

FINAL PERFORMANCE REPORT



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Detecting Native Aquatics Network (DNANet)—A novel biodiversity freshwater monitoring program for native aquatic and semi-aquatic species using Environmental DNA

Oklahoma Department of Wildlife Conservation

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FINAL REPORT

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Executive Summary:

This report represents the fourth and final year of the grant after we completed an approved, one year No Cost Extension (NCE) for the study. The NCE for this award was requested for several reasons, all related to needing more time to finalize our assessment of methodologies for eDNA work, screening and analysis of samples collected as part of the grant and writing up final reports related to the work. Although we confirmed in the early stages of this project that an environmental DNA (eDNA) methodology can work and be applied to wildlife surveys in Oklahoma, we experienced a series of bottlenecks during the first few years of the grant relating to optimizing just how to best employ environmental DNA programs. The biggest issues we have had to try to overcome, or at least better understand, pertain to sampling protocols in the field and DNA extraction protocols in the lab. It is clear now with our studies and others that have been published over the last few years that employing an eDNA approach to biodiversity surveys is not as straight forward as many expect it to be. The concentrations in the wild of targeted species' DNA are incredibly dilute and, in our case, the microhabitats we need to collect and filter water from are often mucky ponds, streams, or temporary pools of water. We spent longer time than expected working to optimize how we were sampling a given environment and what abiotic variables were the best to collect. This was critical to better understanding how best to apply this technique in future studies in the state, but also set us behind our intended timeline for completing our work. Fortunately, the one-year NCE that was approved allowed us to focus our efforts largely on analysis of our eDNA screening results and abiotic environmental data we collected and begin assessing how best to interpret our results.

Overall, the results of this project provide an optimized pipeline for implementing a non-invasive, eDNA monitoring program for tracking the presence of native aquatic and semi-aquatic species in Oklahoma. The methodologies and approaches developed and tested during this project can be applied towards building a **Detecting Native Aquatics Network (DNANet)** in Oklahoma. Although there are considerations for how broadly and easily an eDNA survey approach can be applied to wildlife monitoring in Oklahoma, such an approach has the potential to contribute to statewide databases for occurrence and abundance of native and invasive species for biodiversity monitoring and conservation initiatives.

During the course of this project, we successfully developed and tested primer-probe eDNA assays for four native Species of Greatest Conservation Need (SGCN): three amphibians (*Ambystoma annulatum*, *Amphiuma tridactylum*, *Lithobates areolatus*), and one turtle (*Deirochelys reticularia miaria*), plus one common, widespread species (amphibian *Acris blanchardi*; Table 1). All assays were taken through rigorous *in vitro* testing procedures in the laboratory prior to being employed to screen field-collected eDNA samples collected March–July 2017–2018. Our field sampling schema used the following hierarchy, represented from largest category to smallest: ecoregion (N = 4), counties (N = 11), large-scale sampling sites (N = 25), unique waterbodies within each site (N = 79 total; 1–9 per site) (Figure 1; Table 2–4). Sites included national wildlife refuges (NWR), state parks (SP), state-run wildlife management areas (WMA), The Nature Conservancy preserves (TNCP), and public access points (PUA) affiliated with U.S. Army Corps of Engineers lakes. We also created a negative control for each waterbody by filling a water sample bag with dH₂O, sealing it, and dipping the sealed bag into the waterbody for approximately 30 seconds (resulting in a total of 646 samples to be screened; Table 2). In aquatic environments, eDNA screening can be broken down into the following four steps, for which we developed distinct protocols, that could be used by others across the state: (1) water collection and abiotic factor measurement, (2) DNA concentration via water filtration, (3) DNA extraction, and (4) genetic screening. Due to differences in positive sample sizes, abiotic factor analysis only occurred for the positive control, *A. blanchardi*.

During the first two years of the grant, we also made a first attempt at designing assays for two other amphibian species of conservation concern: *Eurycea multiplicata* and *Eurycea tynerensis*. Given the delays experienced in optimizing our methodologies and primary focus on the four SGCN species mentioned above, we were not able to optimize assays for *E. multiplicata* and *E. tynerensis*. Although both assays showed promise during *in vitro* testing in the laboratory, some cross-amplification issues were observed resulting in the occasional false positive detection associated with the assay amplifying DNA from a closely related species. This means that our first attempt at designing assays for these two salamander species resulted in successful assays, but neither were sensitive enough to exclude picking up DNA occasionally (in only a few of our tested reactions) of closely related species in the same genus *Eurycea*. It is our hope that a future student or researcher can continue working to establish these assays, and others, to add to the state's DNANet toolkit.

During Year 4 of the project, we fine-tuned our eDNA screening methodologies, to ensure that we were maximizing potential positives and minimizing false negatives. This was achieved by re-extracting and rescreening several samples, to confirm that there was no change in results. We also worked closely with graduate student personnel on training to reduce potential errors due to contamination. Three manuscripts have now been submitted to peer-reviewed journals for publication based on data collected or methodologies developed as part of this grant. The first has just been accepted at the journal *Conservation Genetics Resources* and is focused on

describing the four primer-probe assays developed for the target SGCN species (Siler et al., in press). The second manuscript was submitted to the journal *Environmental DNA* and is currently being revised for resubmission following reviewer and editor feedback (Watters et al., in review). Overall, we feel we have made great progress in streamlining and improving methodologies for using environmental DNA approaches in wildlife management and monitoring.

I. BACKGROUND AND NEED:

We continue to face a critical challenge—our planet is experiencing a continuous decline in biodiversity worldwide (Butchart et al., 2010; Thomsen and Willerslev, 2015). Whether our diversity of wild plants or animals is decreasing due to natural or human-mediated disturbances (Barnosky et al., 2011; Dirzo et al., 2014), it is clear that extinctions are increasing at unprecedented rates (Pimm et al., 1995; Barnosky et al., 2011), a pattern that is expected to have direct impacts for human health and resource sustainability (Diaz et al., 2006). A large impediment to this global biodiversity conservation crisis is simple—the lack of knowledge on the presence and distribution of biodiversity—with the majority of species on our planet still undescribed (Goldberg et al., 2015).

Nearly all biodiversity conservation efforts depend on reliable species' distribution patterns and population information resulting from standardized monitoring activities (Goldberg et al., 2015). To date, conservation efforts to study and protect our planet's biodiversity depend largely on monitoring programs aimed at collecting data on species distributions and population sizes (Thomsen and Willerslev, 2015). These programs commonly require time-intensive, often invasive, visual surveys and physical documentation of individual members of a given species. For example, current conservation and research programs tracking the emergence of amphibian infectious diseases require the physical capture of individual frogs or salamanders, collection of cotton swab samples taken from each individual's body, and sample screening in a molecular lab for pathogens. The situation is complicated further for rare species, with presence-absence data often weighted towards higher frequencies of zero observations (MacKenzie et al., 2002; McGrath et al., 2015). Although some of these observations reflect true site absences, studies show that such datasets contain a substantial number of 'false zero' observations for sites where a species is present despite going undetected (McGrath et al., 2015).

As the geographic and taxonomic scale of conservation needs has increased rapidly, so has the need for more efficient and alternative methods for monitoring biodiversity across landscapes and entire ecosystems (Thomsen and Willerslev, 2015; Goldberg et al., 2015). With the survival of many organisms on our planet tied almost entirely to the quality of freshwater resources, these species act as biological indicators and provide critical insight into environmental health (Niemi and McDonald, 2004; Beebe and Griffiths, 2005). Sampling these through traditional survey-based methods has often proven difficult due to cryptic behaviors and/or low densities, thus the failure to detect a given species may not actually indicate an absence of that species from a specific area. However, as individuals interact with the environment, genetic material (DNA) is left behind in the form of urine, feces, hair, and skin, etc (Lydolph et al., 2005), and is termed environmental DNA (eDNA). The ability to sample this eDNA represents a cutting-edge, non-invasive approach for monitoring biodiversity that can be easily standardized and broadly applied (Ficetola et al., 2008; Goldberg et al., 2015; Thomsen and Willerslev, 2015). Empirical and experimental studies have now shown eDNA to degrade at an exponential rate in aquatic environments, with detection probability dropping below 0.5 within the first 72 hours (for

review: Barnes et al., 2014)—therefore, use of such survey methods provide a real-time assessment of species presence in the environment.

Given that every species on our planet, including viruses and fungi, possess unique variation across their genome, similar to a genetic fingerprint, the screening of such variation permits identification of individual species, and at times even populations or individuals (Appendix I). Retrieving genetic information of individual species, populations, or communities from environmental samples has the potential to overcome many of our challenges associated with currently employed biodiversity monitoring programs (Baird and Hajibabaei, 2012; Kelly et al., 2014). The successful screening of environmental samples, particularly water samples for eDNA, has resulted in the rapid growth of studies applying these methods broadly in biodiversity conservation (Rees et al., 2014). In the last three years alone, eDNA has been employed in studies of amphibians (Pilliod et al., 2013a,b; Biggs et al., 2014; Spear et al., 2014), reptiles (Piaggio et al., 2013), endangered and invasive fish (Mahon et al., 2013; Takahara et al., 2013; Wilcox et al., 2013; Laramie et al., 2015), invertebrates (Deiner and Altermatt, 2014; Sigsgaard et al., 2015), marine species (Kelly et al., 2014), aquatic invasive plants (Scriver et al., 2015), and even infectious amphibian diseases (Schmidt et al., 2013; Chestnut et al., 2014).

However, prior to this work, *no studies to date have applied eDNA methods for screening aquatic habitats in Oklahoma or explored the use of eDNA as a tool for biodiversity monitoring in the state.* This project worked to address the following three critical questions: **(1) Can eDNA be used to document the distribution of rare and threatened species in aquatic environments in Oklahoma? (2) Can eDNA be used to quantify population densities of focal species at surveyed sites? (3) Can we develop an eDNA network and protocols for sustained monitoring of Oklahoma's threatened species?**

During the four-year project, we developed and optimized a non-invasive, eDNA monitoring program for rapidly tracking the presence of native aquatic and semi-aquatic species in Oklahoma. For this pilot study, we successfully designed eDNA assays for four species in the east-central and southeastern portions of the state (Appendix II), each designated as “Oklahoma’s Species of Greatest Conservation Need” (SGCN; Appendix E: Oklahoma Comprehensive Wildlife Conservation Strategy [OCWCS]), as well as for a common, widespread amphibian in the state as a positive control for testing our methodologies. The results of this project have established a baseline database and pipeline that can be built upon to further develop a **Detecting Native Aquatics Network** (DNANet) in Oklahoma. Such an approach has the potential to contribute to statewide databases for occurrence and abundance of native and invasive species for biodiversity monitoring and conservation initiatives.

II. OBJECTIVE(S):

This project had three primary objectives:

- **Objective 1:** To estimate the presence and distribution of three species of greatest conservation need in east-central and southeastern Oklahoma by screening freshwater samples for eDNA collected during a series of focused field surveys across 13 counties.
- **Objective 2:** To establish the first phase of development of the OK DNANet, a long-term protocol for sustained non-invasive detection and monitoring of native aquatic and semi-aquatic biodiversity.
- **Objective 3:** To test whether the OK DNANet is a viable method for the quantification of site-specific population densities of native species which would allow for the identification of regions at greatest risk for threatened species.

III. METHODS

Taxonomic focus: We screened eDNA samples for positive genetic signatures of four native SGCN species (three amphibians [*Ambystoma annulatum*, *Amphiuma tridactylum*, *Lithobates areolatus*], and one turtle [*Deirochelys reticularia miaria*]), and one common, widespread species (amphibian [*Acris blanchardi*]).

Assay Development and Testing: The *A. blanchardi* specific qPCR assay was designed for the mitochondrial gene (mtDNA) cytochrome b (cytb). We aligned GenBank sequences for cytb from four *A. blanchardi* individuals from across the geographic range of the species (GenBank accession numbers: Illinois: EF988109; Mississippi: EF988144; Missouri: EF988127; Oklahoma: EF9881260) and four outgroup anuran species whose geographic ranges overlap with *A. blanchardi*. We used the alignment to find single nucleotide polymorphisms (SNPs) in cytb that separate *A. blanchardi* from all other species in the alignment. The consensus *A. blanchardi* sequence from all four individuals (682 bp) was generated in Geneious v9.0 and was imported into the program Primer Express Software v3.01 (Applied Biosystems). At each SNP site, we used Primer Express to find the optimal probe and primer sequences using default optimization settings (probe length = 13–25 base pairs, T_m = 68–70°C, %GC = 30–80%) (Table 1). Because the *A. blanchardi* primer-probe assay was the first one we designed, we tested specificity via three experimental protocols, using a combination of diluted (1/1000 and 1/10000 concentrations; 2×10^{-9} pg/ μ L of DNA) genetic tissue-obtained DNA to mimic natural occurring eDNA dilutions from target species, congeners, and sympatric species (Experiment 1), live frogs from target species, congeners, and sympatric placed into small water containers (Experiment 2), and target species placed in increasingly larger containers to mimic natural conditions (Experiment 3). All frogs used in the experiments were euthanized and prepared as voucher specimens for the Sam Noble Museum Herpetology Collection on the day of the experiment (Simmons, 2015).

For the SGCN species, we designed four species-specific primer and probe sets for qPCR assays targeting either the mitochondrial Cytochrome B (*Amphiuma tridactylum*, *Lithobates areolatus*, and *Deirochelys reticularia*) or Cytochrome Oxidase I (*Ambystoma annulatum*) genes (Table 1). DNA sequences for the focal species, closely related species, and additional species that occur sympatrically with the focal species were obtained from GenBank, and datasets were further supplemented with novel sequence data collected from vouchered tissue samples or blood samples. For novel sequencing efforts, DNA from vouchered tissue samples was extracted via a high salt extraction method (Esselstyn et al. 2008), and from *Deirochelys reticularia* blood samples via a DNeasy Blood & Tissue Kit (Qiagen), with extracts stored at -20°C until used for qPCR screening. Whenever possible, we collected sequence data from individuals from multiple populations across Oklahoma to represent the genetic diversity of the focal species and outgroups. The species *Amphiuma tridactylum* and *Deirochelys reticularia* are poorly represented in museum tissue collections, and so we also included individuals from neighboring states in our datasets.

All four SGCN assays were developed using Primer Express v3.0.1 (Applied Biosystems). For each species, DNA sequences were aligned using Geneious version 9.0.5 (Biomatters), and single nucleotide polymorphism (SNP) sites were identified in which the nucleotide at the site was unique and conserved for the focal species. In doing so, we found that the Cytochrome B sequences of *Deirochelys reticularia* were substantially differentiated (pairwise sequence divergence up to 17%) as expected among the three distinct subspecies recognized in *D. reticularia* (*D. r. chrysea*, *D. r. miaria*, *D. r. reticularia*; Buhlmann et al. 2008), and we were

unable to identify the SNP sites that are identical for all of the *D. reticularia* sequences and distinctive from the other taxa included in the alignment. Therefore, we designed the primers and probe specific only to the *D. reticularia* samples from Oklahoma and surrounding states (Arkansas), so that the assay can be used for the populations of the subspecies *D. r. miaria*. A consensus sequence for each focal species was generated in Geneious and imported into Primer Express for assay design. Each primer-probe set targeted regions of 64–72 base pairs in length. We conducted *in vitro* qPCR tests for each primer-probe assay with DNA extracts from individuals that were not included in the original sequence assay development dataset. For each test, DNA from several ingroup individuals from different populations was tested against several closely related or sympatric outgroup individuals to verify the specificity of each assay (similar to Experiment 1, described above for *A. blanchardi*).

Field Sampling: To minimize any seasonal factors that could negatively impact eDNA detection (De Souza et al., 2016; Takahara et al., 2020), we collected field samples from March–July in both 2017 and March–May in 2018 to coincide with the breeding season of *A. blanchardi* (McCallum et al. 2011) and *L. areolatus* (Heemeyer et al. 2012; Williams et al. 2012), the active larval period for *A. annulatum* (Briggler et al. 2004; Semlitsch et al. 2014), and prior to the dry season aestivation of *D. reticularia* (McKnight et al. 2015). For each waterbody, two to eight samples of 500–600 mL water were collected, 1–2 m from shore at a 5–10 cm water depth, using sterile 36 oz. one-time use Whirl-pak sampling bags (Wineland et al., 2019). Combined with the field-collected negative controls, this resulted in a total of 646 eDNA samples. Samples were then stored cold, but not frozen, in a dark cooler to prevent potential DNA degradation by UV or warm temperatures (Pilliod et al., 2013b; Strickler et al., 2015) and detection difficulties due to freezing (Takahara, et al., 2014). For each individual eDNA sample, we collected the following variables: GPS coordinates, elevation (m), pH, water temperature (°C), conductivity (µS), canopy cover, turbidity, and water flow speed (m/s) to assess the potential impact of environmental factors on eDNA detection of *A. blanchardi* (Pilliod et al., 2013a,b; Barnes et al., 2014; Stoeckle et al., 2017; Strickler et al., 2015). Our final field protocols are outlined in Appendix I.

Water Filtration: We filtered all samples within 24 hours of collection, maintaining strict sterility protocols. Prior to sample filtration, all work surfaces were sterilized with ELIMINase (Decon Labs) or 10% bleach. Water was homogenized in the sample bag, then poured into sterile, one-time use 500 mL polyethersulphone (PES) membrane filters, with a 75 mm filter diameter and a 0.45µm pore size (various vendors: ThermoScientific Nalgene, Fisher Scientific, VWR, Foxx Life Sciences Autofil). We vacuum filtered both the field samples and the negatives until the membranes became clogged or until 500 mL was filtered (whichever came first), cut out of the filter cup using a sterile, one-time use 11-blade scalpel to avoid cross-contamination of samples, and placed into a 10 mL cryovial with 95% ethanol for -20°C freezer storage until the time of extraction (less than six months). Our final water filtration protocols are outlined in Appendix II.

eDNA Extraction: All DNA extraction occurred at the Sam Noble Museum Genomics Core Facility. It is important to note that for each batch of eDNA extractions, a negative control sample was created by placing the same type of filter into sterile molecular grade dissolved water, and then extracted following the same protocol as all field-collected eDNA and control samples. Nitrile gloves were changed between every sample, to reduced concerns related to contamination. We isolated total genomic eDNA from one-half of each vouchered filter membrane, returning the remaining one-half of the filter to the freezer for archival purposes. In order to complete the extraction, we used a modified Qiagen DNeasy Blood and Tissue Kit

protocol, involving Qiagen QIAshredder and Zymo OneStep PCR Inhibitor Removal Kit protocols (Pilliod et al., 2013b; Buxton et al., 2017). We spent much of the first year of the project optimizing the eDNA extraction protocol and our final detailed protocol is described in Appendix III.

eDNA Screening: All eDNA screening and *in vitro* assay testing occurred by quantitative PCR (qPCR), which allows for the detection of extremely small amounts of genetic material, as it normally observed in eDNA samples. The qPCRs reactions were set up in duplicate on an Applied Biosystems MicroAmp Fast 96-well Reaction plate and run on a QuantStudio 3 (Applied Biosystems) using the Presence/Absence experiments option of the QuantStudio Design and Analysis Software v1.4. Our detailed qPCR screening protocols are provided in Appendix IV.

Statistical Analysis: Due to the fact that very few positive samples were detected in any of the SGCN species, we were only able to analyze the effects of ecoregion and abiotic factors as compared to *A. blanchardi* eDNA detection rates.

Univariate Analysis: We first assigned each specific waterbody to a corresponding ecoregion, based on Woods et al. (2005). We then conducted both (1) a nested analysis of variance (ANOVA) determining the effects of *Ecoregion* and *Site*, nested within *Ecoregion*, to influence abiotic variables and the proportion of *A. blanchardi* eDNA detected, (2) a Tukey's HSD post hoc test to determine which *Ecoregions* and *Sites* differed from one another in mean abiotic variables and mean proportion of *A. blanchardi* eDNA detected, and (3) a correlation matrix to determine Pearson pairwise correlation coefficients (e.g., positive or negative associations) between abiotic variables (predictors) and the eDNA variable (proportion of *A. blanchardi* eDNA) through a series of linear regressions. We used JMP 15 for all univariate analyses (SAS Inc, Cary, NC).

Multivariate Analysis: To determine whether *Ecoregion* and/or *Site*, differed in regards to the abiotic microclimate composition (e.g., similarity or dissimilarity in abiotic variable composition), as well as whether *Sites* with varying proportions of *A. blanchardi* eDNA detection differed in their abiotic environment, we conducted two separate non-parametric, permutational multivariate analysis of variance (PERMANOVA; Anderson et al., 2006). We standardized our abiotic variables (canopy cover, elevation, pH, conductivity, turbidity, water temperature, and water flow speed) and then generated a Bray-Curtis similarity matrix. The PERMANOVA approach allowed us to compare the variability in multivariate abiotic composition (e.g., compositional similarity) both *within* treatments (*Ecoregion* or *Site*) and *among* treatments (*Ecoregion* or *Site*) using a pseudo F-ratio to determine whether observed variability across study sites or in the proportion of samples detecting *A. blanchardi* eDNA abundance differed from the variability in abiotic composition using a null distribution (Anderson et al., 2006).

To illustrate the abiotic environment in multivariate space, we performed a series of principal coordinate analyses (PCoA) based on the Bray-Curtis similarity matrix to illustrate in a two-dimensional space the differences in location across *Ecoregions* and *Sites* of microclimate composition. We used the first two PCoA axes, which accounted for a significant proportion of total variation in compositional similarities, to illustrate both *Ecoregions* and *Site* level differences. We also performed a similarity percentage analysis (SIMPER) to determine the contribution of particular abiotic variables to the overall differences in abiotic compositional dissimilarities between *Ecoregions* and *Sites* or across *A. blanchardi* eDNA abundance. We used PRIMER version 6.0 (Plymouth Marine Laboratory, UK) for the multivariate analyses.

IV. RESULTS

In general, our newly developed eDNA protocols resulted in successful species detection; 100% of lab tests were successful in highly specific eDNA detection, with no false positives for any of the five target species. For our four SGCN species, field applications of the assays detected the presence of *A. annulatum* at three distinct waterbodies across two counties (Adair, Cherokee), *L. areolatus* at two waterbodies across two counties (Cherokee, Sequoyah), and *D. reticularia* at a single waterbody in Atoka County (Table 3). Field samples from McCurtain County in Southeast Oklahoma screened for the Three-toed Amphiuma returned a single positive sample; however, repeated screening of the eDNA extraction failed to amplify and all samples were treated as negative (Table 3). Overall, the low detection rates observed in the wild for three of the four focal species and failure to detect *Amphiuma tridactylum* during our field surveys is not surprising, given how rarely these species are observed and the seasonal nature of their activity cycles (Gibbons 1970; Fontenot 1999; Briggler et al. 2004; Heemeyer et al. 2012; Williams et al. 2012; Semlitsch et al. 2014). The positive detection of three of the focal species adds to baseline occurrence data that will aid in spatial analyses and conservation planning. The results of this work support the application of the four designed eDNA assays as a viable biodiversity monitoring method that can aid in more rapid detection of four rare and threatened amphibian and reptile species in North America.

Our results also indicate that the *A. blanchardi* primer-probe assay is able to detect species-specific DNA in a wide variety of field conditions and locations in Oklahoma. For a total of 565 samples (excluding field-based negative controls), *A. blanchardi* DNA was detected in 120 samples (21.2%; Table 2). No field-based negative controls tested positive for *A. blanchardi* DNA. Of the four eastern Oklahoma ecoregions from which we collected samples, *A. blanchardi* DNA was detected in all four and in 48 (60.0%) of 79 waterbodies (Table 4). Live individuals of *A. blanchardi* were positively identified at the time of eDNA sampling at a total of 31 (out of 79) waterbodies (39.2%), and of those, eDNA screening detected their presence only in 15 waterbodies (48.4%; Table 2). However, eDNA screening detected *A. blanchardi* DNA in 33 waterbodies where none of them were visibly observed and/or no field notations were made regarding their presence. The combination of visible observations and eDNA screening detected the presence of *A. blanchardi* in 64 waterbodies (81.0%), which may suggest the importance of using both eDNA and traditional methods to get a complete picture when conducting biodiversity surveys. *Acris blanchardi* eDNA was detected at every large-scale site, with the exception of Johnson Creek Public Use Area (Table 4).

At the ecoregion scale, samples of *A. blanchardi* eDNA was significantly more detected in the Ouachita Mountains/Arkansas Valley/West Gulf Coastal Plains Ecoregion than in Tallgrass Prairie; Crosstimbers and the Ozarks Ecoregions were not significantly different from each other or from the other two ecoregions (Figure 2). All of the following abiotic variables exhibited ecoregion-level differences at p-values <0.0001: canopy light, conductivity, elevation, pH, and temperature (Figure 3). Turbidity (separated by year) and water flow did not show statistical differences by ecoregion alone, but did have differences between sites.

In the pairwise correlation matrix amongst abiotic variables, only temperature had a significant positive association/relationship with the proportion of samples detecting *A. blanchardi* eDNA, explaining approximately 23% of the total variation. Compositional similarity of microclimatic conditions differed across ecoregions and across sites. Specifically, microclimate composition was the most dissimilar between the Crosstimbers Ecoregion than the other three ecoregions: Ouachita Mountains/Arkansas Valley/West Gulf Coastal Plains, Ozarks,

and Tallgrass. At the site level, locations within the Ouachita Mountains/Arkansas Valley/West Gulf Coastal Plains Ecoregion differed the most from each other. Further, dissimilarities in abiotic microclimate composition across sites were mostly driven by differences in conductivity (83–99%) and canopy light (15%) (Figure 4).

In summary, our research revealed that abiotic variables in sampled aquatic environments vary significantly at both ecoregion and site level scales in Oklahoma, with the exception of turbidity and water flow (Figure 3), but that only temperature had a direct positive correlation with *A. blanchardi* eDNA detection rates. While the proportion of samples detecting *A. blanchardi* eDNA varied between ecoregions, statistical differences were only observed between the Ouachita Mountains/Arkansas Valley/West Gulf Coastal Plains Ecoregion and the Tallgrass Prairie Ecoregion (Figure 2). Additionally, the eDNA detection proportions were all fairly low, suggesting that ecoregion differences may not have been the only abiotic factor contributing towards eDNA detection (Figure 2; Table 4). In fact, for the 31 waterbodies at which field crews made concrete notes about *A. blanchardi* presence at the time of eDNA sample collection and/or a specimen was vouchered (on the same date as eDNA sample collection), qPCR screening failed to detect the species 52% of the time (Table 4). In aquatic environments, eDNA screening can be broken down into the following four steps, with potential loss of eDNA at each step and myriad questions that could be posed to resolve the issue, which are unfortunately outside the scope of this particular pilot project: (1) water collection, (2) DNA concentration (How much water was filtered? Was the filter clogged early due to small pore size or high turbidity?), (3) DNA extraction (How much filter was extracted? Was there contamination?), and (4) genetic screening (Were inhibitors present? Did primers and probes perform as expected?) (Guillera-Arroita et al., 2017). Whatever the original cause, these false negatives are of concern when considering that species management decisions may be made based solely on the results of eDNA studies, as has been proposed (Wilson & Wright, 2013; Harper et al., 2018).

RESULTING PUBLICATIONS

Siler, C.D., Freitas, E.S., Yuri, T., Souza, L., Watters, J.L. *In press*. Development and validation of four environmental DNA assays for species of conservation concern in the south-central US. *Conservation Genetics Resources*.

Watters, J.L., Yuri, T., Souza, L., Freitas, E.S., Siler, C.D. *In review*. Development of environmental DNA screening method for the Blanchard's Cricket Frog *Acris blanchardi* and its application to eDNA methodologies in eastern Oklahoma, USA. *Environmental DNA*.

Smith, S.N., Schlupp, I., Higgins, E. D., Watters, J.L., Bennett, K.-A., Bräger, S., Siler, C. D. *In review*. Development and validation of an environmental DNA protocol to detect an invasive Caribbean freshwater guppy (*Poecilia reticulata*). *Environmental DNA*.

V. RECOMMENDATIONS

PRIORITY 1 – REDUCING LIMITATIONS

Amphibians will continue to represent one of the most threatened vertebrate groups on the planet. As such, continued monitoring of amphibian populations across the state are critically needed; however, amphibian survey efforts still often depend on more time-consuming and invasive visual surveys. Despite clear challenges to applying an environmental DNA (eDNA) approach to biodiversity surveying and monitoring, the technique still has great promise as a

toolkit in a growing statewide monitoring program for amphibians and reptiles. However, we believe that there are several areas that must be explored further to better optimize the process.

First, given how dilute DNA left by host species is in the environment, and how quickly it degrades (Stewart 2019), it is important to filter as large of a volume of water as possible across a single filter unit to concentrate as much eDNA as possible from the environment. Unfortunately, given the microhabitats our focal species are often found in, we observed that our filters would clog after about 250–400 mL of water passed through. In fact, approximately one-third of our field samples clogged before reaching the desired filter volume of 500 mL, presumably resulting in less overall captured eDNA. We considered changing to a larger pore size (i.e. 0.8 μm), but filters with a 0.45 μm pore size, such as those used in our research, were the most commonly used at the time of methods development (e.g. Goldberg et al., 2013; Pilliod et al., 2013a,b). More recently, research has shown that there is no statistical difference in eDNA collection between the two pore sizes (Li et al., 2018). Based on the results of our multi-year study, we would recommend researchers try to filter significantly more water than this if at all possible, across each filter unit. Ideally, if it were possible to filter 1,000 mL (1 L) or more of water per filter, this would increase the probability of detecting target DNA in the sample if it was present in the environment. Given the nature of the preferred microhabitats of many amphibian and aquatic reptile species in the state, future studies should investigate how researchers might improve water volume capacities for each filter. Partnering with collaborators in engineering or microbiology fields may present opportunities to develop better techniques for filtering larger volumes of water across individual filter units.

In addition to our concerns regarding filter clogging, the high turbidity and conductivity we observed in sampled environments (Watters et al., in review) could be linked to an increase in PCR inhibitors that can decrease the ability to detect eDNA in a sample at various steps in the extraction and screening process, leading to potential increases in false negatives (Buxton et al., 2017; Williams et al., 2017; Li et al., 2018). These inhibitors tend to build up in stagnant water systems (Harper et al. 2018), such as in those in which we primarily sampled. Inhibitor removal through extract dilution with ddH₂O or buffers is not recommended as it may result in an over-dilution of samples, to the point of eDNA undetectability (Williams et al., 2017). Instead of employing dilution, we incorporated Zymo Research Products OneStep PCR Inhibitor Removal Kits, to remove potential inhibitors due to turbidity and other factors (McKee et al. 2015; Turner et al. 2015; Adams et al., 2019).

Finally, we were surprised at how time-consuming individual components of the eDNA process were. By far, the most time-intensive segment of the process is the DNA extraction of each individual filters. Due to the sensitivity of the process to contamination and equipment and time requirements for each step, we found that a well-trained researcher could only complete a batch of roughly 10–12 filter extractions (including at least one negative control sample) in a two-day period. Despite having dedicated students involved in extracting our field samples throughout the grant, this was the most rate-limiting aspect of this work. Moving forward, future researchers should be aware that larger initiatives would need larger teams available to process samples in the laboratory. Ideally, a statewide initiative, like towards building a Detecting Native Aquatics Network (DNANet) in Oklahoma, would allow for resources to potentially be pooled towards a having a dedicated lab and staff for wildlife management and conservation initiatives involving genetic approaches, such as eDNA, infectious disease, or microbiome studies.

PRIORITY 2 – APPROACHES TO eDNA SAMPLING

The results of this study provide a proof-of-concept framework for implementing an eDNA toolkit for biodiversity monitoring in Oklahoma. A clear result of our work is that species that are rare in the wild may continue to be difficult to survey with these techniques if applied across broad regions. Given how dilute target species DNA is in the environment when water samples are taken, it is likely critical that such surveys for rare or threatened species first develop a strategic environmental sampling approach that targets specific microhabitats expected to be utilized by the focal species. Furthermore, such studies should densely survey regions of highest probability for focal species persistence at the onset of the study before expanding outward from areas of confirmed population presence.

In the context for the application of eDNA technologies to surveying the environment for signatures of rare species, the null hypothesis is that the focal species does not exist currently in the sampled environment. As such, any positive detection result from quantitative PCR (qPCR) analysis would indicate a true rejection of the null hypothesis, indicating that the species' DNA was in fact detected in the environment sampled. On the other hand, failure to detect a species that is known to be present at the site of sampling (negative screening results for DNA presence of the focal species) would result in a Type II error or false negative result. It is clear from our results of screening for our common, positive-control species, *Acris blanchardi*, that even screening filtered water samples for common species can return such false negative results. The good news is that with increased numbers of samples collected and screened at each site with observed or vouchered *A. blanchardi* individuals, we were able to detect DNA signatures of the common species for a proportion of samples from that site. Therefore, we suggest future studies should also greatly increase the number of unique water samples collected, filtered, and screened for target species from each potential site of interest. Researchers now recommend collecting up to 15 water samples for common species and up to 45 for rare species (Goldberg et al. 2018; Akre et al. 2019). With processing time and cost increasing significantly with increased sampling, this recommended approach underscores the need for strategic focus on regions with the highest probability for species persistence in the wild (as discussed in the first paragraph of Priority 2 above).

Related to increasing site-specific sampling efforts, this work also revealed the importance of having positive controls in eDNA studies. Ideally, eDNA efforts would be combined with more traditional survey efforts to allow for the observation of target species in the wild with additional confirmation via eDNA screening at the site of observation and surrounding habitats in close proximity. At the very least, we recommend researchers attempt to conduct pilot studies using any primer-probe assay developed on positive control samples, such as at sites where target species are still known to occur or in a controlled, experimental setting, such as what we completed for *A. blanchardi*. It is also important to include both negative and positive control reactions on each qPCR plate of environmental samples screened to increase confidence in empirical results observed.

Finally, when we started this work, the most common practice with replicate screening of each environmental sample was to repeat qPCR screens in triplicate. However, the results of our work, along with recommendations from findings of recent studies (Erickson et al. 2019; Ficetola et al. 2019), suggest that future researchers should consider screening each sample up to six or more replicate times. This is due to the dilute nature of the target DNA in the wild that is simply difficult to detect even with qPCR methodologies. For example, even if the target species DNA is in fact present in a water sample, it may only be detected in one or two replicate

reactions out of six. Therefore, in addition to increased numbers of unique water samples collected and filtered from individual target sites, additional replicate reactions should be carried out for each eDNA sample to increase confidence in detection events (or in the absence of detection events [negative results]).

PRIORITY 3 – ABIOTIC FACTORS

Our research revealed that abiotic variables in sampled aquatic environments vary significantly at both ecoregion and site level scales in Oklahoma, with the exception of turbidity and water flow (Watters et al., in review). From our analyses, we can also see that both conductivity and canopy light were the primary drivers between site-level microclimate dissimilarity, based on abiotic factors (Watters et al., in review). Since we know from previous research that several aquatic abiotic variables have the potential to impact eDNA detection rates (Pilliod et al., 2013a,b; Strickler et al., 2015; Stoeckle et al., 2017), we feel this is an area worth continued investigation in the future by researchers focused on native Oklahoma species. However, it is clear that our results indicate that predicting eDNA detection rates based on aquatic abiotic factors is not straightforward. For example, we predicted *a priori* that canopy light level, temperature, and water flow would all have a negative effect on *A. blanchardi* eDNA detection as an increase in canopy light level and temperature are thought to increase the rate of DNA degradation (Pilliod et al., 2013b, Strickler et al., 2015), and increased water flow reduces eDNA detection as it moves away from the original source (Deiner & Altermatt, 2014; Pilliod et al., 2013a; Stoeckle et al., 2017). However, temperature was found to be positively correlated with *A. blanchardi* eDNA detection (Watters et al., in review), possibly indicating that the high temperatures needed for DNA degradation may not have been recorded during our March–July sampling. While this degradation may still be actively occurring, a recent review by Stewart (2019) suggests that temperature increases animal metabolism and movement, both of which may result in increased DNA deposition (via increased fecal and urine output, mucous production, and epithelial skin shedding), thereby increasing eDNA detection. This concept is further emphasized when considering shoreline basking behavior of *A. blanchardi*, leading to a temporary increase in DNA deposition at the sampling locations (Smith et al., 2003; Lehtinen & Skinner, 2006). Furthermore, water temperature itself involves multiple factors; our results show a positive correlation with canopy light levels (i.e. lack of tree cover leads to increased warming) and a negative correlation with water flow (i.e. flowing water does not warm as quickly), although we observed no significant relationships between either of these variables and *A. blanchardi* eDNA detection (Watters et al., in review). Therefore, future studies have much that could be investigated on a finer scale with respect to abiotic factors and their impact on the eDNA detectability of species in aquatic environments.

PRIORITY 4 – ADDITIONAL ASSAY DEVELOPMENT

As mentioned in the introduction, we made early attempts at developing primer-probe assays during the first two years of the project for two other amphibian species of conservation concern: *Eurycea multiplicata* and *Eurycea tynnerensis*. Although we were not able to optimize assays for these species as explained above, both assays showed promise during *in vitro* testing in the laboratory, and therefore, it is our hope that a future student or researcher can continue working to establish these assays, and others, to add to the state’s DNANet toolkit. Additionally, after confirming the methodologies can be applied to native amphibians in the state, future research should begin to test eDNA methodologies for other species of conservation interest, including

crayfish, plants, fish, and other semi-aquatic or aquatic vertebrate species. Different taxa have different DNA shedding rates, based on skin type, metabolism, location in water body, etc. and may require eDNA methods modifications (Adams et al. 2019).

VI. SIGNIFICANT DEVIATIONS

There have been no significant deviations.

VII. EQUIPMENT

No equipment exceeding \$5,000 in cost was purchased for this project.

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Figure 1: (Top Left) County map of Oklahoma showing distribution of six recognized ecoregions, with counties included in study outlined in white for reference. (Bottom Left) Statewide distribution of the four SGCN species. (Right) Closeup view of aquatic waterbodies sampled in study (black circles) in eastern Oklahoma (counties outlined in white, ecoregions shown in color), with the statewide distribution of *Acris blanchardi* (positive control) provided above.

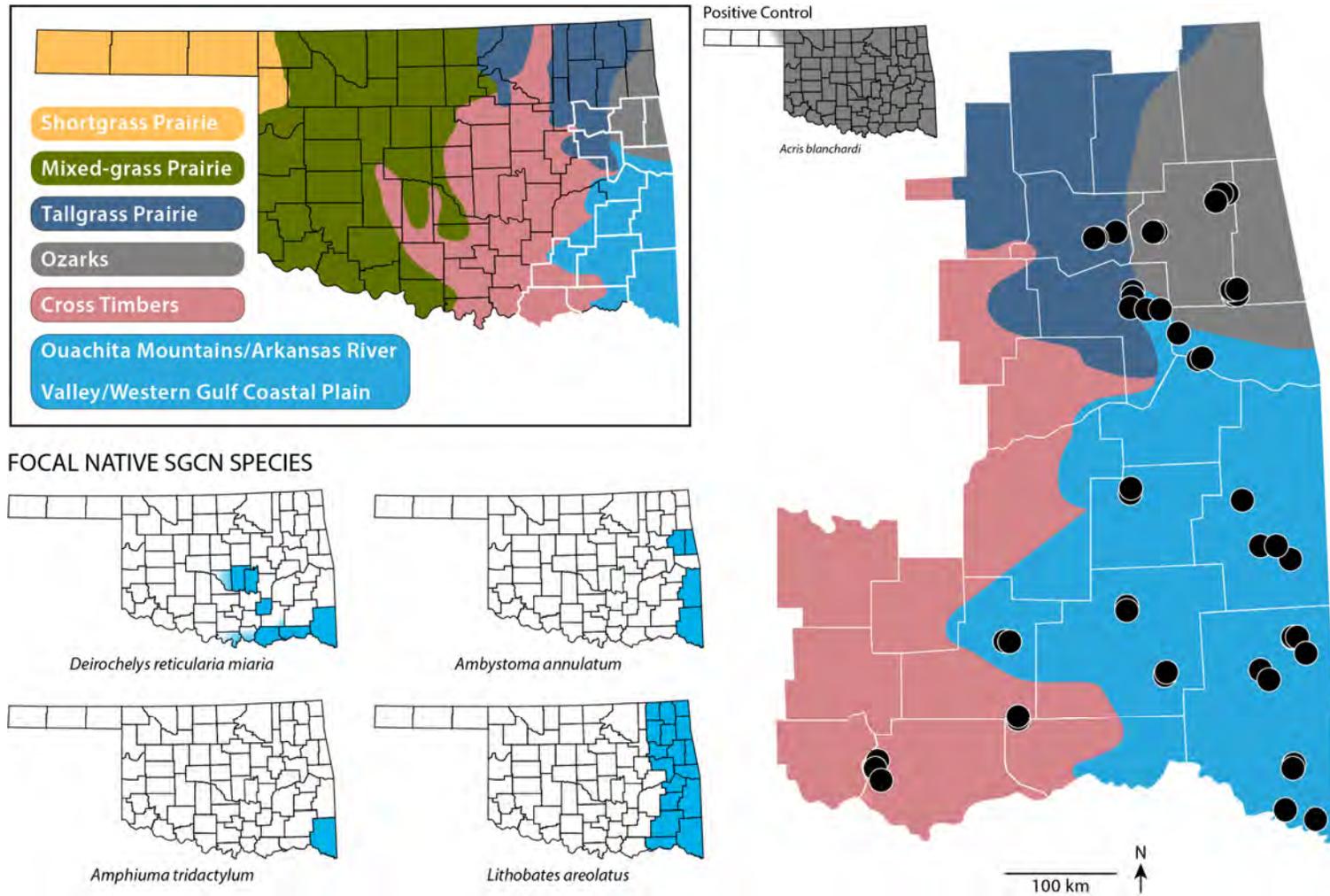


Figure 2: Bar graph showing the mean proportion of samples detecting *Acris blanchardi* (positive eDNA screen) by ecoregion based on a nested ANOVA. Non-overlapping lower-case letters above the bars in each graph indicate statistical differences via a Tukey's HSD post-hoc analysis.

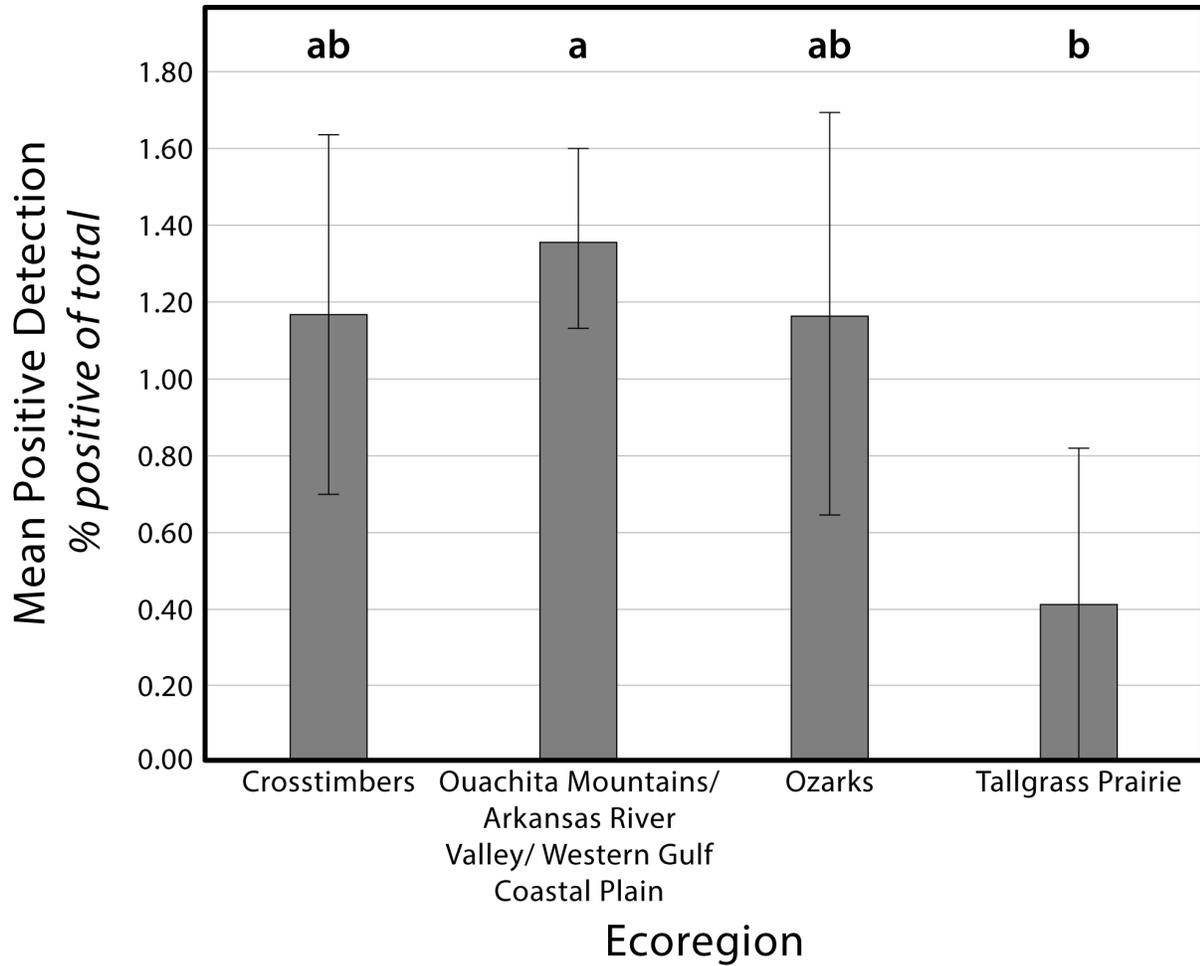


Figure 3: Bar graphs showing results for the statistically significant means of the following abiotic variables by ecoregion based on a nested ANOVA: (A) canopy light, (B) conductivity, (C) elevation, (D) pH, and (E) temperature. Each ecoregion is represented as a different color. Non-overlapping lower-case letters above the bars in each graph indicate statistical differences via a Tukey's HSD post-hoc analysis.

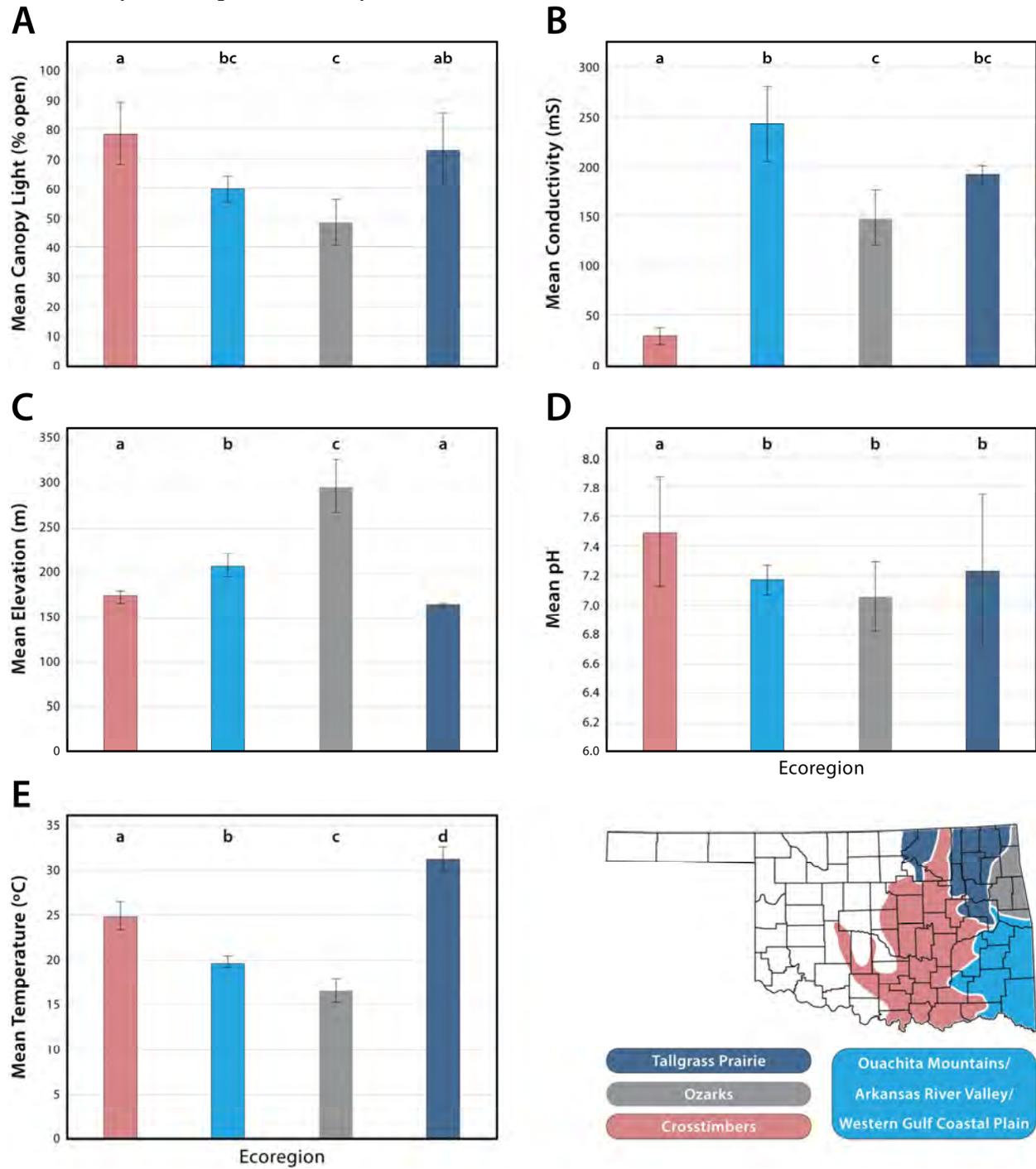


Figure 4: Mean values for principal coordinate ordinate (PCoA) axes one (x-axis) and two (y-axis) representing microclimate composition across the following ecoregions: Crosstimbers, Ouachita Mountains/Arkansas River Valley/Western Gulf Coastal Plain, Tallgrass Prairie, and Ozarks.

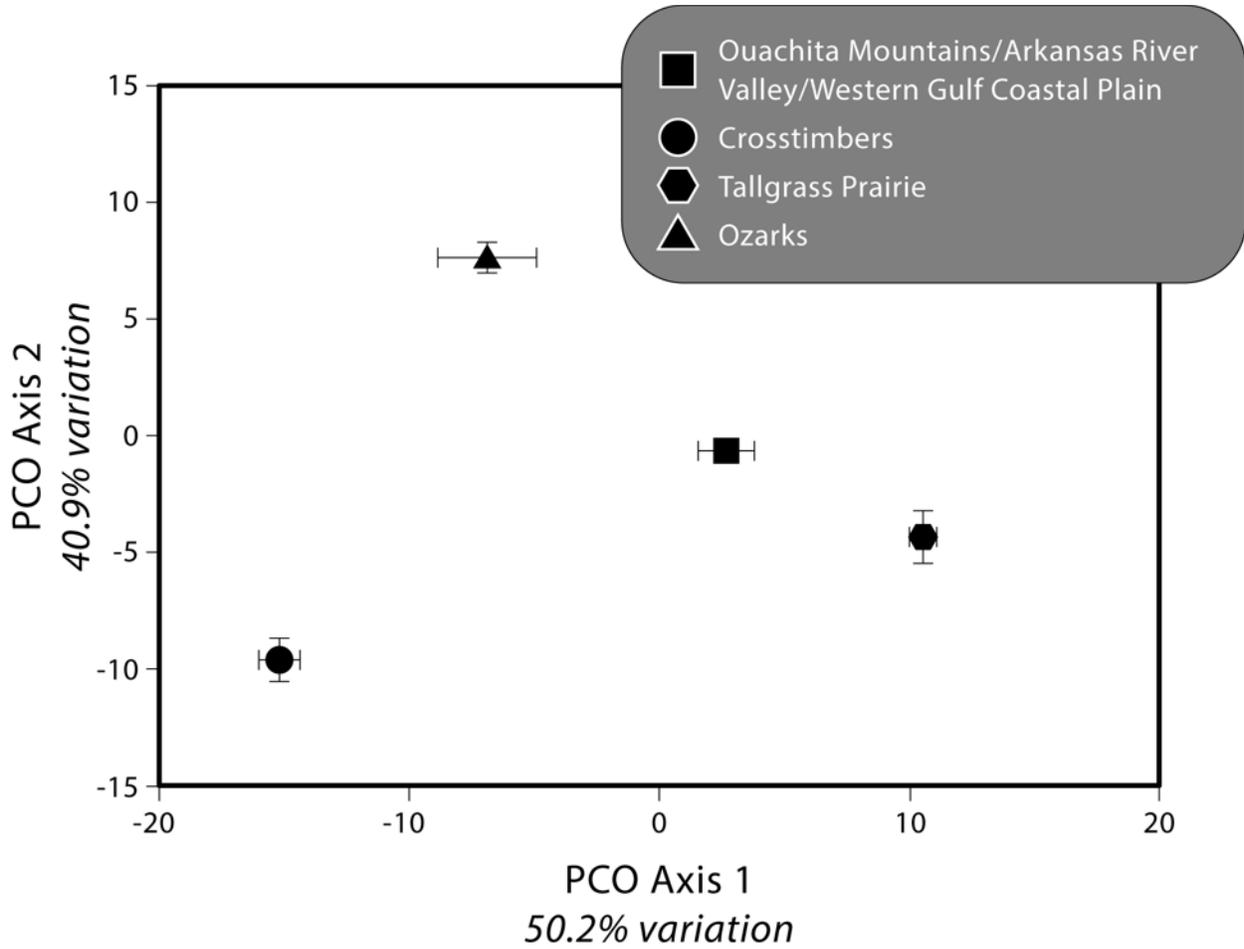


Table 1: Primer and probe information for the five developed eDNA assays.

Assay (Genus/species)	Primer	Primer sequence (5'–3')	Probe	Probe Sequence (5'–3')
<i>Ambystoma annulatum</i> eDNA COI	A_annulatum_COI_F1 A_annulatum_COI_R1	GAGTTGAAGCAGGTGCTGGAA ATGGGCTAAATTACCAGCAAGTG	A_annulatum_COI_probe	TGGACTGTGTACCCG
<i>Amphiuma tridactylum</i> eDNA CytB	A_tridactylum_CytB_F1 A_tridactylum_CytB_R1	AGCCAATCTCACCCGATTCTT TGGATGATACTTGTCCGATAATGA	A_tridactylum_CytB_probe	TTCCATTTTACTCTTCCG
<i>Lithobates areolatus</i> eDNA CytB	L_areolatus_CytB_F1 L_areolatus_CytB_R1	TCGGAACTAACCTTGTCCAATGA CGGGTTAGGGTGGCATTGT	L_areolatus_CytB_probe	TACCGAGAATCCG
<i>Deirochelys reticularia</i> eDNA CytB	D_reticularia_CytB_F1 D_reticularia_CytB_R1	CCTACCATGAGGCCAAATATCC ATATATGGAATGGCTGAGAGGAGATT	D_reticularia_CytB_probe	AGGCGCAACTGTTA
<i>Acris blanchardi</i> eDNA CytB	A_blanchardi_CytB_F2 A_blanchardi_CytB_R1	CCTTTCTGCTGCCCTTA GGTGGCGTTGTCTACTGAA	A_blanchardi_CytB_probe	CTGAGCTAGTCCAATG

Table 2: Summary qPCR results of eDNA field surveys screening using the positive control, *Acris blanchardi*, qPCR assay. The total number of waterbodies and samples by county are shown (including field negative controls), followed by the number and percentage (%) of positive (+) samples detected by county.

County	No. samples (No. waterbodies)	No. + samples (No. waterbodies with +)	% + samples (% waterbodies with +)
Adair	27 (3)	1 (1)	3.7% (33.3%)
Atoka	63 (7)	21 (7)	33.3% (100.0%)
Bryan	22 (4)	2 (1)	9.1% (25.0%)
Cherokee	104 (12)	21 (8)	20.2% (66.7%)
Latimer	27 (3)	5 (2)	18.5% (66.7%)
Le Flore	73 (8)	26 (7)	35.6% (87.5%)
McCurtain	94 (12)	23 (9)	24.5% (75.0%)
Muskogee	71 (8)	3 (3)	4.2% (37.5%)
Pushmataha	28 (6)	6 (4)	21.4% (66.7%)
Sequoyah	114 (13)	10 (4)	8.7% (30.8%)
Wagoner	23 (3)	2 (2)	8.7% (66.7%)
TOTAL	646 (79)	120 (47)	18.6% (59.5%)

Table 3: Summary qPCR results of eDNA field surveys employing the four designed assays for SGCN species. The total number of waterbodies and samples by county are shown (excluding field and lab negative controls), followed by the number of positive samples detected by county for each target species. N/A represents county samples that were not screened for target species in cases where those taxa have never been documented in the counties historically.

County	No. samples / No. waterbodies	<i>Ambystoma annulatum</i>	<i>Amphiuma tridactylum</i>	<i>Lithobates areolata</i>	<i>Deirochelys reticularia</i>
No. + samples (No. waterbodies with +)					
Adair	24 / 3	16 (2)	N/A	0	N/A
Atoka	54 / 7	0	N/A	0	1 (1)
Bryan	18 / 4	0	N/A	0	N/A
Cherokee	92 / 12	1 (1)	N/A	1 (1)	N/A
Latimer	23 / 3	0	N/A	0	N/A
Le Flore	64 / 8	0	N/A	0	N/A
McCurtain	82 / 12	0	0	0	0
Muskogee	64 / 8	0	N/A	0	N/A
Pushmataha	22 / 6	0	N/A	0	0
Sequoyah	102 / 13	0	N/A	1 (1)	N/A
Wagoner	20 / 3	0	N/A	0	N/A
TOTAL	565 / 79	17/565	0/82	2/194	1/158

Table 4: Detailed summary qPCR results of eDNA field surveys screening using the positive control species, *Acris blanchardi*, qPCR assay, by waterbody. Each large-scale site is listed with a number afterward to indicate each individual waterbody located within the site (i.e. Boehler Seeps TNCP 1, 2, 3 are three waterbodies sampled at Boehler Seeps preserve, owned by The Nature Conservancy). Number of eDNA samples collected (excluding field and lab negative controls) is followed by number of samples exhibiting *A. blanchardi* positive (+) detection. The proportion of samples testing positive at each waterbody is also provided (% *Acris* eDNA). Lines designated with an asterisk (*) represent waterbodies in which live *A. blanchardi* were also observed at the time of eDNA sampling. Abbreviations are as follows: NWR = National Wildlife Refuge (US Fish & Wildlife Service); PUA = Public Use Area (US Army Corps of Engineers); SP = Oklahoma State Park; TNCP = The Nature Conservancy Preserve; WMA = Wildlife Management Area (Oklahoma Department of Wildlife Conservation).

Ecoregion	Waterbody name	No. samples (No. + samples)	% <i>Acris</i> eDNA
Crosstimbers	Boehler Seeps TNCP 1	8 (4) *	50.0%
	Boehler Seeps TNCP 2	8 (2) *	25.0%
	Boehler Seeps TNCP 3	8 (2)	25.0%
	Johnson Creek PUA 1	4 (0) *	0.0%
	Johnson Creek PUA 2	4 (0) *	0.0%
	Lakeside PUA 1	4 (0) *	0.0%
	Lakeside PUA 1	6 (2) *	33.3%
	TOTAL	42 (10)	23.8%
Ouachita	Grassy Slough WMA 1	8 (2) *	25.0%
Mountains/ Arkansas River Valley/Western Gulf Coastal Plain	Grassy Slough WMA 2	8 (0) *	0.0%
	Honobia WMA 1	8 (1)	12.5%
	Honobia WMA 2	8 (4)	50.0%
	Little River NWR 1	8 (8)	100.0%
	Little River NWR 2	8 (2)	25.0%
	McClellan-Kerr WMA, Billy Creek/Chouteau Portion 1	4 (1) *	25.0%
	McClellan-Kerr WMA, Billy Creek/Chouteau Portion 2	8 (1) *	12.5%
	McClellan-Kerr WMA, Billy Creek/Chouteau Portion 3	8 (0)	0.0%
	McClellan Kerr WMA, Robert S. Kerr Portion 1	8 (0) *	0.0%
	McClellan Kerr WMA, Robert S. Kerr Portion 2	8 (0)	0.0%
	McClellan Kerr WMA, Robert S. Kerr Portion 3	8 (0)	0.0%
	McClellan-Kerr WMA, Webbers Falls Portion 1	8 (0) *	0.0%
	McClellan-Kerr WMA, Webbers Falls Portion 2	8 (1)	12.5%
	McClellan-Kerr WMA, Webbers Falls Portion 3	8 (1)	12.5%
	McClellan-Kerr WMA, Webbers Falls Portion 4	8 (0)	0.0%
	McClellan-Kerr WMA, Webbers Falls Portion 5	8 (0) *	0.0%
	McCurtain County WMA 1	6 (2)	33.3%
	McCurtain County WMA 2	6 (2)	33.3%
	McGee Creek WMA 1	8 (1)	12.5%
	McGee Creek WMA 2	8 (3)	37.5%
	McGee Creek WMA 3	6 (1)	16.7%
	McGee Creek WMA 4	8 (8)	100.0%
	Ouachita National Forest, Le Flore Unit 1	8 (5) *	62.5%
	Ouachita National Forest, Le Flore Unit 2	8 (0)	0.0%
	Ouachita National Forest, Le Flore Unit 3	8 (1) *	12.5%
	Ouachita National Forest, Le Flore Unit 4	8 (7)	87.5%
	Ouachita National Forest, McCurtain Unit 1	8 (1)	12.5%
	Ouachita National Forest, McCurtain Unit 2	6 (1)	16.7%
	Pushmataha WMA 1	4 (1)	25.0%

	Pushmataha WMA 2	2 (1)	50.0%
	Pushmataha WMA 3	4 (0)	0.0%
	Pushmataha WMA 4	4 (3)	75.0%
	Pushmataha WMA 5	2 (1)	50.0
	Pushmataha WMA 6	6 (0)	0.0%
	Red Slough WMA 1	8 (3) *	37.5%
	Robbers Cave WMA 1	8 (4)	50.0%
	Robbers Cave WMA 2	8 (1)	12.5%
	Robbers Cave WMA 3	7 (0) *	0.0%
	Sequoyah NWR 1	8 (0) *	0.0%
	Sequoyah NWR 2	8 (4) *	50.0%
	Sequoyah NWR 3	8 (0) *	0.0%
	Sequoyah NWR 4	8 (0) *	0.0%
	Sequoyah NWR 5	8 (0)	0.0%
	Sequoyah NWR 6	8 (0)	0.0%
	Sequoyah NWR 7	8 (1) *	12.5%
	Sequoyah NWR 8	8 (1) *	12.5%
	Sequoyah NWR 9	6 (4)	66.7%
	Sequoyah NWR 10	8 (0) *	0.0%
	Three Rivers WMA 1	2 (0)	0.0%
	Three Rivers WMA 2	8 (2) *	25.0%
	Three Rivers WMA 3	6 (0)	0.0%
	Wister WMA 1	8 (7)	87.5
	Wister WMA 2	8 (1)	12.5
	TOTAL	383 (87)	22.7%
Ozarks	Cookson WMA 1	8 (0)	0.0%
	Cookson WMA 2	8 (0)	0.0%
	Cookson WMA 3	8 (1)	12.5%
	Cookson WMA 4	8 (0)	0.0%
	Cookson WMA 5	8 (1) *	12.5%
	Cookson WMA 6	8 (1)	12.5%
	Cookson WMA 7	8 (0) *	0.0%
	Cookson WMA 8	8 (1)	12.5%
	Green Leaf SP 1	8 (1)	12.5%
	Green Leaf SP 2	8 (0) *	0.0%
	Hopewell Park PUA 1	8 (0)	0.0%
	Nickel Family TNCP 1	8 (1)	12.5%
	Nickel Family TNCP 2	8 (7)	87.5%
	Nickel Family TNCP 3	8 (7)	87.5%
	TOTAL	126 (20)	17.9%
Tallgrass Prairie	Fort Gibson WMA 1	4 (1)	25.0%
	Fort Gibson WMA 2	8 (0)	0.0%
	Fort Gibson WMA 3	8 (0) *	0.0%
	Fort Gibson WMA 4	8 (2) *	25.0%
	TOTAL	32 (3)	10.7%
TOTAL		565 (120)	21.2%

APPENDIX I

eDNA Water Sampling Protocol

Lake/pond/wetland/ditch

1. Take an initial GPS point for your “homebase” for this water body.
2. Determine what code name you will call the water body, so that all water samples and measurements follow the code. (Example: your first pond at Pushmataha WMA on March 6, 2017, Quadrante A, sample 1 = Push1-A-1 3/3/17). Be sure all parties know the code before splitting up.
3. Using the same GPA coordinate set at homebase, obtain a control sample: fill up a sterile 50mL falcon tube with DI water, drop the sealed tube in the water, leave for 30 sec., then remove. Label it the same as in #2 but use the word control on your label (i.e. Push1-control 3/3/17).
4. Visually divide the water body into quadrates, as a group, before leaving homebase. If it helps, use a compass (or compass feature on your phone!) and divide the quadrates up along a N-S-E-W gradient. Use natural landmarks like trees and stumps to help you determine the boundaries. Be sure your data sheet accurately reflects your sampling regime. Ideally, there will be eight samples per water body.
5. Split into two groups. One group is responsible for Quadrates A and B; the other group is responsible for Quadrates C and D. Be sure that each water sample occurs a minimum of 10m from another water sample, as measured along the shoreline. If this isn't possible, reduce the number of quadrates and divide the pond in half or thirds instead. Be sure all parties understand which region they are in charge of before splitting up.
6. For each quadrate, measure the following variables, recording ALL information on the provided data sheet. Each water body should have 1–2 data sheets. Do NOT use the same data sheet for any consecutive water bodies.
 - a. Sample 1 (1m from shore) – take water sample (and variables!) at 5cm deep (~2 inches). If this depth is not available 1m from shore, then just record the depth you were able to use.
 - i. GPS
 - ii. pH
 - iii. temperature
 - iv. conductivity
 - v. total dissolved solids (TDS)
 - vi. water flow
 - vii. canopy light
 - viii. water depth
 - b. Sample 2 (2m from shore; 1m closer to center from original Sample 1 point)
 - i. Exact same variables as Sample 1
 - c. Note: Do NOT open your sterile water bag for each sample, until you are physically standing at the point you wish to sample.
7. Determining size of the water body:
 - a. Lakes/large permanent ponds: Do nothing on site. Simply use Google Earth or ARC GIS layers to determine the size of the lake.
 - b. Measure the length of the pond, at its longest point (estimated). Measure the width of the pond, at its widest point (estimated).

Note: if sampling for reptiles and amphibians at the same location, be sure to collect water samples and abiotic variables BEFORE you muck about along the shore too much. This will stir up sediment unnecessarily, which affects our ability to filter water easily and will change the turbidity measurement.

Stream/Creek/River

1. Same as lake/pond/wetland/ditch.
2. Determine what code name you will call the water body, so that all water samples and measurements follow the code. (Example: your first creek at Ozark Plateau NWR on March 6, 2017, Upstream, sample 1 = Ozark1-Up-1 3/3/17). Be sure all parties know the code before splitting up.
3. Using the same GPA coordinate set at homebase, obtain a control sample: fill up a sterile 50mL falcon tube with DI water, drop the sealed tube in the water, leave for 30 sec., then remove. Label it the same as in #2 but use the word control on your label (i.e. Ozark1-control 3/3/17).
4. Determine the accessibility of the water body. Can you cross to both shores? Is it too deep to wade out very far? How far can you go in either direction before you are blocked? Use this info to visually divide the water body into a transect as a group to determine at which points you will sample. Ideally, we want to sample of eight times per water body. This may mean that you head upstream (against water flow) and sample four times, then downstream (with water flow) four times. It may mean you do two upstream on one shore and two upstream on the other shore, etc. Be sure your data sheet accurately reflects your sampling regime.
5. Split into two groups. One group is responsible for Upstream samples and the other for Downstream samples. Be sure that each water sample occurs a minimum of 10m from another water sample, as measured along the shoreline. Be sure all parties understand which region they are in charge of before splitting up.
6. For each Upstream or Downstream sample, measure the following variables, recording ALL information on the provided data sheet. Each water body should have 1–2 data sheets. Do NOT use the same data sheet for any consecutive water bodies.
 - a. Sample 1 (1m from shore) – take water sample (and variables!) at 5cm deep (~2 inches). If this depth is not available 1m from shore, then just record the depth you were able to use. Label the water filter bag with correct full code and date.
 - i. GPS
 - ii. pH
 - iii. temperature
 - iv. conductivity
 - v. total dissolved solids (TDS)
 - vi. water flow
 - vii. canopy light
 - viii. water depth
 - b. Sample 2 – continue another 10m or more up or down stream, based on which group you were assigned. Take the same variables. Label the water filter bag with correct full code and date.
 - c. Sample 3 – continue another 10m or more up or down stream, based on which group you were assigned. Take the same variables. Label the water filter bag with correct full code and date.
 - d. Sample 4 – continue another 10m or more up or down stream, based on which group you were assigned. Take the same variables. Label the water filter bag with correct full code and date.
 - e. Note: Do NOT open your sterile water bag for each sample, until you are physically standing at the point you wish to sample.
7. Same as lake/pond/wetland/ditch.
8. Determining size of the water body:
 - a. Measure the width of the water body, if possible.

Measuring Abiotic Variables Associated with eDNA Water Samples

YSI 63 pH and conductivity probe:

1. Remove the storage container from the tip of the probe.
2. Press the On/Off button
3. Hit the Mode button to select pH (the units to the right of the measurement will read pH)
4. Dip the probe into the water, making sure to cover the entire thing including the two holes on top right below the cable (these holes measure conductivity). Shake briefly to remove any bubbles from the probe, and let the readings stabilize. This may take a couple minutes. DO NOT get the handheld system wet; only place the probe in the water!
5. Both pH and temperature will be visible simultaneously. pH is on the top; temperature is on the bottom. Confirm that it says °C. Record on your data sheet.
6. Press Mode ONCE to switch to conductivity (the units to the right of the measurement will read "µS. Hitting Mode twice will measure specific conductivity, also in µS, which is not the measurement we want!). Record conductivity on your data sheet.
7. Press ON/OFF to turn off the system before putting it away. Do this when you are done at each pond/lake/stream (not between samples).
8. Rinse the probe in DI water and dry before placing the end back into the storage container. [Note: If the sponge in the storage container appears dry, add a small amount of pH 4 standard.]

YSI 1030 pH and conductivity probe:

1. Remove the plastic cover from the end of the probe before starting (but keep the metal protective cage on).
2. Press the power button on the right side below the screen of the handheld system.
3. Dip the probe into the water, making sure to cover the entire thing including the two holes on top right below the cable (these holes measure conductivity). Shake briefly to remove any bubbles from the probe, and let the readings stabilize. This may take a couple minutes. DO NOT get the handheld system wet; only place the probe in the water!
4. Record the results on your data sheet. The pH reading is at the top (pH), followed by pH mV (not recorded), the conductivity (µS) and the temperature.
5. Hold down the power button to turn off the system before putting it away. Do this when you are done at each pond/lake/stream (not between samples).
6. Rinse the probe in DI water and dry before putting the protective cover back on.

2100Q turbidity meter

1. Place the turbidity meter on a flat dry surface.
2. Press the power button located underneath the screen.
3. While filtering the water samples, mix them thoroughly to homogenize sediments, algae, etc. Then pour roughly 15 mL of sample into the glass sample container so that it reaches the line. Screw on the lid.
4. Wipe the sample container with a lint-free cloth or kimwipe to remove any water spots or fingerprints. After this, handle the sample container only by the black lid.
5. Gently invert the sample container several times to make sure the sample is thoroughly mixed (but do not shake).
6. Place the sample container into the meter and close the lid.
7. Push the button under the bottom right of the screen, which corresponds to READ. The meter takes a few seconds to measure the sample.
8. Dump out the water from the glass sample container and rinse it with DI water. Dry with a kimwipe before it is used for the next sample
9. Hold down the Power button to turn off when done for the day.

Water Flow (via wind flow meter)

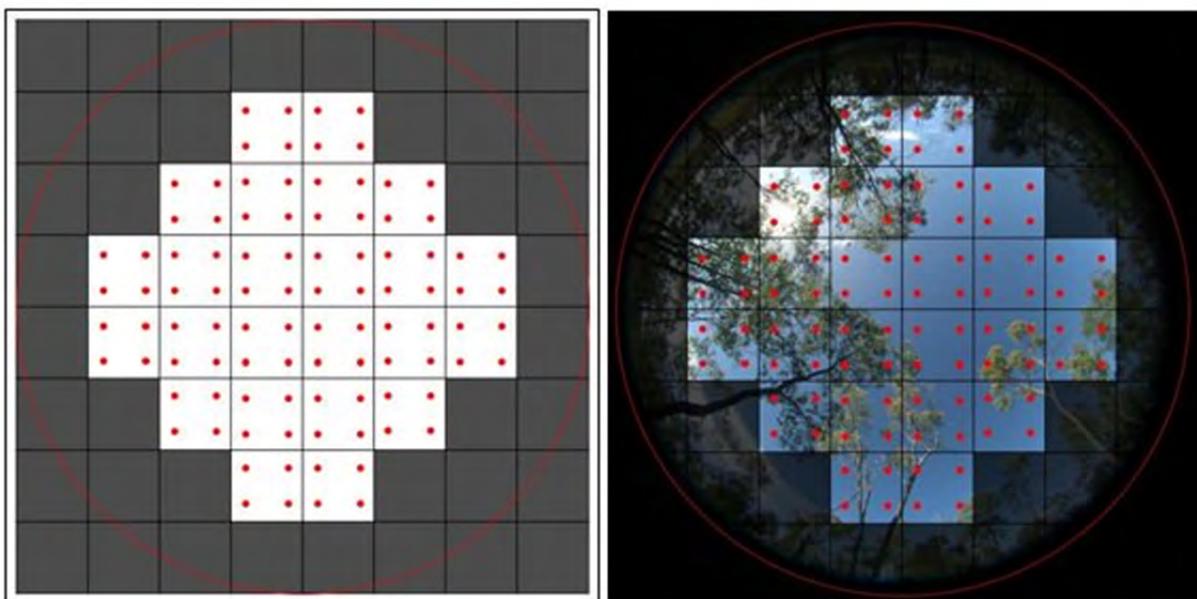
1. Press power button to turn on.
2. Use the < > arrows to scroll to the wind speed measurement (SPd); it looks like this: 
3. Confirm that it is in m/s. If not, simultaneously hold down the power button and the > arrow to select the correct units.
4. Immerse the small impeller in the water; do not immerse the sensors that are just past the impeller.
5. Wait for the reading to stabilize. Record on your data sheet.
6. Hold down the power button to turn off when done.

Canopy Light (via densitometer)

1. While still standing in the exact spot that you took the other measurements, face the center of the pond/lake or the in a stream face upstream or downstream, depending on which direction you sampled.
2. Open the lid on the densitometer (note: the box is wooden and not waterproof!). Hold the instrument level and far enough away from your body such that your head is visible in the mirror, but is just outside the grid.
3. Using the bubble in the lower right corner of the instrument as a guide, hold the densitometer level.
4. Using only your dominant eye (keeping the other one closed), count the number of imaginary dots that are OPEN in the corner of each square on the grid (i.e. not covered with trees); there are a total of 96 imaginary dots. Move systematically across the grid (i.e. left to right, then top to bottom). See Figure 1.
5. Record this number on your data sheet. Once the data is entered back on campus, this will be multiplied by a factor of 1.04 to give us a % of overhead area NOT COVERED by canopy, which therefore provides us with a measurement of the light available.

Figure 1: How to read a densitometer:

- Visualize four small dots in corner of each square (left)
- How many of the dots are OPEN in the picture on the right?



APPENDIX II

eDNA Filtration Protocol

Preparation

Create a wash series to sterilize forceps using a 50mL beaker with a 10% bleach solution (or eliminase) followed by two 50mL beakers with DI water. Use sterile beakers or 50mL “Falcon” tubes.

Protocol

1. Assemble filter tops (sterile, one time use) and bottoms (non-sterile, reuse often) and attach to vacuum system in fume hood or vacuum pump.
2. Invert water sample twice in case particles have settled on bottom. Pour 500mL of water sample into sterile filter then turn on vacuum. (If running multiple samples at once, write the sample name on the lid of filter.)
3. After all water has been filtered, turn off vacuum and record the amount of water filtered on your data sheet. (If running multiple samples at once, disconnect hosing for each unit, as needed.)
 - If water has been pumped for over 30 min., and 500mL is still not filtered, stop filtration and proceed to later steps. Be sure to record the total volume filtered.
4. Using a sterile scalpel, cut the filter from the housing assembly, right along the edge of the plastic.
5. Using sterile filter forceps, roll or fold the filter to fit into a 10,L tube labeled with the sample name/date/volume using sterile forceps.
6. Fill tube with 95% ETOH to preserve DNA on filter. Etch the sample name/date/volume into the tube so the ethanol doesn't remove your data.
7. Water in the base of filter can be disposed and the bottom container can be reused for future samples. Top filter is disposed of.
8. Sterilize utensils by dipping them in the bleach followed by the first DI water, then the second.

*Be sure to change gloves between each water sample filtered.

APPENDIX III

eDNA Extraction Protocol

USE FILTERED TIPS ONLY FOR ALL STEPS.

All stock buffers (AL, ATL, AW1, AW2, and AE buffers, as well as Proteinase K and Ethanol) need to be aliquoted into 5/15/50 ml tubes at the beginning of the procedures. So you dip the pipette tip into the stock buffer only once for a set of extractions. Swirl the bottle gently before pipetting out the aliquot. **DON'T SHAKE** the bottles, especially AL and ATL buffers, as it will cause the solution to bubble.

WARNING BEFORE ALIQUOTING AL BUFFER!: If AL buffer looks cloudy, microwave water in a large beaker for 1-1.5 minutes and swirl the AL buffer bottle in the water until the solution becomes completely clear.

If you have 23 samples (filters) + 1 negative control to extract, you will need the following amounts of buffers and other reagents (add one extra for pipette errors and amount that sticks to the tubes):

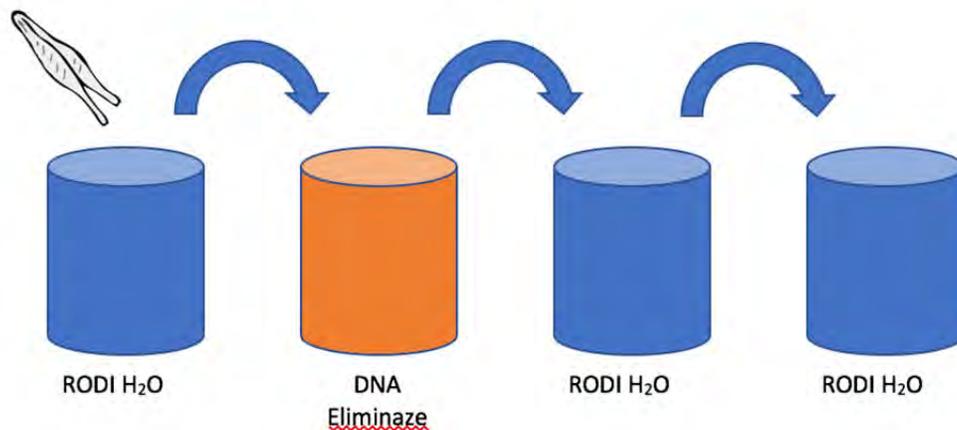
Buffer/ Reagent	Volume per tube (µl)	Number of tubes (Description)	No. of tubes	Total needed (µl)	Total needed (ml)
AL (lysis)	380**	(23 samples + 1 negative + 1 extra) x 2 repeats	50	19,000	19
ATL(tissue lysis)	400**	(23 samples + 1 negative + 1 extra) x 2 repeats	50	20,000	20
200 proof ethanol	400**	(23 samples + 1 negative + 1 extra) x 2 repeats	50	20,000	20
Proteinase K	20	23 samples + 1 negative + 1 extra	25	1,000	1
AW1 (wash 1)	500	23 samples + 1 negative + 1 extra	25	12,500	12.5
AW2 (wash 2)	500	23 samples + 1 negative + 1 extra	25	12,500	12.5
AE (elution)	100	23 samples + 1 negative + 1 extra	25	2,500	2.5

** These volumes may vary (see steps 14, 28, and 30)

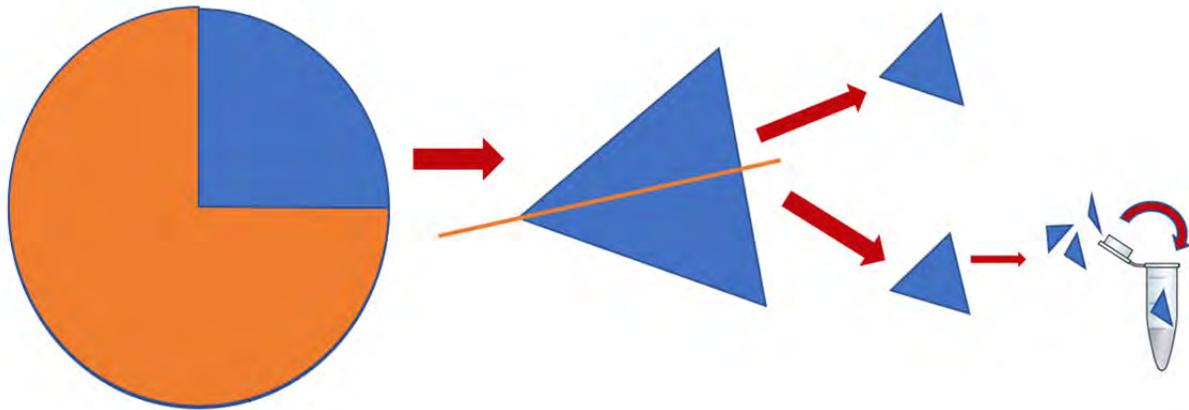
Day 1

1. In a rack, place 2 autoclaved 1.5mL microcentrifuge tubes for each of the samples plus 1 negative control (if you have 12 samples, prepare $[12+1] \times 2 = 26$ tubes). Shake the receptacle to get tubes out onto the lid (this will reduce chance of contaminating other tubes by placing fingers inside of the receptacle). Once on the lid, transfer the tubes onto a rack; close the lids, then label the tubes with sample number and name. Once labeled, place the tubes on a paper towel in the Biosafety cabinet and expose them to UV light for 5 minutes (when you turn on the UV light, the timer will automatically be set for 5 minutes).
2. Clean the work bench with DNA decontaminant (Eliminase or DNA AWAY) and set out a pile of paper towels (at least the number of samples +1).

3. Calculate the amounts of ATL buffer and Proteinase K you need on Day 1 and aliquot out the amounts.
4. Prepare 4 autoclaved beakers. Fill one small beaker with DNA decontaminant (Eliminase or DNA AWAY) about 1/2 (~ 17-20 ml) of the way up, and fill three beakers with deionized (RODI) water. Prepare two pairs of tweezers.
5. Clean two pairs of tweezers using deionized water and DNA decontaminant (see the diagram below). Wash the tweezers in the first beaker of water and then decontaminate them by swishing them in the second beaker with DNA decontaminant for a few seconds. Finally, rinse them in water twice (in the third and fourth beakers). Wipe the water off the tweezers on a piece of kimwipe.



6. Make your negative control by taking out a new piece of filter paper and submerging it in a beaker with deionized H₂O. Let the filter soak in the water. Decant the Deionized H₂O and add 10 ml of 200 proof ethanol into the beaker containing the filter paper.
7. Open the first sample tube. Take out the filter carefully with the tweezers and open it onto your working paper towel (the paper towels will be used to cut the filter paper; replace the paper towel for every new sample).
8. Use 2 pairs of the tweezers and tear a 1/4 piece from the 75 mm filter (a 1/2 piece if it's a 50 mm filter). Put the rest of the filter back in the original tube. Tear the 1/4 piece of filter into 2 pieces, and then tear each piece into smaller pieces to increase surface area (these will go into two separate tubes; replicates) and put them in 2 x 1.5 ml microcentrifuge tubes (see the diagram below). Make sure all the pieces can be air dried (do not overstuff the tubes with filter)– that none is forming a seal at any point in the tube that won't let air in. Shift things around with the tweezers as necessary.



9. Close the top, and replace the paper towel with a new one.
10. Repeat steps 6–8 for all samples in the batch – change gloves at any point you touch a filter or get ethanol from a sample on your gloves (or think you may have).
11. Place the tubes (open lids) in the SpeedVac on manual at low heat for 20 minutes, until no liquid/condensation is seen in the tube or no smell of ethanol exists when you sniff at the tube.
12. Turn on a ThermoMixer C and set it to 55 °C.
13. Add 380 μ l ATL buffer (Qiagen DNeasy kit) to each tube. Replace the pipette tip for every tube.
14. Add 20 μ l Proteinase K (Qiagen DNeasy kit) to each tube individually. Use the tip to swish the filter papers around and make sure that all the materials are fully immersed in the solution. Do not pack the papers so that all the paper surface is in contact with the solution. Vortex the tubes for 20 seconds each. Replace the pipette tip for every tube. If there is not enough solution to cover the filter material, set it aside and add additional 100 μ l of ATL at the end.
15. Incubate at 55 °C in a ThermoMixer C with low speed (500 RPM) overnight.

Day 2

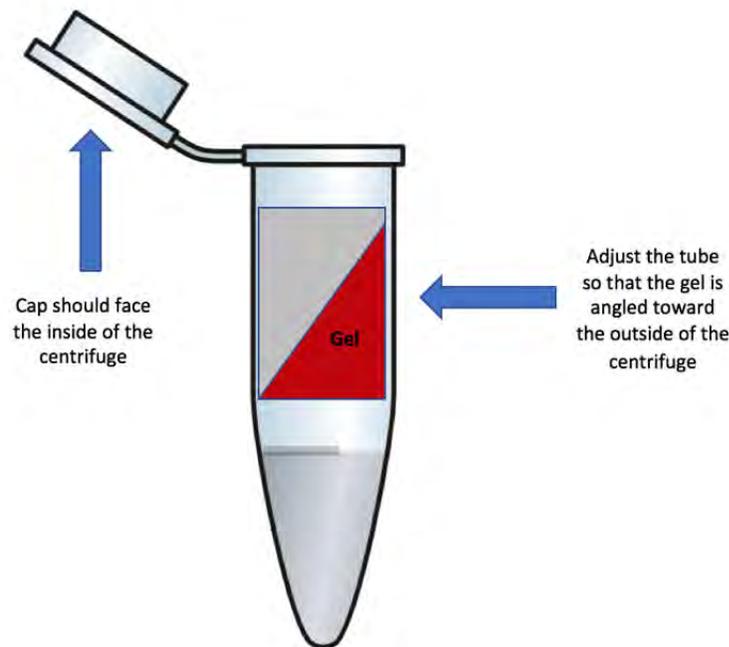
16. Prepare two 1.5 mL microcentrifuge tubes and one 5ml tube for each sample and negative control. Place them under the UV light in BioSafety Cabinet to sterilize for 5 minutes (when you turn on the UV light, the timer will automatically be set for 5 minutes).
17. Remake the Eliminase and water beakers as in Day 1. Prepare 2 pairs of tweezers and decontaminate them. Use a kimwipe for wiping off tweezers.
18. Aliquot all the buffers and 200 proof ethanol that will be used on Day 2 and chill the ethanol in -20 °C freezer until the step 30.
19. Remove samples from ThermoMixer. Turn on the Mini-incubator and set the temperature at 70 °C. Place an aliquot of AE buffer in the incubator until you are ready to use it.
20. Vortex samples for 15 seconds each.
21. Set out one Qiashredder spin column (purple top) for each sample plus negative control and label.
22. Transfer the first tube of each sample (the filter from the sample was divided into two tubes in step 7) to a Qiashredder spin column by transferring all the filter papers with the tweezers and pipetting the rest of the solution into the spin column. If a pellet is formed at the bottom, try breaking it up before transferring the solution. Try to get all of it.
23. Spin Qiashredder spin columns for 5 minutes at 8000rpm.
24. Transfer the filtrate into the labeled 5mL tube. Place the spin column back in the same collection tube.
25. Using a clean pair of tweezers, remove the filter papers in the Qiashredder spin column. Using the same tweezers, repeat the steps in step 20 for the second tube of the same sample (use the same Qiashredder spin column for 2 tubes from the same sample).
26. Spin Qiashredder columns 5 minutes at 8000rpm.
27. Transfer and combine the filtrate from the second set into the 5ml tubes for each respective sample. Discard the spin columns (with pieces of filter paper inside) and save the collection tubes for future use.
28. Add 800 µl AL buffer to each sample, and place caps back on. Vortex. If you added additional 100 µl ATL buffer at step 13, add 1 ml AL buffer instead.
29. Incubate the 5 mL tubes at 70 °C for 10 minutes in the mini-incubator.

30. Add 800 μ l of 200 proof ethanol to each tube and vortex **IMMEDIATELY** for 10 seconds (make sure that the cap is tightly closed). If you added additional 100 μ l ATL buffer at step 13, add 1 ml ethanol instead.
31. Add the mixture to a Qiaamp Spin column (clear top from DNeasy kit). Centrifuge at 8000rpm for 1 minute. Only **600 μ l** of solution can be filtered at a time, so the process needs to be repeated 4-5 times. Pour the filtrate into a waste beaker, remove the old collection tube (save them in a large beaker for future use) and place the spin column in a clean (new or washed and autoclaved) collection tube each time.
32. Place the filter in a **new collection tube**, pour the filtrate into a waste beaker, and save the old collection tube for future use.



33. Add 500 μ l of AW1 buffer to each sample (dispense into the center of the filter). Spin at 8000 rpm for 1 minute.
34. Place the filter in a new collection tube, pour the filtrate into the waste beaker, and save the old collection tube for future use.
35. Add 500 μ l of AW2 buffer and spin at **15,000 rpm** (or maximum speed) for **3 minutes**.
36. Prepare new labeled microcentrifuge tubes and label while waiting for the centrifuge.
37. Take out spin column carefully so no ethanol splashes on the filter. Place the spin column in a clean microcentrifuge tube (prepared in the previous step).

38. Elute the DNA with 50 μ l Buffer AE (preheated to 70 $^{\circ}$ C). Incubate at room temperature for 5 minutes then spin at 8000 rpm for 1 minute.
39. After spinning the samples, add another 50 μ l of AE buffer into the spin column and incubate at room temperature for 2 hours then spin at 8000 rpm for 1 minute.
40. Discard the spin columns and close the lids of the microcentrifuge tubes.
41. Snap off bottoms of Zymo Inhibition kit spin columns (green top), remove caps of the columns, place in collection tubes and spin for 3 minutes at 8000 rpm to remove excess water (**with no sample in the tubes**). Prepare a new set of microcentrifuge tubes and label. **These will be the final tubes, so make sure to LABEL COMPLETELY with the sample name and date.** Place the spin columns (no caps) in the labeled microcentrifuge tubes. Discard the excess water in the collection tubes and save them for future use.
42. Pipette the eluted DNA from the step 40 into the Zymo Inhibition kit spin column. Make sure to dispense the solution in the middle of the gel without touching it. Spin the column for 1 minute at 8000 rpm. Make sure that the deeper side of the gel in the spin column is outside of the centrifuge (See the diagram below for placement of tubes in centrifuge).



43. Discard the spin columns and close the lids of the microcentrifuge tubes. Store the samples in the refrigerator or freezer until ready for qPCR.

APPENDIX IV qPCR eDNA Screening Protocol

Part 1: Creating your qPCR plate (Rm. 316)

A. Preparing PCR reagents – see the qPCR worksheet template (or part B below) for the amounts required for One (1), 96-well plate – Use only filtered pipette tips!

1. Primers (there are two for each target species [F and R]; read names carefully!) – dilute the stock primers (100 μ M) to 1:10 with molecular grade water. For 6 qPCR plates, make 340 μ L of 10 μ M primer solution (34 μ L stock primer with 306 μ L water), and for 8 qPCR plates, make 460 μ L of 10 μ M primer solution (46 μ L stock primer with 414 μ L water).
 - Do this separately for each primer, in separate 1.5mL microcentrifuge tubes
 - Vortex 10 seconds
 - Centrifuge 10 seconds
 - Store temporarily in a drawer and in refrigerator if you don't use it on the same day.
2. Probe (there is for each target species; read names carefully!) – dilute the stock probe (100 μ M) to 1:10 with molecular grade water. For 6 qPCR plates, make 170 μ L of 10 μ M probe solution (17 μ L stock probe with 153 μ L water), and for 8 qPCR plates, make 230 μ L of 10 μ M probe solution (23 μ L stock probe with 207 μ L water).
 - Vortex 10 seconds
 - Centrifuge 10 seconds
 - Store temporarily in a drawer, out of the light. If you don't use it on the same day, wrap it in an aluminum foil and store it in refrigerator.
3. Master Mix (TaqMan Fast Advanced Master Mix) – keep frozen until first use then store in the mini refrigerator – mix in your hand for 10 seconds prior to pipetting.

B. Setting up a qPCR plate

1. Organize reagents for combining into single “PCR Cocktail”. You will need the following reagents to begin the process:

Reagent	Volume PER Reaction Well	x104 Samples (For 1 96-Well Plate)	x208 Samples (For 2 96-Well Plates)
Water	0.75 μ L	78 μ L	156
Master Mix Solution	5.0 μ L	520 μ L	1040
Primer 1	0.5 μ L	52 μ L	104
Primer 2	0.5 μ L	52 μ L	104
Probe	0.25 μ L	26 μ L	52
TOTAL	7.0 μL	728 μL	1456

NOTE: you will lose a small percentage of fluid in general pipetting activities and therefore **MUST account for this by adding in a small volume per reagent extra to account for this loss. As a good general rule, for a 96-well plate cocktail mix, calculate ingredient volume needed for 104 samples (see above table). Total volume:

Cocktail PER Well: 7.0 μ L
eDNA/water PER Well: 3.0 μ L

2. Obtain a new Applied BioSystem MicroAmp Fast 96-well Reaction plate from the blue and white box. Label the plate with the plate name, date, and your name.
3. Use a Rainin or Eppendorf electric pipette to put 7 μ L of the cocktail in each well, including the negative control wells (ask if you don't know how to use) - Use only filtered pipette tips!
4. To put your sample in each well (except for bottom right three wells):
 - Organize your eDNA samples by one of the following two methods:
 - Place the microcentrifuge tubes into the first 3.5 rows of a 96-well rack, with sample name/number going in order vertical A-H (just like on the template).

- Aliquot only the necessary amount of extract into the first 3.5 rows of a 96-well PCR plate or 4 rows of breakaway PCR plates, with sample name/number going in order vertical A-H (just like on the template).
 - Use a red pipette (20 μ L) and a full red pipette tip box (filtered tips) to transfer 3 μ L of extract from your tray/plate as describe above into your qPCR plate. You will repeat this two more times, in each consecutive row (just like on the template) so that the sample samples will be in three vertical rows. Use a new filter pipette tip for each sample and row, to avoid any possible contamination.
 - Note: if your eDNA extractions are organized into PCR plates at the start, you may use a multi-channel pipette to do full rows at a time.
 - No not fill the last three wells on the bottom right! These are set aside for the negative controls (see #5 below).
5. Negatives
- Using a red pipette (20 μ L) with a new pipette tip, transfer 3 μ L of molecular grade water into last three wells on row H (H10, H11, H12). These will be the negative controls.
- C. Cover your wells with a film (MicroAmp Optical Adhesive Film kit by Applied BioSystems)
1. Peel apart, holding only the tabs
 2. Line up carefully on the plate!
 3. Squish down with a roller or scraper very well (scrape side to side and up and down, and remove the air from all side of the plate edges).
 4. Pull off the side tabs
 5. Take the plate out of the tray and carry it to Rm. 308 – use one glove to hold tray and a non-gloved hand to open doors
 - Note: if you do not plan to run your plate right away, cover it with foil, label it (plate name, date, your name), and put it in the fridge in Rm. 308. Do not wait more than 24hrs to run your plates.

Part 2: Running the qPCR machine (Rm. 308)

A. Setting up a qPCR experiment

1. Turn on the Quant Studio 3 by flipping switch on the back (above the power cord plug)
2. Open the Quant Studio Design software on the desktop
3. Open an existing experiment template (ending in .edt) with eDNA in the name.
4. Change the plate name to the current plate name.
5. Hit Next.
6. Experimental set-up should be left as it is. Hit Next.
7. Highlight all data in the first sheet “QS3 plate setup” of your Excel template file and copy into the diagram of the plate on the right side of the screen (Quant Studio software). Make sure that all the data are transferred correctly.
 - Note: The Quant Studio software does not respond to use the keyboard shortcuts for pasting! You must right-click on the mouse to get that option.
8. Hit Next.

B. Placing the qPCR plate in QuantStudio 3

1. Hit the “eject” symbol in upper right hand corner of screen to open
2. Insert your plate, gently (be sure A1 is in upper left corner)
3. Hit the “eject” symbol in upper right hand corner of screen to close – DO NOT PUSH TO CLOSE!!!

C. Hit run on the Quant Studio Design screen – click numbers under the button, once it appears (they will match those on the machine’s screen)

D. The experiment should complete in ~35 minutes. After completion, remove the plate by touching the “eject” symbol on the screen and close the machine by pressing it again. Discard the plate.

- E. Click on the “Export” button at the top of the screen, check all boxes, save the data file with the plate name and date, in the eDNA Results folder on the desktop. Save the same file on the provided USB.