Received: 21 August 2023 | Revised: 20 October 2023 | Accepted: 8 November 2023

DOI: 10.1002/jemt.24460

RESEARCH ARTICLE

MICROSCOPY RESEARCH & TECHNIQUE WILEY

A new, simplified, drying protocol to prepare tardigrades for scanning electron microscopy

Daniele Camarda[1] | Edoardo Massa[2] | Roberto Guidetti[2] | Oscar Lisi[1]

[1]Department of Biological, Geological and Environmental Sciences, University of Catania, Catania, Italy

[2]Department of Life Sciences, University of Modena and Reggio Emilia, Modena, Italy

Correspondence
Edoardo Massa, Department of Life Sciences, University of Modena and Reggio Emilia, via G. Campi 213/D, 41125 Modena, Italy.
Email: edoardo.massa@unimore.it

Funding information
Italian Ministry of University and Research, Grant/Award Number: Project Code CN_00000033; NBFC, National Biodiversity Future Center, Grant/Award Number: CUP=E93C22001090001; Università di Catania; University of Modena and Reggio Emilia

Review Editor: Alberto Diaspro

Abstract

A new protocol for preparation of tardigrades for scanning electron microscope (SEM) analysis is proposed. The more conventional protocols require various steps and a long time to obtain good drying of water bears, together with specific and uncommon instruments (i.e., critical point dryer) or highly volatile toxic compounds (i.e., hexametildisilazane). The new protocol can be performed using few and simple instruments and materials, all easily accessible, and produces a high yield in terms of dried animals in excellent condition for the observation of external morphological structures with SEM. The acquired data exhibit considerable promise, and the proposed methodology shows potential for application to other meiofaunal groups, including small arthropods, nematodes, and rotifers.

Research Highlights

- Cheap, safe, and fast new method for Tardigrada preparation for SEM.
- With the new protocol, the number of animals required for SEM studies is minimized.
- New protocol is potentially applicable to the study of other meiofaunal soft-bodied taxa.

KEYWORDS
dehydration, external morphology, histology, scanning electron microscope, taxonomy

1 | INTRODUCTION

Tardigrades are microscopic animals with dimensions ranging from about 50 to 1000 μm. They are soft-bodied ecdysozoans showing few internal and external rigid structures useful for morphological diagnosis, whose shape and architecture may only slightly vary among different species.

From a morphological point of view, tardigrades are mainly studied through different optical microscopic techniques (phase contrast - PCM and differential interference contrast - DIC microscope, confocal laser scanning microscope - CLSM) and through electron microscopy, namely transmission electron microscope (TEM) and scanning electron microscope (SEM).

The first studies on tardigrades conducted with electron microscopes were investigations through TEM by Baccetti and Rosati (1969, 1971). Shortly after, SEM pictures of tardigrades became frequent (Bertolani et al., 1996; Claxton, 1998; Crowe & Cooper, 1971; Grigarick et al., 1973; Schuster et al., 1980; Toftner et al., 1975). The SEM allowed tardigradologists for the study of the fine morphology of external characters otherwise not identifiable with light microscopy (LM) and, even if SEM does not provide information on depth, it allows a 3D-like visualization of the specimens that can complement

Daniele Camarda and Edoardo Massa are equally contributed.

observation of their morphology, and therefore become a standard in tardigradology. In fact, some morphological features of diagnostic relevance in tardigrades are not well observable with LM (i.e., PCM, DIC), such as peribuccal structures, details of the oral cavity armor, claws annexes, cuticle ornamentations, and eggs morphology. Besides, SEM allows for the observation of other features important to understand the biology and adaptations of these animals, for example, external sensory organs, sperms morphology, or sclerified structures of the feeding apparatus. Therefore, the details acquired with this powerful instrument add value to morphological research and are helpful for a correct diagnosis of any species.

It is worth underlining that SEM images cannot substitute imaging performed with other microscopy techniques. This is because some fundamental diagnostic tardigrade structures are internal and thus impossible to observe through SEM (unless they are extracted via long and cumbersome protocols, as described for the buccal tubes in Eibye-Jacobson, 2001a; also see for example, Eibye-Jacobson, 2001b; Guidetti et al., 2012; Gąsiorek & Michalczyk, 2020; or other ultrastructural analyses, see for example, Michalczyk & Kaczmarek, 2003, 2006), since SEM is only capable of reconstructing images of external surfaces. Nevertheless, SEM remains a useful and, sometimes, essential tool to deepen and enrich the morphological description of specimens.

Currently, a good number of species have been examined through SEM in the last decades and nowadays it is a well-established standard for species description (e.g., Bertolani et al., 2022; Gąsiorek et al., 2018, 2022; Guidetti et al., 2020, 2021; Guidetti, Cesari, et al., 2019; Guidetti, Massa, et al., 2019; Massa et al., 2021; Møbjerg et al., 2020; Pogwizd & Stec, 2022; Stec, 2022; Sugiura et al., 2020; Tumanov et al., 2022), enriching the quality of morphological details.

Limitations in the use of SEM are mainly related to the preparation of specimens, which requires at least two major delicate and essential steps: the fixation and the total drying of the specimens. Considerable difficulties arise in the methods of drying of soft-bodied ecdysozoans, which have a non-rigid exoskeleton, like tardigrades. Air-drying is not possible with these animals due to the severe wrinkling and distortion that would occur, resulting in the loss of their natural shape and in the impossibility of observing useful details of the diagnostic structures, that, together with the eventual production of artifacts, make the mounted material partially or entirely unsuitable for diagnostic purposes.

Fixation and drying, especially for soft-bodied animals, usually require expensive specific instruments and reagents (i.e., toxic fixatives and drying agents). The derived protocols are also time-consuming, due to the significant number of steps (Bray et al., 1993). The main drying techniques used for invertebrates require substitution of fixative (e.g., absolute ethanol or acetone) with high pressure liquid CO_2 and its subsequent evaporation via pressure lowering with a critical point dryer (CPD; an expensive specific instrument; Anderson, 1951; Bray, 2000; Mitchell & Miller, 2008) or with the fast evaporating reagent hexametildisilazane [HMDS] (a flammable, volatile liquid, hazardous for human health and for the environment; Nation, 1983, Hochberg & Litvaitis, 2000; Laforsch & Tollrian, 2000; Barre et al., 2006; Spiers et al., 2013; Haefke et al., 2014). Less used techniques, reported in literature for soft-bodied invertebrates but never used for tardigrades, are freon drying (environmentally hazardous), freeze drying with 100% ethanol (Eisenback, 1986), fluorocarbon compound Peldri II (environmentally hazardous; Bray et al., 1993), tetramethylsilane drying (producing flammable vapors; Dey et al., 1989). Instead, other techniques such as t-butyl alcohol freeze drying (Inoué & Osatake, 1988) and aldehyde-osmium (Halász, 1974; Wieczorkiewicz et al., 2023) have been used but only for biological tissue fixation or TEM analyses.

Concerning tardigrades, the main protocol used to dry specimens for SEM observation is the fixative substitution with liquid CO_2 and its evaporation with CPD (e.g., Dastych, 2019; Gross et al., 2017; Guidetti et al., 2000; Stec et al., 2015; Vecchi et al., 2023).

A few researchers tried to desiccate tardigrades using HMDS (e.g., Bai et al., 2020, 2022; Czerneková et al., 2018; Haefke et al., 2014; Shively & Miller, 2009; Spiers et al., 2013), a drying agent largely used for other invertebrates, or boiling ethanol (Bertolani et al., 2011, 2014; Guidetti et al., 2014, 2022; Guidetti, Cesari, et al., 2019; Guidetti, Massa, et al., 2019). The latter allowed the drying of tardigrades for SEM analysis, but the Authors did not report the detailed protocol which generally led to a low percentage of animals maintaining a natural morphology (Guidetti, personal communication).

In the present study, this drying technique carried out with a single chemical (i.e., absolute ethanol) has been modified with the primary aim to increase the percentage of animals maintaining a natural morphology. The secondary aim was to develop a drying protocol which does not require compounds that are toxic for operators and environment (i.e., HMDS), expensive instruments, and time-consuming protocols.

In addition, comparative data on different protocols to fix and dry animals, including the boiling ethanol drying protocol by Bertolani et al. (2011) and the widely used HMDS drying protocol for invertebrates, are provided. Such comparative analyses do not include protocols requiring specific instruments (e.g., CPD, vacuum evaporator) or harmful chemicals (e.g., osmium tetroxide, aldehydes), and instead include only methods already applied for SEM preparation of tardigrades.

This study proposes a novel and considerably safer, cheaper, and timesaving protocol to dry tardigrades for SEM observation. This novel protocol also resulted in a high rate of successfully dried and naturally looking animals also in comparison to other techniques.

2 | MATERIALS AND METHODS

2.1 | General summary

A total of 60 (10 processed for each of the tested protocols) live specimens belonging to the terrestrial, herbivorous species of eutardigrade *Hypsibius exemplaris* Gąsiorek et al., 2018 reared according to Massa et al. (2023), were extracted with a Pasteur pipette from the culturing Petri dish and rinsed twice in distilled water to clean them from culture algae.

TABLE 1 Different SEM-preparation protocols tested in the present study combining different fixation and drying methods.

Fixation methods	Drying methods	Protocols names	References
Boiling EtOh (A)	EtOH Chamber (1)	A1	Guidetti, Cesari, et al., 2019
	EtOH Drop (2)	A2	Present paper
	HMDS (3)	A3	Present paper[a]
EtOH series (B)	EtOH Chamber (1)	B1	Present paper[b]
	EtOH Drop (2)	B2	Present paper
	HMDS (3)	B3	Barre et al., 2006

[a]Represents the same protocol of B3, already reported in literature, evaluated with a different fixation method (i.e., animals were fixed directly in boiling ethanol instead of the increasing ethanol series).
[b]Represent the same protocol of A1, already partially reported in literature, evaluated with a different fixation method.

In general, preparation for SEM requires a preliminary fixation method followed by a drying method. In the present study, two fixation methods were combined with three possible, subsequent, drying methods resulting in six possible combinations (Table 1), all tested in the present study to compare different SEM-preparations.

The two fixation methods were: (1) animals placed directly in boiling ethanol (indicated in the present paper with the acronym "Boiling EtOH" and with letter "A"); and (2) a series of increasing concentration of ethanol (indicated in the present paper with the acronym "EtOH series" and with letter "B").

The three drying methods were: (1) ethanol chamber (indicated in the present paper with the acronym "EtOH Chamber" and number "1"); (2) ethanol drops (indicated with the acronym "EtOH Drop" and number "2"), which is the newly proposed drying method; (3) drying protocol with HMDS (indicated by the acronym "HMDS" and number "3").

In order to evaluate the results, scores were assigned to each specimen based on its condition observed with SEM (Figure 1). A score of 0 points was given to totally wrinkled animals (defined as "Bad"), 0.5 points was given to partially wrinkled animals with some, but not all, diagnostic structures (e.g., mouth, cavity, claws, cuticle surface) sufficiently visible (defined as "Acceptable"), and the highest score (1 point) was ascribed to animals with a good distension and good visibility and integrity of the diagnostic structures (defined as "Good"). Since 10 specimens were tested for each protocol (Table 1), the maximum possible score of each protocol was 10 points.

The protocols that scored above 4 points were further investigated by testing an additional 90 specimens for each protocol, in order to conduct statistical analyses.

To evaluate the performance of the tested protocols, the following additional parameters were considered: (1) the number of steps required by each protocol, taking into account the risk of animal loss at each step; (2) the time required to complete each protocol; (3) the hazards posed by the reagents used; (4) the amount and cost of the reagents.

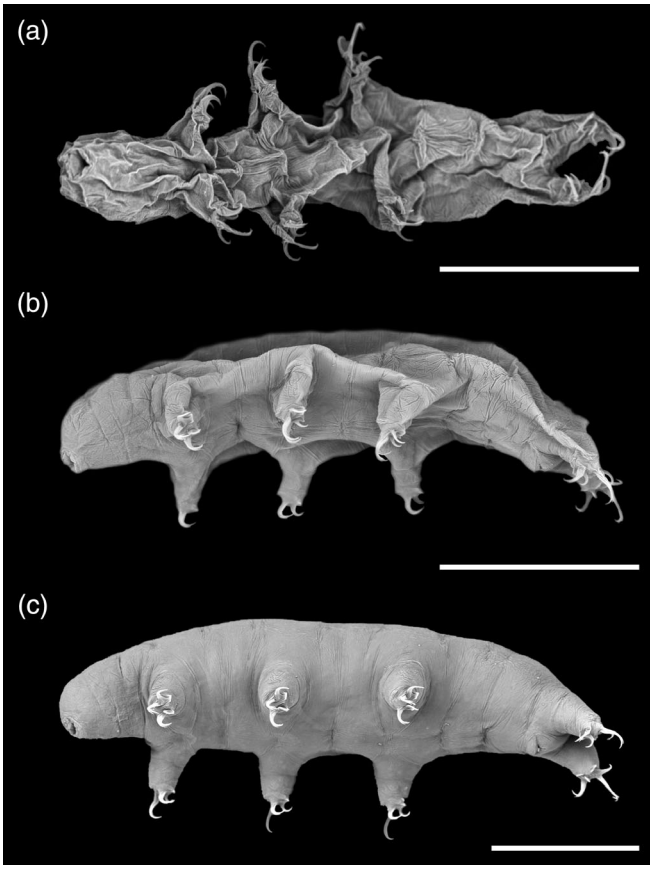

FIGURE 1 Scanning electron microscopy (SEM) pictures of *Hypsibius exemplaris* in toto showing different morphological condition according to the applied score system. (a) 0 point (prepared with B3 protocol). (b) 0.5 point (prepared with A2 protocol). (c) 1 point (prepared with A2 protocol). Scale bars 50 μm.

2.2 | Laboratory reagents and equipment

2.2.1 | Reagents used in protocols

1. Ethanol (EtOH) puriss. p.a. absolute, 99.8% (GC), Molecular formula: C_2H_6O; Producer: Sigma-Aldrich (Product number: UN1170); CAS-No: 64-17-5. Hazard statements according to Regulation (EC) No. 1907/2006: H225, highly flammable liquid and vapor; H319, causes serious eye irritation.
2. Hexamethyldisilazane (HMDS), Molecular formula: $C_6H_{19}NSi_2$; Producer: Sigma-Aldrich (Product number 8.04324); CAS-No: 999-97-3. Hazard statements according to Regulation (EC) No. 1907/2006: H225, highly flammable liquid and vapor; H302, harmful if swallowed; H302 + H332, harmful if swallowed or if inhaled; H311, toxic in contact with skin; H332,

harmful if inhaled; H412, harmful to aquatic life with long lasting effects.
3. Millipore water.
4. Silica gel (see Self-made tools).

2.2.2 | Self-made tools

1. A drying chamber was assembled by using an unsealed, large Petri dish (20 cm in diameter), inside of which a piece of coverslip (placed on a glass support) served as a drying plate. In addition, a smaller Petri dish (3 cm in diameter) filled with silica gel was placed inside the larger dish to accelerate the drying process and prevent the rehydration of the animals once dried.
2. Brush with a single bristle.
3. Microsieve (made according to Figure 2 by Abolafia, 2015).

2.2.3 | Laboratory glasses

1. Borosilicate glass flat surface, that is, small beakers (5 mL) or Boveri capsules (10 mL).
2. Pasteur pipette equipped with propipette.

2.2.4 | Laboratory instruments

1. Stove: Mini Batt 805.
2. Heater plate: VWR hotplate, model 720-HP.
3. SEM: Nova NanoSem 450 available at the "Centro Interdipartimentale Grandi Strumenti" at the University of Modena and Reggio Emilia and Phenom-XL available at the University of Catania.
4. Stereomicroscope: Leica EZ4D.
5. Hood: Biosafety Cabinet Class I.
6. Gold Sputter Coater: Emitech K550.

2.3 | Animals collection and distention

To induce a state of distension before fixation, all live animals were placed in Millipore water at 60°C for 15 min. The animals were then subjected to fixation (see sec. Fixation Method) and drying (see sec. Drying Method) with the protocols listed in Table 1 (see sec. Detailed protocols).

2.4 | Fixation methods

Two following methods, A and B, were used for the fixation:

A. Boiling EtOH (Approximate total time to complete the fixation step -5 min):

This fixation method was applied following Bertolani et al. (2011, 2014) and Guidetti, Cesari, et al. (2019); Guidetti, Massa, et al. (2019); Guidetti et al. (2020, 2021, 2022). Animals were placed directly in boiling ethanol as follows: the heater plate was heated to 150°C; a Boveri capsule with 5 mL of absolute EtOH was placed on the heater plate until ethanol boiling; the capsule with boiling EtOH was removed from the heater plate (using tweezers and/or heat-resistant gloves). Then the animals were immediately transferred into the capsule with about 20–30 µL of water using a Pasteur pipette and kept in the Boveri for 3 min. During this time, the capsule was kept covered with a lid to avoid ethanol evaporation. Subsequently, animals were taken from the Boveri and rinsed three times with absolute EtOH at room temperature.

B. EtOH series (Approximate total time to complete the fixation step 80 min):

Animals were transferred to a microsieve and subjected to increasing ethanol series (30-50-70-80-90-95-absolute) and to a final rinse in absolute ethanol. The eight ethanol steps lasted 10 min each.

2.5 | Drying methods

Three following methods, 1–3, were used for drying.

1. EtOH chamber (approximate total time to complete the drying step 10 min)

This drying method was applied in Bertolani et al. (2011, 2014) and Guidetti, Cesari, et al. (2019); Guidetti, Massa, et al. (2019); Guidetti et al. (2020, 2021, 2022) but never precisely reported.

Fixed animals (with protocol A or B) were placed in a Boveri capsule with about 3 mL of absolute EtOH and covered with a glass lid; then, the capsule was placed on a heater plate preheated to 150°C; right after the ethanol was totally evaporated creating a EtOH saturate chamber the capsule was moved away from the heater plate and the lid was removed (using tweezers and/or heat-resistant gloves).

2. EtOH drop (approximate total time to complete the drying step 3 min)

This represents a newly proposed drying method.

An empty Boveri capsule was placed on the heater plate at a temperature of 180°C, almost 2 min were required for the bottom of the capsule to reach the same temperature, then the fixed animals were dropped in the hot capsule with a single drop of absolute ethanol (about 30–50 µL); right after the drop had evaporated, the Boveri capsule was removed from the heater plate (using tweezers and/or heat-resistant gloves).

It is possible to process a maximum of five animals at time in order to avoid the use of a too large drop of ethanol that would slow

down the drying. It is necessary to gently drop the ethanol close to the hot surface to prevent the dispersion of the animals by the droplets.

3. HMDS (approximate total time to complete the drying step 40 min plus the evaporation time)

This drying method was applied following the protocol described in Barre et al. (2006).

Fixed animals were placed in a EtOH:HMDS solution (1:1) twice for a duration of 10 min each, then placed in a 100% HMDS solution for 10 min twice (caution: EtOH-HDMS solution and HMDS 100% are both very volatile and toxic; it is necessary to work under a biosafety cabinet). Finally, the animals with a drop of HMDS were poured with a glass pipette on a coverslip. The coverslip was placed inside the drying chamber (see above), overnight or until the HMDS was completely evaporated and the animals completely dried (caution: work under a biosafety cabinet).

2.6 | Mounting and coating

After each of the protocols, the dried animals were collected under a stereomicroscope with the single bristle brush and mounted on a stub coated with carbon tape. The stub with mounted animals was sputter coated with an approx. 10 nm layer of gold (60 s at 25 mA).

2.7 | Detailed protocols

We report in this section the descriptions of the six protocols (occurring after the distension step) derived from the combination of the two methods used for fixation (A named "Boiling EtOH" and B named "EtOH series") and the three methods used for drying (1 named "EtOH Chamber", 2 named "Drop", and 3 named "HMDS drying"; Table 1).

2.7.1 | Protocol A1 (approximate duration 15 min plus mounting time)

1. The heater plate temperature was raised up to 150°C;
2. A Boveri capsule in borosilicate glass with 5 mL of absolute EtOH was placed on the heater plate until ethanol started boiling;
3. The animals were taken with about 20–30 µL of water using a Pasteur pipette and placed directly into the boiling ethanol for 3 min, covering the Boveri capsule to avoid total ethanol evaporation;
4. The animals were taken from the Boveri capsule, rinsed with absolute EtOH at room temperature and placed again in the capsule with about 3 mL of absolute EtOH;
5. The Boveri capsule containing animals was subsequently placed on the heater plate (150°C) and left until the ethanol was completely evaporated;
6. As soon as the ethanol had evaporated, the hot capsule was immediately taken (to prevent specimen burning) from the heater plate and observed under a stereomicroscope to find the dried specimens. The specimens were collected using the brush with a single bristle and mounted on the stub.

2.7.2 | Protocol A2 (approximate duration 5 + 3 min plus mounting time; repeat the 3 min step for each group of five animals)

This represents the newly proposed protocol, which allows better results in drying of specimens.

1. The heater plate temperature was raised up to 150°C;
2. A Boveri capsule in borosilicate glass with 5 mL of absolute EtOH was placed on the heater plate until ethanol boiled;
3. The animals were taken with about 20–30 µL of water using a Pasteur pipette and placed directly into the boiling ethanol for 3 min, covering the beaker to avoid the total evaporation of the EtOH;
4. The animals were taken from the capsule and rinsed with absolute EtOH at room temperature;
5. Another empty Boveri capsule in borosilicate glass was placed on the heater plate (180°C) and left until it reached the temperature of the plate (it took approximately 2 min);
6. The animals were taken from the capsule with room temperature ethanol and gently placed with a Pasteur pipette into the empty and already hot capsule/small beaker (use a single drop of ethanol). Gently drop the ethanol with a Pasteur pipette close to the hot surface to prevent the dispersion of the animals by the droplets;
7. As soon as the ethanol had evaporated (it required a few seconds), the hot capsule was immediately taken (to prevent specimen burning) from the heater plate and observed under a stereomicroscope to find the dried specimens (the suggestion is to prepare 3–5 specimens each time). They were collected using the brush with a single bristle and mounted on the stub.

In order to improve the results from this protocol, the following troubleshooting steps are recommended. Verify the relaxed morphology of the animals both after fixation and drying phases with the stereomicroscope. In the case of animals not being in a good condition (e.g., wrinkled), after the fixation ensure the alcohol started boiling before the animal fixation (see points 2–3). In the case of animals in poor condition (wrinkled) after the drying phase, verify the correct heating of the glass surface of the Boveri capsule or increase the temperature of the plate up to 200°C (see point 4).

2.7.3 | Protocol A3 (approximate duration 45 min plus overnight drying and mounting time)

1. The heater plate temperature was raised up to 150°C;

2. A Boveri capsule in borosilicate glass with 5 mL of absolute EtOH was placed on the heater plate until ethanol boiled;
3. The animals were taken with about 20–30 µL of water using a Pasteur pipette and placed directly into the boiling ethanol for 3 min, covering the beaker to avoid the total evaporation of the ethanol;
4. The animals were taken from the beaker and rinsed with absolute EtOH at room temperature;
5. The animals were moved in a solution of EtOH:HMDS (1:1) using a Pasteur pipette. This process was repeated two times;
6. The animals were moved in a solution of 100% HMDS using a Pasteur pipette. This process was repeated two times;
7. The animals were moved, using a Pasteur pipette, with a drop of HMDS on a coverslip that was placed inside a Petri dish containing silica gel to speed up the drying process;
8. After the HMDS had evaporated, the coverslip (or a piece of coverslip) was observed under a stereomicroscope to find the dried specimens; the piece of coverslip was directly mounted on the stub or the animals were collected and mounted on the stub using the brush with a single bristle.

2.7.4 | Protocol B1 (approximate duration 90 min plus mounting time)

(1–8) The animals were fixed in EtOH with water/ethanol series following eight steps, the fixation required about 80 min to be completed (using an increasing series of ethanol at 30-50-70-80-90-95-100-100%; each of the eight steps consists of a 10 min bath) using the microsieve;

(9) The animals were moved in a Boveri capsule in borosilicate glass with about 3 mL of absolute EtOH;

(10) The Boveri capsule containing animals was subsequently placed on the heater plate (150°C) and left until the ethanol was completely evaporated;

(11) As soon as the ethanol had evaporated, the hot capsule was immediately taken (to prevent specimen burning) from the heater plate and observed under a stereomicroscope to find the dried specimens (the suggestion is to prepare 3–5 specimens each time). They were collected and mounted on the stub using the brush with a single bristle.

2.7.5 | Protocol B2 (approximate duration 85 min plus mounting time; repeat the 3 min step for each group of five animals)

(1–8) The live animals were fixed in EtOH with water/ethanol series following eight steps, the fixation required about 80 min to be completed (using an increasing series of ethanol at 30-50-70-80-90-95-100-100%; each of the eight steps consists of a 10 min bath) using the microsieve;

(9) An empty Boveri capsule in borosilicate glass was placed on the heater plate (180°C) and left until it reached the temperature of the plate (it took approximately 2 min);

(10) The animals were taken from the capsule with room temperature ethanol and gently placed with a Pasteur pipette into the empty capsule/small beaker (use a single drop of ethanol). Gently drop the ethanol with a Pasteur pipette close to the hot surface to prevent the dispersion of the animals by the droplets;

(11) As soon as the ethanol had evaporated, the hot capsule was immediately taken (to prevent specimen burning) from the heater plate and observed under a stereomicroscope to find the dried specimens (the suggestion is to prepare 3–5 specimens each time). They were collected and mounted on the stub with the brush with a single bristle.

2.7.6 | Protocol B3 (approximate duration 120 min plus overnight drying and mounting time)

This protocol is already largely used to drying invertebrates. We followed the procedure according to Barre et al. (2006), slightly modified for tardigrades.

(1–8) The live animals were fixed in EtOH with water/ethanol series following eight steps, the fixation required about 80 min to be completed (using an increasing series of ethanol at 30-50-70-80-90-95-100-100%; each of the eight steps consists of a 10 min bath) using the microsieve;

(9) The animals were moved in a solution of EtOH:HMDS (1:1) using a Pasteur pipette. The process was repeated two times;

(10) The animals were moved in a solution of 100% HMDS using a Pasteur pipette. The process was repeated two times;

(11) The animals were moved, using a Pasteur pipette, with a drop of HMDS on coverslips that were placed inside a Petri dish containing silica gel to speed up the drying process;

(12) After the HMDS had evaporated, the coverslip (or a piece of coverslip) was observed under a stereomicroscope to find the dried specimens; the piece of coverslip can be directly mounted on stub or the animals can be collected and mounted on the stub using the brush with a single bristle.

2.8 | Comparative statistical analyses for selected protocols

A total of 100 (10 + 90) scores for each of the selected protocols, that is, those scoring above 4, were processed for statistical analysis in R (R Core Team, 2016).

The scores (i.e., 0, 0.5, 1) of each specimen were recorded for the Pearson's Chi-square test. Post-hoc analysis was performed to investigate pairwise comparisons between the different selected protocols using the *p* values obtained from Fisher's exact test for each comparison on the Pearson's Chi-square results. The *p* values were adjusted for multiple comparisons using the Benjamini-Hochberg correction method (Benjamini & Hochberg, 1995). Raw data, R script, and results are reported in the Supplementary Material (Supporting Information S1).

3 | RESULTS AND DISCUSSION

All the tested protocols allowed us to obtain at least some animals in suitable condition to be studied from a morphological point of view.

TABLE 2 Preliminary comparison of SEM-preparation protocols using 10 animals per protocol and their resulting scores. pt: point of the score system.

Fixing methods	Drying methods	Protocol names	No. of good tardigrades (1 pt each)	No. of acceptable tardigrades (0.5 pt each)	No. of bad tardigrades (0 pt each)	Total (pt)
Boiling EtOH	EtOH CHAMBER	A1	1	0	9	1/10
Increasing EtOH series		B1	2	3	5	3.5/10
Boiling EtOH	DROP	A2	7	3	0	8.5/10
Increasing EtOH series		B2	1	5	4	3.5/10
Boiling EtOH	HMDS	A3	3	5	2	5.5/10
Increasing EtOH series		B3	9	0	1	9/10

However, the overall results differed clearly for each protocol (Table 2). The evaluation of the six protocols, based on the score system (i.e., 0 for the animals considered useless for diagnostic purposes, 0.5 points for animals partially useful for diagnostic purposes, 1 point for animals in perfect shape; Figure 1) applied to the first 10 specimens, allowed the exclusion of some protocols from the subsequent analysis aimed at evaluating the efficacy of these methods through additional replicates due to the very low outcome score (i.e., 3.5 or less).

The sum of the scores calculated on the first 10 mounted specimens gave 1.0, 3.5, and 3.5, for the protocols A1, B1, and B2, respectively. These scores are considered insufficient, and disadvantageous regarding effort, number of sacrificed animals, and average morphological quality of the animals. Additional considerations can be taken into account for these protocols: A1 had very low success rate, and even with larger sample sizes, a high success rate is difficult to predict; this consideration can, at least partially, be extended to B1 and B2, taking into account that B1 represents a protocol already used in the literature, but with low success rates (Guidetti, personal communication), and B2 represents a new proposed protocol, but with an animals fixation method that requires more time and resources and has a higher probability of animal loss during the procedure.

The protocols A2, A3, B3 showed better success rates with the first 10 mounted specimens. Specifically, protocol A2 allowed us to obtain all animals in good or acceptable conditions (Table 2) and B3 produced nine animals in such condition (Table 2). Protocol A3 yielded three animals in good condition and five in acceptable condition (Table 2).

For the protocols that yielded the best results, namely A2, A3 and B3, an additional 90 animals were mounted for each technique in order to obtain more reliable statistical analysis.

Considering the 10 preliminary and the additional 90 specimens mounted and observed with SEM for each of these protocols, the percentage of suitable specimens was 92.5%, 54.5%, and 71.0% for A2, A3, and B3 respectively.

The results of the Pearson's Chi-squared test indicated that there was a significant association between the tested protocol and the categories assigned with the score system. The Pearson's Chi-squared test result was 77.746, with 4° of freedom, and a very low p value ($p < .001$; Supporting Information S1), providing strong evidence to reject the null hypothesis of no association between the tested protocol and the categories.

Post hoc comparisons were then performed to investigate the nature of the significant association. The results of the post-hoc comparisons showed that there were significant differences between all the scores of the three tested protocols ($p < .05$). The comparison between the protocols A2 and A3 had the lowest p value ($p < .001$; Supporting Information S1). The comparison A2–B3 and A3–B3 are significant as well, but with higher p values ($p < .001$; Supporting Information S1). Based on these results and the scores recorded we can conclude that the best protocol is the new proposed protocol A2, that gives better results compared with the protocol B3. The statistical results therefore indicate a better efficiency of the protocol A2 than that of protocols A3 and B3, in terms of dried specimens in good condition for morphological analyses.

The validity of the new protocol A2 is confirmed both by the 10 initially mounted and the 90 additional specimens, whose scores and quality of observed diagnostic characters (Figure 2) were consistent and comparable to those of the B3 protocol, currently considered as the most reliable and widely used for invertebrates in the literature. In addition, protocol A2 proved to be faster and easier for obtaining dried specimens, only requiring approximately half an hour from fixation in ethanol (first boiling) to mounting on stub, while protocol B3 required a much longer time due to the necessity of the increasing series in EtOH, EtOH:HMDS, and HMDS. This protocol took approximately 2 h, to which at least 16 h were added (the sample was left to evaporate overnight) for the complete air-drying of HMDS. Moreover, protocol A2 stands out for the need of simpler and less materials and tools: Boveri capsules in borosilicate glass, thermal plate, Pasteur pipettes, and a single reagent (absolute ethanol, the manipulation of which requires few precautions for its handling); while protocol B3 requires: Boveri capsules, Pasteur pipettes, absolute ethanol, and the hazardous reagent HMDS (a highly toxic, volatile compound) which necessitates numerous precautions for handling, including the need to work under a biological safety cabinet.

Protocols A3 and B3 outcomes differ, with protocol A3 differing from B3 by requiring fewer steps for fixation method ("A" instead of "B"), resulted in worse results. However, considering that A3 is

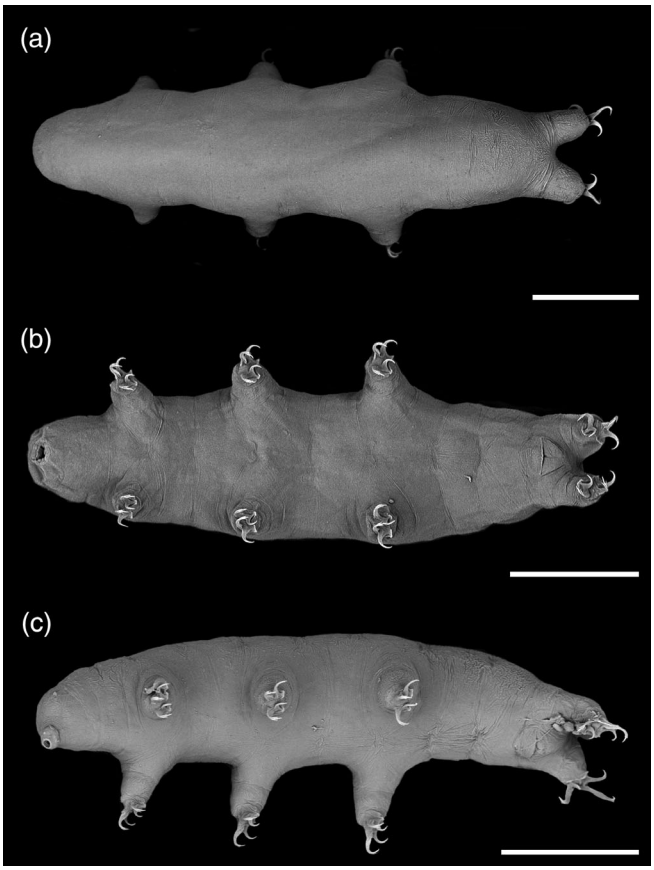

FIGURE 2 Scanning electron microscopy (SEM) pictures of *Hypsibius exemplaris* in toto prepared with protocol A2. (a) Dorsal view. (b) Ventral view. (c) Ventro-lateral view. Scale bars 50 μm.

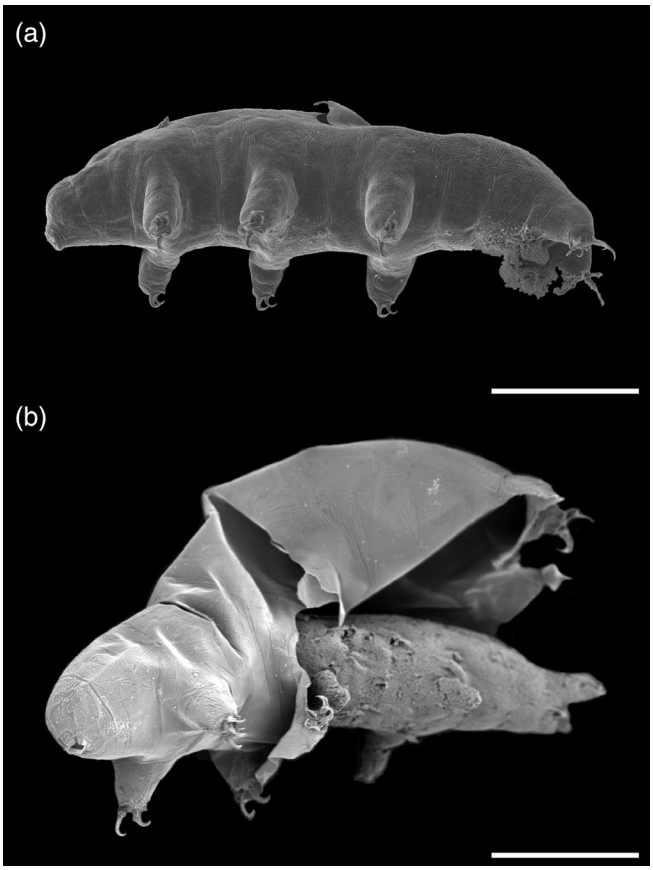

FIGURE 3 Scanning electron microscopy (SEM) pictures of *Hypsibius exemplaris* in toto showing morphological alterations induced by protocol A2. (a) Leakage of the gut content from cloaca and partial disruption of the cuticle. (b) Partial separation of the cuticle from the internal tissues. Scale bars 50 μm.

considerably faster, requires fewer reagents, and reduces the risk of animal loss during the procedure, it can still be considered a valid alternative to the classic protocol (B3) using HMDS for preparing tardigrades for SEM analysis.

Regarding of the animals condition and morphology, observations made using a stereomicroscope during the various steps of the protocols indicate that after fixation, the animals should exhibit a white color with both method "A" (i.e., animals moved into boiling ethanol immediately after distension) and method "B" (i.e., animals subjected to the increasing ethanol series after distension). Differences are instead observed for the internal soft structures that after fixation "A" appear sometimes collapsed and detached from the cuticle, while after "B" seem to be more preserved and adherent to the cuticle.

The observations with SEM showed that the animals treated with protocols A1 and A2 sometimes exhibited material leakage from the cloaca (Figure 3a). Besides, those prepared with A2 showed some additional peculiarities. The cuticle tended to break in some points (e.g., in correspondence of the ventral cuticle between the legs) and, less frequently, it completely or partially detached from the epidermis of the animal (Figure 3b). The animals in the best conditions sometimes showed high and unnatural apparent turgidity of the body which, however, does not compromise a good resolution and condition of the main morphological characters. These inconveniences are likely due to the drying method (i.e., the "drop" method) which results in higher mechanical stress on animals compared to other methods. Our hypothesis is that, after fixation in ethanol, when animals are placed inside the Boveri capsule on the heater plate, the ethanol inside the animals immediately changed its state from liquid to gaseous, resulting in a "popcorn"-like effect (Figure 3). In contrast, with protocol B3, these kinds of alterations are not observed.

Despite this difference between protocols A2 and B3, no other difference was detected in the diagnostic structures of the animals, including the external structure of the cuticle, and the tested protocols did not produce artifacts. Moreover, the diagnostic structures and general morphology of the specimens of *H. exemplaris*, prepared with protocol A2, are comparable to published photographs of specimens of the same species prepared with CPD in Gąsiorek et al. (2018).

4 | CONCLUSION

The new proposed protocol (i.e., A2) proves to be a very simple Scanning Electron Microscope preparation for tardigrade specimens. It is a

protocol that gives very high success rates that minimizes the number of animals devoted for SEM studies and becomes clearly crucial in cases of paucity of animals in a sample. In fact, to provide a good integrative description, there must be an adequate number of specimens for studying morphology using LM as well as for extracting and characterizing the species through DNA sequencing, and the large specimens-consuming protocol for SEM in such cases becomes a side analysis even if it is a well-established standard for tardigrades species description. Furthermore, the A2 protocol requires just a few, easily accessible/obtainable materials and reagents that are minimally toxic for the environment and researchers.

The new drying protocol has also been tested on live and dead adults and eggs of other tardigrade taxa from both Heterotardigrada and Eutardigrada classes with satisfactory results (data in preparation). Moreover, the protocol could be suitable also on small Arthropoda, for example, Collembola, Protura, and Copepoda, or micrometazoans, that is, Nematoda and Rotifera, and further investigations are planned to adjust this newly promising proposed protocol for these taxa.

AUTHOR CONTRIBUTIONS

Daniele Camarda: Conceptualization; methodology; writing – original draft; writing – review and editing; investigation. **Edoardo Massa:** Conceptualization; methodology; data curation; software; writing – original draft; writing – review and editing; visualization. **Roberto Guidetti:** Supervision; funding acquisition; writing – original draft; writing – review and editing; resources. **Oscar Lisi:** Project administration; writing – original draft; writing – review and editing; supervision; funding acquisition.

ACKNOWLEDGMENTS

We would like to extend our sincere gratitude to Sara Brandoli for her invaluable support and assistance during the experiments. Additionally, we are deeply thankful to Prof. Paul Bartels for his meticulous English language revision. We also wish to convey our appreciation to the referees for their valuable comments and suggestions, which greatly contributed to the enhancement of the manuscript.

FUNDING INFORMATION

Project funded under the National Recovery and Resilience Plan (NRRP), Mission 4 Component 2 Investment 1.4 - Call for tender No. 3138 of 16 December 2021, rectified by Decree n.3175 of 18 December 2021 of Italian Ministry of University and Research funded by the European Union - NextGenerationEU; Award Number: Project Code CN_00000033, Concession Decree No. 1034 of 17 June 2022 adopted by the Italian Ministri of University and Research, CUP E93C22001090001, Project Title "National Biodiversity Future Center - NBCF". The project was supported by the University of Modena and Reggio Emilia and University of Catania.

CONFLICT OF INTEREST STATEMENT

The authors report there are no competing interests to declare.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article

ORCID

Daniele Camarda https://orcid.org/0000-0003-2615-4369
Edoardo Massa https://orcid.org/0000-0002-3174-9896
Roberto Guidetti https://orcid.org/0000-0001-6079-2538
Oscar Lisi https://orcid.org/0000-0001-5495-2742

REFERENCES

Abolafia, J. (2015). A low-cost technique to manufacture a container to process meiofauna for scanning electron microscopy. *Microscopy Research and Technique*, *78*(9), 771–776.

Anderson, T. F. (1951). Techniques for preservation of three-dimensional structure in preparing specimens for the electron microscope. *Transactions of the New York Academy of Science-Series II*, *13*, 130–134.

Baccetti, B., & Rosati, F. (1969). Electron microscopy on tardigrades. I. Connective tissue. *Journal of Submicroscopic Cytology and Pathology*, *1*, 197–205.

Baccetti, B., & Rosati, F. (1971). Electron microscopy on tardigrades. III. The integument. *Journal of Ultrastructure Research*, *34*(3–4), 214–243.

Bai, L., Wang, X., Gao, X., Li, Y., & Fontoura, P. (2022). First record of a deep-sea tardigrade from the South China Sea, *Halechiniscus janus* sp. nov. (Arthrotardigrada: Halechiniscidae). *Zootaxa*, *5159*(3), 425–439.

Bai, L., Wang, X., Zhou, Y., Lin, S., Meng, F., & Fontoura, P. (2020). *Moebjergarctus clarionclippertonensis*, a new abyssal tardigrade (Arthrotardigrada, Halechiniscidae, Euclavarctinae) from the clarion-Clipperton fracture zone, North-East Pacific. *Zootaxa*, *4755*(3), 561–575.

Barre, C., O'Neil, D., & Bricelj, V. M. (2006). Preparation of large bivalve specimens for scanning electron microscopy using Hexamethyldisilazane (HMDS). *Journal of Shellfish Research*, *25*(2), 639–641.

Benjamini, Y., & Hochberg, Y. (1995). Controlling the false discovery rate: A practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society B*, *57*, 289–300.

Bertolani, R., Cesari, M., Giovannini, I., Rebecchi, L., Guidetti, R., Kaczmarek, Ł., & Pilato, G. (2022). The *Macrobiotus persimilis-polonicus* complex (Eutardigrada, Macrobiotidae), another example of problematic species identification, with the description of four new species. *Organisms Diversity & Evolution*, *23*, 329–368.

Bertolani, R., Guidetti, R., Marchioro, T., Altiero, T., Rebecchi, L., & Cesari, M. (2014). Phylogeny of Eutardigrada: New molecular data and their morphological support lead to the identification of new evolutionary lineages. *Molecular Phylogenetics and Evolution*, *76*, 110–126.

Bertolani, R., Rebecchi, L., & Claxton, S. K. (1996). Phylogenetic significance of egg shell variation in tardigrades. *Zoological Journal of the Linnean Society*, *116*(1–2), 139–148.

Bertolani, R., Rebecchi, L., Giovannini, I., & Cesari, M. (2011). DNA barcoding and integrative taxonomy of *Macrobiotus hufelandi* C.A.S. Schultze 1834, the first tardigrade species to be described, and some related species. *Zootaxa*, *2997*(1), 19–36.

Bray, D. (2000). Critical point drying of biological specimens for scanning electron microscopy. In J. R. Williams & A. A. Clifford (Eds.), *Supercritical fluid methods and protocols. Methods in biotechnology* (Vol. 13, pp. 235–243). Humana Press.

Bray, D. F., Bagu, J., & Koegler, P. (1993). Comparison of hexamethyldisilazane (HMDS), Peldri II, and critical-point drying methods for scanning electron microscopy of biological specimens. *Microscopy Research and Technique*, *26*(6), 489–495.

Claxton, S. K. (1998). A revision of the genus *Minibiotus* (Tardigrada: Macrobiotidae) with descriptions of eleven new species from Australia. *Records of the Australian Museum*, *50*, 125–160.

Crowe, J. H., & Cooper, A. F. (1971). Cryptobiosis. *Scientific American*, *225*(6), 30–37.

Czerneková, M., Janelt, K., Student, S., Jönsson, K. I., & Poprawa, I. (2018). A comparative ultrastructure study of storage cells in the eutardigrade *Richtersius coronifer* in the hydrated state and after desiccation and heating stress. *PLoS One*, *13*(8), e0201430.

Dastych, H. (2019). *Cryobiotus roswithae* gen. n., sp. n., a new genus and species of glacier-dwelling tardigrades from northern Norway (Tardigrada, Panarthropoda). *Entomologie Heute*, *31*, 95–111.

Dey, S., Basu Baul, T. S., Roy, B., & Dey, D. (1989). A new rapid method of air-drying for scanning electron microscopy using tetramethylsilane. *Journal of Microscopy*, *156*(2), 259–261.

Eibye-Jacobsen, J. (2001a). A new method for making SEM preparations of the tardigrade buccopharyngeal apparatus. *Zoologischer Anzeiger*, *240*(3–4), 309–319.

Eibye-Jacobsen, J. (2001b). Are the supportive structures of the tardigrade pharynx homologous throughout the entire group? *Journal of Zoological Systematics and Evolutionary Research*, *39*(1–2), 1–11.

Eisenback, J. D. (1986). A comparison of techniques useful for preparing nematodes for scanning electron microscopy. *Journal of Nematology*, *18*, 479–487.

Gąsiorek, P., & Michalczyk, Ł. (2020). Phylogeny of Itaquasconinae in the light of the evolution of the flexible pharyngeal tube in Tardigrada. *Zoologica Scripta*, *49*(4), 499–515.

Gąsiorek, P., Stec, D., Morek, W., & Michalczyk, Ł. (2018). An integrative redescription of *Hypsibius dujardini* (Doyère, 1840), the nominal taxon for Hypsibioidea (Tardigrada: Eutardigrada). *Zootaxa*, *4415*(1), 45–75.

Gąsiorek, P., Vončina, K., Bochnak, M., Surmacz, B., Morek, W., & Michalczyk, Ł. (2022). Echiniscidae (Heterotardigrada) of South Africa. *Zootaxa*, *5156*(1), 1–238.

Grigarick, A. A., Schuster, R. O., & Toftner, E. C. (1973). Descriptive morphology of eggs of some species in the *Macrobiotus hufelandi* group (Tardigrada: Macrobiotidae). *Pan-Pacific Entomologist*, *49*(3), 258–263.

Gross, V., Minich, I., & Mayer, G. (2017). External morphogenesis of the tardigrade *Hypsibius dujardini* as revealed by scanning electron microscopy. *Journal of Morphology*, *278*(4), 563–573.

Guidetti, R., Altiero, T., Marchioro, T., Sarzi Amadè, L., Avdonina, A. M., Bertolani, R., & Rebecchi, L. (2012). Form and function of the feeding apparatus in Eutardigrada (Tardigrada). *Zoomorphology*, *131*, 127–148.

Guidetti, R., Cesari, M., Bertolani, R., Altiero, T., & Rebecchi, L. (2019). High diversity in species, reproductive modes and distribution within the *Paramacrobiotus richtersi* complex (Eutardigrada, Macrobiotidae). *Zoological Letters*, *5*(1), 1–28.

Guidetti, R., Cesari, M., Giovannini, I., Ebel, C., Förschler, M. I., Rebecchi, L., & Schill, R. O. (2022). Morphology and taxonomy of the genus *Ramazzottius* (Eutardigrada; Ramazzottiidae) with the integrative description of *Ramazzottius kretschmanni* sp. nov. *The European Zoological Journal*, *89*(1), 339–363.

Guidetti, R., Gneuß, E., Cesari, M., Altiero, T., & Schill, R. O. (2020). Life-history traits and description of the new gonochoric amphimictic *Mesobiotus joenssoni* (Eutardigrada: Macrobiotidae) from the Island of Elba, Italy. *Zoological Journal of the Linnean Society*, *188*(3), 848–859.

Guidetti, R., Massa, E., Bertolani, R., Rebecchi, L., & Cesari, M. (2019). Increasing knowledge of Antarctic biodiversity: New endemic taxa of tardigrades (Eutardigrada; Ramazzottiidae) and their evolutionary relationships. *Systematics and Biodiversity*, *17*(6), 573–593.

Guidetti, R., Rebecchi, L., & Bertolani, R. (2000). Cuticle structure and systematics of the Macrobiotidae (Tardigrada, Eutardigrada). *Acta Zoologica*, *81*(1), 27–36.

Guidetti, R., Rebecchi, L., Cesari, M., & McInnes, S. J. (2014). *Mopsechiniscus franciscae*, a new species of a rare genus of Tardigrada from continental Antarctica. *Polar Biology*, *37*(9), 1221–1233.

Guidetti, R., Schill, R. O., Giovannini, I., Massa, E., Goldoni, S. E., Ebel, C., Förschler, M. I., & Cesari, M. (2021). When DNA sequence data and morphological results fit together: Phylogenetic position of *Crenubiotus* within Macrobiotoidea (Eutardigrada) with description of *Crenubiotus ruhesteini* sp. nov. *Journal of Zoological Systematics and Evolutionary Research*, *59*(3), 576–587.

Haefke, B. J., Spiers, A. I., Miller, W. R., & Lowman, M. D. (2014). Tardigrades of the canopy: *Doryphoribius elleneddiei* nov. sp. (Eutardigrada, Parachela, Hypsibiidae), a new species from eastern Kansas, USA. *Transactions of the Kansas Academy of Science*, *117*(3–4), 299–304.

Halász, N. (1974). Aldehyde-osmium fixation of the rat olfactory bulb: Correlation between synaptic patterns and nature of the transmission. *Acta Anatomica*, *87*(4), 508–533.

Hochberg, R., & Litvaitis, M. K. (2000). Hexamethyldisilazane for scanning electron microscopy of Gastrotricha. *Biotechnic & Histochemistry*, *75*(1), 41–44.

Inoué, T., & Osatake, H. (1988). A new drying method of biological specimens for scanning electron microscopy: The t-butyl alcohol freeze-drying method. *Archives of Histology and Cytology*, *51*(1), 53–59.

Laforsch, C., & Tollrian, R. (2000). A new preparation technique of daphnids for scanning electron microscopy using hexamethyldisilazane. *Archiv für Hydrobiologie*, *149*(4), 587–596.

Massa, E., Guidetti, R., Cesari, M., Rebecchi, L., & Jönsson, K. I. (2021). Tardigrades of Kristianstads Vattenrike biosphere reserve with description of four new species from Sweden. *Scientific Reports*, *11*, 4861.

Massa, E., Rebecchi, L., & Guidetti, R. (2023). Effects of synthetic acid rain and organic and inorganic acids on survival and $CaCO_3$ piercing stylets in tardigrades. *Journal of Experimental Zoology Part A: Ecological and Integrative Physiology*, *339*(6), 578–589.

Michalczyk, Ł., & Kaczmarek, Ł. (2003). A description of the new tardigrade *Macrobiotus reinhardti* (Eutardigrada: Macrobiotidae, *harmsworthi* group) with some remarks on the oral cavity armature within the genus *Macrobiotus* Schultze. *Zootaxa*, *331*(1), 1–24.

Michalczyk, Ł., & Kaczmarek, Ł. (2006). Revision of the *Echiniscus bigranulatus* group with a description of a new species *Echiniscus madonnae* (Tardigrada: Heterotardigrada: Echiniscidae) from South America. *Zootaxa*, *1154*(1), 1–26.

Mitchell, C., & Miller, W. R. (2008). A simple SEM (scanning electron microscope) preparation protocol for tardigrades. *Journal of the Pennsylvania Academy of Science*, *81*(2–3), 86–90.

Møbjerg, N., Jørgensen, A., & Kristensen, R. M. (2020). Ongoing revision of Echiniscoididae (Heterotardigrada: Echiniscoidea), with the description of a new interstitial species and genus with unique anal structures. *Zoological Journal of the Linnean Society*, *188*(3), 663–680.

Nation, J. L. (1983). A new preparation method using hexamethyldisilazane for preparation of soft insect tissues for scanning electron microscopy. *Stain Technology*, *58*, 347–351.

Pogwizd, J., & Stec, D. (2022). An integrative description of a new *Richtersius* species from Greece (Tardigrada: Eutardigrada: Richtersiusidae). *Acta Zoologica Academiae Scientiarum Hungaricae*, *68*(1), 1–21.

R Core Team. (2016). R: A language and environment for statistical computing. https://www.r-project.org/

Schuster, R. O., Nelson, D. R., Grigarick, A. A., & Christenberry, D. (1980). Systematic criteria of the Eutardigrada. *Transactions of the American Microscopical Society*, *99*, 284–303.

Shively, S., & Miller, W. R. (2009). The use of HMDS (hexamethyldisilazane) to replace critical point drying (CPD) in the preparation of tardigrades for SEM (scanning electron microscope) imaging. *Transactions of the Kansas Academy of Science*, *112*(4), 198–200.

Spiers, A. I., Haefke, B. J., Miller, W. R., & Lowman, M. D. (2013). Tardigrades in the canopy: *Doryphoribius dawkinsi* Michalczyk and Kaczmarek, 2010-new records from eastern Kansas, USA. *Transactions of the Kansas Academy of Science*, *116*(3–4), 125–128.

Stec, D. (2022). An integrative description of two new *Mesobiotus* species (Tardigrada: Eutardigrada: Macrobiotidae) with updated genus phylogeny. *Zoological Studies*, *61*, 85.

Stec, D., Smolak, R., Kaczmarek, Ł., & Michalczyk, Ł. (2015). An integrative description of *Macrobiotus paulinae* sp. nov. (Tardigrada: Eutardigrada: Macrobiotidae: *Hufelandi* group) from Kenya. *Zootaxa*, *4052*(5), 501–526.

Sugiura, K., Minato, H., Matsumoto, M., & Suzuki, A. C. (2020). *Milnesium* (Tardigrada: Apochela) in Japan: The first confirmed record of *Milnesium tardigradum* ss and description of *Milnesium pacificum* sp. nov. *Zoological Science*, *37*(5), 476–495.

Toftner, E. C., Grigarick, A. A., & Schuster, R. O. (1975). Analysis of scanning electron microscope images of *Macrobiotus* eggs. *Memorie dell'Istituto Italiano di Idrobiologia*, *32*, 393–411.

Tumanov, D. V., Androsova, E. D., Avdeeva, G. S., & Leontev, A. A. (2022). First faunistic investigation of semiterrestrial tardigrade fauna of north-West Russia using the method of DNA barcoding. *Invertebrate Zoology*, *19*(4), 452–474.

Vecchi, M., Tsvetkova, A., Stec, D., Ferrari, C., Calhim, S., & Tumanov, D. (2023). Expanding *Acutuncus*: Phylogenetics and morphological analyses reveal a considerably wider distribution for this tardigrade genus. *Molecular Phylogenetics and Evolution*, *180*, 107707.

Wieczorkiewicz, F., Sojka, J., & Poprawa, I. (2023). Effect of paracetamol on the storage cells of *Hypsibius exemplaris*—ultrastructural analysis. *Zoological Journal of the Linnean Society*, zlad051.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Camarda, D., Massa, E., Guidetti, R., & Lisi, O. (2023). A new, simplified, drying protocol to prepare tardigrades for scanning electron microscopy. *Microscopy Research and Technique*, 1–11. https://doi.org/10.1002/jemt.24460