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Mongolian Rotifers on Microscope Slides: Instructions to Permanent Specimen Mounts from Expedition Material

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Abstract

We here describe a method for permanently mounting specimens on microscope slides, as we applied it in the newly established rotifer collection in Ulaanbaatar, Mongolia. The liquid photopolymer NOA 61 was used as a primary sealant for pure glycerine mounts. We furthermore outline simple methods of rotifer narcotization and fixation in the field that yield, for the majority of species, adequately preserved specimen material for further preparation and identification purposes.

Key words: Rotifera, narcosis, bulk fixation, glycerine mount, microscope slide preparation, bupivacaine, norland optical adhesive.

Introduction

Difficulties with permanently mounting rotifers in life-like extended state have long been, and often still are, a major hindrance that kept rotifer students from archiving their temporary mounts on durable microscope slides, and from starting collections of type and voucher specimens. Experimenting in that direction only began in the last quarter of the 19th century, over 100 years after the first rotifers have been described (Pelletan, 1878; Rousselet, 1893). Today, the lack of type material and the under-representation of rotifers in natural history collections, impede taxonomic work with this group. Very recently, as stipulated in the 4th edition of the International Code of Zoological Nomenclature (1999; effective 2000), it became mandatory for zoologists who want to establish new species-group names, to also deposit name-bearing types, with the recommendation to integrate these into well-curated institutional research collections.

We recently collected ample specimen materials to inventory rotifer diversity in Mongolia (Jersabek & Bolortsetseg, 2010), with the additional objectives of establishing a local reference collection on microscope slides in Ulaanbaatar, and adding to the Frank J. Myers Rotifera collection (Academy of Natural Sciences) in Philadelphia. We here briefly outline the procedures used to prepare durable specimen mounts, following Taylor (2005), with slight modifications.

Taylor's method (op. cit.) represents a state-of-the-art enhancement of Harring and Myers' (1922, 1928) and Myers' (1936) slide preparation techniques, with particular focus on choice of the most suitable and compatible chemicals, and ease of execution. The first author's experiences, after having studied thousands of slide preparations in historical museum collections, are discouraging for watery fluid mounts, or techniques that used incompatible mounting and sealing media, or brittle protective varnish. Such drawbacks may not be apparent after a few years, but can destroy specimens over the decades, depending on storage conditions. As a rule, watery fluid mounts (mostly formalin) tend to crystallize or dry up completely, and glycerine was often found to be absorbed by paraffin wax, or has been drained off through cracks in cement or brittle ringing compounds (Jersabek *et al*., 2003). The slide preparation technique as described below has already been successfully employed by one of us (C.D.J.) to restore numerous type and high priority specimen preparations that were at threat of deterioration

in the rotifer collections of the Smithsonian Institution (Washington, D.C.) and the Academy of Natural Sciences (Philadelphia).

Fixation of bulk samples in the field

Materials needed:

Bupivacaine hydrochloride, min. 99% (Marcaine®/Sensorcaine®), 0.5% solution (1 g + 200 ml a.d.)

Glutaraldehyde, 2% solution

Formaldehyde, 40% solution (100% Formalin)

Ethanol, 96%

Propane torch

For useful preparations, a basic prerequisite is to obtain specimens with species specific characters still visible after fixation. In species with a stiff lorica this automatically happens if they are allowed to contract on contact with a fixative, and if taxonomy relies on characters that are best shown after the animal has retracted into its lorica. Excessive contraction must be avoided, however, before fixation of soft-bodied forms, as they otherwise contract into unrecognizable lumps (illoricated and some soft-loricated forms). If, ideally, life-like extended specimens are desired, treatment of the sample with an anesthetic prior to fixation, or instant killing in extended condition is required. As different species may show different sensitivity to narcotizing agents, no single anesthetic has yet been shown to be universally effective (Wallace *et al*. 2006, for references). Accordingly, uneven results are generally produced in whole samples, and it is advantageous to employ different techniques to several splits of a single sample.

We chose to split each sample into four fractions for further treatment as follows: (1) Formaldehyde fixation, (2) narcotization with bupivacaine prior to glutaraldehyde fixation, (3) killing in boiling water, and (4) ethanol (96%) fixation. Fractions 1 to 3 are for microscopic studies and specimen preparation, alcohol preserved material is set aside for genetic analyses.

(1) Formaldehyde fixation:

Add 40% formaldehyde dropwise to a final concentration of 4% (= 10% formalin). Note that for longterm-preservation more formaldehyde needs to be added to "thick samples" due to uptake of the fixative by organic material. As a rule of thumb, the amount of the fixative (4% formaldehyde) must be at least 10 times that of organic material present in the sample. If required, adding this excess amount should be not before several hours after killing and fixation to avoid distortions.

(2) Narcotization with bupivacaine prior to glutaraldehyde fixation:

We chose this local anesthetic based on the comparative results of Nogrady and Rowe (1993), who found bupivacaine (BV) to be "by far the most effective compound" in terms of dose, time, and degree of extension of rotifers after fixation. Excellent results were also achieved by subsequent researchers using this drug (e.g., Melone, 1998), but experiences with whole sample-narcotization seem to be limited.

Field experiments with bulk samples and BV-concentrations of 0.2%, 0.16%, and 0.1%, gave best results with the lowest concentration, after 12 minutes of contact (highest average efficacy, as estimated from the degree of extension of illoricate rotifers after glutaraldehyde-fixation).

Procedure (with 25 ml sampling bottle): add 4 ml of 0.5% BV to 16 ml sample volume, shake to distribute the narcotic homogeneously, and leave undisturbed (narcotization is in 0.1% BV). After 12 minutes, add 1 ml of a 2% glutaraldehyde solution (fixation is in 0.1%). It is essential, that the animals are not dead at that point, to prevent post-mortem changes and loss of tissue transparency. A faster fixative, glutaraldehyde is preferred over formaldehyde to immediately "freeze" the narcotized rotifers. For long-term preservation, add formaldehyde after fixation with glutaraldehyde. NB: Some species that are not properly anesthetized by bupivacaine, may react positively to propranolol (Melone, 1998).

(3) Boiling water killing:

By killing and immobilizing specimens instantaneously, the "hot-water fixation technique" (Edmondson, 1959) can give excellent results with species that are notorious as difficult to narcotize (e.g. some bdelloids, sessiles). Reduce the sample volume to the smallest possible amount in a wide mouth bottle (no more than 10 – 20% of bottle volume), and fill it up almost immediately with boiling water. Add formaldehyde to a final concentration of 4%. In the field, a propane torch is very useful to always have boiling water at hand. Caution must be applied in selecting specimens from such samples for further preparation, as artifacts can occur (Melone, 1998).

Specimen preparation, after Taylor (2005)

Materials needed:

(a) Glassware:

- Precleaned microscope slides, 76 x 25 mm, regular
- Cavity slides, 76 x 25 mm, with polished rounddepressions
- Cover glasses, circular, Ø 10-12 mm
- Micropipettes for specimen transfer: draw out from non-heparinized microhematocrit capillary tubes in any flame (even a candle works). Mount in flexible silicone rubber hose with appropriate inside diameter for use with mouth, or disposable polyethylene dropper pipettes for manual use.
- Glass filaments: prepare from Pyrex™ glass rods (Ø 3 mm) by drawing in a bunsen or propane flame down to various diameters (lower limit is ca. 10 µm). Pyrex glass is preferred because of its toughness to allow handling of filaments with a forceps. Draw out filaments of 10 – 20 cm length for later nipping to 7 mm length, and store on dark velvet cloth.
- Glass needles: for arranging and orienting specimens; draw out in gas flame to extremely fine points with relative short taper to ensure sufficient stiffness, mount in stainless steel tubing, e.g. hypodermic tubing.
- Dip rods, with spherical end (Ø 1 – 2 mm), for transferring mounting medium, sealant, and cover slip

(b) Chemicals:

- Glycerine (Glycerol) p.a. (85 – 88 %), with traces of phenol (as bacteri- & fungicide)
- Deionized or distilled water
- Norland Optical Adhesive 61 (NOA 61): liquid photopolymer that cures by exposure to UV. Requires no solvent volatilization for setting and shrinkage is low. Compatible with glycerol, formaldehyde, water, and phenol.
- Dow Corning ® 3140 RTV silicone sealant: non-corrosive, waterproof, flowable, room temperature curing silicone rubber
- Asphaltum varnish: protective ringing compound
- Naphtha (Terpentin-Ersatz)
- Rubber cement

(c) Instruments:

- Dissecting microscope
- Mounting turntable, with underlay for correct location of glass filament supports and coverslip
- Bunsen burner or propane torch
- Dial caliper (e.g., paper micrometer): precision 0.01 mm or higher; for measuring diameter of glass filament supports (determine height of coverslip support)
- Brushes (artist brush # 2), for transferring sealing and ringing components
- Forceps, slender, smooth pointed tips; for seating and removing glass filament supports
- X-acto™ blade (or razor blade)
- Ultraviolet lamp

(1) Dehydration and glycerine infiltration:

For gentle infiltration of formalin-preserved rotifers with glycerine, transfer them individually into a few drops of 5% glycerine (dilute from stock solution with distilled water). For loricated specimens, starting with a 10% solution may suffice. For easier inspection in the compound microscope, cavity slides with polished rounddepressions are useful. Set aside in a dust-free environment to let the water evaporate over a day or two for slow infiltration. Check specimen from time to time for signs of osmotic collapse. If collapse occurred, add a few drops of 50% glycerine to let specimens regain turgidity and natural shape. Repeat process if required. NB: Myers (1936) recommended to add a quantity of the dehydrating agent dioxane, about equal that of glycerine, to support glycerine infiltration with collapsible forms.

(2) Preparation of slides (figures modified from Taylor, 2005):

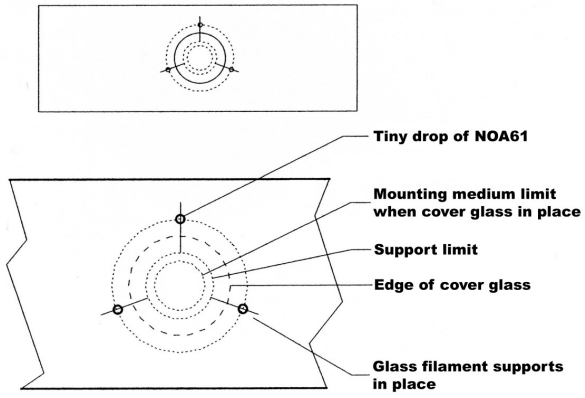

Figure 1. The turntable underlay.

Use the turntable underlay to correctly position glass filament supports, mounting medium, and cover glass as shown in Figure 1. This is most conveniently done by using a mounting turntable with milled recess to receive a 75 x 25 mm slide, with underlay placed

underneath.

Place three tiny drops (Ø < 0.5 mm) of NOA 61 equally spaced 2 mm outside the underlay's cover glass circle as shown in Figure 2 (left). Then select a stored length of filament of the desired diameter (measured with a precision caliper according to specimen size) and nip off three 7 mm-pieces. Place one on each of the NOA 61 points as shown in Figure 2 (right)

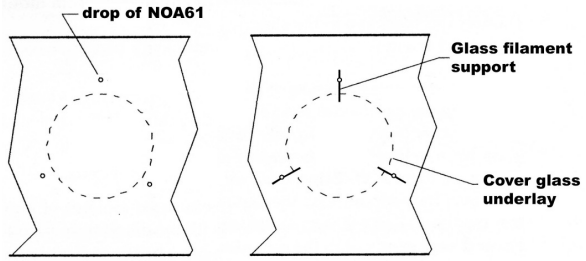

Figure 2. Placement of temporary supports.

and push them down to the surface of the slide. Briefly (15-20 sec) expose to ultraviolet-light for partial setting of NOA 61 not to let it creep along

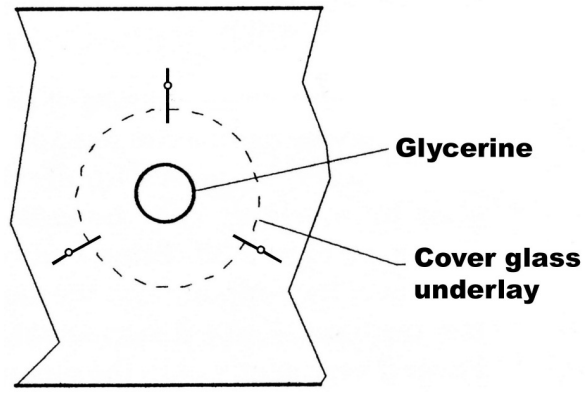

Figure 3. Placement of mounting medium with specimen.

the filaments. NB: choose filament diameter close to maximum specimen height to ensure optimum resolution by thin depth of mountant.

(3) Mounting the specimen in glycerine:

(a) Transfer a small drop of glycerine exactly to the center of the slide as shown in Figure 3.

(b) Transfer the glycerine-infiltrated specimen to the drop, center and orient it.

(c) Pick up a very small amount of rubber

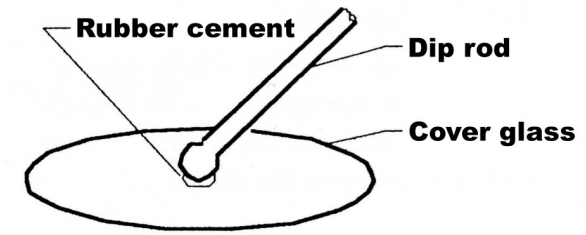

Figure 4. Transferring the cover slip.

cement on the tip of a dip rod and give it a moment to dry. Pick up the cover glass by touching it at the center as shown in Figure 4. Bring the cover glass over the supports and the

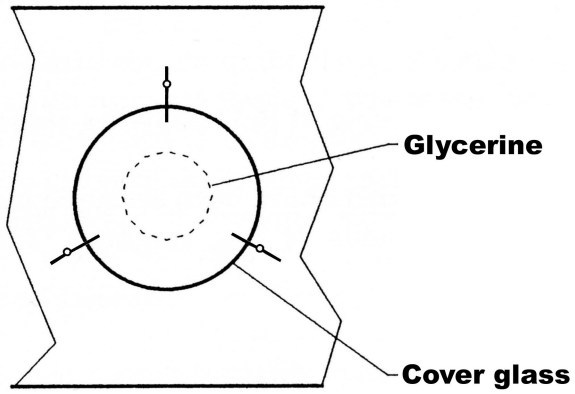

Figure 5. Cover glass in place.

mounting medium. Keep it horizontal and lower it very gently onto the mounting medium and down until it rests on the supports.

(d) Hold the cover glass down with a

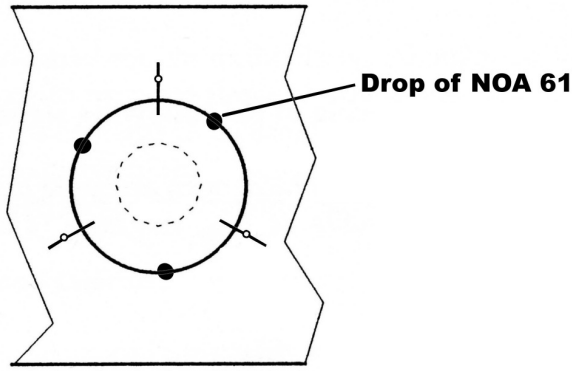

Figure 6. NOA 61 drops in place for taking over support of cover glass.

needle and carefully lift the dip rod off without disturbing the cover glass.

(4) Sealing the slide with NOA 61:

(a) Apply three small drops of NOA 61 on the slide and in contact with the underside of the cover glass (Figure 6).

(b) Expose the slide to UV-light just long enough for the NOA 61 drops to precure so that the cover glass is held in place. This transfers support from the filaments, which now can be removed. Use a X-acto™ blade (or razor blade) to run along each side of the filament supports to free them, and carefully pull them out. NB: no broken fragments of the filaments must remain between slide and coverslip, as this would make the bond between NOA 61 and glass surfaces fail after final cure (NOA 61 has a linear shrinkage of 1.5%; see http://www.norlandproducts.com/

adhesives/noa61pg2.html; accessed January 18th 2010).

(c) Apply NOA 61 equally at three points as shown in Figure 7 until one by one they contact the mounting medium, and eventually fill the whole space between slide and coverslip.

(d) Expose the slide to UV-light to cure the NOA 61 sealing and to complete the curing of

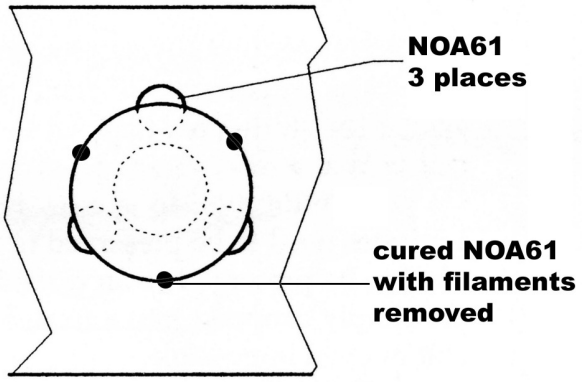

Figure 7. sealing the slide with NOA 61.

the three supports. The length of the exposure depends on the thickness applied and the amount of ultraviolet light energy available, but is usually completed within 30 minutes with a 6 W UV-lamp at 5 cm. If no UV-lamp is available, natural sun light will also do.

(5) Ringing the slide:

When the slide is fully cured, use an X-acto™ or razor blade to remove excessive NOA 61 from slide and coverslip. Then ring it with Dow Corning ® 3140 RTV silicone rubber by applying with a fine brush a circle around the edges of the coverglass and the adjacent area of the revolving slide. Wait at least a week or two to let the silicone rubber ring fully set, before applying a protective ringing. Because the cured 3140 is a bit less than hard, it needs to be ringed with protective Asphaltum Varnish, which is compatible with the 3140. Both, Dow Corning and Asphaltum varnish are thinned to brushing consistency with naphtha (Terpentin Ersatz), and are best applied by using a thin brush (e.g. artist brush #2). NB: Make sure to have the specimen preparation exactly in the center of the slide, otherwise ringing can be difficult and tedious.

(6) Labeling the slide:

We use gummed self-adhesive labels for microscope slides. Labels for permanent mounts must be of "archival" quality, not to become detached or transparent after a few years. A diamond-tipped engraving scribe can be used to write specimen details and accession number directly on the glass slide beneath the labels, to ensure maintenance of the identity of the slide when labels become accidentally detached or the writing on labels fades (Brown, 1997). The following minimum information should accompany each preparation on a slide, ideally on two labels with information on (a) identification, locality, and collecting event on the left, and (b) preparation details on the right side of the slide:

(a) Full species name; type status; locality name; sampling date; geographical details (province, country); collector.

(b) Specimen preparation (whole specimen, trophus, etc.); mounting medium, sealant, and ringing compound; preparation date; repository and catalog number; preparator.

Rotifer trophi can be mounted by the same procedure (steps 2 to 5), after isolation in hypochlorite. For isolating trophi, we followed De Smet's (1998) method.

With some experience, the time effort required to prepare a permanent slide is no more than 20 minutes, not including the time needed for isolation and dehydration of the specimen.

(7) Storage of the slides:

Slides prepared in the above manner don't allow the mountant "creeping", and can be stored either horizontal (e.g. on trays in slide cabinets) or vertical (within slotted boxes or drawers). When not examined, microscope slides should ideally be stored in a controlled environment and total darkness. Given the above choice of mounting, sealing, and ringing components, changes in temperature and relative humidity can be expected, however, to have negligible effects on the longevity of slide preparations.

It is in the nature of things, that track records for proving archival quality are usually short for "latest techniques". Eight years after the first use of NOA 61 with glycerine mounts, the condition of the slides is unchanged, but only with passage of time will it be possible to judge their durability from a historical point of view.

A list of specimen slides so far prepared from Mongolia is given in the Appendix.

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Хураангуй

Энэхүү өгүүлэлд Монгол орны хүрд хорхойн цуглуулгын санг бэлтгэхэд хэрэглэсэн байнгын бэлдмэл хийх аргыг дэлгэрэнгүй танилцуулав. Фотополимер NOA 61 шингэнийг цэвэр глицериний бэлдмэлийг нааж битүүмжлэхэд ашиглана. Түүнчлэн хүрд хорхойг хөдөлгөөнгүй болгох, хээрийн нөхцөлд фиксацлаж хадгалах, зөв горимоор хадгалсан бодгалиудыг цаашдын судалгаанд зориулан бэлтгэх, тодорхойлоход хэрэглэгдэх энгийн аргуудын талаар өгүүлэв.

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Appendix 1. Rotifer species prepared from Mongolian expedition material, with localities and preparation details. Methods were used as outlined above. See Jersabek & Bolortsetseg (2010) for detailed geographic and habitat data for all localities. A total of 170 slides has been prepared for deposit at the National University of Mongolia (NUM), and at the Academy of Natural Sciences of Philadelphia (ANSP). This list does not include specimen material that has been prepared from unidentified and as yet undescribed species. A more detailed catalog for both repositories will be published after all samples have been analysed and newly discovered species have been described.

***Aspelta curvidactyla* Bērziņš, 1949** – Uhegiin Gol wetland, Uvs (1 trophus)

***Asplanchna* cf. *silvestrii* Daday, 1902** – Böön Tsagaan Nuur, Bayanhongor (1 female, 3 trophi)

***Brachionus ibericus* Ciros-Pérez, Gomez & Serra, 2001** – Böön Tsagaan Nuur, Bayanhongor (3 females)

***Brachionus plicatilis* Müller, 1786** – Chuluutyn Tsagaan Nuur, Arhangai (4 females); Böön Tsagaan Nuur, Bayanhongor (12 females); Uvs Nuur, Uvs (9 females)

***Brachionus quadridentatus* Hermann, 1783** – Böön Tsagaan Nuur, Bayanhongor (16 females + loricae)

***Brachionus urceolaris* Müller, 1773** – Böön Tsagaan Nuur, Bayanhongor (1 female); Uvs Nuur, Uvs (1 female)

***Cephalodella balatonica* Zsuga, 1996** – Hugshin Orhon, Arhangai (1 female); Jargalan Gol, Hövsgöl (1 female); Uhegiin Gol, Uvs (1 female, 1 trophus)

***Cephalodella catellina* (Müller, 1786)** – Hövsgöl Nuur, Hövsgöl (3 females); Ih Tunhleg Nuur, Uvs (3 females, 1 lorica, 1 trophus)

***Cephalodella fluviatilis* (Zavadovski, 1926)** – Chuluutyn Tsagaan Nuur, Arhangai (1 female, 1 trophus)

***Cephalodella forficula* (Ehrenberg, 1830)** – Uhegiin Gol, Uvs (1 trophus)

***Cephalodella maior* (Zavadovski, 1926)** – Ih Tunhleg Nuur, Uvs (1 female)

***Cephalodella misgurnus* Wulfert, 1937** – Chuluutyn Tsagaan Nuur, Arhangai (6 females, 1 trophus)

***Cephalodella rotunda* Wulfert, 1937** – Jargalan Gol (backwater), Hövsgöl (1 female, 1 trophus)

***Cephalodella segersi* De Smet, 1998** – Chuluutyn Tsagaan Nuur, Arhangai (7 females, 2 trophi)

***Cephalodella theodora* Koch-Althaus, 1961** – Hövsgöl Nuur, Hövsgöl (3 females, 3 trophi)

***Encentrum algente* Harring, 1921** – Uvs Nuur, Uvs (1 female, 1 trophus)

***Encentrum glaucum* Wulfert, 1936** – Ih Tunhleg Nuur, Uvs (1 females, 1 trophus); Uvs Nuur, Uvs (1 female)

***Encentrum marinum* (Dujardin, 1841)** – Uvs Nuur, Uvs (1 female)

***Encentrum* cf. *armatum* Donner, 1943** – Ih Turuu Gol, Hövsgöl (1 female)

***Encentrum putorius* Wulfert, 1936** – Hövsgöl Nuur, Hövsgöl (1 trophus)

***Encentrum saundersiae* (Hudson, 1885)** – Ih Tunhleg Nuur, Uvs (1 female)

***Encentrum uncinatum* (Milne, 1886)** – Jargalan Gol, Hövsgöl (1 female, 1 trophus)

***Keratella quadrata* (Müller, 1786)** – Hugshin Orhon, Arhangai (1 female, 1 lorica); Ih Tunhleg Nuur, Uvs (4 females, 5 loricae)

***Keratella zhugeae* Segers & Rong, 1998** – Böön Tsagaan Nuur, Bayanhongor (2 females, 24 loricae)

***Lecane crenata* (Harring, 1913)** – Uhegiin Gol, Uvs (6 females)

***Lecane perplexa* (Ahlstrom, 1938)** – Uhegiin Gol, Uvs (1 female)

***Lecane plesia* Myers, 1936** – Ih Tunhleg Nuur, Uvs (8 females)

***Notholca acuminata* (Ehrenberg, 1832)** – Böön Tsagaan Nuur, Bayanhongor (1 female); Hugshin Orhon, Arhangai (4 females); Uvs Nuur, Uvs (3 females)

***Pleurotrocha petromyzon* Ehrenberg, 1830** – Ih Tunhleg Nuur, Uvs (1 trophus); Uhegiin Gol, Uvs (1 female)

***Pleurotrocha robusta* (Glascott, 1893)** – Uhegiin Gol wetland, Uvs (1 trophus)

***Proales minima* (Montet, 1915)** – Ih Turuu Gol, Hövsgöl (2 females); Jargalan Gol, Hövsgöl (6 females)

***Proales theodora* (Gosse, 1887)** – Ih Turuu Gol, Hövsgöl (3 females, 3 trophi)

***Squatinella lamellaris* f. *mutica* (Ehrenberg, 1832)** – Uhegiin Gol, Uvs (3 females)

***Squatinella longispinata* (Tatem, 1867)** – Uhegiin Gol wetland, Uvs (1 female)

***Testudinella emarginula* (Stenroos, 1898)** – Uhegiin Gol, Uvs (6 females)

***Testudinella parva* f. *bidentata* (Ternetz, 1892)** – Uhegiin Gol, Uvs (1 female)

***Trichocerca porcellus* (Gosse, 1851)** – Uhegiin Gol, Uvs (1 trophus)

***Trichocerca taurocephala* (Hauer, 1931)** – Hugshin Orhon, Arhangai (1 female)

***Trichocerca uncinata* (Voigt, 1902)** – Uhegiin Gol, Uvs (2 females)