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A new preparation technique of daphnids for Scanning Electron Microscopy using hexamethyldisilazane

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With 5 figures

Abstract: SEM requires special preparation techniques to avoid artifacts, especially in aquatic organisms. Not all organisms can be prepared equally well for SEM. For example in *Daphnia,* preparation causes major shrinkage of the carapace in most species. We present a new preparation technique for *Daphnia* using hexamethyldisilazane (HMDS), which avoids these problems. We compared this method with the common critical point drying technique (CPD). The routinely used CPD caused shrinkage in the daphnids, predominantly in the region of the neck and the brood chamber. In contrast, all tested *Daphnia* species immersed in hexamethyldisilazane (HMDS) and dried slowly overnight in a vacuum desiccator revealed an excellent surface. The good results, low costs and easy operation without the requirement of special equipment favor HMDS as drying agent for SEM investigations in daphnids.

Key words: *Daphnia*, preparation technique, shrinkage, surface structures, critical point drying technique (CPD), HMDS.

Introduction

Scanning electron microscopy (SEM) is an important tool in many sections of biological research. SEM is the preferred technique for analyzing surface structures of many types of tissues because of its high depth of focus which leads to a three-dimensional impression of the objects. Furthermore, the high resolution of SEM reveals more details of the surface characteristics. This is especially true for transparent organisms such as cladocerans. In ultrastructural examinations of daphnids, usually only a part of the organism has been

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studied. Some authors focused on the surface structures of the ephippia (SCHULTZ 1977, GLAGOLEV 1983, DU & LI 1990). Primarily the filtering structures were investigated to answer ecologically relevant questions; for example, filter screen mesh sizes or the variability of filter structures in different *Daphnia* species (WATTS & PETRI 1981, GOPHEN & GELLER 1984, HESSEN 1985, BRENDELBERGER & GELLER 1985, BRENDELBERGER 1991, VILLALOBOS & GELLER 1997).

The drying process is the critical step in SEM preparation. Various methods have been used to prepare specimens for SEM studies. GOPHEN & GELLER (1984) dried the gut contents and the limbs of daphnids on Nuclepore filters in a heat dryer. A double fixation procedure with 4 % glutaraldehyde and 1 % osmium tetroxide was used by WATTS & PETRI (1981). Subsequently they used a graded acetone series (20–100 %) for dehydration prior to critical point drying.

Critical point drying (CPD) technique is the most common method used to dry organisms for SEM. This technique leads, in most cases, to little or no collapse of softer body parts in contrast to simple air drying which causes major shrinkage and subsequent unnatural images. BOYDE (1980) investigated a variety of vertebrate tissues. He showed that biological samples dried with CPD sometimes exhibit wrinkles due to shrinkage on their surfaces and dimensional changes during the procedure. Therefore, several alternatives have been developed. Freeze drying can result in good preservation of surface morphology of aquatic organisms, but the major disadvantage of this technique is the frequent surface damage caused by ice crystal formation (MAUGEL et al. 1980). Low vacuum SEM freeze drying (SUZUKI et al. 1995) was proposed to prepare aquatic microorganisms. Likewise some chemical agents, such as Peldri II (BROWN 1990), were successfully used for drying small flies. NATION (1983) introduced a new chemical-based drying technique: the evaporation of hexamethyldisilazane (HMDS). This method, originally exerted on soft insect tissues is not frequently used, although good results have been obtained in several taxa (*Gryllus bimaculatus,* BOCK 1987; pollen grains, CHISSOE et al. 1994; Crustacea: *Macrobrachium potiuna,* MORAES & BOUZON 1995; mammals: hepatic endothelial cells, BRAET et al. 1996; demineralized human dentine, CARVALHO et al. 1996; *Sarcocystis,* STOLTE et al. 1996).

We tested HDMS as drying agent for SEM investigations in different *Daphnia* species. We compare this method with the common CPD technique.

Materials and methods

The examined *Daphnia* species were taken from laboratory cultures. *Daphnia lumholtzi* originate from the USA, *Daphnia longicephala* from Australia, *Daphnia magna* from Germany, *Daphnia pulex* from Canada, *Daphnia ambigua* from the USA and

Daphnia cucullata was isolated from Germany. The animals were reared in an artificial medium based on ultrapure water and fed daily with *Scenedesmus obliquus*. Neck teeth, crests and helmets were induced by predator kairomones (GRANT & BAYLY 1981, KRUEGER & DODSON 1981, TOLLRIAN 1990, HANAZATO 1990, TOLLRIAN 1994). The animals were removed from their culture medium and rinsed in filtered newly prepared medium to ensure that the organisms were free of particulate matter. The daphnids were killed in a microwave (4 sec.) and immediately transferred into 70 % ethanol for fixation. Pilot experiments had shown that sugar-formalin is not a suitable agent to fix the daphnids for obtaining good SEM whole body images.

Critical Point Drying

For the CPD method, the daphnids were dehydrated in a graded acetone series (70 %, 80 %, 90 %, 10 min each; 2×10 min 98 %; 2×10 min 100 %) prior to critical point drying (Bio-Rad E 4850, München). The specimens were transferred to a small CPD container (Bio-Rad, München) filled with 100 % acetone. The CPD chamber was flooded with liquid CO_2 under high pressure and low temperature (10–15 °C). After five minutes the CO_2 was slowly vented. Flooding and venting were repeated six times to substitute the acetone. Finally the temperature of the chamber was raised over 10 minutes to 42 °C and 1200 psi for critical point drying. Afterwards the chamber was slowly vented over 20 minutes.

Hexamethyldisilazane Drying

For HMDS drying, the samples were dehydrated in graded acetone solutions (70 %, 80 %, 90 %, 2×98 % and 2×100 %) for 10 min each. Subsequently the specimens were immersed in 1–1.5 ml HMDS (1,1,1,3,3,3 hexamethyldisilazane; Merck-Suchardt, Darmstadt) in 20 ml glass vials. After a soak of 30 minutes, approximately 90 % of the HMDS was removed and the vials were immediately transferred to a desiccator. The bottom of the desiccator was covered by silica gel beads (Merck-Suchardt, Darmstadt) and the desiccator itself was evacuated to avoid water contamination which would cause shrinkage in the specimens. The remaining HMDS was allowed to evaporate overnight under anhydrous conditions. All steps with HMDS were conducted under a fume hood, because HMDS is strongly irritant.

Following CPD or HMDS preparation, the samples were carefully mounted on aluminum stubs (Plano, Wetzlar) with double sticky tabs (Plano, Wetzlar), under a dissecting microscope. Afterwards, the specimens were sputter coated with gold (BIO-RAD SC 510, München) for 135 sec. The samples were examined with a Philips XS – 20 scanning electron microscope at 20 kV.

Results

HDMS dried specimens showed well preserved surfaces. Almost no distortion or shrinkage was observed with this treatment. In contrast, the daphnids prepared with CPD showed partially strong shrinkages (Fig. 1). In some cases, the

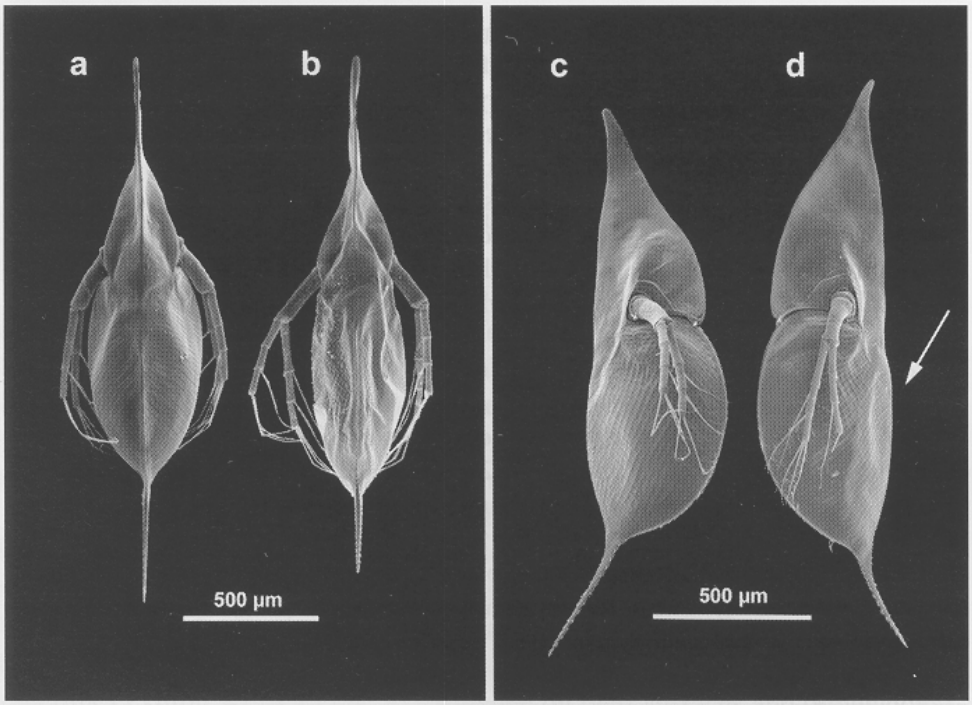

Fig. 1. Dorsal view of *Daphnia cucullata* dried with HMDS **(a)** and CPD **(b)**. Lateral view of *Daphnia cucullata* dried with HMDS **(c)** and CPD **(d)**. Deformations are visible after CPD, while HMDS provides a better surface structure. In particular, the carapace of the daphnids is susceptible for shrinkages in the region of the neck and the brood chamber (arrow).

difference between both treatments was marginal. However, specimens dried with CPD are more susceptible to shrinkage, predominantly in the region of the neck and the brood chamber (Fig. 1 d). The number of very good SEM whole body images was distinctly higher in the HMDS treatment. Drying with HDMS obtained good results both in fragile species such as *Daphnia cucullata* or *Daphnia ambigua* (Figs. 1 a, c, 5) and in more robust species such as *Daphnia magna, Daphnia longicephala* or *Daphnia lumholtzi* (Figs. 2, 4). Even juvenile stages of different *Daphnia* species dried with HMDS show good surface quality (Figs. 2 a, 3 d). The use of HMDS as a drying agent yields good results in the investigation of ultrastructural characteristics in daphnids. Both the neckteeth of *Daphnia pulex* (Fig. 3 c) and the thorn on the tip of the head of *Daphnia ambigua* (Fig. 5 b) showed well preserved surface structure. Likewise, the SEM recordings of the dorsal organ of the second juvenile stage of *Daphnia cucullata* (Fig. 5 a) display a presentable result when the specimens were dried with HMDS. In *Daphnia magna* good results were achieved when morphological details like the shape of the head (Fig. 2 b), the filter appendages or the chemosensillae (Fig. 3 a, b) were examined.

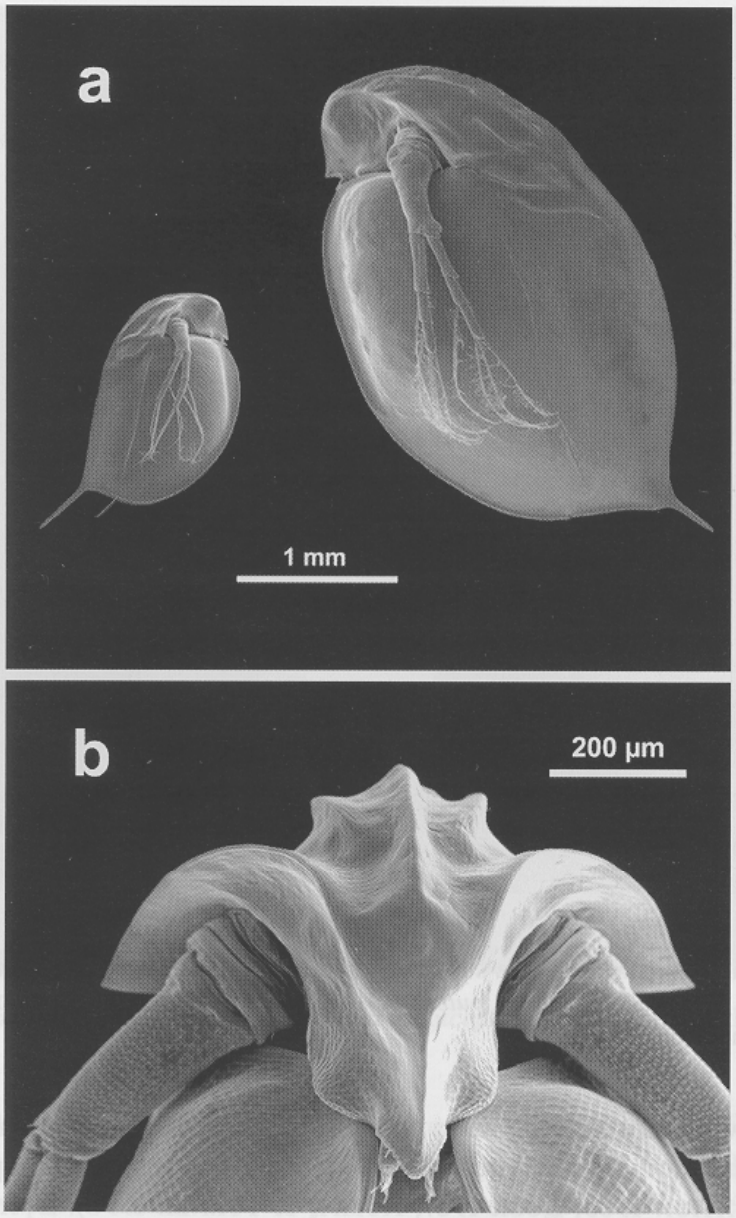

Fig. 2. *Daphnia magna,* second juvenile stage and adult female dried with HMDS **(a)**. The structures which can be seen on the head are no artifacts due to the drying procedure, as can be recognized from the frontal view of the head **(b)**.

Discussion

HMDS seems to be a convenient alternative to the established and widely used CPD method. Both in animals and plants, good results have already been achieved by using this method for SEM investigations. Chissoe et al. (1994) showed that HMDS is an excellent drying agent for pollen grains, especially

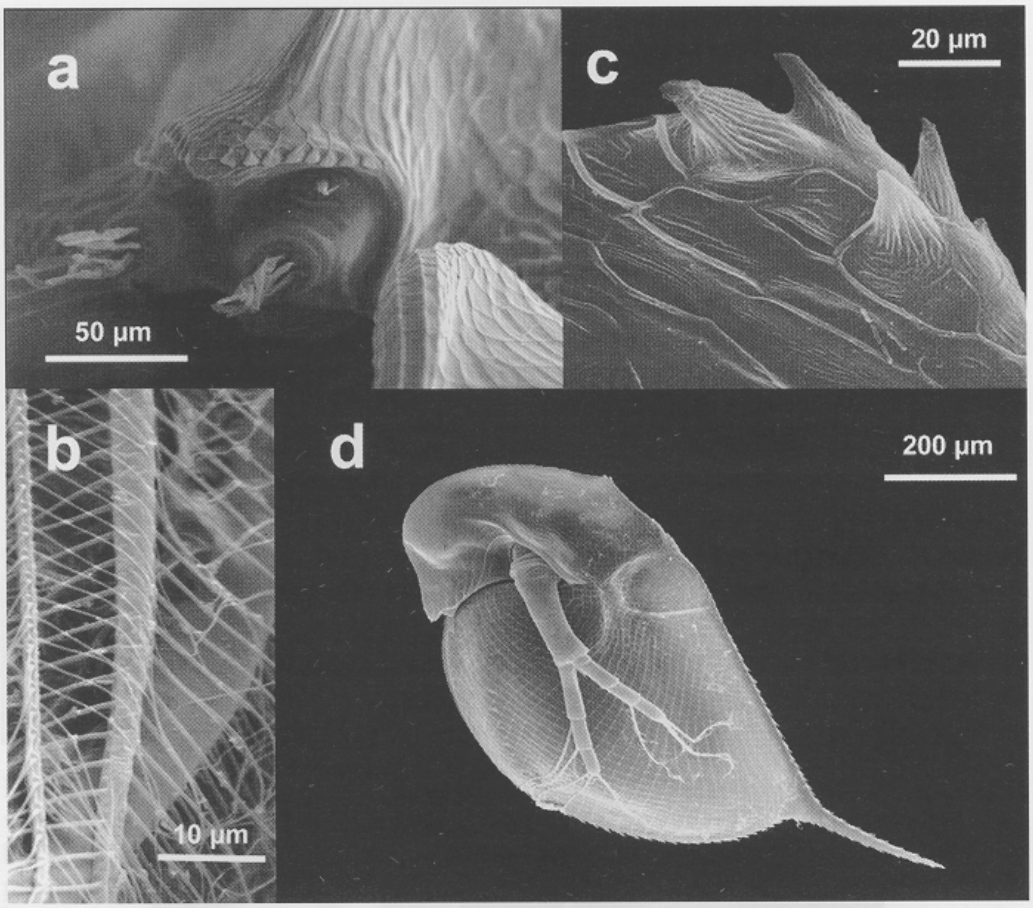

Fig. 3. Fine structure of HMDS prepared daphnids. *Daphnia magna:* **(a)** chemosensillae of the first antenna and fine structure of the carapace (HMDS); **(b)** thoracopodite appendages (HMDS); *Daphnia pulex:* **(c)** neck teeth (HMDS); **(d)** second juvenile stage with neck teeth (HMDS).

for those which are susceptible to collapse in the SEM. Furthermore sensitive internal and surface structures of insects were successfully prepared with HMDS (Nation 1983, Bock 1987, Rumph & Turner 1998). Even the dehydration of soft tissues, like hepatic cells, with HMDS was as satisfactory as the CPD method (Braet et al. 1996). Most authors working with HMDS and CPD pointed out that there is no or just a marginal (Heraty & Hawks 1998) difference between both methods. In a few taxa, such as softbodied hymenopterans, the CPD method might lead to slightly better results (Heraty & Hawks 1998).

In small and fragile species like daphnids the HMDS treatment seems to be more effective for obtaining high qualitative SEM images compared to the CPD method.

HMDS reacts with water to produce hexamethyldisiloxane and ammonia, both of which could evaporate from the object (Heraty & Hawks 1998). Na-

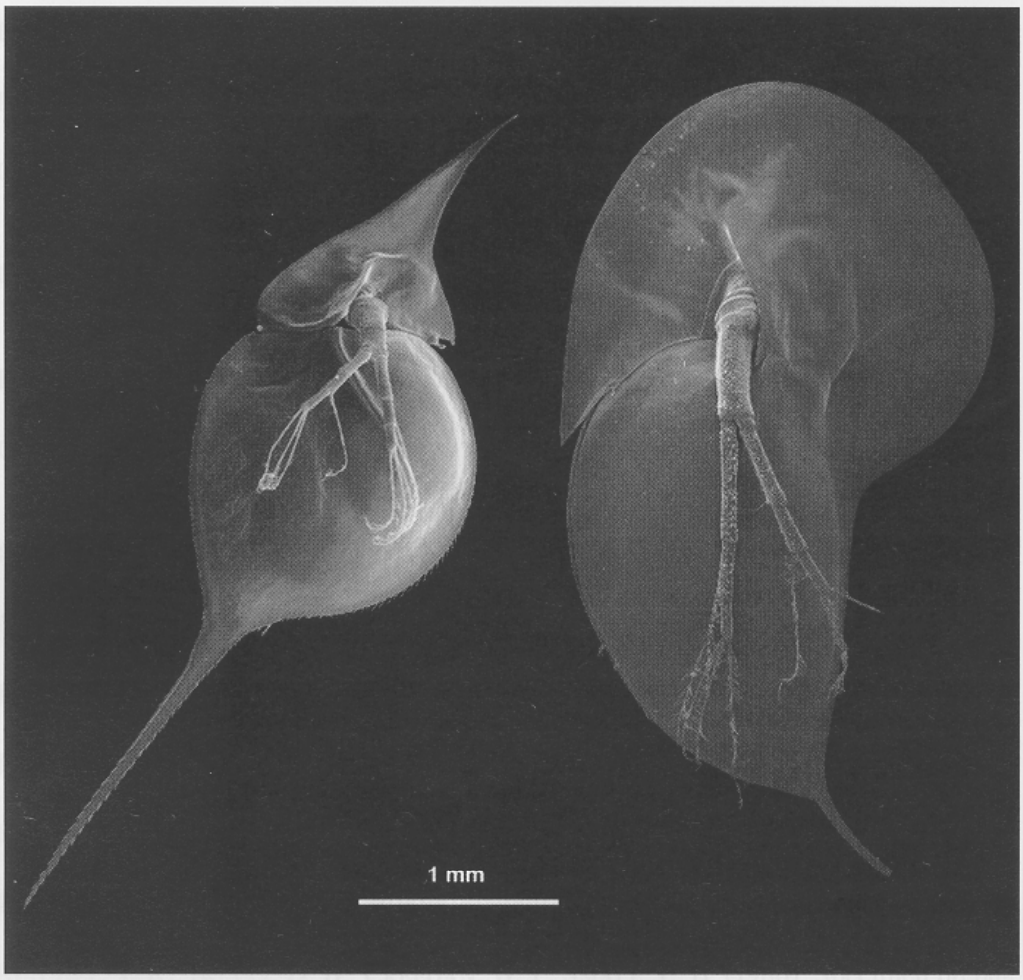

Fig. 4. *Daphnia lumholtzi* (left) and *Daphnia longicephala* (right) dried with HMDS.

TION (1983) suggested that HMDS might crosslink proteins and stiffen the tissue during the drying process. The relatively long period of time HMDS is allowed to evaporate in absolute anhydrous surroundings could be the reason for obtaining an excellent surface structure of the animals in our experiment. The extremely slow vaporizing of HMDS seems to prevent the destructive force of the surface tension during the drying procedure. Astonishingly, there is no difference between the large *Daphnia* species such as *Daphnia magna* and the small, more fragile species such as *Daphnia cucullata,* or between adult and juvenile daphnids (Fig. 2 a). Although there is sometimes a slight difference between both methods, CPD predominantly causes shrinkage in the apparently sensitive dorsal regions of the daphnids. Our results reveal that HMDS drying is successful in preserving ultrastructural features of the daphnids (Figs. 3, 5). Therefore, the HMDS method seems to be a suitable technique for a detailed investigation of the fine structure of morphological traits in daphnids.

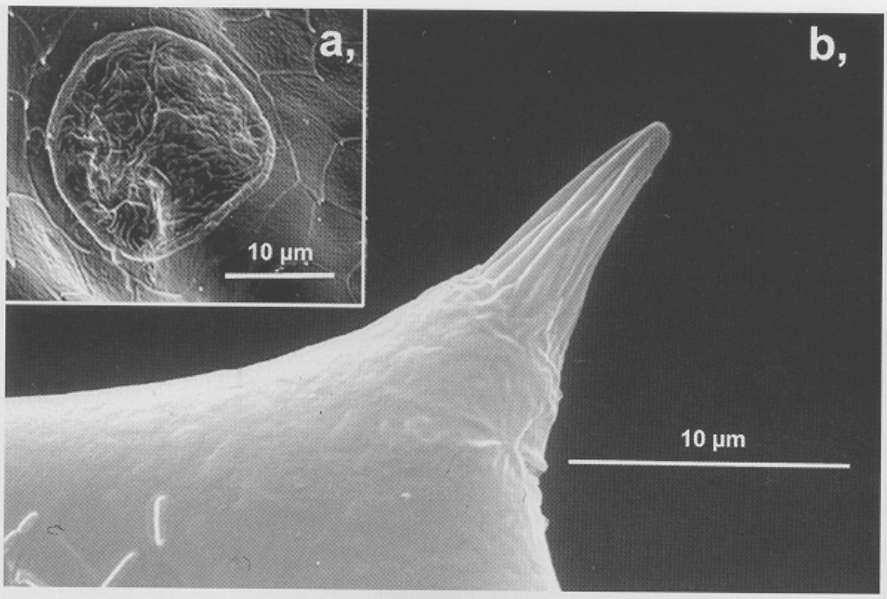

Fig. 5. Dorsal organ of the second juvenile stage of *Daphnia cucullata* **(a)** and tip of the helmet of *Daphnia ambigua* **(b)** dried with HMDS.

Another considerable advantage of HMDS is the simplicity of the method. It requires no specialized equipment, is easy to use and it is inexpensive. We are confident that the good results obtainable with this method will stimulate further investigations and lead to new insights into the link between functional morphology and ecology of cladocerans.

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References

Bock, C. (1987): A quick and simple method for preparing soft insect tissues for scanning electron microscopy using carnoy and hexamethyldisilazane. – Beitr. elektronenmikroskop. Direktabb. Oberfl. **20:** 209–215.

Boyde, A. (1980): Review of basic preparation techniques for biological scanning electron microscopy. – Electron Microscopy **2:** 768–777.

Braet, F., De Zanger, R. & Wisse, E. (1996): Drying cells for SEM, AFM and TEM by hexamethyldisilazane: a study on hepatic endothelial cells. – J. Microsc. **186:** 84–87.

Brendelberger, H. (1991): Filter mesh size of cladocerans predicts retention efficiency for bacteria. – Limnol. Oceanogr. **36:** 884–894.

BRENDELBERGER, H. & GELLER, W. (1985): Variability of filter structures in eight *Daphnia* species: mesh sizes and filtering areas. – J. Plankton Res. **7:** 473–486.

BROWN, B. V. (1990): Using Peldri II as an alternative to critical point drying for small flies. – Fly Times **4:** 6.

CARVALHO, R. M., YOSHIYAMA, M., BREWER, P. D., PASHLEY, D. H. (1996): Dimensional changes of demineralised human dentine during preparation for scanning electron microscopy. – Arch. Oral Biol. **41:** 379–386.

CHISSOE, W. F., VEZEY, E. L. & SKVARLA, J. J. (1994): Hexamethyldisilazane as a drying agent for pollen scanning electron microscopy. – Biotech. Histochem. **69:** 192–198.

DU, N. & LI, Y. (1990): Ultrastructural studies on the ephippium of *Daphnia carinata* (Crustacea; Cladocera). – Acta. Zool. Sin. **36:** 341–344.

GLAGOLEV, S. M. (1983): The structure of the ephippium surface in Daphniidae (Crustacea; Cladocera) based on scanning electron microscopy data. – Zool. Zh. **62:** 1422–1425.

GOPHEN, M. & GELLER, W. (1984): Filter mesh size and food particle uptake by *Daphnia*. – Oecologia **64:** 408–412.

GRANT, J. W. G. & BAYLY, I. A. E. (1981): Predator induction of crests in morphs of the *Daphnia carinata* KING complex. – Limnol. Oceanogr. **26:** 201–218.

HANAZATO, T. (1990): Induction of helmet development by a *Chaoborus* factor in *Daphnia ambigua* during juvenile stages. – J. Plankton Res. **12:** 1287–1294.

HERATY, J. & HAWKS, D. (1998): Hexamethyldisilazane – A chemical alternative for drying insects. – Ent. News **109:** 369–374.

HESSEN, D. O. (1985): Filtering structures and particle size selection in coexisting Cladocera. – Oecologia **66:** 368–372.

KRUEGER, D. A. & DODSON, S. I. (1981): Embryological induction and predation ecology in *Daphnia pulex*. – Limnol. Oceanogr. **26:** 219–223.

MAUGEL, T. K., BONAR, D. B., CREEGAN, W. J., SMALL, E. B. (1980): Specimen preparation techniques for aquatic organisms. – Scan. Electron Microsc. **2:** 57–77.

MORAES, N. & BOUZON, Z. L. (1995): Comparative analysis of the external morphology of *Macrobrachium potiuna* eggs by using hexamethyldisilazane and critical point in scanning electron microscopy. – Arqu. Biol. Tecnol. **38:** 689–692.

NATION, J. L. (1983): A new preparation method using hexamethydisilazane for preparation of soft insect tissues for scanning electron microscopy. – Stain Technol. **58:** 347–351.

RUMPH, J. A. & TURNER, W. J. (1998): Alternative of critical point drying for soft-bodied insect larvae. – Ann. Entomol. Soc. Amer. **91:** 693–699.

SCHULTZ, T. W. (1977): The fine structure of the ephippium of *Daphnia pulex* (Crustacea; Cladocera). – Trans. Amer. Micros. Soc. **96:** 313–321.

STOLTE, M., BOCKHARDT, I. & ODENING, K. (1996): A comparative scanning electron microscopic study of the cyst wall in 11 *Sarcocystis* species of mammals. – J. Zool., Lond. **239:** 821–832.

SUZUKI, T., SHIBATA, M., TANAKA, K. TUCHIDA, K. TODA, T. (1995): A new drying method: low-vacuum SEM freeze drying and its application to plankton observation. – Bull. Plankton Soc. Japan **42:** 53–62.

TOLLRIAN, R. (1990): Predator-induced helmet formation in *Daphnia cucullata* (SARS). – Arch. Hydrobiol. **119:** 191–196.

TOLLRIAN, R. (1994): Fish-kairomone induced morphological changes in *Daphnia lumholtzi* (SARS). – Arch. Hydrobiol. **130:** 69–75.

VILLALOBOS, L. & GELLER, W. (1997): Setular bosses: Report of a new ultrafine structure on the filter appendages of *Daphnia*. – Arch. Hydrobiol. **140:** 565–575.

WATTS, E. & PETRI, M. (1981): A scanning electron microscope study of the thoracic appendages of *Daphnia magna* Straus. – J. Nat. Hist. **15:** 463–473.

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