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NOTE

DIATOM TYPE MATERIAL IN PERMANENT SLIDES DOES NOT NEED TO BE PERMANENTLY UNAVAILABLE FOR ELECTRON MICROSCOPY EXAMINATION[1]

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The inability to thoroughly examine diatom type material existing only in resin-mounted permanent slides is a common frustration for diatomologists. Here, we present an efficient, inexpensive, and straightforward technique to extract siliceous exoskeletons of diatoms from permanent slides prepared with commonly used mounting media. Van Heurck's slide constituting an isotype duplicate of *Proschkinia bulnheimii* var. *belgica*, an entity with an uncertain taxonomic status, was deconstructed to allow thorough examination using scanning electron microscopy. Numerous specimens of the taxon, including intact frustules and specimens with complete areola occlusions, were recovered and documented. The extracted diatom material was sufficient to prepare two scanning electron microscopy specimens and reconstruct the permanent slide. The proposed approach may help overcome many of the taxonomic bottlenecks associated with the low resolution of information provided by older diatom descriptions and permanent slide observations.

Key index words: **diatom identification; diatom morphology; method; mounting medium; permanent slide; *Proschkinia bulnheimii* var. *belgica*; resin; SEM; taxonomy; type material**

While the species concept in diatoms is far from unambiguous, and debate continues, we recognize the importance of accurate morphological identifications. Although recent studies based on molecular analyses of diatom DNA show that the so-called cryptic diversity in diatoms may be high (Pinseel et al. 2019), the extent of ecophysiological variation in cryptic species complexes, and thus the implications of these discoveries for ecological and biomonitoring studies, are yet unclear, and the morphological species concept continues to dominate in diatom research (Cristóbal et al. 2020). It has even been suggested that new species proposals should only be accepted if the observed genetic dissimilarities between known and novel strains are linked with measurable morphological or ecological differences (Guiry 2012). This view does not lack relevance, as detectable genetic differences that do not seem to alter the species morphology or its ecological niche may be virtually meaningless for many diatom-based studies, particularly those using diatoms as bioindicators of environmental conditions regardless of the species identification method used (molecular or morphological), and formal recognition of numerous "microspecies" may be impractical or even undesirable (Mann and Vanormelingen 2013).

Estimations of global diatom diversity vary from 20,000 (Guiry 2012) to about 200,000 species (Mann and Droop 1996), and highly uneven and generally low sampling effort, exacerbated by lack of clarity in the species concept, underlie the current inability to provide a more accurate and reliable assessment. Furthermore, it is uncertain how many validly described diatom taxa exist today. Although multiple attempts have been made to provide catalogues of diatom names (Fourtanier and Kociolek 2009), this goal has not yet been achieved. The latest initiative, the online source DiatomBase (https://www.diatombase.org), states that over 75,000 diatom species names have been proposed, yet the database itself includes only 12,645 diatom species names (including both fossil and extant species), of which 2401 are currently regarded as valid (accessed December 4, 2020; Kociolek et al. 2020).

While information availability and accuracy depend on the joint efforts of the editors and users of such databases, a clear limitation exists in the form of the inability to access or thoroughly

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examine the type material of many diatom taxa described in earlier decades or centuries. Adequate characterizations of discrete features that are now regularly used in diatom taxonomy, as well as micrographs of sufficient quality, are often lacking in older original descriptions, especially those from the 19th century. Although these descriptions are technically not invalid, in practice, a species depicted using line drawings alone is indistinguishable from morphologically similar taxa and cannot be identified with certainty. In such cases, type material re-examination and revisionary work are necessary to properly investigate the identity and phylogenetic position of the name-bearing specimens, and scanning electron microscopy (SEM) has had a significant impact on the diatom taxonomy ever since its first applications to diatom frustule examination (Helmcke 1951). A lectotype is designated when the holotype is lacking or destroyed but the original material is still available, and a neotype can be designated when the original material is missing (Turland et al. 2018). This may not be ideal but often resolves problems of uncertain status of poorly documented taxa. Paradoxically, the situation is more complicated when the type material exists only in the form of a permanent slide. Especially in the case of small taxa with lightly silicified frustules, light microscopy (LM) observations are not sufficient to reveal taxonomically important ultrastructural features such as the raphe system structure, stria and areola shape and density, or the nature of areola occlusions. Thus, it is generally accepted that understanding of the phylogenetic position of many holotype specimens mounted in permanent slides cannot be improved, as such specimens cannot be thoroughly examined with SEM. Or can they?

Here we propose an efficient, inexpensive, and straightforward method to overcome this key limitation and extract intact diatom valves and frustules from permanent slides prepared with standard mounting media. While the example presented involves a slide prepared with Styrax, a modified method can be applied to treat permanent slides prepared with the different resins typically used in diatom studies.

Navicula bulnheimii var. *belgica* was described toward the end of the 19th century (Van Heurck 1885; Fig. 1, A and B). LM observations suggested morphological differences in the valve shape (wider valves) and girdle structure between the new variety and the nominal taxon, yet illustrations were not provided (Van Heurck 1880, 1885). More than a century later, the isotype slides (Types du Synopsis des Diatomées de Belgique; No. 113) were examined by Krammer and Lange-Bertalot (1986) and Cox (1988, 1998), and the first micrographs were produced. These additional examinations warranted the transfer of *Navicula bulnheimii* var. *belgica* to *Proschkinia* (Witkowski et al. 2000). Nevertheless, due to the lack of detailed morphological information provided by the LM observations, the varietal status of the taxon remains unclear. Worse still, it is improbable that without SEM analysis of the frustule ultrastructure those uncertainties will ever be resolved.

Considering the above, we used one (South African National Diatom Collection, Potchefstroom, South Africa; Fig. 1B) of the dozens of isotype duplicates prepared by Van Heurck and hosted by various diatom collections to extract frustules of *Proschkinka bulnheimii* var. *belgica* for examination with SEM. The slide was placed on a hotplate and heated for about 2 min at 80–100°C to soften the resin. The mounting medium and the coverslip were then scraped carefully with a scalpel blade and placed in a beaker filled with ca. 15 mL of solvent. Benzene was used as a solvent for Styrax (Van Heurck 1885). After ca. 30 s, when most of the resin dissolved, the coverslip was carefully removed from the beaker using tweezers. The liquid was poured into a 15 mL Falcon tube[1] and centrifuged for 10 min at 1,400*g*, after which the solvent was decanted, fresh benzene was added, and the tube was vigorously shaken to allow thorough mixing. The cycle was repeated three times until undissolved Styrax (yellowish particles) was undetectable to the naked-eye. A drop of diatom material suspended in benzene was placed on a 12-mm glass coverslip[2] mounted on the SEM stub with carbon tape and left to air-dry under the ventilating hood. The specimen was then sputter-coated with gold–palladium using a Cressington 108Auto sputter-coater (Cressington Scientific Instruments Ltd., Watford, UK) and analyzed with JEOL JSM-7001F (JEOL, Tokyo, Japan) and FEI Quanta Feg 250 (FEI Corporate, Hillsboro, OR, USA) scanning electron microscopes.

The SEM analysis revealed that the cleaning procedure was insufficient and that the organic coating was obscuring the diatom frustules (Fig. 2, A and B). Sulfonation (Kaandorp et al. 1962) was therefore applied to remove the solvent. Benzene-suspended diatom material and concentrated sulfuric acid (98%) were poured into a glass beaker at 1:5 ratio and heated for ca. 3h on a hot plate at 180–200°C, which provided sufficient time to complete the reaction that produced water and water-soluble (and thus easily removable) benzenesulphonic acid. Sulfuric acid was added in excess as the increasing concentration of water in the reaction mixture decreases the rate of the reaction and thus prolongs the time necessary to complete this step. To further simplify and shorten the process, fuming sulfuric acid (oleum) can be used instead of sulfuric acid (Cerfontain et al. 1964). Using fuming sulfuric acid, the reaction will be completed after ca. 30 min of warming at 40°C.

After cooling, the resulting liquid was diluted with distilled water and centrifuged (10 min at 1,400*g*). The supernatant was decanted and replaced with distilled water. The cycle was repeated until near-neutral pH was reached. The diatom material was then resuspended in 70% ethanol and filtered through 1.2-µm Isopore™ (Merck Millipore,

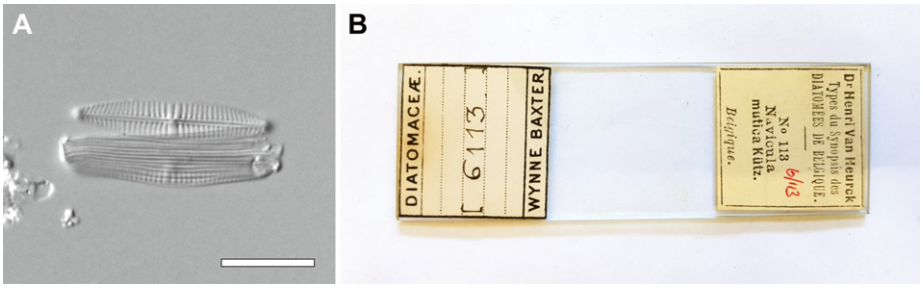

FIG. 1. (A) Frustule of *Proschkinia bulnheimii* var. *belgica* with partially detached valve from Van Heurck's slide No. 113 (Types du Synopsis des Diatomées de Belgique). (B) Van Heurck's slide No. 113 after cleaning (deconstructing). Scale bar: 10 μm. [Colour figure can be viewed at wileyonlinelibrary.com]

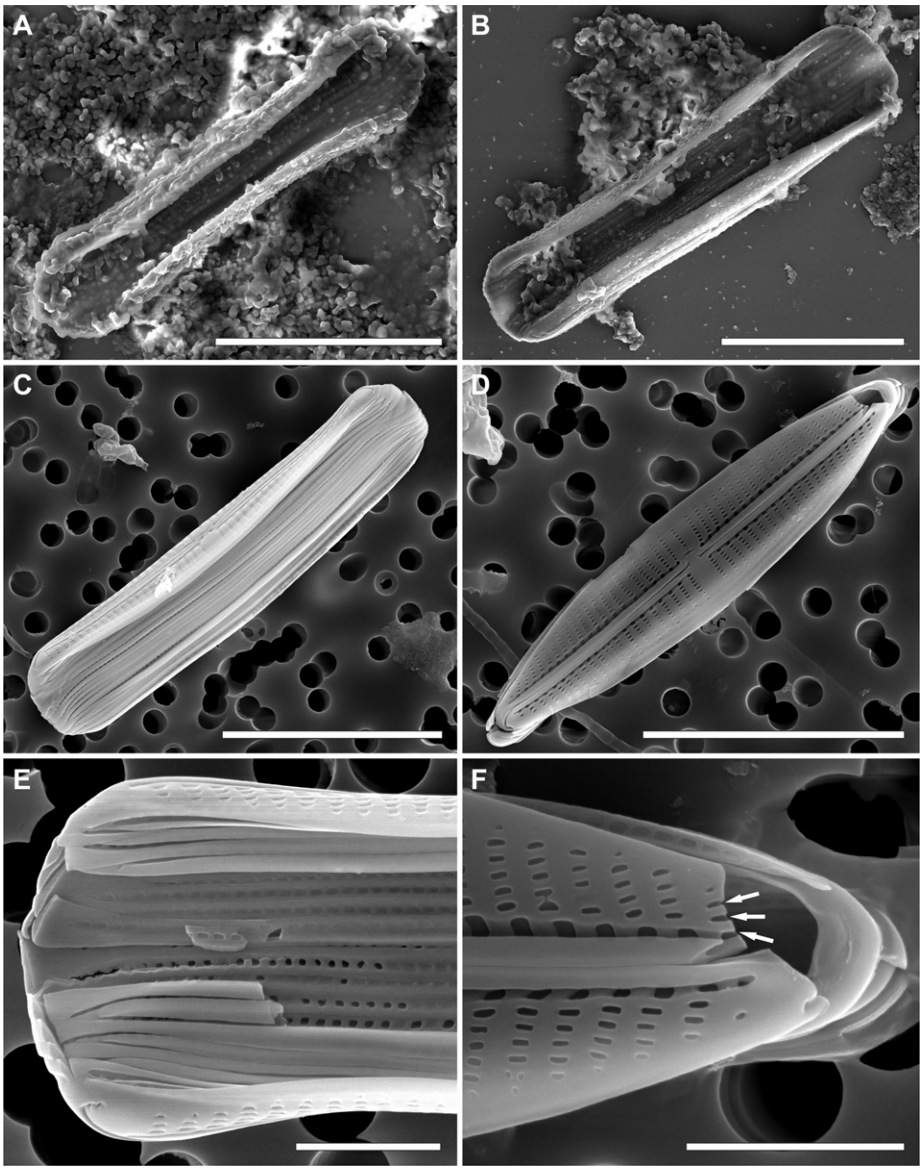

FIG. 2. Scanning electron micrographs of *Proschkinia bulnheimii* var. *belgica* extracted from Van Heurck's slide No. 113 (Types du Synopsis des Diatomées de Belgique). (A & B) Frustules in girdle view before sulfonation was applied. Organic coating indicates the cleaning was not sufficient. (C–F) Frustules after the cleaning procedure was completed. (C) Girdle view. (D) Valve view. (E) Apical part of the frustule (girdle view). (F) Apical part of the frustule (valve view). Arrows indicate areola occlusions (hymenes). Scale bars: A–D = 10 μm; E and F = 2 μm.

Darmstadt, Germany) polycarbonate membrane filters to remove any potential inorganic nondiatom debris that might have been present in the original slide. The filters were subsequently mounted on aluminum SEM stubs, air dried, and sputter coated (as described above).

The results were fully satisfactory and numerous specimens of *Proschkinka bulnheimii* var. *belgica* were recovered and documented in detail with SEM (Fig. 2, C–F). The preparation treatment resulted in no detectable damage to the diatom material, and intact frustules (Fig. 2C) and valves with complete areola occlusions (Fig. 2, E and F, arrows) were present in the prepared SEM specimen. Moreover, only a portion (>50%) of the original diatom material was used to prepare the two SEM stubs, and thus the permanent slide could be reconstructed using the remaining suspension. The SEM analysis revealed significant morphological differences between *P. bulnheimii* and *P. bulnheimii* var. *belgica*. Those differences will be discussed in detail and within an appropriate taxonomic context elsewhere.

Synthetic resins with a high refractive index that are often used in diatom studies, such as Hyrax, Naphrax, and Pleurax, are soluble in, for example, toluene, also known as methylbenzene. Similar to benzene, this solvent can be sulphonated in reaction with either concentrated sulfuric acid or fuming sulfuric acid (Cerfontain et al. 1964). Therefore, slightly modified methods may be used to extract diatoms from permanent slides prepared with different standard mounting media.

Although it must be stressed that any treatment of unique type material should be applied with great care, and a holotype slide deconstruction should only be considered when the lack of original unmounted material is indubitable, the technique described here is simple and efficient, and we are confident that it can be successfully applied by appropriately skilled practitioners in any laboratory. While we recognize the inherent (including historical and cultural) value of the often meticulously prepared and perfectly preserved permanent diatom slides, the scientific value of these specimens may be limited due to the low resolution of the taxonomic information they provide. The proposed method overcomes this limitation, thereby adding new significance and utility to all permanent diatom slide collections. As an added measure of security, various techniques of automatic capturing of high-quality light microscopy images are available today (Cristóbal et al. 2020). To minimize the risk of irreversible loss of type specimens, entire slide scanning and full digitalization of its content could be applied before the slide deconstruction.

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Footnotes

[1]A 15 mL Falcon tube filled with benzene showed deformation after ca. 48 h, indicating that benzene dissolves the tube material. Therefore, we recommend that the centrifugation is completed without unnecessary delay and that the diatom material suspended in benzene is transferred into a glass vial immediately after this step. Alternatively, glass centrifuge tubes may be used.

[2]Benzene dissolves polycarbonate filters often used in diatom studies within a few seconds.

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