Refining the use of environmental DNA (eDNA) as a method to detect presence of the endangered Topeka Shiner (*Notropis topeka)*

FINAL REPORT

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Patrick DeHaan[1], Austin Hannah[1,3], Jenna Bloomfield[2], Louise Mauldin[2], Yu-Chun Kao[2]

[1]Whitney Genetics Laboratory, USFWS Midwest Fisheries Center, Onalaska, WI
[2]La Crosse Fish and Wildlife Conservation Office, USFWS Midwest Fisheries Center, Onalaska, WI
[3]Southeast Conservation Genetics Lab, USFWS Warm Springs Fish Technology Center, Auburn, AL

Summary

Topeka Shiner (*Notropis topeka*) is an endangered fish species that inhabits oxbows, floodplains, and small headwater streams throughout the central United States. Habitat loss and alterations represent the main threats to this species and conservation actions have included restoring oxbows that were historically occupied. Information on species occupancy is critical for evaluating the success of habitat restoration efforts, however, collection efforts can be difficult. Traditionally, sampling for Topeka Shiners has relied on seining. The effectiveness of seine surveys depends largely on habitat characteristics including oxbow size, depth, and substrate. Environmental DNA surveys have emerged as a method to effectively survey across broad landscapes and collect data on species occupancy. In this study our objectives were to: 1) Refine eDNA laboratory methods for Topeka Shiner (specifically increase the sensitivity of existing methods); and 2) Evaluate multiple eDNA field collection methods in order to make recommendations for future Topeka Shiner monitoring efforts. We modified an existing eDNA assay by adding a hydrolysis probe, which resulted in a highly sensitive tool for Topeka Shiner eDNA detection. From 2021 to 2022, we compared three different eDNA collection methods to evaluate their effectiveness: surface water grabs with centrifugation, surface water grabs with vacuum manifold filtration, and on-site surface water filtration using Smith Root filter packs. eDNA sampling at oxbows was followed by seine surveys to confirm species presence/absence. At each site where Topeka Shiners were collected in seine surveys, we also detected Topeka Shiner eDNA. Control sites where Topeka Shiners were presumed absent and no individuals were collected in seine surveys, had no eDNA detections. At one site with unknown Topeka Shiner status, no individuals were collected via seine surveys, but we did detect eDNA. Logistic regression showed that sampling method (i.e., centrifuge, vacuum filtration, Smith Root filtration) was a significant factor predicting detection rate, with centrifugation and Smith Root filtration having higher overall detection rates compared to vacuum filtration. We used linear regression to evaluate which sampling method collected the greatest amount of eDNA. Once again, collection method did have a significant effect on the number of copies per liter collected. At sites sampled in 2021, centrifugation had much higher copies detected than vacuum filtration. In 2022, there was much greater variability in the number of copies detected, and the comparison between vacuum filtration and Smith Root filtration was much more site dependent. For future sampling events, we suggest using Smith Root filters as the most efficient and economical means for collecting eDNA samples for Topeka Shiners.

Introduction

The Topeka Shiner (*Notropis topeka)* is a federally endangered minnow that was once historically widespread throughout prairie stream headwaters in the central United States (U.S. Fish and Wildlife Service 2021). The contemporary decline of this minnow is a consequence of widespread alterations to prairie stream hydrology from conversion of prairie habitats to agricultural use, channelization, habitat fragmentation, small impoundment construction, and other anthropomorphic disturbances (U.S. Fish and Wildlife Service [USFWS] 2021). Most extant Topeka Shiner populations are genetically disjunct and vulnerable to local extirpation by consequence of the species' preference to occupy headwater streams and the presence of anthropogenic barriers preventing site dispersal (Michels 2000; Mammoliti 2002).

Given the broad distribution of Topeka Shiners, collecting accurate landscape-scale distribution data is especially complicated. Prairie streams are naturally harsh, stochastic environments that experience intermittent periods of drought and flooding throughout the year. Topeka Shiners are highly opportunistic and repopulate suitable habitats over fine spatial scales; therefore, local occupancy is highly dynamic (Winemiller and Rose 1992; U.S. Fish and Wildlife Service 2018). Despite the environmental variability of prairie streams, survey data indicates that off-channel oxbows are critically important microhabitats used for spawning, rearing, and overwintering by Topeka Shiners in Minnesota, Iowa, and South Dakota (Hatch 2001; Thomson and Berry 2009; Bakevich et al. 2013). In off-channel pools, Topeka Shiners can be found at significantly higher densities than pools within the adjacent main-channel (Hatch 2001). Restoration of critical off-channel oxbows is the primary conservation action currently utilized by federal, state and county governments, as well as non-governmental organizations. Conservation and future management of Topeka Shiner status necessitates consistent survey data to assess oxbow restoration success as well as range-wide monitoring data to accurately delineate subpopulations, assess range-wide population demographics, and determine the species' movement within a stream complex (USFWS 2021).

Detection of Topeka Shiners in oxbows using traditional methods of seining and electrofishing can be challenging due to oxbow size, depth, vegetation density, substrate type, and water levels. Environmental DNA (eDNA) surveys are widely used to assess the presence and distribution of aquatic species (Ostberg et al. 2019; Beng and Corlett 2020; Dokai et al. 2023). eDNA monitoring allows for determination of a target species' occupancy with high sensitivity and minimal interference to the ecosystem (Takahara et al. 2013; Klymus et al. 2015; Davison et al. 2016) and this method provides an efficient means to sample across large landscapes to determine species distribution (Itakura et al. 2019; Erickson et al. 2019). Despite the advantages offered by eDNA monitoring, its use is not universally advantageous as a monitoring tool for all aquatic species. Environmental factors such as flow rate, pH level, temperature, and sediment composition directly influence the persistence of eDNA in water (Pilliod et al. 2014; Jane et al. 2015; Strickler et al. 2015; Stoeckle et al. 2017). Likewise, characteristics of the focal species such as reproductive state, size, and diet may influence the availability of eDNA for capture (Klymus et al. 2015). Therefore, assessment of sampling methods and confounding environmental factors is fundamental to the development of a species-specific eDNA monitoring plan.

The following study was conducted to: 1) Conduct field validation for a Topeka Shiner eDNA marker developed by the U.S. Fish and Wildlife Service Whitney Genetics Lab and; 2) Compare multiple eDNA water collection and field processing methods in selected oxbows in

Minnesota and Iowa in order to identify the optimal methods for future Topeka Shiner eDNA monitoring efforts.

Study Area

eDNA water samples were collected from restored oxbows located in the Big Sioux and Rock River watersheds in southwest Minnesota and the Boone River watershed in central Iowa in 2021 and 2022 (Figure 1). Oxbows ranged in size from approximately 0.04 acres to nearly 0.4 acres with varying depths. The oxbows intermittently connect to the adjacent stream when stream levels rise in response to rain and snow melt. Specific oxbows were chosen based on previous monitoring and physical sampling data collected by USFWS, which indicated that Topeka Shiners were likely to be present. Sites with likely presence of Topeka Shiners were chosen because the ability to compare methods requires positive detections, therefore we wanted to maximize the likelihood that eDNA was present at our study sites. During each sampling event, we sampled one control oxbow which was newly restored or not connected to the stream and had no history or expectation of Topeka Shiner occupancy. Two sampling periods occurred in 2021, with sites named OX 1-3 during the first period, and OX A-E during the second period (Table 1). In 2022, only one sampling period occurred and sites were named Site 1-6.

Table 1. Site names and locations of oxbows sampled for Topeka Shiner eDNA in 2021 and 2022 in Minnesota and Iowa. Control sites represent oxbows where Topeka Shiners were presumed absent and were sampled to ensure that we did not get false positive detections.

Year (State)	Site Name	Watershed	Stream Name	Control Site
2021 (MN)	OX1	Rock	Rock River	Yes
2021 (MN)	OX2	Big Sioux	Beaver Creek	No
2021 (MN)	OX3	Big Sioux	Beaver Creek	No
2021 (MN)	OXA	Rock	Lower Champepadan Creek	Yes
2021 (MN)	OXB	Rock	Little Rock River	No
2021 (MN)	OXC	Rock	Littel Rock River	No
2022 (IA)	SITE 1	Boone	Lyons Creek	Yes
2022 (IA)	SITE 2	Boone	Otter Creek	No
2022 (IA)	SITE 3	Boone	Prairie Creek	No
2022 (IA)	SITE 4	Boone	Prairie Creek	No
2022 (IA)	SITE 5	Boone	Prairie Creek	No
2022 (IA)	SITE 6	Boone	Prairie Creek	No

Methods

Field Methods

Between the various collection periods of this study, we utilized several methods of water sample collection for comparison (Table 2). eDNA water samples were collected from the perimeter of each oxbow and samples were distributed uniformly around the oxbow when possible. Multiple methods were used side-by-side to collect water at each sampling point. Field

blanks (samples of deionized water) were utilized for quality assurance purposes at all sites. After water samples were collected, each oxbow was seined to determine if Topeka Shiner were present (Figure 2). Topeka Shiners were collected using a 15, 30 or 50-ft bag seine with ¼ inch mesh. The seine was deployed by two people at one end of the oxbow, extending across the width. Then, while extended across the width, the seine was dragged along the length of the oxbow. When possible, the entire length and width of the oxbow was sampled; however, the presence of thick vegetation or depths that exceeded safe conditions for the sampling crew resulted in only a portion of some oxbows being seined. Topeka Shiners collected were verified by field experts, enumerated, and released back into the oxbow.

In 2021, the first round of this project compared two methods of eDNA water sample collection and field processing (Table 1; Figure 3). In the first method, 250mL surface water grab samples, consisting of 5x50 mL centrifuge tubes, were collected. Samples were immediately stored on ice and then concentrated by centrifugation and preserved in 95% non-denatured ethanol within 24 hours of collection. The second method consisted of collecting 2 L surface water grab samples, immediately storing samples on ice, and then filtration through 0.45µm glass fiber filters using vacuum manifolds in a mobile laboratory located near the sample site. Thirty samples were collected with each method. The filter membranes were then stored in plastic tubes containing desiccant beads. During the second round of sampling in 2021, the same general methods were compared (grab samples with centrifugation versus grab samples with vacuum filtration); however, the volume of water collected for filtration was reduced to 500 mL and the total number of samples collected for both methods was reduced from 30 to 14.

In 2022, we compared two methods of filtration, and 14 samples were collected with each method (Table 1; Figure 4). The first method duplicated the filtration methods from round two in 2021; 500 mL grab samples were collected and filtered through 0.45 µm glass fiber filters using vacuum manifolds. The second method collected a target volume of 500 mL passed through a 0.45 µm polyethersulfone (PES) filter housed in a Smith Root self-preserving filter packs (Vancouver, WA). The second method also utilized the Smith Root Citizen Science Sampler, which is a portable battery powered, sampling pump and filtration apparatus. This allowed those samples to be collected and preserved in the field and therefore reduced the overall collection and processing time. Utilizing self-preserving filter packs negated the need to use ice or desiccant to preserve samples in the field. Water samples collected via filter pack and Smith Root sampler were collected until the target volume had been reached or the filter clogged. Actual water volume filtered was recorded if the target volume was not reached. Filter packs remained stable at room temperature for up to 6 months, while the glass fiber filters needed to be kept frozen until lab analysis could take place.

Table 2. Sampling methods used for water sample collection and field processing for Topeka Shiner eDNA detection in 2021-2022. In the Collection Method column, GF = glass fiber filter and PES = polyethersulfone filter.

Year	Sample Type	Target Water Volume (L)	Sample Size/Site	Number of Sites	Collection Method	Preservation Method
2021 (June)	5 x 50 mL centrifuge tube	0.25	30	3	Centrifugation	Ethanol/Freeze
2021 (June)	2 L nalgene bottle	2.00	30	3	Vacuum filtration (0.45µm GF)	Desiccant/Freeze
2021 (Sept)	5 x 50 mL centrifuge tube	0.25	14	5	Centrifugation	Ethanol/Freeze
2021 (Sept)	0.5 L nalgene bottle	0.50	14	5	Vacuum Filtration (0.45µm GF)	Desiccant/Freeze
2022	0.5 L nalgene bottle	0.50	14	6	Vacuum Filtration (0.45µm GF)	Desiccant/Freeze
2022	0.5 µm PES filter	0.50	14	6	Smith Root Citizen Science Sampler Filtration (0.45µm PES)	Self-desiccating filter housing

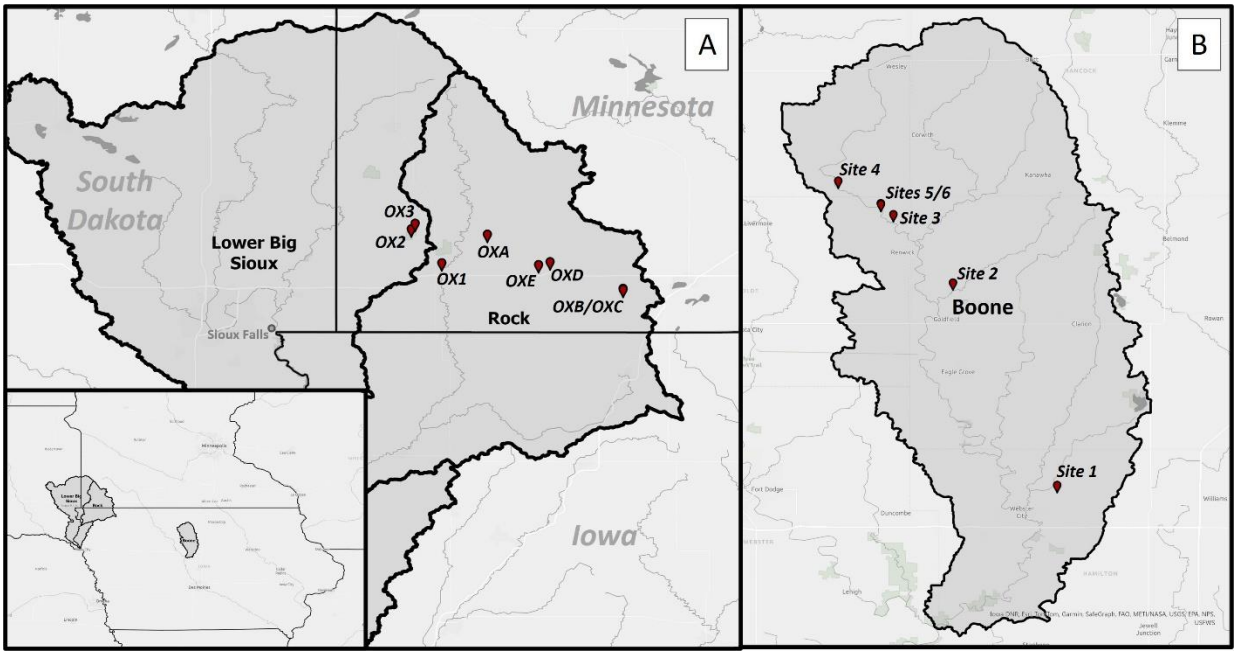

Figure 1. Topeka Shiner seining and eDNA water sample locations within the A) Big Sioux and Rock River watersheds in Minnesota and B) Boone River watershed in Iowa.

Figure 2. Sampling an oxbow by seine, after eDNA water samples have been collected, to check for presence of Topeka Shiner.

Figure 3. A) Side-by-side collection of water grab samples for centrifugation and filtration methods; B) Vacuum manifold filtration apparatus used to filter 2-liter water samples; C) 50 mL centrifuge tube samples being placed into a centrifuge in the mobile lab during field processing.

Figure 4. A) Vacuum manifold filtration apparatus used to filter surface water grab samples; B) Completed filters placed in tubes with desiccant beads; C) Water sample collection and filtration in the field using Smith Root Citizen Science Sampler and filter packs.

Laboratory Methods

Molecular assay development and optimization

We used a qPCR assay that targets an 82 base pair (bp) region of the mitochondrial cytochrome b subunit (CytB) of Topeka Shiner using species-specific forward and reverse primers designed by Everhart (2015). A custom 5' FAM-labelled, double-quenched probe (Integrated DNA Technologies, Coralville, IA) was developed using Geneious (v. 9.3; Biomatters Inc. 2020) and Primer3 software (v. 4.1.0; Untergasser et al. 2012) to establish complementarity to the assay primer set (Table 3). Primers and probes were then aligned to publicly available Topeka Shiner sequences within NCBI's GenBank database and assay specificity was tested *in silico* against all closely related species in NCBI using BLAST. Prior to *in vitro* specificity testing, the assay was optimized in 20 µL reactions containing 1.0 µL forward/reverse primer mix (10 µM), 1.0 µL probe (2.5 µM), 5.0 µL water, 10.0 µL TaqMan® Environmental Master Mix 2.0 (Thermo Fisher), and 3.0 µL DNA template extracted from Topeka Shiner fin clips. Quantitative PCR reaction efficiency was analyzed in CFX Manager software (v3.1; Bio-Rad Laboratories, Inc) and quantified using duplicate reactions of a 5-point standard dilution curve consisting of 6250, 1250, 250, 50, and 10 copies of synthetic double-stranded gBlock ™ gene fragments (Integrated DNA Technologies, Inc).

Table 3. Primer and probe sequences for the qPCR assay used in this study. Primers were originally developed by Everhart (2015).

Oligo Description	Name	Sequence
Forward Primer	NtopCytB579F	5'- AGG CTT TTC GGT GGA TAA CGC GAC G -3'
Reverse Primer	NtopCytB579R	5'- CGT TGC ACC GGC AAT GAC GAA C -3'
Probe	TopShin_cyb_probe	5'- /56-FAM/CGA TTC TTC /ZEN/GCC TTC CAC TTT CTC TTC CC/31ABkFQ/ -3'

Specificity testing of the Topeka Shiner assay was performed *in vitro* using genomic DNA extracted from fin clips of target species and 18 co-occurring species collected by Nebraska Department of Natural Resources in 2021 (Table 4). Target and nontarget genomic DNA was extracted using a modified Chelex/Proteinase K protocol described by Casquet et al. (2012), quantified using a Qubit® dsDNA High Sensitivity Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) on an Invitrogen Qubit™ 4 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA), and subsequently normalized to 1.0 ng/µL prior to qPCR. Four replicates of deionized (DI) water negative controls and two replicates of diluted target DNA extract as positive controls were included on every qPCR plate analyzed. Thermocycler conditions started with a single 10-minute cycle at 95°C followed by 40 cycles of 95°C for 15 seconds and 68°C for one minute. All assay optimization and subsequent field sample qPCR reactions took place on C1000 Touch™ Thermal Cycler with CFX Touch 96-well and 384-well Real-Time PCR Detection Systems and raw qPCR output was analyzed using CFX Manager v3.1(Bio-Rad Laboratories, Inc).

Table 4. Specificity testing species list for the Topeka Shiner qPCR assay used in this study.

Species	**Scientific Name**	**Number Analyzed**
Bigmouth Shiner	*Notropis dorsalis*	8
Bluegill	*Lepomis macrochirus*	2
Brassy Minnow	*Hybognathus hankinsoni*	7
Brook Stickleback	*Culaea inconstans*	3
Common Carp	*Cyprinus carpio*	1
Creek Chub	*Semotilus atromaculatus*	4
Dace (hybrid)	*Chrosomus spp.*	9
Fathead Minnow	*Pimephales promelas*	7
Green Sunfish	*Lepomis Cyanellus*	12
Longnose Dace	*Rhinichthys cataractae*	8
Pearl Dace	*Margariscus margarita*	5
Plains Topminnow	*Fundulus sciadicus*	10
Red Shiner	*Cyprinella lutrensis*	18
Redbelly Dace (hybrid)	*Chrosomus eos spp.*	4
Sand Shiner	*Notropis stramineus*	20
Stonecat	*Noturus flavus*	2
Suckermouth Minnow	*Phenacobius mirabilis*	5
White Sucker	*Catostomus commersonii*	13

We determined the sensitivity of the Topeka Shiner assay using both tissue-extracted genomic DNA of the target species as well as synthetic gBlock™ gene fragments. We serially diluted Topeka Shiner DNA extracts from 10^{-1} ng/µL to 10^{-7} ng/µL and tested 12 replicate qPCR reactions at each dilution step. Similarly, synthetic gBlock™ amplicon fragments were tested using seven serial dilutions from 6250 to 0.4 copies/reaction in 12 replicate qPCR reactions per dilution step. The discrete limit of detection (LOD) and discrete limit of quantification (LOQ) were determined for both sensitivity tests, where the discrete LOD was defined as the lowest concentration of DNA detected in 95% of replicates, and the discrete LOQ was defined as the lowest concentration of DNA with a coefficient of variation below 35% (Klymus et al. 2020). In addition to discrete LOD/LOQ determination, we applied a curve-fitting method to the gBlock™ sensitivity data to estimate an effective LOD/LOQ (Klymus et al. 2020). Lastly, to assess the risk of false-negative amplification of the Topeka Shiner assay, we ran 80 replicate reactions using water as template along with two replicates of a PCR positive and two replicates of a five-step gBlock standard curve as described above.

Field sample processing

Once field samples arrived at the laboratory, they were preserved in one of two ways depending on the collection method. Centrifuged surface water grab samples which were preserved with ethanol were centrifuged again for 5 minutes at 4200 rpm, the ethanol was decanted, and the sample was dried for up to 24 hours in a laminar flow hood to ensure all ethanol had evaporated. Centrifuged samples were then stored at -80° C prior to DNA extraction. All filter samples were stored at -80° C until DNA extraction.

We extracted DNA from both filtered and centrifuged samples using IBI Scientific gMAX Genomic DNA Kits (IBI Scientific, Dubuque, IA). We extracted samples in dedicated DNA extraction rooms separately according to sample site and sample processing method. We followed a modified version of the manufacturer's protocol for IBI gMAX extraction kits (See Appendix); however, for filtered samples containing more than one filter, we incubated replicate filters individually in GSB/proteinase K solution, added ethanol, and consolidated by spinning ethanol from all sample replicates through a single spin column. For centrifuged samples, we soaked sterile, cotton-tipped applicators in GSB/proteinase K solution and swabbed the bottom of each of the five replicate field sample tubes. The head of each cotton swab was broken off and placed back into the GSB solution and then incubated. To ensure that no cross contamination occurred, we changed gloves between handling each sample and included a swab soaked in DI water as an extraction negative in each extraction batch. Additionally, we assessed extraction success by including an extraction positive, a swab soaked in Bluegill (*Lepomis macrochirus*) DNA, in each extraction batch. The extracted DNA from every sample was eluted in 150 µL tris-HCl elution buffer and stored at -20°C until qPCR.

We performed qPCR in quadruplicate (4 qPCR technical replicates) on each water sample in 20 µL reactions described previously. Each qPCR plate contained duplicate PCR positive controls using extracted Topeka Shiner DNA as template and quadruplicate PCR negative controls using DI water as template, as well as a duplicate, 5-point standard curve (6250, 1250, 250, 50, and 10 copies) using <1 week old, previously tested synthetic gBlock™. qPCR plate loading was performed using an EpMotion 5075 automated liquid handling robot (Eppendorf). Cycling conditions and assay performance were analyzed following methods described during assay optimization. After qPCR of all field samples was completed, we tested a final plate containing quadruplicate reactions of extraction negative controls and extraction positive controls from each extraction batch using a bluegill-specific qPCR assay described by Takahara et al. (2013) to rule out the possibility of contamination introduced during the DNA extraction process. If the assay failed to amplify a single replicate of an extraction positive sample or if a single molecular replicate of an extraction negative amplified, detection results from the field samples included in the respective extraction group were omitted. Similarly, if positive controls included on qPCR plates failed to amplify or if negative qPCR controls did amplify, the qPCR results for that plate were omitted and the plate was re-run.

Statistical Analyses

Our standards for declaring a positive detection were a minimum of one qPCR replicate amplifying in a field sample with a concentration greater than or equal to the effective LOD (rounded up to 1 copy/reaction); amplification occurred above a C_T of 15.0; the plate standard curve efficiency was greater than 80% with a $R^2 \geq 0.95$; and lastly, both of the plate PCR positive controls amplified above a C_T of 15.0, and no single PCR negative control amplified. Our criteria for LOD and LOQ were relatively stringent (Klymus et al. 2020); however, other studies using qPCR to detect aquatic species eDNA report the respective assay LOD as the lowest concentration detected, and LOQ as the lowest concentration amplified across all molecular replicates (Tréguier et al. 2014; Ostberg et al. 2018).

We compared sample processing techniques by assessing differences in detection rate between methods at each site and eDNA recovery within positive samples. For each field sampling site, we calculated the detection rate as the percentage of the total number of samples

taken with a positive detection (as defined above). We estimated eDNA recovery as the mean number of DNA copies per liter of water for each sample based on standard curves generated during qPCR analysis.

We compared detection rates among the three different collection methods using the following logistic regression analysis:

$$Detection \sim Method + Site$$

where *Detection* is a binary variable representing whether DNA was detected (1) or not detected (0), *Method* represents the three different collection methods (centrifugation, vacuum filtration, and Smith Root filtration) and *Site* represents the different oxbows that we sampled. Because different sampling methods were compared in 2021 (centrifugation and vacuum filtration) and 2022 (vacuum filtration and Smith Root filtration) and because different sites (presumably with different densities of Topeka Shiners) were sampled each year, we fit this logistic model to the data from the two years of this study separately.

We also compared copies per liter (*Copies*) among the three different sampling methods using a generalized linear mixed model (GLMM) analysis:

$$Copies \sim Method + Site + (1|Sample)$$

where *Method* represents the three different collection methods (centrifugation, vacuum filtration, and Smith Root filtration), *Site* represents the different oxbows that we sampled, and (*1|Sample*) indicates that replicates of the same sample are expected to have the same random intercept. Similar to our analysis of detection rate, we fit this GLMM to the two years of copies per liter data separately. All statistical analyses were conducted in R 4.3.2. (R Core Team 2023), with the "mgcv" package (Wood 2017) for the logistic regression analysis and the "glmmTMB" (Brooks et al. 2017) package for the GLMM analysis.

Results

Field Results

We collected the desired number of environmental samples at each site; we initially collected 30 samples per oxbow during June 2021 sampling, but then reduced the number of samples per oxbow to 14 starting with the September 2021 sampling. Our initial target volume for filtration was two liters and during the June 2021 sampling, one to five filters were required to process that volume of water. During June 2022 sampling, we targeted 500 mL of water for filtration. The amount of water processed with Smith Root filters prior to clogging (at which point sampling was discontinued) ranged from 50 mL to 500 mL. Seine sampling collected no Topeka Shiners at any of the control sites where they were presumed absent. Topeka Shiners were collected at all sites where they were presumed present. The status of Topeka Shiners at Site 2, sampled in 2022, was unknown and no Topeka Shiners were collected via seining at this site.

Assay Optimization

The Topeka Shiner qPCR assay used in our study was modified from an assay optimized by Everhart (2015) by the addition of a 5' FAM-labelled probe. Our qPCR assay maintained high

efficiency throughout lab validation steps, with an average inter-plate standard curve efficiency of 99.025 (SD ± 6.38), average R^2 of 0.997 (SD ± 0.002), and average slope of -3.34 (SD ± 0.15) across 14 standard curve replicates across seven qPCR plates. We found that the addition of a targeted probe increased the assay sensitivity compared to results reported by Everhart (2015). We determined the discrete LOD of the Topeka Shiner assay (defined as the lowest standard concentration detected in at least 11 of 12 molecular replicates) to be 2 copies/reaction and the discrete LOQ as 10 copies/reaction (the lowest standard concentration with a coefficient of variation less than 35%). Using serially diluted DNA extracts, we determined the assay is sensitive enough to amplify as little as 10^{-7} ng/uL of Topeka Shiner DNA in a sample. We subsequently applied a curve-fitting method to the sensitivity test data and estimated the effective LOD as 0.89 copies/reaction (SE = 0.21) and the effective LOQ as 32 copies/reaction. We found the assay to be 100% specific to Topeka Shiner after testing against DNA extracts of 18 sympatric fish species, including 10 samples of the closely related Sand Shiner (*Notropis stramineus)*. Lastly, we found no indication of false-positive amplification after testing the assay using 80 molecular replicates of deionized water as template.

Environmental Samples

A total of 432 environmental water samples were collected from twelve sites in Iowa and Minnesota between June 2021 and May 2022. Topeka Shiner eDNA was not detected in any of the 54 field blanks that were processed among the twelve collection sites. All of our laboratory controls showed the anticipated results (e.g., all qPCR negatives showed no amplification).

At the three negative control sites where Topeka Shiner were presumed absent, Topeka Shiner eDNA was not detected in any of the 116 water samples collected. Seining efforts collected no Topeka Shiners at these sites. For the nine collection sites that Topeka Shiner were presumed to occupy prior to sampling, we detected eDNA in 208 of 316 samples (overall detection rate = 66%; Table 5). All collection methods resulted in Topeka Shiner eDNA detections at nearly every sample site where the species was present. Two sites had 100% detection rates; Site 5 sampled with vacuum filtration in May 2022 and Site 6 sampled in May 2022 with both vacuum filtration and Smith Root filter packs (Table 5, Figure 5). There was one site sampled in May of 2022 with the Smith Root filter packs that produced 0 eDNA detections; however, there was a 7% detection rate for vacuum filtration samples at this same site (Table 5, Figure 5). Topeka Shiner eDNA was detected at all sites where individuals were physically collected via seine survey, as well as a single site where Topeka Shiner presence was presumed but the species was not physically captured (Table 5). In 2021, when we compared centrifugation and vacuum filtration, *Method* was a significant predictor of detection rate ($P = 0.022$, $\alpha = 0.05$) with centrifugation resulting in a higher overall detection rate. In 2022, when we compared the two different filtration methods, there was no significant difference ($P = 0.135$).

Table 5. Topeka Shiner eDNA detection rates for oxbows sampled in 2021 and 2022.

Year	Month	Site Name	eDNA Collection and Preservation Method	Individuals Collected via Seine	eDNA Detection Rate
2021	June	OX2	Centrifuge	Yes	0.833
2021	June	OX2	Vacuum Filtration	Yes	0.967
2021	June	OX3	Centrifuge	Yes	0.833
2021	June	OX3	Vacuum Filtration	Yes	0.667
2021	September	OXB	Centrifuge	Yes	0.857
2021	September	OXB	Vacuum Filtration	Yes	0.571
2021	September	OXC	Centrifuge	Yes	0.786
2021	September	OXC	Vacuum Filtration	Yes	0.214
2022	May	Site 2	Smith Root	No	0.000
2022	May	Site 2	Vacuum Filtration	No	0.071
2022	May	Site 3	Smith Root	Yes	0.429
2022	May	Site 3	Vacuum Filtration	Yes	0.214
2022	May	Site 4	Smith Root	Yes	0.500
2022	May	Site 4	Vacuum Filtration	Yes	0.214
2022	May	Site 5	Smith Root	Yes	0.929
2022	May	Site 5	Vacuum Filtration	Yes	1.000
2022	May	Site 6	Smith Root	Yes	1.000
2022	May	Site 6	Vacuum Filtration	Yes	1.000

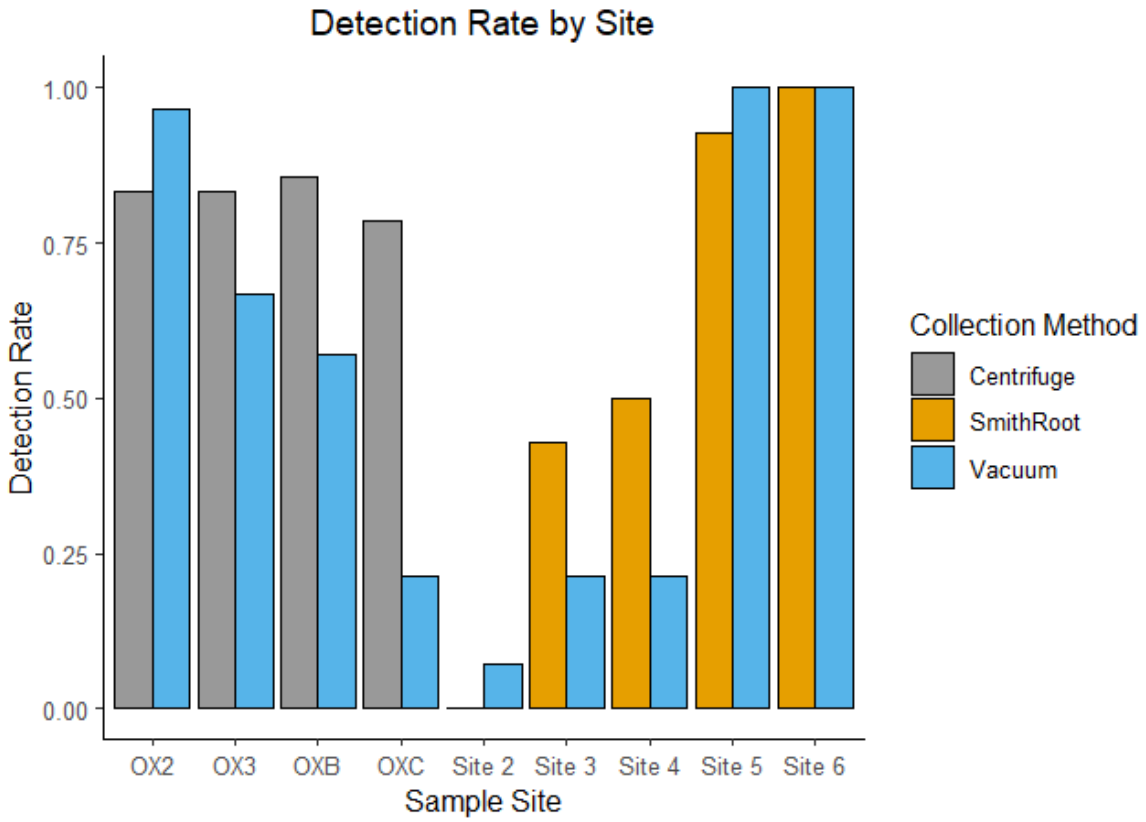

Figure 5. eDNA detection rates for oxbows sampled in this study. The different colors represent the different methods used for eDNA collection and sample preservation.

Copies per liter (an indication of how much DNA each method captured and detected from environmental samples) varied among the different collection methods and among the sample sites (Figure 6). In 2021 when we compared centrifugation and vacuum filtration, the mean copies per liter detected was greater for the centrifuge samples at all four sites (Figure 6). *Method* was a significant factor in the GLMM analysis (P <0.001, $\alpha = 0.05$). In 2022 when we compared vacuum filtration and Smith Root filtration, the mean copies per liter detected was much more variable; at Site 3 and Site 4 Smith Root filtration captured greater amounts of DNA (Figure 6). At Site 5 there was greater amounts of DNA collected with vacuum filtration and at Site 6 the two methods performed nearly equally (Figure 6). GLMM results showed that *Method* was not a significant factor in the analysis of the 2022 data ($P = 0.165$).

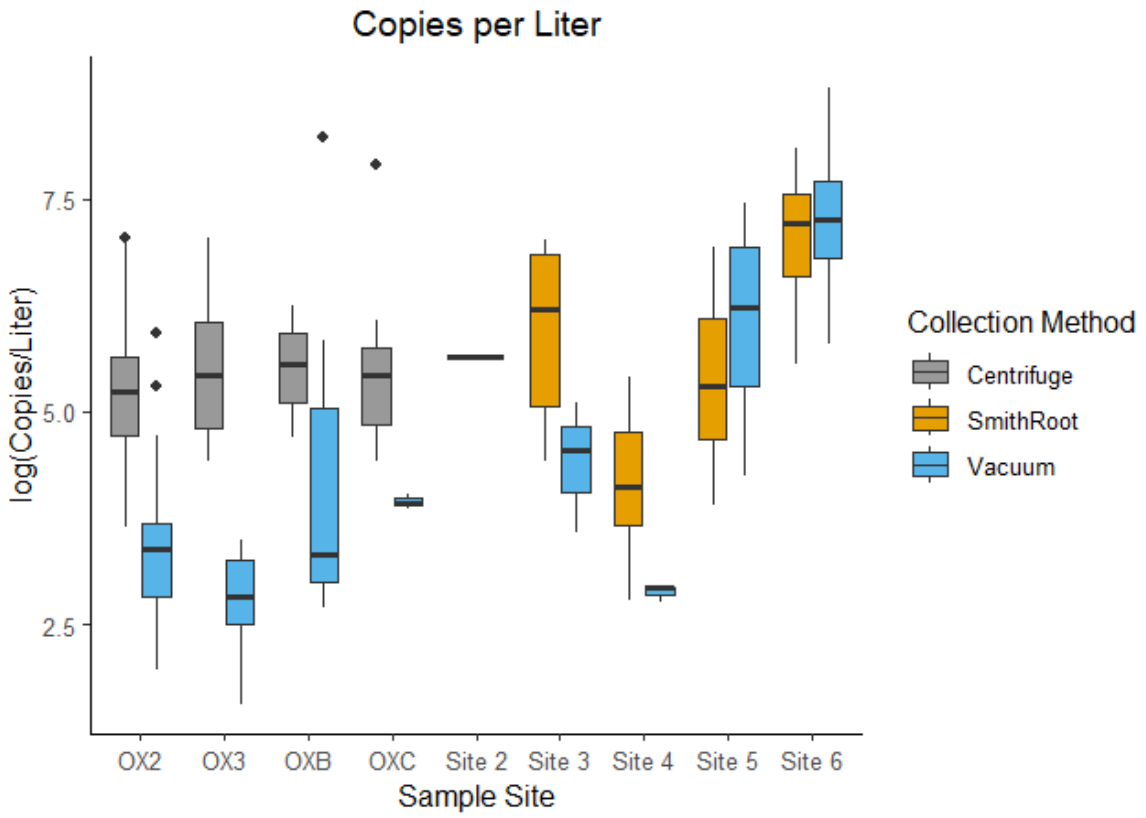

Figure 6. The number of copies per liter collected and detected at each sampling site in this study. The different colors represent the different methods used for eDNA collection and sample preservation. Note: Only one sample collected from Site 2 with vacuum filtration had a positive detection and therefore mean DNA recovery has no variation.

Discussion

The primary objectives of this project were to validate an eDNA assay for Topeka Shiners in the field and to test different eDNA collection and processing methods to determine the optimal sampling approach for Topeka Shiner eDNA monitoring surveys. Adding a hydrolysis probe to the existing Topeka Shiner eDNA marker used in this study resulted in a highly sensitive marker that was able to detect less than one copy of Topeka Shiner eDNA per qPCR reaction. This level of sensitivity is consistent with other eDNA assays reported in the literature (Klymus et al. 2020; Lesperance et al. 2021). The data presented here show that this marker can be used to detect Topeka Shiner eDNA when the species is present in oxbow habitats. At every site where Topeka Shiners were physically present, we detected the species' eDNA, and in all but one site where Topeka Shiners were not collected via seining, we observed no positive eDNA detections. Collectively, these data show that the marker used in this study is an effective method for documenting Topeka Shiner presence in oxbows.

Some of the advantages commonly cited for eDNA sampling are increased detection efficiency and the ability to conduct sampling across large landscapes (Rees et al. 2014; Hinlo et al. 2018; Erickson et al. 2019; George et al. 2021). Traditionally, monitoring efforts for Topeka

Shiners have focused on seining; however, the effectiveness of seining depends on numerous factors including habitat size, water depth, vegetation density, substrate type, water clarity, and seasonality (Holland-Bartels and Dewey 1997; Bayley and Herendeen 2000). Seining may also pose a risk to this endangered species since fish are physically handled during sampling, possibly resulting in increased stress to individuals. eDNA sampling for Topeka Shiners is less invasive since fish are not physically handled and there is a greater ability to detect smaller, juvenile Topeka Shiners not collected by larger mesh sizes of seines. Unlike seining, eDNA sampling causes no habitat disturbance to sediment, vegetation, or water clarity, which can be beneficial in maintaining the integrity of these critical habitats. Furthermore, eDNA sampling is far less labor intensive than seining and could be completed by a single person rather than two to three people required, at minimum, to conduct a seine survey. It is important to note that there are limitations to eDNA sampling; several physical habitat characteristics such as water temperature, flow, substrate type, and microbial community can affect eDNA detection rates, and may also reduce the effectiveness and sensitivity of eDNA sampling under certain conditions (Pilliod et al. 2014; Barnes and Turner 2016). Although high temperatures can impact eDNA detection rates (Strickler et al. 2015; Jo et al. 2019), the methods used in this study successfully detected Topeka Shiner eDNA even at sites where water temperature exceeded 27°C,

Interestingly, one site included in this study, 'Site 2' sampled in May of 2022, had no Topeka Shiners collected via seine survey, however, there was one eDNA detection at this site using vacuum filtration. We do not believe this detection represents contamination or a false positive given that all of our field blanks were negative, but rather an instance where eDNA sampling was able to detect Topeka Shiners in a site with low abundance, or where seining was less effective at capturing individuals. There are multiple instances where eDNA methods could detect species at low densities even though they were not collected with physical sampling methods (Pilliod et al. 2013; Schmelzle and Kinziger 2016; George et al. 2021) and this may be another example where Topeka Shiners did occur at low densities at this site, but they were not collected during seine sampling.

Methods Recommendations

The methods used to collect and process eDNA samples can have a significant effect on detection rates and the amount of eDNA captured (Bockrath et al. 2022). We tested three different eDNA collection methods in this study, and each of these collection methods resulted in positive eDNA detections when Topeka Shiners were physically present. When we compared detection rates, the vacuum filtration method was less effective (e.g., lower detection rates observed) than the centrifugation method and was less or equally effective in three out of four comparison to the Smith Root filtration method. The amount of DNA collected and detected by each method (copies per liter) also varied among methods, with centrifugation resulting in the greatest amount of DNA detected compared to the other two methods. Overall, any of the three methods we tested should be effective for the detection of Topeka Shiner eDNA in oxbows. We summarize the advantages and disadvantages of each collection method below (Table 6). The effectiveness of the different methods appears to be site specific, which presumably reflects differences in the Topeka Shiner densities and habitat characteristics at the different sites we sampled. Although we do not have estimates of Topeka Shiner densities at these sites, seine

surveys in 2022 collected substantially more Topeka Shiners than sites sampled in 2021 (USFWS *unpublished data*), which could mean that there were more individuals (and hence their DNA) present at the 2022 sampling sites. We also did not conduct a quantitative measure of habitat characteristics at each site which may also influence eDNA detections.

Centrifugation

This method was originally developed for invasive carp eDNA monitoring in aquatic systems with high turbidity. With this method, the desired water volume can be collected every time, unlike filters which are prone to clogging, which often results in a variable amount of water being processed. Additionally, this method captures all cells and DNA particles for extraction unlike the filtration methods which selectively remove some of the particles in the sample based on filter pore size. A previous study found that centrifugation methods collected more DNA and had higher detection rates than sampling methods that relied on vacuum filtration (Bockrath et al. 2022).

The primary disadvantages of this method are the costs and the processing time. This method requires the greatest amount of specialty equipment, most notably high-capacity laboratory centrifuges, which can cost upwards of $10,000 each. If samples are not collected within a reasonable distance of a laboratory building with centrifuging equipment, a mobile laboratory may be required to process samples in the field, which adds substantial costs to the process. Specialty equipment was available for this study since it could be "borrowed" from USFWS invasive carp sampling efforts, but we assume most partners would not be able to purchase these items. Samples must be transported to the lab (either a mobile lab in the field or a permanent lab) which could result in DNA degradation if samples cannot be kept cool and free from UV exposure. Current protocols dictate that samples are processed within a 24-hour time period to reduce the risk of DNA degradation. Furthermore, there could be an increased risk of contamination as samples get handled as part of the centrifuging process. The current protocol requires roughly 60 minutes to process a full centrifuge of samples (which equates to about 8 samples in this study), and multiple centrifuges or multiple rounds of centrifugation are required with greater sample numbers.

Vacuum Filtration

Vacuum filtration is a commonly employed method for eDNA collection and processing that has been in use for over a decade. Equipment required for vacuum filtration is much less expensive than centrifugation; the requirements are a manifold pump (less than $500 online), a vacuum manifold (prices ranging from $1500-$2500), several filter funnels/cups ($100-$200 each), and filters (prices depend on filter material, pore size, and quantity). All of these supplies except for the filters can be sterilized and re-used and there is little maintenance required. Although filter prices vary, the filters used for vacuum filtration are typically less than one dollar each, compared to the Smith Root filter packs which are $16 each. Filtration could be set up in the field (although contamination risks exist) if a generator or other power supply was available. This method could be accomplished by a relatively small field crew with one person to collect water samples and one person to do the filtering. It's possible that the same person could do both tasks, however, extreme care would need to be taken to ensure that processing gear was not

contaminated by DNA encountered during water collection (e.g., new gloves, change of clothes after water collection, etc.).

A major disadvantage of this method compared to centrifugation is that filtration does not collect total DNA from a water sample. Depending on the filter pore size, some cells and DNA particles may pass through the filter and be lost from the sample. This could result in lower detection rates compared to centrifugation. Also, filters routinely become clogged in turbid environments and the target sample volume cannot always be reached, or multiple filters per sample may be required. eDNA sampling can be prone to contamination and strict sample handling procedures including the use of clean gloves for every sample, decontaminating processing gear between samples, and careful handling of preserved samples should be followed (Goldberg et al. 2016). The risks of contamination may be increased if samples are being processed in the field as opposed to transporting samples to an offsite, clean lab space. The time required to process samples with vacuum manifold filtration is typically less intensive than centrifugation (although samples with very high turbidity and multiple filters do take much longer to process), but more time consuming than the Smith Root filter packs. As with centrifugation, it is important to keep samples cold and in the dark so that no DNA degradation occurs prior to sample processing.

Smith Root filter packs

This relatively new method offers some advantages over vacuum manifold filtration and centrifugation. Mainly, the time required to process samples is considerably less than the other two methods since water is filtered and preserved on site. The typical time to collect a one-liter water sample can be as little as 1-2 minutes if turbidity is low; but sample processing time can be substantially longer in systems with high turbidity. Since the filter packs are contained within a self-desiccating housing, the risk of contamination in the field is reduced since the actual filters are not handled and preservation requires no additional handling or processing. The filtering procedures are relatively easy to learn and there are a number of online videos available that show how to use this method. Upon collection, filters do not require any special storage and can be preserved at room temperature for up to six months according to the manufacturer. An additional advantage to this method is that sampling could be completed by just a single person, still with minimal risk of contamination.

One main disadvantage of this method are the costs associated with it. As noted above, individual filters packs cost roughly $16 each and are single use. Filters packs need to be used in the field with some type of pump system. In this study we used the Smith Root Citizen Scientist Sampler, which costs roughly $1,800. This is a relatively simple battery-operated pump system. There are additional pump systems available from Smith Root which range in cost from roughly $7,000 to $10,000 depending on the accessories purchased. Other pump systems are certainly available for use with these filter packs and numerous options could be found online. Similar to the vacuum manifold filtration method described above, filters can become clogged which reduces the amount of water and eDNA that can be collected.

General sampling recommendations

Based on our results, we would recommend the use of the Smith Root filter packs as the most efficient and economical method to use for Topeka Shiner eDNA sampling. Sample collection can be completed by a single individual, the risk of contamination is reduced, and there is no need for additional processing in the field to preserve the sample as there is with vacuum manifold filtration and centrifugation. We observed equal or higher detection rates with this method compared to vacuum manifold filtration at three of four sites where we directly compared the two methods; particularly at presumed low density sites. The two main costs associated with this method would be purchasing the filter packs and the pump system. The Citizen Scientist pump system used here was effective, however, it is slower to process samples than other Smith Root eDNA samplers. Multiple pore-sizes are available for the filter packs, and in this study 0.45 µm filters (the smallest size available) worked well; although we hope to test larger pore-sized filters in the future (specifically 5.0 µm) to see if they produce comparable results. Larger pore sized filters may be advantageous for sampling more turbid water in order to process larger volumes of water. Our protocol of collecting 14 filters and a target filtration volume of 0.5 liters produced eDNA detections at all sites where Topeka Shiners were also physically captured. The number of filter samples collected will always be a balance between budgets, timelines, the area that needs to be sampled, and maximizing detection probabilities. Fewer filters per site could be used, particularly for smaller oxbows and for much larger oxbows or habitats than the ones we sampled, additional filters may be required. We would recommend including some level of spatial replication at each sampling site (e.g., a minimum of five filters collected from different locations within an oxbow) and not just collecting a single sample.

The sampling guidelines presented here are meant to be general and could certainly be adjusted based on any of the factors mentioned above (e.g., habitat size, suspected species abundance, turbidity). In order to determine the number of filters required to achieve certain detection probabilities under varying environmental and habitat conditions, eDNA occupancy models could be used (Schmelzle and Kinziger 2016; Strickland and Roberts 2019; Mize et al. 2019). We recommend that partners looking to utilize eDNA sampling as a means to monitor for Topeka Shiners consult with experienced field biologists and laboratory staff to develop a sampling plan prior to collecting eDNA samples and possibly conduct a pilot study specific to their watershed and habitat type before undertaking a larger monitoring effort.

Table 6. Comparison of the different eDNA sampling methods evaluated in this study. Method represents how water samples were collected and processed.

Method	Relative Equipment Costs	Advantages	Disadvantages	Notes
Water grab & centrifugation	$$$$	Target volume is always reached, all DNA is collected	Lots of specialized equipment required, Highest initial investment in equipment, Samples need to be processed offsite or in a mobile lab	High startup costs are likely a barrier for most partners.
Water grab & vacuum filtration	$	Lower equipment costs, many supplies are reusable, Filtration could be set up on site or in a clean lab space, Filter costs are relatively low	Multiple filters may be required to process the target water volume, Risk of contamination when handling samples	Well tested method that many eDNA studies have utilized
Field-based filtration (Smith Root filters)	$-$$	Fewer field crew members required, Self-preserving filter packs reduce contamination risk, No processing or preservation once water is filtered	Cannot achieve target volume if filters clog, Filter packs are expensive and cannot be re-used	Smith Root pumps range from approximately $2,000 to $10,000

Next Steps

To date our efforts have focused on processing samples from oxbow habitats only. Topeka Shiners are also known to inhabit other low-flow habitats in small prairie streams. We have collected eDNA filter samples from several scour pools below culverts for analyses to determine if Topeka Shiners are using those habitats and if we can detect their eDNA when they are present. Additionally, we are experimenting with a larger pore-size filter to determine if eDNA collection and detection rates are comparable between various filter sizes.

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Appendix – DNA Extraction Protocol Used in This Study

IBI gMAX Mini Kit Filter DNA Extractions

Procedure for gMax Mini Kit (IB47282); adapted from the manufacturer's protocol.

- Equilibrate samples to room temperature.
- Bring heating block to 60 °C.
- If a precipitate has formed in GSB Buffer, dissolve by incubating at 56 °C for at least 10 min.
- Add 350 µl GSB Buffer into a lyse and spin column
- Add 35 µl proteinase K into the lyse and spin column.
- Transfer samples to lyse and spin column. **CHANGE GLOVES BETWEEN EACH SAMPLE.**
- Filters: Remove each filter, carefully tear off the entire filter ring so it will fit into the lyse basket. Fold it so that it fits into the lyse and spin basket. Only one filter can be extracted at a time.
- NOTE: If there are multiple filters for a sample, incubate each filter separately in its own column with GSB/ProK and combine at step 11 by spinning liquid through the same GS column.
- Incubate the spin basket and collection tube at 60 °C for 60 min. Use this time to set out GS Column in a collection tube for each sample as well as 3 more collection tubes for each sample and a 2.0 ml Eppendorf MCT. Print labels for the archived filters/swabs and the final extract sample tube. Also, during this time, place the Elution Buffer into the 60 °C bead bath.
- Centrifuge at max speed or 18,000 x g for 1-3 minute to clear the extraction buffer into the collection tube.
- Remove spin basket from the collection tube and throw in garbage.
- Add 500 µl 100% ETOH to sample and mix again by briefly pulse-vortexing.
- Apply up to 790 µl sample to the GS column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 16,000 x g for 1 min. Place the GS column in a clean 2 ml collection tube and discard the tube containing the filtrate.
- Repeat previous step until the whole lysate is loaded.
- Carefully open the spin column and add 400 µl W1 Buffer without wetting the rim. Centrifuge at 16,000 x g for 30 s then discard the flow-through. Place the GS column back in a new 2 ml collection tube.
- Add 600 µl of Wash Buffer to the GS column. Centrifuge at 16,000 x g for 3 min to dry the column matrix then carefully discard the flow-through, being sure not to touch the column to the liquid when removing from the centrifuge.
- Transfer the dried GS column to a clean 2.0 ml Eppendorf MCT. Add 200 µl of pre-heated elution buffer directly into the CENTER of the column matrix. Let stand for at least 1 min at room temperature (15 - 25°C) to allow Elution buffer to be completely absorbed. Centrifuge at 16,000 x g for 30 s to elute the purified DNA.
- Discard the GS column. Store the eluted DNA samples at −80°C.