

PLANKTON SAMPLE COLLECTION PROTOCOLS
for
Dreissenid Veliger Early Detection Monitoring

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1. Sampling Process Design

1.a. Sample Locations

Samples should be collected from a boat, if possible, at a minimum of fifteen sites within each waterbody. Collect two plankton tows at each site. More than fifteen sites can be sampled to increase the likelihood of collecting veligers. Samples from the same area can be composited together (no more than 5 samples per composite). Spread out the sites to further increase the likelihood of collecting veligers. A boat allows the sampling to be independent of land-accessible structures (e.g. docks). In lakes and reservoirs, focus sampling near dams, intakes, outflows, inflows, marinas, boat launches, and in areas that are downwind, downstream, open water and near shore. In large streams and rivers, focus sampling in the main stem, downwind and downstream areas and in near shore areas around boat launches, marinas and other structures that create eddies.

1.b. Sampling Frequency

Veligers can exhibit spatial and temporal patchiness in the water column and high sampling frequency (weekly or biweekly) increases the likelihood of collecting veligers. Additionally, repeated detection reduces the likelihood of a false positive. The optimal time to sample veligers in North America is when peak spawning is occurring, which varies with adult population density and location. On the average, optimal time to sample veligers is when water temperatures are between 16° and 19°C. Until site-specific spawning information is known, we suggest that sampling occur a minimum of three times during spawning season, starting once water temperatures are greater than 9°C. Veliger sampling can be performed anytime during the day but preferably not immediately following a storm event. Storm events can increase water turbidity and hence the time required to process the sample.

1.c. Sample Collection

Vertical and/ or oblique plankton tows are recommended instead of horizontal plankton tows and pumped samples. Veligers have been found throughout the water column, ranging from near the surface to depths greater than 400 feet. The depth where peak veliger density occurs can vary within and between waterbodies as well as between the different Dreissenid species. Most studies have reported greater veliger densities with vertical and/ or oblique tows compared to horizontal tows. For these reasons, vertical plankton tows are recommended to collect a depth-integrated sample. Horizontal tows, however, have yielded greater densities in some cases (e.g. San Justo Reservoir, CA, *Dreissena polymorpha*). Pumped samples are more difficult to collect, requiring more equipment and time. The efficacy of pumped samples, however, is not affected by net clogging as it is with plankton tows (i.e. a pressure wave in front of net).

Do not keep plankton tows that contain large amounts of sediment. If the net is dragged across the lake bottom, large amounts of sediment can be

captured. **Large amounts of sediment interfere with sample analysis, bind up preservative, and may damage sampling equipment.** If your sample contains large amounts of sediment, dump the contents of net back into the lake, thoroughly rinse net and cod-end piece in lake, and then repeat the tow. Some sediment (i.e. suspended solids) may be captured in plankton tows, especially in turbid systems, and this small amount of inorganic debris is acceptable.

If early detection and reduced costs are the primary objectives of sampling, plankton tows collected within the same area may be composited into one sample bottle. A suggestion is to sample at least three different areas in a given waterbody (more for larger waterbodies), and a composite sample can be created for each separate areas. The location of planktonic veligers is not related to the location of adult mussels. Compositing samples may help reduce the likelihood of false negative results in the field (i.e. failing to collect the veligers when they are present). However, care need to be taken not to produce a composite sample with planktonic concentration exceeding normal analytical limits in the laboratories. For this reason, limit the composite to a section or area of the lake having 5 or fewer samples. Otherwise, composite samples may need to be diluted in the laboratories requiring additional time for analysis. With sample compositing, spatial information is lost. The search for the original location of veligers (or possibly adults) is limited due to the loss of spatial information during compositing.

Plankton samples will be split in the laboratory using a Folsom plankton splitter to allow for both molecular and microscopic analysis of the same sample. The sample preparation for molecular methods destroys the veliger shell and it appears the internal tissue containing the genetic material often separates from the veliger shell during sample handling. Sample splits are not the same as duplicate or co-location samples. There is a greater likelihood that co-location plankton samples do not contain the same community of plankton (i.e. one tow captures veligers while the other does not) compared to sample splits.

2. Sampling Methods

2.a. Equipment

- Plankton net (simple, conical plankton tow net, 63 or 64 μm pore size-*the mesh size is critical*, 0.25 m diameter net opening, removable, weighted cod-end piece) (Figure 1)
- Line for deploying the net (30 m on spool or about 100 feet)
- Sample bottles (polyethylene material, 500 mL volume, leak-proof screw lid)
- Decontamination materials: Large bucket (>5 gal), white vinegar or 5% acetic acid solution (>5 gal), tap water (1 gal), and two spray bottles containing 5% acetic acid solution, and tap water (do NOT use lake or river water)
- Hot water power wash ($\geq 140^\circ\text{F}$) OR towels, scrub brush, bottle of household bleach, spray bottle with 5-7% solution of household bleach, another large bucket (>5 gal) and additional tap water (>5 gal)
- Preservative (absolute ethanol (ETOH) OR liquor ≥ 151 proof = 75.5%, e.g. Everclear. Do NOT use denatured ethanol or isopropyl alcohol (rubbing alcohol).)
- Field data sheets (waterproof paper), labels, and waterproof marker and pencils
- Global Positioning Satellite unit (GPS) (*recommended*)
- Tweezers or small spatula (*recommended*)
- Boat (*recommended*)
- Multiprobe water quality instrument (e.g. Hydrolab®) (*recommended*)
- Measuring tape or ruler (*optional*)
- Baking soda (*recommended*)
- Sealable plastic bags (e.g. Ziploc)
- Plastic garbage bags (large enough to hold 4 sample bottles)
- Cooler with cubed/ crushed ice

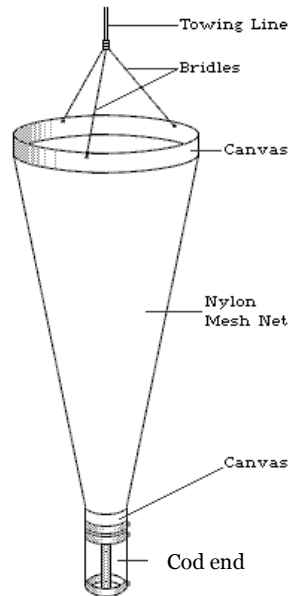


Figure 6: Simple conical plankton-tow net.

2.b. Plankton sample collection

2.b.1. Vertical Plankton Tow

1. Secure the cod-end piece and check that the line is securely attached to plankton net. Secure the other end of the line to the boat. Sometimes vertical tows are easier to do with a two person team, one with the sample cup and one handling the plankton tow net.
2. Lower the net 30 m (100 ft) below water surface, or to 1 m above the sediment, whichever is deeper. Record the depth the net is lowered.
3. Keep net at this depth for 30 seconds and then manually retrieve using a hand-over-hand technique at a rate of 0.5 m/ s (1.5 ft/ s). Slow and steady retrieval is the key to collecting a good plankton tow.
4. Rinse the net by raising the net so that the cod end of the net is at the water surface. Rinse organisms into the cod end of the net by lowering the net back into the water, keeping the opening above the water surface. Then quickly pull net straight up; this action will move collected plankton into the cod-end piece. Repeat this procedure several times to ensure that all the organisms inside the net are in the cod end.
5. A spray bottle, filled with DI water or water from the lake or river, can be used to wash down the outside of the net. Spray the outside of the net starting at the mouth to concentrate veligers into the cod end. Do not use tap water, since residual chlorine may destroy veliger tissue and DNA necessary for sample analysis.

6. Carefully unscrew the cod-end piece without spilling collected water and plankton. Condense the sample as much as possible before pouring into sample bottle. Condense the sample by swirling the cod-end piece. You may need to use tweezers or a spatula to gently clear the mesh netting in the cod-end piece to allow the water to filter through. Rinse the cod-end piece with a spray bottle several times with minimal volume of water and put rinses into the same sample bottle. MARK THE WATER LEVEL ON THE SAMPLE BOTTLE WITH PERMANENT INK (Draw a line).
7. It is important to record the number and length of tows so that the volume of lake water sampled can be estimated.
8. The volume of water sampled is determined using the formula below, assuming a net filtering efficiency of 100% (i.e. no clogging). If clogging occurs, a pressure wave develops, and water will be forced to the surface prior to the net emerging from the water. If clogging occurs, first try reducing the depth of the tow. If it still occurs, estimate the net filtering efficiency and multiple the corresponding percent by the maximum volume of water filtered (e.g. 80% filtering efficiency means $0.80 \times V_m$).

Maximum volume of filtered water, V_m is

$$V_m = \pi * r^2 * d$$

where r = radius of the net opening (0.25 m)
 d = depth to which the net is lowered (30 m)

2.b.2. Horizontal Plankton Tow

1. Attach a weight (1-2 kg or 2-4 lbs) to the line immediately in front of the new opening to keep the net below the water surface.
2. Secure the cod-end piece and check that the line is securely attached to plankton net.
3. Hold the ring of net, which is the metal loop that holds the net mouth open, using thumb and forefinger. Make large loops of the line and hold loosely with the same hand holding the net.
4. Firmly hold the other end of the line with free hand.
5. Throw the net using a sidearm-style, opening your hand upon release to allow line to feed out with the net.
6. Allow net to sink into waterbody. A weighted cod-end piece will aid in pulling the net into the water. If an air bubble gets trapped in the net, retrieve the net and start again.
7. Manually retrieve net using a hand over hand technique at a rate of 0.5 m/ s (1.5 ft/ s). Keep the net off the sediment to avoid both snagging and collecting debris.

8. Follow steps # 4 through # 8 used for vertical plankton tows.

2.b.3. Sampling Inside Facilities

1. Open flow valve and purge system for at least two minutes.
2. Estimate the flow using a flow meter valve or calculate the time needed to fill a 5 gal bucket (gal/ min).
3. Position the plankton net so that the flow of water passes into the mouth of the net. Place the 5 gal bucket underneath the net to collect the water passing through mesh.
4. Record the time that water is entering net so that the volume of water being filtered can be calculated.
5. Follow steps # 5 through # 8 used for vertical plankton tows.

2.c. Labeling and Associated Parameters

Record the following information on both the sample bottle label and the field datasheet. Use a waterproof permanent marker for bottle label and a pencil for datasheet. Be careful because permanent marker ink will smear when in contact with ethanol. For backup, record the sample bottle information on a piece of waterproof paper using a pencil and insert into the sample bottle.

- Date of collection
- Waterbody name
- Sample location (GPS is available or detailed description)
- Number and length of tows
- Type of tow (vertical, horizontal, etc)
- Name and agency of person collecting sample
- Preservative and concentration used (e.g. 50% ETOH)
- Mark sample bottle with two lines using permanent marker, one for the level of sample prior to adding preservative and the final level of preserved sample (sample + preservative).

It is highly recommended to collect the following metadata with plankton samples. Most of these data can be easily measured using a multi-probe unit (e.g. Hydrolab). Calibrate multi-probe units according to their manuals. Record metadata in field datasheets.

- Water temperature (°C) and depths of reading
- pH

- Specific conductance ($\mu\text{S}/\text{cm}$)
- Wind speed (two minute average, MPH)
- Secchi depth reading (ft)
- Turbidity (if available on multi-probe unit)

2.d. Sample Preservation

Preserve samples using absolute ethanol (ETOH) immediately after collection to ensure sample integrity. Samples that cannot be preserved immediately after collection should be placed on ice until preservative can be added. Do NOT wait more than three hours to preserve samples. ETOH is the preferred preservative. Do NOT use denatured ETOH because it appears denatured ETOH dissolves the calcite in shells much faster than absolute ETOH. Do NOT use Lugol's solution as it contains acetic acid and will dissolve shells. Do NOT use isopropyl alcohol because it may interfere with molecular analytical methods.

Preserve samples in a final solution of 25% or 70% ETOH, depending upon the amount of plankton, sediment and other debris in the sample and the sample handling and storage procedures. There are monitoring programs that preserve samples in a final solution of 25% ETOH, but most studies have used between 50% to 70% ETOH. Use more preservative with samples that contain greater amounts of plankton, sediment and other debris. The handling and storage procedures for samples preserved in a final solution of 25% ETOH are different than samples preserved in a final solution of 70% ETOH (Section 2.e.).

To make 70% solution of ETOH (eutrophic and turbid water bodies):

1. Make sure sample bottle is $\frac{1}{4}$ or less full. If needed, pour some sample into another sample bottle. Tighten cap and thoroughly mix sample prior to pouring into another sample bottle.
2. Allow sample to settle until water level is constant.
3. Mark the level of sample on the outside of sample bottle using a permanent marker.
4. Using a stock solution of 95% to 100% ETOH, add three parts ETOH to one part sample to achieve a final concentration of approximately 70% ETOH. A ruler or measuring tape may be placed alongside the sample bottle to help estimate the ratios.

5. Mark the level of final solution containing sample and preservative on outside of sample bottle using a permanent marker.
6. It is strongly recommended, the pH of the preserved sample is measured using a pH probe. If the pH is less than 6.9, add a small amount (1 tsp) of baking soda. Secure lid and shake to dissolve baking soda. Measure pH again to ensure pH is above 6.9.

To make a 25% solution of ETOH:

1. Make sure sample bottle is $\frac{3}{4}$ or less full. If needed, pour some sample into another bottle. Tighten cap and thoroughly mix sample prior to pouring into another sample bottle.
2. Follow steps # 2 and # 3 listed above.
3. Using a stock solution of 95% to 100% ETOH, add one part ETOH to three parts sample to achieve a final concentration of approximately 25% ETOH. A ruler or measuring tape may be placed alongside the sample bottle to help estimate the ratios.
4. Follow step # 6 used for sample pH.

2.e. Sample Handling and Storage

Handle samples while in the field after preserving according to following:

1. Secure sample bottle lid and wrap electrical tape around the bottom of bottle cap and the adjacent edge of the sample bottle to help hold lid firmly against the bottle and prevent leakage.
2. Place sample bottles in sealable plastic bags and then place bottles inside another plastic garbage bag. Sample bottles from the same waterbody may share sealable and garbage bags. Sample bottles are placed into plastic bags to reduce the likelihood of a leaking sample cross contaminating other samples.
3. Place samples preserved in 25% ETOH on crushed ice in a cooler. This is not required for samples preserved in 50% to 70% ETOH.

Samples preserved using a final solution of 70% ETOH may be stored in a cool, dry place up to three months prior to analysis. Veliger densities have been shown to decline after being held for more than three months in 70% ETOH. Avoid placing samples in direct sunlight or freezing conditions. Samples preserved in 70% may be stored for a longer period of time on ice or in the refrigerator.

Samples preserved using a final solution of 25% ETOH must be stored on ice and kept refrigerated prior to and following analysis. Microbial activities will

continue in 25% ETOH and degrade samples. Place sample bottles in a cooler filled with ice while in field and transfer to a refrigerator as soon as possible. Samples preserved in 25% ETOH are shipped using Styrofoam coolers and blue ice or dry ice. It is strongly recommended that samples and coolant are placed in an airtight container that is then placed inside the cooler. This will help maintain low temperatures during transit.

ETOH is a Class 3 flammable liquid and there are restrictions regarding its transport. ETOH can only be transported on the ground/ surface. Do not fly in an airplane with ETOH. Keep preserved samples in a plastic container such as a bin or cooler in the back of the car while in transit. ETOH can be mailed but there are training, certification, labeling and shipping requirements. Ship or mail ETOH-preserved samples to laboratory performing sample analysis via ground or surface mail using USPS and/ or FedEx according to the protocols below, which allow exemptions for training and certification.

USPS Protocols for mailing ETOH:

1. Samples must be in plastic containers with a screw lid. There can be multiple containers but the total volume of the entire package CANNOT exceed 473 mL. Secure screw lids.
2. Place all containers into a sealable plastic bag (e.g. Zip Lock) and then place this bag into another sealable plastic bag.
3. Place sealed bags and sample containers into box and add cushioning material such as grocery bags or scrap paper. Seal this box with clear packing tape. The box does NOT need to be a specific type of box so long as it is sturdy.
4. Place this box into another box and add cushioning material as needed. The outer box does NOT need to be a specific type of box either, so long as it is sturdy. Seal box with clear packing tape.
5. Include a complete return address on the package and also label the address side of box with the information shown below:

Surface Mail Only Consumer Commodity ORM-D Flashpoint = 55.6°F
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6. Mail via USPS domestic surface transport as Standard Mail or Parcel Post.

FedEx Protocols for mailing ethanol:

1. Samples must be in plastic containers with a screw lid. The volume in each container cannot exceed 30 mL. Secure screw lids.
2. A maximum of 16 containers per box. The total volume in all the containers can NOT exceed 500 mL.
3. Place all containers into a sealable plastic bag (e.g. Zip Lock) and then place this bag into another sealable plastic bag.
4. Place sealed bags and sample containers into a box and add cushioning material such as plastic grocery bags or scrap paper. Seal this box with clear packing tape. The box does NOT need to be a specific type of box so long as it is sturdy.
5. Place this box into another box and add cushioning material as needed. The outer box does NOT need to be a specific type of box so long as it is sturdy. Seal box with clear packing tape.
6. Include a complete return address. Label that is placed on address side of box as shown below:

This package conforms to 49 CFR 173.4

7. Mail via FedEx ground transport.

2.f. Decontamination

Field equipment must be decontaminated at the site to prevent transfer of organisms within and between systems and samples. It is strongly recommended that each waterbody being sampled have a dedicated set of equipment. The plankton net, however, will always require decontamination to prevent the cross contamination of other samples. The plankton net, cod-end piece and affiliated rope are decontaminated by totally immersing and soaking in a solution of 5% acetic acid (i.e. white vinegar). Acetic acid dissolves the calcite in the shells of veligers. The ideal soak time is 24 hours and the minimum soak time is two hours. Equipment is thoroughly rinsed with clean water in a spray bottle before and after the vinegar soak. The acetic acid bath may be reused for all sample locations.

Multi-probe sampling units (e.g. Hydrolab) are thoroughly rinsed with fresh water. Mud and debris are rinsed off the unit using tap water in a spray bottle, and then the entire unit is soaked in fresh water and shaken dry. Probes are stored in fresh tap water.

The boat bilge, hull, through-hull fittings, anchor, anchor lines, bow line, and propulsion system are decontaminated using hot water power wash ($\geq 140^{\circ}\text{F}$) and/ or a combination of scrubbing, oxidizing chemicals and towel drying. To make a 5-7% bleach solution (i.e. approximately 0.05 mL of active chlorine per L of water assuming 10% of bleach solution is active chlorine), add 7oz (a little less than 1 cup) of household bleach to 1 gal (16 cups/ 128 oz) of water. The 5 to 7% bleach solution is carefully poured into a spray bottle and applied to hull, propulsion system and through-hull fittings on pavement or concrete a minimum of 200 ft from open water. Scrub surfaces with brush to loosen and remove debris. Rinse with tap water. Fresh towels are used to remove remaining debris and to dry hull, through-hull fittings and propulsion system following the application of the bleach solution and scrub brush. Bleach solution is poured into bilge and allowed to sit for a minimum of 30 minutes. Ropes, anchors and anchor lines should be soaked in a large bucket (>5 gal) containing either a bleach or

acetic acid solution. Bleach and acetic acid are corrosive and equipment must be thoroughly rinsed with tap water following decontamination.