Light Microscopy to Detect and Identify the Bivalve Larvae of *Dreissena polymorpha* and *D. rostriformis bugensis*

Steve Wells and Mark Sytsma

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Portland State University

Scope of Manual

This manual is intended to aid early detection monitoring for shelled, planktonic larvae (veligers) of zebra and quagga mussels (*Dreissena polymorpha* and *D. rostriformis bugensis*) in North America. It is focused on the identification of bivalve larvae using morphology that is visible under light microscopy on preserved specimens collected in the water column of North American freshwater systems. The bivalve species specifically addressed in this manual include *Dreissena polymorpha*, *D. rostriformis bugensis*, *Corbicula* sp., and *Mytilopsis leucophaeata*. Several species of bivalves in the family, Unionidae, are also addressed. This manual is not comprehensive and does not address *Limnoperna fortunei*, *Rangia cuneata*, and many of the species from the families Unionidae and Margaritiferidae.

The following equipment is recommended for use with this manual: a binocular compound light microscope with 10X lens pieces and 4X, 10X, and 20X plan achromatic objectives; polarization kit; mechanical stage; sub-stage condenser; Sedgewick-Rafter counting cells; cover slips; and a trinocular-mounted digital microscope color camera equipped with digital micrometer.

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Table of Contents

Scope of Manual i
Acknowledgements ii
Table of Contents 1
Introduction 1
Larval development 2
Sample processing methods 4
Light microscopy 4
Electron microscopy 5
Molecular methods 5
Standard Operating Procedures: Plankton Analysis 7
Sample Preservation and Storage 7
Sample Preservation 7
Holding Temperature 8
Sample pH 8
Sample Tracking 8
Sample Preparation 9
Filtering 9
Sample concentration 10
Microscopic Evaluation 10
Counting Chambers 10
Microscope 12
Subsampling 12
Aliquot Dilution 13
Scanning Mode 14
Sample Storage 14
Inspecting and Identifying Suspect Specimens 14
Change the Lighting Conditions 15
Increase Total Magnification 15
Reorienting the Specimen 15
Size Measurements 16
Photomicroscopy 16
Quality Assurance/Quality Control 17
Check Standards (Laboratory Control Samples) 18
Internal Cross-Validation 18
External Cross-Validation 18
Laboratory Decontamination 19
Identification Guide 21
Identification Key 21
Online Image Database http://mussels.research.pdx.edu/musselid/ 87
Glossary 88
Bibliography 95
Appendices 102
Appendix A: Sample tracking forms 102
Appendix B: Examples of photomicrographs 104
Appendix C: Form for documenting and standardizing external cross validation of photomicrographs and/or suspect specimens. 105

Introduction

Zebra mussels (*Dreissena polymorpha*) and quagga mussels (*Dreissena rostriformis bugensis*) can cause extensive ecological and economic damage in areas where they are not native. These freshwater mussels are spreading in North America. Watercraft with attached, hitchhiking mussels have been detected in areas outside their known, current distribution. Established populations can be sources of planktonic larvae that can be transported to downstream areas. Monitoring and early detection are key to implementation of rapid response procedures to limit further mussel spread, and prevention and containment efforts are dependent on accurate sample analysis and efficient information dissemination.

Early detection monitoring for dreissenid mussels is difficult. There is a high likelihood of false negative results, i.e., mussels are present but not detected, because incipient populations are inherently rare and have a clumped distribution, it is difficult to observe underwater habitats, and the sample matrix often makes processing samples difficult.

There are multiple monitoring methods for dreissenid mussels that target their different life stages. Settled juveniles and adults can be sampled with artificial and natural settlement substrates, surface scrapings, benthic dredges, SCUBA, remote operated vehicles, and shoreline inspections. This document focuses on the larval stage. Early detection monitoring for mussel larvae is done with plankton samples that are analyzed using light microscopy (e.g., compound microscope, stereo microscope, imaging recognition software), scanning electron microscopy, and molecular techniques (e.g., polymerase chain reaction assays). There are advantages and disadvantages to each method of processing plankton samples for early detection of dreissenid veligers. All methods are affected by specimen integrity.

Light microscopy is an effective, established and relatively inexpensive method for detecting and identifying freshwater bivalve larvae in plankton samples. Morphological features of the larval shell can be used for freshwater bivalve identification (Conn, Lutz, Hu and Kennedy 1993; Kennedy and Hagg 2005; Nichols and Black 1994). For example, Kennedy and Hagg (2005) correctly classified 72 to 79% of bivalve larvae from 21 species in the family Unionidae and Margaritiferidae with discriminant function analysis using shell length, shell height, and hinge length. The use of cross-polarized light microscopy aids in the detection of bivalve larvae in plankton samples (Johnson 1995). Identification of preserved specimens via light microscopy is based on shell dimensions, overall shape, shell surface features and visible internal tissues, e.g., velum and foot (Ackerman, Sim, Nichols and Claudi 1994; Nichols and Black 1994).

Identification of planktotrophic larvae based solely on morphology using light microscopy is easier with freshwater than marine and estuarine species. Indirect larval development in the water column, i.e., planktotrophic development, is common in marine and estuarine bivalves (Dillon 2000; Raven 1958), but only *Dreissena polymorpha*, *D. r. bugensis*, and *Limnoperna fortunei* have planktotrophic development among the freshwater mussels. Larvae and juveniles of other freshwater bivalves are found in the plankton; however, these larvae and juveniles do not develop in the water column, and have morphologies different from planktotrophic larvae. Planktotrophic larvae of different species all have similar larval stages and morphology (Chanley 1969; Chanley and Chanley 1980; D'Asaro 1967; Goodwin, Shaul and Budd 1979; LaBarbera and Chanley 1970; LaBarbera and Chanley 1971).

Larval development

Dreissenid larval development is indirect and occurs in the water column, i.e., planktotrophic, and this is relatively unique in freshwater bivalves. Most other freshwater Lamellibranchiata, e.g., *Sphaerium*, exhibit direct development of larvae without a free-living larval stage, and larvae develop within egg capsules or in a brood-pouch of the adults (Raven 1958). When this happens, the larval stages tend to be suppressed, and there is no metamorphosis (Raven 1958). For example, *Corbicula fluminea* young are typically released from adults as straight-hinge juveniles that have a well-developed foot and siphons, and lack a velum. Nichols and Black (1994), however, noted that *Corbicula* sp. with sway-back hinge lines and velums are collected in the water column in the Midwest; sway-backed *Corbicula* sp. are also collected in the Pacific Northwest (Steve Wells, personal communication, 2012). Larval development in other Lamellibranchiata such as the Unionidae is indirect, but involves a parasitic glochidium (Raven 1958). Glochidia larvae attach to a host and develop in a cyst through a gradual metamorphogensis after which the young mussels break free from the host and fall to sediment (Raven 1958). Most glochidial larvae die without a host, but they may be found in the water column temporarily. Glochodium morphology varies among taxa but can be distinguished from planktotrophic larvae based on shell shape and size, lack of velum and foot, prominent adductor muscle, and in some cases by the presence of barbed teeth and/or hooks on shell margins, and larval threads (Lefevre and Curtis 1912; Pekkarinen and Englund 1995a; Pekkarinen and Englund 1995b; Pekkarinen and Valovirta 1996; Raven 1958; Surber 1912; Tucker 1927).

Planktotrophic bivalve larval development follows a pattern. The trochophore stage is the first larval stage developing from the gastrula (Kume and Dan 1968; Raven 1958). All marine Lamellibranchiata and *Dreissena* sp. exhibit a trochophore stage that is planktonic (Raven 1958). An apical tuft forms at the anterior end, and the shell gland develops from the thickened dorsal ectoderm (Raven 1958). In late trochophore stage, the shell gland and mantle epithelium produces the first larval shell, the prodissoconch I (PI) (Raven 1958). The PI begins as a single shell draped over the dorsal end of the body, and it continues to grow towards both sides and eventually folds into two valves. The PI tends to have a smooth to pitted or punctate surface texture (Carriker and Palmer 1979). Trochophore larvae are damaged during sample preservation and cannot be reliably identified (Nichols and Black 1994). Therefore, early detection efforts are typically focused on the shelled larval stages found in the plankton including the straight-hinge or D-shape, umbonal and pediveliger forms (Figure 1).

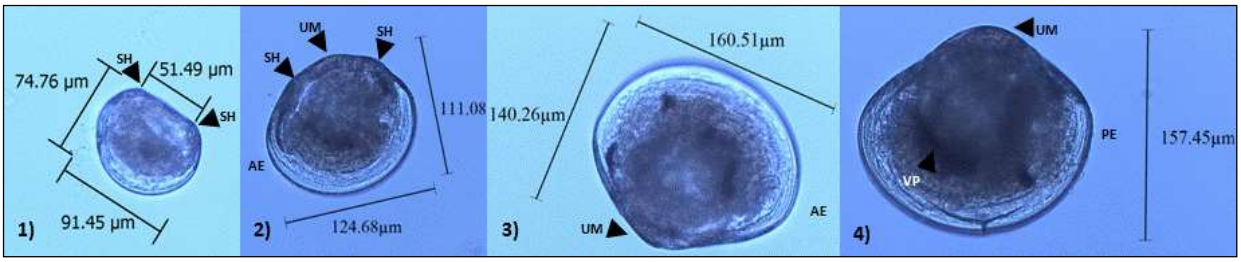


Figure 1: The overall shell shape, general size, and hinge development of the shelled bivalve larval stages found in the plankton that are targeted in early detection efforts including the 1) straight-hinge (D-stage), 2) late-stage straight-hinge/early umbonal (early umbonal), 3) umbonal (umbonal), and 4) late-stage umbonal/pediveliger (pediveliger). The hinge area is marked by the shoulders (SH) and umbo (UM). The velar pigment (VP) is marked. Overall shape includes symmetry of posterior end (PE) and anterior end (AE), umbo height beyond shell margin, and shell outline.

The straight-hinge/D-shape larval stage is the second stage and the first shelled larval stage with a fully developed velum (Figure 2). In this stage, the hinge is straight and the umbo has not developed enough to protrude beyond the hinge line, resulting in a D-shaped shell. The velum develops from the prototroch and resembles a pair of semicircular folds with cilia along outer margin (Raven 1958). Bivalve larvae that have a velum are called veligers.

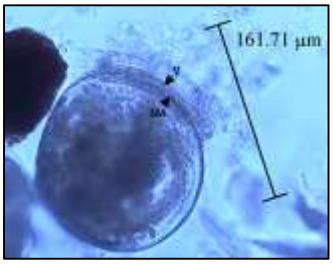


Figure 2: Veliger with velum (V) extended beyond shell margin (SM).

As the veliger grows, the umbo develops and protrudes beyond the straight hinge line, and the overall shape changes from D-shape to round, thus marking the transition to the umbonal larval stage. Sometime around this period, the mantle forms and new growth starts on the peripheral edges of the shell, resulting in commarginal growth lines (Carriker and Palmer 1979; Raven 1958). This new region of shell growth, characterized by growth lines, is the prodissoconch II (PII) (Carriker and Palmer 1979; Raven 1958). The shell then becomes umboned and asymmetrical with the anterior end of the valves becoming more pointed compared to the posterior end (Carriker and Palmer 1979).

The development of a functional foot marks the transition to the pediveliger larval stage. The pediveliger stage is the settling stage and is defined by a distinct umbo and well-developed foot (Carriker and Palmer 1979). Pediveligers still have the velum (Raven 1958), however the velum is broken down and shed during metamorphosis to the plantigrade larval stage. Following metamorphosis, the adult shell, the dissoconch, is secreted by the mantle (Martel, Hynes and Buckland-Nicks 1995), and the mussels are considered juveniles.

Sample processing methods

Light microscopy

Light microscopy is limited to morphology that is visible under magnifications up to 200X[1]. Intact specimens in good condition are required for identification and greatly increase the likelihood of detection using cross-polarized microscopy, i.e., birefringence. Shell sculpture or surface features, e.g., growth lines, can be difficult to positively identify using light microscopy, and this can make it difficult to delineate prodissoconch I from prodissoconch II regions. Hinge structures are important for planktotrophic larvae identification, but may be difficult to observe using light microscopy (Baldwin, Hu, Conn and Kennedy 1994; Garland and Zimmer 2002).

Identification based on morphological characters is complicated by phenotypic plasticity, the morphological similarity of larvae of different species, and genetic variation. The environment can influence planktotrophic bivalve larval shell morphology, but this relationship is complex and not understood (Pechenik 1984; Pechenik, Eyster, Widdows and Bayne 1990). Martel, Baldwin, Dermott and Lutz (2001) reported similar shell morphology between-species and across habitats for early stage larvae of *Dreissena polymorpha* and *D. rostriformis bugensis*, but found significant differences between species and habitat in later stage morphology. Pechenik et al. (1990) reported considerable variation in both the size at which larvae of the marine bivalve, *Mytilus edulis*, developed eyespots and the time required to develop eyespots, with little correspondence between effects of temperature and food concentration on growth rates and physiological differentiation. Although Loosanoff (1959) reported that lamellibranch larvae of the same species had virtually the same shell shape at metamorphosis regardless of the water temperature, he also reported considerable variability at the same temperature. Loosanoff, Davis and Chanley (1966) reported that there was a linear relationship between *M. edulis* shell length and shell height among mussel larvae reared at a given temperature and food concentration. Pechenik et al. (1990) also reported that a linear relationship existed between *M. edulis* shell length and shell height at all tested temperatures and food concentrations. These data suggest that there was no effect of these environmental factors on the ratio of shell height to shell length. Pechenik et al. (1990), however, reported that *M. edulis* larvae were approximately 20-µm wider for a given shell length compared to shell dimensions reported by Loosanoff et al. (1966).

[1] 200X is the maximum total magnification available when using the equipment recommended in this manual. The total magnification available when using a compound light microscope with Sedgewick-Rafter cells is limited by the working distance between the objective lens and the microscope stage.

Shell dimensions and larval size are important for identification, but it can be difficult to compare values reported in the literature. Larval shell dimensions used in this manual follow protocols for measuring bivalves based on the definitions of Bayne (1976), Rees (1950), Loosanoff et al. (1966) and Nichols and Black (1994). Here, shell dimensions are measured from the lateral view, and shell height is the distance between the top of the umbo and the ventral margin of the shell. Shell height is perpendicular to the hinge line. In cases where the umbo does not protrude beyond the hinge, the shell height is measured from the center of the hinge line to the ventral margin of the shell. Shell length is the greatest dimension along the antero-posterior axis that is parallel the hinge line, and perpendicular to shell height dimension. Hinge length is the distance between the shoulders where the bivalve shells join (Figure 3).

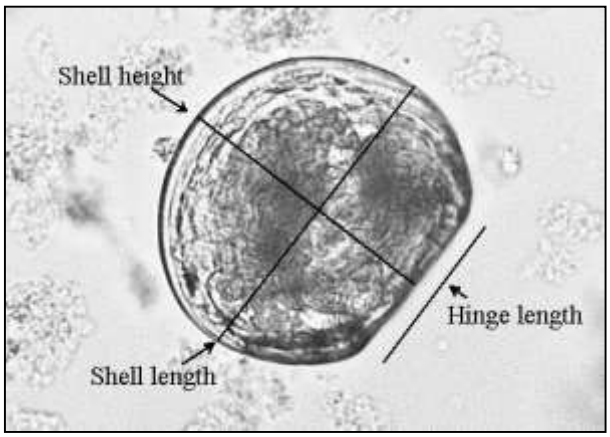


Figure 3: Shell dimensions used in this manual measured from the lateral view.

Intact bivalve larvae, especially in the later stages of development, are not completely parallel with the plane of the cell when lying on the bottom of a counting chamber and viewed from directly above or below. Umbo development increases the shell width disproportionately towards the dorsal end, and this may confound comparisons of shell measurements (Figure 4).

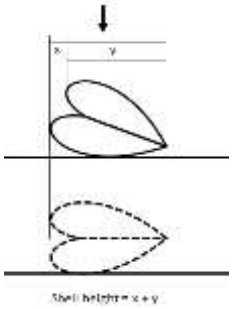


Figure 4: A drawing of intact bivalve shell (lateral view relative to the line of sight indicated by arrow) with one valve lying planar to the counting chamber, and showing the potential error (y) in the shell height on an intact bivalve that is not completely perpendicular to the line of sight versus the height measurement of a shell completely perpendicular to the line of sight (x+y).

Electron microscopy

Scanning electron microscopy (SEM) is an important tool for planktotrophic larvae identification, but is not practical for detection. SEM offers high magnification and resolution and reveals intricate surface details such as the hinge features, prodissoconch I and prodissoconch II regions, commarginal growth lines, and surface sculpture (Baldwin et al. 1994; Garland and Zimmer 2002; Fuller and Lutz 1989). Sample preparation and time, however, usually limit SEM examination of external shell morphology to a limited number of isolated specimens. Shell valves are typically disarticulated, carefully mounted, and may need to be coated with specific metals for analysis. Specific procedures for specimen preparation and measurement, e.g., valve disarticulation, are necessary to minimize the potential for inaccurate shell measurement and distortions of shape (Fuller, Lutz and Pooley 1989).

Molecular methods

Molecular techniques are an important tool for identification that is not dependent upon morphology. Polymerase chain reaction assays (pcr) have been used for early detection of larvae of *Dreissena* sp. veligers (Kevin Kelly, personal communication, 2010; Frischer, Nierzwicki-

Bauer and Kelly 2011), Unionidae (Gerke and Tiedemann 2001), and *Limnoperna fortunei* (Boeger, Pie, Falleiros, Ostrensky, Darrigran, Mansur and Belz 2007; Darrigran, Boeger, Damborenea and Maronas 2009; Endo, Sato and Nogata 2009). Molecular techniques may be able to differentiate closely related species or specimens that cannot be positively identified with morphological features alone (Garland and Zimmer 2002). Challenges in molecular techniques include optimizing the reaction conditions, and understanding the gene sequences of the target organism as well as sympatric species. Reaction conditions such as temperature, pH, and DNA concentration can influence the 'optimization' of the assay (Garland and Zimmer 2002; James Snider, personal communication, 2012). Molecular techniques are affected by the quality and quantity of genetic material, preservation artifacts, and non-target extraneous genetic material. Specific primers must be developed to target unique sites and the uniqueness and stability of these molecules can be a challenge in applying molecular techniques (Garland and Zimmer 2002).

Detecting and identifying rare bivalve larvae in plankton samples is difficult, regardless of the analytical technique. Sediment, algae and other objects in plankton samples create an interfering matrix that confounds bivalve larvae detection. Identification is confounded by degraded or poor specimens lacking sufficient genetic material or morphological features, e.g., moribund specimens. There can also be confusion with morphologically and genetically similar species such as *Corbicula* sp. and *Mytilopsis leucophaeata*.

Accurate veliger identification is important. Incorrect and ambiguous results confuse policymakers and managers, complicate other agency efforts, and compromise trust in the scientific community. Currently, an independent laboratory certification process is lacking for veliger identification laboratories. There are ongoing efforts to develop certification, but this process is complicated and time-consuming. Training in light microscopy analysis can increase the comparability and accuracy of the plankton analyses being performed by multiple laboratories, and it is hoped that this manual advances these efforts.

Standard Operating Procedures: Plankton Analysis

Sample Preservation and Storage

Sample Preservation

Preserve plankton samples using regular ethanol immediately after collection to ensure sample integrity. Regular ethanol is the preferred preservative. Avoid denatured ethanol because denatured ethanol may dissolve the larval shell (Stalvey 2009a). Avoid Lugol's solution because it may contain acetic acid that dissolves shells. Utermohl's modification of Lugol's solution, however, is neutral to alkaline and will not dissolve shells. Avoid isopropyl alcohol because it may interfere with molecular analytical methods; isopropyl alcohol is acceptable for light microscopy analysis. Samples that cannot be preserved immediately after collection should be placed on ice until preservative can be added. Samples held on ice should be preserved within 72 hours of collection (EBMUD 2009; Stalvey 2009). There are many benefits of preserving plankton samples. Preservation greatly extends the time period post collection for sample analysis. It is easier and cheaper to handle preserved versus living specimens. Preserved specimens are easier to examine under the microscope as they do not move on their own accord.

It can be easier to identify living specimens using light microscopy because certain morphological features are more apparent, e.g., foot and velum. Preservation can also distort soft tissues of bivalve larvae, e.g., velar pigment. Living specimens must be held on ice during transit, and at low but not freezing temperatures (greater than 0ºC to 4ºC) prior to and during analysis. Non-preserved plankton samples should be analyzed within 72 hours of collection. Living specimens are not constrained by shipping restrictions associated with the preservative, i.e., ethanol is a Class 3 flammable liquid. The possession, transport and disposal of living specimens of a regulated species such as *Dreissena* sp. are more restrictive, however, than for preserved specimens, and may be prohibited altogether.

Preserve samples in a final solution of 70% ethanol. Use more preservative with samples that contain greater amounts of plankton, sediment and other debris. Plankton samples that are preserved in 70% or greater concentration of regular ethanol are acceptable for both molecular and microscopic analytical methods (Kevin Kelly, personal communication, 2010; Paul Rochelle, personal communication, 2007; James Snider, personal communication, 2011; Steve Wells, personal communication, 2012). Specimen integrity for light microscopy analysis of properly buffered plankton samples preserved in 70% regular ethanol is likely to be maintained for over two years. Repeated measurements were made on three plankton samples collected from Lake Mead, Nevada and preserved in solutions of 70% regular EtOH to determine the effects of holding time on *Dreissena rostriformis bugensis* veliger density. Holding time had no significant effect on veliger density ($F(1.828,3.657) = 6.024$, $p = 0.070$) over a period of 1,310 days in plankton samples preserved in 70% EtOH (Wells and Sytsma 2013). *D. polymorpha* larvae collected from El Dorado Reservoir in Kansas maintained birefringence and shell integrity for over five years when stored in 70% isopropyl alcohol at room temperature (Steve Wells, personal communication, 2013).

Some monitoring programs preserve plankton samples in solutions of 25% to 50% regular ethanol prior to microscopic analysis and later raise the ethanol concentration to 70% for long-term storage (Denise Hosler, personal communication, 2012; EBMUD 2009; Stalvey 2009). Plankton samples preserved in 25% to 50% ethanol should be held on ice during transit, and stored at low but not

freezing temperatures, e.g., refrigerator, prior to and during analysis (Denise Hosler, personal communication, 2012; Stalvey 2009). Reducing the amount of preservative reduces shipping restrictions and may decrease deleterious effects of preservation, but microbial activity and associated sample degradation are not inhibited in plankton samples preserved in a final solution of 25% to 50% ethanol; refrigeration is necessary to delay this degradation.

Holding Temperature

Hold plankton samples preserved in solutions of 70% regular ethanol at room temperature, 20°C to 25°C, and out of direct sunlight for over two years. Samples preserved in 25% ethanol can be stored for up to 30 days prior to light microscopy analysis at temperatures between 0°C and 4°C (Denise Hosler, personal communication, 2012; EBMUD 2009; Stalvey 2009). Note that specimen integrity is not impacted by holding plankton samples at temperatures between 0°C and 4°C in solutions of both 70% and 25% ethanol.

Sample pH

pH may impact the integrity of bivalve larvae in a plankton sample. Low pH (less than 6.8) may dissolve the bivalve larval shell and/or reduce birefringence and detection using cross-polarized light microscopy. High pH (above 9) may disarticulate shells and disintegrate soft tissues (Baldwin et al. 1994; Wells 2010). The pH of a solution of ethanol in water is relatively similar to the pH of water; pure water, however is slightly acidic. Add a small amount of tris(hydroxymethyl)aminomethane (Tris) or sodium bicarbonate (baking soda) to preserved samples collected from typical western US water bodies to raise the pH to an appropriate level (e.g., pH 7.4 to 8.6). Tris is the recommended buffer compared to sodium bicarbonate. Sodium bicarbonate is insoluble in ethanol and may interfere with molecular analyses (James Snider, personal communication, 2013). For example, add 7 drops of 4-M solution of Tris to a 500-mL plankton sample, or add approximately 2-g $NaHCO_3$ per 500-mL sample preserved in solution of 70% regular EtOH. Note that adding too much sodium bicarbonate confounds light microscopy due to the presence of undissolved birefringent crystals. The pH can be lowered by adding acids such as hydrochloric acid (1.0-N HCl).

Sample Tracking

Sample tracking is done to document the handling, analysis and reporting for each sample received by a laboratory in a manner that reduces bias associated with laboratory personnel. Upon receipt into a laboratory, samples should be inspected for leakage, appropriate labeling, and the appropriate sample handling protocol determined according to the type and concentration of preservative. It is recommended that the pH of preserved plankton samples is measured upon receipt into laboratory, and buffer is added as needed to maintain pH above 7. Samples should be entered into a laboratory sample tracking database or spreadsheet noting date of arrival; laboratory turn-around time is measured from the date of receipt to notification of analytical results. A sample tracking number should be assigned to samples during sample preparation. This unique numerical and/or alphabetical code permits tracking and identifying samples, subsamples, sample splits, and/or blind matrix spiked samples during sample analysis.

The database or spreadsheet used to track samples should include fields for date of analysis, sample tracking number, analyst code or name, the type of microscope and counting chamber used,

dilution factor, number of aliquots analyzed, and tallies or notes on bivalve larvae detected as well as other suspect specimens, e.g., ostracods, and the date when analytical results were reported to the responsible party. The sample, in its original sample container, should be archived for storage. An example sample tracking system is provided in Appendix A. Ideally, the sample tracking system is an inventory software system or recorded in bound laboratory notebooks using permanent ink.

Sample Preparation

Filtering

Filters can be used to separate large zooplankton and debris from bivalve larvae. A 500-µm filter (Figure 5a) can be used to remove large zooplankton (e.g., copepods), filamentous algae, and other large debris that contribute to the interfering matrix found in plankton samples. Large debris can reduce bivalve larvae detection, increase analytical time and prohibit the use of certain counting chambers, e.g., 1-mL volume Sedgewick-Rafter cell. The use of 500-µm filters, however, is a source of bivalve larvae loss during sample handling and preparation. Some bivalve larvae are retained with the debris collected on 500-µm filters even though larvae are smaller than 500-µm. The amount of bivalve larvae retained on 500-µm filters is positively related to the amount of inorganic debris and filamentous algae in the plankton sample. Percent recovery of bivalve larvae with or without 500-µm filters is also inversely related to the amount of inorganic debris and filamentous algae in the plankton sample (Wells 2010). Filters can be rinsed/back-washed to reduce bivalve larvae loss, and material removed by 500-µm filters can be sorted using stereo microscopes. Alternatively, one half of the sample can be filtered, and the other half analyzed without filtering. If 500-µm filters are used, the material retained on the filter should be collected and stored in the original sample container.

Filters can also be used to separate bivalve larvae from other debris following centrifugation or settling. Filters of 63-µm mesh or smaller can be used to prevent the unintentional removal of bivalve larvae when siphoning off or decanting supernatant. Filter sleeves (Figure 5b) can be made from 63-µm mesh and placed over the ends of siphon tubes and/or pipettes. These filters can be rinsed/ back-washed to reduce bivalve larvae loss.

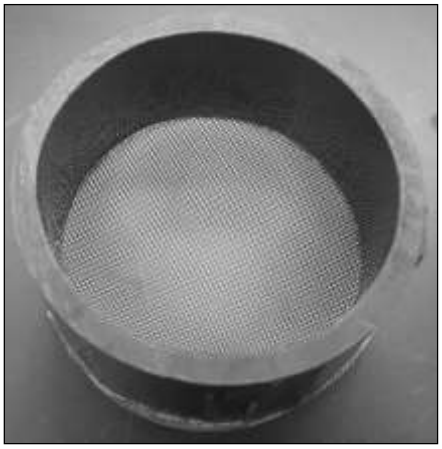
a)  **b)** 

Figure 5: Examples of filters used to separate bivalve larvae from supernatant and other debris in plankton samples, including a) filters made from 500-µm mesh, and b) filter sleeves made from 63-µm mesh.

Sample concentration

Water and ethanol comprise a large portion of a plankton sample, and the bivalve larvae and other particulates must be separated from this liquid. Settling or centrifugation of the raw sample allows the analyst to directly determine the quantity of pelleted particulates, i.e., the concentrated sample volume, that was analyzed, and to control the matrix density encountered in the counting chamber through aliquot dilution. Removing supernatant prior to analysis may result in the potential removal of larvae with the liquid so must be done carefully. It is important to keep enough of the supernatant with the concentrated sample to maintain the target EtOH concentration for preservation.

There are several ways to concentrate bivalve larvae and other dense objects in the raw sample. Bivalve larvae are relatively dense, and preserved bivalve larvae settle to the bottom of the sample container through gravitational settlement. Preserved plankton samples can be gravitationally settled in separation funnels (Figure 6a), Imhoff settling cones (Figure 6b), or by leaving sample containers undisturbed on a level surface for 8 hrs. Separation funnels or settling cones are recommended because the concentrated sample can be selectively removed by opening a valve located at the bottom of the funnel/cone; this method is easy and inexpensive. A centrifuge is an effective way to quickly settle bivalve larvae into the pelleted particulate. The exact centrifuge speed and time are not critical so long as the particulates in centrifuge tubes are pelleted. In general, 1,000 RPM for 10 minutes at 20^{o}C is adequate for centrifugation. If the sample is gravitationally settled in the original sample container, and the supernatant is then selectively removed, e.g., via siphoning, it is recommended that filter sleeves made of 63-µm mesh fitted over the siphon tubing/syringe be used to reduce the likelihood of removing larvae with supernatant. The supernatant can also be decanted. Whichever method is used to remove the supernatant, the supernatant should be retained.

Figure 6: Equipment to concentrate bivalve larvae including a) separation funnels and b) Imhoff settling cones.

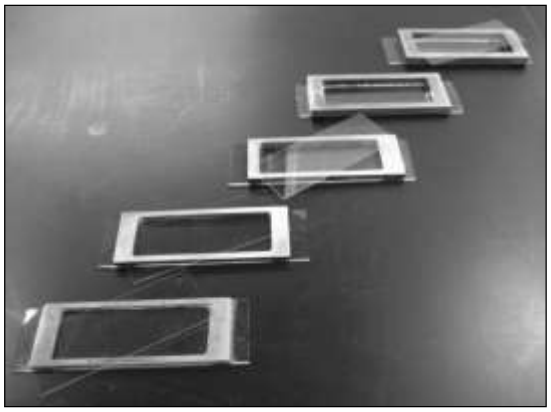
The recommended concentration procedure involves collecting all the particulates gravitationally settled in funnel/cone into several 50-mL centrifuge tubes (approximately 5-mL of concentrated sample with 40-mL of supernatant in each 50-mL centrifuge tube), and then pelleting the particulates through centrifugation. After settlement, the concentrated sample can be selectively removed using siphons/syringes. If centrifugation is used after gravitational settlement, target the pelleted particulates located in the bottom of the centrifuge tube since bivalve larvae are relatively dense. It is recommended that the supernatant in the centrifuge tube not be removed in order to maintain appropriate ethanol concentrations for sample preservation.

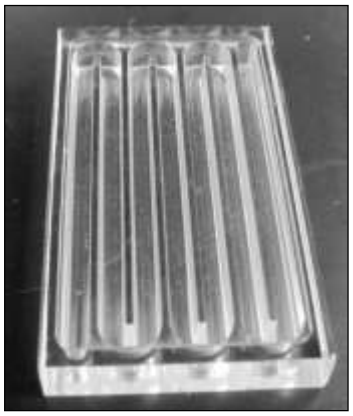
Microscopic Evaluation

Counting Chambers

There are a variety of counting chambers used for light microscopy analysis of plankton samples for early detection of planktotrophic bivalve larvae. Counting chambers include, but are not limited to, Sedgewick-Rafter cells (Figure 7a), zooplankton counting chambers (e.g., Bogorov-Trough

style chamber) (Figure 7b), and petri dishes (Figure 7c). The counting chamber used is largely a function of the type of microscope, aliquot volume, and analyst preference. Sedgewick-Rafter cells are recommended because they are relatively inexpensive ($70/chamber), they hold a known volume of sample, and the chamber is closed with a cover slip, thus eliminating sample movement via convection. Sedgewick-Rafter cells can be used with both compound light microscopes and stereo microscopes. Most commercially available Sedgewick-Rafter cells are 1-mL volume and will work with objective lens up to 20X, i.e., 200X total magnification, on a compound microscope. Sedgewick-Rafter cells can be custom made to hold greater volumes, and thus increase the volume of sample analyzed per slide. Two- or 3-mL volume Sedgewick-Rafter cells are recommended because they maximize efficiency (i.e., samples are typically diluted and settle on chamber bottom, meaning there is a limit to how much sample can be distributed within a given surface area) while allowing use of 10X and 20X objectives on a compound microscope (i.e., microscope stage working distance limits the use of thicker counting chambers with certain objective lens). Zooplankton counting chambers can be used with either compound or stereo microscopes, but these are expensive (e.g., $300/chamber), are typically open chambers, and the edge between the vertical walls and chamber bottom is often curved, which obscures viewing this portion of chamber. Petri dishes that can be used with stereo microscopes, hold a greater sample volume, and allow direct manipulation of the sample (e.g., dissecting probe), but these are always open chambers making specimen identification and photomicroscopy difficult. Additionally, it is difficult to manipulate petri dishes using mechanical stage, meaning the counting chamber is manually moved across microscope stage.

a) 

b) 

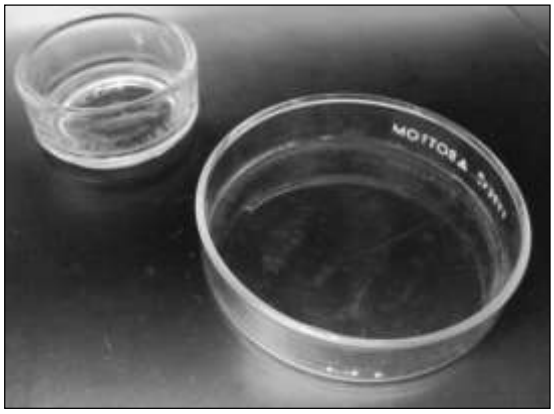
c) 

Figure 7: Examples of counting chambers used for plankton analysis including a) Sedgewick-Rafter cells in 1-mL to 5-mL capacities, b) zooplankton or Bogorov-Trough style chamber, and c) petri dishes.

Microscope

Compound light microscopes are the recommended type of microscope for routine cross-polarized light microscopy for the early detection of planktotrophic veligers. The recommended microscope set-up includes a binocular compound light microscope with 10X lens pieces and 4X, 10X, and 20X plan achromatic objectives with a polarization kit; mechanical stage with Y- and X-axis travel knobs; substage condenser; and a trinocular-mounted digital microscope color camera equipped with digital micrometer.

Stereo microscopes have lower magnification and resolution capacities (e.g., 4X-120X, and up to a numerical aperture of 0.2) compared to compound light microscopes (e.g., 40X-200X, and up to a numerical aperture of 0.4), which affects detection, identification, and photomicroscopy of larvae. Suspect specimens detected using stereo microscopes are often transferred to either compound light or scanning electron microscopes for identification. Scanning electron microscopes are expensive and time-consuming. Different types of microscopes may necessitate the use of different counting chambers, and specimen transfer is time consuming and involves risk of specimen loss.

Subsampling

Subsampling reduces the costs of plankton analysis while allowing a large number of samples to be scanned for the presence/non-detect of bivalve larvae. Analyzing the entire concentrated sample increases the likelihood that even very sparse veliger densities in the sample are detected, but is

often time prohibitive. Most laboratories microscopically scan only a portion of the original plankton sample during routine analysis (Denise Hosler, personal communication, 2009; EBMUD 2009; Stalvey 2009; Steve Wells, personal communication, 2007). Veligers are relatively dense compared to other objects found in plankton samples and subsample aliquots are typically taken from the bottom of the sample container, centrifuge tube, or other container after the particulate has been settled and separated from supernatant.

Subsampling however, can result in false negative results (i.e., larvae present in sample container but not subsampled) because larvae are discrete, small organisms that can be scarce. Increasing the amount of the particulate, i.e., concentrated sample that is analyzed is the best way to decrease the likelihood of false negative results associated with sample analysis. The concentrated sample volume consists of the pelleted particulate found in the bottom of the sample container/centrifuge tube. Subsampling from the bottom of the concentrated sample also reduces the risk of missing a larva due to subsampling. It is important to report the volume of the concentrated sample that was analyzed with sample results.

The volume of the sample that is microscopically analyzed, or subsampled, varies between monitoring programs depending on budget, program objectives, and the matrix density of the plankton sample. It is recommended that at minimum, 20% of the concentrated sample is analyzed. More than 20% of the concentrated sample may be analyzed in samples with suspect specimens that cannot be positively identified and high-risk water bodies of particular interest.

Aliquot Dilution

Aliquots taken from concentrated sample will typically require some dilution within the counting chamber to reduce confounding effects caused by dense matrices that interfere with veliger detection and identification. The ideal aliquot dilution maximizes the volume of sample being analyzed while maintaining the ability to visually inspect all aspects of the objects in the plankton. The recommended aliquot dilution within a Sedgewick-Rafter cell is one that results in most objects being arranged in a single layer on the bottom of counting chamber (Plate 6 and Plate 7). In general, regular ethanol and/or distilled water are added into the counting chamber concurrently with the sample aliquot to achieve the desired matrix density. Covered counting chambers may be shaken to more evenly distribute particulates within the chamber. It is recommended to allow covered counting chambers to sit undisturbed on level surface for several minutes prior to analysis to allow objects to settle to the bottom of the chamber, thus reducing the three-dimensional character of the sample.

Regular ethanol (95-100%) and distilled water are the preferred liquids for dilution of sample aliquots. Ethanol is preferred to water because it reduces surface tension and will not interfere with DNA amplification should molecular tests be warranted in the future. It also maintains an appropriate concentration of preservative for sample archival storage post analysis. Reducing the surface tension of the medium increases the likelihood of debris spreading out within the counting chamber. Ethanol, however, is more expensive than distilled water. Tap water should be avoided because it may contain traces amounts of chlorine and this may deleteriously impact DNA.

Scanning Mode

A magnification of 40X is typically used for scanning plankton samples for bivalve larvae. The detection of suspect specimens is the objective of the scanning mode, and the initial scan of the counting chamber is done under lower total magnifications to capture more of the sample within the field of view and to reduce analytical time. Some morphological features can be obscured at lower magnifications. Higher magnification and greater resolution are used for inspecting and identifying suspect specimens found during scanning mode.

Cross-polarized light microscopy is employed in scanning mode to increase the detection of birefringent objects within an interfering plankton matrix. The presence of birefringence and the characteristic "maltese cross" increases the likelihood of initially detecting veligers (Johnson 1995). Polarization is removed or greatly reduced to increase the background light needed to observe the morphologic features used for identification (Figure 8).

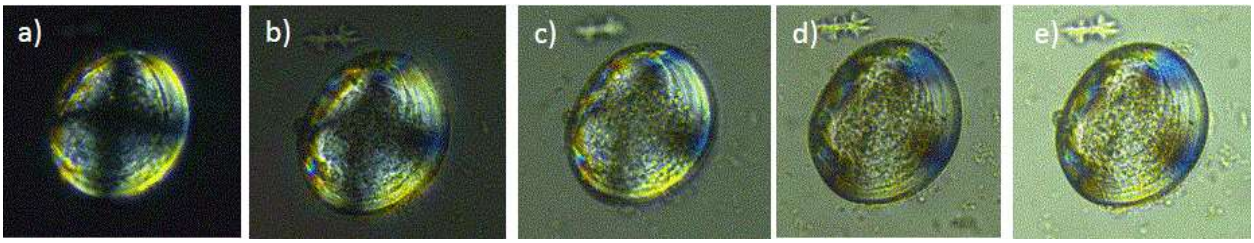


Figure 8: *Dreissena rostriformis bugensis* straight-hinge veliger under different degrees of cross-polarized light, decreasing in degree of cross-polarized light from a) full cross-polarizationa) to e) mostly background light.

Sample Storage

Sample preparation and analysis are non-destructive, and the analyzed and un-analyzed portions of the plankton sample should be retained and archived after light microscopy analysis for a specified time period, e.g., two years. Non-destructive analysis allows for the supernatant and debris removed in sample preparation procedures, as well as the concentrated sample to be re-analyzed at later dates should there be interest. The archival period is determined by the needs of the monitoring program and available storage space. Samples are typically stored in the original sample container immediately after analysis. The analyzed sample should be returned to the sample container, which should also contain the supernatant and any debris removed during sample preparation, such as the debris on the 500-µm filter (if used). Rinse the counting chamber with alcohol and/or distilled water when returning the analyzed aliquot to the sample container to ensure that most organisms are retained. The total sample volume will increase after analysis due to the additional liquid used for aliquot dilution as well as the rinsate used on the counting chambers, so it may be necessary to use additional sample containers to handle the overflow. Alternatively, a portion of the supernatant can be decanted and disposed of. Plankton samples should be stored in solutions of at least 70% regular ethanol. The holding temperatures used for sample storage vary by monitoring program. If space permits, storage at 4^{o}C is preferred, however, storage at room temperature is adequate for at least up to two years.

Inspecting and Identifying Suspect Specimens

There are a variety of steps taken to determine if a suspect specimen is a freshwater bivalve larvae that undergoes larval development in the water column. When the objective of the plankton

analysis is early detection of *Dreissena* sp. larvae, the efforts to identify the suspect specimen stop once it is determined that the specimen is not a planktotrophic bivalve larvae. Suspect specimens that are identified as something other than planktotrophic larvae should be counted and identified to the lowest taxonomic level possible, e.g., *Corbicula* sp., unionid larvae, ostracod, etc.

Change the Lighting Conditions

Changing the lighting of the sample on the microscope is an initial simple and quick step in suspect specimen identification. Appearance of the maltese cross (Plate 2) and birefringence (Plate 3 and Plate Plate 4) under polarized light are not used for identification purposes, but rather serve as a searching tool. Once the birefringent object has been detected, the polarization should be removed or reduced to allow more background light into the field of view, thus allowing greater visibility of morphological features used in identification (Plate 1). Modern compound light microscopes have condensers that can concentrate the light illuminating the specimen, thus changing the optical characteristics. The condenser can be adjusted to help elucidate particular features such as segmented appendages within the carapace of an ostracod (Plate 28, Plate 29, and Plate 30).

Increase Total Magnification

Increasing total magnification and resolving power are critical steps to inspecting and identifying suspect specimens. Increased magnification and resolving power improves the ability to discern key morphological features that, in many cases, are not visible under the 40X scanning level magnification. The ideal magnification and resolution for inspecting suspect specimens using a Sedgewick-Rafter counting chamber under the compound light microscope are provided by using a 20X objective lens, e.g., 200X total magnification, with a numerical aperture of 0.40. The ability to increase the total magnification using stereo microscopes is generally limited to 80X to 120X, and by the resolving power; therefore, suspect specimens detected using a stereo microscope should be transferred to a different microscope for further inspection. If available, a scanning electron microscope can be used for elucidating fine surface features that are sometimes not visible using compound light microscopes, e.g., hinge structures, surface texture differences between the prodissoconch I and II; however, specimen preparation for scanning electron microscopes is especially time consuming.

Reorienting the Specimen

Moving the suspect specimen within the three-dimensional matrix can be an important step when inspecting suspect specimens. Specimens can be in a position that affects the apparent overall shape or obscures key identification features such as the hinge area (Plate 12). Changing the three-dimensional orientation of a specimen can also reveal features that eliminate the possibility of it being a planktotrophic bivalve larva, e.g., segmented appendages, or the lack of relatively symmetrical halves. When a suspect specimen needs to be moved within the counting chamber, it is recommended that the rest of the counting chamber is analyzed before attempting to move the suspect specimen because changing the orientation of a suspect specimen can change the distribution of objects within the counting chamber and introduce air bubbles into a closed chamber. Most compound microscope mechanical stages have x-y numerical markings that allow the counting chamber position to be re-located.

Size Measurements

Size measurements are important to differentiate planktotrophic bivalve larvae from other types of bivalves such as *Corbicula* sp. and unionids. Straight-hinge juveniles of *Corbicula* sp. are commonly encountered in freshwater North American plankton samples. When straight-hinge juveniles of *Corbicula* sp. are released from adults they have an overall shape (D-shape) that is similar to the straight-hinge larval stage of planktotrophic bivalve larvae. *Corbicula* sp. straight-hinge juveniles, however, are approximately 200-µm in shell length, whereas *Dreissena* sp. straight-hinge (D-stage) larvae are approximately 100-µm in shell length. Unionid larvae that have a similar overall shape as the straight-hinge larval stage for planktotrophic bivalves can be differentiated using shell measurements such as the ratio of shell height to shell length and hinge length. The prodissoconch I length is another important shell measurement used for bivalve larvae identification.

Size measurements under the microscope can be made using ocular micrometers, digital micrometers, and measuring the field of view. Ocular micrometers are reticules located in one eyepiece that use a conversion factor for different objective lens. Digital micrometers are available on microscopes fitted with digital cameras and associated computer software. Some digital micrometers require that the objective lens or magnification be specified, whereas other systems automatically calculate the total magnification and resulting scale. Digital micrometers are calibrated by measuring a stage micrometer. The dimensions of the field of view can be measured and used to estimate the size of a particular object within the field of view. Digital micrometers are recommended because they are accurate, precise, easy, and can document size measurements on photomicrographs.

Photomicroscopy

Photomicroscopy capability is critical for light microscopy laboratories that conduct plankton analysis for early detection of *Dreissena* sp. Digital microscope color cameras allow for rapid, accurate and precise photomicroscopy to document suspect specimens and allow for rapid sharing with other experts to confirm identification. Photomicrographs, however, must provide the information necessary to identify suspect specimens. A scale bar or some other means of measuring size, such as calibration grids, must be included on each photomicrograph. Use the highest magnification and resolving power possible and remove or reduce the degree of polarization so that morphological features are clearly visible. Several photomicrographs of each suspect specimen should be provided. Be sure to capture the lateral view and focus on the following areas: hinge and shoulder areas, growth line region near ventral margin, shell surface, and internal organs. An example of acceptable photomicrography is provided in Appendix B. A form documenting procedures and standardizing external cross-validation of photomicrographs is included in Appendix C.

Quality Assurance/Quality Control

Check standards (laboratory control samples) and blind matrix spike samples are used to evaluate quality objectives and to assess bias errors associated with veliger detection via light microscopy. Precision or random error, cannot be evaluated with presence/non-detect data.

Bias error associated with veliger detection using light microscopy analysis includes false positive and false negative results. The sources of false positive and false negative results are identified in Table 1 and Table 2 along with corrective actions and quality control measures. False positive results are caused by misidentification and sample contamination. Misidentification is addressed using appropriate equipment, laboratory control samples and other identification tools (e.g., veliger image database), using multiple experts to confirm identification, increasing the number of subsamples examined to locate additional specimens, and using molecular methods on sample splits. Contamination is addressed by laboratory decontamination procedures. False negative results are caused by analyst error, matrix effects, and low abundances of target specimens. Analyst error is addressed by using check standards (laboratory control samples) and blind matrix spike samples. Matrix effects are addressed by increasing aliquot dilution. Low abundance of veligers is addressed by sample handling procedures in laboratory, sample concentration and increasing subsample volume.

Precision, or random errors, cannot be evaluated with presence/non-detect data and as such are not evaluated for veliger detection for early detection monitoring laboratories.

Table 1: Sources of false positive errors, i.e., absent but detected, with veliger identification using light microscopy, and corrective actions and quality control measures.

	Problem	Corrective Action/ QC Measures
Methods	misidentification	• equipment (increase magnification and resolving power) • training (lab control samples, ID tools) • identification confirmed by lab manager • photomicrograph confirmed by independent experts • molecular analyses on split
	contamination	• field and lab decontamination
Process	other planktotrophic bivalve larvae	• increase subsampling (multiple specimens) • training (lab control samples, ID tools) • identification confirmed by lab manager • photomicrograph confirmed by independent experts • molecular analyses on split
	unusual/poor/limited # of specimens	• increase subsampling (multiple specimens) • identification confirmed by lab manager • photomicrograph confirmed by independent experts

Table 2: Sources of false negative error, i.e., present but not detected, with veliger identification using light microscopy, and corrective actions and quality control measures.

	Problem	Action
Methods	analyst error	• blind matrix spiked samples
	matrix effects	• increase aliquot dilution
Process	unusual/ poor/ limited # of specimens	• preservation/handling (preservative, temperature, pH, turn-around time) • increase subsampling (concentrated sample volume)
	low abundance, clumped spatial distribution	• sample concentration • increase subsampling (concentrated sample volume)

Check Standards (Laboratory Control Samples)

A voucher specimen collection of morphologically similar species can be used for training and as a reference to obtain the most accurate and consistent species identifications possible. Voucher collections should include, at a minimum, bivalve larvae in the genus *Dreissena* (*Dreissena polymorpha*, and *D. rostriformis bugensis*) and members of the Corbiculidae family (*Corbicula* sp.) at various stages of development as well as collections of ostracods. Additional voucher specimens may also include other members of the family Dreissenidae such as *Mytilopsis leucophaeata*, and members of the family Unionidae (e.g., *Gonidea* sp., *Anodonta* sp., *Cyrtonaias* sp., *Lampsilis* sp., *Potamilus* sp., *Toxolasma* sp.).

Internal Cross-Validation

Suspect specimens should be inspected under the microscope by the laboratory manager prior to sending photomicrographs to outside experts for further confirmation. It is easier to identify specimens under a microscope compared to photomicrographs because microscopic examination allows changing the fine focus, lighting, and specimen orientation. It is recommended that suspect specimens are documented with photomicrographs, isolated from the rest of the sample, and retained in solutions of 70% ethanol.

External Cross-Validation

Photomicrographs should be sent to independent experts to corroborate identification made by laboratory staff and the manager. An example of photomicrographs is shown in Appendix B. A form documenting procedures and standardizing external cross-validation of photomicrographs is included in Appendix C. Isolated suspect specimens may also be sent to independent microscopy experts to corroborate identification. Photomicrographs should accompany specimens, when possible.

NOTE: Shipping isolated suspect specimens to another laboratory for confirming identification involves risk of specimen loss and/or degradation.

Sample splits may be sent to other microscopy or molecular laboratories for additional external cross-validation. The value of sample splits is the detection of additional specimens and/or genetic

material to corroborate morphology-based identification. Sample splits regarding discrete organisms, however, may not contain identical species compositions or sufficient viable genetic material, especially with a rare occurrence, i.e., non-detect ≠ absent.

CAUTION: Sample splits used for the detection of rare events regarding discrete organisms such as bivalve larvae are at risk for false negative results.

Laboratory Decontamination

Laboratory equipment and surfaces must be decontaminated using both acid and bleach solutions to prevent the transfer of larvae and genetic material between samples. Acid solutions are used to dissolve the shells of larvae, which are composed of calcium carbonate. A solution of 5% acetic acid, i.e., white vinegar, or 4% hydrochloric acid (HCl) can be used for a decontamination soak. The ideal soak time for acetic acid is 24 hours, and the minimum soak time is 8 hrs. The soak time for a solution of 4% HCl is 4 hours. Equipment such as counting chambers, 63-µm filter sleeves, 500-µm filters, beakers, pipettes, siphon tubing, glass petri dishes, Imhoff settling cones, and centrifuge tubes are thoroughly rinsed with fresh water, and soaked in acid solution to dissolve bivalve shells. Thoroughly rinsing equipment with fresh water prior to decontamination soaks is effective at removing bivalve larvae and genetic material (Denise Hosler, personal communication, 2013), and rinsing equipment with distilled water after decontamination soaks is important for increasing equipment longetivity.

CAUTION: Soaking bivalve larvae in 5% acetic acid solution for 4 hours is not adequate to completely dissolve all bivalve larvae. Soaking bivalve larvae in 5% acetic acid for 4 hours will, at minimum, reduce the birefringence of the shell.

NOTE: Acid solutions such as 4% hydrochloric and 5% acetic acid can be corrosive, and proper precautions must be made to protect people and equipment such as metal and adhesives. Wear proper protective clothing and equipment when handling acid solutions, and follow laboratory safety procedures and appropriate MSDS. Thoroughly rinse all equipment with plenty of distilled water following acid bath. Regularly inspect equipment for failure.

Laboratory decontamination for genetic material in microscopy laboratories is necessary because multiple analytical methods may be used with the same plankton sample to confirm identification made via light microscopy. Contamination can cause false positive results with molecular techniques. Genetic material on laboratory equipment and surfaces is decontaminated using a solution of 10% household bleach (100-mL bleach in 1-L of water) (Prince and Andrus 1992). Equipment such as counting chambers, pipettes, filters, Imhoff settling cones, and centrifuge tubes are soaked in a solution of 10% household bleach for 5 minutes and then thoroughly rinsed with fresh water. Laboratory surfaces in contact with plankton samples (e.g., counters, plastic trays), should be sprayed with a 10% bleach solution and wiped with disposable towels. Solutions of 10% bleach are caustic and it is important to thoroughly rinse equipment with fresh water following decontamination and to wear appropriate safety equipment, e.g., glasses, gloves and lab coat.

Sample preparation and handling, including sample concentration, filtering and adding aliquots into counting chambers, should be done on plastic trays that have a rim. Plastic trays contain spills and are easy to clean.

Field decontamination for both genetic material and bivalve shells is required during plankton sample field collection to allow for multiple analytical methods. Field decontamination protocols are provided in protocols for the field collection of plankton samples, and are not addressed in this manual.

Identification Guide

Identification Key

This key is intended to help determine if suspect specimens are shelled planktotrophic bivalve larvae. It is intended for microscopy laboratories analyzing plankton for the early detection of *Dreissena* sp. veligers. This key was developed by the authors based on their experiences analyzing plankton; there are other sources for descriptions of freshwater mussel larvae, including Conn et al. (1993), Hopkins and Leach (1993), Korniushin and Glaubrecht (2003), Lefevre and Curtis (1912), Martel et al. (1995), Nichols and Black (1994), Pekkarinen and Englund (1995b), Pekkarinen and Valovirta 1996, Surber (1912), and Tucker (1927).

1a. Object displays birefringence. Birefringence ranges from maltese cross **Plate** (Plate 2) to areas of light throughout entire object (Plate 3) or restricted to a portion of the organism such as the peripheral margin (Plate 4 and Plate 6).....2

1b. Object does not display birefringence, appears as darkened object. (**Plate** Plate 5).....2

NOTE: Birefringence and the maltese cross are only search tools. Identification requires background light to see morphology. The degree of polarization influences amount of birefringence and background light (Plate 1). The degree of birefringence produced by veligers may also vary, and may be lacking. Initial search tools should combine birefringence, overall shape, and general size to identify objects for further inspection (Plate 6,**Plate** Plate 7, Plate 19 and Plate 20).

2a. The longest dimension across object is between 40- and 300-µm..... 3

2b. The longest dimension across object is either less than 40-µm or greater than 300-µm.....**STOP**

3a. Overall shape from lateral view is either D-shaped (**Plate** Plate 8), round to oval (Plate 7, Plate 9, Plate 10 and Plate 13), or roundish (Plate 11). The overall shape from the ventral, dorsal, anterior, or posterior view is oval to ovate (Plate 12, Plate 13 and Plate 14) or subtrapezoidal (Plate 15 and Plate 30) 4

3b. Overall shape from lateral view is quadrate, axe-head, rectangular, triangular to subtriangulate (Plate 16), cylindrical, or spiral.....**STOP**

NOTE: Bivalve larvae in counting chambers are most commonly oriented in the lateral view with the face of one valve relatively parallel the plane of counting chamber (Plate 8, Plate 11, Plate 16, Plate 54, Plate 55 and **Plate** Plate 56). In limited cases, however, bivalve larvae are oriented three-dimensionally in manner that may obscure hinge development and the overall shape, and may require reorientation (Plate 12).

4a. Margin is distinct, continuous, curved, and smooth-edged (Plate **Plate** 6, Plate 17, Plate 18, Plate 19 and Plate 20) 5

4b. Margin is fuzzy, angular, jagged, or undulate (Plate 17, Plate 18, Plate 21 and Plate 47) ..... **STOP**

CAUTION: Quality specimens are needed for identification. Bivalve morphology may not be clearly visible or features may appear unusual or different in odd, damaged or old specimens (Plate 22, Plate 23, **Plate** Plate 24, Plate 25 and Plate 26).

5a. Object possesses a distinct head (Plate 27) ..... **STOP**

5b. Object lacks a distinct head 6

6a. Segmented appendages, e.g., legs, are visible and clearly part of object. Segmented appendages are most visible when protruding beyond the margin. Segmented appendages may be curled against the body but still visible through carapace or from different viewpoint (Plate 28, Plate 29,Plate Plate 30) ..... **STOP**

6b. Segmented appendages are not visible 7

7a. Antennae, hair, or setae (bristle, stiff hair) are visibly attached to the outer surface of object. Antennae, hair, and setae are usually difficult to see and are often located near the margin, especially the ventral margin (Plate 28, Plate 29 and Plate 30) ..... **STOP**

7b. Antennae, hair, or setae are not visibly attached to the outer surface of the halves 8

CAUTION: Internal tissues of bivalve larvae may appear similar to segmented appendages curled against the body (Plate 32). Additionally, exogenous objects located inside an open or empty bivalve shell may appear similar to segmented appendages or setae (Plate 32).

8a. Entire margin of object is surrounded by a continuous, peripheral 'ring', appearing as a solid band; hollow band outlined with solid lines, or series of cells appearing like bricks stacked end-to-end (Plate 33 and Plate 34) ..... **STOP**

8b. Object lacks a continuous, peripheral 'ring' that surrounds the entire margin. Concentric or commarginal lines running along margin may or may not be visible but lines do not surround the entire margin. If lines are present, they converge near the shoulder area. The surface of planktotrophic bivalve larval prodissoconch I (PI) shell appears pitted or smooth, while the surface of the prodissoconch II (PII) shell is somewhat pitted but has commarginal or concentric growth lines (Plate 36 and Plate 37). Internal tissues may confound visibility of shell surface features (Plate 38). 9

CAUTION: Bivalve larvae may appear to have hollow or continuous bands along the peripheral shell margin. These bands, however, terminate in the hinge area near shoulders (Plate 35).

9a. Object has two halves that are relatively symmetrical in size and shape. The line dividing halves is continuous and smooth (Plate 39 and Plate 41) 10

9b. Object lacks two halves and dividing line, e.g., one valve 13

10a. Two halves are joined at one location along equatorial line, i.e., hinge. Hinge location is more apparent when two halves are open (Plate 40,Plate Plate 41). Most preserved bivalve larvae have partially closed shells, but specimens collected moribund or dead are often gaping (Plate 15). The margins of both halves converge and join at the shoulders, and the hinge is located between both shoulders, and is the area where two halves are continuously in contact (Plate 39, Plate 40, Plate 41, Plate 42 and Plate 43) 11

10b. Two halves are either joined in multiple locations, or fused throughout equatorial line; no hinge nor shoulders are present (Plate 44 and Plate 45) ..... **STOP**

11a. The overall shape is D-shaped (Plate 8, Plate 49 and Plate 50) 12

11b. The overall shape is not D-shaped 17

12a. Hinge is located along the straight section of the "D" (Plate 8 and Plate 42) 14

12b. Hinge is located along the curved section of the "D" (Plate 30 and Plate 31) ..... **STOP**

NOTE: Ostracods are commonly encountered that lack appendages and are similar in overall shape to the straight-hinge larval stage of *Dreissena* sp. from the lateral view (Plate 31). The shape of different ostracods in the lateral view is variable. Straight-hinged bivalve larvae can be separated from similar 'D' shaped ostracods by overall shape and hinge location. Straight-hinge bivalve larvae are distinctly 'D' shaped, with relatively acute shoulders, and are hinged on the straight section of the 'D' (Plate 8, Plate 42). Whereas, ostracods that are 'D' shaped are somewhat triangulate in overall shape, and the areas corresponding to bivalve shoulders are rounded. Additionally, these 'D' shaped ostracods are hinged on the curved section (Plate 30 and Plate 31).

13a. Overall shape from the ventral, dorsal, anterior, or posterior view is acetabuliform to cymbiform, resembling a shallow saucer or boat that is more flattened or shallow on one end (Plate 15). The shape from the lateral view, however, is D-shaped or round to roundish 14

13b. Object exhibits one continuous outer surface, not divided into halves, that is completely enclosed when viewed from multiple viewpoints and is spherical, or, overall shape from the dorsal or ventral view is orbicular, ovoid, fabiform, triangular, quadrate, rectangular, or spiral-shaped (Plate 44, Plate 45, Plate 46, Plate 47 and Plate 48) ..... **STOP**

NOTE: Specimen will have to be reoriented or moved within the sample matrix to obtain multiple viewpoints.

NOTE: Planktotrophic bivalve larvae internal tissues are generally 1) present throughout shell, 2) absent altogether (Plate 15, Plate 19 and Plate 20), or 3) tissues are concentrated in the dorsal region near hinge (Plate 55). When tissue is present throughout the shell, the tissue is generally distributed in equal amounts towards the anterior, posterior and ventral margins (Plate 42, Plate 43, Plate 51, Plate 54, **Plate** Plate 55, Plate 56, Plate 58 and Plate 59). In some cases, internal tissue protrudes beyond shell margin, e.g., velum. Bivalve tissue rarely appears as a concentrated mass, or nucleus, in the center of the shell (Plate 44, Plate 45 and Plate 47). In some cases, however, bivalve internal tissue may appear as a concentrated mass in the center of the shell (Plate 52 and Plate 60).

14a. The overall shape from the lateral view is 'D' shaped (Plate 8, Plate 49, Plate 50 and Plate 52) 15

14b. The overall shape from the lateral view is round, orbicular or rounded (Plate 9, Plate 10, Plate 11, Plate 54 and Plate 55) 17

15a. Shell length is greater than shell height (Plate 50) 16

15b. Shell length is less than shell height, and/or shell hinge length is greater than 71-µm (Plate 49) ..... **STOP**

16a. Shell length is greater than 160-µm. Growth lines are present but may or may not be visible under 100X to 200X total magnification. Prominent ridges interspaced within, and parallel with growth lines may be visible (Plate 18 and Plate 50). Radial striae or secondary lines running perpendicular to growth lines and shell margin may or may not be visible (Plate 18 and Plate 50). Hinge line may be straight or sway-backed (Plate 51). Internal tissue can be lacking, concentrated in dorsal end, and is often distributed throughout the entire shell valve except the outer peripheral edge within growth line region (Plate 51 and Plate 52). The 'D' shaped bivalve may appear stretched so that the anterior and posterior ends are not symmetrical (Plate 52). Foot is present, and may or may not be visible; in preserved specimens, the foot is often withdrawn within shell (Plate 53). Velum is absent (Plate 58) .... ***Corbicula* sp. (straight-hinge juvenile)**

Shell measurements on *Corbicula* sp. collected from Columbia River in Oregon and Washington): shell length is 194.1 ± 6.521-µm (mean ± SD) (median=193.8-µm, min=171.0-µm, max=216.4-µm, n=240); shell height is 159.8 ± 6.744-µm (mean ± SD) (median=159.2-µm, min=141.0-µm, max=183.5-µm, n=240); and hinge length is 107.5 ± 11.10-µm (mean ± SD) (median=109.7-µm, min=37.99-µm, max=132.6-µm, n=240).

16b. Shell length is less than 160-µm ..... **planktotrophic bivalve larvae (straight-hinge larvae)**

NOTE: It is difficult to positively identify planktotrophic bivalve larvae to species level using only morphology visible on preserved specimens via light microscopy. *Dreissena polymorpha* and *D. rostriformis bugensis* are the only known freshwater bivalves in North America that produce planktotrophic larvae. Many bivalves found in brackish and marine North American waters produce planktotrophic larvae. In limited cases, adult brackish water mussels have been found in freshwater areas,

e.g., *Mytilopsis leucophaeata*. Additionally, *Limnoperna fortunei* is another freshwater mussel that produces planktotrophic larvae; *L. fortunei*, however, is not known to occur in North America. Freshwater mussels that produce planktotrophic larvae are epifaunal and adults develop byssal threads and attach to hard surfaces causing macrofouling. Therefore, the presence of planktotrophic larvae in a freshwater water body is a cause for serious concern, regardless of the species.

Shell measurements on *Dreissena polymorpha* straight-hinge larvae collected from San Justo Reservoir in California: shell length is 96.02 ± 13.1-µm (mean ± SD) (median=92.08-µm, min=73.06-µm, max=149.5-µm, n=192); shell height is 80.54 ± 12.09-µm (mean ± SD) (median=76.62-µm, min=58.3-µm, max=134.1-µm, n=192); hinge length is 51.81 ± 4.225-µm (mean ± SD) (median=51.95-µm, min=36.32-µm, max=60.82-µm, n=189).

Shell measurements on *Dreissena polymorpha* straight-hinge larvae collected from El Dorado Reservoir in Kansas: shell length is 100.2 ± 10.61-µm (mean ± SD) (median=99.27-µm, min=84-µm, max=128.8-µm, n=58); shell height is 82.43 ± 10.79-µm (mean ± SD) (median=82.43-µm, min=63.5-µm, max=107.8-µm, n=58); hinge length is 57.37 ± 3.065-µm (mean ± SD) (median=57.37-µm, min=51.52-µm, max=64.56-µm, n=58).

Shell measurements on *Dreissena rostriformis bugensis* straight-hinge larvae collected from Lake Mead in Nevada and California: shell length is 97.73 ± 8.756-µm (mean ± SD) (median=96.29-µm, min=81.95-µm, max=130.1-µm, n=212); shell height is 81.90 ± 8.875-µm (mean ± SD) (median=80.25-µm, min=65.55-µm, max=117.1-µm, n=212); hinge length is 53.44 ± 3.186-µm (mean ± SD) (median=53.35-µm, min=44.2-µm, max=67.25-µm, n=212).

Shell measurements on *Mytilopsis leucophaeata* straight-hinge larvae collected from Boomtown Lake near Vidor Texas: shell length is 83.04 ± 7.350-µm (mean ± SD) (median=81.86-µm, min=71.88-µm, max=135.4-µm, n=172); shell height is 70.16 ± 7.933-µm (mean ± SD) (median=68.90-µm, min=58.44-µm, max=126.2-µm, n=172); hinge length is 44.77 ± 2.640-µm (mean ± SD) (median=44.91-µm, min=37.93-µm, max=52.73-µm, n=171).

17a. Overall shape from lateral view is round, orbicular to oval. Umbo is developing, but is low and rounded and does not protrude beyond shell line. The anterior and posterior ends are relatively symmetrical with respect to each other; asymmetrical ends are not markedly asymmetrical. Growth lines may or may not be visible (Plate 54 Plate Plate 57) 18

17b. Overall shape of shell from lateral view is rounded and the anterior and posterior ends of shell are markedly asymmetrical. Shell length is less than 360 µm. The umbo is developing and may appear low and rounded (Plate 55) or, umbo may be knobby and protrude beyond shell margin (Plate 56) ..... **planktotrophic bivalve larvae (umbonal or pediveliger larvae)**

NOTE: The functional difference between the umbonal and pediveliger larval stages is the development of the foot, but this can be difficult to see on preserved specimens (Plate 53). The velum can be present in both straight-hinged, umbonal and pediveliger stages (Plate 58, Plate 59 and Plate 60).

18a. Shell length is greater than 220-µm. Prominent growth lines and ridges are visible (Plate 18, Plate 50 and Plate 57). ..... ***Corbicula* sp. (juvenile clam)**

18b. Shell length is less than 220-µm. Growth lines may or may not be visible on shell (Plate 54)..... **planktotrophic bivalve larvae (umbonal larvae)**

Shell measurements on *Dreissena polymorpha* umbonal larvae collected from San Justo Reservoir in California: shell length is 144.1 ± 19.97-µm (mean ± SD) (median=142.1-µm, min=88.14-µm, max=206.6-µm, n=128); shell height is 127.2 ± 19.33-µm (mean ± SD) (median=129.5-µm, min=69.98-µm, max=185.2-µm, n=128); umbo length is 69.04 ± 10.96-µm (mean ± SD) (median=68.43-µm, min=36.04-µm, max=99.60-µm, n=104).

Shell measurements on *Dreissena polymorpha* umbonal larvae collected from El Dorado Reservoir in Kansas: shell length is 169.4 ± 17.92-µm (mean ± SD) (median=166.4-µm, min=121.3-µm, max=206.8-µm, n=86); shell height is 151.1 ± 16.64-µm (mean ± SD) (median=151.0-µm, min=102.0-µm, max=191.1-µm, n=86); umbo length is 66.95 ± 13.31-µm (mean ± SD) (median=70.56-µm, min=29.48-µm, max=87.80-µm, n=61).

Shell measurements on *Dreissena rostriformis bugensis* umbonal larvae collected from Lake Mead in Nevada and California: shell length is 139.4 ± 18.57-µm (mean ± SD) (median=39.4-µm, min=66.26-µm, max=188.3-µm, n=113); shell height is 126.6 ± 17.27-µm (mean ± SD) (median=127.78-µm, min=85.31-µm, max=167.6-µm, n=113); umbo length is 63.66 ± 11.81-µm (mean ± SD) (median=64.97-µm, min=35.17-µm, max=89.64-µm, n=62).

Shell measurements on *Mytilopsis leucophaeata* umbonal larvae collected from Boomtown Lake near Vidor Texas: shell length is 126.8 ± 24.96-µm (mean ± SD) (median=127.6-µm, min=93.81-µm, max=174.6-µm, n=29); shell height is 116.1 ± 24.56-µm (mean ± SD) (median=116.1-µm, min=81.67-µm, max=161.9-µm, n=29); umbo length is 29.73 ± 8.928-µm (mean ± SD) (median=32.90-µm, min=19.65-µm, max=36.64-µm, n=3).

Shell measurements on *Dreissena polymorpha* pediveliger larvae collected from San Justo Reservoir in California: shell length is 188.2 ± 16.25-µm (mean ± SD) (median=186.8-µm, min=158.1-µm, max=228.3-µm, n=57); shell height is 169.9 ± 16.86-µm (mean ± SD) (median=166.2-µm, min=144.1-µm, max=209.6-µm, n=57); umbo length is 84.39 ± 8.599-µm (mean ± SD) (median=83.52-µm, min=64.34-µm, max=108.8-µm, n=57).

Shell measurements on *Dreissena polymorpha* pediveliger larvae collected from El Dorado Reservoir in Kansas: shell length is 210.1 ± 14.93-µm (mean ± SD) (median=209.4-µm, min=178.6-µm, max=257.2-µm, n=84); shell height is 194.2 ± 16.68-µm (mean ± SD) (median=193.8-µm, min=160.1-µm, max=254.3-µm, n=84); umbo length is 83.46 ± 9.303-µm (mean ± SD) (median=83.83-µm, min=53.15-µm, max=107.9-µm, n=79).

Shell measurements on *Dreissena rostriformis bugensis* pediveliger larvae collected from Lake Mead in Nevada and California: shell length is 180.5 ± 25.36-µm (mean ± SD) (median=181.0-µm, min=117.8-µm, max=228.1-µm, n=61); shell height is 168.4 ± 26.25-µm (mean ± SD) (median=172.7-µm, min=102.7-µm, max=235.2-µm, n=61); umbo length is 78.41 ± 10.48-µm (mean ± SD) (median=80.06-µm, min=40.67-µm, max=97.99-µm, n=58).

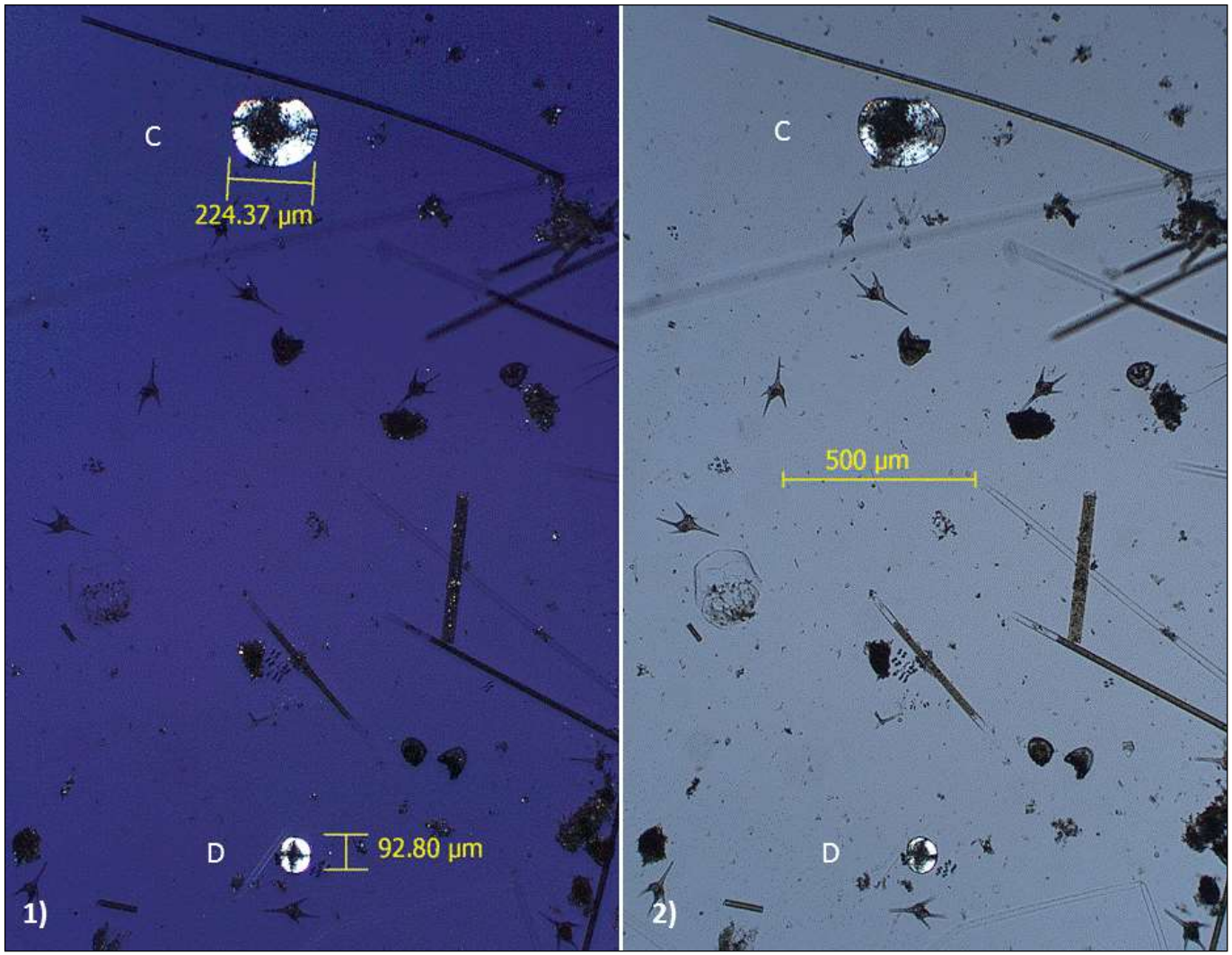


Plate 1: The degree of polarization influences the amount of background light and birefringence. Both *Dreissena* sp. (D) larvae and *Corbicula* sp. (C) straight-hinge juveniles are shown with other non-birefringent planktonic objects under 1) higher degree of polarization versus 2) lower degree of polarization.

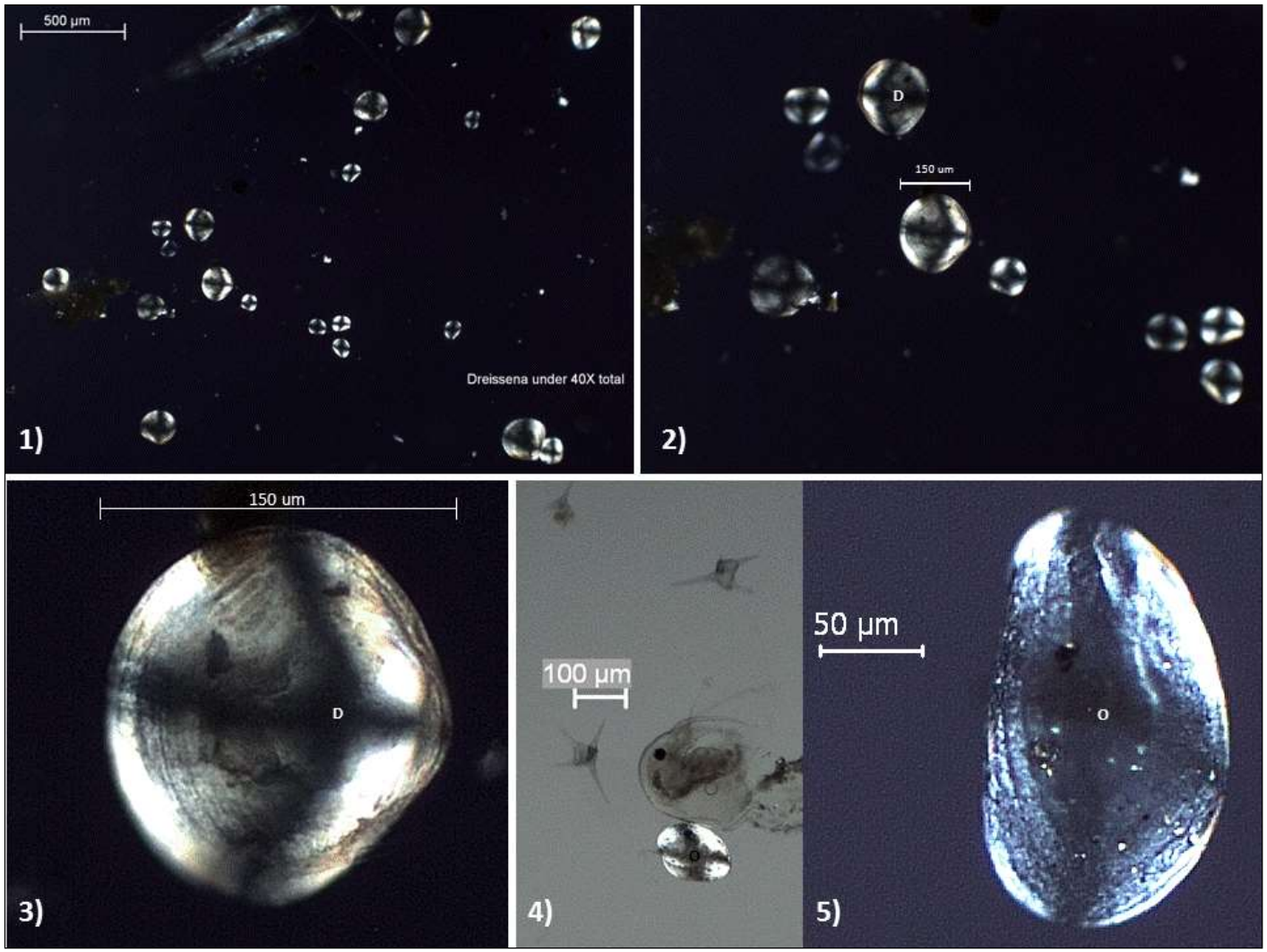


Plate 2: Birefringent objects showing maltese cross, including *Dreissena* sp. (D) under cross-polarized light at 1) 40X total magnification; 2) 100X total magnification; 3) 100X magnification with digital zoom; and ostracods (O) under cross polarized light at 4) 40X total magnification, and 5) 200X magnification.

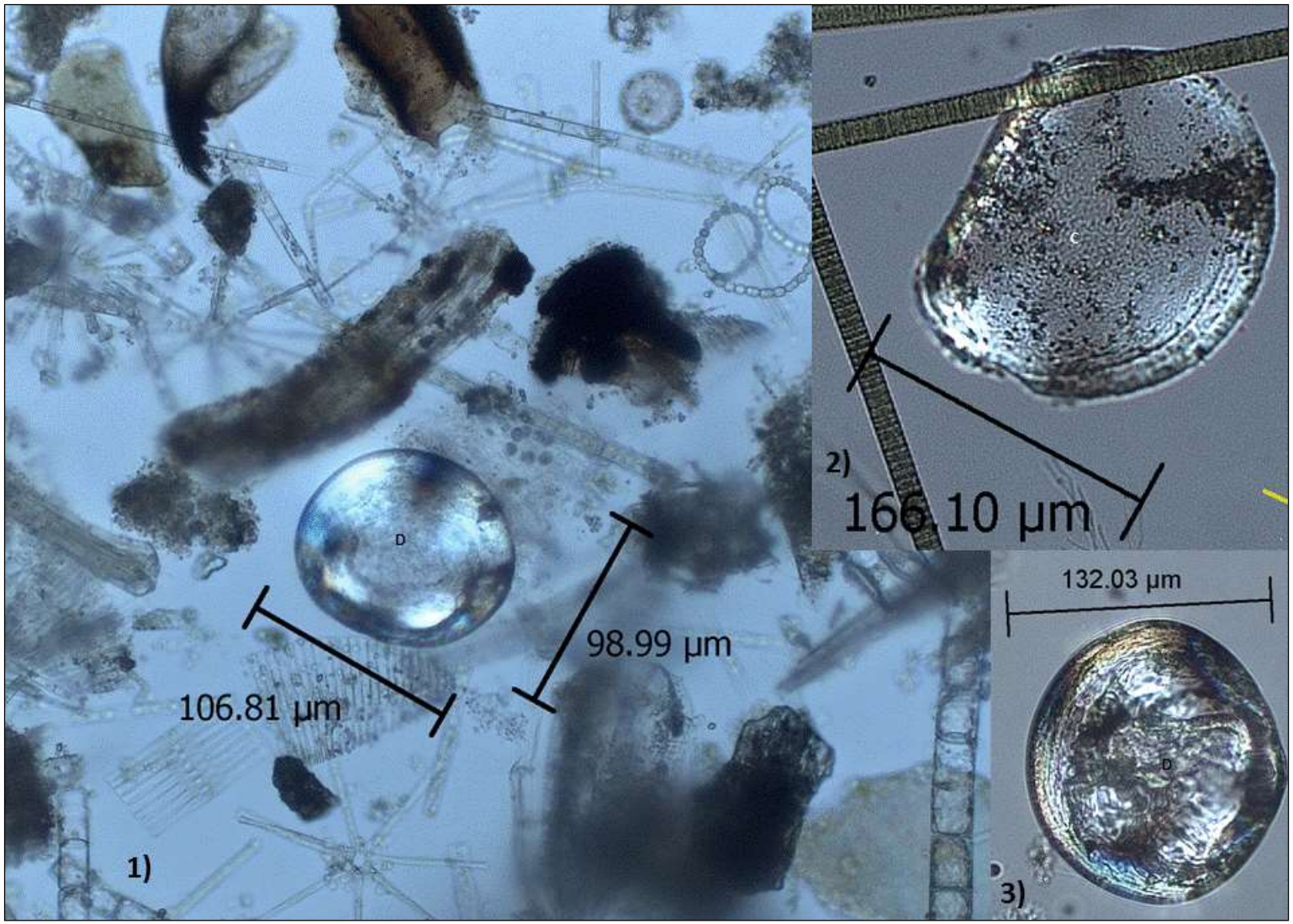


Plate 3: Birefringence seen as light throughout the shells of *Dreissena* sp. (D) larvae (1, 3), and *Corbicula* sp. (C) straight-hinge juveniles (2).

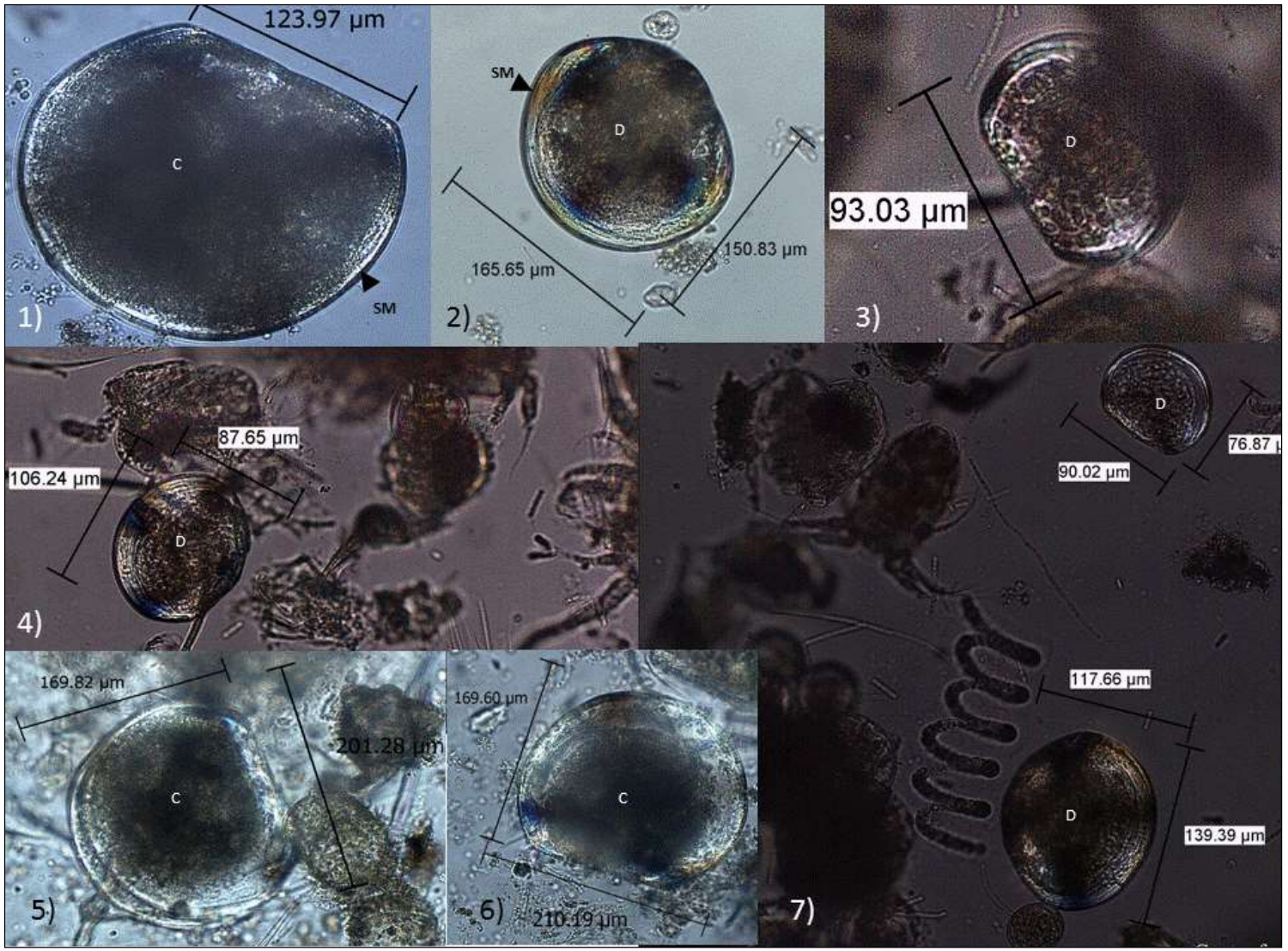


Plate 4: Birefringence concentrated in shell margin (SM) of *Corbicula* sp. (C) straight-hinge juveniles (1, 5, 6), and *Dreissena* sp. (D) larvae in the straight-hinge stage (3, 7) and umbonal stage (2, 4, 7).

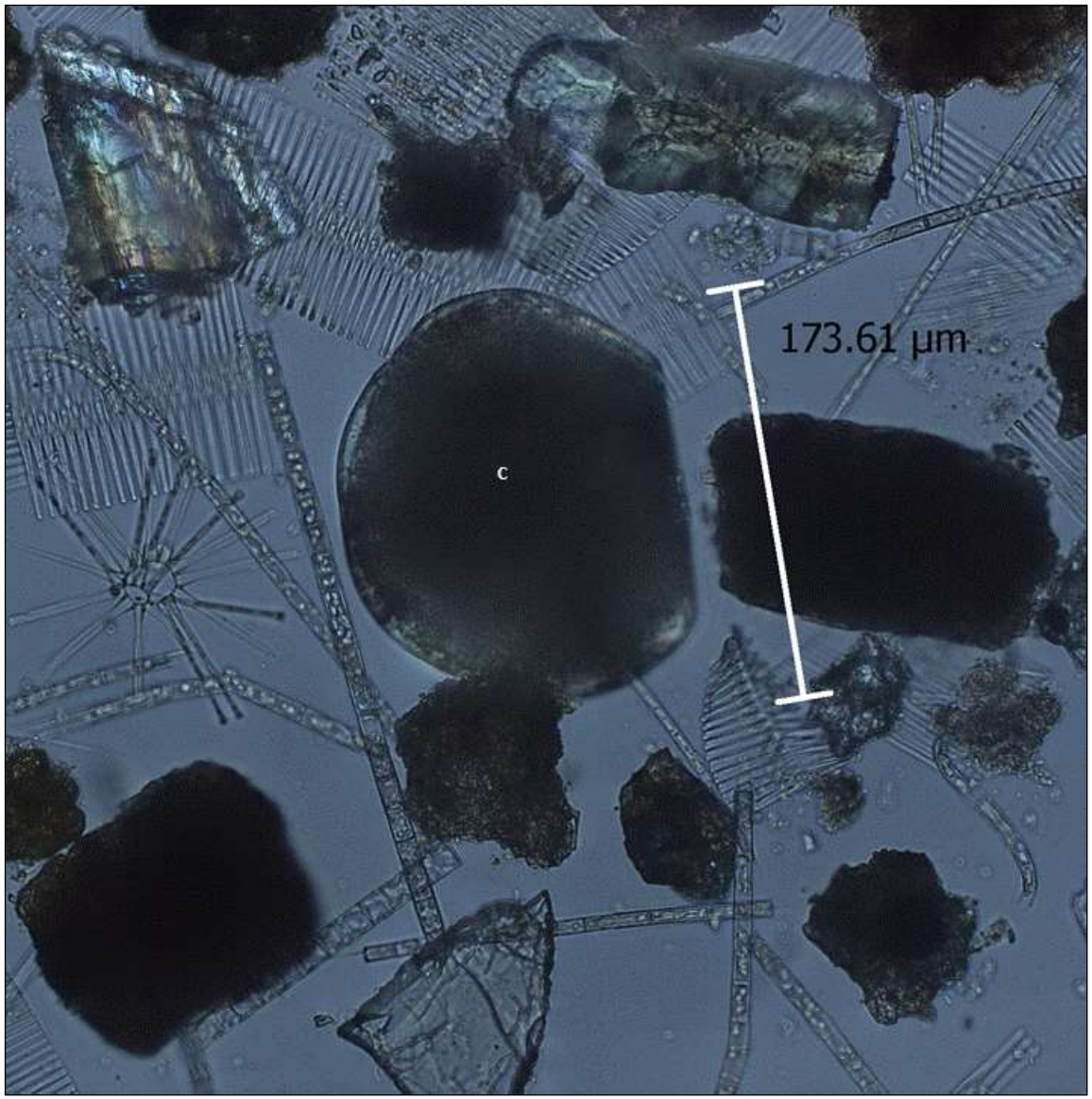


Plate 5: *Corbicula* sp. (C) straight-hinge juvenile that lacks birefringence under polarized light.

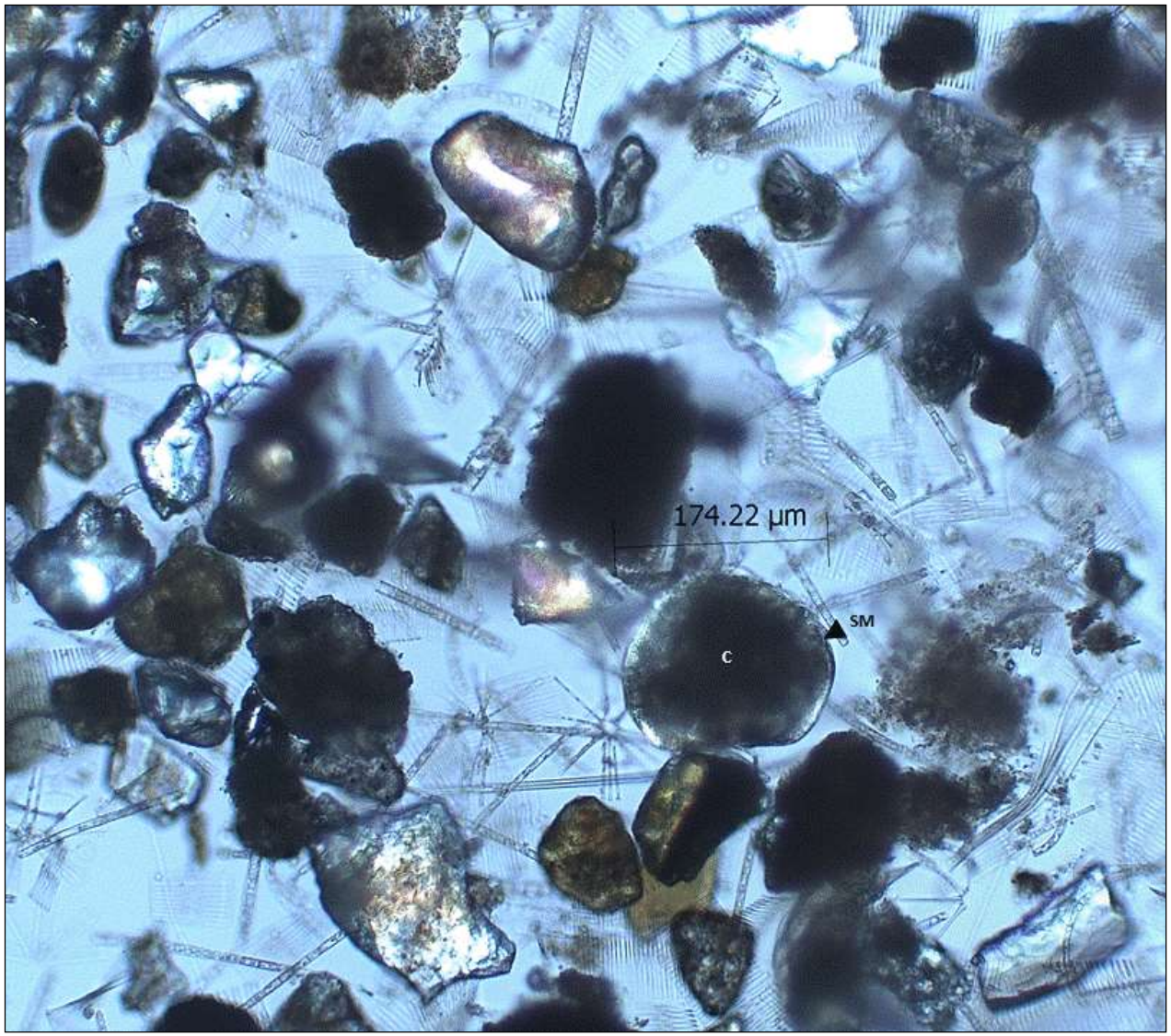


Plate 6: *Corbicula* sp. (C) straight-hinge juvenile that can be detected in the matrix of birefringent inorganic debris by the combination of birefringence located in shell margin (SM) and the overall 'D' shape and size, i.e., 40 – 300 µm. Matrix of organic and inorganic particulates is evenly distributed throughout field of view so that each object can be inspected.

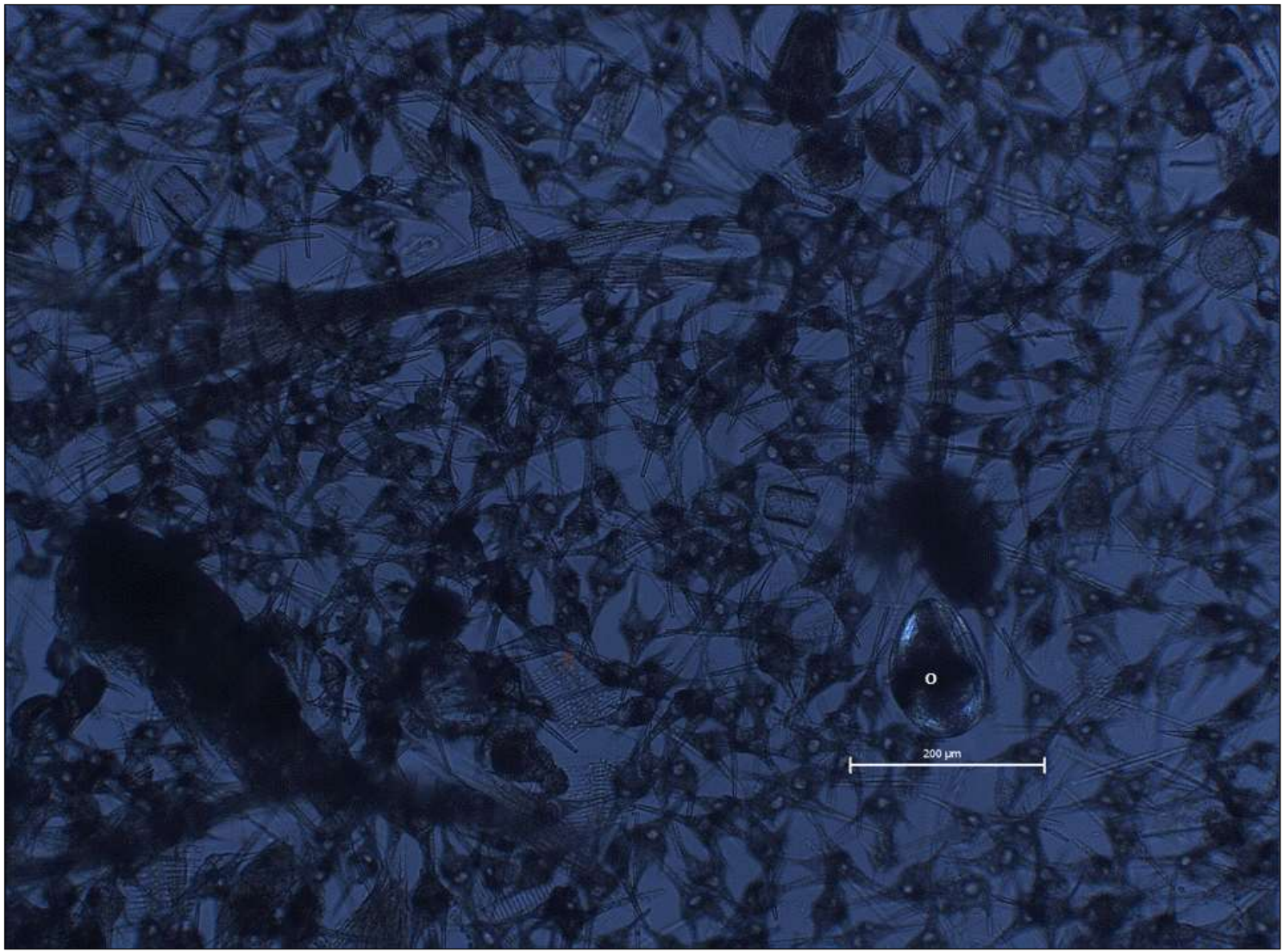


Plate 7: An ostracod (O) that can be picked out from surrounding matrix consisting of non-birefringent plankton, e.g., *Ceratium* sp. dinoflagellates, using birefringence, overall roundish shape, and distinct, smooth continuous margins. Matrix is evenly distributed throughout field of view so that each object can be inspected.

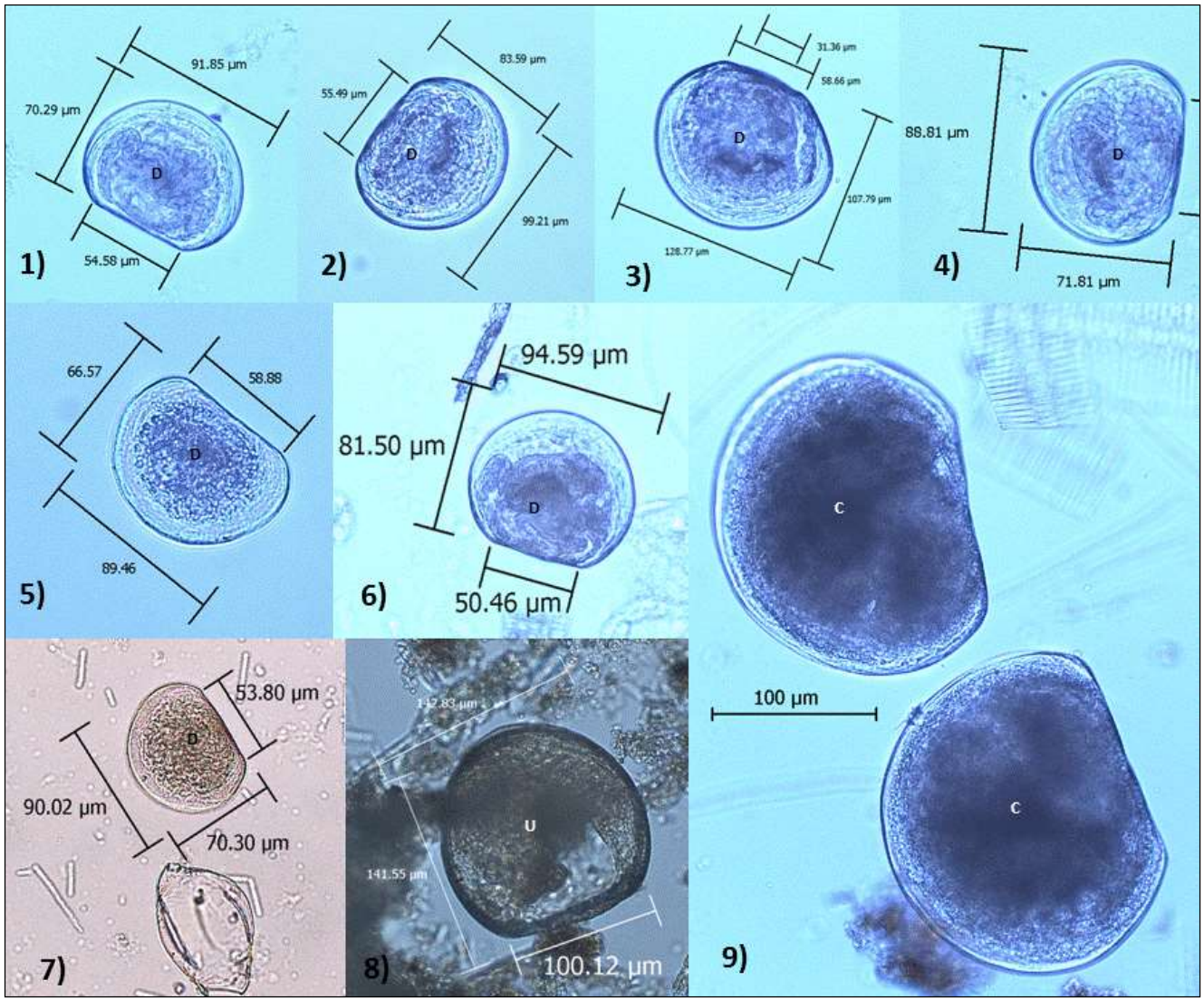


Plate 8: 'D' shaped bivalve larvae in the lateral view including *Dreissena* sp. (D) larvae in the straight-hinge stage (1-7), unionid (U) glochidium larva (8), and *Corbicula* sp. (C) straight-hinge juveniles (9).

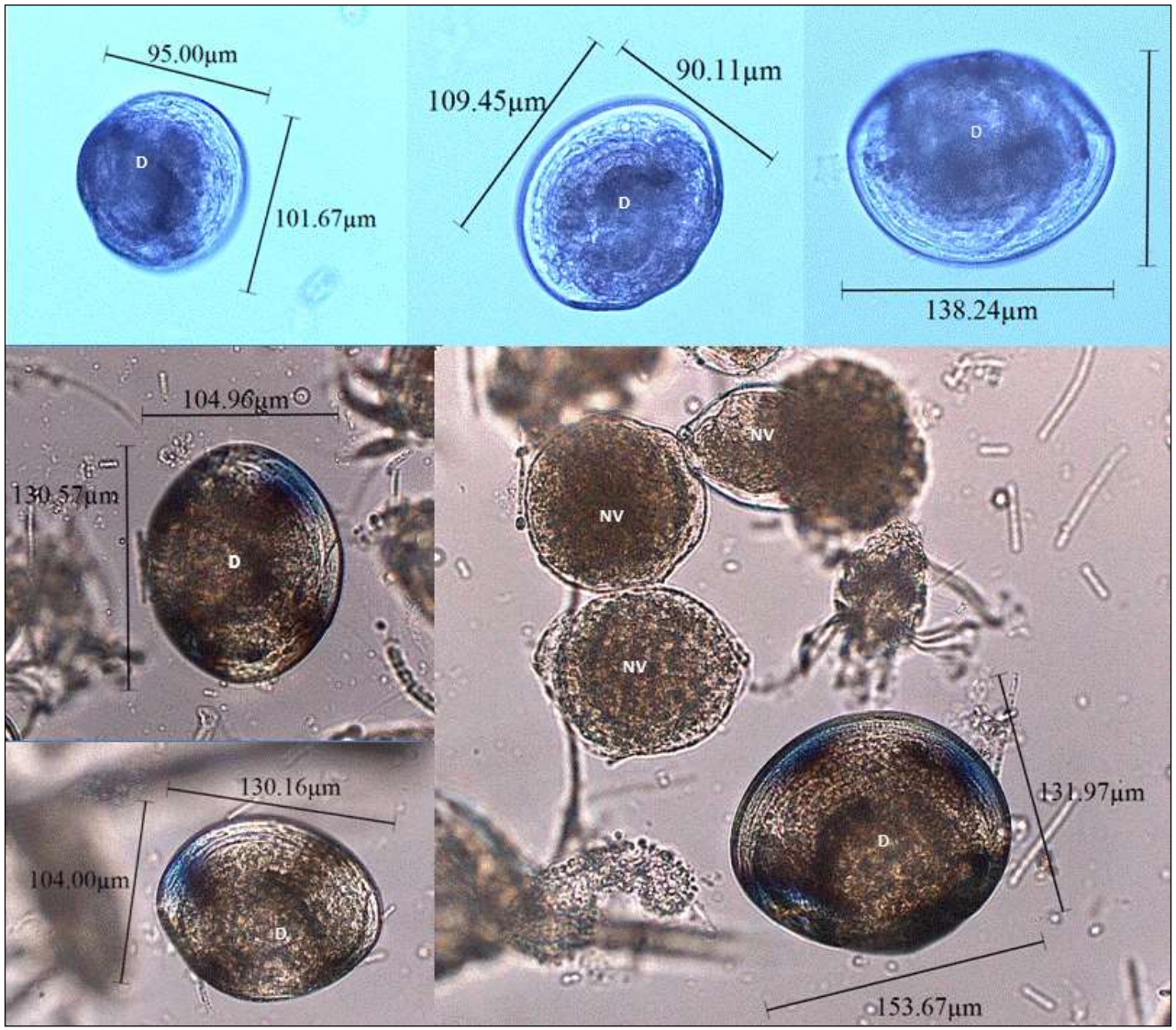


Plate 9: Round to oval shaped objects that have an overall shape, size, and distinct continuous margin warranting further inspection, including *Dreissena* sp. (D) larvae as well as unidentified objects that are not veligers (NV).

Plate 10: Round to oval shaped objects that have an overall shape and size warranting further inspection, including a *Dreissena* sp. (D) larva, *Corbicula* sp. (C) straight-hinge juvenile, and an unidentified object that is not a veliger (NV).

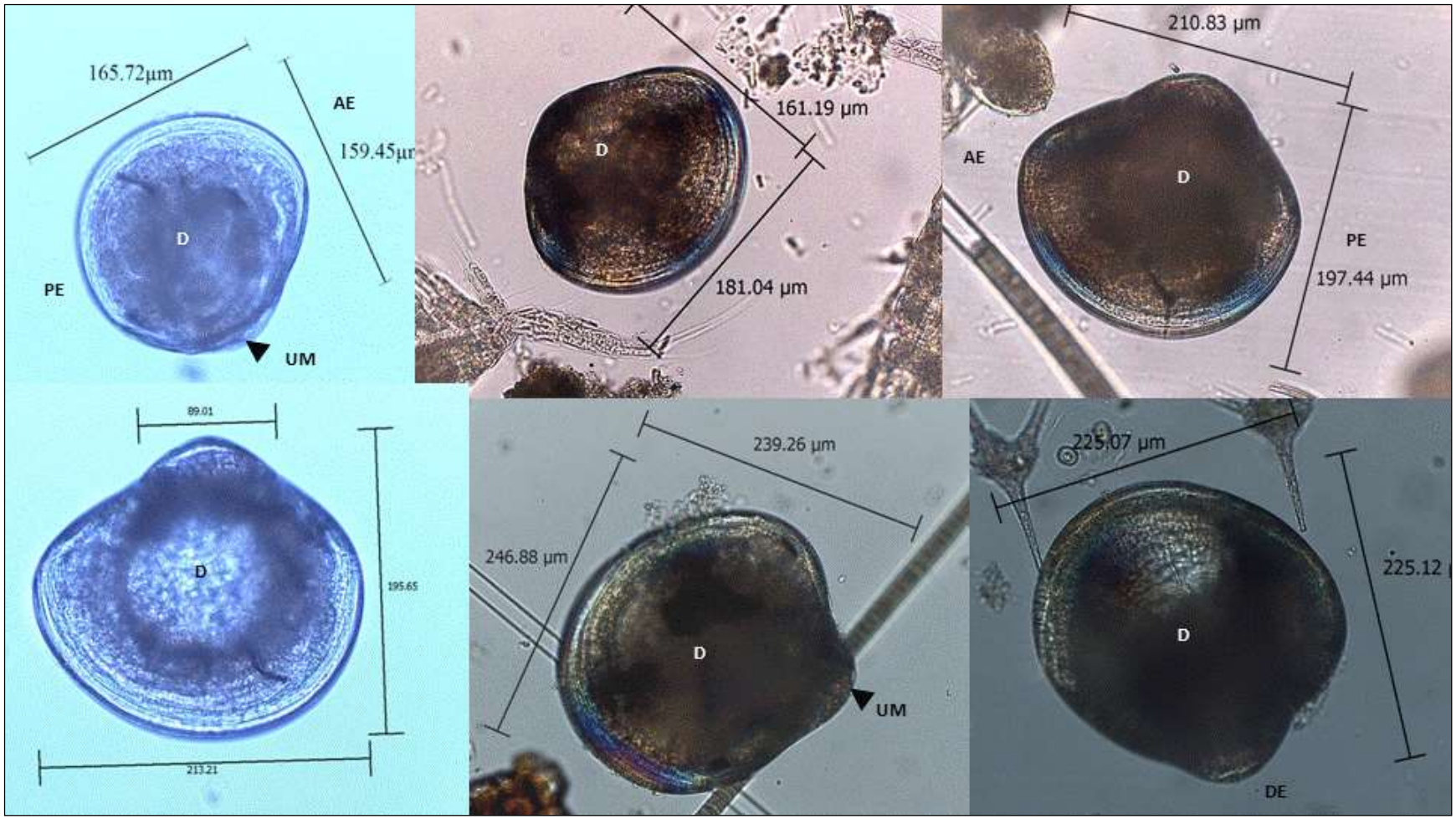


Plate 11: *Dreissena* sp. (D) larvae in various stages of development displaying a roundish overall shape, size, and distinct continuous margins that warrants further inspection. As planktotrophic larvae develop, the overall shape from the lateral view is marked by the development of the umbo (UM) in the dorsal end (DE) and the asymmetry of the posterior end (PE) and anterior end (AE).

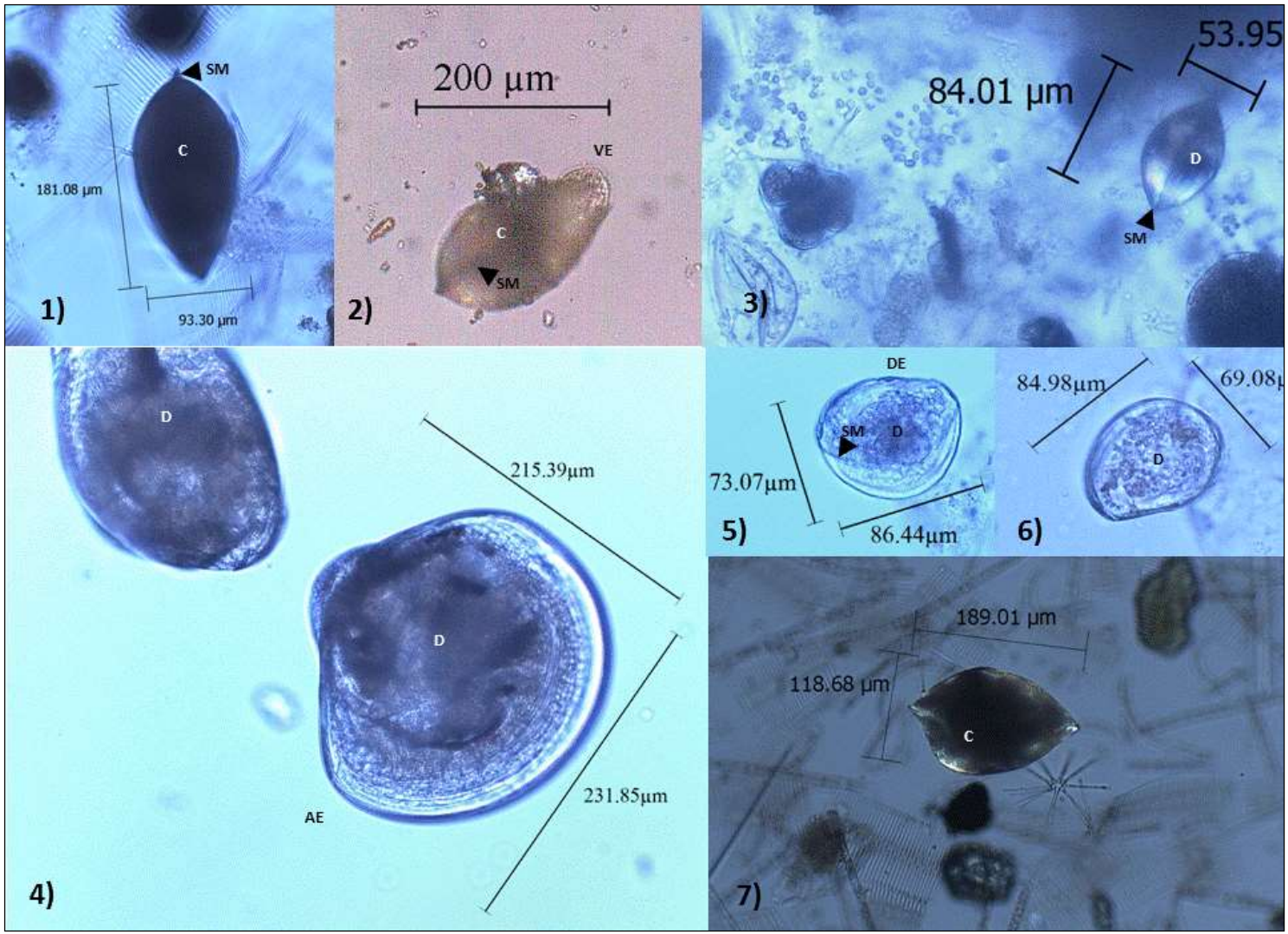


Plate 12: *Dreissena* sp. (D) and *Corbicula* sp. (C) displaying an oval to ovate shape due to orientation. Bivalves are viewed dorsally (1, 3, 4, 7), anterodorsally (2), ventrolaterally (5), and dorsolaterally (6). One *Dreissena* sp. (D) larva is also viewed laterally (4). In some cases, the line separating each shell valve can be seen along shell margin (SM) (2, 3, 5), whereas in others, two valves appear as one structure (1, 4, 7).

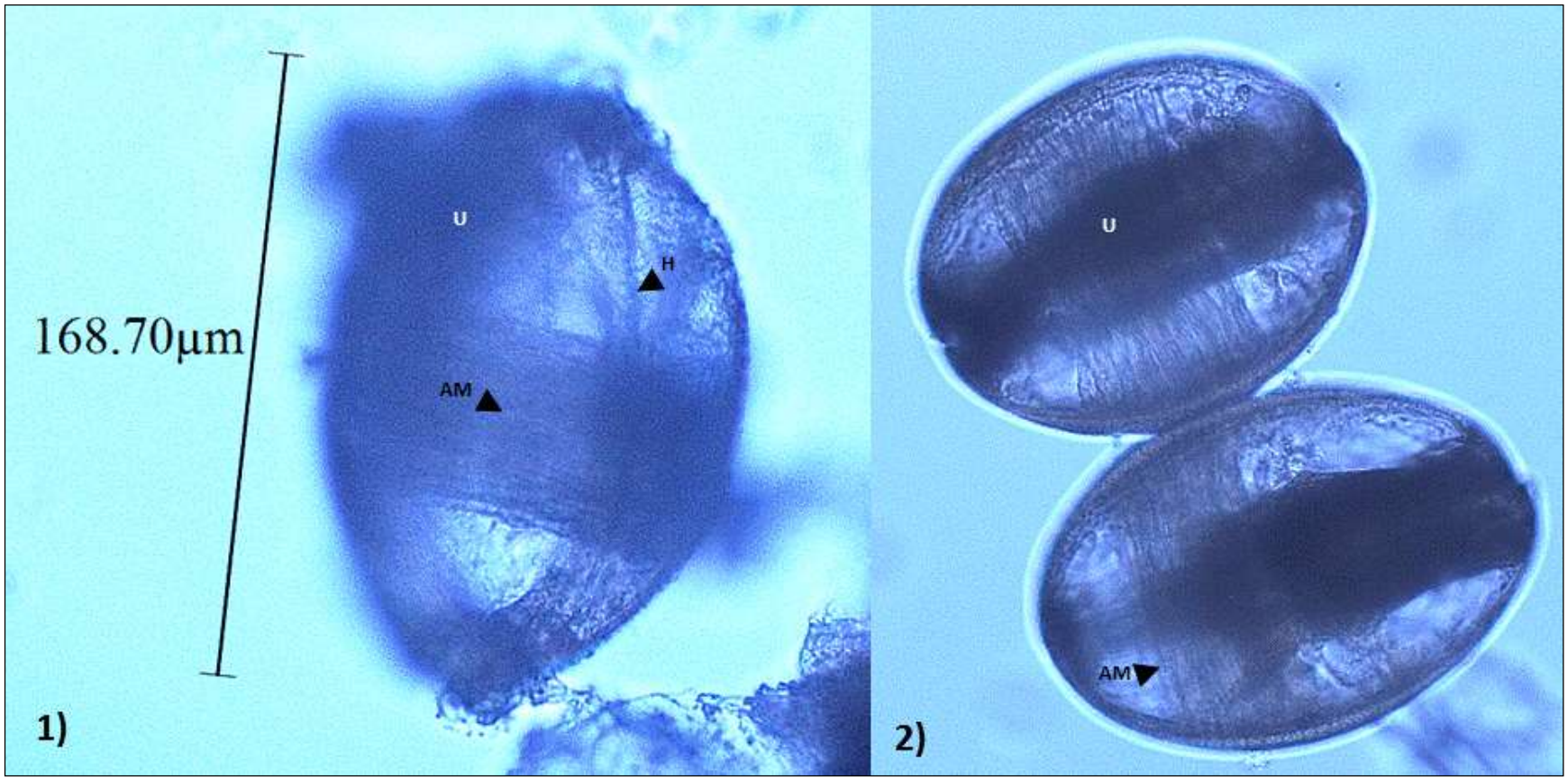


Plate 13: Unionid mussel (U) glochidial larvae showing an oval to ovate overall shape in the dorsal view (1), and ventral view (2). The adductor muscle (AM) is visible (1, 2), as well as the hinge (H) from the dorsal view (1).

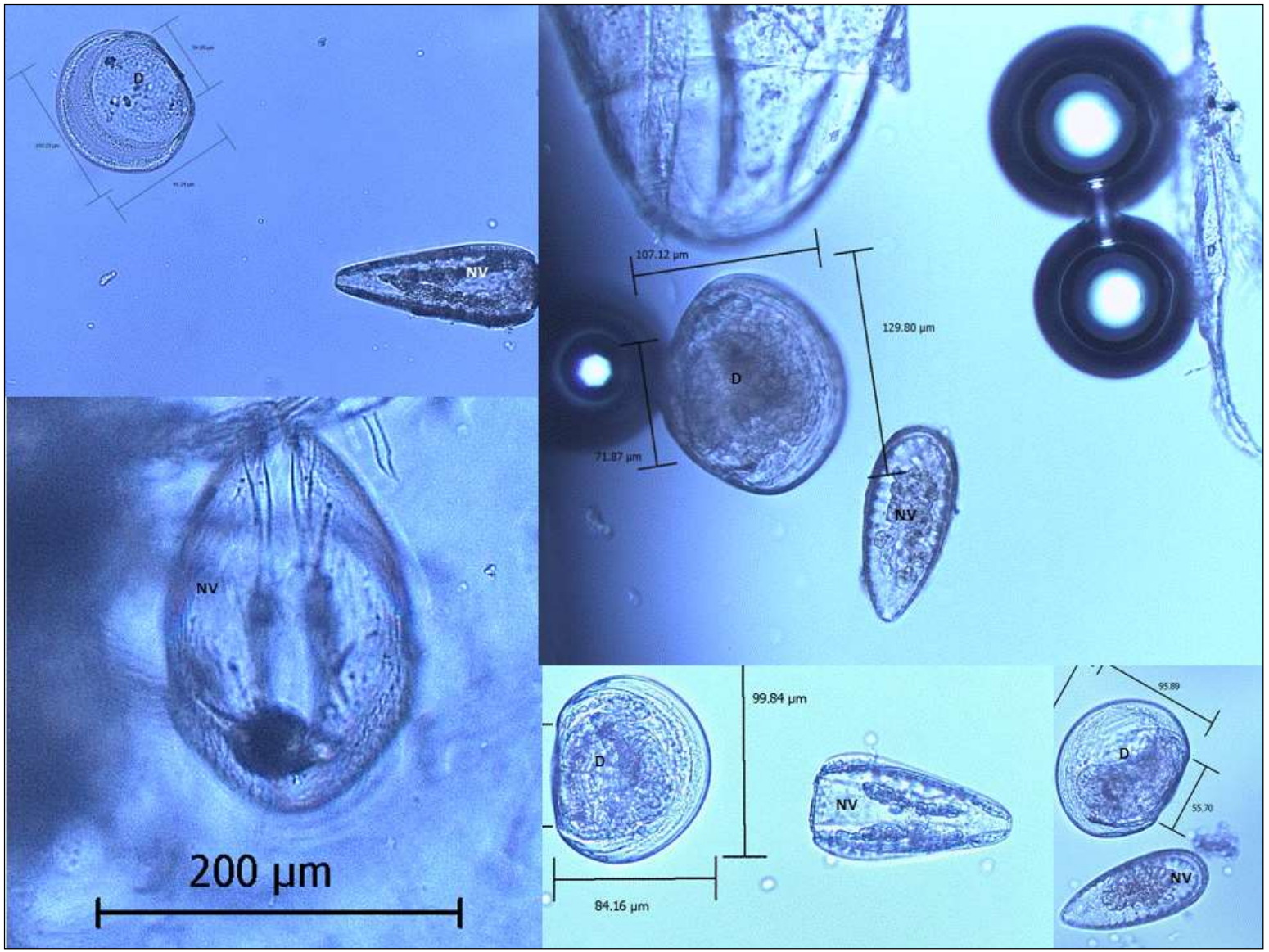


Plate 14: *Dreissena* sp. (D) larvae in relation to oval and ovate shaped unidentified objects that are not veligers (NV).

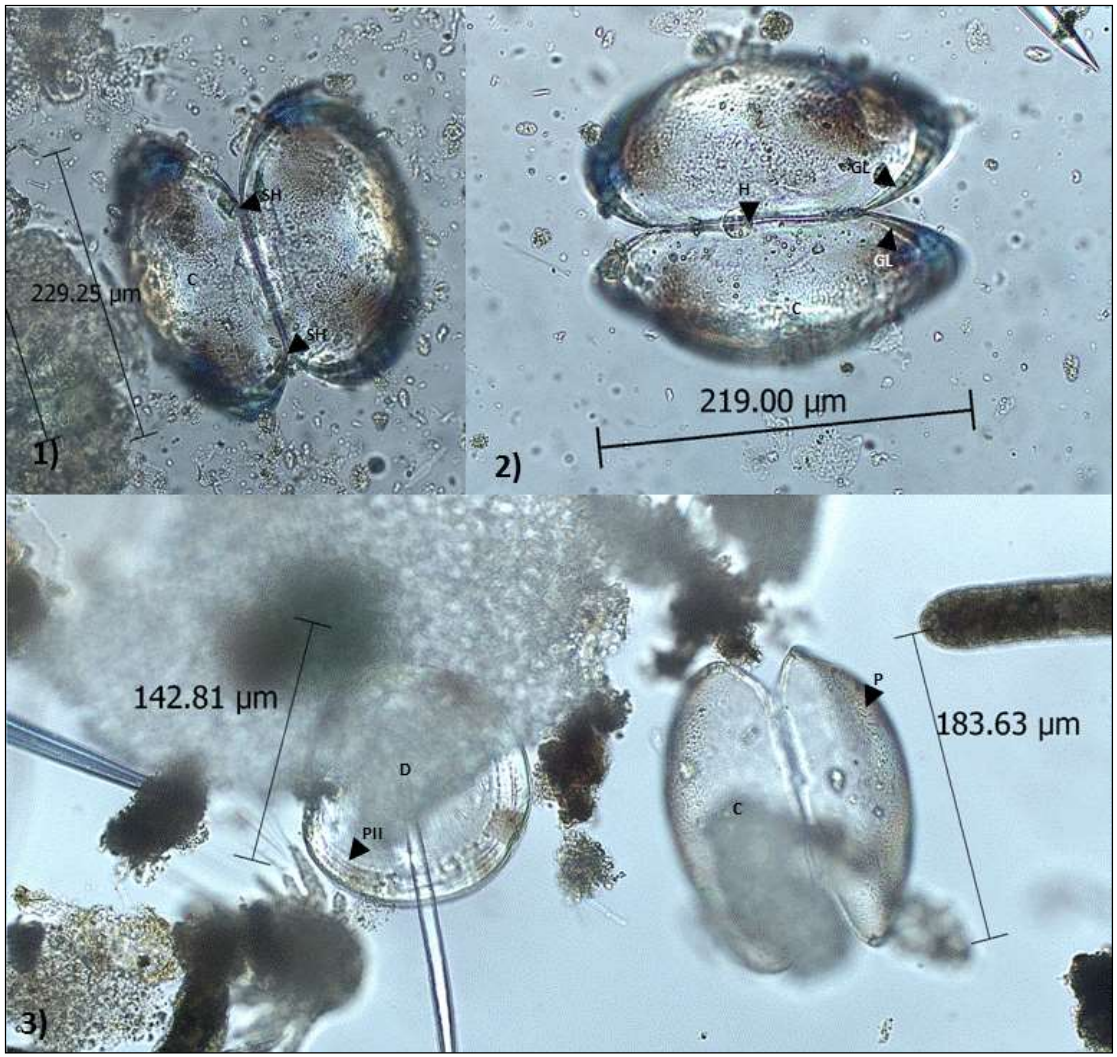


Plate 15: *Corbicula* sp. (C) straight-hinge juveniles in the dorsal view (1-3) where each shell valve has a subtrapezoidal, acetabuliform to cymbiform shape. A *Dreissena* sp. (D) larva in the lateral view is also shown (3). The hinge (H) and shoulders (SH) are visible (1-3), as well as the boundary between the prodissoconch I and prodissoconch II (PII), the pitting surface texture (P) characteristic of prodissoconch I shell, and growth lines located along the shell margin that are converging at the shoulders (GL). All specimens lack internal tissues.

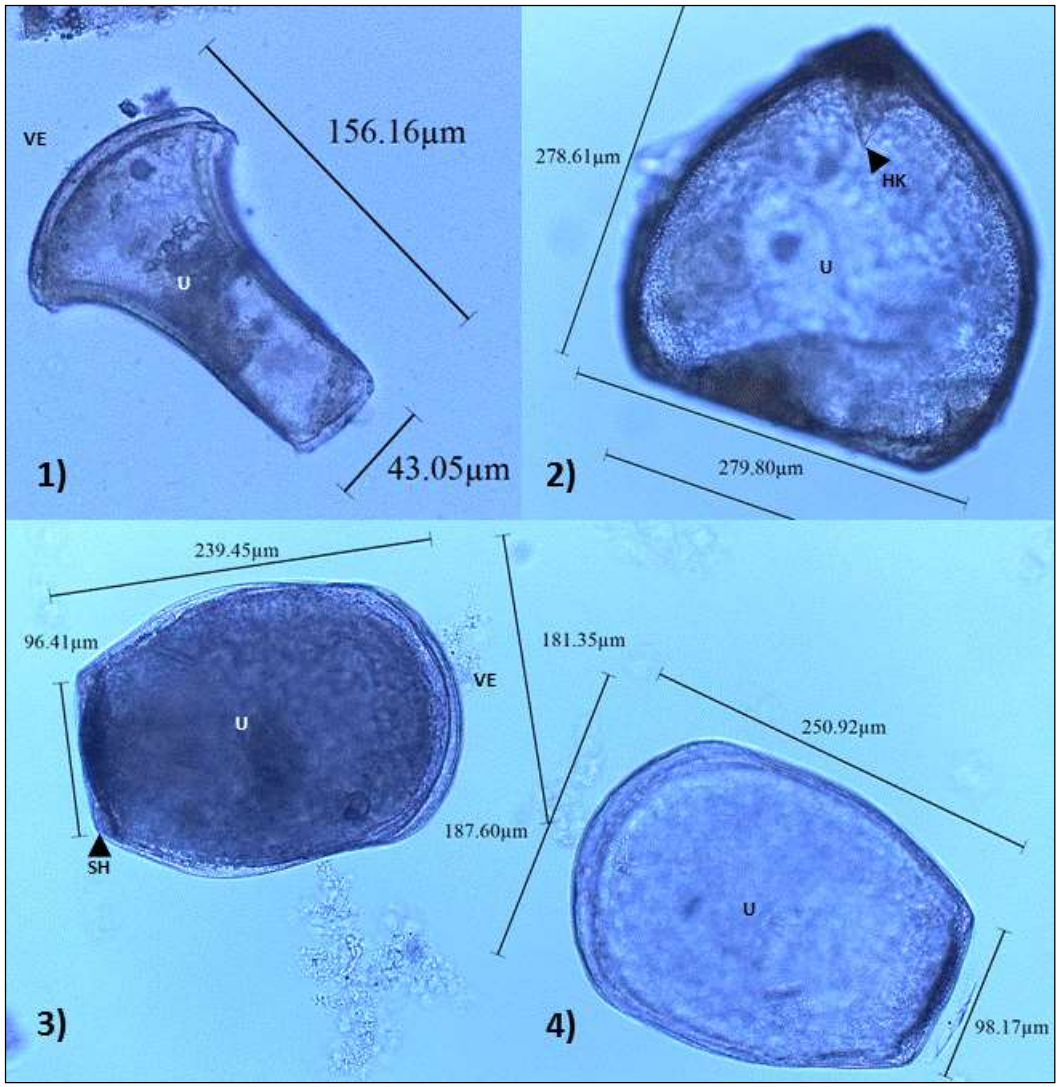


Plate 16: Axe-head, triangular, and quadrate to rectangular shaped unionid mussel (U) glochidial larvae including *Potamilus amphichaenus* (1), *Anodonta suborbiculata* (2), and *Lampsilis hydiana* (3, 4). The shoulders (SH) are visible as is the styliform hook (HK) on the ventral margin of the *Anodonta* larvae. Unionid specimens and identification are courtesy of Robert G. Howells.

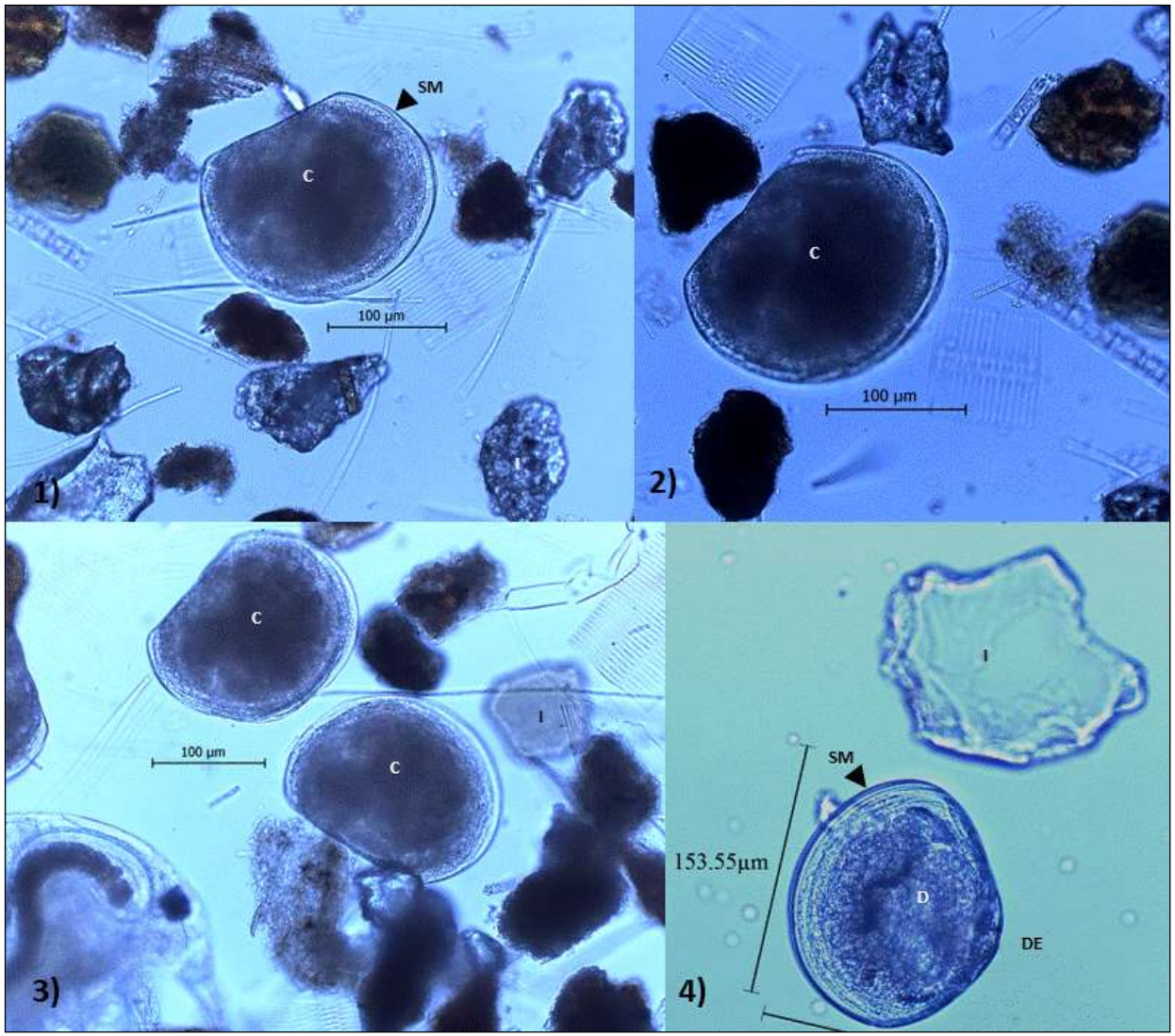


Plate 17: *Corbicula* sp. (C) straight-hinge juveniles and a *Dreissena* sp. (D) larva showing distinct continuous, curved and smooth-edged shell margin (SM) in relation to the angular, indistinct and jagged margin of the surrounding inorganic debris (I).

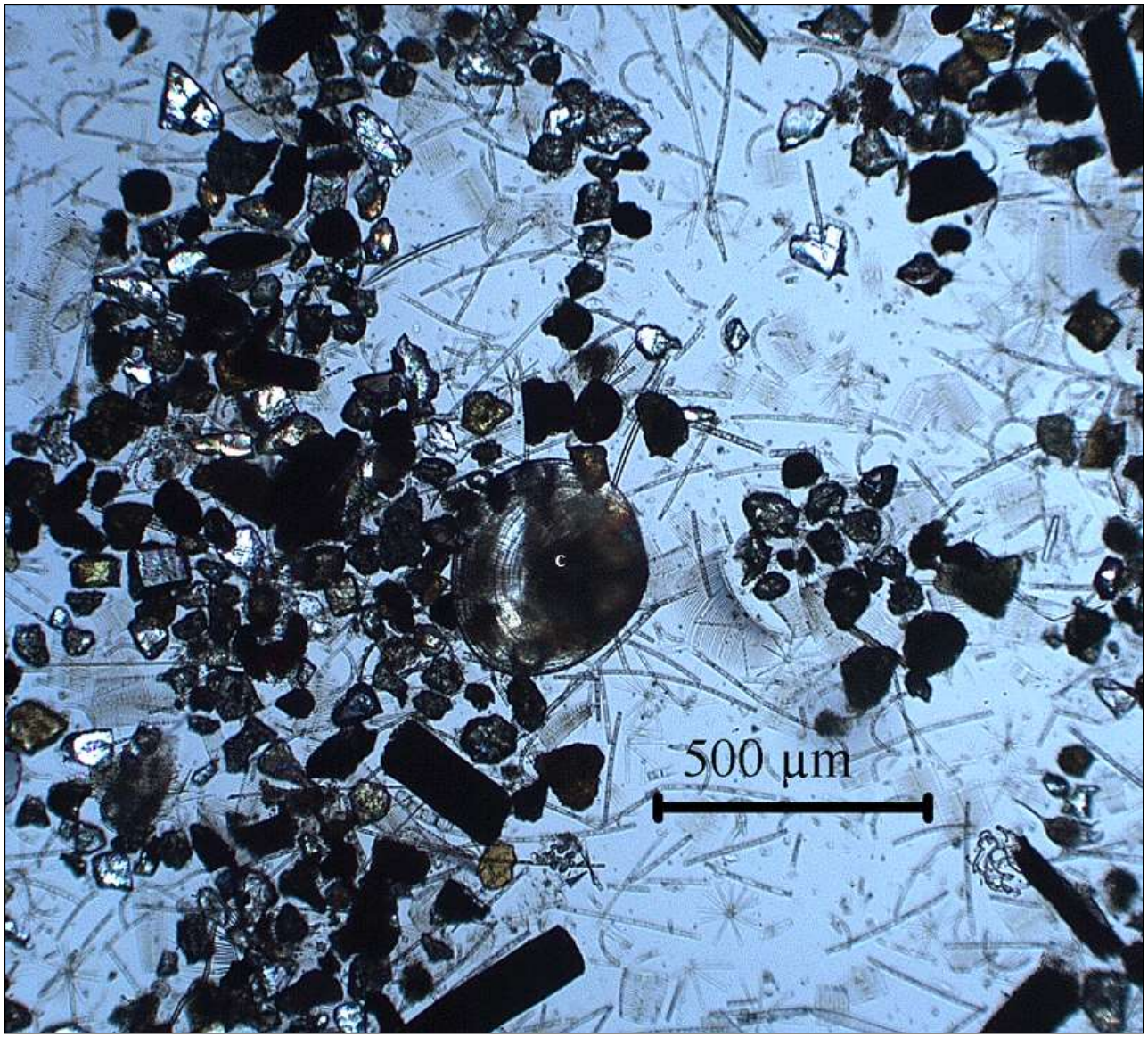


Plate 18: *Corbicula* sp. (C) juvenile showing a continuous, curved, smooth-edged margin in relation to the surrounding matrix of inorganic debris. Note the prominent ridges interspaced within, and parallel the growth lines, as well as the secondary lines that are perpendicular to the growth lines.

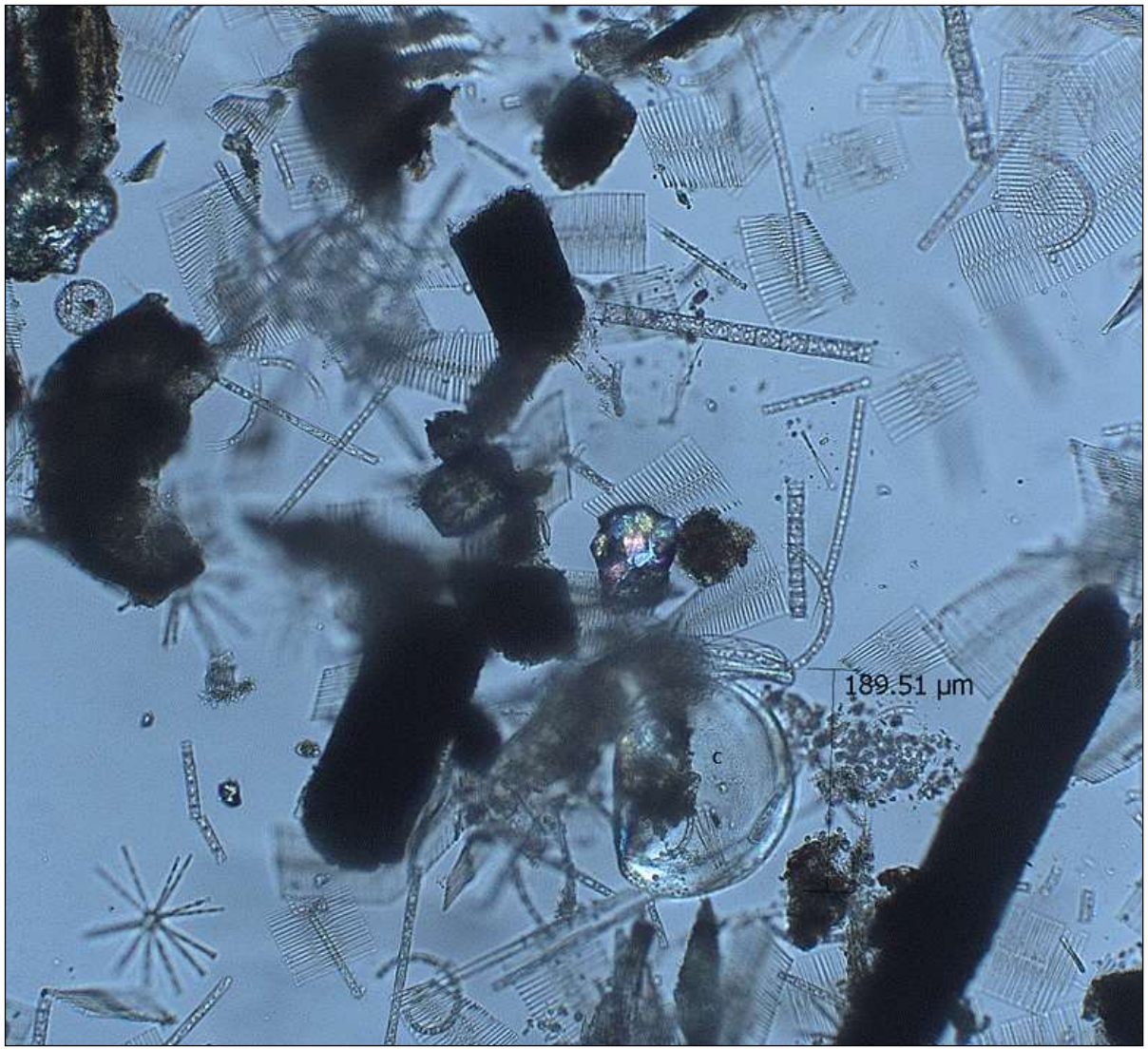


Plate 19: A *Corbicula* sp. (C) straight-hinge juvenile showing a distinct, continuous, curved, and smooth-edged margin in relation to the surrounding plankton matrix. This bivalve lacks internal tissue and is translucent.

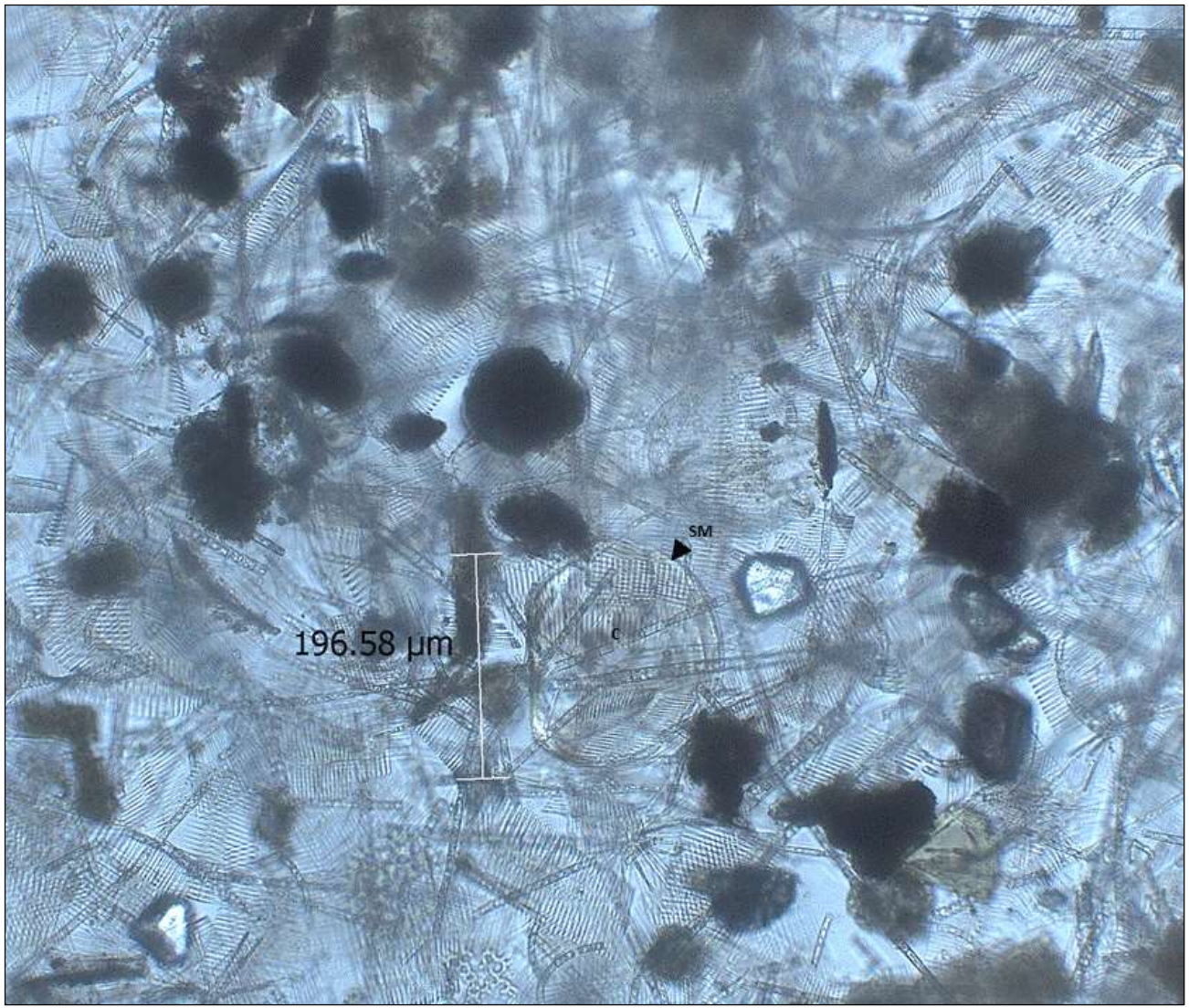


Plate 20: The empty shell of a *Corbicula* sp. (C) straight-hinge juvenile that can be separated from the surrounding plankton matrix by the overall shape, size, and distinct, continuous, curved and smooth-edged margin (SM).

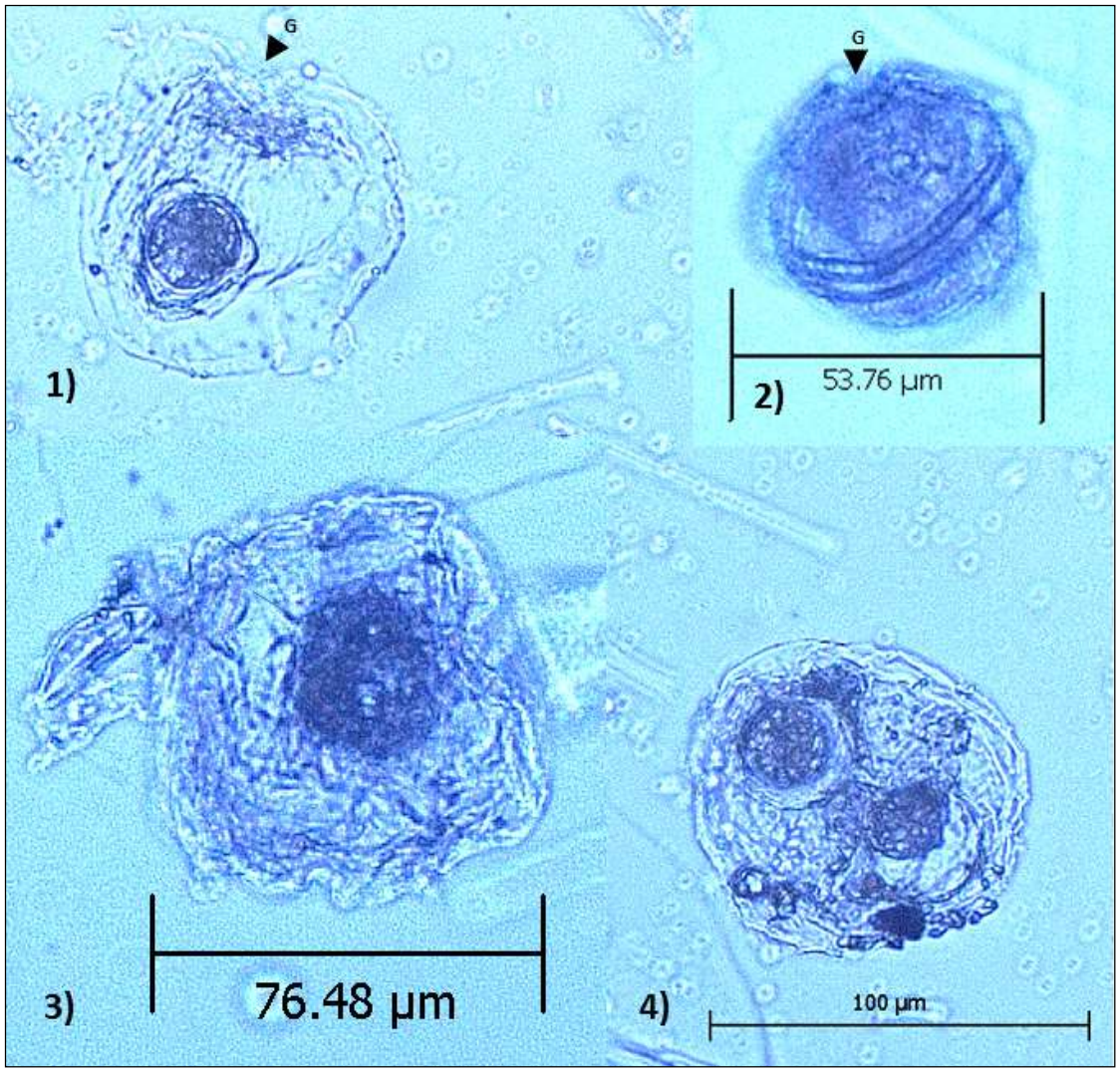


Plate 21: Unidentified objects that are not bivalve larvae (1-4) with angular to undulate, indistinct margins. In addition to angular, undulate, indistinct margins, these non-veliger objects possess other features that separate them from shelled bivalve larvae, including apparent grooves (G), the lack of two relatively symmetrical valves, the presence of apparent plates (3), and the appearance of the internal tissues.

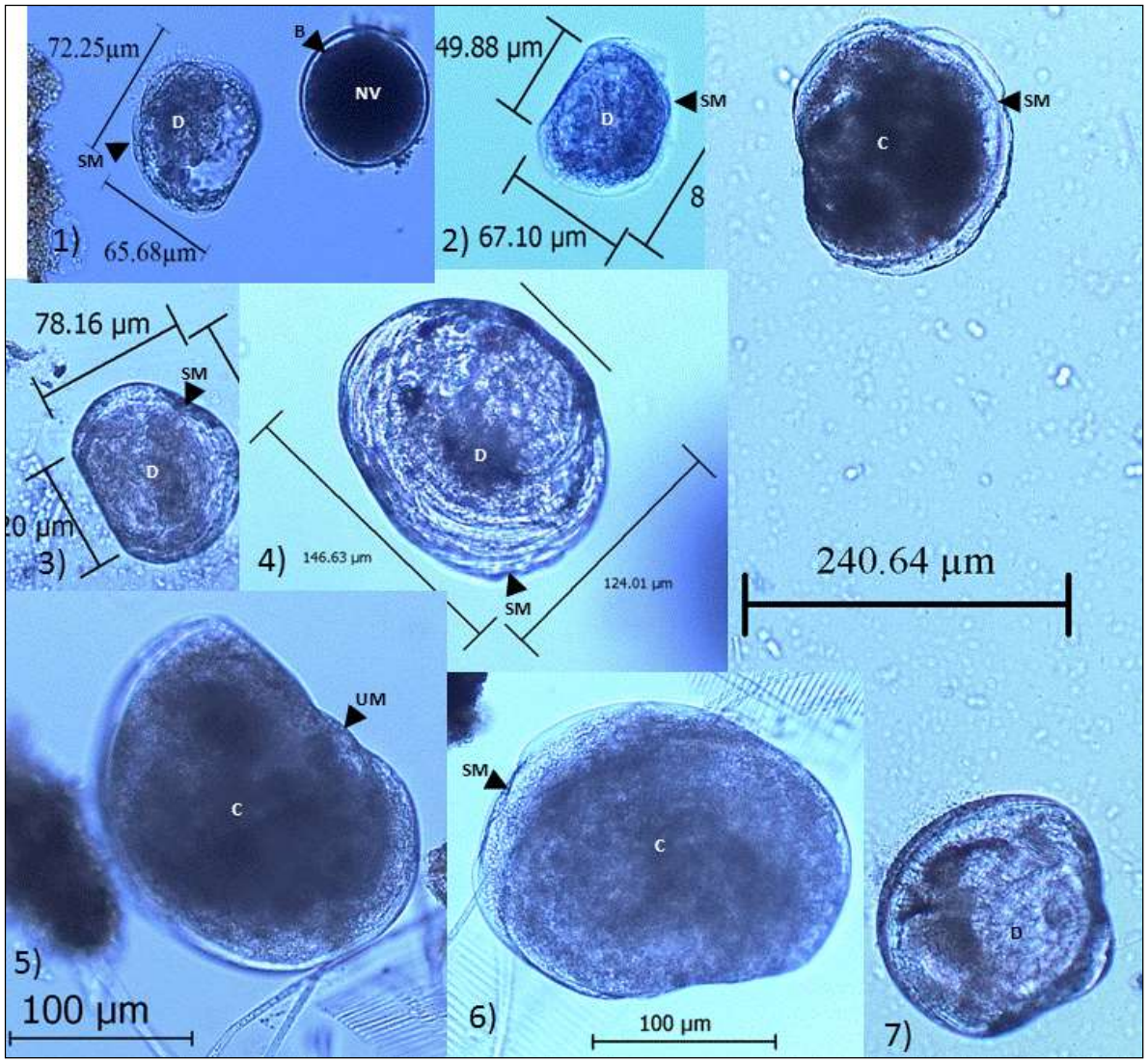


Plate 22: *Dreissena* sp. (D) larvae (1, 2, 3, 4, 7) and *Corbicula* sp. (C) straight-hinge juveniles (5, 6) with morphology that is not clearly visible, or that appears unusual such as the uneven, fuzzy or not continuous shell margins (SM), or an undulate hinge line in the umbo area (UM) (5).

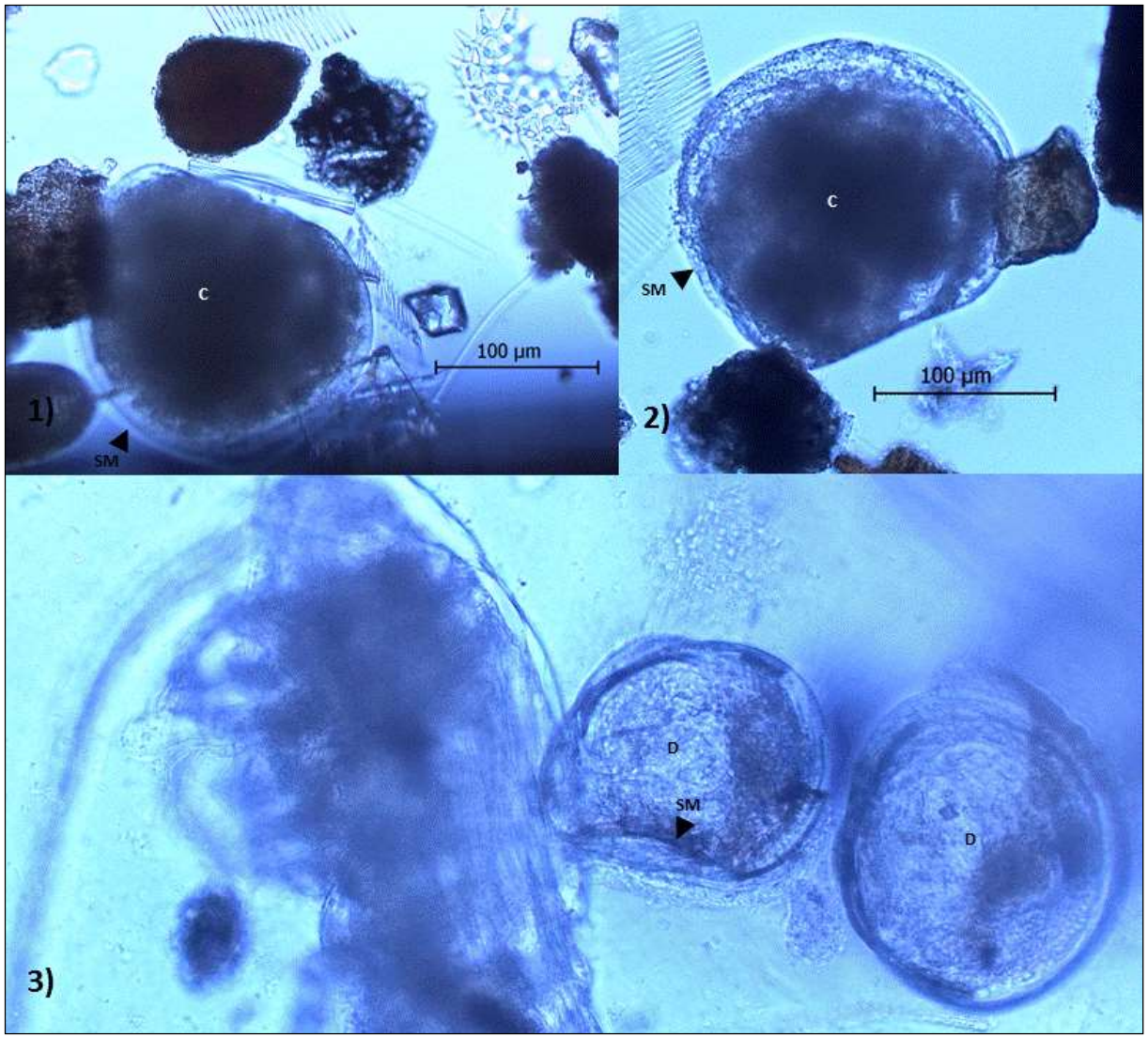


Plate 23: *Dreissena* sp. (D) larvae and *Corbicula* sp. (C) straight-hinge juveniles with unclear or unusual morphology. The shell margins (SM) are indistinct and appear uneven (1, 2), or the shell margin (SM) appears bent or folded upon itself (3).

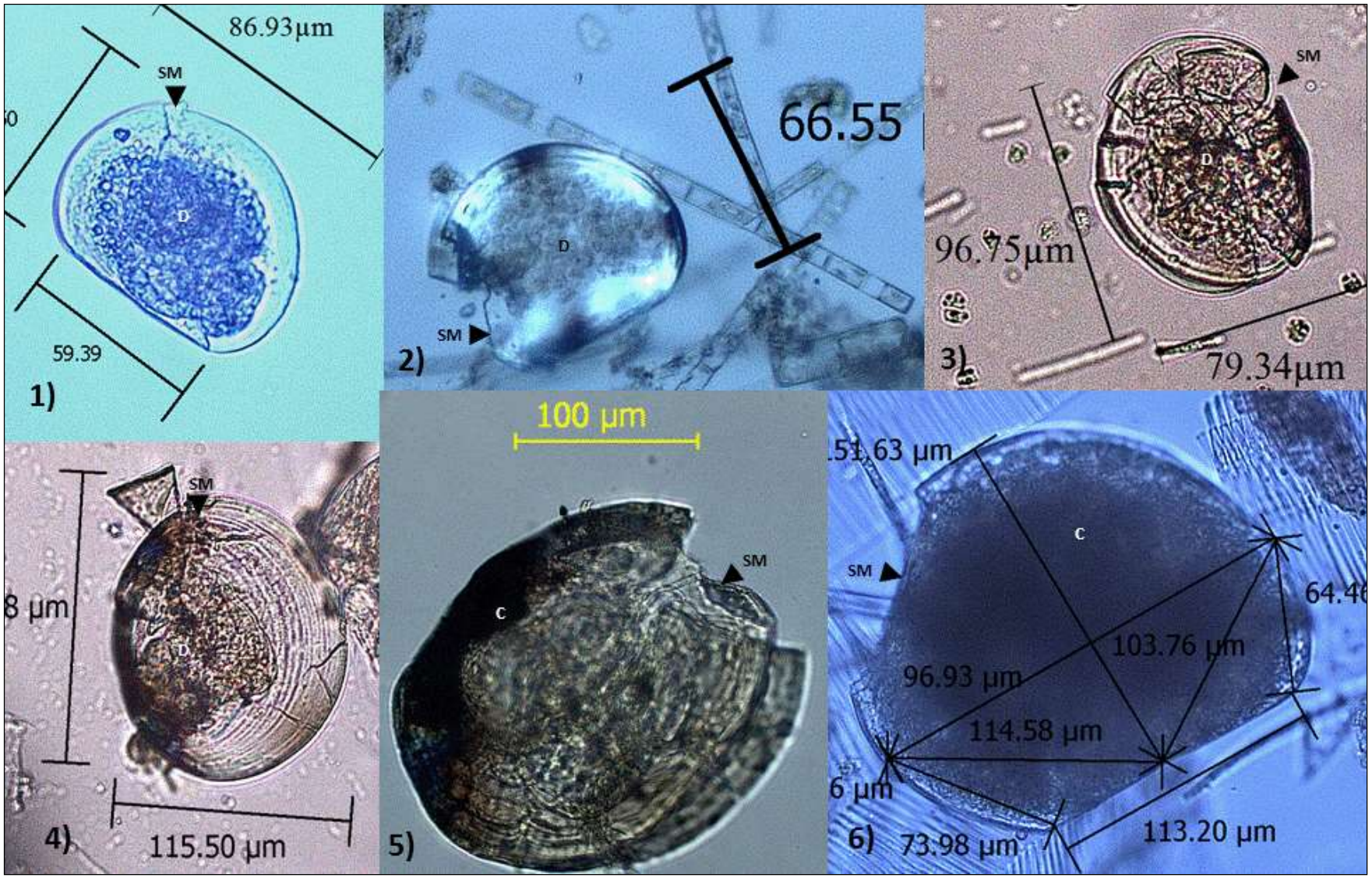


Plate 24: *Dreissena* sp. (D) larvae and *Corbicula* sp. (C) straight-hinge juveniles with uneven or jagged shell margins (SM) due to broken shells.

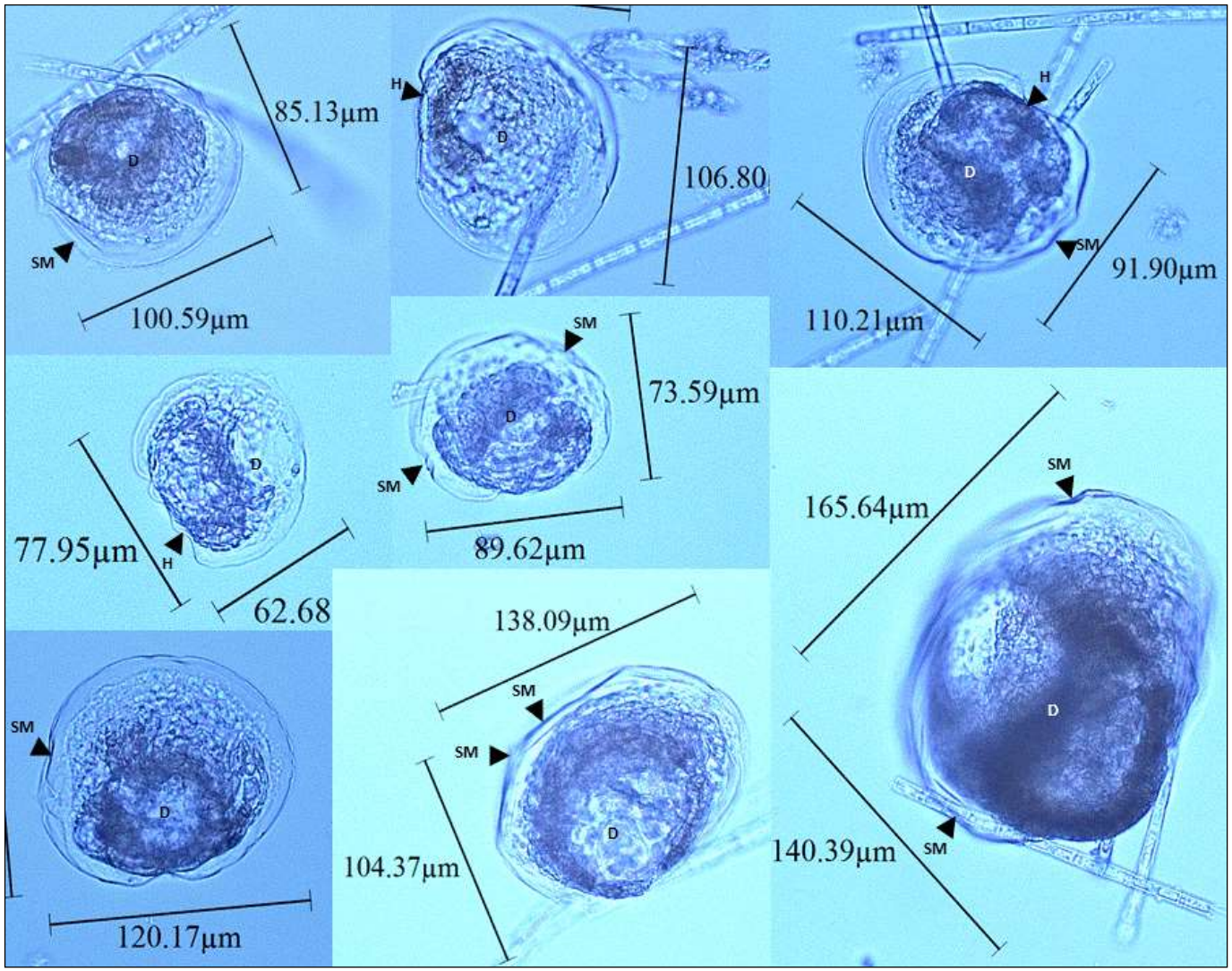


Plate 25: *Dreissena* sp. (D) larvae that are old and degrading. These larvae were collected in August 2006 and have been stored in a solution of 70% ethanol at room temperature for over 65 months. Shell degradation is most apparent in shell margins (SM) (folded, uneven, or not visible), and in the hinge line (H) (undulate, sharply sway-backed).

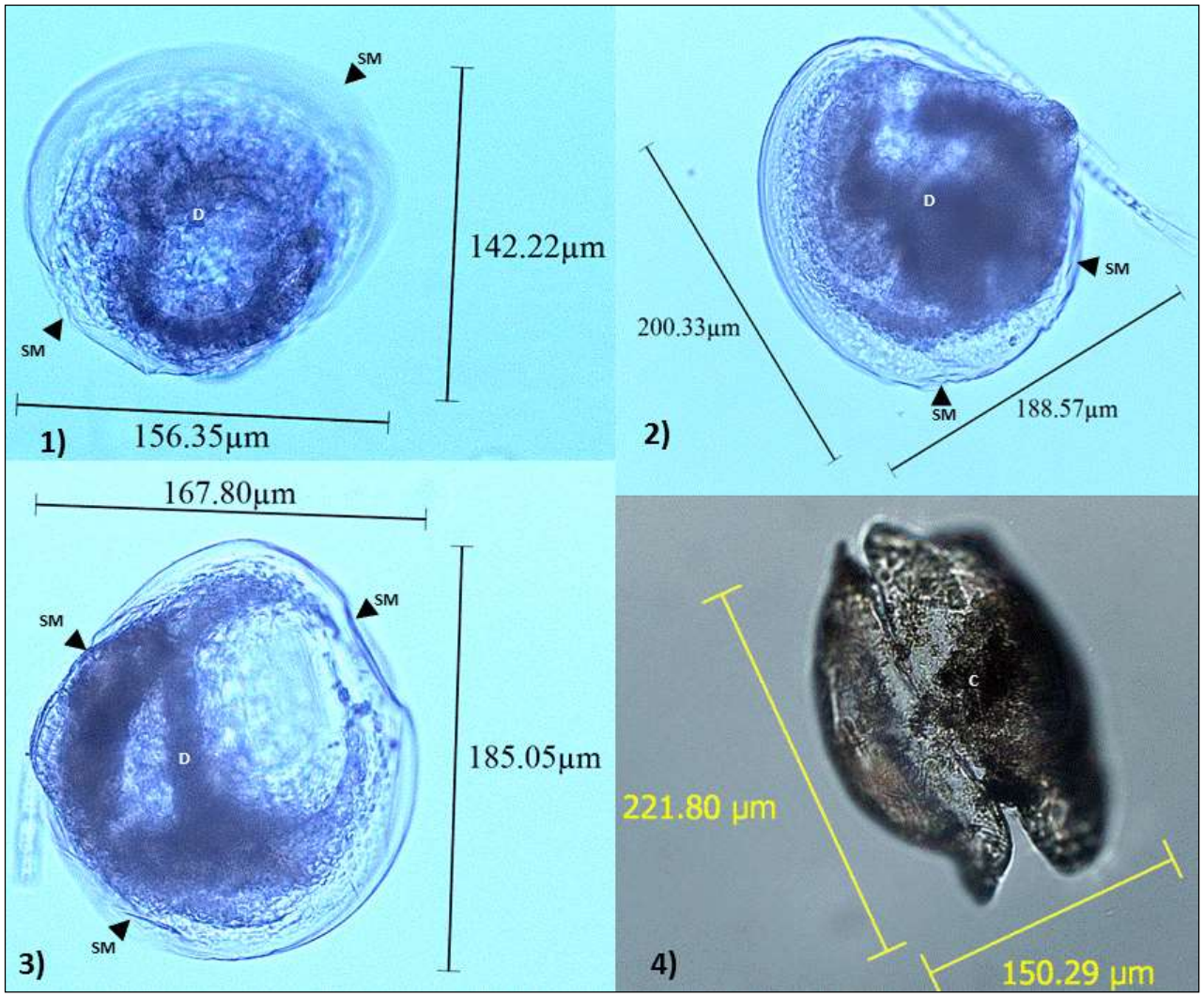


Plate 26: *Dreissena* sp. (D) larvae (1, 2, 3) and a *Corbicula* sp. (C) straight-hinge juvenile (4) that are old and degrading. *Dreissena* sp. larvae were collected in August 2006, and stored in a solution of 70% ethanol at room temperature for over 65 months. The *Corbicula* sp. straight-hinge juvenile was an empty shell upon collection. Degradation is most apparent along shell margin (SM) (uneven, folded, or not visible).

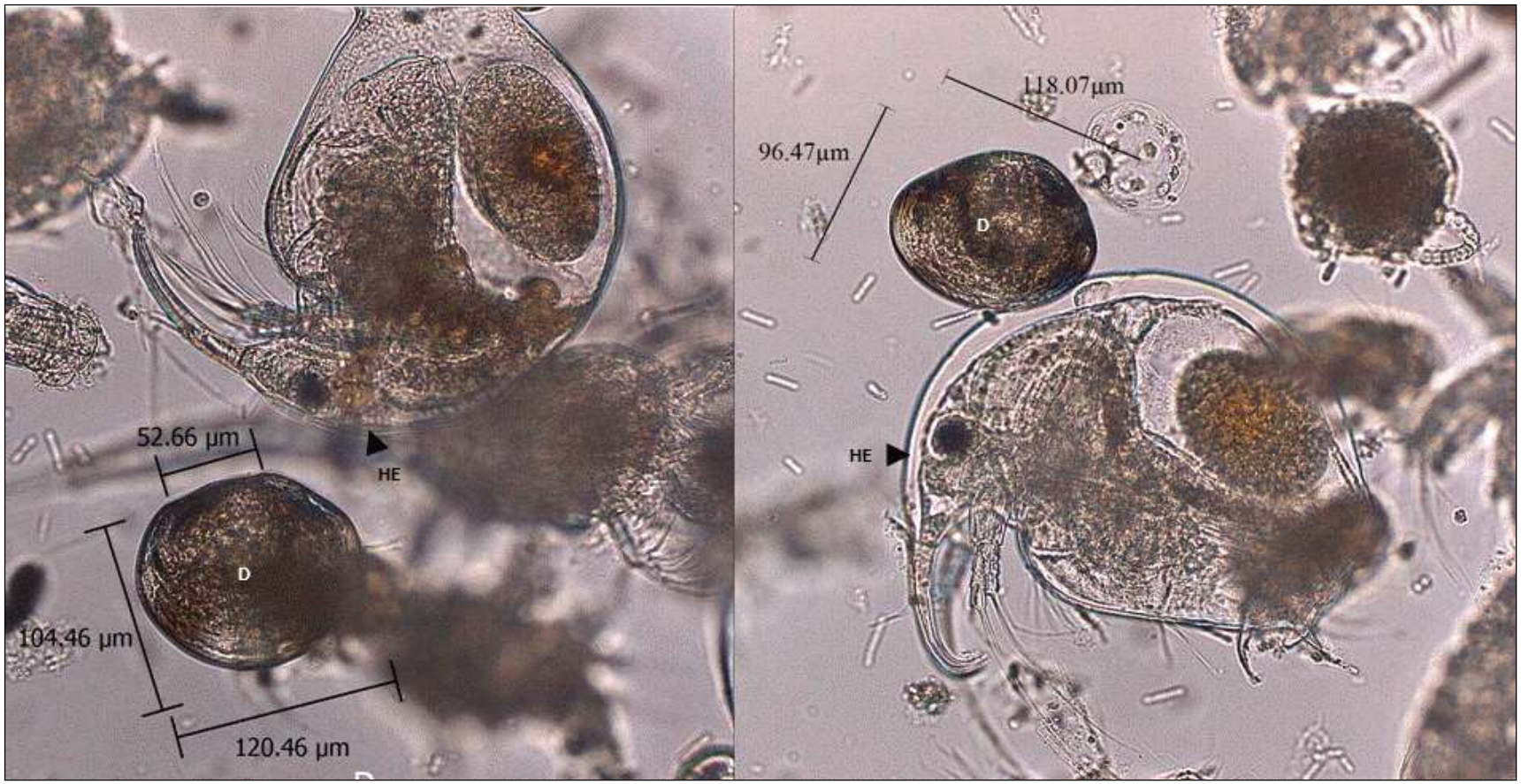


Plate 27: Cladocerans (family Bosminidae) adjacent *Dreissena* sp. (D) larvae. Both have distinct, continuous and smooth-edged margins; bivalve larvae lack a distinct head (HE) and segmented appendages.

Plate 28: Ostracods (O) in the ventral view showing segmented appendages (SA) extended beyond the carapace (1), as well as appendages cured within the carapace against the internal body (1, 2). Ostracod appendages are varied and include legs, antennae, and other projections used for cleaning, reproduction, feeding, etc. Setae (SE) are visible. The ostracod carapace edge is often rounded, whereas bivalve larvae have a more acute shell margin.

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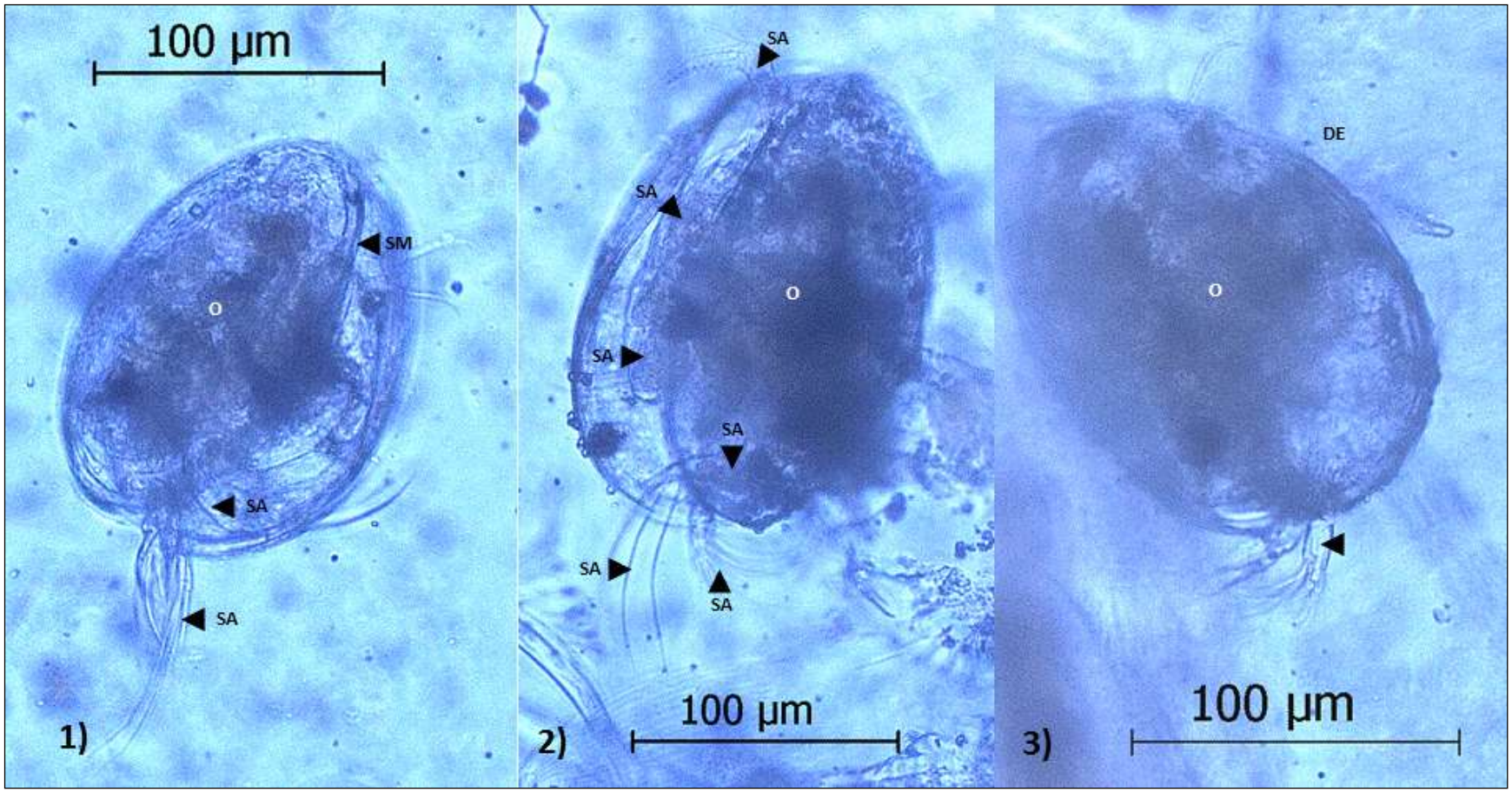


Plate 29: Ostracods (O) in a somewhat ventrolateral view showing segmented appendages (SA) protruding outside the carapace, as well as segmented appendages that are visible through the carapace. The carapace is hinged on the dorsal end (DE), which is opposite the protruding appendages (3).

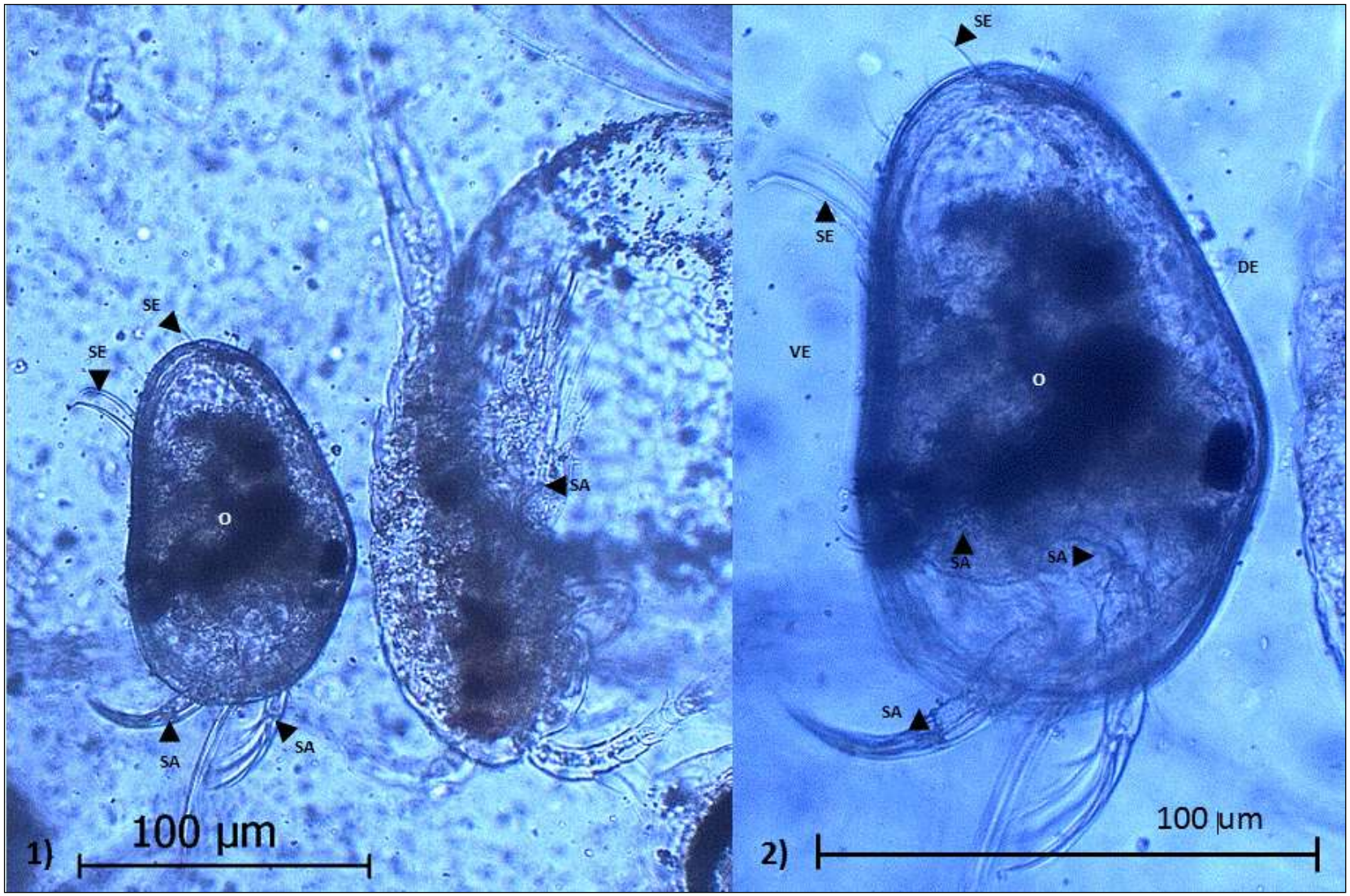


Plate 30: Ostracods (O) and copepods showing segmented appendages (SA) and setae (SE) (1). Ostracods in the lateral view (1, 2) have a bean-like or somewhat subtriangulate overall shape.

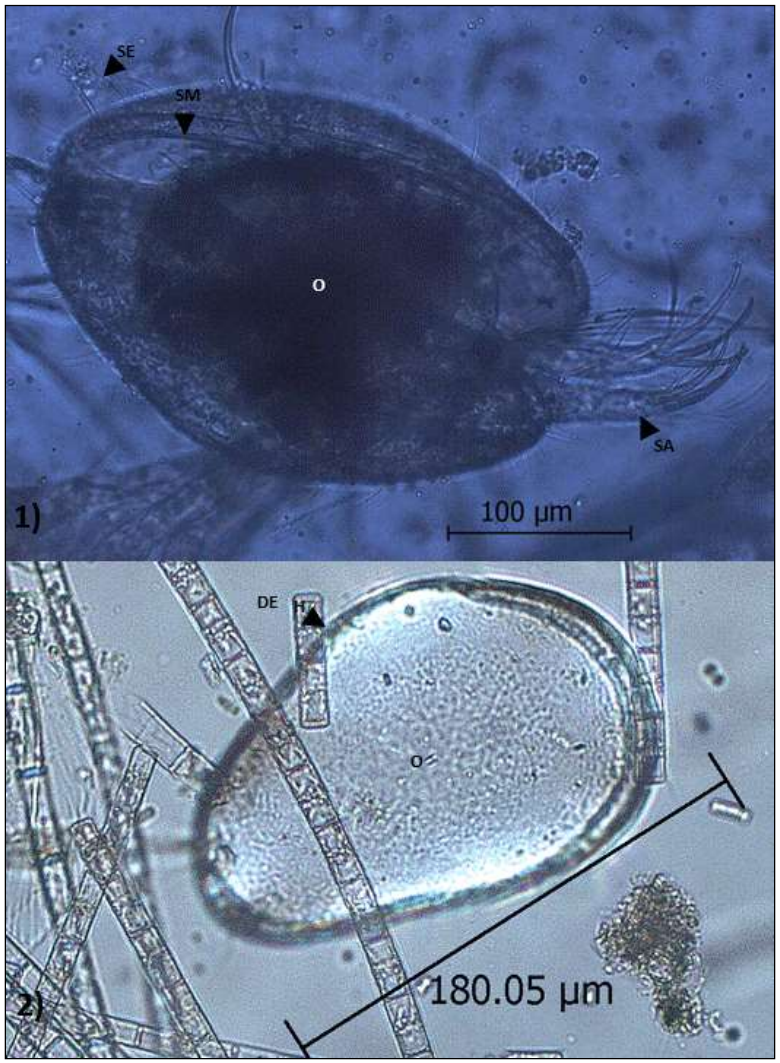


Plate 31: Ostracods (O) in the ventral view (1) and lateral view (2). The internal tissues are present in 1) showing segmented appendages (SA) and setae (SE), whereas the ostracod in 2) lacks internal tissues, but shows the characteristic bean shape, somewhat similar to the 'D' shape of straight-hinge bivalve larvae. The dorsal end (DE) and hinge (H) location for the ostracod is along the curved section of 'D', whereas the bivalve larvae hinge is located on straight section.

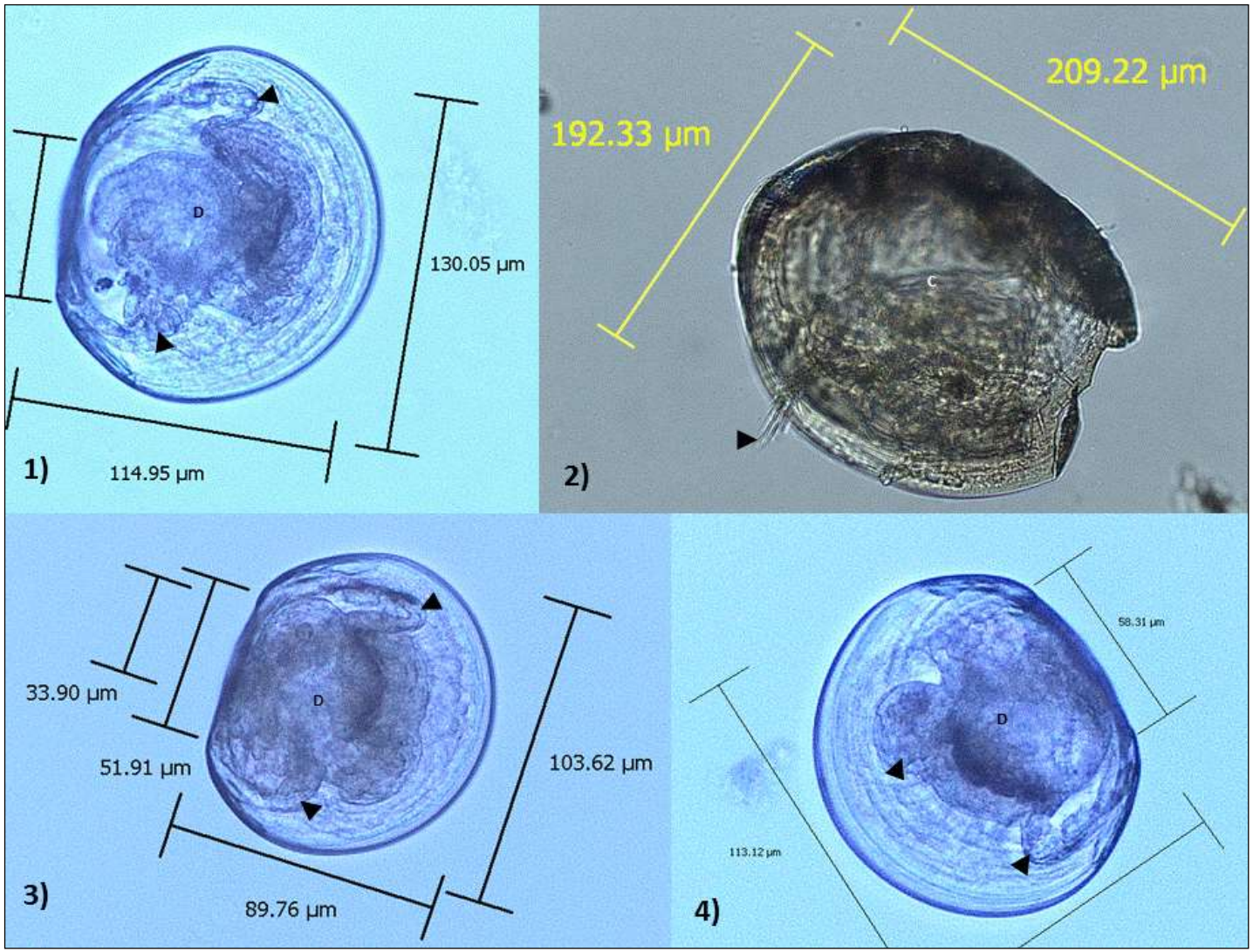


Plate 32: *Dreissena* sp. (D) larvae and a *Corbicula* sp. (C) straight-hinge juvenile with internal tissues (1, 3, 4) or exogenous material (2) that may appear similar to segmented appendages that are withdrawn inside the bivalve shell.

Plate 33: Planktonic objects with similar shapes and size to *Dreissena* sp. larvae that have a continuous peripheral ring around the entire margin (B), including unidentified objects that are not veligers (NV) (1, 2), and unionid (U) mussel larvae (3). The unionid mussel shoulders (SH) are visible in the dorsal end (DE).

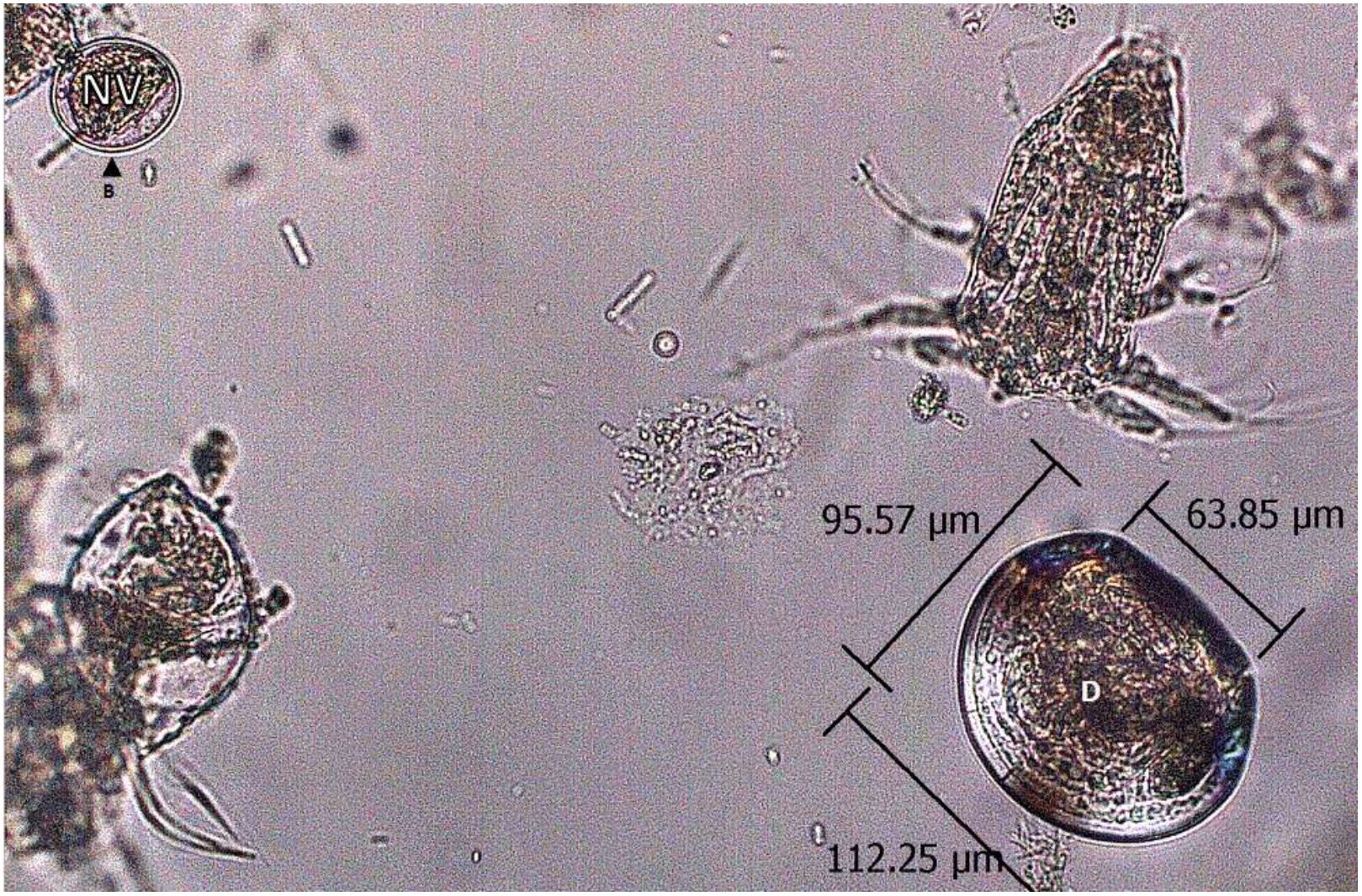


Plate 34: A *Dreissena* sp. (D) larva is shown with an unidentified object that is not a veliger (NV). The entire margin of the non-veliger object has a hollow band (B), whereas, the *Dreissena* sp. veliger margin lacks such hollow band.

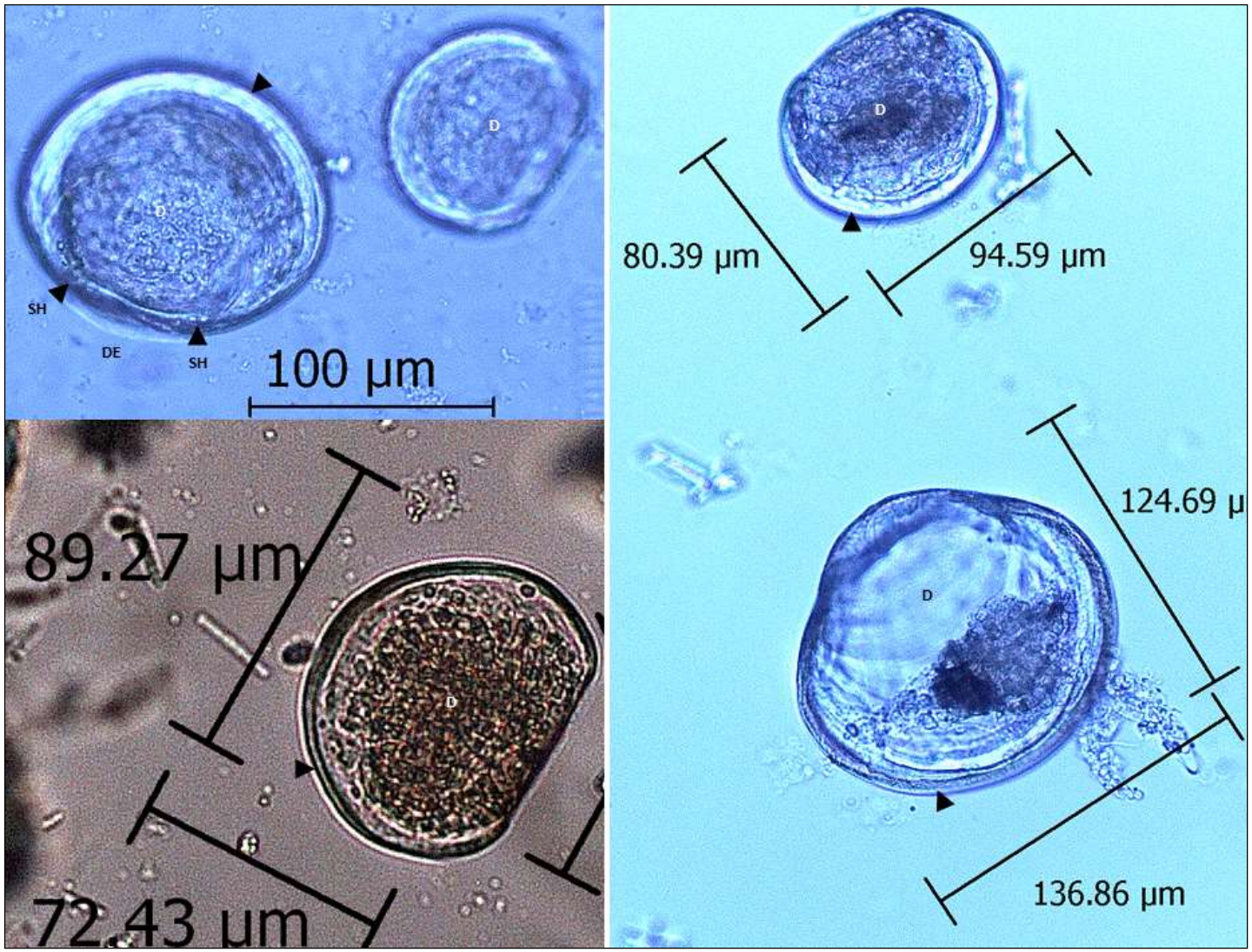


Plate 35: *Dreissena* sp. (D) larvae that appear to have a hollow band in the peripheral shell margin. These apparent hollow bands, however, terminate in the shoulders (SH).

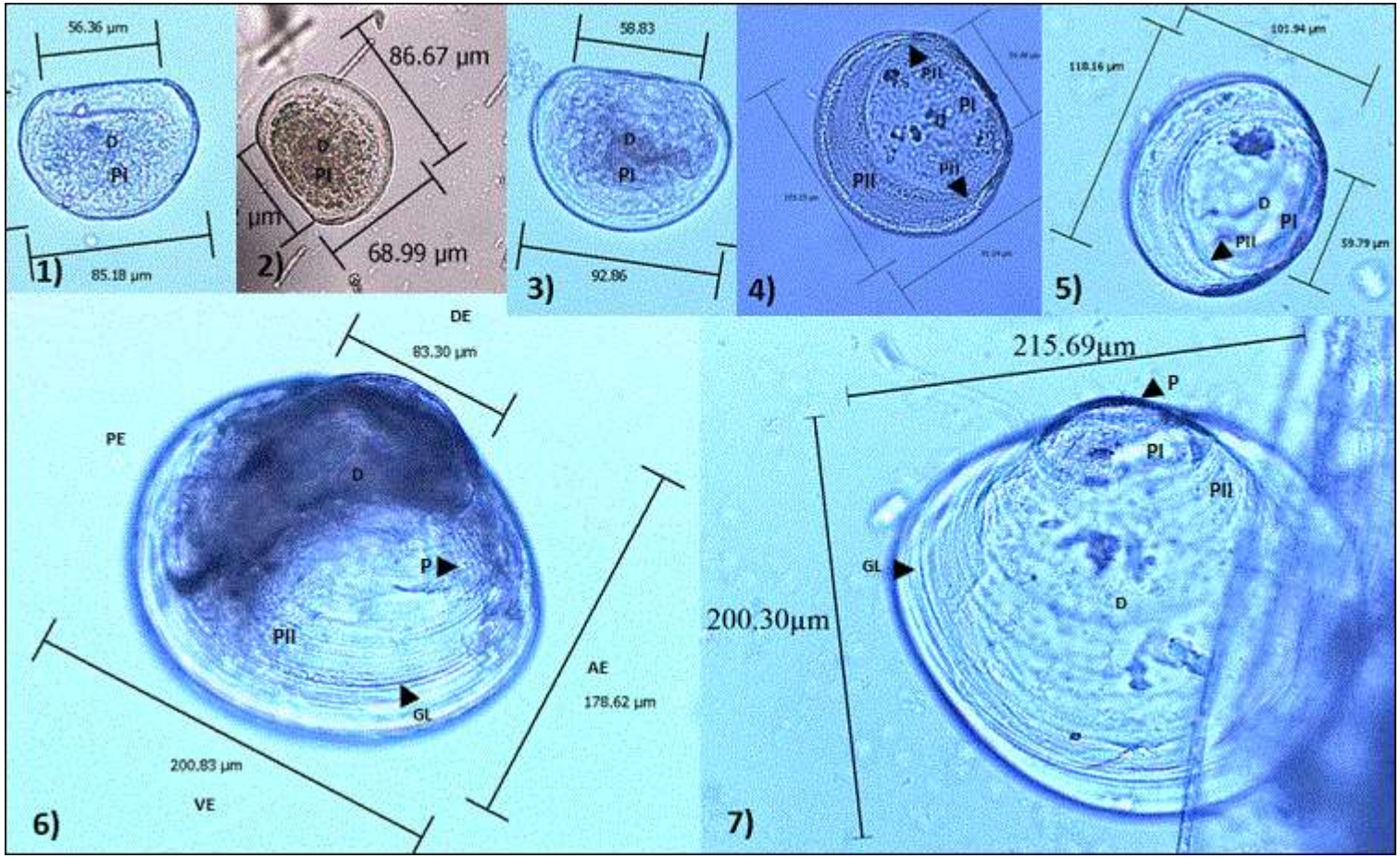


Plate 36: *Dreissena* sp. (D) larvae in various stages of development. The prodissoconch I (PI) is the first larval shell and lacks growth lines (1-3). The PI shell is 'D' shaped and is secreted by the shell gland. The surface of the PI shell is pitted (P) or smooth. Once the veliger develops the mantle, additional shell growth is added to the shell margin in commarginal or concentric growth lines (GL); this growth line region characterizes the prodissoconch II shell (4, 5, 6, 7) and delineates the PI boundary (4, 5). As the shell continues to grow, the PI is retained in the umbo area of the shell, i.e., the oldest part of shell, and the boundary between the PI and prodissoconch II can be seen (4, 5, 7). The surface of the prodissoconch II shell can appear slightly pitted (6), but is best characterized by commarginal or concentric growth lines (7). The dorsal (DE), ventral (VE), posterior (PE) and anterior ends (AE) of the veliger are marked (6).

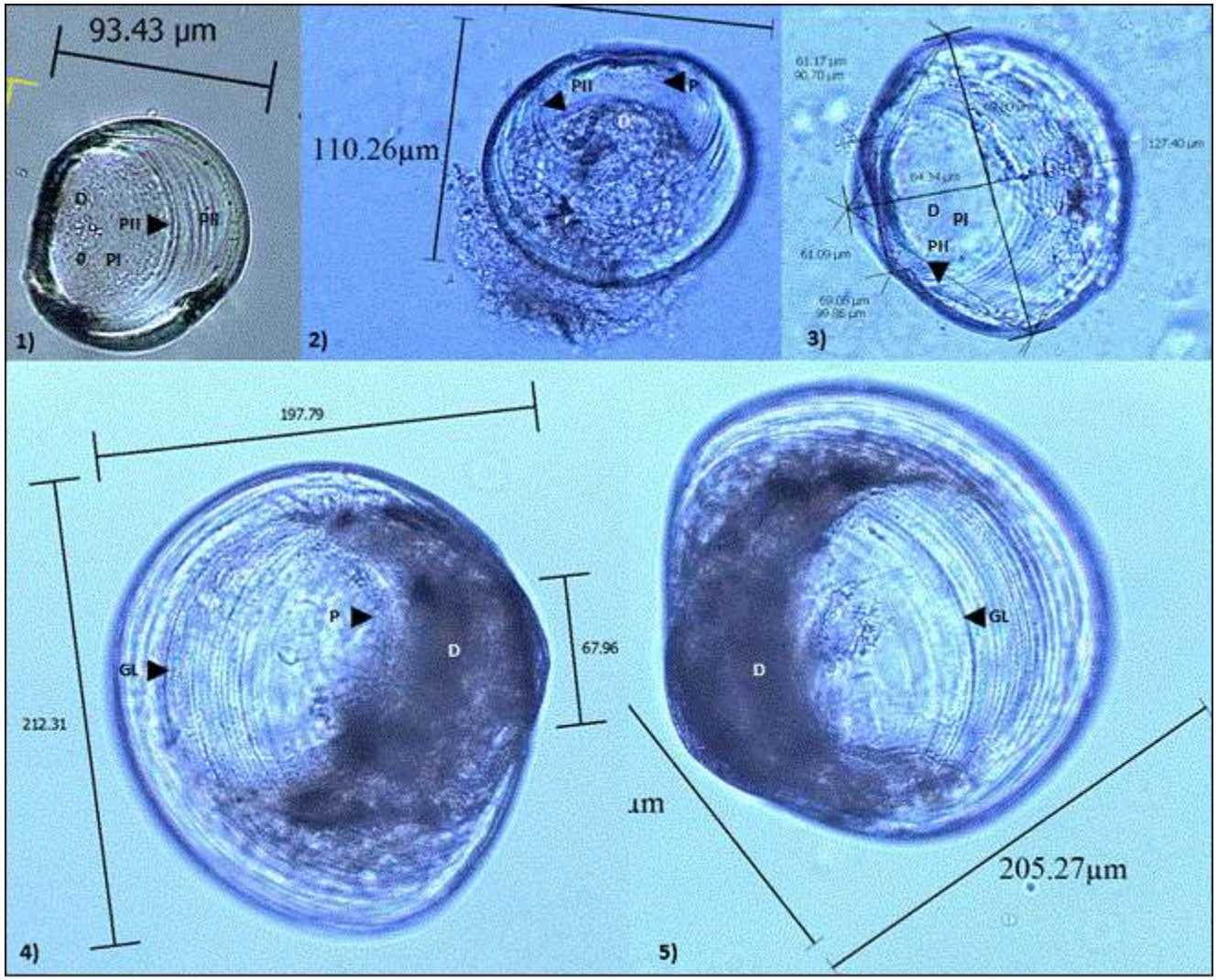


Plate 37: *Dreissena* sp. (D) larvae showing the prodissoconch I (PI) and prodissoconch II (PII) shell regions and surface appearance (1 - 5). The boundary between the prodissoconch I and prodissoconch II regions is shown (1, 2, 3), as well as shell pitting (P) (2, 4), and commarginal or concentric growth lines (GL) (1-5).

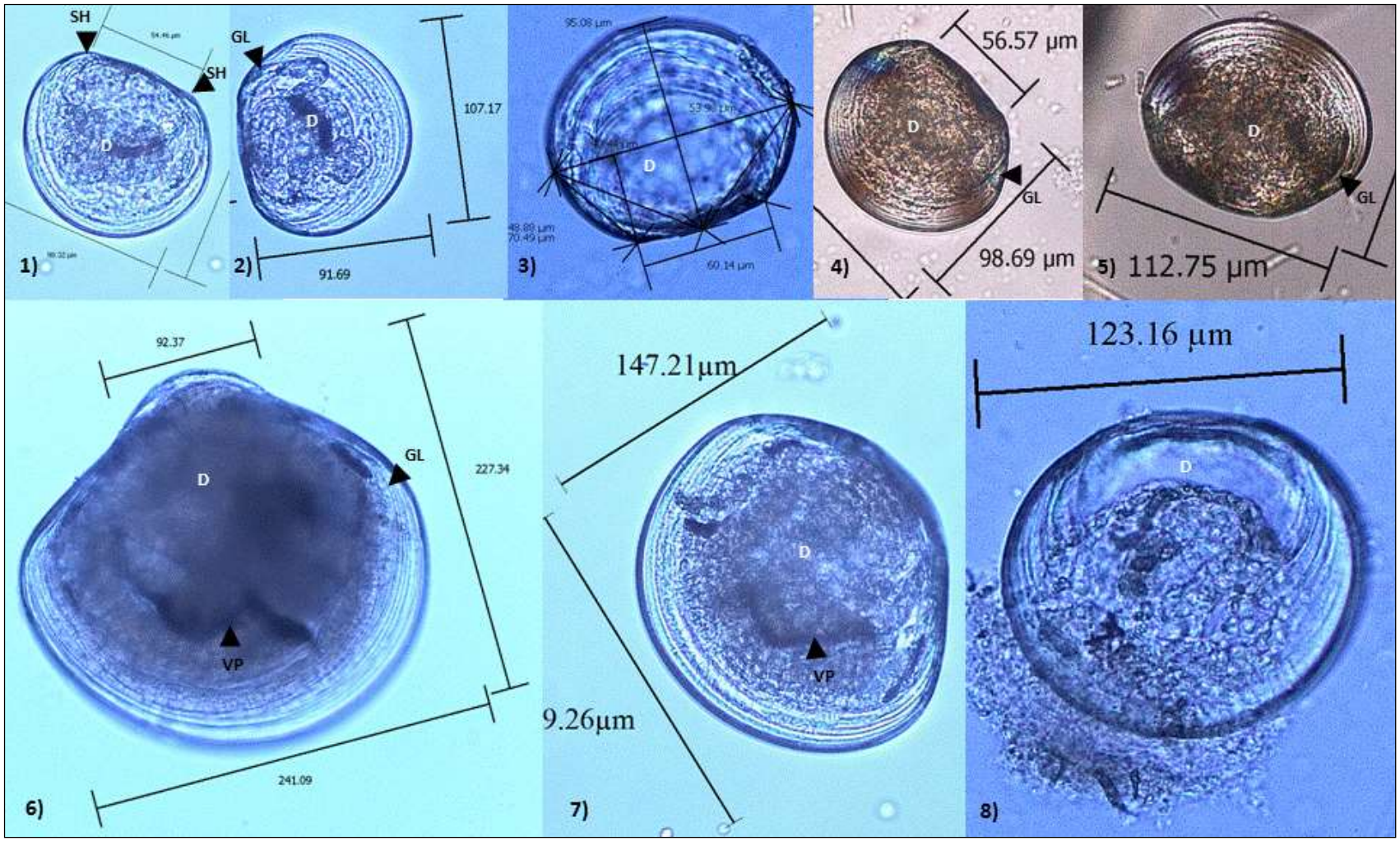


Plate 38: *Dreissena* sp. (D) larvae in various stages of development showing the variability in the appearance of the shell surface. Internal tissues may confound visibility of shell surface features and appear similar to pitted shell surface (1, 2, 4, 5, 7). Commarginal or concentric growth lines (GL) of the prodissoconch II shell are seen along the shell margin and converge at shoulders (SH) (2, 3, 4, 5. 6, 7, 8). Internal tissues such as the velar pigment (VP) may be visible (6, 7).

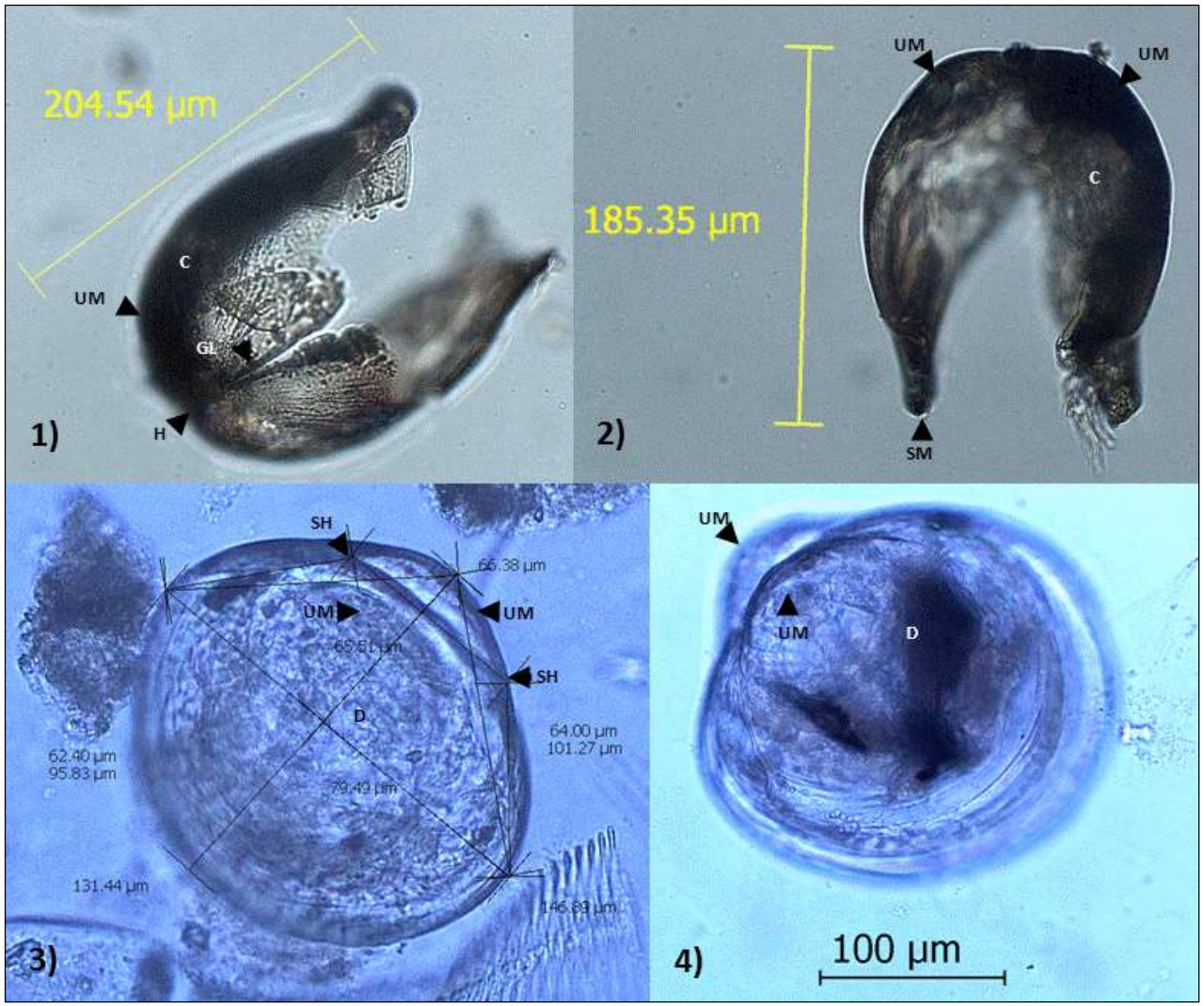


Plate 39: *Corbicula* sp. (C) straight-hinge juveniles in the anterior/posterior view (1,2), and *Dreissena* sp. (D) veligers in the dorsolateral view (3,4) showing shell margins (SM), growth lines (GL) converging at the shoulders (SH), and the hinge area (H) located between the shoulders and umbones (UM) of both shell valves.

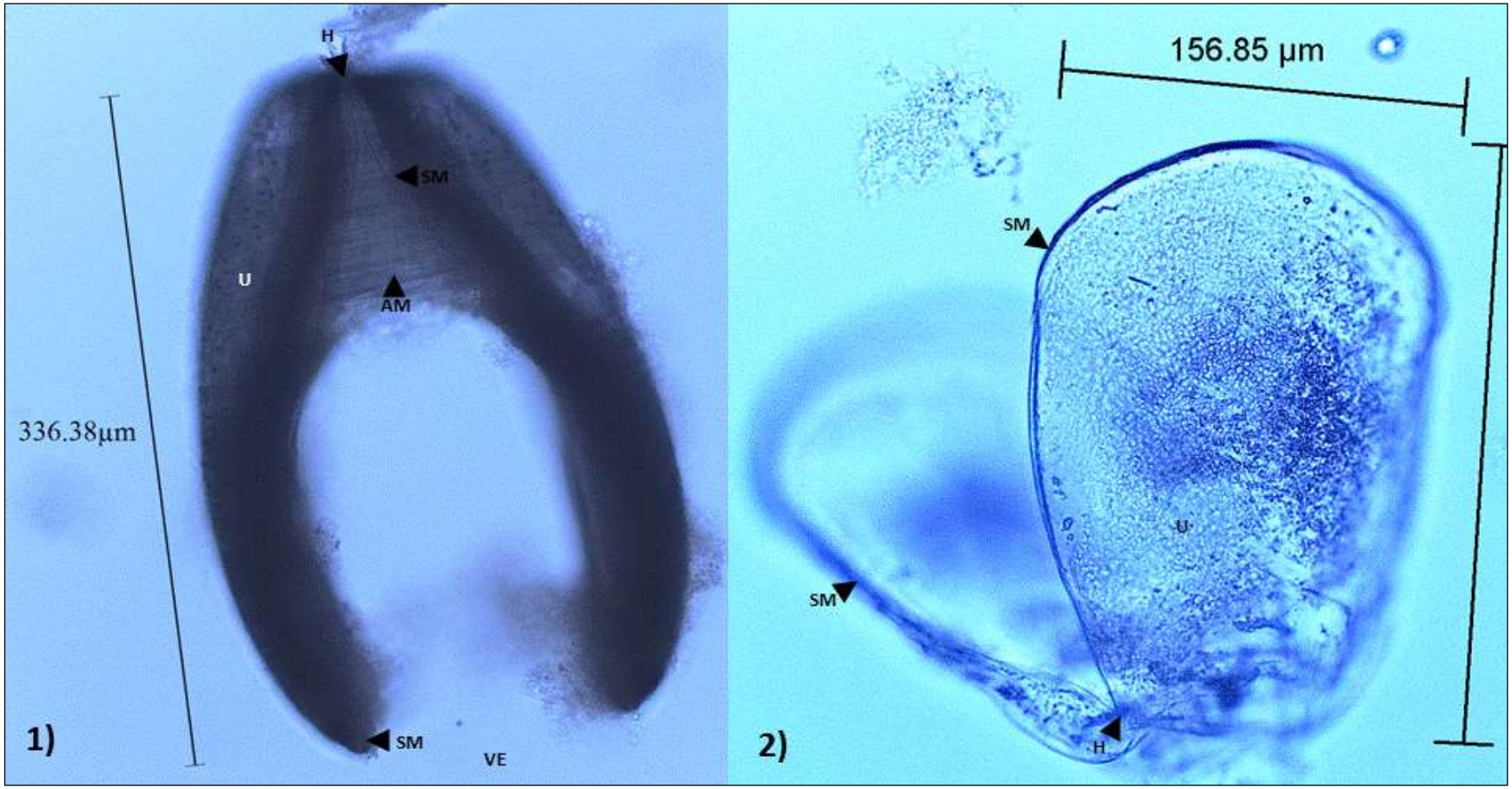


Plate 40: Unionid (U) bivalve larvae in the anterior/posterior view (1), and the dorsolateral view (2) showing shell margins (SM) of both shell valves and hinge area (H). The adductor muscle (AM) is visible (1). Specimens are courtesy of Robert G. Howells.

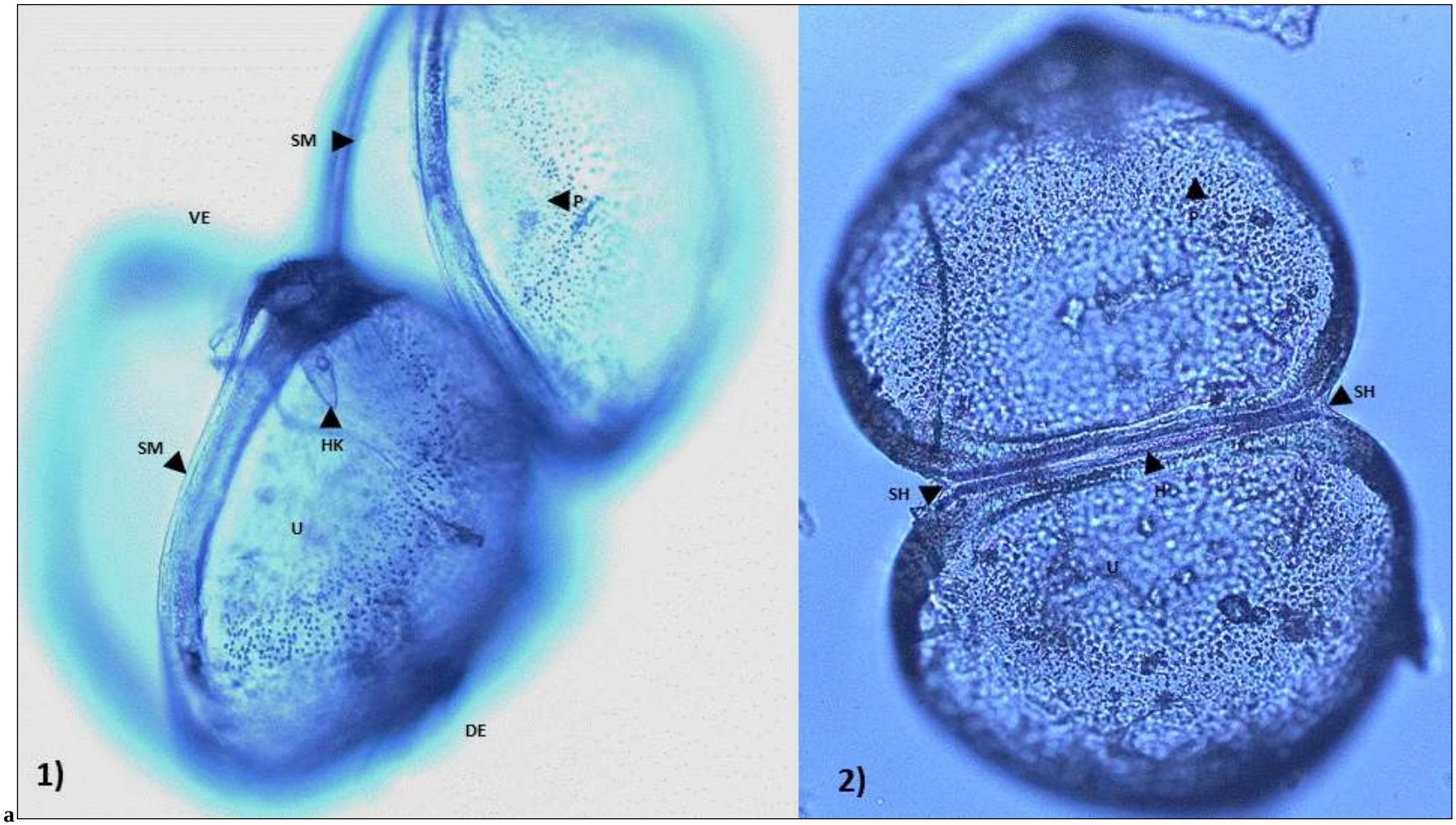
a 

Plate 41: *Anodonta* sp. unionid (U) bivalve larvae in the somewhat ventrolateral view (1) and dorsal view (2) showing two symmetrical valves that are hinged together. The styliform hook (HK) on the ventral shell margin (SM) is visible (1) as is the hinge line (H) between the shoulders (SH) (2). Note the triangulate to subtriangulate overall shape.

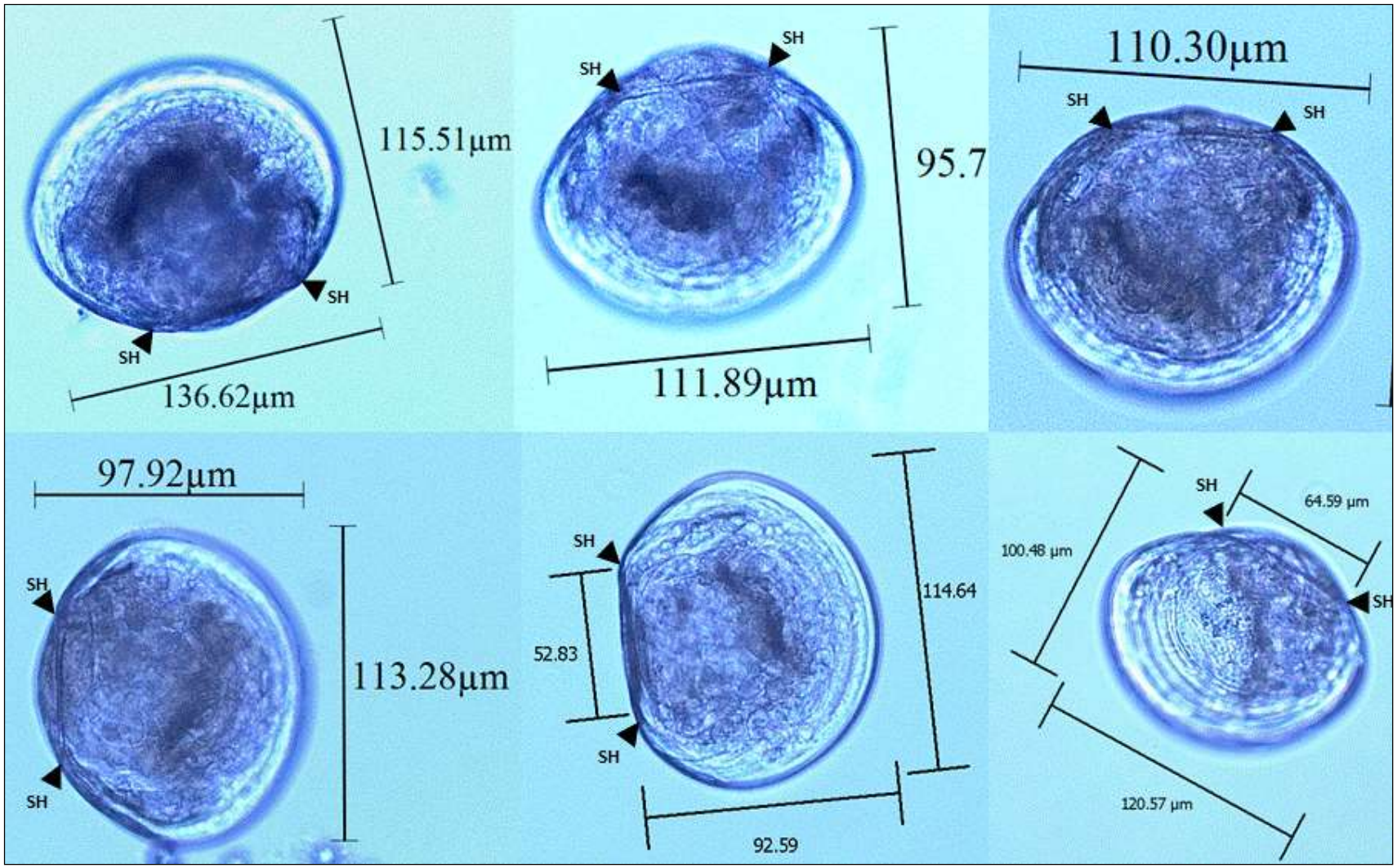


Plate 42: *Dreissena* sp. larvae in the somewhat dorsolateral view showing the hinge area located between the shoulders (SH).

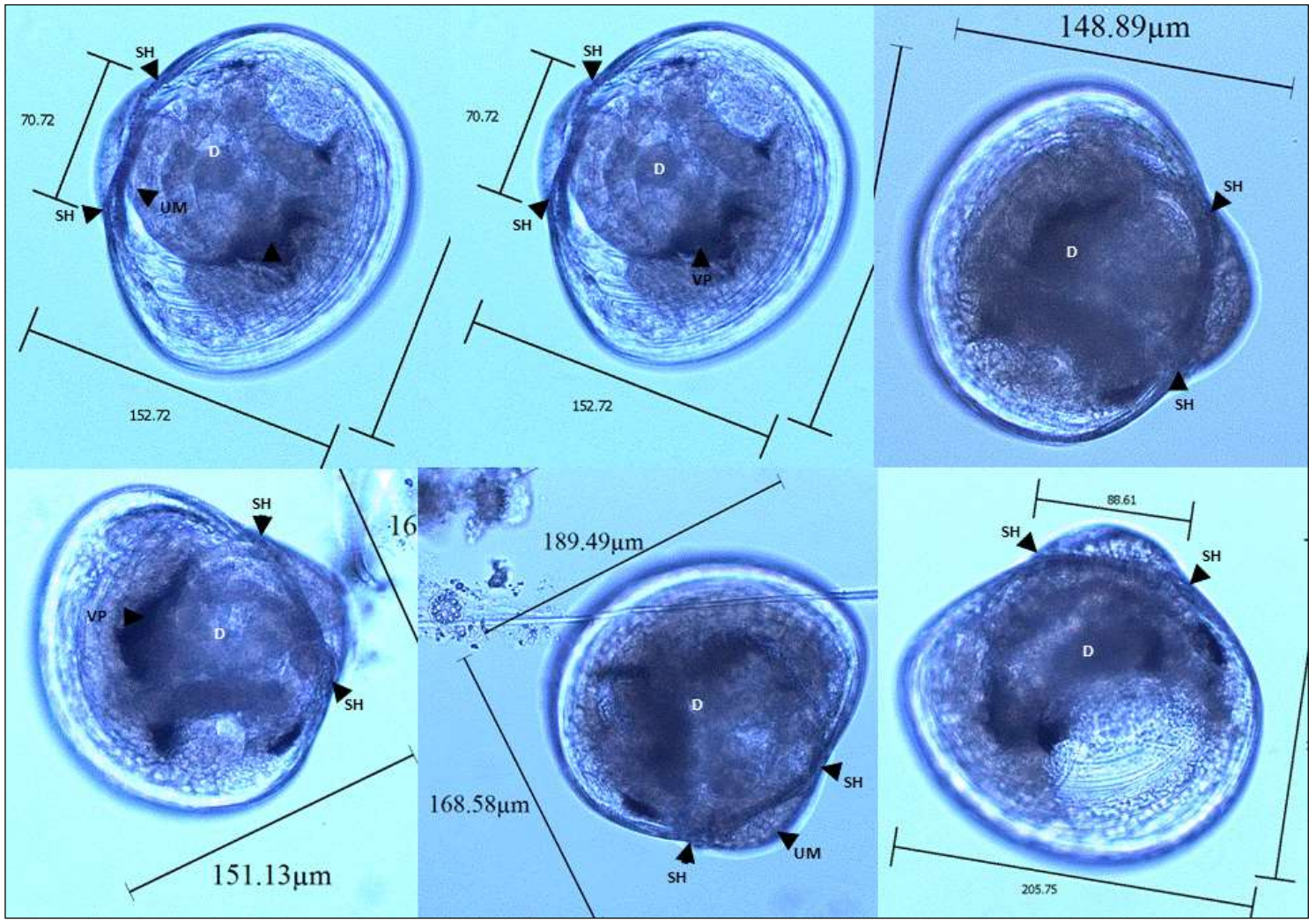


Plate 43: *Dreissena* sp. (D) larvae in the somewhat dorsolateral view showing the hinge area located between shoulders (SH) and developing umbones (UM). The velar pigment is visible (VP).

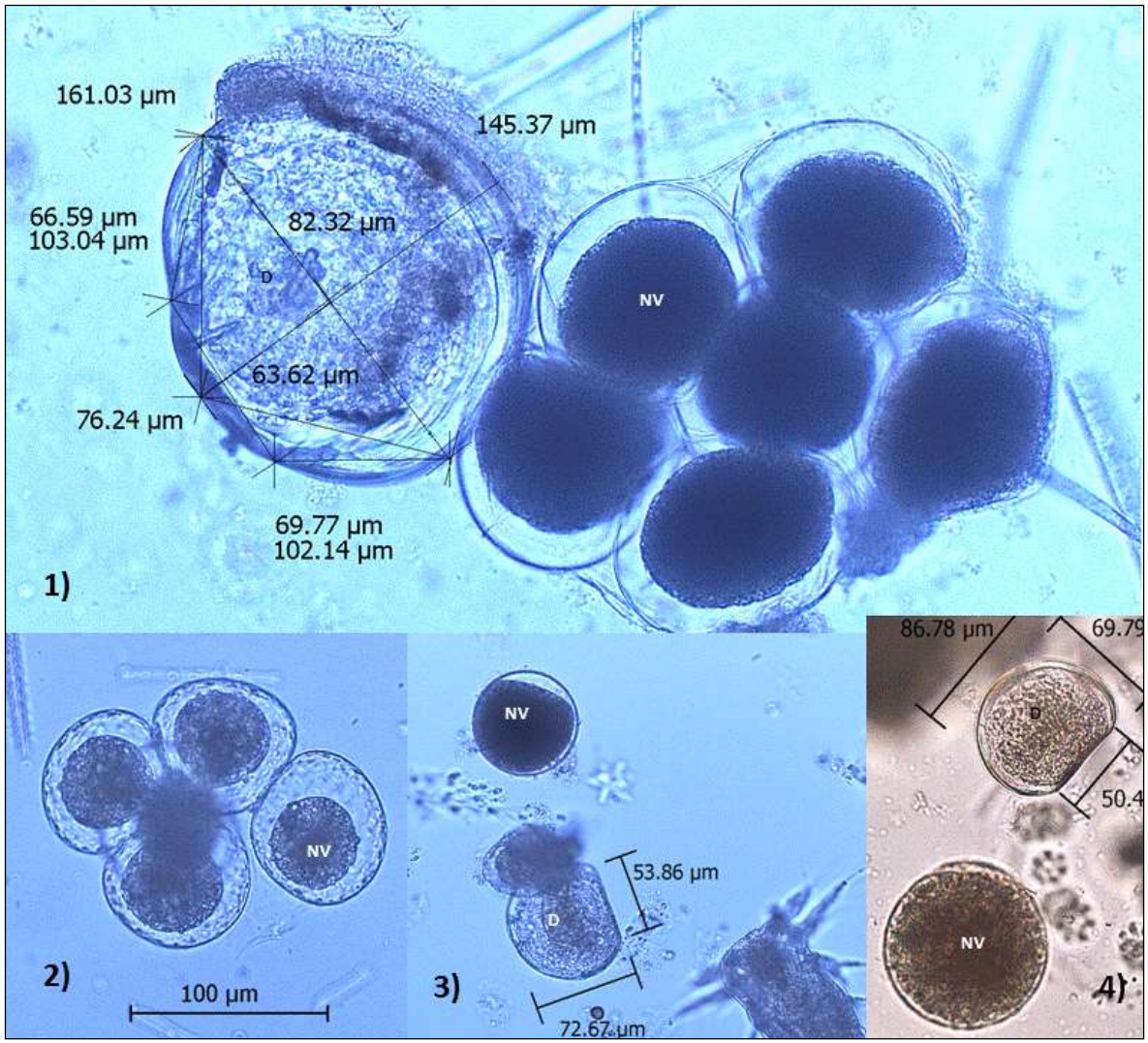


Plate 44: ***Dreissena*** **sp. (D) larvae (1, 3, 4) with unidentified objects that are not veligers (NV) (1, 2, 3, 4). These non-veliger objects have a circular or spherical shape, and one continuous outer surface that is not divided into halves. Bivalve larvae tissues, in general, are distributed throughout shells and extend towards the anterior, posterior and ventral margins instead of being concentrated in the middle like a cell nucleus.**

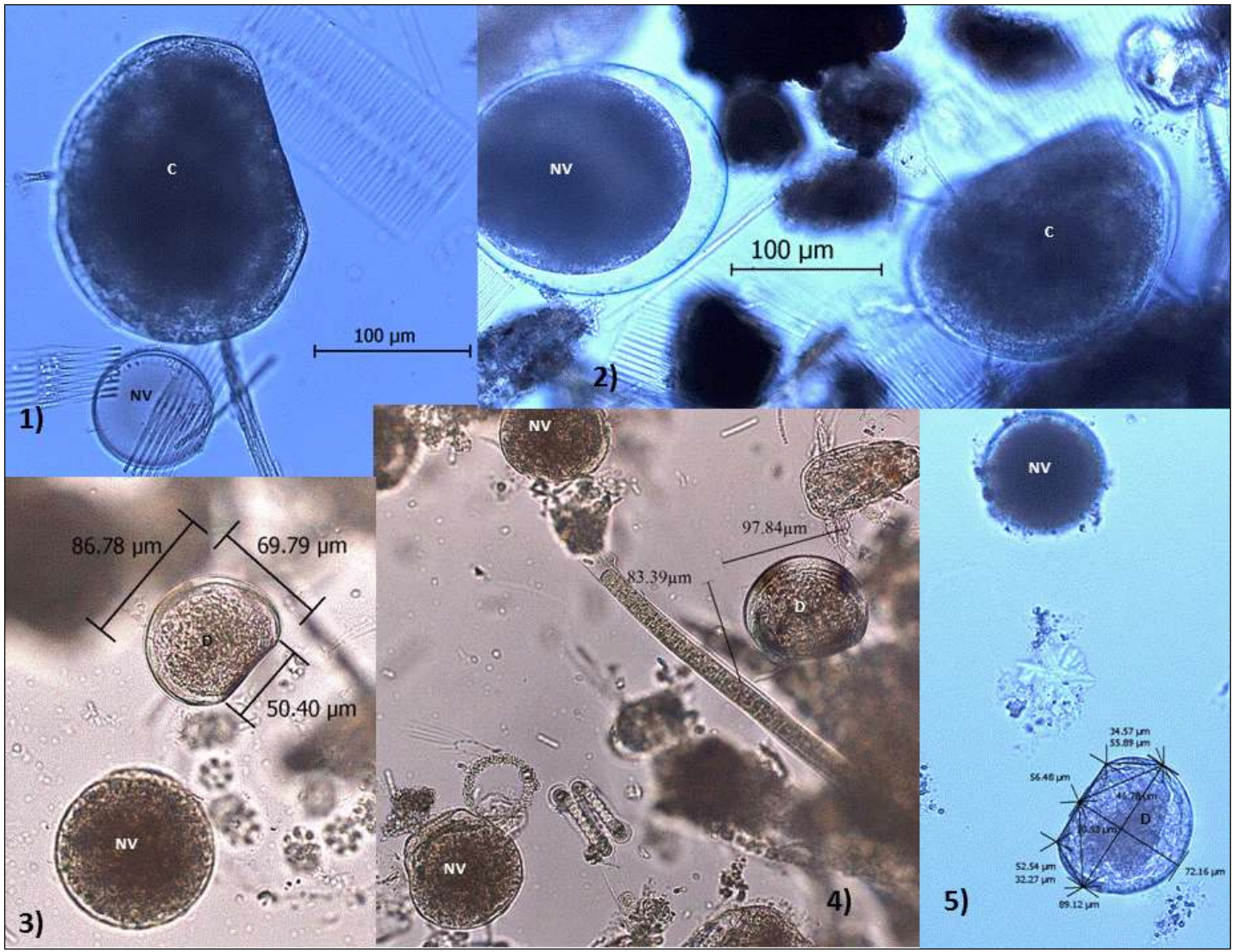


Plate 45: *Corbicula* sp. (C) straight-hinge juveniles (1,2) and *Dreissena* sp. (D) larvae (3, 4, 5) with unidentified objects that are not veligers (NV) (1 - 5). The non-veliger objects have circular shapes and continuous outer surfaces not divided into halves. Note that bivalve internal tissue is distributed throughout cell instead of concentrated in the middle like a cell nucleus (2).

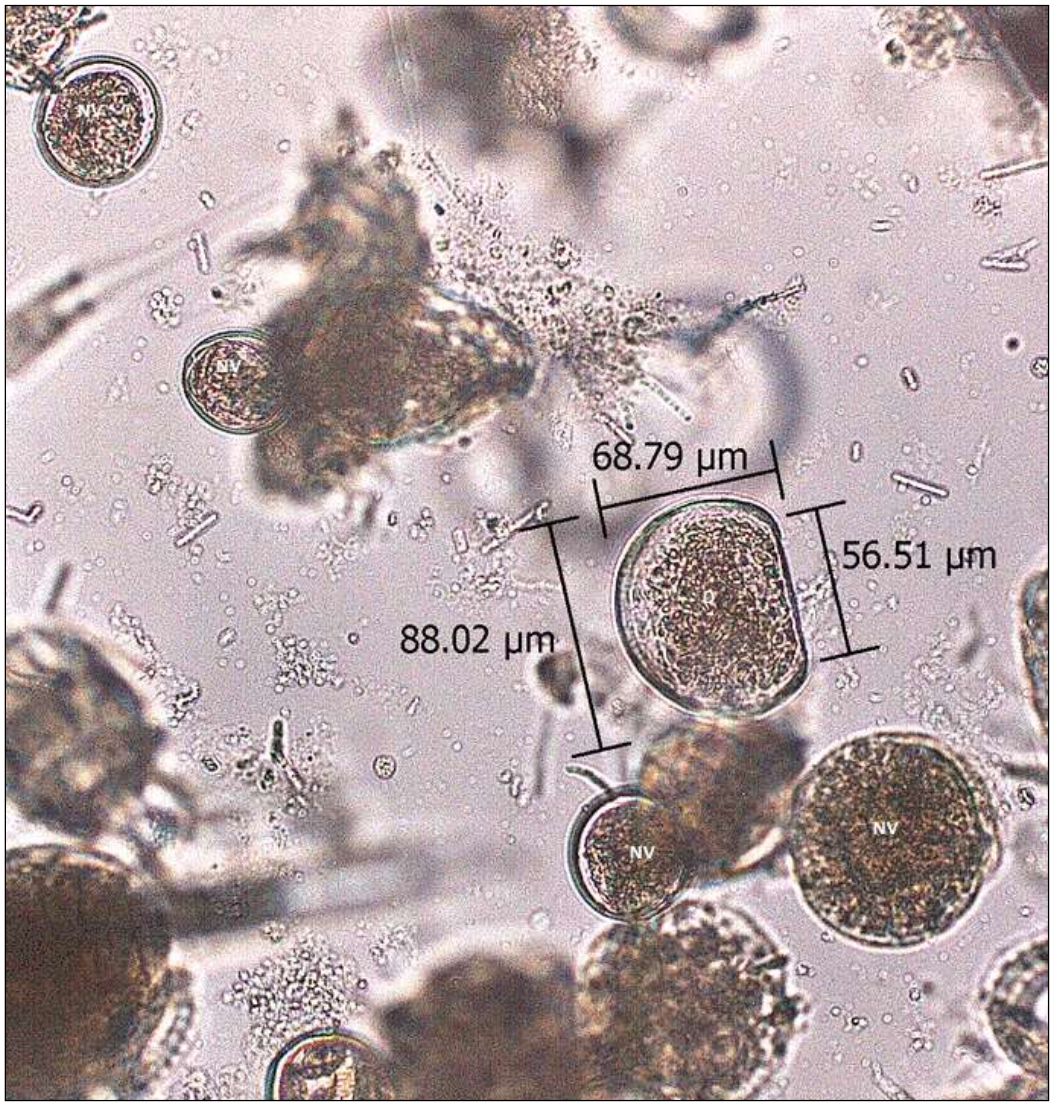


Plate 46: *Dreissena* sp. (D) larva with unidentified objects that are not veligers (NV). These non-veliger objects have a continuous outer surface and a circular shape. Some of these non-veliger objects also have a continuous hollow band around the entire peripheral margin.

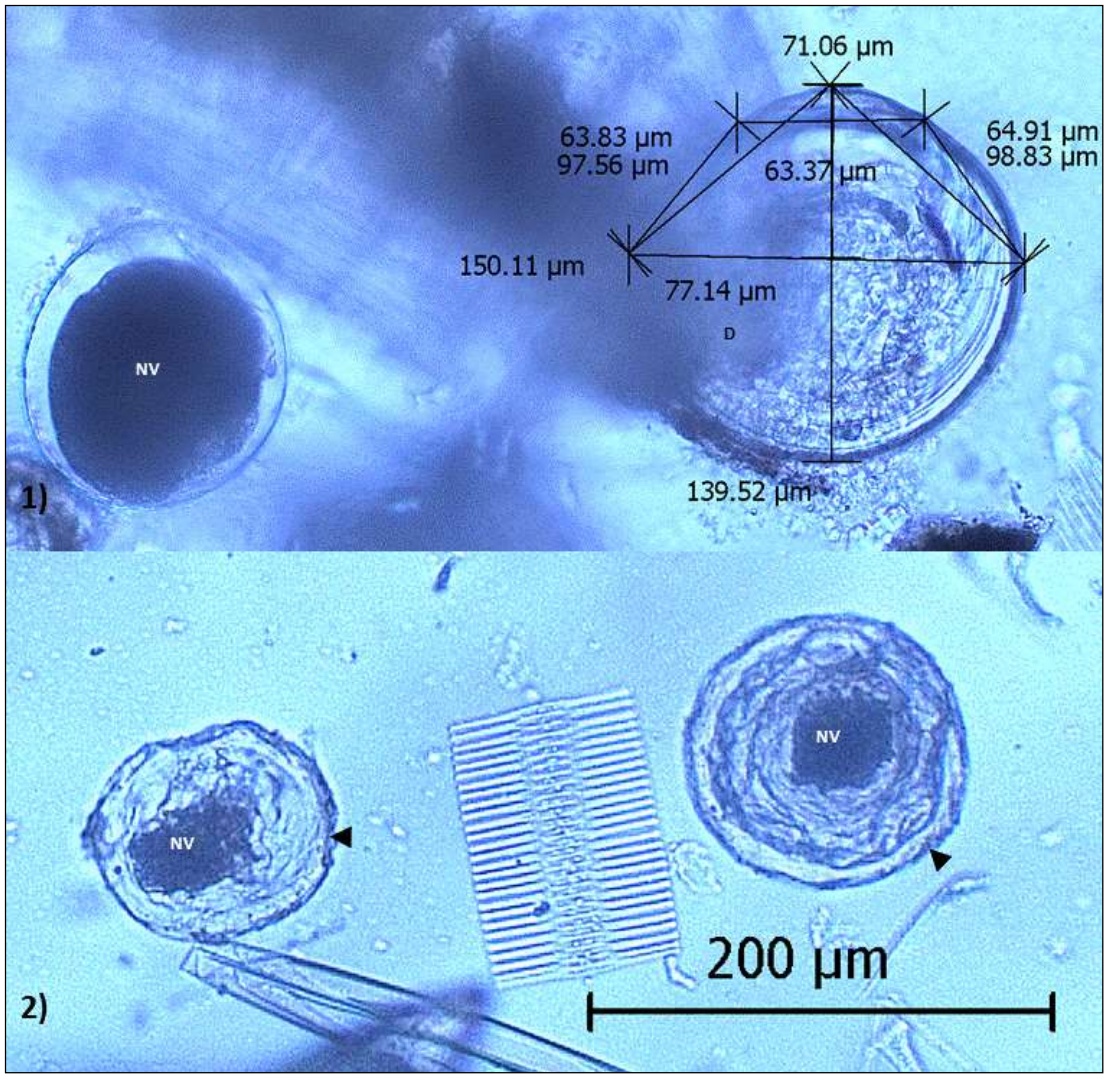


Plate 47: An unidentified non veliger (NV) with a circular shape and tissues concentrated in the center of the object is alongside a *Dreissena* sp. (D) larvae (1). Other non-veliger objects are D-shaped or circular, and have uneven, jagged, and indistinct margins, and tissues concentrated in the center like a cell nucleus (2).

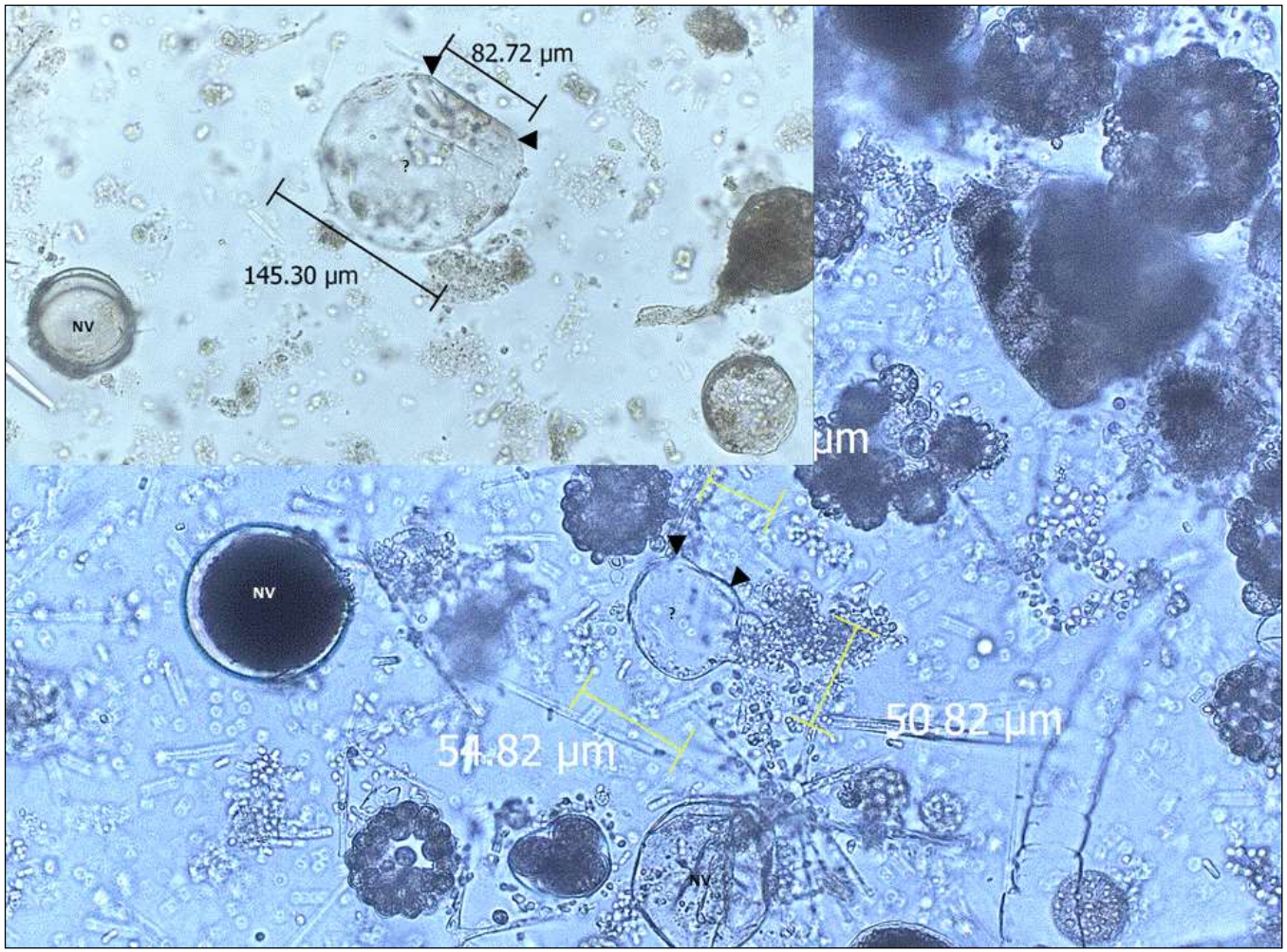


Plate 48: Unidentified objects that include non veligers (NV) and highly suspect objects (?).The non-veliger objects have continuous outer surfaces and are circular in shape. The highly suspect objects lack the morphology to identify, but have overall shapes and sizes that are similar to straight-hinge *Dreissena* sp. larvae, including apparent shoulders (marked) and continuous smooth-edged margins. The suspect objects, however, lack internal tissues and shell features needed to corroborate a definitive identification. In samples with the highly suspect objects, efforts would focus on finding additional specimens.

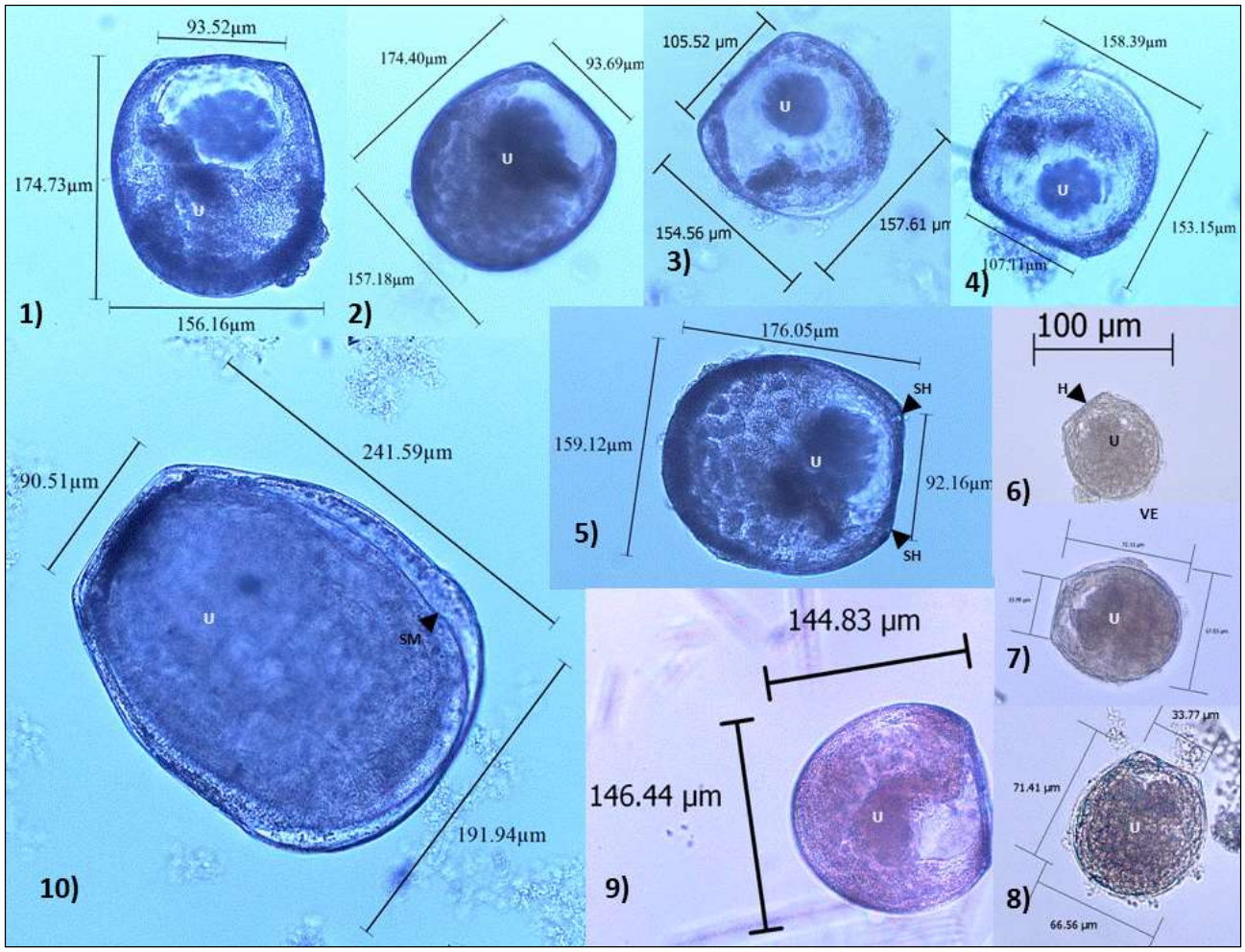


Plate 49: Unionid (U) bivalve larvae that are 'D' shaped and appear similar to *Dreissena* sp. and other planktotrophic bivalve larvae. Unionid mussels include *Cyrtonaias* sp. (1, 2, 3, 4, 5), *Quadrula* sp. (6, 7, 8), *Gonidea* sp. (9), and *Lampsilis* sp. (10). Unionid mussel larvae, however, differ from planktotrophic straight-hinge larvae in shell dimensions. In cases where Unionid glochidial larvae appear D-shaped, the shell length is less than the shell height or close to equal, and hinge length may be greater than 70-µm. Planktotrophic 'D' shaped *Dreissena* sp. larvae in North America have a shell length greater than shell height and hinge length less than 70-µm.

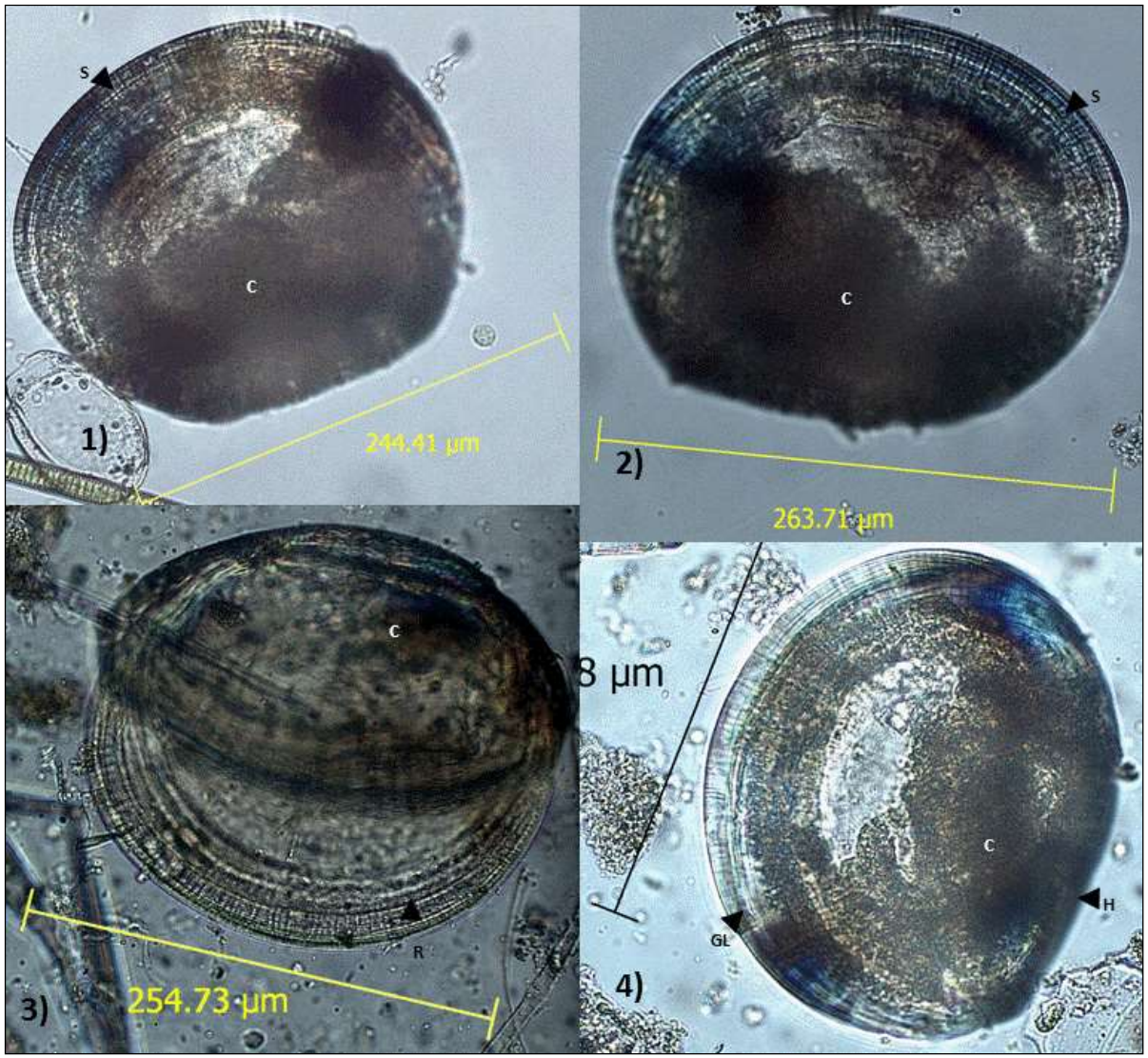


Plate 50: *Corbicula* sp. (C) straight-hinge juveniles in the lateral view (1, 2, 4) and ventrolateral view (3) showing commarginal or concentric growth lines (GL) and perpendicular secondary lines (S). Prominent ridges are also visible (R).

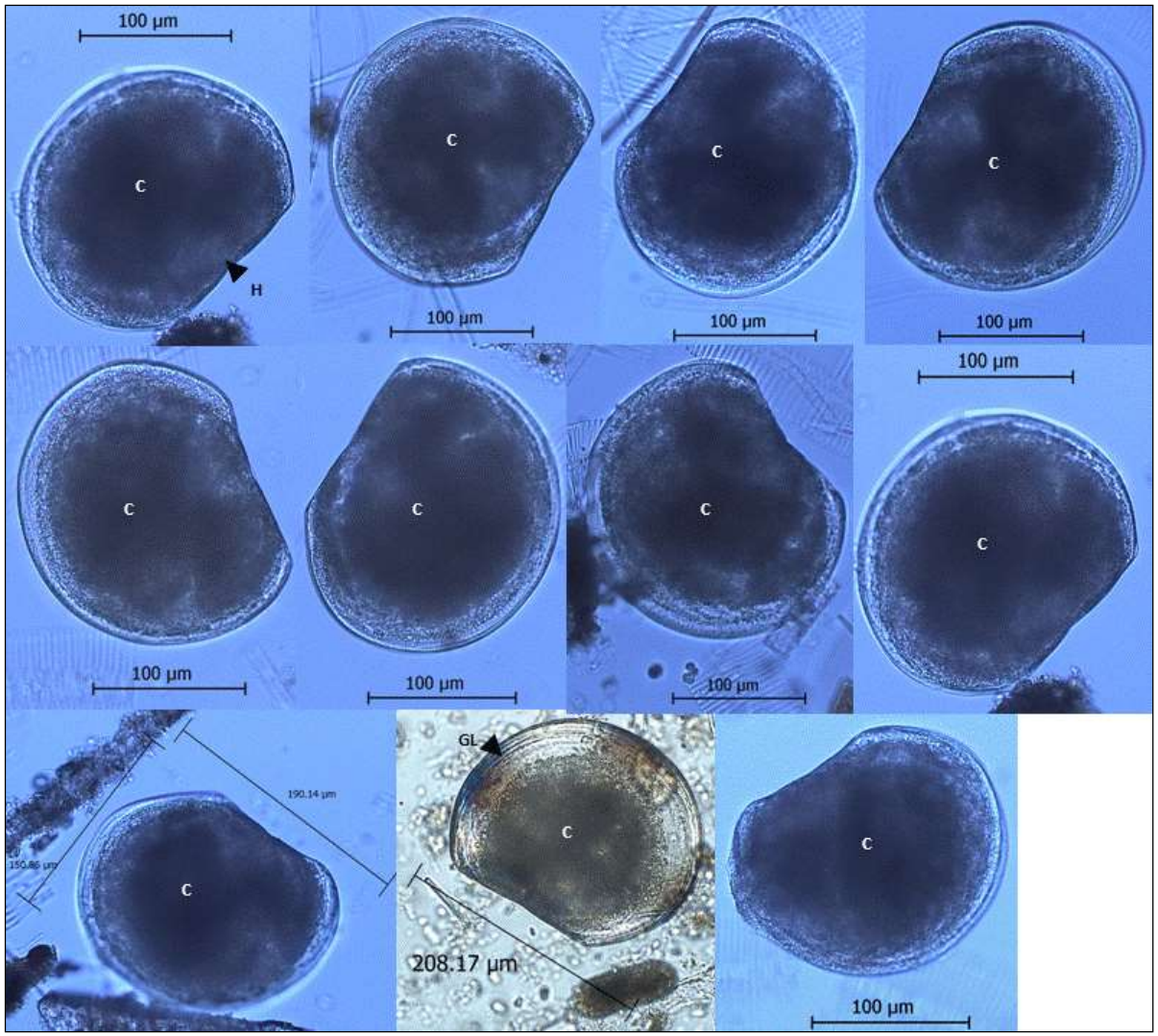


Plate 51: *Corbicula* sp. (C) showing sway-back hinges (H). Some reports have indicated that *Corbicula* sp. with a sway-back hinge are younger and still possess a velum as well as a foot. The growth lines (GL) on many of these bivalves are not visible.

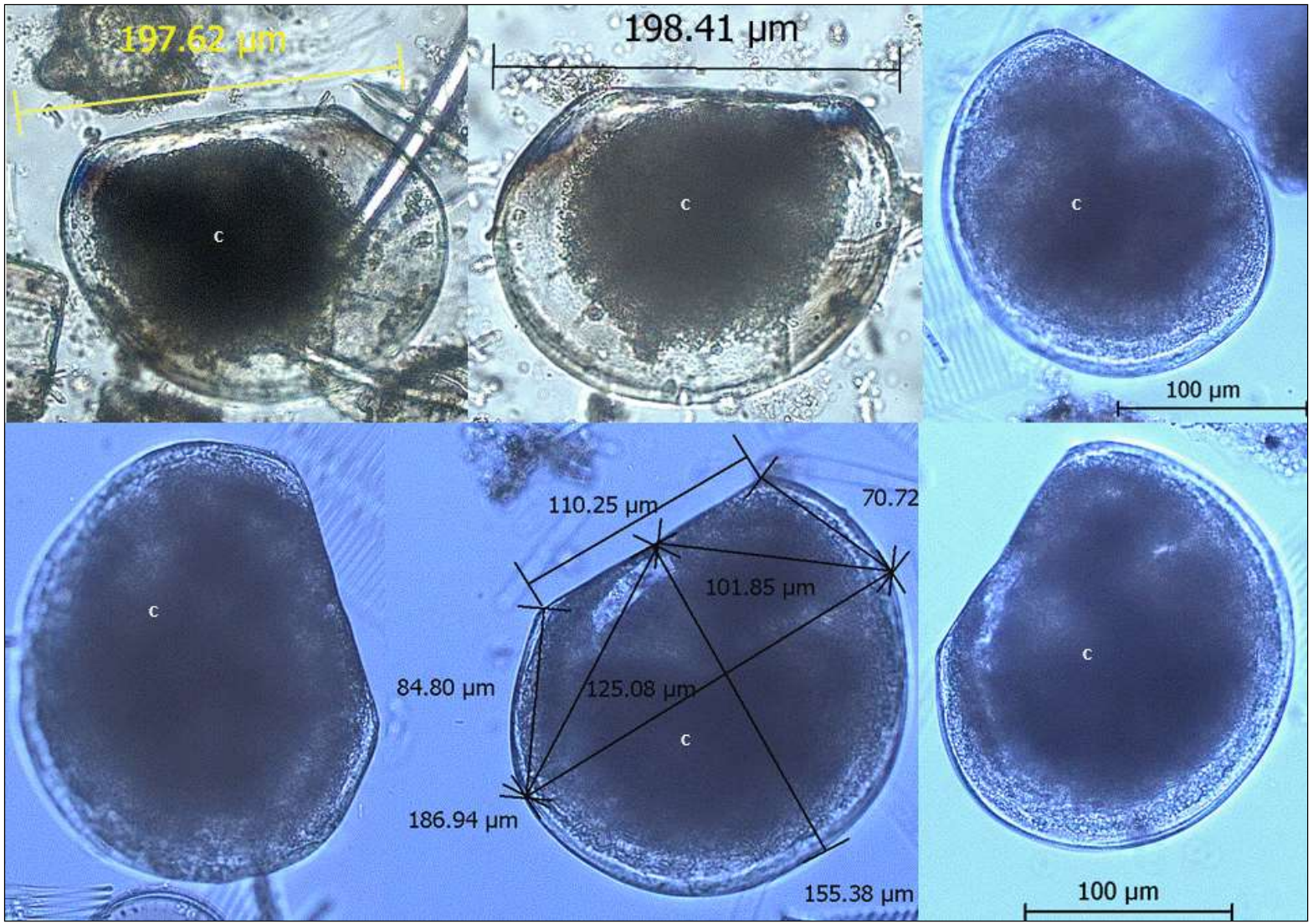


Plate 52: *Corbicula* sp. (C) straight-hinge juveniles that appear stretched so that the anterior and posterior ends are not symmetrical.

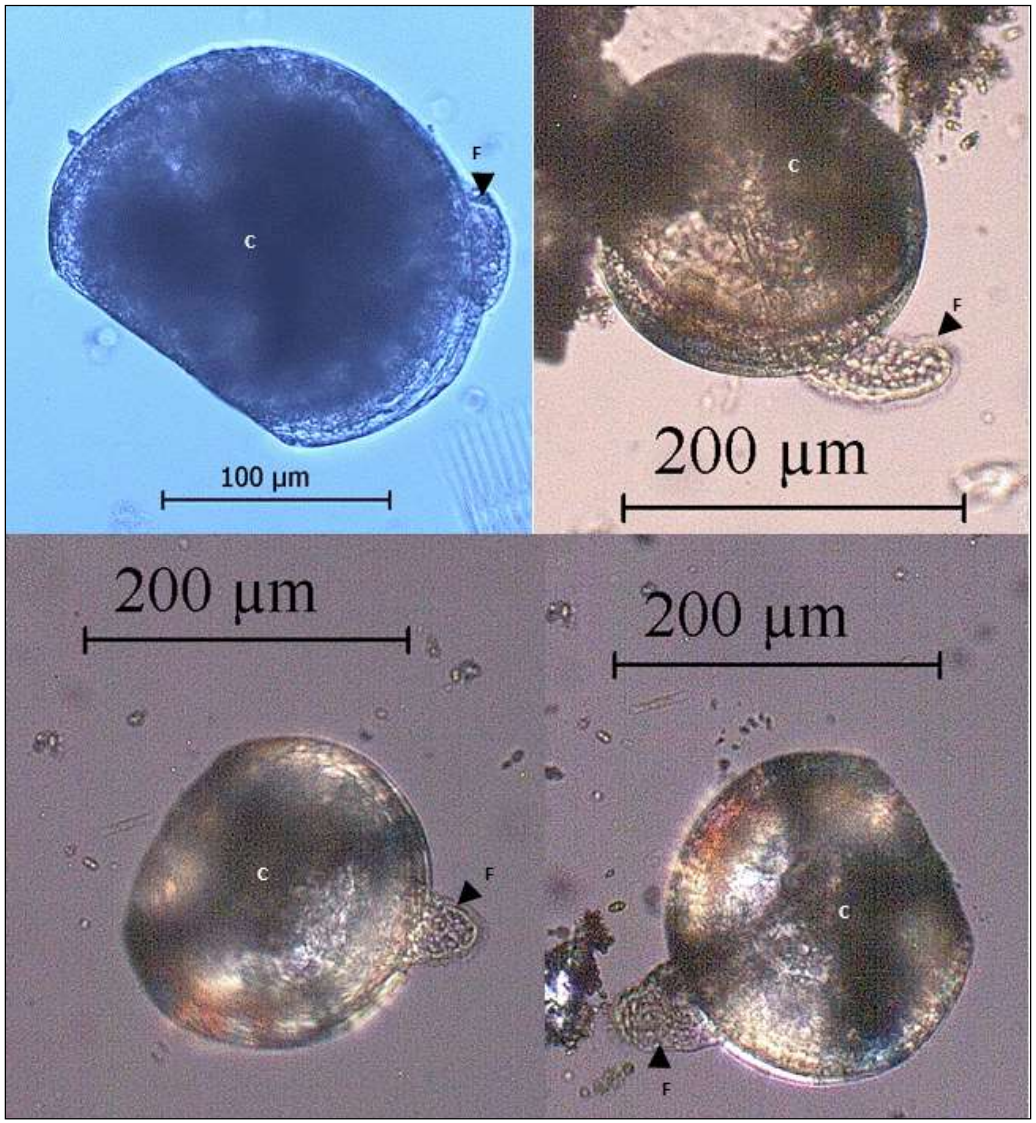


Plate 53: *Corbicula* sp. (C) straight-hinge juveniles with foot (F) protruding beyond shell margin.

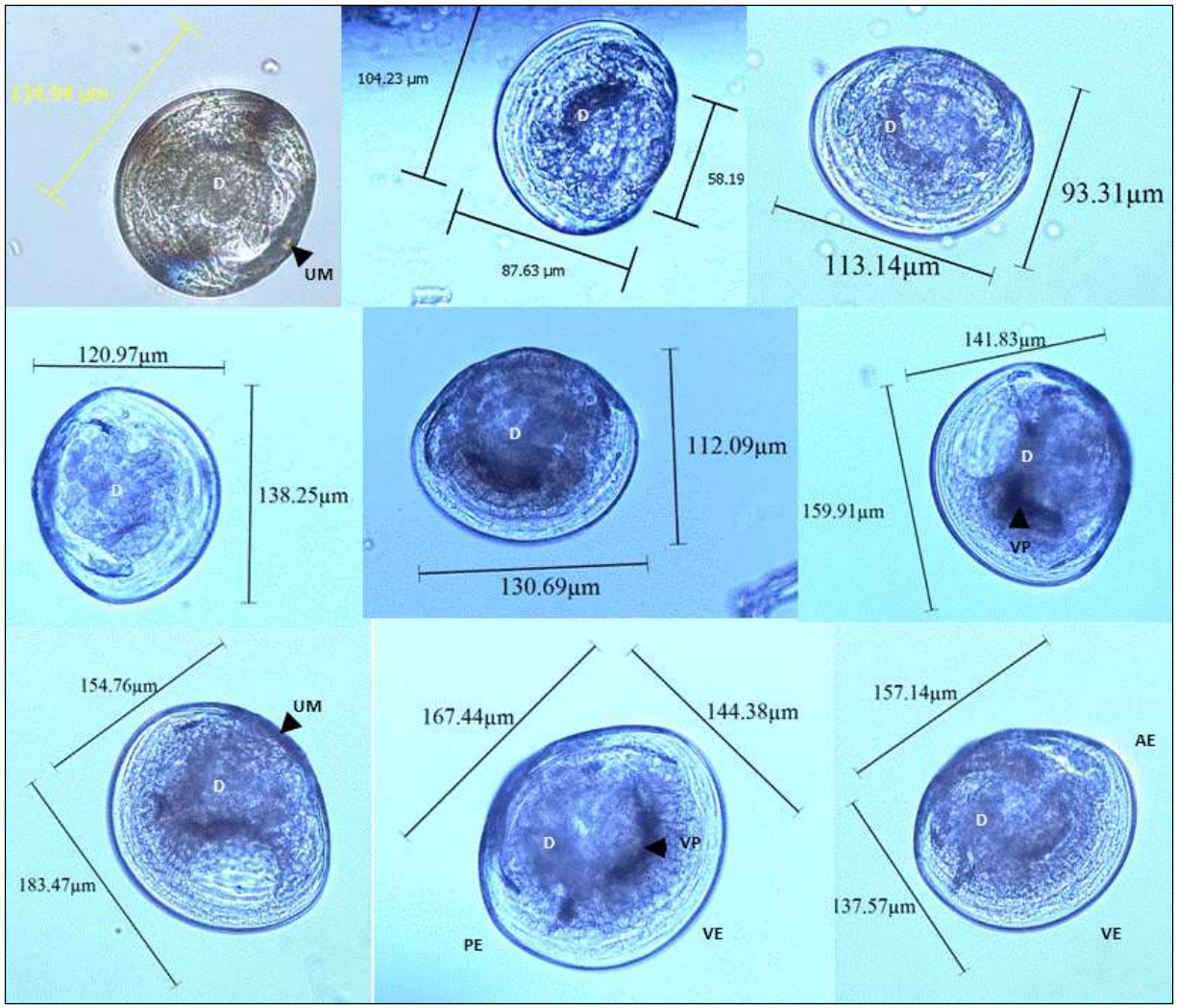


Plate 54: *Dreissena* sp. (D) larvae in the lateral view that are round, orbicular to oval shaped with a developing umbo (UM) that is low, rounded, and does not protrude beyond the shell line. The posterior (PE) and anterior ends (AE) of these larvae are relatively symmetrical. Velar pigment (VP) is visible.

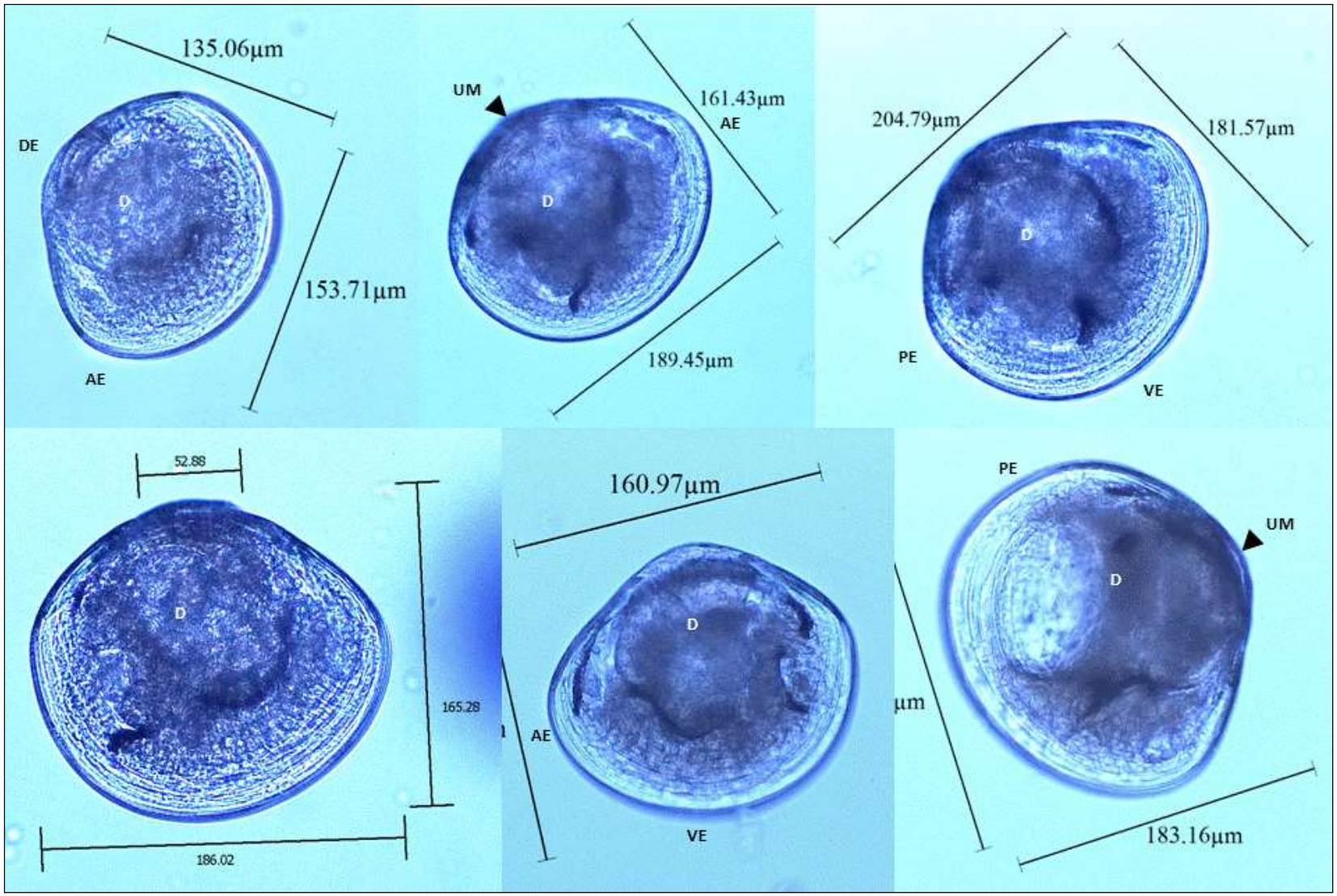


Plate 55: *Dreissena* sp. (D) larvae in the lateral view with an oval to roundish shape, asymmetrical anterior (AE) and posterior ends (PE), and low developing umbo (UM) just beginning to protrude beyond shell line. Dorsal (DE) and ventral ends (VE) are indicated.

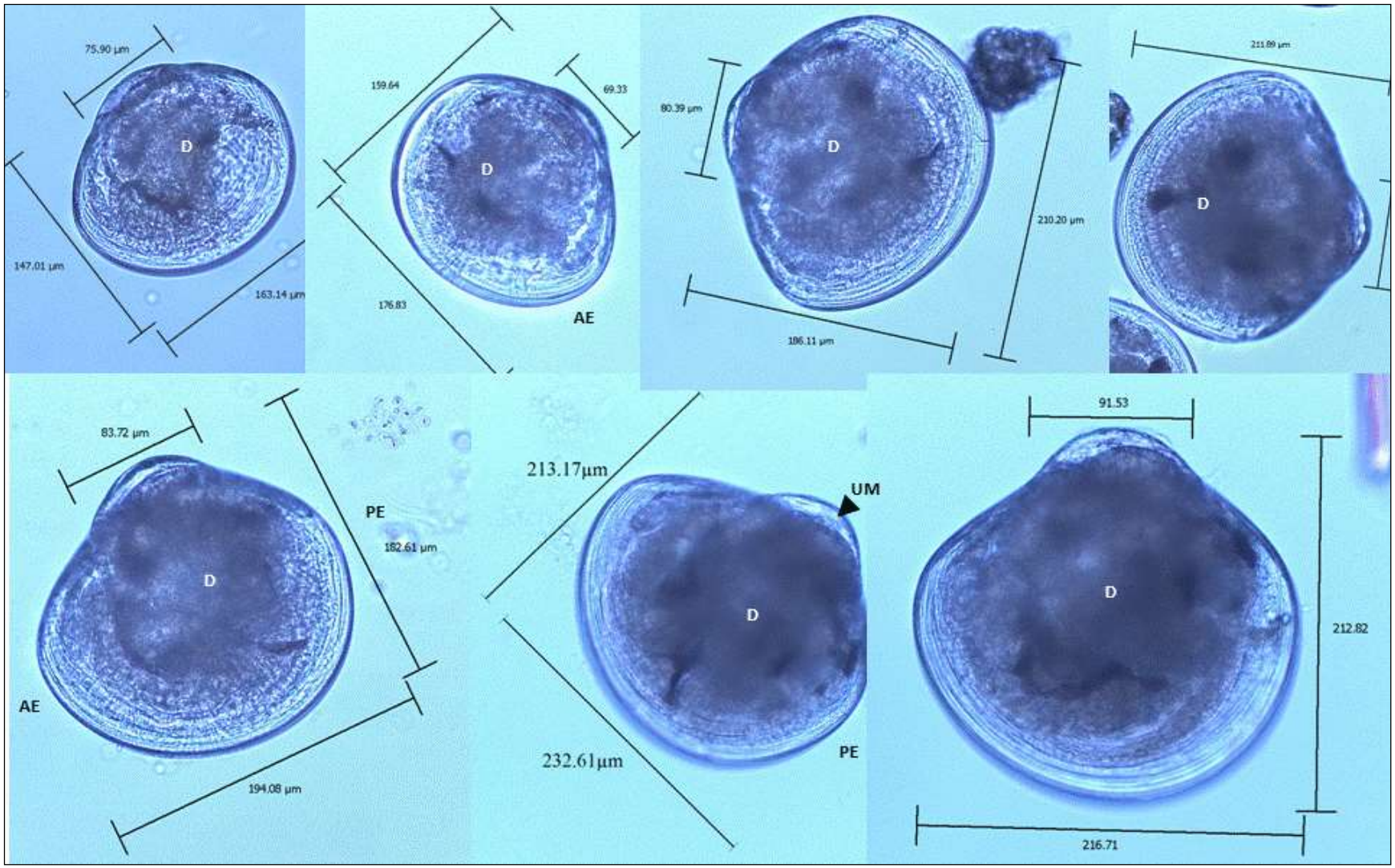


Plate 56: *Dreissena* sp. (D) larvae in the lateral view with an oval to roundish shape, asymmetrical anterior (AE) and posterior ends (PE), and a knobby umbo (UM) that protrudes beyond shell line.

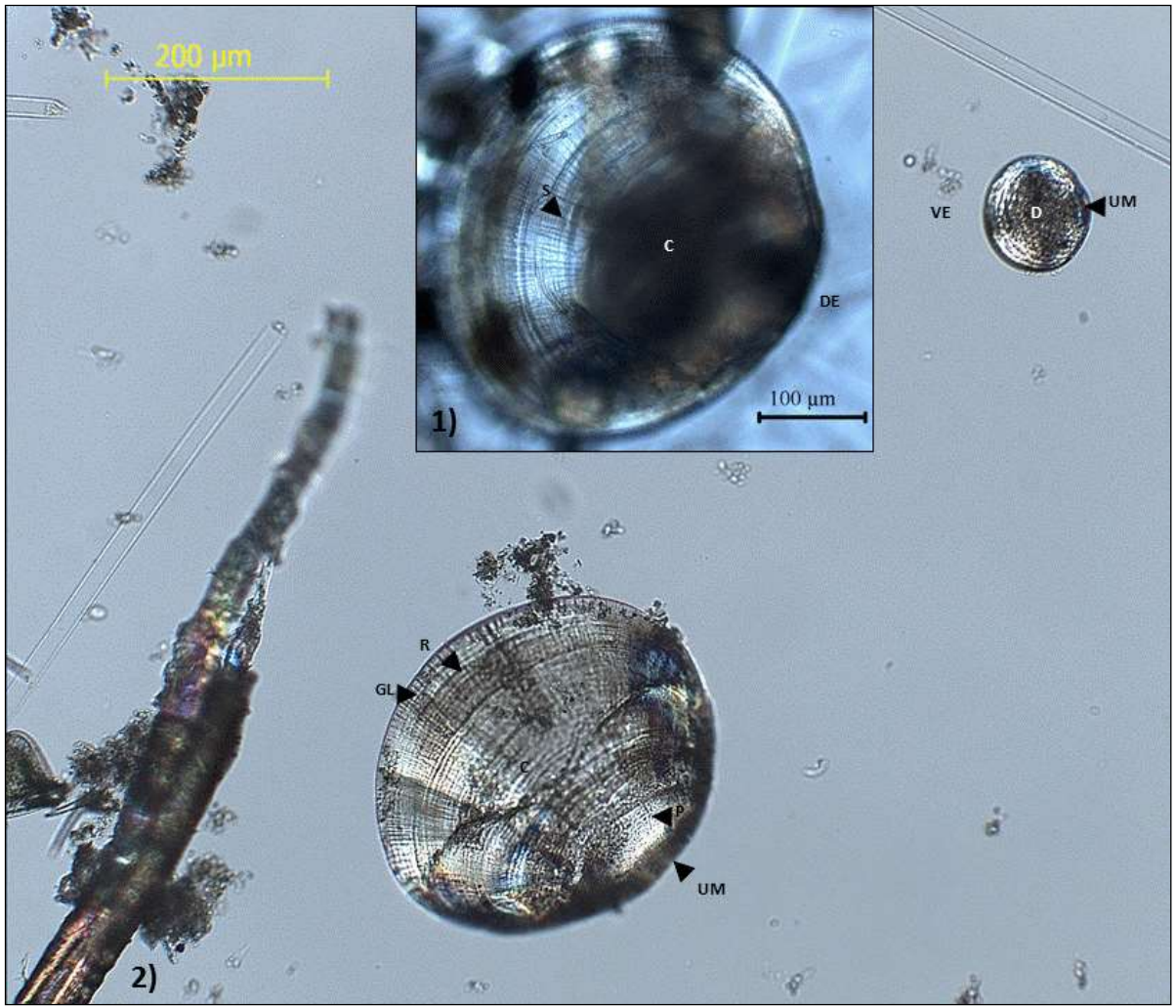


Plate 57: *Corbicula* sp. (C) juveniles (1, 2) in comparison to a *Dreissena* sp. (D) larva in the early umbonal larval stage. *Corbicula* sp. juveniles have clearly visible growth lines (GL), ridges (R), and secondary lines (S) that are perpendicular to growth lines. Both types of bivalves have low, rounded umbones (UM) that do not protrude beyond shell line in the dorsal end (DE) of veliger. The pitted (P) surface of the prodissoconch I shell is visible on the *Corbicula* sp. juvenile (2). Note the size difference, however, between *Corbicula* sp. juveniles and the *Dreissena* sp. umbonal larva.

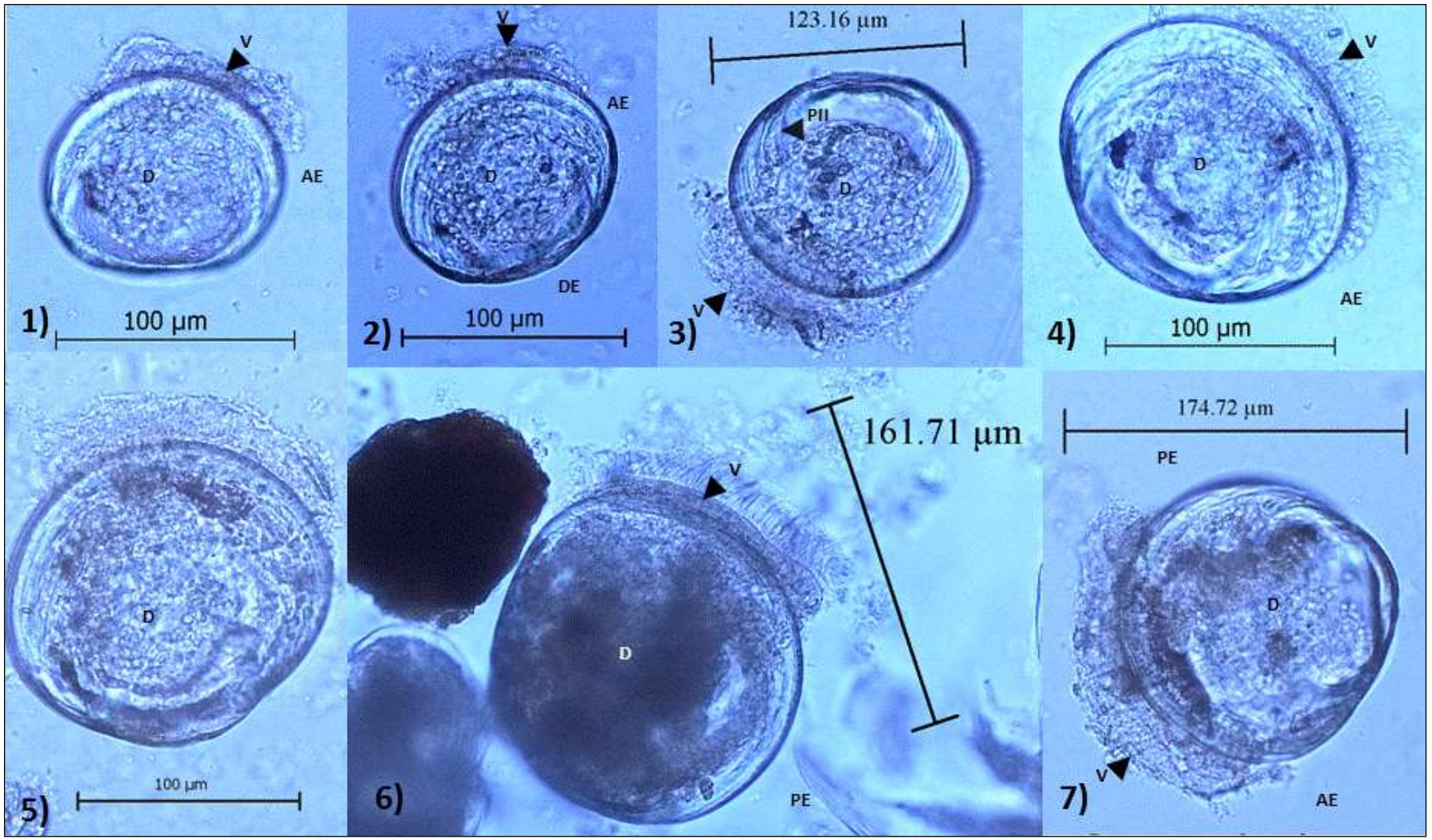


Plate 58: *Dreissena* sp. (D) larvae in the lateral view showing the velum (V) extended beyond the shell margin (1 – 7). The velum is a lobed ciliated mass located along ventral margin from the center towards the anterior end (AE) of shell. Velum appearance is affected by preservation. The boundary between the prodissoconch I and prodissoconch II shell (PII) is visible (3).

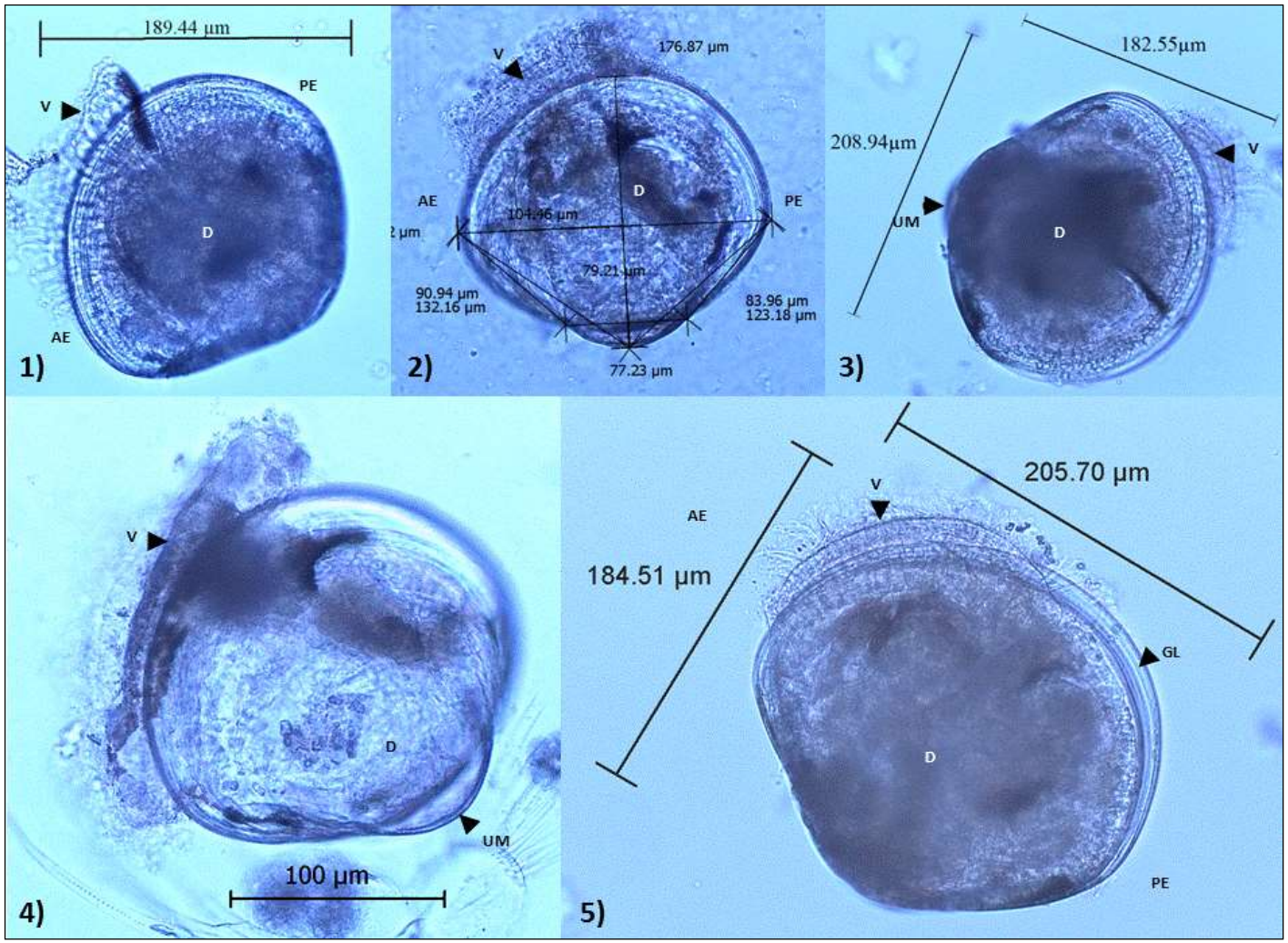


Plate 59: *Dreissena* sp. (D) larvae in the lateral view showing the velum (V) extended beyond the shell margin (1 – 5). Velum is located along ventral shell margin from center towards the anterior end (AE) of shell. An umbo (UM) that is low but protruding beyond shell line is visible (3) in contrast to a knobby umbo (UM) protruding beyond shell line (4). Commarginal or concentric growth lines (GL) are visible (5).

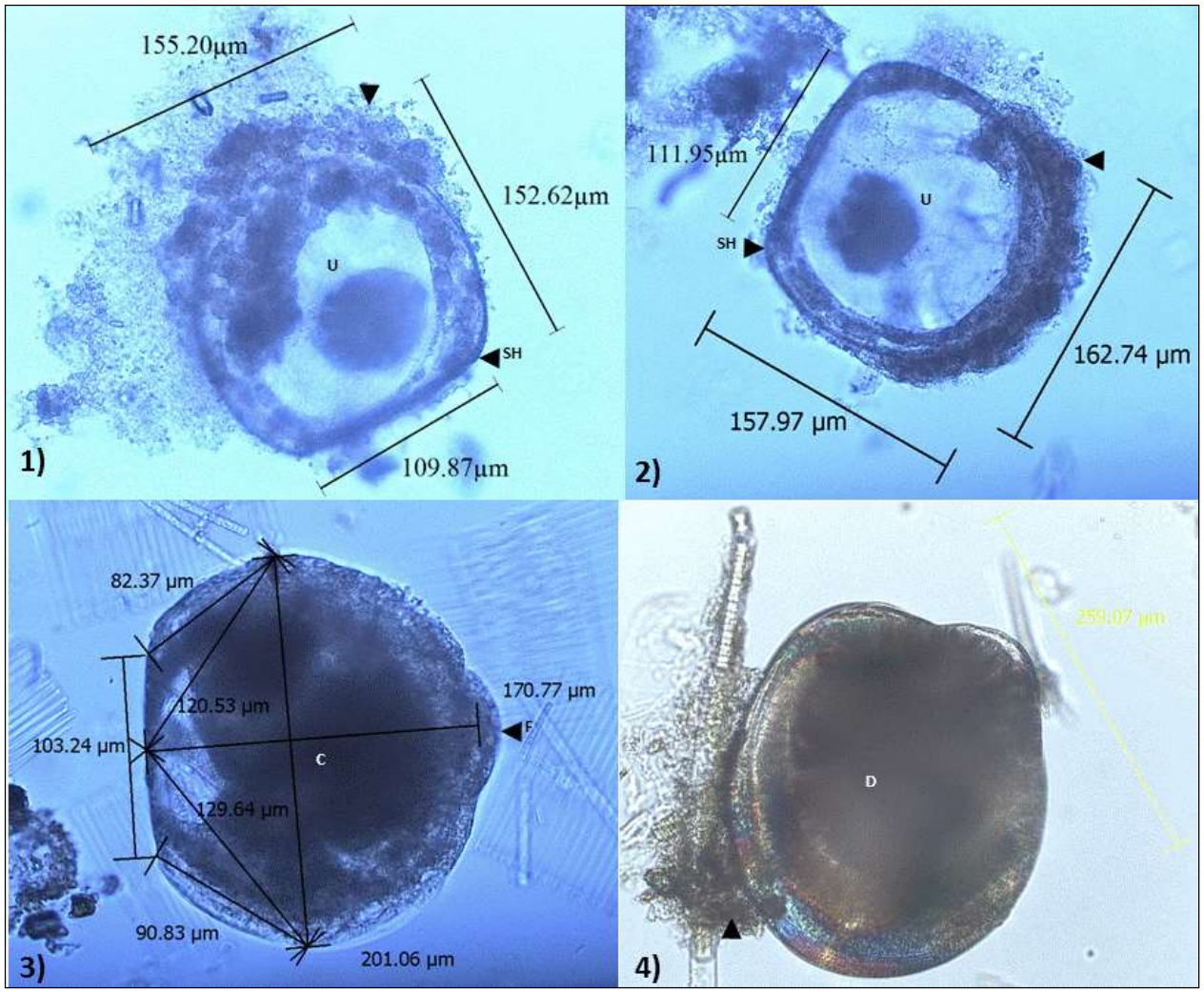


Plate 60: Bivalve larvae with material near the ventral shell margin that appears similar to a velum extended beyond shell margin. *Cyrtonaias* sp. unionid (U) mussel larvae lack a velum but material appears similar to velum (1, 2). A foot (F) is visible on a *Corbicula* sp. straight-hinge juvenile that appears similar to velum (3). *Corbicula* sp. straight-hinge juveniles lack a velum. A *Dreissena* sp. (D) larva may have velum extended beyond shell margin but it is difficult to distinguish from exogenous material (4).

Online Image Database http://mussels.research.pdx.edu/musselid/

An online image database is available at http://mussels.research.pdx.edu/musselid/ to provide reference digital microphotographs of bivalve larvae encountered in freshwater plankton samples during light microscopy analysis. The bivalve larvae that are currently represented in the database include the following: *Dreissena polymorpha*, *D. rostriformis bugensis*, *Corbicula* sp. *Mytilopsis leucophaeata*, *Anodonta suborbiculata*, *Cyrtonaias tampicoensis*, *Lampsilis hydiana*, *Potamilus amphichaenus*, and *Toxolasma texasense*. Images in the database can be queried by genus and species name, larval stage, larval size, and prominent features. Images can be copied and pasted for use as laboratory control references. Multiple photographs are included in the database for each species to provide clear documentation of the variation encountered regarding morphology, specimen orientation and condition as well as general visibility. Relevant images will be added as they become available.

Glossary

acetabuliform: *adj*. shaped like a saucer or shallow cup.

acetic acid: *n*. (synonyms: ethanoic acid, methane carboxylic acid) a weak carboxylic acid used to dissolve the calcium carbonate bivalve larval shells and remove birefringence when viewed under polarized light. White vinegar is 5% acetic acid.

adductor muscle: *n*. the muscles extending between two bivalve shell valves used to close valves when muscle is contracted.

antennae: *n*. elongated sensory appendage.

anterior end: *n*. the frontal end of a bivalve where the foot or byssus threads protrudes from the shell, and the beak is located. In planktotrophic bivalve larvae, an imaginary line perpendicular to the hinge from the center of umbo to the ventral margin divides the anterior end and posterior end. In *Dreissena* sp., the anterior end has a higher shoulder, is more elongated and less rounded than the posterior end, and is the half of the shell from which the velum protrudes from the shell.

aragonite: *n*. a common crystal form of calcium carbonate that differs from calcite in its crystal lattice.

axe-head: *adj*. overall shell shape characteristic of some glochidial larvae that resembles the head of an axe.

birefringence: *n*. double refracted light. It appears as white light when light is passed through the crystalline lattice of calcite when calcite is located between two polarizing filters that are oriented perpendicular to each other.

blind matrix spike sample (BMS): *n*. a matrix sample that is spiked with the analyte of interest, i.e., bivalve larvae, and then submitted to a laboratory disguised as a field sample to determine matrix effects and reliability of analytical processes and equipment.

brood-pouch: *n*. in bivalves, chambers within adults used for developing embryonic stages. For example, *Corbicula fluminea* develop larvae in interlamellar spaces of the gills that lack other specialized brooding structures. Spaeriids have specialized brooding chambers formed by evaginations of the gill filaments into the interlamellar space.

byssal threads: *n*. proteinaceous threads produced from a byssal gland at the base of the foot, and used to attach juvenile and adult bivalves to hard surfaces.

carapace: *n*. a dorsal section of a hard shell that protects most of the body region (e.g., head and thorax) of some arthropods.

cilia: *n*. slender protuberances or hair-like structures attached to a larger structure that function to move liquid past the larger structure.

clam: *n*. non-specific term applied to many bivalve families.

compound light microscope: *n*. a light microscope that uses multiple lens to collect light from a specimen and then focus the light into the eyepiece.

concentrated sample: *n*. pelleted particulate in plankton sample from which aliquots are taken for light microscopy analysis for planktotrophic bivalve larvae.

concentric: *adj*. nearly commarginal, having a common center, such as a series of ridges or lines radiating from the shell umbo.

condenser: *n*. a lens that concentrates light from an illumination source into a cone that illuminates the specimen. Adjustments to the size of the diaphragm affect the intensity and angle of the light cone.

commarginal lines: *n.* lines parallel to the ventral shell margin.

cross polarized microscopy: *n*. microscopy technique that uses two polarizing filters to investigate materials that change the polarization of light, e.g., calcite crystals. The specimen is placed between two polarizing filters that are rotated perpendicular to each other. Each filter blocks any light not polarized parallel to the axis of the filter, and when filters axes are perpendicular to each other, all light is extinguished, except the light that is refracted by a specimen, which will appear as a light object in a dark background.

cylindrical: *adj*. having the properties of a cylinder.

cymbiform: *adj*. shaped like a boat.

decant: *v*. to pour off.

denatured ethanol: *n.* (synonyms: methylated spirits) ethanol that has additives to make it inedible and prevent human consumption thereby making a cheaper alternative to regular ethanol for preservation of biological specimens. Additives vary but include 10% methanol, acetone, and methyl ethyl ketone.

digital micrometer: (see micrometer)

dissecting light microscope: (see stereo microscope)

dissoconch shell: *n*. adult bivalve shell.

dorsal end: *n*. the hinge area of bivalves.

dorsal view: *n*. looking directly at the dorsal end or hinge area of bivalve.

dorsolateral view: *n*. the view of a bivalve so that part of the hinge structure is visible as well as the face of one shell valve.

D-shape (see straight-hinge)

duplicate sample: *n.* (synonym: collocated sample, replicate sample) sample taken from virtually the same place and time used to estimate the precision of sample collection. A distinction is made between duplicate and split samples in this document, but some authors define duplicate samples as split samples.

fabiform: *adj*. shaped like a bean.

false negative: *n.* failing to detect something that was actually present.

false positive: *n*. detecting something that was actually absent.

foot: *n*. muscular organ used for anchoring to substrate and crawling. Formed on ventral side of body between the mouth and the anus in the latter part of veliger development.

gaping: *v*. open shell valves.

glochidium/glochidia: *n*. a modified veliger stage that undergoes indirect development and is adapted to a parasitic life, developing in a cyst on host, e.g., fish gills or fins. Characteristic of freshwater Unionidae, which are the freshwater Lamellibranchiata besides *Dreissena* sp., as well as some marine Lamellibranchiata. Glochidia have a bivalve shell, partly covering the larval mantle, which serves as an organ of nutrition. The two shell valves are connected by prominent adductor muscle, and the shell margin may or may not have a barbed tooth/ hook. Glochidia larvae lack a velum, foot and mouth. Glochidia of *Anodonta* and *Unio* have a long larval thread in the center for larva, but this is lacking in other species.

gravitational settlement: *n*. the use of gravity to passively settle or concentrate particulate in a liquid.

growth line region: *n*. area on the external surface of a shell valve characterized by growth lines, usually most apparent along the peripheral shell margin.

growth lines: *n*. concentric lines on the external surface of a shell valve that show growth annulations. Periods of extended rest are manifested as darker and/or thicker lines.

growth rings (see growth lines)

hinge: *n*. the structures located in the dorsal part of bivalve shell where the left and right valves attach.

hinge length: *n*. (synonym: hinge line) the measurement along the antero-posterior axis of the hinge indicated by the area between each shoulder.

Imhoff settling cones: *n*. a settling cone with continuous graduations and a valve at the bottom. Traditionally used for measuring sand and other settled matter.

isopropyl alcohol (IPA): *n*. (synonyms: isopropanol, rubbing alcohol) a secondary alcohol that is used for preserving biological specimens. DNA is insoluble in isopropyl alcohol.

laboratory control samples (LCS): *n*. samples similar in composition to real plankton samples with concentrations of all the analytes of interest, and that undergo similar preparatory and analytical procedures as real samples. In this document, LCS are used as reference samples to provide examples of target organisms and matrix during training, and to improve accuracy and performance of microscopy analysis.

lamellibranchiata: *n*. (synonym: pelecypods) a subclass of Bivalvia that is characterized by ciliary filter feeders with elongated gill filaments held together in parallel series to form folded lamellae, or thin raised ridges.

lateral view: *n*. a view looking at the shell valve face with valves oriented parallel to the surface on which they are lying.

light microscopy: *n*. (synonym: optical microscopy) type of microscope that uses visible light and lenses to magnify and view specimens.

lugol's solution: *n*. (synonym: Lugol’s iodine) a preservative solution commonly used for phytoplankton. Many forms of Lugol’s are acidic and are made by dissolving 20 g of KI and 10 g iodine crystals in 200 mL distilled water containing 20 mL glacial acetic acid. Utermohl’s modification of Lugol’s solution is neutral to alkaline and substitutes sodium acetate for the acetic acid.

maltese cross: *n* . birefringent property of bivalve larvae and other organisms that appears as a light cross against a dark background.

mantle: *n*. soft tissue enclosing the bivalve body that secretes the prodissoconch II and dissoconch shell.

micrometer: *n*. ruled scale used to measure magnified objects. Physical length of the marks on the scale depends on magnification.

moribund: *adj*. at the point of death.

mussels: *n*. non-specific term used for many bivalve families.

ocular micrometer (see micrometer)

orbicular: *adj*. (synonym: sub circular) circular in outline, or almost circular.

ostracod: *n*. mostly free-living microfauna in class Ostracoda that have body, including segmented appendages, suspended from the dorsal region and covered with a chitinous and calcite exoskeleton forming a bivalved carapace.

oval: *adj*. resembles the outline of an egg.

ovate: *adj*. egg-shaped, or having the outline of an egg.

ovoid: *adj*. oval-shaped.

pediveliger: *n*. (synonym: settling veligers) the larval stage for planktotrophic bivalve larvae characterized by a well-developed foot that can be used for crawling on substrate. A velum is still present but may be diminished in size.

perpendicular lines: (see secondary lines)

phenotypic plasticity: *n*. ability of an organism to change its phenotype in response to changes in the environment.

photomicrograph: *n*. (synonyms: micrograph) a photograph or digital image taken through a light microscope to capture image of a magnified specimen.

pitted: *adj*. having hollows or indentations on the surface.

plan achromat objective lens: *n*. a type of objective lens used with compound light microscopes that has been corrected for color dispersion effects and corrected for field flatness over the entire field of view. Used for standard applications in visual spectral range, and photomicroscopy.

planktonic: *adj*. refers to those microscopic aquatic organisms having little or no resistance to the currents that live free-floating and suspended in open or pelagic waters.

planktotrophic: *adj*. larval development that occurs in the water column.

plantigrade: *n*. the post veliger stage. Pediveligers that have settled and undergone metamorphosis.

polarization: (see cross-polarized microscopy)

posterior end: *n*. the end of the bivalve where the siphons protrude from the shell. In planktotrophic bivalve larvae, a line perpendicular to the hinge from the center of umbo to

the ventral margin divides the anterior end and posterior end. The posterior end is more rounded and blunt than the anterior end.

prodissoconch: *n*. (synonyms: larval shell, shell rudiment) bivalve larval shell.

prodissoconch I shell (PI): *n*. the first larval shell region secreted by planktotrophic bivalve larvae in the late trochophore stage, and thus marking the veliger stage. It is secreted by the shell gland and mantle epithelium, and usually has a smooth to pitted or punctate surface. It begins as an unpaired shell draped over the dorsal edge of the body, and continues to grow out towards both sides and eventually folds into two valves. This shell region is common early in the straight-hinge or D-shaped larval stage, and is said to be composed of dahllite.

prodissoconch II shell (PII): *n*. the second larval shell region secreted by planktotrophic bivalve larvae. It is secreted by the mantle along the peripheral shell edge, and is characterized by concentric lines, rings or commarginal growth annulations. This shell region develops during the straight-hinge or umbonal larval stage, and is mainly composed of calcium carbonate (calcite), similar to the adult shell.

punctate: *adj*. surface with minute pits.

quadrate: *adj*. square, or nearly square in outline.

radial striae: (see secondary lines)

rectangular: *adj*. having a shape of a rectangle.

regular ethanol: *n*. (synonyms: ethyl alcohol, grain alcohol) ethanol with low water content (2-4%) used for preservation of biological specimens.

replicate sample: (see duplicate sample)

reticules: *n*. a network of intersecting elements or lines.

ridge: *n*. an elongate elevation on the outer surface of a shell valve.

rinsate: *n*. liquid used to rinse containers or equipment.

sample split: *n*. in this document, a sample that is divided into equal parts after collection, and analyzed separately. Used to assess the reliability of analytical processes and equipment.

secondary lines: *n.* (synonyms: radial striae) shell sculpture appearing as lines on the outside of the bivalve shell valve that run perpendicular to the commarginal lines.

setae: *n*. bristle-like or hair-like structures.

shell gland: *n*. secretes the first larval shell, prodissoconch I, in planktotrophic bivalve trochophores, and is characterized by a thickened area of the ectoderm. It is located opposite the mouth.

shell height: *n*. the greatest dorso-ventral dimension perpendicular to the hinge line.

shell length: *n*. the dimension along the antero-posterior axis parallel the hinge line.

shell margin: *n*. the outer edge or circumference of a shell valve when laid flat.

shell valve: *n*. the right or left half of bivalve shell.

shell width: *n*. distance from the outer surface of the left shell valve through the bivalve body to the outer surface of the right shell valve.

stereo light microscope: *n*. optical microscope that uses incident light and a separate objective and eyepiece lens for each eye to produce a three-dimensional image of specimen. Typically used for low magnification observation.

straight-hinge/D-shape: *n*. first shelled larval stage for planktotrophic bivalve larvae involving a straight-hinge and 'D' shaped shell.

styliform hook: *n*. structures on ventral margin of glochidial shells in *Anodonta* that assist with firm attachment to host.

subtrapezoidal: *adj*. outline with four sides with length being greater than height, similar to rounded rectangle.

subtriangulate: *adj*. outline roughly three sides, similar to triangle.

supernatant: *n*. liquid lying above pelleted particulate following sedimentation or centrifugation.

sway-backed: *adj*. a hinge line that is bent inward towards the organism at the center of the hinge line.

sympatric species: *n*. different species, from a common ancestral origin, that occupy the same geographical area but do not interbreed.

triangular: *adj*. shaped like a triangle, and having three sides.

trochophore: *n*. the first larval stage developing from the gastrula in most planktotrophic bivalves. All marine Lamellibranchiata and *Dreissena* sp. trochophore larvae are free living, but others occur within egg capsules.

umbo length: *n*. the distance measured across the umbo taken from each side of umbo where the umbo breaks across the hinge line.

umbonal: *adj*. situated near or relating to the umbo. Also the second shelled planktotrophic bivalve larval stage that is characterized by the development of the umbo so that it protrudes beyond the hinge line.

umbone: *n*. (synonym: beak) the raised portion of the dorsal area that is the oldest part of the shell.

undulate: *adj*. waved or wavy.

valves: (see shell valves)

velar cilia: *n*. fine hair-like projections associated with the velum.

velar pigment: *n*. crescent-shaped band of black or dark pigment in anterior end of *Dreissena* sp. larvae associated with the velum.

veliconcha: *n*. term used to describe fully-developed veliger stage immediately after metamorphosis when larvae can swim using the velum but also crawl using the foot.

veliger: *n*. bivalve larval stage characterized by a ciliated velum and bivalve shell.

velum: *n*. in bivalves, a ciliated bilobate organ used for swimming, feeding, and respiration that is formed from the prototroch. Each lobe may be subdivided into two or three lappets. The velum may be partially or completely withdrawn into the shell.

ventral end: *n*. the lower or bottom edge of the shell opposite the dorsal end and hinge.

ventral view: *n*. the view when looking directly into the ventral end, which often involves looking at the velum, foot and other soft tissue extended beyond the shell margin.

ventrolateral view: *n* the view of a gaping bivalve where the face of one valve is parallel with the plane of the counting chamber, and the other valve is perpendicular to it.

ventrolaterally: (see ventrolateral view)

voucher specimen: *n*. identified specimens retained and stored for reference.

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Appendices

Appendix A: Sample tracking forms

Sample Receiving and Handling Tracking Form

Laboratory:_____

Point of contact:_____

Zebra/ Quagga Mussel Early Detection Monitoring **FORM_____ of _____**

Water body	Site	Date Collect	Preservative	Holding Temp.	Total Sample Vol. (mL)	Date Rec'd	Leakage/ proper temp? (y/n)	Lead Agency	Date conc'd	Time conc'd	Filter'd (y/n)	Sample tracking number(s)	Date Results Out

Sample Analysis Tracking Form **Laboratory:_____**

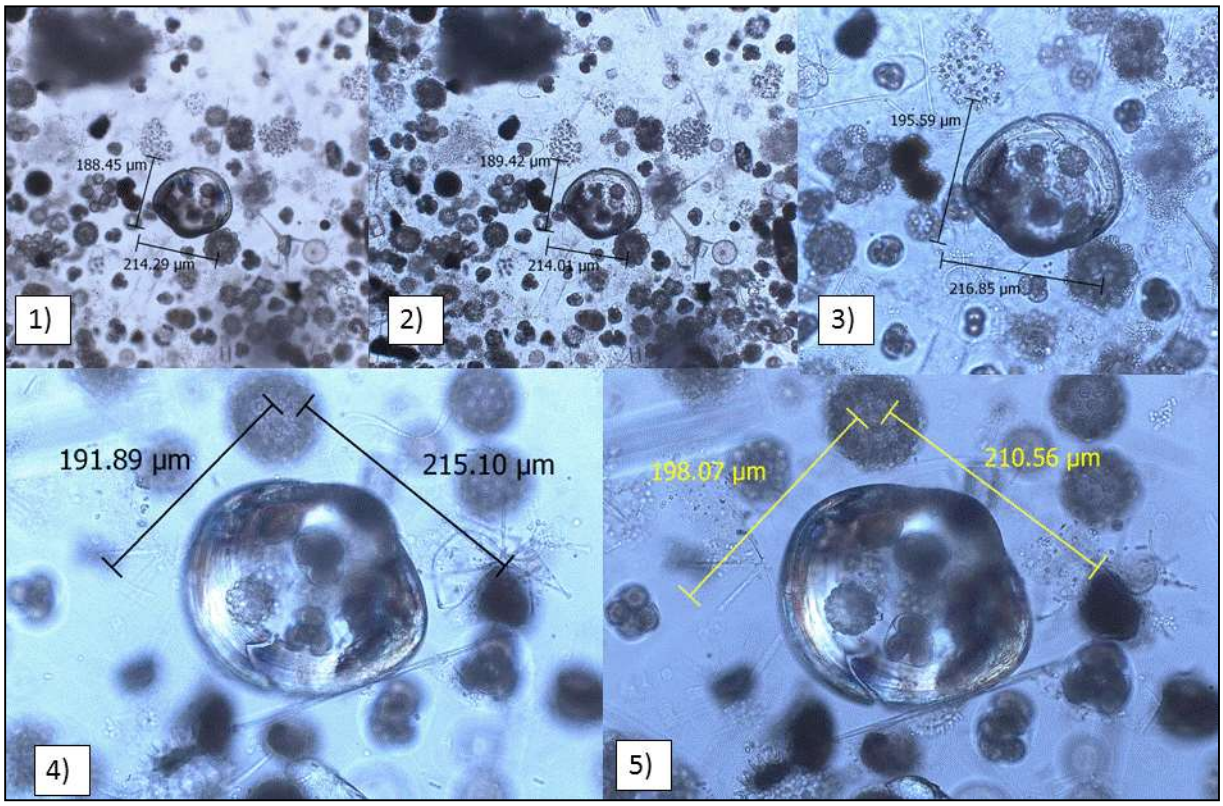
Point of contact:_____

Zebra/ Quagga Mussel Early Detection Monitoring **FORM_____ of _____**

Date	Analyst initials	Scope #	Sample tracking number	Counting cell volume	Dilution ratio	# aliquots analyzed	# ZQM larvae	# Corbicula larvae	# ostracods	Notes

+ abundance records are stopped after cumulative total exceeds 40 organisms

Appendix B: Examples of photomicrographs



Example of photomicrographs of a suspect specimen to send to other microscopy experts for verification. Photomicrographs are taken with limited polarization to allow enough background light to see morphology. Multiple photomicrographs are taken to focus on different features. For example, the external shell sculpture around the break in the shell valve is focused in photo 4), while top of umbo is focused on photo (5). Photomicrographs were taken with a Leica DFC290 microscope camera using 10X eyepiece and 4X HI PLAN objective (1-2), 10X HI PLAN objective (3), and 20X HI PLAN objective (4-5).

Appendix C: Form for documenting and standardizing external cross validation of photomicrographs and/or suspect specimens.

Photomicrograph Form for Independent Expert Identification Cross-Validation

WARNING: Sensitive information. Do not share enclosed images unless written permission from "Submitter" listed below.

Date:_____ **Form _____ of _____**

Instructions for Independent Expert for Identification Cross-Validation

1. Review instructions on this form. If you are not willing to participate, please inform Submitter and discard images.

2. Review attached images. Direct any questions to Submitter via indicated preferred method of contact.

3. Respond to Submitter via indicated method regarding your opinions on suspect specimen identification based on photomicrographs and supplementary information provided below. Please provide rationale for opinions.

4. Please respect sensitivity of this information, and do not share images with anyone unless written permission from Submitter.

Submitter Information

Laboratory
Name:_____ Point of Contact:_____
Mailing Address:_____ City/ State/ Zip Code:_____
Telephone: _____ Fax:_____ Email:_____
Preferred method of contact:_____

Sample Information

Date of detection:_____ Number of photomicrographs:_____
Number of suspect specimens:_____ Total magnification of image(s):_____
Specimen size (microns):_____ Type of microscope:_____
Preliminary identification(s):_____
Rationale for preliminary identification(s):_____
_____