Using Environmental DNA to Monitor Vernal Pool Organisms in California

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ABSTRACT. In the last ten years environmental DNA sampling has grown rapidly in popularity. By sampling an organism's environment, rather than the organism itself, environmental DNA can provide sight-unseen detection of the presence of a target organism. It has gained popularity by conservationists and land managers to monitor both a wide variety of targets, including listed organisms, invasive species and species that are extremely difficult to monitor with other means. This report provides an overview of environmental DNA and presents an example of the design of a project which utilizes environmental DNA assays to survey a number of target species present in California's sensitive vernal pool habitats.

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INTRODUCTION

What is Environmental DNA?

Environmental DNA (eDNA) is a popular novel sampling method for detecting the presence of a target organism. Rather than needing to physically locate a specimen for observational or tissue sampling, eDNA methods sample the environment in which the organism lives and seek to detect its shed genetic material. Although aquatic environments are the most common, eDNA has now been used to detect targets in soil, ice, air, and pollen (McKelvey et al., 2016; Fahner et al., 2016; Zielińska et al., 2017; Kinoshita et al., 2019; Johnson et al., 2019).

All organisms, including humans, leave genetic material (DNA) behind in their environments through their day-to-day living. In freshwater environments like vernal pools, this DNA can come from skin cells, mucous, blood, reproductive tissue, exoskeletons, and waste, among other sources (Goldberg et al., 2016). DNA is generally captured in whole cells but still has a relatively short persistence time in the water, from days to weeks depending on many biotic and abiotic factors. This means that an eDNA detection generally represents the recent presence of the organism at the site (Dejean et al., 2011).

California's vernal pools are vital ecosystems that contain a number of endemic and protected species including plants, invertebrates, and amphibians listed as either threatened or endangered by the United States or California Endangered Species Acts. These species require a significant monitoring effort by land managers. Some monitoring is done by reserve managers to track the status of species in their pools, while other surveys are undertaken by conservation banks that need to establish the presence of species. Currently, monitoring these pools for listed animals involves trained experts wading out into the pools with nets and visually identifying the species present (U.S. Fish and

Wildlife Service, 2015). This visual process is expensive and requires significant expertise especially for differentiation of morphologically similar crustacean species. The example project described in this work seeks to determine if eDNA may be an appropriate method for monitoring two California vernal pool species: the Vernal Pool Tadpole Shrimp, (VPTS) *Lepidurus packardi* and the Central Valley distinct population segment of California Tiger Salamander (CTS), *Ambystoma californiense*.

Lepidurus packardi is a federally endangered (United States Fish and Wildlife Service, 1994) omnivorous crustacean. It hatches immediately after winter rains inundate California's vernal pools, emerging as a metanauplius and molting into a larval form within hours. It matures over the course of 4-7 weeks (Ahl, 1991). Adults breed and deposit fertilized eggs onto sediment and plant matter. These can survive the summer dry period and the next generation hatches with the next wet season (Alexander and Schlising, 1998).

The California Tiger Salamander is an endemic mole salamander. It is listed as threatened rangewide under the California Endangered Species Act (Califonria Fish and Game Commission, 2010). Federally, the Sonoma County and Santa Barbara County Distinct Population Segments are listed as endangered (U.S. Fish and Wildlife Service, 2000, 2003) while the Central Valley Distinct Population Segment is threatened (U.S. Fish and Wildlife Service, 2004). Like many salamanders, adult CTS is mostly terrestrial, living underground in small burrows and transitioning into vernal pools and other small bodies of freshwater to breed in winter. Larval salamanders hatch in spring and are aquatic until metamorphosis in early summer as the pools begin to dry (Trenham et al., 2006). Dip-net monitoring is commonly used to survey for both CTS and VPTS.

Designing an eDNA Monitoring Study

There are important considerations when designing a study or program using environmental DNA. The major decisions to make in advance are when and how frequently to collect samples in order to maximize the chance of successful detection. One important question to consider when making these decisions is which biotic and abiotic factors might affect eDNA persistence and abundance. For instance, in freshwater, bacterial community, temperature, pH, oxygen level and total dissolved solids (TDS) can all increase or decrease how quickly DNA degrades (Barnes et al., 2014). For our example project, vernal pools are shallow and usually exposed, so the temperature, pH, TDS and oxygen level can vary dramatically even within a single day (Rogers, 2014). This may limit the ability to estimate the recency of occupation from a single eDNA detection in a vernal pool, so samples should be taken when the organism is expected to be present.

Another important consideration is when, how long, and for what purpose the target organism inhabits the sampling environment. Organisms that pass through an environment and quickly leave will be much harder to detect than a full-time resident. In our example, VPTS hatches almost immediately after the pool inundates in the winter and persists until late spring water temperatures increase so much that the population dies off or the pool dries. So we can assume that VPTS genetic material will be detectable shortly after inundation, with its abundance increasing as the larvae grow until the end of the wet season. While DNA is likely to degrade more quickly in warm temperatures (Eichmiller et al., 2016), DNA abundance is not likely to decrease sharply, since the vernal pool will begin to shrink as it dries down, concentrating the remaining DNA, although we cannot assume this will happen proportionally.

For other species, the "peak" DNA timing may be different. CTS is likely to have very abundant DNA early in the winter as adults breed in the pond, releasing huge amounts of free genetic material and depositing fertilized eggs. The adults then leave the pools and the remaining genetic material decays over time, leaving a potential low point for eDNA recovery between breeding and larval emergence. After larval hatch, DNA is likely to increase again through the end of the wet season, as the larvae will persist and grow until metamorphoses. Life stage (egg, juvenile, adult) and activity (reproduction, growth, diapause) can all affect the release of DNA into the environment (Maruyama et al., 2014; Sassoubre et al., 2016; Vörös et al., 2017; Goldberg et al., 2016).

In vernal pool environments, released DNA is trapped, as water does not flow except in flood conditions. This eliminates the need to worry about DNA transport, although it may be possible for DNA to be moved by birds, cattle, or humans between nearby ponds, as individuals and eggs are believed to be moved this way (Brendonck et al., 2017; Cottenie et al., 2003; Hathaway and Simovich, 1996; Bohonak and Whiteman, 1999). For vernal pools, we believe that it is reasonable to sample for the immediate or very-recent presence of a target organism directly. While our study sampled the same pools multiple times to compare results and validate our methods, a monitoring study may not need to repeatedly sample the same vernal pool to establish the presence of the target organism, as long as the timing of the sample collection accounts for when the organism is likely to be inhabiting the vernal pool and releasing DNA.

THE FOUR STEPS OF EDNA ANALYSIS

Once a monitoring study has been designed and target ecosystems have been chosen, eDNA monitoring includes four key steps: collecting the sample, processing the sample, extracting the DNA from the sample, and analyzing the sample.

Collection

Collecting eDNA is relatively simple. In aquatic environments, water is collected and subsequently filtered either in the field or later in a laboratory in order to isolate DNA from the water. The volume of collected water varies with the system with volumes between 250 mL and 1L typically reported with some studies taking higher volumes of 5 or more liters (Goldberg et al., 2016; Gorički et al., 2017). To prevent contamination, the water is generally collected by someone wearing protective gear and collected into a sterile, sealable canister, which is submerged into water by hand or using a pole sampler. Alternately, a much smaller volume (generally 15 mL) of water can be collected into a sterile 50 mL tube or single-use Whirl-Pak and treated with sodium acetate and ethanol to precipitate out the DNA before extraction (Forsström and Vasemägi, 2016). For soil/sediment, a sediment sample or soil core is taken and deposited into a sterile canister or single-use Whirl-Pak. Then the sediment is homogenized before DNA extraction (Eichmiller et al., 2014).

Processing

For aquatic sampling, processing to separate the DNA from the substrate generally involves pouring the collected sample over a filter. This can be done in the field (Minamoto et al., 2017; Goldberg et al., 2011; Bergman et al., 2016) or in a processing laboratory (Balasingham, 2016; Gorički et al., 2017; Simpfendorfer et al., 2016; Newton et al., 2016). If processing in the laboratory, samples must be kept cold (Takahara et al., 2013; Balasingham, 2016) until filtration. Filter materials vary and include cellulose nitrate (Takahara et al., 2013; Goldberg et al., 2011), glass fiber (Balasingham, 2016;

Minamoto et al., 2017), polyethersulfone (PES) (Bergman et al., 2016; Gorički et al., 2017), nylon, (Simpfendorfer et al., 2016) and polyvinylidene fluoride (PVDF) (Newton et al., 2016) among others. To facilitate filtration, pressure is applied to the filter using a vacuum pump, peristaltic pump or syringe. Pore size of the filter can vary, but generally pores are between 0.22 μm (Kelly et al., 2014; Bergman et al., 2016; Port et al., 2015) and 1.5 μm, though larger pores up 20 μm have been reported (Simpfendorfer et al., 2016). When filtering fieldside, field negatives are necessary to ensure that samples are not contaminated during the filtration process. In the laboratory, similar "equipment" blanks are used. Both involve the filtration of pure water as an extra sample.

Extraction

Most eDNA extraction is done in a clean laboratory. Because the concentration of DNA from target organisms is very low in an eDNA sample, additional contamination controls are introduced during extraction to minimize the possibility of a contaminated sample. The clean laboratory usually contains no amplified or tissue-derived DNA from a target organism, and bleach and UV are used to sterilize consumables and equipment before and after use (Goldberg et al., 2016; Deiner et al., 2015).

Extraction methods vary. Most eDNA studies opt for a commercially-available DNA extraction kit, such as the Qiagen DNEasy Blood and Tissue Kit (Ikeda et al., 2016; Kelly et al., 2014; Civade et al., 2016; Goldberg et al., 2011; Dogdu and Turan, 2016), or the PowerWater Kit (Handley et al., 2016; Gorički et al., 2017; Jerde et al., 2011; Banks et al., 2015; Port et al., 2015). However, some studies use the phenol-chloro-isopropanyl (PCI) method (Balasingham, 2016; Renshaw et al., 2015; Deiner et al., 2015). Because both water and soil contain chemicals that may inhibit downstream analysis, samples are frequently purified to remove these inhibitors (Eichmiller, 2014; Hashizume et al., 2017; Hartman et al., 2005) using commercial kits or centrifugation.

Analysis

There are two main ways to analyze an eDNA sample: using a species-specific assay to test for a single target organism, or using a multi-species, high-throughput sequencing approach to describe an entire community. The same sample can be used for both analyses if desired.

Species-specific eDNA assays test for the presence of a single target species in a sample. They are specially designed to amplify a short sequence of DNA specific to that organism. Usually, these are Taqman quantitative PCR (qPCR) assays, which use two short primers and a fluorescent probe to amplify and detect the target genomic content within the samples. qPCR is more sensitive and more specific than traditional PCR in most cases, although traditional PCR assays have been successfully used to detect a species' eDNA (Dejean et al., 2011; Newton et al., 2016). In either case, if the target stretch of DNA (usually between 50 and 100 base pairs of mitochondrial DNA) is present in the sample, that DNA molecule is replicated and amplified. In qPCR, the fluorescent signal is increased with every PCR cycle until a designated threshold is surpassed and the sample is considered positive for the target. Due to the low concentration of target DNA, in most eDNA qPCR studies each sample is run in replicate, usually between 3 and 12 replicates (Furlan and Gleeson, 2016; Stewart et al., 2017; Thomsen et al., 2012; Schneider et al., 2016; Ikeda et al., 2016). There is no standard for how many replicates need to amplify before a sample is considered positive, and it is recommended to make that determination based on the results from a small number of test samples with varying replicate numbers (Goldberg et al., 2016).

Multispecies metabarcoding analysis uses high-throughput sequencing to sequence eDNA from every species present in the sample at a particular gene. Instead of using highly-specific, specially designed primers, a very general universal primer set is used. There are no truly "universal" primers due to the genetic variation present across the tree of life. However, there are many primer sets that can capture a wide range of the life on earth. The products of these primer sets is sequenced on a very large scale using an Illumina Miseq (Elbrecht and Leese, 2015; Ushio et al., 2017; Valentini et al., 2015) sequencing platform, producing millions of short sequences. These sequences are compared to a database of known organisms and categorized taxonomically to produce a snapshot of the community present within the sample (Bohmann et al., 2014; Ushio et al., 2018; Lim et al., 2016; Civade et al., 2016; Lopes et al., 2017).

TESTING EDNA IN CALIFORNIA'S VERNAL POOLS

Experimental Design

In our example study we designed two species-specific qPCR assays for the target species using a combination of Sanger sequences from tissue-derived DNA and publicly-available DNA sequences from Genbank (Sayers et al., 2019). Primer and probe candidates were generated using both Primer3 (Untergasser et al., 2007) and IDT's Primer-Quest. The best options were chosen from among these candidates by visual alignment to the target, ensuring as many species-specific SNPs and as little within-species variation as possible while maintaining optimal melting temperatures and reducing potential secondary structure formation. Assays were tested using DNA derived tissue from both the target organism and from any closely-related species (within the same genus) that are found swimming with it anywhere in its range according to historical reports (Eriksen and Belk, 1999), to ensure no cross-amplification which might lead to false positives.

Over three years (the 2016, 2017 and 2018 wet seasons), we carried out side-by-side eDNA and dip-net sampling on 80 vernal pools from five locations in California: the UC Merced Vernal Pool Reserve in Merced County, the Dutchman Creek Conservation Bank in Merced County, the Jepson Prairie Reserve in Solano County, and two private preserves in Sacramento County. Pools were sampled between 1 and 5 times across those three years for a total of 159 sampling events. Many pools were sampled only once. Sometimes this was because a pool dried down during a subsequent visit, sometimes it contained no target organisms. We tried to strike a balance which included enough "negative" pools to establish that the assay does not deliver false positives while surveying as many pools as possible at each complex to capture the variation in temperature, turbidity, vegetation, inundation timing, and other factors that might affect detection. Forty-eight percent of our pools were sampled only once. A "sampling event" is defined here as the collection of eDNA samples from a single pool on a single visit. During each sampling event, three replicate eDNA samples were taken plus a field negative control, followed by dip-net sampling using the USFWS Survey Guidelines (U.S. Fish and Wildlife Service, 2015). When branchiopods were present, voucher specimens were collected in 95% ethanol for independent species identification.

We collected samples using the sterile container method described above. We systematically filtered some samples immediately in the field, and others in the laboratory. For laboratory-filtered samples, water samples were transported from the field in a cooler on ice and kept at 4°C until filtration, which was carried out within 72 hours. We filtered up to 1L of collected water per sample. In 2016, we used

0.45 μm glass fiber filters (VWR) and filtered up to 500 mL of water. In 2017, we used 0.45 μm cellulose nitrate filters (Millipore Sigma) which clogged more quickly. While we still filtered up to 500 mL of collected water, most filters clogged between 250 and 350 mL, with very turbid ponds clogging the filter almost immediately (< 50 mL). In 2018 we trialed a larger, 1.0 μm glass fiber filter (Whatman) and filtered up to 1L of water. Although some very turbid pools still clogged these filters between 400-700 mL, this is still a 10x improvement over the cellulose nitrate filters. It was also considerably faster, taking less than 10 minutes rather than the 10-15 minutes common in both 2016 and 2017. In 2016, we stored filters dry in silica desiccant at room temperature. We found that this storage method worked well and we were able to recover DNA from the filters both immediately and more than two years later in 2018, using ¼ of the filter for each extraction. However, we found that the volume of extraction reagents required to effectively extract DNA from these filters was high due to their absorbency, and so we switched to short-term-only wet storage during 2016 and 2017, when we stored whole filters in Qiagen extraction buffer ATL immediately after filtration. We used 2 mL microcentrifuge tubes (Eppendorf) for wet filter storage, kept filters at 4°C and extracted the entire filter within five days.

We chose the Qiagen DNEasy Blood and Tissue Kit for our filter extractions. We made two changes to the standard Qiagen procedure: We incubated filters overnight on a rotisserie rather than for only a few hours, and we increased the length of the incubation period during elution from 1 minute to 15 minutes, as well as incubating at 55°C rather than at room temperature. We performed two elutions of 60 μL each for a total elution volume of 120 μL. Samples were treated for inhibition with the OneStep Inhibitor Removal Kit (Zymo) following the manufacturer's protocol.

Each sample, including each field negative, was analyzed for the presence of at least one of the two target organisms using the designed qPCR assays. For analyzing CTS eDNA, biological replicate samples (not including the field negative) were combined into a single sample after testing a subset of these samples separately and finding high consistency between them. For VPTS, each biological replicate was run separately. Four PCR replicates were analyzed from each sample. A sample was considered a "positive detection" if two or more of the four PCR replicates amplified. If only one replicate of the sample amplified, the sample was re-run. If none of the replicates amplified, it was considered a "non-detection."

Initial Results and Discussion

Although data analysis is still ongoing, early results suggest that eDNA monitoring may be a good method for detecting the presence of the target species in vernal pools. We present these early results from our example project but these results may be subject to change as analysis continues.

For California Tiger Salamander (CTS), the assay detected the species 14/14 (100%) times it was found in dip-nets. It also detected the presence of the species 14 times when it was not found in dip-nets, but was found later that same year in the same pool. Because CTS does not hatch until February or March, a significant amount of eDNA sampling was done before this time. In these instances, the eggs and reproductive material of breeding adults are present in the vernal pool even though the larvae are not, meaning this assay can detect the presence of CTS even when salamanders are not visible in the pool.

For the Vernal Pool Tadpole Shrimp, the assay detected 44/47 (93.6%) instances when the organism was found in the dip-net. It also detected 8/8 instances when it was not found in

dip-nets but was found later that same year in the same pool, and five instances where either no later survey was done or the species was not found in the dip-nets at all. While these five instances (4.5% of the 111 sampling events assayed so far for this species) may represent false positives, they may also represent a biologically real detection that nonetheless does not correspond to a population detectable by a dip-net. These three samples represent repeated detections at a single pool, from three separate visits over two years. It is unlikely but possible that dip-nets missed the population three visits in a row, but it is also possible that there has been the movement of VPTS genetic material into that pool from established populations at neighboring pools which may be connected during flood years or from which DNA may be transported via a dispersal vector such as cattle hooves or bird feathers.

Both of these assays may offer a reasonable alternative to visual monitoring efforts, although no eDNA method will replace the trained eye of an expert, who can do things eDNA cannot, including track abundance. There may be some vernal pool systems that are more amenable to eDNA testing than others; smaller, less turbid pools are faster to sample and easier to filter. However, eDNA's real benefit may lie in larger, more turbid pool systems, which are the most difficult and time-consuming to manually monitor.

While the exact cost-per-sample of eDNA analysis is not yet established, it certainly does not require the time commitment and training cost of manual monitoring. Training monitors to identify vernal pool organisms to species, and obtaining or adding monitors to permits can cost upwards of $1000. Meanwhile, collecting and filtering water samples requires no special permit and can be done with very little specialized equipment, while processing the DNA requires basic lab equipment and a lab technician level of expertise.

This study is an example of one way to deploy eDNA in vernal pools. The qPCR assays used in this study, as well as assays for related vernal pool crustaceans in California, will be released to the public in 2020. Studies using proven assays will not need the same level of field validation and can be deployed rapidly and with little testing using the methods described earlier in this paper.

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