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IMAGING OF AQUATIC ORGANISMS USING VARIABLE-PRESSURE SEM

OBRAZOWANIE ORGANIZMÓW WODNYCH ZA POMOCĄ SEM ZE ZMIENNĄ PRÓŻNIĄ

Abstract

Examples of the use of a scanning electron microscope (SEM) to study organic matter in variable vacuum (VP) were presented. The organisms growing in water (e.g. *Chironomidae*), carried by water/wastewater (e.g. *Protozoa*, *Amoebozoa*) or inhabiting reservoirs (e.g. *Algae*), were investigated. The results indicate a good agreement between the predictions in source literature and the actual imaging quality in our VP-SEM experiments.

Keywords: scanning electron microscope, biological material, preparation

Streszczenie

Przedstawiono przykłady wykorzystania skaningowego mikroskopu elektronowego (SEM) do badań materii organicznej w warunkach zmiennej próżni (VP). Próbkami były organizmy rozwijające się w wodzie (np. *Chironomidae*), niesione przez wodę/ścieki (np. *Protozoa, Amoebozoa*), zasiedlające zbiorniki (np. *Algae*). Wyniki obrazowania uzyskane w naszych eksperymentach z użyciem VP-SEM wskazują zgodność z danymi literaturowymi.

Słowa kluczowe: elektronowy mikroskop skaningowy, materiał biologiczny, preparatyka

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1. Introduction

The use of scanning electron microscope (SEM) in studies of biological, moist and wet samples requires its right settings of imaging conditions. Traditional SEMs operate with vacuums in the range of 10^{-3}–10^{-5} Pa, so naturally moist samples become dried out in a SEM chamber environment and lose important morphological features. Another serious problem is that the sample had to be electrically conductive in order to prevent charging under electron beam bombardment (electron beam accelerating voltage of 0.3–30 kV). There are only two options for biological sample studies [1, 2]: either modifying the environment inside the microscope column or stabilizing the specimens to make them sufficiently robust to withstand the SEM imaging conditions. Fig. 1 shows a diagram of preliminary preparation steps of biological sample for the SEM.

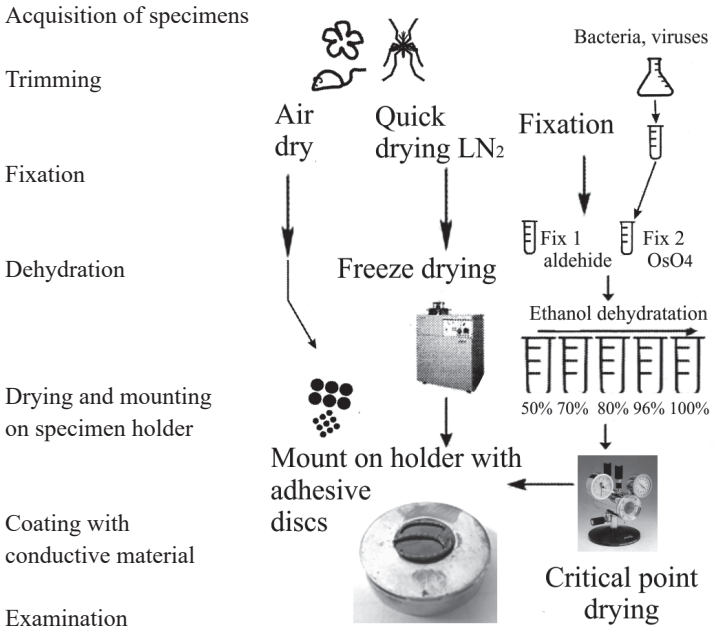

Fig. 1. Preparation methods of biological material for the SEM (according to [3, 4])

In ref. [1], there are numerous sample preparation principles suggested, concerning various impact stages: trimming, fixation, dehydration and different approaches to drying specimen, and final coating. However, structural damage and artifacts can occur prior to any microscopy analysis. It can happen that the end product is quite different from the original specimen. Thus, the design of *ab-initio* preparation protocols remains an empirical process around the sample concerned [1, 4].

Current SEMs are versatile in being able to operate in elevated gas pressure and low voltage, combined with sample cooling, together with specialized electron detectors

(to explore the high-pressure SE signals) etc. [5–11]. That gives new opportunities for examining specimens, which otherwise would be difficult to examine. The new environmental SEM generation (ESEM) permits also "*in-situ* experiments", i.e. the closest replication of the conditions associated with the problem under consideration [12].

The goal of the present work was to examine aquatic organisms exhibiting different humidity and dispersion in a variable-pressure scanning electron microscope (VP-SEM). A common fundamental step in sample preparation was the incorporation of the wet material on a conductive carbon substrate. This is a double side adhesive disk, used to support the sample on a standard flat specimen holder (Fig. 1). Then, samples were air-dried for varying periods of time. Some of them were vapor-deposited from the top with a thin gold layer. The incompletely dried samples were inserted in VP-mode without any coating. The capabilities of Peltier cooling device were used where necessary [11].

2. The specimens investigated

2.1. *Chironomidae*

Diptera is one of the major insect orders which is of great importance in terms of the environment. The *Chironomidae* are a family of nematoceran flies, class *Insecta*, order *Diptera*, suborder *Nematocera*, with a global distribution. It is characterized by high species diversity and is the major and very important component of zoobenthos in all freshwater ecosystems. *Chironomidae* may be a predator, algae eater, filter feeder, parasite or commensal of other invertebrates. It happens that *larvae* are predominant in the leaves and stems of aquatic plants. They are relatively rare in the semi-aqueous environment or in the seas [13]. The insect develops via complete metamorphosis from egg to adult, including larval stages and pupa.

A big role in bioindication of aquatic environment, e.g. in typology and biomonitoring of lakes, bioavailability and bioaccumulation of metals, is played by the *Chironomidae* larva (Fig. 2). Based on the quantitative and qualitative occurrence of particular *Chironomidae* species, the quality of the aquatic environment, its fertility and acidity can be evaluated [13, 16–23]. Due to the contamination of aquatic environment, the *Chironomidae* larva is susceptible to deformities of mouthparts (such as the asymmetry of the teeth or their displacement, the absence, addition or exacerbation of teeth or reducing the tooth surface), but also the changes in the DNA (there have been cases of changes in the structure and number of chromosomes) [24–27].

For research purposes, the larvae of species *Chironomus riparius* and *Glyptotendipes glaucus* were grown in the laboratory. The breeding aquatic environment consisted of 1 kg pure fine quartz sand (120–250 μm), to which a 1 g diet and 1 liter dechlorinated tap water was added. The culture medium was prepared two weeks in advance and placed in an aquarium under aeration. Next, the *Chironomidae* footbridge eggs were transferred from the natural water Reservoir Goczałkowice and inserted gently in aquarium under the water table surface. The larvae hatching from the eggs were fed with a suspension of fish food in dechlorinated water in concentration of 4 g/l. After the predetermined time (*Chironomidae* species have

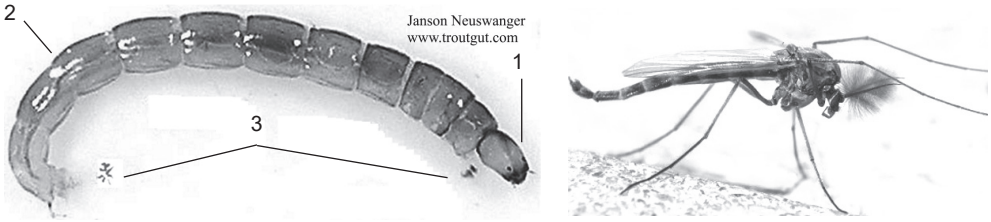

Fig. 2. Images of *Chironomidae* larva (left) and imago (right): 1 – capsule-like head distinct from the thorax; 2 – elongated body with distinct segments; 3 – two pairs of prolegs [14, 15]

different lengths of a life cycle, from a few days up to a year and even longer), the larva were collected by sieving the sediment through 1 mm mesh. The fished out individuals were further rinsed: first in tap water, than for 5 min. in deionized water, than for 10 min. in 0.001M of EDTA solution and finally, again for 5 minutes in deionized water. After the complete sequence of washing, specimens were preserved in formalin or alcohol.

Selected *Chironomidae* larvae, pupal exuvium and an imago were preserved from laboratory growth for SEM imaging. After few days of air-drying on a carbon tape, larvae were coated with conductive gold layer for high-vacuum SEM. Instead, pupal exuvium and an imago were dropped wet on a carbon tape of a specimen holder after rinsing, than put directly in a specimen chamber for VP-SEM.

2.2. Activated sludge

Activated sludge is a complex biological material, produced during sewage and wastewater treatments [28]. The granules (flocks) of activated sludge consist of various microorganisms, such as heterotrophic bacteria, algae, flagellate and amoebas [29, 30]. The quantitative relationship between bacteria, protozoa and some eumetazoa present in the flocks indicates the current condition of activated sludge. For VP-SEM, specimens were collected from an aeration tank of a sewage treatment plant. Single drops of sludge were air-dried on carbon discs direct on a specimen holder.

2.3. Protozoan intestinal parasites

Protozoan intestinal parasites are single cell microorganisms that cause infection of the gastrointestinal tract of humans and animals through the consumption of potable water containing live cysts/oocysts or through the contact with infected carriers (excrements) [31]. The most dangerous for humans are small intracellular parasites of epithelial cells of the gastrointestinal and respiratory tracts, which belong to vertebrates species *Cryptosporidium parvum* and *C. hominis* (phylum *Apicomplexa*, class *Coccidea*, order *Eucoccidiorida*, family *Cryptosporidiidae*, genus *Cryptosporidium*) or to flagellate species *Giardia intestinalis* (= *G. lamblia*, = *G. duodenalis*) [32–35]. In fluorescence light microscope, *Cryptosporidium* oocyst looks like a slightly oval object with a diameter about 5 μm, while *Giardia* cyst exhibit clearly elliptical shape with a typical width to length size approximately 8 to10 μm.

The differences in the size of individual oocyst yield maximum 1–2 μm regardless of the type of species. The wall of infective oocysts has typical thickness about 0.5 μm; it is multi-layered and resistant to mechanical forces and physicochemical factors. Inside the cysts, there are four sporozoites (with visible nuclei) and residual body; the visualization of them require special staining techniques for fluorescent microscope or different interference contrast microscopy [36, 37].

Due to the emission of huge amounts of oocysts from the host (up to several million oocysts per gram of feces) and the significant resistance to environmental factors (including traditional methods of water treatment that cannot destroy those parasites), oocysts are extremely widespread in surface waters and soil. The small dimensions of the invasive forms of the protozoa allow them to pass through some filters used in water treatment plants. The biology and epidemiology of waterborne outbreaks caused by parasitic protozoa, e.g. cryptosporidiosis, giardiasis or toxoplasmosis, are well documented in scientific literature [38, 39]. On the other hand, the standard techniques for detection of parasites in water are laborious and are not always guaranteed to succeed. Moreover, the dead and empty oocysts are detectable, making it difficult to determine their actual number in the sample.

The detection protocol for SEM involved:

- compaction of the biological material on membrane filters;
- purification of cysts/oocysts by gradient centrifugation;
- applying one drop of as-cleaned suspension on the carbon substrate;
- air drying on SEM specimen holder;
- coating with a thin gold layer (eventual);
- imaging in a high-vacuum mode of VP-SEM (SE and BSE detectors).

3. The advantage of VP-SEM

The essential idea of the VP-technique is shown in Fig. 3. The pumping differential aperture, placed inside the objective lens, enables lower pressure conditions in a specimen chamber of SEM. In a poor vacuum, any gas including water vapor may be present, thus gas molecules interact with incident and emerging electrons. These become ionized and produce a cloud of cations that neutralizes any charge of electrically insulating samples.

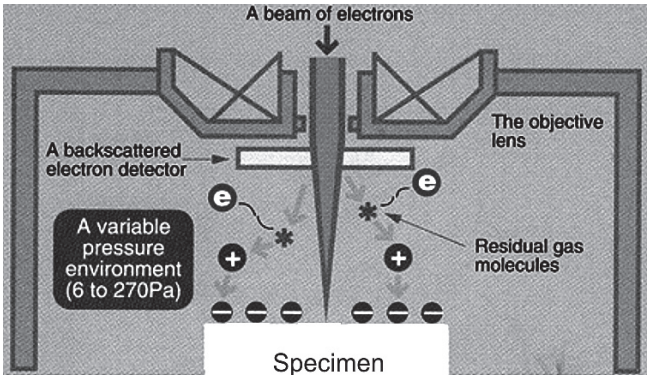

Fig. 3. Schema of the variable pressure operation mode in VP-SEM [Hitachi S-3400N]

The SEM used in this work was the HITACHI S-3400N at the Laboratory for Study Aqueous Suspensions of the Institute of Water Supply and Environmental Protection at the Cracow University of Technology (CUT). The microscope has a conventional tungsten pin thermionic electron gun and can be operated with high vacuum of 1.5×10^{-3} Pa or in VP pressure from 6 Pa to 270 Pa, according to the operator selection. The microscope is equipped with detectors for secondary electrons (SE) and back-scattered electrons (BSE). In VP-mode, the image signal comes from BSE, as having gas inside a vacuum chamber presents a problem for the traditional SE detector. With a help of a standard Peltier cool-stage, temperatures of down to –30°C at maximum pressure of 270 Pa could be obtained [11].

4. Example analysis

4.1. Imaging of *Chironomidae*

As shown in Fig. 2, the *Chironomidae* larva has narrow and elongated body with distinct segments and a capsule-like head. Two pairs of prolegs help larva to catch on: one pair is on the last segment of the abdomen and the other – on the first segment of the thorax.

The images of conductive coated samples were recorded under high vacuum mode using BSE signal in low beam accelerating voltage of 3 kV. The results show that a soft larval body is more sensitive to shrinkage during air-drying than a harder larval head (Fig. 4a),

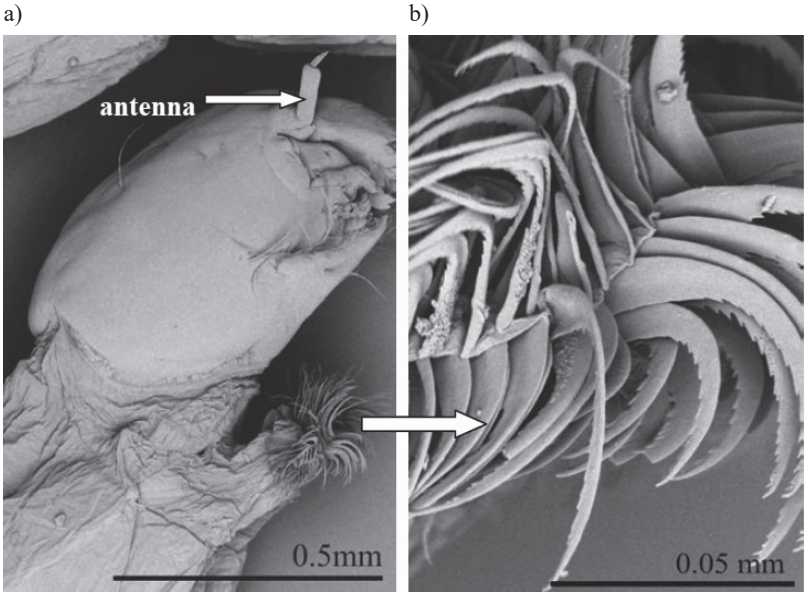

Fig. 4. Images of *Chironomidae* larva observed with BSE detector at 3 kV accelerating voltage, high-vacuum mode: a) the head, lateral view, nominal magnification 100×; b) the claws of anterior parapod, nom. mag. 1000×

on which, in lateral view, a typical antenna [40] and a few fine hears are visualized. Figure 4b shows the tiny hooks of anterior proleg (a parapod found immediately behind the head) in magnification of 1000×. The topology details observed may suggest the presence of particles of dirt (e.g. mud, debris, etc.), collected on the tiny claws. However, the composition differences from eventual surface impurities could not be resolved because of the uniform metal coating of *larvae* sample. A masking of the real structure when coated with gold is a real problem of biological sample studies in high-vacuum SEM [4], whichever detector is used.

Instead, a semi-chemical contrast due to BSE signals allows visualization of adhered particles and other surface artefacts on the biological samples when uncoated (Figs. 5 and 6). Experiments on fresh samples (conserved in alcohol) were performed under constant operation settings: a voltage of 10 kV, a pressure of 15 Pa and a working distance of 9 mm in VP-mode. Fig. 5 shows parts of *Glyptotendipes glaucus*. The satisfactory image resolution and contrast achieved without air-drying of bulk specimens indicate that the less drastic process of dehydration takes place in the specimen chamber environment. Figure 5a shows the hypopygium (a modified 9th abdominal segment) which the copulatory apparatus of many insects, especially dipterous, is associated with. The examples that follow confirm that in VP-mode, magnifications up to 1000× are achieved, insulating wet materials without any problem.

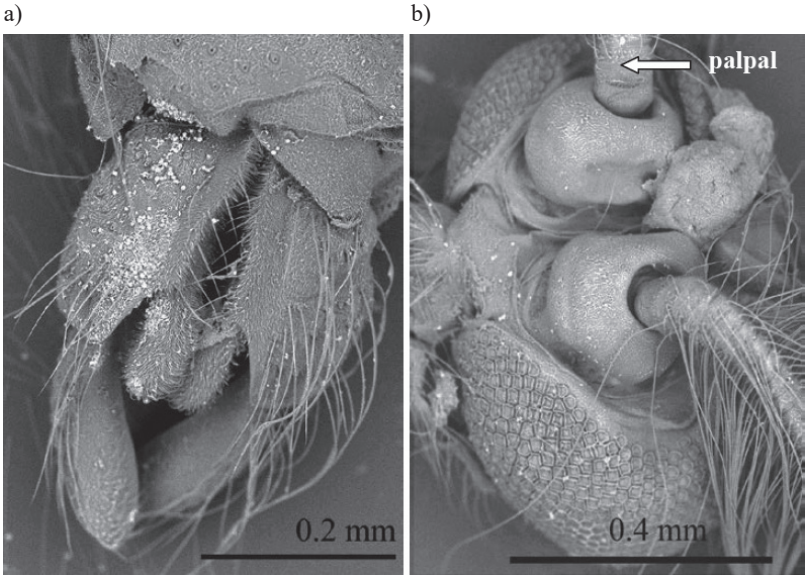

Fig. 5. Images of *Glyptotendipes glaucus*: a) the hypopygium, nom. mag. 200×; b) the head, nom. mag. 140× (sample courtesy of Dr A. Kownacki, PAN)

Figure 6 shows parts of pupal exuvium of *Glyptotendipes glaucus*. For description of the pupal structures of *Chironomidae*, the previously reported SEM and light microscopy (LM) images and entomological drawings [4, 40] were useful. The correlative light-electron microscopy helps to identify new morphological features of various parts of the organisms

studied [4]. For large samples, the most informative surface morphology and structural details are usually found in the magnification range of 100–1500×.

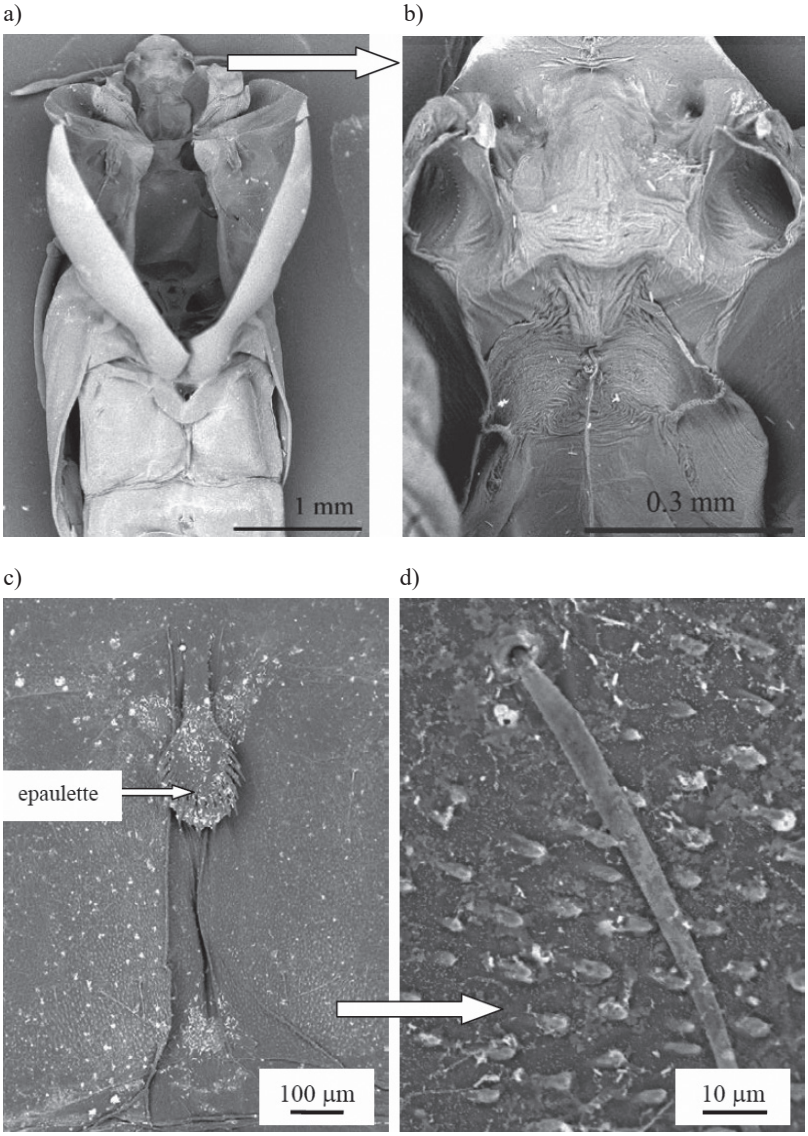

Fig. 6. Images of pupal exuvium of *Glyptotendipes glaucus*: a) anteroventral part of cephalotorax; b) the head in nominal magnification of 1500×; c) tergite in dorsal view, nom. mag. 110×; d) shagreen of tergite, nom. mag. 1000× (sample courtesy of Dr A. Kownacki, PAN)

4.2. Specimen of activated sludge

Images of activated sludge specimen are shown in Figures 7 and 8. The first experiments were performed in high-vacuum, showing the resolution advantage of SE detector at low accelerating voltage. By imaging of bulk structures in order of tenth micrometers (e.g. *Euglenoid algae*, Fig. 7a), charging of the sample occurred at 10 kV immediately. However, lowering a voltage of bombarding electrons to 3 kV permitted many fine particles to be detected in activated sludge (Fig. 7b) and images were taken at magnification of 15 000× (for a short time before next specimen charging). Next, the operation settings were changed to VP-mode after the procedure described in [11].

Figures 7c, d and Figure 8 were recorded at 10 kV and 25 Pa using the BSE signal in VP-mode. The different approach used in this experiment was that specimens were first

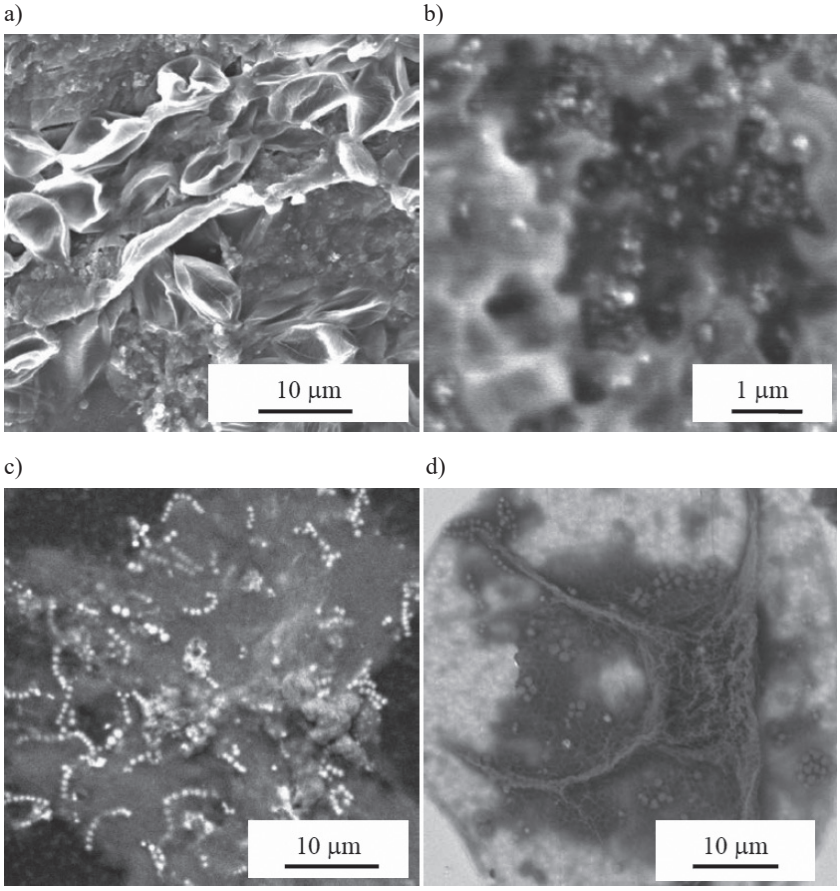

Fig. 7. Images of activated sludge: a) *Euglenoid algae*, SE, 10 kV, nom. mag. 2000×; b) other area, SE, 3 kV, nom. mag. 15000×; c) chains of bacteria attached to the flock, BSE, 10kV, 25 Pa, nom. mag. 2000×; d) *Amoebae* on a gold-coated glass holder, BSE, 10kV, 25 Pa, nom. mag. 2000× (sample courtesy of Prof. A.M. Anielak, CUT)

tested at room temperature and the carbon disc holder was replaced by glass one, priory gold coated (Figs. 7c, d). Results showed that in both cases, fine microstructure of activated sludge appear in good contrast and resolution (nominal magnification of 2000×). It is possible that the same glass holder, with partial surface area made conductive, could provide a useful tool for correlative light-electron microscopy studies in VP-SEM.

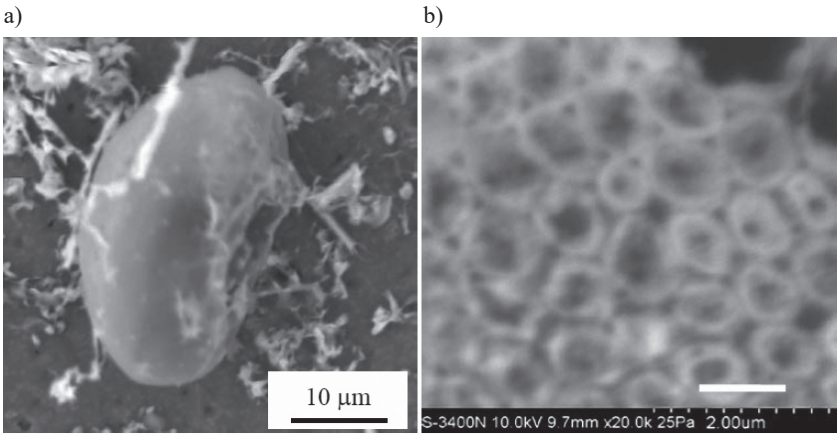

Fig. 8. Images of *Amoebae* cooled down to –25°C in VP-mode: a) the shell of testate *Amoebae*, nom. mag. 2000×; b) surface structure, nom. mag. 20000×, scale bar of 1 μm (sample courtesy of Dr T. Woźniakiewicz)

Figure 8 shows results obtained with the application of a Peltier cooling device. The study confirmed our previous results [11] that settings of 10 kV, 25 Pa and –25°C were enough for imaging less-wet samples with nominal magnification up to 10 000×. For insulating samples, however, the resolution of images acquired at nominal magnifications above 10 000× was not satisfying (Fig. 8b). The resolution of traditional BSE detector in VP-mode (4 nm at 30 kV) is not much less than of SE detector in high-pressure mode (3 nm at 30 kV). However, higher beam voltage could destroy a soft tissue.

4.3. Intestinal protozoan parasites

SEM images of protozoan parasites are presented in Fig. 9. Although the composition of samples under study was known, an unambiguous identification of single parasite cells was difficult.

The BSE image of *G. intestinalis* sample (Fig. 9a) reveals that the base of suspension was not sufficiently removed after one-step spin in distilled water. Single oval forms, which seem "transparent" at accelerating beam voltage of 10 kV, were observed. Apart the appropriate ovoid shape observed, that feature is most probably an artefact, because the too small size of the imaged object. The image of residual suspension constituents is ten times smaller, than the typical dimensions of *Giardia sp.* cysts [38].

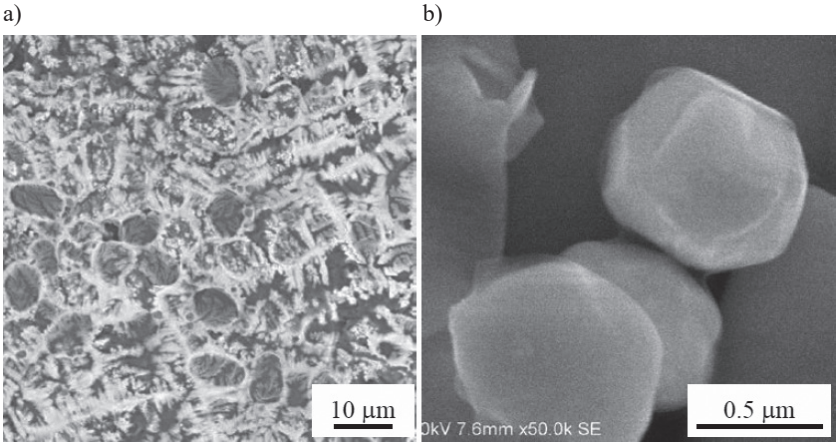

Fig. 9. Images of protozoan species in high-vacuum SEM: a) crystallized residual suspension with *Giardia intestinalis*, BSE, 10 kV, nom. mag. 1000×; b) single organisms in a *C. parvum* sample, SE, 25 kV, nom. mag. 50 000×

Assuming higher analytical depth of BSE for organic matter, the images might show the internal structure of the *Giardia sp.* cysts, which was not the case. To eliminate crystallization effects of residual suspension for SEM studies, much extensive/multistep centrifuging of the specimen is required.

The SE image of the *C. parvum* sample (Fig. 9b) was taken at nominal magnification of 50 000, the highest achieved with satisfactory resolution for biological samples in our VP-SEM. That sample was thoroughly centrifuged and finally conductive coated. A few particles with globular morphology raised the question if the object observed could be protozoan clod plasma or an external armor? Unfortunately, the size of the detected particles adds to the uncertainty as to their nature. Typical *Cryptosporidium* oocysts are ten times larger than the objects of 0.5µm observed in that sample.

Our experiments revealed, that parasite oocysts/cysts are very difficult to detect and diagnose in SEM. Despite its superior image resolution and depth of field, SEM could be used rather for routine microstructural investigations only. It should not be treated as a diagnostic tool for these species, mainly because of laborious sample preparation and many non-characteristic structural features, confirmed by means of immunofluorescent antibody (IFA) technique [39].

5. Conclusions

The variable pressure scanning electron microscope (VP-SEM) is particularly well adapted for observation and experimentation on small and highly hydrated biological specimens. The application of VP-SEM was illustrated by several cases of aquatic organisms, which play an important role in monitoring of water and sewage qualities. Examples demonstrate

that the insulating bulk samples show sufficiently good resolution and contrast in semi-chemical BSE signal for magnifications up to 2000×. The standard carbon disc holder as well as gold evaporated glass (example presented) could be used as conductive specimen substrate in room temperature studies. For magnifications up to 10 000, the best solution would be the use of VP in combination with a Peltier cool-stage.

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