ERDC/CERL SR-24-1

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Environmental DNA Sampling for At-Risk and Invasive Species Management on Military Ranges

Guidelines and Protocols for Installation Biologists and Land Managers

Aron D. Katz, Mark D. Johnson, and Jinelle H. Sperry

March 2024

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Final Report

Distribution Statement A. Approved for public release: distribution is unlimited.

Prepared for Headquarters, US Army Corps of Engineers
Washington, DC 20314-1000

Under National Defense Center for Energy and the Environment (NDCEE), Project Number 20-10146, Environmental DNA Surveillance of Threatened/Endangered Species on Military Ranges

Abstract

Environmental DNA (eDNA) analysis, or the detection of trace DNA shed by organisms into their environment, has the potential to transform Army capabilities for threatened and endangered species (TES) and invasive species management by providing a rapid, noninvasive, and cost-effective option for monitoring wildlife. Despite these benefits, eDNA analysis is underutilized on military installations as limited access to guidance materials, protocols, training opportunities, and support from eDNA scientists makes it difficult for installation biologists and military land managers to design and execute eDNA surveys, let alone identify management questions that may benefit from eDNA monitoring. Therefore, the aim of this resource is to increase awareness of the benefits and limitations of eDNA monitoring and provide eDNA study design guidelines and field sampling protocols for nonexperts to make this tool more accessible to installation biologists and land managers and help facilitate the adoption of eDNA-based approaches for wildlife management on military ranges.

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Preface

This study was conducted for Headquarters, US Army Corps of Engineers, under Project Number 20-10146, "Environmental DNA Surveillance of Threatened/Endangered Species on Military Ranges." The technical monitor was National Defense Center for Energy and the Environment (NDCEE) Program Manager Mr. Clayton Ferguson, US Army Environmental Command, Joint Base San Antonio–Fort Sam Houston.

The work was performed by the Training Lands and Heritage Branch of the Operational Science and Engineering Division, Engineer Research and Development Center, Construction Engineering Research Laboratory (ERDC-CERL). At the time of publication, Ms. Angela Rhodes was chief, Training Lands and Heritage Branch; Dr. George Calfas was chief, Operational Science and Engineering Division; and Mr. Jim Allen was the technical director for Operational Science and Engineering. The deputy director of ERDC-CERL was Ms. Michelle Hanson, and the director was Dr. Andrew Nelson.

COL Christian Patterson was commander of ERDC, and Dr. David W. Pittman was the director.

1 Introduction

The Army has identified hundreds of threatened, endangered, proposed, and candidate species on, or contiguous to, its installations. Endangered Species Act (ESA) and Sikes Act restrictions caused by threatened and endangered species (TES) and their critical habitat can negatively impact the military's mission of readiness. However, proactive management can have positive impacts for vulnerable species populations, reducing the risk of potential future listing decisions. Therefore, rapid, efficient, and accurate assessments of wildlife are imperative to inform adaptive management strategies. Many installations currently implement biodiversity monitoring programs, but conventional survey approaches can be ineffective, cost prohibitive, and time intensive, especially for species that are numerically rare, elusive, cryptic, or have life histories that complicate survey efforts (e.g., nocturnal, fossorial, parasitic, or cave-limited species), and they often require seasonal contracting of permitted personnel with specific taxonomic expertise.

Environmental DNA (eDNA) analysis has the potential to completely transform Army capabilities for wildlife management by providing a rapid, noninvasive, and cost-effective option for detecting and monitoring wildlife on military installations. eDNA is trace DNA shed by organisms into their environment (e.g., soil, water, or air), which can be sampled and analyzed to detect species without requiring efforts to observe, capture, handle, or identify them at the time of survey. Numerous species have been successfully detected with eDNA sampled from terrestrial (e.g., Thomsen and Sigsgaard 2019; Katz et al. 2021), aquatic (e.g., Deiner et al. 2016; Strickland and Roberts 2019), marine (e.g., McClenaghan et al. 2020), airborne (e.g., Johnson et al. 2019; Lynggaard et al. 2022), and subterranean (Niemiller et al. 2018; Saccò et al. 2022) environments, including parasites (Bass et al. 2015; Katz et al. 2023), pathogens (Wittwer et al. 2018), invasive species (Morisette et al. 2021), pollinators (Thomsen and Sigsgaard 2019), and TES (Niemiller et al. 2018; Strickland and Roberts 2019; Katz et al. 2021). Other studies have used eDNA data in novel ways to predict the impacts of climate change (Wilcox et al. 2018), reconstruct ancient communities and infer how their diversity has changed over time (Bálint et al. 2018), and reveal potential species interactions (Banerjee et al. 2022; Johnson et al. 2023). Moreover, compared to conventional methods, eDNA analysis can expand survey coverage and sampling frequency

(Evans et al. 2018), offer greater detection sensitivity (Fediajevaite et al. 2021), and sample otherwise inaccessible habitats (Saccò et al. 2022).

Despite the potential benefits and diverse applications of eDNA analysis, this state-of-the-art technology remains underutilized as a tool for wildlife management on military installations. Limited access to guidance materials, protocols, training opportunities, and support from eDNA scientists makes it difficult for land managers to design and execute eDNA surveys, let alone identify management questions that may benefit from eDNA monitoring (Nagarajan et al. 2022). Therefore, the aim of this report is to help facilitate the adoption of eDNA analysis on military ranges by increasing the awareness and understanding of its benefits and limitations and how and when it can be implemented on installations. We provide examples of its use for wildlife management, guidance on eDNA study design, and step-by-step eDNA field sampling guidelines, including example protocols for sampling eDNA from water and soil to detect species or characterize communities of interest.

2 Study Design Guidelines

2.1 Identifying Management Questions

The first step for implementing eDNA monitoring is to identify management questions that can benefit from eDNA analysis as they will serve as the basis for guiding experimental design. eDNA monitoring is most useful when conventional methods are inefficient (e.g., expensive, time or labor-intensive, or low detection rates), problematic (e.g., invasive, destructive, or require specialists), or unavailable (e.g., otherwise inaccessible habitat). For example, it can provide enormous benefits when there is a need for noninvasive and nondestructive sampling for TES; expanding survey coverage and sampling frequency to detect rare or low-density species (Evans et al. 2018); rapid screening for TES (Katz et al. 2021), pathogens (Wittwer et al. 2017), parasites (Bass et al. 2015; Katz et al. 2023), and invasive species (Morisette et al. 2021); or to survey otherwise inaccessible habitats or communities, such as caves or groundwater ecosystems (Saccò et al. 2022). Oftentimes, eDNA analysis is most effective when used in conjunction with conventional survey approaches. For example, eDNA detections can be leveraged to establish probabilities of TES presence at more sites, more frequently, and across larger areas compared to conventional approaches alone. Habitats or sites can then be prioritized based on eDNA detections for follow-up conventional surveys to assess population status and health and enable land managers to deploy rapid management actions if needed (Evans et al. 2018). See Table 1 for additional examples.

Unlike most conventional methods, eDNA analysis is not currently practical for routine inferences regarding population abundance (but see Sigsgaard et al. 2020), demographics, or health, so it may not be appropriate for management questions that require certain population-level information. However, the field of environmental RNA (eRNA) analysis, though still in its infancy, shows promise in assessing species populations at resolutions far beyond the capacity of eDNA (Yates et al. 2021). eRNA may be able to monitor the physiological status and health of populations and communities by targeting RNA transcripts that are differentially expressed when exposed to stressors (e.g., heat stress, disease, etc.), and the relative abundance of RNA profiles associated with sex, life stage, and phenotype, may provide information on population demographics. Also, because eRNA degrades at a faster rate compared to eDNA, detections via

eRNA analysis may offer increased confidence for inferences of recent species presence (e.g., within hours of sample collection).

Table 1. Examples of management questions that could benefit from environmental-DNA (eDNA) analysis.

Example Management Questions	How eDNA Analysis Can Help
Where is this species occurring? What is its distribution? Does site use change seasonally?	eDNA analysis offers increased detection sensitivity for elusive or rare species that are difficult or impossible to detect with conventional survey methods. Compared to conventional surveys, eDNA analysis is generally more cost-efficient, providing land managers with more flexibility to sample more frequently throughout the year and across larger spatial scales.
How has the community composition changed after the habitat has been disturbed or restored? Was restoration successful?	eDNA metabarcoding for community-wide snapshots of diversity can be used before, during, and after disturbance or restorations. Changes in patterns of diversity identified via eDNA metabarcoding can indicate ecological disruption, help direct restoration efforts, and gauge restoration success.
Where should we direct our management resources? Which sites should be prioritized for follow-up survey, control, or conservation efforts?	eDNA sampling can be used as a cost-effective initial step to determine whether continued monitoring or additional analysis is necessary. This preliminary sampling can increase efficiency and save time and resources.
Are these conventional survey methods adequately detecting species of interest? What species are we missing?	eDNA detection of species not observed or captured during paired conventional surveys may indicate a need to modify conventional sampling protocols to increase detection probability.
Is this population infected with parasites and/or pathogens? Has this invasive species reached our installation? Where should we focus our prevention, control, and treatment efforts?	eDNA sampling can be used for parasite and pathogen screening or monitoring of invasive or nuisance species that could affect TES management actions. eDNA analysis excels at detecting invasive species before establishment, at a time when control is both the cheapest and most likely to succeed. eDNA detections can be leveraged to establish infection and invasion risk for sites and be conducted more frequently across larger areas compared to conventional surveys alone. High-risk sites, informed by eDNA analysis, can then be better prioritized for follow-up conventional surveys to determine infection prevalence and intensity or invasive or nuisance species density and abundance, assess host population/habitat status and health, and deploy rapid prevention, control, or treatment actions if needed.
Which species are providing pollination or seed dispersal services for this endangered plant species or at-risk plant community?	eDNA can be isolated from most environmental substrates and surfaces. In this case, eDNA isolated from the surface of plants, animals, or from fecal material can be used to establish species interactions such as pollination or seed-dispersal networks.
What does this endangered species eat? Where should we direct management efforts to conserve prey species of TES? Is this invasive species consuming TES?	Though not technically eDNA analysis, diet analysis via fecal DNA metabarcoding uses the same methodological approach and can reveal species consumed by animals. For example, metabarcoding can help inform management strategies by identifying important TES prey species or determining whether invasive species (e.g., feral pigs) are consuming TES.
What lives in this spring or cave? Do any TES or species of concern use this spring or cave?	eDNA can be sampled from sites that are too difficult or impossible to survey with conventional methods (e.g., subterranean habitats such as springs or caves), which can be used to characterize entire communities or target species of interest whose presence would have otherwise remained unknown.
How will climate change impact TES on the installation? Which species or habitats can we anticipate needing additional management focus in the future?	Comprehensive eDNA sampling paired with environmental data (e.g., habitat quality, temperature, etc.) can be used to model how TES distributions may respond to ecological change (e.g., warming temperatures). Predictions based on modelling efforts can be used to identify at-risk species or critical habitat for management focus.

Source: adapted from Nagarajan et al. (2022).

2.2 Interpreting Environmental DNA (eDNA) Detections

eDNA analysis and conventional approaches each have distinct advantages and limitations, but most importantly, they measure different things (i.e., eDNA presence and concentration versus species presence and abundance, respectively). Unlike conventional methods that sample physical specimens, inferences of species presence based on eDNA detections are more nuanced because the origin, transport, and age of eDNA is nearly always impossible to initially determine (Barnes and Turner 2016; Harrison et al. 2019): it may have originated from a live or dead animal or its feces; traveled vast distances by air or water or not at all; or been deposited recently or long ago and has remained preserved. These inherent uncertainties related to the ecology of eDNA have caused a considerable level of distrust amongst the management community concerning the utility of eDNA in wildlife management (Darling 2019; Jerde 2021). Although some skepticism is beneficial, it is important to have realistic expectations about what eDNA data can offer and recognize that there are ways to reduce uncertainty (i.e., the probability of false negative and false positive detections) and thereby increase the confidence for inferences of species presence based on eDNA detections (Jerde 2021). For example, the risk of false negatives (i.e., species was not detected but is actually present) can be reduced with an increased sampling effort, better sample preservation protocols to prevent eDNA degradation, inhibitor removal steps during eDNA extraction to prevent polymerase-chain-reaction (PCR) failure, optimized assay conditions, or increased sequencing depth for metabarcoding to better detect low-abundance eDNA. The risk of false positives (i.e., species was detected but is actually absent) can be reduced with decontamination protocols that minimize the contamination risk, the use of negative controls to recognize contamination, assay validation testing and optimization steps to assess and reduce the risk of nontarget species detection, sequence PCR amplicons to confirm target species identity, and a robust reference sequence library for metabarcoding taxonomic assignments. With the appropriate levels of replication, precaution, control, optimization, and testing, eDNA detections, especially those that can be replicated consistently, occur repeatedly over time, are present across all replicates, or are correlated with known species observations at the same sample site, offer a high degree of confidence in species presence (Jerde 2021).

2.3 Additional Considerations for Sampling Strategy and Analytical Approach

Different management goals will require different methodological choices that consider a wide range of interdependent factors that can influence both sampling strategy and analytical approach (Table 2). For example, the impact false positives and false negatives have on management outcomes will help determine the appropriate level of sample replication, contamination precaution, controls, and assay optimization and testing. Target species characteristics (e.g., ecology, biology, and behavior) can also inform sampling strategy, such as sample type (e.g., water versus soil), target habitat (e.g., ponds versus streams), and survey scope (e.g., sampling period, spatial extent, and replication). However, different sample types or habitats will require different collection and processing methods, and the survey time, coverage, and level of replication can impact the probability of detections and costs. See field-sampling guidelines in Section 4 for additional guidance on sampling strategy and establishing field protocols.

Determining the appropriate analytical approach depends primarily on whether management is focused on detecting a single species (e.g., TES, invasive species, parasite, or pathogen) or characterizing the biodiversity of communities (e.g., broad screening for parasites, pathogens, or invasive species; at-risk communities; diet analysis; or ecological networks), but additional factors also need to be considered (e.g., budget and assay/reference sequence availability). Quantitative polymerase chain reaction (qPCR) is the most commonly used approach for single-species eDNA detection, which uses specific qPCR assays (i.e., primers, probe, and reaction chemistry) that have been designed to detect and quantify eDNA for only the species of interest. This approach can be extremely sensitive, simple to perform in the lab, and does not require expensive high-throughput sequencing services like multispecies methods. However, its relative affordability depends on whether a qPCR assay is available (i.e., developed and validated) for the target species. Novel or custom qPCR assays will require multiple stages of research and development prior to their application in the field, including the generation of target (and nontarget) reference sequence data, assay design and optimization steps, and various steps to validate assay specificity, sensitivity, and performance (Klymus et al. 2020; Thalinger et al. 2021)—all of which can significantly increase costs.

eDNA metabarcoding may be a more cost-effective option for single-species targets in cases when species-specific qPCR assays are unavailable or if detection sensitivity is less of a concern, but this approach is typically more expensive than qPCR; thus, it is primarily used when there is a need to survey multiple species or entire communities. eDNA metabarcoding assays are designed to detect all eDNA for a particular taxonomic group (e.g., insects, mammals, plants, bacteria, fungi, etc.). Metabarcoding leverages generalized (nonspecific) PCR primers and high-throughput DNA sequencing to generate sequence data for all target-species eDNA present in samples, which can then be taxonomically identified by comparing eDNA sequences against a reference DNA-sequence database. This approach excels at rapidly screening biodiversity in environmental samples, but compared to qPCR, the laboratory and data-analysis steps are more complicated and time consuming, and the availability of sufficient computing resources must be considered. Sample and environmental conditions (e.g., presence of PCR inhibitors or recent precipitation) can affect the performance of DNA extraction and PCR—steps that are required for both qPCR and metabarcoding. Metabarcoding also requires high-throughput DNA sequencing, but this can be very expensive, and data quality can vary depending on the sequencing platform, sample quality, chemistry, and vendor. Moreover, federal spending regulations and requirements can complicate and limit sequencing options available to federal agencies. Efforts to establish service contracts with commercial or academic sequencing facilities are usually required. See Appendix D for an example performance work statement (PWS) used for contracting sequencing services.

Table 2. Factors influencing eDNA study design, including questions and guidance for considering each factor.

Factor	Questions	Guidance
Purpose	What are the management questions and goals? What sampling and analytical approaches are needed?	Different goals require different sampling and analytical approaches (e.g., routine biomonitoring versus ecological analysis versus survey methods comparison).
Budget	Is the budget sufficient to achieve intended purpose?	Most costs are associated with laboratory analysis, not sample collection. Maximize user needs within budget constraints.
Spatial scale	Single site versus large scale? Fine versus coarse sampling?	Consider a minimum number of sites and samples to reach sufficient spatial coverage and statistical power.
Sampling frequency	Single time point versus long-term sampling?	Consider whether repeated, long-term sampling efforts are necessary to meet project goals.
Replication	What level of replication is sufficient to meet project goals?	More replication is needed for goals that require higher levels of stringency (e.g., legal ramifications), statistical power (e.g., for modeling), or sensitivity (e.g., for rare, patchy, or low-density species or samples with low eDNA concentrations).
Environmental factors	How will environmental conditions impact our study design? Are any sample or site metadata required to meet project goals?	Sample and ecosystem conditions (e.g., temp, UV, pH, terrestrial versus aquatic, precipitation, or disturbance) can influence eDNA production, transport, and decay and require specific field and laboratory methods. Consider the minimum number of metadata requirements to meet project goals.
Sample type	What is the sample type (e.g., water, soil, feces, swabs, or airborne dust) and target volume and mass per sample?	Different sample types depend on study goals (e.g., biomonitoring versus diet analysis) and target species (e.g., ecology and behavior) and require specific field collection and laboratory protocols (e.g., eDNA extraction).
Controls	What types of controls should be implemented and at which stage of the process?	Controls can identify false positive or negative detections and determine where and how they occurred in the process (e.g., contamination or PCR inhibition), and their use depends on study goals and sampling strategy.
Target species	What is the target species or community? Where and when is their eDNA likely to occur and at what levels? What is the risk of false positives?	Study design should consider the biology, ecology, life history, or behavior of target species and any co-occurring potential nontarget species, as these factors can impact sampling and analysis. Preliminary *in silico* testing can identify the risk of nontarget species detections.
Fieldwork restrictions	How will field conditions impact contamination risk and sample processing, preservation, and storage options?	Consider needs for sample processing (e.g., electricity for vacuum filtering versus syringe filtering in field) and preservation and storage (e.g., freezer versus cooler versus dry or in buffer) given fieldwork restrictions.
Laboratory processing	What laboratory protocols and supplies are required for eDNA extraction?	Extraction methods can vary in DNA yield and quality and their use often depends on sample type and preservation method.
Analytical approach	What is the appropriate analytical approach (i.e., qPCR versus metabarcoding) to meet project goals?	Consider the advantages and limitations of qPCR versus metabarcoding given the project's target (e.g., species versus communities), sensitivity requirements, and budget.
Assay and reference sequences	Are eDNA assays developed and validated for the target species? Are relevant reference sequence data available?	If validated eDNA assays or reference sequence data for target species are not available, then consider the costs and expertise needed to develop them.
Training needs	What is the experience level of field personnel? Are they capable of performing tasks following protocol?	Ensure all field personnel are adequately trained and provided with necessary protocols. If needed, collaborate with eDNA scientists for training opportunities.

Source: adapted from De Brauwer et al. (2022).

There are several other emerging technologies currently being explored that may offer increased sensitivity and quantitative abilities (e.g., droplet digital polymerase chain reaction [ddPCR]) (Doi et al. 2015) or specificity (e.g., clustered regularly interspaced short palindromic repeats [CRISPER]) (Williams et al. 2019) for single-species eDNA detection, PCR-free approaches for species and communities (e.g., DNA sequence capture) (Giebner et al. 2020), in-field sequencing (e.g., Oxford Nanopore MinION), and extraction- and PCR-free DNA amplification (e.g., loop-mediated isothermal amplification [LAMP]) (Williams et al. 2017). However, these methods are either in active development and not ready for routine application or require relatively expensive equipment or reagents compared to standard qPCR and eDNA metabarcoding approaches.

Last, all these methodological considerations need to balance project needs given budget constraints. Generally, eDNA analysis is highly flexible and can usually be scaled up or down in various ways, including many cost-saving options to choose from, but each comes with important trade-offs (Bruce et al. 2021). For example, pooling field sample replicates or eDNA extracts from each site can greatly reduce costs, allowing more sites to be sampled or analyzed, respectively, but this will reduce the spatial resolution, decrease detection probabilities, and limit available options for analysis (e.g., occupancy modelling or power analysis). Table 2 provides questions and guidance for considering these important factors, along with others, when conceptualizing eDNA experiments. Also, see Appendix A for a list of recent eDNA literature that includes general reviews and guidelines for eDNA analysis and specific topics, including example eDNA applications, eDNA ecology and other critical considerations, and eDNA assay validation guidelines.

3 eDNA Laboratory Support

Installation biologists and land managers interested in eDNA monitoring may not have the facilities, equipment, or expertise required to process and analyze eDNA samples and, instead, must rely on downstream services provided by experienced and well-equipped eDNA research labs. We recommend partnering with government, academic, or commercial eDNA research labs that can provide these services, especially those that can offer additional collaborative support to installations interested in implementing eDNA monitoring programs. For example, collaborating with eDNA research labs can do the following:

1. Help identify existing (or new) management questions and concerns that could benefit from eDNA analysis.
2. Help communicate clear and realistic data expectations for eDNA analysis.
3. Facilitate the pursuit of collaborative funding.
4. Assist with eDNA study design.
5. Provide field training and support in eDNA sampling and processing.
6. Provide laboratory services including eDNA extraction, assay development, reference sequence generation, qPCR analysis, metabarcoding library preparation, and DNA sequencing.
7. Assist with data analysis and interpretation.
8. Produce reports and publish results.

4 Field Sampling Guidelines

These guidelines cover aspects of eDNA sampling, such as establishing field protocols, preparing supplies and equipment, sampling water or soil, minimizing contamination risks, and sample preservation, storage, and shipment. Examples are provided but specific study design is dependent on study objectives and site-specific considerations. We provide a brief outline of laboratory workflow for users, but it is not the focus of this resource.

4.1 Field Sampling Preparation

1. Determine sampling locations and number of field sites, including number of samples to be collected at each site, well ahead of time. Certain supplies can take weeks to arrive after ordering, so it is important to establish the location and number of field sites early to determine the type and quantities of supplies needed and to provide ample time to order and receive them. In aquatic systems typically, at least four samples, including one negative control (e.g., distilled water), are collected at each site.
2. Consider mapping software (e.g., Google Earth, Avenza, or ArcGIS Field Maps) that can be used to map prospective sampling sites, label coordinates with predetermined site IDs, and preload relevant maps, coordinates, and site IDs into hand-help GPS or mobile device (e.g., cellphone).
3. Create a daily field sampling schedule that includes sites to visit each day with estimated survey and travel times to ensure all prospective sites can be sampled within the proposed sampling period.
4. Clean and decontaminate all reusable supplies that come into contact with samples (e.g., vacuum manifold, bottles, tubes, filter funnels, forceps, and shovels). See Section 4.2. for an equipment decontamination protocol.
5. Use a checklist to confirm that all required field supplies are packed, prepared, and in working condition. See section Appendix B for an example field packing checklist.
6. Ensure field crews are sufficiently trained in relevant field protocols. See Sections 4.2–4.6 for example protocols.

4.2 Equipment Decontamination Protocol

1. If possible, decontamination should be performed in a room designated for low-copy DNA (e.g., eDNA extracts) only (i.e., no high-copy DNA such as PCR products, tissue DNA extracts, or synthetic DNA standards).
2. Put on gloves and scrub and rinse off all visible dirt on equipment with 20% bleach solution (preferred) or soapy tap water.
3. Submerge equipment in 20% bleach solution for at least 30 minutes.
4. Put on new gloves then triple rinse supplies using distilled water (preferred) or tap water, taking care to rinse off all the bleach solution.
5. Let equipment air-dry upside down on clean paper towels.
6. Once equipment is dry, put on new gloves and store supplies inside sealed Ziploc bags, labeled “sterile.”

4.3 Establishing Field Protocols

4.3.1 Sample Collection, Preservation, and Storage

It is important that sample collection (e.g., number of sample replicates per site, volume of sample, controls, etc.), preservation (e.g., frozen, dry, or in buffer), and storage (e.g., in freezer or cooler) procedures consider contamination risk, field conditions (e.g., temperature and sun exposure), site access (e.g., short walk or long hike), and available facilities (e.g., lab, field station, hotel room, or camp). For example, remote field sites that require camping overnight may necessitate samples be stored dry or in a buffer preservative (e.g., soil) or processed in the field by hand (e.g., filtering water with syringes or hand pumps) if there is no electricity or access to ice.

4.3.2 Contamination Prevention

1. Avoid situations that increase the likelihood of cross contamination between multiple independent samples:
   a. Always use new supplies for each sample and immediately discard any supplies after contact with samples.
   b. Reduce the likelihood of reusing contaminated supplies by clearly labeling any supplies that have already been used or are suspected to be contaminated as “dirty” and make sure they are discarded or set aside until decontaminated.

c. Always wear disposable gloves and change frequently and in between samples and sites to reduce the risk of sample cross contamination or introducing your own DNA into the sample.
d. Only put on gloves immediately prior to sample collection. Once gloves have been put on, do not touch anything except the bottle used to collect water. Immediately remove and discard gloves after the sample has been capped and placed into Ziploc bag.
e. In lotic (flowing water) systems, always enter the water downstream of sampling area and collect water facing upstream.
f. In lentic (nonflowing water) systems, avoid entering the sampling area prior to or during sample collection to reduce the risk of transferring DNA from footwear or clothing. If the water must be entered for collection, enter slowly, try not to disturb the sediment or splash water, and sample ahead of you in an area that has not been disturbed.

2. If performing both conventional and eDNA sampling, minimize the potential for cross contamination between specimens and environmental samples:

   a. Make sure environmental samples are collected prior to collecting and handling specimens at each site.
   b. Minimize specimen handling as much as possible.
   c. Wear disposable gloves when handling specimens, change gloves frequently, and remove gloves after handling specimens before touching anything.
   d. Implement decontamination protocols between surveys and sampling sites.
   e. Do not share equipment or supplies between surveys.
   f. If possible, separate personnel by task, that is, into two separate teams: one to perform eDNA sampling, the other to perform conventional sampling.

3. Include a negative field control for each site sampled, especially when reusing equipment that has been decontaminated to collect or store samples.

4.3.3 Sample Tracking, Naming Conventions, and Data Management

For most eDNA studies, samples are often collected by multiple teams at different times and locations, which are then provided to other independent teams for downstream laboratory work and data analysis. Therefore, it is important to establish strict data collection protocols and naming

conventions (i.e., for sites, samples, and eDNA extracts) before field work begins to ensure effective sample tracking.

1. Site, sample, and extraction IDs should be consistent, unique, and informative to facilitate sample tracking from collection through PCR and data analysis. The following is an example:
   a. Site Code = four-letter code to identify survey site. For example, Site Code LR12 refers to the 12th sampling site along the Little River.
   b. Site ID = a unique ID for each site that incorporates a site code and survey date. For example, Site ID LR12-2022Jun06 refers to the 12th sampling site along the Little River, which was surveyed on 6 June 2022.
   c. Sample Replicate ID = a letter designating a sample replicate type or location (e.g., for stream samples, L = left bank, M = middle channel, and R = right bank). Sample replicates in other systems (e.g., ponds or soil) can be labeled "X, Y, Z," which may each be associated with specific GPS coordinates if samples are far enough apart within a given site. Negative field controls should also be named consistently, for example, with a "B" for "Blank."
   d. Sample ID = a unique ID for each sample collected that incorporates a site code, survey date, and sample replicate ID. For example, a sample collected along the right bank of the Little River site LR12 on 6 June 2022 would be named LR12-2022Jun06-R.
   e. Extraction ID = a unique ID for a purified eDNA extract. Because eDNA could be extracted multiple times from the same sample, it is important to be able to track each individual eDNA extract via a unique ID. All extractions performed within a given day are sequentially numbered (e.g., 1–10 for 10 extractions). Therefore, each extract can be identified by combining the extraction date and its sequential number—a unique combination that can be associated with sample collection metadata and tracked through PCR or metabarcoding library preparation. For example, extraction ID 2022Aug18-07 represents the 7th eDNA extraction performed on 18 August 2022.
2. The types of sample metadata needed can vary depending on study goals and methods, but minimum data requirements, like those listed in Appendix C, are generally recommended for most eDNA studies. Standardized

field data templates can help maintain data and naming consistency throughout the project. See Appendix C for an example field data template.

3. It is important to establish data management protocols to minimize the risk of data loss.

    a. Taking photographs of all field notes and data sheets after each site, which can be accomplished using a cell phone camera, is a simple and effective way to secure and backup field data.
    b. All field notes and data sheets should be digitized (e.g., in Excel spreadsheets) at the end of each day, and if possible, backed up on a local computer hard drive and uploaded to cloud storage.
    c. Spatially referenced digital data collection software (e.g., ArcGIS Field Maps on mobile phones) can provide a more efficient alternative to field notebooks; they do not require efforts to digitally transcribe hard-copy field data, can promote standardized data collection, and may help prevent data loss.
    d. Data loss (e.g., via lost datasheets, missing notebooks, failed hard drives, and accidental deletions) can be prevented with sufficient backup redundancy. At least three types of data backups (e.g., as hard paper copies, on local hard drives, and in cloud storage) are recommended.

4.4 Example Water Sampling Protocol

4.4.1 Before Water Sampling

1. Prelabeling each sample bottle ahead of time (following the labeling convention described below) will speed up field work but requires prospective sampling sites to be predetermined.

2. Clearly differentiate negative controls from samples. For example, use blue labeling tape or tough tags for sample bottles and red for negative control bottles.

3. Ensure that each sample has a unique identification that can be linked to site, date, and replicate location (see Section 4.3.3 for more information on naming conventions). The following is an example:

    a. For lotic systems, the sample ID can include “site name-sample date (YYYYMonDD)-replicate location (L = left bank, M = middle channel, R = right bank, and B = blank).” For example, a sample collected at site ER6 on 22 May 2022 from the left bank when facing upstream would be ER6-2022May22-L. The associated negative control would be ER6-2022May22-B.

   b. For lentic systems, the sample ID can include "site name-sample date-replicate location (X, Y, Z)." For example, the first sample collected at site ER6 on 22 May 2022 would be ER6-2022May22-X. The associated negative control would be ER6-2022May22-B.

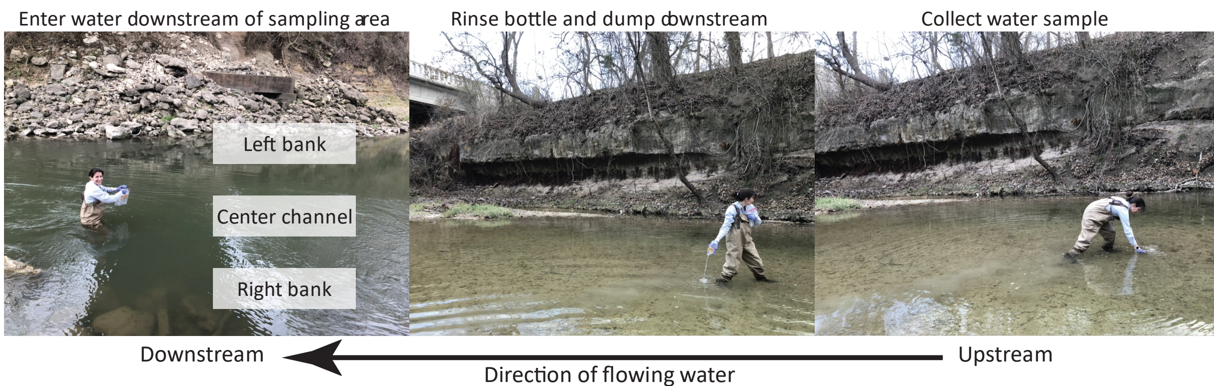
4. Fill negative controls: put on gloves, add label tape to negative control bottles, rinse bottles three times, then fill to top with distilled (preferred) or tap water, and cap and place in gallon Ziploc bag(s).
5. Fill cooler with ice. Add the negative controls.
6. Confirm that all required supplies are prepared and packed for the field. See Appendix B for an example field supplies checklist.

4.4.2 At the Field Sampling Site

1. Before samples are collected at each site, put on gloves and open the lid of the negative control bottle to expose the distilled water to the air for 5–10 seconds, then close the lid and put back on ice.
2. An example of sampling water from lotic (flowing) systems follows (Figure 1):
   a. Put on new gloves, grab the Ziplock bag with the sample bottles, and enter the water downstream of the sampling area.
   b. Immediately prior to sample collection, rinse the bottle with upstream surface water and dump downstream. Repeat one or two times.
   c. Facing upstream, collect samples (1 L) on the left bank, middle of the channel, and right bank, always sampling upstream to minimize the potential for cross contamination from footwear or clothing.* Repeat this until all site replicates (3 L total) are collected and place in bag.

* For a full list of the spelled-out forms of the units of measure used in this document, please refer to *US Government Publishing Office Style Manual*, 31st ed. (Washington, DC: US Government Publishing Office, 2016), 248–52, https://www.govinfo.gov/content/pkg/GPO-STYLEMANUAL-2016/pdf/GPO-STYLEMANUAL-2016.pdf.

Figure 1. Diagram illustrating the water sampling procedure in lotic systems, including sample replicate locations and orientation.



3. An example of sampling water from lentic (nonflowing) systems follows:
    a. Put on new gloves, grab the Ziplock bag with the sample bottles, and either avoid entering the water during sample collection or enter slowly, trying not to disturb the sediment.
    b. Immediately prior to sample collection, rinse the bottle with water from an undisturbed area and dump behind you. Repeat one or two times.
    c. Collect samples (1 L) from undisturbed areas around the waterbody, as access allows. If the sediment is easily disturbed and walking makes water turbid, try to sample areas where the sediment has not been disturbed or where the water is still clear. The number and distribution of sample replicates per water body will depend on the specific research goals and accessibility, for example, 3–5 replicates per small pond, dispersed at cardinal points, or 10–12 replicates per larger lake, distributed nearshore and offshore. It is recommended to take GPS coordinates at each sample replicate location in larger water bodies.
4. Place all water sample bottles in cooler with ice.
5. Record any required sample, location, collection, and environmental data. Note any information that may be relevant to sample collection (i.e., high flow, recent rain, target species observed, etc.). An example field datasheet with recommended sample metadata is provided in Appendix C.
6. Repeat this procedure at each site, making sure to change gloves between sites.

4.4.3 Example Water Filtering Procedure

1. Prior to field work, confirm that all the required supplies are prepared, in working condition, and packed for water filtering. See Appendix B for a water filtering supplies checklist.
    a. Load the buffer and label filter sample tubes: add 900 µL of Cetyltrimethylammonium bromide (CTAB) buffer using a pipette or syringe to the 1.5–2 mL tubes prelabeled with sample IDs on color-coded tough tags (e.g., blue for samples and red for controls).
    b. Preload the sterile filter funnels with 0.8 µM cellulose nitrate filters:
        (1) Put on gloves and use a 20% bleach solution to decontaminate the counter space in a clean room designated for handling low-copy DNA only.
        (2) Lay out paper towels on a bleached counter and pull apart funnels.
        (3) Using forceps, lay the white filter (the blue papers sheets are spacers NOT filters) on top of the blue-filter funnel base, then gently snap on the filter funnel cup without twisting it (Figure 2). Cellulose nitrate filters are very fragile and can easily tear. If a filter is torn, cracked, or has a hole, do not use and discard.
        (4) Repeat the procedure. Once enough funnels have been packed, seal them in Ziploc bags labeled "clean" and "0.8 µM" to indicate filter pore size.

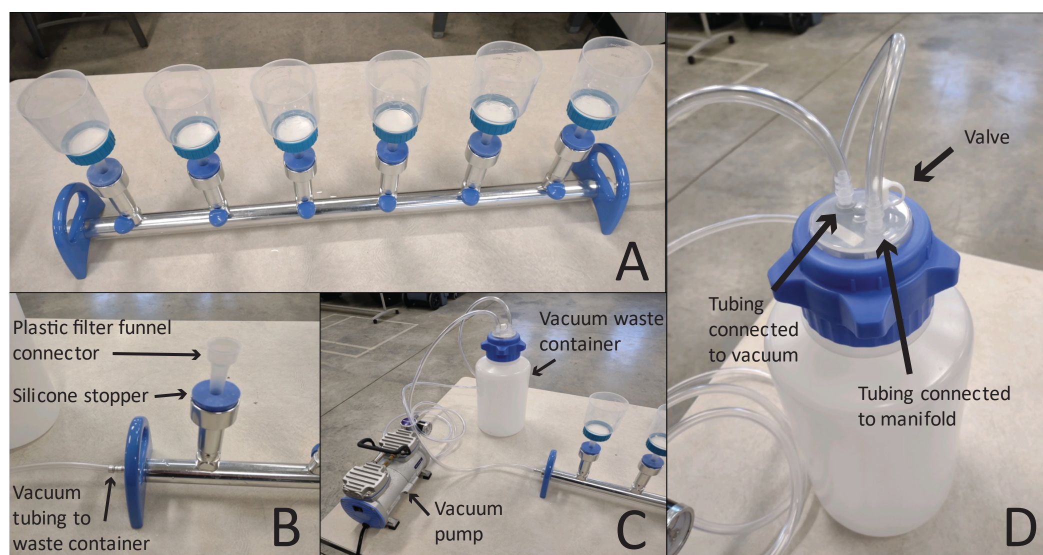
Figure 2. Example of the filter funnel components and how to differentiate the cellulose nitrate filters from the blue paper spacers.

2. Setting up all the filtering equipment in the hotel, field station, or lab space as soon as you arrive, prior to collecting samples, is recommended. Having

everything ready to go can speed up filtering after a long day in the field. Refer to Figure 3 for the water-filtering setup.

a. Put on gloves and wear a lab coat or apron to protect clothing from the bleach.
b. Liberally spray and wipe down countertops with the 20% bleach solution.
c. Use vacuum tubing to connect (1) the vacuum pump to the vacuum port on the vacuum waste container and (2) the water port on the vacuum waste container to the side of the filtering manifold. Confirm that the vacuum waste container is connected between the vacuum output and the manifold (so that water does not go from the manifold into the vacuum).
d. Place the silicone stoppers with the plastic filter funnel connectors into the manifold openings.
e. The filter funnel should be snapped into the manifold connectors immediately prior to filtering.
f. Make sure the waste container valve is closed when you are ready to begin filtering.

Figure 3. Example of a filtering set up, showing (*A*) the full manifold with the silicone stoppers, plastic filter funnel connectors, and filter funnels attached—valves at the bottom of each opening provide independent on/off vacuum control for each filter funnel; (*B*) a close-up of the silicone stoppers, plastic filter funnel connectors, and vacuum tubing to waste container; (*C*) the vacuum pump and waste container; and (*D*) a close-up of the waste container tubing connectors and valve.



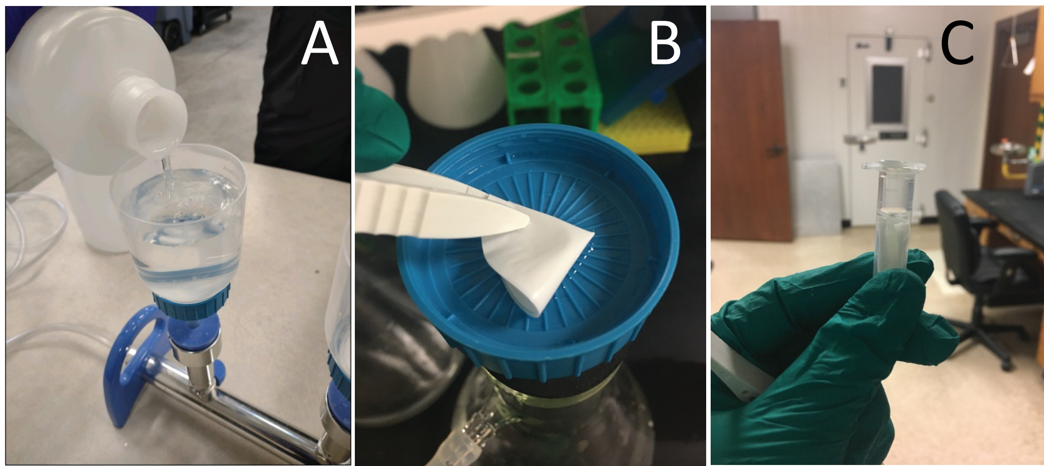
3. Try to filter all the samples the same day after collecting from the field. If filtering the same day is not possible, then store samples in a cooler on ice

(preferred) or refrigerate and filter within 24 hours. Record the time and date at the start of filtering.

4. Filter the samples, negative controls first.
   a. Put on new gloves, remove sample bottles from cooler, and spray and wipe down each sample bottle and decontaminate the workspace with a 20% bleach solution.
   b. Change gloves and place new filter funnels on the manifold (Figure 3*A*)
   c. Pour each sample to the 250 mL line on the filter funnel cups (Figure 4*A*) and place each labeled sample bottle directly in front of their corresponding filter to help you remember which sample goes to which filter.
   d. Change gloves after pouring. Note: remember to change gloves frequently. For example, after pouring samples, between each sample site, after decontaminating between filtering runs, before handling filters, and before reaching into bags containing sterile supplies (i.e., funnels, forceps, etc.).
   e. Turn on the vacuum and open the manifold valves. Keep the manifold valve closed if the filter funnel is not attached.
   f. After 250 mL of water has been filtered, pour another 250 mL of the same sample into the same filter funnel, and repeat until the entire 1 L sample has been filtered.
   g. Remember to empty the vacuum waste container periodically while filtering to prevent overflow into the vacuum pump.
   h. After the sample is finished filtering, remove the filter funnel cups by gently pulling them off from blue base, and discard them in a garbage bag for contaminated supplies.
   i. Using forceps and the blue funnel base as support, repeatedly fold the filter in half to form “a pie slice” (Figure 4*B*).
   j. Transfer the folded filter into the appropriately labeled 1.5–2 mL tube filled with CTAB buffer, make sure that the filter is fully submerged in the CTAB solution using forceps, and confirm the cap is on tight (Figure 4*C*). Store the tubes with filters in a labeled freezer box at room temperature.
   k. Remove the blue funnel base from the manifold and discard it into a garbage bag for contaminated supplies along with all forceps, filter funnels, and other contaminated equipment used in this round of filtering.

l. Record all required filtering data in a filtering worksheet or lab notebook.
m. Repeat for the rest of the negative controls then all the field samples.

Figure 4. Example of (*A*) pouring a sample into the filter funnel cup; (*B*) how to fold filters using forceps and the funnel base as support (filters can be folded in half again after what is shown here); and (*C*) a folded filter, fully submerged in cetyltrimethylammonium bromide (CTAB).



5. Avoid contamination during filtering:
   a. Always decontaminate workspaces with a 20% bleach solution before processing each batch of samples, including the outside of each sample bottle before opening them.
   b. If filtering in a laboratory space, ensure that it has not been exposed to high-copy DNA (i.e., PCR products, tissue DNA extracts, or synthetic DNA standards).
   c. Filter all the negative controls before the field samples to minimize the risk of contaminating the negative controls.
   d. Change gloves frequently: between samples, after decontamination, after pouring samples, and after handling filters.
   e. After putting on new gloves, do not touch anything before handling filter funnels, samples, filters, or reaching into bags containing sterile supplies.
   f. Pour samples into filter funnels slowly, taking care not to splash.
   g. Have two garbage bags set up: one for trash, the other for contaminated supplies that will be cleaned and reused.

6. Additional water filtering tips are as follows:

a. The amount of sample water that will be filtered will depend on the turbidity of the water. Ideally, the entire 1 L will be filtered, but that is occasionally not feasible. For most projects, we limit filtering time to 30–60 minutes per sample and strive for a minimum of 500 mL filtered.
b. If the water is clearly turbid (tea colored), start with 125 mL and see if it filters through. If necessary, split the sample across two filters. You can either place the filters in the same tube if you can fit them or into two separate 1.5–2 mL tubes and record that the volume was split between different filters.
c. If filters break during vacuuming, handle as normal and document in a lab notebook.
d. If a filter is damaged when you set it up (before you add water), discard and use an intact filter.
e. While filtering, water may be dripping from the bottom of the filter funnel cups. This means that the filter funnel cup is not tightly sealed, but these leaks generally do not cause any problems. Place towels along the manifold to soak up the water and continue filtering.
f. Sample filters preserved in CTAB can be stored at room temperature up until shipment to an eDNA lab. Samples should be shipped within 1–2 weeks to a laboratory for eDNA analysis.

4.5 Example Soil Sampling Protocol

4.5.1 Before Soil Sampling

1. Prepare the negative field controls:

   a. If targeting a single species of interest (e.g., via qPCR), negative field controls for each site can consist of a tube with soil collected outside the range of the target species, soil that has been sterilized, or tubes containing the lysis buffer used for eDNA extraction (no soil). For eDNA metabarcoding, a tube with sterilized soil or a lysis buffer for each site is preferred.
   b. Fill the 50 mL centrifuge tubes (one for each site) with soil from outside the range of the target species (e.g., someone's backyard), sterilized soil, or a lysis buffer.
   c. Put on gloves, add labeling tape (e.g., red for control) to a tube and label with unique sample ID (e.g., SITE-YYYYMonDD-B), add soil (or buffer) to the 25 mL mark on the tube, secure the lid on the tube, and place in sealed Ziploc bags.

d. After preparing the negative field controls, make sure they are stored in a cooler (with ice, ice packs, or dry ice) or in a lab-grade freezer (−20°C or −80°C, with manual defrost).
e. Note: the field controls must be taken into the field even though they are prefilled in the lab.

2. Prepare the soil sample collection tubes:
   a. Put on gloves, add labeling tape (e.g., blue for sample) to empty 50 mL centrifuge tubes, label each with a unique sample ID (e.g., SITE-YYYYMonDD-Rep#), and place in sealed Ziploc bags.
   b. Pack plenty of extra, unlabeled tubes in case they are needed.
3. Fill the cooler(s) with ice packs (or dry ice packs) and add the Ziploc bag(s) containing the empty sample tubes and field controls for each site that will be sampled.
4. Confirm that all supplies are prepared, in working condition, and packed using a field supplies checklist list like the example provided in Table 3.

Table 3. Example field supplies checklist for soil sampling.

☐ Coolers and ice to store field samples (2 minimum)	☐ Soil analyzer, cables, sensors, batteries, manual (e.g., RS-TRREC-N01-1, renkeer.com)
☐ Empty 50 mL tubes (e.g., fishersci.com, Cat. No. 50-189-7796)	☐ Weather meter, cables, batteries, manual (e.g., Kestrel 5500, kestrelinstruments.com)
☐ Pencils	☐ Nitrile disposable lab gloves
☐ Ziploc bags, 1–2 gal.	☐ Laptop with cables and charger
☐ Lab tape (e.g., red and blue)	☐ Cellphone and charger
☐ Sharpies	☐ GPS unit and extra batteries
☐ Paper towels	☐ Car charging adaptors
☐ Data sheets and clipboards	☐ ID/Common access card
☐ Storage bins to keep field equipment clean and organized	☐ Large garbage/contractor bags for trash and contaminated supplies
☐ Data sheets and clipboards	☐ Styrofoam cooler with ice (or dry ice) packs for shipping sample

4.5.2 At the Field Sampling Site

1. At each sampling location, carefully collect soil with gloved hands (Figure 5) or a sterilized tool (e.g., soil core, shovel, or scoop) and fill a sample collection tube to 50 mL. Note: depending on the study goals, there are likely to be specific requirements for soil sample collection. For example, in

Figure 5 the surface soil is being carefully removed to access and sample soil inside a gopher mound entrance hole, which would be more protected from UV, high temperatures, and more likely to come into contact with the target species, Louisiana pine snake.

Figure 5. Example of soil collection. The surface soil is being carefully removed to access and sample soil inside a gopher mound entrance hole.

2. Collect at least three sample replicates for each site.
3. At each site, open the field control tube and expose it to air for 10–30 seconds.
4. Place the sample replicates and field control together in a Ziploc bag labeled with the site ID (e.g., SITE-YYYYMonDD).
5. Limit the samples' exposure to sunlight and high temperatures by storing them in a dark cooler with ice immediately after collection.
6. Record all the site and sample metadata before sampling the next site. See Appendix C for an example of the minimum required sample metadata.
7. Store the soil samples in a cooler on ice or in a lab-grade freezer (with manual defrost) until ready to ship to a laboratory for eDNA analysis.

4.5.3 Tips for Avoiding Contamination when Sampling Soil

1. Always put on new gloves before collecting soil, do not touch anything except soil and the tube it is going into, and immediately after, remove and dispose gloves in a trash bag.
2. When collecting soil, if possible, have someone else (wearing clean gloves) hold the tube, secure the cap, place the sample into a Ziploc bag, and label the Ziploc bag. This will speed up the process and reduce the likelihood of accidentally fumbling and dropping sample tubes before, during, or after collection.

4.6 Sample Preservation and Storage

1. In the field, water samples should be preserved on ice and stored in a dark cooler.
2. After filtering, water sample filters can be preserved in CTAB buffer and stored at room temperature for up to 14 days (30 days max), after which they can be stored in a lab-grade freezer (−20°C or −80°C, with manual defrost) until eDNA extraction.
3. Soil, fecal, or surface swab samples can be stored in a laboratory-grade freezer (preferred) or at room temperature with desiccants (e.g., silica beads) or buffer preservatives (e.g., CTAB) until eDNA extraction.
4. Note that many freezers (especially those in hotel rooms) are not adequate for eDNA preservation because the high temperatures and frequent temperature fluctuations caused by autodefrost cycles can degrade eDNA. Therefore, if a laboratory-grade freezer (with manual defrost) is not available, we recommend using a large cooler, with ice changed daily, to store samples in the hotel room, lab space, or field station for the duration of the field work.

4.7 Shipping Samples to a Lab for Downstream Analysis

1. Samples, organized in freezer boxes, should be carefully packed into a cooler with ice packs (or dry ice packs) and sufficient padding (e.g., paper towels or packing peanuts), sealed, and shipped overnight to eDNA lab partners.
2. It is recommended that samples are shipped early in the week to ensure they arrive Mon–Fri, rather than over the weekend, so that someone will be on-site to receive them and store them appropriately.

3. Write on the cooler that it contains frozen samples so that shipping and receiving personnel will know to expedite the delivery to the lab.
4. Inform eDNA lab partners that the samples have been shipped via email. Include the tracking number and an Excel spreadsheet listing all samples, including any associated metadata.

5 Laboratory Considerations and Recommendations for eDNA Extractions

1. eDNA needs to be isolated from environmental samples and purified prior to subsequent laboratory analysis.
2. Extractions should be performed in rooms that have not been exposed to high-copy DNA.
3. Physical separation of the workspaces for different stages of eDNA analysis should be maintained. For example, (1) a dedicated clean room for working with low-copy DNA (e.g., only environmental samples and eDNA extracts), equipped with UV lights and a laminar flow hood with HEPA filters for performing eDNA extractions and setting up PCR reactions, (2) a separate room for high-copy DNA (e.g., only PCR products, tissue DNA extracts, and synthetic DNA standards) where all post-PCR activities (e.g., running gels, bead cleanups, etc.) will be performed, and (3) a room to store and process field supplies that is separate from wet lab areas
4. Equipment should not be shared between rooms.
5. Gloves should be worn and changed frequently to prevent contamination.
6. Negative controls should be implemented during each step of eDNA analysis (i.e., sample collection, extraction, PCR, and sequencing).
7. Extraction methods should be carefully considered given sample types and budget. For example,
    a. Phenol chloroform isoamyl-alcohol (PCI) DNA extraction (Renshaw et al. 2015) is relatively cheap and simple and excels at removing PCR inhibitors without sacrificing DNA yield, but it requires a chemical fume hood.
    b. Mu-DNA is a do-it-yourself, universal DNA extraction protocol that can be applied to most environmental sample types (e.g., water, soil, or feces) (Sellers et al. 2018). Although extremely cost effective, it requires users to purchase all reagents independently and make their own solutions.
    c. DNA extraction kits can be purchased from a variety of commercial vendors (e.g., Qiagen). These kits include all premade solutions and supplies required for DNA extraction protocols that are usually very simple to follow. However, they can vary in quality (depending on vendor) and can be relatively expensive.

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Appendix A: A Selection of Recent Environmental DNA (eDNA) Literature

A.1 General Reviews and Guides

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A.4 Assay Validation Guidelines

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Appendix B: Example Field Supplies Packing Checklist for Water Sampling

Water sampling supplies	*Miscellaneous field supplies*
☐ Coolers (2 minimum)	☐ Water meter, cables, sensors, batteries, manual (e.g., ProDSS, ysi.com, SKU 626870-1)
☐ Nitrile disposable lab gloves (2 boxes minimum)	
☐ 1 L plastic bottles, sterile (4 per site minimum) (e.g., fishersci.com, Cat. No. 02-912-324)	☐ Weather meter, cables, batteries, manual (e.g., Kestrel 5500, kestrelinstruments.com)
☐ Field notebook	☐ Laptop with cables and charger
☐ Pencils	☐ Cellphone and charger
☐ Ziploc bags, 1–2 gal. (4 per site minimum)	☐ GPS unit and extra batteries
☐ Lab tape (2 colors, e.g., red sample, blue control)	☐ Car charging adaptors
☐ Sharpies, black or blue	☐ ID/Common access card
☐ Paper towels (4 rolls/boxes minimum)	☐ Large garbage/contractor bags for trash
☐ Waders, boots	☐ Storage bins to keep field equipment clean
☐ Distilled water (1 L per site minimum)	☐ Data sheets and clipboards
Water filtering supplies	
☐ Filter funnels, with filters preloaded (4 per site minimum) (e.g., Nalgene single use analytical filter funnels, fishersci.com, Cat. No. 09-740-30K) (e.g., Whatman nitrocellulose filters [0.8 μM, 47 mm], sigmaaldrich.com, SKU WHA7188004)	☐ Plastic tubes (1.5–2 mL), sterile, gasket screw cap, preloaded with 900 µl CTAB (4 per site minimum) (e.g., Axygen self-standing screw cap tubes [2 mL], fishersci.com, Cat. No. 14-222-626) (e.g., Promega CTAB buffer [100 mL], fishersci.com, Cat. No. PRMC1411)
☐ Nitrile disposable lab gloves (2 boxes minimum)	
☐ Forceps, sterile (4 per site minimum) (e.g., fishersci.com, Cat. No. NC0256707)	☐ Freezer boxes, preloaded w/ tubes & 1 empty (e.g., fishersci.com, Cat. No. 03-395-464)
☐ Vacuum manifold with silicone stoppers and plastic funnel connectors (e.g., MultiVac 610-MS, sterlitech.com, SKU 180610-01)	☐ Bleach (1 gal. minimum)
	☐ Plastic bin to bleach-sterilize equipment if needed
☐ Vacuum waste container (4 L) (e.g., PP waste bottle 4000mL, sterlitech.com, SKU 197200-53)	☐ Spray bottle for bleach (20% solution)
	☐ Tube Rack to hold 2 mL vials (e.g., fishersci.com, Cat. No. 21-402-18)
☐ Connective vacuum filter tubing (3/8 in.)	☐ Lab coats
☐ Vacuum pump (e.g., Rocker 400, sterlitech.com, SKU 16700	☐ Tough tags/tube sticker labels (2 colors, e.g., red sample, blue control)
☐ Extension cord for vacuum pump	☐ Lab markers, fine tip, alcohol-proof
☐ Paper towels (4 rolls/boxes minimum)	☐ Large garbage/contractor bags for used supplies

Pre-trip supplies check
☐ Water filtration setup (i.e., pump, manifold, tubing, waste container, extension cord) performs as expected.
☐ GPS unit/mobile devices are in working condition, have been preloaded with relevant maps, site IDs, and site coordinates, and packed with batteries, cables, and chargers.
☐ Weather and water-quality handheld meters have been properly calibrated, perform as expected, and packed with cables, batteries, sensors, and manuals.
☐ All reusable supplies (e.g., bottles, forceps, filter funnels) are bleach-sterilized and sealed in Ziploc bags.
☐ All plastic 2 mL tubes (at least 4/site) have been preloaded with 900 µl CTAB, prelabeled with sample ID (e.g., on colored tough tags; blue for samples, red for negative controls) and stored in freezer boxes.
☐ All filter funnels are clean, preloaded with 0.8 µM cellulose nitrate filters and stored in sealed Ziploc bags.
☐ Data sheets & protocols printed on water-resistant paper (e.g., rite-in-the-rain) and packed with clipboards.

Appendix C: Example Field Site Datasheet with Metadata for Water eDNA Sampling and Filtering

Unique Site ID (SiteID-YYYYMonDD)	LR12-2022May06
Collector(s)	Names and contact info
Collection date	2022Jun06
Collection time	16:00
State	Texas
County	Milam County
Location	SE of Cameron, Rt. 77
Waterbody	Little River
Coordinates	30.835669, -96.947474
Sample type	water
Sample description	clear (other examples: yellow, brown, murky, full of sediment or algae)
Sampling depth (m)	surface
Sample volume (mL)	1,000
Number of sample replicates	3 + control
Sample IDs (SiteID-YYYYMonDD-Rep#)	LR12-2022May06-L, -M, -R, -B
Control type	negative control, distilled water
Preservation method	stored in dark cooler on ice until filtered
Additional fields for environmental data (optional)	Can include additional data fields to record water (e.g., temp, pH, flow, etc.), weather (e.g., solar radiation, air temp., precipitation, etc.), or habitat (e.g., % plant cover, dominant plant species, erosion, disturbance, etc.) information at each sampling site.
Field sampling notes and observations	e.g., heavy storm prior to sampling, light rain during sampling, target species observed or evidence of recent presence, other species observed, forgot gloves, samples exposed to high temps and direct sunlight for 2 hours, accidentally entered water upstream of sample collection, etc.
Filtering date	2022Jun06
Filtering time	19:00
Filtering method	Rocker 400 vacuum pump
Number of filters used/sample	2 for each sample replicate, 1 for negative control
Total filtered water volume/sample (mL)	500
Filter type	Cellulose nitrate

Unique Site ID (SiteID-YYYYMonDD)	LR12-2022May06
Filter pore size (µM)	0.8 µM
Filter preservation	CTAB, 900 µL, room temp
Water filtering notes and observations	e.g., filter damage observed, sample spillage, potential contamination from splashing sample, forgot to change gloves or decontaminate bottles, possible sample mix-up, unable to process sufficient volume due to filter clogging, etc.

Appendix D: Example Performance Work Statement (PWS) for Contracting Metabarcoding Library Sequencing Services Provided to US Army Corps of Engineers (USACE)

PERFORMANCE WORK STATEMENT (PWS)

DNA Sequencing and Data Interpretation for Environmental DNA Metabarcoding Libraries

1. **INTRODUCTION:** The Engineer Research and Development Center–Construction Engineering Research Laboratory (ERDC-CERL) is leading several projects that use molecular tools, specifically environmental DNA (eDNA), to survey for endangered, at-risk, and invasive species on military lands. ERDC-CERL seeks support for DNA sequencing of DNA metabarcoding samples, to include quality control and data interpretation. ERDC-CERL will be responsible for sample collection and preparation of sequence libraries. The Contractor shall provide determination of optimal sequencing strategy/platform, quality control of sequenced libraries, DNA sequencing and demultiplexing of sequence libraries, and data interpretation and trouble shooting.

2. **OBJECTIVE:** The objective of this effort is to derive DNA sequence data for a suite of environmental DNA projects that survey for endangered, at-risk, and invasive species on military lands. The Contractor shall participate in study design, quality control, DNA sequencing, and data interpretation. The resulting data will be incorporated into reporting to installation and DoD sponsors regarding distribution of focal species on military installations.

3. **MAJOR REQUIREMENTS:** To accomplish the above objective, the Contractor shall complete the following tasks:

   a. **Task 1. Determination of optimal sequencing strategy.** The Contractor shall determine the optimal sequencing strategies needed to meet ERDC-CERL project objectives. Any information pertaining to sequence libraries (e.g., amplicon length, DNA concentration, target loci, indexes, preparation method, sample-size) needed for the Contractor to complete this task shall be provided by ERDC-CERL. Planning-level sequencing strategies shall be discussed and decided via e-mail, phone, and/or in-person meetings between the Contractor and ERDC-CERL.

   b. **Task 2. Quality check of prepared sequence libraries.** The Contractor shall perform a quality check for each library provided prior to DNA sequencing. Quality checks shall include a fragment analysis and qPCR to validate fragment length distribution and Illumina adaptors, respectively. The Contractor shall provide a report detailing the results of the quality check to ERDC-CERL prior to DNA sequencing. Any abnormalities identified in quality-check report and subsequent changes to sequencing strategies shall be discussed via e-mail, phone, and/or in-person meetings between the Contractor and ERDC-CERL.

   c. **Task 3. DNA sequencing and demultiplexing of validated sequence libraries.** After quality check validation (Task 2), the Contractor shall perform DNA sequencing on validated libraries. Four independent sequencing runs shall be performed with the Illumina NovaSeq 6000 platform using the SP flow cell with 2×150 bp ($n = 3$) and 2×250 bp ($n = 1$) reagent chemistry. All resulting sequencing reads shall be demultiplexed by the Contractor and provided to ERDC-CERL along with a report detailing sequencing results (general statistics, read counts, quality scores, duplication, read lengths, adaptor content, etc.).

d. **Task 4. Data interpretation and troubleshooting solutions for DNA sequence products.** The Contractor shall provide an interpretation and/or potential explanation of any suboptimal sequence products. Any issues regarding sequence products and potential troubleshooting solutions shall be discussed via e-mail, phone, and/or in-person meetings between the Contractor and ERDC-CERL.

4. **GOVERNMENT-FURNISHED SUPPORT/INFORMATION/PROPERTY**: The government will provide sequence libraries that were constructed for previously collected environmental/biological samples. Government project team members will be available to discuss optimal sequence strategies and data interpretation.

5. **CONTRACTING OFFICER'S REPRESENTATIVE (COR):** The ERDC CERL COR for this task order will be appointed in accordance with DFARS 252.201-7000, and a copy of the designation letter will be provided to the Contractor. Although other Government personnel may clarify technical points or supply relevant information to the Contractor regarding this task order, only a Contracting Officer has the authority to revise any requirements in these specifications, including those resulting from verbal clarification. Only a warranted Contracting Officer (either a Procuring Contracting Officer [PCO], or an Administrative Contracting Officer [ACO]), acting within their delegated limits, has the authority to issue modifications or otherwise change the terms and conditions of this contract. If an individual other than the Contracting Officer attempts to make changes to the terms and conditions of this contract, you shall not proceed with the change and shall immediately notify the Contracting Officer.

   The Government and the Contractor understand and agree that the services to be delivered under this contract by the Contractor to the Government are nonpersonal services, and the parties recognize and agree that no employer-employee or master-servant relationship exists or will exist under the contract between the Government and the Contractor's employees.

6. **MEETINGS AND REVIEWS:** The contractor shall attend the following:

   a. A kickoff meeting, either in person or via telephone, shall be held no later than 30 working days after award.
   b. Speak by telephone with the ERDC-CERL Contracting Officer's Representative (COR) as necessary, at mutually agreeable times and dates, to discuss progress to date and anticipated problems and to ensure that the Contractor's work is compatible with ERDC-CERL work on other aspects of the project. Periodic progress meetings via telephone shall be scheduled, based on sample delivery schedule, throughout the contract performance period.

7. **TRAVEL REQUIREMENTS**: N/A

8. **REPORTS/DELIVERABLES**: The Contractor shall submit the following reports/deliverables to the ERDC-CERL COR, or to the required reporting system specified, within the time specified:

   a. Status Reports–Reports describing progress on the project, results obtained, and lessons learned shall be submitted to the COR via email within 30 days of completion of DNA sequencing for each sequence library (anticipate a total of 4 sequencing runs).

      Invoices for partial payment shall be submitted to coincide with submission of the status reports, and a copy shall be included as an attachment in WAWF (see Section G). No partial payment will be approved unless the government has received all status reports which are due.

   b. A final report documenting the results of all work performed under this award shall be submitted to the COR at the end of the period of service. This report shall contain DNA sequencing and demultiplexing results and data interpretation. The report shall document all sequencing issues identified and potential explanation of any suboptimal sequence products.

a. DD Form 882: The Contractor shall submit DD Form 882(s) in accordance with Section I, Clause No. 252.227-7039, "Patents–Reporting of Subject Inventions" of the basic contract. If the period of service of this task order is not in excess of eighteen (18) months, an interim DD Form 882 is not required.

b. Accounting for Contractor Services: The contractor shall report ALL contractor labor hours (including subcontractor labor hours) required for performance of services provided under this contract for the US Army via a secure data collection site. The contractor is required to completely fill in all required data fields using the following web address: https://www.ecmra/mil.

   Reporting inputs will be for the labor executed during the period of performance during each Government fiscal year (FY), which runs from October 1 through September 30. While inputs may be reported any time during the FY, all data shall be reported no later than October 31 of each calendar year, beginning with 2016. Contractors may direct questions to the help desk at http://www.ecmra.mil/. The Contractor shall provide this input not later than the end of the period of service. However, if the period of service for this task order expands over more than one Government fiscal year, it shall be provided by October 31 of the calendar year for each fiscal year of performance as well as at the end of the period of service.

CMRA DATA	
UIC	W2R205
FSC	Enter applicable FSC Code
Contract Number	Complete with the award number listed in Block 2 of the Standard Form 26 or 33
Contract Fiscal Year	2014
Form of Contract Action	BPA Order under Federal Schedule BPAs and BPA Calls Definitive Contract Definitive Contract (IDC) Definitive Contract (Non IDC) Order under Indefinite Delivery Contract
Contract Type	Cost Cost Plus Award Fee Cost Plus Fixed Fee Cost Plus Incentive Fee Cost Sharing Firm Fixed Price Firm Fixed Price–Level of Effort Fixed Price Ceiling–Retro Price Redetermin. Fixed Price Award Fee Fixed Price Incentive Fixed Price with EPA Fixed Price with Prosp. Price Redetermin. FP-EPA for Telecom Labor Hours Letter No Cost Time and Materials

CMRA DATA	
PBSA	Yes or No
Non-DoD Purchasing Agency	(leave blank)
Competed Action	Competed under SAT Competitive Delivery Order Follow on the Competed Action Full and Open Competition Full and Open Comp. After Excl. of Sources Noncompetitive Delivery Order Not Available for Competition Not Competed Not Competed under SAT
If Competed, number of offerors	2 (enter correct number)
Small Business	Yes or No
Small Disadvantaged Business	Yes or No
Women-Owned Small Business	Yes or No
DoD Administering Component	USA-USACE CERL, Champaign
Contracting Organization	USA-USACE CERL, Champaign

9. SCHEDULE:

10. QUALIFICATIONS: The contractor shall be an Illumina-Certified Service Provider and have access to Illumina NovaSeq 6000 for ultrahigh throughput sequencing.

11. ANTI-TERRORISM/OPERATIONAL SECURITY:

12. PERIOD OF SERVICE: All work to be performed shall be completed no later than twelve (12) months after award.

Abbreviations

CERL	Construction Engineering Research Laboratory
CRISPER	Clustered regularly interspaced short palindromic repeats
CTAB	Cetyltrimethylammonium bromide
ddPCR	Droplet digital polymerase chain reaction
eDNA	Environmental DNA
ERDC	Engineer Research and Development Center
eRNA	Environmental RNA
ESA	Endangered Species Act
LAMP	Loop-mediated isothermal amplification
PCI	Phenol chloroform isoamyl-alcohol
PCR	Polymerase chain reaction
PWS	Performance work statement
qPCR	Quantitative polymerase chain reaction
TES	Threatened and endangered species
USACE	US Army Corps of Engineers

REPORT DOCUMENTATION PAGE

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED	
		START DATE	END DATE
March 2024	Final Report	FY20	FY23

4. TITLE AND SUBTITLE
Environmental DNA Sampling for At-Risk and Invasive Species Management on Military Ranges: Guidelines and Protocols for Installation Biologists and Land Managers

5a. CONTRACT NUMBER	5b. GRANT NUMBER	5c. PROGRAM ELEMENT
5d. PROJECT NUMBER	**5e. TASK NUMBER**	**5f. WORK UNIT NUMBER**

6. AUTHOR(S)
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2902 Newmark Drive
Champaign, IL 61822

8. PERFORMING ORGANIZATION REPORT NUMBER
ERDC/CERL SR-24-1

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Headquarters, US Army Corps of Engineers
Washington, DC 20314-1000

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Distribution Statement A. Approved for public release: distribution is unlimited.

13. SUPPLEMENTARY NOTES
National Defense Center for Energy and the Environment (NDCEE), Project Number 20-10146, Environmental DNA Surveillance of Threatened/Endangered Species on Military Ranges

14. ABSTRACT
Environmental DNA (eDNA) analysis, or the detection of trace DNA shed by organisms into their environment, has the potential to transform Army capabilities for threatened and endangered species (TES) and invasive species management by providing a rapid, noninvasive, and cost-effective option for monitoring wildlife. Despite these benefits, eDNA analysis is underutilized on military installations as limited access to guidance materials, protocols, training opportunities, and support from eDNA scientists makes it difficult for installation biologists and military land managers to design and execute eDNA surveys, let alone identify management questions that may benefit from eDNA monitoring. Therefore, the aim of this resource is to increase awareness of the benefits and limitations of eDNA monitoring and provide eDNA study design guidelines and field sampling protocols for nonexperts to make this tool more accessible to installation biologists and land managers and help facilitate the adoption of eDNA-based approaches for wildlife management on military ranges.

15. SUBJECT TERMS
DNA--Analysis; DNA--Sampling; Environmental monitoring; Endangered species--Monitoring; Environmental management; Military bases

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES
a. REPORT	b. ABSTRACT	C. THIS PAGE	SAR	56
Unclassified	Unclassified	Unclassified		

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