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Evaluating the Sensitivity and Reproducibility of Environmental DNA Field Sampling Workflows for Detection of Dreissenid Mussels

Science and Technology Program
Research and Development Office
Final Report No. ST-2022-19009-01

U.S. Department of the Interior

September 2022

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
30-09-2022	Research	2019-2022

4. TITLE AND SUBTITLE
Evaluating the Sensitivity and Reproducibility of Environmental DNA Field Sampling Workflows for Detection of Dreissenid Mussels

5a. CONTRACT NUMBER
RR4888FARD1904201/ F053A

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER
1541 (S&T)

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5d. PROJECT NUMBER
Final Report ST-2022-19009-01

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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8. PERFORMING ORGANIZATION REPORT NUMBER
EcoLab-F053A-2022-08

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Science and Technology Program
Research and Development Office
Bureau of Reclamation
U.S. Department of the Interior
Denver Federal Center
PO Box 25007, Denver, CO 80225-0007

10. SPONSOR/MONITOR'S ACRONYM(S)
Reclamation

11. SPONSOR/MONITOR'S REPORT NUMBER(S)
Final Report ST-2022-19009-01

12. DISTRIBUTION/AVAILABILITY STATEMENT
Final Report may be downloaded from https://www.usbr.gov/research/projects/index.html

13. SUPPLEMENTARY NOTES

14. ABSTRACT

There is a need to develop and evaluate eDNA dreissenid mussel field sampling protocols. We compared two common eDNA field sampling methods, sub-surface grab samples and plankton tow samples, in 12 waterbodies with known or potential dreissenid mussel infestations spanning the coterminous United States. To mimic early detection sampling scenarios, we sampled waterbodies or areas within waterbodies that had lower densities of dreissenid mussels or were suspect for dreissenid mussels. The two eDNA sampling methods resulted in similar dreissenid mussel DNA detection patterns. Both methods detected target DNA at the same 7 waterbodies and both methods failed to detect target DNA at the same 5 waterbodies. At one of the lower density sites, more mussel DNA was amplified in plankton tow samples than from grab samples.

15. SUBJECT TERMS
Environmental DNA, eDNA, dreissenid mussels, quagga mussel, zebra mussel, early detection

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**THIS PAGE**			Sherri Pucherelli
U	U	U		**27**	**19b. TELEPHONE NUMBER** *(Include area code)* **303-445-2015**

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Acknowledgements

The Science and Technology Program, Bureau of Reclamation, sponsored this research. We thank Allison Zach, Kris Stahr, Alison Watts, Denise Blanchette, Kimberley Jensen, Meg Modley, and Steven Pearson for collecting both grab and tow samples that facilitated this study.

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Peer Review

Bureau of Reclamation
Research and Development Office
Science and Technology Program

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Executive Summary

Multiple field sampling methods are currently being used for environmental DNA (eDNA) surveillance of dreissenid mussels and there is no guidance designating which field sampling method is optimal for early detection. eDNA sampling methods must not only be sensitive and specific, but they must also be repeatable and reproducible when results are used for decision-making. To support the acceptance of eDNA surveys for dreissenid mussel surveillance, it is necessary to assess the quality of methods employed and to ultimately promote method standardization.

We compared two common eDNA field sampling methods, sub-surface grab samples and plankton tow samples, in 12 waterbodies with known or potential dreissenid mussel infestations spanning the coterminous United States. To mimic early detection sampling scenarios, we sampled waterbodies or areas within waterbodies that had lower densities of dreissenid mussels or were suspect for dreissenid mussels.

The two eDNA sampling methods resulted in similar dreissenid mussel DNA detection patterns. Both methods detected target DNA at the same 7 waterbodies and both methods failed to detect target DNA at the same 5 waterbodies. Saguaro Lake has the smallest veliger population of dreissenids relative to other infested lakes that we sampled in Arizona. This was the only site with a notable difference between grab and tow sample detections, with 20% of grab samples amplifying and 60% of tow samples amplifying for dreissenid mussel DNA. Additional sampling at low-density waterbodies is needed to determine if our initial result was random chance or indicative that tow samples have higher detection probabilities when dreissenid mussels are rare.

1. Introduction

The Western Regional Panel on Aquatic Invasive Species, the Western Governors' Association's Biosecurity and Invasive Species Initiative, and the Department of Interior (DOI) Safeguarding the West Initiative identified development and evaluation of eDNA dreissenid mussel (quagga (*Dreissena rostriformis bugensis*) and zebra mussel (*Dreissena polymorpha*)) field protocols as a need. eDNA sampling methods must not only be sensitive and specific, but they must also be repeatable and reproducible when results are used for decision-making. To support the acceptance of eDNA surveys for dreissenid mussel surveillance, it is necessary to assess the quality of methods employed and to ultimately promote method standardization.

Multiple field sampling methods are currently used for eDNA surveillance of dreissenid mussels and there is no guidance clarifying which field sampling method is optimal and provides repeatable, reproducible results. Field sampling methods include sub-surface, mid-water, and near-bottom grab samples isolated using either filtration or centrifugation (e.g., Amberg et al. 2019, Sepulveda et al. 2019) and plankton tows (e.g., Schabacker et al. 2020).

We compared two common eDNA field sampling methods, sub-surface grab samples and plankton tow samples, in 12 waterbodies (Table 1) with known or potential dreissenid mussel infestations spanning the coterminous United States. To mimic early detection sampling scenarios, we sampled waterbodies or areas within waterbodies that had lower densities of dreissenid mussels or were suspect for dreissenid mussels.

Table 1. Sampled waterbodies and their approximate surface-areas.

State	Waterbody	Mussel status	Area (km^2)	Sample date
Nebraska	Carter Lake	Suspect	0.13	May, Jun 2021
	Offutt Base Lake	Infested	0.46	Apr, Jun 2021
	Zorinsky Lake	Delisted	1.03	Apr 2021
New York/ Vermont	Lake Bomoseen (VT)	Infested	9.71	Apr, May, Jun, Jul 2021
	Lake Champlain (NY/VT)	Infested	1,331	May, Jun, Jul 2021
	Lake George (NY/VT)	Infested	120	Apr, May, Jun 2021
Arizona	Apache Lake	Infested	10.39	Nov 2019
	Bartlett Reservoir	Suspect	11.39	Nov 2019
	Canyon Lake	Infested	3.84	Nov 2019
	Lake Pleasant	Infested	40.47	Nov 2019
	Theodore Roosevelt Lake	Suspect	86.98	Nov 2019
	Saguaro Lake	Infested	5.12	Nov 2019

2. Methods

We collected or worked with partners to collect eDNA samples from the 12 waterbodies. For the waterbodies with known infestations, samples were collected from sites thought to have lower mussel densities. For the waterbodies with suspected (but unconfirmed) mussel status, samples were collected from sites most likely to be introduction points from motorized watercraft vectors (e.g., marinas and docks). Multiple sampling events occurred at the waterbodies listed in Table 1 to evaluate if field sampling method detection rates varied seasonally. At Lake George, we double sampled to provide insight about detection probabilities for a confirmed low density zebra mussel population.

Sub-surface grab samples ('grab samples') consisted of five, 1-L samples collected from the subsurface (~ 20 cm depth) in sterile, Whirl-Pak plastic bags (Nasco Corporation) (Appendix A). One field negative control, that consisted of 250 mL distilled water, was collected at each site during each sampling event. Filled bags were filtered immediately or placed on ice inside coolers until filtration. Water samples were filtered through a 47 mm, 1.2 μm Whatman glass-fiber filter (GE Healthcare) held within a filter cone and attached to a peristaltic pump (Geotech Environmental Equipment Inc.). Filters were placed in individual, sterile Whirl-Pak bags filled with silica desiccant and shipped to the USGS Northern Rocky Mountain Science Center (NOROCK). Upon their receipt, samples were frozen at -20° C until DNA extraction. DNA was extracted from frozen filter samples using the Investigator Lyse & Spin Basket Kit (Qiagen) in concert with the DNeasy Blood & Tissue Kit (Qiagen) and eluted in 400 μL buffer.

Plankton tow samples ('tow samples') followed Reclamation's standard operating procedure (SOP) for field sampling methods for invasive mussel early detection (Bureau of Reclamation, 2022, excerpt in Appendix B). Tow sampling involved collection of five composite plankton tow samples, where each composite sample consisted of five tows. One field negative control was collected at each site per sampling event that consisted of 1-L distilled water poured through a plankton tow net prior to the start of sampling. Composite tow samples were preserved in ethanol and Tris buffer and shipped to Reclamation's Ecological Research Laboratory. Upon their receipt, water samples followed Reclamation's SOP for quantitative polymerase chain reaction (qPCR) for the early detection of invasive mussel eDNA. In brief, the water sample is shaken, and 40 mL of this sample is poured into a conical tube and then centrifuged. DNA is extracted from the resulting pellet using the Quick-DNA Fecal/Soil Microbe Microprep Kit (Zymo Research).

DNA extract from grab and tow samples was analyzed using the Dreissenid 16S rRNA assay (Gingera et al. 2017), which is genus-specific, so it amplified both quagga and zebra mussels. Four replicate PCR reactions were run per sample and each reaction included an internal positive control to test for inhibition. No-template controls (NTCs) were run on each plate. A subset of DNA extract was exchanged between labs to evaluate reproducibility of results. qPCR data were evaluated for both the sample detection rate (i.e., the proportion of samples from a site (5 samples per site) for which at least one qPCR technical replicate resulted in a detection, and the replicate detection rate (i.e., the proportion of all qPCR replicates (20 qPCR replicates per site) from a site that resulted in a detection).

3. Results

The two eDNA sampling methods resulted in similar dreissenid mussel DNA detection patterns (Figure 1). Both methods detected target DNA at the same 7 waterbodies and both methods failed to detect target DNA at the same 5 waterbodies. Neither method detected target DNA at Lake George despite doubling effort from 5 to 10 samples per method. At 6 of the 7 waterbodies where target DNA was detected, detection rates were very high using either method — target DNA was detected in 60% to 100% of samples (Figure 1) and in 30% to 100% of PCR technical replicates (Figure 2). At the 7th waterbody (Saguaro), detection rates were much lower for both methods — target DNA was detected in 20% to 60% of samples (Figure 1) and in 5% to 60% of PCR technical replicates (Figure 2).

No sampling method was consistently more sensitive than the other. Grab samples had slightly higher detection rates than tow samples at 4 of 7 waterbodies, but this difference was not meaningful since tow samples also detected the target DNA. At 2 of the 7 waterbodies, performance was identical. At the 7th waterbody (Saguaro), tow samples had a higher detection rate, but this difference was also not meaningful since grab samples also detected target DNA.

Sample and PCR replicate-level results from the same grab or tow sample did not differ by lab (Figure 3; Wilcoxon signed rank test, $p > 0.37$ grab samples, $p > 0.44$ tow samples).

Figure 1. Proportion of samples where dreissenid mussel DNA was detected in ≥ 1 PCR replicate using grab or tow field sampling methods. eDNA samples were collected at (a) three waterbodies in Nebraska, (b) three waterbodies in New York/Vermont, and (c) six waterbodies in Arizona.

Figure 2. Proportion of PCR replicates that amplified for dreissenid mussel DNA using grab or tow field sampling methods. eDNA samples were collected at (a) three waterbodies in Nebraska, (b) three waterbodies in New York/Vermont, and (c) six waterbodies in Arizona.

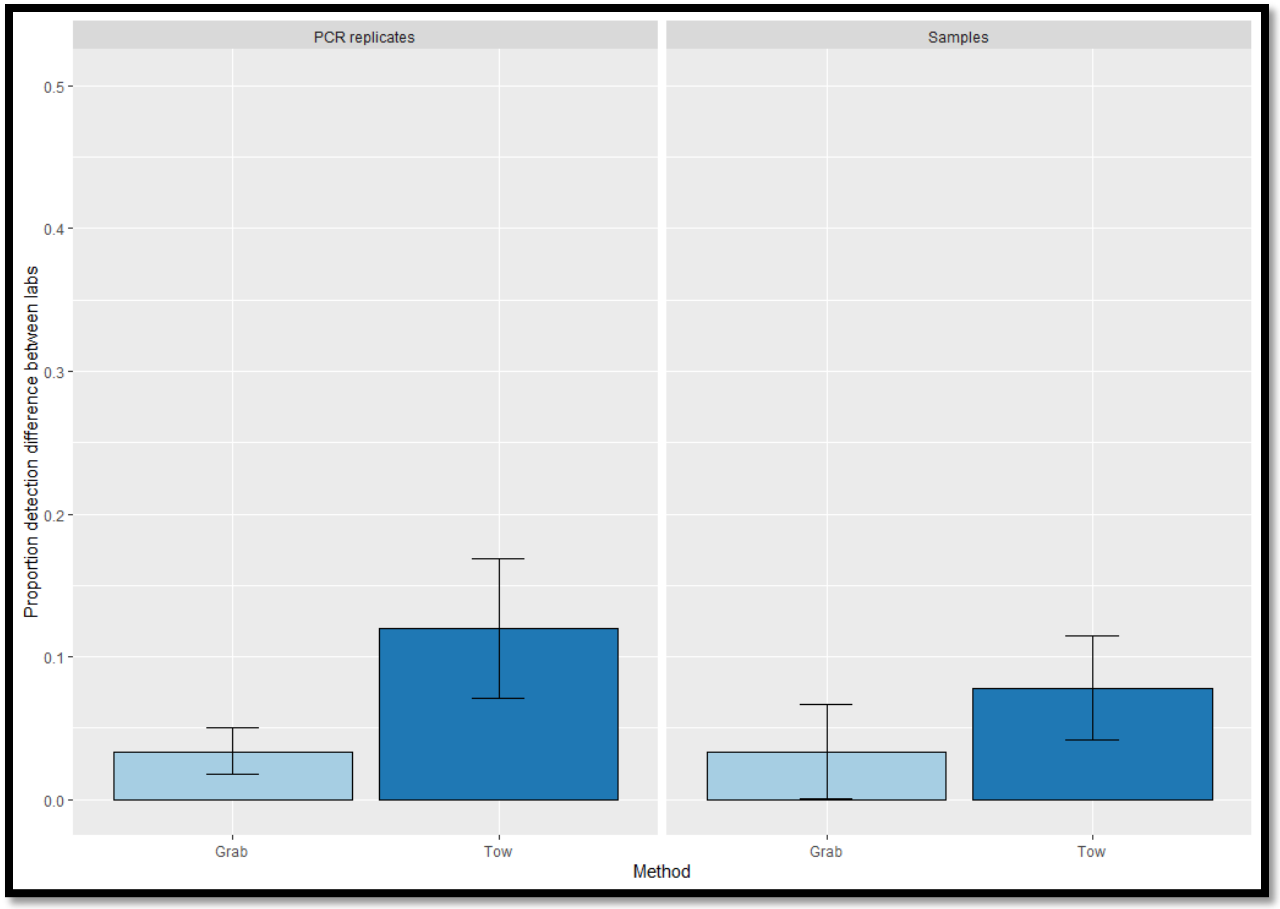

Figure 3. Mean (± 1 SE) proportional difference among labs of PCR replicates and samples that amplified for dreissenid mussel DNA using grab or tow field sampling methods. DNA extract from the same sample was analyzed by both Reclamation and USGS labs.

4. Discussion

We found that grab and tow samples resulted in similar dreissenid mussel DNA detection patterns at 12 waterbodies across the country. We also found that positive and negative results were reproducible between USGS and Reclamation labs. Based on these results, managers can select the eDNA field sampling method that best fits the current program structure.

It is unclear which sampling is optimal for early detection of nascent invasions. Saguaro Lake was the only site with a notable difference between grab and tow sample detections, with 20% of grab samples amplifying and 60% of tow samples amplifying for dreissenid mussel DNA. Saguaro Lake has the lowest population numbers of dreissenid veligers relative to other infested lakes that we sampled in Arizona. Additional sampling at Saguaro Lake or at other low-density waterbodies is

needed to determine if our initial result was random chance or indicative that tow samples have higher detection probabilities when dreissenid mussels are rare.

A desired use of eDNA sampling is for early detection of nascent invasions when the invader is at densities too low to be detected by other sampling methods. Except for Lake George, identifying waterbodies where dreissenid mussel presence is confirmed yet mussels are at very low densities typical of nascent invasions was challenging. Dreissenid mussel confirmation generally occurs once mussels are at higher densities. Our four 'suspect' sites likely did not have mussels. More useful eDNA-early detection sampling method evaluations will likely require experimental approaches, where mussel abundance can be manipulated, and close collaboration with land managers to sample waterbodies early in their invasion trajectories or following control actions.

- Data Availability:
    - Share Drive folder name and path where data are stored: \\bor\do\TSC\Jobs\DO_NonFeature\Science and Technology\2019-PRG-eDNA Field Collection Methods
    - Data Contact: Sherri Pucherelli, spucherelli@usbr.gov, 303-445-2015
    - Description of the data: Data and final report

5. References

Amberg J.J., Merkes C.M., Stott W., Rees C.B., Erickson R.A. (2019). Environmental DNA as a tool to help inform zebra mussel, Dreissena polymorpha, management in inland lakes. Management of Biological Invasions 10(1): 96–110, https://doi.org/10.3391/mbi.2019.10.1.06

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Appendix A: Environmental DNA (eDNA) Filter Sampling Protocol for Field Filtered Samples (desiccant bead preservation)

General Field Supplies

- Small cooler or container to store samples
- Small trash bag
- Distilled or lab water
- Spray bottle containing 10% bleach
- Small bucket with a 1L line marked inside*
- Geopump peristaltic pump*
- Geopump battery (ensure it has been charged)*
- Power cable connecting pump and battery*
- Peristaltic tubing with filter adaptor at one end*
- Waders- if not sampling from a boat*
- Container to carry equipment*
- Sharpie marker for labeling bags*
- Pump tubing and connector*
- Extra gloves

*Clean with 10% bleach between sites.

Per site supplies

- Data collection sheet
- 250 ml of reverse osmosis water in a sterile container
- 1 sample kit per reach; gallon size bag containing …
    - 6 filter assemblies
    - 6 small whirl-pak bags of silica gel beads
    - 1 empty gallon bag for samples

Before Sampling

Label the 6 sample whirl-pak bags of silica gel beads and fill out the sample sites on field sheets. Check field supplies.

Step 1. Sampling

a. Designate one person as the 'Sampler'. This person will collect the sample and will have direct contact with the water.
b. A second person will be designated as 'Handler'. This person will assist the Sampler but will not have direct contact with water (this helps minimize transfer of contamination from the sample to the unused supplies).

Step 2. Setting up for on-site filtration (adapted from Carim et al. 2016)

a. Prepare sampling area by setting up peristaltic pump, tubing, and sample kit (FIGURE 1); choose a bank that allows you to lay out all the sampling equipment without risk of it being blown away or falling into the lake (the flatter, the better). .
b. To prepare peristaltic pump
    a. Place the pump and battery near the lake but in a dry area.
    b. Connect the pump to the battery using the power cord
    c. Lift the quick-release lever to load the supplied tubing with adaptor into the pump head (FIGURE 2). Center the tubing in the track and lower the quick-release level. Once the tubing has been loaded, thread the outflow end of the tubing from the pump into a bucket with 1 L graduations to collect the outflowing water.
    d. Ensure that you have enough length of tubing to reach into the lake and that the direction of flow on the pump is consistent with inflow at the adaptor and outflow at the bucket (FIGURE 3).

Step 3. Collect Field Blank sample (250 ml)

a. Put on one pair of sterile nitrile gloves.
b. Retrieve a filter assembly (FIGURE 4) and a silicon bag from the sample kit bag and reseal the kit bag.
c. Set the silicon bag next to the pump/battery and then snap the filter assembly onto the adaptor of the peristaltic tubing (FIGURE 5).
d. Pour ~250 mL of the field blank control water into the cup
e. Turn the pump on and filter the blank. You don't have to measure outflow.
f. Keep the pump on and remove the clear cup from the filter holder.
g. Allow the pump to run for an additional minute or so to help dry the filter. During this time, you can fill out information on the data sheet and label the silicon bag with the reach and sample names.
h. Turn the pump off and open the silicon bag.
i. Remove the blue filter holder (with filter) from the adaptor and place it into the silica bag.
j. Roll the top of the silica bag down, like a dry bag, as tightly as you can and then twist the metal wires together to seal.
k. Place the silicon bag with the sample into the supplied gallon size bag and make sure the gallon bag is labeled with the reach information.

Step 4. Collect field samples (1 L)

a. Retrieve a filter assembly (FIGURE 4) and a silicon bag from the sample kit bag and reseal the kit bag.
b. Check that there are no visible holes, cracks, or gaps in the filter that water can easily flow through. If there are, you may need to reseat or replace the filter from the supplied bag of extra filters (use clean forceps to handle replacement filters).
c. Set the silicon bag next to the pump/battery and then snap the filter assembly onto the adaptor of the peristaltic tubing (FIGURE 5).
d. Turn the pump on.

e. Collect the eDNA water sample at an arm's reach into the lake while standing/kneeling on dry ground. It is best to continuously hold the cup during filtration, as opposed to letting it move on its own in the lake. You can simply turn the filter assembly upside down and let a vacuum form in the cup and continue the entire filtrations with the cup upside down at the surface of the water.
f. Once 1 L have been filtered through, gently remove the cup from the lake by tipping it right side up without disturbing the surface of the filter in the assembly.
g. Keep the pump on and remove the clear cup from the filter holder.
h. Allow the pump to run for an additional minute or so to help dry the filter. During this time, you can fill out information on the data sheet and label the silicon bag with the reach and sample names.
i. Turn the pump off and open the silicon bag.
j. Remove the blue filter holder (with filter) from the adaptor and place it into the silica bag.
k. Roll the top of the silica bag down, like a dry bag, as tightly as you can and then twist the metal wires together to seal.
l. Place the silicon bag with the sample into the supplied gallon size bag and make sure the gallon bag is labeled with the reach information.
m. Repeat four more times for a total of five, 1 L samples.

*If glove or supplies become soiled or contaminated (e.g., drop filter cup), replace with spare gloves or supplies rather than attempting to clean in the field. When in doubt error on the side of caution and replace gloves or supplies.

Step 5. Cleanup

a. Remove used tubing from peristaltic and cable from pump and pack these items for transport to the next site.
b. Place all used forceps, filter cups, and filter holders into a "trash" bag for expended materials (this will be shipped back to USGS NOROCK).
c. Discard used gloves.

Step 6. Store and ship samples

a. Place samples in a refrigerator until all samples are ready to ship.
b. Use my FedEx account for shipment (######)
c. Ship eDNA samples FedEx express (overnight) within 48 hrs. to:

    USGS Northern Rocky Mountain Science Center
    Adam Sepulveda
    2327 University Way, Suite 2
    Bozeman, MT 59715 USA
    (406) 994-7975

d. FedEx ship used supplies and peristaltic pump to same address using cheaper option (e.g., ground or 2-day).
e. Email tracking number to Adam Sepulveda (asepulveda@usgs.gov) and Patrick Hutchins (phutchins@usgs.gov)

Notes: Bleach destroys DNA, so be careful not to let it contact your sample or filter!

Sample Collection

Location:_____ **Date:**_____

Sampling Team:_____

Weather/comments:_____

Site ID:_____ **Other ID:** _____

Water Temp:_____ **Sample Depth:**_____

Samples collected:

Sample ID:	Volume:	Comments

Notes:_____

Figure 1. Example setup of on-site filtration equipment.

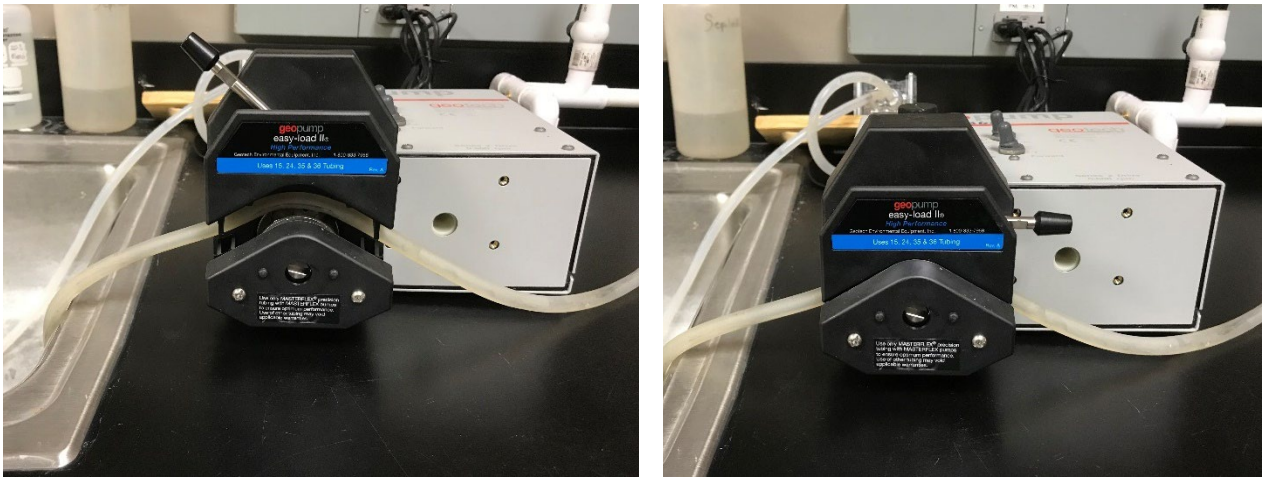


Figure 2. (Left) Example of how to load tubing into peristaltic pump with quick-release lever open. (Right) Once tubing is loaded, close the quick-release lever.

Figure 3. Set the direction of the pump (forward or reverse). In this photo, I am pulling water from the sample (on the right) and spilling the water in the sink (on the left); so, the pump is set in "Reverse".

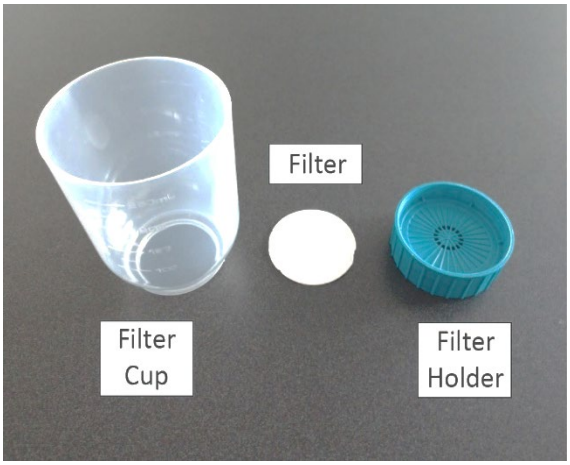


Figure 4. Components of filter assembly.

Figure 5. Snap the filter cup assembly to the adapter.

Appendix B: Plankton Tow Sample Protocol

Supplies

- Personal floatation devices (PFDs)
- Chain of custody (COC); provided at end of protocol
- Plankton net (64-µm mesh) with weighted cod-end (64-µm mesh)
- Rope, marked in 1-meter increments up to 50-meters
- Labeled sample bottles (new bottles only – do not re-use old bottles)
    - ≥ 500 mL size
    - 6 per sampling trip
- Secchi disk or some other means of measuring depth
- Permanent marker
- Distilled water
- Decontamination buckets for plankton tow nets
- Disposable droppers
    - 6 droppers per sampling trip
- GPS
- Tape measure or ruler

Reagents/Chemicals

- Alcohol (90% or greater ethyl [ethanol] or isopropyl [isopropanol])
- Tris buffer
- Vinegar

Shipping

- Electrical tape to seal sample bottles
- Cooler
- Ice packs
- Garbage bags
- Ziplock bags (for COC)
- Packing tape to seal coolers
-

Step 1. Field Sample Preparations

- Print out Chain of Custody (COC) datasheet, provided at the end of this protocol
- Prepare bottle labels as follows
    - Date collected
    - Waterbody
    - Field Sample or Field Blank
    - Number of tows in the bottle
    - Length of tows

- Inspect plankton tow net
    - Check for holes, rips or tears
    - Check metal/PVC collar to ensure cod-end is not broken and screw on securely
    - Check mesh is attached to inside of cod-end with no gaps
    - Check that weight on bottom of cod-end is secure
    - Make sure all knots are securely tightened
    - For May and June trips, make sure plankton tow net and cod-end have been decontaminated in vinegar and rinsed.
- Gather supplies, reagents and chemicals needed for the field

Step 2. Site selection

- Target sampling near areas where dreissenid mussels are known/thought to be present but are at lower densities.
- Areas where mussels are likely to be present include areas of high use such as boat docks, boat launch ramps, marinas.
- If in a boat, drop anchor or turn boat so there is the least amount of drift.

Step 3. Collect a field-blank

- This should be done at the sampling site before anyone comes into direct contact with the lake water.
- Sampler: Pour a gallon of distilled water through the opening of the net. While pouring the water through the net be sure that water passes over most of the inside of the net before it passes through the cod-end.
- Sampler: Pour the water that is retained in the cod-end into a labeled sample bottle and buffer and preserve in the same manner as all other samples (see Step 5. Sample Preservation section below for details).

Step 4. Determine water depth at site

- Handler: Lower Secchi disk (or equivalent) with a rope marked at 1/10-meter increments until it hits bottom or there is no more rope.
- Handler: Record this depth as "Total Depth "Step
    - If depth is ≥ 4m, please follow the vertical plankton tow sampling procedure.
    - If depth is < 4m, please follow the horizontal plankton tow sampling procedure.

Step 5a. Vertical Plankton Tow

- Sampler: Lower plankton tow net vertically from dock or boat to 1 meter above the "Total Depth", pre-determined by using the Secchi disk (or some other means of measuring depth

like a boat's depth finder). If "Total Depth" is deeper than the length of the plankton tow net rope, lower the plankton tow net to the deepest depth possible.

- Sampler: Slowly (~ 1 meter per second) pullup the net, hand-over-hand, to the surface. If cod-end is filled with sediment, discard sample, rinse cod-end in lake, and re-sample by raising the sample depth by half a meter.
- Handler: Record the length of tow, in meters, on the sample bottle and on the COC.
- Proceed to **Step 6. Sample Completion**

Step 5b. Horizontal Plankton Tow

- Sampler: Throw net as far as possible and estimate the distance thrown.
- Handler: Record this distance, in meters, on the sample bottle and on the COC.
- Sampler: Slowly reel in the net, making sure the entire opening of the net is submerged and that the net is not dragging along the bottom. . If cod-end is filled with sediment, discard sample, rinse cod-end in lake, and re-sample.
- Proceed to **Step 6. Sample Completion**

Step 6. Sample Completion

- Sampler: After each tow is complete, rinse the contents of the net into the cod-end by dunking the net into the water three-times without submerging the opening of the net.
- Sampler: Unscrew the cod-end, gently swirl the sample around to drain excess water, and pour the remaining sample into the appropriate pre-labeled sample bottle.
- Sampler: Using a wash-bottle containing distilled or DI water, rinse the cod-end and pour the rinse water into the sample bottle, repeating this step three times.
- Sampler: Rinse the cod-end and the net (with the cod-end unscrewed) in the lake water between each tow.
- Sampler: Reattach cod-end to the net and prepare for the next tow.
- Collect 5 tows and combine all 5 tows into a single sample bottle.
- Sampler: Leave enough space for the addition of the alcohol preservative (3x the sample volume). If sample water volume in the bottle exceeds ¼ of the bottle volume, pour the unpreserved sample back into the cod-end and swirl gently to drain excess water. Return sample to the bottle and rinse cod-end with distilled or DI water as above.
- Handler: Label bottle with the number of tows and length of tows. Record on COC.

Step 7. Sample Preservation

- Handler: Add 6 drops of Tris buffer per every 100 mL of sample.
- Handler: Gently shake the bottle to mix.
- It is important to add the Tris buffer before the alcohol.
- Handler: Calculate the amount of 90% or higher alcohol to add by:
    - Measure the height of the collected sample (in cm or in) and multiply by 3.0.

    - The result of this calculation is the amount of alcohol, in cm or in, that should be added to the sample.
    - Add alcohol to the sample until the height of liquid increases by the calculated number.
    - Total volume of the sample and alcohol should not exceed the shoulder of the sample bottle.
- Handler: Gently shake the sample to mix, then release built up pressure in the bottle by opening the lid of the bottle.
- Handler: Close the lid tightly and seal the bottle with electrical tape around the base of the lid and the top of the bottle to prevent leaking.
- Handler: Ensure any writing or labeling on the bottle was not inadvertently rinsed off while adding the alcohol. Re-label if necessary.
- Handler: Place sample in a cooler with ice.

Step 8. Do it again 4 more times

- Repeat steps 5a/5b – 7 four more times, for a total of 5 single sample bottles.
    - 25 tows total, with 5 tows per sample bottle.

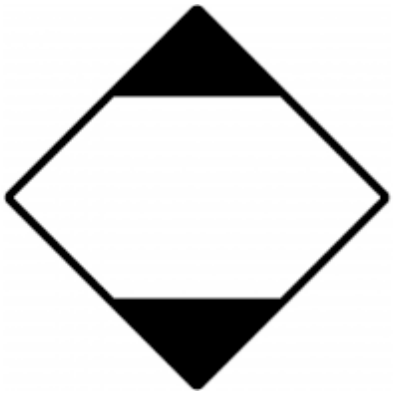
Step 9. End of Day Procedures

- Store samples on ice in cooler or keep samples refrigerated until they are shipped.
- Decontaminate the plankton tow net and the cod-end by submerging them in a bucket with vinegar for at least 10 min.
    - Rinse with tap, well, DI or distilled water.
    - Air dry before storing.
- Rinse the Secchi disk and other equipment that came into contact with water.
    - Air dry before storing

Step 10. Shipment

- If your cooler has a drain valve, make sure it is closed and sealed with tape. Make sure cooler is clean and dry. Open a trash bag and lay it in the cooler to contain any leaks that may occur. **DO NOT USE ICE**.
    - Coolers that leak, sweat, or are wet in any way will be rejected by all shipping companies and may end up lost. Instead, use frozen ice packs to keep samples cool during shipping.
- Once all samples and ice packs are inside, close garbage bag by tying a tight knot to prevent spills during shipping.
- Confirm that all information has been added to the COC. Put the COC into a plastic bag and place on top of the samples. Tape lid and sides of cooler securely closed.

- Due to the increased volume of ethanol required to preserve samples, coolers must be shipped via Ground transport only, and must be labeled with a "Limited Quantity" label, shown below. This label indicates that each individual sample bottle contains less than 1L/1000 mL of ethanol. The label can be printed from the internet and taped to the cooler.
    - Note that the center of this label is blank – a similar label with a "Y" in the center is for air transport.
    - Coolers shipped via UPS may contain no more than 11 – 500 mL sample bottles and must be labeled with the statement "Contains Ethyl Alcohol".
    - Coolers shipped via FedEx must weigh less than 66 lbs. when loaded with samples and icepacks. Additional questions regarding shipping requirements should be directed to your chosen shipping company.



- Ship samples as soon as possible after collection, ideally within 2 days of collection. Confirm that samples will not arrive in the Denver mailroom on Friday afternoon or over a weekend, even if that means holding samples for an additional day or two.
- Ship samples to the following address:

    US Bureau of Reclamation
    Attn: ECO-LAB (86-68560)
    1 Denver Federal Center
    Denver, CO 80225

- If you would like email confirmation of sample arrival at the lab, email the tracking number to the Eco-Lab at: **bor-sha-ecolab@usbr.gov**.

BUREAU OF RECLAMATION

APPENDIX B - CHAIN OF CUSTODY

ECOLOGICAL RESEARCH LABORATORY
INVASIVE MUSSELS FIELD SAMPLING LOG

Sample Collector and Agency:_____ Net Diameter: _____

Phone Number: _____ Type and % Alcohol Added: _____

Email: _____ Baking Soda Added (circle): Y / N | Tris Buffer Added (circle): Y / N

Date Collected	Water Body	Sample Location	Tow Type (V or H)	Number of Tows	Length of Tows (M)	Total Water Depth (M)	Secchi Depth (M)	Coordinates (decimal degrees preferred)

Special Instructions: _____

Address to Return Cooler: _____ Page _____ of _____