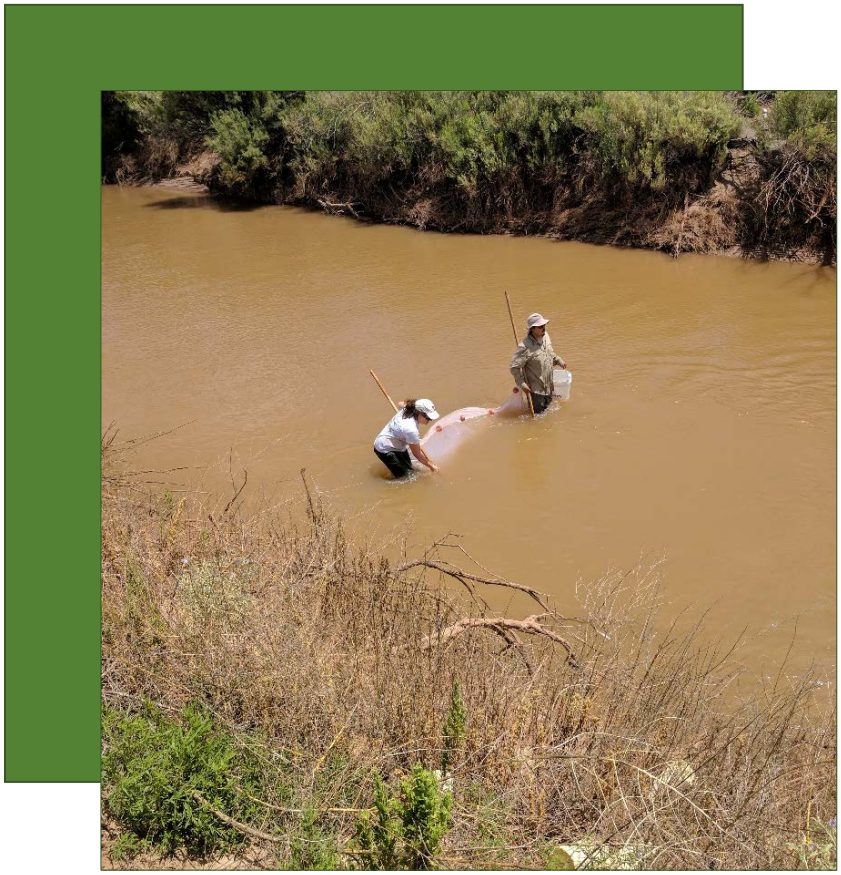
eDNA detection of species of greatest conservation need in the Lower Pecos River System



Kelbi Delaune
kelbi.delaune@ttu.edu

Dr. Matthew A. Barnes
matthew.a.barnes@ttu.edu | 806-834-2122

Dr. Allison Pease
allison.pease@ttu.edu | 806-834-0463

Department of Natural Resources Management
Texas Tech University, P.O. Box 42125
Lubbock, Texas 79409-2125, USA

TEXAS TECH UNIVERSITY
Department *of* Natural Resources Management™

SUMMARY

Successful environmental management depends on accurate knowledge of species distributions. One emerging detection technique is the collection and analysis of environmental DNA (eDNA), which refers to genetic material collected not through targeted methods, such as trapping individuals or collecting fresh feces, but extracted from bulk environmental samples, such as soil or water. The objective of our research was to design and apply eDNA assays for the detection of species of greatest conservation need (SGCN) in the Lower Pecos River system and its tributaries in New Mexico. Due to water quality issues caused by a combination of anthropogenic and natural influences, much of the Lower Pecos River system is considered biologically impaired, yet the system contains 9 fish species considered SGCN in New Mexico. We developed eDNA assays for a subset of these fish SGCN, including Blue Sucker (*Cycleptus elongatus*), Gray Redhorse (*Moxostoma congestum*), Arkansas River Shiner (*Notropis girardi*), and Bigscale Logperch (*Percina macrolepida*), as well as the federally listed Texas Hornshell mussel (*Popenaias popeii*).

We developed eDNA assays through a two-stage process: first, assays were designed *in silico*, and then they were optimized using tissue samples under controlled laboratory conditions. Once species-specific primers had been identified for each focal species, we collected water samples from the Pecos River system to validate *in situ* performance. We collected samples for eDNA analysis while concurrently sampling fish assemblages with traditional survey techniques across three seasons (i.e., spring, summer, and fall) in 2017. We collected eDNA samples from eight study sites in the Pecos River basin: 1) Pecos River at Bitter Lake National Wildlife Refuge, 2) Pecos River at Hwy 82 crossing near Artesia, 3) Brantley Lake, 4) Lake Avalon, 5) Lake Carslbad, 6) Black River (subdivided into three locations), 7) Pecos River at McDonald Road crossing near Malaga, and 8) Delaware River.

Species-specific assays developed for 5 SGCN in this study were all able to successfully detect focal species eDNA in the field, suggesting eDNA surveillance may represent a useful addition to the toolbox of managers and other stakeholders seeking to increase understanding of these species within the Pecos River system. However, eDNA detections notably varied across time and space. Additionally, across all sampling times and survey locations, we used traditional survey methods (i.e., seining) to collect a total of 1,915 fishes, including 44 individuals representing our focal species. However, discrepancies between our eDNA and traditional survey results suggest that there is still much to learn about the application of eDNA in the Pecos River system. For example, *N. girardi* was collected via seining, and its eDNA was detected at the Pecos River at Bitter Lake National Wildlife Refuge during sampling trips 2 and 3, representing agreement between our two surveillance methods. However, *C. elongatus* was never physically collected during our surveys, while eDNA from this species was identified across three sites and two sampling events. Similarly, *P. macrolepida* was never collected, but its eDNA was detected at three sites and on all three sampling trips. On the other hand, *M. congestum* was successfully collected with seining in the Delaware River on all three sampling trips, but it's eDNA was never detected at that site.

We cannot say with certainty that eDNA results reflect the presence of living individuals at each site. Indeed, eDNA detection could be the result of biological materials, such as mucous or feces,

flowing downstream or resuspension of old genetic material within the sediment. Several apparent plumes of eDNA corresponded with summer sampling following a high-discharge event. Better understanding of SGCN population dynamics, as well as eDNA dynamics, in lotic environments could aid efforts to use eDNA methodologies to understand and manage conservation targets in such environments. Future studies should consider a larger range of sites across broader spatial scales to look for patterns in eDNA presence and detectability.

Our results do suggest that, even where it was detected with relatively high frequency, eDNA occurred at low overall concentrations and was heterogeneously dispersed in the environment. Furthermore, eDNA demonstrates complex relationships in which it is both influenced by, and influences, its surrounding environment. This phenomenon is termed "the ecology of eDNA." Recent work has demonstrated that eDNA transport in lotic systems is particularly difficult to characterize, as eDNA interacts with inorganic substrates and local biota rather than flowing like a conservative tracer. The Pecos River's SGCN exhibit varying life-history strategies and habitat use, which may explain variability in our detections using both sampling methods. For the immediate future, a monitoring strategy that combines traditional sampling and eDNA analysis may represent the most successful strategy for detection of SGCN.

INTRODUCTION

The Pecos River and its tributaries contain 9 fish species considered species of greatest conservation need (SGCN) in New Mexico (NMDGF 2006 and 2016). However, due to water quality issues caused by multiple anthropogenic impacts, much of the Lower Pecos River system is considered biologically impaired (NMED 2007). Fully understanding the influence of degraded conditions on SGCN is hindered by the fact that sampling rare SGCN populations is methodologically and logistically challenging (Thompson 2004). For instance, given the degradation of habitats in the mainstem of the Pecos River, SGCN may retreat to tributaries or reservoirs as refuges during periods of low discharge, harmful algal blooms, and other unfavorable conditions (NMDGF 2006 and 2016). The Black River represents one such retreat, as it is a large tributary that lacks major impoundments, has a less altered watershed, and provides aquatic habitat with higher ecological integrity and greater local fish diversity (Cowley and Sublette 1987; Zymonas and Propst 2007). However, the Black River watershed increasingly suffers impacts from irrigation, grazing, and oil and gas development. Reservoirs in the lower Pecos, including large storage reservoirs like Brantley Lake and run-of-river reservoirs like Lake Carlsbad, may also serve as refuges for SGCN during periods of low discharge, but the use of these habitats by lower Pecos SGCN is poorly understood (NMDGF 2006 and 2016). Overall, improved methods to facilitate SGCN population monitoring would enhance conservation and management within the Pecos River system.

One emerging detection technique is the collection and analysis of environmental DNA (eDNA), which refers to genetic material collected not through targeted methods, such as trapping individuals or collecting fresh feces, but extracted from bulk environmental samples, such as soil or water (Thomsen and Willerslev 2015). Rapid advancements in eDNA application have resulted in the unprecedented ability to detect species for conservation, management, and research, especially in scenarios where collection or observation of organisms is impossible, impractical, or challenging, as in the Pecos River system. Within the Pecos River system, eDNA techniques could be particularly useful in determining presence, and potentially abundance, of SGCN. However, eDNA analysis likely does not represent a panacea, especially since SGCN in the Pecos River exhibit varying life-history strategies, and temporal variation in behavior related to reproduction and ontogenetic shifts could influence the "ecology of eDNA" (*sensu* Barnes and Turner 2016). The "ecology of eDNA" is its interaction with the environment and associated detectability across space and time. For example, de Souza *et al*. (2016) found that detection probability of two threatened aquatic species was strongly influenced by seasonal changes due to altered behavior and organismal activity. Thus, thoughtful development of eDNA methodologies for SGCN within the Pecos River system, including comparison between eDNA monitoring and more traditional survey methods, represents a critical need for conservation and management of this impaired system.

The Blue Sucker (*Cycleptus elongatus*), Gray Redhorse (*Moxostoma congestum*), Arkansas River Shiner (*Notropis girardi*), and Bigscale Logperch (*Percina macrolepida*) are fish SGCN in the Pecos River system, and these species represent a variety of life-history strategies and habitat associations which affect how they are monitored and managed (Table 1). *C. elongatus* and *M. congestum* are apparently extirpated in the Rio Grande in New Mexico and are increasingly rare in

the Pecos River (Sublette *et al*. 1990; Propst 1999; Zymonas and Propst 2007). Recent monitoring suggests that in New Mexico, both species are currently limited to the lower Pecos below Brantley Dam, with occurrences also documented in the Black River (Zymonas and Propst 2007). *N. girardi* has experienced extensive declines in its native range in the Arkansas River basin and is currently federally listed as threatened. An introduced population of this species persists in the Pecos River below Fort Sumner Dam and above Brantley Lake (Hoagstrom and Brooks 2005). Developing an eDNA monitoring assay for this species in the Pecos could provide a valuable management tool for use in its native range. *P. macrolepida* is currently rare in the mainstem of the Pecos River, but common in Pecos reservoirs (Sublette *et al*. 1990) and in the Black River (Propst 1999; Archdeacon and Davenport 2009). Declines of this species in the mainstem Pecos may be related to degraded water quality and reduced availability of deep-water habitats (Archdeacon and Davenport 2009).

The Pecos Pupfish (*Cyprinodon pecosensis*; Table 1), which has nearly been extirpated in Texas, is another SGCN that remains in some reaches of the lower Pecos and associated springs in New Mexico. A primary threat for *C. pecosensis* is hybridization with the nonindigenous Sheepshead Minnow (*C. variegatus*; Table 1) (Wilde and Echelle 1992; Echelle and Echelle 1997). Hybridization presents a fundamental challenge to the development of species-specific eDNA assays. Specifically, the mixing of genes between species potentially violates a critical requirement of eDNA methodologies: that diagnostic DNA sequences can be identified from environmental samples and assigned to their contributing species.

The Texas Hornshell (*Popenaias popeii*; Table 1) is an aquatic invertebrate SGCN in the Pecos River system. The only known occurrences of this newly federally-listed mussel in New Mexico are in a small reach (~14m) of the Black River. The Black River *P. popeii* population is isolated from the Texas populations as a result of increased salinity and poor habitat availability in the mainstem lower Pecos River (Inoue *et al*. 2013).

Thus, the objective of this research was to design and apply eDNA assays for the detection of SGCN in the Lower Pecos River system and its tributaries in New Mexico. Furthermore, we repeated sampling efforts through time and conducted *in situ* comparisons between eDNA results and both expected distributions and traditional survey methods (i.e., seine netting) to elucidate relationships between eDNA concentration, species abundance, and species behavior to refine eDNA methodology and maximize the ability of emerging methods to promote conservation and management.

Table 1. Life history, habitat use, and expected distribution of focal species within our study region.

Species	Life History	Habitat Use	Expected Distribution within Study Area
Cycleptus elongatus (Blue Sucker)	Up to 825 mm standard length (SL), long-lived, high fecundity, demersal adhesive eggs, breed March-May	Deep river main channels, and reservoirs; cobble or bedrock	Mainstem below Brantley Dam, Black River
Moxostoma congestum (Gray Redhorse)	Up to 514 mm SL, long-lived, high fecundity, demersal adhesive eggs, breed March-May	Stream pools and deep runs; sand, gravel, or rock	Mainstem below Brantley Dam, Black River, Delaware River, run-of-river reservoirs
Notropis girardi (Arkansas River Shiner)	Up to 65 mm SL, high fecundity, pelagic broadcast spawner, breed May-August	Shallow main channels, side channels, and backwaters; silt and shifting sand	Mainstem above Lake Avalon (non-native population)
Percina macrolepida (Bigscale Logperch)	Up to 95 mm SL, medium fecundity, demersal adhesive eggs, breed March-May	Large river channels, backwaters, and reservoirs; soft or rocky substrate	Reservoir sites, Black River
Popenaias popeii (Texas Hornshell mussel)	Up to >110 mm, long-lived, generally immobile as adults, fish host required for reproduction (catostomids; Red Shiner are known hosts), breed March-August	Narrow main channels; sand, clay, gravel and bedrock; prefer undercut banks and other structure that slows the current	Middle Black River
Cyprinodon pecosensis (Pecos Pupfish)	Up to 50 mm SL, low fecundity, spawn over guarded territory, breed May-September	Backwaters and pools; euryhaline	Mainstem above Malaga
Cyprinodon variegatus (Sheepshead Minnow)	Up to 70 mm SL, low fecundity, spawn over guarded territory, breed April-October	Backwaters and pools; euryhaline	Mainstem below Malaga

METHODS

eDNA Assay Development

The development of eDNA markers for focal species represents the creation of sensitive, specific, rapid, and easily-implementable detection tools that will aid in the conservation and management of SGCN in the Lower Pecos River system and throughout New Mexico. Specifically, our objective was to develop eDNA assays for detection of each of seven focal species (Table 1). Early in project development, we determined that detection of hybrids (specifically, of the focal species *C. pecosensis* and *C. variegatus*) with a single eDNA assay would not be feasible due to the heterogeneous nature of environmental sample collection (i.e., environmental samples represent a mix of genetic material from multiple species and likely multiple individuals per species). However, development of assays for potential paternal species may contribute to efforts to determine hybrid status of collected individuals. Development of species-specific eDNA assays for *C. pecosensis* and *C. variegatus* continues at the time of submission of this report. Final, validated assays will be submitted as an addendum to this report. The remainder of the present report focuses on development and application of eDNA assays for *C. elongatus*, *M. congestum*, *N. girardi*, *P. macrolepida*, and *P. popeii*. Development of eDNA assays is a two-stage process: first, assays are designed *in silico* and then they are optimized using tissue samples under controlled laboratory conditions.

To begin *in silico* development of species-specific genetic markers for our focal fish species, we generated a list of fish species in the Pecos River system based on previously published reports (Cowley and Sublette 1987; Propst 1999; Cheek and Taylor 2015) and the Biota Information System of New Mexico Database (www.bison-m.org). The complete list of fish species is available in Appendix Table A1. Following the generation of this list, mitochondrial COI-5p sequences were downloaded for each species from the Barcode of Life Data Systems (http://boldsystems.org/). In total, 2,237 sequences were accessed, and BioEdit software was used to align sequences and truncate them to a common length. Using the DECIPHER Online Software Toolset for Biological Sequence Management (Wright 2016), we designed forward and reverse oligonucleotide primers of approximately 20 base pairs in length that overlapped genetic mismatches between species. Thus, primers were designed to amplify each of the focal species and no other taxa. Candidate primers produced by DECIPHER were assessed for species-specificity using the Primer-BLAST feature of NCBI GenBank (Ye *et al*. 2012; https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and the Refseq Representative Genomes database.

Initial *in silico* design of primers for detection of *P. popeii* proceeded similarly to fish primer development. We began by accessing all COI-5p sequences for *P. popeii* (N = 29) and the closely related Threeridge *Amblema plicata* (Campbell and Lydeard 2012; N = 27) from the Barcode of Life Data Systems followed by alignment and truncation in BioEdit. We again used the DECIPHER Online Software Toolset for Biological Sequence Management to identify multiple candidate primers and tested them for species specificity using the Primer-BLAST feature of NCBI GenBank and the Refseq Representative Genomes database.

Candidate primers that were confirmed to be species-specific using Primer-BLAST were ordered (Integrated DNA Technologies, Skokie, Illinois) for laboratory confirmation with quantitative PCR

(qPCR). Candidate primers for SGCN fishes were tested against tissue-derived genomic DNA for all candidate species (Table 1), as well as the closely-related and/or co-occurring Red Shiner (*Cyprinella lutrensis*), Sand Shiner (*Notropis stramineus*), Rio Grande Shiner (*Notropis jemezanus*), and Fathead Minnow (*Pimephales promelas*). Candidate *P. popeii* primers were assessed using tissue-derived genomic DNA from *P. popeii* as well as Asian Clam (*Corbicula fluminea*) and zebra mussel (*Dreissena polymorpha*). All possible combinations of forward and reverse primers were first tested to confirm successful qPCR amplification of multiple individuals of their intended species. Next, candidate primer pairs that demonstrated consistent, robust amplification of all focal individuals were tested to confirm no cross-amplification of non-targets. For each focal species, the most successful primer combination was identified as the one that demonstrated the most robust and consistent amplification of targets and absence of reactivity with non-target species.

During candidate primer identification, qPCR was performed on a QuantStudio 3 Real-Time PCR System (Applied Biosystems). Each 20 µL reaction included 10 µL 2x PowerUp SYBR Green Master Mix (Applied Biosystems), forward and reverse primer concentrations of 200 nM, and 5 µL tissue-extracted genomic DNA. Reactions for all species began with a 10-minute activation phase at 95°C. Assays for *M. congestum*, *P. macrolepida*, and *P. popeii* then proceeded with 50 cycles of 95°C for 15 seconds and 60°C for 1 minute, with fluorescence data collected at each 60°C step. To improve primer specificity by creating more prohibitive annealing conditions, assays for *N. girardi* instead proceeded with 50 cycles of 95°C for 15 seconds and 67°C for 1 minute, while assays for *C. elongatus* proceeded with 50 cycles of 95°C for 15 seconds and 68.5°C for 1 minute. All assays concluded with a melting curve analysis to identify spurious PCR products. Negative controls featuring ultrapure H_2O in place of DNA extract were included on each plate of reactions to detect contamination during qPCR setup.

Field Sampling

Once species-specific primers were identified for each focal species, we collected samples from the Pecos River system to validate *in situ* performance. Specifically, we collected water samples for eDNA analysis and sampled fish assemblages with traditional survey methods across three seasons (i.e., spring, summer, and fall) in 2017. Spring sampling occurred 29 April - 1 May; summer sampling occurred 19 July - 21 July; and fall sampling occurred 15 September - 18 September. We collected eDNA samples from eight study sites in the Pecos River system: 1) Pecos River at Bitter Lake National Wildlife Refuge, 2) Pecos River at Hwy 82 crossing near Artesia, 3) Brantley Lake, 4) Lake Avalon, 5) Lake Carlsbad, 6) Black River (subdivided into three locations), 7) Pecos River at McDonald Road crossing near Malaga, and 8) Delaware River (Figure 1). To collect eDNA at each site, surface water samples were collected by a researcher wearing gloves using 1-liter polypropylene bottles that had previously been sterilized with 10% bleach and autoclaved. Sampling protocols varied slightly between sites and sampling dates (Table 2). In stream sites, the furthest downstream water samples were collected prior to fish surveys and then we moved upstream of fish survey sites to reduce potential for researcher-mediated disturbance of the in-stream environment to influence eDNA results. In streams, distance between upstream and downstream water sample sites were typically ~200 m (details and exceptions in Table 2). Once samples were taken, they were placed on ice and transported to the laboratory for filtration (see Laboratory Analysis).

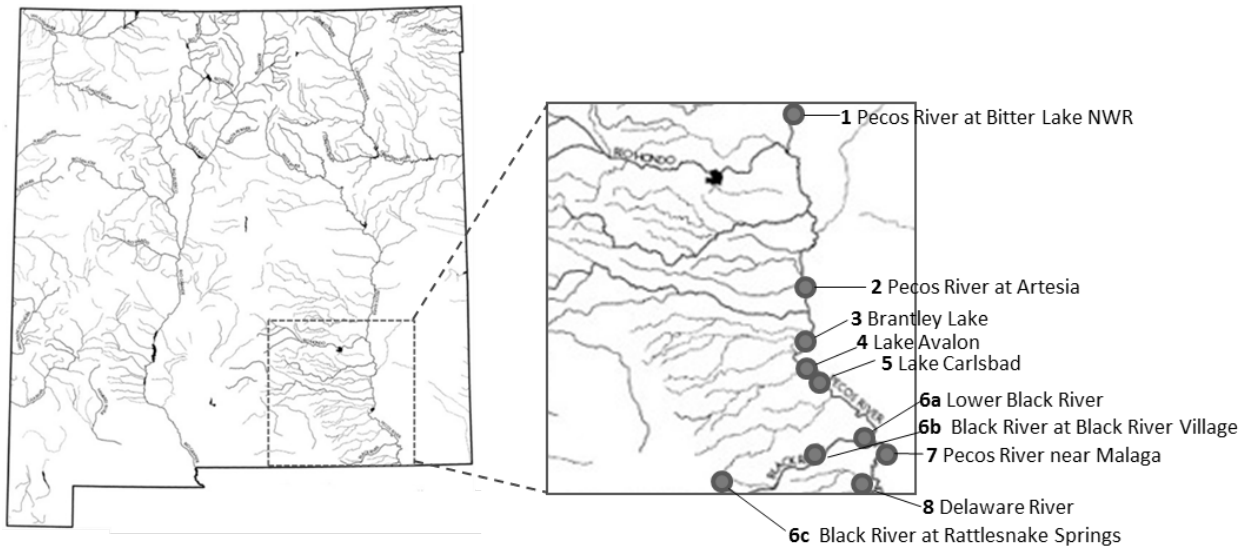


Figure 1. Map of lower Pecos study area within Eddy and Chaves Counties, New Mexico. Paired eDNA collections and traditional fish surveys occurred at Sites 1, 2, 7, and 8. At sites 3, 4, 5, and 6, only eDNA sample collection occurred.

Fishes were collected using a 2m x 3m, 0.5mm mesh seine, with seine hauls conducted across all representative mesohabitat types, at sites 1, 2, 7, and 8 (Figure 1). A minimum of six seine hauls were performed per site. If the sixth haul yielded any species not detected in the five previous hauls, additional seine hauls were made until no new species were captured. Seining effort was quantified based on the distance of each haul so that abundance could be calculated and compared across sites and seasons. Fishes were identified immediately (using diagnostic characteristics described in Sublette *et al*. 1990) and released alive. Seine sampling was initiated only after the furthest downstream eDNA samples had been collected, and were all conducted below the location of the furthest upstream eDNA sampling, to avoid stirring up sediments that may interfere with eDNA analysis. Seining did not occur at sites 3, 4, and 5 (i.e., lentic sites unsuitable for the method) or site 6 (i.e., the Black River sites with known *P. popeii* populations where disturbance was undesirable), although eDNA samples were collected at these sites. Habitat measurements including specific conductivity, water temperature, and dissolved oxygen were also collected at each site before seining events using a YSI Pro2030 handheld probe (YSI Inc., Yellow Springs, OH) and Hach FH950 velocity flow meter (Hach Co., Loveland, CO).

Table 2. Descriptions of location and water sampling protocols at each site.

SITE	SAMPLING LOCATION
Pecos River at Bitter Lake National Wildlife Refuge (33°27'8.82"N 104°22'45.96"W)	The 200m sampling reach started near the Scout Camp access point on the refuge. In spring, 15 samples were taken from the downstream and upstream portions of the reach, and five additional water samples were taken from a backwater habitat. The backwater habitat was dry in summer and fall sampling events, so 20 samples were collected at only two sites, directly downstream and upstream from the traditional fish sampling reach. Total N = 35 in spring; 40 in summer and fall.
Pecos River at Hwy 82 Crossing, near Artesia, NM (32°50'24.93"N 104°19'24.53"W)	In spring, 15 samples were taken from each of three sites occurring downstream, in the middle, and upstream from the sampling reach. In summer and fall, 20 samples were collected at each of two sites, directly downstream and upstream from the traditional fish sampling reach. The sampling reach at this site was ~200m, with the lowest part of the reach located near the south side of the Highway 82 bridge. Total N = 45 in spring; 40 in summer and fall.
Brantley Lake (32°33'28.17"N 104°23'29.07"W)	Samples were taken from the two boat ramp access points at Brantley Lake State Park, with 10 samples taken from the west side and 10 samples taken from the east side of the reservoir. Total N = 20 in spring, summer, and fall.
Lake Avalon (32°29'40.13"N 104°14'46.94"W)	Samples were collected from the shore at the Lake Avalon boat ramp, with 10 samples collected north of the ramp, and 10 samples collected south of the ramp. Total N = 20 in spring, summer, and fall.
Lake Carlsbad (32°26'23.33"N 104°13'57.57"W) (32°26'8.48"N 104°13'18.31"W)	We collected 10 water samples from each of two locations. The first location was near a public access point near boat docks on the southwest portion of the lake. The second location was on public land near a residential access point on the lake. Total N = 20 in spring, summer, and fall.
Black River (32°14'9.53"N 104° 4'26.45"W) (32°14'8.58"N 104°11'49.98"W) (32° 5'43.93"N 104°28'0.93"W)	Fifteen water samples were collected at each of three locations on the Black River: 1) upstream at Rattlesnake Springs recreation area; 2) middle near the Black River Village Road crossing (river reach with known *Popenaias popeii* presence); and 3) downstream of the Onsurez Road crossing. Total N = 45 in spring, summer, and fall.
Pecos River at McDonald Road Crossing, near Malaga, NM (32°11'20.97"N 103°58'39.65"W)	The designated sample reach was ~200m, with the most downstream portion just below the bridge. Twenty samples each were collected upstream and downstream from the study reach. Total N = 40 in spring, summer, and fall.
Delaware River (32° 0'44.42"N 104° 6'18.81"W)	Twenty water samples were collected from below and 20 from above a ~250m designated study reach just west of U.S. Hwy 285. Total N = 40 in spring, summer, and fall.

Laboratory Analysis

Each water sample was stored on ice and filtered in the laboratory within 48 hours of collection using 1.2 µm Isopore membrane filters (EMD Millipore). Total genomic DNA was extracted using protocols described by Barnes *et al.* (2014). Briefly, DNA extraction began by combining 700 µL cetrimonium bromide (CTAB), a cell lysis buffer, with the filter. Next, we added 700 µL 24:1 chloroform:isoamylalcohol for DNA purification. Samples were agitated for ~5 minutes and centrifuged at 14,000 rpm for 15 minutes. Next, we transferred 500 µL of supernatant liquid into a new, sterile tube. We added 500 µL isopropanol and 250 µL 5 M NaCl to the new tube and incubated samples at - 20 °C for at least 24 hours. After incubation, samples were centrifuged at 14,000 rpm for 10 minutes, resulting in the formation of a DNA pellet at the bottom of the tube and allowing the supernatant to be decanted. We then rinsed the DNA pellet with 150 µL 70% ethanol twice. Finally, pellets were suspended in 100 µL TE buffer and stored at 4 °C until qPCR was performed. A "blank" negative control consisting of an unused, sterile filter was also extracted with each batch of field samples. Previous research has found that certain chemicals and other factors co-extracted from environmental samples can cause PCR inhibition and misinterpretation (especially determination of false-negatives) during eDNA analysis (Jane *et al*. 2015). To avoid artefacts of PCR inhibition in our generally turbid water samples, all samples were diluted 1:10 using ultrapure H_2O prior to qPCR analysis.

For each focal species, excluding *C. pecosensis* and *C. variegatus*, individual species-specific qPCR reactions (i.e., not multiplexed) were performed on each eDNA sample using an Applied Biosystems QuantStudio 3 Real-Time PCR System. Samples were tested in triplicate reactions, and each 20 µL reaction included 10 µL 2x PowerUp SYBR Green Master Mix (Applied Biosystems), forward and reverse primer concentrations of 200 nM, and 4 µL 1:10 diluted eDNA. Duplicate negative controls consisting of ultrapure H_2O in place of DNA extract and positive controls with tissue-derived genomic eDNA were included on each plate. Optimized reaction conditions described previously were then used. An environmental sample was considered positive for a given focal species if any of the three triplicate reactions amplified.

Special precautions must be taken to manage contamination and other unique challenges in PCR labs (Mifflin 2007). These challenges are particularly acute in labs which focus on diagnostic analysis of samples containing potentially trace amounts of DNA (e.g., Knapp *et al*. 2012). PCR data integrity was ensured by generous incorporation of negative controls ("blanks") handled as "real samples" at multiple stages of the eDNA collection and analysis process (i.e., field collection, eDNA extraction, and qPCR). Furthermore, a rigorous schedule of decontamination with bleach and/or UV radiation was maintained throughout the project, and laboratory procedures were partitioned across two separate laboratories to isolate pre- and post-PCR processing.

RESULTS

eDNA Assay Development

For each focal species, the most successful primer combination was identified as the one that demonstrated the most robust and consistent amplification of targets and absence of reactivity with non-target species. Primer sequences are listed in Table 3.

Table 3. Primers for eDNA detection of focal SGCN.

Species	Forward Primer (3' – 5')	Reverse Primer (3' – 5')
Cycleptus elongatus	CACCCTCATTCCTCCTACTACTG	TGGCTCCAAGGATTGATGAAACA
Moxostoma congestum	ACTGACTCGTACCATTAATAATCGGG	TCCTGAAGAGGCTAATAGTAGCAGG
Notropis girardi	AACAACATGAGCTTCTGACTTCTA	ACCTGCTAGGTGAAGAGAGAAAATTG
Percina macrolepida	ACCGTCTACCCGCCTTT	AATGGCCCCTAGAATTGAAGAAATG
Popenaias popeii	GGTGCTTCTTCTATTTTAGGGGCC	TGTAAGCAACATCGTAATAGCACCA

Sampling Sites

Physicochemical, habitat, and discharge data were variable across both sampling dates and sites. During spring sampling, water temperature was considerably lower than it was in the summer and fall samples, increasing more than ten degrees from spring to summer and fall at all four eDNA-traditional sampling sites (Table 4). Additionally, specific conductivity varied between sites and seasons, ranging from 1,775µS/cm at Bitter Lake National Wildlife Refuge in the summer to 7,227µS/cm at the Pecos River site at Malaga in the summer and fall (Table 4). During the spring sampling at Bitter Lake National Wildlife Refuge, we were able to sample a backwater habitat that was not present in later seasons. Discharge in lotic sites varied seasonally across our study period (Figure 2), with particularly high flows in July and August for the mainstem Pecos River sites above Brantley dam.

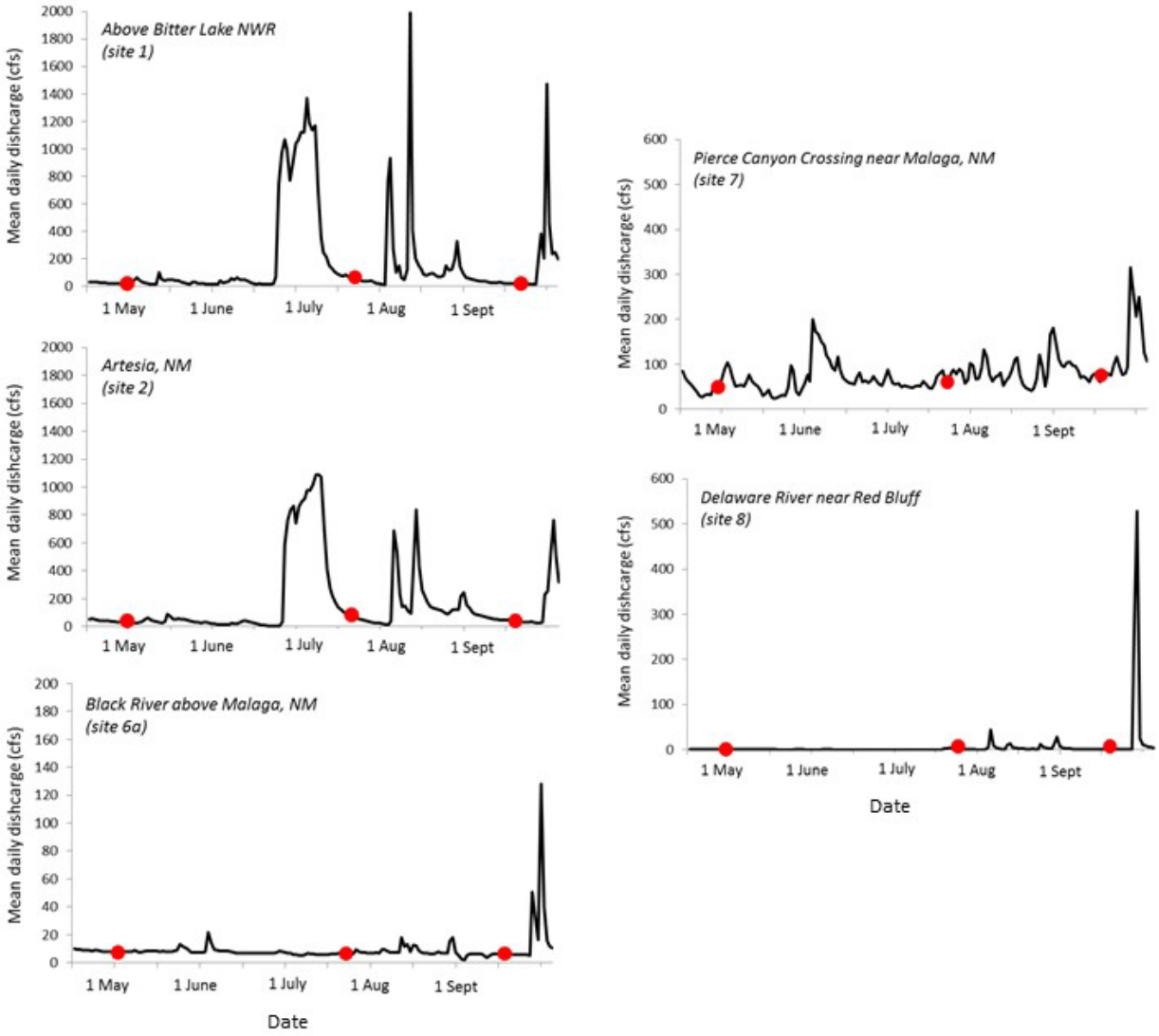


Figure 2. Hydrographs depict mean daily discharge based on United States Geological Survey gage data at available sample sites. Bitter Lake National Wildlife Refuge data collected from gage #8386000; Pecos River at Artesia data collected from gage #8396500; Black River data collected from gage #8405500; Pecos River at Malaga data collected from Pierce Canyon Crossing gage #8407000; and Delaware River data collected from gage #8408500. Red circles indicate survey dates.

Table 4. Physicochemical data collected during seining events across sites and dates.

Date[a]	Measurement	Site[b]			
		1	2	7	8
Spring	Specific Conductivity (µS/cm)	3,725	7,043	5,290	2,437
	Water temperature (°C)	15	14	16	16
Summer	Specific Conductivity (µS/cm)	1,775	3,418	7,227	2,682
	Water temperature (°C)	27	29	27	29
Fall	Specific Conductivity (µS/cm)	3,585	2,758	7,227	2,633
	Water temperature (°C)	24	26	27	25

[a] Date: Spring = 28 April-1 May; Summer = 19-21 July; Fall = 15-18 September

[b] Site: 1 = Pecos River at Bitter Lake National Wildlife Refuge; 2 = Pecos River at Artesia; 7 = Pecos River near Malaga; 8 = Delaware River

Traditional sampling

Across all sampling times and survey locations, we collected a total of 1,915 fishes, including 44 individuals representing our focal species (focal species catch per unit effort shown in Table 5; counts of all encountered species available in Appendix Tables A2-A13). In the spring sampling event, we captured a total of 505 individuals, representing 12 species across all sites. Eleven individuals of three of our focal species were detected; five *C. pecosensis* collected from Bitter Lake National Wildlife Refuge, one *C. variegatus* from the McDonald Road Crossing, and five *M. congestum* from Delaware River. In summer, we captured a total of 1,125 individuals, representing 18 species across all sites. Twenty-one individuals of three of our focal species were caught; two *N. girardi* collected from Bitter Lake National Wildlife Refuge, 13 *C. variegatus* from McDonald Road Crossing, and six *M. congestum* from Delaware River. In the fall, we captured a total of 285 individuals, representing 17 species across all sites. Three of our focal species were caught for a total of 12 individuals; one *N. girardi* from Bitter Lake National Wildlife Refuge, eight *C. variegatus* from the McDonald Road Crossing, and three *M. congestum* from the Delaware River.

eDNA Detection

Species-specific assays developed for 5 SGCN in this study were all able to successfully detect focal eDNA *in situ* (Table 6). All positive controls and all levels of negative controls (i.e., cooler and extraction "blanks" as well as qPCR no-template controls) performed as expected throughout the analysis. Several environmental samples were accidentally lost due to technical errors during the extraction process. Of the remaining processed samples, eDNA detections notably varied across time and space. For example, traces of *C. elongatus* eDNA were detected in Bitter Lake National Wildlife Refuge and Delaware River sites in the spring, but not summer or fall. Conversely, *C. elongatus* eDNA was identified in the Black River, but only during our summer sampling. *M.*

congestum eDNA was only detected in a single sample from the Black River in the fall. We observed relatively frequent detections of *N. girardi* eDNA among samples collected at Bitter Lake National Wildlife Refuge in the summer, but only observed this species in a single sample, also at Bitter Lake National Wildlife Refuge, in the fall. We detected *P. macrolepida* eDNA in samples from all three sampling events. *P. macrolepida* represented the only species that was consistently detected across all three sampling events at a single site, the Black River. It was also detected at Bitter Lake National Wildlife Refuge and Lake Avalon in the summer. *P. popeii* was not detected in the spring, but we did identify its eDNA from multiple Pecos River (Malaga) samples in the summer, multiple Black River samples in the fall, and samples from the Delaware River in both summer and fall. No eDNA from any of our focal species was identified in the Pecos River (Artesia), Brantley Lake, or Lake Carlsbad during any sampling event.

Table 5. Catch per unit effort (number per 10 m seine haul) of each focal fish species across sites and dates. Non-zero numbers shown in bold. Orange cell shading denotes sites with eDNA detections (see Table 6)

Date[a]	Focal species	Site[b]			
		1	2	7	8
Spring	*Cycleptus elongatus*	0	0	0	0
	Moxostoma congestum	0	0	0	**0.54**
	Notropis girardi	0	0	0	0
	Percina macrolepida	0	0	0	0
	Cyprinodon pecosensis	**0.43**	0	0	0
	Cyprinodon variegatus	0	0	**0.22**	0
Summer	*Cycleptus elongatus*	0	0	0	0
	Moxostoma congestum	0	0	0	**0.88**
	Notropis girardi	**0.24**	0	0	0
	Percina macrolepida	0	0	0	0
	Cyprinodon pecosensis	0	0	0	0
	Cyprinodon variegatus	0	0	**2.54**	0
Fall	*Cycleptus elongatus*	0	0	0	0
	Moxostoma congestum	0	0	0	**0.54**
	Notropis girardi	**0.16**	0	0	0
	Percina macrolepida	0	0	0	0
	Cyprinodon pecosensis	0	0	0	0
	Cyprinodon variegatus	0	0	**1.70**	0

[a] Date: Spring = 28 April-1 May; Summer = 19-21 July; Fall = 15-18 September

[b] Site: 1 = Pecos River at Bitter Lake National Wildlife Refuge; 2 = Pecos River at Artesia; 7 = Pecos River near Malaga; 8 = Delaware River

Table 6. Number of eDNA detections of each focal fish species across sites and dates. Detections shown in bold. Orange cell shading denotes sites with seine captures (see Table 5)

Date[a]	Focal species	Site[b]							
		1	2	3	4	5	6	7	8
Spring	*Cycleptus elongatus*	**1/29**	0/30	0/20	ND[c]	ND	0/30	0/30	**1/29**
	Moxostoma congestum	0/29	0/30	0/20	ND	ND	0/30	0/30	0/29
	Notropis girardi	0/29	0/30	0/20	ND	ND	0/30	0/30	0/29
	Percina macrolepida	0/29	0/30	0/20	ND	ND	**4/30**	0/30	0/29
	Popenaias popeii	0/29	0/30	0/20	ND	ND	0/30	0/30	0/29
Summer	*Cycleptus elongatus*	0/40	0/40	0/20	0/20	0/20	**2/45**	0/40	0/29
	Moxostoma congestum	0/40	0/40	0/20	0/20	0/20	0/45	0/40	0/29
	Notropis girardi	**15/40**	0/40	0/20	0/20	0/20	0/45	0/40	0/29
	Percina macrolepida	**2/40**	0/40	0/20	**1/20**	0/20	**14/45**	0/30	0/29
	Popenaias popeii	0/40	0/40	0/20	0/20	0/20	0/45	**2/30**	**2/29**
Fall	*Cycleptus elongatus*	0/38	0/25	0/12	0/4	0/20	0/42	ND	0/27
	Moxostoma congestum	0/38	0/25	0/12	0/4	0/20	**1/42**	ND	0/27
	Notropis girardi	**1/38**	0/25	0/12	0/4	0/20	0/42	ND	0/27
	Percina macrolepida	0/38	0/25	0/12	0/4	0/20	**18/42**	ND	0/27
	Popenaias popeii	0/38	0/25	0/12	0/4	0/20	**2/42**	ND	**1/27**

[a] Date: Spring = 28 April-1 May; Summer = 19-21 July; Fall = 15-18 September

[b] Site: 1 = Pecos River at Bitter Lake National Wildlife Refuge; 2 = Pecos River at Artesia; 3 = Brantley Lake; 4 = Lake Avalon; 5 = Lake Carlsbad; 6 = Black River; 7 = Pecos River near Malaga; 8 = Delaware River

[c] "ND" = no data collected.

DISCUSSION

Successful environmental management depends on accurate knowledge of species distributions. Increasing sophistication and decreasing costs of genetic methodologies have contributed to the recent emergence of techniques that use traces of organisms left in the environment, such as fur and feces, to provide clues regarding species presence (Beja-Pereira *et al*. 2009). One particularly promising technique is the use of eDNA. Rapid advancements in eDNA application have resulted in unprecedented ability to detect species for conservation, management, and research, especially in scenarios where collection or observation of organisms is impractical or impossible. In the present report, we have described the development and testing of eDNA assays for detection of four SGCN fish species and a federally-listed mussel SGCN in the Lower Pecos River system, New Mexico. Comparison of our developed eDNA assays and traditional survey results often conflicted with one another through time and space, so we conclude this report by with a discussion relative to the management of our five focal organisms, as well as speculating about broader lessons to benefit further development of eDNA applications in the Pecos River and other systems.

Species of Interest

The Pecos River SGCN exhibit a spectrum of life-history strategies and habitat use, which may explain temporal and spatial variability in detections across sampling methods. For example, *C. elongatus* was detected in two sites (Delaware River and Bitter Lake National Wildlife Refuge) using eDNA techniques during our spring sampling, but the species was not collected at either site during fish sampling. The positive eDNA detection within the Bitter Lake National Wildlife Refuge is surprising, as this is considerably further north than this species is expected to be found based on historic distribution data. Summer detection in the Black River was in agreement with its remaining known range in New Mexico (Zymonas and Propst 2007). Notably, eDNA detection of this species in both the Delaware River and Bitter Lake National Wildlife Refuge in the spring surveys coincides with its expected spawning season (March through May). *C. elongatus* is known to undergo spawning migrations in other systems, and movement in the spring may involve migrating from the mainstem river to tributaries (Neely *et al*. 2009), which would explain the Delaware River detection. Increased movement in the spring could also have led to expanded detection ability across the river network (i.e., as far north as Bitter Lake National Wildlife Refuge; Zymonas and Propst 2007; de Souza *et al*. 2016).

M. congestum eDNA was only detected in the fall within the Black River; we did not have eDNA detections in the Delaware River where the species was readily captured across seasons in our fish surveys. One possible explanation for this discrepancy is that individuals were netted only within a distinct pool mesohabitat at the Delaware River site. The low discharge of the site in general (Figure 2) combined with the fact that pool habitats represent channel units with the slowest local discharge, could have caused eDNA to settle out of the water column into the sediments and be retained within the pools, thereby decreasing eDNA detection probability. Furthermore, this may attest to the variability in detection probability with eDNA and the influence of the environment on the ecology of eDNA (Barnes and Turner 2016). The Black River detection of *M. congestum* came from the Village Road sampling location, which is near many large pools and known habitat for the species (Zymonas and Propst 2007).

N. girardi was collected in seine surveys on the Pecos River at Bitter Lake National Wildlife Refuge in the summer and fall sampling. Correspondingly, we had 16 positive detections for eDNA for this species across these two seasons. Detection with both methods at this site confirmed expectations that the species occurs in the Pecos River mainstem only in the northern part of our study area. The high frequency of detection in the summer using eDNA could be a result of increased activity due to spawning, which is expected to occur May-August (Bestgen *et al*. 1989; Hoagstrom and Brooks 2005).

Within the Black River, *P. macrolepida* was consistently detected in eDNA samples across seasons, aligning with previously collected fish sampling data (Archdeacon and Davenport 2009). The species was also detected in our summer sampling at Lake Avalon; however, it was not found in Brantley Reservoir where it is known to occur in high abundance in the littoral zone (Archdeacon and Davenport 2009). A positive eDNA detection of *P. macrolepida* at Bitter Lake National Wildlife Refuge is surprising, as Archdeacon and Davenport (2009) did not collect any individuals of this

species between Fort Sumner and Brantley Lake in recent comprehensive surveys, and none were collected at Bitter Lake National Wildlife Refuge during our own seining efforts.

P. popeii eDNA was detected only in the summer and fall sampling events. Environmental DNA detections in the Black River were consistent with known locations of mussel beds (Dan Trujillo, NMGF, personal communication). The two detections in the Pecos River at the McDonald Road crossing could be a result of eDNA flowing downstream from the Black River population and being detected in the mainstem. Indeed, it is unlikely that live *P. popeii* occur in this reach of the mainstem Pecos, as salinity levels are expected to be too high for freshwater mussels below the confluence with the Black River (USFWS 2016). We detected *P. popeii* eDNA at the Delaware River in summer and fall, which is most likely the result of recent reintroduction efforts to restore a population in this system.

Implications for designing monitoring protocols

Species-specific assays developed for 5 SGCN in this study were all able to successfully detect focal eDNA in the field, suggesting eDNA surveillance may represent a useful addition to the toolbox of managers and other stakeholders seeking to increase understanding of these species within the Pecos River system. However, discrepancies in our study between eDNA and traditional survey results suggest that there is still much to learn about this emerging application. For example, *N. girardi* was collected via seining, and its eDNA was detected, at the Pecos River at Bitter Lake National Wildlife Refuge during sampling trips 2 and 3, representing agreement between our two surveillance methods. However, notable differences occurred in survey results for other species that make it difficult to determine if one method is superior to the other. For example, *C. elongatus* was never physically collected during our surveys, while eDNA from this species was identified across three sites and two sampling events. Similarly, *P. macrolepida* was never collected, but its eDNA was detected at three sites and on all three sampling trips. On the other hand, *M. congestum* was successfully collected by seine in the Delaware River on all three sampling trips, but its eDNA was never detected at that site.

The fact that detection via eDNA could not be reliably repeated across sampling events or samples within a sampling event suggests that even where it was detected with relatively high frequency across samples from a given site, eDNA occurred at low overall concentrations and was heterogeneously dispersed in the environment. This pattern has been observed frequently in natural systems (e.g., Turner *et al*. 2014, Wilcox *et al*. 2016, Davidson *et al*. 2017). Furthermore, eDNA demonstrates complex relationships in which it is influenced by and influences its surrounding environment, termed "the ecology of eDNA" (Barnes and Turner 2016). Recent work by Jerde *et al*. (2016) and Shogren *et al*. (2016, 2017) has demonstrated that eDNA transport in lotic systems is particularly difficult to characterize, as eDNA interacts with inorganic substrates and local biota rather than flowing like a conservative tracer.

Hydrologic conditions can greatly influence the ability to detect eDNA (Jane *et al.* 2015). For example, low flow conditions can increase the detectability of eDNA as materials are more concentrated. High flow conditions may dilute eDNA and decrease detectability, or flood pulses may resuspend eDNA from sediment into the water column, influencing detection probability

(Eichmiller *et al.* 2011, Shogren *et al.* 2017). The latter could provide insight into the number of detections at sites 1 and 2 during the summer sampling, which followed high flow conditions above Brantley dam. Although our single-gage depictions of streamflow represent oversimplifications of hydrology, given factors such as mesohabitat heterogeneity, the complexity of lotic systems influences transport, retention, resuspension, and other eDNA dynamics within the system (Shogren *et al.* 2017), which in turn influences detection.

Based on our current results, we cannot say with certainty that our eDNA results reflect the presence of living individuals at each site. Indeed, eDNA detection could simply be the result of biological materials such as mucous and feces flowing downstream (Deiner *et al*. 2016). Better understanding of SGCN population dynamics (e.g., population and individual size variations as well as seasonal movements of population concentrations), as well as eDNA dynamics in lotic environments, could aid efforts to use eDNA methodologies to understand and manage species of interest in such environments. Based on our results, reproductive seasons represent potentially fruitful periods for eDNA sampling, although the relationship between species presence (according to traditional sampling methods) and eDNA detection was less definitive. Future studies should consider a wider range of sites across larger spatial scales to look for patterns in eDNA presence and detectability. For the immediate future, a monitoring strategy that combines traditional sampling and eDNA analysis may represent the most successful strategy for detection of these five focal SGCN.

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APPENDIX

Appendix Table A1. Fish species in the Pecos River System

Species Name	Common Name
Ambloplites rupestris	Rock Bass
Ameiurus melas	Black Bullhead
Anguilla rostrata	American Eel
Astyanax mexicanus	Mexican Tetra
Campostoma anomalum	Central Stoneroller
Carassius auratus	Goldfish
Carpiodes carpio	River Carpsucker
Catostomus commersonii	White Sucker
Cycleptus elongatus*	**Blue Sucker***
Cyprinella lutrensis	Red Shiner
Cyprinella proserpina	Proserpine Shiner
Cyprinella venusta	Blacktail Shiner
Cyprinodon pecosensis*	**Pecos Pupfish***
Cyprinodon varigatus*	**Sheepshead Minnow***
Cyprinus carpio	Common Carp
Dionda episcopa	Roundnose Minnow
Dorosoma cepedianum	Gizzard Shad
Etheostoma grahami	Rio Grande Darter
Etheostoma lepidum	Greenthroat Darter
Fundulus grandis	Gulf Killifish
Fundulus zebrinus	Plains Killifish
Gambusia affinis	Western Mosquitofish
Gambusia nobilis	Pecos Gambusia
Gila pandora	Rio Grande Chub
Herichthys cyanoguttatus	Rio Grande Cichlid
Hybognathus amarus	Rio Grande Silvery Minnow
Hybognathus placitus	Plains Minnow
Ictalurus furcatus	Blue Catfish
Ictalurus lupus	Headwater Catfish
Ictalurus punctatus	Channel Catfish
Ictiobus bubalus	Smallmouth Buffalo
Lepisosteus osseus	Longnose Gar
Lepomis auritus	Redbreast Sunfish
Lepomis cyanellus	Green Sunfish
Lepomis gulosus	Warmouth
Lepomis macrochirus	Bluegill
Lepomis megalotis	Longear Sunfish
Lucania parva	Rainwater Killifish
Macrhybopsis aestivalis	Speckled Chub
Menidia beryllina	Tidewater Silverside

Micropterus punctulatus	Spotted Bass
Micropterus salmoides	Largemouth Bass
Morone chrysops	White Bass
Moxostoma congestum*	**Gray Redhorse***
Notropis amabilis	Texas Shiner
Notropis braytoni	Tamaulipas Shiner
Notropis girardi*	**Arkansas River Shiner***
Notropis jemezanus	Rio Grande Shiner
Notropis simus pecosensis	Pecos Bluntnose Shiner
Notropis stramineus	Sand Shiner
Oncorhynchus clarki virginalis	Rio Grande Cutthroat Trout
Oncorhynchus mykiss	Rainbow Trout
Oreochromis aureus	Blue Tilapia
Percina macrolepida*	**Bigscale Logperch***
Pimephales promelas	Fathead Minnow
Pimephales vigilax	Bullhead Minnow
Platygobio gracilis	Flathead Chub
Pomoxis annularis	White Crappie
Pylodictis olivaris	Flathead Catfish
Rhinichthys cataractae	Longnose Dace
Sander vitreus	Walleye
Semotilus atromaculatus	Creek Chub

***Focal species**

Appendix Table A2. Fish species collected from Bitter Lake National Wildlife Refuge in Roswell, New Mexico, 1 May 2017.

Fish species	**Number Collected**
Cyprinodon pecosensis*	5
Macrhybopsis aestivalis	24
Cyprinella lutrensis	92
Gambusia affinis	1
Lucania parva	1
Hybognathus placitus	20
Notropis jemezanus	3

***Focal species**

Appendix Table A3. Fish species collected from the highway 82 crossing in Artesia, New Mexico, 29 April 2017.

Fish species	**Number Collected**
Macrhybopsis aestivalis	3

***Focal species**

Appendix Table A4. Fish species collected from the McDonald Road crossing near Malaga, New Mexico, 30 April 2017.

Fish species	Number Collected
Pimephelas promelas	3
Cyprinodon variegatus*	1
Cyprinella lutrensis	2

***Focal species**

Appendix Table A5. Fish species collected from the Delaware River upstream from the Hwy 285 crossing, New Mexico, 30 April 2017.

Fish species	Number Collected
Micropterus salmoides (Juvenile)	1
Moxostoma congestum*	5
Cyprinella lutrensis	325
Lepomis cyanellus	4
Pimephelas promelas	15

***Focal species**

Appendix Table A6. Fish species collected from Bitter Lake National Wildlife Refuge in Roswell, New Mexico, 21 July 2017.

Fish species	Number Collected
Carpiodes carpio	14
Cyprinella lutrensis	160
Hybognathus placitus	42
Macrhybopsis aestivalis	7
Notropis girardi*	2
Notropis jemezanus	8
Notropis simus pecosensis	6
Notropis stramineus	1
Gambusia affinis	249
Fundulus zebrinus	14
Morone chrysops	1

***Focal species**

Appendix Table A7. Fish species collected from the highway 82 crossing in Artesia, New Mexico, 19 July 2017.

Fish species	Number Collected
Cyprinella lutrensis	21
Hybognathus placitus	4
Macrhybopsis aestivalis	3

Appendix Table A8. Fish species collected from the McDonald Road crossing near Malaga, New Mexico, 20 July 2017.

Fish species	Number Collected
Cyprinella lutrensis	164
Cyprinus carpio	1
Pimephelas promelas	13
Menidia beryllina	17
Gambusia affinis	2
Cyprinodon variegatus*	13
Fundulus grandis	2
Morone chrysops	1

***Focal species**

Appendix Table A9. Fish species collected from the Delaware River upstream from the Hwy 285 crossing, New Mexico, 20 July 2017.

Fish species	Number Collected
Cyprinella lutrensis	301
Pimephelas promelas	7
Moxostoma congestum*	6
Astyanax mexicanus	1
Lepomis cyanellus	2

***Focal species**

Appendix Table A10. Fish species collected from Bitter Lake National Wildlife Refuge in Roswell, New Mexico, 17 September 2017.

Fish species	Number Collected
Lepisosteus osseus	1
Cyprinella lutrensis	88
Hybognathus placitus	42
Macrhybopsis aestivalis	28
Notropis girardi*	1
Notropis jemezanus	13
Notropis simus pecosensis	2
Pimephelas promelas	1
Gambusia affinis	104
Fundulus zebrinus	1

***Focal species**

Appendix Table A11. Fish species collected from the highway 82 crossing in Artesia, New Mexico, 15 September 2017.

Fish species	Number Collected
Cyprinella lutrensis	9
Hybognathus placitus	6
Macrhybopsis aestivalis	5
Notropis jemezanus	1
Notropis simus pecosensis	1
Gambusia affinis	1

Appendix Table A12. Fish species collected from the McDonald Road crossing near Malaga, New Mexico, 18 September 2017. No fishes were collected in the main channel; a fish kill was suspected. Fishes were collected only from a small, isolated backwater.

Fish species	Number Collected
Cyprinella lutrensis	18
Menidia beryllina	2
Cyprinodon variegatus*	8

***Focal species**

Appendix Table A13. Fish species collected from the Delaware River upstream from the Hwy 285 crossing, New Mexico, 16 September 2017.

Fish species	Number Collected
Cyprinella lutrensis	7
Dionda episcopa	37
Moxostoma congestum*	3
Lepomis cyanellus	5
Micropterus salmoides	1

***Focal species**