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ORIGINAL ARTICLE

Two for the price of one: eDNA metabarcoding reveals temporal and spatial variability of mussel and fish co-distributions in Michigan riverine systems

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Abstract

Freshwater mussels (family Unionidae) are among the world's most endangered taxa, with almost 75% of North American taxa classified as a species of concern, threatened, or endangered. Despite the critical importance of comprehensive distributional data for the conservation of unionids and fishes, these data are often lacking because of the labor and resources associated with traditional survey methods. During their larval stage, unionid mussels use various fish species as obligate hosts, making native fish species vital to unionid persistence and an understanding of host distribution similarly important. Here, we utilized an eDNA metabarcoding approach to evaluate patterns of co-distribution of unionid mussels and fishes along ~362 km of the densely sampled Grand River network as well as the outlets of 19 tributaries along the eastern shore of Lake Michigan, USA. We detected a total of 21 mussel and 40 fish taxa, with distinctive composition of both mussel and fish assemblages across tributaries and differences in fish taxa between sampling periods. Notably, we detected more mussel taxa within the Grand River watershed than at the outlets of all 20 rivers combined. Within the Grand River network, two fish taxa (*Pylodictus olivaris* and *Cyprinella*) were found more frequently in areas of high mussel diversity, and three fish taxa more frequently in areas of low mussel diversity (*Umbra*, *Leuciscidae*, and *Etheostoma*). There was little difference between eDNA detections of mussels from samples collected in June versus August, but we detected significantly more fish taxa in August compared to June. Taken together, our findings demonstrate the value of eDNA metabarcoding for evaluating co-distribution of ecologically connected taxa. The use of eDNA as a tool for determining distributions of mussels and their obligate hosts may facilitate conservation efforts for these imperiled taxa.

KEYWORDS
12S, COI, eDNA metabarcoding, species co-distribution, unionid mussels

1 | INTRODUCTION

The successful conservation of aquatic species requires extensive data regarding their geographical distribution as well as continuous monitoring to determine how these distributions may change in response to global climate change and anthropogenic impacts. Conventional surveys to quantify and monitor species distributions are labor-intensive and often limited by financial constraints (Bottrill et al., 2008). Even well planned and executed surveys can lead to an underestimation of species occupancy when conducted on populations of cryptic or small species (Macdonald & Willis, 2013). This underestimation can have serious conservation implications (Gu & Swihart, 2004). Environmental DNA (eDNA) methodologies represent an affordable alternative to conventional surveys that have been shown to be more expensive, less sensitive, and detect fewer species (Fediajevaite et al., 2021).

North America is host to the largest diversity of freshwater mussels in the world with the highest species richness concentrated in the southeastern United States (Williams et al., 1993). Of the 293 North American unionid species described to date, 213 are considered endangered, threatened, or of special concern, while 37 species are presumed extinct (Bogan, 2008; Williams et al., 1993, 2017). While geologically young due to recent glaciation, the Laurentian Great Lakes are important habitat for many mussel species (family Unionidae) and fishes of significant ecological, economic, and recreational value (Bogan, 2008; Jude & Pappas, 1992; Lupi & Hoehn, 1998). However, distributional data for these species are often imprecise or outdated (Freshwater Mollusk Conservation Society, 2016; Landsman et al., 2011). Further, many Great Lakes tributaries are difficult to survey using traditional methods due to their large size and inaccessibility (Clapp & Horns, 2008). This deficiency of data represents an important gap in knowledge that is critical to address for the successful management of these species.

Habitat loss has been widely implicated as one of the primary causes in the decline of the abundance and diversity of unionids (Allan & Flecker, 1993; Downing et al., 2010), particularly in the Laurentian Great Lakes due to the invasions of dreissenid mussels (e.g., zebra mussels; Nalepa et al., 1991, 1996). Unionid mussels are also sensitive to changes in the abundance and diversity of the coinciding fish community, largely because they are dependent upon fish to serve as a host for their parasitic larvae called glochidia (Strayer et al., 2004). Glochidia attach to the gills or fins of the host fish, encyst, and grow before releasing. This parasitic life-history stage is vital to unionid development, and also enables these relatively sessile species to disperse over long distances (Newton et al., 2008). While some mussel species are generalists in their requirements for hosts, others have only a few or a single known host fish species (Haag, 2012; Watters, Hoggarth, & Stansbery, 2009). Due to this parasitic life-history trait, the distribution of host fishes has been shown to strongly influence the distribution of unionids (Haag, 2012; Schwalb et al., 2013; Vaughn, 1997; Watters, 1992).

Because mussels require fish to complete their life cycles and the two groups share broadly similar environmental requirements, it is important to monitor fish communities where mussels of conservation concern reside. Distributional data for mussels are typically collected with quadrat, snorkel, or SCUBA sampling, while electrofishing and trap netting are common for fish. Alternatively, environmental DNA (eDNA) is a non-invasive method that can produce similar data with substantially less time and effort in the field (Darling & Mahon, 2011; Ficetola et al., 2008). Single-species qPCR-based eDNA studies have been used to determine the rates of production, transport, and decay of mussel and fish eDNA (Deiner & Altermatt, 2014; Sansom & Sassoubre, 2017) and the presence or absence of a single species (Dysthe et al., 2018). In contrast, eDNA metabarcoding enables the simultaneous detection of multiple species within a sample using high-throughput sequencing and primers that amplify a broad group of taxa (Evans et al., 2017; Gehri et al., 2021; Klymus et al., 2021; Sard et al., 2019). Previous research has demonstrated the ability of eDNA to efficiently and accurately determine the presence or absence of a wide range of species in aquatic habitats, including freshwater mussels and fish (Civade et al., 2016; Currier et al., 2018; Gehri et al., 2021; Klymus et al., 2021; Preece et al., 2021; Sard et al., 2019). Despite the effectiveness of eDNA for mussel and fish surveys, and the parasitic relationship of mussel taxa and their fish hosts, there has not been a previous eDNA metabarcoding effort that simultaneously examined both mussels and fish.

The Grand River is the longest river in Michigan and the second largest river basin in the state by area, encompassing approximately 14,439 km^2. The Grand River was the main focal point of our sampling effort because it is known habitat for 32 of the 45 mussel species native to Michigan, including numerous species of conservation concern (Badra & Goforth, 2002; Hanshue & Harrington, 2017). In addition to mussels, the Grand River is also habitat for 108 species of fish (Hanshue & Harrington, 2017). Although our main focus was to sample 50 locations along the Grand River and its tributaries, we also sampled 19 other tributaries of Lake Michigan for eDNA, many of which do not have well-characterized mussel community distribution data. These 19 tributaries encompass an area of approximately 41,000 km^2, and when combined with the Grand River, cover about half of the drainage area of Michigan's Lower Peninsula.

In this study, we validated fish and unionid mussel metabarcoding with the goal of applying eDNA methodologies to the conservation and management of these taxa. We sampled the Lake Michigan tributaries and the Grand River and its tributaries on two occasions (June and August). Our primary objectives were to (1) validate eDNA as a detection method for unionid mussels and fish across a large area of Michigan, (2) determine whether mussel and fish communities vary between the Grand River watershed and other Lake Michigan tributaries, and (3) investigate whether detections of certain fish species are associated with certain mussels or differential levels of mussel diversity.

2 | METHODS

2.1 | Study area

Two geographic regions in the lower peninsula of Michigan, USA were sampled on two occasions: 8–17 June and 7–17 August of 2020 (Figure 1; Table S1): (1) Lake Michigan tributaries and (2) the Grand River and tributaries. The sampling locations of 20 Lake Michigan tributaries encompassed a geographic area along ~560 km of the eastern shore of Lake Michigan from near the Indiana border north to Indian River, Michigan (Table S1). The sampling locations of the Grand River, 17 tributaries and 33 mainstem locations, stretched ~362 km from Grand Haven East to Center Lake, near Michigan Center (Figure 1).

2.2 | Collection and filtration of water samples

Three replicates of water samples and one negative field control (NFC) were collected during each sampling event, a particular river location in either June or August, yielding a total of 429 water samples and 143 NFCs. Prior to collection of water samples, all 1-L Nalgene HDPE bottles (ThermoFisher Scientific, Waltham, MA) were sterilized by full immersion in a 20% by volume bleach solution for 10 min, and then triple rinsed with distilled water. Each NFC consisted of a 1-L bottle filled with laboratory grade water, brought to the field, opened for 30 seconds during sample collection, and subsequently handled like all other samples.

Each 1-L water sample was collected by submerging the bottle just subsurface, less than 1 m from the riverbank, using a new latex glove for each sampling location. Bottles were then sealed and the exteriors of the bottles were wiped with 20% bleach solution. Water samples were placed in a cooler on ice until filtered. All water samples were filtered in their entirety within 12 h of collection using disposable 47 mm diameter 0.45 µm pore nitrocellulose Nalgene analytical test filter funnels (ThermoFisher Scientific). Filters were preserved in Buffer ATL (Qiagen) following recommendations by Majaneva et al. (2018) for recovering biodiversity in eDNA samples, and a 20% bleach solution was used to sterilize all tools, and working surfaces.

2.3 | Extraction of DNA

All extractions were performed in an eDNA-dedicated laminar flow UV hood system to minimize contamination. All surfaces and equipment were frequently wiped with 20% bleach solution, and all pipetting was done with sterile, aerosol barrier pipette tips.

DNA extractions were performed within 3 months of sample collection using Qiagen DNeasy Blood and Tissue kits (Qiagen), according to the manufacturer's protocol as modified per Gehri et al. (2021), except that nitrocellulose filters were preserved in Qiagen Buffer ATL to eliminate the ethanol evaporation step. After extraction of eDNA from the filters, all samples were treated with Zymo OneStep™ PCR inhibitor (Zymo Research) to remove potential PCR inhibitors.

2.4 | Development of metabarcoding primer for freshwater mussels

In communication with the U.S. Fish and Wildlife Service, 16 species of native Michigan mussels were identified as species of interest for an increase in monitoring effort (Table S3). Sequences of the mitochondrial cytochrome oxidase subunit I (COI) for the 16 target species were downloaded from NCBI and imported into Geneious

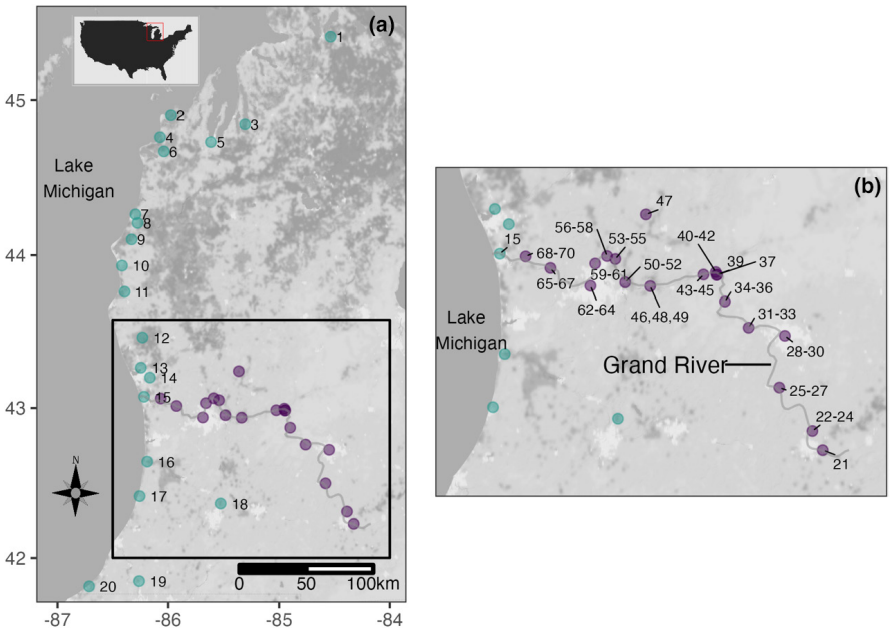

FIGURE 1 Map of 70 eDNA collections within Michigan Rivers. Numbered sampling locations found in Table S1 (a) Tributary outlets into Lake Michigan (sites 1–20) with inset box of the Grand River watershed (sites 21–70). (b) Sampling locations in the Grand River watershed. Points on the map with multiple site numbers correspond to the tributaries of the Grand River and locations on the mainstem Grand River that were sampled upstream and downstream of each tributary confluence.

Prime (v 2019.2.3; Biomatters, Inc). Tissue samples of voucher specimens were acquired for which COI sequences were unavailable, extracted using a DNeasy Blood and Tissue extraction kit (Qiagen), and an ~800 bp fragment of COI was amplified using a universal primer set (Folmer et al., 1994). PCR products were Sanger sequenced at the Molecular Conservation Genetics Laboratory at the University of Wisconsin-Stevens Point using a 3730xl DNA Analyzer (ThermoFisher Scientific). Sequencing data for each species were visualized, processed, and aligned using Geneious Prime to generate consensus sequences. Consensus sequences from each species were then aligned and compared for identification of potential priming sites.

Within Geneious Prime, we determined that a primer sets previously developed by Cho et al. (2016) for eastern Canadian unionid mussels and Klymus et al. (2021) contained key mismatches in the priming site and were unlikely to amplify all of the 16 target species. However, with the substitution of degenerate bases at locations of interspecific variability, a primer set based on Cho et al. (2016) was developed: Forward: 5′ cgacaggttcagagttctacagtccgacgatcAGNCTTYTVATYCGDGCYGA 3′, Reverse: 5′ gtgactggagttcagacgtgtgctcttccgatctCCRGTHCCNACACCHCTCTC 3′, with Illumina small RNA and TruSeq sequences as lowercase, respectively. Despite substitution of degenerate bases, these primers likely only amplify the female mitotype, which is present in both somatic and gonadal tissue, whereas the male mitotype is restricted to male gonadal tissue only. The male and female mussel mtDNA genomes are thus highly divergent and do not consistently or evenly amplify in both sexes (see Klymus et al., 2021; Table 1).

2.5 | Amplicon PCR

All PCR reactions were performed in 10 µl volumes with 3 µl of extracted eDNA or positive control DNA and 7 µl of PCR master mix. The master mix for COI consisted of 1 µl of New England Biolabs 10X Standard Taq Reaction Buffer, 0.2 µl of 10 mM dNTPs, 0.8 µl of 25 mM $MgCl_2$, 0.5 µl of 20 mg/mL bovine serum albumin (BSA), 0.3 µl of 1.25 U/µl NEB Taq, 3.4 µl of molecular grade water, and 0.4 µl of 10 µM of each forward and reverse primer [see above]. The master mix for amplifying the 12S locus in fish was similar to that for COI, except 2.6 µl of molecular grade water and 0.8 µl of 10 µM of each forward and reverse primer were used. The primers used to amplify the 12S mitochondrial gene were developed by Riaz et al. (2011): Forward: 5′-cgacaggttcagagttctacagtccgacgatcACTGGGATTAGATACCCC-3′, Reverse: 5′-gtgactggagttcagacgtgtgctcttccgatctTAGAACAGGCTCCTCTA-3′ with Illumina small RNA and TruSeq sequences as lowercase, respectively.

Each 96-well plate included 90 eDNA samples, three positive controls and three negative controls. For the COI-positive control, we used DNA extracted from tissue samples of Eastern Floater *Pyganodon cataracta*, a species not found in Michigan. Extracted DNA from Pacific Cod *Gadus macrocephalus*, a marine fish species not found in Michigan, was used as a positive control for the 12S locus. Molecular grade water was used in place of extracted DNA for the PCR negatives.

The thermal cycler profile for both amplicons consisted of a 2-min hold at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 57°C, 30 s at 72°C and a final extension of 5 min at 72°C. Primer dimer and excess primers were removed from PCR products with a single-sided bead cleanup at 1X concentration (Beckman-Coulter AMPure XP beads) in 10 µl volumes. Following the clean-up, each sample was eluted in 20 µl of TLE.

2.6 | Library preparation for metabarcoding

Libraries for fish 12S metabarcoding were prepared using the barcoding, normalizing, pooling, and bead size selection steps of the "genotyping in thousands by sequencing" (GT-Seq) protocol by Campbell et al. (2015) following Bootsma et al. (2020) and Gehri et al. (2021). Briefly, samples were ligated with Illumina-specific barcoding oligonucleotide sequences in a barcoding PCR reaction. Next, products of the barcoding PCR were normalized using SequelPrep DNA normalization kits (Invitrogen). Normalized DNA was then pooled and cleaned using AMPure XP beads (Beckman-Coulter) in a double-sided 0.5X and 1.2X concentration protocol.

Preparation of libraries for mussel metabarcoding differed from the preparation of fish libraries in several ways. For the barcoding PCR, the Illumina i07 and i05 primers concentrations were reduced by half in order to reduce the formation of primer dimer that was observed during primer optimization. The barcoded PCR products were then pooled without normalization, and a single-sided cleanup using AMPure XP beads (Beckman-Coulter) at 0.5X concentration was performed in order to target the removal of primer dimer in the libraries.

All prepared libraries were then visualized using E-gel EX 2% agarose cassettes on a Power Snap Electrophoresis Device (ThermoFisher Scientific) and quantified using a Qubit 1x dsDNA HS Assay Kit and Qubit 2.0 fluorometer (ThermoFisher Scientific). All sequencing was performed on an Illumina MiSeq. The fish and mussel libraries were prepared separately and run on separate Illumina flow cells. Because the 12S fish and COI mussel amplicons differ in size, libraries for fish 12S used a single 150-cycle lane run with 2x75 base pair chemistry, while the mussel COI libraries were run with 300-cycle and 2x150 base pair chemistry.

2.7 | Data processing

Data were processed using methods similar to Larson et al. (2022). Raw sequencing reads were trimmed of primers using Cutadapt (Martin, 2011). Paired-end sequences were then merged with the software FLASH 2 (Magoc & Salzberg, 2011) using the default parameters except that the maximum overlap was set to 15 bp for COI and 10 bp for 12S. Merged reads were processed using DADA2 (Callahan et al., 2016) within R version 4.1.0 (R Core Team, 2021)

TABLE 1 Mussel taxa that were detected in eDNA samples, their known host species, and their Michigan state conservation status.

Subfamily	Tribe	Taxa detected in eDNA	Common name	Host fish	Michigan conservation status
Anondontinae	Anodontini	*Lasmigona* (*L. costata*, *L. complanata*, *L. compressa* are extant within study region)	Fluted Shell White Heelsplitter Creek Heelsplitter		
		Pyganodon (*P. grandis and P. lacustris* are extant within study region)	Giant Floater Lake Floater		
		Strophitus undulatus	Creeper	*Semotilus atromaculatus*, *Luxilus cornutus*	Not listed
		Utterbackia imbecillis	Paper Pondshell	*Semotilus atromaculatus*, *Lepomis macrochirus*, *Lepomis marginatus*, *Lepomis gulosus*, *Gambusia affinis*	Not listed
Ambleminae	Amblemini	*Amblema plicata*	Threeridge	Generalist	Not listed
	Lampsilini	*Ortmanniana ligamentina*	Mucket	Generalist	Not listed
		Lampsilis cardium	Plain Pocketbook	*Sander canadensis*, *Pomoxis annularis*	Not listed
		Lampsilis siliquoidea	Fatmucket	Generalist: Centrarchidae, Percidae, Ictaluridae	Not listed
		Ligumia recta	Black Sandshell	*Anguilla rostrata*, *Lepomis macrochirus*, *Pomoxis annularis*, *Sander canadensis*	Endangered
		Potamilus alatus	Pink Heelsplitter	*Aplodinotus grunniens*	Not listed
		Potamilus fragilis	Fragile Papershell	*Aplodinotus grunniens*	Not listed
		Toxolasma parvum	Lilliput	*Lepomis gulosus*	Endangered
		Truncilla truncata	Deertoe	*Aplodinotus grunniens*, *Sander canadensis*	Special Concern
		Venustaconcha ellipsiformis	Ellipse	Genaralist: Etheostoma, Percina, Cyprinidae	Special concern
		Cambarunio (*C. iris* and V. fabalis are extant within study region)	Rainbow Rayed Bean		*C.iris*: Special Concern *V.Fabalis* Endangered
	Pleurobemini	*Eurynia dilatata*	Spike	Generalist: Ictaluridae, Centrarchidae, Percidae, Clupeidae	Not listed
		Fusconaia flava	Wabash Pigtoe	*Pomoxis annularis*, *Pomoxis nigromaculatus*	Not listed
		Pleurobema sintoxia	Round Pigtoe	*Lepomis macrochirus*	Special concern
	Quadrulini	*Quadrula quadrula*	Mapleleaf	*Pylodictis olivaris*, *Ameiurus natalis*	Not listed
		Cyclonaias pustulosa	Pimpleback	Generalist: Ictaluridae, Acipenseridae, Centrarchidae	Not listed
		Cyclonaias tuberculata	Purple Wartyback	Ictaluridae*	Threatened

Note: Known host fishes listed are natural glochidia infestations, except those denoted with an *, which are laboratory infestations (Freshwater Mussel Host Database, 2017). *Lasmigona* and *Pyganodon* genera were not resolved to the level of species, and listed are the possible species extant within Michigan.

with a minimum length of 200 bp for mussels and 142 bp for fish, a maximum number of expected errors specified as two, and using the consensus method to remove chimeric sequences. The resulting amplicon sequence variants (ASVs) were queried to the full National Center for Biotechnology Information (NCBI) database using BLASTn (Altschul et al., 1990). Only ASV sequences with greater than or equal to 98% similarity to NCBI sequences were retained. All ASVs that fell below that threshold were blasted to a custom database constructed with the 16 target species. The remaining ASVs assigned to non-target species were removed from the dataset. For example, ASVs that assigned to terrestrial mammals such as *Marmota monax*, *Sorex alpinus*, and *Bos taurus* were removed as non-target species. Other ASVs that were assigned to fish or mussel species not present in Michigan based on distributional information from the FishBase (https://www.fishbase.org) and Nature Explorer databases (https://explorer.natureserve.org) were removed. Full taxonomy of the remaining ASVs was determined from NCBI taxonomy using custom scripts (Larson et al., 2022). Any ASVs that matched a single species at ≥98% similarity was assigned to that species. Next, if an ASV matched multiple species, it was assigned to the lowest shared taxonomic level for that species group. For example, a group of fish ASVs assigned to *Salmo trutta*, *Salvelinus* spp., and *Oncorhynchus tshawytscha* was assigned to the subfamily Salmoninae. To control for contamination, the maximum number of reads detected in the positive and negative controls for each taxon was subtracted from the samples and only taxa that had more than four reads after subtraction were counted as present in a sample. Water filtration for multiple sites was conducted at the same time, so we conservatively called positive detections by subtracting the maximum read counts for a particular taxon from the controls across all water samples in the study. Finally, remaining read counts were converted into presence or absence data.

2.8 | Statistical analyses

A variety of data visualization and statistical analyses were conducted to assess potential differences in mussel and fish communities across the study area. Taxa accumulation curves were produced in the R package *vegan* (Oksanen et al., 2020) using the "exact" method, in order to visualize the rates of taxa detection across cumulative sampling replicates and locations. Curves were produced for groups of sites in the Grand River watershed, the other tributaries of Lake Michigan, and for the June and August sampling events to visualize any differences in detections among those groups. Heatmaps were produced to visualize the number of replicate water samples with positive detections for each taxon at each sampling location.

Non-metric multidimensional scaling (NMDS) plots were also produced in *vegan* using the Bray–Curtis dissimilarity matrix to visualize any potential differences in fish and mussel communities between and within the Grand River watershed and the tributaries of Lake Michigan, as well as between the June and August sampling events. Because three ordination axes for the NMDS plots generally captured much of the variation within the groups and minimized stress values, three axes were used for all plots for consistency.

Analysis of Similarities (ANOSIM) was performed within *vegan* to test for significant differences ($\alpha = 0.05$) in mussel and fish taxa detected between the Grand River watershed and tributaries of Lake Michigan and between the June and August sampling events. Wilcoxon-Rank Sum tests were used to test for differences ($\alpha = 0.05$) in the mean number of taxa detected between the June and August sampling events and between Grand River watershed and tributaries of Lake Michigan.

Indicator species analyses were performed using the R package *indicspecies* (Cáceres & Legendre, 2009) to explore whether any taxa of mussels or fish were found significantly more frequently in samples in the Grand River watershed versus Lake Michigan tributaries, between June versus August samples, or among areas of differing mussel diversity. An indicator species analysis aims to determine if any taxa are detected significantly more frequently within an a priori-determined group of samples, which might include different habitat or community types (Cáceres & Legendre, 2009).

3 | RESULTS

3.1 | Sequencing and taxa detection

For the COI mussel sequencing run, a total of 9,386,090 paired-end reads were produced, 3,851,141 of which were successfully merged, with an average of 6272 reads per sample. Visualization of the libraries on a TapeStation (Agilent) suggested that the low proportion of merged reads was because of extensive primer-dimer. Despite usable data being obtained for this study, further optimization of PCR conditions is recommended. After filtering and chimera removal, 923 ASVs remained. After quality filtering, removal of non-mussels, and removal of mussel taxa not extant within our study region, 644 ASVs remained, which assigned to 21 unique mussel taxa. There were two NCBI blast results for male mussels of the 8930 ASV matches where sex or mitotype was reported. Both of these matches were for adductor muscle and likely reflect the female mitotype as the male mitotype is generally restricted to gonads and sperm (Venetis et al., 2006). Of the 21 mussel taxa we detected, 18 were resolved to a single species and three were assigned to a genus (Table 1). The number of mussel taxa detected per site ranged from 0 to 14, with a mean of 3.9 and a median of three.

One species of mussel, *Epioblasma triquetra*, was present early in the bioinformatic analysis, but was removed completely during quality filtering. Of the 775 ASVs originally detected, 131 were specific to *E. triquetra*. The subtraction of the maximum number of read counts detected across positive and negative controls (negative field controls and no template PCR controls) from all samples resulted in the loss of detection of *E. triquetra*. High read counts (often >100) of *E. triquetra* in positive controls and negative laboratory and field controls were likely the result of laboratory contamination during our primer and lab workflow development, where tissue samples

of *E. triquetra* were repeatedly PCR-amplified. No other species of mussels for which we PCR-amplified tissue samples generated high read counts across positive and negative controls. As a result, we are confident that the contamination was limited to *E. triquetra*, and that we successfully controlled for this contamination with bioinformatic filtration. Most sources of contamination were attributed to the negative field controls rather than positive and negative PCR controls suggesting contamination occurred prior to or during filtration. Removal of positive detections after controlling for contamination was more frequent for the June sampling event (66.1% of removals) compared to the August sampling event (33.9%).

For the 12S fish sequencing run, a total of 8,078,616 paired end reads were merged successfully from 16,081,991 total pairs of reads, with an average of 13,157 reads per sample. After filtering for size and chimera removal, 1,613,992 reads (15.47%) remained, corresponding to 485 ASVs. After quality filtering, removal of non-fish, and removal of fish not known to be extant within our study region, 232 ASVs remained, which assigned to 40 unique fish taxa. Of the 40 unique fish taxa we detected, 18 were resolved to a single species, 14 were assigned to a genus, seven to family, and one to order (Table S2). Across all study sites, the number of fish taxa detected ranged from 4 to 23, with a median of 14 and a mean of 13.6 taxa detected per sampling site.

3.2 | Taxa detections by study region

Mussel taxa detections per sampling location were higher in the Grand River watershed compared to Lake Michigan tributaries (Figures 2, S1, and S3). The mean number of mussel taxa detected per sampling location was 5.1 (±4.4 SD) in the Grand River watershed and 0.75 (±1.3 SD) in the tributaries of Lake Michigan, which were significantly different (Wilcoxon-Rank sum $W = 776$ $p < 0.001$). Mussel taxa detected per site ranged from 0 to 14 within the Grand River watershed and from 0 to 4 in Lake Michigan tributaries. Within the Lake Michigan tributaries, 13 sites had zero detections of mussels, and seven sites (site numbers 2, 20, 16, 7, 13, 10, and 6) had at least one taxon detected. The Kalamazoo had the most mussel taxa detections (4) of the Lake Michigan tributaries. Within the Grand River watershed, the Grand River upstream of Plaster Creek had the most mussel taxa detected (14), while eight sites had zero mussel taxa detected (sites 26, 31, 32, 33, 45, 47, 54, and 60; Table S1).

We detected 4–23 fish taxa per sampling location within the tributaries of Lake Michigan and 8–20 fish taxa within the Grand River watershed (Figures 2, S1, and S2). Within the tributaries of Lake Michigan, the site with the fewest fish taxa detected was the Dowagiac River (site 19, four taxa) and the site with the most fishes detected was the Muskegon River (site 13, 23 taxa). Within the Grand River watershed, the site with the fewest fish taxa detected was the Grand River upstream of Looking Glass River (site 34, eight taxa), and site with the most fish taxa was the Grand River upstream of Deer Creek (site 65, 20 taxa). The mean number of fish taxa detected per sampling location in the Grand River watershed was 13.6 (±2.6 SD), while the mean in the other Lake Michigan tributaries was 13.7 (±4.5 SD), which were not significantly different (Wilcoxon-Rank Sum $W = 476.5$, $p = 0.862$; Figure 2).

3.3 | Differences in communities across sampling events

There was no evidence of differences in mussel community composition between sampling events. There was no distinct separation of groups within the NMDS plot (Figure S6), and the ANOSIM provided no support for differences in the mussel communities ($R = 0.019$, $p = 0.095$). The Wilcoxon-Rank Sum test also did not indicate any differences in the mean number of taxa detected, with 2.93 (±3.7 SD) mussel taxa detected in June and 2.46 (±3.1 SD) in August ($W = 2496$, $p = 0.61$). There was not a distinct pattern of separation visible in the NMDS plot of fish taxa detections over all sampling sites between June and August sampling events (Figure S4), although an ANOSIM test revealed a significant difference in the fish taxa detected between June and August ($R = 0.124$, $p = 0.0001$). A Wilcoxon-Rank Sum test revealed that the mean number of fish taxa detected in August was significantly higher than in June (Wilcoxon Rank Sum $W = 951.5$, $p < 0.0001$), with a mean of 7.88 (±3.8 SD) taxa detected in June and 11.93 (±3.5 SD) in August; however, taxa were disproportionately removed for the June sampling event when controlling for contamination (see above).

3.4 | Distribution of mussels and fishes in the Grand River watershed

Throughout the Grand River watershed, the diversity of mussel taxa detected was relatively low in both the farthest upstream and farthest downstream locations, with the highest diversity in the middle to middle-lower sampling locations (Figure 2). Some of the most commonly detected mussels included *Lampsilis cardium*, *Amblema plicata*, *Fusconaia flava*, *Ortmanniana ligamentina*, and *Eurynia dilitata* (Figure 3b). Fish diversity was relatively consistent across the watershed, and some of the most commonly detected fish taxa included Catostomidae, *Cyprinella*, *Lepomis cyanellus*, *Micropterus dolomieu*, and *Etheostoma* (Figure 3a).

We identified 22 sampling locations in the Grand River watershed that we considered as high diversity sites, with five or more mussel taxa detections across the two sampling events (the median number of mussel taxa detected within the Grand River watershed was four). There were 22 sampling locations classified as low diversity sites that had 1–3 mussel species detected (Figure S7a,b). An indicator species analysis was performed between the high-diversity sites and low-diversity sites to determine if any fish species were associated with either group of sites. Two fish taxa were found significantly more frequently in the high mussel diversity sites (*Cyprinella* $p = 0.037$, and *Pylodictis olivaris* $p = 0.0072$), while three fish taxa were found significantly more frequently in the low diversity mussel

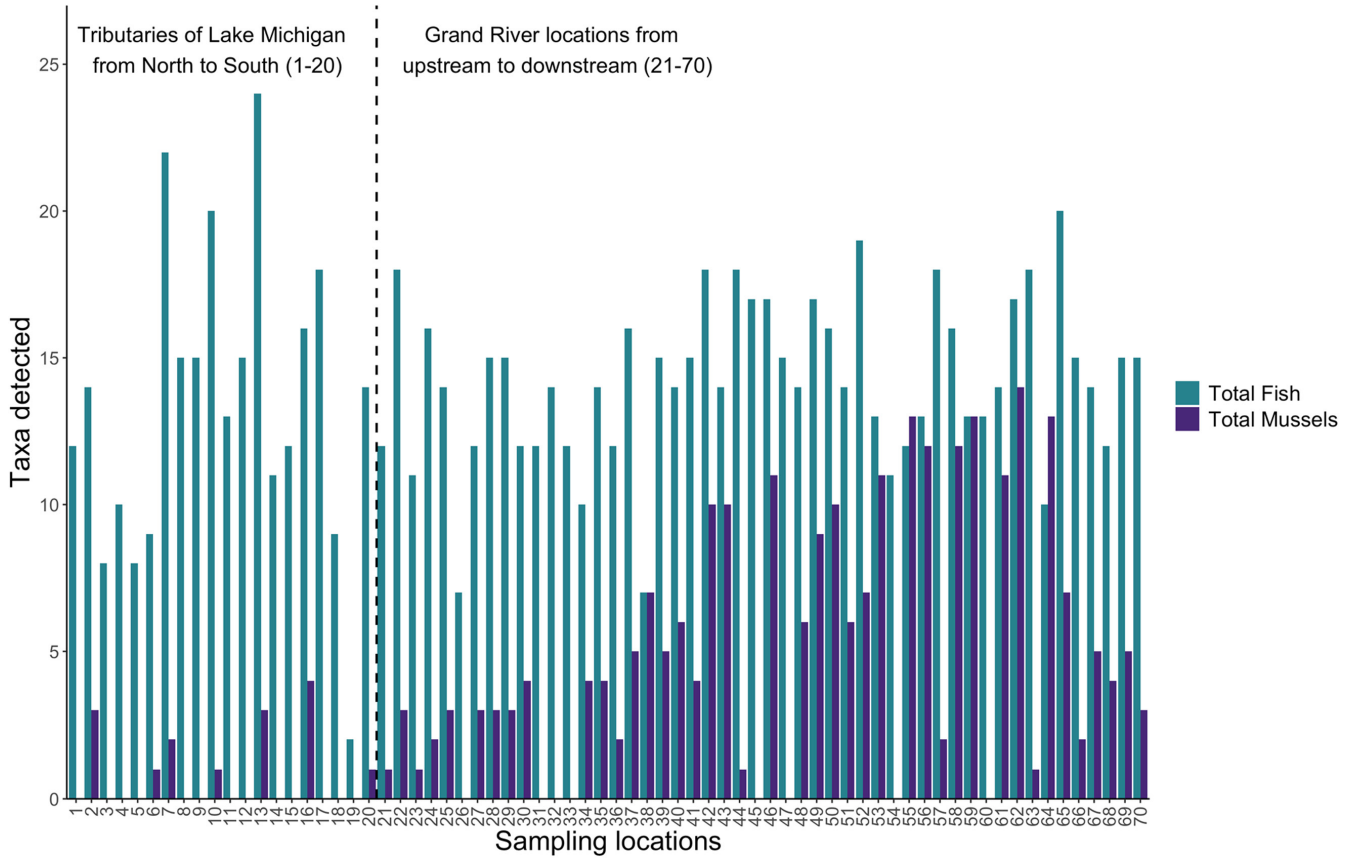

FIGURE 2 Bar plot of fish and mussel taxa detections, combined over replicate samples and sampling event, by sampling location. Tributary outlets of Lake Michigan are sites ordered from farthest North (site 1) to farthest South (site 20). Grand River sites are ordered from farthest upstream (site 21) to farthest downstream (site 70). Note that the lowest location on the Grand River is site 15, presented here with the tributary outlets of Lake Michigan.

sites (*Umbra* $p = 0.012$, *Leuciscidae* $p = 0.0034$, and *Ethesotoma* $p = 0.044$). The taxa *A. plicata*, *L. cardium*, *Cyclonaias pustulosa*, *F.flava*, *Quadrula quadrula*, *O. ligamentina*, *Ligumia recta*, *Pleurobema sinotxia*, *Strophitus undulatus*, *Cyclonaias tuberculata*, and *Lasmigona* spp. were found significantly ($p < 0.05$) more frequently in the high diversity sites, while no mussel taxa were detected significantly more often in low diversity sites.

4 | DISCUSSION

4.1 | Validating eDNA as a tool for detecting mussels and fishes

Multi-marker eDNA metabarcoding is emerging as a valuable and affordable tool for conservation and management in aquatic systems (Cordier et al., 2019; Robinson et al., 2022). Our study is an excellent demonstration of the utility of eDNA as an effective method for detecting both unionid mussels and fish across a variety of habitats within a large area of Michigan's lower peninsula. The multi-marker eDNA metabarcoding approach was sensitive enough to effectively detect differences in mussel and fish communities across sampling locations and between sampling events, as well as differences in fish communities between areas of high and low mussel diversity. The ability to simultaneously survey mussels and the hosts of their parasitic larvae with this methodology suggests that it can supplement or replace conventional surveys and is likely a useful tool for informing conservation strategies.

eDNA metabarcoding results were consistent with conventional surveys and often detected more mussel and fish species. We expected to detect higher diversity in the Grand River compared to the Lake Michigan tributaries, because 32 of the 45 mussel species native to Michigan have been documented in the Grand River, while fewer species have been documented in the other Lake Michigan tributaries we sampled (Badra & Goforth, 2002). While we detected 14 total mussel taxa within the Grand River, and eight mussel taxa significantly more frequently compared to Lake Michigan tributaries, this may have been a result of unbalanced sampling between the two systems. Among the Lake Michigan tributaries, the Kalamazoo River is known to be relatively biodiverse, with a previous survey identifying 19 species of unionid mussels being present, either live or as shells (Badra & Goforth, 2002). We detected four mussel taxa in the Kalamazoo River, but only sampled one location in the lowest reach of the river, compared to Badra and Goforth (2002) who sampled four locations with multiple SCUBA transect surveys per location, two of which were in close proximity to our sampling location.

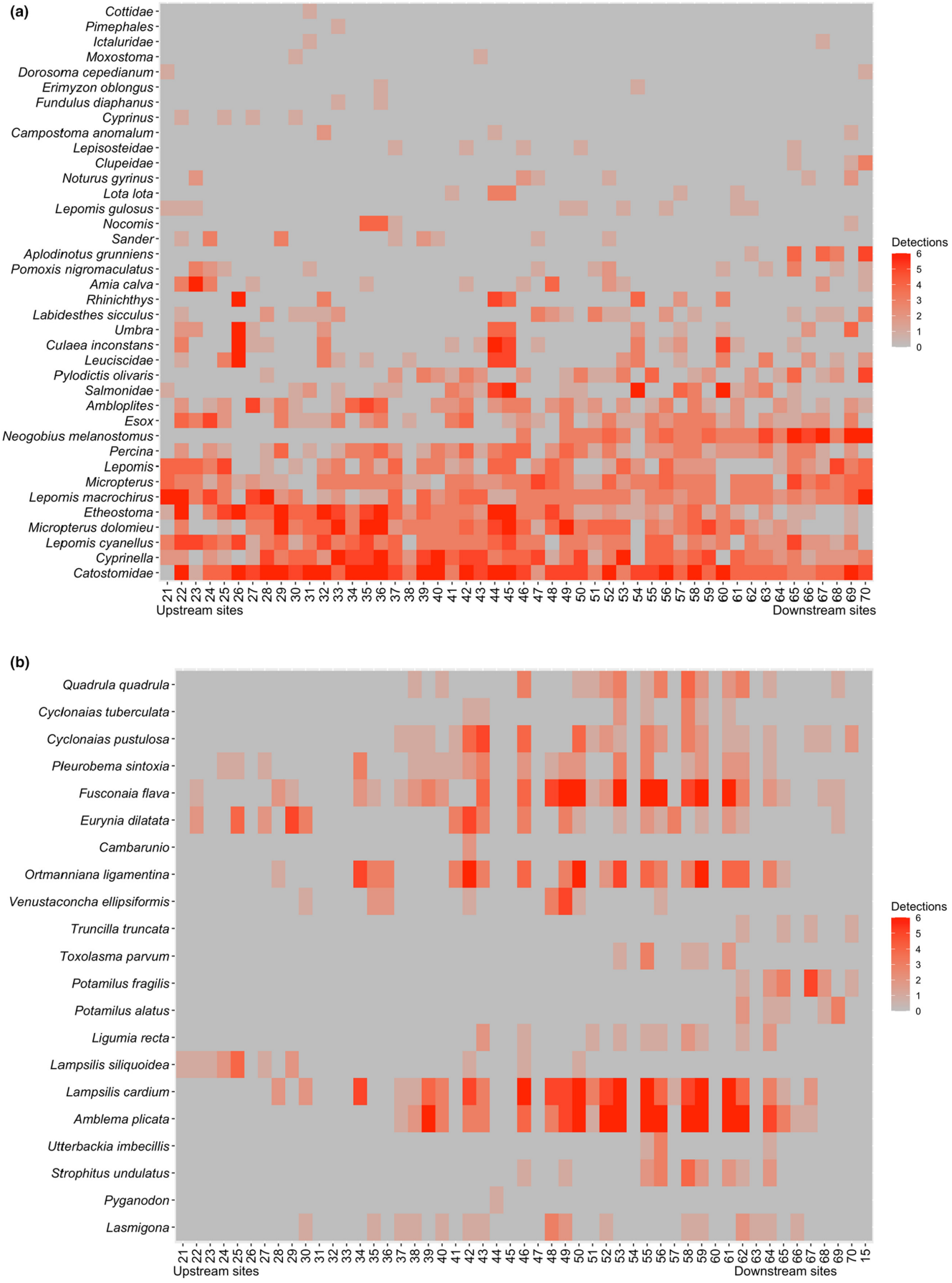

FIGURE 3 Fish (a) and mussel (b) taxa detected at sites within the Grand River watershed combined over replicate samples and the June and August sampling events. Sampling site numbers are ordered from left to right as farthest upstream (site 21) to farthest downstream (site 15).

Of the four mussel taxa we detected in the Kalamazoo River all have been detected in traditional mussel surveys: *Toxolasma parvum* and *Fusconaia flava* (Badra & Goforth, 2002) and *Cyclonaias pustulosa* and *Potamilus fragilis* (D. Woolnough https://cmumussels.shinyapps.io/KalamazooRiverSurveys/).

The Lake Michigan tributaries we detected the most mussels in are among the largest watersheds in Michigan, with the exception of the Crystal River. Using traditional survey methods, Haag (2012) found that in the upper Mississippi River basin, headwater streams generally had low diversity and small populations of mussels, but diversity and abundance both rapidly increased with increasing size of rivers. Because smaller streams are subject to highly variable streamflow and are more susceptible to disturbances, these systems typically have short lived, smaller mussel species, with lower overall diversity (Haag, 2012). As river size increases, habitat heterogeneity increases, and the habitats that support mussels become more abundant and diverse, and mussel diversity typically increases as a result (Haag, 2012). We observed a similar trend within the Grand River, where the headwater sampling locations had a lower diversity of mussels, and the number of species detected increased as we sampled locations farther downstream, with the highest number of species detected in the middle-lower sections of the river. We also observed a trend of declining mussel diversity in the very lowest downstream sampling sites within the Grand River. We hypothesize that the lowest reaches of the Grand River are less supportive of diverse mussel species assemblages due to more homogenous and lacustrine habitat, as this section of the river is generally slow moving, deep, and channelized.

Our sampling locations for the tributaries of Lake Michigan were all near Lake Michigan in the lowest reaches of the rivers, where environmental conditions may be more similar to the lowest reaches of the Grand River. It is possible that if we had sampled farther upstream in the Lake Michigan tributary watersheds, we might have detected areas of higher mussel diversity, as we did in the Grand River farther from Lake Michigan. Finally, it should be noted that for this project, our sampling effort was highly focused on the Grand River watershed, with many locations within the Grand River compared to a single location on each of the tributaries of Lake Michigan.

4.2 | Fishes and mussels associated with areas of high and low mussel diversity

The fish communities we detected in areas of high mussel diversity were different from those at areas of low mussel diversity. Fish taxa detected significantly more frequently in areas of high mussel diversity included *Pylodictis olivaris* and *Cyprinella*. Which are documented potential hosts for glochidia of at least eight, and nine Michigan mussel species, respectively (Boyer et al., 2011; Cliff et al., 2001; Coker et al., 1921; Hove et al., 1995a, 1995b, 1997, 2014; Hove & Kurth, 1997; Howard, 1914; Howard & Anson, 1922; Howells, 1996; McGill et al., 2002; Weiss & Layzer, 1995). We detected three fish taxa (*Umbra*, *Leuciscidae*, and *Etheostoma*) significantly more frequently in locations of low mussel diversity. In natural or laboratory conditions, members of *Umbra*, *Leuciscidae*, and *Etheostoma* have been documented as hosts for the glochidia of at least three, 14, and 20 native Michigan unionid species, respectively (Bloodsworth et al., 2013; Clarke & Berg, 1959; Cliff et al., 2001; Ehlo & Layzer, 2014; Fuller, 1980; Gibson et al., 2014, 2015; Gibson & Watters, 2011; Hove et al., 1997, 2013, 2014, 2016; Howells, 1997; Marr et al., 2016; O'Dee & Watters, 2000; Riusech & Barnart, 2000; Schroeder et al., 2014; Watters, 1996; Watters et al., 2005; Watters, Gibson, & Kelly, 2009; Watters & O'Dee, 1997; Watters, O'Dee, & Chordas, 1998; Watters, O'Dee, Chordas, & Rieger, 1998; Wilke et al., 2021).

It is likely that differences in habitat preference explain the increased detection of these unionid larvae host fish in areas of low mussel diversity. Though host fish must be present concurrent with brooding in order for mussels to successfully reproduce, the host fishes and respective mussel species may have different habitat preferences, if only seasonally. Both fish species richness as well as abiotic variables such as land use, upstream dam density, and stream discharge contributed significantly to the habitat suitability of 11 Michigan unionid mussels (Daniel et al., 2018). As much as 44% of the variation in mussel species composition within southwestern Ontario Great Lakes tributaries was attributed to host fish distributions; however, watershed identity and environmental factors were also important, explaining 28% and 23% of mussel species composition, respectively (Schwalb et al., 2013). The importance of environmental factors on mussel distribution may be an underestimate as several environmental variables that may influence mussel distribution, such as substrate stability and water chemistry, were not included in their model (Schwalb et al., 2013).

River size was likely a main driver in determining diversity of mussel taxa detected. We found that 11 mussel taxa were detected significantly more frequently in the areas of high mussel diversity, while no mussel taxa were found significantly more frequently in areas of low mussel abundance. Larger rivers typically support higher diversity and abundance of unionids and fishes (Ford et al., 2016; Watters, 1992). Generally, the sampling locations in which we detected high mussel diversity were in the mainstem of the Grand River, which is a much larger river compared to the tributaries of the Grand River, where we often found low mussel diversity.

4.3 | Differences in taxa detections across sampling events

The relative mobilities of mussels and fish may explain the differences in taxa detections between sampling events. We generally detected fewer fish taxa in June than in August, while there was no difference in mussel detections between the two sampling events. Several of the fish species in Michigan exhibit seasonal movements between different habitats (Chorak et al., 2019; Daugherty & Sutton, 2005; DePhilip et al., 2005). Phenological patterns of species occupancy, relative abundance, and activity affect detection probabilities. eDNA

monitoring has accurately detected seasonal shifts in fish occupancy in freshwater and marine environments (Erickson et al., 2017; Sigsgaard et al., 2017; Stoeckle et al., 2021); however, seasonal activity patterns can influence shedding rates and thus the probability of detection (de Souza et al., 2016; Troth et al., 2021). Among the fish taxa, we detected with eDNA, Walleye (*Sander vitreus*), Yellow Perch (*Perca flavescens*), and Flathead Catfish (*Pylodictis olivaris*), all exhibit seasonal movements within Michigan rivers and between drowned river mouth habitats and Lake Michigan (Chorak et al., 2019; Daugherty & Sutton, 2005; DePhilip et al., 2005). While differences in fish detections between June and August likely reflect the seasonal movements of these fishes in the study area, we cannot rule out the possibility that the removal of nearly twice the positive detections for the June sampling event while controlling for contamination contributed to the seasonal differences detected. If shedding rates decrease or either eDNA degradation or water flow increases, total read counts may have been lower in June even if no distributional differences existed. Mussels, on the other hand, are relatively immobile organisms, which are only capable of short-distance movements, except as glochidia larva attached to fishes (Schwalb & Pusch, 2007; Strayer, 1999). While seasonal variation in eDNA detection of mussels, attributed to reproductive timing, has been observed late spring to late summer (Wacker et al., 2019), our sampling occurred over a much smaller timescale. The short time period between sampling events likely precludes any substantial changes in environmental factors (e.g., temperature, turbidity, salinity, and pH) that have been associated with the alteration of eDNA decay rates (Harrison et al., 2019; Strickler et al., 2015). While the mussels and fishes we detected may be present in different locations at points in the season, mussels only require access to fish during brooding, which can occur throughout the season for different species.

The differences in fish detection across sampling events highlight the importance of multiple sampling events at different points in time to maximize the detection of species that may be present only during a portion of the year. In addition, the lack of differences we found in the detection of mussel species between sampling events is encouraging, because the June sampling event occurred when traditional survey methods would likely not have been possible due to swift and highly turbid water.

5 | CONCLUSIONS AND RECOMMENDATIONS

We used a multiple marker eDNA metabarcoding approach to simultaneously detect a diverse group of mussel and fish taxa and found that it was highly effective and will likely be beneficial for monitoring and conservation efforts. We were able to validate the methodology while simultaneously exploring differences in the mussel and fish communities within and among major drainages and between two sampling events. In addition, we were able to identify fish species associated with areas of different mussel diversities. Our study does, however, reinforce the need for comprehensive and geographically broad sampling across a river watershed to maximize the probability of detecting all of the species present. Additionally, the collection of environmental covariates along with eDNA samples will potentially give insights into mechanistic relationships driving differences in taxa distributions and help inform management decisions.

The use of multiple metabarcoding assays will enable future researchers to efficiently monitor many biotic components of an ecosystem in a single sampling effort. In addition to multi-marker eDNA, extracted eDNA collected for one project can easily be re-purposed for a myriad of research questions (see Dysthe et al., 2018).

AUTHOR CONTRIBUTIONS

WAL, PBM, and WKD designed the study. DTZ provided mussel tissue samples for primer development and consulted on the ecology and regional distribution of mussels throughout the study. KMG adapted the original eDNA sampling and laboratory protocols, oversaw Sanger sequencing, and designed the unionid COI primers. WKD conducted the laboratory analysis. WKD, PDB, and WAL performed the data analyses. All authors contributed in drafting and editing the manuscript.

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CONFLICT OF INTEREST

No conflicts of interest were declared.

DATA AVAILABILITY STATEMENT

Raw sequence reads (fastq format) used in this research along with corresponding metadata are archived with the Dryad Digital Repository (https://doi.org/10.5061/dryad.1c59zw3zs).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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