A Field Protocol for Preparing, Collecting, and Storing Aquatic eDNA Samples

COLORADO
Parks and Wildlife
Department of Natural Resources

Contents

Cover photos (clockwise): Breeding Boreal Toads by F. Boyd Wright III; Brook Trout by Charles G. Summers Jr.; Greenback Cutthroat Trout by Charles G. Summers Jr., wildimages.biz

Background

Over the past decade, the use and scientific relevance of environmental DNA (eDNA) as an additional sample method to detect and quantify aquatic organisms has increased considerably (Ficetola et al. 2008; Thomsen et al. 2011; Carim et al. 2016). Environmental DNA has shown particular promise in monitoring organisms that are otherwise difficult to sample, and has been used in early detection of invasive species (Goldberg et al. 2013), distribution mapping of rare endemic species (McCelvey et al. 2016), occupancy modeling (Pilliod et al. 2013; Bailey et al. 2019), and even to estimate abundance of brook trout (Baldigo et al. 2016). Colorado Parks and Wildlife (CPW) has used eDNA for early detection of Aquatic Nuisance Species, surveying potential habitat for boreal toads, and evaluating chemical reclamation projects.

Several protocols exist for eDNA collection (e.g. Carim et al. 2016; Smith-Root 2020); however, CPW biologists usually use equipment and protocols developed by Pisces Molecular LLC (Boulder, CO). Their method uses a modified Nalgene bottle as a reservoir for water collection. A bike bump (either hand or battery powered) is then connected to the Nalgene bottle to pressurize water through a filter medium (Figure 1). Samples are sent to Pisces Molecular, where DNAs are first isolated, then species specific target fragments representing the organism of interest are amplified (if present) with quantitative Polymerase Chain Reactions (qPCR) using either published or novel primers.

Although Pisces Molecular provides a general field protocol that is useful to getting started with eDNA sampling using their equipment, a more specific uniform statewide protocol would be beneficial for enhancing eDNA sample quality and fostering more consistent results. The occurrence of false positive results from sample contamination in the field is of particular concern, as they decrease confidence and utility in eDNA sample results. Additionally, equipment decontamination procedures may also result in residual chlorine that can degrade DNA in samples and result in false negatives. Thus, this field protocol is provided in an effort to minimize the occurrence of false negative and false positive samples, and yield more consistent results across comparable projects around the state.

Figure 1. A bike pump connected to the modified Nalgene bottle and filter medium used in collection of eDNA samples, following the Pisces Molecular protocol.

Definitions

In this document, "**clean**" refers to materials and equipment that are sealed and not previously used (e.g. a Ziploc bag fresh out of the box), or reusable items that have been decontaminated (e.g. Nalgene bottles) following procedures herein. In some cases, "**clean**" may be used interchangeably with "**decontaminated**." The term "**dirty**" refers to equipment and materials that have been used or potentially exposed to contamination.

Equipment Decontamination and Preparation

Supplies and Materials

Item	Use
Nitrile gloves	Handling and preparing sampling equipment
10% Bleach Solution	Decontaminating "dirty" Nalgene bottles
Schrader valve core tool[1]	Removing valve core from Schrader valve stem
Replacement Schrader valve cores[1]	Install in Schrader valve stem following decontamination and drying
Bottle drying rack	24 hours of UV and drying of decontaminated bottles
Used Nalgene sample bottles and lids[2]	Sampling equipment to be decontaminated
10 gallon tub for storing/cleaning used bottles	Used bottles are kept in a sealed container until cleaning
10 gallon tub for storing bottles	Sample bottles are kept in a separate tub once decontaminated
Preloaded 25 mm Swinnex filter holder[2]	Hold the filter medium for filtering water samples
Snack size Ziploc backs	Filter assemblies are pre-bagged (wearing gloves) prior to going into the field
Heavy duty Gallon size Ziploc bags	Sample bottles are bagged (wearing gloves) before going into the field
Teflon tape	Threaded neck of sample bottle is wrapped before going into the field
Distilled water	Final rinse of sample bottles

[1]Available: https://www.grainger.com/search?searchBar=true&searchQuery=schrader+valve+core
[2]Available: Pisces Molecular, 1600 Range St, Boulder, CO 80301, 303-546-9300

Procedures- Decontamination

1. Use one "dirty" designated closeable container for storage of used sample bottles and another for storage of "clean" decontaminated bottles ready for use. Keep these containers in separate areas of the workspace. Ideally, the "clean" container is kept far away from any fish sampling gear. The "dirty" container can be used to create a bath for decontamination (see #3).
2. Wearing nitrile gloves, use the Schrader valve core tool to remove the Schrader valve core from "dirty" bottle lids. Dispose of the used valve cores in the trash and place the lids back in the tub (not fastened to bottles).
3. Fill the storage container with enough water to submerge all bottles and lids. Either measure water volume as it is being added, or designate a marked "fill-to" line of known volume. Add bleach and thoroughly mix to create a 10% bleach solution, accordingly. Allow bottles to soak for 15 minutes. With the lid on the container, vigorously rock it back in forth to ensure that all

surfaces inside the container come into contact with bleach solution. You may also need to manually rotate the bottles to ensure that all surfaces have a minimum contact time with the bleach solution of 15 minutes.

4. Wearing a new pair of nitrile gloves, remove bottles and lids from the decontamination bath and thoroughly triple rinse with tap water. **It is critically important to ensure all residual bleach is removed.** Once all bottles have been rinsed in tap water, give them a final light rinse with distilled water (removes residual tap water which may contain chlorine that could degrade DNA in future samples).
5. Place rinsed bottles and lids on a large drying rack outdoors in a sunny location, protected from wind, where they should dry for at least 24 hours.

Procedures- Preparing Equipment for Future Use

1. Once bottles and lids are completely dry, having aired out in a sunny location for at least a day, they are ready for packaging and preparation for sampling use.
2. Wearing nitrile gloves, install new Schrader valve cores into all of the decontaminated lids.
3. Wrap Teflon tape around the threaded neck of the bottle, with at least four clockwise revolutions around the neck, and replace a lid on each bottle.
4. Place each bottle and lid assembly into an individual "clean" heavy duty 1-gallon Ziploc storage bag.
5. The preloaded Swinnex filter holders will either come from Pisces Molecular individually bagged in small Ziploc baggies, or in bulk together in one larger storage bag. If the latter, individually place each filter holder assembly into snack size Ziploc bag and seal the bag.
6. It is ideal to keep all "clean", bagged eDNA sampling bottles and filter assemblies in a closed container, away from any conventional fish sampling equipment or any other sources for potential contamination.

Collecting Samples

Supplies and Materials

Item	Use
Nitrile Gloves	Enough for two pairs per sample, per person
Nalgene bottle w/ Teflon tape, lid, new Schrader valve core, in a sealed Ziploc	Collecting water to sample; 1 per sample
Bike pump with Schrader valve attachment	Pressurizing sample bottle to force water through filter
1 gallon Ziploc bags	To place all individual, double-bagged filter assemblies in after use (grouped by waterbody and sample date): plus 1 for used gloves. A few extra bags is advisable as well.
Swinnex filter assemblies (pre-bagged)	At least one per sample location to filter water
Snack size Ziplocs (minimum 1/sample)	To double bag Swinnex filter assembly after use
Sharpie marker (2)	Label samples
GPS Unit (1)	Record coordinates of sample site
Camera (1)	Record image of site
Data sheet (1/water body) and pencil (2)	Record sample metadata
(2) day use backpacks	Transporting sample equipment
Cooler with ice/ice packs	Store samples for transport back to office

Procedures- Preparing Equipment to go into the field

1. Having two people collect samples is ideal. In addition to the "buddy system" being safer, the presence of two people allows a more efficient and cleaner sampling process, in which one person handles all "clean" equipment and labels samples, while the other actively collects the samples.
2. Prior to heading out into the field, allocate a day pack to one person, which will contain all "clean" sampling equipment, extra Ziploc baggies, nitrile gloves, GPS unit, camera, datasheets, and labeling and data recording supplies. **Each sample requires the use of a new bottle and filter,** so it is helpful to know how many samples will be collected so that the appropriate number of bottles and filters are loaded in the backpack. The second backpack is assigned to the sample collector. For heading out into the field, it will only have the bike pump with Schrader valve attachment, but will also be loaded with used sample bottles or any other "dirty" items once samples are collected.
3. Since waders can be a likely path of sample contamination, it is ideal if both people collecting the samples do not wear waders, instead wearing clothing that has not been used to sample or process the target organism. If waders must be worn, be sure to take extra precautions to avoid contamination, and stay out of the water to the greatest extent possible.

Procedures - Collecting Samples

1. Select a sample site in flowing water (but not a riffle or cascade).
2. The sample collector is provided a bagged bottle from the second person. The sample collector removes the lid from the bottle, and both lid and bottle are triple rinsed with water from the water body of interest, near the sample site (i.e. within a few feet), but downstream so as not to disturb the actual sample site. Water needs to be dumped away from the collectors and gear,

with care taken so that splashes do not contaminate either. Make sure to not place lid or bottle in contact with equipment or other surfaces that may be potential contamination pathways.

3. The second person removes the Swinnex filter unit from the Ziploc and provides it to the sample collector. The Swinnex filter unit is then fastened to the bottom of the bottle by the sample collector. Take extra care not to overtighten the Swinnex unit; simply apply light finger tightening pressure until the unit reaches a stopping point.
4. The sample collector scoops 1 liter (i.e. fill the sample bottle) of water from the sample site, being careful not to stir up and collect sediment, if possible. The cap is screwed tightly on the bottle to seal, then the bicycle pump is attached to the air valve and pumped to pressurize the bottle to between 30 to 40 PSI. Pressure is maintained around 30 PSI until all of the water has passed through the filter, or until the filter clogs.
5. While pumping, care should be taken to prevent contact with sources that could compromise the integrity of the sample. It is advised that the individual pumping does not hold the bottle between their legs, especially if they are wearing waders. Care must also be taken to prevent the exiting stream of water from the filter splashing on individuals or equipment.
6. Repeat the process of collecting water, pumping to pressurize, and filtering until the filter clogs or a maximum of 10 liters has been filtered. The filter should be considered clogged once it no longer passes a steady stream, but rather changes to a series of droplets, while under 30 PSI of pressure. Any remaining water should be carefully dumped out at the sample site, again away from collectors or equipment.
7. The sample collector then unscrews the Swinnex unit and places it into the snack size Ziploc baggie from which it came. Ideally, the second person holds the bag open so the sample collector does not have to handle the bag. The second person then seals the bag and places it inside a second, new snack size bag and seals it. This baggie is labeled by the second person with a unique sample identifier, volume of water filtered (to the nearest tenth of a liter), and any other pertinent information. (note: it may be desirable to keep the lab "blind", in which case using a non-descriptive identifier and not recording other details on the bag is advised, but be sure to cross-reference this information on a data sheet somewhere else).
8. The bagged and sealed Swinnex unit should then be placed in a 1 gallon Ziploc that will hold all samples collected for the outing, and then place in the second person's backpack in a compartment separate from the unused sampling equipment.
9. The sample collector places the emptied Nalgene bottle and lid into the 1 gallon Ziploc from which it came and places the sealed Ziploc into their backpack. At this point nitrile gloves may be removed, inside out, and placed into a 1 gallon Ziploc designated for used gloves and "dirty" materials and waste, which will also go into the sample collector's backpack.
10. Once all other information is collected at the site, personnel are ready to travel to the next sample site.
11. The samples need to be kept cool, dry, and out of the sun. In cool conditions, samples are fine kept in a backpack for the day, but a "clean" soft sided cooler with an ice pack is advised for holding samples in the field. Upon return to the vehicle, the samples should be placed in "clean" cooler on ice (i.e. decontaminated with 10% bleach between uses, following same protocol for bottles). Upon return to the office/shop, the samples should be stored in the freezer until ready. Samples should not be stored in freezers which also contain specimens, especially those species which are being evaluated through the eDNA sampling.
12. NEGATIVE CONTROL SAMPLE - This optional step may be desired to test for any contamination in the field and provide additional confidence in the data. A negative control sample using distilled water should be the last sample collected for the batch. Filter the distilled water streamside, following the above protocol and filtering a volume similar to that of the other

samples collected. Label samples in such a way that they are not easily discernable as control samples to the laboratory.

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