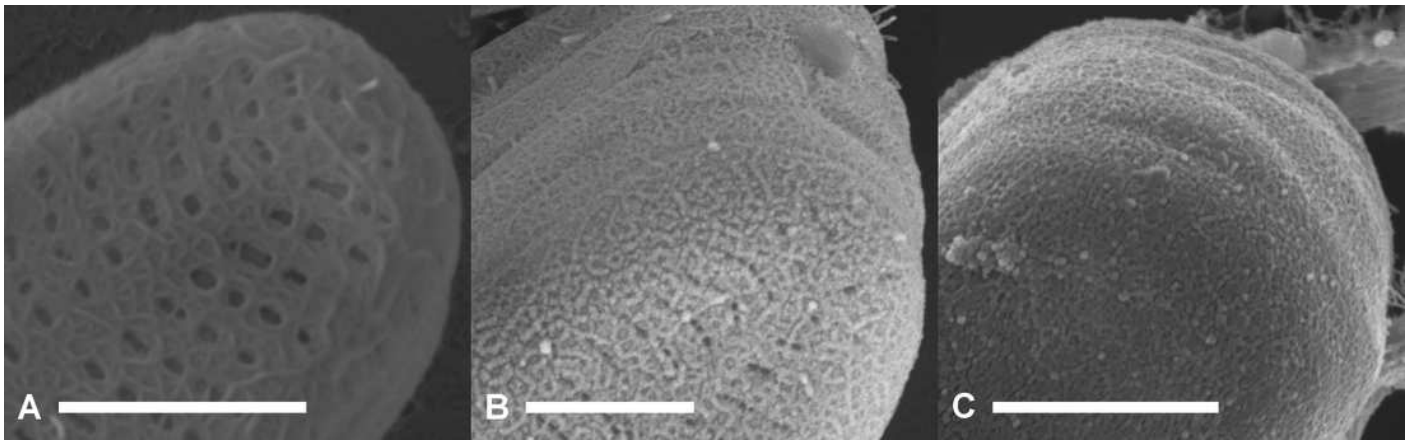
Comparison of microstructures on *A. minutissimum* cell surfaces in early stationary, xenic culture.

A (scale: 2 µm): Capsule material is sometimes stretched between cells and/or towards the substrate (arrows). Asterisks denote magnified areas B and C. **B** (scale: 1 µm): Non-encapsulated cells possess a fibrillar mesh of varying degrees of density. Frustule pores are only partially covered and in some cases, fibrils stick out from the frustule. **C** (scale: 1 µm): Encapsulated cells are completely covered with a material that lacks clearly discernible structure, despite some granularity.



4

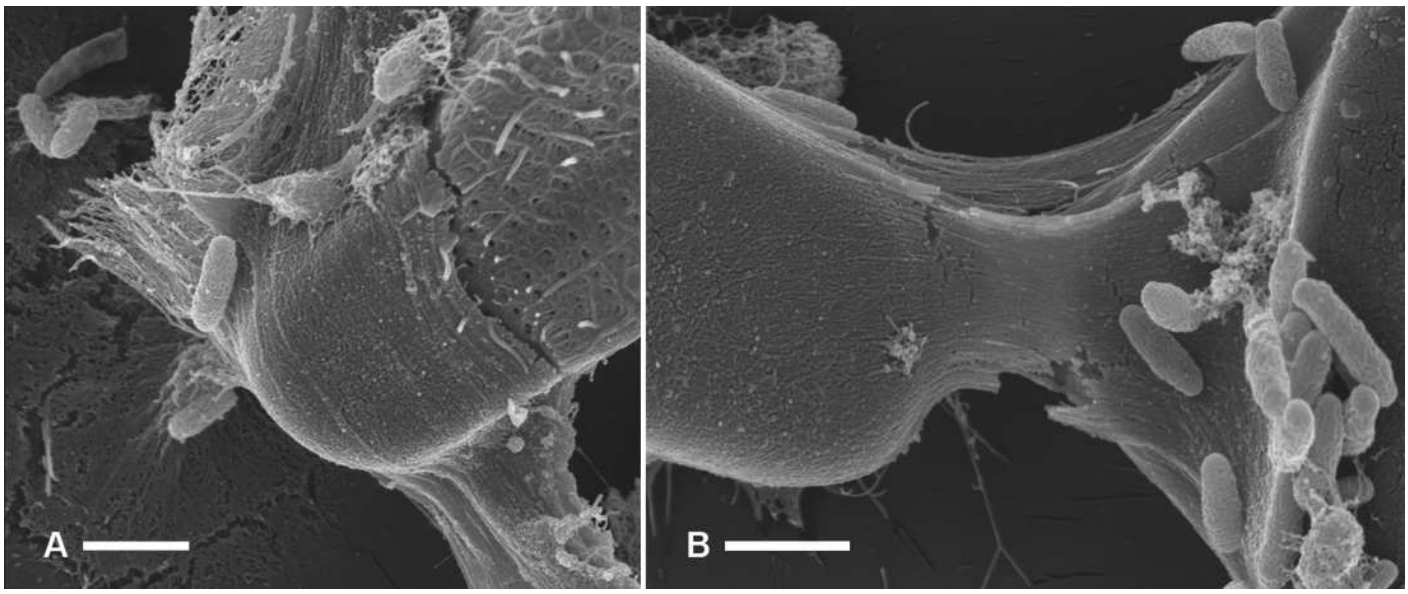
Fibrillar meshes (A) may form capsule material (C) by denser growth and cross-linking (B) of fibrils in xenic *A. minutissimum* cultures (scales: 1 µm).



5

Capsule material reveals fibrillar composition under mechanical stress (scale bars = 1 µm).

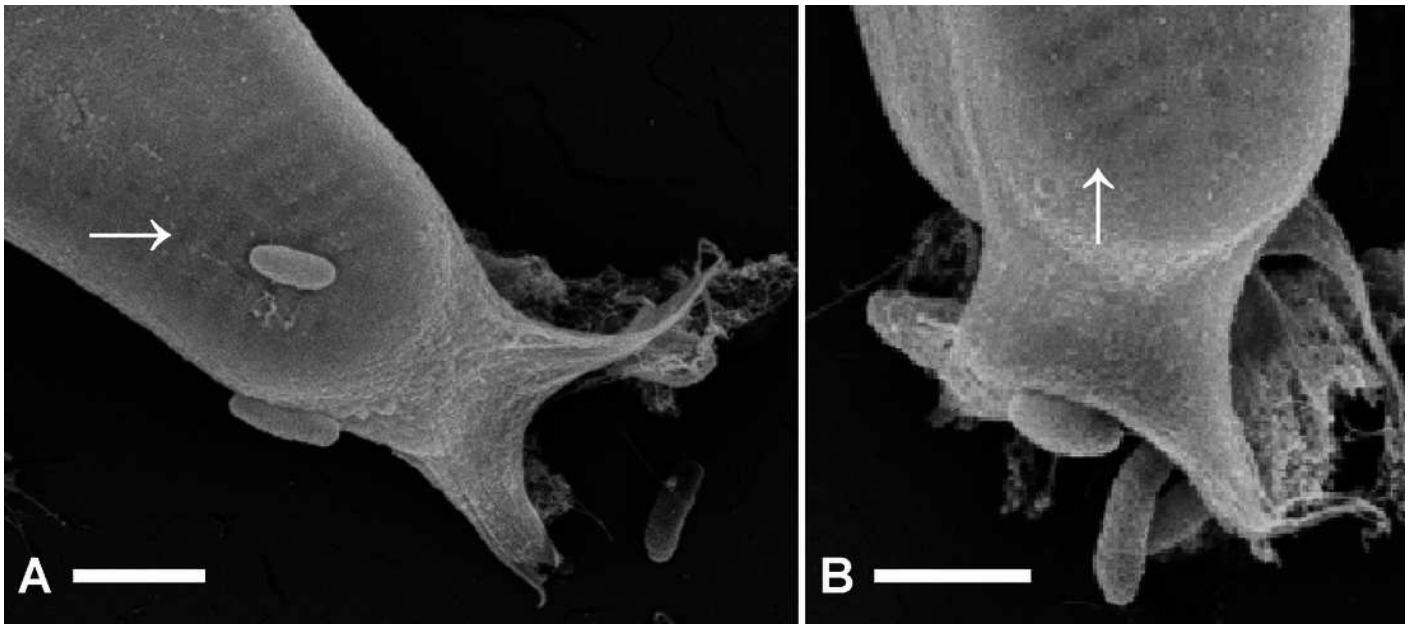
A: Tip of a partially encapsulated *A. minutissimum* cell with continuous fibrillar substructures within the capsule material. **B:** Capsule material stretched between cells reveal fibrillar microstructures.



6

Capsule material around xenic *A. minutissimum* cells becomes translucent at increased excitation voltages (scales = 1 µm).

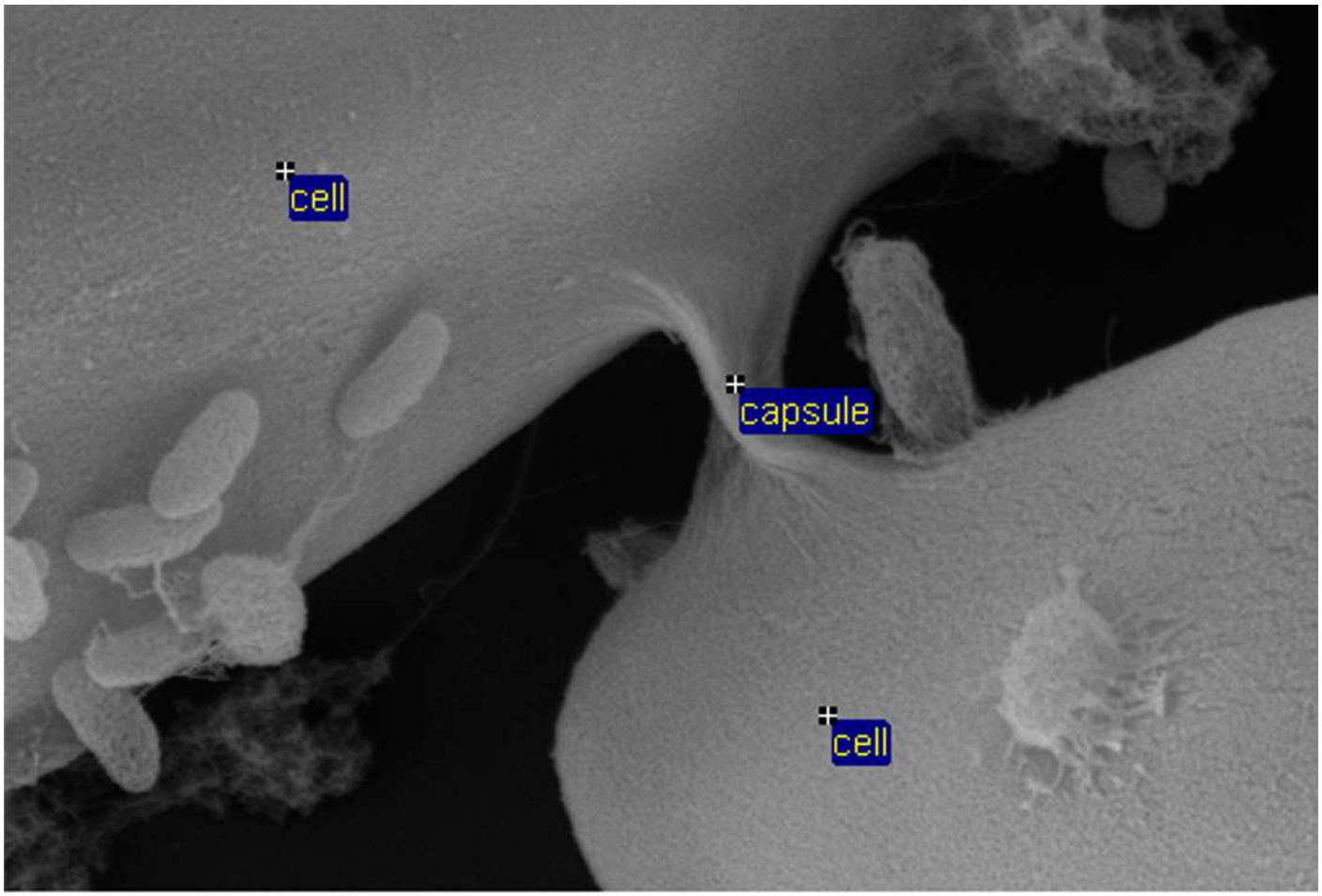
Frustule pores are visible below the capsule material (arrows).

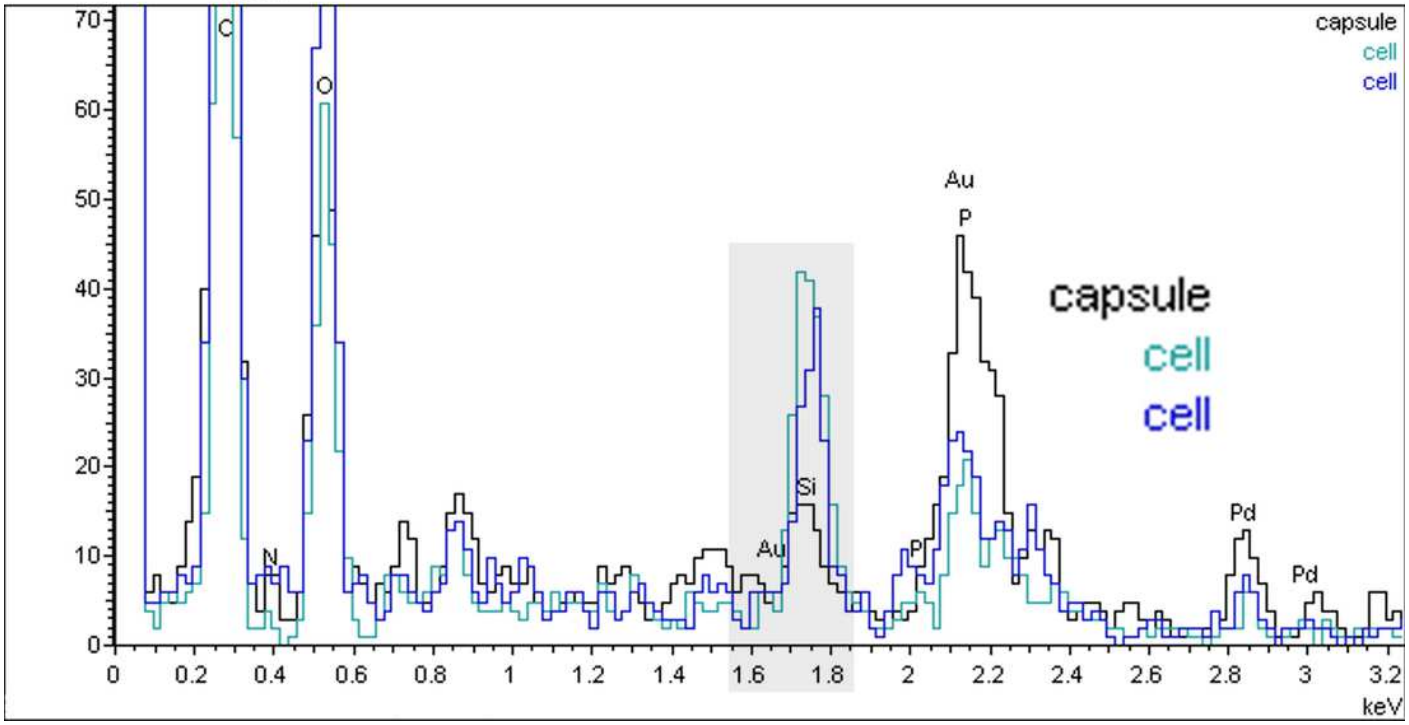


7

Scanning electron micrograph and energy-dispersive X-ray spectrum of early stationary, xenic *A. minutissimum* cells.

Less silicon (highlighted signal around 1.75 keV) is found in the capsule material (black trace) compared to the frustules (blue and turquoise traces; axis: x = keV, y = counts).





8

Box plot of the number of attached bacteria cells per frustule or capsule in stationary, xenic *A. minutissimum* cultures.

Dark grey boxes represent first and third quartile. White lines in box centres represent medians. Diamond symbols represent means. Dots represent individual counts of attached bacteria per diatom (larger dots are outliers). Bacteria were counted, if they were in direct, visible contact with the valve face of either a frustule, or a completely encapsulated diatom cell (see figures 2B and 3A for illustration). More capsules than frustules were counted, because more encapsulated diatom cells were present in our samples.