PRELIMINARY ANALYSIS SUGGESTS FRESHWATER INVERTEBRATE ENVIRONMENTAL DNA IS MORE CONCENTRATED IN SURFACE WATER THAN IN BENTHIC SEDIMENTS

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Abstract.–The collection, identification, and census of freshwater invertebrates helps to increase understanding of the ecological function of lakes and streams. However, this work can be time-consuming and laborious because invertebrate identification often requires considerable taxonomic training and expertise. The collection and analysis of environmental DNA (eDNA), the genetic material that organisms shed into their surrounding environment, represents a potentially revolutionary approach for rapid and accurate invertebrate surveillance in freshwater environments. Previous studies have demonstrated that fish eDNA tends to be more abundant in freshwater lake sediments than the water column above, so we conducted an experiment to examine whether this pattern holds true for freshwater invertebrates. We collected paired samples from benthic sediments and the water column at ten sites around an urban playa lake in Lubbock, Texas. Based on cycle threshold values from quantitative PCR (qPCR) amplification with universal invertebrate primers targeting the COI gene, a paired Wilcoxon signed-rank test and Spearman rank-order correlation suggested that invertebrate eDNA quantities were correlated between the sediment and water column but consistently more concentrated in the water compared to the sediment below, directly contrasting with previous studies of fish eDNA. Future work combining eDNA detection and high-throughput sequencing (i.e., metabarcoding) will increase understanding of how eDNA signals relate to local invertebrate pools and increase the utility of eDNA sampling for freshwater invertebrates.

Keywords: aquatic, bioassessment, bioindicator, eDNA, macroinvertebrate, monitoring, playa lake

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Invertebrates fulfill multiple critical ecological roles across diverse ecosystems, including acting as drivers of carbon and nutrient transfer

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though food webs (Covich et al. 2004; Lavelle et al. 2006; Prather et al. 2013). Furthermore, invertebrates represent valuable targets for environmental study and management as bioindicators of both terrestrial and aquatic habitat quality (reviewed by Burger 2006). Thus, collection, identification, and census of freshwater invertebrates are foundational for ecological understanding of wetlands, lakes, and streams. However, collection of freshwater invertebrates can be time-consuming and laborious, and identification often requires considerable taxonomic training and expertise.

Collection and analysis of environmental DNA (eDNA), the genetic material that organisms shed into their surrounding environment, represents a potentially revolutionary approach for rapid and accurate invertebrate surveillance in freshwater environments. Indeed, multiple studies have applied eDNA analysis to diverse invertebrate taxa. For example, eDNA analysis has been used to detect invasive New Zealand mudsnails in freshwater sites in both the western (Goldberg et al. 2013) and eastern (Woodell et al. 2021) USA, as well as other invasive invertebrates including crayfish (Agersnap et al. 2017), and quagga and zebra mussels (Barnes & Patiño 2020). Threatened and endangered invertebrates have also been the target of eDNA analysis, including mussels (Klymus et al. 2020) and insects (Doi et al. 2017; Mauvisseau et al. 2019). With advancing technology, recent research has begun to combine eDNA collection with high-throughput sequencing (i.e., metabarcoding) to enable whole-community characterization rather than just single-species detection (e.g., Brantschen et al. 2021; Ficetola et al. 2021).

As eDNA applications and technology have increased, so too has the knowledge that biotic and abiotic factors in the environment influence the origin, state, transport, and fate of eDNA (collectively referred to as “the ecology of eDNA”), which in turn influence information gained through eDNA analysis (Barnes & Turner 2016). For example, Turner et al. (2015) found that fish eDNA was 8–1800 times more concentrated in aquatic sediments compared to the water column directly above. It is unknown whether invertebrate eDNA follows a similar trend, but this knowledge could lead to more efficient

and sensitive eDNA detection of invertebrates in aquatic systems. Therefore, our objective was to compare the concentration of invertebrate eDNA present in paired benthic sediment and surface water samples.

MATERIALS & METHODS

Study site.—Sample collection took place within a freshwater urban playa lake at Clapp Park in Lubbock, Texas (33.555611, -101.864917) on 29 Sep 2021. We selected ten sites approximately equally spread out around the perimeter of the lake (Figure 1). At each site, we collected paired sediment and water samples and conducted a traditional invertebrate survey as described in the following sections.

Environmental DNA collection.—At each site, we collected paired benthic sediment and surface water samples for eDNA analysis. First, we collected 1 L of surface water from the shore using a sterile bottle. Next, we obtained a core of approximately 40 mL benthic sediment (core depth = approximately 5 cm) immediately below where we collected surface water using a sterile 50-mL conical vial. Water and sediment samples were stored on ice for transport to the laboratory. Clean gloves were worn during all sample collections to avoid contact with the water, and we changed gloves between sites to prevent site-to-site contamination.

Traditional invertebrate sampling.—To provide preliminary data on the invertebrate diversity and abundance across sites, we conducted traditional invertebrate sampling at each site. Dip net surveys using a 500-µm D-frame net occurred only after collection of surface water and sediment samples for eDNA analysis to avoid stirring up benthic materials, artificially mixing the aquatic environment, or otherwise disrupting the environment in a way that could influence eDNA collection. At each site we performed five dip net passes, each pass lasting approximately 10 sec, and preserved collected invertebrates in 70% ethanol. In the laboratory, we used a dissecting microscope as necessary to identify each collected invertebrate to the most specific taxonomic level possible based on Thorp & Rogers (2016).

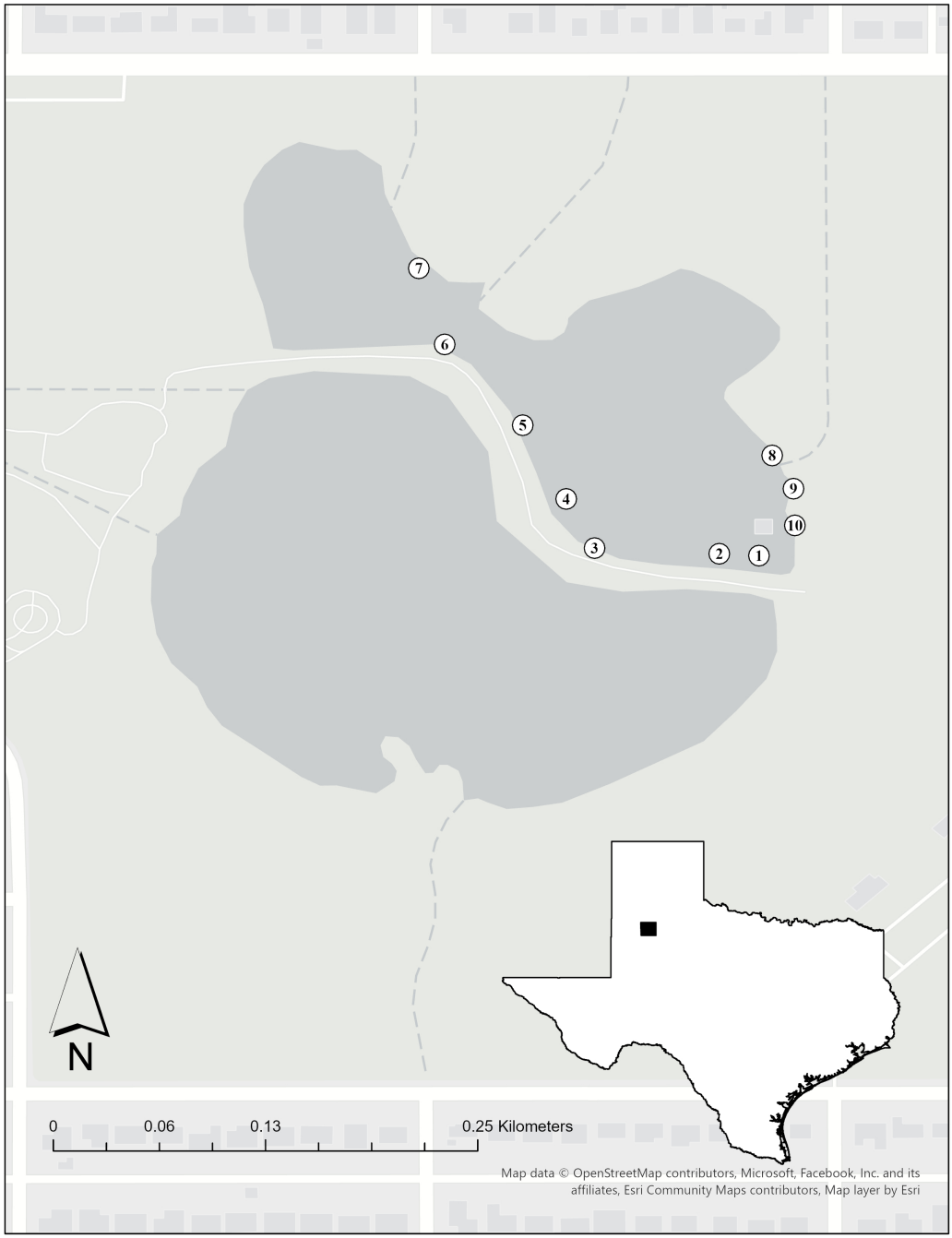

Figure 1. Paired sediment and surface water samples, as well as traditional invertebrate sampling, occurred at ten sites (numbered 1–10) approximately equally spaced around the perimeter of an urban playa lake at Clapp Park, Lubbock, Texas. Map produced using *Google Earth*, earth.google.com/web/.

Table 1. Volume of water (mL) filtered in 5 min for each sampling site.

Sample	1	2	3	4	5	6	7	8	9	10
Water volume filtered (mL)	100	75	75	415	160	75	65	65	50	80

DNA Extraction.—Upon returning to the laboratory, we vacuum-filtered water samples using 1 µm PCTE membranes. Due to differences in turbidity, samples filtered at different rates, so to avoid clogging (Turner et al. 2014), we filtered each sample for a maximum of 5 min and recorded filtered volume (Table 1). Filters and sediment samples were stored at -20°C until DNA extraction.

We extracted eDNA from filters (= surface water samples) using a CTAB-chloroform method (Barnes et al. 2020). Briefly, we began by adding 500 µL CTAB cell lysis buffer to each sample. We then added 500 µL chloroform:isoamyl alcohol to dissolve the PCTE filter and separate DNA from cellular debris and other non-DNA components. Solutions were vortexed for 5 min, then centrifuged at 15,000x g for 15 min. We then pipetted 500 µL of the supernatant layer containing extracted DNA to a new microcentrifuge tube. We added 500 µL isopropanol and 250 µL 5M NaCl to the solution and incubated at -20°C overnight. The next day, we centrifuged at 15,000x g for 15 min to concentrate DNA in a pellet at the bottom of the tube, then decanted liquid from the tube. Next, we rinsed the pellet with 150 µL 70% ethanol centrifuged at 15,000x g for 5 min before again decanting liquid from the tube. This rinse step was repeated once more before we air-dried residual ethanol and resuspended the extracted DNA in 100 µL low-TE buffer. The DNA was stored at 4°C until further analysis.

To extract eDNA from the sediment samples, we followed a modified protocol based on Taberlet et al. (2012). First, we added 30 ml phosphate buffer to each sediment sample and vigorously homogenized the mixture for 20 min. We centrifuged the solution at 4100x g for 40 min, then transferred 500 µL supernatant into a new microtube while the pellet and residual buffer were discarded. Finally, DNA was extracted using a NucleoSpin Soil kit (NucleoSpin Soil;

Macherey-Nagel, Düren, Germany) following manufacturer instructions and eluting the DNA into 100 µL buffer.

PCR amplification.—We measured total invertebrate eDNA in each sample using quantitative polymerase chain reaction (qPCR) and primers LCO1490 and HCO2198 of Folmer et al. (1994), which target an approximately 700-bp fragment of the mitochondrial cytochrome oxidase subunit I gene (COI) across most invertebrate taxa. Based on experience with PCR inhibition in previous studies at Clapp Park (Barnes et al. 2020), we diluted extracted DNA 1:10 using ultrapure water. Each 20-µL qPCR reaction included 1x Perfecta Tough Mix (Quantabio, Massachusetts), 200 nM forward and reverse primers, and 4 µL diluted DNA template. Reaction conditions included an activation step of 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. In a qPCR run, each sample was prepared in three technical replicates, including three non-template control reactions with TE buffer in place of template DNA, which served to ensure no contamination occurred during qPCR setup. We conducted qPCR in this manner twice, resulting in six total technical replicates per sample. We assigned a value of 40 to any qPCR reactions that did not show amplification. The average cycle threshold (Ct) value of six technical replicates was recorded as an indicator of invertebrate eDNA quantity.

Statistical analysis.—Cycle threshold data, the number of cycles it takes to detect target DNA, are not normally distributed because they are artificially bound by the maximum number of cycles in a PCR protocol (i.e., 40 cycles in our experiment). Thus, we analyzed Ct data using nonparametric analyses. First, because filtered water volume differed between samples (Table 1) and because there was not an analogous variability in sample size in sediment sample processing, we used a Spearman Rank correlation to confirm that volume filtered did not affect the Ct value of surface water samples. We ran a paired Wilcoxon signed-rank test to assess differences in Ct value between sediment and water samples. We used separate Spearman rank correlations to ask whether the Ct values of sediment or water samples related to the number of individual invertebrates collected at each site. We also applied a one-tailed Spearman rank-order correlation to

determine whether a positive relationship existed between Ct values in sediment and water samples. Statistical analyses were conducted using R version 4.1.2 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

The number of invertebrate species and total number of individuals collected with net-based surveys varied among sites, ranging from two to eight taxa and 3–22 individuals per site (Table 2). Overall, we collected specimens representing eight taxonomic orders (Acoela, Araneae, Coleoptera, Diptera, Gastropoda, Hemiptera, Odonata, and Trichoptera). Coleopterans were the most represented taxon (36.7% of all individuals collected), followed by Diptera and Hemiptera (26.6% each). Odonata and Trichoptera (1.3% each) were the least represented taxa. Total invertebrate abundance differed among sites, with Site 10 (27.5% of all collected individuals) demonstrating the highest abundance and Sites 4, 6, and 8 (3.8% each) representing the lowest (Table 2).

A Spearman rank correlation indicated that surface water Ct values were not related to volume filtered ($p = 0.428$). Spearman rank correlations also indicated that the number of individual invertebrates collected at a site did not predict the Ct of surface water ($p = 0.1368$) or sediment ($p = 0.1136$) eDNA samples (Table 3). However, a one-tailed Spearman rank correlation did indicate that the amounts of invertebrate eDNA in benthic sediment and surface water samples were positively related ($p = 0.037$), and a paired Wilcoxon signed-rank test indicated that the amount of invertebrate eDNA in surface water samples exceeded the amount of eDNA in benthic sediment at the same site ($p = 0.002$; Figure 2).

Table 2. Identification (to order and family, where possible) and count (n) of all invertebrates collected at each site using traditional netting methods.

Site	Order	Family	n
1	Diptera	Tabanidae	4
	Hemiptera	Notonectidae	1
2	Acoela	Mecynostomidae	2
	Coleoptera	Dytiscidae	1
		Noteridae	1
	Diptera	Tipulidae	2
		undetermined	1
	Hemiptera	Notonectidae	1
		undetermined	2
3	Coleoptera	Sphaeriusidae	1
	Diptera	Tabanidae	5
		Ceratopogonidae	1
		Dixidae	1
	Hemiptera	undetermined	3
4	Araneae	Pisauridae	1
	Hemiptera	Notonectidae	2
5	Coleoptera	Dytiscidae	2
	Gastropoda	Planorbiodea	1
	Hemiptera	undetermined	2
6	Araneae	Pisauridae	1
	Coleoptera	Dytiscidae	1
	Gastropoda	undetermined	1
7	Coleoptera		
		Dytiscidae	5
		undetermined	1
	Diptera	Ceratopogonidae	1
	Hemiptera	undetermined	3
		undetermined	1
		undetermined	1
	Odonata	Lestidae	1
	Trichoptera	undetermined	1
8	Coleoptera	Curculionidae	1
		Dytiscidae	2
9	Coleoptera	Dytiscidae	1
	Diptera	Tipulidae	1
	Gastropoda	undetermined	1
	Hemiptera	Notonectidae	1
		undetermined	2
10	Coleoptera		1
		Dytiscidae	0
		Notonectidae	2
		Hydraenidae	1
	Diptera	Tipulidae	1
		undetermined	4
	Hemiptera	undetermined	4

DISCUSSION

As eDNA applications continue to develop, research is needed that advances the understanding of the ecology of eDNA and how it influences our inferences in eDNA detection studies (Barnes et al. 2021). Although previous literature has shown that fish eDNA is more abundant in sediment samples than surface water (Turner et al. 2014), our study found more invertebrate eDNA present in the water column than in the sediments below. Understanding why different taxonomic

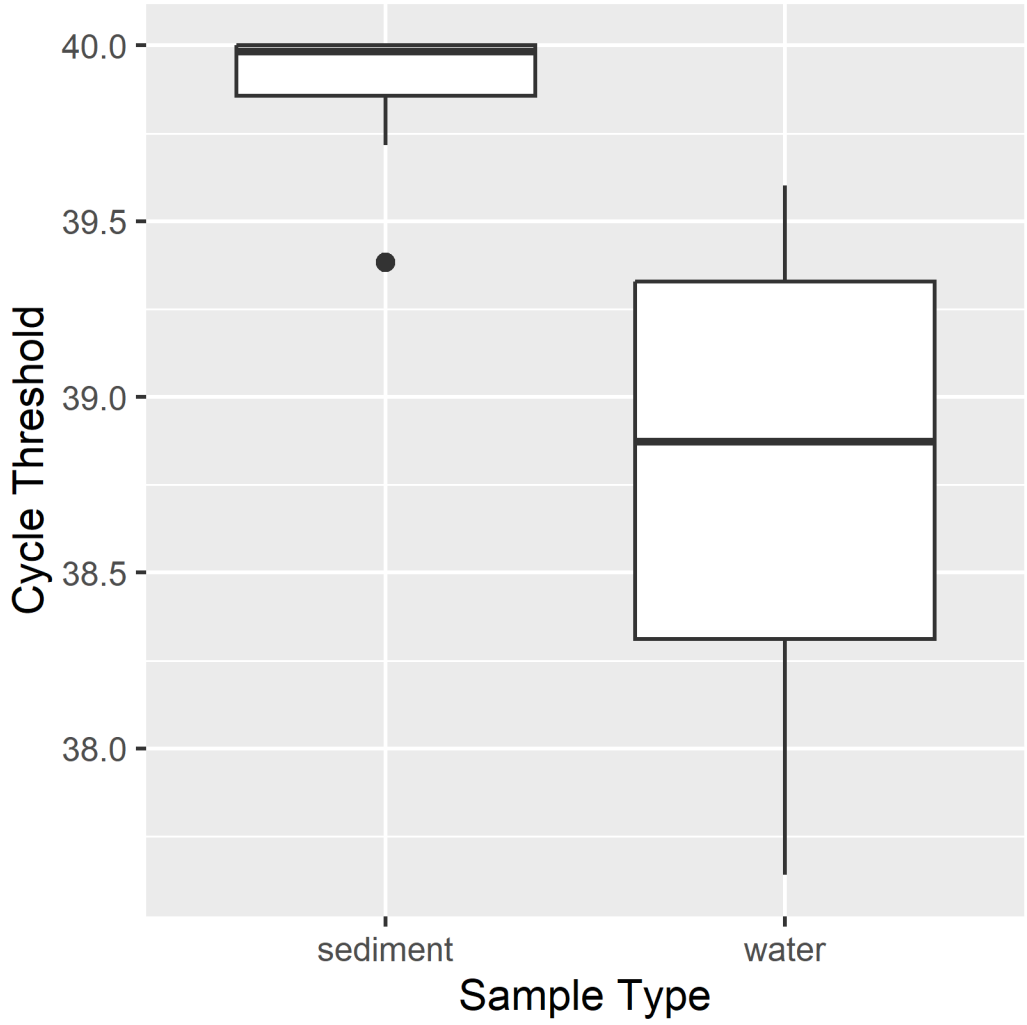

Figure 2. Cycle threshold (Ct) was significantly later in sediment samples than paired surface water samples (Wilcoxon signed-rank p = 0.002), indicating more invertebrate eDNA present in water compared to sediment. In box and whisker plot, the bold line identifies the median, boxes extend to the first and third quartiles, and whiskers depict the 95% confidence interval. The black point represents a statistical outlier beyond 1.5 times the interquartile range over the 75th percentile.

groups may display different eDNA trends will be critical to expanding the utility of eDNA analyses and generalizing across studies.

We suspect that the higher quantities of invertebrate eDNA collected in the water column as compared to the sediment could be the result of the capture of whole micro-invertebrates, such as rotifers and

Table 3. Cycle threshold (Ct) values for each qPCR replicate of each surface water or benthic sediment sample from ten sites. Lower Ct values correspond to higher quantities of eDNA. Reactions that failed are indicated by a dash (–) and were assigned a Ct value of 40 for statistical analyses.

Site	Sample Type	Cycle Threshold Value (Ct)					
1	surface water	–	–	–	–	37.612	–
2	surface water	–	–	–	34.090	37.590	–
3	surface water	–	–	–	–	35.978	–
4	surface water	–	–	36.617	–	–	–
5	surface water	37.019	–	–	36.937	37.797	–
6	surface water	–	–	–	34.885	37.470	36.908
7	surface water	–	–	–	39.289	33.284	33.972
8	surface water	–	–	–	–	35.922	–
9	surface water	–	–	–	–	34.714	–
10	surface water	–	–	–	34.200	38.943	32.698
1	benthic sediment	–	–	–	–	–	–
2	benthic sediment	–	–	–	–	–	–
3	benthic sediment	38.962	–	–	–	–	–
4	benthic sediment	–	–	–	–	–	–
5	benthic sediment	–	–	–	–	–	–
6	benthic sediment	39.694	–	–	–	–	–
7	benthic sediment	–	38.297	–	–	–	–
8	benthic sediment	–	–	–	–	–	39.794
9	benthic sediment	–	–	–	–	–	–
10	benthic sediment	36.302	–	–	–	–	–

small copepods, within our surface water samples. Such organisms are unlikely to be detected with traditional methods such as our net-based survey, but they can easily be collected within bulk water samples. Unfortunately, by the conclusion of our laboratory analyses, there was not enough DNA template remaining to explore this hypothesis via sequencing. Combination of eDNA-based methods and high-throughput sequencing (e.g., Brantschen et al. 2021) in future studies will test this hypothesis, but our results suggest that eDNA may provide a valuable complement to traditional methods due to its ability to efficiently document the smallest invertebrates that may go undetected by more traditional methods. Mis-priming (e.g., both failure to amplify

invertebrate DNA from some taxa as well as undesired amplification of non-invertebrate taxa) has been noted with the primers we used in our experiment (e.g., Geller et al. 2013). If present, we anticipate that errors associated with mis-priming would be stochastically distributed across our study, so it is unlikely that mis-priming influenced the overall pattern in our results, but sequencing-based approaches will explore this possibility in future studies.

Our results indicate that eDNA does not exist as a homogenous mixture across an entire system, even in a relatively small lake, and even considering sediments vs. surface waters. This is supported by our net-based results, which demonstrated differences in number of species and individuals across sites. Critically, the amount of eDNA did not vary in a predictable way with the number of invertebrates collected at each site, suggesting other aspects of the ecology of eDNA (origin, state, transport, and fate) also influenced our results. This is further supported by our finding that filtered water volume was unrelated to Ct in water samples. Critically, due to the use of two different extraction methods for sediment vs. water samples, the comparison of eDNA concentration per unit volume (i.e., mL water or g sediment) is also murky, and the correlation as well as sample-to-sample variation observed in our study deserves continued study. Such variation represents a critical consideration for future eDNA-based studies and stresses the importance of collection and analysis of replicate samples as well as samples from across substrate types (e.g., water vs. sediment) and possibly across space and habitat types (e.g., open water, shorelines, backwaters, and eddies) to maximize the representativeness of eDNA surveys.

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