FINAL REPORT

Environmental DNA as a Tool for Inventory and Monitoring of Aquatic Vertebrates

ESTCP Project RC-201204

JULY 2017

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ESTCP

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
24-12-2016	Final Report	03/06/2012 – 12/24/2016

4. TITLE AND SUBTITLE

Environmental DNA as a Tool for Inventory and Monitoring of Aquatic Vertebrates

5a. CONTRACT NUMBER
14-C-0049

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Goldberg, Caren S.
Strickler, Katherine M.
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5d. PROJECT NUMBER
RC-201204

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Washington State Univ., 1228 Webster Hall, Pullman WA 99124

8. PERFORMING ORGANIZATION REPORT

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

Environmental Security Technology Certification Program
4800 Mark Center Drive, Suite 17D03
Alexandria, VA 22350-3605

10. SPONSOR/MONITOR'S ACRONYM(S)
ESTCP

11. SPONSOR/MONITOR'S REPORT NUMBER(S)
RC-201204

12. DISTRIBUTION / AVAILABILITY STATEMENT

Distribution Unlimited; public release.

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The project demonstrated the effectiveness of environmental DNA techniques for monitoring sensitive aquatic vertebrate species and their invasive threats at Department of Defense (DoD) installations. We developed and validated eDNA sampling protocols for a variety of aquatic species, including frogs, salamanders, fish, and disease-causing pathogens. Working with field crews at Eglin AFB, Florida, Fort Huachuca, Arizona, and Yakima Training Center, WA, we compared the results of our eDNA protocol to traditional field sampling with respect to sensitivity, detection probabilities, and cost efficiency. Compared to field surveys, eDNA sampling generally resulted in higher probability of detecting species when the species were present. Environmental DNA sampling for Sonora tiger salamander and flatwoods salamander had higher costs than field surveys in the initial year of test development for each species, but lower costs for ongoing sampling in subsequent years.

15. SUBJECT TERMS
Environmental DNA, species detection, monitoring, genetics, amphibians, fish, amphibian disease, aquatic invasive species, aquatic species at risk

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
Final Report				176	509-335-8689

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Project: RC-201204

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ACRONYMS AND ABBREVIATIONS

AZGFD	Arizona Department of Game and Fish
AFB	Air Force Base
AMBI	*Ambystoma bishopi*; reticulated flatwoods salamander
AMTI	*Ambostyma tigrinum*; tiger salamander
ATV	Ambystoma tigrinum virus
Bd	Batrachochytrium dendrobatidis
CI	Confidence interval
CRITFC	Columbia River Inter-Tribal Fish Commission
DoD	Department of Defense
eDNA	Environmental DNA
ENRD	Environmental and Natural Resource Division
ERDC	US Army Corp of Engineers Engineer Research and Development Center
ESA	Endangered Species Act
ESU	Evolutionarily Significant Unit
IDFG	Idaho Department of Fish and Game
INRMP	Integrated Natural Resource Management Plan
LICA	*Lithobates catesbeianus*; American bullfrog
MCO	Marine Corps Order
NMFS	National Marine Fisheries Service
NOAA	National Oceanic and Atmospheric Administration
ONMY	*Oncorhynchus mykiss*; rainbow trout
ONTW	*Oncorhynchus tshawytscha*; Chinook salmon
PCR	Polymerase chain reaction
PES	Polyethersulfone
PSOR	*Pseudacris ornata*; ornate chorus frog
qPCR	Quantitative polymerase chain reaction
RACH	*Rana chiricahuensis*; Chiricahua leopard frog
SACO	*Salvelinus confluentus*; bull trout
SAFO	*Salvelinus fontinalis*; brook trout
SNP	Single nucleotide polymorphism

TAGR	*Taricha granulosa*; rough-skinned newt
UI	University of Idaho
USAF	United States Air Force
USFWS	United States Fish and Wildlife Service
USGS	United States Geological Survey
UV	Ultraviolet
WDFW	Washington Department of Fish and Wildlife
WSU	Washington State University
YTC	Yakima Training Center

ACKNOWLEDGEMENTS

This demonstration was funded by the DoD Environmental Security Technology Certification Program (ESTCP). We thank Dr. John Hall, former ESTCP Resource Conservation and Climate Change Program Manager, for his guidance and support during this project, and SERDP-ESTCP program staff (Cara Patton, Kristen Lau, Stephanie Lawless, Andrew Blackmore, and others) for facilitating administration of the project.

This project was a collaborative effort among the principal investigators, installations, universities, and agencies. We are grateful to the following individuals for valuable information, data, feedback, coordination, and field sampling support:

Eglin AFB Jackson Guard:
Bruce Hagedorn Kathy Gault

Fort Huachuca ENRD:
Sheridan Stone Debbie Brewer

Yakima Training Center ENRD:
Colin Leingang Jennifer Lannoye

Arizona Game and Fish Department:
Tom Jones Mike Sredl Jeff Sorenson
Christina Akins Abigail King

Virginia Tech:
Carola Haas Tom Gorman Kelly Jones
Brandon Rincon

Florida Fish and Wildlife Department:
Paul Moler Kevin Enge

Washington Department of Fish and Wildlife:
Eric Anderson

U.S. Fish and Wildlife Service:
Cat Crawford Judy Neibauer Jeff Krupka

Mid-Columbia Fisheries Enhancement Group:
Cassandra Weekes Garrett Brenden

Yakima Basin Fish and Wildlife Recovery Board:
Alex Conley Yuki Reiss

Columbia River Inter-Tribal Fish Commission:
Shawn Narum

Idaho Fish and Game Department:

Joel Sauder	Brett Bowersox	Rob Ryan

Caldwell Design:

Dennis Caldwell

The Nature Conservancy:

Brooke Lebow

University of Idaho:

Lisette Waits	Jennifer Adams	Bill Seybold
Scott Williams	Andy Gygli	Kevin Lewallen

Washington State University:

Kody Cochrell	Josh Morman	Matt Smith
Mallory Bedwell	Hanna Davis	

Sample analysis for this project was conducted in the Goldberg eDNA laboratory at Washington State University and the Laboratory for Ecological, Evolutionary and Conservation Genetics at the University of Idaho. Laboratory equipment at the LEECG was supported in part by the U. S. Army Research Laboratory and the U. S. Army Research Office under contract/grant number W911NF1310266.

EXECUTIVE SUMMARY

Six threatened or endangered amphibian species and 23 threatened or endangered fish species are known to occur on Department of Defense (DoD) lands, creating potential constraints on readiness training and resource management. These and other aquatic species pose unique challenges for inventory and monitoring due to the difficulty of thoroughly surveying aquatic environments. Current technologies for detection include dip nets, seining, electrofishing, audio surveys, and visual surveys, which all can have low detection probabilities and/or require high inputs of field time of trained technicians and potentially disturbance to the aquatic environment. An alternative to conventional survey methods is a recently developed technique that uses environmental DNA (eDNA) extracted from water to detect the presence of aquatic species. Animals deposit cells with their DNA into the environment regularly (through the shedding of skin, mucous, gametes, and excrement). Collection and identification of this eDNA is a highly-efficient, low-impact, low-cost alternative to time-intensive field surveys.

For elusive species, such as many amphibians and fishes, lack of reliable monitoring data can lead to an underestimate of the species' distribution. Accurate information about the locations of aquatic vertebrates is needed to effectively and efficiently manage these populations and their habitat, prevent constraints on military training, and reduce the potential for listing of at-risk species under the Endangered Species Act (ESA). Environmental DNA provides a technique for detecting aquatic species that would improve monitoring programs by lowering costs, reducing impacts to sensitive species, and improving the quality of data produced.

OBJECTIVES OF THE DEMONSTRATION

The overall goal of this project was to produce an efficient, broadly applicable set of protocols for the use of eDNA techniques for monitoring sensitive aquatic vertebrate species and their invasive threats at Department of Defense (DoD) installations. Specifically, we sought to develop and validate eDNA protocols for a variety of aquatic target species (both species of concern and invasive species that threaten their persistence) across a range of environmental conditions. This project was a field-scale demonstration of eDNA techniques.

Performance objectives for this project included demonstration of an eDNA sampling and analysis protocol that 1) has a high probability of detecting species when they are present, 2) has a higher probability of detecting rare species than field surveys, 3) can detect amphibian disease when present, 4) minimizes the probability of contamination, 5) is cost-effective, 6) is easy to use by technician-level workers, 7) is applicable to different aquatic species, and 8) reduces permitting requirements for surveys of protected species. An additional performance objective was to demonstrate the ability of an empirical model to predict success of eDNA methods based on correlation of environmental factors with probability of detecting eDNA.

TECHNOLOGY DESCRIPTION

Environmental DNA methods use the trace DNA that all organisms leave in their environments to draw inference to the presence of target species. For aquatic species, a water sample can be collected from waterbodies in which the target species may occur. DNA is then extracted from the sample and analyzed using quantitative polymerase chain reaction (qPCR) to determine whether and how much of the target species' DNA is present in the water sample.

Detection of target species involves two phases: eDNA test development and eDNA test application. Test development requires several steps: 1) identifying the target species for the test; 2) collecting sequence data from either databases or DNA sequencing; 3) creating the test and verifying its specificity and sensitivity. Environmental DNA tests for target species are species-specific, and test development occurs once for every new species to be monitored.

Once the test has been developed, the application phase involves the collection and analysis of water samples. Replicate samples are collected from each monitoring location and analyzed with the validated eDNA qPCR test to detect target species. The resulting data constitute the results for a survey or monitoring program. Replicate samples are used to estimate detection probabilities, which are used as a measure of uncertainty in an occupancy modeling framework. This framework allows researchers to detect changes in occupancy over time, as well as providing detailed data on the probability that the target species was present but not detected at each location. Additionally, covariates of the probability of occupancy and detection can be included to provide more accurate estimates of occupancy. If detection probabilities are not high enough to provide necessary levels of certainty, the results of these models can also be used to adjust sampling design spatially and temporally to maximize detection probability. This latter approach was taken during this demonstration project to improve initial occupancy estimates. We incorporated these findings in sampling recommendations that can be applied across systems, reducing the need for this optimization step in many systems.

DEMONSTRATION RESULTS

We demonstrated environmental DNA methods for detecting aquatic vertebrates and pathogens at three DoD installations that host an array of at-risk aquatic species and invasive threats: Fort Huachuca, Arizona; Eglin Air Force Base, Florida; and Yakima Training Center, Washington. Each site hosts aquatic species of immediate management concern to site managers and represents a different type of aquatic system. This demonstration showed that environmental DNA can be a sensitive and cost-effective technology for monitoring aquatic species under a range of conditions that included factors expected to limit eDNA detection (e.g., low pH). We fully or partially met seven of our eight quantitative and qualitative performance objectives.

Target species detection with eDNA protocols

In the oak woodland and desert grassland wetlands at Fort Huachuca, we developed and applied eDNA protocols for detecting the federally endangered Sonora tiger salamander (*Ambystoma tigrinum stebbinsi*) and federally threatened Chiricahua leopard frog (*Lithobates [Rana] chiricahuensis*). We also developed and applied eDNA tests for threats to these species, including the invasive American bullfrog (*L. catesbeianus*), amphibian pathogenic chytrid fungus (*Batrachochytrium dendrobatidis*; Bd) and iridovirus. We collected water samples for eDNA analysis concurrently with ongoing Arizona Game and Fish Department field surveys for Sonora tiger salamanders and Chiricahua leopard frogs. In the first year of sampling we identified different limiting factors for detection of each species (sample volume for Sonora tiger salamanders, wetland area for Chiricahua leopard frogs, and some evidence of water temperature for American bullfrogs). In the second year, we adjusted sampling protocols to increase sample volume for salamanders and increase the spatial coverage sampling at larger wetlands for leopard frogs. With improved sampling designs, we detected target species at almost all sites where field crews detected them, plus 19 additional detections with eDNA methods only.

Our eDNA tests for ephemeral wetlands at Eglin Air Force Base included federally endangered reticulated flatwoods salamanders (*Ambystoma bishopi*), the amphibian pathogen Bd, and ornate chorus frogs (*Pseudacris ornata*), a Florida Species of Greatest Conservation Need. We teamed with researchers from Virginia Tech to conduct concurrent eDNA water sample collection and field surveys for flatwoods salamanders and ornate chorus frogs. Initial results indicated that pH was the limiting factor for detecting flatwoods salamanders and ornate chorus frogs in Eglin's acidic, spatially complex wetlands. We adjusted our sampling protocol for these species by increasing sample volume and, at sites with pH < 5, sampled more locations within the wetland. With the adjusted protocol we detected flatwoods salamanders and ornate chorus frogs at almost all sites with field detections and two additional sites where field crews did not detect them, but did not detect target species at two sites in which field crews found them at very low densities.

In streams on and near Yakima Training Center, our eDNA tests included the federally threatened bull trout (*Salvelinus confluentus*), its non-native threat brook trout (*Salvelinus fontinalis*), and Chinook salmon (*Oncorhynchus tshawytcha*). For samples in which Chinook salmon were detected, we applied additional tests targeting single nucleotide polymorphisms to determine the probability that the Chinook salmon present were from the federally endangered Upper Columbia Spring Chinook salmon evolutionarily significant unit (ESU), rather than the non-listed Upper Columbia Summer-/Fall-run Chinook Salmon ESU. We detected bull trout in all sites at which field crews detected the species. Our eDNA tests were successful for detecting brook trout in five sites, but because no field surveys were conducted during the demonstration, we were unable to compare detection probabilities for this species. We detected spring-run Chinook salmon in all but one location where they were thought to be located.

Field sample collection protocol

We developed, tested, and iteratively revised a field protocol for collecting water samples for eDNA analysis. We evaluated the ability of field staff to correctly follow the field protocol using a qualitative survey of experienced and technician-level biologists working in a range of lentic and lotic settings in Idaho, Washington, Florida, Arizona, and New Mexico. The response scores for the final version of the protocol were 4.5 or greater on a 5-point Likert scale for ease of use and versatility, demonstrating that the protocol is easy to follow in field settings and applicable across different types of aquatic systems.

Cost comparison

We successfully demonstrated the cost-effectiveness of eDNA surveys for Sonora tiger salamanders at Fort Huachuca and flatwoods salamanders at Eglin AFB, the only species for which both field costs and field detection probabilities were available. Environmental DNA sampling includes front-end costs for developing and validating qPCR assays for target species in the initial year of sampling, and ongoing costs of collecting and analyzing samples in subsequent sampling periods.

- For Sonora tiger salamanders, costs for the initial season of eDNA sampling, including all front end costs, was $12,774 for a single survey of 20 sites, compared with $5,582 per survey for current seining surveys. One eDNA survey or two seining surveys would be needed each year to achieve a detection probability of ≥0.95. Ongoing surveys for tiger salamanders after the initial year would cost $5,351 per year for eDNA sampling or $11,164 per year for seining surveys.

- For flatwoods salamanders, the initial year of sampling cost $16,092 for one eDNA survey and $4,460 for one survey using current dipnetting methods. One eDNA survey or four dipnet surveys would be needed each year to for detection probabilities ≥0.90. Costs for ongoing flatwoods salamander surveys after the initial year are estimated at $2,843 per year for eDNA sampling or $3,960 per year for dipnet surveys.

IMPLEMENTATION ISSUES

The following technology implementation issues were encountered during this demonstration and may be anticipated to affect future applications of eDNA methods:

- **Regulatory issues –** Environmental DNA sampling requires only collection of water samples, similar to water sampling for other types of monitoring such as water quality parameters. We expected that permits under the Endangered Species Act would not be required for an eDNA-based monitoring program for listed species. However, permitting requirements are species-specific and may vary across regions. Although U.S. Fish and Wildlife Service staff in several states concurred that permits would not be required for eDNA water sampling, we were not able to receive consensus that eDNA surveys would have lower permitting requirements than conventional field surveys for aquatic species.
- **Spatial issues –** The presence of a species' eDNA at a location may not indicate the presence of the species. There are two main examples of this: 1) in streams, eDNA may flow into an installation from an upstream source; 2) eDNA may be deposited from an allochthonous source such as animal movements. Environmental DNA samples should always be collected and analyzed as replicates to be able to understand the strength of evidence for presence of a species at a site. The detection of a large amount of eDNA in a sample is stronger evidence for species presence than one sample with a small amount of eDNA detected of the target species. For some applications (e.g., the detection of aquatic invasive species), several strongly positive eDNA samples may need to be found to trigger management actions if field surveys cannot locate the species.
- **Temporal issues –** Environmental DNA degrades quickly in water and we demonstrated that degradation rates are strongly influenced by temperature, UV radiation, and acidity. The period of time eDNA remains detectable likely ranges from a few days in warmer, sunnier systems to a few weeks in sites with colder water and less UV exposure. The sampling design should target the period in which the species is most likely to be present at the highest density. Because eDNA in sediment can persist up to thousands of years, sediment samples should only be analyzed if the question of interest is whether the target species has ever been present at the site.
- **Procurement issues –** Midway through our demonstration, the filter funnel we used for water collection was discontinued by the manufacturer. We tested other available filters with different combinations of filter pore size and material to see what types of filters would be adequate for capturing eDNA from freshwater samples. In our test, filters made of mixed cellulose ester, cellulose nitrate, or polyethersulfone materials had similar effectiveness at capturing and efficiently filtering eDNA, while polycarbonate track-etched filters had lower eDNA capture and efficiency. Filter pore size did not influence the amount of eDNA captured, but smaller pores were generally associated with longer filtration times, and filters with larger pores are likely to be more efficient in systems where smaller pore size filters are likely to become clogged.

An additional major issue remains for installations wishing to implement targeted eDNA sampling of species. There is still a very limited number of labs offering analysis services for these samples, and no certification program to ensure the quality of the analysis. To address this concern, we developed guidelines for practitioners interested in partnering with laboratories to analyze eDNA samples. Additionally, we worked with eDNA researchers from around the world to develop a set of best practices for eDNA laboratory analyses.

- **Sampling considerations -** We found that spatial sampling design, sample volume, and filter characteristics can have a strong effect on detection probabilities. Practitioners who are implementing eDNA sampling for a novel species or system should consider these factors carefully in determining an appropriate sampling approach. For situations in which eDNA sampling approaches have been successfully demonstrated for similar species and aquatic systems, practitioners can use the sampling design from existing applications to inform sampling, which will likely result in similar detection probabilities. For applications in new types of systems, a pilot survey can help practitioners evaluate whether the eDNA sampling strategy detects the target species with sufficient accuracy and sensitivity to meet survey objectives. Concurrent field sampling can be useful but is not necessary for initiating an eDNA sampling program. This comparison allows managers to select the most efficient and reliable survey method, whether that may be field surveys, eDNA surveys, or an integration of the two methods.

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1.0 INTRODUCTION

1.1 BACKGROUND

Fish and amphibians are among the most imperiled vertebrate taxa, declining more rapidly than birds and mammals, due in part to their dependence on freshwater systems that are often highly modified (Ricciardi and Rasmussen 1999, Stuart et al. 2004). These species pose unique challenges for inventory and monitoring due to the difficulty of thoroughly surveying aquatic environments. Current technologies for detection include dip nets, seining, audio surveys (for amphibians), and visual surveys, which all can have low detection probabilities and/or require high inputs of field time of trained technicians and potentially disturbance to the aquatic environment. Electrofishing can yield high detection probabilities yet requires time, equipment, and trained technicians, and may pose health risks to sensitive species. An alternative to conventional survey methods is a recently developed technique that uses environmental DNA (termed eDNA) extracted from water to detect aquatic species.

Animals deposit cells with their DNA into the environment regularly (through the shedding of skin, mucous, and excrement). Although this DNA is subject to a number of degrading forces, including ultraviolet light, heat, and hydrolysis, recent studies have shown that enough DNA stays suspended in natural water bodies to allow for detection of aquatic species at low densities using DNA analysis of water samples (Ficetola et al. 2008, Goldberg et al. 2011, Jerde et al. 2011, Thomsen et al. 2012). Collection and identification of this eDNA is a highly-efficient, low-impact, low-cost alternative to time-intensive field surveys.

For elusive species, such as many amphibians and fishes, lack of reliable monitoring data can lead to an underestimate of the species' distribution. Accurate information about the locations of aquatic vertebrates is needed to effectively and efficiently manage these populations and their habitat, prevent constraints on military training, and reduce the potential for listing of at-risk species under the Endangered Species Act (ESA). Environmental DNA provides a technique for detecting aquatic species that would improve monitoring programs by lowering costs, reducing impacts to sensitive species, and improving the quality of data produced.

1.2 OBJECTIVE OF THE DEMONSTRATION

The overall goal of this project was to produce an efficient, broadly applicable set of protocols for the use of eDNA techniques for monitoring sensitive aquatic vertebrate species and their invasive threats at Department of Defense (DoD) installations.

The specific objective of the demonstration was to develop and validate eDNA protocols for a variety of aquatic target species (both species of concern and invasive species that threaten their persistence) across a range of environmental conditions. This project was a field-scale demonstration of eDNA techniques. The demonstration included three DoD installations that host an array of at-risk aquatic species and invasive threats: Fort Huachuca, Arizona; Eglin Air Force Base, Florida; and Yakima Training Center, Washington.

Developing an installation-specific protocol for using eDNA to identify species presence involved 1) selecting a codified list of species of management concern (target species), 2) identifying short species-specific DNA sequences (to design primer/probe sets) from established databases, 3) developing quantitative polymerase chain reaction (qPCR) protocols to target and copy DNA for each species, 4) confirming assay sensitivity and specificity (detection of target species and non-detection of co-occurring relatives), and 5) evaluating environmental and ecological variables that could influence detection probabilities to develop sampling protocols.

We compared the results our eDNA protocol to traditional field sampling by demonstrating the following:

- Sensitivity of our eDNA protocol to target species presence and absence
- High detection probabilities for target species
- Robustness of field and laboratory protocol to contamination
- Cost effectiveness
- Usability by field technicians with a minimum of training
- Applicability to a range of aquatic systems

In addition to our primary objective of demonstrating eDNA methods across a range of environmental systems, we also sought to better understand factors that affect eDNA detection. We conducted field and lab experiments with the objectives of 1) quantifying eDNA persistence over time across a range of environmental conditions, 2) describing the transport properties of eDNA in stream ecosystems, and 3) identifying filter types with the greatest eDNA capture efficiency. These experiments are described in Appendices C, D, and E, respectively.

1.3 REGULATORY DRIVERS

At least 22 at-risk amphibian species and over 40 at-risk fish species are known to occur on DoD lands, many of which are difficult to detect using conventional survey methods. The need for accurate information about the abundance and location of aquatic vertebrates is driven primarily by the ESA and the Sikes Act. The ESA requires that DoD and other federal agencies ensure that their actions do not jeopardize the existence of endangered or threatened species, and directs agencies to maintain reliable population data on listed species. The Sikes Act requires military installations to prepare, implement, review, and revise Integrated Natural Resource Management Plans (INRMPs) to provide for conservation of species at risk and other natural resources. Additional DoD guidance related to management of listed species and biological diversity include DoD Instruction 4715.03, Army Regulation 200-3, U.S. Air Force Instruction 32-7064, U.S. Navy Instruction OPNAVINST 5090.1B, and U.S. Marine Corps Order (MCO) P5090.2A.

2.0 TECHNOLOGY/METHODOLOGY DESCRIPTION

2.1 TECHNOLOGY/METHODOLOGY OVERVIEW

2.1.1 Technology description

Animals shed cells with their DNA into the environment regularly (through the shedding of skin, mucous, and excrement). By sampling this shed DNA, we can infer a species' presence in the sampled environment using existing genetic methods. The first study proving that vertebrate species could be detected through eDNA found in water samples focused on the American bullfrog (*Lithobates catesbeianus*), an invasive species in much of the northern hemisphere (Ficetola et al. 2008). This study showed that it is possible to detect even very small populations of vertebrates by analyzing eDNA in water samples. Subsequently, a number of studies have produced similar results over a range of aquatic environments and taxa (Goldberg et al. 2011, Jerde et al. 2011, Thomsen et al. 2012). Collection and identification of this eDNA is a highly-efficient, low-impact, low-cost alternative or complement to time-intensive field surveys. Additionally, sampling of eDNA avoids potential harm of handling and disturbance to habitat of protected and at-risk species.

The process of collecting and analyzing eDNA samples to draw inference to the presence of target aquatic species involves collecting water samples, extracting the DNA from the sample, and conducting quantitative PCR analysis. Field and laboratory protocols have been established to minimize the probability of contamination and maximize the probability that contamination will be detected if it occurs (Goldberg et al. 2016). As part of this technique, negative controls are collected in the field and created at each step of the laboratory analysis (DNA extraction and PCR). Field crews also follow protocols to ensure no contamination from their clothing between field sites (this involves standard decontamination with bleach, as is done by field crews to prevent the spread of aquatic pathogens).

The development of an eDNA protocol for a new species involves several steps: 1) identify the target species for the test; 2) collect sequence data from either databases or DNA sequencing; 3) create and verify the specificity and sensitivity of the test (Figure 2-1). Once the test has been developed, the application involves the collection and analysis of water samples.

During the application phase, replicate samples are collected from each monitoring location and analyzed to detect target species. These data are the basis for the monitoring program, with replicate samples being used to estimate detection probabilities. These probabilities are used as a measure of uncertainty in an occupancy modeling framework (Mackenzie et al. 2002). Replicate samples are used as repeat surveys in the analysis. This framework allows researchers to detect changes in occupancy over time, as well as providing detailed data on the probability that the target species was present but not detected at each location. Additionally, covariates of the probability of occupancy and detection can be included to provide more accurate estimates of occupancy. If detection probabilities are not high enough to provide necessary levels of certainty, the results of these models can also be used to adjust sampling design spatially and temporally to maximize detection probability. This latter approach was taken during this demonstration project to improve initial occupancy estimates. However, many of the findings of this demonstration project have been incorporated in sampling recommendations that can be applied across systems, potentially negating the need for this optimization step in many systems.

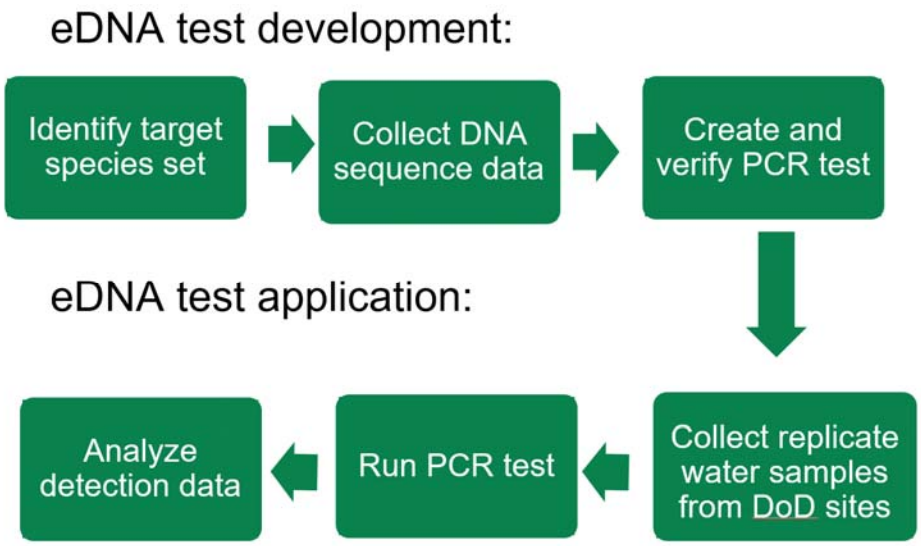

Figure 2-1. Flow Diagram for Application of eDNA Monitoring

Test development occurs once for every new species to be monitored, while test application occurs on a monitoring schedule.

2.1.2 Chronological summary of eDNA technique development

Researchers have been using DNA from extracted feces, urine, hair, feathers, shed skin, and eggshells to detect terrestrial vertebrate species for the past decade (Waits and Paetkau 2005). These low-quantity DNA sources are similar to aquatic eDNA in that they are exposed to heat and light and mixed with non-target DNA as well as soils that may contain PCR inhibitors (Watson and Blackwell 2000). The field of non-invasive genetic sampling has grown quickly and techniques have been developed for purifying DNA samples and analyzing these low-quantity and often low-quality samples. These techniques include both new chemistries (e.g., Qiagen Multiplex PCR mix) and standards for replication to ensure accurate data collection (Taberlet et al. 1999). These techniques are directly applicable to aquatic eDNA projects.

In one of the earliest studies to use aquatic eDNA to detect vertebrate species, Ficetola et al. (2008) found that the invasive American bullfrog could be detected in wetlands, even at low densities (1-2 individuals per km^2 surface area). Following this work, a large study by the Center for Aquatic Conservation developed a test to detect Asian carp (*Hypophthalmichthys molitrix* and *H. nobilis*) in the canals of the Great Lakes system (Jerde et al. 2011). We expanded on this technique by testing multiple DNA extraction and PCR protocols to detect Idaho giant salamanders (*Dicamptodon aterrimus*) and Rocky Mountain tailed frogs (*Ascaphus montanus*) in headwater streams (Goldberg et al. 2011). These studies laid the groundwork necessary for widespread application of aquatic eDNA techniques to detect and monitor species of concern. Shortly after these demonstrations, Thomsen et al. (2012) showed that eDNA detection in water samples is possible for crustaceans, aquatic insects, and aquatic mammals as well as birds and terrestrial mammals.

Since 2012, there has been a large increase in publications using eDNA to detect vertebrates, including several reviews (Barnes and Turner 2015, Bohmann et al. 2014, Rees et al. 2014) and a special issue of Biological Conservation (Goldberg et al. 2015). However, field applications of this approach are still limited, with most studies reporting on development of assays, pilot studies, and aquarium experiments. Important developments for the design of field studies include: the discovery that vertebrate eDNA particles may mostly be in the range of 1–10 µm (Turner et al. 2014), which indicates that larger pore size filters may be used, increasing sample volumes; the localization of eDNA signals in lentic systems (Eichmiller et al. 2014); and the long transport distance of eDNA in a river system (9.1 km; Deiner and Altermatt 2014).

Most early studies of eDNA methods have used targeted PCR to detect one or several species of interest. This approach (with standard PCR replaced by qPCR; Goldberg et al. 2016) continues to be a robust, sensitive tool for detecting target species and is well-suited for species monitoring efforts. Another approach to eDNA surveys involves sequencing all or a phylogenetically targeted (e.g., all fish) set of components of DNA in each sample, which could be used as a biodiversity monitoring tool. This technology, which uses high-throughput sequencing, is at an earlier stage of development and currently cost-prohibitive except in large sample numbers. Additionally, the library of DNA sequences linked with known species of origin is currently not complete enough to identify each species. Unless its sequences were represented in the library, we would not be able to identify which species produced most of the recovered sequences. Therefore, this method is not yet optimal for species monitoring. However, eDNA samples extracted for targeted PCR can be preserved for future use with this high-throughput technology as databases are expanded and costs are reduced.

2.1.3 Expected applications

At least 22 at-risk amphibian species and over 40 at-risk fish species are known to occur on DoD lands, creating potential constraints on training and resource management. The number of invasive aquatic vertebrate species is difficult to estimate, but a number of invasive aquatic species have had demonstrated impacts on DoD operations and natural resources. For rare and elusive species such as many amphibians and fish, lack of reliable monitoring data can lead to an underestimate of the species' patch occupancy and local distribution. Accurate information on the presence of aquatic vertebrates is needed to effectively manage these populations and habitats, prevent constraints on military training, and reduce the potential for unnecessary listing of at-risk species under the ESA.

Environmental DNA can be used to detect any vertebrate species with a significant aquatic stage, but is best applied to species that are difficult to detect using conventional survey methods. The technology could thus be deployed at any DoD site with sensitive or invasive aquatic vertebrate species that are rare or elusive.

With this report, we provide protocols for designing field sampling programs, developing and validating qPCR tests for new systems, and evaluating the quality of laboratory tests, informed by this multi-site demonstration project. The end benefit to military natural resource managers will be a technique for monitoring aquatic vertebrates that allows managers to implement endangered species management plans efficiently, reliably, and with minimal impacts to sensitive species.

For species where sampling is destructive to the target organism, managers may want to replace standard surveys with eDNA surveys; for species that are sometimes non-invasively and easily directly observed, eDNA may better serve as a supplementary technique, with samples collected only from sites where the target species is not detected through other means (e.g., visual surveys).

2.2 ADVANTAGES AND LIMITATIONS OF EDNA TECHNOLOGY

Current technologies for aquatic species detection for population monitoring include field surveys with dip nets, seining, audio surveys (for amphibians), and visual surveys. Each of these survey methods can have low detection probabilities and/or require high inputs of field time of trained technicians and potentially disturbance to the aquatic environment. Although electrofishing can yield high detection probabilities for some species, it requires time, equipment, and trained technicians, and may pose health risks to sensitive species (Snyder 2003). Detection probabilities using eDNA sampling should greatly exceed that of traditional field surveys for rare or secretive species, but likely are equal with higher costs for common or easily-observable species. Detecting species using eDNA avoids potential harm of handling and disturbance to habitat of protected and at-risk species. Endangered species permits should not be required under most circumstances and technicians need to be trained only in collection of eDNA samples. The major cost considerations for current protocol surveys are the payment of trained technicians (which can exceed $100/hour if the surveyor needs to hold a U.S. Fish and Wildlife Service permit for endangered species surveys), transportation to study sites, and field equipment. Protocol surveys for aquatic species can take several hours at a site, require multiple site visits, and require sizable field crews (≥2 technicians).

Limitations of the eDNA approach include the possibility of false positives, which should be negated by appropriate field and lab protocols. Another potential source of error is allochthonous DNA consisting of trace amounts of target species' DNA carried into the sampling area from outside sources (e.g., by predators or scavengers moving carcasses from one water body to another, or consuming the target species in one area and depositing feces in the sampling area). In this case, however, the amount of eDNA detected would be small and inconsistent through time (Goldberg et al. 2016). Replicate sampling through space and time should be employed to increase the strength of evidence that positive eDNA detections indicate species presence when very low or inconsistent positive results are obtained.

Cost may be a limiting factor for implementation of eDNA technology in some cases. The up-front cost of developing an eDNA protocol for a target species can be several thousands of dollars. However, the cost of developing and validating a new protocol is incurred only at the outset of a new eDNA monitoring program. For ongoing monitoring, primary costs include laboratory analysis (currently estimated at $37/sample for two target species, including only labor and consumables), field technician wages and transportation to study sites, and field equipment (about $500). Higher costs associated with laboratory sample analysis may be offset by reduced technician time, smaller field crews, and lower species-specific expertise compared with field surveys. In many cases, replacing or augmenting field sampling with eDNA techniques for rare and elusive species should result in improved detection probabilities. An analysis of the comparative costs of these approaches can be found in Section 7.

3.0 PERFORMANCE OBJECTIVES

Table 3-1. Performance Objectives

Performance Objective	Metric	Data Requirements	Success Criteria	Result
Quantitative Performance Objectives				
1. Demonstrate an eDNA sampling and analysis protocol that has a high probability of detecting species when they are present	Presence/ absence of eDNA in samples	• Replicate eDNA samples at each site • Field surveys for target species	eDNA tests detect species at every site where they are detected through field surveys	• Successful for 3 of 7 target species. • Remaining 4 target species were each not detected at one low-density site.
2. Demonstrate an eDNA sampling and analysis protocol that has a higher probability of detecting rare species than field surveys	Presence/ absence of eDNA in samples	• Replicate eDNA samples at each site • Field surveys for target species	Replicate eDNA samples indicate species presence consistently (at least 3 of 4 samples indicating presence) at some sites when field surveys do not detect the target species	• Successful for 5 of 6 species. • Criteria could not be evaluated for the remaining species due to limited field surveys.
3. Demonstrate an eDNA sampling and analysis protocol that can detect amphibian disease when present	Presence/ absence of eDNA in samples	• Replicate eDNA samples at sites where disease testing occurs • Field surveys of disease status of individuals	eDNA tests detect diseases at all sites where direct sampling of amphibians detects disease	• Successful, pathogens detected at all sites. .
4. Demonstrate an eDNA sampling and analysis protocol that minimizes the probability of contamination	Presence/ absence of eDNA in negative controls	• Field negative controls: eDNA analysis of filter samples taken of clean water before sampling each site • Lab negative controls: negative control run through the extraction process alongside samples, DNA of co-occurring closely-related species	<1% of negative controls test positive using standard disease testing qPCR criteria	• Successful, no detections in negative controls.
5. Demonstrate cost-effectiveness of eDNA approach	Cost-effectiveness of eDNA techniques over traditional field sampling	• Personnel and supply costs for field sampling in relation to detection probability for target species • Personnel and supply costs for eDNA sampling in relation to detection probability for target species	Cost to reach equal detection probabilities for each target species less for eDNA sampling than for traditional field sampling	• Successful for Sonora tiger salamander and flatwoods salamanders. • Data not available for additional species.
6. Demonstrate ability of an empirical model to predict success of eDNA technologies	Correlation of environmental variables with probability of detecting eDNA	• Water pH, temperature, volumetric, and canopy cover data collected with each eDNA sample • eDNA test results from each sample	Validation tests of model predictions have misclassification error rates <20%	• Technically passed success criteria, but models did not accurately predict rare missed detection events.

Table 3-1. Performance Objectives (Continued)

Performance Objective	Metric	Data Requirements	Success Criteria	Result
Qualitative Performance Objectives				
7. Ease of use	Ability of a technician-level individual to follow the field sampling protocol	• Results from qualitative survey of project field technicians on usability of field protocol • Results from qualitative survey of participants in field trials	Responses to qualitative survey of field technicians and field trial participants result in average Likert scores ≥4 out of 5	• Successful for 7 out of 7 items for clarity and 7 out of 8 items for usability.
8. Versatility	Applicability of field sampling protocol in different aquatic systems	• Results from qualitative survey of technician-level individuals on usability of field protocol in forested wetland, desert wetland, and lotic systems	Responses to qualitative survey of technicians result in average Likert scores ≥4 out of 5 in each aquatic system	• Successful in all 3 aquatic systems.
9. Reduce permitting requirements	ESA permit requirements for collection of eDNA water filter samples	• Written confirmation from USFWS Ecological Services staff regarding need for incidental take permits for collection of water filter samples in aquatic systems where listed species occur	Incidental take permits not required for collection of water filter samples for eDNA	• Did not pass success criteria; conversations with USFWS are ongoing.

3.1 DESCRIPTION OF EACH PERFORMANCE OBJECTIVE

1. **Demonstrate an eDNA sampling and analysis protocol that has a high probability of detecting species when they are present.** Success criteria defined for this objective were set at perfect detection when compared to field crews. Like any sampling process, however, eDNA sampling occasionally misses low numbers of individuals when they are present. If an installation's goal is occupancy monitoring, this uncertainty is explicitly accounted for in the occupancy modeling framework. If the goal is perfect detection at all sampled sites, installations will have to increase the number of samples selected at sites, as recommended in Section 8.6.

2. **Demonstrate an eDNA sampling and analysis protocol that has a higher probability of detecting rare species than field surveys.** Success criteria for this objective was set at 0.75 probability of detection per sample. The one species where this was not met had a detection rate of 0.50 per sample at one site, which is still enough to have a high probability of detection for four samples (0.94). Given the sampling recommendations in Section 8.6, we conclude that eDNA sampling has at least as high of a detection probability as standard field sampling.

3. **Demonstrate an eDNA sampling and analysis protocol that can detect amphibian disease when present.** Successful.

4. **Demonstrate an eDNA sampling and analysis protocol that minimizes the probability of contamination.** Successful.

5. **Demonstrate cost-effectiveness of eDNA approach.** Successful.

6. **Demonstrate ability of an empirical model to predict success of eDNA technologies.** Technically met success criteria, but model failed to predict rare missed detections, making it unusable.

7. **Ease of use.** Successful with the exception of the question ranking being able to filter the required volume of water. Details on our experiments to identify filters to accommodate more difficult water samples can be found in Appendix E.

8. **Versatility.** Successful.

9. **Reduce permitting requirements.** We found variable willingness among Ecological Services staff from different regions to confirm that incidental take permits were not needed for eDNA sampling. USFWS staff in the Arizona and Florida state field offices were more sure that permits were not needed than staff in the Washington state field office. We were not able to obtain a blanket statement from the agency regarding this issue, as permitting is generally handled at the regional level and potential impacts may vary across systems.

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4.0 SITE DESCRIPTION

4.1 SITE LOCATION AND HISTORY

We used the following criteria to select suitable sites for successful demonstration of eDNA technology:

- Site had at least one aquatic at-risk species that is difficult to detect using traditional survey methods and/or is sensitive to disturbance and handling;
- Site aquatic system and ecoregional setting was unique relative to other selected demonstration sites; and
- Site natural resource managers were supportive of the technology's implementation.

Using these criteria, we selected three sites: Fort Huachuca, Arizona; Yakima Training Center, Washington; and Eglin Air Force Base (AFB), Florida (Figure 4-1). Each site hosts aquatic species of immediate management concern to site managers and represents a different type of aquatic system. Of particular concern for site selection were the environmental factors influencing eDNA degradation, including water temperature, UV (UV) radiation, and pH (Ravanat et al. 2001). For this reason, sites were situated in different latitudinal, land cover, and water chemistry settings. Our sites included a desert grassland/oak woodland wetland system, an acidic forested wetland system, and a lotic anadromous fish system (Table 4-1).

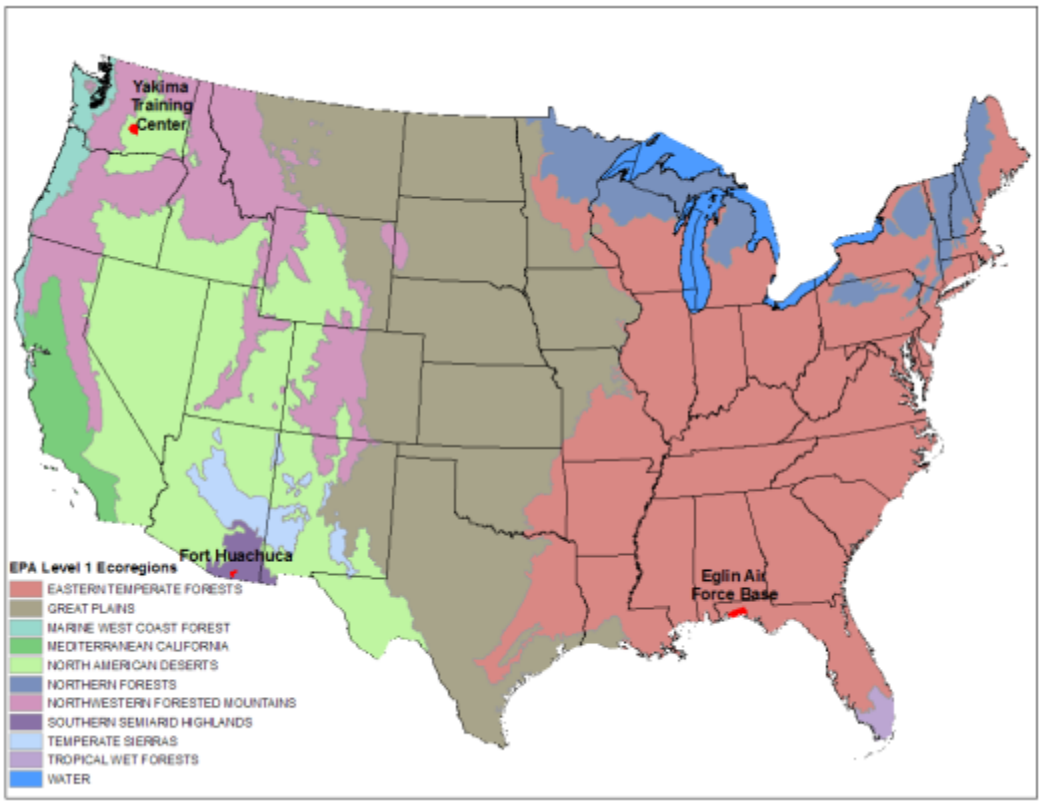

Figure 4-1. Map of Demonstration Sites

Table 4-1. Description of Selected Demonstration Installations

Installation	Ecoregion	Aquatic habitat	Sampling focus
Fort Huachuca, AZ	Southern Semiarid Highlands	Desert grassland and oak woodland wetlands, highly modified	Threatened and endangered amphibians and their invasive threats (vertebrates and disease)
Eglin AFB, FL	Eastern Temperate Forest	Acidic ephemeral forest wetlands	Threatened and sensitive amphibians and their invasive disease threat
Yakima Training Center, WA	North American Deserts	Arid and semi-arid river system	Threatened migratory salmonids and an invasive vertebrate threat

4.2 SITE CHARACTERISTICS

4.2.1 Fort Huachuca, Arizona

The Fort Huachuca Military Reservation lies adjacent to Sierra Vista, Arizona, in the San Pedro River Valley and Huachuca Mountains 75 miles southeast of Tucson, Arizona and 8 miles north of the Mexican border. First established as Camp Huachuca in 1877, Fort Huachuca is currently a major communications and intelligence center for the military. The installation is home to testing and training units such as the Army Intelligence Center, the Army Information Systems Command, and the Joint Interoperability Test Command. It also provides field test facilities and test ranges for communications systems and equipment, including an electronic proving ground complex, associated with White Sands Missile Range and Aberdeen Proving Ground.

Intelligence and electronic warfare trainers and testers require realistic placement of intelligence systems over wide areas just as they can expect to experience while supporting combat situations. The variety of ecological systems at Fort Huachuca provides these groups with a range of opportunities that represent realistic training settings in relatively undisturbed areas. Thus, maintaining the environment at Fort Huachuca in as natural condition as possible is necessary to support the military mission.

At 73,272 acres, Fort Huachuca consists of the cantonment area and open/operational areas, including a C-5A aircraft training mockup, a leadership reaction course, short and long land navigation courses, a rappelling tower and cliffs, and live-fire ranges. Libby Army Airfield is located in the northernmost corner of the cantonment area.

The Fort supports an active outdoor recreation program and provides public access to most areas of the site, notably in the canyons of the mountainous southern and western portions. Recreational access has presented challenges to management of aquatic species on the Fort through unauthorized transport and release of non-native fish and salamanders, collection of native amphibians, and off-road vehicle effects on streams and ponds (Fort Huachuca ENRD 2001).

Fort Huachuca lies within in the basin and range region of southeastern Arizona and supports a variety of species adapted to life in dry environments as well as species requiring the more mesic slopes of the Huachuca Mountains. The area ranges from mountainous terrain with steep slopes in the southwestern portion of the installation to the relatively flat terrain of East Range.

The area typically receives about 14 inches of precipitation a year at the base of the mountains, but it is distributed bimodally, with most precipitation occurring during summer monsoons and winter rains (Fort Huachuca ENRD 2001).

Fort Huachuca has about 4.5 miles of perennial streams in four canyons (Garden, Huachuca, McClure, and Blacktail canyons). Most surface water features are ephemeral streams, consisting of dry washes that only flow in response to significant precipitation events. There are 16 ponds and stock tanks on the installation, ranging in size from < 1 to 5 acres (Fort Huachuca ENRD 2001).

Fort Huachuca and surrounding areas support populations of the federally endangered Sonora tiger salamander (*Ambystoma tigrinum stebbinsi*) and the federally threatened Chiricahua leopard frog (*Lithobates [Rana] chiricahuensis*). These species persist primarily in cattle tanks and restoration sites and are threatened by encroachment of two invasive species: the American bullfrog (*L. catesbeianus*), a voracious predator, and the non-native barred tiger salamander (*A. t. mavortium*), which hybridizes with the Sonora tiger salamander (Storfer et al. 2004). Additionally, Chiricahua leopard frogs are threatened by the amphibian pathogenic chytrid fungus (*Batrachochytrium dendrobatidis*; Bd) (Bradley et al. 2002) and the Sonora tiger salamander is affected by an iridovirus (Ambystoma tigrinum virus, ATV; Jancovich et al. 1997).

Surveys for Sonora tiger salamanders and Chiricahua leopard frogs in the vicinity of Fort Huachuca are currently conducted by the Arizona Game and Fish Department (AZGFD). The department conducted annual surveys for both species throughout the species' ranges in southeastern Arizona during the course of this demonstration. Methods currently used include seining, dipnetting, and visual and audio encounter surveys, which may cause capture stress to individual animals or underestimate occupancy if animals are present but not detected. Our eDNA tests for Fort Huachuca included tiger salamander (both), Chiricahua leopard frog, American bullfrog, Bd, and ATV.

4.2.2 Yakima Training Center, Washington

Yakima Training Center (YTC) is a U.S. Army installation located in central Washington that provides military training facilities and logistical support for cross-country maneuvers and live-fire training. Beginning in the early 1940s and continuing into the early 1950s, the U.S. Army acquired 261,451 acres of land for the original installation. The original portion of the training center has been used for military training since its establishment in 1942 as an anti-aircraft firing range. In 1992, Congress approved acquisition of the northern expansion lands, forming the current configuration of YTC, which includes 327,242 acres (YTC ENRD 2002).

Currently, major military land uses at YTC include the cantonment area with residential, administrative, commercial, light industrial, and open space uses; training areas with maneuver, impact, firing ranges, and other special uses; and the Selah Airstrip and Vagabond Army Airfield (YTC ENRD 2002).

Training activities at YTC have resulted in increased frequency of wildfires, which remain the primary source of perturbation for aquatic and upland resources. A livestock grazing program existed at YTC from 1960 to 1995 but was terminated to reduce conflicts with training and natural resource management (YTC ENRD 2002).

Access to the Central Impact Area in the center of YTC is not permitted. However, this restriction will not limit the planned eDNA demonstration because the fish-bearing reaches of perennial streams do not extend into the Impact Area.

The Yakima Training Center (YTC) lies within a sagebrush-steppe landscape characterized by rolling hills and flats. Chief characteristics of the climate are its relatively low precipitation, seasonal cycle of cool, moderately rainy and snowy winters, wet springs, hot dry summers, and warm, predominantly dry autumns. Topography at YTC varies from low plains to rocky escarpments. Elevations vary from about 152 m at the eastern boundary of the site along the Columbia River to 1277 m in the southeast portion of YTC (YTC ENRD 2002).

The YTC drains into two major basins: the Columbia River to the east and the Yakima River to the west. There are six streams with perennial flow, four that support fish populations (Johnson, Hanson, Alkali, and Lmuma Creeks) and two that do not (Cold and Selah Creeks).

The site lies within the range of the federally endangered Upper Columbia Spring Chinook Salmon (*Oncorhynchus tshawytscha*) evolutionarily significant unit (ESU) as well as the Upper Columbia Summer-/ Fall-run Chinook Salmon ESU, which in 1998 was determined to be not warranted for listing (NMFS 1998). This installation also lies within the range of the federally threatened bull trout (*Salvelinus confluentus*). Chinook salmon fry were found in YTC's perennial streams (Johnson, Hanson, and Alkali Creeks), as well as the intermittent Corral Creek, in the 1980s and 1990s (YTC ENRD 2002), but the extent to which Chinook salmon or bull trout currently use these streams is not known. There are several road culverts that could act as partial or complete barriers to Chinook passage from the Columbia River to YTC streams. Based on the habitat of YTC's fish-bearing streams and the distribution of nearby Chinook salmon populations, it is likely that streams draining directly into the Columbia River (Johnson, Hanson, and Alkali Creeks) would be most likely to have fall Chinook present, while salmon occurring in Lmuma Creek, which drains into the Yakima River, are most likely to be spring Chinook.

Other fish species known to occur in YTC streams include brook trout (*Salvelinus fontinalis*), which are non-native and may hybridize with bull trout (Kanda et al. 2002), and mountain sucker (*Catostomus platyrhynchus*), a Washington State Candidate species.

To meet the requirements of the ESA, and to implement management activities laid out in YTC's Salmonid Endangered Species Management Plan, it is critical that YTC managers have accurate information about if and where spring Chinook and bull trout occur on the site. Traditional surveys for these species include electroshocking and snorkel surveys, which can be stressful for fish and are often inconclusive, as juveniles from the spring and fall Chinook ESUs are morphologically similar. The eDNA test for YTC occurred in two steps. The initial procedure tested for presence of bull trout, brook trout, and Chinook salmon. For samples in which Chinook salmon were detected, we applied additional tests targeting single nucleotide polymorphisms to determine the probability that the Chinook salmon present were from the spring run ESU.

4.2.3 Eglin Air Force Base, Florida

The largest forested military reservation in the United States, Eglin AFB and Reservation contains 464,000 acres of land area in the Florida Panhandle. The main reservation is located within Santa Rosa, Okaloosa, and Walton counties, east of Pensacola, Florida. Eglin AFB was established in 1935 as a small bombing and gunnery range, but the U.S. Forest Service ceded the 800 square mile Choctawhatchee National Forest to Eglin AFB in 1940, allowing the base to expand its operations to include aircraft and tactical training, underwater operations, and missile launching and handling (USAF 2012). The base is currently designated as an Air Integrated Weapons and Armaments Research, Development and Acquisition, Test and Evaluation Center and is the Air Force Materiel Command's primary center for air-delivered weapons.

Mission activities at the Eglin Reservation today fall into four broad categories: weapons system research, development, test, and evaluation; training; space operations; and base and Reservation support. Land areas on the Reservation are used for the testing, development, and evaluation of weapons systems and methods of warfare, as well as for live-fire ranges and military tactical maneuvers. There are five operational airfields within the Eglin Reservation.

Recent increases in ground training operations have begun to limit access for natural resources management (USAF 2012). The Eglin Natural Resources Section works actively with operational units and military tenants to ensure access to important areas for listed species and provide for monitoring and compliance with requirements from Section 7 consultations.

Eglin AFB supports four major ecological associations: Flatwoods Matrix, Wetlands/Riparian Matrix, Sandhills Matrix, and Barrier Islands Matrix (USAF 2012). Approximately 1150 km^2 of the Eglin AFB are managed as a Core Conservation Area (CCA) to prioritize management of habitat for endangered species, particularly red-cockaded woodpeckers (*Picoides borealis*) and reticulated flatwoods salamanders (*Ambystoma bishopi*). Most of the habitat types are fire-dependent ecosystems that require periodic fires to maintain structure and diversity, and prescribed fire is used as a primary management tool for maintaining habitat for these species within the CCA (USAF 2012).

Wetlands on Eglin AFB provide extensive habitat for reticulated flatwoods salamanders, a federally and state listed endangered species. Optimum habitat for the species is open, mesic woodlands that contain shallow, ephemeral wetlands that are maintained by frequent fires. These wetlands have pH values ranging from 3.6 to 5.6 (Palis 1997), providing an opportunity to test the performance of detection using eDNA testing under acidic conditions. Occupancy of the ephemeral wetlands by salamanders is often difficult to determine, and all potential salamander habitat must currently be treated as occupied with respect to habitat management (USAF 2012).

Researchers from Virginia Polytechnic Institute (Virginia Tech) have conducted monitoring and research for flatwoods salamanders on Eglin lands since 2001, but the dipnet surveys primarily used to detect larval salamanders may fail to detect very small larvae or those that are active primarily at night (C. Haas, pers. comm.). We worked with Virginia Tech crews to collect water samples during ongoing surveys in early spring of each year. Our eDNA tests for ephemeral wetlands at Eglin included flatwoods salamanders, the amphibian pathogen Bd, and ornate chorus frogs (*Pseudacris ornata*), a Florida Species of Greatest Conservation Need.

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5.0 TEST DESIGN

5.1 CONCEPTUAL TEST DESIGN

Demonstration of eDNA at DoD installations consisted of two phases for each installation: qPCR assay development and validation (presented in Section 2) and field testing. Field testing consisted of collecting replicate eDNA samples from multiple sites at each installation per year and analyzing these samples using the described qPCR assays. For comparison, we worked with field crews using standard field methods (visual surveys, dipnetting, audio surveys) to conduct surveys for target species after eDNA samples were collected. We collected site covariate data (area, pH, conductivity, water temperature, and UV exposure) associated with each eDNA sample and analyzed these data to inform improvements in sampling design between Year 1 and Year 2 of this demonstration. To ensure no cross-contamination between samples, we collected field blanks with every set of field samples (distilled water filtered with the same equipment and methods as field samples) as well as creating extraction and qPCR blanks with every extraction set and qPCR plate.

5.2 BASELINE CHARACTERIZATION AND PREPARATION

The reference condition for monitoring fish and amphibians at DoD installations consists of a variety of field techniques requiring high levels of effort and having imperfect detection probabilities. At Fort Huachuca, amphibian presence is sometimes monitored using seining of wetlands, which could disturb vegetation and habitat. Surveys for the endangered Sonora tiger salamander using seines showed differing results for occupancy at 23 of 29 sites between 2001 and 2006 (USFWS 2007b). This variation could be due to changes in occupancy or imperfect detection. Recent analysis estimates per-survey detection probability for this species at 0.79 (Hossack et al. 2017). Protocol surveys for threatened Chiricahua leopard frogs involve night visual encounter surveys conducted by a permitted surveyor that must attend USFWS and State approved certification training (USFWS 2007a). These surveys involve walking through the aquatic habitat whenever possible and are believed to have a 90% detection probability (USFWS 2007a). However, Chandler et al. (2015) found this species to have a 0.69 (95% CI 0.60-0.78) per-survey detection probability in similar habitat using protocol surveys.

Currently implemented monitoring for reticulated flatwoods salamanders at Eglin Air Force Base consists of three repeated dipnet sampling visits per year during larval period (C. Haas, pers. comm.). These surveys have been estimated to have a detection probability averaging 0.46 (for two 5-min surveys (Gorman et al. 2009); however, if the sampling occurs when larvae are too small, they can remain undetected as they slip through the net (C. Haas, pers. comm.).

Monitoring for salmonids at YTC has been sporadic and has typically relied on using baited minnow traps to identify species occurring in fish-bearing streams (C. Leingang, pers. comm). Minnow trap sampling can have detection probabilities as low as 1%, depending on sampling frequency, species rarity, and trap size relative to size of the target fish species (Jackson and Harvey 1997).

5.3 DESIGN AND LAYOUT OF TECHNOLOGY AND METHODOLOGY COMPONENTS

5.3.1 qPCR protocol development

Once target species have been identified, the next step of the eDNA PCR protocol development process was to collect sequence data to identify short species-specific DNA fragments that could be used to detect the target species using qPCR techniques. These data were found for all target species in the demonstration in the National Institutes of Health repository of all publicly available DNA sequence data (GenBank; http://www.ncbi.nlm.nih.gov/genbank/). We compiled these sequences and used sequence analysis software (Sequencher) to create a consensus sequence that included all known variation in the sequence for the target species. This ensured that the test developed would detect all known genotypes for the species.

We then used qPCR-specific primer design software (PrimerExpress, Applied Biosystems) to design optimal primers and probes for the species, with fragment lengths between 80 and 120 base pairs. Each of these sets was compared again to the GenBank database using a primer search (Primer BLAST; http://www.ncbi.nlm.nih.gov/tools/primer-blast/) to make sure they would not identify a non-target species. This check was also done with mixed sets with any set of primer pairs that were used together in a test (i.e. where multiple target species are being detected in the same reaction). The number of mismatches on the primers and probe for each non-target species was maximized, ideally exceeding 2 for the primers and 1 for the probe at the most influential location (3' end for primers, center for probes). Probes were ordered in different fluorescent colors to allow for maximum multiplexing (6FAM, NED, Quasar 670, Cal Fluor Red 610). We then demonstrated this lack of cross-amplification using DNA samples from non-target species as negative controls when running the test in the lab (species lists and reaction information can be found in Appendix B). Quantitative PCR was conducted using an Applied Biosystems 7500 Fast Real-Time PCR System (Figure 5-1) for all samples collected 2012-2013. For 2014, an upgraded machine with the same technology was used that was obtained with a QuantStudio 7 Flex Real-Time PCR System (Life Technologies). Starting in 2015, samples were analyzed with a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Controls, assays, and protocols were kept constant across machines.

For distinguishing anadromous fish runs, we took a similar approach, using SNPs from the set characterized by Hess et al. (2011). From the set of 96 used in that study, we identified those that were found to be the most distinct between interior stream-type and ocean-type lineages (i.e., allele frequencies within both lineages furthest from 0.50 and in different directions from each other) and tested them against a panel of co-occurring salmonids. From these results, we identified 5 SNPs that were unique to Chinook and used the known allele frequencies from Hess et al. to calculate the probability that spring Chinook were present in the sample given analysis results.

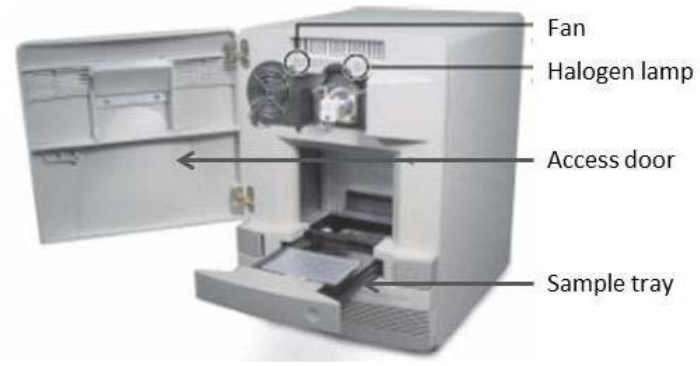

Figure 5-1. The Applied Biosystems 7500 Fast Real-Time PCR System, where Most of the Samples for the Project Were Analyzed

5.3.2 eDNA collection equipment

Environmental DNA was collected in the field using a filter and pump (Goldberg et al. 2011; Figure 5-2). Water was added to the filter using a single-use whirlpak or a bottle that was decontaminated between samples by soaking in 50% bleach. Filters were in single-use filter funnels and the baseline for each demonstration site was to use 0.45 µm cellulose nitrate filters (Whatman). Filters were preserved in 95% ethanol and stored away from excessive heat and light until DNA extraction. Any equipment and field clothing came into contact with the water at a sampling site was decontaminated before continuing to another site. Negative samples (using distilled water) were collected in the field at each new field location to ensure sampling procedure was not introducing DNA into the samples. At each sampling site, we collected data on area (wetlands) or velocity and width (streams), pH, temperature, conductivity, and canopy cover.

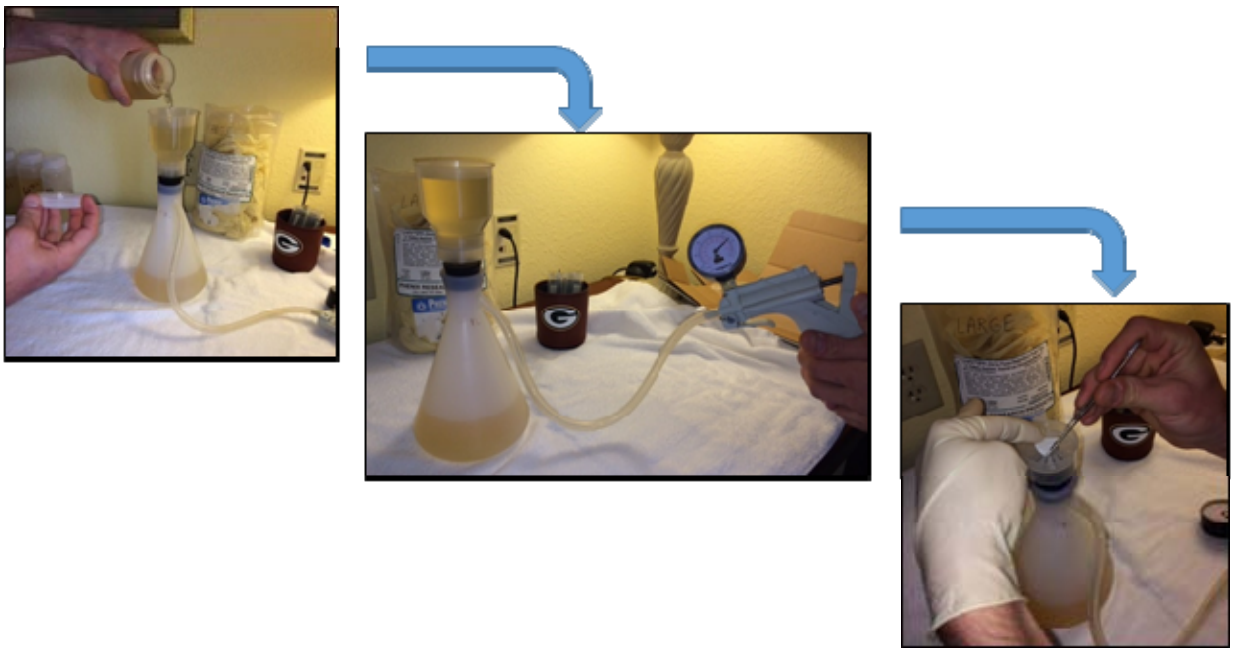

Figure 5-2. Field Collection Equipment for eDNA Sampling

5.4 FIELD TESTING

We collected field samples for this project in collaboration with agencies conducting standardized field surveys whenever possible. Field surveys were conducted when species are at their most detectable life stage (high densities in aquatic systems), maximizing the probability of detection for both field and eDNA surveys. We conducted field surveys according to the Table 5-1. No equipment or material was left in place.

Table 5-1. Dates of Field Sampling in Association with Each Installation

Associated site or subproject	Dates	Field survey agency	Samples collected (inclusive)	Negative controls collected	Target species	Project personnel
Fort Huachuca	August 20 - 25th, 2012	AZGFD	15	3	RACH, LICA	CG, KS
Fort Huachuca	September 12-16, 2012	AZGFD	85	17	RACH, LICA	AF, CG, KS
Fort Huachuca	February 11-14, 2013	AZGFD	70	14	AMTI, LICA	CG, KS
Fort Huachuca	February 24, 2013	DoD	5	1	AMTI	CG
Fort Huachuca	April 1-4, 2013	AZGFD	65	14	RACH, LICA, AMTI	AG, CG, KS
Fort Huachuca	September 6-8, 2013	AZGFD	43	7	RACH, LICA	CG, KS
Fort Huachuca	October 1-3, 2013	AZGFD	35	6	RACH	KS
Fort Huachuca	March 3-6, 2014	AZGFD	73	4	AMTI, LICA	KC, KL, KS
Eglin AFB	March 3-5, 2013	Vtech	100	20	PSOR, AMBI	CG, KS
Eglin AFB	February 17-20, 2014	Vtech	103	20	PSOR, AMBI	CG, KC
Eglin AFB	February 23-26, 2015	Vtech	85	11	PSOR, AMBI	KC, KS
Yakima TC	September 4, 2012	WDFW	4	1	SACO, SAFO	KS
Yakima TC	September 20-22, 2012	WDFW	25	5	SACO, SAFO, ONTS	KS
Yakima TC	October 4-5, 2012	DoD	15	3	SACO, SAFO, ONTS	CG, KS
Yakima TC	November 13, 2012	WDFW	10	2	SACO, SAFO, ONTS	KS

Table 5-1 (*continued*). Dates of Field Sampling in Association with Each Installation

Associated site or subproject	Dates	Field survey agency	Samples collected (inclusive)	Negative controls collected	Target species	Project personnel
Yakima TC	June 10-12, 2013	DoD	40	8	SACO, SAFO, ONTS	KS
Yakima TC	September 23-24, 2013	WDFW	35	7	SACO, SAFO, ONTS	CG, KS
Yakima TC	October 10, 2013	WDFW	25	5	SACO, SAFO, ONTS	KS
Yakima TC	October 14-15, 2013	IDFG	20	4	SACO, SAFO	KS
Yakima TC	October 26-November 1, 2013	IDFG	93	3	SACO, SAFO	CG, KS
Yakima TC	August 12, 2014	IDFG	17	1	ONTS	KC
Yakima TC	October 23-25, 2015	WDFW	125	5	SACO, SAFO	CG, KS, etc.
Disease testing, ID	August 31, 2013		27	1	Bd	JB, etc.
Disease testing, ID	April 7-May 8, 2014		81	3	Bd	KC
Disease testing, AZ	August 5-6, 2014	AZGFD	54	2	Bd	KC, KS
Disease testing, ID	September 11, 2015		27	1	Bd	CG
Disease testing, ID	September 20, 2015		27	1	Bd	KC
Disease testing	June 10, 2014		53	1	Ranavirus	EH
Disease testing	June 24, 2014		65	1	Ranavirus	EH
Degradation	April 8 – June 5, 2013		481	45	LICA	KS, etc.
Transport	August 18 – October 1, 2015		175	14	Sturgeon	AF, KS, etc.
Filter test	October 23-29, 2014		365	15	LICA, TAGR, ONMY	KC, KS, etc.

*Agencies: AZGFD – Arizona Game and Fish Department, DoD – Department of Defense, IDFG – Idaho Department of Fish and Game, Vtech – Virginia Tech Eglin AFB crew, WDFW – Washington Department of Fish and Wildlife. Species: AMBI – reticulated flatwoods salamander, AMTI – Tiger salamander, Bd – *Batrachochytrium dendrobatidis*, LICA – American bullfrog, ONMY – rainbow trout, ONTS – Chinook salmon, PSOR – ornate chorus frog, RACH – Chiricahua leopard frog, SACO – bull trout, SAFO – brook trout, TAGR – rough-skinned newt. Personnel aside from report authors: EH – Emily Hall, JB – Jesse Brunner, KC – Kody Cochrell, KL – Kevin Lewellan, etc. – additional students and technicians.

5.5 SAMPLING PROTOCOL

This project had five components, each with its own sampling design, goals, and sample collection schedule (Table 5-2): eDNA detection of target species; confirming eDNA detection of pathogens; degradation influences on eDNA; eDNA transport in lotic systems; and comparison of collection methods for eDNA. The first two comprise the main part of the original project and are presented in detail here. Details on sampling and analysis of the following three are presented in Appendices C, D, and E. Details of equipment calibration are presented in Appendix F.

Table 5-2. Description of Samples Collected During Each Phase of the Project

Specific months for sampling at each installation are as described in Section 5.4.

Phase	Number of Samples	Description
1) PCR protocol development	245	DNA samples (tissue) from target and co-occurring closely-related non-target species in the vicinity of the installation. See section 2.2.
2) Field Collection - eDNA detection of target species	927	eDNA water filter samples.
2) Field Collection - Confirming eDNA detection of pathogens	68	eDNA water filter samples.
2) Field collection	171	eDNA field negative samples.
2) Field Collection - Confirming eDNA detection of pathogens	80	Tadpoles for ranavirus testing.
2) Field Collection - Confirming eDNA detection of pathogens	176	Body swabs for Bd testing.
S1) Degradation study	436	eDNA water filter samples.
S1) Degradation study	45	eDNA filter negative samples.
S2) Transport study	161	eDNA water filter samples.
S2) Transport study	14	eDNA filter negative samples.
S3) Filter test study	360	eDNA water filter samples.
S3) Filter test study	15	eDNA filter negative samples.

5.5.1 Environmental DNA detection of target species

In Year 1 (2012-2013), we collected samples from lentic sites in Arizona and Florida using a standard design of four replicate samples collected at one location in each wetland. For this sampling, we targeted the shallowest area with vegetation as the area most likely to contain tadpoles. For sample collection, we used 0.45 µm cellulose nitrate single-use sterile filter funnels (Whatman) and concentrated eDNA in 250 mL of water per filter, or the amount that could be filtered before clogging. For lotic sites, we collected four replicate samples of 1L each in all years.

For each set of samples collected, a negative field control of distilled water was also collected with the same protocol and instruments. Sites focused on detection of Chiricahua leopard frogs and those sampled for salmonids in 2012-2014 were primarily sampled on site; remaining sites were collected using grab bottles and kept cold until filtering within 24 hours. Standardized sampling protocols can be found in Appendix G.

For each site where we collected eDNA samples, we also collected data on factors that may affect eDNA detection through degradation, adsorption, and dilution. Specifically, we measured pH, water temperature, and conductivity at each site using a multimeter meter (Oakton, Inc.) and canopy cover as a measure of exposure to UV with a densiometer. In Arizona, we mapped the area of wetlands by walking the perimeter with a Trimble XT (Trimble). In Florida, we primarily used area data from Eglin Air Force Base biological crews and supplemented with GPS mapping with the Trimble XT in the final year (2015).

We extracted filter samples using the Qiashredder/DNeasy method described in Goldberg et al. (2011). All filter sample extractions and qPCR set up was conducted in a lab dedicated to low-quantity DNA samples. Researchers are required to shower and change clothes before entering this room after being in a high-quality DNA or post-PCR laboratory, and no tissue samples have been handled in this room. A negative extraction control was included with each set of extractions and an additional negative qPCR control was run with each plate of samples. Reactions were run using QuantiTect Multiplex PCR Mix (Qiagen, Inc.) with recommended multiplexing concentrations (1X Quanti-Tect Multiplex PCR mix, 0.2 µM of each primer, and 0.2 µM probe). Reactions were 15 µl in volume and each included 3 µl of sample. Cycling began with 15 min at 95 °C followed by 50 cycles of 94 °C for 60 s and 60 °C for 60 s, except for bull trout and brook trout (final temperature 68 °C), Chinook (final temperature 70 °C), and identification of Chinook run using SNPs. Chinook run detection was conducted using the Type-it Fast SNP Probe PCR Kit (Qiagen, Inc.). These reactions were 10 µl in volume with 1X Type-it Fast SNP Probe PCR Master Mix, 0.2 µM of each primer, and 0.2 µM probe and each included 2 µl of sample. Cycling began with 15 min at 95 °C followed by 50 cycles of 95 °C for 15 s and 60 °C for 30 s. Samples collected in 2012-2014 were analyzed at the University of Idaho's Laboratory for Ecological, Evolutionary, and Conservation Genetics on an Applied Biosystems 7500 Fast Real-Time PCR System (2012-2013) or a Quantstudio 7 Flex Real-Time PCR System (ThermoFisher; 2014). Samples collected in 2015 and run analysis for Chinook were analyzed at the eDNA Lab at Washington State University using a CFX96 Touch Real-Time PCR Detection System.

We used a multi-tube approach for analysis, where multiple reactions were conducted for each sample (Taberlet et al., 1999). We analyzed each sample in triplicate and included an internal positive control (IC; Qiagen or IPC; Applied Biosystems) in each well. A positive sample was defined as any sample that showed exponential amplification in all three wells the first time it was tested or in one or more wells from two separate reactions (samples were rerun whenever the original triplicate wells yielded inconsistent results). Quantitative standards consisted of diluted DNA samples derived from tissue from external skin to best represent what eDNA may be sourced and quantified on a Nanodrop spectrophotometer and diluted 10^{-3} through 10^{-6}, run in duplicate. The exception to this was for the bull trout and brook trout samples collected in 2015, which were run with synthetic genes (gblocks; IDT) in duplicate serial dilutions from 10,000 to 10 copies.

We analyzed data from each year in an occupancy modeling framework (Mackenzie et al. 2002) and used results to inform sampling design in the following year. Because the goal of the analysis was to analyze factors affecting detection rather than occupancy, we analyzed the subset of data for each species where there was evidence of occupancy (psi = 1) from either field crews or eDNA detection in at least one filter sample. We treated each filter sample as a replicate at a site, producing four 'occasions' at each site in Year 1 for each species in Year 1. We tested multiple hypotheses of influences on detection probability and used evidence weights to determine the most likely explanation for variation in this measure. We used results to adapt sample designs to increase detection probabilities in the following year(s). Year 2 and 3, we focused on sampling sites that were the most challenging for detection, so average detection probabilities reported may be biased low compared with a random sample of all occupied sites.

Detecting spring-run Chinook salmon

We additionally investigated whether spring-run Chinook salmon could be differentiated from fall run using markers described in Section 2.2. Because our dataset did not include many samples testing positive for Chinook, we obtained an additional 11 samples from Laramie et al. (2015) from both mixed run (fall and spring) and spring-only locations. For samples from this study (more dilute than those of Laramie et al.), alleles had to be seen in at least two of the four sampling replicates to be counted in the analysis. We used overall genotype frequencies from Hess et al. (2011) to calculate the probability of each genotype being produced by each run of salmon and considered the run with the higher frequency to be the one producing the genotype. This assumes that only one run was dominant enough to produce the genotype detected. In a true mixed-stock run, additionally analytical processes will need to be developed to calculate the probability that each run was contributing to the sample but may not account for all alleles detected.

5.5.2 Confirming eDNA detection of pathogens

We conducted surveys at eight additional sites for each of the target pathogens in the study (Bd; *Batrachochytrium dendrobatidis* and iridovirus (*Ranavirus* spp.)) using both eDNA and direct sampling of amphibians to confirm detection of each pathogen in filter samples when infected individuals were detected at each site.

For Bd, we sampled 22 amphibians at each site by collecting and analyzing body swabs using the protocol standardized by Hyatt et al. (2007). For this project component, we sampled five ponds in north Idaho and two ponds in Arizona. At each site, we also collected four 250 mL water filter samples using the protocol in Appendix G. These filter samples were analyzed using the same protocol as above with the qPCR assay of Boyle et al. (2004). A field negative sample was collected with each site.

For iridovirus, we collected 10 wood frog (*Lithobates sylvaticus*) tadpoles at each site and extracted liver tissue for qPCR detection of ranavirus (Hall et al. 2016). This sampling was done at the Yale Myers Forest, Connecticut, where ranavirus die-offs are regularly observed. Just prior to tadpole collection, three water filter samples were taken from different locations around the edges of the wetland. For the first set of collections, 0.22 µm filters were used (cellulose nitrate; Nalgene Analytical Filter funnels). For the second set, both 0.22 µm and 0.45 µm filters were used. A field negative sample was collected with each set of filters per sampling day.

5.6 SAMPLING RESULTS

5.6.1 Environmental DNA detection of target species

Modeling amphibian detection with eDNA – Year 1

As this was one of the first widespread applications of eDNA techniques, we found that results in Year 1 indicated that efficiencies could be gained by improving sampling designs. As our understanding of these processes increases (through this study, for example), we believe that these efficiencies can be built into designs from the beginning, reducing the need for this kind of optimization. However, a pilot season with eDNA sampling paired with standard field sampling is recommended for all novel applications.

Results of Year 1 data indicated different limiting factors for detection of each species, with area as the driving factor for Chiricahua leopard frogs, volume filtered as the limiting factor for tiger salamanders, temperature as the limiting factor for bullfrog detection, and pH as the limiting factor for both Florida amphibians (Table 5-3, Figure 5-3). There was not enough variation in detection of fish species to model detection probabilities. For Florida species, flatwoods salamanders were not present in 2013 (failed breeding season) and eDNA detected ornate chorus frogs at only four sites. Because of this outcome, we added a sampling season at this site (2015), increased volume filtered to 500 mL per filter and combined water from four sites around the wetland in equal volumes for the middle season (2014) and used results from this season to inform sampling design the following year.

Table 5-3. Weights of Evidence for each Factor Influencing eDNA Detection Probability for Each Amphibian Species: Chiricahua leopard frogs (RACH), Tiger Salamanders (AMTI), and American Bullfrogs (LICA), Ornate Chorus Frogs (PSOR) and Flatwoods Salamanders (AMBI)

– indicates not enough variation in the data to run the model. Sample sizes are in parenthesis after each species name. Only sites with known occupancy were used in the analysis.

Model	RACH (10)	AMTI (12)	LICA (16)	PSOR (6)	AMBI (10)
Area	**0.97**	**0.00**	**0.05**	**0.00**	**0.00**
Canopy cover	**0.00**	**-**	**-**	**0.00**	**0.00**
Conductivity	**0.00**	**0.00**	**0.07**	**0.00**	**0.00**
Grab sample	**0.00**	**-**	**-**	**-**	**-**
Null	**0.00**	**0.00**	**0.10**	**0.00**	**0.00**
pH	**0.00**	**0.00**	**0.04**	**1.00**	**1.00**
Sampling occasion	**0.00**	**0.00**	**0.01**	**0.00**	**0.00**
Temperature	**0.00**	**0.00**	**0.69**	**0.00**	**0.00**
Volume	**0.02**	**1.00**	**0.04**	**0.00**	**0.00**

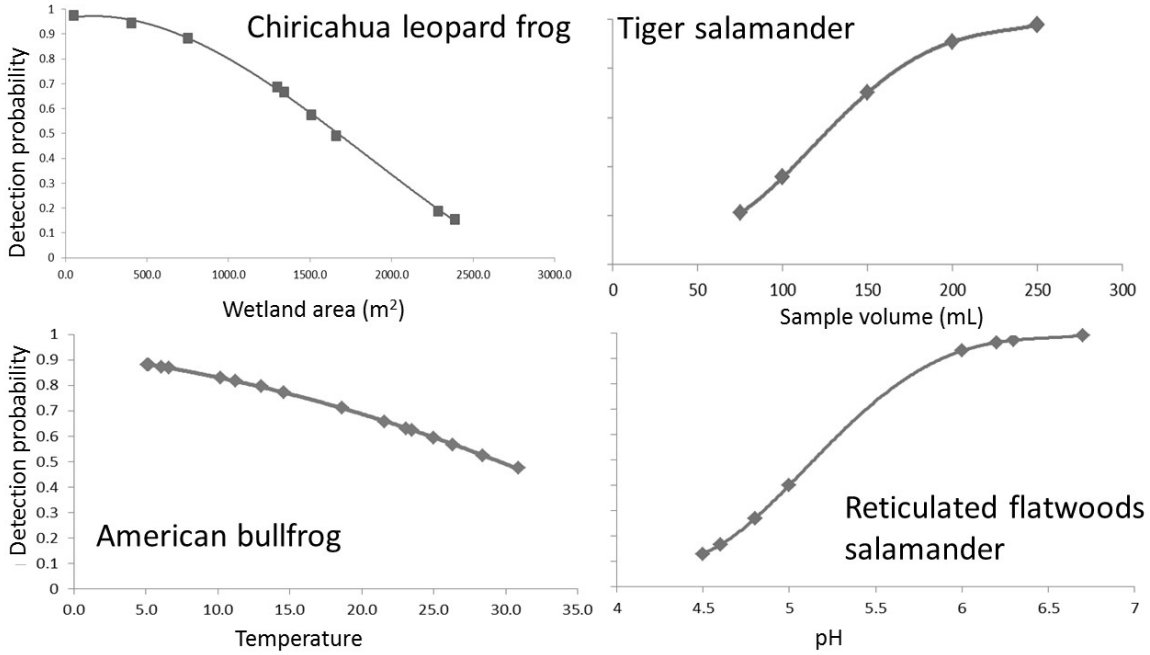

Figure 5-3. Models of per Sample Detection Probabilities for Each Species as Sampled by eDNA Prior to Sample Design Optimization

The prediction for ornate chorus frogs did not converge as all samples with pH > 5 had perfect detection (1.0) and all sites with pH < 5 had no detection (0/4). Points indicate measures at sampled sites.

Improving efficiency of sampling designs for amphibians

Based on the findings from the first analysis of each site, we adjusted protocols by target species. For Chiricahua leopard frogs, we added an extra sampling site at each wetland if that wetland was larger than 1200 m^2 (field estimated at 40 m diameter measured by rangefinder). At each sampling site, we collected three replicate samples to bring the overall probability of detection above 0.95. For tiger salamanders, we changed the filter used to a 6 µm cellulose filter so that the goal of 250 mL of water could be reached in these sites, which mostly consisted of cattle ponds. Bullfrogs at sites targeted for each species were detected with these adjusted protocols. In Florida, we adjusted protocol in the final year by changing filters to a 5 µm polyethersulfone (PES) filter (it had taken several hours per filter to concentrate 500 mL using the 0.45 µm cellulose nitrate filters the previous year) and sampling eight locations instead of four at sites with pH < 5. We then repeated modeling to detect limiting factors for detection probabilities.

Four survey sites for Chiricahua leopard frogs had to be discarded because field crews observed the related species lowland leopard frog (*R. yavapaiensis*) but eDNA tests detected Chiricahua leopard frog sequences (and not lowland leopard frog sequences). Three survey sites for bullfrogs were discarded, two because eDNA assays indicated American bullfrogs, but southern leopard frogs (a newly introduced species) were later found at the sites and testing indicated that this species could produce false positives in the American bullfrog assay as designed and one because sampling was conducted at only two locations in Year 2 when the improved protocol called for at least four (the area of the wetland was not estimated correctly in the dark when sampling occurred).

With improved sampling designs, we detected target species at almost all sites where field crews detected them, plus 21 additional detections with eDNA methods only (Table 5-4). Average per sample detection probabilities ranged from 0.62-0.83, indicating that 2 to 4 samples would be needed per site to achieve on average a 0.95 probability of population detection given the improved sampling design targeted to each species that compensates for limiting factors. Practitioners should note, however, that detection probabilities will always vary across a system and that these are average values that are not applicable at the per-wetland level.

Table 5-4. Comparison of Field and eDNA Detections of Target Species after Application of Improved Sampling Designs

95% confidence limits are below each average detection probability.

	Sites surveyed	Sites with both field and eDNA detections	Additional eDNA detections	Field detections only	Average per sample eDNA detection probability	# samples for >0.95 detection probability
RACH	**21**	**4**	**2**	**1 calling frog**	**0.62 (0.43-0.81)**	**4 (2-6)**
AMTI	**20**	**10**	**3**	**0**	**0.77 (0.61-0.93)**	**3 (2-4)**
LICA	**31**	**6**	**14**	**1 site**	**0.75 (0.60-0.90)**	**3 (2-4)**
PSOR	**11**	**3**	**1**	**1 site**	**0.83 (0.51-1.00)**	**2 (1-5)**
AMBI	**11**	**4**	**1**	**1 site**	**0.75 (0.44-1.00)**	**3 (1-6)**

Simultaneous pathogen detection

Iridovirus. We detected iridovirus at five sites during tiger salamander surveys, with an average detection rate of 0.75 (95% CI: 0.22 – 0.97), given that the pathogen was detected in at least one eDNA sample. This sample size was too small to model for detection probabilities, and was collected across both pore sizes. Detection rates with 0.45 µm pore size filters were 0.75-1; those with 5 µm pore size filters were 0.5-1.

Bd. We detected the pathogen Bd at 17 sites in the course of the project, 10 in Florida and seven in Arizona. Because of the different sampling designs, we could not use occupancy modeling for detection rate, so linear regression was used instead, with an arcsin square root transformation of the response variable to better meet assumptions of normality. All sites modeled together indicate strong evidence for a relationship between detection rate and temperature, which is driven by samples collected in Arizona (Table 5-5; Figure 5-4). Overall average detection rate was 0.83 (95% CI 0.71-0.95), given that the pathogen was detected in at least one eDNA sample.

Table 5-5. Weights of Evidence for Hypothesized Limiting Factors for eDNA Detection of the Amphibian Pathogen Bd.

Model	All sites (17)	Florida (10)	Arizona (7)
Area	0.01	0.09	0.00
Canopy	0.01	0.09	0.00
Conductivity	0.01	0.07	0.00
Null	0.02	0.19	0.00
pH	0.01	0.13	0.00
Samples collected	0.03	0.08	0.00
Temperature	0.92	0.16	1.00
Volume	0.01	0.19	0.00

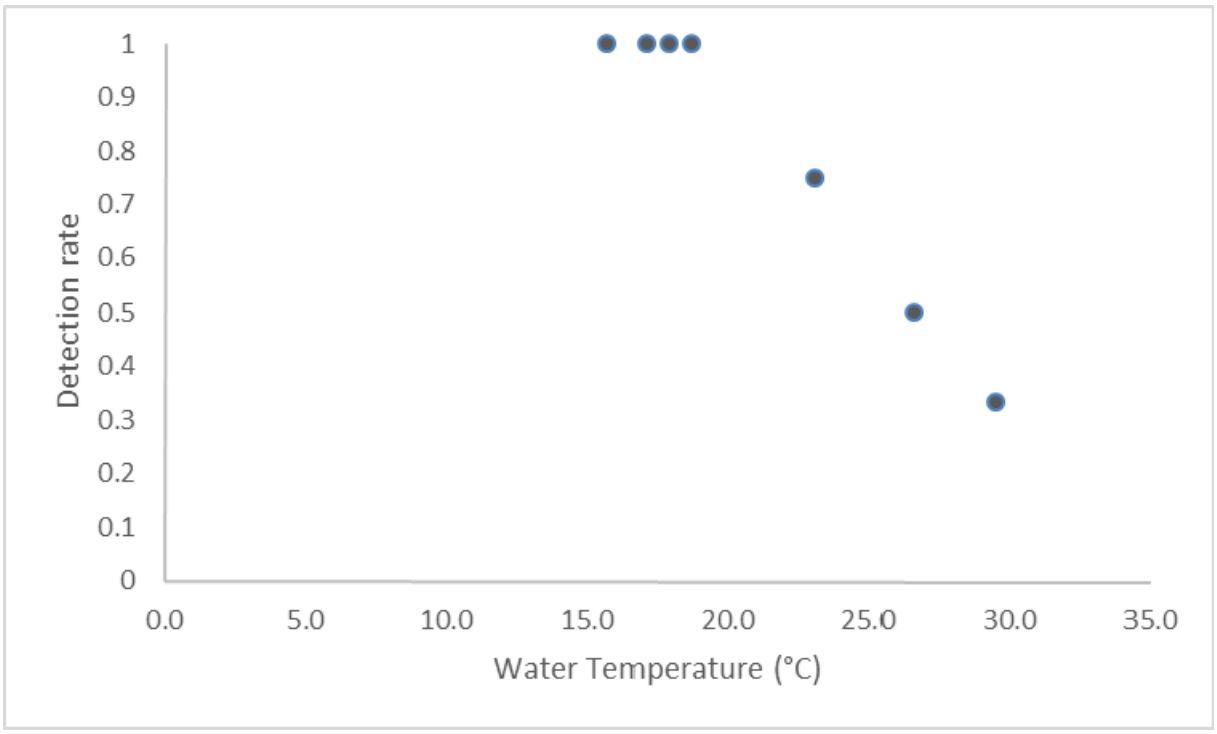

Figure 5-4. Relationship between Water Temperature of Collected eDNA Samples at Sites in Arizona and per Sample Detection Rate for the Amphibian Pathogen Bd

Factors affecting detection probability of salmonids

Field surveys for salmonids in the study area focused on bull trout redds during this demonstration. We conducted these surveys at three scales: among drainages, in cross transects within drainages, and within a basin. For the among-drainage samples (7 sites), there was only one set of sites with less than 1.0 detection rate; this was the site with the lowest observed density of bull trout redds. Therefore, we did not do a statistical analysis of covariates of detection. In the cross-transect study, we collected three 1L samples from the thalweg and a slow-flowing area at the same point longitudinally at five locations each in three bull trout streams and compared the quantitative values of the results. We found that the slow water samples had a mean of 3.3X (95% CI 1.4-7.7X) the amount of bull trout eDNA than the paired fast water samples (paired t = -3.00, p = 0.009; program R (R Core Team 2016)). However, when we analyzed these samples for Rocky Mountain tailed frog (*Ascaphus montanus)* eDNA (assay in Pilliod et al. 2013), we found no evidence of a difference in eDNA concentration between fast and slow samples (paired t = 0.39, p = 0.70). In the basin-wide study, we had a 1.0 detection rate at all sites (25 mainstem plus four tributaries). In a simultaneous study further north, we found in collaboration with United States Geological Survey (USGS) surveys that eDNA detection of Chinook salmon increased in August compared with June due to lower flows, increases in population density, or both (Laramie et al. 2015).

Detecting spring-run Chinook salmon

We obtained enough genotype information (≥4 markers) to calculate probabilities of spring-run Chinook presence at eight sampled locations (Figure 5-5). Within this set, we detected spring-run Chinook salmon in all but one location where they were thought to be located (Figure 5-6).

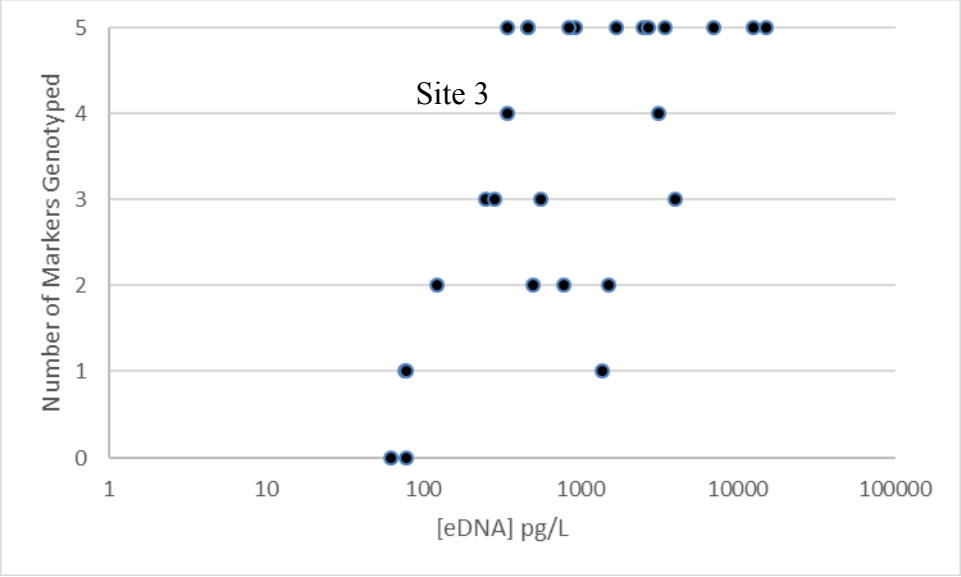

Figure 5-5. Relationship between Chinook eDNA Concentration in Sample Replicate and the Number of Single Nucleotide Polymorphism Markers that Generated Data for Run-specific Analysis.

A minimum of 4 markers was required for run identification.

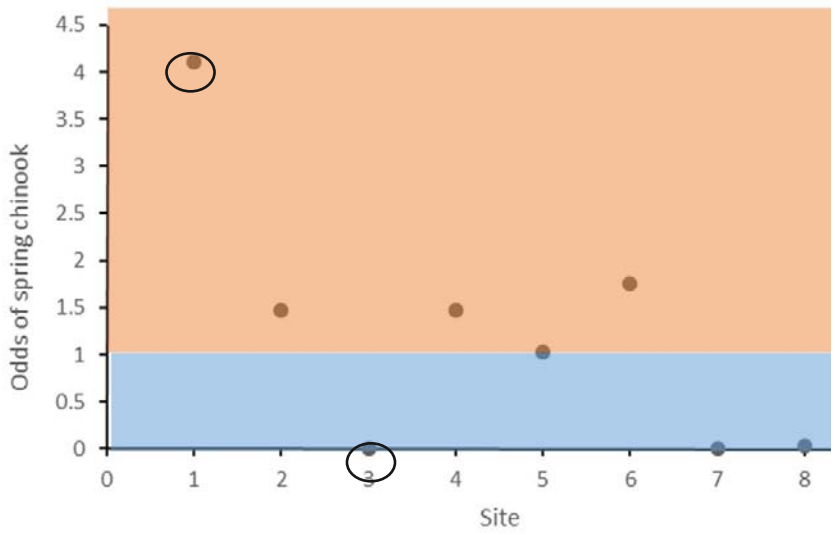

Figure 5-6. Odds of Genotype from eDNA Sample of Chinook being Sourced from Spring Rather than Fall-run Chinook

1 = even odds, orange area indicates spring Chinook as the dominant genotype in the sample, blue area indicates fall-run Chinook as the dominant genotype. Circled sites are those with only 4 loci producing results. Site 3 is a spring Chinook site misidentified, likely due to allelic dropout. Sites 7 and 8 were the only sites known to be dominated by fall run Chinook in the dataset.

Confirming eDNA detection of pathogens

Ranavirus. We detected ranavirus in all eDNA samples collected as well as in 62/80 tadpoles. Filter pore size did not affect detection (1.0 in both cases) or the amount of eDNA collected ($F_{2,58}$ = 0.968, p = 0.39; Figure 5-7).

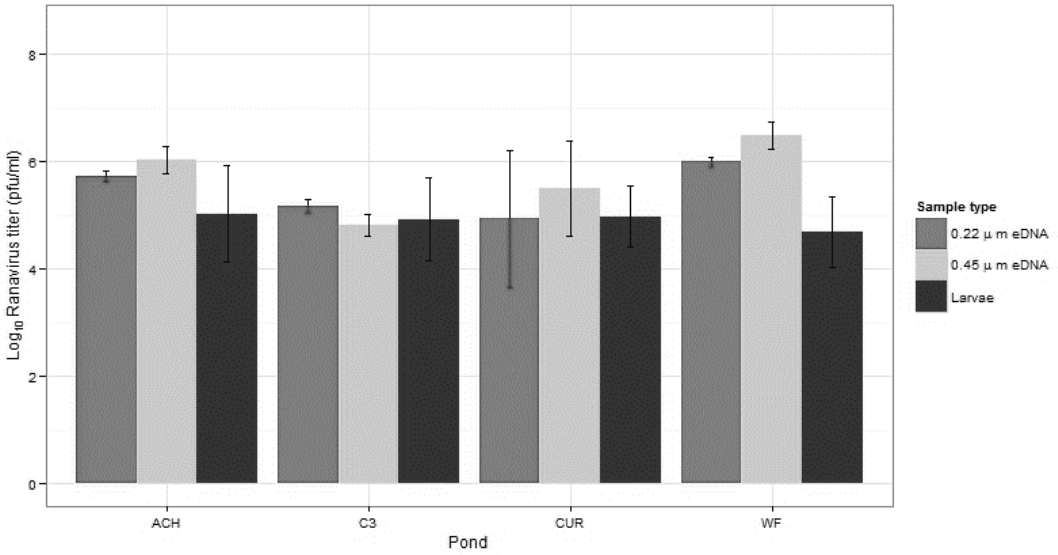

Figure 5-7. Comparison of Ranavirus Titers between Filter Pore Sizes and Found in Livers of Infected Larvae

Courtesy of Emily Hall.

Bd. We detected Bd in all eDNA samples collected and in 156/176 frogs swabbed (mean prevalence = 0.89, 95% CI 0.83-0.94). Detection rates ranged from 0.125-1 per sample and increased with infection intensity in the population (Figure 5-8).

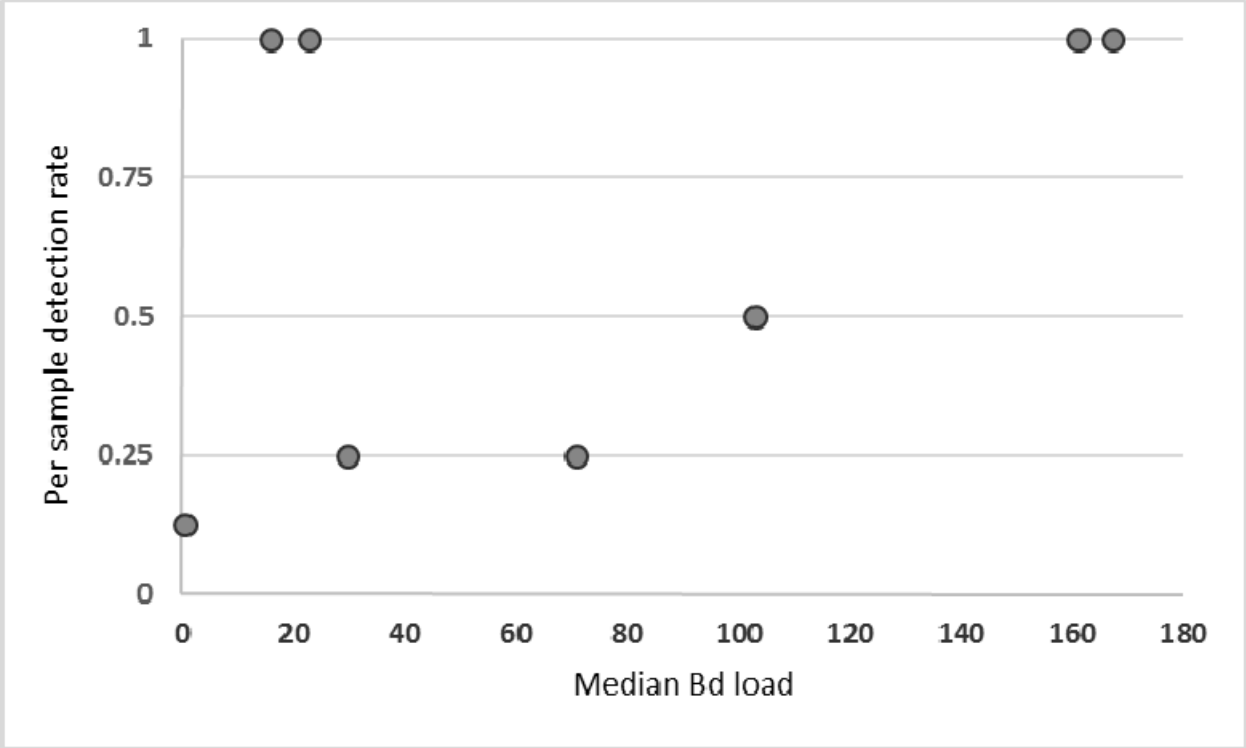

Figure 5-8. Relationship between Median Bd Load per Frog in each Population in Zoospore Equivalents and Detection Rate of Bd Using eDNA Sampling

One data point to the upper left had individuals with very high Bd loads (>100,000 copies).

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6.0 PERFORMANCE ASSESSMENT

1. Demonstrate an eDNA sampling and analysis protocol that has a high probability of detecting species when they are present.

Demonstrating that the eDNA approach has a high probability of detecting species when present was the first major goal of this project. This performance objective evaluated whether the eDNA technique is able to detect species at least as well as field surveys, setting the baseline for demonstrating the cost-effectiveness of this technique. Conducting this test across a range of species also allowed for the detection of differences among species in detection probability compared with field samples. The metric for this assessment was the presence/absence of eDNA detected in samples collected at each site where field crews detected the species. To accomplish this, we collected replicate samples at each field location (wetland or stream reach) in coordination with standard field surveys. Evaluation of samples was conducted according to the following decision rules: all samples were tested in triplicate; if all three test wells agreed (all positive or all negative), that was considered the final result for that sample; if the three test wells disagreed (one or two tested positive), the sample was rerun; if at least one tested positive, the sample was considered positive, if all tested negative, the sample was considered negative. Field sampling was conducted according to standardized protocols for target species. For success, the eDNA tests needed to detect the species (in at least one of the samples taken at the site) at every site where they were detected through field surveys.

Fort Huachuca

Chiricahua leopard frog: We sampled 20 sites in each year in coordination with expert field crews. In total, field crews detected the species during 12 surveys, of which we detected the species in 10 (83%). In Year 2, we increased the number and spatial distribution of samples in larger wetlands. However, in each year, eDNA sampling missed detection at a single site where one frog was observed visually (Year 1) or by audio cue (Year 2).

Tiger salamander: We sampled 23 sites in Year 1 and 20 sites in Year 2 in coordination with expert field crews. In total, field crews detected the species during 21 surveys, of which eDNA sampling detected the species in eight of 11 in Year 1 (73%) and 10 of 10 in Year 2 (100%). For these sites, we changed the filter we used to collect eDNA in Year 2 to accommodate more volume.

American bullfrog: We conducted 43 surveys in Year 1 (2012-2013) and 32 surveys in Year 2 (2013-2014) in coordination with expert field crews. In total, field crews detected the species at 36 sites, of which eDNA samples detected the species in 34 (94%). The two sites where the species was missed by eDNA were both large (>0.3 ha) and samples were collected at only one location in the wetland.

Yakima Training Center

Bull trout: We conducted 62 surveys over three years in coordination with field crews where possible. Field crews detected bull trout at seven sites, of which we detected the species in all seven (100%).

Brook trout: We conducted 64 surveys over three years; no field surveys were conducted in the study area for this species during the demonstration.

Chinook: We conducted 28 surveys over two years in coordination with field crews where possible. Four sites had known presence from previous surveys or concurrent field surveys; of these we detected the species in all four (100%). Additionally, we collaborated on a larger project with this species north of the Yakima Training Center (Laramie et al. 2015). In this study, this method yielded a 0.77 detection probability per sample, which would be equivalent to >0.99 detection with this sampling design (4 replicates).

Eglin AFB

Flatwoods salamander: We sampled 20 sites in 2014 and 11 sites in 2015 in coordination with expert field crews. We also sampled this area in 2013, but there were no detections of this species using either method, indicating a failed breeding year. In total, field crews detected the species during 10 surveys of these same sites in 2014, of which we detected the species in seven (70%). In 2015, we increased the number and spatial intensity of sampling at sites with pH < 5. This led to detections at four of the five sites (80%) where field crews detected them. The site where eDNA did not detect the species was a large wetland (>2 acres) with low pH (4.8). Field crews detected eight larvae at this site.

Ornate chorus frog: We sampled 20 sites in each year for two years (2013 and 2014) and 11 sites in 2015 in coordination with expert field crews. In the first year, we detected the species at one of nine sites where they were detected by field crews (11%). In the second year, we increased volume and spatial distribution of samples and detected the species at three of five sites (60%) where they were detected by field crews. In the final year, we again increased the number and spatial intensity of sampling at sites with pH < 5. This led to detections at 3 of the four sites (75%) where field crews detected the species. The site where eDNA did not detect the species was a large wetland (>2 acres) with low pH (4.6). Field crews detected two larvae at this site.

Evaluation of success criteria: We collected samples for this project in two or 3 seasons at each site. For amphibians (Fort Huachuca and Eglin AFB), we adapted sampling design in each year based on modeling results from the previous year of each survey. For success criteria, we evaluated detection only in the final year, when final sampling designs were implemented. For salmonids, sampling design did not change between the years and results are cumulative. Demonstrated methodology detected target species at every site where field crews detected the species for tiger salamanders, bull trout, and chinook salmon. Remaining target species (Chiricahua leopard frogs, American bullfrogs, flatwoods salamanders, and ornate chorus frogs) were each missed at a single site where they were detected by field crews. These sites were all low density (1-8 individuals detected by field crews). There were no active surveys for brook trout in the study area at the time of this sampling.

Implications: Many efficiencies we found during the first year of sampling are widely applicable and may negate the need for modeling during a pilot season. However, we recommend a pilot season of eDNA sampling paired with standard field sampling when possible for applying eDNA techniques in new systems. Whether eDNA techniques can be used instead of field methods for detection in a system (rather than as a complement) depends on the outcome of this pilot work as well as the goals of the survey (e.g., age structure and disease status cannot be inferred from eDNA).

Like any sampling method, there is some probability that eDNA techniques will fail to detect a species that is very rare at a site. While eDNA can improve probabilities of detection, it should be noted that there is always some uncertainty involved and lack of detection should not be taken as definitive absence. However, by increasing detection probability, eDNA techniques can greatly reduce the probability of a false absence over current methods. Recommendations for sampling larger wetlands (e.g., sampling every 50 m rather than taking a fixed number of samples) can be found in Appendix G. Samples required to reach >0.95 probability of detection at a site can be found in Table 5-4.

2. Demonstrate an eDNA sampling and analysis protocol that has a higher probability of detecting rare species than field surveys.

To be cost-effective for widespread implementation, eDNA techniques need to be more sensitive for detecting rare species than field surveys. To demonstrate this, we compared the presence/absence of eDNA in each sample to presence data obtained through standard field surveys. These data were collected as described in Performance Objective 1. To demonstrate the lack of false negatives and additional sensitivity of the eDNA technique, success for this objective is demonstrated by replicate eDNA samples indicating species presence when field surveys did not detect the target species. For full success, the detection at these sites using eDNA samples would be consistent across replicate samples (at least three of the four samples indicating presence).

Fort Huachuca

Chiricahua leopard frog: We detected this species at two sites in Year 1 and 2-6 sites in Year 2 where field crews did not detect them (the uncertainty in Year 2 is because of a phylogenetic issue – a similar species was detected by the field crews and may be carrying the mitochondrion of the target species; the population appears to have gone extinct shortly after with no direct genetic samples archived). Excluding the phylogenetically uncertain sites, per site detection in Year 2 at these sites was 1.0.

Tiger salamander: We detected this species at one site in Year 1 and three sites in Year 2 where field crews did not detect them. Per site detection at these Year 2 sites ranged from 0.5 – 1 with a mean of 0.83 (95% CI 0.51 – 1).

American bullfrog: We detected this species at four sites in Year 1 and 14 sites in Year 2 where field crews did not detect them. Per site detection at these Year 2 sites ranged from 0.17 – 1 with a mean of 0.73 (95% CI 0.58 – 0.88).

Yakima Training Center

Bull trout: We detected this species at one site where field crews conducting redd surveys did not detect them.

Brook trout: No field surveys for comparison.

Chinook: We did not detect this species in additional sites.

Eglin AFB

Flatwoods salamander: We detected this species at one site in Year 2 where field crews did not detect them. Detection at this site was 0.75.

Ornate chorus frog: We detected this species at one site in Year 2 where field crews did not detect them. Detection at this site was 1.0.

Evaluation of success criteria: Demonstrated methodology detected target species at ≥1 site where field crews did not detect the species for all amphibian species (5) and bull trout. We were not able to compare detection probabilities for chinook and brook trout, which had limited and no field surveys, respectively, and thus no sites where eDNA detected the species where field crews did not. For all sites where amphibian species were detected using eDNA only, replicate samples detected the target species on average for ≥73% of samples, which we consider to have met success criteria. For bull trout, the one site detected with eDNA and not field crews had a 50% detection rate.

3. Demonstrate an eDNA sampling and analysis protocol that can detect amphibian disease when present.

An advantage of eDNA surveys for aquatic vertebrates over field sampling is the ability to detect disease organisms simultaneously through qPCR analysis. We simultaneously collected water samples (4 replicates) and samples of host amphibians and tested for the presence of Bd (*Batrachochytrium dendrobatidis*). We sampled 22 individuals at each site through body swabs, providing a ≥90% probability of detecting the pathogen if 10% or more of the population was infected. We also compared detection of ranavirus through direct sampling (which involves sacrificing the tadpole) and eDNA methods. For this pathogen, we collected 10 tadpoles at each sampling event at eight wetlands for seven sampling events simultaneously with collection of three water filter samples to test for eDNA of the virus.

Batrachochytrium dendrobatidis

We sampled eight sites for *Batrachochytrium dendrobatidis* directly by swabbing 22 amphibians and indirectly through collection of four 250 mL eDNA filter samples. We detected Bd on swabs at all eight sites and in eDNA samples at seven sites using standard protocols. This number was increased to eight after analysis of the second filter half (usually archived) for the site testing negative on the standard analysis.

Iridovirus

Samples were collected as part of a Ph.D. project (Emily Hall, Washington State University) at ranavirus-positive sites in the Yale Myers Forest, Connecticut. Ten wood frog (*Lithobates sylvaticus*) tadpoles were collected at each of eight sites and liver tissue was analyzed for qPCR detection of ranavirus (Hall et al. 2016). Just prior to tadpole collection, three water filter samples were taken from different locations around the edges of the wetland. For the first set of collections, 0.22 µm filters were used (cellulose nitrate; Nalgene Analytical Filter funnels). For the second set, both 0.22 µm and 0.45 µm filters were used. Ranavirus was detected at all sites with eDNA (both pore sizes) and tadpole sampling.

Evaluation of success criteria:

We detected both pathogens at all sites in at least one water sample per site, although at one Bd site both filter halves had to be analyzed for success.

4. Demonstrate an eDNA sampling and analysis protocol that minimizes the probability of contamination.

A critical component of the eDNA demonstration is to confirm the low probability of false positives in eDNA samples. This performance objective focuses on demonstrating the lack of false positives in the eDNA collection and analysis protocol through the analysis of negative control samples at several stages. Negative control samples serve to indicate at what stage contamination has taken place so that samples or results that may be a result of contamination can be discarded. This is a costly process in that field samples may have to be re-collected or lab analyses re-run. Therefore, success for this objective involves demonstrating that this occurs very rarely. To demonstrate this low probability of contamination, we analyzed negative control samples from each step of the eDNA collection and analysis process. In the field, negative controls were collected by taking a filter sample of distilled water on each sampling occasion. In the laboratory, we processed an extraction negative control with each set of eDNA extractions that included all extraction reagents and was processed in parallel with eDNA samples. Additionally, with every PCR we included a "no template control," where water is substituted for the eDNA sample, to ensure detection of any contamination occurring at that step. For success, we needed to demonstrate a sampling and analysis protocol where <1% of all these negative controls tested positive.

We collected and tested 245 field negatives in the course of the study, all of which tested negative. We also created 106 extraction negatives and 279 PCR negatives, all of which tested negative.

Evaluation of success criteria:

All negative controls tested negative at all stages of this demonstration.

5. Demonstrate cost-effectiveness of eDNA approach.

The eDNA technique needs to be cost-effective to be an improvement over standard protocol field surveys. In this performance objective, we sought to demonstrate that the eDNA approach costs less than standard field surveys to reach the same detection probability. Here, we collected four replicate eDNA samples at each site, analyze them as described in Performance Objective 1, and calculate a detection probability for each target species based on how many of the four samples test positive, averaged over all field sites. For each method (field and eDNA sampling and analysis), we estimated the costs of personnel and supplies to achieve equal detection probabilities for each target species. Success of this performance objective for each species is indicated by the cost to reach an equal or greater detection probability for eDNA surveys being lower than the cost of equivalent protocol field surveys.

We estimated costs for eDNA sampling and compared them with costs for field surveys from two of our collaborating groups who were conducting dipnet or seine surveys concurrently. Cost types that were common to both methods included 1) salaries and wages for technicians and project biologists to travel between and survey sites and 2) costs associated with travel between sites (vehicle charges, fuel, etc.). For field surveys we included costs of equipment purchase and maintenance. For eDNA sampling, we included costs of consumables and sample analysis for ongoing eDNA sampling, after the steps associated with qPCR test development had been completed.

We compared per-site costs for labor, travel, equipment, sample collection, and sample analysis for field and eDNA surveys for Sonora tiger salamanders at Fort Huachuca and flatwoods salamanders at Eglin AFB, the only species for which both field costs and field detection probabilities were available. We used Arizona Game and Fish Department (AZGFD) and Virginia Tech rates, respectively, for labor and travel to estimate costs for ongoing field and eDNA surveys. For Sonora tiger salamander, we obtained probabilities directly from AZGFD surveys. We used detection probabilities from Gorman et al. (2009) for flatwoods salamander surveys.

Per-site average detection probability for seining surveys of tiger salamanders was estimated to be 0.79 and the estimated cost of surveying a single site one time was $279. To achieve a detection probability of ≥0.95, two seining surveys would be required for each site, for a total estimated survey cost of $558 per site and $11,164 per year for 20 sites. For ongoing eDNA sampling, average per-sample detection probability was 0.77. One survey per year with three samples at each site would be needed to achieve a >0.95 detection probability. Per-site cost for three eDNA samples is estimated at $268, and one annual survey of 20 sites would cost $5,351.

For flatwoods salamanders, per-site detection probability of a single dipnetting survey was estimated to be 0.46 and the estimated cost of surveying a single site once was $49. Current annual dipnetting effort of four surveys per year yields an overall detection probability of 0.91, for an estimated survey cost of $198 per site and $3,960 per year for 20 sites. For ongoing eDNA sampling, average per-sample detection probability was 0.75, with two samples needed at each site to achieve a >0.91 detection probability. Per-site costs for two eDNA samples is estimated at $142. One eDNA survey would be required per year, with annual cost of about $2,843.

The higher costs of Sonora tiger salamander sampling compared with flatwoods salamander sampling can primarily be attributed to the increased costs of travel among sites in Arizona. Detailed information on cost calculations can be found in Section 7.

Evaluation of success criteria:

To exceed 0.95 probability of detection for Sonoran tiger salamanders, we estimated that three seining surveys ($935 per site) or three eDNA samples on one survey ($268) would be needed. To exceed 0.95 probability of detection for flatwood salamanders, we estimated that four dipnet surveys ($198) or three eDNA samples on one survey ($142).

6. Demonstrate ability of an empirical model to predict success of eDNA technologies.

A major challenge in the application of eDNA techniques is to determine the limits of application of this technology for new target species and systems. This can inform both sampling design (sample sizes) and what monitoring programs may benefit the most from this technology. In this performance objective, we attempted to demonstrate an empirical model that can predict the detection probability of a species using eDNA in untested systems. In the field, we measured covariates of DNA degradation, including pH, water temperature, and area at each wetland site where eDNA samples were collected. We then built predictive models using parametric and non-parametric statistical models to determine which one best predicted eDNA detection probability for sites using cross-validation.

We compiled data from Year 2 of all wetland sites to model how per-sample probability of detection at a site could be predicted by the covariates of water temperature, pH, conductivity, canopy cover, and wetland area.

We used three modeling methods for these data, each with site as the sampling unit: generalized mixed-effect models with species as the random effect on continuous per sample detection probabilities; generalized mixed-effect models with species as the random effect and a binomial link function for detection of the species at a site; random forests algorithms to predict binomial detection at a site. Canopy cover and pH were negatively correlated (-0.78) in this dataset and were not used in the same mixed models.

Evaluation of success criteria: Generalized linear models produced predicted values with low explanatory power of detection probabilities. Random forest models had low misclassification error (13.33%), but did not correctly identify any of the sites where detection was missed.

7. Ease of use.

A potentially strong advantage of eDNA-based monitoring programs is rapid collection of samples that may reduce field labor costs and require less specialized personnel than other field methods. For this advantage to be realized, the monitoring program requires a protocol for collecting and preserving samples in the field that has unambiguous, explicit instructions and is easy to follow in field settings. We evaluated the ability of field technicians to correctly follow the field protocol using a Likert-type qualitative survey as an assessment tool. The objective of the survey was to identify any impediments to implementation of the protocol. Participants were asked rate their degree of agreement with a series of statements, which addressed protocol clarity (lack of confusing or ambiguous language), comprehensiveness (applicability to all field situations encountered), and general usability (no difficulties following procedures for collecting samples, preventing contamination, or meeting quality assurance requirements). The survey consisted of seven scorable items for clarity and comprehensiveness (on a 5-point Likert scale), eight scorable items for usability (yes or no), and four additional questions, which allowed us to compare scores among categories representing different aquatic systems and levels of technician experience, and to assess whether supplemental instructions were needed to successfully complete eDNA sampling procedures.

Two groups of individuals were asked to participate in the qualitative survey. The first group consisted of field technicians collecting water samples at or near each demonstration site, and included both technician-level and more experienced biologists who were naïve to the sample collection and preservation procedures. The second group consisted of minimally trained students in the School of the Environment at Washington State University. These individuals traveled to aquatic systems in the vicinity of the university to test the usability of the field protocol in non-target systems. The field protocol was revised iteratively until overall survey results met the success criteria of a median Likert score of at least 4 on a 5-point Likert scale for clarity and comprehensiveness, and at least 1.5 on a 2-point scale for usability.

We revised the eDNA field sampling protocol several times internally before releasing it for two rounds of evaluation, then revised it following each round of testing and evaluation. The qualitative survey for the second version of the protocol (November 2015) scored the protocol at 4.77 (out of 5) for clarity and comprehensiveness and 1.92 (out of 2) for usability. The final version is included as Appendix G.

Evaluation of success criteria: We surveyed users of two versions of the field protocol. For version 1, released in May 2014, the median Likert score for clarity/comprehensiveness was 4.58. The clarity/comprehensiveness score increased to 4.77 for version 2, released in November 2015. Median usability scores were 1.67 for version 1 and 1.92 for version 2. The score for only one usability item, difficulty in filtering the recommended volume of water, was below 1.5.

8. Versatility.

In addition to being clearly written and easy to follow, our field protocol must be suitable for use in a variety of different aquatic systems. We assessed the versatility of the protocol using the qualitative survey described above, with survey results from field technicians collecting samples in each of the three unique aquatic systems (forested wetland, desert grassland wetland, and desert river systems) represented by our demonstration sites. This performance objective was successfully met when survey results in each aquatic system had a median Likert score of at least 4 on a 5-point Likert scale for clarity and comprehensiveness, and at least 1.5 on a 2-point scale for usability.

Evaluation of success criteria: Median Likert scores for clarity/comprehensiveness were above 4.5 for each of the three aquatic systems. Median scores for usability were above 1.7 for each system. There were no differences among aquatic systems for either assessment category (clarity/comprehensiveness: $F = 1.92$, $p = 0.18$; usability: $F = 1.32$, $p = 0.29$).

9. Reduce permitting requirements.

Because eDNA techniques avoid handling or disturbing individual animals, we expected that "take" permits under the Endangered Species Act would not be required for an eDNA-based monitoring program for listed species. We consulted with USFWS Ecological Services staff, who are tasked with evaluating impacts to listed species and providing incidental take permits, to assess the need for permits for collection of water filter samples in aquatic systems where listed species occur.

We assessed the need for incidental take permits for eDNA water sample collection by consulting USFWS Ecological Services staff from the Washington, Arizona, and Florida field offices. We were unable to obtain concurrence among the staff that permits would not be required for eDNA sampling, and are pursuing concurrence at the national level of USFWS. More detailed information can be found in Section 8.

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7.0 COST ASSESSMENT

7.1 COST MODEL

Operational costs of implementing an eDNA-based monitoring program include both front end and per-sample costs. Front end costs are required for developing and validating qPCR assays for target species and are thus specific to installations where the species occurs. Per-sample costs represent the ongoing costs of collecting and analyzing water samples for a monitoring program. In the cost model detailed below and in Table 7-1, cost elements 1 through 4 represent one-time, front end costs, while cost elements 5 through 7 represent per-sample costs that would be required in an ongoing monitoring program.

1. **Primer/probe set development for each target species**. For each target species, DNA sequence data were obtained at no cost from GenBank, aligned, and a consensus sequence was created that included all the known sequence variation within the species. We then completed primer and probe designs (qPCR assay designs) that were species-specific. Labor costs for creating these designs formed the data tracked for this cost element. Cost were tracked per target species for the eight species included in this demonstration.

2. **Sample collection from target and closely related species**. We collected samples from target and closely-related co-occurring non-target species for *in vitro* validation of the qPCR assays (Goldberg et al. 2016). Samples were obtained from existing sources (e.g., collaborating researchers, agencies, or archives) where available. When existing samples were not readily available, we collected tissue samples in the field. The data used to track this cost element included shipping and materials costs to obtain existing samples and/or travel and labor costs to collect samples in the field. Costs for this element were tracked as average cost for sample collection per species for the eight species in this demonstration.

3. **qPCR test validation.** We used qPCR assay designs from element 1 with DNA samples from target and non-target species collected in element 2 to validate species-specific assays for each target species. Cost data tracked for this cost element included labor and laboratory costs required to validate qPCR tests for the suite of target species at each installation. The total cost of qPCR test development depended on the number of species targeted and whether assays for co-occurring target species could be analyzed in multiplex reactions. Costs for this element were tracked as average cost for qPCR test validation per species in this demonstration.

4. **Sampling equipment procurement**. Collection of water samples requires equipment to pump water through a filter that concentrates DNA. We tested a battery-powered peristaltic pump for filtering samples, but found that it was not more efficient than handheld manual vacuum pumps. The peristaltic pump was also heavier, less portable, and required more accessory equipment than hand vacuum pumps. We thus tracked the cost of procuring hand vacuum pumps. We also estimated the cost of repair or replacement for sampling equipment. For example, the inexpensive silicone tubing used in the pumps should be replaced every other year. Properly maintained handheld pumps likely have a life expectancy of one to two years, depending on level of use. Filters tend to clog more quickly when water samples have high loads of organic material.

Handheld pumps used in aquatic systems with more organic matter thus experience more stress and have a shorter life expectancy compared with oligotrophic systems with clearer water. Other equipment required for water sample collection and filtration (vacuum flasks, stoppers, forceps, and grab bottles) are durable and we tracked these costs as one-time purchases. Costs for this element were tracked per target species for the eight species in this demonstration.

5. **Water filter sample collection.** Water filter samples can be collected relatively quickly in the field but require labor and other expenses for operating a field crew to collect samples. Data tracked for this cost element included the labor needed to collect a set number of samples at each installation (e.g., five samples at each of 20 sites) as well as travel expenses required to travel among sites. Travel costs between the general demonstration area and WSU or UI were not included. Because the number of target species and sampling sites was different for each demonstration site, we tracked this cost element as costs for sampling each site per target species.

6. **Per-sample consumable cost.** Consumables needed to filter each water sample in the field included 1 water filter per sample as well as gloves, vials and medium (e.g., ethanol) for preserving each water filter. For this cost element, we tracked the cost of a single filter, vial, gloves, and appropriate amount of preservative for 1 sample.

7. **Water filter sample analysis.** Analysis of each water filter sample requires extracting DNA from the filter and conducting qPCR to identify DNA from target species in the sample. We tracked the labor and lab costs for these procedures to estimate the analysis cost per sample for a single-species analysis. These costs are unique to our own lab and are likely to differ from the cost of sample analysis at commercial or governmental labs.

Table 7-1. Cost Model for Environmental DNA Technology

Cost Element	Data Tracked During the Demonstration	Estimated Costs
Primer/probe set development for 8 target species	Labor costs to analyze available sequence data and create sets of diagnostic primer/probe sets.	$305
Sample collection from target and closely related species	Cost to obtain samples from researchers or field collection	$1045
qPCR test validation	Labor and lab costs for optimization of site-specific qPCR tests	$1118
Sampling equipment procurement	Cost of periodic (estimated 2 years) replacement of handheld vacuum pump and tubing. Equipment for decontamination of equipment and boots between sampling sites. Annual labor costs for procuring equipment.	$259
Water filter sample collection	Labor and on-site travel costs required for sample collection for each demonstration site	Fort Huachuca: $15.2K labor + 11.8K travel 78 sites, 3 species $168 mean cost/site/species Eglin AFB: ($4.9K labor + $3.9K travel) 58 sites, 2 species $113 mean cost/site/species Yakima TC: ($14.7K labor + $11.2K travel) 87 sites, 3 species $147 mean cost/site/species
Per-sample consumable cost	Rate of consumables used for collecting and filtering each sample	$6
Water filter sample analysis	Per-sample cost of analyzing samples using qPCR tests	$30 without field supplies accounted for in previous element

7.2 COST DRIVERS

Cost elements 1-4 are front-end costs that must be completed for each species. Cost drivers for these elements include the availability of existing 1) primer/probe sets or sequence data for target species in the installation area, 2) Tissue samples for target and closely related non-target species, and 3) adequate qPCR protocols for target species. For cost element 1, per-species costs could be substantially reduced if existing species-specific primer/probe sets could be used and would be substantially increased if new sequence data need to be developed. For cost element 2, costs would be lower if DNA tissue samples were already owned by or easily obtained by the operating laboratory and increases with the number of samples that need to be collected in the field.

If an adequate qPCR test is available for a target species, costs for elements 1-3 would be significantly reduced, although for each new laboratory there are some costs involved in validating assay performance.

Costs for most elements may depend on the number and characteristics of target species included in a monitoring program. For cost element 2, if monitoring includes more than one target species that are closely related, they may share the same set of non-target species, and sequence/sample acquisition costs would be lower than if they are phylogenetically distant. This is the case for target species at the YTC demonstration site, where all three target species share the same set of non-target species, as described in section 2.2. However, if target species are very closely related to each other or to a non-target species, this could be problematic for validating species-specific assays, as with the tiger salamander subspecies at the Fort Huachuca demonstration site.

Equipment procurement costs (element 4) may be lower when monitoring programs include more than one species, even when the target species sampling seasons differ, because equipment could be shared for collecting and filtering water samples for each target species.

For the initial year of our demonstration, we collected our eDNA water filter samples (element 5) concurrently with collaborators conducting seine or dip netting surveys for target species. This approach allowed us to directly compare detection probabilities between methods and assess the accuracy of eDNA methods. This comparison is useful but not essential for effective implementation of new eDNA sampling programs (see section 8.6). Concurrent sampling in the initial season of a new program provides information that can be used to revise sampling protocols to improve efficiency in subsequent years. In this demonstration, the first sampling season at each site functioned as a pilot season. Our cost analysis is based on actual costs tracked during the demonstration, including any additional cost for assessing and improving sampling efficiencies. However, in Table 7-2 we included estimated costs of concurrent field surveys during the first season of eDNA sampling. When concurrent surveys are not conducted, or when eDNA sampling can be conducted in collaboration with other groups, the estimated costs of the first season of eDNA sampling can be lower than those provided in Table 7-2.

Considerable cost savings could be realized for cost elements 5 and 6 if two or more target species could appropriately be sampled in the same season and at the same sampling sites. In this case, all target species could potentially be detected from each water sample, substantially reducing the cost of labor, travel among sites, and field consumables. In our demonstration, this was the case for the two target species at Eglin AFB (flatwoods salamander and ornate chorus frog), which may occur at the same sites within the same timeframe. At Fort Huachuca, however, sampling for two target species (Chiricahua leopard frogs and Sonora tiger salamanders) occurs in different seasons and generally at different sites, and costs associated with water sample collection and filtration could not be combined for these species.

7.3 COST ANALYSIS AND COMPARISON

7.3.1 Cost comparison with current surveys for target species

Fort Huachuca – Sonora tiger salamander

We conducted concurrent sampling for Sonora tiger salamanders with field crews from Arizona Game and Fish Department in the San Rafael Valley adjacent to Fort Huachuca. The AZGFD crews were using conventional seine netting for Sonora tiger salamanders following an established capture-based survey protocol. We collected water samples for eDNA analysis immediately prior to seining. We compared costs for the two survey methods by tracking costs for eDNA sampling during three sampling sessions in 2013 and 2014 and comparing those costs with field survey cost estimates provided by AZGFD (Table 7-2). For comparison purposes, we based cost estimates on survey costs for one principal investigator/principal biologist and two field technicians sampling 20 sites in one week. Because we tested each filter sample for American bullfrogs as well as Sonora tiger salamanders, eDNA costs are higher than they would be had filter samples been tested solely for Sonora tiger salamanders.

Front end costs: Costs for cost element 2 (target and non-target sample collection) were relatively low for the Sonora tiger salamander, in part because some existing samples were available, and because only one closely related non-target species was relevant to development of the eDNA protocol (section 2.2). However, costs for element 3 (qPCR test development) was high due to the difficulty of validating a test for Sonora tiger salamander that excluded the barred tiger salamander. Equipment costs (element 4) were similar between field and eDNA surveys.

Per sample costs: Costs for sample collection/field surveys (element 5) were higher for seine netting than for eDNA sample collection, but this difference was solely attributable to higher burdened labor rates for AZGFD personnel compared to UI/WSU personnel. Per-sample consumables (element 6) were very low for seine netting compared to the filters and other supplies required for each eDNA water filter sample. There were no post-survey costs associated with seine netting surveys, while eDNA sampling required laboratory costs for sample analysis (element 7) of approximately $30 per sample.

Overall, the extensive front end costs for eDNA sampling resulted in total implementation costs that were about twice those for seine netting. However, front end costs (elements 1-4) were incurred only in the first year of eDNA sampling. For subsequent years, costs of one eDNA sampling session would be about 8% less than one seining survey. However, to achieve equivalent detection probabilities ≥ 0.95 as described in Section 6, ongoing eDNA sampling would require one survey per year with three samples per site, while two seining surveys would be needed per year for each site. Annual costs of ongoing sampling would be $5,351 for eDNA surveys and $11,164 for seining surveys.

Eglin AFB – Reticulated flatwoods salamanders

We conducted concurrent sampling for reticulated flatwoods salamanders with field crews from Virginia Tech at Eglin AFB. The Virginia Tech crews were using conventional dipnetting for flatwoods salamanders following an established capture-based survey protocol.

To prevent contamination between sites, we collected water samples for eDNA analysis immediately prior to dipnetting, and at least two weeks after any previous dipnet survey. We tracked costs for eDNA sampling during three sampling sessions in 2013, 2014, and 2015. We adapted our sampling strategy in the final year to improve detection probabilities and collected eight eDNA water samples when wetland pH was less than five and four samples when pH was greater than 5. For this comparison we assumed that, for a typical 20-site sampling season, 10 sites would have pH < 5 and 10 would have pH > 5, resulting in 120 samples collected during the season. We compared costs for eDNA sampling at 20 sites with field survey cost estimates provided by VT (Table 7-2). For comparison purposes, we based cost estimates on survey costs for one principal investigator/principal biologist and one field technician sampling 20 sites.

Front-end costs: Costs for sample collection for target and closely related non-target species (cost element 2) was high due to the number of species for which tissue collection was required and the cost of travel to Eglin AFB from Idaho. Costs for qPCR test validation (element 3) were moderate and benefitted from collaboration with USGS researchers (McKee et al. 2015). As in Arizona, equipment costs (element 4) were similar between field and eDNA surveys.

Per-sample costs: Water collection/field survey costs (element 5) were comparable for eDNA and dipnetting surveys for a single sampling session. However, the flatwoods salamander field survey protocol used at Eglin AFB calls for multiple surveys during the breeding season, with the number of surveys ranging from one survey at little-used sites to eight surveys at core sites, and an average of four surveys per site per season. If a single session of eDNA sampling can yield similar detection rates for flatwoods salamanders as multiple sessions of dipnetting, eDNA water sample collection costs could be up to one-third the cost of field surveys under the current survey protocol. Flatwoods salamander field surveys do not require consumables or water sample analysis, but eDNA surveys incurred a combined cost of about $43 per sample for per-sample consumables (element 6) and sample analysis (element 7). Because we tested each filter sample for ornate chorus frogs as well as flatwoods salamanders, eDNA costs are higher than they would be had filter samples been tested solely for flatwoods salamanders.

The overall costs for implementing an eDNA sampling program were about 3.5 times higher in the first year than annual dipnetting surveys, based on four eDNA samples per site and four dipnetting surveys per year. For ongoing eDNA monitoring beyond the first year, annual costs were limited to cost elements 5-7. In subsequent years eDNA surveys would require only two samples per site, and a single eDNA survey of 20 sites would be about 3 times higher than a single session of dipnetting, due to costs for per-sample consumables and water sample analysis for eDNA sampling. After the initial year of eDNA surveys, costs for a single session of eDNA sampling per year would be about 25% lower than for repeated dipnetting surveys.

Table 7-2. Comparison of Costs for Conventional Netting and eDNA Surveys for Sonora Tiger Salamanders (AZ) and Flatwoods Salamanders (FL)

Costs were tracked for concurrent surveys using both methods in 2013-2014 for Sonora tiger salamanders and in 2013-2015 for flatwoods salamanders.

Cost Element	Data Tracked During the Demonstration	Estimated Costs (AZ)[1]	Estimated Costs (FL)[2]
Primer/probe set development for each target species	Seine or dip netting NA eDNA Labor costs to analyze available sequence data and create sets of diagnostic primer/probe sets.	Seine netting NA eDNA $610	Dip netting NA eDNA $610
Sample collection from target and closely related species	Seine or dip netting NA eDNA Cost to obtain samples from researchers or through field collection.	Seine netting NA eDNA $841	Dip netting NA eDNA $6,169
qPCR test development	Seine or dip netting NA eDNA Labor and lab costs for optimization of site-specific qPCR tests.	Seine netting NA eDNA $2,092	Dip netting NA eDNA $1,247
Sampling equipment procurement	Seine or dip netting Periodic replacement of nets, waders, and buckets. eDNA One-time purchase of equipment for collecting and filtering water samples and decontaminating equipment and boots between sampling sites.	Seine netting $450 eDNA $518	Dip netting $500 eDNA $518
Field surveys: Seine or dip netting **eDNA: Water filter sample collection**	Seine or dip netting Average labor and travel costs required for sample collection for each survey session. Costs are based on 20 sites per survey session. eDNA (first year, if comparing eDNA sampling with concurrent field surveys) Average labor and travel costs required for sample collection for each survey session *plus* additional labor costs for conducting concurrent seine or dipnetting survey. Costs are based on 20 sites per survey session. eDNA (subsequent years) Average labor and travel costs required for sample collection for each survey session. Costs are based on 20 sites per survey session.	Seine netting $6,162 for one survey of 20 sites eDNA (first year) $5,292 ($3,612 for one eDNA survey of 20 sites *plus* $1,680 for concurrent seine netting survey of 20 sites) eDNA (subsequent years) $3,612 for one survey of 20 sites	Dip netting $990 for one survey of 20 sites $3,960 for season (4 surveys per site) eDNA (first year) $2,419 ($1,817 for one eDNA survey of 20 sites *plus* $602 for concurrent dip netting survey of 20 sites) eDNA (subsequent years) $1,817 for one survey of 20 sites

Table 7-2. Comparison of Costs for Conventional Netting and eDNA Surveys for Sonora Tiger Salamanders and Flatwoods Salamanders (Continued))

Cost Element	Data Tracked During the Demonstration	Estimated Costs (AZ)[1]	Estimated Costs (FL)[2]
Per-sample consumable cost	Seine or dip netting Quaternary ammonia used for disinfecting boots and equipment. Cost is per site for 20 sites. eDNA Rate of consumables used for each sample.	Seine netting $3 per site $60 for 20 sites eDNA $6 per sample $460 for 20 sites	Dip netting NA eDNA $6 per sample $690 for 20 sites
Water filter sample analysis	Seine or dip netting NA eDNA Per-sample cost of analyzing samples using qPCR tests.	Seine netting NA eDNA $37 per sample $2,960 for 20 sites	Dip netting NA eDNA $37 per sample $4,440 for 20 sites
TOTAL COST OF INITIAL SURVEY SEASON	Seine or dip netting Combined cost of labor, travel, equipment, and consumables for one field survey season for 20 sites. eDNA Combined cost of all eDNA cost elements for one sampling season for 20 sites including cost for concurrent seine or dip net survey.	Seine netting $5,582 for one survey of 20 sites; $22,328 for season (4 surveys of 20 sites) eDNA $12,774 for one survey of 20 sites, one concurrent seine netting survey of 20 sites, and all front-end costs	Dip netting $4,460 for season (4 surveys per site of 20 sites) eDNA $16,092 for one survey of 20 sites, one concurrent dip netting survey of 20 sites, and all front-end costs (4 samples per site)
TOTAL COST OF ONGOING SURVEYS	Seine or dip netting Combined cost of labor, travel, equipment, and consumables for one field survey season for 20 sites. eDNA Combined cost of labor, travel, equipment, and consumables for sample collection and sample analysis for one sampling season for 20 sites.	Seine netting $5,582 for one survey of 20 sites; $11,164 for year (2 surveys of 20 sites) eDNA $5,351 for one survey of 20 sites (3 samples per site)	Dip netting $4,460 for season (4 surveys per site of 20 sites) eDNA $3,361 for one survey of 20 sites (2 samples per site)

[1] Arizona sampling included field and eDNA surveys targeted for Sonora tiger salamanders and American bullfrogs.

[2] Florida sampling included dip netting and eDNA targeted for reticulated flatwoods salamander and ornate chorus frogs.

7.3.2 eDNA water filter sample analysis costs

While we can estimate the unit cost of sample analysis in our own lab or a similar academic or governmental lab, the cost of sample analysis in a commercial lab is likely to be greater. Where data were available, we compared our sample analysis costs with estimated costs from one commercial and one governmental lab (Table 7-3). Because different labs use different approaches for qPCR test development and cost tracking, available cost data for comparison are limited to the costs of laboratory analysis of water filter samples. Additionally, costs provided by external labs likely include room for errors that use time and supplies (common to any lab), while costs calculated for this demonstration are the raw costs of time and supplies used to analyze samples. Additionally, machines for the demonstration were provided by the hosting universities and under the Defense University Research Instrumentation Program, and no per-sample charges were incurred on this project to use the machines. Therefore, estimated costs include costs for labor and consumables for eDNA extraction, qPCR tests, and quality controls only. They do not include water sample collection or filtration.

Table 7-3. eDNA Water Filter Sample Analysis Costs for this Demonstration Compared with Estimated Per-sample Costs from Other eDNA Labs to Detect a Single Species

** WSU/UI Labs cost provided is only the direct cost of sample analysis and does not include overhead, equipment depreciation, labor for general lab maintenance, or margin of error, while costs for a commercial company are expected to include all of these elements. Therefore, per-sample costs incurred during this project are not equivalent to per-sample charges that would include the elements listed above.*

Environmental DNA sample processing laboratory	Estimated Per-sample Costs/Charges
WSU/UI Labs	$30*
ERDC[1]	$43-107 (depending on number of samples)
GENIDAQS[2]	~ $150 (depending on number of samples)

[1] Laboratory costs for the US Army Corp of Engineers Engineer Research and Development Center (ERDC) provided by Dr. Richard Lance.

[2] Laboratory charges for Genidaqs, a commercial aquatic monitoring lab (www.genidaqs.com), provided by Dr. Scott Blankenship.

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8.0 IMPLEMENTATION ISSUES

8.1 REGULATORY ISSUES

Most field surveys for species that are listed as threatened or endangered under the ESA require "incidental take" permits or "recovery" permits from USFWS or NOAA Fisheries (USFWS 2013). These permits can take anywhere to several months to over a year to receive, and may delay implementation of surveys and monitoring for listed species. Unlike many conventional survey methods for aquatic surveys, eDNA sampling is non-invasive and does not disturb the target species or its habitat. Collection of eDNA water samples is similar to water sampling for other types of monitoring such as collection of water samples for water quality monitoring or microbial sampling. We thus expected that take or recovery permits under the Endangered Species Act would not be required for an eDNA-based monitoring program for listed species.

We consulted with USFWS Ecological Services staff, who are tasked with evaluating impacts to listed species and providing incidental take permits, to assess the need for permits for collection of water filter samples in aquatic systems where listed species occur. At the level of the state Ecological Services Field Office, USFWS staff in Arizona and Florida concurred that permits would not be required for eDNA water sampling. In Washington, however, USFWS staff were unable to agree that, as a general policy, permits would not be required for collecting eDNA water samples in streams that support bull trout populations. They were primarily concerned that technicians collecting water samples might step on and disturb bull trout redds during spawning season.

Because we could not receive consensus about permitting requirements at the state level, we have initiated conversations with USFWS staff at the national level to develop a framework for permitting requirements for eDNA sampling. These conversations are ongoing, but at this time we have not established that eDNA surveys would have lower permitting requirements than conventional field surveys for aquatic species.

8.2 ENVIRONMENTAL DNA CONCEPTUAL MODEL

The overall goal of our ESTCP project was to improve the understanding and ease by which DoD managers could apply the eDNA method on individual installations and surrounding lands. We specifically kept our scientific approach broad and process-based in an effort to improve the general understanding of the application of eDNA. To do this, we created a conceptual and mathematical model on the production, fate, and transport of eDNA along with SERDP project RC-2240 and ESTCP project 201205. Although we did not directly test the model, we used a conceptualization of the important processes to guide our work. We published an early version of the model in 2015 (Figure 8-1; Strickler et al. 2015). An updated version of the model will be added to the manuscript on eDNA transport in streams, currently in preparation (Appendix D).

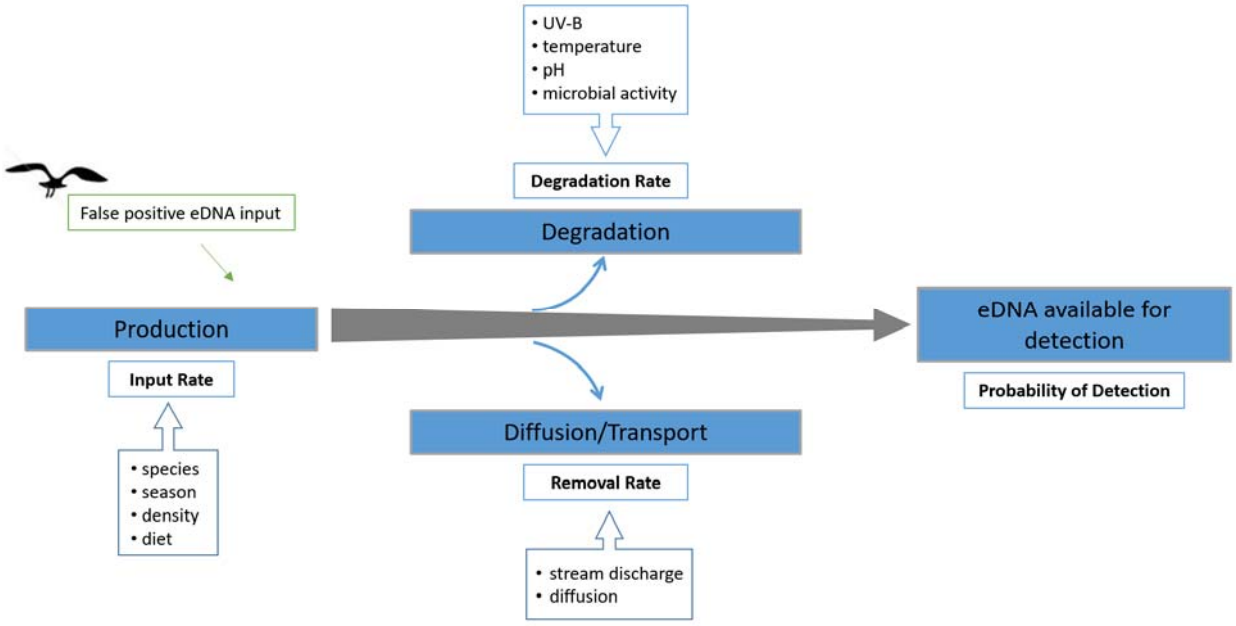

Figure 8-1. Conceptual Model of Factors Affecting eDNA Detection

The conceptual model outlines the processes that influence eDNA detection in aquatic system (production, degradation, transport, and uptake). For each process, we describe the factors that might influence the rate at which eDNA lost or gained in the system, which ultimately influences detection. In this project, we did not directly analyze production (the production of eDNA input into the water body), but we did find that it varied greatly among bullfrog tadpoles in microcosms under identical conditions (Strickler et al. 2015). We conducted two experiments to test the influence of environmental conditions on degradation and on uptake, measured as loss during transport. The goal of both these studies were to measure empirically the main degradative and uptake processes in order to generalize understanding to a broader set of waterbodies. For degradation, we tested the influence of pH, UV radiation, and temperature on rate of loss of eDNA (Strickler et al. 2015, Appendix C). For uptake, we tested the influence of stream complexity on eDNA loss downstream (mean residence time; Appendix D). In both cases, we will be able to calculate the expected loss rate of eDNA across many (but not all) systems with varying degrees of environmental variability.

Given the process-based framing of the project, our individual studies help inform a general understanding the biophysical processes control eDNA transport and fate, and ultimately detection across environmental heterogeneity. Future work expanding the range and breadth of processes controlling eDNA in aquatic environments will help improve the eDNA method of detecting rare species.

8.3 SPATIAL AND TEMPORAL ISSUES

8.3.1 Spatial issues in eDNA survey implementation

Unlike direct observations of species presence, the presence of eDNA at a location, especially in a lotic system, may not indicate the presence of the species. There are two main examples of this: 1) eDNA may flow into an installation from an upstream source; 2) eDNA may be deposited from an allochthonous source such as waterfowl feces. Because of these issues, the presence of a species should not be directly inferred from a single eDNA detection, especially if that detection indicates a very low level of eDNA in the sample. Thresholds for management action (i.e. what constitutes an actionable positive) will have to be established by system and consider the consequences of Type I and Type II errors (Darling and Mahon 2011). This is an active area of research and debate in the eDNA field (reviewed in Goldberg et al. 2016) and will require additional experimentation to distinguish the signal of low-density populations from allochthonous or non-viable sources (e.g., Dunker et al. 2016) and the development of risk management strategies to put results in appropriate context for regulation and management action.

Environmental DNA samples should always be collected and analyzed as replicates to be able to understand the strength of evidence for presence of a species at a site. The amount of eDNA collected at a site can be highly variable among sample replicates (Pilliod et al. 2013), and the detection of a large amount of eDNA in a sample is stronger evidence for species presence than one sample with a small amount of eDNA detected of the target species. For some applications (e.g., the detection of aquatic invasive species), several strongly positive eDNA samples may need to be found to trigger management actions if field surveys cannot locate the species.

For installations that do not include the headwaters of the lotic systems they contain, samples collected upstream of the installation should be collected if the location of species along a lotic system is in question. The longest reported distance for eDNA transport is 9.1 km (Denier and Altermatt 2014). If positive samples begin on the installation but not above, it can be concluded that the species is present on the base (assuming the source is not allochthonous; see next paragraph). Quantification of eDNA in samples can help determine whether additional individuals are adding eDNA to the system below the boundary; however, more research is required to understand the relationship between eDNA removal and transport in lotic systems to form predictions of expected values if additional individuals are present. Over whole stream reaches (1 km), the quantity of eDNA in a sample can be correlated with population density or biomass for amphibians, but within a stream reach eDNA quantities can be uncorrelated with local sampling (Pilliod et al. 2013). Environmental DNA does not solely accumulate with downstream distance (Laramie et al. 2015, this study), but may reflect population density at some scale. Determining this scale requires additional research; until then, eDNA detections within 10 km of installation boundaries on the upstream end of lotic systems should be viewed with caution.

Environmental DNA can also be transported by wildlife and added to systems (allochthonous eDNA). This eDNA is expected to be at low concentrations and potentially be irregular through time (although the habitual use of an area such as a rookery may cause routine allochthonous inputs). While this has not been documented in natural systems yet, it is telling that prey species eDNA can be detected in the feces of a predator for many days after ingestion (Merkes et al. 2014).

8.3.2 Temporal issues in eDNA survey implementation

Environmental DNA in water samples has a short half-life and likely becomes undetectable within a few weeks (Dejean et al. 2011; Thomsen et al. 2012), depending on original concentration and degradative forces (Strickler et al. 2015). However, eDNA in sediments can last much longer, up to thousands of years (Giguet-Covex et al. 2014). Therefore, it is important to recognize the temporal inference of the sample collected; sediment samples should only be analyzed if the question of interest is whether the target species has ever been present at the site. For aquatic sampling, eDNA can extend the detection time frame over visual surveys by a few weeks, but sampling design should target the timing where the species is most likely to be present at the highest density at the life stage of interest for the study, and err on the side of being a little late rather than a little early. The exception to this is when wetlands may dry for the season, making water collection impossible.

8.4 INFLUENCE OF DENSITY IN RELATION TO DEGRADATIVE FORCES

Density of species likely has the largest influence on detection using eDNA and may also be correlated with factors that influence eDNA detection through degradation rate, such as acidity. The wetlands at Eglin Air Force base are particularly challenging in this regard, as they vary from highly acidic (3.8) to neutral (7.0). As density of individuals is by design unknown when using eDNA sampling, differentiating between these two causes of low detection rate is not possible. However, when occupancy (presence/absence) is the goal of the study, it is not necessary to distinguish between the two factors; they both lead to the same sampling design. That is, whether lower detection rates at more acidic ponds was caused by high degradation rate, low densities, or both, the solution was the same: sample more locations in a wetland to increase the probability of detection. This method is effective whether eDNA is not diffusing as far from an individual because of high degradation rate or whether there are fewer individuals producing eDNA. Additionally, it accounts for within-wetland heterogeneity of acidity by increasing the probability of sampling more alkaline areas. The results at this site in the final year of study indicate that additional sampling locations within a wetland should be included when wetlands are both large (>2 ac) and acidic (pH < 5.0).

8.5 PROCUREMENT ISSUES

During the course of this demonstration, manufacture of the filter cup that we used for all collections was discontinued. Recognizing the reliance of this method on filter materials, we conducted an experiment (Appendix E) to see what materials would be adequate for this application. We compared the quantity of eDNA captured from freshwater samples using four filter materials in three nominal pore sizes (0.45 µm, 1.2 µm, and 5 µm). Because an optimal filter would be one that maximizes the quantity of eDNA captured while minimizing filter clogging and the time required for filtration, we also evaluated the filters' efficiency by comparing the sample volume that could be filtered through each and the time required to filter the final volume.

We found that polycarbonate track-etched (PCTE) filters consistently captured less eDNA relative to the three other materials (cellulose nitrate [CN] mixed cellulose ester [MCE], and polyethersulfone [PES]). The polycarbonate filters were also less efficient, often yielding lower sample volumes and requiring longer filtration times compared to other filter types.

The other filter materials performed comparably well, with similar eDNA yields, sample volumes, and filtration times. Filter pore size did not influence the amount of eDNA captured, but smaller pore sizes were generally associated with longer filtration times.

The reduced eDNA capture and filtering efficiency of PCTE filters suggests that PCTE may be a poor choice for eDNA-based research and monitoring. Our results indicate that MCE, CN, or PES filters would be preferable to PCTE and have similar effectiveness in capturing eDNA.

Larger sample volumes, which generally result in higher eDNA capture and detection, often require extensive filtration times and/or limited total volume with small pore sizes. Therefore, in systems where smaller pore size filters are likely to become clogged, the most effective filter is likely to be one that efficiently filters high sample volumes.

An additional consideration for filter selection is ease of use in the field. Although larger filter pore sizes may allow greater sample volumes in systems with high turbidity or organic matter, filters with larger pore size are generally not available in sterile, disposable filter cups. Using these filters requires dismantling a disposable filter cup and replacing the existing filter with the selected filter. This process not only requires an extra task in the field; it may also create an additional chance for cross-contamination of samples.

An additional major issue remains for installations wishing to implement targeted eDNA sampling of species. There is still a very limited number of labs offering analysis services for these samples, and no certification program to ensure the quality of the analysis. To address this concern, we have developed guidelines for practitioners interested in partnering with laboratories to analyze eDNA samples (Appendix I). Additionally, we have worked with eDNA researchers from around the world to develop a set of laboratory guidelines in the peer reviewed literature (Goldberg et al. 2016).

8.6 INITIATING AN ENVIRONMENTAL DNA SAMPLING PROGRAM

Environmental DNA methods successfully detected each of the target species in this demonstration under a range of conditions that included factors expected to limit eDNA detection (e.g., low pH). The results can help guide implementation of eDNA sampling for inventory and monitoring programs.

We found that spatial sampling design, sample volume, and filter characteristics can have a strong effect on detection probabilities. Practitioners who are implementing eDNA sampling for a novel species or system should consider these factors carefully in determining an appropriate sampling approach. When initiating eDNA sampling programs for situations in which eDNA sampling approaches have been successfully demonstrated for similar species and aquatic systems, practitioners can use the sampling design from existing applications to inform sampling, which will likely result in similar detection probabilities.

For applications in new types of systems, a pilot survey is useful for identifying environmental and sampling factors that might limit detection, which can then be used to adjust sampling strategies to improve detection probabilities. This may be particularly important when high detection probabilities are needed to provide greater certainty about the presence of target species at particular sites, as is the case for most endangered or threatened species. A pilot survey can help practitioners evaluate whether the eDNA sampling strategy detects the target species with sufficient accuracy and sensitivity to meet survey objectives.

In a pilot survey, three or more samples are collected at each site so that detection probabilities can be estimated from replicate samples. Occupancy modeling can be used to determine factors limiting detection (e.g., area, water quality parameters, current velocity, sample volume) if those factors are measured at the same time pilot samples were collected. If detection probabilities are low and are strongly influenced by environmental or sampling factors, it may be possible to improve detection probabilities by adapting the sampling strategy to address the influence of those factors. For example, in this demonstration, detection of flatwoods salamanders was influenced by pH, and detection probabilities were improved by modifying the spatial arrangement and volume of samples at acidic wetlands.

Concurrent field sampling can be useful but is not necessary for initiating an eDNA sampling program. In novel systems, it is helpful to compare error rates (sampling events in which the target species was detected with eDNA sampling but not with field surveys, and vice versa) to determine which method produces consistently higher detection rates. This comparison allows managers to select the most efficient and reliable survey method, whether that may be field surveys, eDNA surveys, or an integration of the two methods.

8.7 SPECIES-SPECIFIC RECOMMENDATIONS

8.7.1 Lentic systems

Integration with standard survey methods. If a species can be quickly detected through visual or audio surveys (e.g., Chiricahua leopard frogs, American bullfrogs, ornate chorus frogs), it may be most beneficial to conduct those surveys prior to eDNA sample collection. If the species is not detected, water samples can be collected using whirlpaks and placed in the shade while dipnet surveys are conducted (note that dipnets should not enter the water before samples are collected as they are likely to carry eDNA). If the species is not detected using dipnets, the water samples can then be filtered for eDNA analysis.

Arizona – moderately degradative environment (pH: 6.7-10.1, temperature: 10.7-30.8°C, area: 50-3409 m^2.)

Filter pore size and material. At sites where 250 mL samples can be collected using 0.45 µm cellulose nitrate filters, this can provide a useful level of detection (e.g., four samples for >0.95 detection of Chiricahua leopard frogs). For sites where these filters clog before reaching the goal volume, 5 or 6 µm cellulose or mixed cellulose ester filters can be used. For Sonora tiger salamanders, three of these larger pore size filters were necessary for >0.95 probability of detection. For American bullfrogs, a mix of these filter types depending on area also produced an estimation of three samples for >0.95 probability of detection.

Spatial integration of samples. If wetlands are small (<40 m diameter), samples collected from one targeted location seem to be sufficient for high detection probabilities. This location is most likely to be successful if the most likely area being used by the species (shallow and sunny with emergent vegetation) is selected. For wetlands > 40 and < 55 m in diameter, samples integrated from two locations at maximal distances can be collected. If the wetland is larger, sample locations can be located every 50 m around the perimeter.

Florida – highly degradative environment (pH: 4.1-6.7, temperature: 9.2-24°C, area: 726-35,612 m^2)

Filter pore size and material. In these wetlands, larger pore size (5 µm PES) filters were required to filter the larger volumes (500 mL) necessary for detection. Given this design, three samples were required to reach > 0.95 detection probability for flatwoods salamanders. This number was two for ornate chorus frogs, which are not the main target of surveys but should be highly detectable using this design when they are present (i.e. during the breeding season).

Spatial integration of samples. For neutral wetlands (pH 6-8), samples integrated in equal amount from four locations around the wetland were sufficient. For more acidic (pH < 5), eight locations were required, except at sites with very low densities. For large (>8000 m^2) acidic sites, more sampling locations need to be included to ensure detection. This could be by increasing sampling density or targeting areas based on habitat characteristics.

8.7.2 Lotic systems

Environmental DNA in lotic systems is mixed by the action of the stream and therefore not limited by the diffusion processes that complicate sampling in lentic systems. Studies have found very high detection probabilities for eDNA sampling of stream amphibian populations (0.92-0.97 for 1L;(Pilliod et al. 2013) and chinook (0.93 for 1 L at base flow;(Laramie et al. 2015) with 0.45 µm cellulose nitrate filters. Recent work has estimated that detection of brook trout is >0.99 at population-level densities (≥3 fish per 100 m) and 0.50 for low densities (1 fish/100 m) in 5 L samples taken with 1.5 µm glass fiber filters ((Wilcox et al. 2016).

Results from this study indicate very high detection for streams with known bull trout populations for the demonstrated protocol (1 L samples collected using 0.45 µm cellulose nitrate filters). Even at the lowest density site (5 redds in 150 m transect), half of the samples tested positive, indicating that two 1 L samples would be sufficient. Therefore, for salmonids, we generally recommend collecting duplicate samples of 1 L each using 0.45 µm cellulose nitrate filters. For sites where densities are expected to be very low, we recommend increasing this volume to 5 L and using a peristaltic (rather than a vacuum) pump. Timing should be targeted when eDNA densities will be highest, for example at base flow as demonstrated by Laramie et al. (2015). Our cross-transect study (Section 5.6), which was conducted in streams with 6-11 m average width, indicates that detection probabilities for bull trout may be highest in slower-moving waters, likely because of their habitat use. For bull trout, other salmonids, and stream species with similar habitat use, sampling in areas with slower-moving water may improve detection.

For distinguishing fall from spring-run chinook in eDNA samples, we found that the minimum amount of eDNA required for a correct assignment was only found in samples >338 pg chinook DNA in a 1 L sample but that samples up to 4014 pg chinook DNA/L may not produce enough information for assignment (< 4 SNPs genotyped). False assignment was found for a sample at 341 pg chinook DNA/L and equivocal results for a sample at 925 pg chinook DNA/L, so criteria for sample testing should include both a minimum concentration (e.g., 1000 pg chinook DNA/L) and a minimum number of SNPs genotyped (4). For reference, the site in Laramie et al. (2015) just below a hatchery was estimated at 15170 pg chinook DNA/L.

To increase the overall yield for eDNA samples where the goal is to distinguish between the runs, we recommend sampling additional volume (i.e. 5 L instead of 1 L per sample). Assuming a linear relationship between sample volume filtered and eDNA yield, that would have brought most of the samples up to close to or above the threshold. For these measurements, we note that standards were measured on a NanoDrop spectrophotometer and samples were extracted without removing RNA, so more accurate measures of just DNA (e.g., those taken with a Qubit) would be about half (Goldberg and Cochrell, unpublished data).

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APPENDIX A POINTS OF CONTACT

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APPENDIX B TARGET SPECIES QUANTITATIVE PCR ASSAYS

Fort Huachuca

Species: Chiricahua leopard frog (*Lithobates chiricahuensis*)

Dataset: Goldberg, C. S., K. J. Field, and M. J. Sredl. 2004. Mitochondrial DNA sequences do not support species status of the Ramsey Canyon Leopard Frog *(Rana subaquavocalis*). Journal of Herpetology 38:313-319.

Assay design:

Forward Primer	GGTACCGCTCATATCATGACTACTTG
Reverse Primer	TCCAGTTGGACTCACTTAGGAATG
Probe	TAGGACCTTCGCTTGTTAT-MGB

Publication: Unpublished

Validated species:

American bullfrog (*Lithobates [Rana] catesbeianus*)
Arizona treefrog (*Hyla wrightorum)*
Barking frog (*Craugaster augusti*)
Canyon treefrog (*Hyla arenicolor*)[1]
Chiricahua leopard frog (*Lithobates [Rana] chiricahuensis*)
Couch's spadefoot (*Scaphiopus couchi*)[2]
Great Plains toad (*Anaxyrus [Bufo] cognatus*)[2]
Lowland leopard frog (*Rana yavapaiensis*)
Mexican spadefoot (*Spea multiplicata*)
Red-spotted toad (*Anaxyrus [Bufo] punctatus*)
Sonoran Desert toad (*Incilius [Bufo] alvarius*)
Tarahumara frog (*Lithobates [Rana] tarahumarae*)[3]
Western narrow-mouthed toad (*Gastrophryne olivacea*)[4]
Woodhouse's toad (*Anaxyrus [Bufo] woodhousii)*

[1]Collected by Meryl Mims, [2]Collected by Lauren Chan, [3]Collected by Mike Sredl, [4]Provided by UC Berkeley MVZ, 3 samples only

Species: American bullfrog (*Lithobates catesbeianus*)

Dataset: Austin, J.D., Lougheed, S.C., Boag, P.T., 2004. Controlling for effects of history and nonequilibrium conditions in gene flow estimates in northern bullfrog (*Rana catesbeiana*) populations. Genetics 168, 1491–1506.

Assay design:

Forward Primer	TTTTCACTTCATCCTCCCGTTT
Reverse Primer	GGGTTGGATGAGCCAGTTTG
Probe	TTATCGCAGCAGCAAGT-MGB

Publication: Strickler, K. M., A. K. Fremier, and C. S. Goldberg. 2015. Quantifying the effects of UV, temperature, and pH on degradation rates of eDNA in aquatic microcosms. Biological Conservation 183:85-92.

Validated species:

North Idaho
American bullfrog (*Lithobates [Rana] catesbeianus*)
Sierra treefrog (*Pseudacris sierra*)
Western toad (*Anaxyrus [Bufo] boreas*)
Columbia spotted frog (*Rana luteiventris*)

Arizona
American bullfrog (*Lithobates [Rana] catesbeianus*)
Arizona treefrog (*Hyla wrightorum)*
Barking frog (*Craugaster augusti*)
Canyon treefrog (*Hyla arenicolor*)[1]
Chiricahua leopard frog (*Lithobates [Rana] chiricahuensis*)
Couch's spadefoot (*Scaphiopus couchi*)[2]
Great Plains toad (*Anaxyrus [Bufo] cognatus*)[2]
Lowland leopard frog (*Rana yavapaiensis*)
Mexican spadefoot (*Spea multiplicata*)
Red-spotted toad (*Anaxyrus [Bufo] punctatus*)
Sonoran Desert toad (*Incilius [Bufo] alvarius*)
Tarahumara frog (*Lithobates [Rana] tarahumarae*)[3]
Western narrow-mouthed toad (*Gastrophryne olivacea*)[4]
Woodhouse's toad (*Anaxyrus [Bufo] woodhousii)*

[1]Collected by Meryl Mims, [2]Collected by Lauren Chan, [3]Collected by Mike Sredl, [4]Provided by UC Berkeley MVZ, 3 samples only

Species: Tiger salamander (*Ambystoma [tigrinum] mavortium* and *Ambystoma tigrinum stebbinsi*)

Dataset: Storfer, A., Mech S. G., Reudink, M. W., Ziemba, R. E., Warren, J., Collins, J. P. 2004. Evidence for introgression in the endangered Sonora tiger salamander, *Ambystoma tigrinum stebbinsi* (Lowe). Copeia 2004:783-796.

Assay design:

Forward Primer:	GGCAGATAGTTGGATGCACGATAG
Reverse Primer:	ACTACCTCTTGTCCTGGTTTTCCT
Probe:	CATAATATGTTGCCACGCTACT-BHQPlus

Publication: Unpublished

Validated species:

Sonora tiger salamander (*Ambystoma tigrinum stebbinsi*) [1]
Barred tiger salamander (*Ambystoma mavortium*)[1]

[1]Some samples provided by Andrew Storfer

We were unable to validate an assay for the subspecies *Ambystoma tigrinum stebbinsi* that excluded the invasive barred tiger salamander. We are working with the Arizona Game and Fish Department to develop additional genetic resources for this subspecies.

Yakima Training Center

Species: Bull trout (*Salvelinus confluentus*)

Dataset: Pleyte, K. A., Duncan,S. D., Phillips, R.B. 1992. Evolutionary relationships of the salmonid fish genus *Salvelinus* inferred from DNA sequences of the first internal transcribed spacer (ITS 1) of ribosomal DNA. Mol. Phylog. Evol. 1:223-230.

Assay design:

Forward Primer:	CCGCTATTCCTTTTGCCTAGGGTAG
Reverse Primer:	GCTTCACAATTGGAGACCGTTTCG
Probe:	CACACGGCGCACCTATGGGAG-BHQ

Publication: Unpublished

Validated species:[1]

Brook trout (*Salvelinus fontinalis*)
Bull trout (*Salvelinus confluentus*)
Chinook (*Oncorhynchus tshawytscha*)
Brown trout (*Salmo trutta*)
Coho salmon (*Oncorhynchus kisutch*)
Cutthroat trout (*Oncorhynchus clarkii*)
Lake trout (*Salvelinus namaycush*)
Mountain whitefish (*Prosopium williamsoni*)
Rainbow trout/steelhead (*Oncorhynchus mykiss*)
Sockeye salmon (*Onchorhynchus nerka*)

[1]Samples provided by CRITFC and Matthew Laramie

Species: Brook trout (*Salvelinus fontinalis*)

Assay design (from publication below):
Forward Primer: CCACAGTGCTTCACCTTCTATTTCTA
Reverse Primer: GCCAAGTAATATAGCTACAAAACCTAATAGATC
Probe: ACTCCGACGCTGACAA-MBG

Publication: Wilcox, T. M., McKelvey, K. S., Young, M. K., Jane, S. F., Lowe, W. H., Whiteley, A. R., Schwartz, M. K. 2013. Robust detection of rare species using environmental DNA: the importance of primer specificity. PLoS One 8:e59520.[1]

Validated species:[2]

Brook trout (*Salvelinus fontinalis*)
Bull trout (*Salvelinus confluentus*)
Chinook (*Oncorhynchus tshawytscha*)
Brown trout (*Salmo trutta*)
Coho salmon (*Oncorhynchus kisutch*)
Cutthroat trout (*Oncorhynchus clarkii*)
Lake trout (*Salvelinus namaycush*)
Mountain whitefish (*Prosopium williamsoni*)
Rainbow trout/steelhead (*Oncorhynchus mykiss*)
Sockeye salmon (*Onchorhynchus nerka*)

[1]Note that the assay validated at a higher temperature than published, likely due to change in enzyme.

[2]Samples provided by CRITFC and Matthew Laramie

Species: Chinook (*Oncorhynchus tshawytscha*)

Dataset: Multiple

Assay design:

Forward Primer	CTGGCACMGGGTGAACAGTCTACC
Reverse Primer	AATGAAGGGAGAAGATCGTYAGATCA
Probe	CTCCTGCGTGGGCTAG-MBG

Publication: Laramie, M. B., D. S. Pilliod, C. S. Goldberg. 2015. Characterizing the distribution of an endangered salmonid using environmental DNA analysis. Biological Conservation 183:29-37.

Validated species:[1]

Brook trout (*Salvelinus fontinalis*)
Bull trout (*Salvelinus confluentus*)
Chinook (*Oncorhynchus tshawytscha*)
Brown trout (*Salmo trutta*)
Coho salmon (*Oncorhynchus kisutch*)
Cutthroat trout (*Oncorhynchus clarkii*)
Lake trout (*Salvelinus namaycush*)
Mountain whitefish (*Prosopium williamsoni*)
Rainbow trout/steelhead (*Oncorhynchus mykiss*)
Sockeye salmon (*Onchorhynchus nerka*)

[1]Samples provided by CRITFC and Matthew Laramie

We used markers Ots-171, Ots-271, Ots-180, Ots-321, and Ots-371 from Hess et al. (2011) to identify spring-run Chinook salmon. These were chosen because of lack of amplification in these other species as analyzed by Shawn Narum and staff at Columbia River Inter-Tribal Fish Commission (CRITFC). Eleven additional eDNA samples were provided by Matt Laramie for this test.

Eglin AFB

Species: Flatwoods salamander (*Ambystoma bishopi* and *A. cingulatum*)

Dataset: Pauly, G. B., Piskurek, O., Shaffer, H. B. 2007. Phylogeographic concordance in the southeastern United States: the flatwoods salamander, *Ambystoma cingulatum*, as a test case. Mol. Ecol. 16:415-429.

Assay design:

Forward Primer	GGCCCGTCAACTTTCCTCTAA
Reverse Primer	TGGTCCAGGTAAATCAATTGCA
Probe	TACGGTAATATGTCTGGTACTAC-MGB

Publication: McKee, A. M., D. L. Calhoun, W. J. Barichivich, S. F. Spear, C. S. Goldberg, T. C. Glenn. 2015. Assessment of environmental DNA for detecting presence of imperiled aquatic amphibian species in isolated wetlands. Journal of Fish and Wildlife Management 6:498-510.

Validated species:[1]

Frosted flatwoods salamander (*Ambystoma cingulatum*)
Spotted salamander (*Ambystoma maculatum*)
Marbled salamander (*Ambystoma opacum*)
Mole salamander (*Ambystoma talpoideum*)
Reticulated flatwoods salamander (*Ambystoma bishopi*)
Tiger salamander (*Ambystoma tigrinum*)

[1]Provided by Anna McKee

Species: Ornate chorus frog (*Pseudacris ornata*)

Dataset: Degner,J. F., Silva, D. M., Hether, T. D., Daza, J. M., Hoffman, E. A. 2010. Fat frogs, mobile genes: unexpected phylogeographic patterns for the ornate chorus frog (Pseudacris ornata). Mol. Ecol. 19:2501-2515.

Assay design:

Forward Primer	TRGGTGTCTGCCTCATTCTTCA
Reverse Primer	GGCYACGGATGAGAAGGCTAT
Probe	ATCGCCACTGGCCTATTT-BHQPlus

Publication: Unpublished

Validated species:

Barking treefrog (*Hyla gratiosa*)[1]
Bird-voiced treefrog *(Hyla avivoca*)
Florida (southern) cricket frog (*Acris gryllus*)
Gray treefrog (*Hyla chrysocelis*)[2]
Green treefrog (*Hyla cinerea*)
Little grass frog (*Pseudacris ocularis*)[3]
Northeastern cricket frog (*Acris crepitans*)
Pine barrens treefrog (*Hyla andersonii*)
Pine woods treefrog (*Hyla femoralis*)[4]
Southern chorus frog (*Pseudacris nigrita*)[3]
Spring peeper (*Pseudacris crucifer*)[3, in part]
Squirrel treefrog (*Hyla squirella*)

[1]Provided by Todd Pierson, [2]3 of 5 provided by Paul Moler, [3]Provided by Emily Lemmon-Moriarty, [4]Provided by Anna McKee

APPENDIX C QUANTIFYING ENVIRONMENTAL DNA DEGRADATION

Strickler, K.M., A.K. Fremier, and C.S. Goldberg. 2015. Quantifying effects of UV-B, temperature, and pH on eDNA degradation in aquatic microcosms. Biological Conservation 183:85–92.

Biological Conservation 183 (2015) 85–92

Contents lists available at ScienceDirect

Biological Conservation

journal homepage: www.elsevier.com/locate/biocon

Special Issue Article: Environmental DNA

Quantifying effects of UV-B, temperature, and pH on eDNA degradation in aquatic microcosms

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ARTICLE INFO

Article history:
Received 18 April 2014
Received in revised form 10 November 2014
Accepted 21 November 2014
Available online 31 December 2014

Keywords:
Environmental DNA
eDNA
Degradation
Species detection
UV-B radiation
pH
Temperature

ABSTRACT

Environmental DNA (eDNA) degradation is a primary mechanism limiting the detection of rare species using eDNA techniques. To better understand the environmental drivers of eDNA degradation, we conducted a laboratory experiment to quantify degradation rates. We held bullfrog (*Lithobates catesbeianus*) tadpoles in microcosms, then removed the tadpoles and assigned the microcosms to three levels each of temperature, ultraviolet B (UV-B) radiation, and pH in a full factorial design. We collected water samples from each microcosm at six time steps (0 to 58 days). In all microcosms, most degradation occurred in the first three to 10 days of the experiment, but eDNA remained detectable after 58 days in some treatments. Degradation rates were lowest under cold temperatures (5 °C), low UV-B levels, and alkaline conditions. Higher degradation rates were associated with factors that contribute to favorable environments for microbial growth (higher temperatures, neutral pH, moderately high UV-B), indicating that the effects of these factors may be biologically mediated. The results of this experiment indicate that aquatic habitats that are colder, more protected from solar radiation, and more alkaline are likely to hold detectable amounts of eDNA longer than those that are warmer, sunnier, and neutral or acidic. These results can be used to facilitate better characterization of environmental conditions that reduce eDNA persistence, improved design of temporal sampling intervals and inference, and more robust detection of aquatic species with eDNA methods.

1. Introduction

Effective conservation of imperiled species depends on our ability to reliably detect individuals and quantify uncertainties in detection rates. Similarly, control of invasive species is most successful when the species are found while they are still rare. For species that are difficult to find or identify, a recently developed approach using environmental DNA (eDNA) has been found to improve detection rates for aquatic species (Jerde et al., 2011; Dejean et al., 2012; Pilliod et al., 2013). Environmental DNA in aquatic systems is DNA released into water by aquatic and terrestrial organisms, which can be sampled and used as an effective tool for identifying the presence and distribution of target species. As with any method of surveillance, the reliability of eDNA monitoring requires an understanding of factors that improve or detract from accurate detection (Lodge et al., 2012). Although eDNA methods have shown to be reliable, we lack a quantified understanding of the environmental processes that effect eDNA detection (Díaz-Ferguson and Moyer, 2014).

In concept, three processes determine the availability of detectable DNA in environmental samples: (1) eDNA production, (2) transport and removal of eDNA, and (3) eDNA degradation (Fig. 1). For aquatic eDNA, production, the rate at which DNA is released in the water, is a highly variable function influenced by population density and species-specific characteristics, such as metabolic rates and aquatic habitat use. After eDNA is present in water, it is removed from the source by hydrologic processes (diffusion and advection), which vary in time, space, and type of aquatic system (e.g., lentic, lotic, or marine), by other sources of removal such as binding to and settling with sediment, and by *in-situ* degradation.

Degradation of DNA in water is considered a primary agent for reducing detectability over time (Dejean et al., 2012; Barnes et al., 2014; Pilliod et al., 2014), and thus limits the temporal and related spatial inference of eDNA detection results. Understanding the rates and environmental factors controlling degradation is essential to understanding this scope of inference and improving sampling strategies for eDNA monitoring. DNA is broken down in

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http://dx.doi.org/10.1016/j.biocon.2014.11.038
0006-3207/

water by chemical hydrolysis, primarily through exposure to acid or by enzymatic hydrolysis. Microbial activity in water contributes directly to enzymatic hydrolysis by producing exogenous nucleases that break down DNA into its components (Lindahl, 1993). Although temperature can directly degrade DNA when very high temperatures (>50 °C) cause denaturation, most temperature-related eDNA degradation is likely indirect, as moderately higher temperatures stimulate microbial metabolism and exonuclease activity (Hofreiter et al., 2001; Zhu, 2006; Corinaldesi et al., 2008; Poté et al., 2009; Fu et al., 2012). Exposure to high levels of ultraviolet radiation, particularly ultraviolet B (UV-B) light, can photochemically damage DNA (Ravanat et al., 2001, Häder et al., 2003) to the point where DNA amplification (polymerase chain reaction [PCR]) fails. Naturally-occurring levels of solar radiation can have variable effects on exonuclease activity, and thus eDNA degradation, depending on the type of bacteria present. Ultraviolet radiation can inhibit growth of heterotrophic bacteria or stimulate growth of autotrophic bacteria (Sommaruga, 2001; Häder et al., 2003), consequently decreasing or increasing exonuclease production, respectively. Thus, these factors (pH, solar radiation, and temperature) are likely to interact, either directly or mediated through the biological community, to influence the process of eDNA degradation in aquatic systems.

Recent research has provided experimental evidence that eDNA degrades quickly in water (Dejean et al., 2011; Thomsen et al., 2012a,b; Goldberg et al., 2013; Piaggio et al., 2014). Degradation rates, estimated by measuring eDNA over time following removal of target animals from experimental microcosms or mesocosms, have varied across species and experimental conditions. Dejean et al. (2011) reported that eDNA persisted for 25 days for American bullfrog (*L. catesbeianus*) tadpoles in laboratory microcosms, and Goldberg et al. (2013) found New Zealand mudsnail (*Potamopyrgus antipodarum*) eDNA was detected in laboratory containers for at least 21 days following removal of the organism. In experiments conducted in outdoor containers or ponds, Piaggio et al. (2014) detected Burmese python (*Python bivittatus*) eDNA for at least 2–7 days after removal. Thomsen et al. (2012b) found that eDNA of two larval amphibian species was detectable for 7–14 days (Thomsen et al., 2012b), and Dejean et al. (2011) were able to detect eDNA of Siberian sturgeon (*Acipenser baerii*) for up to 21 days. The lowest eDNA persistence reported to date was for two species of marine fish held in laboratory microcosms, where eDNA was undetectable in about 1–7 days (Thomsen et al., 2012a). None of these studies, however, measured the extent to which environmental conditions influenced eDNA persistence.

Two studies have specifically quantified the effects of particular environmental factors on eDNA degradation. Persistence of Idaho giant salamander eDNA (*Dicamptodon aterrimus*) in outdoor containers was detectable until 8 days under ambient light and temperature conditions, at least 11 days under ambient temperature and reduced light, and at least 18 days in a refrigerated treatment without light (Pilliod et al., 2014). Common carp (*Cyprinus carpio*) eDNA in laboratory mesocosms was estimated to be undetectable at 95% probability after approximately 4 days (Barnes et al., 2014), but was detected in extreme cases as long as 14 days. Degradation rate was negatively correlated with indices of physiochemical factors associated with microorganisms expected to influence degradation (chlorophyll *a*, biochemical oxygen demand (BOD), pH, and total eDNA concentration from any organism in the water sample). Together, these studies illustrate that environmental factors can affect eDNA degradation in different ways. However, none of these studies, nor any others of which we are aware, have explicitly isolated and quantified the primary factors likely to control degradation of eDNA.

In this study, we set out to evaluate eDNA degradation in a controlled setting to better understand the drivers of eDNA degradation and the persistence of eDNA over time. We set up a full factorial study to measure degradation rates of eDNA in laboratory microcosms under different treatments of UV-B, pH, and temperature. Our primary goal was to quantify the effect of these factors, independently and interactively, on the persistence of eDNA. Our second objective was to develop a regression model to help inform eDNA sampling strategies by quantifying variable degradation rates across environments. With this work, we will be better prepared to identify potential areas of high and low degradation and recommend sampling intervals to maximize likelihood of detection under different environmental conditions.

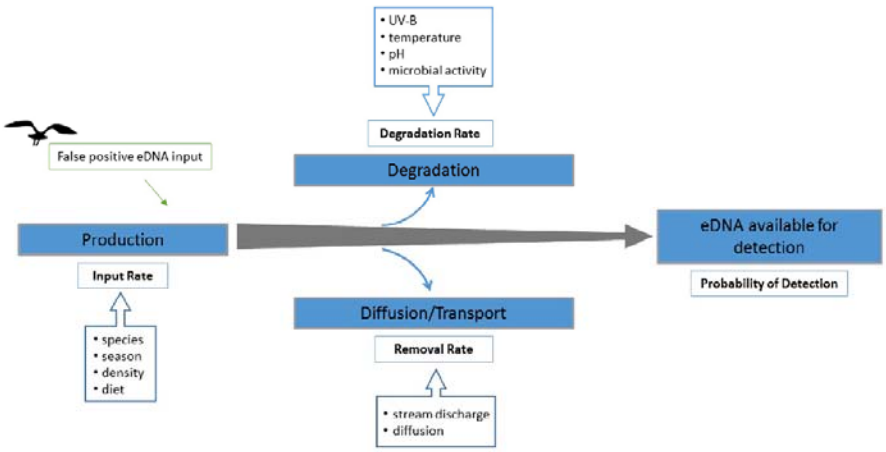

Fig. 1. Conceptual model of factors affecting eDNA detection. Following input of eDNA into an aquatic system, eDNA is removed from the system through degradation and by diffusion and transport processes, reducing the amount available for eDNA detection. Arrow boxes identify some of the biotic and abiotic factors that influence the production, degradation, and diffusion/transport processes. In some situations, it is possible that DNA brought to the sampling site from external sources (including movement by humans, vehicles, or animals) can interfere with estimates of true production by the target species and thus lead to false positive detections.

2. Materials and methods

2.1. Experimental design

We held American bullfrog tadpoles in microcosms for two weeks, then removed the tadpoles and exposed the microcosms to three levels each of temperature, UV-B, and pH in a full factorial design (27 treatments) with three replicates in each treatment. We added nine control microcosms (one to each temperature/pH combination, distributed among UV-B treatments) to monitor potential contamination among treatments, for a total of 90 experimental units. Control microcosms had never been exposed to tadpoles but were otherwise treated the same as experimental microcosms.

We placed large (5–10 cm) tadpoles in polypropylene microcosms at a density of two tadpoles per 3.79-L microcosm. Tadpoles were purchased from Connecticut Valley Biological Supply, where they were sourced from outdoor ponds. Prior to introduction of tadpoles, microcosms were filled with tap water mixed with a mild salt solution (Holtfreter's solution; Cold Spring Harbor Protocol 2009) to prevent infection in experimental animals. Tadpoles were fed twice each day with frozen bloodworms. Uneaten bloodworms were removed after feeding with a clean, disposable pipette. We removed bullfrog tadpoles from microcosms after 14 days (ACUC protocol: University of Idaho 2012-40). Microcosms were then randomly assigned to treatments and placed in growth chambers at the University of Idaho greenhouses. After Day 0 sampling and treatment assignment, we wrapped each microcosm lightly with plastic wrap to prevent contamination.

2.2. Experimental variables

2.2.1. Temperature

We assigned microcosms to one of three temperature levels: 5°, 20°, and 35 °C. Temperature levels were selected to represent the range of temperatures found in streams and ponds in a range of environmental settings. Each treatment temperature level was held in a separate growth chamber automated to maintain constant temperature.

2.2.2. UV-B

We exposed microcosms to three levels of UV-B radiation exposure: 2 kJ/m^2/day, 25 kJ/m^2/day, and 50 kJ/m^2/day. The radiation exposure treatments were selected on the basis of UV-B intensities because, among the wavelengths most likely to be present in terrestrial sunlight, UV-B wavelengths are most harmful to aquatic organisms (Diffey, 2002). Levels of UV-B radiation were selected to represent a range of ambient exposures measured at United States (U.S.) Department of Agriculture UV-B Monitoring Network stations across most of the continental United States (USDA, UV-B-Monitoring and Research Program. Colorado State University, CO, USA; UV-B daily sum data downloaded from http://uvb.nrel.colostate.edu/UVB). We used UVA 340 fluorescent light bulbs (Q-Lab Corporation) to simulate direct solar UV radiation. These bulbs emit light in both the UV-B (315–280 nm) and UV-A (400–315 nm) regions but have no UV output below 295 nm, which is the lower cutoff wavelength for terrestrial sunlight, and do not produce detrimental UV-C radiation (<280 nm) (Q-Lab, 2011). Radiation exposures for each treatment were achieved by varying the height of the lamps over each microcosm.

We monitored UV-B levels in each microcosm every 2–5 days using a hand-held digital Solarmeter radiometer (Model 6.2 UVB, Solar Light Company) to measure UV-B at the water surface, and adjusted lamp height as needed to maintain treatment UV-B levels; water levels declined following removal of water for each eDNA sample. Our radiometers measured irradiance, or the amount of radiation reaching the water surface, measured in W/m^2 (Diffey, 2002). We then controlled radiant exposure, measured in J/m^2/day, in each treatment by adjusting the daily on–off cycles of the UV lamps to maintain the target daily exposure dose. We confirmed that UV-B levels within the microcosms were not affected by plastic wrap covers.

2.2.3. pH

We used levels of pH 4, pH 7, and pH 10 to represent a range of acidic, neutral, and alkaline water found in temperate freshwater systems (Allan and Castillo, 2007). Water in all microcosms was neutral when the experiment began; therefore, microcosms in the pH 7 treatment were not manipulated. Acidic and alkaline treatments were achieved using sterile 1 M HCl or 0.5 M NaOH, respectively. We monitored pH levels to confirm they were stable throughout the experiment.

2.3. Microorganisms

Because we hypothesized that bacteria can play an important role in DNA degradation, we inoculated the microcosms with three bacterial species (*Staphylococcus epidermidis*, *Pseudomonas fluorscens*, *Corynebacterium xerosis*) after tadpole removal. These bacteria are ubiquitous in freshwater systems and have been detected on the skin of American bullfrogs in natural ponds (Culp et al., 2007). We incubated each bacterium overnight and used a spectrophotometer to obtain a turbidimetric measurement of cell density. We then inoculated each container with 10 mL of the bacterial solution after microcosms had been placed in pH, UV-B, and temperature treatments. In addition to the quantified concentrations of bacteria we introduced into microcosms, it is likely that bacteria and other microorganisms were also present on the skin of the experimental tadpoles, and the amount may have varied among microcosms. Although we could not control for experimental changes in the microbial community, we created an index of microbial activity over the course of the experiment by quantifying the total amount of DNA (from any organism) present in each microcosm at Days 0, 21, and 58 and subtracting from it the amount of bullfrog eDNA measured at each time step.

2.4. Sample collection

We collected 250 mL water samples from each microcosm immediately after removal of tadpoles (Day 0) and at 3, 10, 21, 35, and 58 days following removal. Samples were filtered on-site using 0.45 µm cellulose nitrate filters in disposable filter funnels (Whatman product number 1920-7001), and filters were preserved in 95% ethanol. Because extracellular DNA is able to pass through filters with pores larger than 0.2 µm (Matsui et al., 2001, Turner et al., 2014), our filters likely captured DNA that persisted within cells or mitochondria. Filtrate was disposed of following filtration.

2.5. Laboratory analysis

We developed and validated a species-specific quantitative PCR (qPCR) assay for the American bullfrog from previously generated mitochondrial sequence data (Austin et al., 2004) (Table 1).

Table 1
Quantitative PCR assay developed for American bullfrogs (*Lithobates catesbeianus*).

Species	Primer/Probe name	Sequence
American Bullfrog	BullfrogF	TTTTCACTTCATCCTCCCCTTT
	BullfrogR	GCGTTGGATGAGCCAGTTTG
	Bullfrog Probe	NED-TTATCGCAGCAGCAAGT-MGB

Reactions were run using QuantiTect Multiplex PCR Mix (Qiagen, Inc.) with recommended multiplexing concentrations (1X QuantiTect Multiplex PCR mix, 0.2 μM of each primer, and 0.2 μM probe) on an Applied Biosystems 7500 Fast Real-Time PCR System. Reactions were 15 μl in volume and each included 3 μl of sample. Cycling began with 15 min at 95 °C followed by 50 cycles of 94 °C for 60 s and 60 °C for 60 s. We validated this assay *in silico* using PrimerBLAST to confirm that the assay was species specific. We also confirmed that non-target species DNA from a subset of co-occurring species in the western U.S. (Supplementary material, Table A1) did not produce a positive result, defined by any exponential increase in signal from a non-target sample. It is possible that, because these tadpoles were sourced from an outside pond, DNA of eastern U.S. species (e.g., the green frog, *Lithobates clamitans*) was present at the beginning. However, because of time lag between arrival of the tadpoles and the start of the experiment, this DNA is likely to have degraded.

Laboratory analyses were conducted at the University of Idaho's Laboratory for Ecological, Evolutionary, and Conservation Genetics. We extracted filter samples using the Qiashredder/DNeasy method described in Goldberg et al. (2011). All filter sample extractions and qPCR set up was conducted in a lab dedicated to low-quantity DNA samples. Researchers are required to shower and change clothes before entering this room after being in a high-quality DNA or post-PCR laboratory, and no amphibian or reptile tissue samples have been handled in this room. A negative extraction control was included with each set of extractions and an additional negative qPCR control was run with each plate of samples. We used a multi-tube approach for analysis, where multiple reactions were conducted for each sample (Taberlet et al., 1999). We analyzed each sample in triplicate and included an internal positive control (IC; Qiagen) in each well. A positive sample was defined as any sample that showed exponential amplification in all three wells the first time it was tested or in one or more wells from two separate reactions (samples were rerun whenever the original triplicate wells yielded inconsistent results; 6% of samples met this criterion). Quantitative standards consisted of diluted tissue-derived DNA quantified on a Nanodrop spectrophotometer and diluted 10^{-2} through 10^{-6}, run in duplicate. Mean r^2 was 0.99 and mean efficiency was 101.64%.

We quantified the total amount of DNA from all organisms in samples from each microcosm at Days 0, 21, and 58 using a Nanodrop (Thermo Fisher Scientific). We subtracted estimates of bullfrog eDNA from total eDNA to obtain an index of changes in microbial activity over time.

2.6. Statistical analysis

Degradation rate was estimated by the overall change in eDNA detected in each microcosm over the duration of the experiment, as calculated by the slope of the line through all sampling events. Prior to analysis, eDNA values were log-transformed to achieve normality. We used a one-way ANOVA to verify that bullfrog eDNA concentrations at the start of the experiment were not different between treatments. Because starting concentration of eDNA varied among microcosms, we also used linear regression to test whether starting bullfrog eDNA concentration was related to degradation rate. To test for differences in bullfrog eDNA degradation rates among levels of temperature, UV-B, and pH, we used a one-way ANOVA for each factor. We developed a predictive model with main and interaction effects using multiple linear regression. We estimated an overall degradation rate by fitting an exponential decay model to the raw data across all treatments using the exponential decay model $N(t) = N_0 e^{-rt}$, where $N(t)$ is the concentration of bullfrog eDNA measured at time t, N_0 is the bullfrog eDNA concentration at time 0, and r is the degradation or decay rate. We used the estimates for r from the exponential model to calculate the time it would take for eDNA in each treatment to fall below 5% of the mean initial concentration for that treatment. We examined the rate of total eDNA growth over time using linear regression. All statistical analyses were conducted using SAS version 9.3 software (SAS Institute Inc.).

3. Results

Estimated concentration of eDNA in microcosms declined rapidly, with 80–90% of the observed degradation occurring in the first three days after tadpole removal (Fig. 2); however, appreciable amounts of eDNA (>5% of initial concentration) were detected in 53 (of 81) microcosms at Day 3, 35 at Day 10, 21 at Day 21, 13 at Day 35, and 3 at Day 58.

Total eDNA from all sources at Day 0 ranged from 12.55 to 164.53 ng per 250 ml, while starting concentrations of bullfrog eDNA ranged from 0.06 to 5.83 ng DNA per 250 ml water sample. Initial (Day 0) concentrations of bullfrog eDNA did not differ among levels of temperature, pH, and UV-B (Temperature: $F_{(2,80)} = 0.47$; $P = 0.63$; pH: $F_{(2,80)} = 1.65$, $P = 0.20$; UV-B: $F_{(2,80)} = 0.06$, $P = 0.94$). We found no evidence that starting concentration of bullfrog DNA affected degradation rate ($R^2 = 0.02$, $P = 0.13$).

Negative control microcosms (6 of 9) tested positive at 1–3 time steps for minute amounts of tadpole eDNA. This apparent cross-contamination was at very low levels (max = 0.49 pg DNA/250 ml sample) and short-lived (6 microcosms at Day 0, 4 microcosms at Day 3, 1 microcosm at Day 10, and 0 microcosms throughout the remainder of the experiment). Our microcosms were in close proximity to one another (0.1–2 m) within each growth chamber and it is possible that small water droplets were carried between microcosms through aeration, most likely at the outset of the experiment before microcosms were covered with plastic wrap. This issue also occurred in a similar eDNA degradation experiment (Barnes et al., 2014). Because the amounts of eDNA in control microcosms were extremely small, disappeared within the first three time steps, and were detected in all levels of the three treatment factors, it is unlikely that cross-contamination affected the observed patterns of eDNA degradation throughout the experiment.

Across all treatments, temperature exhibited a strong influence on the amount of eDNA detected over time (Fig. 3a; one-way ANOVA $F_{(2,78)} = 9.85$, $P = 0.002$). Degradation rate was significantly lower in the 5 °C treatment compared with 20° and 35 °C

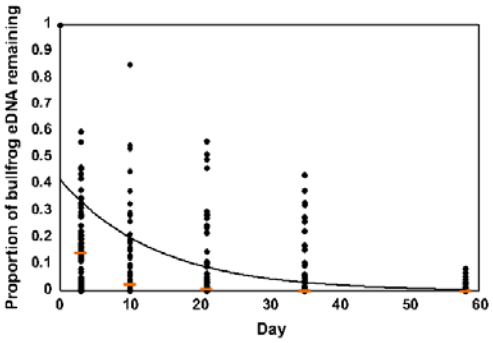

Fig. 2. Proportion of American bullfrog (*Lithobates catesbeianus*) eDNA remaining for 81 microcosms from 0 to 58 days following bullfrog tadpole removal. Horizontal lines represent the median proportion remaining in the sample. Day zero represents the initial amount of detectable bullfrog eDNA in each microcosm.

treatments. Overall degradation rate was higher under pH 4 compared with pH 10 (Fig. 3b; one-way ANOVA $F_{(2,78)} = 3.88$, $P = 0.025$). UV-B did not affect degradation rate when considered across all treatments of pH and temperature (Fig. 3c; one-way ANOVA $F_{(2,78)} = 1.86$, $P = 0.162$).

The full linear regression model with all main effects and interactions (Table 2) was fit to the data ($R^2 = 0.52$, $F = 6.67$, $P < 0.001$). All factors and interactions except pH significantly influenced degradation rate; however, pH had an interactive effect on degradation rate with both temperature and UV-B. The influence of interactions in the model indicate that, while temperature and UV-B affect degradation rate, their effects differ depending on the levels of the other factors. Independently, temperature and UV-B had positive effects on degradation rate, but the direction of the effect reversed when interactions with the other factors were included.

We found that the exponential decay model represented a high level of variation in the data ($F_{(2,480)} = 377.76$, $P < 0.001$). The degradation rates (r) estimated from the exponential decay model ranged from 0.05 to 0.34 and were lowest for 5 °C for all levels of pH and UV-B (Fig. 4). Estimated time to reach 5% of initial eDNA concentrations was correspondingly highest in low temperature treatments, and ranged from <1 day to 54 days over all treatments. Across all treatments, we estimated a degradation rate of 0.243 ± 0.070, indicating that bullfrog eDNA concentrations decreased by approximately 24% per day.

Total eDNA from non-bullfrog sources ranged from 1.87 to 256.09 ng eDNA/µl (compared to 0 to 5.83 ng bullfrog eDNA/µl) and did not significantly change over the course of the experiment (Fig. 5; $R^2 = 0.004$, $F = 0.10$, $P = 0.75$).

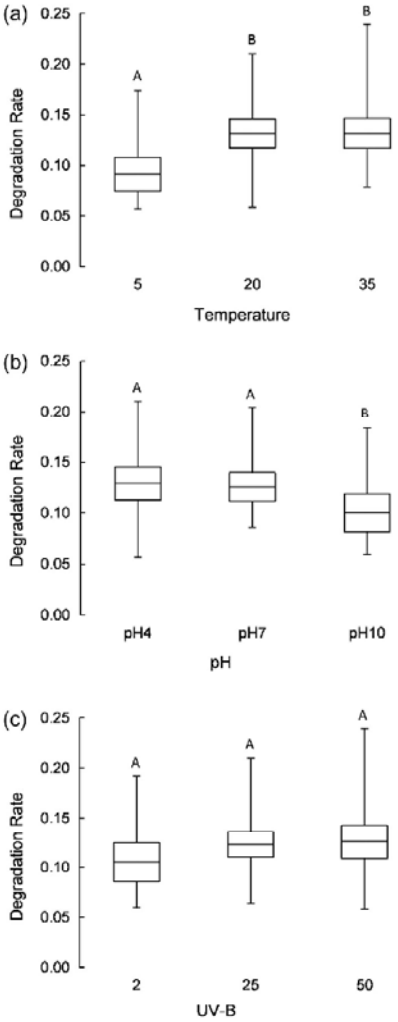

Fig. 3. Effects of (a) temperature, (b) pH, and (c) UV-B radiation on rate of eDNA degradation. Degradation rate represents the rate of change in eDNA detected from Day 0 to Day 58. Horizontal bars in box plots represent mean degradation rates, and upper and lower ends of boxes represent upper and lower 95% confidence intervals. Factor levels with the same letter are not significantly different. Temperature is measured in degrees Celsius (°C). UV-B is measured in kilojoules per square meter per day (kJ/m^2/day).

4. Discussion

For eDNA to be a useful tool for monitoring imperiled or invasive species, researchers must be able to focus sampling efforts to periods in which eDNA of the target organism is likely to be present. This requires knowledge about how long eDNA can be detected after the species has left or been removed from the water body of interest. Under controlled conditions, where other forms of eDNA removal (e.g., diffusion, advection, adsorption to particles) were not factors, degradation reduced the mean amount of detectable eDNA by 78% within 3 days and by almost 90% within 10 days of tadpole removal; though eDNA was still detectable at very low concentrations in several microcosms after 58 days.

DNA has been shown to degrade exponentially over long time periods (Willerslev et al., 2004; Allentoft et al., 2012), and we found that bullfrog eDNA degradation followed an exponential pattern of decline, irrespective of experimental treatment. Our estimated degradation rate (0.243) was within the range of similarly estimated rates in other studies (0.322 and 0.701, Thomsen et al., 2012; 0.105, Barnes et al., 2014). The congruence of degradation patterns among studies with a range of environmental covariates (Dejean et al., 2011; Thomsen et al., 2012a,b; Barnes et al., 2014) provides evidence that the initially sharp decrease in eDNA is common across systems and environmental conditions. It is likely that the effects of specific covariates are initially minor but gain influence over time as eDNA becomes more scarce (Fig. 2).

Table 2
Results of linear regression model of the influence of pH, temperature, and UV-B radiation on eDNA degradation rate ($R^2 = 0.52$, $F = 6.67$). pH is treated as a categorical variable with pH 4 as the reference level.

Predictor	Parameter estimate	Standard error	p-Value
Intercept	0.0571	0.0194	0.004
pH 7	0.0370	0.0275	0.190
pH 10	0.0168	0.0275	0.569
Temperature	0.0037	0.0008	<0.001
UV-B	0.0005	0.0006	0.001
pH 7 * UV-B	−0.0005	0.0009	0.038
pH 10 * UV-B	−0.0007	0.0008	0.002
pH 7 * Temperature	−0.0030	0.0012	0.014
pH 10 * Temperature	−0.0032	0.0012	0.008
Temperature * UV-B	−0.00002	0.00003	<0.001
pH 7*Temperature * UV-B	0.00004	0.00004	0.001
pH 10 * Temperature * UV-B	0.00005	0.00004	<0.001

pH4	UV-B (kJ/m²/day) 2	25	50
Temperature (°C) 5	0.05 / 53	0.13 / 22	0.13 / 17
20	0.14 / 14	0.17 / 9	0.14 / 1
35	0.20 / 10	0.15 / 1	0.34 / <1

pH7	UV-B (kJ/m²/day) 2	25	50
Temperature (°C) 5	0.07 / 23	0.12 / 12	0.12 / 12
20	0.14 / 7	0.12 / 1	0.12 / 1
35	0.11 / 8	0.13 / 1	0.15 / 3

pH10	UV-B (kJ/m²/day) 2	25	50
Temperature (°C) 5	0.05 / 53	0.05 / 54	0.05 / 41
20	0.06 / 39	0.12 / 13	0.14 / 13
35	0.09 / 27	0.15 / 11	0.14 / 8

Fig. 4. Estimated within-treatment degradation rate (above diagonal) and estimated number of days for eDNA to fall below 5% of initial quantity (below diagonal) for each full factorial treatment of pH, temperature, and UV-B radiation. Degradation rate and days to 5% were estimated from exponential decay function for bullfrog eDNA.

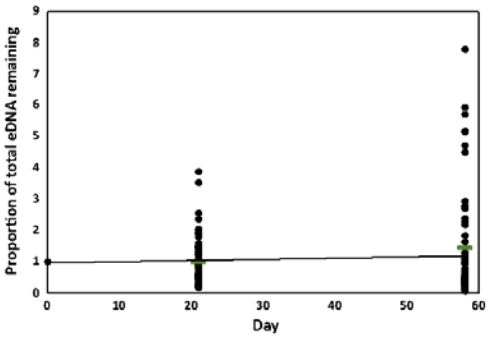

Fig. 5. Proportion of total non-bullfrog eDNA remaining for 81 microcosms at 0, 21, and 58 days following bullfrog tadpole removal. Horizontal lines represent the median proportion remaining in the sample. Day zero represents the initial amount of detectable eDNA in each microcosm.

In our study, temperature had a stronger effect on degradation rate than any other factor, with a greater proportion of bullfrog eDNA remaining at 5 °C than at 25 °C or 35 °C at time steps 10 through 58. Total eDNA concentration in 5 °C microcosms was similar to 25 °C or 35 °C microcosms at the outset of the experiment, but decreased to levels below the other temperatures by Day 58 (Supplementary material, Fig. A1), indicating that microbial growth was lower in the 5 °C microcosms during the later time steps and could have slowed the biotic eDNA degradation process. Regardless of the mechanism, our results provide clear evidence that low temperatures lengthen persistence of eDNA in freshwater and that the impact of temperature is influenced by UV-B and pH.

Solar radiation was also positively associated with increased degradation rate in this study, although this pattern was obscured when considered univariately. Time until <5% of eDNA remained dropped consistently more between UV-B levels 5 kJ/m²/day and 25 kJ/m²/day than between the higher levels of UV-B, suggesting a non-linear effect. Penetration of UV-B in the water column depends on the wavelength of the light and optical qualities of the water (Hargreaves, 2003), and can vary from a few centimeters in humic lakes to 10–20 m in oligotrophic lakes (Kirk, 1994). The water in our microcosms was less than 25 cm deep and likely to be low in humic acids, dissolved organic carbon (DOC), and other substances that may attenuate UV-B radiation (Hargreaves, 2003), suggesting that UV-B penetrated throughout water in the microcosms. In practice, direct effects of UV-B on eDNA degradation are probably limited to shallow or oligotrophic water bodies with low levels of phytoplankton, DOC, and organic matter. This means, since geographic characteristics such as latitude and altitude are primary determinants of UV-B radiation (Godar, 2005), that aquatic environments at higher elevations or closer to the equator are more likely to experience increased effects of UV-B radiation on eDNA degradation rate.

Acidic conditions catalyze hydrolytic processes that degrade DNA (Lindahl, 1993; Alaeddini et al., 2010). Because of this, DNA tends to persist longer in samples with neutral or slightly alkaline pH (Lindahl, 1993) and we expected to find high degradation rates in our acidic microcosms. Overall, our mean degradation rate was higher in pH 4 compared with pH 10 treatments, but in the full model pH was only significant in interactions with other variables and we continued to detect eDNA in some pH 4 microcosms until the end of the experiment. The low pH in our study is at least three units lower than has been reported in other eDNA degradation studies (Zhu, 2006; Barnes et al., 2014), but is similar to the pH of natural forested wetlands in Florida, USA where we have successfully detected eDNA of target amphibians (Goldberg, Strickler, and Fremier, unpublished data). These results show that, by itself, pH as low as 4 does not accelerate eDNA degradation to the point that it limits detection – an encouraging outcome for the use of eDNA in highly acidic aquatic environments. Additionally, systems with higher pH levels, such as alkaline or saline wetlands, may extend the period of time eDNA is detectable. Unexpectedly, neutral conditions in our study were not associated with eDNA persistence, and in fact had generally shorter time to <5% eDNA remaining compared to the other pH levels. We found that total eDNA (which includes eDNA from bullfrog and non-target organisms such as bacteria and algae) increased throughout the study in pH 7 treatments but declined in pH 4 and 10 treatments, suggesting that neutral conditions were more favorable for microbial growth and led to these higher rates of degradation. We note, however, that the bacterial species used in our study were most likely adapted for more neutral environments, which has implications for extrapolating to other environments. In natural settings, bacterial communities adapted to the local pH may result in greater production of exonucleases and correspondingly higher degradation rates than we observed in our acidic and alkaline treatments.

This study was designed to specifically test the influence of degradation on eDNA detection, without consideration of other processes (production and diffusion/advection) that also affect detection rates. Interestingly, however, our experimental setup revealed highly variable rates of DNA production (Fig. 1). Our tadpoles were of similar age and size, housed and fed identically, yet Day 0 DNA levels differed widely across treatments. High levels of individual variability in eDNA production have been documented in other species of amphibians (Pilliod et al., 2014) as well as fish (Klymus et al., 2015), and may be due to differences in individual physiology and behavior. Variability in the production term is a large source of uncertainty in the detection of species using eDNA and warrants further study. Although we found that starting concentration of eDNA did not affect degradation rate, we would expect that the time to reach the threshold of detectability would be shorter when starting concentrations are lower, such as for semi-aquatic or terrestrial species visiting aquatic systems, narrowing the temporal inference for detection of such species. Another unexpected result was the detection of small amounts of eDNA (<0.5 pg/250 mL) in negative control microcosms at the start of the experiment. Detection of this very low level of contamination demonstrates the high level of sensitivity of this detection method.

The results of this experiment indicate that aquatic habitats that are colder, more protected from solar radiation, and more alkaline are likely to hold detectable amounts of eDNA longer than those that are warmer, sunnier, and neutral or acidic. As single factors, warmer temperatures and acidity also degraded eDNA faster while relationships with UV-B were more difficult to discern independently. Factors that contribute to favorable environments for microbial activity (higher temperatures, neutral pH, moderate UV-B) were related to higher degradation rates, lending support to the conclusion that some effects of these factors are mediated in part by biological processes. Although our experiment was conducted under controlled conditions, the levels of temperature, pH, and UV-B we selected are representative of a wide range of aquatic systems. We expect that the general patterns we found in the laboratory for the relative effects of each factor on eDNA persistence will extend to natural environments.

Within the environmental conditions in this study, the variation in the length of time eDNA is predicted to be detectable after the target organism was removed was <1 to 54 days. Because effective eDNA sampling depends on identifying the window of highest probability of detecting the target species, the wide range of predicted persistence times underscores the importance of characterizing degradative conditions to best design sampling intervals and understand the temporal inference of positive and negative detections in each study system. We believe that, with improved understanding of factors influencing eDNA production, transport, degradation, and other mechanisms of removal, eDNA will be an increasingly powerful tool for accurate, robust detection and monitoring of sensitive, rare, or elusive species.

Acknowledgments

This work was funded by the Department of Defense Environmental Security Technology Certification Program under ESTCP Project RC-201204. Use of experimental animals was permitted under the University of Idaho Institutional Animal Care and Use Committee Protocol 2012-40 and Idaho State Department of Agriculture Invasive Species Possession Permit. We appreciate the work of Kody Cochrell and Andy Gygli in the laboratory and the greenhouse and Matthew Modlin and Jamie Jarulimek in the greenhouse on this study. Philip Anderson and Audrey Harris of the University of Idaho provided essential support for our research. We are grateful for the insight and support of Lisette Waits and the Laboratory for Ecological, Evolutionary, and Conservsation Genetics at the University of Idaho. Insightful comments from three anonymous reviewers substantively improved the manuscript.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocon.2014.11.038.

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APPENDIX D DOWNSTREAM RETENTION OF ENVIRONMENTAL DNA CONTROLLED BY SIMPLE STREAM CHANNEL METRICS

Draft Manuscript

Downstream retention of environmental DNA controlled by simple stream channel metrics

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KEYWORDS: eDNA transport, eDNA uptake, environmental DNA, field experiment, lotic, streams, residence time, transient storage

Introduction

The application of environmental DNA (eDNA) to detect species in water bodies has rapidly increased since its primary inception in 2008 (Ficetola et al. 2008). The eDNA method confirms species DNA in water to infer species presence/absence, and abundance in some cases, of a particular set of species (Goldberg et al. 2015). The eDNA method is highly sensitive and is considered a viable parallel method to traditional field sampling of rare species. However, because the method confirms species presence by proxy of DNA in water, the processes influencing eDNA removal becomes critical to its efficacy. In situ degradation and removal of eDNA from the aquatic system through absorption to the benthos are the likely cause (Jerde et al. 2016). These process are likely highly variable between systems and depend on a set of interacting physical and chemical processes (Strickler et al. 2014, Turner et al. 2015a). Of particular recent focus is the removal of eDNA in stream ecosystems with the downstream movement of water and concomitant in situ degradation.

Multiple removal processes control the abundance of eDNA in a water body, be it a lentic or lotic system (Figure 1). Species physiology, species life stage, consumption rate, temperature, and a host of other factors can influence the shed rate of DNA into water (termed here as production); only a few studies have examined abiotic and biotic controls on production and results illustrate high variability (Jerde et al. 2016). Once DNA is deposited into a water body it begins to degrade, be moved laterally or transported downstream, or absorbed into an adjacent environment (e.g. stream or lake bed) (Strickler et al. 2014, Turner et al. 2015a, Jane et al. 2015). Biotic and abiotic processes of the aquatic system control the importance of each factor, and these variables interact across different scales (reach to stream, and hours to season). The scale and rate of removal influences detection, and ultimately, the strength of inference of species presence.

Across multiple studies, degradation after deposition into the waterbody is rapid and rates are contingent on environmental conditions (Strickler et al. 2015). In a replicated, controlled experiment, Strickler et al (2015) found that water temperature, pH and UV light control degradation rates. They found degradation to be detectable within hours after deposition, but eDNA remained detectable for up to two months after inoculation. Degradation rates appear to be consistent over time, but illustrate exponential decline. They suggest biological consumption to be important and related to temperature, both in the water body (in situ) and post-sampling (in the sampling container). Most studies show degradation to be an important factor driving detection, be it in situ or post-sampling. For instance, field protocols for sampling water bodies call for immediately getting the sample on ice or stabilized in reagents to reduce degradation post sampling (Goldberg et al. 2016).

Transport of eDNA way from the organism increases the area where detection is possible. This improves our ability to detect species, particularly highly cryptic species. However, this dispersion process increases uncertainty of detection, most notably in lotic systems where water transports eDNA downstream. Removal of eDNA by absorption to benthic surfaces or other submerged material along the transport path likely reduces the abundance of eDNA, thereby influencing detection probability (Pilliod et al. 2013). These uptake rates are presumably highly variable between aquatic type (lentic versus lotic) but also within type with strong differences in degradation and uptake forces.

Movement of eDNA in ponds and lakes depends on water movement and diffusion, although it is unclear if DNA actively diffuses in water as a chemical diffusion process. Empirical studies suggest relatively short movement distances from the deposition location. Transport and uptake of constituents (nutrients, sediment, etc.) in streams are complex processes (Webster et al. 1975). eDNA transport in streams might be similar. Multiple studies has shown that eDNA transport and removal is rapid (Pilliod et al. 2013, Deiner and Altermatt 2014, Laramie et al. 2015). Transport rapidly moves eDNA away from the production location (Jane et al. 2015) and detection distances are relatively short (100-300 m in the case of small cold water streams using caged animals placed upstream from the sampling location (Jane et al. 2015, Pilliod et al. 2013). These studies provide evidence that transport and uptake directly influence the concentration of eDNA. However, our understanding of the specific production, transport and uptake rates limits our ability to quantify detection probabilities.

Here, we define transport as the movement of eDNA downstream but not a direct removal process. DNA transported downstream can still be detected in the water sample given high enough concentration and/or sample amount. We define dilution as a process influencing detection, as low levels of DNA are difficult to capture with sample amount (250ml-2 liters). The identification of eDNA, if in the sample, has shown to be highly sensitive (Goldberg et al. 2016). As eDNA travels downstream, abiotic removal by the streambed or other materials in the water (e.g. wood, vegetation) is presumably the dominant removal process, although one study has showed difficulty in quantifying this process (Jerde et al. 2016). Other studies point to this absorption to the bed as the main removal process downstream (Pilliod et al. 2013, Jane et al. 2015). Additionally, Turner et al. (2015) found significant eDNA in sediment of ponds, further associating biotic uptake as an important removal process; yet, they also report resuspension as a possible confounding process. However, no study has quantified uptake rates or the controlling factor of eDNA uptake in streams (Jerde et al. 2016).

Several studies have attempted to relate nutrient uptake with transient storage in streams. Nutrient uptake is a function of several abiotic and biotic factors including water residence time, stream size, water temperature, benthic leaf litter, periphyton growth, and stream metabolism (Valett, Crenshaw, and Wagner 2002, Hall and Tank 2003). These studies often show conflicting conclusions because there is high variability between measurements of transient storage and nutrient uptake. Often, the variability between samples from the same site is greater than the variability between sites being measured which prevents relationships between transient storage and uptake from being statistically significant (Lautz and Siegel 2007). Relating eDNA uptake to transient storage and geomorphic characteristics may be more appropriate because eDNA removal is more heavily controlled by abiotic than biotic variables.

For eDNA studies where eDNA travels longer distance than a single reach, measuring residence time is not feasible. For this reason, correlative geomorphic measures with residence time would enable us to estimate the uptake potential for long stretches of streams. Gooseff et al (2007) found correlations between a set of simple geomorphic measures (sinuosity, bed slope and bed topography) and transient storage across multiple stream types. These studies suggest that direct transient storage controlled by geomorphic complexity will regulate the uptake of eDNA in stream ecosystems. Quantifying this relationship for eDNA would improve our understanding of the specific processes influencing eDNA removal, and thereby detection to help inform sampling protocols and strength of inference.

The goal of this study was to better understand the transport properties of eDNA in stream ecosystems. To quantify the relationship between channel morphology and eDNA uptake, we performed a tracer experiment with sturgeon eDNA (novel to the stream) combined with rhodamine (RWT) in five reaches of varying channel complexity in a small, simple, small stream (< 3m average width). We extracted DNA from grab sampling using quantitative PCR (qPCR) to quantify concentration changes. We then quantified the relationship between channel complexity, transient storage and eDNA uptake rate, controlled for in-situ degradation during the experiment (~12 hours). We asked: 1) Does mean hydrological residence time explain eDNA uptake rate? 2) What measures of geomorphic complexity best correlate with eDNA uptake rates? We discuss the eDNA transport properties but also in relation to how they will influence sampling strategies and scale of interference in studies using the eDNA method.

Site Description

We performed the tracer experiment in five 200-meter reaches on Paradise Creek (4^{th} order) between Moscow (ID) and Pullman (WA) (Figure 1). Paradise Creek is a small headwater stream draining mostly a dryland agricultural watershed with the Palouse Region of the Northwest portion of the United States. Reed canary grass (*Phalaris arundinacea L.*) lines the edges of each reach and extends into the water during the low flow period. Channel banks are comprised deep loamy soils. The creek is incised within inconsistent pool riffle sequences and loose cobble to gravel substrate. Multiple reaches have short sections of emergent Columbia basalt bedrock. See Tables 1 for the detail geomorphic calculations and description of each reach.

Methods

Channel Surveys

We initially selected reaches based on access and visual indicators of geomorphic complexity (sinuosity, topography) and then, quantified more in-depth measures of hydraulic and geomorphic complexity after the experiment (Table 1). We performed topographic surveys of the streambed thalweg to characterize channel morphology, including channel dimensions, topographic complexity and substrate. Down the thalweg of each reach, we made measurements approximately every two meters and at slope breaks of topography (lat, long, elevation) along the streambed thalweg using an RTK GPS. We fixed RTK accuracy at its highest precision (~1 cm). We measured channel width at five positions along the channel. We performed Wolman pebble counts of 100 randomly selected particles through each reach to calculate the median grain size (D_{50}).

We calculated multiple channel complexity metrics from this dataset (Table 1). Channel length is the curvilinear distance of the channel. Slope is the curvilinear distance divided by the change in elevation between endpoints. Following Gooseff et al. (2007), we calculated three other channel complexity metrics – longitudinal roughness (s), the Average Water Surface Concavity (AWSC) (Anderson et al. 2005), and the product s, slope and sinuosity (χ). Table 2 describes the four complexity metrics. We lost the fixed positioning in two reaches (LP and WXP) of about 40 meters each due to canopy cover. For these two reaches, we averaged the longitudinal roughness (s) and AWSC of the two sections with high vertical precision.

eDNA Tracer Experiment

Hours before the eDNA tracer experiment, we performed salt slug injections to estimate discharge at 0 m and 200 m, and calculate both transient storage and residence time (Runkel 2002). We placed YSI sondes at 0 m and 200 m continuously (every 10 seconds) measuring electrical conductivity and temperature. For each reach, we injected 12 L of salt water more than 20 channel widths above the upstream and downstream reach boundaries to calculate discharge using dilution gaging. We then injected a third slug above the upstream reach boundary to calculate transient storage. We also used break through curves from this third injection to estimate when to initiate eDNA sampling (based on tracer travel time) and at what frequency to sample in order capture the entire tracer slug.

For the eDNA tracer experiment, we pulled water from a recirculating system of two tanks (1250 liter each) with seven adult white sturgeon (*Acipenser transmontanus*) from the Aquaculture Center at the University of Idaho, Moscow, Idaho. There were approximately 30 kg of fish between the two tanks and the fish were not mature. Water was pulled from the tank early in the morning before the tanks were cleaned to increase the eDNA concentration. Sturgeon DNA is novel to Paradise Creek, with no white sturgeon nor does the Center drain water into the creek. For each reach, we collected 200 liters of the sturgeon water as injectate for the tracer experiment and 18 liters for the degradation experiment. We kept the injectate as cool as possible and limited the time from pulling the sturgeon water to dropping the slug, in an effort to limit degradation within the experiment. We added 500 milliliters of Rhodamine (RWT) to the slug to describe conservative solute transport and allow for calculation of eDNA uptake. We preferred to use RWT over salt, as salt most likely influences eDNA degradation rates and inhabitation during amplification.

For each eDNA tracer experiment, we dropped 200 liters of the eDNA and RWT slug approximately 40 channel widths above the study reach. With estimated sampling initiation and frequency from the salt slug, we collected 20 two liter samples at roughly equal intervals as the eDNA slug past at the end of each reach; intervals varied based on the duration of the salt slug break-through curves. Sampling frequencies ranged from 2-5 minutes with a total sampling times ranging between one to two hours. We took grab samples at 200 m downstream from reach start. We took concurrent RWT samples just downstream from the eDNA samples to avoid contamination. We immediately placed samples on ice and labeled RWT samples in opaque 250ml bottles. The experiment took place over a two-week period of no rain and a constant stream discharge.

We accounted for in-situ (in-stream and within experiment) degradation by concurrently sampling the saved 18 liters of injectate. This injectate remained in covered buckets in the stream to simulate stream temperatures. After the experiment, we brought the buckets back to the lab and placed them in a 5°C refrigerator to mimic the on-ice samples. We took two-liter samples from the buckets just before dropping the slug, right after the last tracer sample, half way through the filtration and at after the last filtration. We assumed degradation was minimal in the stream, low with in the cooled environment, and stopped when filtered and placed in ethanol.

eDNA Extraction and qPCR

We extracted filter samples using the Qiashredder/DNeasy method described in Goldberg et al. (2011). All filter sample extractions and qPCR set up was conducted in a lab dedicated to low-quantity DNA samples. Researchers are required to shower and change clothes before entering this room after being in a high-quality DNA or post-PCR laboratory, and no tissue samples have been handled in this room. A negative extraction control was included with each set of extractions and an additional negative qPCR control was run with each plate of samples. Reactions were run with the white sturgeon assay of Brandl et al. (2015) with a 6FAM label, using QuantiTect Multiplex PCR Mix (Qiagen, Inc.) with recommended multiplexing concentrations (1X Quanti-Tect Multiplex PCR mix, 0.2 µM of each primer, and 0.2 µM probe). Reactions were 15 µl in volume and each included 3 µl of sample. Cycling began with 15 min at 95 °C followed by 50 cycles of 94 °C for 60 s and 60 °C for 60 s, using a CFX96 Touch Real-Time PCR Detection System. All samples were analyzed in triplicate and each well included an internal positive control (IC, Qiagen). A quantitative standard was diluted from a synthetic gene (gblock; IDT, Inc.) in duplicate serial dilutions from 1,000,000 to 1 copies. For each plate, results were accepted only if all levels of the standard curve amplified with $r^2 > 0.98$ and efficiency was between 95-105%. Results were analyzed using the Bio-Rad CFX Manager 3.1 (Bio-Rad). When any samples from a site were inhibited, all samples from that site were treated using a OneStep™ PCR Inhibitor Removal Kit (Zymo Research) and reanalyzed. Triplicate quantitative values (including zeros) were averaged to create estimates of eDNA concentration at each time point and multiplied to infer that average to the total water sample.

Stream Tracer Simulations in OTIS

Transient storage zones are areas in the stream where solutes are temporarily detained and slowed relative to faster moving water in the center of the channel. We hypothesize that transient storage zones increase eDNA removal compared to the main channel because they have slower moving water and increase the probability of absorption to bed and bank materials (periphyton, vegetation, substrate). We modeled transient storage by using the One-Dimensional Transport with Inflow and Storage (OTIS) model (Runkel 1998). OTIS uses the advection-dispersion equation to characterize fluvial solute transport and transient storage. Breakthrough curves from the salt slug injection were imported into OTIS and transient storage metrics were computed. The fraction of median travel time due to transient storage (F_{med}) was calculated for each site according to the following equation:

$$F_{med} = \left(1 - e^{-L\left(\frac{a}{u}\right)}\right)\frac{A_s}{A + A_s} \qquad \text{Eq. 1}$$

where L is reach length (m), a is the storage zone exchange coefficient, u is advective velocity (m/s), A_s is the storage zone cross sectional area (m^2), and A is main channel cross sectional area (m^2) (Runkel 2002). L was set to 200 meters for each site to allow comparisons between reaches of slightly different lengths (Runkel 2002). F_{med} is the preferred metric for describing transient storage because unlike other metrics, it combines storage zone size, exchange rate, and advective velocity. We also hypothesize that mean reach velocity approximates the passive uptake process. Mean reach velocity is the quotient of the reach discharge and cross-sectional area.

eDNA Uptake Modeling

eDNA uptake experiments were performed using slug injections of a reactive solute (eDNA) and a conservative tracer (RWT) as described in Tank et al. (2008) and Covino et al. (2010). One background RWT sample was taken before the first injection to background correct all grab samples. eDNA concentrations were assumed to be 0 because sturgeon are absent from Paradise Creek. We measured both eDNA and RWT concentration of the slug by taking three samples prior to injection. Tracer mass recovery of both RWT and eDNA were calculated by integrating downstream grab sample concentrations over time and dividing by downstream discharge. We compared the eDNA:RWT ratio of the injectate to the tracer mass recovery ratio to calculate eDNA uptake.

Spiraling length (S_w) is the distance traveled by the average eDNA particle before it is removed from the water column. Spiraling length was calculated by plotting the natural log of injected and recovered eDNA:RWT ratios versus distance downstream. k_w is the slope of the best fit line between these two points, and Sw is equal to the negative inverse of k_w according to:

$$S_w = -\frac{1}{k_w} \qquad \text{Eq. 2}$$

Areal uptake (U) is defined as the rate of eDNA removal from the water column per area of streambed per second, as defined as:

$$U = \frac{Q*[eDNA_{add-int}]}{S_w*w} \qquad \text{Eq. 3}$$

where Q is discharge (m^3/s), $eDNA_{add-int}$ is the geometric mean of observed and conservative breakthrough curve integrated eDNA concentrations ($parts/m^3$), and w is mean stream width (m).

The mass transfer coefficient (V_f) is defined as the downward velocity of an average eDNA particle towards the streambed. V_f is the preferred metric for describing eDNA uptake because it allows for comparison of uptake between sites with different discharge. The mass transfer coefficient was calculated as defined as:

$$V_f = \frac{U}{[eDNA_{add-int}]} \qquad \text{Eq. 4}$$

Data Preparation and Statistical Analysis

Prior to analysis, we performed an outlier analysis to remove data points with higher eDNA:RWT ratios than expected. We assume that eDNA does not actively and evenly diffuselike other tracers or chemicals because it is contained within a cell or groups of cell. Therefore, eDNA concentrations in individual samples can be highly variable, with high eDNA readings from clumps of cells. For this reason, we removed grab samples with a eDNA:RWT higher than that of the slug eDNA:RWT. We assumed that sturgeon DNA was not getting produced within the reach

and RWT was mostly likely not taken up more rapidly than eDNA. Temporary storage and resuspension might be possible (Turner et al. 2015b), although unlikely given the short duration of our experiment. It is therefore reasonable to assume that grab samples with eDNA:RWT higher than the slug occurred when a sample contained a larger "chunk" of eDNA as it traveled downstream. These are not incorrect readings, but illustrate the difficulty in non-diffusive, colloid transport. All grab samples with eDNA:RWT greater than the slug were removed. Seven samples of the 89 were removed (roughly two per site). These samples were always at the beginning or tail end of the pulse where the eDNA:RWT ratios were low. As RWT:eDNA approaches that of the slug, k_w begins to vary widely due to small changes in RWT or eDNA.

We applied simple linear regression to compare relationships among geomorphic, hydrological and uptake data. All statistics were performed in the R Project statistical package (version 3.1.3). We considered a p-value less than 0.05 as a significant result.

Results

Channel reaches showed distinct channel morphology metrics related to transport properties (Table 2). W.XP and T.V. reaches had higher slopes and D_{50} values compared to the other reaches. W.XP had higher channel sinuosity and T.V had the lowest sinuosity of all reaches. Sinuosity and slope did not strongly correlate most likely because of the small range of sinuosity in the section of stream. W.XP site had both a higher sinuosity and slope, where the T.V. site had high slope but a low sinuosity. W.XP had the highest channel roughness, followed by L.POE and T.V. W.XP showed a positive concavity and L.POE and negative. The μ metric integrates the influence of slope and sinuosity with higher values suggesting longer hydrologic residence times (lower slope and higher sinuosity). W.XP had the highest μ and T.V the lowest.

Our eDNA slug injections recovered 89% of the RWT and only 28% of the eDNA released. Each break through curve of the expected versus measured eDNA concentrations through the experiment showed a similar pattern as illustrated in Figure 2; the curves have a similar shape with the observed concentrations lower than the expected. We removed points where the observed was higher than the expected (see outlier procedure above). As expected, the observed concentrations are variable due to the clumped nature of eDNA in clumps of cells. The standard deviations here are calculated from the replicate filter samples (Figure 2).

We calculated both spiraling length and uptake velocity for each sample and accumulatively for each reach (Table 3, Figures 3-4). Figure 3 shows the calculated spiraling length for each sample plotted on to eDNA concentration. The observed pattern shows that the uptake process is concentration-dependent, with S_w increasing with concentration. All but the L.POE site show significant results (Figure 3). Table 3 shows that all sites except for W.XP were slight gaining streams with some discharge differences among reaches due to position but also because the experiments were not performed on the same day. Because the we removed water directly from the tank with sturgeon, we could not control for eDNA concentrations in the 200 L slug. For this reason, the YOTA site had higher concentrations of eDNA (637 part/m^3) than the other sites which hovered between 100-300 parts/m^3.

We performed simple linear regression among both S_w and V_f against the five geomorphic variables (Figure 4). We present only the significant relationships. The other relationships were

not significant at the $p < 0.05$ level. Slope, sinuosity and μ all showed significant relationships with both S_w and V_f. The r-squared values indicate variability in the precision ($R^2 = 0.2 - 0.3$).

Discussion

The method of using eDNA to confirm species presence or abundance rests on our understanding of the how DNA moves through the aquatic environment. To date, only one study has considered using methodologies from tracer studies in stream to identify the important controlling factor of eDNA removal from the streams. Although they did not find a significant correlation with sediment type (Jerde et al. 2016), their evidence and the evidence of eDNA in sediment (Turner et al. 2015a), suggest uptake to be an important factor. In the study, we provide a strong evidence from a tracer study that suggests that potentially reach scale geomorphology could be applied to predict eDNA removal. In particular, we found that channel slope and channel sinuosity are the best predictors, and potentially μ which might approximate the residence time of water in a stream reach. However, like Jerde et al. (2016), our view and data indicator that a conservative tracer does not match eDNA properties, and a more stochastic framework is necessary.

Our data suggest the reach scale geomorphology can inform the frequency and scale of eDNA studies. Long residence times or the more DNA has the potential to interact with the stream bed, the higher the uptake rates. The uptake process is most likely passive through adsorption to the biofilms on the streambed, rather than active biological uptake. We did not observe significant degradation of DNA is our samples in situ or during the experiment, to suggest significant biological consumption or UV-driven degradation (Strickler et al. 2014). Given this, we suggestion that uptake in streams is the dominant 'loss' of eDNA from the aquatic systems and therefore should be incorporated into field designs for species monitoring.

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Tables

Table 1. Calculated Channel Complexity Metrics Shown to Correlate with Transient Storage (see Anderson et al. 2005 and Gooseff et al. 2007 for details).

Based on results, we define μ to posit that reach scale morphology controls uptake.

	Symbol	Description	Equation
Slope	S	Quotient of curvilinear distance (L) and the vertical drop (h) between endpoints	$\frac{L}{h}$
Sinuosity	s	Quotient of the curvilinear distance (L) and the straight-line distance (L_s) between endpoints	$\frac{L}{L_s}$
Longitudinal roughness	ε	The average of the absolute value of the differences between the observed elevation (z_{obs}) and elevation predicted by the mean slope (z_{pred}). n = number of points	$\frac{1}{n} * \sum_{i=1}^{n} \lvert z_{obs,i} - z_{pred,i} \rvert$
Average water surface concavity	AWSC	The average of the absolute value of concavity measured at each point, where z is elevation and x is downstream distance	$\frac{1}{n} * \sum_{i=1}^{n} \lvert d^2 z_i / d x_i^2 \rvert$
Reach-scale form	μ	Product of the inverse of slope and sinuosity	$(1 - S) * s$

Table 2. Geomorphic Characteristic of each Reach on Paradise Creek, Washington.

	Reach Length (m)	Slope, S (m/m)	Sinuosity s (m/m)	Long. Roughness ε, (m)	AWSC $*10^{-3}$ (m^{-1})	μ (m/m)	Width (m) (n=10)	D_{50} (mm) (n = 100)
W.XP	209	0.011	1.20	1.78	11	1.87	2.6 ± 0.2	9.3 ± 118
L.POE	226	0.005	1.13	1.25	-15.5	1.12	3.0 ± 0.7	1.5 ± 50
YOTA	217	0.003	1.11	0.37	-2.3	1.11	1.4± 0.3	0.2 ± 73
U.POE	230	0.003	1.09	0.33	-3.1	1.09	2.3 ± 0.3	0.2 ± 61
T.V.	173	0.010	1.04	1.19	-0.2	1.03	2.1 ± 0.3	>256 ± 123

Table 3. Hydrology and eDNA Uptake Metrics of each Reach on Paradise Creek, Washington.

Site	UpS Q (m^3/s)	DS Q (m^3/s)	k_w	U $*10^{-3}$ parts/m^2/sec	Obs [eDNA] parts/m^3	S_w (m)	vf $*10^{-3}$ m/s
W.XP	0.0935	0.0917	-0.004	73.2	215.4	227.3	0.16
L.POE	0.0575	0.0723	-0.006	73.1	161.2	138.9	0.17
YOTA	0.0807	0.0902	-0.003	206.8	637.9	303.0	0.21
U.POE	0.0453	0.0500	-0.006	97.7	279.3	161.3	0.14
T.V.	0.0578	0.0590	-0.002	18.5	199.5	400.0	0.07

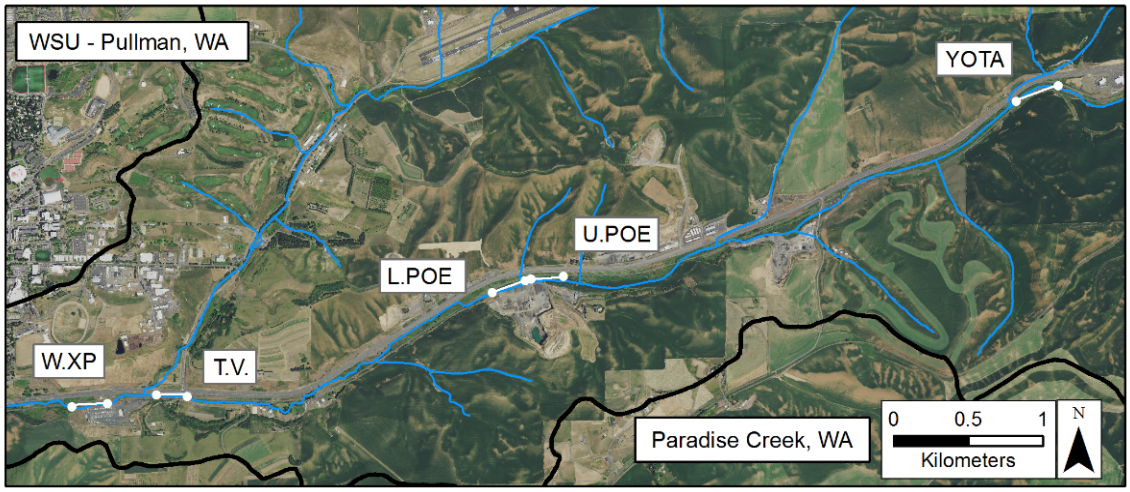
Figures

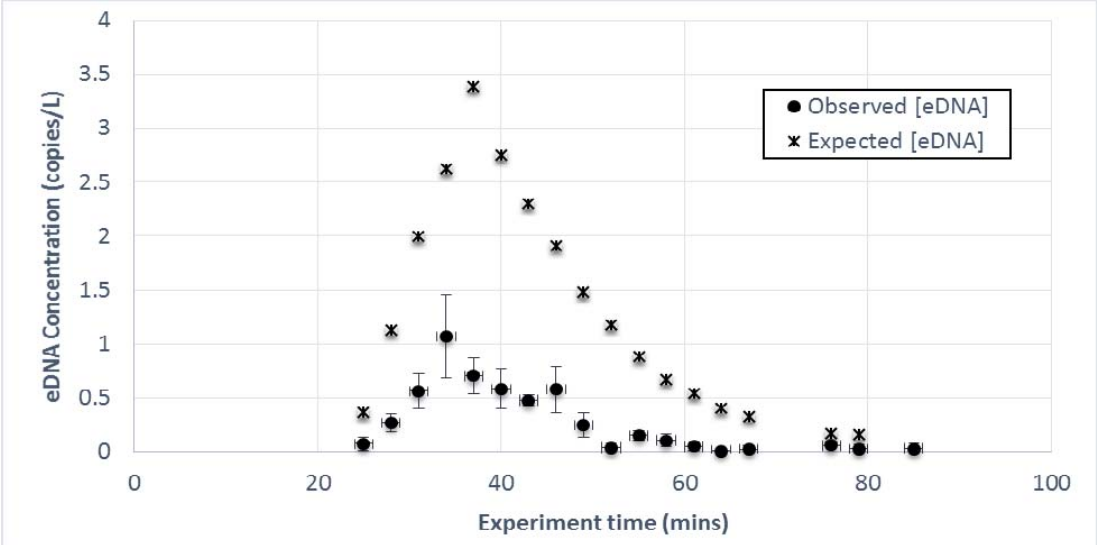
Figure 1. Study site map. The five approximately 200-meter study reaches on Paradise Creek in Washington State.

Figure 2. Expected and observed eDNA concentration through the length of the tracer pulse for the W.XP reach. Expected concentrations are calculated using the ratio of RWT to eDNA in the slug and the sample RWT values. Error bars for the [eDNA] are calculated using the three lab replicate samples from one two-liter field sample, both for the grab samples and the slug.

Figure 3. Calculated spiraling length plotted onto the concentration of eDNA for each sample at each reach.

Figure 4. Relationships between spiraling length and uptake velocity and multiple geomorphic metrics. The other geomorphic variables are not shown because they were not statistically significant.





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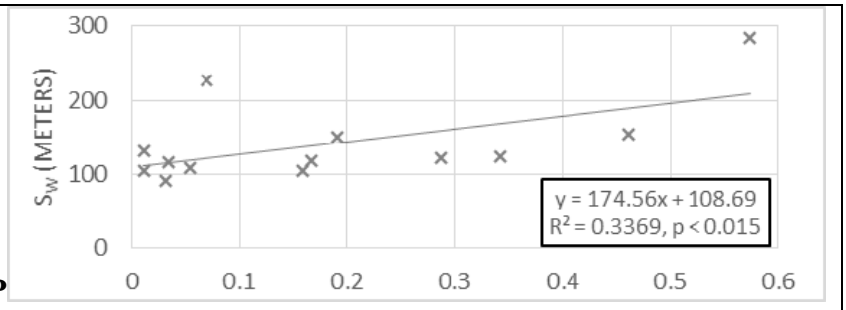

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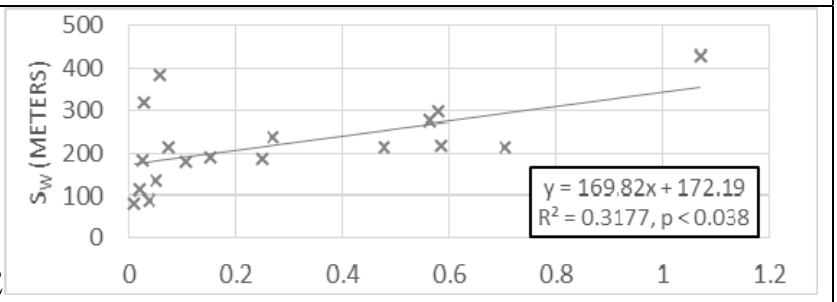

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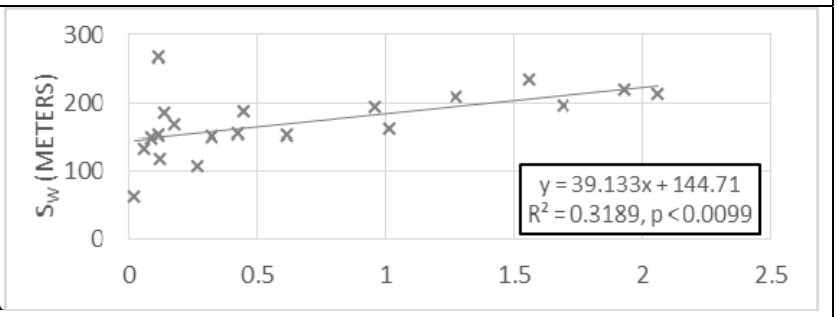

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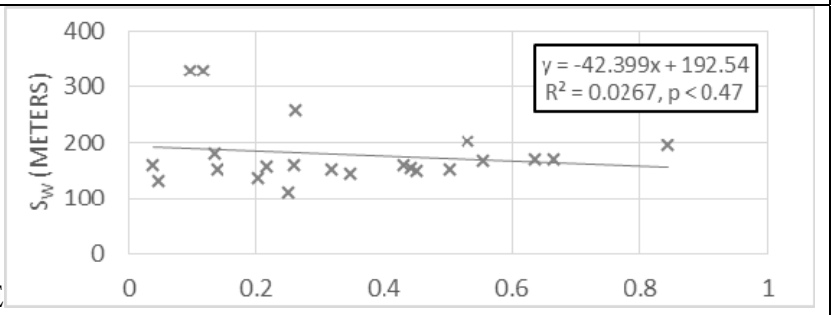
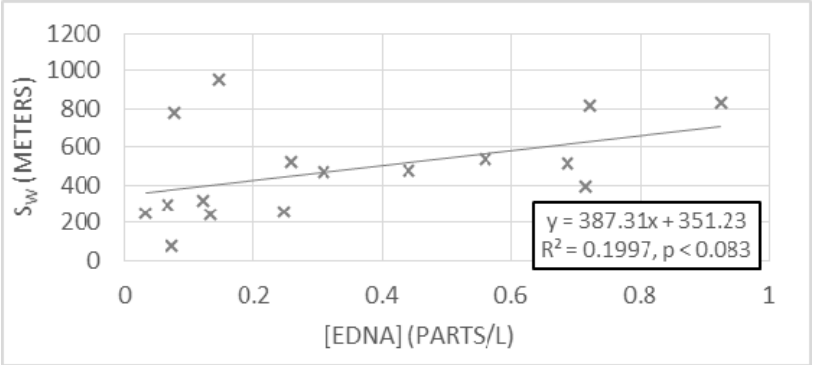
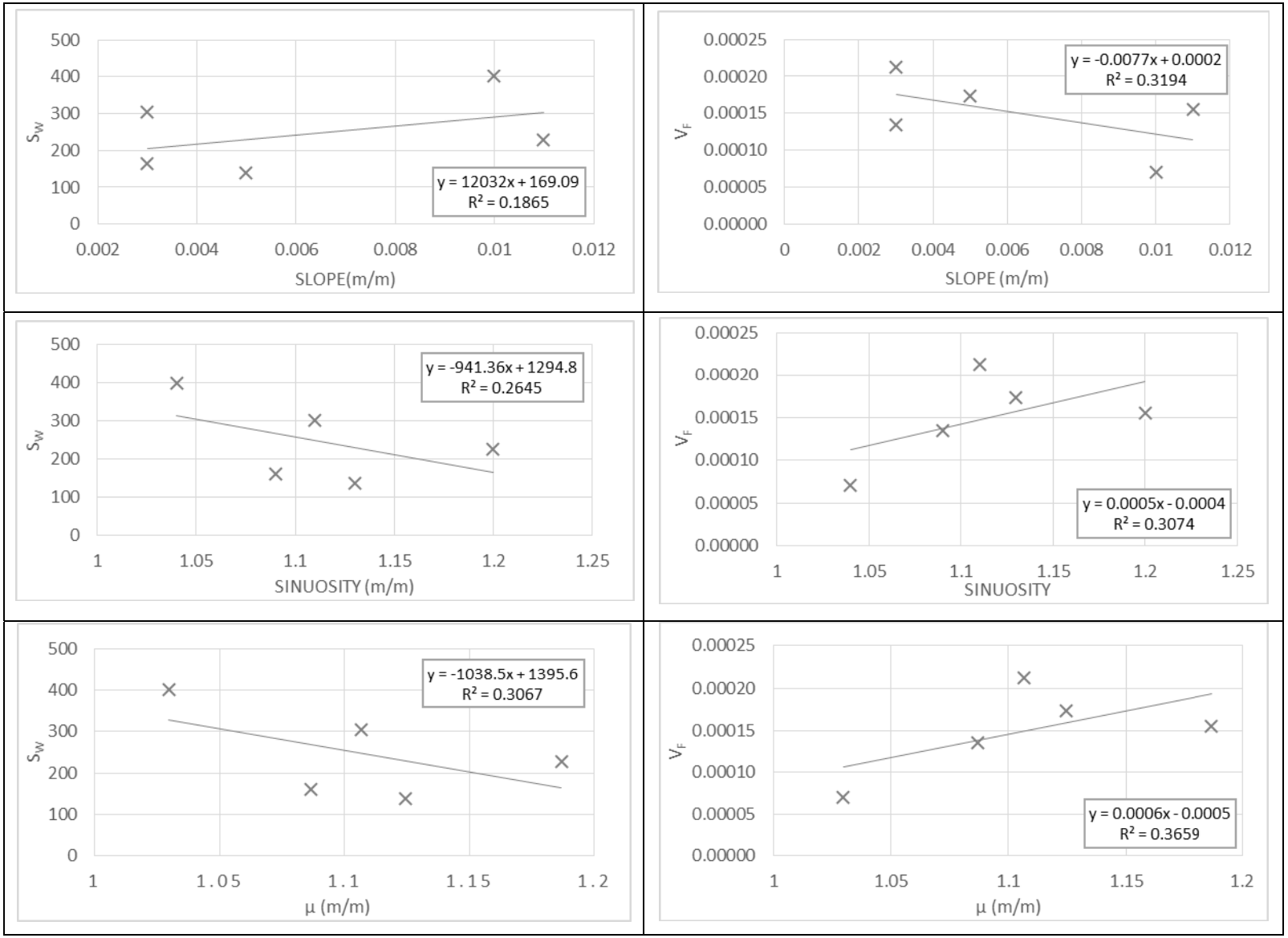


T.V.




500
400
300
200
100
0
S_W
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0.004
0.006
0.008
0.01
0.012
SLOPE(m/m)
0.00025
0.00020
0.00015
0.00010
0.00005
0.00000
V_F
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R² = 0.3194
0
0.002
0.004
0.006
0.008
0.01
0.012
SLOPE (m/m)
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R² = 0.2645
1
1.05
1.1
1.15
1.2
1.25
SINUOSITY (m/m)
y = 0.0005x - 0.0004
R² = 0.3074
SINUOSITY
y = -1038.5x + 1395.6
R² = 0.3067
μ (m/m)
y = 0.0006x - 0.0005
R² = 0.3659
μ (m/m)

APPENDIX E EFFECT OF FILTER MATERIAL AND PORE SIZE ON CAPTURE OF ENVIRONMENTAL DNA FROM WATER SAMPLES

Effect of filter material and pore size on capture of environmental DNA from water samples

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Introduction

Filtration of environmental water samples is one of the most commonly used methods for capturing and concentrating environmental DNA (eDNA) for detection of aquatic organisms. Sensitivity of eDNA detection depends in part on optimizing the capture of eDNA with water filters. This becomes particularly important when eDNA concentrations in water samples are low, and the effectiveness of eDNA capture from the filter may be the difference between detecting or not detecting the target species' eDNA.

Filter material and pore size can have dramatic effects on eDNA recovery. Environmental DNA studies have used a multitude of filter types, with various combinations of filter materials and pore sizes. Commonly used materials include glass microfiber (Jerde et al. 2011, Wilcox et al. 2013, Piaggio et al. 2014, and others), polycarbonate (Takahara et al. 2012, Turner et al. 2014a, Minamoto et al. 2015, Eichmiller et al. 2016), cellulose acetate (Takahara et al. 2013), mixed cellulose ester (Goldberg et al. 2013), and cellulose nitrate (Goldberg et al. 2011, 2013, Pilliod et al. 2013, 2014).

Pore sizes regularly used in eDNA field studies have ranged from 0.2 to 3.0 μm nominal pore diameter. Optimal pore size represents a balance between eDNA capture efficiency and filter volumes, as small pore sizes may maximize eDNA capture, but can clog easily and limit the amount of water sample that can be filtered (Turner et al. 2014a, 2014b). The volume of water filtered can strongly influence the quantity of eDNA captured (Goldberg et al. unpublished data). Additionally, as filters clog, filtration rates decline, and the amount of time required to filter each sample increases, resulting in reduced efficiency and higher costs. Clogging problems are frequently more pronounced in samples from water bodies with high levels of turbidity and organic matter.

An optimal filter would be one that maximizes the quantity of eDNA captured while minimizing filter clogging and the time required for filtration. From a practical standpoint, it's also important that the filter type can be easily purchased from major suppliers. The cellulose nitrate filters used in many early eDNA studies is no longer commercially available, underscoring the need for a comparison of alternative filter types.

In this study, we tested the effects of different filter materials and pore sizes on the quantity of eDNA captured from freshwater samples. Our objective was to identify filter types with the greatest eDNA capture efficiency across a gradient of environmental conditions.

Methods

Experimental design

We compared the eDNA recovery of 10 filter types across four filter materials (cellulose nitrate [CN], polycarbonate track-etched [PCTE], polyethersulfone [PES], and mixed cellulose ester [MCE]). We compared each material in three nominal pore sizes (0.45, 1.2, and 5.0 μm) with the exception of cellulose nitrate, which was available only in 0.45 pore size at the time of the test. We tested 0.45 CN μm filters from two manufacturers, Whatman and Nalgene. Earlier studies (Goldberg et al. 2011, 2013, Pilliod et al. 2013, 2014)

Although many studies have used glass fiber (GF) filters for capturing eDNA from water samples, GF filters have a number of disadvantages, including limited range of pore sizes available and reduced extraction efficiency (Turner et al. 2014a). Moreover, GF filters have been found to yield lower quantities of eDNA compared to other materials in several studies (Turner et al. 2014a, Renshaw et al. 2015) *but see* (Eichmiller et al. 2015). Because of these issues, we did not include GF filters in our test.

We preserved the filters in ethanol and followed a standard extraction protocol using a Qiagen extraction kit for all filter types. As additional treatments, we also compared a cetyl trimethyl ammonium bromide (CTAB) preservation and extraction protocol found to yield high detection probabilities in laboratory and field tests (Turner et al. 2014a), as well as a protocol for drying filters as an alternative to preservation in ethanol. The combinations of filter materials, pore sizes, and preservation/extraction methods resulted in 13 treatments (Table 1).

Sample collection and filtration

We collected water from four ponds (Table 2) near Moscow, ID in October 2014. We assumed that turbidity was a suitable measure of the amount of potentially filter-clogging suspended solids, and the gradient of turbidities at the ponds (8-185 NTU) provided a range of conditions for which pore size might affect eDNA capture. Each site contained a species for which a qPCR assay was developed: American bullfrog (*Lithobates catesbeianus*), rainbow trout (*Oncorhynchus mykiss*), and rough-skinned newt (*Taricha granulosa*). We collected water from each pond in five 1-gallon containers, placed the containers on ice, and took them into the lab. We drew 250 mL samples from each container for each treatment, resulting in five replicate samples per treatment for each pond. Filters were held in disposable funnels that were discarded after a single use to prevent cross contamination among samples. We applied vacuum pressure to filters by attaching filter funnels to 1 L polypropylene vacuum flasks, which were connected with silicone tubing to laboratory vacuum lines. We recorded the length of time required for each sample to pass through the filter and, when a filter clogged (defined as less than one drip per 5 seconds) prior to filtering the full 250 mL sample, we recorded the final volume of sample water filtered. We swirled the water in the containers prior to taking each sample to minimize heterogeneity among samples and rotated filter types during filtering to factor out potential time effects. Following filtering, we preserved each filter in a 2 mL screw-top tube partially filled with ethanol or CTAB buffer, or dried it in a small paper envelope.

Laboratory methods

We extracted all filter samples using the Qiashredder/DNeasy method described in Goldberg et al. (2011). Sample extractions and qPCR set up were conducted in a lab dedicated to low-quantity DNA samples. Researchers are required to shower and change clothes before entering this room after being in a high-quality DNA or post-PCR laboratory, and no tissue samples have been handled in this room. A negative extraction control was included with each set of extractions and an additional negative qPCR control was run with each plate of samples. Samples from Tunnel Pond were analyzed using the American bullfrog assay in (Strickler et al. 2015), samples from Hordemann Pond were analyzed using the rainbow trout assay of Brandl et al. (2015), and samples from the remaining sites were analyzed using a new test for rough-skinned newt developed using sequence data from Kuchta and Tan (2005) (Table 3). All assays were run using QuantiTect Multiplex PCR Mix (Qiagen, Inc.) with recommended multiplexing concentrations (1X Quanti-Tect Multiplex PCR mix, 0.2 µM of each primer, and 0.2 µM probe). Reactions were 15 µl in volume and each included 3 µl of sample. Cycling began with 15 min at 95 °C followed by 50 cycles of 94 °C for 60 s and 60 °C for 60 s. For Tunnel Pond and Hordemann Pond, analysis was conducted using a QuantStudio 7 Flex Real-Time PCR System and QuantStudioTM 6 and 7 Flex Real-Time PCR System Software (Life Technologies). The remaining sites were analyzed using a CFX96 Touch Real-Time PCR Detection System and the Bio-Rad CFX Manager 3.1 (Bio-Rad). All samples were analyzed in triplicate and each well included an internal positive control (IC, Qiagen). A quantitative standard was diluted from a tissue sample (fin or toe clip) in duplicate serial dilutions 10-3 to 10-6. For each plate, results were accepted only if all levels of the standard curve amplified with $r^2 > 0.98$ and efficiency was between 95-105%. Samples from Tunnel Pond originally tested as inhibited and were subsequently treated using a OneStep™ PCR Inhibitor Removal Kit (Zymo Research) and reanalyzed. Triplicate quantitative values (including zeros) were averaged to create estimates of eDNA concentration for each sample.

Statistical analyses

We used a linear mixed model to identify the relationship between eDNA capture and filter types across all sites, using site as a random factor and filter pore size and material as fixed factors. We used the z-score standardization of quantity of eDNA per mL of filtered water as our measure of eDNA capture. The quantity of eDNA captured at two of the sites was several orders of magnitude less than the other two sites (Table 2). For these sites, we also used a mixed effects logistic regression models to compare eDNA detection (0 = no detection, 1 = detection) with site as a random factor and filter pore size, material, and volume filtered as fixed factors. Pore size and volume were modeled as continuous variables and material modeled as a categorical variable. Because sample volume can influence eDNA capture, we also analyzed results using a mixed effects logistic regression model with eDNA detection as a binomial response variable, site as a random factor, and volume as the sole fixed factor.

Results

At all sites, the CTAB treatment captured about one-tenth of the amount of eDNA captured by other treatments, and we excluded it from further analyses. Drying the filter following filtration yielded similar amounts of eDNA as the same filter preserved in ethanol. We thus excluded this treatment as well, allowing subsequent analyses to focus directly on filter material and pore size without considering preservation and extraction methods.

For the full dataset of all four ponds and all ethanol/Qiagen treatments, we found no effect of pore size on eDNA capture (Table 4). The amount of eDNA captured was similar for MCE, CN, and PES filter materials, but significantly lower for all PCTE filters.

The mixed effects model for the low quantity sites indicated a positive effect of pore size on eDNA detection (Table 5). The PCTE filters were again less effective for eDNA capture, with lower probability of detection for PCTE filters compared with the MCE reference filters. This result was significant at a 0.10 threshold for *p*. However, when volume was included in the model as a fixed effect, there were no significant effects of pore size, material, or volume on probability of detection (Table 6). The volume of water that could be passed through the filter had a small but significant effect on detection probability (Table 7).

Treatments with PCTE filters typically yielded less eDNA per minute of filtration time compared with other filter materials (Figure 1a), as did treatments with 0.45 µm pores compared with other pore sizes (Figure 1b).

The relationships between final sample volume and filtration time were similar across sites (Figure 2). Compared to other filter materials, MCE filters yielded greater sample volumes relative to the time required to reach that volume, while PCTE filters had the highest filtration times relative to sample volume.

Across all treatments, filtration time increased with increasing turbidity (Table 2; linear regression, $R^2 = 0.21$, $F = 60.59$, $P < 0.001$), while sample volume was not affected by turbidity (linear regression, $R^2 = 0.009$, $F = 2.16$, $P = 0.14$).

Discussion

Filter material

Polycarbonate filters consistently performed poorly in our tests. Not only did they capture less eDNA at all pore sizes relative to other materials, they had lower filtration efficiency in terms of both filtration time and sample volume. The PCTE filters either yielded less eDNA for similar filtration times or took longer to yield similar amounts of eDNA compared to other materials. At all sites, we were able to filter less of the water samples through the PCTE filters than other filter materials, and longer filtration times were required to reach the final sample volume. Therefore, lower detection using these filters may be explained in part by the lower sample volumes collected using these filters for all sites.

The other filter materials captured similar amounts of eDNA across all sites. At the two low-quantity sites (Phillips Farm and Pond 9), detection rates were slightly higher for CN filters compared to MCE and PES filters. The MCE, CN, and PES filters captured eDNA with similar efficiency, with no relationship between amount of eDNA captured and the time required to filter the sample. The MCE filters generally resulted in larger final sample volumes than the other filters, and required less time to filter the final volumes.

Our results are consistent with filter material comparisons by Liang and Keeley (2013) and Renshaw et al. (2014). Liang and Keeley (2013) tested recovery of extracellular DNA in water samples (likely a small component of the eDNA analyzed in this study) using multiple filter materials (polyvinylidene fluoride, PES, PC, and MCE) and found that MCE filters always recovered more eDNA than other filters of the same pore size, while PC filters recovered the least eDNA.

Similarly, in a comparison of CN, PES, and PCTE filters by Renshaw et al. (2014), PCTE and GMF filters yielded less eDNA than CN and PES filters, which captured similar amounts of eDNA.

Extraction methods may also play a large role in eDNA capture. Turner et al. (2014a) found higher eDNA capture in PCTE filters with a CTAB/PCl extraction compared to GF filters with a PowerWater DNA extraction kit. The authors suggest that the different amounts of eDNA recovered is due to the different extraction methods, rather than filter material. We tested the CTAB/PCl extraction using PES filters, which, like PCTE filters, are chemically dissolved during the CTAB extraction. In contrast to Turner et al., our CTAB extraction captured very little eDNA compared to PES filters preserved in ethanol and extracted with a Qiagen kit, regardless of filter pore size.

Pore size

Filter nominal pore size was not a predictor of quantity of eDNA per mL captured across all of our sites. This finding is in contrast with other studies (Liang and Keeley 2013, Turner et al. 2014b, Renshaw et al. 2015), which have found that the amount of DNA extracted from filters decreases as pore size increases. However, Liang and Keeley (2013) measured only extracellular DNA and the latter two papers used only PCTE membranes, which may perform more precisely with respect to pore size due to their structure. There was some evidence that eDNA detection increased with increasing pore size in our study, which was explained by the increase in volume associated with these larger pore sizes.

Conclusions

Although we found equivocal results for the efficacy of eDNA capture per volume sampled across pore sizes and most materials (CN, MCE, PES), differences in filtering time and total volume that can be sampled indicate that some combinations are more efficient. The reduced eDNA capture and filtering efficiency of PCTE filters suggests that PCTE may be a poor choice for eDNA-based research and monitoring, even if other extraction methods are used (Renshaw et al. 2014). Combined with the findings of other studies (Liang and Keeley 2013, Renshaw et al. 2015) our results indicate that MCE, CN, or PES filters would be preferable to PCTE and have similar effectiveness in capturing eDNA.

Optimal filters for eDNA sampling need to balance effective capture of eDNA with filtration efficiency. Higher eDNA capture and detection is generally associated with larger sample volumes (Turner et al. 2014b), Goldberg et al. unpublished data), but larger water volumes often require extensive filtration times and/or limited total volume with small pore sizes. In this study, detection at low density sites that most closely resemble those where eDNA will be applied in the field was related to volume sampled. Therefore, the most efficient high-volume filter is likely to be the most effective for eDNA sampling in systems where smaller pore size filters become clogged.

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Table 1. Filter type and preservation/extraction methods for each experimental treatment.

Treatment	Filter material*	Filter pore size (µm)	Manufacturer	Preservation method	Extraction method
MCE45	MCE	0.45	Millipore	Ethanol	Qiagen
MCE12	MCE	1.2	Millipore	Ethanol	Qiagen
MCE5	MCE	5.0	Millipore	Ethanol	Qiagen
NCN45	CN	0.45	Thermo Scientific	Ethanol	Qiagen
WCN45	CN	0.45	Whatman	Ethanol	Qiagen
WCN45D	CN	0.45	Whatman	Drying	Qiagen
PCTE4	PCTE	0.4	Pall	Ethanol	Qiagen
PCTE12	PCTE	1.2	Millipore	Ethanol	Qiagen
PCTE5	PCTE	5.0	Millipore	Ethanol	Qiagen
PES45	PES	0.45	Pall	CTAB	PCl
PES45	PES	0.45	Pall	Ethanol	Qiagen
PES12	PES	1.2	Sterlitech	Ethanol	Qiagen
PES5	PES	5.0	Sterlitech	Ethanol	Qiagen

* MCE = Mixed cellulose ester, CN = cellulose nitrate, PCTE = polycarbonate track etched, PES = polyethersulfone

Table 2. Site and Site-specific Sample Characteristics.

Site name	Turbidity (NTU)	Target species*	Mean volume filtered (mL)	Mean filtration time (minutes)	Mean eDNA quantity (pg)
Tunnel Pond	8	LICA	241	15	96.64
Hordemann Pond	185	ONMY	231	61	12.49
Phillips Farm Pond	9	TAGR	222	26	0.61
Pond 9	30	TAGR	234	35	0.17

* LICA = *Lithobates catesbeianus*, ONMY = *Oncorhynchus mykiss*, TAGR = *Taricha granulosa*

Table 3. Quantitative PCR Assay for Rough-skinned Newts (*Taricha granulosa*).

Assay confirmed to be species-specific in silico as well as when tested against tissue samples of tiger salamanders (Ambystoma mavortium) and long-toed salamanders (A. macrodactylum), the only other salamanders in the area.

Forward Primer	GGAATCCCATCGAACCAAGAC
Reverse Primer	AGTATCAGGAGGAAGCCAAGGATA
Probe	6FAM-TCATTCCACCCGTACTT -MGB

Table 4. Estimates of Fixed Effects Produced by a Linear Mixed Model of Filter Characteristics Across all Sites, with Site as a Random Effect and Mean eDNA Quantity/mL (± standard error) as the Continuous Response Variable.

MCE was considered the reference category for material type in the model. Filter material codes follow Table 1.

Variable	Coefficient	*t*	*P*
Intercept	0.132 ± 0.152	0.873	0.384
Pore size	0.001 ± 0.037	0.033	0.974
Material:CN	0.148 ± 0.213	0.695	0.488
Material:PCTE	-0.369 ± 0.182	-2.032	**0.043**
Material:PES	0.115 ± 0.181	0.637	0.525

Table 5. Estimates of Fixed Effects Produced by a Mixed Effects Logistic Regression of Filter Characteristics for Phillips Farm and Pond 9, with Site as a Random Effect and "Detection" (0 or 1) as the Binomial Response Variable.

MCE was considered the reference category for material type in the model. Filter material codes follow Table 1.

Variable	Coefficient	*z*	*P*
Intercept	0.659 ± 0.842	0.783	0.434
Pore size	0.278 ± 0.138	2.019	**0.044**
Material:CN	1.255 ± 0.819	1.533	0.125
Material:PCTE	-1.092 ± 0.620	-1.762	**0.078**
Material:PES	0.213±0.650	0.327	0.744

Table 6. Estimates of Fixed Effects Produced by a Mixed Effects Logistic Regression of Filter Characteristics for Phillips Farm and Pond 9, with "Detection" (0 or 1) as the Binomial Response Variable, Site as a Random Effect, and Pore Size, Material, and Sample Volume as Fixed Effects.

MCE was considered the reference category for material type in the model. Filter material codes follow Table 1.

Variable	Coefficient	*z*	*P*
Intercept	-0.315 ± 1.652	-0.191	0.849
Pore size	0.231 ± 0.153	1.512	0.131
Material:CN	1.174 ± 0.830	1.414	0.157
Material:PCTE	-0.925 ± 0.665	-1.391	0.164
Material:PES	0.266 ± 0.653	0.408	0.684
Volume	0.004 ± 0.006	0.689	0.491

Table 7. Estimates of Fixed Effects Produced by a Mixed Effects Logistic Regression of Sample Volume for Phillips Farm and Pond 9, with "Detection" (0 or 1) as the Binomial Response Variable, Site as a Random Effect, and Sample Volume as a Fixed Effect.

Variable	Coefficient	*z*	*P*
Intercept	-1.848 ± 1.366	-1.353	0.176
Volume	0.013 ± 0.005	2.434	0.015

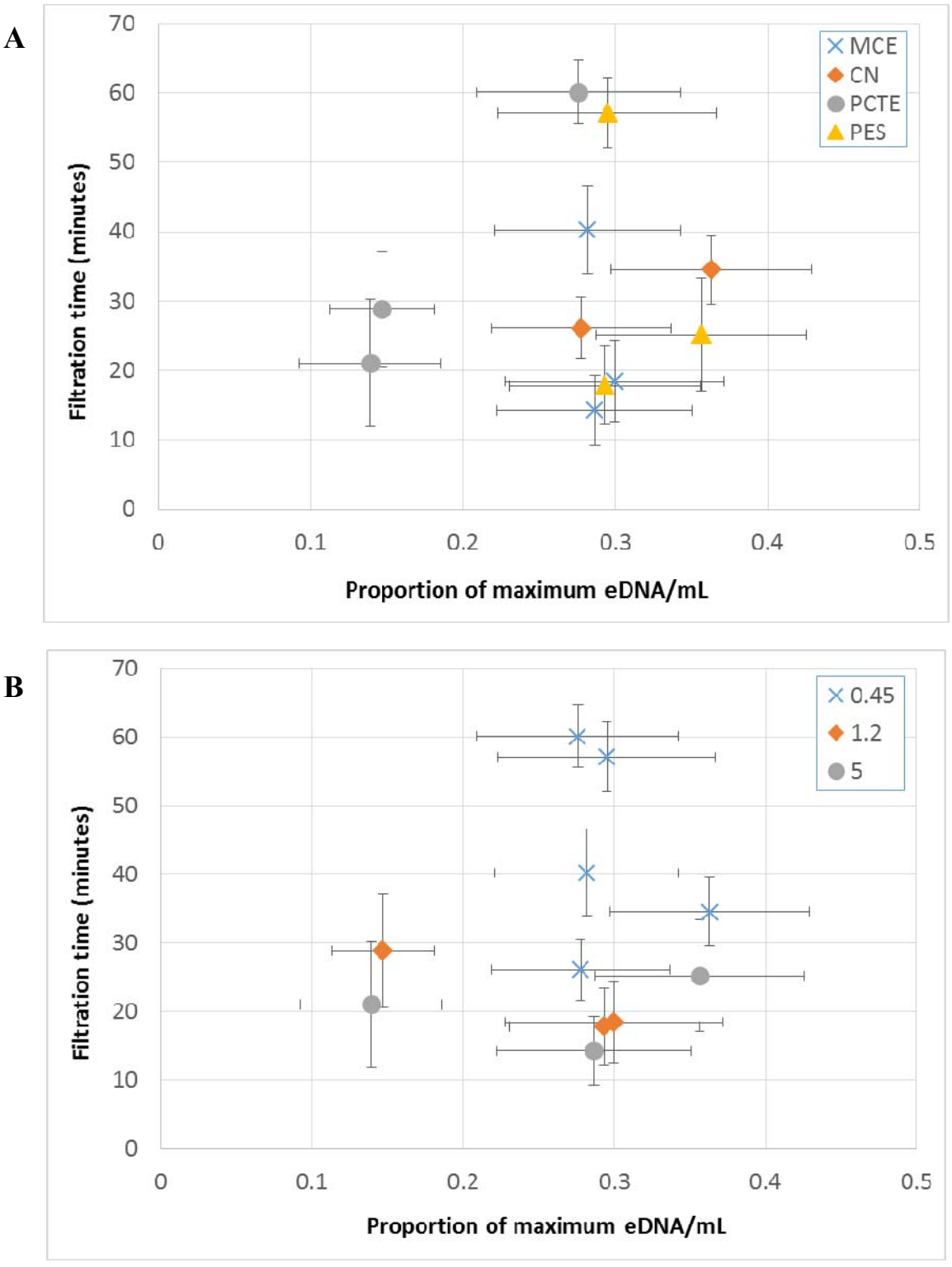

Figure 1. Mean Proportion of Maximum eDNA Yield Compared with the Mean Time Required to Filter the Final Sample Volume for each A) Material Type and B) Pore Size.

Because eDNA yields were very high for a small number of samples, overall proportions were low for all treatments.

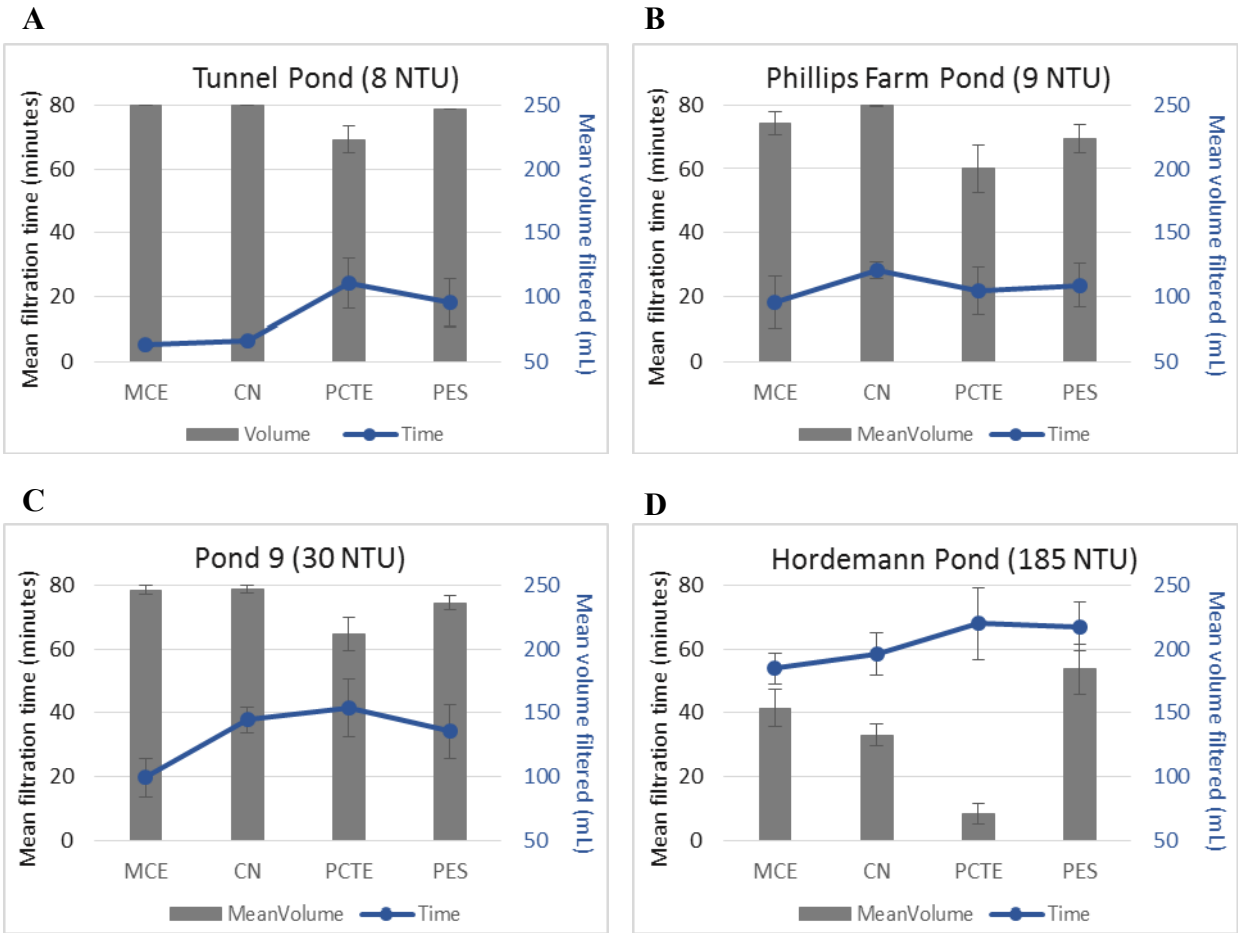

Figure 2. Mean Time (in minutes, blue line) to Filter the Final Volume of Water and Mean Volume of Water Filtered (in mL, gray bars) for Each Filter Material Type at A) Tunnel Pond, B) Phillips Farm Pond, C) Pond 9, and D) Hordemann Pond.

Turbidity for each pond is listed in nephelometric turbidity units (NTUs). Filter materials: MCE = mixed cellulose ester, CN = cellulose nitrate, PCTE = polycarbonate track etch, and PES = polyethersulfone.

APPENDIX F CALIBRATION AND DOCUMENTATION

F-1.0 CALIBRATION OF EQUIPMENT

Calibration of Applied Biosystems 7500 Fast Real-Time PCR System and QuantStudio 7 Flex Real-Time PCR System (Life Technologies) were performed according to manufacturer recommendations. The CFX96 Touch Real-Time PCR Detection System (Bio-Rad) does not require calibration. Calibration of the multiparameter water meter was performed according to the operations manual.

F-2.0 QUALITY ASSURANCE SAMPLING

All field samples were taken in sets of 3-8 replicates to quantify and model the uncertainty of detecting a species given that it was present (detection probability). A field blank was collected at each filtering event to ensure no equipment contamination occurred. A negative extraction control was created with each set of DNA extractions. A blank (water) sample was run with each qPCR reaction to ensure no contamination occurred during the preparation for that reaction. Positive samples for each target species were analyzed alongside every test and an exogenous internal control was included in every PCR reaction to ensure no false negatives. Samples testing as inhibited were cleaned with a Zymo One-Step Cleanup Kit (Zymo, Inc.) and retested; samples still presenting with inhibition were diluted and rerun until the internal control tested within 3 Cq of the no template control.

F-3.0 SAMPLE DOCUMENTATION

Each DNA and eDNA sample was collected in an O-ring tube (Sarstedt, Inc.) and labeled with a unique name on both the top and side of the tube, as well as sample collection date and collector on the side of the tube, with an ethanol-proof marker. For DNA samples, this name reflected the species sampled and for eDNA samples this name reflected the site sampled and replicate number. All field information, including GPS data, was recorded in a Trimble GPS, which was downloaded, checked for accuracy, and backed up at the end of every field day. Sample labeling was also double-checked against the field-recorded data for accuracy at the close of every field day. A data dictionary for each site was developed for accurate data recording (Figure F1). Results of eDNA PCR analysis was be archived in raw form (Figure F2) as well as in a spreadsheet where sample name and results of each analysis were documented (Figure F3).

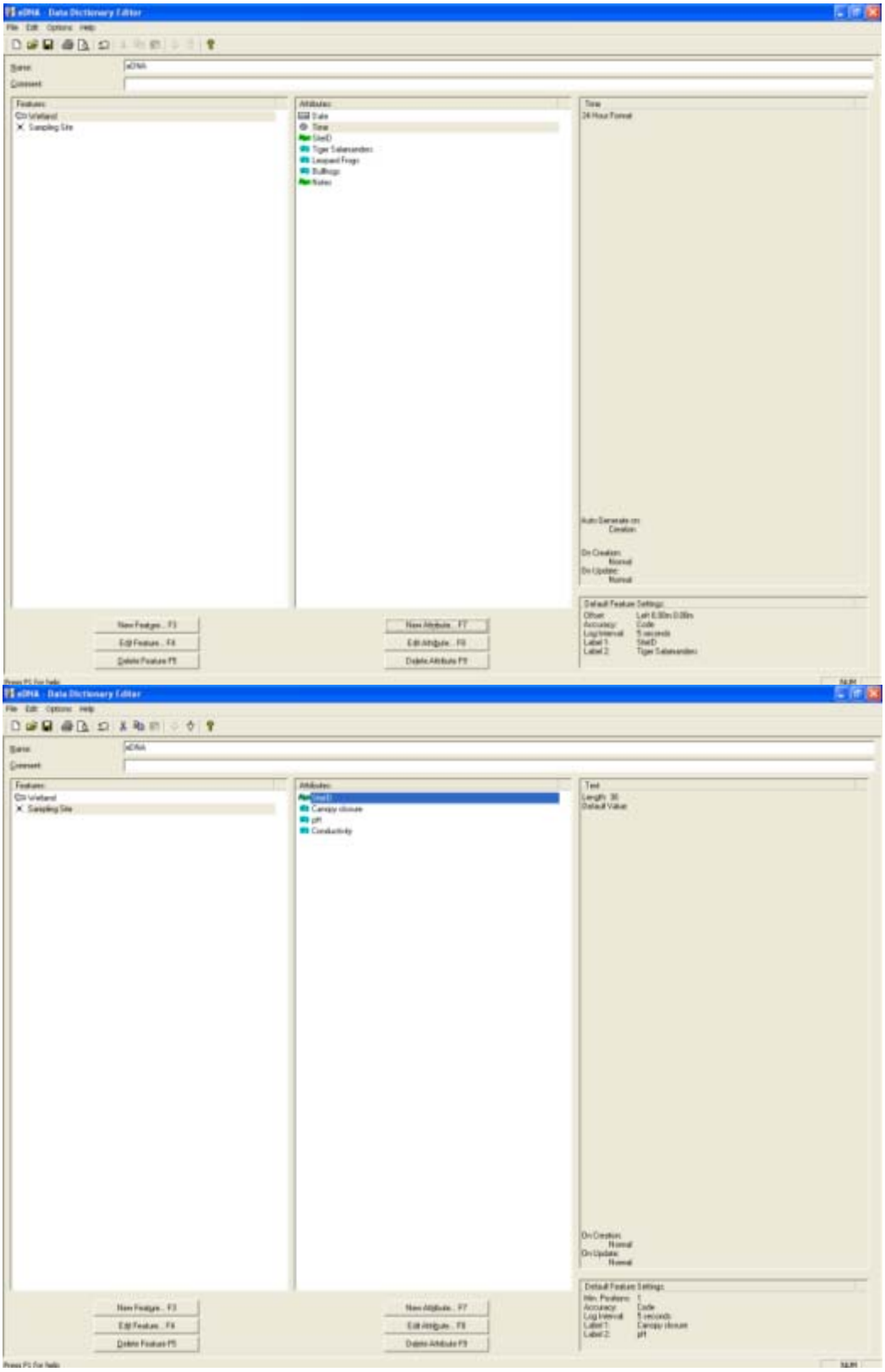

Figure A1. Example Data Dictionary for Ft. Huachuca.

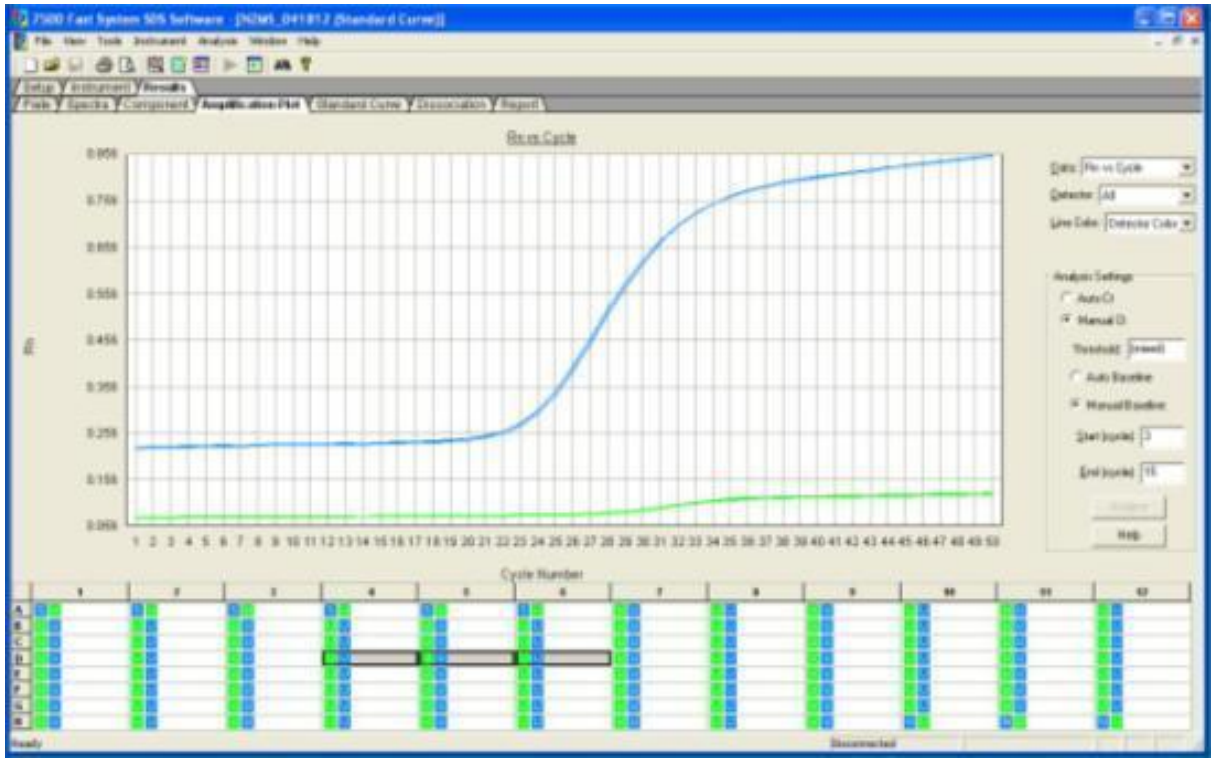

Figure A2. Example Spectral Data for eDNA Raw Data Storage.

Blue lines indicate detection of target species in three replicate samples. Green lines indicate the detection of exogenous internal control in those same reactions.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
1	Sample Name	Result	Date extracted	Extracted by	PCR1 date	PCR1 # positives	PCR1 mean	PCR1 SD	PCR1 r2	PCR1 slope	PCR2 date	PCR2 # positives	PCR2 mean	PCR2 SD	PCR2 r2	PCR2 slope
38	20082	0.00482	2-Feb-12	CG/AG	18-Apr-12	3	0.00481962	8.11E-04	0.98	-2.68						
39	200C2	0.001189	10-Apr-12	AG	16-Apr-12	3	0.00118882	1.30E-04	0.99	-2.68						
40	100A2	0.001129	10-Apr-12	AG	16-Apr-12	3	0.00112877	6.13E-05	0.99	-2.68						
41	100B2	0.004572	10-Apr-12	AG	16-Apr-12	3	0.00457159	2.93E-04	0.99	-2.68						
42	100C2	0.006588	10-Apr-12	AG	16-Apr-12	3	0.00658768	3.63E-04	0.99	-2.68						
43	50A2	0.001438	9-Apr-12	AG	16-Apr-12	3	0.00143777	3.57E-04	0.99	-2.68						
44	50B2	0.002519	9-Apr-12	AG	16-Apr-12	3	0.0025193	4.50E-04	0.99	-2.68						
45	50C2	4.49E-04	9-Apr-12	AG	16-Apr-12	3	4.49E-04	4.12E-05	0.99	-2.68						
46	10A2	7.08E-05	10-Apr-12	AG	16-Apr-12	3	7.08E-05	1.25E-05	0.99	-2.68						
47	10B2	1.48E-05	10-Apr-12	AG	16-Apr-12	3	2.58E-05	1.48E-05	0.99	-2.68						
48	10C2	1.35E-05	10-Apr-12	AG	16-Apr-12	3	1.35E-05	2.49E-06	0.99	-2.68						
49	1A2	1.56E-08	10-Apr-12	AG	16-Apr-12	1	4.07E-07		0.99	-2.68	18-Apr-12	1	1.36E-08		0.98	-2.68
50	1B2	1.02E-06	10-Apr-12	AG	16-Apr-12	2	6.85E-07	3.65E-07	0.99	-2.68	18-Apr-12	1	2.01E-06		0.98	-2.68
51	1C2	5.25E-07	10-Apr-12	AG	16-Apr-12	1	5.90E-07		0.99	-2.68	18-Apr-12	1	4.61E-07		0.98	-2.68
52	CA2	neg	10-Apr-12	AG	16-Apr-12	0										
53	CB2	neg	10-Apr-12	AG	16-Apr-12	0										

Figure A3. Example Spreadsheet Recording of eDNA PCR Results.

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APPENDIX G ENVIRONMENTAL DNA SAMPLE COLLECTION PROTOCOL

eDNA PROTOCOL
SAMPLE COLLECTION

Caren Goldberg and Katherine Strickler, Washington State University
Revised October 2016

MATERIALS

1. Cellulose nitrate disposable filter funnels or other field-tested, disposable filter funnels
2. Vacuum flask (1L)
3. Silicone tubing
4. Vacuum hand pump (from auto parts store)
5. Rubber stopper with hole for funnel stem
6. Latex or nitrile gloves (non-powdered)
7. Forceps, either stainless steel or disposable plastic (flat-ended filter forceps if possible)
8. *If using steel forceps:* 50 mL tubes with 30 mL of 50% bleach solution (15 mL household bleach and 15 mL distilled water) in a holder to stabilize tubes (a foam drink holder such as a koozie works well)
9. High quality, o-ring screw cap 2mL tubes (e.g., Sarstedt brand) with 1mL 100% molecular-grade ethanol (<u>not</u> denatured)
10. Ethanol-proof laboratory pen (do not use a regular Sharpie marker)
11. Polypropylene grab bottles and cooler with ice (for off-site filtering) or Whirl-Pak® bags (for on-site filtering)
12. Water, bleach, scrub brush, and tubs (for decontaminating between sites)

This protocol is adapted from
Protocol Version 04/12/2012 (D.S. Pilliod, R.S. Arkle, and M.B. Laramie)
USGS Snake River Field Station

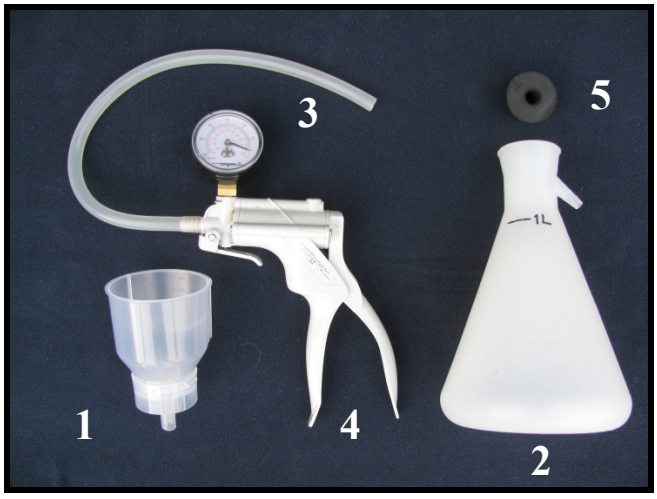

Figure 1. Filter funnel (1), vacuum flask (2), silicone tubing (3), vacuum pump (4), and rubber stopper (5).

Figure 2. Latex or nitrile gloves (6), forceps (7), 2 mL o-ring tubes with 1 mL ethanol (8), ethanol-proof lab marker (9), and 2 50 mL tubes with 50% household bleach/distilled water solution (10).

Figure 3. Polypropylene grab bottles (11a) and Whirl-Pak® bag (11b).

Figure 4. Water (12a), bleach (12b), scrub brushes (12c), and tubs (12d) for decontaminating boots and equipment between sites.

CONTAMINATION PREVENTION

Avoid cross-contamination between samples! Contamination can result from a variety of factors at every step in the sample collection process. Be vigilant.

1. Be careful with gloves and other supplies. Do not leave them unprotected and do not toss them in a backpack. Keep everything clean and in plastic bags. Keep grab bottles and Whirl-Paks in clean bags.
2. Guidelines for wearing gloves:
   - Wear new gloves when pulling Whirl-Paks or grab bottles from bags and collecting water for sampling unless hands have been decontaminated with bleach while decontaminating boots and other gear between consecutive sites.
   - Wear clean gloves when removing filter and placing in ethanol storage tubes. Do not touch anything other than the filter or decontaminated tips of the forceps before you handle the filter. If your gloves touch anything that you're not certain is clean, replace them with clean gloves.
   - No gloves are needed when handling the outside of the filter funnel, vacuum flask, and rubber stopper, as these are downstream from the filter (that is, they are below the filter and do not come into contact with sample water before it is filtered).
   - Use non-powdered gloves only.
3. Open filter funnel package from bottom (stem end) and keep closed between sites.
4. When filtering samples, be careful not to touch the top or inside of the filter cup.
5. *If reusing forceps:* Decontaminate forceps in 50% bleach for at least 1 minute between each sample. Rinse well with distilled or deionized water (Figure 5). *Other methods do not remove DNA and will cross-contaminate your samples!*
6. *If using disposable forceps*: use new forceps for each sample, discarding after use. Remove disposable forceps from plastic wrapper by the hinged end, careful not to touch the tips.
7. Clean boots thoroughly between sites. Remove all dirt, pebbles, etc. from soles and sides of boots. Decontaminate in 10% bleach if they came in contact with water or mud during sampling. Rinse well in tap water (not water from the site) (Figure 6).
8. Bleach vacuum flask and stopper in 10% bleach between sites to prevent disease transport. If vacuum pump and tubing got wet during sampling or filtering, bleach them as well. Submerge equipment in 10% bleach for at least 1 minute, then rinse thoroughly with tap water.
9. To re-use Nalgene grab bottles, bottles must be decontaminated prior to collecting new samples. Submerge bottles in 50% bleach/50% tap water solution for at least 1 minute. Rinse thoroughly with clean tap water (fill, cap, shake, and rinse; repeat at least 3 times). At the sampling site, rinse again with water from the water body 3 times (shaking with cap on each time) before collecting sample to make sure there is no bleach residue in the bottle.
10. To test for field contamination, collect 1 field negative per site. The field negative is distilled water that is filtered and preserved using the same equipment and procedures as the water samples. Fill collection receptacle (Whirl-Pak or bottle, whichever is being used for the samples) with distilled water. Using methods for filtering samples as described in Step 3 below, filter the same volume of distilled water as the volume of samples. Remove and preserve filter as described in Step 4 below.

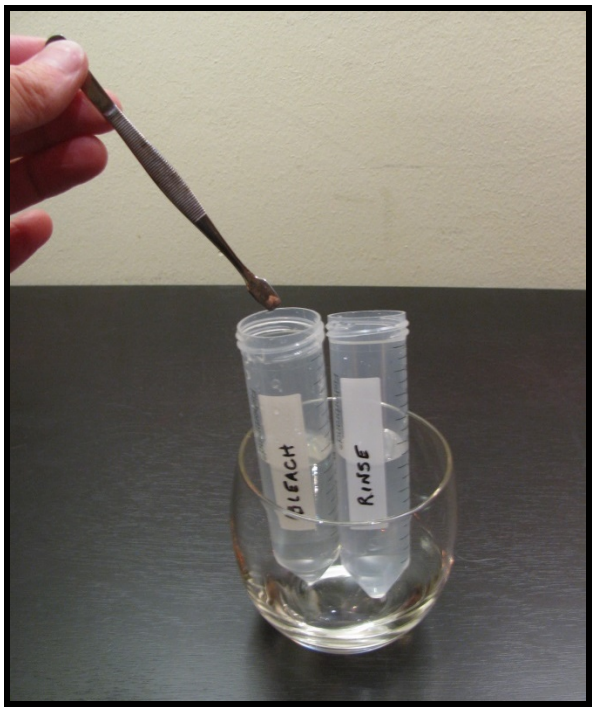

Figure 5. Decontaminate forceps in 50% bleach for at least 1 minute between each sample. Rinse well with distilled water.

Figure 6. Clean boots thoroughly between sites. Decontaminate with 10% bleach and rinse well with tap water.

SAMPLE COLLECTION

Step 1. Sample Site Selection

1. Environmental DNA is not homogenously distributed within lentic aquatic systems, so within-site sample location can be important for detection. Samples can be collected in association with particular habitat characteristics or evenly spaced. It is easiest to sample from the edge of aquatic sites, but space use by species may indicate that sampling from a (decontaminated) boat will increase detection.

   Distribution of eDNA in streams is also likely to be heterogeneous. Knowledge of the target species' ecology can be used to select sampling locations in the stream habitats likely to be used by the species. Samples can be collected from the stream margin, thalweg, or, in larger streams, from a decontaminated boat. In all cases, collect samples upstream of your position and equipment. When sampling multiple sites on the same stream, always begin sampling at the site that is furthest downstream and sample other sites sequentially as you move upstream.

Step 2: Filter Assembly (Figure 7)

1. Attach rubber stopper to top of the vacuum flask.
2. Attach disposable filter funnel to rubber stopper by inserting stem of funnel into hole in stopper, creating airtight seal.
3. Attach vacuum pump to tube on vacuum flask using silicone tubing.

Figure 7. Filter assembly. Note pressure release lever on underside of vacuum pump near the nozzle.

Step 3. Water Collection and Filtration

If filtering on-site:

1. Collect water in new Whirl-Pak for filtering (Figure 8a). Wear new gloves when touching Whirl-Pak and collecting sample.
2. Pour sample slowly into filter funnel, tracking water volume with gradations on filter funnel (Figure 9). Pause several times to swirl water in Whirl-Pak or bottle before pouring remaining water into funnel.
3. Engage vacuum pump to begin filtration (Figure 10). During filtering, make sure vacuum pressure is sustained (monitor pump gauge if available, or watch water level to make sure water is flowing between the funnel and vacuum flask).
4. If > one filter funnel of volume is being collected, disengage vacuum pump when adding more volume if you are using the funnel to measure volume. Otherwise, use mark on flask to determine when target volume has been reached. Do not use the pressure release lever on the vacuum pump or water from hose may contaminate the filter sample. (The pressure release is the small plastic lever located on underside of the pump, below the pressure gauge.)
5. In some aquatic systems, the filter may clog before the target water volume has been filtered. The filtering rate may slow to individual drips separated by several seconds. Consider setting a cutoff time or drip rate for ending filtering. For example, you might end filtering when the drip rate slows to 3 drips every 10 seconds.
6. Make note of the volume of water filtered, whether samples were collected using Whirl-Paks or grab bottles, and any unusual events, conditions, or problems. Be sure to make a note if you suspect there might have been any sort of contamination of the sample.

If taking grab samples for later filtering off-site:

1. Collect water in decontaminated Nalgene bottle (Figure 8b). Wear new gloves for removing bottle from bag and collecting sample.
2. Rinse grab bottle 3 times with water from sample site. Cap and shake water during each rinse. Dispose of rinse water away from spot where you'll collect water sample.
3. Fill grab bottle with water away from where rinsing occurred, while standing in one place to the extent possible. Avoid stirring up sediment while collecting sample.
4. Cap firmly, label with site name and sample number, and place in a cooler with ice.
5. Filter as soon as possible (within 12 hours) using steps 2-6 described above for filtering on-site. Keep grab samples refrigerated or in a cooler filled with ice until they can be filtered.

Figure 8. Collect water in (a) disposable Whirl-Pak® bag or (b) decontaminated Nalgene bottle.

Figure 9. Pour sample slowly into filter funnel.

Figure 10. Engage hand pump to begin filtration.

Step 4. Filter Membrane Removal

1. Decontaminate forceps by soaking in 50% bleach solution for at least 1 minute and then in deionized or distilled water, each stored in a 50 mL tube. Replace water frequently to ensure that forceps are free of bleach before touching filter. After decontamination, the tips of the forceps should not come into contact with anything other than the filter or clean gloves.
2. Remove silicone tubing from the vacuum flask to release vacuum pressure on the filter.
3. Remove funnel cup. Grasp funnel cup in one hand and the funnel base in the other. Gently squeeze and lift funnel cup to disconnect the funnel cup from the base, exposing filter membrane (Figure 11). Remember that the outside of the funnel cup and flask may be contaminated. Gloves are not needed for this step, and if worn, gloves must be replaced for the following step.
4. Open 2 mL o-ring tube to prepare for filter.
5. Put new glove on one hand. Do not touch anything other than the filter membrane with gloved hand.
6. Using decontaminated (or disposable) forceps and gloved fingers, fold filter membrane as described below. In Nalgene cellulose nitrate and some other filter funnels, the filter membrane sits on top of a paper disc. Discard this thicker paper disc and preserve the thinner, uppermost filter membrane. Fold the filter membrane in quarters by folding it in half and then in half again.
7. Roll the folded filter membrane into a cylinder that fits easily into the ethanol tube (Figure 12). Keep filter stable and prevent it from unrolling by using gloved finger. Place filter in 2 mL vial filled with 1 mL ethanol (Figure 13).
8. Cap vial firmly and label with sample ID and date, using an ethanol-proof marker. Label cap with sample ID. Remove glove.
9. Remove filter funnel from rubber stopper and discard funnel.
10. Repeat filtration and filter preservation for each sample and field negative, making sure to empty vacuum flask between samples to prevent it from overfilling. For each sample, wear clean gloves whenever touching filter or forceps tips.
11. Store sample vials at room temperature or colder, and away from light.

Note for shipping samples: Ethanol is prohibited in some methods of shipping. Check with your carrier.

Figure 11. Remove funnel cup.

Figure 12. Fold filter.

Figure 13. Place filter in 2 mL o-ring tube of ethanol.

APPENDIX H SPECIES-SPECIFIC SAMPLING RECOMMENDATIONS – LENTIC SYSTEMS

Optimizing environmental DNA sampling designs to detect rare amphibians

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Introduction

Accurate knowledge of species presence at a site is critical to understanding the drivers of species distributions and identifying effective management actions; however, this local inference can be difficult for species that are rare or elusive (Chadés et al. 2008, Lahoz-Monfort et al. 2014). A new method for detecting presence using environmental DNA (eDNA) in water samples has recently gained traction as a powerful tool (Rees et al. 2014), surpassing traditional field sampling in many cases (Pilliod et al. 2013, Biggs et al. 2015). As with any sampling method, however, detection of species by eDNA is imperfect, with probability <1 of each sample detecting animals that occupy a site (Goldberg et al. 2016). Recent eDNA studies have applied occupancy modeling to incorporate this uncertainty when predicting occupancy of a species at a site (e.g., Hunter et al. 2015, Schmelzle and Kinziger 2015, De Souza et al. 2016). In this framework, the probability of detection is modeled simultaneously with the probability of occupancy of a species at a site given model covariates; higher detection probabilities lead to more precise estimates of occupancy (Mackenzie et al. 2006). Therefore, finding ways to optimize sampling designs to maximize detection probabilities is essential for realizing the contribution eDNA analysis can make to ecology and conservation.

The probability of detecting a species given that the organism is present using eDNA is likely influenced by several processes, including production, degradation, adsorption, and transport (Barnes and Turner 2015). Production rate varies greatly across individuals and within individuals through time (Klymus et al. 2015, Wilcox et al. 2016), and may be influenced by water quality (e.g., conductivity). Degradation rate is likely the result of an interaction between the microorganismal community and abiotic conditions (Barnes et al. 2014, Strickler et al. 2015); because the microbial community is largely uncharacterized, making precise predictions of the impact of abiotic conditions at sites is challenging. In lentic systems, eDNA has been shown to stay local to sources (Takahara et al. 2013, Eichmiller et al. 2014, Yamamoto et al. 2016), with detection dropping off with distance from the source (Dunker et al. 2016), indicating that dispersion is the primary process of transport for eDNA from sedentary individuals. The same pattern from tidal and lotic systems (Port et al. 2016, Wilcox et al. 2016) suggests that forces such as adsorption (and subsequent dropping out of the water column) are acting quickly to limit the transport of these molecules away from the source.

Designing a sampling scheme for an eDNA study requires numerous decisions. These include collection method (filter or precipitation), filter material and pore size (if using filters), preservation method, sample volume, spatial sampling design, number of field replicates, extraction method, and analysis method (e.g., qPCR, metabarcoding). Some of these are determined by the goals of the study or logistical constraints, but some must be chosen by the researcher.

Many filter materials have now been vetted (reviewed in Goldberg et al. 2016), but their effectiveness may vary depending on extraction method (Renshaw et al. 2015). Larger sample volumes may be prohibited by logistical constraints or the pore size of a filter, and small sample volumes may miss eDNA present in clusters (Furlan et al. 2016). However, increasing the pore size may cause a loss of the smaller particles of eDNA (Turner et al. 2014). In addition to the logistical constraints, understanding among-site variation in production, degradation, and dispersion that may limit eDNA detection is critical to designing efficient sampling schemes.

To address the issue of how eDNA sampling design can be optimized to maximize detection probabilities, we tested hypotheses of which covariates most affected detection probability for six amphibian species in two wetland systems presenting a gradient of degradative challenges to eDNA persistence (temperature, pH, canopy cover), dispersion (area, sample volume), and production (conductivity). Our objectives were 1) to understand limiting factors to eDNA detection for each species given environmental conditions and 2) to investigate how sampling designs could be optimized to compensate for those limitations. To accomplish these objectives, we collected eDNA samples over ≥2 seasons for each species simultaneously with crews conducting standard field detection surveys and analyzed the data in an occupancy framework.

Study areas

Arizona Perennial Wetlands. The Arizona Perennial Wetlands study area consisted of wetlands in the Huachuca and Pajarito Mountains and the San Rafael Valley of southeastern Arizona, U.S. Chiricahua leopard frogs (*Lithobates [Rana] chiricahuensis*; federally threatened) persist in upland wetlands in this area and valley wetlands provide habitat for endangered Sonoran tiger salamanders (*Ambystoma mavortium stebbinsi*). Wetlands in this area were primarily cattle tanks (ponds) and restoration sites. The invasive American bullfrog (*L. catesbeianus*), a threat to both native species, was also found throughout this area. These wetlands were a range of sizes and temperatures (Table 1), providing a gradient of challenges for eDNA detection.

Arizona Intermittent Wetlands. The Arizona Intermittent Wetland area consisted of wetlands in the Huachuca Mountains that filled during the summer rainy season, providing habitat for Arizona treefrogs (*Hyla wrightorum*), a species recently considered as a candidate under the ESA. This species spends a limited amount of time at temporary wetlands after they fill with summer rains and can be difficult to detect (Mims et al. 2016). These wetlands were small and warm, providing a challenge to eDNA persistence and therefore detection.

Florida panhandle. The Florida study site was located at Eglin Air Force Base (AFB) in the long-leaf pine forests of the Florida Panhandle. Wetlands on Eglin AFB provide extensive habitat for reticulated flatwoods salamanders (*A. bishopi*), listed as endangered under the ESA, and ornate chorus frogs (*Pseudacris ornata*), a sensitive species. These wetlands are large and acidic (Palis 1997), providing multiple challenges for eDNA detection.

Methods

Assay development. We designed and validated species-specific qPCR assays for four target species (Table 2, Appendix S1) and applied two previously-published assays (Strickler et al. 2015, McKee et al. 2015; Appendix S1). For assay design, we compiled sequence data for each target species from Genbank (NCBI) and created an inclusive consensus sequence using Sequencher version 5.2.4 (GeneCodes Corp., Ann Arbor, MI). We used Primer Express 3.0.1 (Applied Biosystems, Foster City, CA) to design potential qPCR assays using that sequence and then tested those assays *in silico* using the Primer- BLAST algorithm (Ye et al. 2012), set to indicate any sequence matches with less than 2 base pairs changes total with at least 1 located within 4 base pairs from the 3' end on each primer. If any species with an overlapping geographic range with the range of the target species was indicated, the design was discarded and another was tested. Each assay that met criteria was then tested against a panel of tissue samples from the target species (minimum 10 samples) and co-occurring closely related non-target species (minimum 5 samples each). Tissue samples collected for this validation (Appendix S1) were under University of Idaho Animal Care and Use Committee Protocol 2012-40 and permitted by the Florida Fish and Wildlife Conservation Commission and Arizona Game and Fish Department.

Reactions were run using Quantitect Multiplex PCR Mix (Qiagen, Inc, Hilden, Germany) with recommended multiplexing concentrations (1X QuantiTect Multiplex PCR mix, 0.2 µM of each primer, and 0.2 µM probe) on an Applied Biosystems 7500 Fast Real-Time PCR System at the University of Idaho Laboratory for Ecological, Evolutionary and Conservation Genetics (2012-2013 samples) or a CFX96™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA) at Washington State University (2014-2015 samples). Reactions were 15 µl in volume and each included 3 µl of sample. Cycling began with 15 min at 95°C followed by 50 cycles of 94°C for 60 s and 60°C for 60 s.

Field sample collection. We worked with field crews applying established protocols to collect eDNA samples matched with field detection data in each study area. In the baseline year(s), we sampled 20 wetlands in each study area using filter funnels with hand pumps (Goldberg et al. 2011), following clean protocols (Goldberg et al. 2016). Following eDNA sample collection, standard field surveys were conducted by field crews. For each site, we also collected data on factors hypothesized to affect eDNA detection through production, degradation, adsorption, and dilution. Specifically, we measured pH, water temperature, and conductivity using a multiparameter meter (OAKTON Instruments, Vernon Hills, IL) and canopy cover as a measure of exposure to UV with a densiometer. In Arizona, we mapped the surface area of wetlands by walking the perimeter with a Trimble XT (Trimble Navigation Limited, Sunnyvale, CA). In Florida, we primarily used area data from Eglin Air Force Base biological crews and supplemented with GPS mapping with a Trimble XT of some wetlands in the final year (2015).

In the first sampling year (2012-2013), we collected samples using a standard design of 4 replicate samples collected at one location in each wetland in all study sites. For this sampling, we targeted the shallowest area with vegetation as the area most likely to contain larvae. For sample collection, we used 0.45 µm cellulose nitrate single-use sterile filter funnels (Whatman™, GE Healthcare, Pittsburgh, PA) and concentrated eDNA in 250 mL of water per filter, or the amount of water that could be filtered before clogging.

For Florida species, flatwoods salamanders were not present in the first year (due to a failed breeding season) and we detected ornate chorus frogs with eDNA at only 4 sites. Because of this outcome, we increased volume filtered to 500 mL per filter and combined water from 4 sites around the wetland in equal volumes for the following season (2014; considered as baseline sampling for this study area).

For each set of samples collected, a negative field control of distilled water was also collected with the same protocol and instruments to test for cross-contamination among samples. Sites focused on detection of Chiricahua leopard frogs and Arizona treefrogs in 2012 were primarily sampled on site; remaining sites were collected using polypropylene grab bottles and kept on ice until filtering within 24 hours. Grab bottles were soaked in 50% household bleach after use, triple rinsed in with tap water, and triple rinsed with wetland water before sampling from each wetland. Samples were collected from just under the water surface. Filters were preserved in 95% ethanol and stored away from excessive heat and light until DNA extraction (within 6 months). After the baseline sampling year, sampling designs were optimized for each system as described below.

Laboratory analysis. We extracted DNA from filter samples using the Qiashredder/DNeasy method described in Goldberg et al. (2011). All filter sample extractions and qPCR set up was conducted in a lab dedicated to low-quantity DNA samples. Researchers are required to shower and change clothes before entering this room after being in a high-quality DNA or post-PCR laboratory, and no tissue samples have been handled in this room. A negative extraction control was included with each set of extractions and an additional negative qPCR control was run with each plate of samples. Reactions were as described under assay design. We used a multi-tube approach for analysis (Taberlet et al. 1999), in that we analyzed each sample in triplicate. To test for inhibition we included an internal positive control (IC; Qiagen or IPC; Applied Biosystems) in each well. A positive sample was defined as any sample that showed exponential amplification in all three wells the first time it was tested or in one or more wells from two separate reactions (samples were rerun whenever the original triplicate wells yielded inconsistent results). Samples testing as inhibited were cleaned using a OneStep^{TM} PCR Inhibitor Removal Kit (Zymo, Inc., Irvine, CA). Quantitative standards consisted of diluted DNA samples derived from tissue from external skin to best represent what eDNA may be sourced, quantified on a Nanodrop spectrophotometer, diluted 10^{-3} through 10^{-6}, and run in duplicate.

Modeling amphibian detection with eDNA (baseline sampling). To investigate factors influencing detection of species using eDNA, we analyzed data from the baseline sampling year in an occupancy modeling framework (Mackenzie et al. 2002) applied in PRESENCE 10.0 (Hines 2006) and used results to inform sampling design in the following year. The exception to this was for the Arizona treefrogs, where occupancy was relatively unknown and we conducted our own dipnet surveys; it took all three years of sampling to have enough data (N>10) to model detection for this species. In the final year, we used 0.45 µm cellulose nitrate single-use sterile filter funnels from Nalgene (Rochester, NY) instead of the originals as the original filter funnels had been discontinued. Because the goal of the analysis was to analyze factors affecting detection rather than occupancy, we analyzed the subset of data for each species where there was evidence of occupancy (psi = 1) from either field crews or eDNA detection in at least one filter sample and fixed psi at 1 in the models.

We treated each filter sample as a replicate at a site, producing four sampling 'occasions' at each site in the baseline year for each species, and z-scored all continuous covariates. We assumed that the threshold of evidence applied to PCR replicates removed all false positives. Although this conservative threshold may have decreased our detection probability per filter sample, occupancy model structure assumes that detection probabilities are < 1, while false positives can cause large biases and are more difficult to account for (Chambert et al. 2015, Lahoz-Monfort et al. 2016). We tested hypotheses of which covariates most affected detection probability and used evidence weights to determine the most likely explanation for variation in this measure. Due to small sample sizes, all hypotheses included only a single variable.

Applying adaptive sampling designs. To investigate factors influencing detection of species using eDNA, we analyzed data from the baseline sampling year in an occupancy modeling framework (Mackenzie et al. 2002) applied in PRESENCE 10.0 (Hines 2006) and used results to inform sampling design in the following year. Because the goal of the analysis was to analyze factors affecting detection rather than occupancy, we analyzed the subset of data for each species where there was evidence of occupancy (psi = 1) from either field crews or eDNA detection in at least one filter sample. We assumed that the threshold of evidence applied to PCR replicates removed all false positives. Although this may have decreased our detection probability per filter sample, these false negatives are accounted for in the model structure, while false positives can cause large biases and are much more difficult to account for (Lahoz-Monfort et al. 2015, Chambert et al. 2015). We treated each filter sample as a replicate at a site, producing four 'occasions' at each site in the baseline year for each species, and z-scored all continuous covariates. We tested hypotheses of which covariates most affected detection probability and used evidence weights to determine the most likely explanation for variation in this measure. Due to small sample sizes, all hypotheses included only a single variable. We used results to adapt sample designs to increase detection probabilities in the following year. Sampling design in these follow-up years focused on sites that were the most challenging for detection (low density, high degradation rate). Lastly, we analyzed factors influencing detection probability in the final data collection year to see if further improvements could be made.

Results

Modeling amphibian detection with eDNA (baseline sampling). We collected 891 field samples at sites that ranged across several variables hypothesized to be important to detection, including temperature, acidity, and area (Figure 1; Table 1). All negative controls tested negative and standard curves had $r^2 > 0.96$ and efficiencies between 80 and 120%. All Florida samples were treated for inhibition before final analysis; inhibition was uncommon from Arizona sites and was treated only for samples where the internal positive control failed to amplify. Models indicated that in the original sampling design detection of most species was limited by factors influencing degradation rate, specifically pH and temperature (Table 3, Figure 2). The two exceptions were Chiricahua leopard frogs, which were limited by dispersion at a wetland scale, and tiger salamanders, which were limited by the amount of water that could be filtered, a measure of dispersion at a micro scale.

Applying adaptive sampling designs. Based on the findings from the baseline year of sampling at each site, we adjusted protocols by target species. At Chiricahua leopard frog sites, if the wetland was larger than 1200 m2 (field estimated at 40 m diameter measured by laser rangefinder), we added an extra sampling location at the farthest points distant around that wetland.

At each sampling location, we collected 3 replicate samples to bring the overall probability of detection above 0.95 (based on model predictions; Figure 2). At tiger salamander-targeted locations, we changed the filter used to a 6 µm cellulose filter so that the goal of 250 mL of water could be reached in these sites, which mostly consisted of cattle ponds. In Florida, we sampled 8 within-wetland locations instead of 4 at wetlands with pH<5. At Florida sites, filter samples consisted of an equal amount of water from each sampling location at a site, with the total number of filters equaling the number of sampling locations. Additionally, at Florida sites we adjusted protocol in the final year by changing filters to a 5 µm polyethersulfone (PES) filter to reduce filtration time (which had taken several hours per filter to concentrate 500 mL using the 0.45 µm cellulose nitrate filters the previous year).

With adaptive sampling designs, we detected target species at almost all sites where field crews detected them, plus 26 additional sites with eDNA methods only (Table 4). Average per-sample detection rates ranged from 0.62-0.86, indicating that 2 to 4 samples would be needed per site to achieve on average a 0.95 probability of population detection given the adaptive sampling design targeted to each species to compensate for limiting factors. Because sampling design in these follow-up years focused on sites that were the most challenging for detection (low density, high degradation rate), average detection probabilities may be biased low compared with a random sample of all potentially occupied sites.

Analysis of final year detection probability data for species where adaptive sampling was applied indicated no strong evidence that detection probabilities were related to covariates ($\Delta AIC < 4$ from null model) for species with large enough sample sizes to model (tiger salamanders and American bullfrogs). For American bullfrogs, there was some indication that applying additional sampling locations at larger sites could further improve detection (evidence weight = 0.61), and for Arizona treefrogs models indicated that additional sampling at warmer sites (>25°C) could improve detection, although per filter detection was estimated to be 0.71 even at the warmest site sampled (30.3°C). Field data from sites where target species were not detected consisted of: one calling Chiricahua leopard frog estimated to be in the water approximately 50 m from sampling location; one calling Arizona treefrog approximately 15 m from the water (27.6°C water temperature); two American bullfrogs in a 3266 m^2 wetland sampled at one location; two ornate treefrogs in an 33,386 m^2 wetland with pH 4.6 sampled at 8 locations; and 8 flatwoods salamanders in an 8292 m^2 wetland with pH 4.4 sampled at 8 locations.

Discussion

Detection of species using eDNA is still a new approach and although the standard recommendation is to conduct a pilot study (Goldberg et al. 2016), there are few examples of how to use the findings of that pilot study to improve sampling design. We found evidence that degradation and dispersion strongly influence the detection of amphibians in wetlands, and that these forces can be compensated for through adaptive sampling to maximize efficacy of eDNA monitoring approaches. Additionally, we found that the eDNA lost by increasing filter pore size from 0.45 µm to 6 µm in a high-particulate system was more than made up for by the increased volume of water that could be filtered. This is consistent with fractionation studies that showed that the modal size for fish eDNA was between 1 and 10 µm (Wilcox et al. 2015, Turner et al. 2014), but how this would work in a field setting had not been previously documented.

Contrary to our original expectations, we found that dispersion was a limiting force in eDNA detection even for small wetlands (>1200 m^2) and that sampling from multiple locations could ameliorate this issue. Additionally, sampling at more locations in space was critical to raising detection probabilities in a highly acidic environment, even when sample volume was fixed. This suggests an interaction between dispersion and degradation important to informing sampling design. We propose that the eDNA shed by an organism surrounds that individual in an exponential distribution, where the density of eDNA is highest just outside of the organism and drops off with distance away from the organism (source of production), such that the probability of sampling a particle from the organism becomes exponentially smaller with distance (Figure 3). The distance from the organism at which the density of eDNA can be detected in a water sample is then inversely related to the removal rate (dispersion, adsorption and degradation), and positively correlated with production. Using this model, detection probability at a lentic site would be maximized when production is highest at the individual level, due to activity or feeding patterns, or the population level, reflecting abundance. Additionally, the larger the space use of an individual, the more distributed, but also sparse, the target eDNA molecules would be. The optimal spatial density of sampling therefore depends on space use, population density, and removal rate. One applicable inference from this model is that more degradative environments would require denser spatial sampling.

Implications for occupancy modeling. After optimization, we found that eDNA sampling still had imperfect detection. Some of this variation could be designed out through further optimization, but some factors influencing eDNA detection cannot be ameliorated through sampling design. For example, the space use of target organisms will influence the optimal spatial distribution of eDNA samples, with the most challenging species those that are sedentary in large lentic systems where spatial patterns are difficult to predict. Additionally, wetlands can have heterogeneous flows (Lightbody et al. 2008) and the dispersion of eDNA particles can influence the probability of a particle being collected in a given volume of water (Furlan et al. 2016). Therefore, eDNA samples should be analyzed in an occupancy modeling framework that accounts for lack of detection, even after optimization.

One challenge for occupancy modeling is that replicate surveys need to be conducted within a defined sampling unit. For population monitoring of wetland species, the wetland is generally used as this sampling unit, while in lake and streams determining an appropriate unit can be more challenging. Because eDNA is dispersion limited and therefore patchy across wetlands, it is important that each filter sample collected be a replicate of the same points in space and therefore share a probability of detection. This can be accomplished by integrating water samples collected from around wetlands in equal amounts through each filter. In this design, the ultimate number of samples (=filters) analyzed can be different than the number of spatial locations where water is collected, a cost-saving measure. Where it possible to control for contamination, continuous sampling (e.g., Civade et al. 2016) may be the best method for collecting samples across space.

Implications for biomass estimation. If the scale of the distribution of eDNA around an organism varies as a function of degradative forces, then these forces need to be accounted for both when sampling for occupancy as well as in efforts to estimate biomass. For example, Yamamoto et al. (2016) used the relationship between an index of fish biomass and surface eDNA to demonstrate that eDNA quantities generally reflected fish biomass within 150 m of the sample. If this distance varies with degradative forces, parameterizing the effect of these processes will be critical to interpreting the spatial inference of eDNA quantification.

Conclusions

By sampling across systems with different limiting factors, we explored the space in which each factor (pH, area, temperature) affected detection through extreme values. Based on these results, we recommend increasing pore size up to 6 μm if smaller pore sizes clog in a system and combining samples from across wetlands to create replicate samples. Our results indicate that 60 m (assuming a perfect circle of 1200 m^2) may be a good sampling interval in moderately degradative systems, and is consistent with eDNA detection of milfoil in a lake setting (Newton et al. 2016). However, the optimal sampling interval likely varies with degradation rate and species space use and may be an underestimate for sedentary individuals; detection of caged fish in Dunker et al. (2016) dropped off between 1 and 10 m and the signal was similar to background levels at 40 m.

While we did not find that conductivity or grab sampling affected detection probability, this may be because none of our systems contained extreme values for these variables. For example, recent work has shown that holding grab samples at ambient temperature for six hours or longer than two days refrigerated can reduce yields (Yamanaka et al. 2016, Hinlo et al. 2017), and yields may even be reduced by spending 6.5 hours on ice (Yamanaka et al. 2016). Within the values included in this study, we found that three or four 250 mL samples were sufficient for a 0.95 probability of detection of amphibians given a dispersed sampling design for larger and more acidic ponds. Additionally, increased sampling when water temperatures are >20ºC may be warranted. These results provide a baseline for sampling design for additional systems.

Optimizing sampling designs for eDNA surveys is an important step in this developing field. As our understanding of the processes of eDNA production, removal, and transport increases, we will further be able to determine the most effective ways to sample to infer to a targeted spatial unit. In the meantime, incorporating pilot studies and adaptive sampling into large eDNA sampling efforts can help increase the contribution of this technique on conservation and ecology and further elucidate how environmental conditions influence the detection of species using eDNA.

ACKNOWLEDGEMENTS

We are grateful for the fieldwork of Arizona Game and Fish biologists, including Tom Jones, Christina Akins, Mike Sredl, Abigail King, and Jeff Sorenson, and of Kelly Jones, Brandon Rincon, Paul Moler, Kevin Enge, and Meryl Mims. This project was made possible by Bruce Hagedorn, Kathy Gault, Carola Haas, Tom Gorman, Sheridan Stone, and Debbie Brewer. Lisette Waits provided invaluable support and advice at the instigation of this project. The Nature Conservancy provided field site housing. Field and laboratory work was conducted by Andy Gygli, Kody Cochrell, and Kevin Lewallen. Illustration by Lisa Waananen Jones. This work was funded by the Department of Defense Environmental Security Technology Certification Program under ESTCP Project RC-201204, Legacy Resource Management Program 12-616 and 14-616, and in part by the U. S. Army Research Laboratory and the U. S. Army Research Office under contract number W911-13-1-0266.

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Ye, J., G. Coulouris, I. Zaretskaya, I. Cutcutache, S. Rozen, and T. Madden. 2012.

Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics. 13:134.

Table 1. Range of Variation for Sites Where Each Species was Detected by Field or eDNA Methods.

Chiricahua leopard frogs (RACH), Arizona treefrogs (HYWR), tiger salamanders (AMTI), and American bullfrogs (LICA), ornate chorus frogs (PSOR) and flatwoods salamanders (AMBI).

Hypothesized Limiting Process	Model	AMTI	LICA	RACH	HYWR	AMBI	PSOR
Degradation	Canopy cover (%)	0-2	0-25	0-25	0-19	0-79	0-79
	Grab sample (Y/N)	Y	Y/N	Y/N	Y/N	Y	Y
	pH	7.8-10.7	6.5-10.0	6.7-10.1	6.6-9.6	4.2-6.7	4.1-6.7
	Temperature (ºC)	5.1-23.2	5.1-30.9	17.1-30.8	20.6-30.8	9.2-24.0	9.6-24.0
Dispersion	Area (m^2)	185-3409	68-7354	50-2388	10-5025	728-121810	728-59084
	Volume (mL)	75-250	100-250	100-250	250	500	500
Production	Conductivity (mS/cm)	58-660	23-619	91-586	52-390	24-99	22-99

Table 2. Quantitative PCR Assay Designs Created for this Study.

Assays were validated per Goldberg et al. 2016 (Appendix S1).

Species	Primer	Sequence
Chiricahua leopard frog (*Lithobates chiricahuensis*)	RACHF	GGTACCGCTCATATCATGACTACTTG
	RACHR	TCCAGTTGGACTCACTTAGGAATG
	RACHProbe	6FAM- TAGGACCTTCGCTTGTTAT-MGB
Arizona treefrog (*Hyla wrightorum*)	HYWRF	CGCTCCATTCCAAATAAGCTAGGA
	HYWRR	AGGCGGTGGTTCGTTGGTTAG
	HYWRProbe	CAL Fluor Red 610- AGTCCTCGCCCTCCTCTTCTCCAT-BHQ2
Tiger salamander (*Ambystoma mavortium*)	AMSTF	GGCAGATAGTTGGATGCACGATAG
	AMSTR	ACTACCTCTTGTCCTGGTTTTCCT
	AMSTProbe	CalFluorRed- CATAATATGTTGCCACGCTACT-BHQPlus
Ornate chorus frog (*Pseudacris ornata*)	PSORF	TRGGTGTCTGCCTCATTCTTCA
	PSORR	GGCYACGGATGAGAAGGCTAT
	PSORProbe	Quasar670- ATCGCCACTGGCCTATTT-BHQPlus

Table 3. Weights of Evidence for Each Factor Influencing eDNA Detection Probability for Each Amphibian Species Based on Baseline Sampling Design: Chiricahua Leopard Frogs (RACH), Arizona Treefrogs (HYWR), Tiger Salamanders (AMTI), and American Bullfrogs (LICA), Ornate Chorus Frogs (PSOR) and Flatwoods Salamanders (AMBI).

The [–] symbol indicates not enough variation in the covariate for the model to converge for that species. Sample sizes are in parentheses after each species name. Only sites with known occupancy were used in the analysis.

Hypothesized Limiting Process	Model	AMTI (12)	LICA (16)	RACH (10)	HYWR (16)	AMBI (10)	PSOR (6)
Degradation	Canopy cover	-	-	0.00	0.03	0.00	0.00
	Grab sample	-	-	0.01	-	-	-
	pH	0.00	0.04	0.00	0.12	**1.00**	**1.00**
	Sam. Occasion	0.00	0.01	0.00	0.01	0.00	0.00
	Temperature	0.00	**0.69**	0.00	**0.51**	0.00	0.00
Dispersion	Area	0.00	0.05	**0.97**	0.15	0.00	0.00
Filter clogging	Volume	**1.00**	0.04	0.02	**-**	0.00	0.00
Production	Conductivity	0.00	0.07	0.00	-	0.00	0.00
	Null	0.00	0.10	0.00	0.19	0.00	0.00

Table 4. Comparison of Field and eDNA Detections of Target Species after Application of Adaptive Sampling Designs.

95% confidence limits are in parentheses below each average detection probability. For all sites with field detections only, a low density of individuals was estimated (max: 1 individual/1000 m^2).

	Sites surveyed	Sites with both field and eDNA detections	eDNA detections only	Field detections only	Average per-sample eDNA detection probability	# samples per site for >0.95 detection probability
AMTI	20	10	3	0	0.77 (0.61-0.93)	3 (2-4)
LICA	31	6	14	1	0.75 (0.60-0.90)	3 (2-4)
RACH	21	4	2	1	0.62 (0.43-0.81)	4 (2-6)
HYWR	43	12	5	1	0.86 (0.79-0.94)	2 (2-2)
AMBI	11	4	1	1	0.75 (0.44-1.00)	3 (1-6)
PSOR	11	3	1	1	0.83 (0.51-1.00)	2 (1-5)

Figure legends

Figure 1. Principal component analysis of potential factors limiting detection across sampling sites where target species were found. Blue shading indicates where area and acidity become limiting, red shading where temperature becomes limiting. Created using package factoextra 1.0.4 (Kassambara and Mundt 2017) in Program R 3.3.1 (R Core Team 2016). Chiricahua leopard frogs (RACH), Arizona treefrogs (HYWR), tiger salamanders (AMTI), and American bullfrogs (LICA), ornate chorus frogs (PSOR) and flatwoods salamanders (AMBI).

Figure 2. Models of per-sample detection probability for each species as sampled by eDNA prior to sample design optimization. The model for ornate chorus frogs did not converge as all samples with pH > 5 had perfect detection (1.0) and all sites with pH < 5 had no detection (0/4). Points indicate explanatory variable measures at sampled sites.

Figure 3. Conceptual model of the influence of degradative processes on the distribution of environmental DNA in water. Panel A represents the dispersion of eDNA particles in a system without extreme degradative conditions, while Panel B indicates that the same process occurs at a smaller extent when temperatures, UV, and acidity are increased.

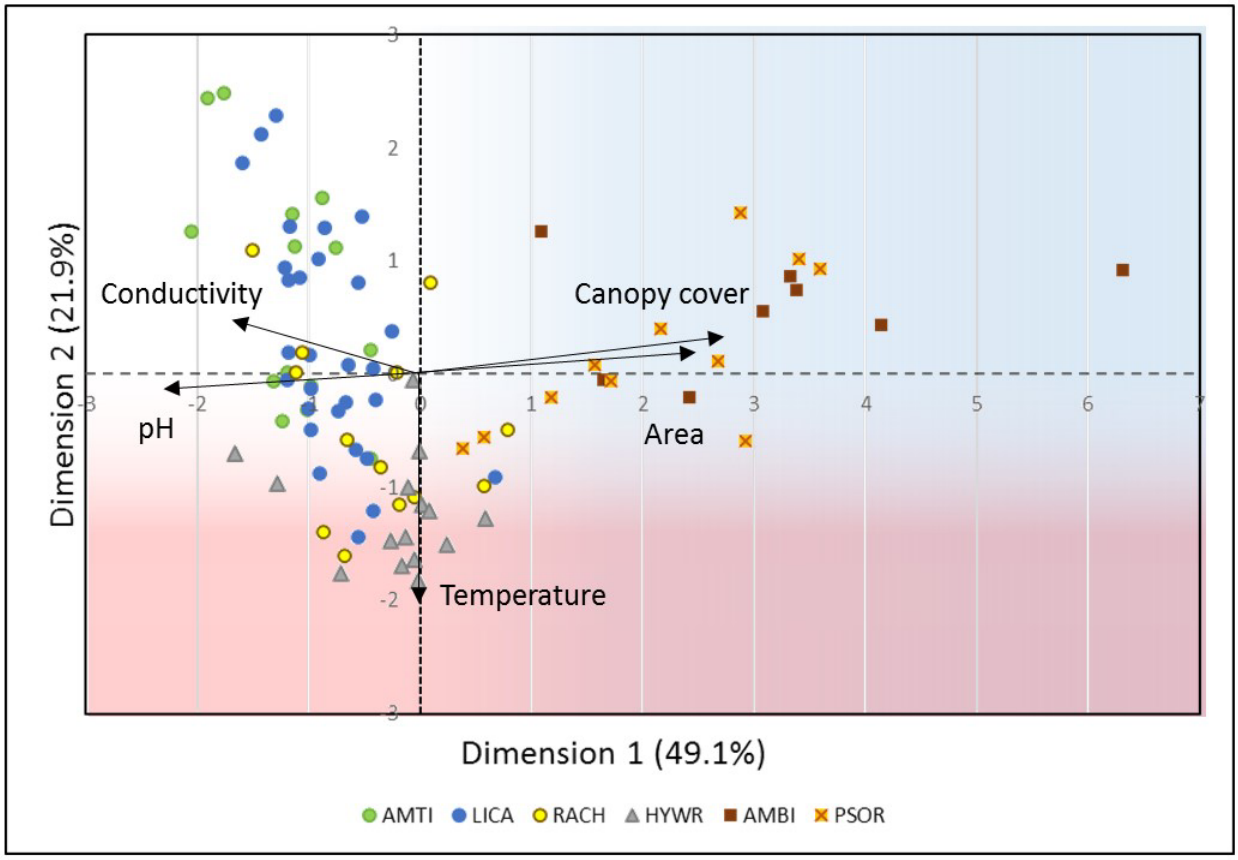

Figure 1

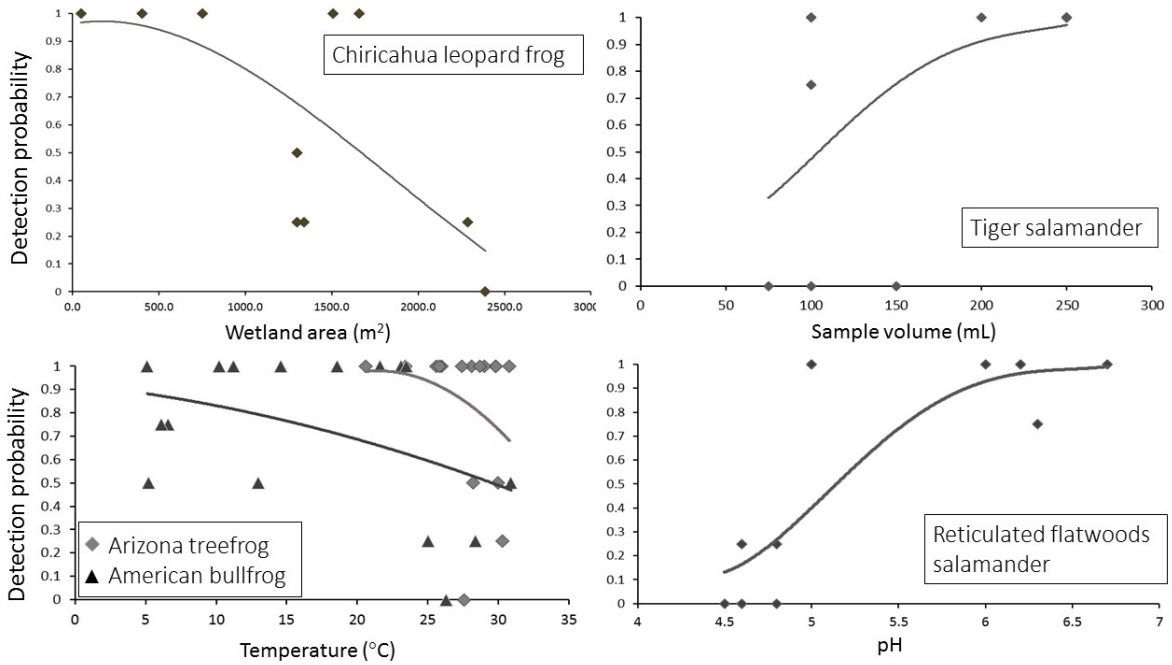

Figure 2

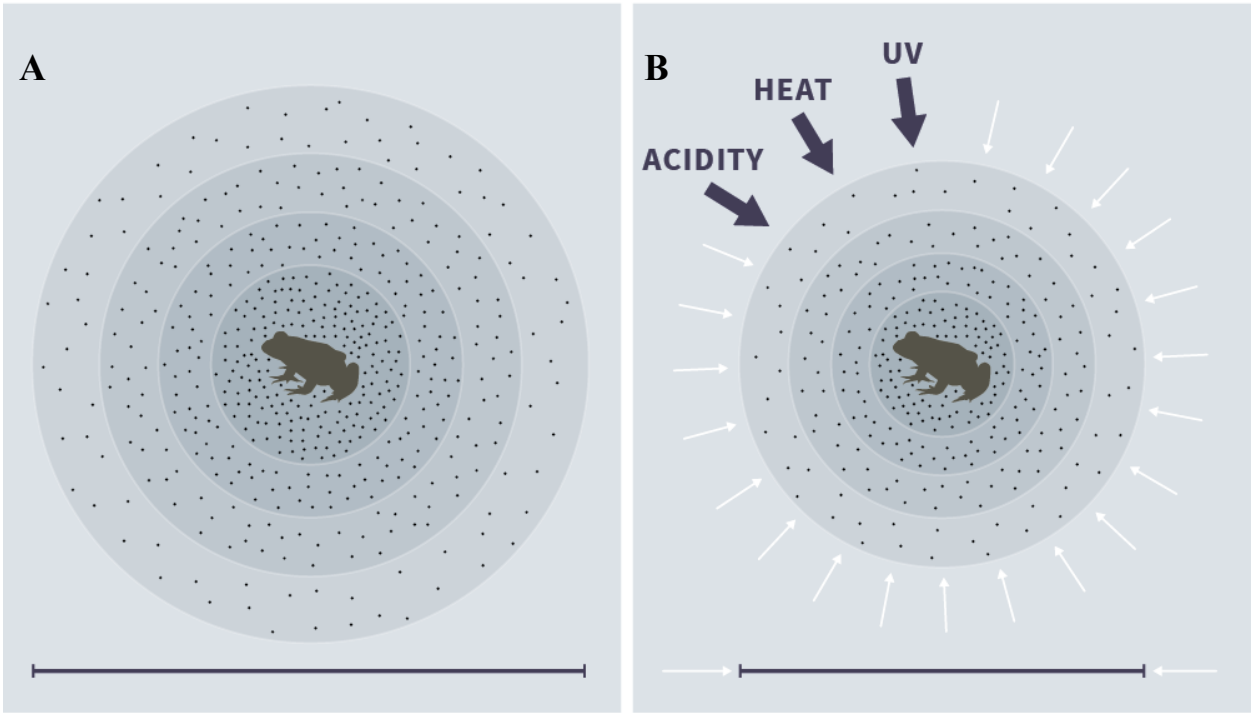

Figure 3

Appendix S1

Species: Chiricahua leopard frog (*Lithobates chiricahuensis*) and Arizona treefrog (*Hyla wrightorum*)

Datasets: Goldberg, C. S., K. J. Field, and M. J. Sredl. 2004. Mitochondrial DNA sequences do not support species status of the Ramsey Canyon Leopard Frog *(Rana subaquavocalis*). Journal of Herpetology 38:313-319.

Gergus, E, Reeder, T. W., Sullivan, B.K. 2004. Geographic variation in *Hyla wrightorum*: advertisement calls, allozymes, mtDNA, and morphology. Copeia 2004:758-769.

RACH assay design (84 bp):

Forward Primer	GGTACCGCTCATATCATGACTACTTG
Reverse Primer	TCCAGTTGGACTCACTTAGGAATG
Probe	TAGGACCTTCGCTTGTTAT-MGB

HYWR assay design (105 bp):

Forward Primer	CGCTCCATTCCAAATAAGCTAGGA
Reverse Primer	AGGCGGTGGTTCGTTGGTTAG
Probe	AGTCCTCGCCCTCCTCTTCTCCAT-BHQ2

Publication: Unpublished

Validated species:

American bullfrog (*Lithobates [Rana] catesbeianus*)
Arizona treefrog (*Hyla wrightorum)*
Barking frog (*Craugaster augusti*)
Canyon treefrog (*Hyla arenicolor*)[1]
Chiricahua leopard frog (*Lithobates [Rana] chiricahuensis*)[2]
Couch's spadefoot (*Scaphiopus couchi*)[3]
Great Plains toad (*Anaxyrus [Bufo] cognatus*)[3]
Lowland leopard frog (*Rana yavapaiensis*)
Mexican spadefoot (*Spea multiplicata*)
Red-spotted toad (*Anaxyrus [Bufo] punctatus*)
Sonoran Desert toad (*Incilius [Bufo] alvarius*)
Tarahumara frog (*Lithobates [Rana] tarahumarae*)[4]
Western narrow-mouthed toad (*Gastrophryne olivacea*)[5]
Woodhouse's toad (*Anaxyrus [Bufo] woodhousii)*

[1]Collected by Meryl Mims, [2]Collected by Tom Jones, [3]Collected by Lauren Chan, [4]Collected by Mike Sredl, [5]Provided by Museum of Vertebrate Zoology, University of California, Berkeley, 3 samples only (MVZ 228-274 through 276)

Species: American bullfrog (*Lithobates catesbeianus*)

Dataset: Austin, J.D., Lougheed, S.C., Boag, P.T., 2004. Controlling for effects of history and nonequilibrium conditions in gene flow estimates in northern bullfrog (*Rana catesbeiana*) populations. Genetics 168, 1491–1506.

Assay design (83 bp):

Forward Primer	TTTTCACTTCATCCTCCCGTTT
Reverse Primer	GGGTTGGATGAGCCAGTTTG
Probe	TTATCGCAGCAGCAAGT-MGB

Publication: Strickler, K. M., A. K. Fremier, and C. S. Goldberg. 2015. Quantifying the effects of UV, temperature, and pH on degradation rates of eDNA in aquatic microcosms. Biological Conservation 183:85-92.

Validated species:

North Idaho
American bullfrog (*Lithobates [Rana] catesbeianus*)
Sierra treefrog (*Pseudacris sierra*)
Western toad (*Anaxyrus [Bufo] boreas*)
Columbia spotted frog (*Rana luteiventris*)

Arizona
American bullfrog (*Lithobates [Rana] catesbeianus*)
Arizona treefrog (*Hyla wrightorum)*
Barking frog (*Craugaster augusti*)
Canyon treefrog (*Hyla arenicolor*)[1]
Chiricahua leopard frog (*Lithobates [Rana] chiricahuensis*)
Couch's spadefoot (*Scaphiopus couchi*)[2]
Great Plains toad (*Anaxyrus [Bufo] cognatus*)[2]
Lowland leopard frog (*Rana yavapaiensis*)
Mexican spadefoot (*Spea multiplicata*)
Red-spotted toad (*Anaxyrus [Bufo] punctatus*)
Sonoran Desert toad (*Incilius [Bufo] alvarius*)
Tarahumara frog (*Lithobates [Rana] tarahumarae*)[3]
Western narrow-mouthed toad (*Gastrophryne olivacea*)[4]
Woodhouse's toad (*Anaxyrus [Bufo] woodhousii)*

[1]Collected by Meryl Mims, [2]Collected by Lauren Chan, [3]Collected by Mike Sredl, [4]Provided by UC Berkeley MVZ, 3 samples only

Species: Tiger salamander (*Ambystoma mavortium*)

Dataset: Storfer, A., Mech S. G., Reudink, M. W., Ziemba, R. E., Warren, J., Collins, J. P. 2004. Evidence for introgression in the endangered Sonora tiger salamander, *Ambystoma tigrinum stebbinsi* (Lowe). Copeia 2004:783-796.

Assay design (79 bp):

Forward Primer:	GGCAGATAGTTGGATGCACGATAG
Reverse Primer:	ACTACCTCTTGTCCTGGTTTTCCT
Probe:	CATAATATGTTGCCACGCTACT-BHQPlus

Publication: Unpublished

Validated species:

Sonora tiger salamander (*Ambystoma mavortium stebbinsi*) [1]
Barred tiger salamander (*Ambystoma mavortium mavoritum*)[1]

[1]Some samples provided by Andrew Storfer

Species: Flatwoods salamander (*Ambystoma bishopi* and *A. cingulatum*)

Dataset: Pauly, G. B., Piskurek, O., Shaffer, H. B. 2007. Phylogeographic concordance in the southeastern United States: the flatwoods salamander, *Ambystoma cingulatum*, as a test case. Mol. Ecol. 16:415-429.

Assay design (133 bp):

Forward Primer	GGCCCGTCAACTTTCCTCTAA
Reverse Primer	TGGTCCAGGTAAATCAATTGCA
Probe	TACGGTAATATGTCTGGTACTAC-MGB

Publication: McKee, A. M., D. L. Calhoun, W. J. Barichivich, S. F. Spear, C. S. Goldberg, T. C. Glenn. 2015. Assessment of environmental DNA for detecting presence of imperiled aquatic amphibian species in isolated wetlands. Journal of Fish and Wildlife Management 6:498-510.

Validated species:[1]

Frosted flatwoods salamander (*Ambystoma cingulatum*)
Spotted salamander (*Ambystoma maculatum*)
Marbled salamander (*Ambystoma opacum*)
Mole salamander (*Ambystoma talpoideum*)
Reticulated flatwoods salamander (*Ambystoma bishopi*)
Tiger salamander (*Ambystoma tigrinum*)

[1]Provided by Anna McKee

Species: Ornate chorus frog (*Pseudacris ornata*)

Dataset: Degner,J. F., Silva, D. M., Hether, T. D., Daza, J. M., Hoffman, E. A. 2010. Fat frogs, mobile genes: unexpected phylogeographic patterns for the ornate chorus frog (Pseudacris ornata). Mol. Ecol. 19:2501-2515.

Assay design (91 bp):

Forward Primer	TRGGTGTCTGCCTCATTCTTCA
Reverse Primer	GGCYACGGATGAGAAGGCTAT
Probe	ATCGCCACTGGCCTATTT-BHQPlus

Publication: Unpublished

Validated species:

Barking treefrog (*Hyla gratiosa*)[1]
Bird-voiced treefrog *(Hyla avivoca*)
Florida (southern) cricket frog (*Acris gryllus*)
Gray treefrog (*Hyla chrysocelis*)[2]
Green treefrog (*Hyla cinerea*)
Little grass frog (*Pseudacris ocularis*)[3]
Northeastern cricket frog (*Acris crepitans*)
Pine barrens treefrog (*Hyla andersonii*)
Pine woods treefrog (*Hyla femoralis*)[4]
Southern chorus frog (*Pseudacris nigrita*)[3]
Spring peeper (*Pseudacris crucifer*)[3, in part]
Squirrel treefrog (*Hyla squirella*)

[1]Provided by Todd Pierson, [2]3 of 5 provided by Paul Moler, [3]Provided by Emily Lemmon-Moriarty, [4]Provided by Anna McKee

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APPENDIX I ENVIRONMENTAL DNA LABORATORY PROTOCOL AND GUIDELINES FOR SELECTING A LAB FOR PROCESSING ENVIRONMENTAL DNA SAMPLES

ENVIRONMENTAL DNA LABORATORY PROTOCOL

AND

GUIDELINES FOR SELECTING A LAB FOR PROCESSING ENVIRONMENTAL DNA SAMPLES

January 2017

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Environmental DNA lab protocol: designing species-specific qPCR assays

Species-specific surveys should use quantitative polymerase chain reaction (qPCR) assays that are designed and validated for each species. Quantitative PCR provides estimates of the amount of the target species' DNA in the eDNA sample. Conventional PCR, which simply screens eDNA samples for the presence of DNA from the target species, is not recommended because it is typically less sensitive than qPCR, and may cross-amplify and provide false-positive results.

Species-specific qPCR assays need to be validated to ensure that they are both sensitive (that is, detect the species' DNA when it's present in the sample) and specific (do not detect the DNA of closely related non-target species). It's important that qPCR assays are designed and validated in adherence to the following steps:

1. Create an inclusive consensus sequence that incorporates all within-species variability for a species in a well-known region of DNA. Mitochondrial DNA is generally preferred because it is more abundant than nuclear DNA, and more sequence data are available. However, nuclear regions can also be used. Sequences can be from GenBank (National Center for Biotechnology Information, https://www.ncbi.nlm.nih.gov/genbank/), or obtained by sequencing tissue samples of target species. If closely related species co-occur in the area where the test will be applied, it is helpful if sequences for those species in the targeted region of DNA are also present in the database, so that cross-amplification can be avoided in the design stage. It is important that the data incorporated include adequate sampling in the geographic area where the test will be applied.
2. For the selected probe chemistry (e.g., MGB-NFQ), set appropriate qPCR primer software to design primers and probe. Alternatively, identify unique primers and probe from an alignment of target and co-occurring non-target species sequences, meeting quality-control criteria for the chosen chemistry (e.g., melting temperature, lack of GC-clamp, product length). Optimal probe length will differ by chemistry. These primers and probe will allow for amplification and detection of the target sequence.
3. Compare the resulting design to sequences in GenBank using PrimerBlast or other software to determine if the sequences are likely to cross-amplify with other species. Try to incorporate as many differences as possible (at least 2 on each primer and 2 on the probe, including 1 toward the 3' end of the primers and 1 in the middle of the probe) between the primer/probe design for the target species and any other species occurring in the area where the assay will be applied.
4. For validation, test the resulting assay against tissues of target (at least 10 from across the area where the test will be applied) and non-target species (at least 5 of each species from across the area where the test will be applied). It is important that these tissues were collected with clean implements so that target species DNA does not appear in non-target species samples (this can be confirmed by sequencing any non-target species amplicons). Additionally, the assay should be validated against eDNA samples from known positive and negative locations in the study area.

Additional information and consensus about validation can be found in Goldberg et al. (2016).

Guidelines for selecting a laboratory to process environmental DNA samples

When laboratory analysis of eDNA samples can't take place in-house, samples will need to be sent to a commercial, academic, or government laboratory for analysis. This process is most likely to be successful if this relationship is established prior to sampling so that the collaborating laboratory can advise on sampling design, materials, and preservation. Workflows differ between labs in ways that may affect detection probability from samples collected using different filter materials and preservatives.

Environmental DNA laboratories should follow a set of practices and procedures designed specifically for handling eDNA samples. When selecting a lab for processing samples from eDNA monitoring, make sure the following practices are part of the lab's operating procedures. This information can also be found in Goldberg et al. (2016).

1. Environmental DNA samples are very low quality and quantity compared with DNA samples collected directly from organisms. They therefore need to be handled in a separate room (clean room) from high-quality samples and the products of polymerase chain reactions (PCR). This clean room needs to have dedicated equipment, including pipettors, centrifuges, and any other item that is needed for sample processing. Technicians should be required to shower and change clothes or go through equivalent decontamination procedures before entering this room after having been in a lab containing PCR products.

2. For assays to be specific enough to detect only target species in eDNA samples, quantitative PCR or next-generation sequencing is required. Conventional PCR tests are not adequate unless every sample is sequenced.

3. Filter tips should be used at all times when handling samples or reagents pre-PCR to prevent cross-contamination.

4. Fifty percent household bleach should be used to clean all items that come into contact with samples (e.g., forceps) between uses and at least 10% household bleach for regular decontamination by wiping, or UV directly in contact with surfaces. Autoclaving, ethanol, and products such as DNA Away are not sufficient to destroy DNA.

5. A negative control should be extracted with each batch of extractions and tested in all downstream processes.

6. A test for inhibition should be incorporated with each sample analysis. This consists of an assay that should always amplify at a known concentration (sold as IC or IPC by several companies). Environmental DNA samples are often inhibited and false negatives reported if this control is not included.

7. The laboratory should be able to archive samples after processing (preferably at -80°C) for future analysis, if that is requested by the agency (it is reasonable to expect an additional fee for this service).

8. The agency should collect a series of samples from known positive and negative sites and send them for a blind test to the laboratory (it is reasonable to expect to pay for this service, although some laboratories may waive this cost). All sites with the species should test positive and without should test negative. However, detection probabilities may not be perfect at positive sites and sometimes field crews can introduce small amounts of DNA into samples when first learning techniques (or if clean field practices are not applied absolutely). This testing should be an iterative process that involves working with a lab to understand where errors are occurring and fix problems during a pilot phase. Laboratories should be willing to work collaboratively with the agency during this phase and produce accurate data from blind samples to the satisfaction of the agency before embarking on extensive sample processing.

At this time, there is a small number of laboratories that analyze eDNA samples, but the number is likely to increase as eDNA sampling becomes more widely used. A list of eDNA processing labs is available at https://labs.wsu.edu/edna/edna-labs/ and will be updated periodically.