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Environmental DNA Metabarcoding as a Tool for Fish Community Assessment in Wetlands

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ENVIRONMENTAL DNA METABARCODING AS A TOOL FOR FISH COMMUNITY ASSESSMENT IN WETLANDS

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ABSTRACT

Environmental DNA (eDNA) metabarcoding is a genetically based method of assessing biodiversity in aquatic environments. While the efficacy of eDNA surveys has been well documented in riverine and marine systems, it has been relatively underemployed in freshwater wetland environments. In this study, we conducted an eDNA metabarcoding survey of fish diversity and its seasonal variation in a wetland along the Mississippi River in the Missouri Bootheel. Samples were collected from both permanent and seasonal water bodies including oxbow lakes, a shallow, man-made lake, a ditch, and a slough. For each of the 28 sites in this study, three water samples were collected in late May. The area was revisited in early October and sites that still held water were resampled. A combination of two, universal fish primer sets were used to amplify fragments of the mitochondrial 12s and 16s rRNA genes and Illumina sequencing was used to generate DNA sequences. A total of 54 species representing 37 genera and 17 families were detected between both markers among all samples. Our results indicated that the detected fish communities among different water bodies were distinct from one another despite periodic connectivity between them. We detected 20 species with eDNA metabarcoding that have not been previously observed at our study site, 5 of which are species of conservation concern. Our results add to the evidence that eDNA metabarcoding is an effective method of assessing species diversity and contributes to our understanding of fish community structure in complex wetland environments.

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1. INTRODUCTION

Monitoring the distribution and composition of fish communities is critical to inform management efforts, but traditional monitoring techniques can often be labor intensive, costly, and invasive. Traditional techniques that rely on capture also often underestimate species richness as some species may not be suited to the gear used (Evans et al., 2017). Environmental DNA metabarcoding is a genetically based, non-invasive biomonitoring tool that can be used to estimate the diversity of species in an area of interest (Mächler et al., 2019). Environmental DNA (eDNA) is DNA that is released into the environment via shed skin, saliva, blood, waste, or gametes of living or decomposing organisms (Rees et al., 2014). eDNA-based surveys have become increasingly popular over the past decade, particularly in aquatic systems, and can be used in addition to or as an alternative to traditional sampling techniques (Cilleros et al., 2019). eDNA can be filtered and extracted from a water sample, amplified, sequenced, and aligned to a reference database to infer what species are present. eDNA surveys are particularly useful in areas that are difficult to reach or transport gear into due to the minimal amount of equipment necessary to collect eDNA samples in the field.

1.1. eDNA METABARCODING

eDNA metabarcoding is a multi-species sampling approach that allows for the characterization of entire species assemblages. This approach involves using universal oligonucleotide primers that will amplify a target sequence across an entire taxonomic

group within a single PCR. Universal primers are designed to target a hypervariable region of the mitochondrial genome that contains enough taxonomic resolution to identify sequences to a species level (Miya et al., 2015). Unique sequences are identified by matching them to a database of known DNA sequences and assigned to a species. The ability of eDNA metabarcoding to detect species assemblages compared to traditional methods has been well documented, particularly in marine and riverine environments (García-Machado et al., 2022; Lecaudey et al., 2019).

1.2. CHALLENGES OF eDNA SAMPLING IN WETLANDS

While the efficacy of eDNA surveys has been well documented in riverine and marine systems compared to traditional sampling methods, few studies have implemented this technique in freshwater wetlands (Kačergytė et al., 2021). Wetlands possess a unique set of habitats and water parameters that necessitate the evaluation and potential modification of established eDNA metabarcoding protocols to successfully sample these environments. High concentrations of suspended organic and inorganic matter can interfere with water filtration, speed up the degradation of suspended eDNA in the water column, and inhibit subsequent PCR amplification (Kumar et al., 2021). Additionally, periodic flood events may homogenize eDNA from the main river channel and various floodplain habitats making it difficult to distinguish which habitats certain fish actually occupy (Sales et al., 2021). The inundation of floodplains may also dilute eDNA concentrations making it more difficult to effectively capture species diversity (Curtis et al., 2021).

1.3. WETLAND HABITATS IN MISSOURI'S BOOTHEEL

In this study, we set out to investigate the efficacy of eDNA metabarcode sampling on fish communities in a wetland complex in Missouri's Bootheel. Prior to colonization, the area was dominated by bald cypress and tupelo forests and wetlands. The majority of this land was granted to Missouri under the Swamp Land Act of 1850 to encourage land cultivation and the majority of standing timber was harvested. Beginning in 1914, the Little River Drainage District began construction of a series of canals and levees to drain 1.2 million acres of bottomland forest and wetlands (Pierce et al., 2012). Today, about 96% of Missouri's Bootheel has been drained and few wetlands remain in a relatively untouched state (Olson et al., 2016b).

The endemic fish communities of the remnant wetlands are some of the most unique in the lower Mississippi River basin and contain species that may have once been common but have been negatively impacted by the alteration and destruction of their habitats. Additionally, efforts to convert the floodplain to agricultural land have largely eliminated the seasonal floods that many large river species depend on for reproduction and early life stages (Humphries et al., 1999; Olson et al., 2016a). Furthermore, the flood-pulse concept suggests that periods of annual flooding in large rivers are the most biologically productive as water, nutrients, and organisms are exchanged between the main river channel and floodplain habitats (Junk et al., 1989). Species that utilize littoral habitats such as the black basses and sunfishes particularly benefit from annual floods as the littoral zone grows and shifts due to rising and falling water levels (Bartels et al., 1999).

Traditional fish community sampling in these habitats is infrequent due in part to the difficulty of access and transportation of gear and the risk of encountering dangerous wildlife. Fyke nets, which are commonly used to sample fish communities in this region by management agencies, often trap cottonmouth snakes during the warmer months of the year which creates a hazard to field crews and limits the effective sampling window. Despite sampling difficulties, efforts to assess the composition of the communities present in these remnant habitats is critical for effective management. Floodplain habitats often support a greater diversity of species than can be reliably observed in main river channels and traditional sampling methods may be inadequate to effectively capture that diversity (Phelps et al., 2015; Wahl et al., 2011). The addition of eDNA based methods to sample fish and other taxa in wetlands could greatly improve our ability to monitor the communities present in these habitats.

Our primary objectives were to verify the effectiveness of eDNA metabarcoding in freshwater wetlands as well as evaluate fish community composition in these habitats. More specifically, we sought to determine the extent that eDNA metabarcoding could discriminate unique fish communities in wetland complexes. Additionally, we wanted to investigate how community composition and detection ability changed between seasons as well as the amount of sampling effort required to effectively capture fish diversity in these habitats. Lastly, we sought to compare the fish communities detected with eDNA metabarcoding to the historical records of the fish species that are known to occur within our sample area.

2. METHODS

eDNA sampling was conducted at 28 sites in May 2022 within Black Island Conservation Area, a wetland complex along the Mississippi River in Missouri's Bootheel (Figure 2.1). A second sampling event took place in October 2022 where 19 of the initial sites were resampled. Sample sites were allocated among four habitat types, broadly defined as a bayou (oxbow lake), shallow lake, slough, or ditch. The Bayous are represented by Wolf, Hosner, and Samples Bayous which make up a chain of oxbow lakes that are connected by narrow channels. Robinson Lake and the ditch are both man-made features that were constructed to provide wildlife habitat and direct water across the landscape. An earthen dam and spill way was constructed on a small stream to retain water in Robinson Lake. The ditch was made by digging a relatively straight and narrow channel out from the slough. Five sites within Robinson Lake were not revisited during the fall due to difficulty of access or because they were dry. All three ditch sites and one slough site were not sampled a second time as they were dry during the fall sampling period. Water quality parameters including standard conductivity, dissolved oxygen, and temperature were logged at each sample location using a YSI probe. A secchi disk was used to measure water clarity and the depth at each sample site was recorded.

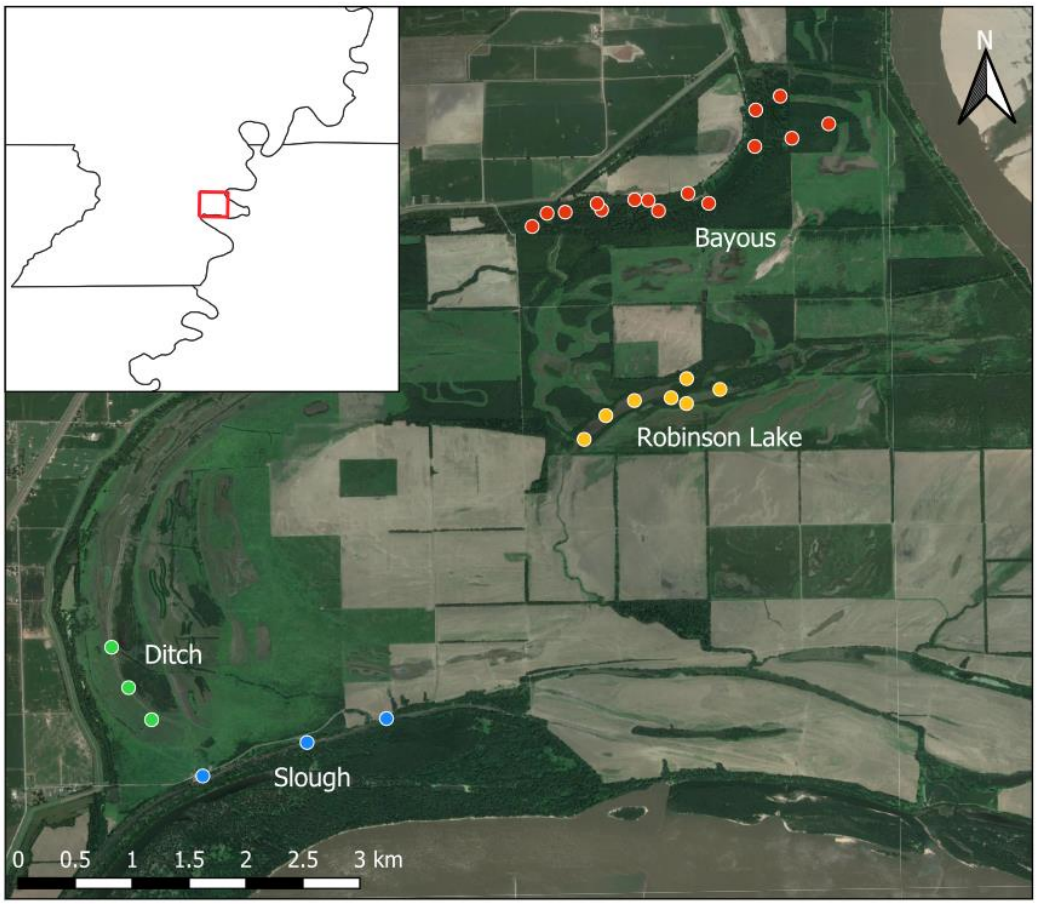

Figure 2.1 Locations of the 28 sample sites at Black Island Conservation Area. A total of 15 sites were sampled in the bayous (red), 7 sites were sampled in Robinson Lake (yellow), and 3 sites were sampled in both the ditch (green) and slough (blue).

2.1. SAMPLE COLLECTION

Three 500 ml water samples were collected at each site in sterile bottles and immediately pressure filtered through enclosed 0.45 µm polyvinylidene fluoride (Millipore Sigma) filters using clean 50 ml leur-lock syringes until the volume reached 500 ml or the filter clogged. The mean volume of water filtered was 275 ml with a range of 15- 500 ml. Field negatives consisting of 500 ml of deionized (DI) water were filtered alongside field samples to monitor potential field contamination. Following filtration, the

filter housing was flooded with 95% molecular grade ethanol, sealed with parafilm, and stored on ice to preserve eDNA prior to extraction (Williams et al., 2016). Disposable gloves were worn during filtration and changed between sites to minimize the risk of contamination.

2.2. DNA EXTRACTION AND PCR AMPLIFICATION

Extraction of eDNA from the filters was conducted within 7 days of sample collection using a modified protocol of the Qiagen Blood and Tissue Kit (Qiagen) for enclosed filters outlined in Spens et al. (2017). To minimize cross contamination, extractions were carried out in a dedicated clean lab and all work surfaces and equipment were decontaminated using bleach between extractions (Goldberg et al., 2016). Additionally, a lab negative consisting of DI water was included in each round of extractions to monitor cross contamination during this step. Following eDNA extraction, PCR inhibitors were removed using a OneStep PCR Inhibitor removal Kit (Zymo).

Extracted eDNA was PCR amplified using two universal fish mitogenome primer sets: Mifish 12s primers (Miya et al., 2015) and the 16s rRNA primers Chord 16s F TagA and Chord 16s R short primers (Deagle, Kirkwood, & Jarman, 2009). Both primer sets were 5’ tagged with sequences to provide binding sites for the Illumina sequencing primers. eDNA samples were amplified using AmpliTaq Gold 360 DNA polymerase (Thermo Fisher Scientific) in 50 µL total volume reactions divided into six independent reactions in order to minimize amplification bias between PCR replicates (Ruppert et al., 2019). Cycling conditions for Mifish were denaturation at 95° C for 5 min, then 33 cycles

of 95° C for 20 sec, 65° C for 20 sec, 72° C for 1 min, then a final extension step of 72° C for 5 min, and a final hold of 4° C. Cycling conditions for Chord 16s were denaturation at 95° C for 5 min, then 45 cycles of 95° C for 25 sec, 55° C for 30 sec, 72° C for 1 min, then a final extension step of 72° C for 6 min, and a final hold of 4° C. A PCR negative was included in each PCR run. A positive control consisting of DNA from exotic fishes was included periodically. The replicate PCR products were then merged and verified using gel electrophoresis. Amplification of extraction negative controls and PCR negative controls was not observed, so they were not sequenced. Field negatives and 2 positive controls were included in the following steps and were treated in the same manner as field samples. PCR cleanup and concentration normalization was carried out using the SequalPrep Normalization Plate Kit (Thermo Fisher Scientific). Samples were then sequenced at the University of Missouri Genomics Technology Core using the Illumina MiSeq v2 platform with 150 bp paired- end reads.

2.3. BIOINFORMATICS

Raw sequencing reads were processed using the Barque metabarcoding analysis pipeline, which included steps to remove primer sequences, merge forward and reverse reads, and filter chimeric sequences (Mathon et al., 2021). Taxonomic identification was performed using a reference database consisting of published sequences in GenBank. This database was curated to include only species known to occur in the lower-Mississippi drainage basin to filter out erroneous identifications (Appendix). Sequences were identified using a 97% sequence similarity as a threshold for species assignment and

a 95% sequence similarity to assign sequences to a genus level (Deiner et al., 2017). Reads detected in the negative controls were subtracted from each field sample to reduce the risk of false positive detections (Sakata et al., 2021). Species with a read count less than 5 in a given sample were discarded from that sample. All samples were then rarefied to 40,000 reads to normalize library sizes between samples and primer sets (Cameron et al., 2021). Read counts from both primer sets were then merged and reduced to presence/absence data for statistical analysis.

2.4. STATISTICAL ANALYSIS

Statistical analyses were carried out using R version 4.2.2 and the *vegan* community ecology package (Oksanen et al., 2019; R Development Core Team, 2008). Differences in community compositions between habitat types and sampling seasons were visualized using nonmetric multidimensional scaling (NMDS). The community dissimilarity was calculated using incidence- based Jaccard indices. Community differences between habitat types were evaluated using a permutational multivariate analysis of variance (PERMANOVA) using the *adonis2* function in the *vegan* R package. If the analysis returned significant results, a pairwise adonis test was used using the pairwiseAdonis package and similarity percentage analysis (SIMPER) was then utilized to identify which species were most responsible for the differences between habitats (Martinez Arbizu, 2020). Shifts in community structure between seasons in each habitat were evaluated using an analysis of similarities (ANOSIM). An analysis of variance (ANOVA) was used to determine if there were significant differences in the number of

species detected per sample in each habitat and season. A Tukey-Kramer Honest Significant Difference (HSD) post hoc test was then used to determine which groups were significantly different from one another. Species accumulation curves were generated for each sampling event using the specaccum function.

3. RESULTS

A total of 54 species were detected in the spring between both markers representing 37 genera and 17 families. Of these, 28 species were detected ubiquitously across all habitat types. Robinson Lake had the greatest diversity with 44 species followed closely by the bayous with 43 (Figure 3.2). A total of 37 species were detected in the slough and 31 were detected in the ditch. Centrarchidae was the most represented family, making up 23% of the total species detected, followed by Ictaluridae and Percidae (both 11%) and Leuciscidae (9%). The most commonly detected species (detected in at least 90% of samples) were *Lepomis macrochirus, Cyprinus carpio, Lepisosteus platostomus, Hypophthalmichthys molitrix, Ctenopharyngodon idella,* and *Hypophthalmichthys nobilis*. Negative controls showed minimal read counts and subtracting them from field samples resulted in the loss of *C. Idella* from one sample. Removing species with a low read count resulted in the loss of *Ictalurus furcatus* from the single bayou sample it was detected in. No species included in the positive controls were detected in any field samples.

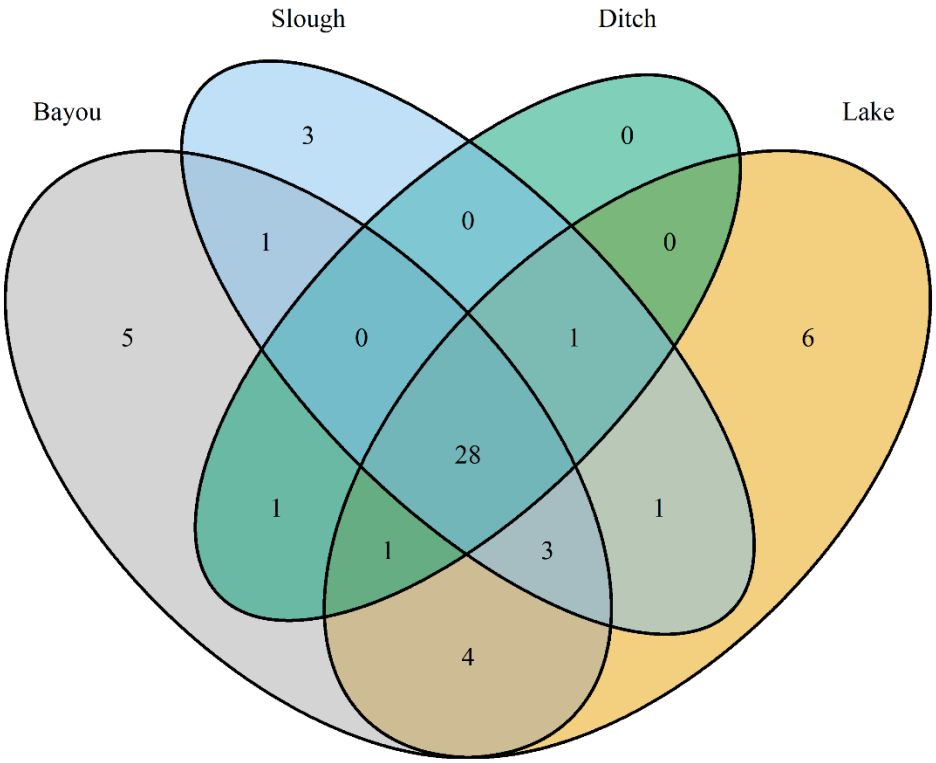

Figure 3.1 Venn diagram of the number of species detected in each habitat type. Overlapping regions represent species that were detected in two or more habitat types.

3.1. WATER QUALITY PARAMETERS

The bayous showed the most stability in water quality parameters between seasons. Water clarity was also the greatest in these habitats with about a meter of visibility during both sampling events (Table 3.1). The standard conductivity of the bayous was also the lowest of the four habitats and remained around 300 µS/cm between seasons. Both the average and maximum recorded depth were greater in the bayous than all other habitats. The water clarity in the lake, slough, and ditch were considerably lower than the bayous with half a meter of visibility during spring sampling. Conductivity was also much higher

in these habitats ranging from 400- 500 µS/cm during the spring. In the fall, water clarity in the lake and slough dropped to 0.2 m and conductivity fell by about 120 µS/cm. Changes in temperature were also much more apparent in these habitats compared to the bayous as shallow waters lose heat more quickly than deep waters.

Table 3.1 Summary of water quality data collected at each sampling location. The mean and standard deviation for each parameter is displayed. The max depth represents the deepest point at which a water sample was collected.

Habitat	Season	Secchi Depth (m)	Std. Conductivity (µS/cm)	DO (mg/L)	Temperature (°C)	Max Depth (m)	Volume Filtered (mL)
Bayou	Spring	1.0 (0.3)	290.4 (25.8)	6.9 (1.23)	25.2 (1.4)	7.92	305.4 (89.3)
	Fall	0.9 (0.3)	306.9 (5.2)	5.6 (0.9)	21.8 (3)	6.71	353.3 (22)
Lake	Spring	0.5 (0.0)	464.8 (16.3)	5.5 (1.49)	27.7 (1.6)	1.22	316.3 (50.5)
	Fall	0.2 (0.0)	338.7 (0.9)	5.9 (0.1)	15.5 (0.2)	0.56	15 (0.0)
Slough	Spring	0.5 (0.0)	484.6 (49.9)	7.7 (1.17)	26.3 (0.9)	1.37	303.1 (40.3)
	Fall	0.2 (0.0)	361.4 (2)	6.6 (0.8)	16.1 (0.2)	0.61	20 (0.0)
Ditch	Spring	0.5 (0.1)	405.7 (3.3)	5.8 (0.39)	24.2 (0.3)	1.67	273.8 (15.1)

3.2. COMMUNITY COMPOSITION ACROSS HABITATS

The NMDS ordination showed that samples from the same habitat largely cluster together with some overlap between the four habitat types (Figure 3.2). PERMANOVA analysis with habitat type, secchi depth, depth, and standard conductivity as environmental factors was performed on the spring community data. Differences in the detected fish communities were dependent mostly on habitat type ($R^2 = 0.415$, $p = 0.001$), but also on secchi depth ($R^2 = 0.086$, $p = 0.001$) and depth ($R^2 = 0.022$, $p = 0.009$). The subsequent pairwise adonis test showed that the community composition in all four habitats were significantly different from one another ($p < 0.05$). SIMPER

analysis showed several key species that account for the differences detected between habitats (Table 3.2). *Morone chrysops* consistently ranked as the first or second largest contributor to community differences between the ditch and slough versus the bayous and lake. *Pylodictis olivaris* was commonly detected in all habitats except Robinson Lake. The two silverside species detected in this survey were also significant contributors to the differences between the bayous and the other three habitats. *Labidesthes sicculus* was commonly detected in the bayous whereas *Menidia beryllina* was more frequently detected in the lake, ditch, and slough. Despite direct connectivity between the ditch and slough, *E. chlorosomum* and *O. emiliae* were only detected from the slough and were responsible for 23.6% of the dissimilarity between these two habitats.

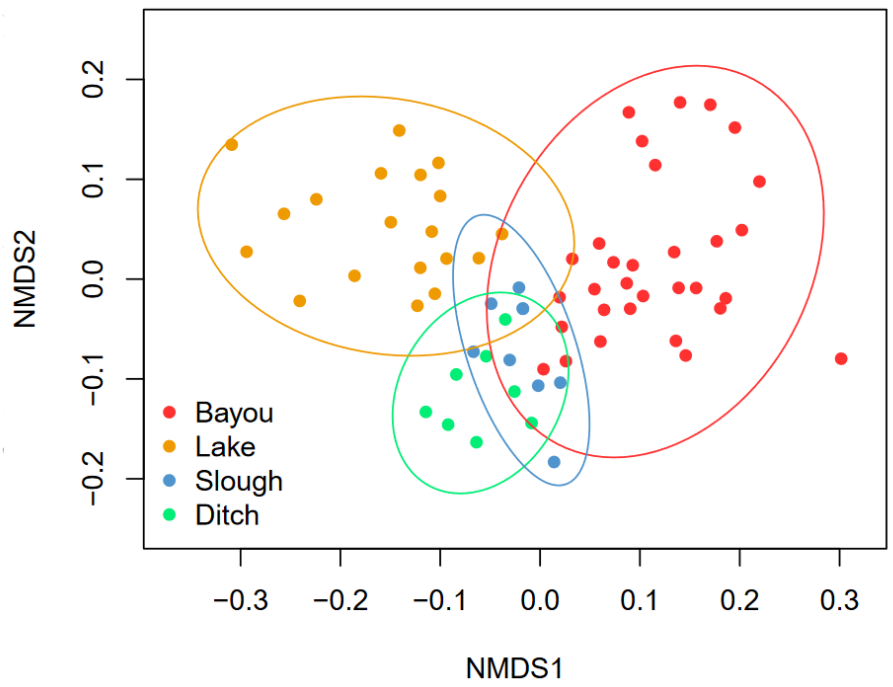

Figure 3.2 Nonmetric multidimensional scaling (NMDS) ordination of fish community composition. Points show the community detected between both markers of all spring eDNA samples grouped by habitat type. The ellipses show the 95% confidence level based on the centroid calculated for each habitat type.

Table 3.2 Results of SIMPER analysis on spring fish community data. Comparisons between the Bayous, Lake, and Slough are displayed. The contribution percentage of the species that contributed the most to the dissimilarity between pairs of habitats are listed.

Contrast	Species	Avg. Abund in Group 1	Avg. Abund in Group 2	Contrib. %	Cumulative %	P-Value
Bayou vs Lake	*Aphredoderus sayanus*	0.00	0.95	6.7	6.7	0.001
	Pylodictis olivaris	0.97	0.11	6.1	12.8	0.001
	Micropterus punctulatus	0.71	0.00	5	17.8	0.001
	Menidia beryllina	0.03	0.74	5	22.8	0.001
	Labidesthes sicculus	0.71	0.00	4.9	27.7	0.001
	Etheostoma chlorosomum	0.74	0.26	4.3	32.0	0.001
	Fundulus olivaceus	0.61	0.00	4.3	36.3	0.001
	Gambusia affinis	0.48	1.00	3.7	40.0	0.002
	Etheostoma asprigene	0.77	0.47	3.7	43.7	0.006
	Lepomis symmetricus	0.16	0.53	3.5	47.2	0.001
	Lepisosteus osseus	0.58	0.47	3.6	50.8	0.079
Lake vs Slough	*Pylodictis olivaris*	0.11	1.00	8	8.0	0.001
	Morone chrysops	0.11	0.89	7.3	15.3	0.001
	Etheostoma chlorosomum	0.26	1.00	6.7	22.0	0.002
	Menidia beryllina	0.74	0.22	5.6	27.6	0.005
	Etheostoma asprigene	0.47	1.00	4.8	32.4	0.039
	Lepisosteus osseus	0.47	0.89	4.7	37.1	0.167
	Lepomis symmetricus	0.53	0.00	4.6	41.7	0.026
	Opsopoeodus emiliae	0.58	0.78	4.2	45.9	0.95
	Lepomis cyanellus	0.89	0.56	4.1	50.0	0.029
Bayou vs Slough	*Morone chrysops*	0.10	0.89	7.5	7.5	0.001
	Micropterus punctulatus	0.71	0.00	6.5	14.0	0.001
	Labidesthes sicculus	0.71	0.00	6.5	20.5	0.001
	Aphredoderus sayanus	0.00	0.67	6.1	26.6	0.003
	Fundulus olivaceus	0.61	0.00	5.6	32.2	0.006
	Gambusia affinis	0.48	1.00	4.8	37.0	0.027
	Pomoxis annularis	0.55	0.89	4.4	41.4	0.509
	Opsopoeodus emiliae	0.58	0.78	4.2	45.6	0.988
	Dorosoma petenense	0.35	0.33	4.2	49.8	0.786
	Lepomis cyanellus	1.00	0.56	4.1	53.9	0.031

NMDS ordination of the three bayous showed that Wolf Bayou had the most variation in community composition between samples (Figure 3.3). PERMANOVA analysis with water body, secchi depth, depth, and standard conductivity as environmental factors was performed on the spring community data from the bayous. Water body was identified as a significant factor contributing to the differences among bayous (R^2 = 0.158, p = 0.001). Secchi depth (R^2 = 0.213, p = 0.001) and depth (R^2 = 0.063, p = 0.009) were also determined to be significant factors while standard conductivity was not. The subsequent pairwise adonis test showed that community composition was only significantly different between Hosner Bayou and Samples Bayou (R^2 = 0.259, p = 0.001).

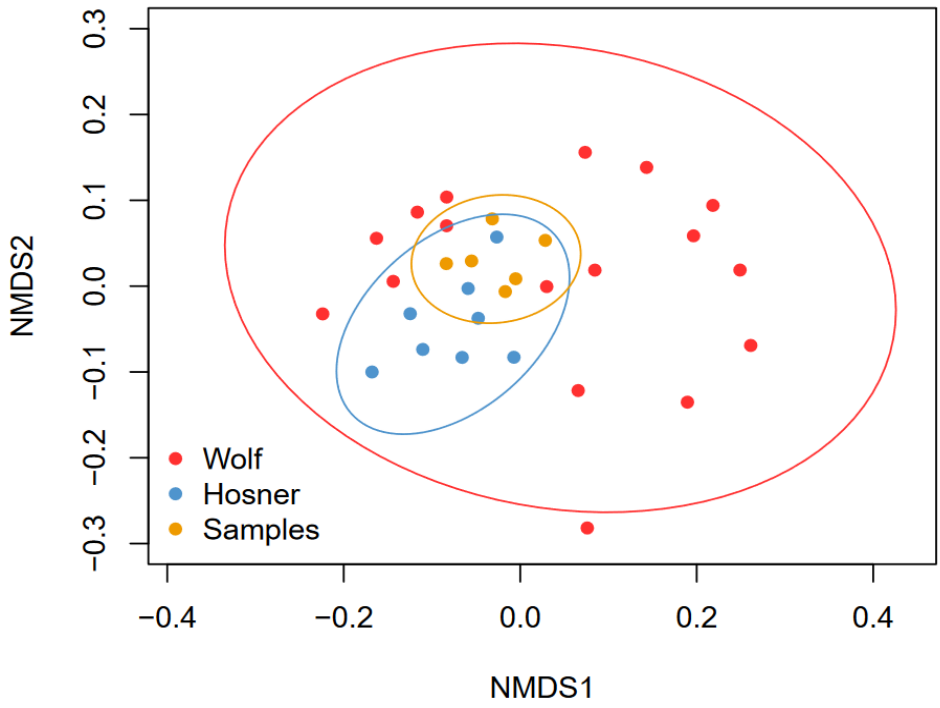

Figure 3.3 NMDS ordination of fish community composition detected between both markers of all spring eDNA samples from each of the three bayous. The ellipses show the 95% confidence level based on the centroid calculated for each bayou.

3.3. SEASONAL eDNA VARIATION

In total, 19 sites were sampled in both the spring and fall. None of the ditch sites still held water during the fall so they were not resampled. The ordination of the detected communities in each habitat was visualized with a NMDS of Jaccard dissimilarities (Figure 3.4). ANOSIM analysis suggested that there was a significant difference in the detected fish communities between seasons for Robinson Lake and the slough, but not for the bayous. The proportion of variance that can be attributed to seasonal effects was different between Robinson Lake and the slough.

The bayous showed no significant variation between seasons ($R = 0.015$, $p = 0.24$) and only three species (*Lepomis microlophus, Lythrurus fumeus,* and *Morone mississippiensis)* were detected in the spring but not in the fall. *Aphredoderus sayanus* was detected in the fall in two samples, but not at all in the spring. Robinson Lake showed moderate variation between seasons ($R = 0.5111$, $p = 0.004$). Twelve species were detected there in the spring that were absent from the fall samples. The slough sites showed the highest variation in fish community composition between seasons ($R = 0.9851$, $p = 0.001$). Eighteen species were missing from the fall samples that were detected in the spring.

SIMPER analysis showed that of the twelve species missing from the fall Robinson Lake samples, only *Micropterus salmoides* made a significant contribution to the dissimilarity between seasons (Table 3.3). All but one species, *Lepisosteus osseus*, were detected in a greater proportion of spring samples compared to the fall. In the slough, eleven of the eighteen species missing from the fall samples made significant

contributions to the dissimilarity between seasons. All species detected in the slough were present in a greater proportion of spring samples compared to the fall.

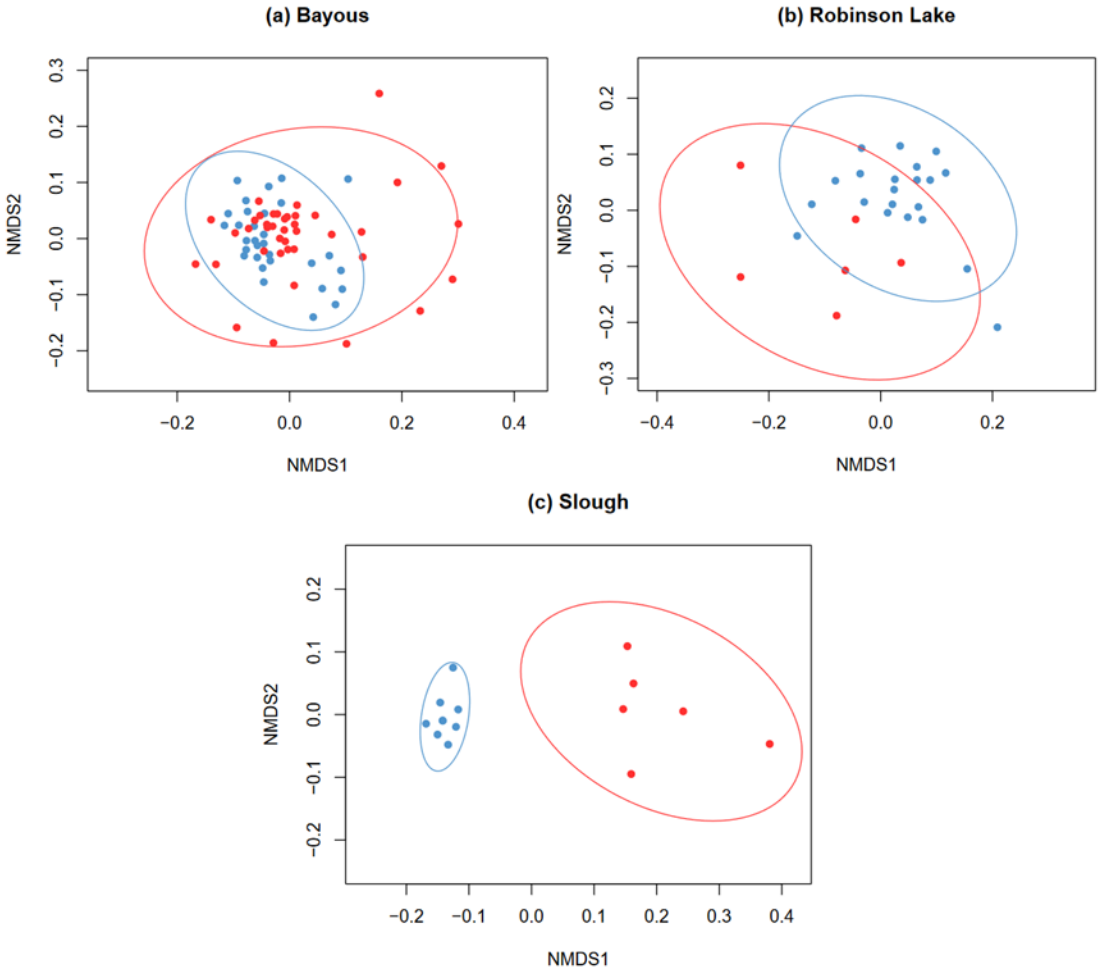

Figure 3.4 NMDS ordination comparing spring (blue) and fall (red) eDNA samples. The (a) Bayous, (b) Robinson Lake, and (c) Slough are shown. The ellipses show the 95% confidence level based on the centroid of each sampling event.

Table 3.3 Results of SIMPER analysis on spring fish community data. The contribution percentage of the species that contributed the most to the dissimilarity between pairs of habitats are listed

Contrast	Species	Avg. Abund in Group 1	Avg. Abund in Group 2	Contrib. %	Cumulative %	P-Value
Lake: Spring vs Fall	*Aphredoderus sayanus*	0.86	0.17	6.2	6.2	0.001
	Menidia beryllina	0.76	0.17	5.6	11.8	0.002
	Fundulus notatus	0.95	0.33	5.4	17.2	0.006
	Micropterus salmoides	0.62	0.00	5.1	22.3	0.002
	Lepisosteus osseus	0.43	1.00	4.9	27.2	0.007
	Opsopoeodus emiliae	0.57	0.17	4.5	31.7	0.046
	Pomoxis annularis	0.90	0.50	4.3	36.0	0.013
	Lepomis symmetricus	0.52	0.17	4.3	40.3	0.101
	Ictalurus punctatus	0.76	0.50	4.3	44.6	0.053
	Lepomis megalotis	0.57	0.50	4.2	48.8	0.196
	Lepomis marginatus	0.67	0.67	3.8	52.6	0.299
Slough: Spring vs Fall	*Micropterus salmoides*	1.00	0.00	6.0	6.0	0.001
	Dorosoma cepedianum	1.00	0.00	6.1	12.1	0.001
	Pylodictis olivaris	1.00	0.00	6.0	18.1	0.001
	Etheostoma asprigene	1.00	0.00	6.1	24.2	0.001
	Mylopharyngodon piceus	1.00	0.00	6.0	30.2	0.001
	Morone chrysops	0.89	0.00	5.4	35.6	0.001
	Ctenopharyngodon idella	0.89	0.00	5.4	41.0	0.001
	Lepomis macrochirus	1.00	0.17	5.1	46.1	0.002
	Etheostoma chlorosomum	1.00	0.17	5.0	51.1	0.002

3.4. SAMPLING EFFORT

By comparing the number of species detected in all samples, we found that there was a significant interaction effect between habitat and season on the number of species detected per sample (ANOVA, $p < 0.001$) (Figure 3.5). Habitat type did not have a significant effect on the number of species detected per sample (ANOVA, $p = 0.291$) while season was a significant factor (ANOVA, $p = 0.034$). A Tukey-Kramer HSD post hoc test showed that the fall slough samples detected significantly fewer species per

sample than all others except the fall Robinson Lake samples. The fall Robinson Lake samples detected significantly fewer species than only the fall Samples Bayou and spring slough samples.

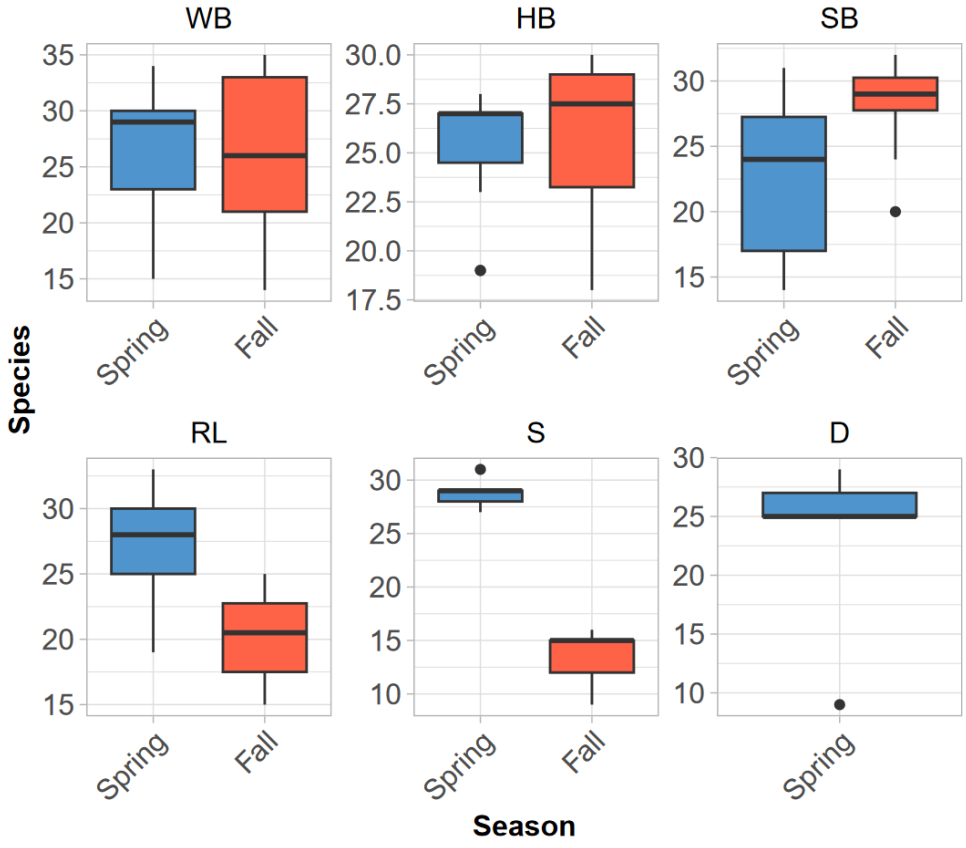

Figure 3.5 Boxplots of the number of species detected per sample from each water body sampled.

The species accumulation curves from the three bayous appear to approach the asymptote after about 4 water samples (approximately 1.1 L of water) (Figure 3.6). There was no substantial difference in the number of samples necessary to capture 95% of the species inferred to be present between spring and fall in these sites. The accumulation

curve for the lake showed that about 12 water samples (approximately 3.3 L of water) were necessary to capture 95% of the detected species in spring. The lake and slough sites both showed a decrease in the number of new species detected per sample in the fall compared to the spring. The accumulation curve for the fall lake samples did not appear to reach the asymptote after 6 samples.

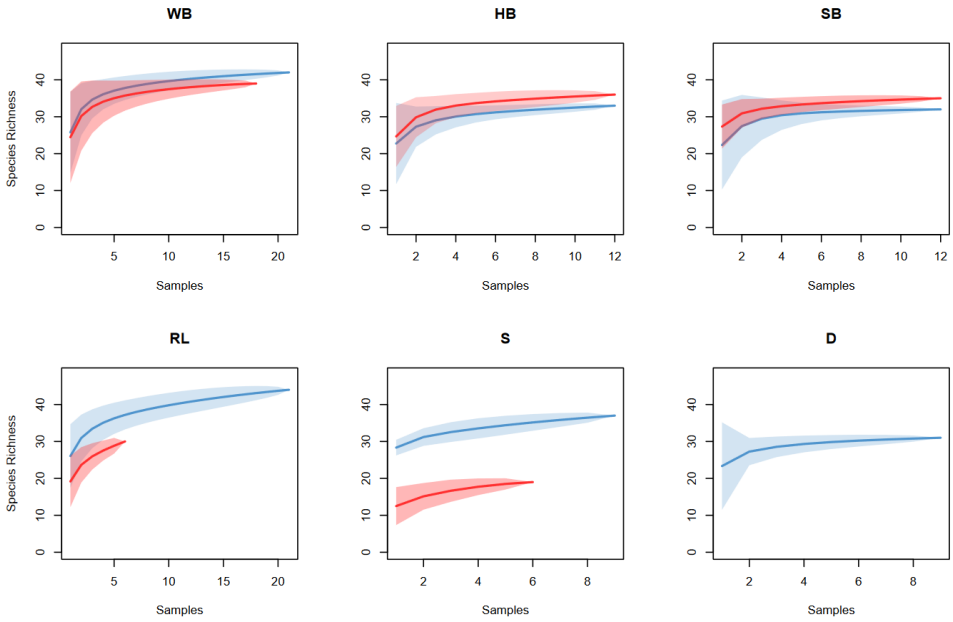

Figure 3.6 Species accumulation curves for each water body sampled. Spring samples are represented in blue and fall samples are represented in red.

3.5. COMPARISON TO TRADITIONAL METHODS

Black Island Conservation Area has been sampled five times by MDC between 1940 and 2022. Various traditional sampling methods have been used including fyke nets, electroshocking, and seining and a total of 44 fish species have been observed there. We detected 34 of the species with eDNA metabarcode sampling that were known to

occur there as well as 20 species that have not been collected there previously (Figure 3.7). Centrarchids made up 20% of new detections followed by Leuciscids with 15%. Two invasive Asian Carp species (*H. nobilis* and *M. piceus*) were also detected only with eDNA metabarcoding and have not been captured there previously. Two *Carpiodes* and three *Ictiobus* species were detected with traditional methods that were only able to be identified to a genus level using eDNA metabarcoding.

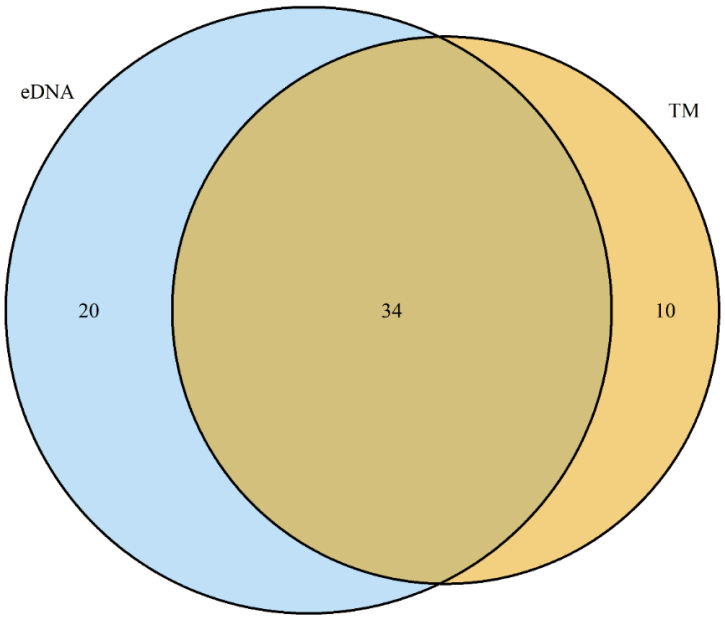

Figure 3.7 Comparison of the number of species detected by eDNA metabarcoding and species captured using traditional sampling methods (TM).

3.6. NON-TARGET TAXA

Despite the universal primers used in this study being designed to amplify fish DNA, they are also effective on a number of other vertebrate taxa (Kumar et al., 2022). A total of 24 non-fish species were detected in our samples representing 21 genera and 18

families (Table 3.4). Mammalia was the most represented class with 9 species detected followed by Amphibia with 7 species, Reptilia with 5 species, and Aves with 3 species. No snake species were detected with eDNA despite frequent visual observations of them during sample collection.

Table 3.4 Non-fish species detected by eDNA among all spring and fall samples.

Class	Family	Species	# of Samples Detected
Amphibia	Bufonidae	*Anaxyrus americanus*	10
	Ranidae	*Lithobates sphenocephalus*	9
	Bufonidae	*Anaxyrus fowleri*	6
	Hylidae	*Dryophytes cinereus*	2
	Ranidae	*Lithobates clamitans*	2
	Hylidae	*Acris blanchardi*	1
	Ranidae	*Lithobates catesbeianus*	1
Aves	Ardeidae	*Ardea cinerea*	15
	Phalacrocoracidae	*Phalacrocorax auritus*	10
	Icteridae	*Quiscalus quiscula*	8
Mammalia	Hominidae	*Homo sapiens*	123
	Castoridae	*Castor canadensis*	68
	Bovidae	*Bos taurus*	44
	Procyonidae	*Procyon lotor*	24
	Felidae	*Felis catus*	22
	Cricetidae	*Peromyscus leucopus*	19
	Cervidae	*Odocoileus virginianus*	17
	Cricetidae	*Neotoma floridana*	12
	Suidae	*Sus scrofa*	11
Reptilia	Emydidae	*Trachemys scripta*	69
	Emydidae	*Pseudemys concinna*	59
	Emydidae	*Graptemys ouachitensis*	41
	Trionychidae	*Apalone spinifera*	21
	Chelydridae	*Macrochelys temminckii*	7

4. DISCUSSION

Although the use of eDNA metabarcoding to assess fish communities has significantly increased since its inception, few studies have applied this technique to freshwater wetlands. In this study, we demonstrate the efficacy of eDNA metabarcoding in these environments with few modifications to existing protocols. An impressive diversity of species was detected using eDNA metabarcoding among all sites. Furthermore, our data shows that distinct habitats within a wetland complex display unique eDNA signatures. In particular, Robinson Lake held the greatest diversity and the most unique species which highlights the value of artificially created water bodies for restoring habitat and promoting biodiversity in wetlands (Thiere et al., 2009). Although the majority of all species detected are native to the region, several invasive species were among the most commonly detected across all habitats. Three of the four invasive Asian carp were present in at least 90% of the samples collected and all four were detected in each habitat.

4.1. SEASONAL COMMUNITY COMPARISONS

The bayous were the most stable habitat throughout both sampling events and showed the least change in water clarity, standard conductivity, and temperature. The amount of water that could be filtered before clogging was also similar between seasons. Although there was a significant difference in the detected fish communities between seasons, only four species were detected in one season from these habitats. It seems most

likely that these species were not detected during one of our sampling events due to their rarity as three of them only appeared in one sample each. The remaining species was detected in two samples from the bayous. Fish can only move in or out of the bayous during flooding events and with the absence of any significant flooding between sampling events to facilitate movement in or out of these habitats, as was the case during our study, fish community composition will likely remain relatively stable (Appendix).

The available habitat and environmental qualities of Robinson Lake and the slough both changed significantly between seasons. Water levels had dropped, isolating these habitats from each other and the Mississippi River. Turbidity was also much greater during the fall than in spring. A number of species were detected in these habitats during the spring but not in the fall. There are three plausible explanations for the disappearance of any of these species from our fall samples. The first is that a given species was too rare, and their DNA was not recovered during the fall despite the species being present. Alternatively, the species was truly absent and had moved out of these habitats before they became disconnected from the Mississippi River. Lastly, there exists the possibility that the species remained after the habitats became disconnected but were not able to survive and died out before the fall samples were collected.

In Robinson Lake, there were twelve species that were only detected in the spring. Of these, five were detected in only a single spring sample each and may have been missed in the fall due to their rarity and our smaller sample size. Three *Etheostoma* species were missing from the fall samples where only *E. gracile* was detected. Detections of these species may have been inflated during the spring due to spawning activity (Gehri et al., 2021). *Micropterus salmodies* (largemouth bass) and *Notemigonius*

crysoleucas were both commonly detected in Robinson Lake during the spring but were absent from fall samples. While the fall slough samples detected the fewest species per sample, the species accumulation curve suggests that few, if any, additional species would be detected with a larger sample size. In the slough, 18 species were detected in spring that were absent in the fall. Five of these species were detected in a single spring sample each. Eight species, *C. idella, Dorosoma cepedianum, E. asprigene, L. gulosus, M. salmoides, M. chrysops, Mylopharyngodon piceus,* and *P. olivaris* were frequently detected (present in >75% of samples) in spring.

Although significant differences in fish community composition were detected between seasons, caution must be used when interpreting these results, particularly in Robinson Lake. The species accumulation curve for the fall in this habitat did not reach the asymptote which suggests that species diversity at that time was not completely captured by the six water samples collected (Bessey et al., 2020). Water levels dropped and turbidity increased between sampling events which lowered the amount of water that could be filtered during the fall. With an increased sample size during the fall in this habitat, it is likely that more species would have been detected which could reduce the dissimilarity between seasons. Additionally, using filters with a larger pore size (≥ 2 μm) in more turbid habitats could increase the volume of water samples while still effectively capturing diversity as eDNA is largely bound to suspended particulate matter (Barnes et al., 2021; Díaz et al., 2020).

Many species of fish, particularly nest-builders such as *Lepomis* spp., are known to take advantage of backwater habitats of large rivers for spawning and nursery areas (Slipke et al., 2005). Others considered to be main-channel species still make use of

backwaters during their juvenile stages and shift towards the main-channel as they develop (Sheaffer & Nickum, 1986). Largemouth bass, for instance, are known to move into shallow backwater habitats during the spring and seek out deeper waters to overwinter (Karchesky & Bennett, 2011). As the year progressed and water levels fell, Robinson Lake and the slough were likely not suitable habitats for largemouth bass, and they evacuated before they became disconnected from the Mississippi River. Both the total amount and varieties of habitat available were decreased due to receding water in the fall. Whereas the water extended into the tree line during the spring, it receded away from the shore during the fall reducing the amount of woody structure and vegetation available to fish. While water levels had fallen enough to disconnect both Robinson Lake and the Slough from each other and the Mississippi River during our fall sampling period, this is not the case every year. In some years past, water levels remained high enough to maintain connectivity between these habitats throughout the fall and winter. Seasonal shifts in the fish communities detected by eDNA metabarcoding in these habitats may vary depending on whether these connections are maintained or broken throughout the year (Fullerton et al., 2010).

4.2. RARE AND INVASIVE SPECIES DETECTIONS

Using eDNA metabarcoding, we were able to detect 77% of the fish species historically known to occur within our study site. Additionally, 20 species that had not been observed there previously were detected using eDNA. These included five species of conservation concern (*Anguilla rostrata, L. marginatus, L. symmetricus, Alosa*

chrysochloris, and *Hybognathus nuchalis*) and two invasive species (*H. nobilis* and *M. piceus*). These findings contribute to the growing body of evidence that eDNA metabarcoding is well suited for the detection of rare or cryptic species that traditional sampling methods may not be well suited for (Bylemans et al., 2019). Expanding the use of eDNA metabarcoding across the region may be useful in refining our knowledge of the distribution of these and other species of conservation concern as well as determining potential habitat preferences.

Our results indicated that eDNA metabarcoding is a useful tool for detecting invasive species as demonstrated in previous studies (Pukk et al., 2021). All four invasive Asian carp were detected ubiquitously across the four habitats sampled in this study, two of which did not have records within the study area previously. Although *Channa argus* was not detected, this species has been observed in southeastern Missouri and is well suited to habitats like those sampled in this survey (Resh et al., 2018). eDNA based sampling techniques may prove useful for monitoring the distribution of this species as it continues to spread through the Mississippi River basin. Monitoring the distribution of these and other invasive species will be critical to inform management efforts and conserve natural fish populations.

4.3. SAMPLING EFFORT

We found no significant difference in the number of species detected per sample and only a slight difference in community composition between seasons in the bayous. Water clarity and the volume of water filtered per sample in these habitats was similar

between seasons. While the number of species detected in the fall Robinson Lake samples was lower on average compared to the spring, the difference was not significant. The volume of water filtered per sample at this site was about 5% of the volume filtered per sample in spring due to increased turbidity. Despite this, an average of 20 species per sample were detected in the fall. This finding is consistent with previous studies which have found that eDNA yield is greater from the same volume of turbid water compared to clear water (Kumar et al., 2021). This is because eDNA in aquatic environments is most commonly bound to suspended particulate matter so a smaller volume of more turbid water can contain sufficient eDNA to capture species diversity (Díaz et al., 2020; Turner et al., 2014).

Similarly in the slough, the volume filtered in the fall was about 6% of the volume filtered in the spring. At these sites, however, there was a significant decrease in the number of species detected per sample in the fall. Nearly half of the species absent in the fall samples were commonly detected in the spring and we suspect that these species were truly absent from the site, which contributed to the decrease in the number of species detected per sample.

4.4. CHALLENGES AND LIMITATIONS

Despite many studies demonstrating the value of eDNA metabarcoding as a tool to assess fish communities, the technique does have some limitations (Ruppert et al., 2019). Current eDNA metabarcoding techniques do not provide any information about the age or size structure of a population (Pont et al., 2021). There were ten species that

had been captured at our study site using traditional methods that were not detected with eDNA metabarcoding. Five of these were because our markers were only able to identify the three *Ictiobus* and two *Carpiodes* species to the genus level. *H. hayi* and *Notropis shumardi* have been captured there using traditional methods, but we did not have reference sequences for these species and did not detect them. *Moxostoma macrolepidotum, Centrarchus macropterus,* and *Ameiurus melas* have also been observed there in several past surveys but were not detected with eDNA despite having reference sequences for these species.

Despite using both a 12s and 16s marker to increase taxonomic resolution, we were unable to distinguish the three *Ictiobus* and two *Carpiodes* species from one another. Other mitochondrial DNA regions may be more suited to distinguishing these particular species from one another if they are of interest in future studies. These genera and several others detected in this survey are capable of producing hybrid offspring (Avise & Saunders, 1984; Bart et al., 2010). However, maternally inherited mitochondrial markers like those used in this study are typically not capable of distinguishing hybrids (Hallam et al., 2021). Mitochondrial DNA is still more suitable for eDNA based techniques than nuclear DNA because it is present in higher copy numbers per cell and thus has a higher chance of being captured in an eDNA sample (Thomsen & Willerslev, 2015).

5. CONCLUSIONS

The application of eDNA metabarcoding to aquatic environments provides a quick and cost-effective method for the collection of information about fish community structure. We found evidence that eDNA metabarcoding is capable of distinguishing unique eDNA signals from distinct habitats within a wetland complex as well as identifying key species that contribute to the community dissimilarity between habitats. Seasonal community differences were most apparent in the slough and to a lesser extent in Robinson Lake. The bayous did not show any significant changes, likely because the Mississippi River did not flood the area between sampling events. Data collected in this study also identified new records for several species of conservation concern as well as invasive species that were previously unknown to occur at our study site. Expanding the use of eDNA metabarcoding may be particularly useful in monitoring the distribution of these species across their respective ranges. While traditional fish surveys still provide valuable information to inform conservation and management decisions, such as life history data and the confirmation of species presence, eDNA metabarcoding provides a useful complementary or alternative method for collecting fish community and distribution data. In future studies, the choice of whether to employ traditional methods, eDNA based methods, or both will depend on the objectives of the study.

APPENDIX

Table A.1. All sample IDs and locations. Fall sample locations are displayed only for sites that were collected at different coordinates.

Sample Site	Season	Latitude	Longitude
WB-1A	Spring	36.321179	-89.637457
WB-1B	Spring	36.32095	-89.637597
WB-1C	Spring	36.320836	-89.637292
WB-2A	Spring	36.322342	-89.635176
WB-2B	Spring	36.322336	-89.635078
WB-2C	Spring	36.322142	-89.634879
WB-3A	Spring	36.319357	-89.630186
WB-3B	Spring	36.319454	-89.629895
WB-3C	Spring	36.319522	-89.630612
WB-4A	Spring	36.31881	-89.633148
WB-4B	Spring	36.318446	-89.633685
WB-4C	Spring	36.318015	-89.633809
WB-5A	Spring	36.317826	-89.637024
WB-5B	Spring	36.317281	-89.637446
WB-5C	Spring	36.316688	-89.637884
WB-6A	Spring	36.311945	-89.641786
WB-6B	Spring	36.311807	-89.642043
WB-6C	Spring	36.311714	-89.642457
WB-7A	Spring	36.31116	-89.646963
WB-7B	Spring	36.311313	-89.647086
WB-7C	Spring	36.311234	-89.647117
HB-1A	Spring	36.312046	-89.653247
HB-1B	Spring	36.311971	-89.652949
HB-1C	Spring	36.312111	-89.652911
HB-2A	Spring	36.312289	-89.649599
HB-2B	Spring	36.312287	-89.649266
HB-2C	Spring	36.312285	-89.648752
HB-3A	Spring	36.31216	-89.648477
HB-3B	Spring	36.312205	-89.64795
HB-3C	Spring	36.312258	-89.647375
HB-4A	Spring	36.312668	-89.644377
HB-4B	Spring	36.312808	-89.644046
HB-4C	Spring	36.312681	-89.643804
SB-1A	Spring	36.311543	-89.652454
SB-1B	Spring	36.311347	-89.652519
SB-1C	Spring	36.311463	-89.652167
SB-2A	Spring	36.259901	-89.681857
SB-2B	Spring	36.259863	-89.682201
SB-2C	Spring	36.259646	-89.683161
SB-3A	Spring	36.256752	-89.692046
SB-3B	Spring	36.256772	-89.692441
SB-3C	Spring	36.256351	-89.693502
SB-4A	Spring	36.262064	-89.6744
SB-4B	Spring	36.262136	-89.673894
SB-4C	Spring	36.262049	-89.674581

Sample Site	Season	Latitude	Longitude
RL-1A	Spring	36.294736	-89.644501
RL-1B	Spring	36.294703	-89.644258
RL-1C	Spring	36.29465	-89.644632
RL-2A	Spring	36.293455	-89.640691
RL-2B	Spring	36.293627	-89.641267
RL-2C	Spring	36.293554	-89.64174
RL-3A	Spring	36.292496	-89.642985
RL-3B	Spring	36.292298	-89.644559
RL-3C	Spring	36.29242	-89.646322
RL-4A	Spring	36.292822	-89.645809
RL-4B	Spring	36.292891	-89.64605
RL-4C	Spring	36.293096	-89.646678
RL-5A	Spring	36.293101	-89.64845
RL-5B	Spring	36.292684	-89.649639
RL-5C	Spring	36.292293	-89.650576
RL-6A	Spring	36.29158	-89.651823
RL-6B	Spring	36.291453	-89.65228
RL-6C	Spring	36.291256	-89.652452
RL-7A	Spring	36.290194	-89.652656
RL-7B	Spring	36.289723	-89.653296
RL-7C	Spring	36.288979	-89.654613
S-1A	Spring	36.309698	-89.659006
S-1B	Spring	36.309649	-89.659252
S-1C	Spring	36.309845	-89.659331
S-2A	Spring	36.310953	-89.658384
S-2B	Spring	36.311109	-89.657855
S-2C	Spring	36.311205	-89.657449
S-3A	Spring	36.311121	-89.656153
S-3B	Spring	36.311191	-89.656081
S-3C	Spring	36.311271	-89.655596
D-1A	Spring	36.269499	-89.701136
D-1B	Spring	36.269204	-89.70065
D-1C	Spring	36.270093	-89.701107
D-2A	Spring	36.267165	-89.70054
D-2B	Spring	36.265521	-89.699544
D-2C	Spring	36.263933	-89.698275
D-3A	Spring	36.261915	-89.697785
D-3B	Spring	36.262354	-89.697353
D-3C	Spring	36.262603	-89.696931
RL-1A	Fall	36.290214	-89.654168
RL-1B	Fall	36.290294	-89.654008
RL-1C	Fall	36.290407	-89.653812
RL-2A	Fall	36.290658	-89.653743
RL-2B	Fall	36.290779	-89.653592
RL-2C	Fall	36.290866	-89.653397

Table A.2. All species that were included in our database for the identification of unique sequences.

Family	Species
Acipenseridae	*Acipenser fulvescens*
	Scaphirhynchus albus
	Scaphirhynchus platorynchus
Amiidae	*Amia calva*
Anguillidae	*Anguilla rostrata*
Aphredoderidae	*Aphredoderus sayanus*
Atherinopsidae	*Labidesthes sicculus*
	Menidia beryllina
Catostomidae	*Carpiodes carpio*
	Carpiodes cyprinus
	Cycleptus elongatus
	Erimyzon claviformis
	Erimyzon oblongus
	Erimyzon sucetta
	Ictiobus bubalus
	Ictiobus cyprinellus
	Ictiobus niger
	Minytrema melanops
	Moxostoma anisurum
	Moxostoma carinatum
Centrarchidae	*Centrarchus macropterus*
	Lepomis cyanellus
	Lepomis gulosus
	Lepomis humilis
	Lepomis macrochirus
	Lepomis marginatus
	Lepomis megalotis
	Lepomis microlophus
	Lepomis miniatus
	Lepomis symmetricus
	Micropterus punctulatus
	Micropterus salmoides
	Pomoxis annularis
	Pomoxis nigromaculatus
Clupeidae	*Alosa alabamae*
	Alosa chrysochloris
	Dorosoma cepedianum
	Dorosoma petenense
Cyprinidae	*Cyprinus carpio*
Esocidae	*Esox americanus*
	Esox niger
Fundulidae	*Fundulus chrysotus*
	Fundulus dispar
	Fundulus notatus
	Fundulus olivaceus
Hiodontidae	*Hiodon alosoides*
	Hiodon tergisus
Ictaluridae	*Ameiurus melas*
	Ameiurus natalis
	Ameiurus nebulosus
	Ictalurus furcatus
	Ictalurus punctatus
	Noturus flavus
	Noturus gyrinus
	Noturus nocturnus
	Pylodictis olivaris
Lepisosteidae	*Atractosteus spatula*
	Lepisosteus oculatus
	Lepisosteus osseus
	Lepisosteus platostomus

Family	Species
Leuciscidae	*Campostoma anomalum*
	Cyprinella lutrensis
	Cyprinella venusta
	Cyprinella whipplei
	Hybognathus nuchalis
	Hybognathus placitus
	Hybopsis amnis
	Lythrurus fumeus
	Lythrurus umbratilis
	Macrhybopsis gelida
	Macrhybopsis hyostoma
	Macrhybopsis meeki
	Macrhybopsis storeriana
	Notemigonus crysoleucas
	Notropis atherinoides
	Notropis blennius
	Notropis dorsalis
	Notropis hudsonius
	Notropis maculatus
	Notropis nubilus
	Notropis stramineus
	Notropis texanus
	Notropis volucellus
	Notropis wickliffi
	Noturus eleutherus
	Opsopoeodus emiliae
	Phenacobius mirabilis
	Pimephales notatus
	Pimephales promelas
	Pimephales vigilax
	Platygobio gracilis
	Semotilus atromaculatus
Moronidae	*Morone chrysops*
	Morone mississippiensis
Mugilidae	*Mugil cephalus*
Percidae	*Elassoma zonatum*
	Etheostoma asprigene
	Etheostoma caeruleum
	Etheostoma chlorosomum
	Etheostoma fusiforme
	Etheostoma gracile
	Etheostoma histrio
	Etheostoma proeliare
	Percina caprodes
	Percina sciera
	Percina shumardi
	Percina vigil
	Sander canadensis
	Sander vitreus
Petromyzontidae	*Ichthyomyzon castaneus*
Poecilliidae	*Gambusia affinis*
Polyodontidae	*Polyodon spathula*
Sciaenidae	*Aplodinotus grunniens*
Umbridae	*Umbra limi*
Xenocyprididae	*Hypophthalmichthys nobilis*
	Hypophthalmichthys molitrix
	Ctenopharyngodon idella
	Mylopharyngodon piceus

Table A.3. All species detected among spring and fall samples. The proportion of samples in which a species was detected from a given habitat is displayed.

	Wolf Bayou		Hosner Bayou		Samples Bayou		Robinson Lake		Slough		Ditch
Species	Spring	Fall	Spring	Fall	Spring	Fall	Spring	Fall	Spring	Fall	Spring
Amia calva	0.90	0.62	0.75	0.83	0.75	0.92	1.00	1.00	1.00	1.00	1.00
Anguilla rostrata	-	-	-	-	-	-	-	-	0.11	-	-
Aphredoderus sayanus	-	-	-	-	-	0.17	0.86	0.17	0.67	-	0.22
Labidesthes sicculus	0.57	0.71	0.42	0.92	0.83	0.83	-	-	-	-	-
Menidia beryllina	0.05	0.14	-	0.17	-	-	0.76	0.17	0.22	-	0.67
Lepomis cyanellus	1.00	0.76	0.92	0.83	0.83	1.00	0.95	0.83	0.56	-	0.33
Lepomis gulosus	0.95	0.62	0.83	0.67	0.75	1.00	1.00	0.67	0.78	-	0.78
Lepomis humilis	0.95	0.76	0.92	1.00	0.92	0.92	1.00	0.83	1.00	1.00	0.89
Lepomis macrochirus	0.95	0.86	0.92	1.00	0.92	1.00	1.00	1.00	1.00	0.17	0.89
Lepomis marginatus	0.86	0.86	0.92	1.00	0.92	1.00	0.81	0.67	1.00	0.83	0.89
Lepomis megalotis	0.86	0.62	0.83	0.92	0.67	1.00	0.52	0.50	1.00	0.33	0.78
Lepomis microlophus	-	-	-	-	0.08	-	0.05	-	-	-	-
Lepomis miniatus	-	-	-	-	-	-	0.05	0.17	-	-	-
Lepomis symmetricus	0.24	0.10	0.08	-	-	-	0.52	0.17	-	-	-
Micropterus punctulatus	0.62	0.52	0.33	0.50	0.50	0.50	-	-	-	-	-
Micropterus salmoides	0.76	0.67	0.92	0.67	0.92	1.00	0.62	-	1.00	-	0.56
Pomoxis annularis	0.52	0.62	0.58	0.50	0.42	0.67	0.90	0.50	0.89	0.33	0.33
Pomoxis nigromaculatus	0.62	0.33	0.92	0.42	0.33	0.58	0.95	0.67	1.00	0.67	1.00
Alosa chrysochloris	0.10	-	-	0.08	-	-	-	-	0.22	-	-
Dorosoma cepedianum	0.95	0.81	0.92	1.00	0.58	1.00	1.00	1.00	1.00	-	0.89
Dorosoma petenense	0.52	0.62	-	0.75	-	0.42	0.29	-	0.33	-	0.56
Cyprinus carpio	1.00	0.81	0.92	0.92	0.75	1.00	1.00	1.00	1.00	0.33	1.00
Esox americanus	-	-	-	-	-	-	0.10	-	-	-	-
Fundulus notatus	0.76	0.43	0.58	0.67	0.50	1.00	0.95	0.33	0.78	0.33	0.44
Fundulus olivaceus	0.57	0.43	0.17	0.58	0.42	0.83	-	-	-	-	-
Ameiurus natalis	-	-	-	-	-	-	0.38	0.33	-	-	-
Ictalurus punctatus	0.67	0.62	0.42	0.58	0.42	0.58	0.76	0.50	0.89	0.50	0.89
Noturus gyrinus	0.14	0.19	-	0.08	0.33	0.25	0.05	-	-	-	-
Noturus nocturnus	-	-	-	-	-	-	0.05	-	-	-	-
Pylodictis olivaris	0.86	0.57	0.67	0.67	0.67	0.83	0.10	0.17	1.00	-	0.89

Table A.3. All species detected among spring and fall samples. The proportion of samples in which a species was detected from a given habitat is displayed. (Cont.)

	Wolf Bayou		Hosner Bayou		Samples Bayou		Robinson Lake		Slough		Ditch
Species	Spring	Fall	Spring	Fall	Spring	Fall	Spring	Fall	Spring	Fall	Spring
Atractosteus spatula	0.19	0.05	-	-	-	-	-	-	-	-	-
Lepisosteus oculatus	1.00	0.57	0.92	0.83	0.58	0.92	0.90	1.00	1.00	1.00	0.89
Lepisosteus osseus	0.67	0.76	0.42	0.50	0.42	0.92	0.43	1.00	0.89	0.83	1.00
Lepisosteus platostomus	0.90	0.81	0.83	0.83	0.83	1.00	1.00	1.00	1.00	0.83	1.00
Hybognathus nuchalis	-	-	-	-	-	-	-	-	0.11	-	-
Lythrurus fumeus	0.05	-	-	-	-	-	-	-	-	-	-
Notemigonus crysoleucas	-	0.10	0.08	0.08	-	-	0.33	-	-	-	-
Notropis atherinoides	-	-	-	-	-	-	0.14	-	0.11	-	-
Opsopoeodus emiliae	0.43	0.43	0.58	0.83	0.50	0.83	0.57	0.17	0.78	0.17	-
Morone chrysops	0.10	0.14	0.08	0.25	-	-	0.10	-	0.89	-	0.89
Morone mississippiensis	0.05	-	-	-	-	-	0.05	-	-	-	-
Etheostoma asprigene	0.57	0.48	0.92	0.42	0.83	0.83	0.57	-	1.00	-	1.00
Etheostoma caeruleum	-	-	-	-	-	-	0.05	-	-	-	-
Etheostoma chlorosomum	0.71	0.43	0.58	0.83	0.50	0.83	0.33	-	1.00	0.17	-
Etheostoma gracile	0.24	0.05	-	-	-	-	0.33	0.17	0.11	-	-
Percina caprodes	0.24	-	-	-	-	0.08	-	-	-	-	0.11
Percina shumardi	-	-	-	-	-	-	-	-	0.11	-	-
Gambusia affinis	0.62	0.24	0.33	0.58	0.17	0.58	0.95	1.00	1.00	1.00	1.00
Polyodon spathula	0.43	0.38	-	-	-	0.08	0.14	-	-	-	0.11
Aplodinotus grunniens	0.90	0.76	0.83	0.83	0.67	1.00	1.00	0.83	1.00	1.00	0.89
Ctenopharyngodon idella	0.86	0.71	0.92	1.00	0.92	0.83	1.00	0.67	0.89	-	0.89
Hypophthalmichthys molitrix	0.86	0.86	0.92	1.00	0.92	1.00	1.00	1.00	1.00	1.00	1.00
Hypophthalmichthys nobilis	0.90	0.86	0.92	1.00	0.92	1.00	1.00	1.00	1.00	1.00	0.89
Mylopharyngodon piceus	0.86	0.81	0.92	1.00	0.75	0.92	0.90	0.83	1.00	-	0.89

Table A.4. Results of PERMANOVA analysis of all spring fish community data depending on habitat type, secchi depth, depth, standard conductivity, and their interactions.

Factor	Df	SumOfSqs	R2	F	Pr(>F)
Habitat	3	1.613	0.417	18.522	0.001
Secchi	1	0.332	0.086	11.447	0.001
Depth	1	0.086	0.022	2.972	0.009
Conductivity	1	0.033	0.008	1.130	0.331
Habitat:Secchi	1	0.028	0.007	0.959	0.435
Habitat:Depth	3	0.101	0.026	1.163	0.273
Secchi:Depth	1	0.035	0.009	1.192	0.298
Habitat:Conductivity	3	0.110	0.028	1.259	0.188
Secchi:Conductivity	1	0.052	0.013	1.797	0.089
Depth:Conductivity	1	0.053	0.014	1.827	0.077
Habitat:Secchi:Depth	1	0.030	0.008	1.047	0.403
Habitat:Depth:Conductivity	3	0.055	0.014	0.628	0.899
Secchi:Depth:Conductivity	1	0.034	0.009	1.159	0.302
Residual	45	1.306	0.338		
Total	66	3.867	1		

Table A.5. Results of pairwiseAdonis analysis on all habitat comparisons of the spring fish community data.

Pairs	Df	SumsOfSqs	F	R2	p-value
Ditch vs Bayou	1	0.197	15.108	0.290	0.001
Ditch vs Lake	1	0.162	13.683	0.354	0.001
Ditch vs Slough	1	0.044	8.257	0.355	0.002
Bayou vs Lake	1	0.482	32.480	0.404	0.001
Bayou vs Slough	1	0.139	10.711	0.220	0.001
Lake vs Slough	1	0.140	11.823	0.313	0.001

Table A.6. Results of PERMANOVA analysis of spring fish community data from the three bayous depending on water body, secchi depth, depth, standard conductivity, and their interactions.

Factor	Df	SumOfSqs	R2	F	Pr(>F)
Body	2	0.199	0.158	3.683	0.001
Secchi	1	0.269	0.213	9.925	0.001
Depth	1	0.080	0.063	2.952	0.009
Conductivity	1	0.036	0.029	1.345	0.245
Body:Depth	2	0.053	0.042	0.984	0.475
Secchi:Depth	1	0.059	0.046	2.161	0.058
Body:Conductivity	2	0.041	0.032	0.751	0.729
Secchi:Conductivity	1	0.039	0.031	1.451	0.192
Depth:Conductivity	1	0.019	0.015	0.711	0.647
Body:Depth:Conductivity	2	0.055	0.044	1.017	0.458
Secchi:Depth:Conductivity	1	0.003	0.003	0.122	0.99
Residual	15	0.406	0.322		
Total	30	1.260	1		

Table A.7. Results of pairwiseAdonis analysis on all comparisons between Wolf Bayou (WB), Hosner Bayou (HB), and Samples Bayou (SB).

Pairs	Df	Sum Of Sqs	F	R2	p-value
HB vs SB	1	0.024	4.185	0.259	0.001
HB vs WB	1	0.045	2.885	0.111	0.029
SB vs WB	1	0.030	1.911	0.083	0.095

Table A.8. Results of ANOVA analysis on the number of species detected per water sample among habitats and seasons.

Factor	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Habitat	5	147.4	29.49	1.248	0.2906
Season	1	108.4	108.37	4.588	0.0341
Habitat:Season	4	1122.3	280.58	11.879	0.0001
Residuals	126	2976.2	23.62		

Table A.9. Species with historical records from our sample sites.

Family	Species
Amiidae	Amia calva
	Aphredoderus sayanus
Atherinopsidae	Labidesthes sicculus
	Menidia beryllina
Catostomidae	Carpiodes carpio
	Carpiodes cyprinus
	Ictiobus cyprinellus
	Ictiobus bubalus
	Ictiobus niger
	Moxostoma macrolepidotum
Centrarchidae	Centrarchus macropterus
	Lepomis cyanellus
	Lepomis gulosus
	Lepomis humilis
	Lepomis macrochirus
	Lepomis megalotis
	Micropterus salmoides
	Pomoxis annularis
	Pomoxis nigromaculatus
Clupeidae	Dorosoma cepedianum
Cyprinidae	Cyprinus carpio
Esocidae	Esox americanus
Fundulidae	Fundulus notatus
	Fundulus olivaceus
Ictaluridae	Ameiurus melas
	Ictalurus punctatus
	Noturus gyrinus
Lepisosteidae	Atractosteus spatula
	Lepisosteus oculatus
	Lepisosteus platostomus
Leuciscidae	Hybognathus hayi
	Notemigonus crysoleucas
	Notropis shumardi
	Opsopoeodus emiliae
Moronidae	Morone chrysops
Percidae	Etheostoma asprigene
	Etheostoma chlorosomum
	Etheostoma gracile
	Percina shumardi
Poeciliidae	Gambusia affinis
Polyodontidae	Polyodon spathula
Sciaenidae	Aplodinotus grunniens
Xenocyprididae	Ctenopharyngodon idella
	Hypophthalmichthys molitrix

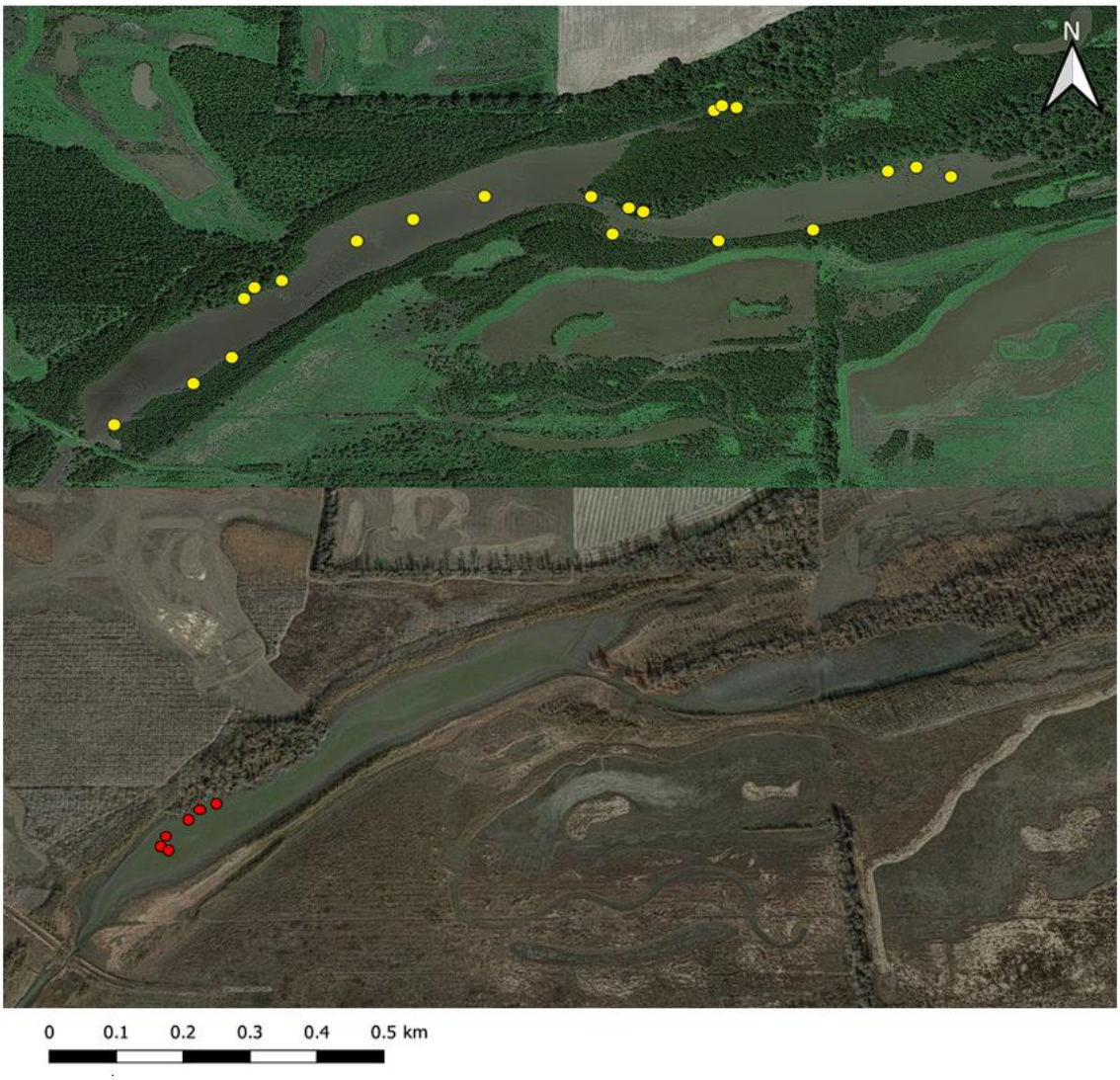

Figure A.1. Aerial imagery of Robinson Lake during water levels similar to when (a) spring samples and (b) fall samples were collected. Spring sample locations are displayed in yellow and fall sample locations are displayed in red.

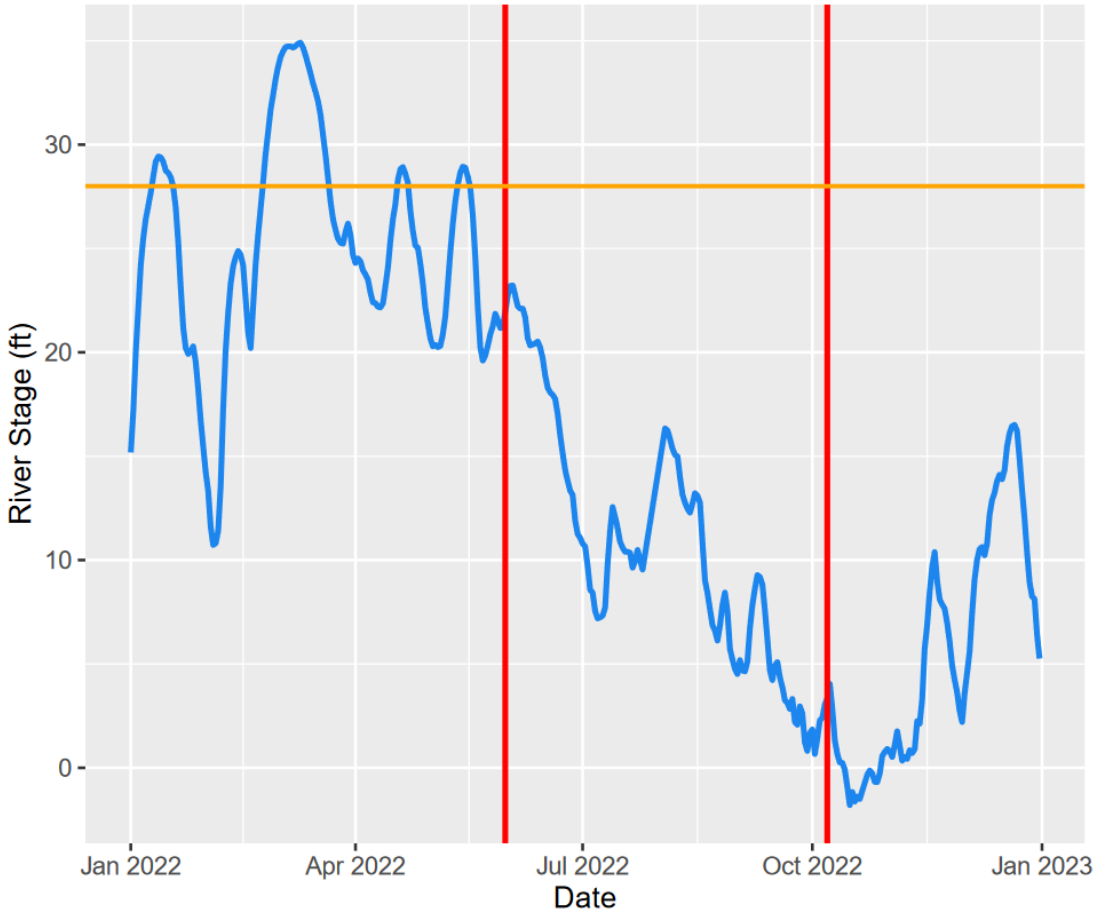

Figure A.2. Gauge height of the Mississippi River at Caruthersville, MO in 2022. Red lines show when spring and fall sampling events took place. The orange line at 28 ft represents the stage at which public access is prohibited except by boat.

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