eDNA Sampling Protocol:

This protocol has been adapted from the Montana Fish Wildlife & Parks (MT FWP) plankton tow protocol that is based on the standard United States Bureau of Reclamation (USBR) veliger plankton tow protocol which has been accepted/used by Western Regional Partnership (WRP) and the United States Geological Survey (USGS.) We integrated the Flathead Lake Biological Station (FLBS) standard eDNA protocol to show additional steps useful for collecting an eDNA sample along with the standard veliger sample. Biologists often take multiple replicate tow samples (N=5) at each site and pool them into one container. This protocol describes the collection of one of these replicates with the addition of a supplemental eDNA sample.

Crucial Considerations

1. **Gloves must be always worn and changed periodically** as directed in the protocol. Change gloves whenever you might have contaminated them (e.g., touched an old sample, local water, dock, boat, etc.)

2. It is crucial to have **decontaminated** your net and collection bucket before sampling (**see detailed protocol below**). We recommend storing decontaminated nets and collection buckets in individual garbage bags, labeling and adding the buffer in advance and creating individual kits for sterile sampling supplies and equipment for each site. Once on site, a sterile working area should be established, and sampling equipment and sample collection supplies should be separated and organized ahead of time. Note: Nets must be decontaminated between different water bodies. In mussel positive and suspect waterbodies, nets must be decontaminated between each sample collected. We recommend having enough decontaminated nets to have 1 per replicate and to do DI blanks for each net.

3. A deionized water **(DI) blank** is required to check for equipment contamination for each net/ collection bucket. You can skip this but then cannot test for potential contamination of samples or equipment used at a site.

4. When collecting an eDNA sample, we recommend doing a **final wash with 95-100% EtOH** to rinse materials down into the bottom of the collection bucket. Then pour 10 mL of concentrated sample into a 50 mL tube and top off with 40 mL of 95-100% ETOH. larger volume samples can be collected as long as it is preserved at 80%. (Note: Additional tubes may be necessary to collect entire sample)

5. **80% final EtOH concentration** is recommended. eDNA samples should be **stored away from light in a cooler with ice** immediately after collection. UV light degrades DNA. Make sure the lid is screwed on flush and tightly.

6. **Labels should include Date, Location/Site ID, Sample # or ID and be consistent** for all samples. Cover labels with clear tape. (Even pens that say EtOH-proof will wash and smear). It is crucial to have permanent labels. To help prevent cross-sample contamination, consider storing samples from each site in a separate additional container (e.g., large Ziplock).

7. Compositing (pooling) of samples requires homogenization and then the eDNA sample can be taken. **We recommend taking a 10 mL aliquot from each tow in the field before compositing and preserving in a 50 mL tube with 95-100% EtOH per instructions for eDNA below**. Composite samples can only be used for presence/absence detection especially if they come from multiple sites. **Avoid compositing (pooling together) samples from different sites (locations)**. Consider keeping multiple samples (different tows) separate from one site and let the lab composite samples (if the lab might want to test each sample (tow net) separately for increased sensitivity or to test "repeatability" of detection from different tow samples.

8. **Bleach is dangerous!!!** Wear eye protection and gloves!!!

Equipment and Supplies:

Equipment/Disposables	**Specs/Description**	**Count/Amount Needed**
Personal COVID protection equipment	Masks, sanitizer, etc.	Per agency requirements
Gloves	Latex or Nitrile	3 pairs per person per sample
Plankton net	30 cm diameter x 90 cm length	1 per site
Collection bucket	61-64 μm mesh	1 per site
pole/ rope and bridle	Varies per site/study	1 per site
Screw cap tubes	50 mL	1 veliger + 1 eDNA per tow and 1 DI blank per net used
Ethanol (EtOH)	95-100%	~ 40 mL per eDNA sample + ~40 mL per veliger sample and extra for rinsing
Deionized (DI) water	N/A	1 gallon per Blank + 1 gallon per tow for rinsing
Tris pH Buffer	N/A	1 x 50 mL tube
Tube Racks	For 50 mL tubes	2
Squirt bottles	500 mL	2
Cooler	N/A	1
Freezer Packs	N/A	Varies

Clear Packing Tape	N/A	1
Pens, Pencils and Sharpies	N/A	1
Tubs	Containers for decontamination	3
White vinegar	5% acetic acid	~6 gallons
Bleach	~6% sodium hypochlorite	~3 gallons
Gloves for Decon	Dishwasher Rubber Gloves	1 pair per person
Protective Eyewear	Lab Goggles	1 per person
Garbage bags	N/A	2 per site

Work Area Setup/ DI Blank Collection

1. **Put on a clean pair of latex or nitrile gloves**
2. Prepare a clean surface. (Note: A small tarp or the lid of a decontamination tub can be used.)
3. Separate sampling equipment from sample storage supplies (tubes, EtOH etc.) and designate an area for contaminated supplies and a disposal container for single-use materials.
4. Pre-label tubes and caps and cover label on side of the tube with clear tape (1 per DI Blank; 1 per Veliger Sample; 1 Per eDNA Sample)
5. Fill EtOH squirt bottles.
6. Place your 50 mL tube rack and labeled tubes on the clean surface.
7. Use a sponge to wipe down your pole with 10% Bleach solution and rinse thoroughly with DI. (Note: Change gloves after using bleach.)
8. Remove your decontaminated net and collection bucket from the storage bag (**See detailed decontamination procedure below**).
9. Attach net to the pole. (**Note: If using a rope or winch**, we recommend doing the DI blank before attaching the rope or cable.)
10. With one person holding the pole with the net off the ground, screw the collection bucket to the net adapter.

11. With the pole and the net off the ground, thoroughly rinse all inner and outer surfaces of the net and collection bucket with 1-gallon of DI water. (Note: Take care that water does not splash back up and contaminate the blank or your other sampling gear.)

12. Allow water to drain below the connection between the collection bucket/net adapter and the collection bucket. (water will drain quickly, but blank should be mostly EtOH anyway)

13. Detach the collection bucket and set the pole and net aside in a clean area. (Note: The net and collection bucket from DI blank should be used for the sample and must not contact contaminated surfaces.)

14. Remove as much residual water as possible. Sample should be concentrated to **10 mL**.

15. Turn the collection bucket so the screen is facing up to stop remaining material from leaking out.

16. Rinse collection bucket screens with 95-100% EtOH (Note: Tip of squirt bottle must not touch or be placed inside the collection bucket.)

17. Remove the cap from the 50 mL tube designated for your blank.

18. Transfer material to the 50 mL tube (Should be about 10 ml. Top off with 40 mL of 95-100% EtOH to reach at least 80% preservation concentration.)

19. Store samples in a cooler out of direct sunlight, on ice.

20. Discard gloves.

eDNA/Veliger Sample Collection

1. **Put on fresh gloves.** (It helps to have one person do the collection, sample concentration and pour the sample into the tube and have a second person manage the tubes, add final preservation and store the samples.)

2. Make sure the EtOH bottle is full, tubes are labeled and organized, and you have your buffer solution made.

3. Reattach the collection bucket to the net. (Note: The same net and collection bucket from DI blank should be used to take the sample)

4. Collect samples per your agencies' standard plankton veliger tow procedure. (We recommend a 100-meter tow using a 30 cm diameter 64-micron net and collection bucket with the same 64-micron mesh screen)

5. Allow the water to fully drain down through the net and through the collection bucket screens. (To help wash material down into the collection bucket, the net can also be sprayed down with DI water.)

6. Before unscrewing the collection bucket, make sure the water level is below the connection point between the net adapter and the collection bucket. (Note: The net can be held just above the collection bucket and swirled to assist with draining the liquid to this point)

7. Unscrew the collection bucket without spilling contents. (Note: Water will be draining out of the screen at this point. To avoid contamination, do this away from other sample collection supplies.)

8. Concentrate the sample as much as possible by swirling the contents in the collection bucket and allowing water to drain through the screens. Final samples should be no more than 20 mL if splitting or 10 mL for eDNA-only **(Note: Samples containing lots of sediment or organic material may block the screens and it will be necessary to gently tap the screens to assist with draining.)**

9. Do a final rinse with 95-100% EtOH to flush remaining material from sides and screens. (Note: Tip of the squirt bottle must not touch or be placed inside the collection bucket.)

10. Turn the collection bucket so the screen is facing up to stop remaining liquid from leaking out. (Note: Samples should not be excessively large, and you may have to drain some of the EtOH used for rinsing through the screen.)

11. Gently swirl to homogenize (be careful not to swirl the sample out of the cup).

12. Remove the cap from the 50 ml tube designated for your eDNA sample.

13. Tilt the collection bucket with the screen facing up. (Note: The collection bucket must not be contaminated while collecting samples and should be held until the eDNA sample is collected.)

14. Pour 10 mL of the homogenized sample into the designated eDNA tube. **If doing eDNA only, skip to step 17.**

15. Remove the cap from your Plankton tow tube.

16. Pour 10 mL of the homogenized sample into the veliger tube.

17. Top off the eDNA sample with 40 mL 95-100% EtOH. Final EtOH concentration should be at least 80%. (Note: It is crucial to close the lid very tightly because EtOH leaks).

18. Store eDNA samples in a cooler out of direct sunlight, on ice. **If doing eDNA only, skip to step 20.**

19. Preserve the veliger sample by adding 6 drops of Tris buffer solution and topping off with 95-100% EtOH.

20. Rinse the collection bucket and net in the waterbody or with a backpack sprayer to remove any leftover material.

21. Place used nets in a designated garbage bag or tub for contaminated non-disposable supplies.

22. Decontaminate all sampling equipment or if not taking independent samples rinse both the interior and exterior of the net and cod end with fresh, clean water.

Decontamination Background

All gear should be decontaminated between water bodies. Ideally, each waterbody should have its own set of sampling gear. But when this is not possible, decontamination of gear is very important. (Note: all nets can be stored and transported in a plastic (garbage) bag so that no debris or insects will get into the nets between decontamination and sampling).

The bleach solution must be made up fresh immediately prior to use, as bleach will quickly oxidize. This will kill organisms and denature DNA should any eDNA testing need to be conducted on samples collected with that net. (**Note:** for eDNA 30% bleach is recommended (e.g., Goldberg et al. 2016), with agitation 3-4 times during the 10-minute soak; be sure to wash off the bleach very thoroughly immediately after soaking to prevent net degradation from bleach; see FLBS training video). Items can also be thoroughly sprayed with 10% bleach solution and left to sit for 10 minutes. Bleach may be mixed in two strengths for spraying: 10% solutions require 10-minute contact time, but 20% solutions require 5-10 seconds contact time. Please use some common sense when spraying bleach – wear goggles and avoid inhaling vapors. Open windows when spraying large volumes indoors.

Not all nets are made the same so chemicals may destroy rubbers and glues. Bleach will cause the net to degrade so keep a close eye on the net for small pinholes that can be repaired before they turn into large tears that can't be repaired.

For decontaminating equipment used in DNA analyses (eDNA, PCR, qPCR, etc.) and/or to ensure organisms are not transferred between sites/waterbodies, the following protocol should be used:

Decontamination:

1. First rinse the net as clean as you can get it. If necessary, scrub surfaces to remove dirt, biofilm, or debris. (Rinsing and removal of material from nets at the sampling site prior to it drying will aid in this process)
2. Soak net, collection buckets and rope (if used) in vinegar (acetic acid (5%)) for 24 hours (or a minimum of 4 hours if working in the field). Be sure to test vinegar with a pH testing meter or strip to ensure pH level remains around 2-3. This will dissolve any shells and allow the bleach to penetrate and degrade any DNA present.
3. Rinse the net with clean water. (Note: It is okay to use tap water.)
4. Soak net, collection bucket and rope in a 30% bleach solution and agitate every 3 minutes for 10 minutes.
5. Rinse net, collection bucket, and rope in freshwater. (We recommend an additional post bleach soak in fresh water with periodic agitation for an additional 10 minutes)
6. Hang to dry away from sources of target DNA.
7. Store in containers provided for CLEAN and DRY nets only or in site specific sterile kits.