2019 WNERR eDNA Manual

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A Note on eDNA

Environmental DNA (eDNA) is a developing tool in conservation that allows scientists to establish the presence or absence of specific species in a system. This emerging technique relies on collaboration between ecologists, geneticists, and data scientists. One such group, the UNH-NERR Science Collaborative, explains its significance:

"Biological monitoring programs are essential foundations for effective management of estuaries and coasts, but they can be expensive, labor intensive, and intrusive on target species. Advancements in DNA methods now make it possible to identify organisms in an area by the DNA they leave behind. This residual or environmental DNA (eDNA) may be generated from feces, gametes, scales, bodily fluids, and cells that an organism sheds, and is easily collected from water and sediment samples. Rapid reductions in analytical costs now allow scientists to analyze eDNA in water samples and identify dozens of species without having to capture live animals or plants and reduce logistical challenges associated with traditional monitoring approaches."

This manual will serve as a guide for eDNA practices at the Wells National Estuarine Research Reserve, and our methods will vary depending on the question we ask. In each case, DNA from many species will be present within one water grab. We might choose to target DNA sequences for all species under an umbrella taxon to obtain a snapshot of biodiversity at our site (***metabarcoding:*** **Figure 1**). Alternatively, we might target the DNA sequence for one particular species to establish its presence or absence in the system (***qPCR*** or ***barcoding:*** **Figure 2**).

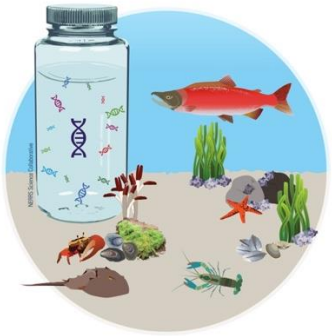


Figure 1: We may choose to isolate, amplify, and sequence the DNA for many species under an umbrella taxon to take a snapshot of biodiversity in the system. **Metabarcoding** allows us to sequence multiple species at once.

Figure 2: Alternatively, we may choose to target the DNA of a single species, which could be helpful for establishing the presence of an endangered or invasive species in a system. This can be achieved by **barcoding** or **qPCR**.

In general, the process will proceed in the following order:

(1) collection fieldwork of water or sediment samples,
(2) vacuum filtering for water samples,
(3) DNA extraction using the Qiagen DNeasy kit,
(4) Polymerase Chain Reaction (PCR) or quantitative PCR (qPCR) using primers for the desired taxa, and/or
(5) Illumina sequencing to obtain genomic sequences for bioinformatic analysis.

Steps **1** and **2** will be performed at WNERR. While WNERR does have the ability to extract DNA, steps **3–5** will typically be outsourced to genomic centers such as the Hubbard Center for Genome Studies at UNH.

Best Practices

- Change gloves when the protocol requires **AND** when you suspect they may have become contaminated. This includes touching non-sterile surfaces, spilling a sample/reagents, and leaving the room. It may feel wasteful, but it is crucial to the integrity of the results. The little things matter!

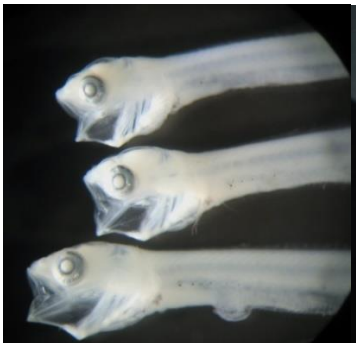
- Aliquot your materials whenever possible, namely glassfiber filters, microcentrifuge tubes, and chemical reagents for DNA extraction. This decreases the chances of contaminating a bulk package – if a small aliquot is contaminated, you will always have more.

- Keep a lab notebook with detailed notes. This allows you and your collaborators to notice small details that may help to explain a hiccup in later lab processes or in the final results.

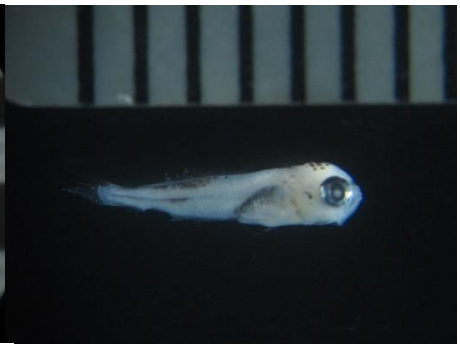
- Ask questions. Lots of little tricks are out there – reach out to collaborators at UMaine or UNH with any questions about materials or methods.

Larval Fish eDNA Sampling Protocol

Protocol based on "Water Bottle Sampling for Environmental DNA Analysis," Kinnison (University of Maine); "Wells NERR Larval Fish Sampling Protocol," Miller, J. (WNERR)



Larval Atlantic herring (2009)



Larval red hake (2013)

Purpose: To detect fish species in the Webhannet River Estuary by extracting eDNA from water and captured specimen biomass. This will be executed in coordination with our long-term larval fish and zooplankton monitoring initiative.

Location: Wells Harbor, off the main dock (site code: WHAR).

Sampling frequency: 60 minute deployments taken 1.5 hours before listed high tide, 1x/month, July-October.

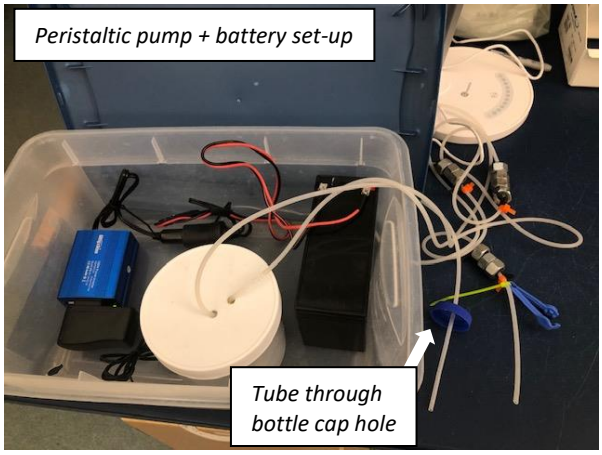
Equipment and materials for one collection visit:

eDNA Prep / Collection Materials

- Disposable exam gloves
- Indelible ink marker
- Three sterile 1.5 or 2.0 mL microcentrifuge tubes
- 2 sterile disposable pipettes
- 6 1-gallon Ziplock bags
- 1 snack-sized Ziplock bag
- 2 unscented kitchen trash bags
- Duct tape
- 1 roll paper towels
- Clorox bleach spray (1.84% NaClO)
- 1 ½ gallons concentrated bleach solution
- 1 large plastic bin with lid
- 1 cooler + ice packs
- 4 1-L Nalgene bottles
- 1 1-gal water bottle
- Peristaltic pump + battery set-up in lidded bin
- Bottle cap with a hole attached to pump tubing
- eDNA notebook with pencil

Larval Tow Equipment (1 set for eDNA tow + 1 set for regular fish tow)

- 0.5 m diameter plankton net (500 µm mesh)
- Flow meter (attached to net bridle)
- Cod-end
- Tow rope (attached to net)
- Depressor (anchor)
- Notebook with pencil



Sample Descriptions

Label	Description	Vol	Location
WHAR B1	Blank #1, tap water run through sterile net	1 L	LAB
WHAR B2	Blank #2, tap water opened & closed at harbor	1 L	FIELD
WHAR B3	Blank #3, tap water run through sterile pump	1 L	LAB
WHAR W1	Water #1, net rinse water grab sample post-deployment	1 L	LAB
WHAR W2	Water #2, continuous harbor water sample	2.6 L	FIELD
WHAR LAR	Larval biomass sample from cod-end: x1 pre-blender, x2 post-blender	1.5 uL x3	LAB

Preparing eDNA Kits

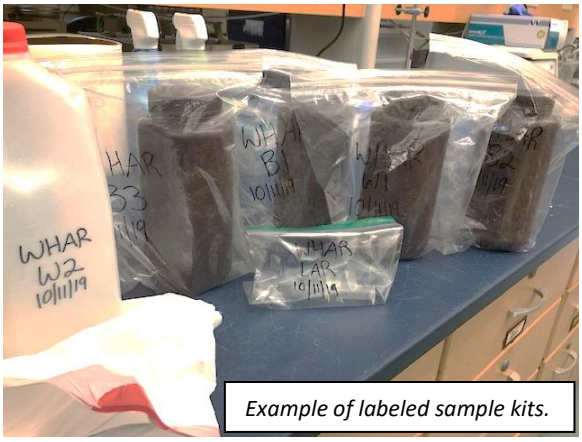
1. Wearing gloves, wipe down a surface with Clorox spray and cover with paper towels. Sterilize cooler, ice packs, and indelible ink marker with Clorox bleach spray.

2. With clean gloves, uncap the 1-gallon water bottle, empty, and recap. On sterile surface, label the bottle **WHAR W2 *date***. Place in a sterile trash bag.

3. On sterile surface, label 5 1-gallon Ziploc bags with the following:
   i. **WHAR B1 *date***
   ii. **WHAR B2 *date***
   iii. **WHAR B3 *date***
   iv. **WHAR W1 *date***
   v. **TRASH**

4. Label the snack-sized Ziplock bag **WHAR LAR *date.*** Label three 1.5-uL microcentrifuge tubes with **WR *MMDDYY* LAR A**, **WR *MMDDYY* LAR B**, and **WR *MMDDYY* LAR C** (ex: WR 071919 LAR A). Place labeled tubes in the bag.

5. Place at least 8 pairs gloves and 4 paper towels in a Ziploc bag.



Example of labeled sample kits.

Preparing the eDNA Tow Net

1. Prep flow meter as usual: make sure it spins freely, fill with water, replace the screw, and attach meter to net bridle with bungee cord and cable tie. Attach the tow rope and cod-end bottle to net, but keep the anchor detached.
2. Wearing gloves, fill large plastic bin with 10% bleach solution (1 gallon bleach + rest of bin filled with tap water). Fully immerse for at least 15 minutes: net, anchor, tow rope, cod-end, 4 Nalgene bottles with caps detached, food processor cup, blade, and top. Submerge as much of the pump tubing as you can (both input and output ends) with the bottle cap with hole in it attached, and run the pump for at least 15 minutes.

3. Meanwhile, bring a cart outside and bleach the surface to act as a sterile outdoor work station. Wipe down bin lid with bleach spray and place on sterilized cart. Hang up a sterile trash bag on the nail outside and use duct tape to keep the

bag lying flat against the wall. This creates a sterile location to hang the net.

4. *Thoroughly* rinse all bleached materials with tap water, including the plastic bin and lid, to ensure no bleach remains. Nalgene bottles should be filled, capped, and shaken to rinse, repeating at least 3 times. The net should be hung on the nail so nothing touches the ground (hanging parts can be tucked into the net) and the trash bag separates it from the wall, and hosed down thoroughly.

5. After it has been thoroughly rinsed, fill bin with tap water, submerge tubing again, and let pump continue to run for a few minutes.

6. Fill 1 sterilized Nalgene bottle with tap water, cap, and place in the **WHAR B2** bag.

7. Set food processor items inside for later use. Place 1 sterilized Nalgene bottle in each of the remaining labeled 1-gallon Ziploc bags. Place **WHAR B1** and **WHAR B3** kits in sterilized cooler and bring outside.

8. Collect sample **WHAR B3:** Dump out and refill bin with fresh tap water. Place weighted end of tubing in bin and other end in the **WHAR B3** bottle. Let pump run until bottle is full (~20 minutes), keeping the bottle in the cooler. Check on sample occasionally to ensure the bottle does not overflow and tubing stays securely in the bottle. Cap the bottle and close the Ziploc. Place sterile tubing in a clean Ziploc bag to keep tubing separate from the non-sterile components of the pump and battery set-up.

9. Collect sample **WHAR B1**: Empty bin and place under sterile net. Rinse net again with tap water so it collects in the bin. Submerge the **WHAR B1** bottle in the net rinse water to fill. Cap and place in its Ziploc bag.

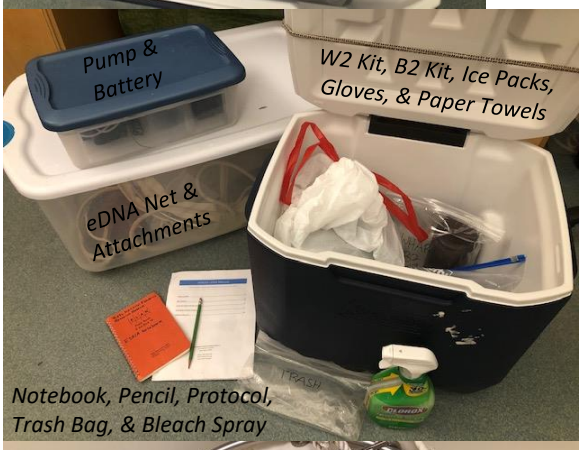
10. Place these samples immediately in the fridge if vacuum filtering within 3 days, or in freezer if storing long-term. If you believe the inside of the cooler may have become contaminated, re-sterilize it with bleach spray.

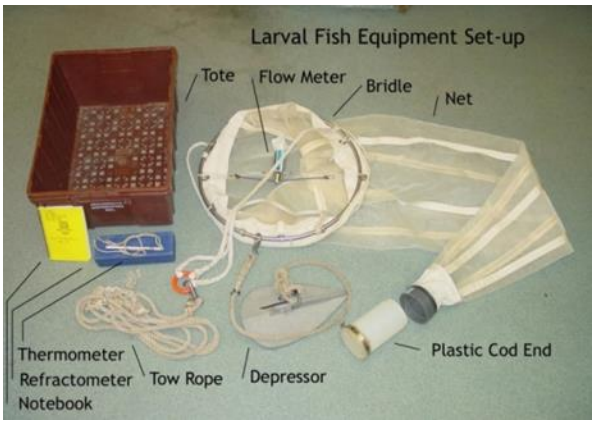
11. Empty tap water from bin, place net with attachments in sterile bin, attach the anchor to the net bridle, and close the lid.

<u>Field Supplies Checklist:</u> (see next page for photos)

1. Bin containing eDNA net with attachments
2. Cooler with ice packs containing:
    a. **WHAR W2** (bottle in trash bag)
    b. **WHAR B2** (bottle in Ziploc bag)
    c. Bag of gloves and paper towels
3. Bin containing pump and battery set-up, with sterilized tubing and attached bottle cap contained within a Ziploc bag.
4. Bottle of bleach spray
5. Ziploc bag labeled **TRASH**
6. eDNA Protocol
7. eDNA Notebook
8. Pencils
9. Bin containing regular tow net with attachments, YSI, regular tow notebook, eDNA fish tow notebook, and pencils.

Cooler containing W2 kit, B2 kit, ice packs, and bag of gloves and paper towels.





Deployment & FIELD Sample Collection

1. Arrive at harbor at least 1.5 hours before high tide. Set up where you will have about 10-15 ft of dock space. Regular tow net will go in front (upstream) of the eDNA net.

2. Wear gloves. Have Sampler collect the samples and Assistant touch any non-sterile surfaces (bags, cooler, etc.).

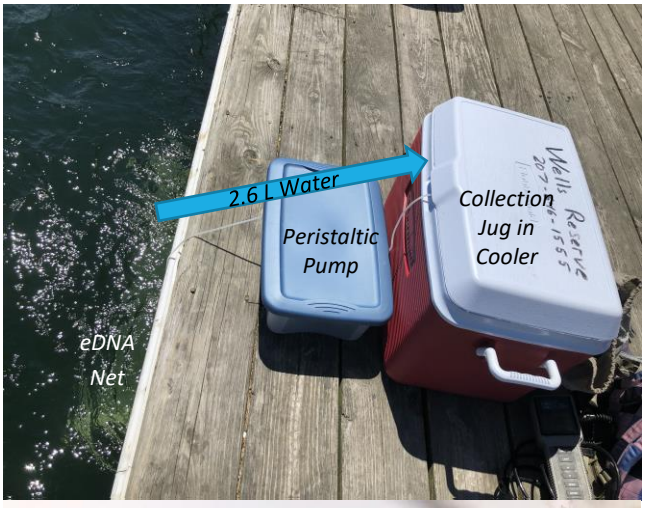
3. Assistant opens eDNA bin. Wearing gloves, Sampler reads the Meter # and Flow Start reading on the flow meter. Assistant records these numbers.

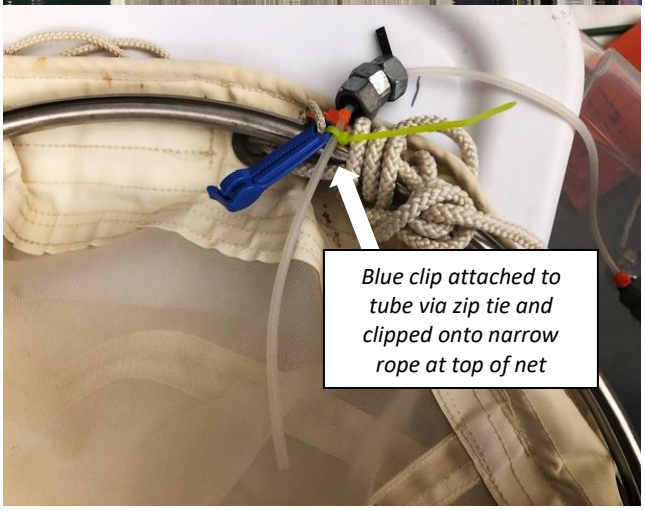
4. Assistant and Sampler both wear gloves to work together to lower the anchor and net into the water 1 meter down (rope is marked). Make sure the net does not touch the dock and the ropes are untangled and tie the longer rope to the dock cleat. Make sure the open end of the net is facing the incoming current. Once attached to the cleat, have one person hold the net at the surface (so top of net ring is out of the water) while the other person attaches the weighted pump tubing: the blue clip attaches to the loop on the top of the net ring. Release the net down and record the Start Time.

5. Collect sample **WHAR W2*:*** Open cooler and trash bag. Replace the regular bottle cap with the cap with a hole so pump tubing empties into the bottle. Keep the original cap inside the bottle's trash bag. Turn on pump and make sure water is being pumped into the bottle.

6. Collect sample **WHAR B2**: Open the bottle (DON'T pour out water), wait a few seconds, and close it. Store immediately in cooler with ice. Close the cooler (but don't pinch the tubing shut!).

7. Deploy the regular fish tow net per usual so cod end of regular net is slightly in front of





eDNA net opening *but not touching*. Record meter number, start flow, start time, temperature, and salinity. Make sure YSI does not touch eDNA net.

8. Monitor net during deployment: Make sure the open end stays facing the incoming current and stays deployed at 1 meter. Watch for incoming boats and be prepared to move the net to another cleat if needed. Keep equipment in a safe location and out of walking path. Make sure pump bin does not get pulled into the water. Record detailed notes about positioning of net, currents, weather, activity near the net (fishing, crabbing, boats, etc.), and whether sterilized items touch the docks or other "contaminated" surfaces.

9. After 60 minutes, put on a new pair of gloves and retrieve the eDNA net. Have one person hold the net at the surface while the other unclips the tubing. While holding the bridle firmly, gently dip the net in and out of the water to flush sample material down through the cod end. Untie the rope from the cleat, bring the entire net out of the water, and place directly into plastic tote without touching anything else.

10. Record number on flow meter and time when net was retrieved. Fasten the lid onto the bin.

11. Turn off pump, remove tubing from bottle, and replace the original bottle cap. Close the trash bag and the cooler.

12. Retrieve the regular fish tow net as per usual. Record end time, end flow, and any notes, including a note that eDNA was collected during this tow.

<u>Post-Deployment & Lab Sample Collection</u>

1. Back at the lab, put on new gloves and open the plastic bin. Remove the net from the bin and hang it on the outdoor hook with the trash bag separating it from the wall. Place the plastic bin directly underneath to collect rinse water.

2. Rinse the net thoroughly with tap water from a hose to lead all biomass into the cod-end. Rinse water will collect in the bin.

3. Collect sample **WHAR W1**: Submerge Nalgene bottle in rinse water, stir around to mix, fill, cap, and place back in Ziploc bag.

4. Screw off cod-end and transfer contents to the food processor cup. You can use a squirt bottle of tap water to rinse the cod end, but avoid adding too much water to the sample as this will make pureeing messier. With a clean gloved hand and sterile pipette, transfer 1.5 mL of sample to the **LAR C** microcentrifuge tube. Cover food processor and puree to obtain homogenous mixture. One after another, dip the **LAR A** and **LAR B** microcentrifuge tubes into larval mixture and fill. You can use a sterile pipette to help transfer samples to the tubes as well. Try to collect the samples from various places throughout the mixture.

5. Transfer all samples (from field and lab) to fridge if vacuum filtering within 3 days, or in freezer if storing long-term. Try to filter on the same day if possible.

6. Detach and empty eDNA flow meter. Clean and store all equipment. Process regular fish tow net as per usual, but label jar with "eDNA" in red Sharpie for *priority processing.*

<u>eDNA Filtering Prep:</u>

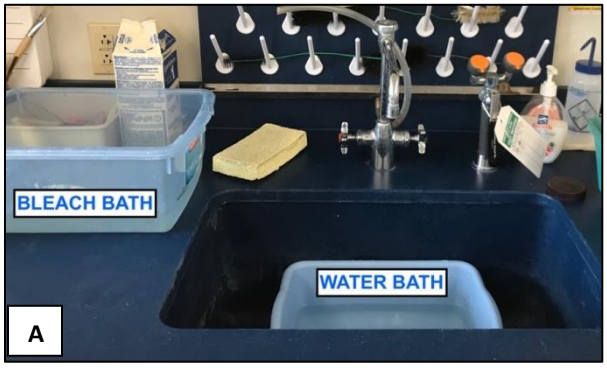
1. Sterilize counter space with bleach spray and wear clean gloves while labeling.
2. Label 1.5 mL microcentrifuge tubes: **WR *MMDDYY SAMPLE ID*** (ex: WR 071919 B1). Add "A, B, C, D, …" to the end of the label if a sample will be split between multiple filters (each filter is placed in its own tube). Place in microcentrifuge tube holder. *NOTE: The **W1** sample usually needs to be split between 4 filters (A, B, C, D).*
3. Label snack-sized Ziploc bags: **Wells NERR eDNA, *DATE, SAMPLE ID***

eDNA Water Filtration Protocol

WNERR Protocol based on UMaine Kinnison Lab Protocol (2018).

Equipment needed

- Gloves, nitrile
- 2 plastic bins for 50% bleach bath and water bath
- Sterile cooler with ice
- Magnetic filter cups & stoppers
- Bleached table top
- 2 Erlenmeyer filtering flasks, 4 L, along with tubing and plugs for spout
- Vacuum pump
- Power strip with on/off flip switch to control pump by stepping on it (avoid hand contamination)
- Glassfiber filters, 47 mm diameter
- Filter tweezers
- Paper towels
- Beaker
- Prelabeled 1.5 ml conical microcentrifuge tubes
- Indelible markers
- Bag for recycling used bottles



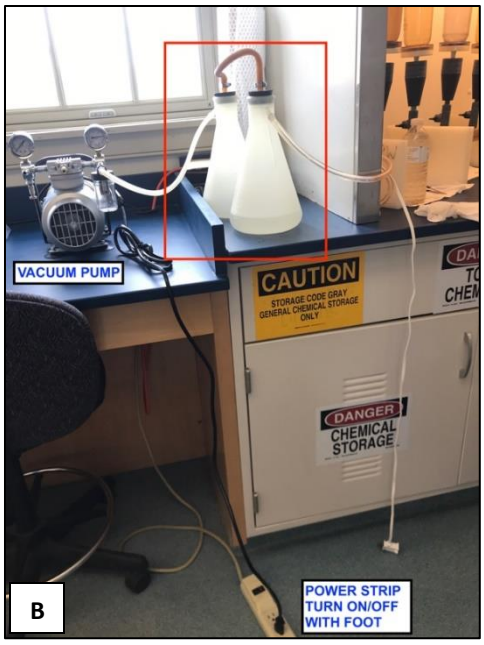
1) Wear Gloves

2) Bleach necessary surfaces, including lab bench inside and next to fume hood.

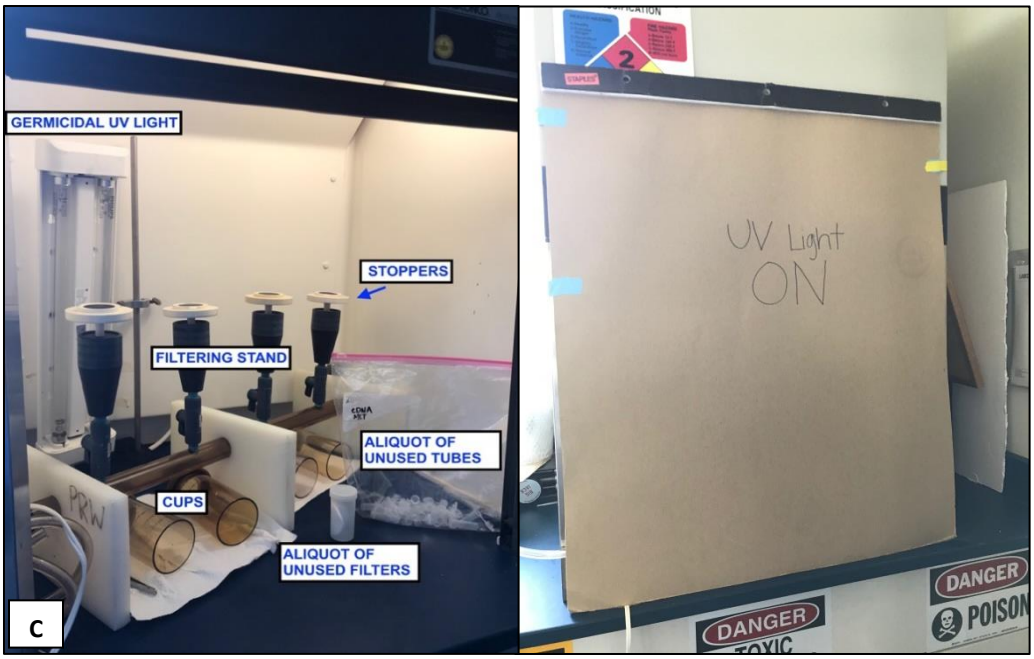
3) Prepare bleach bath (50% bleach) and water bath: Sterilize water bath bin and thoroughly rinse before filling with tap water. *(Image A)*

4) Bleach magnetic filter cups, stoppers, filter tweezers, and beaker: Soak in bleach bath 1 min, rinse thoroughly with tap water, and air dry on clean table.

5) Assemble the filtering equipment: Set up pump and Erlenmeyer filtering flasks on bleached table to left of fume hood. Connect: (1) pump to first Erlenmeyer flask with the shorter tubing; 2) rubber black stopper to mouths of both Erlenmeyer flasks; (3) filtering stand to second Erlenmeyer flask with the longer tubing. Plug vacuum pump into power strip with switch that you can step on to turn on/off. *(Image B)*



6) Prepare for UV. Place all bleached filter cups, stoppers, filter tweezers, and beaker in fume hood for UV. place aliquots of filters and tubes in hood at start of the day OR per every 10 samples, whichever comes first. Cover the fume hood with cardboard and plug in germicidal UV light. Leave for at least 15 minutes. *(Image C)*

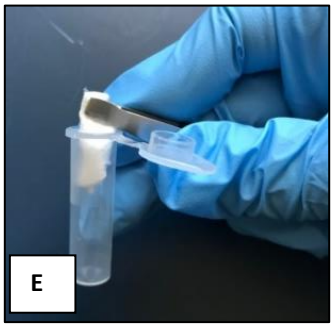


7) After UV: Unplug light and remove cardboard barrier. With gloved hands, place stoppers into filtering stand. With sterilized tweezers, place a clean filter onto each stopper and attach magnetic cup. *(Image D)*

8) Turn on pump by stepping on power switch (no hands!). Assistant: open cooler, pull out and open sample bag. Sampler: reach inside sample bag, pull out and open bottle, and pour sample into cup.
    a. Filter lab blank first: Fill sterilized beaker with 1 L tap water and pour into filter cup. Run at the start of each day OR per every ten samples, whichever comes first.
    b. Filter water samples, including field blanks:
        i. Change gloves when pouring separate samples.

ii. If water is high in particulates or flow is slow, use an additional filter (max 2 filters/sample) *Note: In 2019, net rinse samples were split between 4 filter cups (~250 mL each) due to excessive filtering times.*
iii. Run pump a bit longer than it takes for the water to run through to "dry" the filter.
iv. Turn off pump with your foot, remove filter cups, and change gloves.

9) Roll up filter: Use clean filter tweezers to roll filter up and clean left gloved pinky finger to help if needed. Use tweezers to place rolled filter into pre-labeled microcentrifuge tube. Place tube/s in pre-labeled Ziploc bag. Store tubes of filters on ice bath until moving to freezer. *(Image E)*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10) When finished with a round of samples, place filter cups, stoppers, and tweezers in bleach bath and then proceed to change gloves, grab a clean filter cup and stopper for the next round of samples.

11) Repeat beginning with step 7 until finished filtering for the day. Keep an eye on the Erlenmeyer flasks and empty when needed to ensure water doesn't enter the pump.

12) Disconnect tubing from vacuum pump and run the pump for 10 minutes to ensure it stays dry.

13) Store samples in -80 freezer if not extracting for DNA soon.

14) Put equipment away and wipe down surfaces.

Additional Resources

eDNA Overview:

Laramie, M. B., Pilliod, D. S., Goldberg, C. S., & Strickler, K. M. (2015). *Environmental DNA sampling protocol — filtering water to capture DNA from aquatic organisms* (No. 2-A13). US Geological Survey.

Schmelzle, M. C., & Kinziger, A. P. (2016). Using occupancy modelling to compare environmental DNA to traditional field methods for regional-scale monitoring of an endangered aquatic species. *Molecular ecology resources*, *16*(4), 895-908.

Thomsen, P. F., & Willerslev, E. (2015). *Environmental DNA–An emerging tool in conservation for monitoring past and present biodiversity.* Biological Conservation*,* 183*, 4-18.*

Larval Fish eDNA Studies:

Bréchon, A. L., Coombs, S. H., Sims, D. W., & Griffiths, A. M. (2012). Development of a rapid genetic technique for the identification of clupeid larvae in the Western English Channel and investigation of mislabelling in processed fish products. *ICES Journal of Marine Science*, *70*(2), 399-407.

Loh, W. K. W., Bond, P., Ashton, K. J., Roberts, D. T., & Tibbetts, I. R. (2014). DNA barcoding of freshwater fishes and the development of a quantitative qPCR assay for the species-specific detection and quantification of fish larvae from plankton samples. *Journal of Fish Biology*, *85*(2), 307-328.