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METHOD

Dead-end hollow fiber ultrafiltration capture of environmental DNA for freshwater mussel (Unionidae) species detection with metabarcoding

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Abstract

Insufficient water sample volumes can be a limiting factor for detecting species with environmental DNA (eDNA) from aquatic habitats. We compared detections of freshwater mussel (Unionidae) communities using large water sample volumes and dead-end hollow fiber ultrafiltration (D-HFUF or DEUF) with traditional eDNA filtration methods that use relatively small water sample volumes. Unionid species were detected in approximately 50-L D-HFUF eDNA samples with two mitochondrial DNA metabarcoding markers (COI and ND1) and compared to species detection results from eDNA captured from commonly used 1-L samples filtered with polyethersulfone (PES) filters at three lotic sites in Georgia and Missouri. Of the 431,560 COI and 1,035,472 ND1 reads from all environmental samples of both filter types that passed quality control, 95% (410,755 reads) of COI reads and 85% (883,472 reads) of ND1 reads were assigned to a unionid species. Nineteen different freshwater mussel species were detected across all D-HFUF samples, and 11 species were detected across all PES samples. Reads assigned to the genus *Elliptio* could not be resolved beyond the genus level with either marker. From D-HFUF samples, 15 and 16 mussel species were detected with the COI and ND1 markers, respectively. From PES samples, nine and seven species were detected with the COI and ND1 markers, respectively. More mussel species were detected at each site in D-HFUF samples than in PES samples regardless of whether results from both markers were combined or evaluated separately. Our results demonstrate the merit of further exploration and optimization of D-HFUF for capturing eDNA from high-volume water samples to facilitate detection of unionids and likely other aquatic organisms.

KEYWORDS
DNA barcoding, eDNA, environmental DNA, metabarcoding, ultrafiltration, Unionidae

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1 | INTRODUCTION

Environmental DNA (eDNA) is often described as a sensitive tool for detecting rare or difficult-to-detect species. However, the suitability of eDNA as a tool for detecting species that expel, excrete, or shed low levels of DNA or occur at low densities may be limited by the volume of water that can be filtered. Water filtration volumes for eDNA studies are generally 6 L or less (Mächler et al., 2016) because of constraints such as filter pore size and particulate matter in water bodies (Kumar et al., 2020). For species that do not produce large amounts of eDNA or occur at very low densities, low water volumes may not contain sufficient quantities of DNA for detection of the species of interest.

Studies have indicated that larger water sample volumes can increase the concentration of target DNA in a sample and improve detection with eDNA (but see Mächler et al., 2016). Hunter et al. (2015) used eDNA to detect Burmese pythons (*Python bivittatus*) with multiscale occupancy modeling. Detection probabilities from eDNA samples with varying water volumes suggested that increasing the volume of water sampled would improve eDNA-based detection. Hunter et al. (2019) compared the concentrations (target copies per μL) of eDNA extracted from 200 mL of water on a single polyethersulfone (PES) filter to the concentration of target copies from 800 mL of water on four PES filters. They found that the target concentration from the four filter samples was 4.4× the concentration from the single filter samples. Schabacker et al. (2020) compared a high-volume (>3000 L) eDNA sampling method using a tow net to eDNA sampling with 1 L of water filtered through 0.45 μm nitrate cellulose filter (47 mm diameter) for the detection of an aquatic plant species (Northern watermilfoil, *Myriophyllum sibiricum*) and aquatic mollusks in Montana waterbodies (including *Helisoma anceps*, *Margaritifera falcata*, *Lampsilis siliquoidea*, and possibly others). The aquatic plant was detected more frequently in high-volume tow net eDNA samples compared to the 1-L eDNA samples. When eDNA samples were diluted 25-fold to mimic a low target concentration scenario, mollusks were detected more frequently in the high-volume tow net eDNA samples compared to the 1-L eDNA samples.

Dead-end hollow fiber ultrafiltration (D-HFUF or DEUF) is a water filtration method that uses hemodialysis filters adapted from the medical industry for capturing and concentrating microbial indicators and pathogens in environmental samples from large volumes of water (e.g., 100 L to 200 L) via size exclusion (Francy et al., 2013; Mull & Hill, 2012). Similar to standard eDNA filtration approaches, D-HFUF often uses a peristaltic pump to move water through the filter (e.g., Wu et al., 2023). D-HFUF has not been widely tested in eDNA studies for metazoan taxa but could help address the issue of low detectability caused by insufficient sample volume and low DNA shed rates or low abundance of target organisms.

Because of their cryptic nature, levels of imperilment, and high degree of taxonomic uncertainty, freshwater mussels (Bivalvia: Unionoida) are an exemplary taxon for the application of eDNA. Traditional surveys for freshwater mussels generally require physical surveys of stream and river substrate, which can require snorkeling or SCUBA surveys by teams of researchers (Obermeyer, 1998). Species can be difficult to differentiate due to intraspecific variability and interspecific similarity in morphological characters, leading to the possibility for species misidentification (Shea et al., 2011) or inaccurate accounts of species-level diversity (Johnson et al., 2018; Smith et al., 2018). Field methods for eDNA-based surveys can be relatively simple, rapid, and non-invasive, while improving detection sensitivity and differentiation of morphologically similar species (Lor et al., 2020). Furthermore, in some cases, timing of traditional mussel surveys may be limited to specific seasons or field conditions, such as low flows in lotic systems, whereas it may be possible to conduct eDNA surveys under a broader range of field conditions (but see Curtis et al. (2021)) for an example of high-flow limitations for eDNA.

Attempts to detect freshwater mussels with eDNA have had varying successes, which may be related to differences in shedding rates and the ecology of eDNA in different aquatic systems. In some studies, positive detections occurred far downstream of known mussel occurrences. Sansom and Sassoubre (2017) used experimental estimates of eDNA shedding and degradation along with incorporation of stream flows to predict that eDNA from *Lampsilis siliquoidea* should be detectable up to 36.7 km downstream. They conducted field testing up to 1 km from a known mussel bed and detected eDNA from duplicate 100 mL samples as predicted by the model. Similarly, Preece et al. (2021) detected eDNA from 20 *Gonidea angulata* up to 8 km downstream from enclosures using duplicate 1-L samples. The authors did note that they observed glochidia shed from the caged mussels during transport, which likely increased their detection rate. In contrast to these studies, an experimental study also filtering 1 L of water was able to detect the mussel *Lampsilis fasciola* only at the cage location and not further downstream as little as 10 m away (Gasparini et al., 2020). A study focused on the critically endangered *Lasmigona decorata* was able to detect the mussel at a known native site during pilot validation with duplicate 2-L samples, but later detection was unsuccessful using eDNA sampling with duplicate 2-L samples at the same location (Schmidt et al., 2021). These examples demonstrate the uncertainties and variability in eDNA monitoring of freshwater mussels.

Shedding rates are unlikely to be constant and mussels may be buried in the substrate when sampling occurs. Furthermore, opportunities to sample eDNA during high shedding events (such as spawning events or release of glochidia) can be difficult to predict precisely (Landis et al., 2012) and may happen over short time periods (Barnhart et al., 2008; Beaver et al., 2019). When the optimal time to survey is unknown or varies among mussel species or across local habitats, one strategy to increase detection would be to sample greater volumes of water to increase the likelihood of sampling mussel eDNA. However, increased water volume could lead to greater levels of polymerase chain reaction (PCR) inhibitors, which is a common problem in freshwater mussel eDNA studies (Gasparini et al., 2020; Sansom & Sassoubre, 2017; Schmidt et al., 2021).

Early freshwater mussel eDNA studies primarily used species-specific assays, although more recently, studies have emerged

using metabarcoding approaches to identify unionid mussel communities (Coghlan et al., 2021; Klymus et al., 2021; Marshall et al., 2022; Prié et al., 2021). These metabarcoding studies have included development of multiple metabarcoding primers and have demonstrated that high proportions of the known mussel community can be detected using eDNA (e.g., >80% (Coghlan et al., 2021) and 91% (Marshall et al., 2022)). Both species-specific and metabarcoding approaches face similar challenges, including the need for numerous field samples and replicates to obtain sufficient detection rates when typical eDNA filtering approaches are employed (Marshall et al., 2022). In this study, we tested whether increasing filtration volume during eDNA sampling for freshwater mussels results in improved species detections. We used a set of recently developed mussel metabarcoding markers (Klymus et al., 2021) to compare detection rates from D-HFUF filtration and a more typical eDNA filtering approach for samples across three watersheds with known unionid mussel assemblages. We also provide novel DNA sequences representing mussel species absent from public repositories to facilitate assigning metabarcoding results and provide comprehensive DNA libraries for future studies.

2 | METHODS

2.1 | Study locations

The southeastern United States is the epicenter of freshwater mussel diversity in North America (Graf & Cummings, 2021), including numerous threatened and endangered species (Haag, 2012). Water samples were collected from three sites near U.S. Geological Survey (USGS) stream gages with mussel community data from previous physical surveys: Spring Creek near Colquitt, GA (near the USGS stream gage 02356638; 31°10′26.7″ N 84°44′41.7″ W), Flint River near Newton, GA (near the USGS stream gage 02353000; 31°18′25.0″ N 84°20′20.0″ W), and the Big Piney River in Pulaski County, MO (near the USGS stream gage 06930060; 37°45′36.9″ N 92°03′28.7″ W). The Flint River and Spring Creek are in the Apalachicola–Chattahoochee–Flint (ACF) River Basin, which has high mussel endemism (Williams et al., 2014). These sites were selected because of known mussel diversity and availability of historical records in the basins from traditional mussel surveys and museum collections (Brim-Box & Williams, 2000; Georgia Department of Natural Resources, 2022; Williams et al., 2014; Wisniewski et al., 2013, 2014). A site on the Big Piney River in Missouri was selected because of the ongoing mussel eDNA research at that location, with species survey data for the Big Piney River from the Missouri Department of Conservation (MDC) between 1979 and 2008 (unpublished data, Missouri Mussel Database, MDC). Overall, the Spring Creek basin has 24 known species, the lower Flint River basin has 25 known species, and the Big Piney River has 27 known species (Table 1). We followed the updated nomenclature of Williams et al. (2017).

2.2 | Dead-end hollow fiber ultrafiltration setup and DNA extractions

Ultrafiltration methods generally followed those outlined in Francy et al. (2013) with several modifications. For samples collected from the Flint River and Spring Creek in Georgia, 50 L of water was filtered per D-HFUF filter using a Geopump peristaltic pump with an EZ-Load pump head (Geotech, Denver, CO) and silicone tubing (Figure 1) as described in Durigan et al. (2020). Due to equipment availability, a different peristaltic pump (Masterflex® E/S® Portable Sampler (Cole Parmer)) was used for collecting and filtering samples from the Big Piney River in Missouri. An attempt was made to filter 50 L of water from the Big Piney River with the D-HFUF; however, filtration was stopped after 46 L of water was filtered because of clogging. Water sample influent tubing was attached to the red port of a hollow fiber ultrafilter Rexeed-25S (Asahi Kasei Medical Co., Dial Medical Supply) (Francy et al., 2013), a single-use polysulfone hemodialyzer filter that has a molecular cutoff of 30 kDa, an effective surface area of 2.5 m^2, and an internal diameter of the hollow filter of 185 μm. Adapters to connect tubing to the filters were used as described in Durigan et al. (2020). Influent tubing was attached to the hollow fiber ultrafilter with custom-fitting DIN adapters (Molded Products Corporation). Effluent tubing was also attached to the permeate port at the blue port end of the hollow fiber ultrafilter. A flowmeter (Omega™ Economical Turbine Flowmeter) was attached to the effluent tubing to track the filtered sample volume and flow rate. Tubing and adapters were cleaned with soap and tap water, rinsed with deionized (DI) water, and sterilized in an autoclave at 15 PSI at 121°C for 15 min prior to use. One sterile set of D-HFUF tubing and adapters were used at each site. Two D-HFUF samples were collected at both the Flint River and Spring Creek sites. At the Flint River site, downstream and upstream samples were collected from the eastern bank approximately 80 m apart. At the Spring Creek site, downstream and upstream D-HFUF samples were collected approximately 30 m apart. For downstream and upstream D-HFUF samples collected from the Flint River and Spring Creek, tubing and adapters were not sterilized between downstream and upstream samples. The filtration rate for the Flint River and Spring Creek D-HFUF samples ranged from approximately 2.3 to 2.7 L/min. One D-HFUF sample was collected at the Big Piney River site. D-HFUF filters were stored in individual Ziploc bags and kept in a cooler on ice until elution the following day (described below). The filtration rate for the D-HFUF sample from the Big Piney site was approximately 0.6 L/min.

Water samples of approximately 1 L (0.9 L to 1.2 L; Table S1) were also collected for eDNA analysis and were filtered with 1.2 μm pore size (47 mm diameter) PES filter membranes (Smith-Root) as a reference to compare to the eDNA results from D-HFUF samples (Table S1). For each downstream and upstream sample location at the Spring Creek and Flint River sites, after D-HFUF sample collection, approximately 1 L of environmental water was collected in sterile 1-L sample bottles using the same D-HFUF

TABLE 1 Unionidae species occurrence at the study sites based on historical survey data and the number of COI and ND1 sequences available from GenBank (https://www.ncbi.nlm.nih.gov/genbank/).

Species	Big Piney, MO	Flint River, GA	Spring Creek, GA	COI	ND1
Actinonaias ligamentina	Y	N	N	+	+
Alasmidonta marginata	Y	N	N	+	+
Alasmidonta triangulata	N	Y	N	+	1
Amblema neislerii[a]	N	Y	Y	1[b]	–[b]
Amblema plicata	Y	N	N	+	+
Cyclonaias infucata	N	Y	Y	+	+
Cyclonaias pustulosa	Y	N	N	+	+
Cyclonaias tuberculata	Y	N	N	+	+
Elliptio arctata	N	Y	Y	+	2
Elliptio crassidens	Y	Y	Y	+	+
Elliptio fumata	N	Y	Y	+	–
Elliptio nigella	N	Y	N	3	–
Elliptio pullata	N	Y	Y	+	2
Elliptio purpurella	N	Y	Y	+	1
Elliptoideus sloatianus[a]	N	Y	N	2	3
Eurynia dilatata	Y	N	N	+	+
Fusconaia flava	Y	N	N	+	+
Hamiota subangulata[a]	N	Y	Y	+	3
Lampsilis brittsi	Y	N	N	–	–
Lampsilis cardium	Y	N	N	+	+
Lampsilis floridensis	N	Y	Y	+	+
Lampsilis reeveiana	Y	N	N	–	–
Lampsilis siliquoidea	Y	N	N	+	+
Lampsilis straminea	N	Y	Y	+	1
Lampsilis teres	Y	N	N	+	+
Lasmigona costata	Y	N	N	+	+
Leptodea fragilis	Y	N	N	+	+
Ligumia recta	Y	N	N	+	+
Ligumia subrostrata	Y	N	N	1	2
Margaritifera monodonta[a]	Y	N	N	+	+
Medionidus penicillatus[a]	N	Y	Y	–[b]	–[b]
Megalonaias nervosa	N	Y	Y	+	+
Obliquaria reflexa	Y	N	N	+	+
Pleurobema pyriforme[a]	N	Y	Y	+	2
Pleurobema sintoxia	Y	N	N	+	2
Potamilus alatus	Y	N	N	+	+
Pyganodon grandis	Y	Y	Y	+	+
Strophitus radiatus	N	Y	Y	+	+
Strophitus undulatus	Y	N	N	+	+
Theliderma metanevra	Y	N	N	+	+
Toxolasma parvum	N	N	Y	+[c]	+
Toxolasma paulum	N	Y	Y	+[d]	+[e]
Tritogonia verrucosa	Y	N	N	+	+
Uniomerus columbensis	N	Y	Y	–	–
Utterbackiana heardi	N	N	Y	+[c]	+[c]
Utterbackia imbecillis	Y	Y	Y	+	+[f]

TABLE 1 (Continued)

Species	Big Piney, MO	Flint River, GA	Spring Creek, GA	COI	ND1
Utterbackia peggyae	N	Y	Y	+	+[g]
Venustaconcha ellipsiformis	Y	N	N	+	+
Villosa lienosa	N	Y	Y	+	+
Villosa vibex	N	Y	Y	2	+
Villosa	N	Y	Y	+	4

Abbreviations: −, no sequence available in GenBank; +, more than four sequences available in GenBank; N, no presence recorded in the basin; Y, presence recorded in the basin.

[a] On the U.S. Fish and Wildlife Service Threatened and Endangered Species list.

[b] Novel DNA sequence data provided herein and published on GenBank.

[c] Primer mismatches with all available sequences.

[d] 15 sequences with no forward primer and portion of product not present.

[e] 17 sequences with first 6 bp of forward primer not present.

[f] 18 sequences with first 6 bp of forward primer not present.

[g] 11 sequences with first 6 bp of forward primer not present.

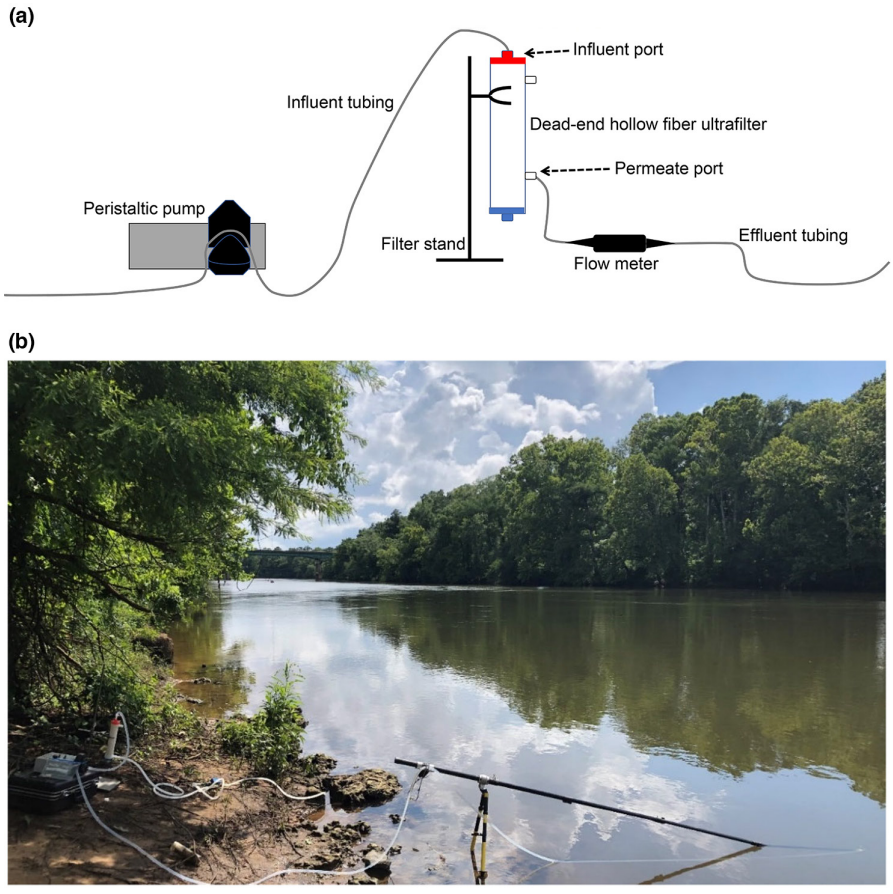

FIGURE 1 Dead-end hollow fiber ultrafiltration diagram (a) and setup at the upstream Flint River sample site (b). Photo credit: Chris Goodson.

inflow tubing and peristaltic pump. These 1-L water samples were stored on ice until filtration using an eDNA Sampler (Smith-Root, Vancouver, WA) the following day in the U.S. Geological Survey South Atlantic Water Science Center (SAWSC) laboratory in Norcross, GA. At the Big Piney River site, four 1-L samples were filtered using an eDNA Sampler (Smith-Root) in the field. Sterile DI (autoclaved at 15 PSI and 121°C for 15 min per 1 L of DI water) cooler blanks were also filtered with PES filters to test for field and lab-based contamination (from here on referred to as field blanks).

D-HFUF filtrate was eluted using Innovaprep FluidPrep Tris High Volume Elution Fluid (HVEF; 0.075% Tween 20, 25 mM Tris) canisters with the large volume concentration can interface (Innovaprep catalog number HC08018-T) attached at the red port with elution expressed from the blue port. The Big Piney D-HFUF was eluted with one HVEF cannister and Spring Creek and Flint River D-HFUFs

with two HVEF cannisters. Approximate volumes of expressed eluate per HVEF cannister ranged from 40 to 170 mL (Table S1). Eluates were centrifuged in 85-mL vials for 30 to 60 min at 3500 rpm. Eluates greater than 85 mL were split across multiple centrifuge vials (Table S1). Sterile phosphate buffered saline was added to sample vials as needed for centrifugation balance. Centrifuged supernatant was poured off and eluate pellets were stored at −80°C until DNA extraction. DNA was extracted using the Qiagen DNeasy PowerLyzer PowerSoil DNA Isolation Kit (Qiagen) following the manufacturer's instructions. Pelleted eluates were resuspended in 750-μL PowerBead solution and 60-μL C1 solution and transferred to PowerBead tubes. Eluate suspensions were split across two PowerBead tubes as needed if the suspension volume exceeded that allowed by the tube. Extracted DNAs from these split samples were processed independently for downstream metabarcoding (i.e., each DNA extraction was sequenced individually) but data were combined for analysis after species assignments. One DNA extraction blank (no eluate added) was processed for each round of DNA extractions to test for contamination during the DNA extraction process.

2.3 | Metabarcoding library preparation

Metabarcoding library preparation and sequencing occurred in the molecular laboratory at the USGS Upper Midwest Environmental Sciences Center (UMESC) in La Crosse, WI. For each sample, two Unionida markers were used, one marker targeting the NADH dehydrogenase subunit 1 (ND1) gene and the other targeting the cytochrome c oxidase subunit 1 (COI) gene (Klymus et al., 2021). Included in the library preparation were two mock communities comprised of DNA from seven freshwater mussel species (*Actinonaias pectorosa*, *Anodonta californiensis*, *Epioblasma brevidens*, *E. capsaeformis*, *E. triquetra*, *Lampsilis fasciola*, and *Villosa iris*) which served as positive controls. These mock species were chosen because they are not known to occur within the study areas so tag jumping could be assessed. Mock communities included both variable percentage (Mock A) as described in Taylor et al. (2016) and equal percentage (Mock B) of DNA concentrations from each species to address if relative concentration influenced amplification in the mock species. Additionally, no-template controls (NTCs) were included in each PCR plate to ensure no sample contamination occurred throughout the library preparation process (Table S1).

For each marker, DNA samples were initially amplified in quadruplicate 25-μL PCR reactions to increase DNA concentration of the eDNA samples. Each reaction contained 12.5 μL of Quantabio PerfeCTa qPCR ToughMix (Quantabio, Beverly, MA, USA), 1 μL of 0.4 μM of either ND1 or COI forward and reverse primers each, and 2 μL of DNA template. The COI PCR thermal profile had an initial denaturation at 98°C for 2 min followed by 35 cycles of denaturation at 98°C for 20 s, annealing at 50.4°C for 30 s, and extension at 72°C for 30 s, followed by a final extension at 72°C for 5 min. The thermal profile for ND1 was the same as the COI thermal profile previously mentioned with the exception that the annealing temperature was slightly higher (51°C).

A second round of PCR amplifications was done to attach the Illumina Nextera overhang adapter sequence (OAS) tags (Illumina, Inc.) to the target amplicons according to the Illumina 16S metagenomic library preparation protocol (Illumina, 2013; Klymus et al., 2021; Taberlet et al., 2018). Each PCR reaction had the same volume and reagents mixture as mentioned in the previous paragraph with the exception that the amplicons from the first PCR were used as the DNA template. The COI PCR thermal profile had an initial denaturation at 95°C for 3 min followed by 35 cycles of denaturation at 95°C for 5 s, annealing at 50.4°C for 15 s, and extension at 72°C for 15 s, followed by a final extension at 72°C for 5 min. Again, the ND1 thermal profile was the same as COI with the exception that the annealing temperature was slightly higher (51°C). The quadruplicate OAS-tagged amplicons for each sample were pooled and then bead purified using Quantabio sparQ PureMag Beads (Quantabio) according to the manufacturer's instructions. Next, amplicons were analyzed on an Agilent 4200 TapeStation System (Agilent Technologies, Inc.) to check for target amplicons and sample concentration. No amplification was detected in the DNA extraction blanks and they were therefore excluded from further processing and sequencing. Samples were then normalized and dual indexed using the Illumina Nextera XT Index Kit v2 (Illumina) according to the manufacturer's instructions. The indexed libraries were purified and analyzed again as previously described to confirm that indices had been attached to the target amplicons. The concentration of each library was measured in triplicate using the KAPA Library Quantification Kit (Roche Sequencing) following the manufacturer's instructions and then normalized to 4 nM. Normalized libraries were pooled, then denatured and diluted to 4.5 pM loading concentration, and sequenced on an Illumina MiSeq System using a MiSeq Reagent Kit v2 (300 cycles) with a 15% PhiX spike-in to help increase the diversity of the sequencing run.

2.4 | Bioinformatic filtering and species assignment

Raw FASTQ files were exported from the Illumina MiSeq System and imported into the Quantitative Insights Into Microbial Ecology (QIIME) 2 (https://qiime2.org/) version 2022.2 as QIIME artifacts for bioinformatics analysis (Bolyen et al., 2019). Specifically, the FASTQ files were imported into QIIME2 using the qiime tools plugin with the options "—type 'SampleData[PairedEndSequencesWithQuality]' —input-format PairedEndFastqManifestPhred33". Next, the qiime cutadapt trim-paired plugin (Martin, 2011) was used to trim primer sequences twice, once for each primer set, and any reads less than 80 bp were discarded. Default options were used except "--p-match-read-wildcards --p-discard-untrimmed --p-minimum-length 80". The paired-end sequences were denoised and dereplicated using the qiime dada2 denoise-paired plugin (Callahan et al., 2016) using default options and zero for --p-trunc-len-f/r and --p-trim-left-f/r parameters to generate an amplicon sequence variant (ASV) table and sequence file. Read thresholds were applied to remove ASVs with less than five total reads and to remove ASVs

with less than five reads per sample prior to taxonomy assignment. Next, the ASVs sequence file was exported using the qiime tools export plugin and taxonomy assignment was done using the National Center for Biotechnology Information (NCBI) executable BLAST v2.12.0+ (Camacho et al., 2009) against the NCBI nt database (Benson et al., 2012; Sayers et al., 2021). The classify-consensus-blast plugin (Camacho et al., 2009) was used for taxonomy assignment using supplemental sequences for two species (*Amblema neislerii* and *Medionidus penicillatus*) listed under the U.S. Endangered Species Act (ESA) that were previously absent from public databases. We generated supplemental DNA sequences for *A. neislerii* and *M. penicillatus* using previously collected tissue samples from the ACF basin (Table 1; Table S2). These tissue samples were processed at the U.S. Geological Survey Wetland and Aquatic Research Center in Gainesville, FL, and DNA was extracted and sequenced following Johnson et al. (2018). Any ASVs that did not result in a species hit after blastn or classify-consensus-blast plugin were deemed as true unassigned hits.

After taxonomic assignment, read filtering was implemented again based on the mock communities and blanks (including the NTC) to account for tag jumps and contamination. We treated any contaminant or tag jump species reads present in the mock communities and the blanks as baseline threshold reads for species presence. Specifically, for a species to be considered "present" in the field samples, it must have had a read count greater than the baseline threshold reads established by the mock communities and blanks. For the COI marker, no mussel species from the mock communities or environmental samples were detected in any of the blanks (Table S10). For the ND1 marker, over 99% of the mussel sequence reads in the blanks were assigned to species from the mock communities (*Epioblasma capsaeformis* and *Ortmanniana pectorosa*, Table S11). Environmental sample-based contamination detection in the blanks for the ND1 marker was predominantly assigned to *Elliptio* sp. with the exception of five reads assigned to *Lampsilis cardium* in one of the field blanks. *Lampsilis cardium* was detected in only two environmental sample DNA-sequencing replicates with sequence read counts of more than 100× the number detected in the blank (Table S11). The large difference in the number of read counts for *L. cardium* between the blank and environmental samples and the fact that *Elliptio* results were removed from our analyses suggest our results were unlikely to be affected by environmental sample-based contamination or tag jumps.

After accounting for contaminant and tag jump reads, species identified in field samples for which there were at least five reads were considered present in that sample. To investigate the difference in species detections between filter types regardless of site, we calculated the mean number of species detected per sample for each marker. Downstream and upstream filters from the Flint River and Spring Creek were considered distinct samples for this analysis and the mean was calculated from the DNA-sequencing replicates for each sample. Matched pair analyses that compared the mean number of species per sample by marker with paired t-tests were conducted in JMP (v. 14.2.0, SAS Institute Inc.). Five matched pairs were included in the analysis for each marker (one mean value per filter type for Big Piney and two mean values per filter type for the Flint River and Spring Creek). We acknowledge that the lack of independence between the downstream and upstream samples violates the assumption of this statistical test. However, any bias caused by this lack of independence between downstream and upstream samples would be expected to be applicable to both the D-HFUF and PES filters as the samples were collected from the same location, and the same tubing was used for the collection of both types of filtered samples.

3 | RESULTS

We generated COI and ND1 sequences from 22 eDNA samples and tissue samples representing 2 species absent from public DNA databases. The eDNA samples included twelve D-HFUF samples (representing five individual D-HFUF filters; Table 2), eight 1-L PES filter samples (Table 2), and two 1-L PES field blanks (Lor et al., n.d.). One NTC and two mock community samples were also included (Table S1). After sequencing and onboard instrument base call conversion to FASTQ file format using Local Run Manager v3 (Illumina), 14,406,632 total reads were demultiplexed to samples and 1,703,328 reads were undetermined (Table S3), meaning that the reads were not assigned to any of the barcodes used in this study. Number of reads remaining after subsequent bioinformatic filtering is available in Tables S3–S9. All COI and ND1 sequences generated for *A. neisleri* and *M. penicillatus* were published on GenBank (GenBank accessions OQ954343, OQ979616, and OQ979617; Table S2).

After denoising sequences and filtering tag jumps, the number of COI reads at the expected amplicon length (235 base pairs) that remained were 571,142 (431,560 from environmental samples, 139,560 from mock communities, and 22 from the field blanks and

	D-HFUF			PES		
Site	*N*	COI	ND1	*N*	COI	ND1
Big Piney River	1 (1)	311 (22%)	1181 (15%)	4 (4)	56 (1%)	6315 (31%)
Flint River	2 (5)	33,976 (99%)	330,311 (93%)	2 (2)	37,139 (99%)	71,032 (99%)
Spring Creek	2 (6)	332,661 (97%)	424,922 (86%)	2 (2)	6612 (89%)	49,711 (57%)

Note: Includes species assigned to the genus, *Elliptio*.

Abbreviations: D-HFUF, dead-end hollow fiber ultrafiltration; PES, polyethersulfone.

TABLE 2 Number of filters collected (N; number of DNA extractions in parentheses) and number of DNA sequence reads assigned to mussels by site, filter type, and marker (percentage of total reads at the expected amplicon size for the respective site, filter type, and marker are presented in parentheses).

NTC; Tables S8 and S9). Nearly all COI reads from environmental samples were assigned to mussels (410,755 reads, 95% of COI environmental sample reads; Table 2). The number of ND1 reads that remained at the expected amplicon length (235 base pairs) after denoising sequences and filtering tag jumps was 1,254,125 (1,035,472 from environmental samples, 180,232 from mock communities, and 38,421 from blanks; Tables S8 and S9). A smaller percentage of ND1 reads were assigned to mussel species for the environmental samples than with the COI reads (883,472 reads, 85% of ND1 environmental sample reads; Table 2).

No pattern emerged in terms of which filter type produced more total reads at the expected amplicon size. More total reads at the expected amplicon size were obtained from the PES filters than D-HFUF at both markers for Big Piney River samples (4.7× and 2.6× as many reads for PES filters than D-HFUF for COI and ND1, respectively; Table S9), whereas more total reads at the expected amplicon size were obtained from D-HFUF than PES filters at both markers for Spring Creek (46.4× and 5.7× more reads for D-HFUF than PES filters for COI and ND1, respectively; Table S9). Results were mixed for samples from the Flint River (Table S9). The proportion of total reads assigned to mussel species per site–filter type–marker combination ranged from 1% to 99% (Table 2). A higher percentage of total reads were assigned to mussel species for samples collected from the Flint River and Spring Creek compared to Big Piney River (range: 57%–99% and 1%–31%, respectively; Table 2), but neither filter type consistently produced a higher percentage of reads assigned to mussel species. Big Piney River has experienced severe mussel declines over the last 20 years (unpublished data, M.C. Barnhart, Missouri State University, written communication July 3, 2023), which suggests the discrepancy in the number of mussel reads and percentage of reads assigned to mussels between samples from the Big Piney site and the other two sites could be caused by the difference in

TABLE 3 Detection results by site for unionid mussel species across filtration type and marker (COI and ND1).

		D-HFUF		PES	
Site	**Species**	**COI**	**ND1**	**COI**	**ND1**
Big Piney River	*Actinonaias ligamentina*	−	+	−	−
	Cyclonaias pustulosa	−	+	−	−
	Fusconaia flava	−	+[a]	−	−
	Lampsilis cardium	+[b]	+	−	+
	Theliderma metanevra	+	+	−	−
	Utterbackia imbecillis	+	+	−	+
	Venustaconcha ellipsiformis	+	+	−	−
Flint River	*Cyclonaias infucata*	+	+	+	−
	Elliptoideus sloatianus (TH)	+	+	+	+
	Hamiota subangulata (EN)	+	+	−	−
	Lampsilis floridensis	+	+	+	+
	Megalonaias nervosa	−	+	−	−
	Toxolasma paulum	+	+	−	−
	Villosa lienosa	−	−	+	−
	Villosa vibex	+	−	+	−
Spring Creek	*Hamiota subangulata* (EN)	+	+	+	+
	Lampsilis floridensis	+	+	+	−
	Pleurobema pyriforme (EN)	+	+	−	+
	Pyganodon grandis	+	+	−	−
	Toxolasma paulum	+	+	+	+
	Utterbackia imbecillis	+	+	+	+
	Utterbackia peggyae	+	−	+	−
	Villosa lienosa	+	−	+	−
	Villosa vibex	+	−	+	−
	Villosa villosa	+	+	−	−

Abbreviations: −, not detected; +, detected; D-HFUF, dead-end hollow fiber ultrafiltration; EN, endangered; L, liter; PES, polyethersulfone filtration; TH, threatened.

[a]Taxonomic assignment to Fusconaia sp. Based on known distributions, we assigned the sequence to *F. flava*.

[b]Assignment to a sequence identified as either Lampsilis satura or *L. cardium*. Based on known distribution, we assigned the sequence to *L. cardium*.

concentrations of mussel eDNA between sites such that the majority of reads from Big Piney were non-target reads or dimer product. Of the reads that were assigned to unionids, the majority of ASVs could be assigned with confidence to a single species. However, neither COI nor ND1 was able to resolve members of the genus *Elliptio* to the species level. Therefore, *Elliptio* assignments were not included in subsequent analyses.

3.1 | Mussel species detections

All species detected with eDNA were known to occur in the respective basins. Nineteen different mussel species (not including species from the genus *Elliptio*) were detected in D-HFUF samples, and 11 species were detected in PES samples (Table 3). From D-HFUF samples, 15 and 16 mussel species were detected with the COI and ND1 markers, respectively (Table 3; Tables S10 and S11). From PES samples, nine and seven species were detected with the COI and ND1 markers, respectively (Table 3). At each site, more mussel species were detected in D-HFUF samples than PES samples when results from both markers were combined (Table 3; Figure 2). From the Big Piney River, seven and two species were detected in D-HFUF and PES samples, respectively; from the Flint River, seven and five species were detected in D-HFUF and PES samples, respectively; and from Spring Creek, ten and eight species were detected in D-HFUF and PES samples, respectively (Figure 2). Only one species was detected in PES samples which was not detected with D-HFUF samples at the corresponding site (*Villosa lienosa* from the Flint River; Figure 2, Table 3). In contrast, five, three, and two species were detected in D-HFUF samples but not in PES samples from the Big Piney River, the Flint River, and Spring Creek, respectively (Figure 2; Table 3). When results were separated by marker, the total number of species detected in D-HFUF samples was greater than the total number of species detected in PES samples for both markers at each site (Figure 2; Table 3). Similarly, the mean number of species detected per filter was greater for D-HFUF than PES for both markers across all sites (Table S12). Paired t-tests indicated more species were detected with D-HFUF samples than PES samples for both COI and ND1 ($p = 0.027$ and $p = 0.016$, respectively; Figure 3).

Historic survey records indicated 26, 19, and 19 non-*Elliptio* mussel species in the Big Piney River, Flint River, and Spring Creek basins, respectively (Table 1; Figure 2). However, across both filter types and markers, we detected 7, 8, and 10 species at the Big Piney River, Flint River, and Spring Creek sites, respectively

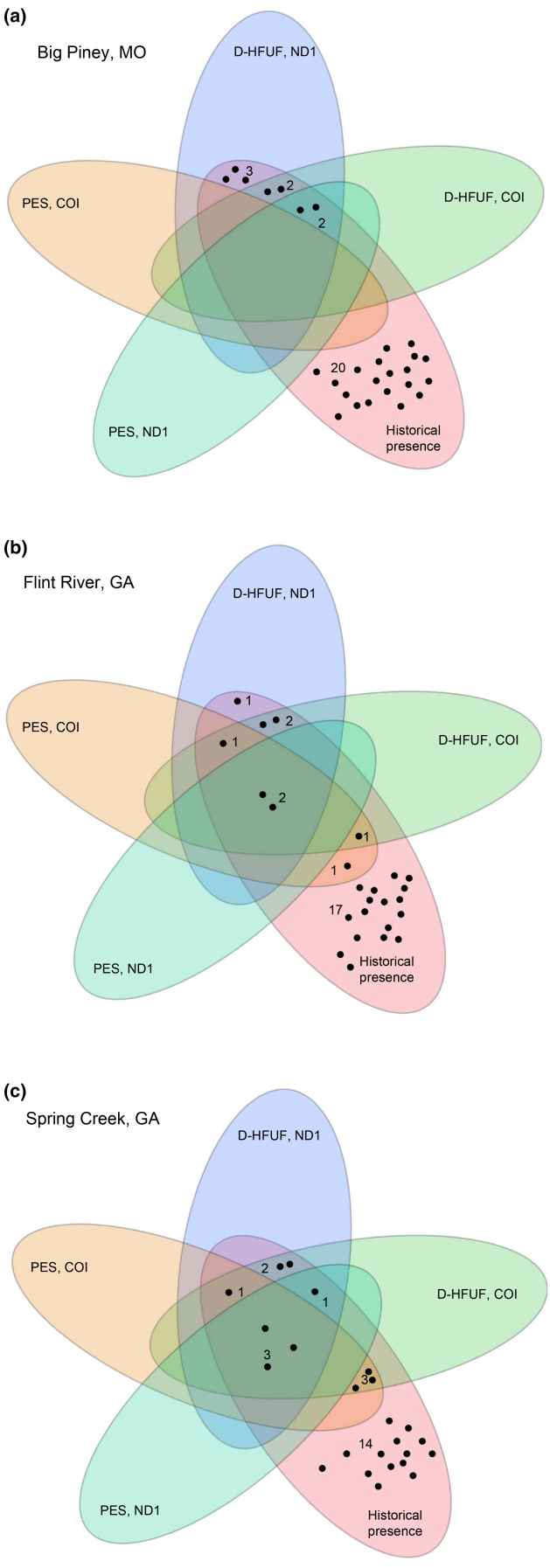

FIGURE 2 Venn diagram of species detected by site (a: Big Piney River, MO; b: Flint River, GA; c: Spring Creek, GA), marker (COI: cytochrome c oxidase subunit 1; ND1: NADH dehydrogenase subunit 1), and filter type (D-HFUF: dead-end hollow fiber ultrafiltration; PES: polyethersulfone) in comparison with historical surveys. Dots represent species detection with the associated method(s). Numbers indicate the number of species detected with the associated method(s).

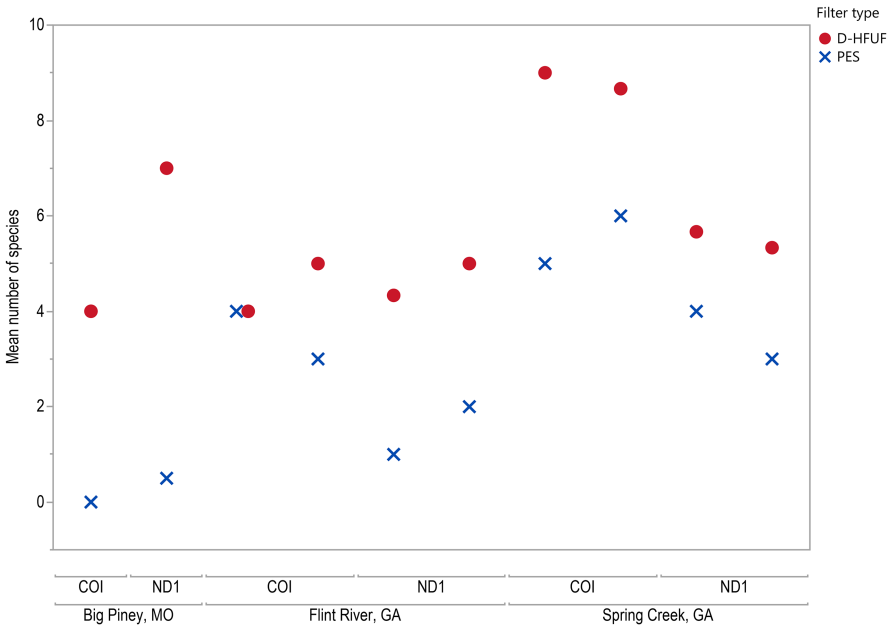

FIGURE 3 Mean number of species detected by sample and filter type. Paired *t*-tests results: $p = 0.027$ for cytochrome c oxidase subunit (COI) and $p = 0.016$ for NADH dehydrogenase subunit 1 (ND1). D-HFUF, dead-end hollow fiber ultrafiltration; PES, polyethersulfone.

(Figure 2). False negatives are one possible explanation for the discrepancy between eDNA results and historical survey results. Alternatively, the historic survey results were based on basin-wide records and may not be representative of the mussel community composition at our study sites. For the Spring Creek and Flint River sites, annual surveys are conducted as weather conditions, flow conditions, and logistics permit by the Georgia Department of Natural Resources and during annual ACF Freshwater Mussel Workshops. Results from our eDNA surveys compared to 2019 annual survey data for the Spring Creek and Flint River sites (the closest preceding year of annual surveys with suitable survey conditions) show strong similarities in species detections between the two survey methods (Andrew Hartzog, U.S. Fish and Wildlife Service, written communication April 27, 2023; Matthew Rowe, Georgia Department of Natural Resources, written communication May 8, 2023). For the Flint River site, non-*Elliptio* species detections aligned identically between the 2019 annual survey data and the species detected with eDNA with the exception of *Amblema neislerii*, which was detected during the annual survey but not with eDNA. At Spring Creek, six species were detected in both our eDNA survey and in the 2019 annual survey, one non-*Elliptio* species was detected during the 2019 survey that was not detected in our eDNA survey (*Uniomerus columbensis*), and four species were detected with eDNA that were not detected during the 2019 annual survey (*Pyganodon grandis*, *Utterbackia imbecillis*, *Utterbackia peggyae*, and *Villosa villosa*). Missouri Department of Conservation traditional mussel surveys at the Big Piney River study site detected the presence of four Unionidae species (unpublished data, Missouri Mussel Database, MDC), two of which were also detected in our eDNA survey (*Actinonaias ligamentina* and *L. cardium*). Five of the species detected at the Big Piney site with our eDNA survey, while known to occur in the basin (Table 1), had not been detected by the Missouri Department of Conservation during their traditional surveys at the study site. Species detection with eDNA but not with traditional surveys could indicate overlooked mussels, downstream transport of eDNA, or release of DNA from dead shell material.

3.2 | Threatened and endangered species detection

Among the six mussel species protected under the ESA known to occur in the study basins (Table 1), three were detected in this study (*Elliptoideus sloatianus*, *Hamiota subangulata*, and *Pleurobema pyriforme*; Table 3). Each of these three species was detected at sites with known occurrence in the respective basins (Table 1) and was detected in both filter replicates and with both markers for D-HFUF samples. PES sample results were less consistent across filter replicates and markers. *Elliptoideus sloatianus* was detected using both markers but in only one PES filter replicate from the Flint River (the only known site in this study; Table 3). *Hamiota subangulata* was detected at both markers in both PES filter replicates from Spring Creek; however, it was not detected in either of the PES filter replicates from the Flint River (Table 3). *Pleurobema pyriforme* was detected in only one of two PES filter replicates from Spring Creek (the only known site in this study) and only with the ND1 marker (Table 3).

3.3 | Non-mussel species detection

The two metabarcoding markers used in this study were developed to be specific to freshwater mussels (Unionida). However, the COI marker also yielded four non-mussel species-level detections against the NCBI nt database: one gastropod species (*Callinina georgiana*; less than 1% of COI reads from environmental samples), two mayfly

species (*Ephoron album* and *Hexagenia limbata*; less than 2% of COI reads from environmental samples), and one ribbon worm species (*Prostoma* sp. RiverLostock; less than 1% of COI reads from environmental samples) (Lor et al., n.d.). *Callinina georgiana* was detected in a Spring Creek downstream sample with the PES filter (Lor et al., n.d.). The two mayfly species were only detected in the Big Piney River samples; *Ephoron album* was more prevalent and detected in the one D-HFUF and all of the PES filters, and *Hexagenia limbata* was detected in fewer read counts in three of the PES filter samples (Lor et al., n.d.). There was no clear pattern with the detection of the ribbon worm species; it was found in both D-HFUF and PES filters for the Big Piney River, in both D-HFUF filter replicates for Spring Creek, and only in one PES filter replicate for the Flint River (Lor et al., n.d.). The ND1 marker did not yield any non-mussel species-level detections.

4 | DISCUSSION

Our study provides evidence that D-HFUF may be an effective method for capturing eDNA from large water sample volumes and for detecting freshwater mussels. An equivalent number or more PES filters were collected per site than D-HFUF filters yet more species were detected with the D-HFUF filters than PES filters for all sites at both markers. This suggests performance of the D-HFUF method is at least comparable to traditional PES filters for detecting freshwater mussels with eDNA in terms of the total number of mussel species detected per filter. Further testing and refinement of the D-HFUF method for detecting freshwater mussel species are warranted, including analysis in an occupancy modeling framework to estimate detection probability across a range of collection scenarios (e.g., Dorazio & Erickson, 2018; Preece et al., 2021).

Results from studies that have used D-HFUF for the detection of pathogenic organisms in environmental water suggest that D-HFUF may be an effective method for capturing eDNA across a range of environmental systems. The direct detection of pathogenic organisms in environmental waters, as opposed to culture-based methods for detecting pathogenic organisms, presents some of the same considerations as the detection of low-density species with environmental DNA; namely the rare and patchy nature of pathogenic organisms in environmental waters can require high volumes of water to be sampled for their detection. D-HFUF has been employed for the detection of a variety of viruses, bacteria, and protozoa (Holowecky et al., 2009) across a range of environmental water body types including rivers (Rhodes et al., 2016), lakes (McMinn et al., 2017), marine systems (Korajkic et al., 2021), groundwater (Morales-Morales et al., 2003; Olszewski et al., 2005), and wastewater (Gyawali et al., 2015; Wu et al., 2023); and has been successfully used to detect microbes in environmental waters across a range of water quality conditions (Korajkic et al., 2021; Mull & Hill, 2012; Smith & Hill, 2009). The success of D-HFUF for detecting eDNA of pathogenic organisms at low concentrations in a variety of aquatic environments indicates D-HFUF filtration may be a useful tool for capture and detection of eDNA from metazoan taxa with low abundance or low eDNA shedding rates across a wide range of habitats.

The benefits of increased sample volume from D-HFUF relative to standard eDNA filtration approaches may not outweigh the additional time, sample processing steps, and financial cost associated with D-HFUF samples. Wittwer et al. (2018) compared the time involved in collecting eDNA samples with glass fiber and D-HFUF filters and concluded that for their target (the crayfish plague agent, *Aphanomyces astaci*), eDNA collection with glass fiber filters resulted in better detection with less sampling collection and processing time and at a lower cost per sample. Their estimation of the time involved in D-HFUF sample collection (60 min for 100 L) and processing prior to DNA extraction (50 min for elution and centrifugation) was similar to our experiences, with the exception of D-HFUF filtration at Big Piney, where the collection of 46 L took over an hour. For our study, at the time that supplies were purchased (Spring 2021), the cost per D-HFUF filter was approximately $21USD and the cost for elution supplies was $26 USD per cannister and $7 USD per cannister adapter (one to two cannisters were used per D-HFUF and one adapter was used per DHFUF), totaling $54 USD to $80 USD per D-HFUF sample for the filter and elution supplies alone. In contrast, the cost per PES filter in Spring 2021 was $15 USD.

To address the issue of filter clogging from suspended particulate matter in water samples, some studies have used prefilters to exclude larger suspended particles from filtration with smaller-pore-size filters. For example, Ma et al. (2016) used medical-grade gauze wrapped over the filter inlet to prefilter 1-L water samples from aquaria and surface waters prior to filtration through 0.45-μm-pore-size mixed cellulose ester filters. Wilson et al. (2014) prefiltered their 2-L samples of lake water with 120 μm mesh prior filtration through 1.2 μm glass microfiber filter.

Other high-volume filtration methods have been tested for the capture and isolation of eDNA from environmental waters. These methods have included tow nets (Fernandez et al., 2021; Schabacker et al., 2020), in-situ samplers (Brandt et al., 2021; Fernandez et al., 2021), and passive collection (Bessey et al., 2021). In general, these methods have been effective for detecting eDNA of target taxa with PCR (Schabacker et al., 2020) and metabarcoding (Bessey et al., 2021; Brandt et al., 2021; Fernandez et al., 2021), and these methods may be more appropriate than D-HFUF for high-volume eDNA concentration, such as when repeated passive sampling applications used over a longer timeframe better fits the study objectives (e.g., Brandt et al., 2021; Sepulveda et al., 2020). However, D-HFUF offers several advantages over most other large water volume eDNA concentration methods that may make D-HFUF easier to implement for some studies. Field equipment for D-HFUF are portable, do not require more than one person to operate, and the in-field filtration setup for D-HFUF resembles common in-field eDNA filtration methods that use an electric vacuum pump, such as the eDNA sampler (Thomas et al., 2018).

Our inability in this study to distinguish species from the genus *Elliptio* with the two previously developed markers demonstrates that the markers are not able to resolve all North American freshwater mussels to the species level, particularly closely related species. The species resolution of these assays should be tested in other assemblages for different watersheds. Previous studies have also shown limited ability of mitochondrial DNA (mtDNA) to distinguish between members of the genus *Elliptio* (e.g., Inoue et al., 2018), suggesting further investigation is needed to determine whether mtDNA variation is insufficient to separate valid species or if taxonomic revision is warranted. Similarly, available mtDNA sequences show low interspecific divergence between *L. cardium* and *L. satura*; however, a recent phylogenomic study using single-nucleotide polymorphisms (SNPs) supported recognizing both as distinct, monophyletic taxa (Hewitt et al., 2021). The reliance of eDNA studies on comprehensive DNA libraries and accurate taxonomy amplifies the need for further refinement of unionid systematics and taxonomy; this was identified as the top issue in a national strategy for the conservation of native freshwater mussels by the Freshwater Mollusk Conservation Society (2016).

Although we detected mussel eDNA using the described method of filtration (influent in through red port and filtrate out through the blue side port) and elution (HVEF connected to the red port and eluate out through the blue port), other studies have successfully detected target organisms captured with D-HFUF using different filtration and elution configurations and methods. For example, we used the HVEF canisters to elute the filters. Other methods have used clockwise and counterclockwise rinsing of the D-HFUF with 400 mL of elution solution (0.01% Tween 80, 0.01% sodium hexametaphosphate, and 0.001% Antifoam Y-30) (McMinn et al., 2017). Liu et al. (2012) tested the recirculation of 500 mL of elution solution (500 mL elution solution consisting of 0.01% Tween 80, 0.01% sodium polyphosphate, and 0.001% Antifoam Y-30 Emulsion) through an ultrafilter for 5 min. In their D-HFUF method for detecting *Cyclospora cayetanensis*, Durigan et al. (n.d.) filtered their samples with D-HFUF through a different port configuration than the study herein. Holowecky et al. (2009) tested different UF cartridges, not including the Rexeed 25 s filter, and found no difference among filter types for detecting pathogenic organisms in drinking water. However, our study provides an initial outline for how D-HFUF samples could be collected in eDNA projects for which large water sample volumes are needed to effectively detect eDNA. Future eDNA studies seeking to employ D-HFUF may benefit from further optimization of the methods presented.

AUTHOR CONTRIBUTIONS

A.M., S.S., K.K., and M.C. conceived and designed the study design and field sampling. A.M., C.G., M.C., K.K., and N.J. collected samples. T.T., M.K., and N.J. performed laboratory work. L.Y. and A.M. analyzed the data. A.M., K.K, Y.L, M.K., T.T., N.J, S.S., and M.C. wrote and edited the manuscript. All authors provided feedback on the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Associated metabarcoding data are available as a United States Geological Survey Data Release (DOI: 10.5066/P9G9B6M2). Sanger sequences are archived on GenBank (Accession OQ979616, OQ979617, and OQ954343).

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