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VALIDATION OF eDNA METABARCODING:

A COMPARISON TO TRADITIONAL SURVEY METHODS IN OZARK STREAMS

by

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ABSTRACT

Environmental DNA (eDNA) sampling provides a method for assessing fish communities that has potential as a supplement to traditional sampling methods due to its ability to save time as well as its non-invasive nature. This is a method in which from just one sample, eDNA from multiple individual species are able to be sequenced in tandem and the resulting reads identified to describe a community. In order to evaluate this technique and its efficacy for monitoring fish community diversity, we collected water samples alongside surveys performed by the Missouri Department of Conservation sampling program in summers 2020-21. DNA were extracted from these samples and amplified via polymerase chain reaction (PCR), using several targeted mitochondrial gene markers, which were then cleaned and sequenced. We investigated the variation in species detection among different gene markers. We also sought to determine environmental factors involved in variation of eDNA results between summer and winter. We compared the species detected by eDNA to traditional survey detection. Using a variety of statistics including NMDS, ANOSIM, UPGMA clustering, and others, we provide support for the implementation of eDNA metabarcoding techniques to supplement traditional sampling as a robust technique able to provide optimal coverage of fish communities in the Ozarks. We found an average detection rate of 2 species identified by eDNA metabarcoding for every 1 identified by traditional methods.

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For my parents, my sister Samantha and her family, and my little nephew Arlo, I am grateful for the continuous encouragement and love. To my Grandma Sharon for always being one phone call away to talk for hours. To my friends, old and new, who've offered countless support, inspiration, and laughs. And all my love to Andrew, my best friend, my partner in everything, who has and always will stand with me at my side.

This research has been a driving force for me to keep going during the ongoing tragedy that has been the Covid-19 pandemic. I express my sorrow for this time.

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NOMENCLATURE

Term	Description
Environmental DNA (eDNA)	Any DNA that enters the environment surrounding an organism, either by sloughed off skin or cells, waste, gametes, blood, or even decaying deceased organisms, hereafter eDNA.
Polymerase chain reaction	PCR is an assay in which a targeted gene region is amplified to produce amplicons, either used for downstream sequencing, or for quantification during quantitative PCR (qPCR).
Barcoding	The method of identifying species with a short sequence of DNA that can then be compared to a reference library.
Metabarcoding	The barcoding of DNA that allows for the identification of many different taxa present within the same sample.
Next-generation sequencing	NGS refers in general to the more recent available sequencing technology in which millions of reads can be sequenced in parallel and then de-multiplexed, allowing researchers to be able to sequence entire communities and identify species matched to references.
Operational Taxonomic Unit	OTU refers to a classification of a similar group of organisms or DNA into one specific unit, and may be used to define clusters of similar sequences, and is generally used as a more broad or applicable term than ‘species.’
Rapid Assessment Monitoring Team	The RAM team is the group deployed by the Missouri Department of Conservation to conduct traditional sampling in wadable streams across Missouri. These surveys include methods like electrofishing and seining to count fish community members.

1. INTRODUCTION

Characterizing the composition of fish communities is crucial for conservation and management efforts, and current traditional methods can be labor intensive and have inherent limitations (Antognazza et al., 2019; Jerde et al., 2011; Shaw et al., 2016; Shelton et al., 2016). An emerging technology in biomonitoring is the use of environmental DNA metabarcoding. Environmental DNA (eDNA) is any DNA that is released into the environment, whether by living or decomposing organisms, and can be shed from sources including skin, blood, saliva, waste, and gametes (Bohmann et al., 2014). In aquatic environments it is available in the water column for collection, and it has the potential to provide biologists with a powerful tool for monitoring and detecting organisms (Darling and Mahon, 2011) as a complement to current traditional sampling techniques. In the past decade, eDNA-based sampling methodology has been predicted to become the future of biodiversity monitoring techniques and has subsequently been investigated and discussed as tools have improved and protocols have been field-tested and validated (Baird & Hajibabaei, 2012; Bush et al., 2019; Ficetola et al., 2008; Keck et al., 2017; Mathon et al., 2021; Pawlowski et al., 2018).

The aims and scope of this thesis encompass the review, practice, and recommendation for the use of eDNA metabarcoding as a method to complement or enhance traditional fish community surveys conducted by the Missouri Department of Conservation every year in streams by the Rapid Assessment Monitoring (RAM) program. We will also contribute our findings to the field of eDNA at large for other systems similar to the Missouri Ozarks in order to help support land managers in the use of this

methodology. A literature review is included to provide general background and context for the scope of the project.

Our objectives were:

1) To review published universal primer assays in the efficacy of amplification by PCR for an optimal level of species detection and minimal taxonomic bias within Missouri Ozark fish communities.
2) To understand the level of community characterization provided by eDNA metabarcoding, and whether this technique can uniquely describe the similarities and differences of species in different habitats.
3) To compare the findings of eDNA metabarcoding to established traditional methods.
4) To evaluate potential seasonal or environmental factors which may cause variation in eDNA results.

The process of metabarcoding utilizes PCR to amplify targeted segments of mitochondrial DNA among organisms of interest. Published studies have included a variety of optimized assays targeting several gene regions for surveying fish taxa. We conducted a survey of Ozark fish communities using five different published assays in order to evaluate efficacy of each for metabarcoding in this system. In sumer 2020, we collected water samples from four different river drainages, extracted the eDNA, and then PCR amplified five different mitochondrial gene markers. Three assays targeted segments of the 12s rRNA gene region, one targeted a 16s rRNA gene region, and one a portion of the COI gene region. PCR products were sequenced, and the raw sequence data were processed using the Barque v1.7.3 (https://github.com/enormandeau/barque) metabarcoding analysis

pipeline with a curated reference database of available Missouri fish DNA sequences. We conducted an empirical evaluation of the number of species detected by each, overall resolution of closely related taxa, and accuracy of species identification in order to determine which primers were most effective for our studies of Ozark fish communities. Our results support an approach with a combination of primers targeting the 12s rRNA and 16s rRNA gene region for an optimal representation of Ozark fish communities.

In order to evaluate the eDNA metabarcoding technique and its efficacy for monitoring fish community diversity, we collected water samples in summers 2020 and 2021. DNA were extracted from these samples and amplified via PCR using our chosen 12s and 16s rRNA mitochondrial gene markers, which were then cleaned and sequenced. We investigated the difference in species detection found by eDNA metabarcoding among streams within a drainage. We compared the detection of species provided by eDNA to traditional RAM detection across a total of six drainages in the Missouri Ozarks. To determine the optimal number of samples needed to reach a desired threshold of species represented in a community sampled, we generated species accumulation curves. To visualize the number of species captured, we generated graphs to display the number of species detected by eDNA compared to RAM. We also analyzed the effect that certain seasonal and environmental factors had on eDNA metabarcoding results. We provide support for the implementation of eDNA metabarcoding techniques to supplement traditional sampling as a robust technique able to provide optimal coverage of fish communities in the Ozarks.

2. LITERATURE REVIEW

The scientific understanding and implementation of eDNA sampling to detect and monitor the presence of species has advanced substantially in the last fifteen years (Corlett, 2017; Jerde et al., 2011). There are two general strategies to implement eDNA surveying. The first approach is a single-species targeted approach, and the second a multi-species metabarcoding approach. The former method involves the design of a polymerase chain reaction (PCR) assay with primers and probes designed to target a specific gene segment, typically in the mitochondrial DNA (mtDNA) for eukaryotes or the chloroplast genome for plants, the sequence of which is unique to a single species. The resulting PCR product can either be assessed with quantitative PCR (qPCR) or then sequenced to provide additional data if applicable. This single-species approach is the most sensitive method for detecting presence or absence of specific rare species of interest, such as invasive species entering a habitat or the detection of cryptic or low abundant species that are difficult to observe with previous traditional methods (Anglès d'Auriac et al., 2019; Jerde et al., 2011).

There are considerations that must be made for both the single- and multi-species metabarcoding approaches. Single-species approaches can be used to target a known organism, such as an endangered or invasive species of interest. This technique may benefit from being able to target a sampling event to correspond with known seasonal changes that may include higher metabolic rates or species activity, which may lead to more eDNA being captured (Takahara et al., 2012). In addition, the single-species approach generally requires life history data to be known about the target organism, as well as the genetic sequence and those of sympatric species to be available in order to design primers for PCR.

This may also come at the upfront cost of generating this data in order to establish this monitoring technique (Smart et al., 2016).

For the multi-species approach, a highly conserved gene region for a group of taxa is used as a template for universal PCR primers to amplify, which will then be identified with metabarcoding. These universal primers most often target the mtDNA genome across a wide taxonomic range that may include target organism (e.g., all teleost fishes or all batrachian amphibians, (Valentini et al., 2016). The PCR products are then processed using Next Generation Sequencing techniques, typically generating hundreds of thousands of individual DNA sequence reads per water sample. Highly similar sequences are grouped together using a computer algorithm, usually with a cutoff designated at 97% similarity match, and each unique sequence cluster, described as operational taxonomic units (OTUs), can be identified by matching to a database of known DNA sequences, curated from available sequences pulled from a reference database such as Genbank. This method has the potential to detect and identify all species within a targeted taxonomic group in a given environment, and thus the ability to characterize a community.

With its versatility and potential, eDNA metabarcoding can be used in any system, albeit through a plethora of different study designs and applications. There have been studies conducted using available eDNA within soil, air, and water. While a few examples of soil and air studies will be mentioned here, the bulk of this review will pertain to eDNA metabarcoding and its use in aquatic systems.

2.1. eDNA FROM ITS ORIGIN TO NOW

The earliest eDNA methods were pioneered in the study of soil microbial communities (Ehrlich et al., 2015). These methods generally included the utilization of the bacterial 16s rRNA gene region in PCR amplification, sequencing the amplicons and clustering the results within OTUs matching at 97% or higher threshold. For soil microbes, these results would often lead to ~50% of reads being novel sequences. This was proven a useful method, especially for gene discovery and characterization in the diverse world of soil microorganisms. As the technique became more established and the availability of Next-Generation Sequencing increased, soil sampling was used in other studies investigating eukaryotes. For a more recent example, Andersen et al. (2012) sampled soil from zoological parks to investigate the efficacy of vertebrate 16s mtDNA matching with the known animals present. Their results reflected the overall taxonomic richness and abundance, as well as preliminary affects on DNA deposition rates for the animals studied.

Common uses of eDNA sampling of the air include sampling for pollen, fungal spores, and air-suspended microbes. A study with the use of aerobiological sampling was conducted by Banchi et al. (2018) to characterize fungal spores and mycelium fragments in the air. They found a tenfold increase in taxonomic diversity compared to results of traditional microscopy techniques, and they note that their workflow can be extremely beneficial to routine, high-throughput monitoring.

An emerging and prominently investigated application of eDNA barcoding and metabarcoding is within aquatic systems, which will entail the bulk of this literature review. Many studies have investigated the use of eDNA as a supplement or replacement to traditional methods, which have included invasive strategies like electrofishing, seining,

and setting traps. As Next Generation Sequencing has become more affordable and accessible, aquatic eDNA sampling is likely to become a large area of focus, demanding a thorough understanding of the variables that affect eDNA capture across a wide range of systems.

2.2. AQUATIC eDNA SAMPLING

Aquatic eDNA sampling can either be performed by collecting water for vacuum filtration or sediment from aquatic ecosystems, then extracting eDNA from the filter or directly from the sediment, respectively. There are many variables to consider that have been known to affect the persistence and quality of eDNA, especially when suspended in the water column, such as pH, UV light penetration, and temperature (Deiner & Altermatt, 2014; Hinlo et al., 2017). Sediment may also sequester eDNA, prolonging its persistence (Deiner & Altermatt, 2014), and in some cases, researchers have found the eDNA in sediment to be more concentrated than in the water column (Turner et al., 2015). Therefore, the establishment of protocols that are best suited to each system are needed, and recent reviews have emphasized the need for a pilot test of a system before full-scale monitoring (Beng & Corlett, 2020; Goldberg et al., 2016). There is a growing demand for aquatic systems to employ similar or standard protocols as the field rapidly expands in order to draw comparisons and ecological conclusions from these techniques.

One example of eDNA monitoring in aquatic systems was the study designed to detect an invasive aquatic plant species, *Elodea canadensis*, by Anglès d'Auriac et al. (2019) in Norway. They found that their targeted eDNA method detected the plant chloroplast DNA in a variety of turbid conditions with suspended clay in the water column,

and was more affected by season than other factors, peaking in concentration during the fall. In another study, a species of European mudsnail, *Peringia ulvae*, was detected in ballast water after cross-latitudinal voyages even up to two weeks, and this early detection may yet prevent this species from becoming a threatening invasive in that system (Ardura et al., 2015). Sampling directly from the water column for eDNA is quickly becoming one of the most popular uses of eDNA, both for single-species as well as metabarcoding approaches.

2.3. AREAS OF BIAS

Bias and error may enter the eDNA metabarcoding process at every step (Figure 2.1). First, the true biotic community may not be represented accurately or evenly in a water sample due to factors that influence the synthesis, transport, persistence and degradation of eDNA (e.g. Dejean et al., 2011; Strickler et al., 2015). Synthesis of eDNA is connected to overall species biomass and metabolic rates and is influenced by a variety of factors that include temperature, breeding condition, stress, developmental stage, and diet (Klymus et al., 2015; Valentini et al., 2016). While eDNA may persist for extended periods in the protection of sediments (Turner et al., 2015), the persistence time of eDNA in the water column is generally much shorter, often on the order of hours or days (Li et al., 2019), and dependent on factors including UV-B exposure, pH, temperature and microbial activity (Strickler et al., 2015). Transport in aquatic environments, and homogeneity of eDNA across spatial scales is dependent on flow and mixing in the aqueous medium coupled with persistence time. In general, eDNA sampled in the water column

should be connected, in most circumstances, to contemporaneous species distributions (Pilliod et al., 2014).

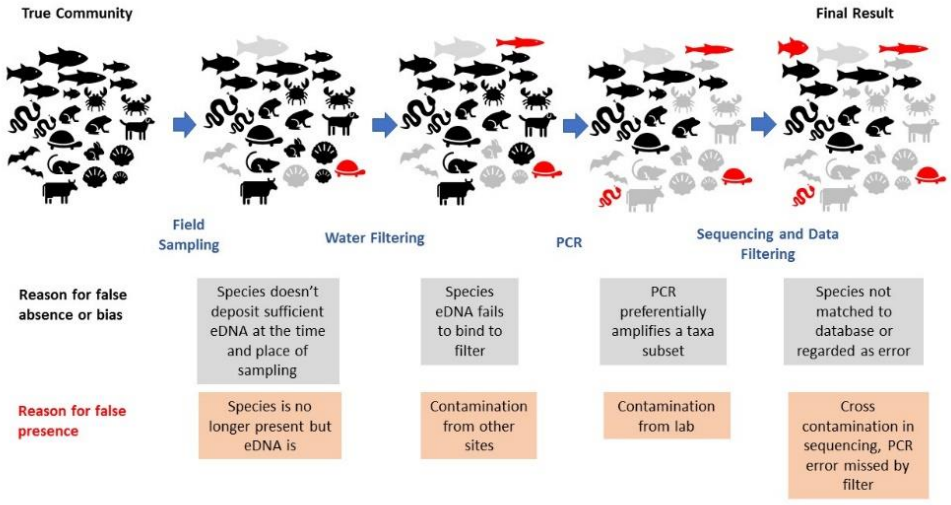


Figure 2.1. eDNA community bias. (Credit: Dr. Leah Berkman, MDC). Schematic diagram of how bias, false absences (grey), and false presences (red) enter an eDNA metabarcoding analysis during each step (blue) of the process. Correctly detected species are depicted in black.

One technical challenge of eDNA sampling is the generation of false positive outcomes resulting from sample cross contamination. In other words, the power of the PCR technique to detect and amplify rare templates is also a technical liability of the method. Contamination occurs in even the most carefully executed eDNA studies (Klymus et al., 2017), and must be managed through adherence to best practices and monitored by effective use of positive and negative controls. These practices were recently reviewed by (Evans & Lamberti, 2018; Ruppert et al., 2019; Thomsen & Willerslev, 2015; Zinger et al., 2019).

An area that would benefit from more thorough investigation and standardization is in the downstream analysis of sequencing data generated by metabarcoding (Corlett, 2017). There are many different packages and programs readily available, and these pipelines all may vary slightly in their function at certain steps in the process. A byproduct of PCR with universal targets is the formation of PCR chimeras, which are the products of two different species erroneously amplified together, and analysis pipelines should have a chimera removal step. Bioinformatics platforms and optimal filtering parameters can be assessed, and errors identified by analyzing known communities and utilizing a reference library curated to the focal community that is as complete as possible (Olds et al., 2016). It can be beneficial to conduct a comparison of different pipelines, especially if handling sequencing data that contains sequences of closely related species, in order to compare the ability of pipelines to characterize populations (Macé et al., 2022).

Several pipelines have emerged for eDNA metabarcoding sequence data analysis. Some widely cited pipelines include Barque (https://github.com/enormandeau/barque), Anacapa (Curd et al., 2019), Mitofish for use with Mifish primers (Miya et al., 2015), and many others. Each of these has their own benefits and challenges, some being more user friendly, while others may allow for more customization to suit a project, but require more specialization in bioinformatics to use. Overall, the decision on pipeline choice should come down to which is best suited to answer the research questions, and available resources of bioinformatic experts (Mathon et al., 2021).

It is difficult to standardize many aspects of eDNA metabarcoding across different systems. Different sampling techniques, DNA extraction methods, sequencing, and even bioinformatic pipelines will have varying affects on the outcome. Some freshwater samples

extract better from the water column directly, while for some samples it may be more prudent to extract DNA from the sediment. Some cells may lyse properly and expel their DNA more easily than others. Different bioinformatics pipelines may have different OTU clustering thresholds or chimera filtration scripts that change the final data output. These inconsistencies make it difficult to understand all the factors that go into a published result, and leaves gaps when attempting to replicate studies to the best of our ability (see Pawlowski et al., 2020; Ruppert et al., 2019).

Shelton et al. (2016) suggested statistical framework in which to compare traditional and eDNA metabarcoding methods, detailing the assumptions made by both techniques and considerations to be made when attempting to infer biomass of species detected. One observation of note is that it is impossible to make inferences about biomass with eDNA sampling alone, similar to estimates of biomass by fisheries are assumed to be indices of abundance rather than absolute abundance (Shelton et al., 2016). There are many different factors that go into both the rate at which eDNA is shed by organisms (Klymus et al., 2015; Takahara et al., 2012) as well as different downstream PCR amplification and sequencing analysis factors that affect how final sequencing read counts change (Ficetola et al., 2015; Goldberg et al., 2016; Kanagawa, 2003). Researchers are cautioned not to directly correlate sequencing read counts or qPCR *ct* curves as absolute abundance in a similar way, though correlations may still be valid points of further ecological research (Goldberg et al., 2016).

One other consideration is the potential for eDNA metabarcoding to provide seasonally affected results. There has been an effort to understand if the variations in these results are ecological and reflect community changes (Buxton et al., 2017; Djurhuus et al.,

2020; Tillotson et al., 2018), or if they are in part due to the persistence and condition of eDNA changing in the environment under different conditions (Dejean et al., 2011; Lawson Handley et al., 2019; Strickler et al., 2015), and how to quantify these influences.

2.4. eDNA AND TRADITIONAL METHODS

In general, most traditional methods of surveying freshwater communities were designed to detect as many of the organisms present as possible. These methods could be invasive, destructive, as well as still not applicable to cryptic and rare species (Olson et al., 2012; Shelton et al., 2016). Traditional methods may also not be the most effective for some species. For example, as Antognazza et al. (2019) investigated the European shads, *Alosa alosa* and *A. fallax*, which can be hard to sample with standard methods as they are sensitive to handling and anesthesia. But the use of qPCR in a targeted approach allowed them to successfully reveal positive detection across the known range of the *Alosa* spp. spawning reach, and the authors expressed interest in further use of this technique for the management of species traveling across barriers in the stream in the future.

One of the most investigated aspects of eDNA metabarcoding is how well the method compares to traditional methods. Fortunately, this area has been investigated extensively in recent years, across many different systems (Polanco Fernández et al., 2021; Shelton et al., 2016; Valentini et al., 2016). While eDNA barcoding and metabarcoding may posit their own challenges and biases, most often these issues have parallels to traditional methods (Shelton et al., 2016). In a marine system, Polanco Fernández et al. (2021) surveyed two Colombian tropical reefs, comparing their eDNA metabarcoding and underwater visual census. They found in one of the two sites that eDNA performed

similarly to visual census, and that eDNA detected broader phylogenetic diversity as well as more smaller species compared to the visual census. However, they mention that a more robust reference database is needed for that system to optimize this technique, which can be a common pressure point to eDNA surveying (Mathieu et al., 2020). Many studies have arisen recently that have found eDNA metabarcoding and barcoding to perform as well as or better than previously employed traditional methods in a variety of aquatic systems (Gehri et al., 2021; Hallam et al., 2021; McColl-Gausden et al., 2021).

One study was performed by Doi et al. (2021) in freshwater streams in Japan. This group found more taxa at a site with eDNA metabarcoding than any other visual or capture surveying consistently across their study area of five different sites. These authors also describe the potential for eDNA metabarcoding to be a suitable method for characterizing communities, with the caveat that it may offer less distinction between groups in an upstream to downstream orientation than traditional surveys. This, they suggest, is because the flow of eDNA suspended in the water column from the upstream sites down to the downstream site may cause the eDNA metabarcoding results to be more similar between sites, where traditional surveys directly capture species in the locations they reside.

The group of researchers Cilleros et al (2019) discussed the difficulty of sampling tropical South American freshwater rivers by traditional methods, explaining their invasive and commonly destructive nature. They looked at the potential for eDNA metabarcoding to replace these methods. They found eDNA to provide a similar number of species to traditional methods at each site, but which had a very different profile of matching taxa in common. They suggest for these systems that eDNA can be helpful as an assessment of

large-scale biodiversity, and a complement to traditional sampling in which the two methods together provide a more complete characterization of biodiversity overall.

Another group in Japan, Nakagawa et al. (2018), surveyed 51 rivers at a total of 102 sites, and compared their results to existing observational data. They reported consistent results from eDNA metabarcoding compared to existing visual survey observational data, finding 38/44 known species as well as two new ones, with a reported decrease in overall time and cost compared to traditional methods.

There is a growing consensus that eDNA sampling offers the potential to enhance the results of traditional community survey methods across a variety of aquatic systems, providing the opportunity to detect cryptic species or those which are difficult to monitor historically, as well as an overall picture of biodiversity within a study site, with careful implementation and consideration of site characteristics needed for drawing sound ecological conclusions.

3. AN EMPIRICAL COMPARATIVE ASSESSMENT OF ASSAY EFFICACY FOR eDNA METABARCODING OF OZARK FISH COMMUNITIES

3.1. BACKGROUND

We set forth to investigate the potential of primer bias on eDNA metabarcoding of fish communities in Missouri Ozark streams, comparing eDNA results side-by-side with traditional methods as a point of reference. These traditional methods consisted of electrofishing followed by seining, performed annually in the summer by the Missouri Department of Conservation's Resource Assessment and Monitoring (RAM) teams.

Primer bias and efficacy can affect our ability to accurately characterize a community with eDNA metabarcoding (Ficetola et al., 2021; Kelly et al., 2019; Stadhouders et al., 2010). We evaluated five different published primer assays, including Teleo 12s rRNA primers (Valentini et al., 2016), Mifish 12s rRNA primers (Miya et al., 2015), the COI primers mlCOIintF and dgHCO2198 (Leray et al., 2013), 16s rRNA primers Chord_16s_F_TagA and Chord_16s_R_short (Deagle et al., 2009), and 12s rRNA primers Am12s_F and Am12s_R (Evans et al., 2016). Hereafter these are referred to as the Teleo, Mifish, COI, Chord16s, and Am12s primer pairs, respectively (See Figure 3.1).

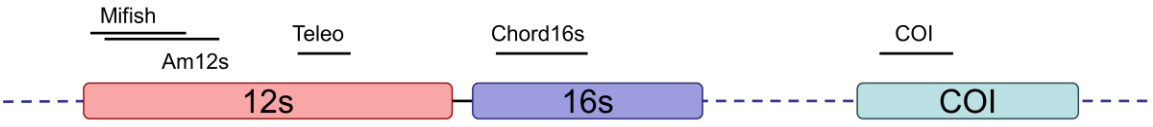


Figure 3.1. Approximate primer gene region locations.

We sought a combination of primer assays which amplified a conserved region of DNA sequence at the forward and reverse binding sites, but still provide a sufficient region within of variable sequence which would discriminate between closely related taxa. To that end we included samples from four different drainages in order to understand what composition of community is detected by these primer sets across different assemblages (Figure 3.2). We evaluated species richness and overall composition relative to RAM results as a point of reference for each of the primer pairs.

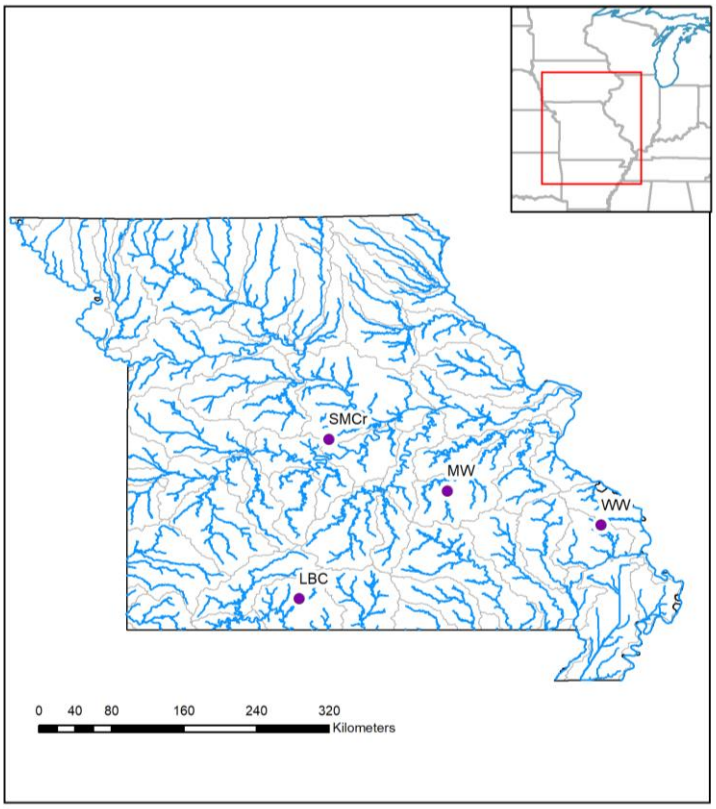


Figure 3.2. Map of our four study sites, South Moreau Creek (SMCr), Meramec River at Wescoe (MW), Little Beaver Creek (LBC), and Whitewater River (WW).

3.2. PRIMER PANEL EVALUATION METHODS

In summer of 2020, we sampled alongside the MDC's RAM fish monitoring team as they collected community data using previously established traditional methods, including backpack electroshocking and seining. We sampled four Missouri Ozark sites, which were on the Meramec River (MW), the Whitewater River (WW), South Moreau Creek (SMCr), and Little Beaver Creek (LBC), each in a separate drainage offering a different composition of species to assess primer assay bias. We collected 500 mL water samples in triplicate at three different sections along the stream reach of the RAM site, sampling at the downstream-most location, center, and upstream-most location, with an effort to sample a variety of microhabitats at these points. One field negative was included at each site to monitor contamination, for a total of ten samples. Samples were transported on ice and stored in a refrigerator within the same day of sampling.

These samples were filtered within 48 hours following a modified protocol similar to the methods described by Valentini et. al (2016) using a 0.45 μm cellulose nitrate filter under vacuum. We extracted the eDNA from the membranes using Qiagen DNeasy Blood and Tissue Extraction kits following manufacturer's instructions with modifications following Hinlo et al. (2017).

All surfaces of equipment were decontaminated between processing of successive samples, using bleach, which destroys DNA molecules on contact (Goldberg et al., 2016). This included field equipment, water collection vessels, filter apparatus, as well as lab equipment and workspaces that were in contact with water samples. Sample cross-contamination was monitored by periodically processing de-ionized water samples (negative control) (Goldberg et al., 2016; Ruppert et al., 2019) and exotic species samples

taken from water in the tanks of tropical fish at a local pet store (positive control) (Klymus et al., 2017; Nakagawa et al., 2018). These controls were used to establish baseline expectations for contamination levels in sample data, as well as to establish minimum detection criteria for validation of species detection in field-collected water samples (Klymus et al., 2017; Valentini et al., 2016).

3.2.1. PCR. PCR product contamination was managed by conducting all pre-PCR processing steps in a separate "clean" research lab space. DNA extractions and PCR reactions were conducted in the clean room with separate dedicated equipment. All post-PCR steps, including PCR product quantification, normalization, and gel electrophoresis, were conducted in a separate post-PCR lab space. Equipment, samples, and reagents were used with minimal transport between labs.

Primer sets were 5' tagged with sequences providing binding sites for Illumina sequencing primers. The extracted eDNA samples were PCR amplified using high-fidelity AmpliTaq Gold 360 DNA polymerase (Thermo Fisher Scientific) in 30 µL total volume reactions divided into triplicate independent reactions for each primer pair in order to minimize reaction-to-reaction heterogeneous template amplification bias. Cycling conditions were as follows: for Mifish, denaturation at 95º C for 5 min, then a cycle repeating 45 times of 95º C for 20 sec, 65º C for 20 sec, 72º C for 1 min, finishing with an extension step of 72º C for 5 min and before a final hold of 4º C. For the COI, Am12s, Teleo, and Chord 16s primers, an initial denaturation of 95C for 5 min was followed by a cycle repeating 45 times of 95º C for 25 sec, 55º C for 30 sec, and 72º C for 1 min, with an extension step of 72º C for 6 minutes before a final hold of 4º C. PCR products were verified with gel electrophoresis, then cleaned using AMPure XP magnetic beads following

manufacturer protocols (Beckman Coulter). Samples were quantified with Qubit 3.0 High Sensitivity dsDNA assay (Thermo Fisher Scientific) and concentrations were normalized to an acceptable range for library indexing and MiSeq sequencing (Illumina) services provided by the University of Missouri Genomics Technology Core.

3.2.2. Database Curation. To rapidly filter erroneous identifications, we curated our databases for each of the drainages sampled to only contain references of species known to inhabit them (Pflieger & Smith, 1997; Hrabik, 2022, in press). To provide a comparable assessment across our primer gene regions, we did not include any reference sequences in this analysis that weren't available across all of the 12s, 16s, and COI gene regions. Most reference sequences were retrieved from Genbank. Additional references were derived from our own unpublished sequences.

3.2.3. Sequencing Analysis Pipeline. We processed our raw sequencing read files through the Barque v1.7.3 (https://github.com/enormandeau/barque) metabarcoding analysis pipeline. This pipeline includes steps to trim primer sequences, pair forward and reverse reads, and filter chimera sequences. We set the parameters to identify fish at the species level with a 97% match to reference sequences. We subtracted the number of reads in the negative controls from the field samples as a precaution against possible contamination, though instances of contamination were so few that this did not cause any species detected to be removed from our analysis.

3.2.4. Species Identification. There were a small number of taxa among the five primer sets which could not be distinguished to the species level. Any instance of ambiguity in which a portion of sequencing reads matched to multiple taxa, but the species involved were also identified at the species level, the ambiguous multiple hit reads were removed.

Any results that were both unidentified to the species level and contained less than 100 total reads across all samples were removed, as well as any single sample that had less than 5 reads.

3.2.5. Statistical Analysis. In order to evaluate the performance of the primer sets within the Missouri Ozarks, we generated species accumulation curves in the iNEXT software (https://chao.shinyapps.io/iNEXTOnline/, Chao et al., 2016), with the set parameter of incidence (presence/absence) values. We did this in order to visualize the number of species identified among the different primer sets within the same community, also plotting a line against the curves to reference the number of species detected by RAM. To further understand the number of unique and common species detected by primers in comparison to RAM species, we also plotted the number of species detected by RAM in bar graphs for each primer set and site. We used RAM as a baseline for evaluation of the varying primer assay results, and the reported RAM results exclude any species that were detected by RAM but not available in the reference databases for eDNA. To determine if the different primer assays detected species in similar proportions, a non-metric multidementional scaling (NMDS) analysis was generated using sequencing reads transformed via Hellinger matrix (Borcard et al., 2018).

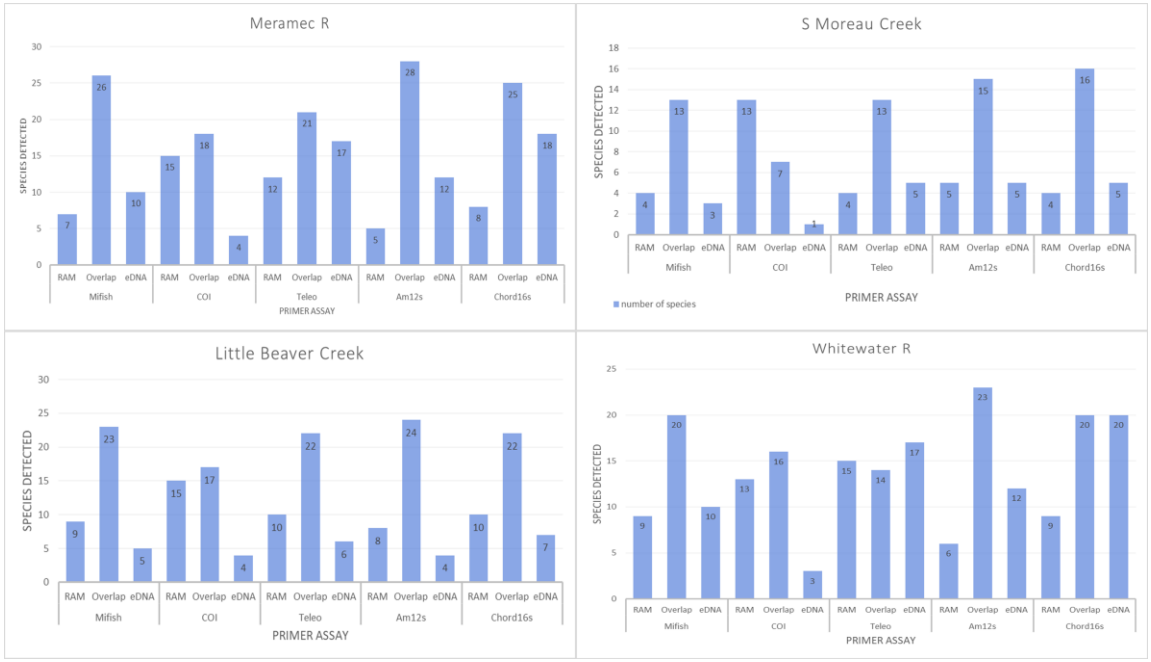
3.3. PRIMER PANEL RESULTS

Across all sites, with eDNA metabarcoding, we detected 48 species with MiFish, 50 species with Am12s, 52 with Chord16s, 51 with Teleo, and 33 with COI. RAM traditional sampling found a total of 53 species. Consistently, the COI primers resulted in

fewer species which were also detected by RAM, in addition to fewer unique identifications compared to those found by the other primer assays.

The overlap between detection with the different primer sets and RAM are displayed in bar graphs containing the number of species detected at each of the four sites broken into groups for each primer assay (Figure 3.3a). A set of Venn diagrams display the same information condensed across all four sites (Figure 3.3b). By drainages (Figure 3.3a), the overlap of species detected by both eDNA and RAM ranged from the lowest value of 7 species in common with RAM detected by COI primers in South Moreau Creek. The highest value of 28 species in common with RAM detection was found by Am12s primers in the Meramec River. The proportion of species detected only by eDNA was highest in the Whitewater River, with the Chord16s primers detecting 20 unique species in that drainage. Chord16s primers consistently found the highest number of unique species detected which were not found by RAM in each drainage. Across all sites and species detected (Figure 3.3b), COI had the lowest number of species detected uniquely from RAM, at a total of 3, as well as the lowest total overlap of 30 species. The highest number of species detected by eDNA not found by RAM was 18, which were found by the Teleo primer assay. The highest amount of overlap between RAM and eDNA across all sites was found with the MiFish primers at a total of 39 common species.

Sampling effort needed and estimations of the resulting diversity captured were plotted by using species accumulation curves (Figure 3.4). Most of the eDNA were able to capture the same species diversity found with RAM within three sampling units, however for every site, the COI primer assay results did not reach the reference number of species found by traditional sampling.



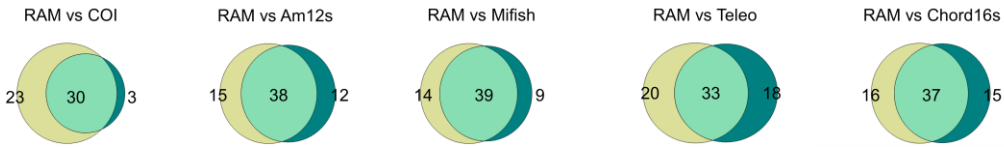


Figure 3.3. The number of species found by eDNA, RAM, or both methods. A) (top) Bar graphs which include species detected within our four sites for all five tested primer assays. B) (bottom) Venn diagrams of the total number of species detection overlap across the four sites and RAM summed together for the five primers.

For site Little Beaver Creek (LBC), eDNA results did not surpass the value of RAM results, even though the RAM comparison only includes species which are available in our reference databases. We extrapolated further than our 9 sampling units for LBC site to demonstrate that at no point does it surpass our point of reference RAM line. The Chord16s primers provided the highest estimation of species richness across all four sites, followed closely by the 12s primer assays.

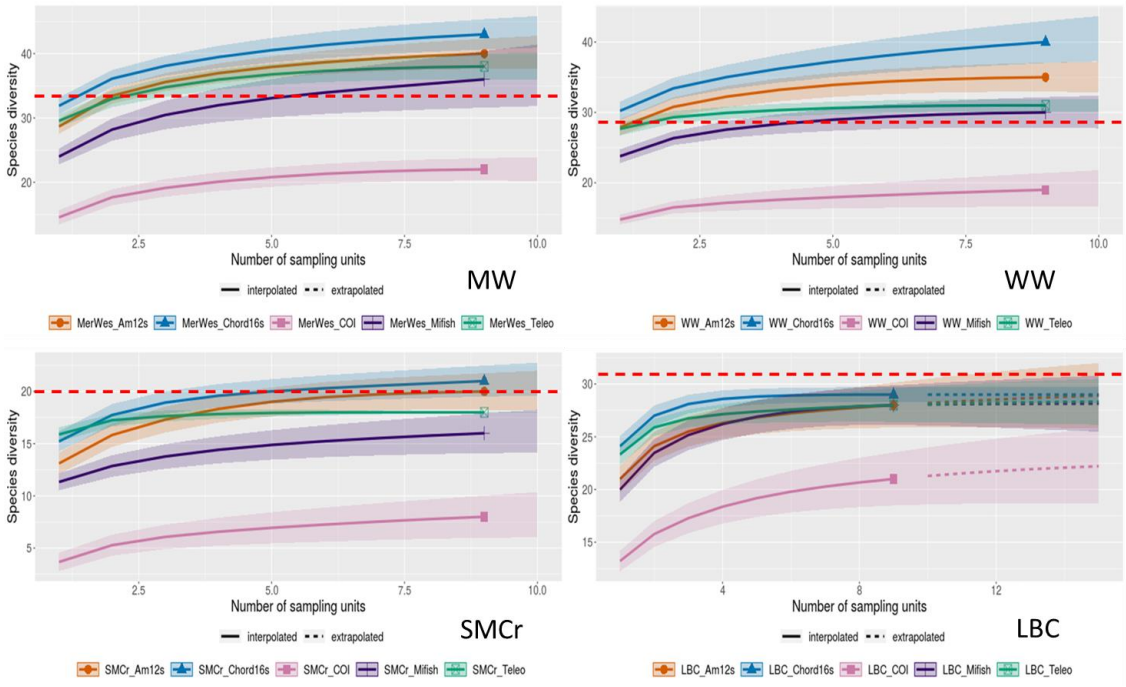


Figure 3.4. Species accumulation curves. The red dashed line provides a reference for the number of species detected by the RAM team at these four sites.

The results of our NMDS analysis demonstrate that primer assays produced different community profiles as a result of different spcies detection, with the exception of site SMCr. This may be explained by the lower overall species diversity in the South Moreau Creek relative to the other drainages (Figure 3.5).

3.4. PRIMER PANEL DISCUSSION

We sought to compare the efficacy of published primer assays in our own system of fish communities within Missouri Ozark streams. Our key objective was to find a primer set which produced conserved sequences for amplification, but enough variation within the amplicon for discrimination between closely related taxa. Our results show that primers

targeting the 12s and 16s rRNA mitochondrial gene region which exceed a minimum of 100 bp of amplified sequence offer the best amount of taxon discrimination.

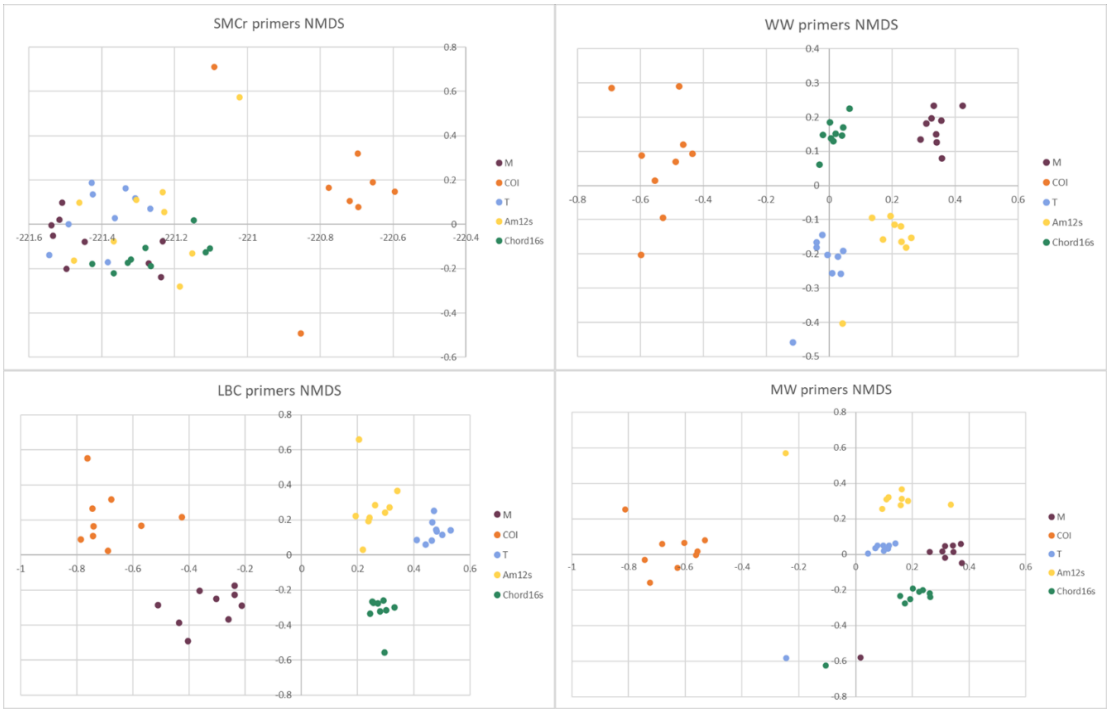


Figure 3.5. NMDS analyses of the four sites with the five primers, Mifish (M), COI, Teleo (T), Am12s, and Chord16s.

The Chord16s primers were designed to target prey items, at a size of ~250 bp, with a blocking primer to stop amplification of predator DNA (Deagle et al., 2009). This provided them with very promising results as universal primers that are echoed here by our own in which this primer assay offered a robust representation of the Ozark fish communities. This primer assay had the highest species accumulation curves across all drainages, and amplified the greatest number of unique species which were not found by RAM in each drainage.

The Mifish primers by Miya et al. (2015) were designed to be an optimal size of less than 200 bp but still provide taxonomic resolution of closely related species. They selected an area on the 12s gene region found to have hypervariability between two sections of highly conserved material, providing an ideal circumstance for amplification and discrimination of taxa. Mifish and Am12s had a considerable amount of overlap in sequence in common in the gene region, and our results indicate similar species detection between the two primer assays. While Am12s was originally designed to target amphibian species (Evans et al., 2016), these primers performed well for detection of fish as well.

The Teleo primers were designed to assess fish diversity (Valentini et al., 2016), and they worked well for our study. However, the Teleo primers amplify a region of only approximately 65 bp, and we found that this short segment did not provide a large enough length of variable sequence to have as much taxonomic resolution as other, larger regions targeted by the other 12s rRNA primer sets.

Our results support the recent findings that COI primers do not offer enough resolution between closely related taxa to be ideal candidates for eDNA metabarcoding surveys (Collins et al., 2019; Hajibabaei et al., 2019; Zinger et al., 2019). Many have discussed that while there may be a benefit provided by the amount of reference data available for the COI gene region, lending this to be an attractive site to target for universal amplification, some argue that the conserved regions of COI are not suitable for most amplicon-based metabarcoding applications (Collins et al., 2019; Deagle et al., 2014; Ficetola et al., 2021). The COI primers mlCOIintF and dgHCO2198 designed by Leray et al. were employed to categorize a wide range of phyla that were prey items in guts of fish, amplifying a size of ~310 bp using a degenerate reverse primer which may amplify more

easily across many diverse species (2013) but does not appear to be an ideal method for closely related taxa in Ozark fish communities.

The two sites, SMCr and LBC, were smaller than the other two river sites, MW and WW. It would be expected that overall, these sites would have a lower species richness in general compared to WW and MW. However, eDNA metabarcoding had a lower species richness relative to the RAM baseline than eDNA provided for MW and WW, which is likely to be explained by further exploration into different ecological or physical conditions that affect eDNA dispersal. Since the results are across all of the primer sets, it is likely an issue specific to eDNA metabarcoding and not any indication of PCR assay efficacy for these primers. The sites which have a higher species diversity captured by eDNA also have a higher number of species detected by RAM.

Further studies looking in more detail at family bias on a broader range of drainages and communities will help to provide more insight on the bias of these primer assays (Kanagawa, 2003; Kelly et al., 2019).

While we used only those species whose sequences had available references shared in common between all targeted gene regions, in actual practice, employment of these primer assays may be swayed by whatever published sequence references are available. There is a benefit to utilizing more than one to target different gene regions in an eDNA metabarcoding survey to optimize likelihood of detection across a variety of degraded fragments of eDNA as well as to maximize reference data when possible.

In the case of the Missouri Ozarks, an optimal combination of primer sets would be one targeting the 12s rRNA gene region, and one targeting the 16s rRNA region. Many eDNA metabarcoding studies use a similar combination of two primer sets and manage a

broad range of coverage for the species in their respective systems (Ficetola et al., 2021; Valdivia-Carrillo et al., 2021). As eDNA often exists in some state of degradation, having two primer sets targeting different gene regions allows for an increased likelihood of being able to amplify fragments of eDNA captured upon sampling (Deiner et al., 2017; Deiner & Altermatt, 2014).

4. VALIDATION OF eDNA METABARCODING: A COMPARISON TO TRADITIONAL SURVEY METHODS IN OZARK STREAMS

4.1. BACKGROUND

Many recent studies have investigated questions relating to the performance of eDNA metabarcoding techniques compared to traditional methods (Antognazza et al., 2019; Gehri et al., 2021; Hallam et al., 2021; McColl-Gausden et al., 2021; Polanco Fernández et al., 2021). Several of these studies have found eDNA sampling techniques to perform as well as or better than previously employed traditional methods in a variety of aquatic systems. However, there are areas within different aquatic systems in which further investigation is needed (Mathieu et al., 2020; Zinger et al., 2019). There are unique characteristics in different systems that may pose challenges to the efficacy of eDNA metabarcoding as a surveying technique (Mathieu et al., 2020).

We set forth to investigate the potential of eDNA metabarcoding of fish communities in Missouri Ozark streams, comparing eDNA side-by-side with traditional methods. These traditional methods consisted of electrofishing and seining, performed annually in the summer by the Missouri Department of Conservation's Resource Assessment and Monitoring teams, or RAM teams.

Our primary objective for this study was to determine if eDNA metabarcoding would provide enough sensitivity to capture sample heterogeneity among small stream environments within one drainage. We also wanted to know how eDNA metabarcoding performed overall directly compared to RAM traditional methods. And lastly, we endeavored to categorize potential seasonal or environmental factors which may affect eDNA metabarcoding performance, including site-level and sample-level variables.

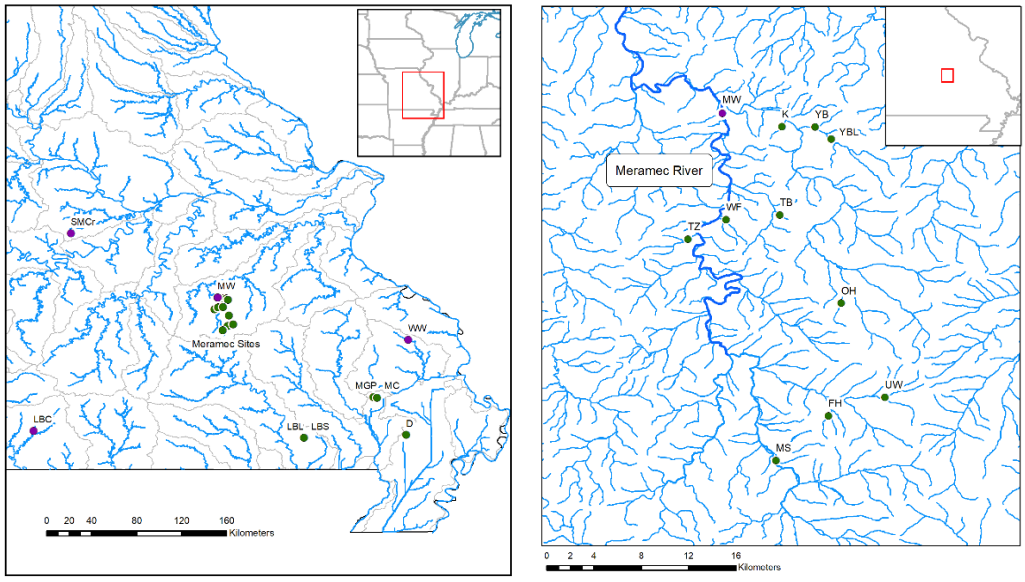


Figure 4.1. Maps of our study sites from 2020-21, the points in purple denote 2020 and green denote 2021.

4.2. eDNA AND TRADITIONAL METHODS

We collected 500 mL water samples in triplicate at three different sections along the stream reach of the RAM sites, including samples at the downstream-most, central, and upstream-most locations, with an effort to sample a variety of microhabitats. Between summers 2020-21, we visited a total of 19 Missouri streams (Figure 4.1). One field negative was taken for each site to monitor contamination, for a total of ten samples. Each sample was transported on ice and stored in a refrigerator within the same day of sampling.

We also sought to collect different site-level and sample level variables for statistical analysis, and to this end, depth and flow were measured at each point of sampling. We also collected the total cumulative drainage area for each site with GIS software ArcGIS. In addition, in ArcGIS software (Esri) we also calculated the percentage

of land use types (i.e., percentage of forest coverage) within that drainage area using public data from USGS (Sohl, 2018).

4.2.1. Extraction. Samples were filtered within 48 hours of collection following a modified protocol similar to the methods described by Valentini et al. (2016) using a 0.45 µm cellulose nitrate membrane in a vacuum filter. The samples were vacuum filtered through nitrocellulose membranes (0.45 µm pore size) and eDNA was extracted from membranes using Qiagen DNeasy Blood and Tissue Extraction kits following manufacturer's instructions with modifications following Hinlo et al. (2017).

All surfaces of equipment were decontaminated in the field as well as in the lab between processing of successive samples, using bleach, which destroys DNA molecules on contact (Goldberg et al., 2016). This included field equipment, water collection vessels, filter apparatus, as well as lab equipment and workspaces that were in contact with water samples. Sample cross-contamination was monitored by periodically processing de-ionized water samples (negative control) (Goldberg et al., 2016; Ruppert et al., 2019) and exotic species samples (positive control) (Klymus et al., 2017; Nakagawa et al., 2018) in place of field-collected water samples. These controls were used to establish baseline expectations for contamination levels in sample data, as well as to establish statistical minimum detection criteria for validation of species detection in field-collected water samples (Klymus et al., 2017; Valentini et al., 2016). We managed PCR product contamination by conducting all pre-PCR processing steps in a separate, PCR-product-free research lab space. DNA extractions and PCR reactions were conducted in the clean room with separate dedicated equipment. All post-PCR steps, including PCR product

quantification, dilution and gel electrophoresis, were conducted in a separate post-PCR lab space. Equipment, samples, and reagents were used with minimal transport between labs.

4.2.2. PCR. Using a high fidelity AmpliTaq Gold 360 (Thermo Fisher Scientific) polymerase, the extracted eDNA were PCR amplified with published universal fish mitogenome primers, Mifish 12s rRNA primers (Miya et al., 2015) and 16s rRNA primers Chord_16s_F_TagA and Chord_16s_R_short (Deagle et al., 2009), tagged at the 5' end with binding sites for Illumina sequencing primers. We employed these primers following their respective published cycling protocols modified slightly for optimal amplification with increased cycle numbers. We amplified extracted eDNA samples with PCR reactions in a total of 50 µL volume divided into six independent replicate reactions with each primer pair. PCR products were pooled and verified with gel electrophoresis, then cleaned using AMPure XP magnetic beads following manufacturer protocols (Beckman Coulter). Samples were quantified with Qubit 3.0 High Sensitivity dsDNA assay (Thermo Fisher Scientific) and concentrations were normalized to an acceptable range for library indexing and MiSeq sequencing (Illumina) services provided by the University of Missouri Genomics Technology Core.

4.2.3. Species Identification. Illumina sequencing performed at the University of Missouri DNA Core, which provided raw forward and reverse reads. We investigated a few options of metabarcoding analysis pipelines, and settled on using the Barque v1.7.3 (https://github.com/enormandeau/barque) pipeline for DNA sequence alignment and identification due to this program's flexibility and usability. Sequences were aligned, trimmed, filtered, and identified following the author's recommendations, customizing the scripts to our two different primers and their respective PCR product sizes. Most reference

sequences were retrieved from Genbank. Additional references were derived from our own unpublished sequences. We compiled a curated reference database of available Missouri fish DNA sequences for each drainage with a list containing known species in that area (Pflieger & Smith, 1997; Hrabik, 2022, in press). This allowed for filtration of any ambiguous reads that could be ruled out by location. Separate analyses were conducted using reference databases which contained the exotic species of the positive control to monitor for potential sequence error. Any sequence reads that appeared in the field or lab negative results were subtracted out from the overall detection to address possible contamination, though this process did not remove any species from our analyses. Species were identified with 97% sequence match to references. There were a small number of taxa between the two primers that could not be distinguished to the species level, including *Campostoma oligolepis* and *C. anomalum.* In some instances, the bass species *Micropterus salmoides, M. dolomieu,* and *M. punctulatus* would be called in the same multiple hit identification (i.e., that one sequencing read was matched with 97% identity between multiple closely related species). Where these instances cannot be ruled out by location due to habitat range overlap, these are reported as *Campostoma sp.* and *Micropterus sp.*, respectively, however, we do retain the RAM identification of these to the species level. Any instance of ambiguity in which a portion of sequencing reads matched to multiple results, but the species involved were also identified at the species level, the former reads were removed. Any ambiguous results that were both unidentified to the species level and contained less than 100 total reads across all samples were removed, as well as any single sample that had less than 10 reads was removed. Any sample that had fewer than 100 reads total across all species were removed as outliers of poor quality, although this only occurred

for one sample and didn't appear to affect overall trends. Upon these filtrations, both the Mifish 12s and Chord16s primer results were combined for downstream analysis.

4.2.4. Statistical Analysis. A major objective of this project was to determine whether estimates of fish community composition were influenced by sampling method, spatial location, or other environmental variables. In order to compare how each sampling method (12s and 16s primers for eDNA combined, electrofishing and seining methods for RAM combined) surveyed the fish communities within a drainage, we evaluated community similarity with NMDS based on a Jaccard-dissimilarity transformation (Borcard et al., 2018) of the raw clustered OTU sequence reads as well as non-transformed RAM results, and an analysis of similarities (ANOSIM) in the R vegan package (Oksanen et al., 2022). We generated UPGMA clusters also based on a Jaccard-dissimilarity where differences in OTUs were measured using the pvclust R Package (Suzuki & Shimodaira, 2006). This UPGMA analysis provided approximately unbiased probability values, which are the values assigned by the multiscale bootstrap resampling, as well as the bootstrap p-values which are the frequency that a cluster appears on the boostrap replicates. To observe changes in species richness, we used the species accumulation function with the Vegan R package (Oksanen et al., 2022), similar in methods to McColl-Gausden et al (2021) and following modified R scripts from Valdivia-Carrillo et al (2021). Another NMDS was generated based on Jaccard-dissimilarity to evaluate differences in summer and winter samples to observe overall trends and identify potential species which could drive the changes in community composition.

We developed a mixed model in JMP Pro 16.1 to investigate which environmental variables were significantly associated with species richness. We also evaluated whether

there were significant differences in species richness reported between summer and winter. We determined the predictor variables of season, velocity, and depth to be fixed effects, land use and watershed area as site-level environmental fixed effects, and the site itself as a random effect. Keeping the site random accounted for the hierarchical structure of the data with multiple samples collected within each site that are likely more similar to each other than to samples collected at different sites. A significance level of $\alpha = 0.05$ was used for all hypothesis tests.

Any statistics performed on seasonal variation included the Meramec sites FH, K, MS, OH, TZ, UW, WF, YB, and YBL which were sampled in the summer and following winter. The seasonal UPGMA clustering analysis also included the site MW which was sampled in the summer of 2020, the following fall and spring, but this site was not included in any other seasonal models.

We also compiled the number of each species detected in the most common families across all of our drainages to evaluate eDNA community bias. We also generated a heat map of the most common detections of both RAM and eDNA results (see Appendix).

4.3. COMPARISON OF SAMPLING METHODS RESULTS

With our field and lab negative controls, we found negligible amounts of cross contamination within samples, typically under 10 reads in any instance, but most often zero. It was observed that in the rare instance of contamination, reads correlated with the other highest read counts of species for a site, suggesting that the most common species in a system would be most likely to have amplification in our controls. We had several detections in field samples of the invasive carp species, *Hypophthalmichthys nobilis* and

Ctenopharyngodon idella, which were removed from sites which would be too small to support these species. We categorize these results as possible contamination or the unlikely possibility of run-off from nearby stocked ponds in fields. The results of our exotic species controls also did not indicate any underlying instances of contamination occurring within the lab steps; no raw exotic DNA reads above our minimum cutoff of 10 were found in any field samples.

We found a total of 94 species across all drainages with eDNA sampling, and RAM detected a total of 87. Within the Meramec drainage specifically, eDNA found 68 species where RAM found 38. Overall, RAM detected 15 species that weren't described by eDNA. Conversely, our eDNA results found 15 unique species not found with RAM. For every 1 species detected with RAM, we detected approximately 2 with eDNA metabarcoding on average across all of our sites from 2020 and '21. In just the Meramec drainage, for every 1 RAM species, we detected approximately 2.5.

For the state-wide results, eDNA detected more species in most of the families present than RAM surveying, particularly for the family Percidae (Figure 4.2). Per site, on average we had approximately 2.4 more Percidae species detected than RAM. On average, eDNA metabarcoding found approximately 2.2 more Centrarchidae species, and 1.4 more Leuciscidae species per site than were found by RAM.

4.3.1. Community Clustering of Meramec Sites. Our analyses which clustered the different representation of species present within streams in the Meramec drainage by either eDNA metabarcoding or RAM methods are shown in Figure 4.3. We found that a similar cluster forms within the two analyses for the sites MS and MW as separate from the remaining sites. However, the au p-value for the RAM clusters is 96, and the au p-value

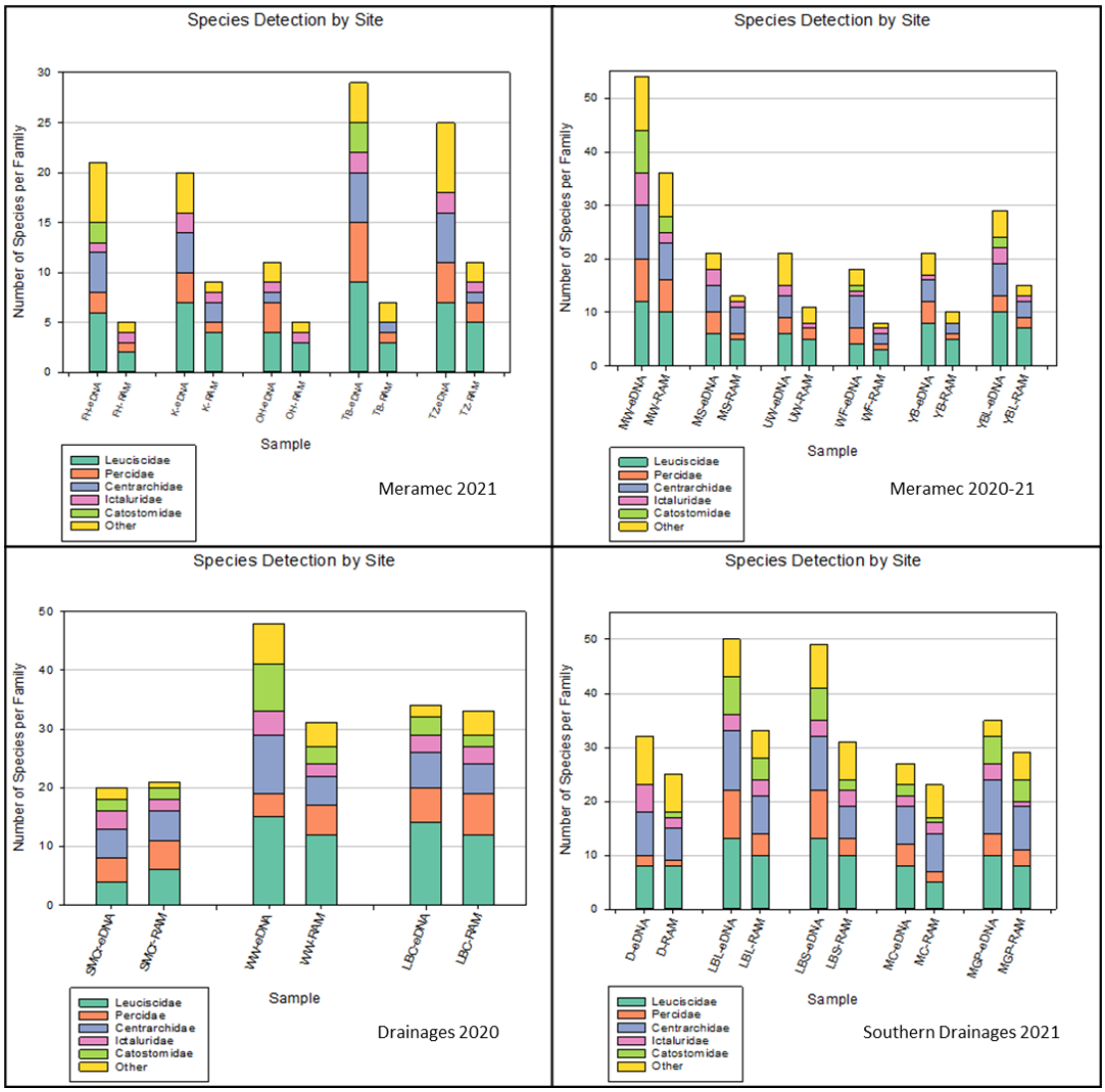


Figure 4.2. Species detection across all sites grouped by number of species detected by the most common families for both eDNA results and RAM results for each site. Note the y-axis is scaled differently for each analysis for visual clarity.

for this branch with eDNA is 87. There is also a separation of site WF from the other streams remaining in the eDNA UPGMA cluster, where WF clusters more closely with YB and YBL for RAM.

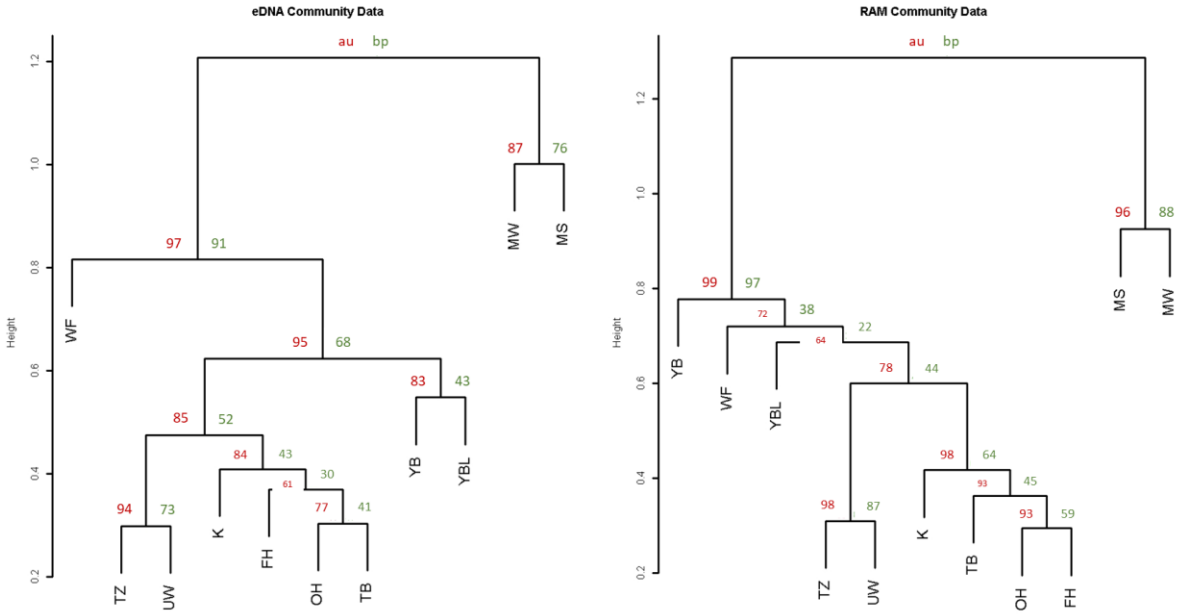


Figure 4.3. UPGMA clustering figures for eDNA and RAM communities based on Hellinger transformed OTU dissimilarities. Red approximately unbiased (au) p-values are on the left of each branch, and green bootstrap (bp) p-values on the right of each branch.

Our analysis similarity of communities using NMDS and ANOSIM statistics (Figure 4.4) display a significant clustering by similarity of Meramec sites by either RAM or eDNA methodology. Similar results were obtained by analyzing all species (Figure 4.4a) or by analyzing only species detected by both RAM and eDNA methods (Figure 4.4b). Either way of including or excluding species on the basis of being shared between methods, they each cluster significantly according to our ANOSIM test.

4.3.2. Species Richness for eDNA vs RAM. Nearly all sites could capture the same number of species detected by RAM within as few as two sampling units according to our species accumulation curves (Figure 4.5). Only two Ozark sites, SMCr and MC, required more than that sampling effort to capture the species richness managed by traditional methods.

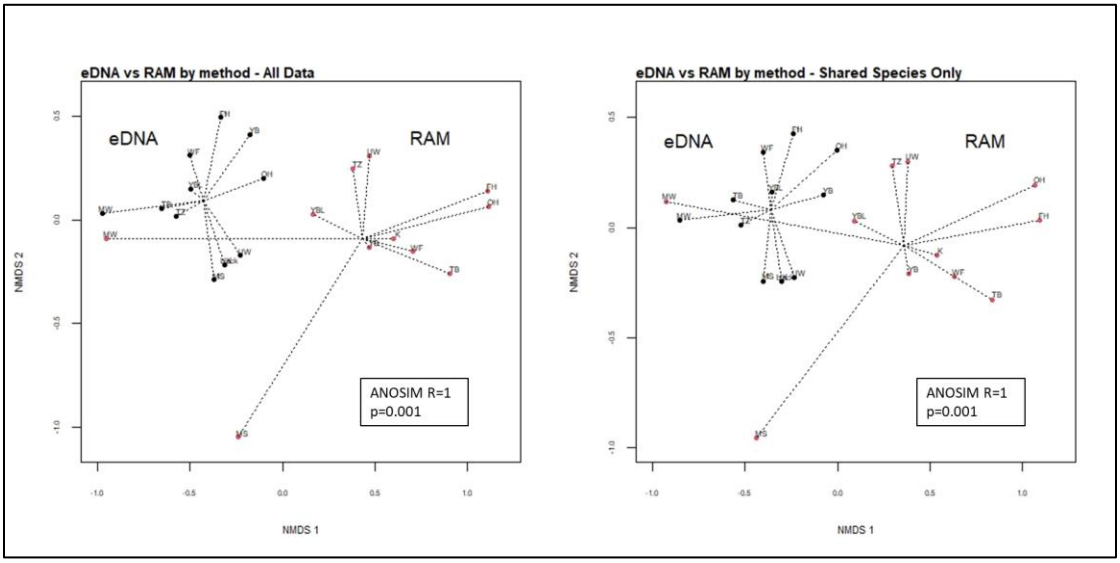


Figure 4.4. NMDS plots of the communities in the Meramec drainage compared by our two primers combined and the RAM methods combined. A) (left) All species included and B) (right) with only the species detected by both methods. An ANOSIM value of R=1 and p-value of 0.001 was reported for both analyses.

4.3.3. Seasonal eDNA Variation. We were unable to obtain a winter sample for site TB, one of the tributary sites in the Meramec drainage. Altogether, we sampled 9 sites in the summer and winter. We were interested in testing for seasonal variation in species detection. Seasonal variation might result if communities vary by season, or if species detectability varies by season. We compared eDNA metabarcoding communities between summer and winter with UPGMA clustering (Figure 4.6). The large river sites, MS and MW, clustered together in the summer for MS and all seasons for MW. In general, a similar community composition was captured for both summer and winter for a majority of the other sites. One exception to note is MS in the winter, which had a more similar species composition to the other sites WF, YB, and YBL in the winter than summer. Site MW was sampled during summer and the subsequent fall and spring rather than summer and winter

like the other Meramec sites, so it is only included in the seasonal UPGMA cluster analysis, but not in any analyses which look at overall species richness between summer and winter.

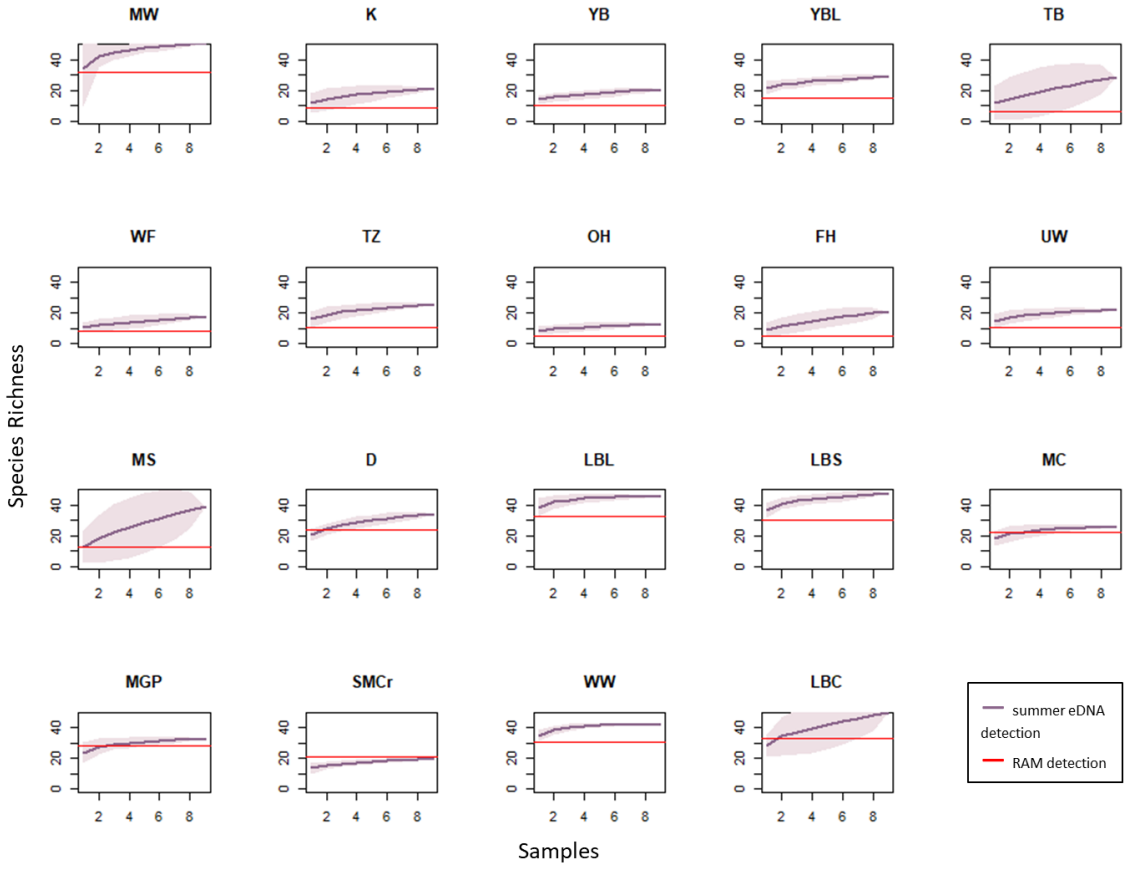


Figure 4.5. Species accumulation curves for all sites. The eDNA curves are the results of the combination of our two primers, and the red line is overall RAM detection with both electrofishing and seining.

We generated species accumulation curves for the species richness captured in the summer vs winter eDNA samples (Figure 4.7). These also represent an overall trend of higher species richness detected in the summer over winter, with a few exceptions in MS which was the opposite, as well as YB and YBL which were relatively the same.

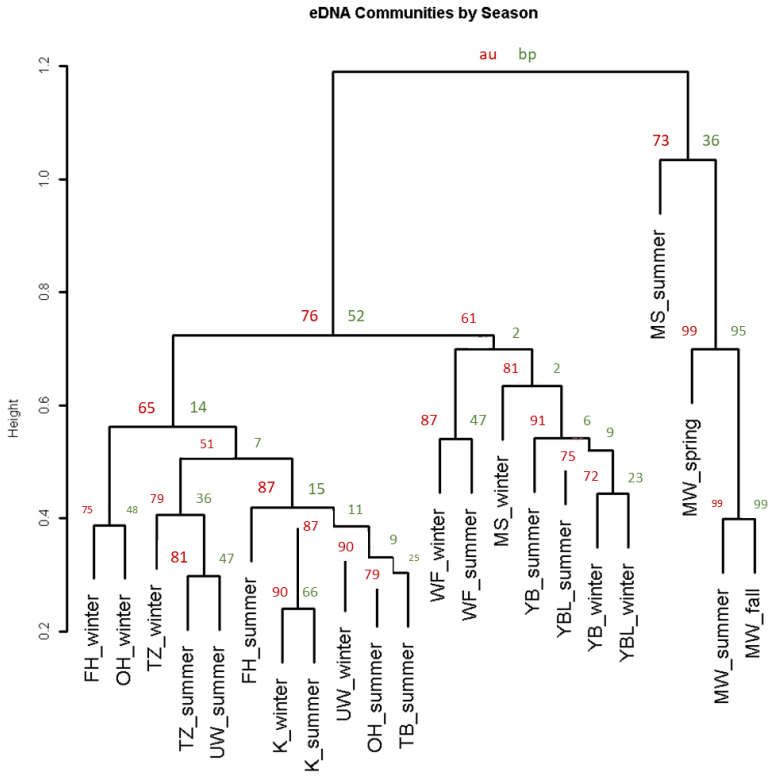


Figure 4.6. Clustering of eDNA results of Meramec drainage sites sampled in the summer and winter of 2021. Most fall into tight clusters, with some exceptions like MS, UW, and others. One Meramec site, MW, was sampled in summer of 2020, with resamples in the following fall and spring, and these seasonal results are reported here. Red approximately unbiased (au) p-values are on the left of each branch, and green bootstrap (bp) p-values on the right of each branch.

From our mixed model JMP analysis, we found a significant difference in average species richness between summer and winter (p=0.0035), after accounting for the environmental variables. Average species richness was estimated to be 0.6146 units higher (95% CI: between 0.2059 to 1.023 units higher) in summer compared to winter (Figure 4.8). The estimated average species richness is 33.9 in winter and 34.5 in summer. This trend was found significantly positive across all of our sites, despite site MS having a negative correlation.

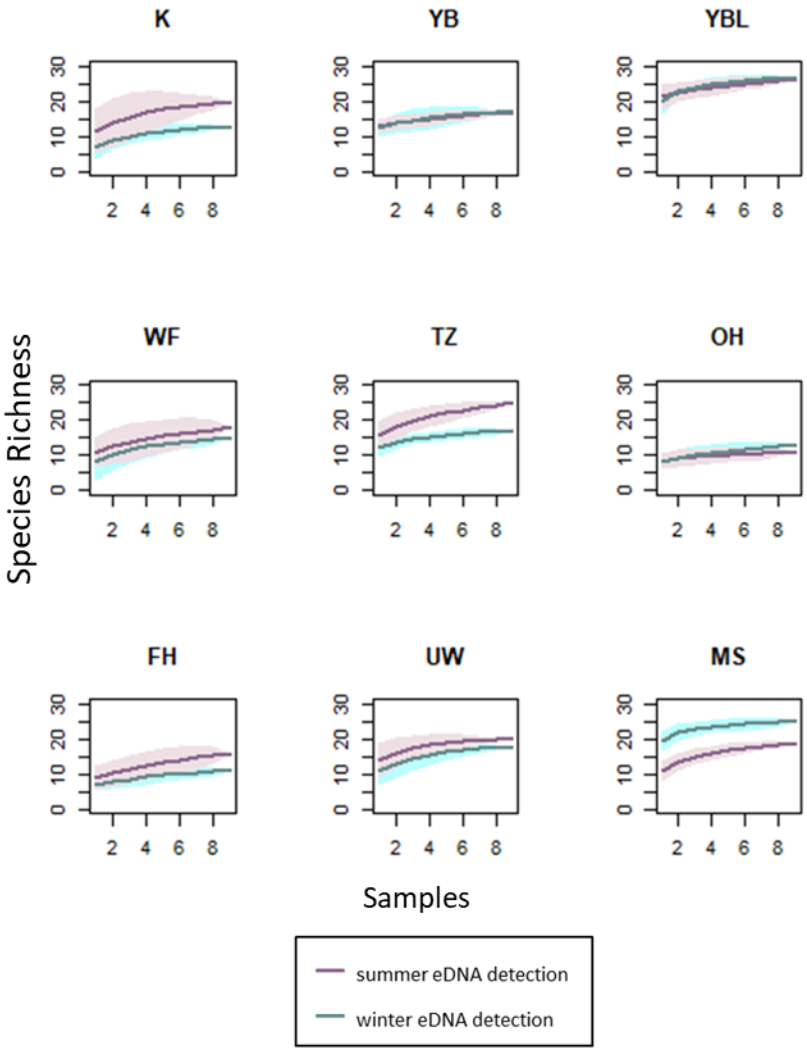


Figure 4.7. Species accumulation curves for summer and winter eDNA. Generally, higher richness coincided with summer sampling for most sites.

For the environmental variables that have a significant effect on the species richness, we found depth (p=0.0127) and velocity (p=0.0105) had a positive significant association with species richness after adjusting for other variables (Figure 4.9), whereas

site-level land use and the cumulative drainage area were not significantly associated with species richness (Table 4.1).

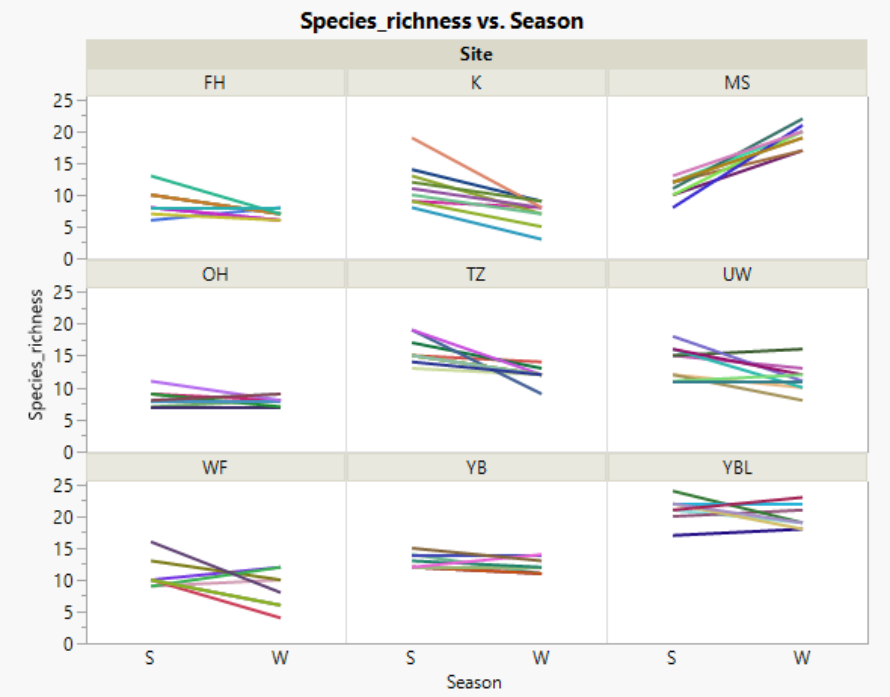


Figure 4.8. Species richness vs season. Each colored line represents one of the nine samples and their trends for each site. MS is the only site where overall species richness increased in the winter.

The intercept field describes the average species richness in the winter, and the average in the summer is the intercept estimate plus the estimate for the season [S] term (Table 4.1).

The NMDS analysis of our species detected between summer and winter show a subtle cluster at each individual site that is different between seasons (Figure 4.10). However, when all sites are combined, the overall composition is not distinctly clustered across the two seasons. It is likely that these clusters at the site level are driven by an overall

drop in sequencing reads which may cause a few species to no longer be detected, but which are still detected to some level across all sites and both seasons. In this NMDS analysis, where there are fewer than 9 points displayed for a certain season, our analysis found them to be so similar that they are placed in the same position, i.e., no samples are missing or dropped as outliers.

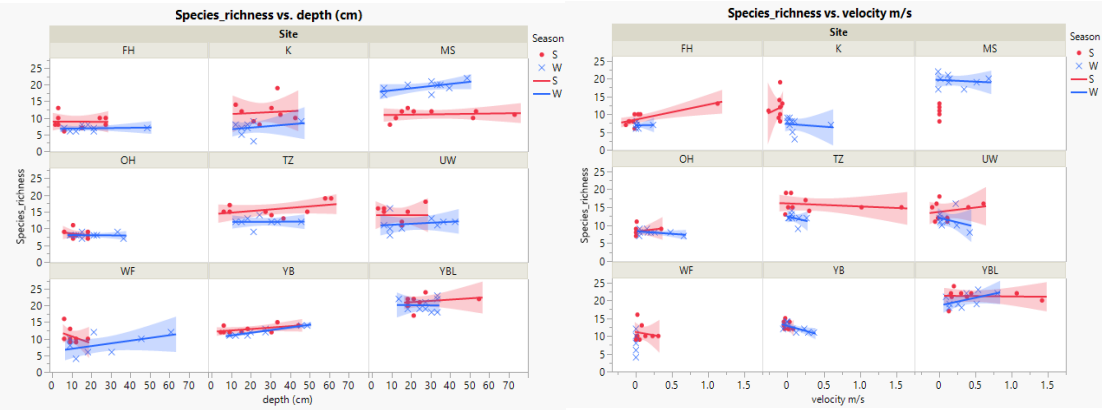


Figure 4.9. Species richness vs depth (left) and species richness vs flow (right) for both seasons across each site.

Table 4.1. Significant variables in species richness for eDNA variance across the sites with summer and winter sampling. The estimate is relative to the baseline, which is set to the winter season results, so reported values are the difference between those results.

Term	Estimate	Std Error	DFDen	t Ratio	Prob>|t|	95% Lower	95% Upper
Intercept	33.896063	12.132071	5.9	2.79	0.0319*	4.0974477	63.694677
Season[S]	0.614631	0.2068606	150.0	2.97	0.0035*	0.2058946	1.0233675
depth (cm)	0.043125	0.0171089	152.7	2.52	0.0127*	0.0093243	0.0769256
velocity m/s	2.3758401	0.9167186	153.7	2.59	0.0105*	0.5648483	4.1868318
land use (forest cover %)	-0.28056	0.1285988	5.9	-2.18	0.0728	-0.596714	0.0355953
log(area)	0.9798729	0.7202654	5.9	1.36	0.2231	-0.787652	2.7473981

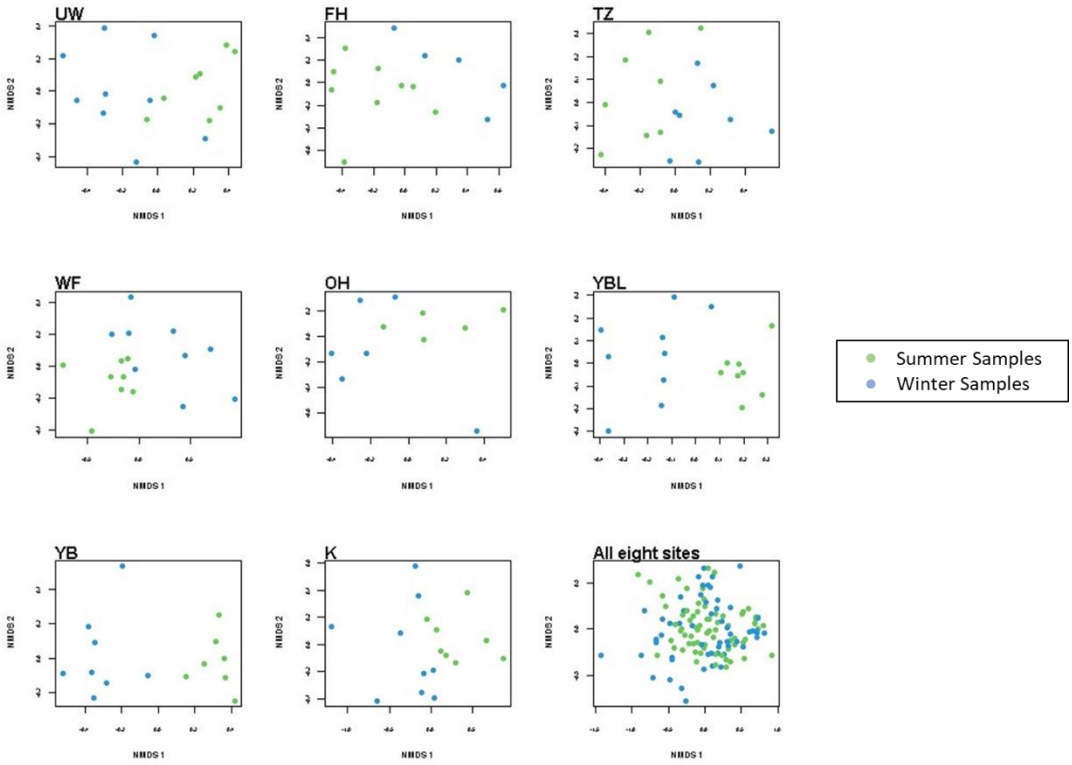


Figure 4.10. NMDS plots of summer and winter community distributions in the Meramec Drainage.

4.4. eDNA PERFORMANCE DISCUSSION

Our primary objective of this study was to investigate the potential for eDNA to be sensitive enough to detect sample heterogeneity among small streams within one drainage. Our results indicate that eDNA can provide a similar insight into overall communities present compared to RAM, driven by key indicator species. We found optimal representation of taxa present in Missouri Ozark streams with a combination of Mifish 12s primers and Chord16s 16s primers. We also wanted to know how eDNA metabarcoding performed overall directly compared to RAM traditional methods, and on average, found that eDNA metabarcoding was able to offer more species richness on per site than RAM.

And lastly, we endeavored to categorize potential seasonal or environmental factors which may affect eDNA metabarcoding performance, including site-level and sample-level variables. In this effort, we found significant factors in eDNA performance include season, flow, and depth, with further work needed for other site-wide conclusions. Given that there were unique species detected by both of the methods, a combination of RAM and eDNA metabarcoding may offer a more complete composition of fish asssemblages in the Ozarks.

Further investigation into family bias is ongoing, though these results do not seem to indicate any significant areas of underrepresentation compared to RAM results. However, our reference database is still incomplete for a few remaining Missouri fish, and it has been noted as an important consideration for ecological conclusions (Polanco Fernández et al., 2021; Valdivia-Carrillo et al., 2021). However, our most complete reference database is the Meramec Drainage.

4.4.1. Key Contributor Species. When taxa were found by the RAM team but not by eDNA metabarcoding, these species tended to be fairly rare or cryptic species, like the American eel (*Anguilla rostrata)* or a lamprey (*Icthyomyzon sp.)*, which were only found once at the sites they occurred. It's possible that the physiology or ecology of these organisms make them less optimal for detection by eDNA metabarcoding, and for these two specifically. If an organism is fairly recluse or not very active in the water column, it is not surprising that their eDNA could have not accumulated in sufficient volume for capture at the point of sampling. There is also a chance that species such as these have mitochondrial genomes which are far more divergent that other fish which are detected by our primers. Nester et al. (2020) found that for cryptic seashorse taxa in marine environments, they had to modify their 12s and 16s primer sets to capture members of this

family specifically. The sets which they modified were a similar combination of primers to our experiment, Mifish-U (Miya et al., 2015) and 16s Fish (Berry et al., 2017; Deagle et al., 2007), the first of which we employed, the second being also in the 16s gene region. Therefore, further exploration on possible modifications to the primers used in our study may be beneficial to capture any rare taxa of interest that were not detected by eDNA metabarcoding.

For the clusters formed by sites in the Meramec drainage, there were a handful of species detected which were likely key contributors to how different sites were from one another. For example, for the eDNA UPGMA results, site WF branched out singularly, where it was grouped more closely with other sites with RAM sampling. An obvious difference with eDNA metabarcoding for WF is that we did not detect the fantail darter, *Etheostoma flabellare*, at the same scale of all other Meramec sites. Most sites found *E. flabellare* in every sample, 9/9 times, but for the summer we only found WF one time, 1/9 (See Appendix Table 2 for the heatmap of RAM detection, and Appendix Table 3 for the heatmap of eDNA detection). This species which likely provided interesting clustering in the UPGMA analysis of eDNA results was not detected at all by RAM in the same sites. Therefore, this species may also have contributed to the different clusters of RAM and eDNA within our NMDS analysis. Interestingly, we also had a difference in the MS site for the summer with that *E. flabellare,* only detecting it in 2/9 samples, but in the winter, it was detected 9/9 times. MW had instances of *E. flabellare* in 6/9 samples in the summer. These observations support that this species may be a key driver in some of the clustering with our UPGMA analysis. Another species to note was *Fundulus catenatus*, which was only found in the larger river sites of MW and MS. Some species were found common

across all samples within the smaller tributaries but not as common for MW and MS during summer, including *Chrosomus erythrogaster, Cottus bairdii*, and *Cottus carolinae*. In addition to differences, there were also species like *Etheostoma spectabile,* which was detected in almost 9/9 samples across all eDNA as well as found at nearly all RAM for all Meramec sites.

4.4.2. Site Interconnection. Drainage systems are comprised of bifricating branch networks. Sites on the same branches might be expected to have similar species communities by virtue of eDNA or species movement between them. For example, YB and YBL occur on the same branch, Yankee Branch, and so will likely have many of the same species traveling back and forth between the sites. However, we still did capture difference between the sites, likely based on the difference in average size and depth, for example we detected the northern hogsucker, *Hypentelium nigricans,* in YBL both in the summer and winter, 9/9 and 7/9 respectively, but was not found in either season for YB. MS was our most upstream site of the drainage, and MW the furthest downstream, both of which occur on the main stem of the Meramec River. There was a high amount of species richness found overall for the MW site. However, we can have a certain degree of confidence that the influence of community composition is not always influenced by downstream positioning, because while the species *Chrosomus erythrogaster* was detected at all tributaries upstream of the site MW, it was not detected at MW itself. It could be beneficial to have more investigation on the differences in community members observed along the Meramec River, and to see if eDNA metabarcoding will capture an interesting gradient of species.

There did not seem to be any specific species which dropped out between summer and winter that would be the main source of influence on the significant difference in

season. In general, there is an overall drop in rates of detection across the nine samples in the winter, across all species. The results of our NMDS (Figure 4.10) seem to suggest that there may be some species within one site which may vary in detection rates between seasons, but the overall trend within the Meramec sites is that there are similar species detected across the drainage. Species richness significantly decreasing in the winter is likely due to an overall drop in sequencing reads and detection rates across all sites.

Within our modeling of environmental factors, we found that flow is a positively significant variable in relation to species richness. This is supported by the species richness exhibited by the site MS which measured a zero m/s flow rate at every sampling location during the summer, but averaged 0.2 m/s across all points during the winter, and was one of the only sites to display this swap as well as a higher richness during the winter. However, one other site, WF, had intermittent dryness upon sampling in the winter, and sampling was focused on the remaining upstream half of the site, with flow being observed to be lower than during the summer sampling. Both of these observed differences appear to influence our results in which they cluster differently across the different UPGMA clusters as well as their species richness changing with the decrease in flow for the respective season. It is unclear if this result is better explained ecologically or rather that the increase of flow at a site allows for a greater transport of eDNA suspended in the water column from a greater distance, and the increase in richness accounts instead for a broader range of eDNA travel. This question has been suggested by others as an area needing further consideration when using eDNA to interpret ecological results (Barnes & Turner, 2016; Deiner & Altermatt, 2014). Another interesting observation is that the au and bp values for the seasonal UPGMA clustering tend to be lower than those for the summer

eDNA and RAM UPGMA clusters. This lower value may imply an overall higher level of similarity across all sites with eDNA compared to the difference provided within just one season.

It is likely that the statistical significance of depth follows a similar line of reasoning as flow, where there is a question of whether the significance is driven by ecological or environmental factors. Species richness increasing with depth may either correspond with larger sites because they support a higher number of species, or that perhaps an increase in depth has the potential to provide more optimal conditions for eDNA transport from a larger distance. This could be factors like lower temperatures or potential cover from UV light degradation, which are known to negatively impact eDNA lingering in the water column (Dejean et al., 2011). While we also evaluated the size of drainage area as another possible variable which could affect species richness, we did not observe a significant correlation in our analyses of the Meramec drainage. This could mean that we need a larger variety of sites in order to draw meaningful conclusions on the relationship between drainage area and species richness.

5. CONCLUSIONS

Our research contributes to and supports the results of similar studies which suggest that eDNA metabarcoding provides a powerful supplement or alternative to traditional survey methods in the identification of species present in freshwater fish communities (Deiner et al., 2017; Doi et al., 2021; Ficetola et al., 2008; McColl-Gausden et al., 2021; Nakagawa et al., 2018). The use of MiFish 12s rRNA (Valentini et al., 2016) and Chord16s rRNA (Deagle et al., 2009) universal primers worked well for an optimal level of taxon discrimination for fish species in the Missouri Ozarks. Our findings suggest that eDNA can provide a community composition that is sensitive enough to be shaped by key contributor species. We also found that eDNA detected a greater number of species per site than RAM, with an overall average of 2 species detected by eDNA for every 1 found with RAM. In the Meramec drainage, this ratio was as high as 2.5 for every 1. In our work to understand which environmental factors affect the species richness detected by eDNA metabarcoding, we found significance in season sampled, flow, and depth, with further work needed for other site-wide conclusions. We feel the data support a combination of RAM and eDNA metabarcoding to offer a more complete composition of fish asssemblages in the Ozarks. Further work is ongoing to investigate possible family bias in detection with eDNA metabarcoding, as well as the addition of reference sequences for Missouri fish species.

APPENDIX

Table A.1 Our sample IDs, the full name we gave to each site, drainage, year sampled, as well as if seasonal sampling results were reported. *MW had a smaller subset of seasonal sampling done the following fall and spring, rather than a replicate of 9 in the winter like the other sites. *WF was sampled the following winter, however, half of the downstream site was too dry to sample, and the upstream portion remaining was sampled more heavily to reach the full 9 samples.

Sample ID	Site Name	Drainage	Lat/Long Coordinates	Year	Seasonal Sampling
LBC	Little Beaver Creek	Jamesfinley	36.803117, -92.909002	2020	
SMCr	South Moreau Creek	Moreau	38.385195, -92.610372	2020	
WW	Whitewater River	Headwater Diversion	37.534965, -89.915346	2020	
MW	Meramec at Wescoe	Meramec	37.873906, -91.437700	2020	X*
FH	Fortune Hollow	Meramec	37.6435940, -91.3576177	2021	X
K	Kallumbah Creek	Meramec	37.8634368, -91.3922524	2021	X
MS	Meramec at Salem	Meramec	37.6093184, -91.3980909	2021	X
OH	Onion Hollow	Meramec	37.729728, -91.347766	2021	X
TB	Taff Branch	Meramec	37.797195, -91.39533	2021	
TZ	Taft Ziske	Meramec	37.7783664, -91.4638965	2021	X
UW	Waterfork Creek	Meramec	37.657800, -91.314855	2021	X
WF	Wurdack Farm	Meramec	37.7934503, -91.4357526	2021	X*
YB	Yankee Branch	Meramec	37.8625516, -91.3763287	2021	X
YBL	Yankee Branch Litz	Meramec	37.8537531, -91.353040	2021	X
LBL	Little Black Long	Current	36.7484057, -90.7405203	2021	
LBS	Little Black Short	Current	36.7483216, -90.7459328	2021	
D	Ditch at 724 Dexter	St. Francis	36.774151, -89.929972	2021	
MC	McGee Creek	St. Francis	36.773551, -89.930546	2021	
MGP	McGee Creek Pollock	St. Francis	37.0705115, -90.1639829	2021	

Table A.2 Heat map of detection by RAM methods, both electrofishing and seining combined. Color scale relative to each site to account for variation in total biomass of different streams. The teal color in the species names indicates species found only by RAM methods.

Species RAM	SMCr	WW	LBC	MW	FH	K	MS	OH	TB	TZ	UW	WF	YB	YBL	LBL	LBS	D	MC	MGP
Ambloplites_ariommus	0	2	0	0	0	0	0	0	0	0	0	0	0	0	2	9	0	0	0
Ambloplites_constellatus	0	0	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ambloplites_rupestris	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ameiurus_melas	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0
Ameiurus_natalis	4	1	0	1	0	0	8	0	0	0	0	0	0	0	3	4	22	9	2
Anguilla_rostrada	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Aphredoderus_sayanus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	7	9	6
Campostoma_anomalum	1292	29	448	67	0	10	25	3	14	3	30	26	3	4	71	59	0	22	82
Campostoma_oligolepis	1	91	343	0	0	0	0	0	0	0	0	0	0	0	36	39	0	3	47
Catostomus_commersonii	15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Centrarchus_macropterus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	3
Chrosomus_erythrogaster	0	0	0	0	90	150	0	208	150	119	146	23	20	27	0	0	0	0	0
Cottus_bairdii	0	0	0	4	26	8	0	31	75	24	49	5	4	24	0	0	0	0	0
Cottus_carolinae	0	3	19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cottus_hypselurus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	1	0	0	0
Cottus_immaculatus	0	0	72	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cyprinella_galactura	0	0	18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cyprinella_lutrensis	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cyprinella_venusta	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	58	0	0
Cyprinella_whipplei	0	61	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cyprinus_carpio	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0
Erimystax_harryi	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Erimyzon_oblongus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	20	0
Erimyzon_sucetta	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	49
Esox_americanus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7	8
Esox_niger	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	3	0
Etheostoma_blennioides	8	0	18	14	0	0	0	0	0	0	0	0	0	0	3	4	0	0	0
Etheostoma_caeruleum	0	23	83	18	0	0	0	0	0	1	2	0	0	1	23	5	0	0	0
Etheostoma_chlorosoma	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Etheostoma_erythrozonum	0	0	0	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Etheostoma_euzonum	0	0	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Etheostoma_flabellare	35	13	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Etheostoma_gracile	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Etheostoma_nigrum	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Etheostoma_proeliare	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	2	0
Etheostoma_spectabile	255	12	21	1	4	13	11	0	3	1	9	5	4	7	0	0	0	28	11
Etheostoma_uniporum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	22	0	0	0	0
Etheostoma_zonale	0	6	1	4	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0
Fundulus_catenatus	0	44	24	31	0	0	0	0	0	0	2	0	0	0	2	1	0	0	0
Fundulus_dispar	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Fundulus_olivaceus	3	5	1	4	0	0	0	0	0	1	2	0	14	6	34	31	12	72	33
Gambusia_affinis	0	1	0	17	0	0	26	0	0	0	0	0	0	0	0	0	14	0	0
Hybopsis_amblops	0	176	20	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hypentelium_nigricans	2	18	68	9	0	0	0	0	0	0	0	0	0	0	11	13	0	0	0
Ichthyomyzon_sp	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0

Table A.2 Heat map of detection by RAM methods, both electrofishing and seining combined. Color scale relative to each site to account for variation in total biomass of different streams. The teal color in the species names indicates species found only by RAM methods. (Cont.)

Species RAM	SMCr	WW	LBC	MW	FH	K	MS	OH	TB	TZ	UW	WF	YB	YBL	LBL	LBS	D	MC	MGP
Labidesthes_sicculus	0	5	0	3	0	0	0	0	0	0	0	0	0	0	26	14	2	0	0
Lepisosteus_oculatus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9	0	0
Lepisosteus_platostomus	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lepomis_cyanellus	62	10	3	10	0	9	156	0	10	0	0	1	5	6	5	11	8	8	17
Lepomis_gulosus	0	0	0	1	0	0	0	0	0	0	0	0	0	0	2	1	2	1	2
Lepomis_macrochirus	54	0	2	36	0	1	48	0	0	0	0	13	13	6	7	0	6	16	43
Lepomis_megalotis	4	27	72	189	0	0	78	0	0	1	0	0	0	0	100	99	29	57	145
Lepomis_microlophus	0	1	0	0	0	0	240	0	0	0	0	0	0	0	0	0	0	1	0
Lepomis_miniatus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	4	22	0	11
Luxilus_chrysocephalus	0	11	2	35	0	0	0	0	0	0	0	0	0	1	70	31	0	0	5
Luxilus_pilsbryi	0	0	521	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Luxilus_zonatus	0	375	0	97	0	7	0	0	0	73	54	0	1	25	56	20	0	0	0
Lythrurus_umbratilis	0	0	0	0	0	0	31	0	0	0	0	0	0	0	42	16	0	901	491
Micropterus_dolomieu	0	5	29	22	0	0	0	0	0	0	0	0	0	0	5	12	0	0	0
Micropterus_punctulatus	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Micropterus_salmoides	8	0	0	2	0	0	26	0	0	0	0	0	0	2	0	0	0	1	3
Minytrema_melanops	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	2
Moxostoma_duquesnei	0	12	0	17	0	0	0	0	0	0	0	0	0	0	4	4	0	0	11
Moxostoma_erythrurum	0	3	10	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7
Nocomis_biguttatus	0	0	46	3	0	0	0	0	0	8	3	0	4	3	18	0	0	0	0
Notemigonus_crysoleucas	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	55
Notropis_atherinoides	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	6	0	0
Notropis_boops	0	156	0	129	0	0	286	0	0	0	0	0	0	0	11	105	0	25	137
Notropis_buccatus	0	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Notropis_greenei	0	0	0	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Notropis_nubilus	16	219	90	29	0	0	0	0	0	0	0	0	0	6	10	22	0	0	0
Notropis_percobromus	0	5	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Notropis_telescopus	0	4	9	0	0	0	0	0	0	0	0	0	0	0	1	129	0	0	0
Notropis_texanus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0
Notropis_volucellus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	16	0	0
Noturus_albater	0	0	4	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0
Noturus_exilis	38	19	8	3	4	4	0	8	0	8	24	1	0	35	5	2	0	0	0
Noturus_flavater	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Noturus_gyrinus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	17	0	0
Percina_caprodes	0	0	48	4	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0
Percina_maculata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10
Phenacobius_mirabilis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Pimephales_notatus	0	133	2	38	0	0	43	0	0	0	0	0	0	0	0	0	8	0	61
Pimephales_promelas	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pimephales_vigilax	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Pomoxis_nigromaculatus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Semotilus_atromaculatus	98	0	0	0	18	39	1	100	57	13	20	52	5	54	5	23	0	14	16

Table A.3 Heat maps of detection by eDNA methods, both primer (12s and 16s) results combined. Values are based on presence and absence, summed for each site to provide a scale of detection rates from 0-9, where 9 is the most commonly detected. Orange highlight indicates species found only by eDNA methods. Table A (top) is the sites from 2020 and 2021 Meramec Drainage, and Table B (bottom) is the southern 2021drainages. The list of species is the same between the two.

Species eDNA	LBC-S	SMCr-S	WW-S	MW-S	FH-W	FH-S	K-W	K-S	MS-W	MS-S	OH-W	OH-S	TB-S	TZ-W	TZ-S	UW-W	UW-S	WF-W	WF-S	YB-W	YB-S	YBL-W	YBL-S
Ambloplites_ariommus	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ambloplites_constellatus	7	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Ambloplites_rupestris	1	0	0	8	0	1	0	0	0	0	0	0	1	0	0	5	0	0	0	0	1	0	0
Ameiurus_melas	1	2	5	2	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	2
Ameiurus_natalis	6	8	9	7	0	0	0	3	7	9	0	0	2	2	3	0	2	3	0	2	0	2	8
Amia_calva	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Aplodinotus_grunniens	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Campostoma_sp	9	9	9	7	0	3	5	5	9	4	7	9	9	9	9	9	9	9	9	9	9	9	9
Catostomus_commersonii	0	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Chrosomus_erythrogaster	2	0	3	0	9	9	9	9	8	0	9	9	9	9	9	9	9	9	9	9	9	9	9
Cottus_bairdii	1	0	0	5	9	9	5	9	9	0	9	9	9	9	9	9	9	8	9	9	9	9	9
Cottus_carolinae	8	0	9	6	9	9	3	8	9	1	9	9	9	8	9	9	8	6	7	9	8	9	9
Cyprinella_galactura	4	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Cyprinella_spiloptera	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Cyprinella_venusta	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cyprinus_carpio	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dorosoma_cepedianum	0	0	3	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Erimystax_harryi	6	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Erimyzon_claviformis	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Esox_americanus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Etheostoma_blennioides	9	6	7	8	1	0	0	0	1	2	5	0	1	0	2	0	0	0	0	0	1	1	0
Etheostoma_caeruleum	9	0	9	8	0	0	0	3	5	2	0	1	1	8	8	1	7	0	1	0	0	3	9
Etheostoma_erythrozonum	0	0	0	8	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Etheostoma_euzonum	7	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Etheostoma_flabellare	0	9	9	6	9	9	9	9	9	2	8	9	9	9	9	9	9	0	1	9	9	9	8
Etheostoma_gracile	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Etheostoma_nigrum	0	3	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Etheostoma_proeliare	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Etheostoma_spectabile	9	9	9	1	7	8	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
Etheostoma_stigmaeum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Etheostoma_uniporum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Etheostoma_zonale	0	0	8	8	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Fundulus_catenatus	8	0	9	7	0	0	0	0	6	0	0	0	0	1	1	1	1	0	0	0	0	2	0
Fundulus_notatus	1	0	0	8	0	2	0	0	0	0	0	0	3	1	5	0	7	0	0	0	9	4	8
Fundulus_olivaceus	1	0	9	5	0	2	0	0	0	0	0	0	1	0	4	0	5	0	0	1	9	3	5
Gambusia_affinis	1	1	7	5	0	0	0	1	9	8	0	0	0	0	0	0	2	0	0	0	0	9	0
Hybognathus_nuchalis	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hybopsis_amblops	8	0	9	6	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	2
Hybopsis_amnis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hypentelium_nigricans	8	1	9	8	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	7	9
Ictalurus_punctatus	0	0	0	3	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Labidesthes_sicculus	1	1	5	8	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Lepisosteus_oculatus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0

Table A.3 Heat maps of detection by eDNA methods, both primer (12s and 16s) results combined. Values are based on presence and absence, summed for each site to provide a scale of detection rates from 0-9, where 9 is the most commonly detected. Orange highlight indicates species found only by eDNA methods. Table A (top) is the sites from 2020 and 2021 Meramec Drainage, and Table B (bottom) is the southern 2021drainages. The list of species is the same between the two. (Cont.)

Species eDNA	LBC-S	SMCr-S	WW-S	MW-S	FH-W	FH-S	K-W	K-S	MS-W	MS-S	OH-W	OH-S	TB-S	TZ-W	TZ-S	UW-W	UW-S	WF-W	WF-S	YB-W	YB-S	YBL-W	YBL-S
Lepisosteus_platostomus	1	0	0	7	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lepomis_cyanellus	8	9	9	8	0	0	5	8	9	9	0	0	9	0	3	2	4	4	8	9	9	9	9
Lepomis_gulosus	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lepomis_humilis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lepomis_macrochirus	9	9	9	8	0	1	0	2	9	8	0	0	5	3	6	2	2	5	9	7	9	9	9
Lepomis_megalotis	9	9	9	8	0	1	0	3	9	9	1	1	1	6	6	4	6	0	2	7	0	7	9
Lepomis_microlophus	1	0	0	7	0	0	0	1	9	8	0	0	0	0	0	0	2	0	0	0	6	5	6
Lepomis_miniatus	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lepomis_symmetricus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Luxilus_chrysocephalus	9	0	7	8	0	0	1	3	2	1	0	0	0	2	9	4	4	0	0	4	1	7	9
Luxilus_pilsbryi	8	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Luxilus_zonatus	1	0	9	8	1	4	6	9	9	1	3	3	1	9	9	6	9	1	3	9	2	9	9
Lythrurus_umbratilis	0	0	0	0	0	0	0	0	5	5	0	0	0	0	0	0	0	0	0	1	0	0	0
Micropterus_dolomieu	9	1	9	8	0	0	0	0	0	1	0	0	1	0	4	0	0	0	1	0	0	0	1
Micropterus_punctulatus	4	0	9	8	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0
Micropterus_salmoides	1	7	6	7	0	1	0	0	5	4	0	0	0	0	0	0	0	1	9	0	0	7	9
Minytrema_melanops	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Moxostoma_anisurum	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Moxostoma_carinatum	0	0	4	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Moxostoma_duquesnei	9	0	9	8	0	1	1	0	0	1	0	0	0	0	0	0	0	0	1	0	0	1	1
Moxostoma_erythrurum	8	0	9	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Moxostoma_macrolepidotum	1	0	9	8	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Nocomis_biguttatus	9	0	8	8	0	2	0	2	0	1	0	0	1	9	9	7	9	0	0	2	7	8	9
Notemigonus_crysoleucas	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Notropis_atherinoides	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Notropis_boops	1	0	9	8	0	0	0	0	7	6	0	0	1	0	0	0	0	0	0	0	0	0	0
Notropis_greenei	7	0	0	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Notropis_heterolepis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Notropis_nubilus	9	6	9	8	0	0	0	1	0	1	0	0	1	0	1	0	0	0	0	0	0	7	9
Notropis_percobromus	4	0	6	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Notropis_telescopus	7	0	8	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Notropis_texanus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Notropis_volucellus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
noturus_albater	8	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Noturus_exilis	9	9	9	8	4	9	2	9	7	2	2	6	6	4	8	4	9	5	6	2	7	7	8
Noturus_flavus	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Noturus_gyrinus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Percina_caprodes	8	0	2	8	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0
Percina_evides	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Percina_maculata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

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Species eDNA	LBC-S	SMCr-S	WW-S	MW-S	FH-W	FH-S	K-W	K-S	MS-W	MS-S	OH-W	OH-S	TB-S	TZ-W	TZ-S	UW-W	UW-S	WF-W	WF-S	YB-W	YB-S	YBL-W	YBL-S
Percina_phoxocephala	0	0	0	0	2	0	0	0	0	0	1	0	0	0	0	3	0	0	0	0	0	0	0
Percina_sciera	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Phenacobius_mirabilis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pimephales_notatus	2	6	9	8	0	0	0	0	9	5	0	0	1	0	1	0	0	0	0	0	0	0	0
Pimephales_promelas	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pimephales_tenellus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pimephales_vigilax	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Pomoxis_nigromaculatus	0	0	9	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8	0
Pylodictis_olivaris	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Semotilus_atromaculatus	6	9	9	0	9	9	8	9	9	3	9	9	9	9	9	9	9	9	9	9	9	9	9

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Species eDNA	LBL-S	LBS-S	D-S	MC-S	MGP-S
Ambloplites_ariommus	9	9	0	0	0
Ambloplites_constellatus	0	0	0	0	0
Ambloplites_rupestris	0	0	0	0	0
Ameiurus_melas	9	6	2	2	2
Ameiurus_natalis	9	9	9	9	9
Amia_calva	0	0	2	0	0
Aplodinotus_grunniens	0	0	1	0	0
Campostoma_sp	9	9	0	9	7
Catostomus_commersonii	0	0	0	0	0
Chrosomus_erythrogaster	1	1	0	0	0
Cottus_bairdii	9	9	0	0	0
Cottus_carolinae	3	4	0	0	0
Cyprinella_galactura	0	0	0	0	0
Cyprinella_spiloptera	0	0	0	0	0
Cyprinella_venusta	0	0	9	0	0
Cyprinus_carpio	0	0	8	0	0
Dorosoma_cepedianum	0	0	2	0	0
Erimystax_harryi	0	0	0	0	0
Erimyzon_claviformis	8	9	0	9	7
Esox_americanus	4	1	0	3	7
Etheostoma_blennioides	9	9	0	0	0
Etheostoma_caeruleum	9	9	0	0	0
Etheostoma_erythrozonum	0	0	0	0	0
Etheostoma_euzonum	0	0	0	0	0
Etheostoma_flabellare	9	9	0	0	0
Etheostoma_gracile	0	0	0	1	1
Etheostoma_nigrum	5	5	0	0	0
Etheostoma_proeliare	0	0	9	9	8
Etheostoma_spectabile	0	0	0	9	7
Etheostoma_stigmaeum	0	1	0	0	0
Etheostoma_uniporum	3	6	0	0	0
Etheostoma_zonale	6	7	0	0	0
Fundulus_catenatus	5	1	0	0	0
Fundulus_notatus	0	0	0	0	0
Fundulus_olivaceus	6	6	9	9	9
Gambusia_affinis	2	4	9	1	5
Hybognathus_nuchalis	5	1	0	0	3
Hybopsis_amblops	0	0	1	0	0
Hybopsis_amnis	0	0	1	0	0
Hypentelium_nigricans	9	9	0	0	0
Ictalurus_punctatus	0	0	9	0	0
Labidesthes_sicculus	9	9	6	0	0
Lepisosteus_oculatus	0	0	9	0	0
Lepisosteus_platostomus	0	0	2	0	0
Lepomis_cyanellus	9	9	9	9	9
Lepomis_gulosus	6	8	7	9	7
Lepomis_humilis	0	0	2	0	0
Lepomis_macrochirus	9	9	8	9	9
Lepomis_megalotis	9	9	9	9	9

Table A.3 Heat maps of detection by eDNA methods, both primer (12s and 16s) results combined. Values are based on presence and absence, summed for each site to provide a scale of detection rates from 0-9, where 9 is the most commonly detected. Orange highlight indicates species found only by eDNA methods. Table A (top) is the sites from 2020 and 2021 Meramec Drainage, and Table B (bottom) is the southern 2021drainages. The list of species is the same between the two. (Cont.)

Species eDNA	LBL-S	LBS-S	D-S	MC-S	MGP-S
Lepomis_microlophus	9	9	7	2	9
Lepomis_miniatus	9	9	8	8	7
Lepomis_symmetricus	0	0	0	0	1
Luxilus_chrysocephalus	9	9	0	0	4
Luxilus_pilsbryi	0	0	0	0	0
Luxilus_zonatus	9	9	0	2	1
Lythrurus_umbratilis	7	8	0	9	9
Micropterus_dolomieu	9	9	0	0	0
Micropterus_punctulatus	9	8	0	0	0
Micropterus_salmoides	7	6	6	7	5
Minytrema_melanops	9	7	0	3	9
Moxostoma_anisurum	0	0	0	0	8
Moxostoma_carinatum	9	3	0	0	0
Moxostoma_duquesnei	9	9	0	0	0
Moxostoma_erythrurum	9	9	0	0	9
Moxostoma_macrolepidotum	0	0	0	0	0
Nocomis_biguttatus	9	9	0	0	0
Notemigonus_crysoleucas	0	0	0	7	9
Notropis_atherinoides	0	0	1	0	0
Notropis_boops	9	9	0	8	8
Notropis_greenei	0	0	0	0	0
Notropis_heterolepis	0	0	0	0	0
Notropis_nubilus	9	9	0	0	0
Notropis_percobromus	9	6	0	5	4
Notropis_telescopus	8	9	0	0	0
Notropis_texanus	0	0	1	0	0
Notropis_volucellus	0	0	9	0	0
noturus_albater	0	0	0	0	0
Noturus_exilis	9	9	0	0	0
Noturus_flavus	0	0	0	0	0
Noturus_gyrinus	0	0	6	0	6
Percina_caprodes	9	8	0	0	0
Percina_evides	0	1	0	0	0
Percina_maculata	0	0	2	4	8
Percina_phoxocephala	0	0	0	0	0
Percina_sciera	1	0	0	0	0
Phenacobius_mirabilis	0	0	9	0	0
Pimephales_notatus	9	8	7	0	9
Pimephales_promelas	0	0	1	0	0
Pimephales_tenellus	0	0	3	0	0
Pimephales_vigilax	0	0	0	0	0
Pomoxis_nigromaculatus	0	0	0	0	1
Pylodictis_olivaris	0	0	3	0	0
Semotilus_atromaculatus	9	9	0	9	9

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