ENVIRONMENTAL DNA (eDNA) ANALYSIS AS A SURVEY TOOL IN NATURAL SPRING AND STOCK TANK BIOMONITORING

By

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ABSTRACT

Recent research has shown the viability of environmental DNA (eDNA) in identifying the presence of organisms in freshwater ecosystems. Environmental DNA includes the DNA from an organism that is collected from environmental samples, instead of from the organism itself; by collecting water samples and analyzing this eDNA through metabarcoding techniques, the presence of species in a given ecosystem can be established. However, there is limited research indicating the feasibility of eDNA as a survey tool in water sources such as springs and stock tanks. Because of the scarcity of water in the high desert landscape of central Arizona, natural springs and man-made stock tanks, used as water sources for open range cattle, play an important role in the functioning of the ecosystem, and are a stopping point for many vertebrate species. Therefore, we address the question of whether eDNA analysis is a viable survey tool in monitoring vertebrate species presence in springs and stock tanks within the Prescott National Forest in the Arizona central highlands. Water samples were collected in 250 mL and 500 mL containers, and were filtered to capture fine particles and cells greater than 0.45 μm. These particles were then amplified with universal primers for 16S sections, which are conserved in vertebrates as part of the rRNA gene. Current methodologies have shown that the 16S rRNA gene is effective in recognizing vertebrate DNA. The 16S rRNA metabarcoding technique used in this project detected the presence of species that are common to the Prescott National Forest, including species of bear, deer, and squirrel. Additionally, this eDNA metabarcoding technique detected the American bullfrog, which is an invasive species in the Southwestern United States. Our detection of the invasive American bullfrog represents a next step in better monitoring presence and removing the species from ecosystems throughout the region. Our results suggest eDNA metabarcoding is an effective tool in assessing species presence at natural springs and stock tanks, and should continue to be used for further research focused on biomonitoring in other water sources.

INTRODUCTION

The rate of species extinction is continuing to increase, and the spread of invasive species is intrinsically changing ecosystem function and structure (Doherty et al., 2016). Biomonitoring plays an important role in establishing species presence in ecosystems, and is invaluable in implementing conservation, management, and restoration (Derocles et al., 2018). Traditional surveying methods require direct observation conducted through field work to assess specific taxonomic groups (Soroye et al., 2018), but traditional biomonitoring techniques can be invasive and potentially harmful to the tracked species, and require trained professionals to conduct surveys to ascertain the presence or absence of specific species in an area. These wildlife survey techniques, including observation counts, roadside counts, and infrared-triggered cameras ("trail cameras") are useful in understanding species presence, but are often expensive and time-consuming to implement (Skalski & Robson, 2012).

Recent research has shown the viability of environmental DNA (eDNA) in identifying the presence of organisms in freshwater ecosystems (Davy et al., 2015). Environmental DNA includes the DNA from an organism that is collected from environmental samples such as water, soil, and snow, instead of from the organism itself. By collecting water samples, for example, and analyzing eDNA through metabarcoding techniques, the presence of a species in a given ecosystem can be established (Taberlet et al., 2012). The use of this emerging technology can augment biomonitoring objectives in environmental management and aid in forestry and wildlife management, especially in identifying invasive or endangered species (Robson et al., 2016). Furthermore, results from eDNA analysis can quantify biodiversity in a system through a means that is less expensive and less stressful for the wildlife being observed (Sigsgaard et al., 2015).

eDNA in biodiversity monitoring

Environmental DNA collection and metabarcoding is still a novel technology in the realm of biomonitoring (Coble et al., 2018). Although eDNA has been implemented as a biomonitoring tool since the early 2000s, most laboratories focus on quantitative polymerase chain reactions (qPCR), which only addresses the presence of a single species, and costs roughly $100 per sample to assess (U.S. Forest Service Rocky Mountain Research Station, 2018). Environmental DNA metabarcoding, on the other hand, can provide results that are representative of numerous species (Klymus et al., 2017). Although eDNA metabarcoding provides results on species richness, it does not provide the number of each species that are present.

Past eDNA research has focused almost exclusively on fish, invertebrates, reptiles, and aquatic vegetation (Coble et al., 2018). While observational surveying may indicate the presence of various larger mammals, eDNA biomonitoring may provide a more accurate depiction of which species are visiting various water sources, and when they are visiting. Although eDNA metabarcoding has continued to grow in application, there are still few laboratories that contract metabarcoding services, and metabarcoding assays are still under development (Schwartz, et al., 2017).

Despite the novelty of the technology, applications of eDNA metabarcoding for biomonitoring are extensive. In a literature review of eDNA metabarcoding for biomonitoring, Ruppert et al. (2018) illustrate the current applications of eDNA metabarcoding in terrestrial, marine, estuarine, and freshwater settings (Figure 1).

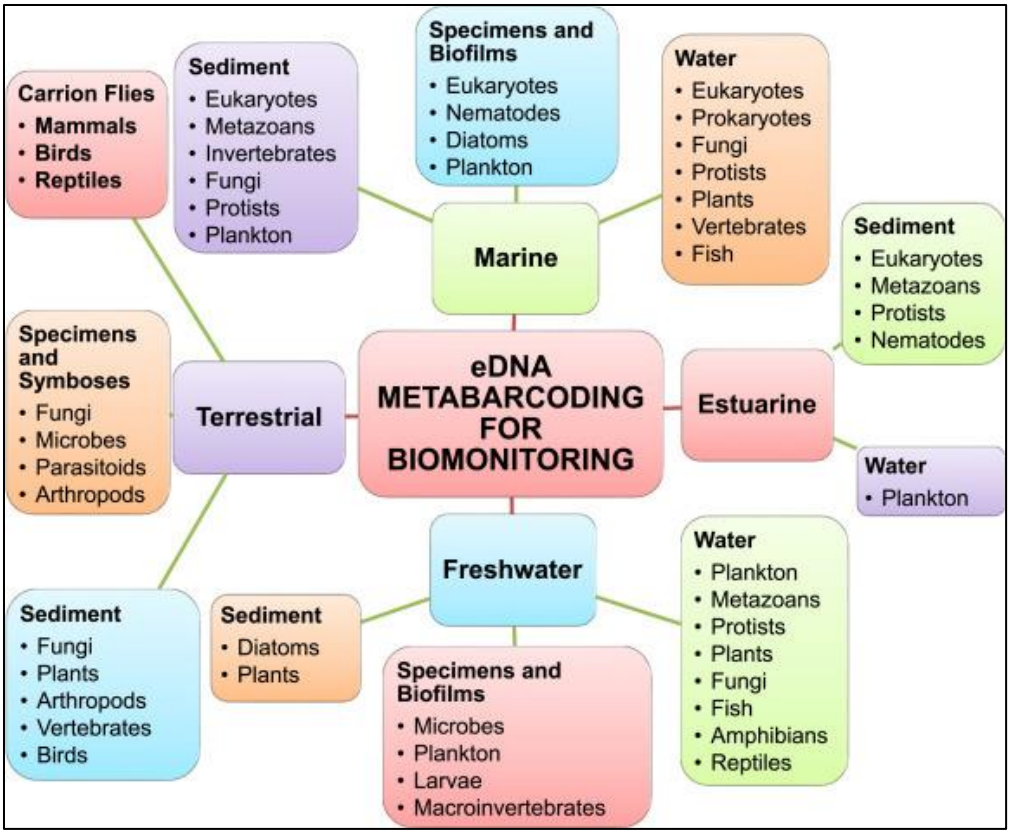

Figure 1. eDNA metabarcoding for biomonitoring (from Ruppert et al., 2018).

Although the applications of eDNA metabarcoding for biomonitoring are diverse, there is limited application of metabarcoding to vertebrate biomonitoring, as the majority of research has focused on the detection of invertebrates and other specimens (Ruppert et al., 2018). However, Ushio et al. (2017) demonstrated the viability of mammalian biomonitoring in freshwater ponds with the use of eDNA metabarcoding. There is a need, however, for more research on the use of eDNA as a tool in vertebrate biomonitoring in freshwater environments.

Verde River Research

Beginning in the fall of 2018, a research team from the Wildlife Science and Forensic Biology departments at Embry-Riddle Aeronautical University (ERAU) in Prescott, Arizona began collecting water samples and analyzing eDNA from the Verde River in north-central Arizona. Dr. Catherine Benson, Assistant Professor of Wildlife Science, and Matt Valente,

Biology Instructor and Lab Manager, spearheaded this research. The goal of the study was to establish the viability of eDNA biomonitoring in this river system, and to further examine the use of metabarcoding in establishing presence of vertebrate species. In addition to demonstrating the use of eDNA analysis in detecting vertebrate organisms in the river, the results of the study illuminated the presence of the redeye bass (*Micropterus coosae*), a species of fish not known to be present in the region (Benson et al., 2018). The research, therefore, highlighted the need for further studies of eDNA analysis in other bodies of water.

The ERAU research began as a pilot project in two ecologically important watersheds in central Arizona, the Verde River and Fossil Creek. Water sampling and DNA extraction techniques were created based on best practices found in current peer-reviewed methodologies. In following established protocol, the Forensic Biology laboratory at ERAU extracted DNA from water samples at these sources. These samples were then amplified with universal primers for 16S sections, which are conserved in vertebrates as part of the rRNA gene. Current methodologies have shown that the 16S rRNA gene is effective in recognizing vertebrate DNA (Vences et al., 2016). Using a methodology based on Vences et al. (2016) work with the 16S rRNA sequencing technique, the ERAU research team detected 41 vertebrate species from 16 sample sites, including the Northern American beaver (*Castor canadensis*), river otter (*Lontra canadensis*), coyote (*Canis latrans*), mountain lion (*Puma concolor*), and Arizona gray squirrel (*Sciurus arizonensis*). Throughout the data collection and analysis in this pilot study, I aided in field work with the Department of Wildlife Science, but was not involved in the development of the 16S primer methodology with the Department of Forensic Biology.

Due to the efficacy of the pilot study, the research team has continued to increase the number and types of sampling areas, with concentrations on other regions of the Verde River and

other bodies of water. Environmental DNA sampling has proven to be an effective means of biomonitoring, and we hope to continue to show its application in various bodies of water and at different temporal occasions. In addition to the samples collected for the purpose of this professional paper, we have collected samples from local lakes and reservoirs, to include all major water bodies in the Prescott Basin. These data collections and analyses displayed robust results of which species are in the water, but provided limited information on the presence of transient users of these riparian environments. Consequently, these findings led to the current project focused on species presence in springs and stock tanks.

Research Problem – Springs and Stock Tanks

The ERAU research team found water sampling and metabarcoding to be a viable approach for detecting species in river samples, but there is limited research on the feasibility of eDNA as a survey tool in other types of water sources, such as natural springs and man-made stock tanks. Stock tanks, used as water sources for open range cattle, are common throughout the Prescott National Forest, and serve as a resource for a variety of local wildlife (Sheridan, 2001). With the guidance of Dr. Catherine Benson and Matt Valente, we were able to employ a methodology to assess eDNA biomonitoring in natural springs and stock tanks. For this project, we addressed the question of whether eDNA analysis is a viable survey tool in monitoring species presence in springs and stock tanks. As a pilot study in monitoring natural springs and man-made stock tanks, the goal of this project was to further demonstrate the functionality of eDNA sampling as a tool in biomonitoring. We hypothesize that eDNA samples collected from stock tanks and natural springs will accurately represent the species that visit these water sources.

To test this hypothesis, we gathered water samples at eight separate locations within the Prescott National Forest, including natural springs and man-made stock tanks. Environmental DNA analysis was conducted in the Forensic Biology laboratory at ERAU, but the primary focus of this professional paper is on the collection of data in the field and the analysis of species detection results. Within the scope of the LRES professional paper, this research seeks to specifically demonstrate the capacity of eDNA metabarcoding in assessing which species are using the springs and stock tanks in the Prescott National Forest, and provide a scientifically supported approach to pursuing further research in vertebrate biomonitoring through eDNA collection. Although there have been some studies that address biomonitoring in freshwater systems, there is limited peer-reviewed literature that addresses springs and stock tanks, and limited research that focuses on the presence of vertebrates. Results from this research, therefore, will be useful in providing a segue into future research on the application of eDNA analysis in a variety of freshwater sources.

MATERIALS AND METHODS

Following the methodology of previous eDNA collections and analyses, samples were collected in 250 mL and 500 mL water samples. These samples were then filtered to capture fine particles and cells greater than 0.45 μm, and DNA was extracted from the collected material (Coble et al., 2018). Testing for target species was implemented through the use of eDNA metabarcoding, with a focus on the 16S rRNA gene to detect vertebrate species (Vences et al., 2016). Details on sampling design, collection, and preparation are discussed below.

Study Area - Prescott National Forest

All sampling was conducted within the Prescott National Forest in the north-central Arizona highlands (Figure 2).

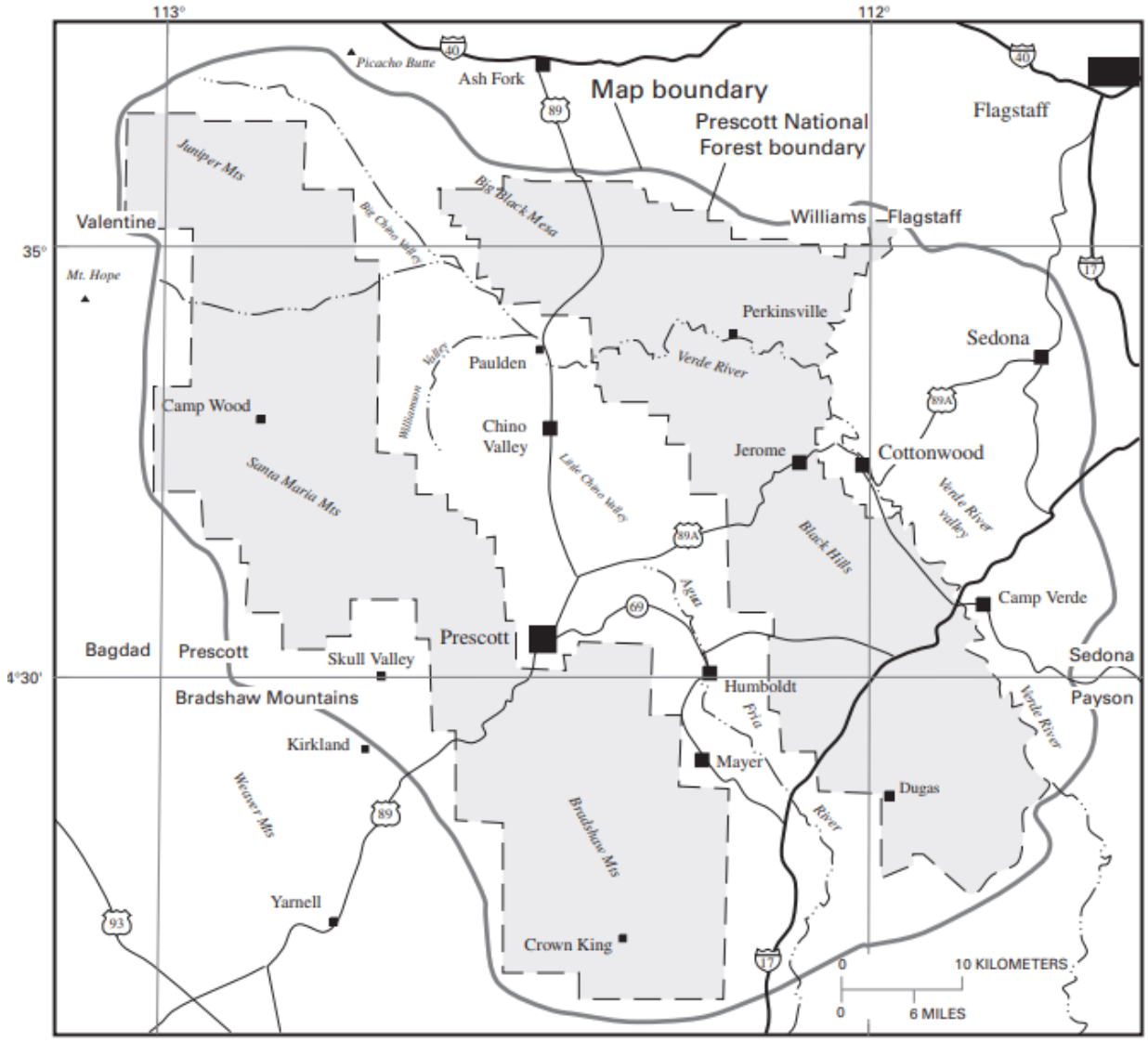

Figure 2. Prescott National Forest boundaries (DeWitt et al., 2008).

The Prescott National Forest, located in central Arizona, encompasses roughly 4,900km^2, including timberland, woodland, and non-forest areas. Within this characterization, timberland is dominated by Ponderosa pine (*Pinus ponderosa*) and Douglas fir (*Pseudotsuga menziesii)*, and woodland areas include pinyon pine (*Pinus edulis*), juniper (*Juniperus osteosperma*), and Arizona white oak (*Quercus arizonica*) (Figure 3).

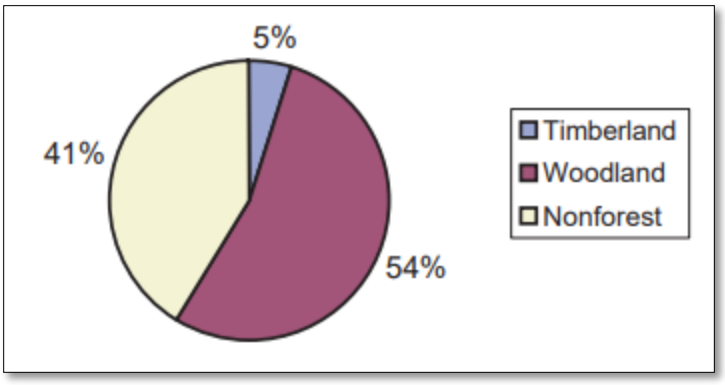

Figure 3. Dominant land cover types of the Prescott National Forest (Rogers, 2003).

Much of the Prescott National Forest encompasses non-forest land; a majority of the area is covered by chaparral *(Quercus turbinella)* and other brush that are typical of high desert ecosystems and native to the Sonoran Desert (DeBano, Baker & Overby, 1999) (Figure 4).

Figure 4. Prescott National Forest ecosystems (Rogers, 2003).

Within the Prescott National Forest, only 8% of the land is designated as wilderness areas (Rogers, 2003). Much of the land is leased to local ranchers, and open range grazing is common (Sheridan, 2001). Because of the scarcity of water in this high desert landscape, natural springs and man-made stock tanks play an important role in the functioning of the ecosystem, and are a stopping point for many vertebrate species throughout the Prescott National Forest. While cattle are commonly seen on leased land, wildlife common to the area also include elk (*Cervus*

elaphus), American black bear (*Ursus americanus*), javelina (*Pecari tajacu*), pronghorn antelope (*Antilocapra americana*), mule deer (*Odocoileus hemionus*), cougar (*Puma concolor*), bobcat (*Lynx rufus*), coyote (*Canis latrans*), bald eagle (*Haliaeetus leucocephalus*), roadrunner (*Geococcyx californianus*), turkey (*Meleagris gallopavo*), several species of rattlesnake (*Crotalus* spp.), and antelope jackrabbit (*Lepus alleni*) (Jaworski & Unit, 2011).

Sample sites

Sites within the Prescott National Forest were chosen based on accessibility of the water sources, and stratified between natural springs and human-made tanks. Proper permissions and permits were obtained through the Prescott National Forest before collecting samples.

All natural springs and human-made stock tanks used for sampling in this research were either found by the research team through recreational hiking or recommended as sample sites by Francisco Anaya, an ecologist for the Prescott National Forest. Diversity in the combination of natural springs and stock tanks for this research was important in establishing which species are visiting specific water features, and how species are dispersed throughout the Prescott National Forest.

The *Land and Resource Management Plan for the Prescott National Forest Yavapai and Coconino Counties, Arizona* (Jaworski & Unit, 2011) incorporates seven different management areas within the Prescott National Forest, including Agua Fria, Crown King, Prescott Basin, Upper Verde, Verde Valley, Williamson North, and Williamson South. The sites used for sampling (Table 1) are dispersed throughout the Prescott National Forest, and incorporate all management areas with the exception of Agua Fria and Crown King (Figure 5).

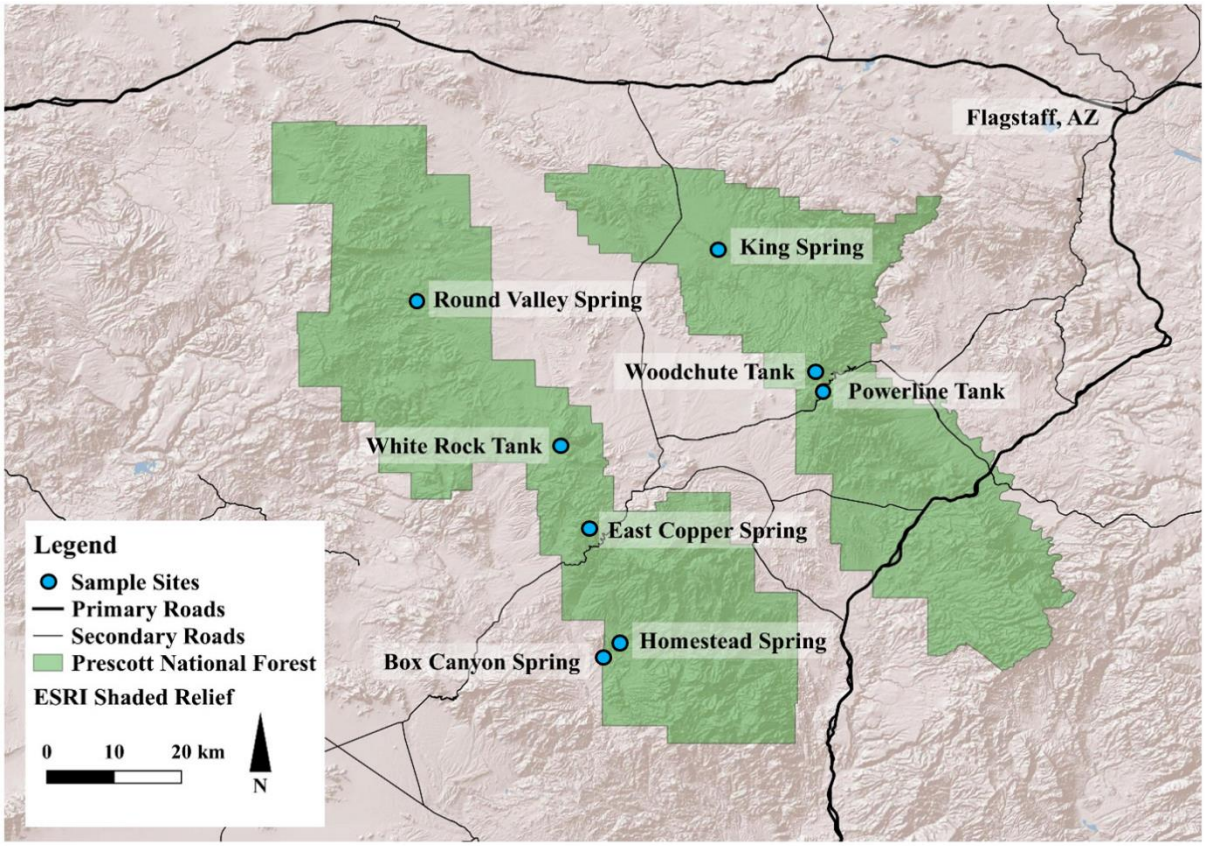

Figure 5. Sample Site Locations.

These sample sites are representative of a variety of different ecosystems within the Prescott National Forest, ranging from canyons populated by chaparral and prickly pear cacti (*Opuntia engelmannii*) to mountainous terrain in Ponderosa pine stands. These sites are differentiated by their site number, site name, differentiation between spring and stock tank, and Universal Transverse Mercator (UTM) coordinates (Table 1).

Table 1. Sample sites

Site number	Site Name	Spring / Tank	UTM Coordinates: Zone 12S	
			Easting	Northing
1	White Rock Tank	Spring	354749	3832082
2	East Copper Spring	Tank	358813	3816971
3	King Spring	Spring	378829	3867874
4	Round Valley Spring	Spring	333659	3859188
5	Woodchute Tank	Tank	393012	3844847
6	Powerline Tank	Tank	393851	3841858
7	Box Canyon Spring	Spring	362802	3795810
8	Homestead Spring	Spring	360132	3793238

Site 1, White Rock Tank, is considered a natural spring, but has been diverted into a man-made tank. Site 2, East Copper Spring, may originate as a spring, but the water itself is stagnated in a human-made concrete tank. The natural springs are differentiated from the stock tanks based on their hydrological behavior, and the springs are defined by their natural renewal of subterranean water (Vashisht & Sharma, 2007). The springs found at sites 2, 4, 7, and 8 are all naturally occurring and untampered by people. Site 2, King Spring, is located near the Verde River, and is surrounded by southern cattails (*Typha domingensis*) and Arizona white oak. Site 4, Round Valley Spring, is located on the western edge of the Prescott National Forest at Camp Wood near a stand of Arizona white oak. There are indications at this site that the spring has been previously used by people, as there is a small dam built at the head of the spring. Sites 7 and 8 are both located in the southern area of the Prescott National Forest along a rural and unmaintained Forest Service road. When the research team collected water samples, Site 7, Box Canyon Spring, indicated previous habitation of the American black bear due to the presence of a bedded area in a cave and significant amounts of scat. Site 8 is located near the remains of an old homestead. This site is populated by a large stand of Arizona cypress (*Cupressus arizonica*). The population of Arizona cypress in this area is unique to the Prescott National Forest and the

sampling sites, as they generally grow best at elevations less than 3,000 feet (Parker, 1982), which is much lower than the first six sampling sites. Site 2, East Copper Spring, is contained within a small concrete tank and is located in the Sierra Prieta Mountains outside the city of Prescott, Arizona. As a very accessible tank, it is located along a designated trail and serves as a water source for open-range cattle in the area. Sites 5 and 6 are located near one another in the Woodchute Wilderness area in the eastern region of the Prescott National Forest. As human-made ponds designated for cattle, these sites are located in a mountainous region among Ponderosa pine stands. During sample collection, the research team observed mule deer visiting both stock tanks.

Field Work

Water samples were collected at eight locations throughout the Prescott National Forest between April and September 2019. Three water samples were collected at each site, and a field negative was also analyzed each day in the field. Ultra-pure deionized water from the ERAU Forensic Biology laboratory was transported to the field every day as a field negative. By processing this water sample in the field, we were able to account for potential contamination from the laboratory that could appear later in data processing.

During each day of field work, the research team took Whirl-Pak® bags, Nalgene cellulose nitrate filters, latex gloves, sterile forceps, a bleach solution, a cooler with ice, and a field negative from the laboratory. A separate filter was used for each sample, and gloves were bleached between sample collection. One field negative was analyzed during each day of field work, and new gloves were bleached and utilized by all members of the research team before handling the field negative in order to avoid potential cross-contamination.

Water Sampling Protocol

Two separate rounds of samples were assessed in this research that incorporate different methods of water sampling. The first round of samples was collected in spring of 2019, before the monsoon season, and included sites 1 and 2. The second round of samples was collected in summer of 2019, during the central-Arizona monsoon season, and included sites 3 through 8. All samples were collected from a downstream to upstream direction to mitigate the potential for between-site contamination from the researcher. We used an aseptic technique to collect samples, including wearing gloves that were cleaned with 10% bleach and allowed to air-dry.

Round 1 Sampling Protocol – Sites 1 and 2

Three water samples of 500 mL were collected in immediate succession at each site with Whirl-Pak® bags (Nasco, Fort Atkinson, WI). Each day, a field-negative Whirl-Pak® bag with ultra-pure deionized water from the ERAU lab was transferred to a sterile Whirl-Pak® bag at the field site to account for potential laboratory contamination. These samples were then wiped with 10% bleach solution and stored on ice in a cooler that had also been sterilized with 10% bleach solution. The samples were then transported back to the ERAU lab at 4 °C and were processed within 24 hours to collect eDNA. Vacuum filtration was used to pull 250 mL of collected water through a sterile Nalgene cellulose nitrate (CN) filter of 0.45-µm pore size (ThermoFisher Scientific, Waltham, MA). Vacuum filtration took place in a Biological Safety Cabinet Class II (Envirco Corporation, Albuquerque, NM) to prevent contamination of the water samples with DNA from the air in the laboratory. Filter housings were opened and dry filters were torn into four equal-sized pieces with flame-sterilized forceps, placed into a sterile 2 mL screw-capped microcentrifuge tube, and stored at -20 °C until processed for DNA extraction. Gloves were

cleaned with 10% bleach and allowed to air dry and DNA & DNase Away (RX Biosciences, Gaithersburg, MD) was used to clean the workspace between processing each sample.

Round 2 Sampling Protocol – Sites 3 through 8

As the process of collecting samples became more streamlined, we vacuum filtered samples from sites 3 through 8 in the field. We found that there was not significant contamination in the field setting, meaning that the use of the Biological Safety Cabinet Class II was redundant for this research. However, the sampling protocol remained the same. We continued to clean and air-dry gloves with 10% bleach, and tested a field negative each day. Before collecting samples, we established a sampling area, in which a shaded, flat area was established as the data procurement area. Exposure to heat may result in the degradation of DNA, so finding a shaded area for the duration of sample collection was imperative (Shokere, Holden & Jenkins, 2009). Before handling any materials, we ensured that skin was covered and gloves were cleaned with a 10% bleach solution to prevent contamination (Goldberg & Stickler, 2017). Samples were then collected at the edge of each water source (Figure 6).

Figure 6. Sample collection at Box Canyon Spring, Prescott National Forest.

At each site, we collected samples with the same filter we had used in Round 1, a sterile Nalgene cellulose nitrate filter with a 0.45-µm pore size (ThermoFisher Scientific, Waltham, MA). Three samples were collected in immediate replication. These filters were attached to a hand-pump vacuum, and water was drawn through the filter (Figure 7).

Figure 7. Sterile Nalgene analytical test filter funnel (RX Biosciences, Gaithersburg, MD).

After data samples were collected in the Nalgene filter and the water sample had passed through the filter, the filter was removed from the funnel with sterile forceps. The protocol for moving the filters was the same as in the lab. We used two sterile forceps to fold the filters into four equal-sized filters and place into a sterile 2 mL screw-capped microcentrifuge tube. These tubes were transported on ice in a cooler at 4°C back to the ERAU laboratory, where they were stored at -20 °C until they were processed for DNA extraction.

eDNA SAMPLE PROCESSING

In 2018, the ERAU Department of Biology and Chemistry developed a 16S rRNA metabarcoding process that can be used to address the presence of vertebrates in the freshwater environment, based on a protocol established by Vences et al. (2016)

In the metabarcoding methodology of extracting eDNA, amplified fragments of DNA are sequenced; then, these fragments are classified against a reference database of DNA to determine the fragments' taxonomic group (Deiner et al., 2017). In doing this, the presence of specific species in a sample can be confirmed. As eDNA research progresses and more species are inputted into DNA databases, previously unidentified species can be classified, aiding in biomonitoring efficiency (Elbrect et al., 2017).

DNA extraction

The ERAU research team used a modified version of the Vences et al. (2016) protocol for eDNA metabarcoding protocol to develop a 16S rRNA sequencing process to amplify the DNA samples. In this protocol, polymerase chain reaction (PCR) universal primers are used to target the section of the 16S rRNA gene that is present in vertebrate species and can be used for species identification (Vences et al., 2016).

Before DNA extraction, work-spaces and equipment were disinfected and/or sterilized. Environmental DNA was extracted from filters using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol for blood and tissue with some modifications (Renshaw, Olds, Jerde, McVeigh, & Lodge, 2015). All DNA extractions included an extraction negative to check for contamination. All extracted DNA samples were then cleaned to remove enzymatic inhibitors with the OneStep ™ PCR Inhibitor Removal Kit (Zymo Research, Irvine, CA) according to the manufacturer's protocol.

DNA SEQUENCING

Sequence analysis

The Illumina MiSeq FGx Forensic genomics system was used to sequence the eDNA samples (Jäger et al., 2017). These sequences were then grouped into relevant operational taxonomic units (OTUs). The results of these sequences were then compared with the National Center for Biotechnology Information (NCBI) GenBank, a genetic sequence database that contains all DNA sequences that are publicly available.

The process of data extraction ranges from water filtration to data analysis with bioinformatics (Figure 8). During this project, however, my focus was primarily on completing field work, as I do not have the training or expertise necessary to complete accurate DNA assessments in the forensic biology laboratory.

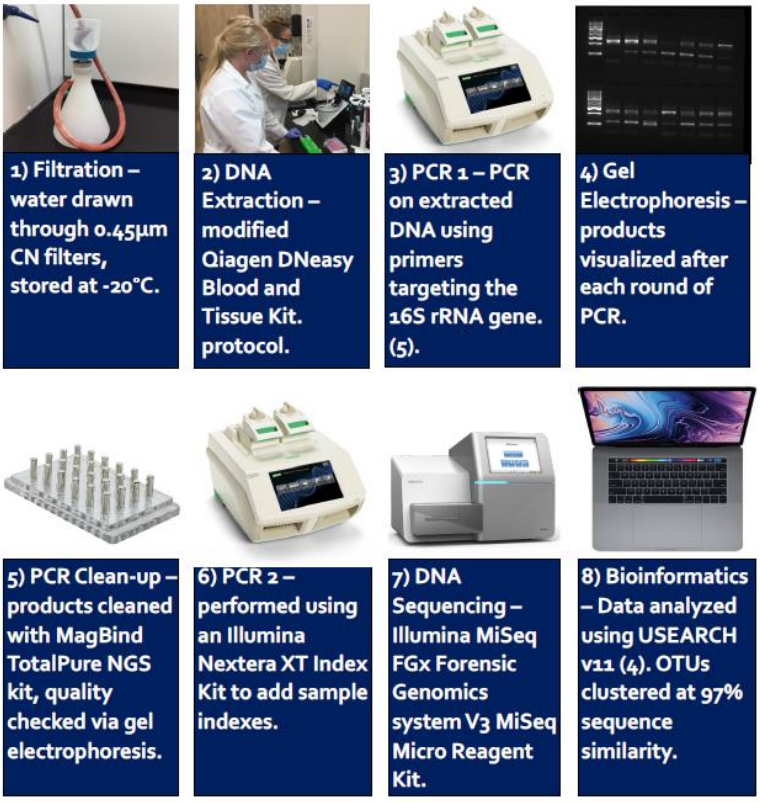

Figure 8**.** ERAU Forensic Biology eDNA Extraction Process (Turner-Rathbone, Eaton, Benson & Valente, 2018).

DNA sequencing in the Illumina MiSeq FGx Forensic Genomics system resulted in paired-end reads, which were exported as fastQ files using Illumina's BaseSpace fastQ generation pipeline. The reads were then processed using several algorithms available in USEARCH v11 (Edgar, 2018). We identified OTUs by comparing the consensus sequence for each OTU with sequences in the NCBI GenBank using the Basic Local Alignment Search Tool (BLAST; version 2.9.0+). BLAST results were sorted based on e-values, where the reference sequence with the lowest e-value was used to assign sequence identity. We used the following parameters to refine our BLAST results: percent match = 97% and e-value = 1e-20 (Andruszkiewicz et al., 2017). The reference sequence with the lowest e-value was used to assign a species-level identity to each OTU. In the case that OTUs matched entries for multiple reference sequences, we used the lowest common taxonomic grouping (family or genus) to assign identity and identify each OTU.

RESULTS

Genera identified via eDNA metabarcoding

Sample site results detected a variety of bird, mammal, and invertebrate species at Sites 1 through 8 (Table 2).

Table 2. Sample Sites (1-8) Results: GenBank Matches and Hypothesis

GenBank Match			Hypothesis	
Common name	**Scientific name**	**% Identity**	**Common name**	**Scientific name**
Cattle	*Bos taurus*	100	Cattle	*Bos taurus*
Elk	*Cervus elaphus*	100	Elk	*Cervus elaphus*
Human	*Homo sapiens*	100	Human	*Homo sapiens*
Crab-eating Macaque	*Macaca fascicularis*	100	Crab-eating Macaque	*Macaca fascicularis*
Rhesus Macaque	*Macaca mulatta*	99	Rhesus macaque	*Macaca mulatta*
White-tailed Deer/Mule Deer	*Odocoileus hemionus/virginianus*	100	Mule Deer	*Odocoileus hemionus*
Fox Squirrel	*Sciurus niger*	94	Arizona Gray Squirrel	*Sciurus arizonensis*
American Black Bear	*Ursus americanus*	100	American Black Bear	*Ursus americanus*
American Bullfrog	*Lithobates catesbeiana*	100	American Bullfrog	*Lithobates catesbeiana*
Mallard	*Anas platyrhynchos*	100	Mallard	*Anas platyrhynchos*
Wild Turkey	*Meleagris gallopavo*	100	Wild Turkey	*Meleagris gallopavo*
Western Shovelnose Snake	*Sonora occipitalis*	91	Western Groundsnake	*Sonora semiannulata*

In Table 2, “GenBank Matches” signifies the species that were identified through the comparison of DNA results with those found in GenBank. However, GenBank is a working database, and is still incomplete. Therefore, the column “Hypothesis” serves to further define which species we believe were represented if the GenBank results did not seem accurate. As stated in the sequence analysis section of this paper, a match of 97% and higher was considered significant, and accurate for species identification. However, matches as low as 94% and 91% are included in this analysis, as these species, the Arizona gray squirrel and western groundsnake (*Sonora semiannulata*) are found in the Prescott National Forest (Jaworski & Unit, 2011).

The use of the differentiation between “GenBank Match” and “Hypothesis” is important for this study due to disparity between species representation, as the GenBank database is not complete, and sometimes does not contain DNA sequences for species. For example, the falcated teal (*Mareca falcada*) is native to Asia (Melnikov, 2000). However, the American widgeon

(*Mareca americana)*, is common in the Southwest United States, and is a member of the same genus as the falcated teal (Peters et al., 2008). Therefore, we believe that we detected American widgeon.

Additionally, we believe that the Arizona gray squirrel and mule deer were detected instead of the fox squirrel (*Sciurus niger*) and white-tailed deer (*Odocoileus virginianus*). As the two species of squirrel and deer are members of the same genus, respectively, it is difficult to differentiate between the two with the data provided by GenBank. GenBank BLAST provides feedback that may make it difficult to discern which species is being detected. If both the white-tailed deer and mule deer are represented at 100% match, researcher's discretion and knowledge of the ecosystem must be used to determine which species was detected (Figure 9).

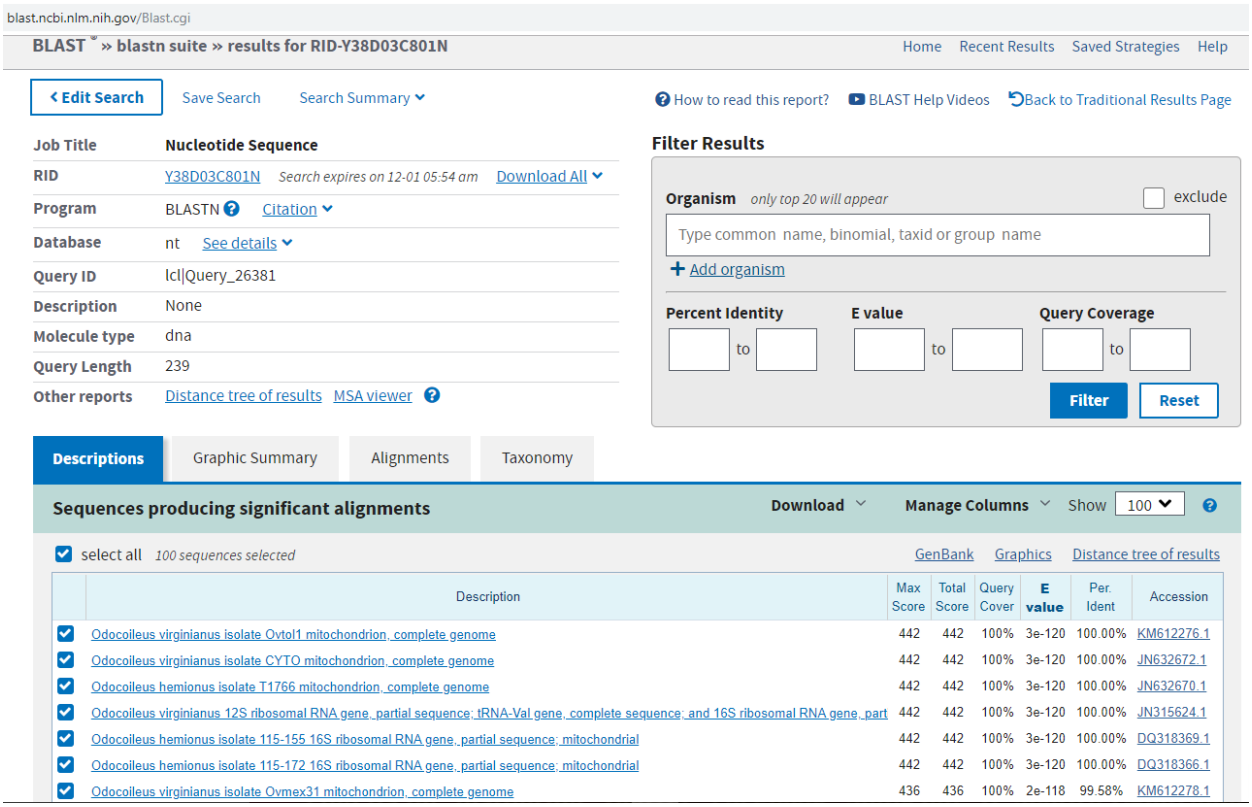

Figure 9. GenBank BLAST: white-tailed deer (*Odocoileus virginianus*) and mule deer (*Odocoileus hemionus*).

Multiple invertebrate species were represented in the sampling data, but, given the lack of robustness of DNA data on waterflea and mayfly species, it is difficult to verify which species were detected. For the purpose of this project, only detected vertebrates are presented and described due to the large number of invertebrate species that were detected in the samples. Further analysis of the detection of invertebrate species can be found in eDNA metabarcoding limitations in the discussion section of this paper.

The hypothesized species listed above in Table 2 were found throughout the sample sites in the Prescott National Forest (Table 3).

Table 3. Detected Species in Sites 1-8, Prescott National Forest.

Common name	Scientific name	Total Seq.	Extraction Negative	Field Negative 1	Field Negative 2	Field Negative 3	Positive Control	Site 1 - East Copper Spring	Site 2 - White Rock Tank	Site 3 - King Spring	Site 4 - Round Valley	Site 5 - Woodchute Wilderness	Site 6 - Powerline Meadow Tan	Site 7 - Box Canyon Spring	Site 8 - Homestead Spring
American Black Bear	*Ursus americanus*	5861	0	0	0	0	0	4824	0	20	297	720	0	0	0
American Bullfrog	*Lithobates catesbeiana*	586	0	0	0	0	0	0	0	0	0	586	0	0	0
American widgeon	*Mareca americana*	16522	236	1654	1999	0	0	0	8699	968	1330	1445	53	0	138
Arizona Gray Squirrel	*Sciurus arizonensis*	895	0	0	0	0	895	0	0	0	0	0	0	0	0
Cattle	*Bos taurus*	3	0	0	0	0	3	0	0	0	0	0	0	0	0
Crab-eating Macaque	*Macaca fascicularis*	188	0	0	0	0	0	188	0	0	0	0	0	0	0
Elk	*Cervus elaphus*	18843	0	0	0	0	0	0	18843	0	0	0	0	0	0
Human	*Homo sapiens*	519	0	0	0	0	0	0	519	0	0	0	0	0	0
Mallard	*Anas platyrhynchos*	218	8	0	0	0	0	0	0	32	161	17	0	0	0
Mule Deer	*Odocoileus hemionus*	6	0	0	0	0	0	0	0	0	0	0	6	0	0
Rhesus macaque	*Macaca mulatta*	48	0	0	42	0	0	0	0	0	3	3	0	0	0
Western Groundsnake	*Sonora semiannulata*	4778	0	0	0	0	0	0	4778	0	0	0	0	0	0
Wild Turkey	*Meleagris gallopavo*	9617	0	0	1772	0	0	0	0	1	3922	3922	0	0	0

DISCUSSION

eDNA metabarcoding provides realistic census of species' presence in stock tanks and springs

eDNA metabarcoding was shown to be a realistic means of demonstrating species' presence in natural springs and human-made stock tanks, and displays species detection results that are consistent with observational surveying data in the Prescott National Forest (Jaworski & Unit, 2011). The results of this project represent a next step forward in further employing eDNA metabarcoding as a biomonitoring tool, and encompass a series of lessons-learned and best practices in field work and in the laboratory, which are further discussed in the recommendations section of this paper.

The combination of different departments and individuals involved in this research is unique. As a primarily undergraduate institution, many of ERAU's research assistants are undergraduates who are also learning different processing techniques. While eDNA metabarcoding and data analysis could have been completed by sending raw samples to an external laboratory, all analysis in this project was completed on campus and by university employees and students.

Field negatives were not sequenced during Round 1 of sampling (Sites 1 and 2) due to the lack of species presence; however, during Round 2 (Sites 3-8), species were detected in the field negatives, indicating the need to process these samples. One field negative was collected during each day of sampling. Therefore, Field Negative 1 coincides with samples collected at Sites 3 and 4 (King Spring and Round Valley Spring), Field Negative 2 coincides with Sites 5 and 6 (Woodchute Wilderness Tank and Power Meadow Tank), and Field Negative 3 coincides with Sites 7 and 8 (Box Canyon Spring and Homestead Spring).

The detection of the rhesus macaque (*Macaca mulatta*) and crab-eating macaque (*Macaca fascicularis*) is consistent with the positive control, which was used to ensure that there was not contamination from the laboratory. While a field negative was collected during each day of field work to account for potential contamination in the field, the extraction negative was prepared in the laboratory to account for any laboratory contamination or cross-talk between the samples. Sample cross-talk signifies the potential for samples to share DNA while in the sequencer, thus making it seem as though a species was detected in a sample that it did not originate from (Vences et al., 2016).

Rhesus macaque DNA was purchased from Zyagen, Inc.; if a sample other than the positive control displayed rhesus macaque DNA, we knew that the sample had been contaminated in the laboratory. However, upon sequencing the data for the extraction negative, we found another species of macaque, the crab-eating macaque (*Macaca fascicularis*). Although the DNA for rhesus macaque originated from a liver sample from one organism, the detection of the crab-eating macaque signifies either that there is hybridization of the species in the laboratory setting, or that the sample was contaminated before it reached our laboratory.

We used the results from the positive control to calculate a detection threshold that would eliminate potential false positives due to lab contamination or cross-talk between samples that could have occurred during sequencing. To determine this threshold, we first calculated the number of sequence reads assigned to the positive control across the sequencing run, or the number of total samples that detected crab-eating macaque and rhesus macaque. We then divided the maximum number of reads found in an experimental sample, 11, by the total number of reads assigned to the positive control, 910, to determine the detection threshold. This resulted in a threshold of 0.012 or 1.2%. We then applied this threshold to data from the entire sequencing run

to eliminate potential false-positives from samples where the total number of reads for a species in a sample represented less than 1.2% of the total reads for that species.

This project detected several invertebrate species, but it is difficult to know the viability of this representation. Additionally, we were primarily focused on the availability of vertebrate detection data. The species we found at each site were representative of those that are found in the Prescott National Forest. Site 1 showed the presence of cattle and mule deer, which is unsurprising for this area. The location of East Copper Spring is in an open range leased area in a Ponderosa pine stand, and the tank itself is roughly 6 ft^2. However, this site only includes one sample for analysis, as the other two were contaminated. Therefore, other vertebrates could have been found in those two samples, which is a limitation of this analysis.

Site 2 detected a variety of birds and mammals that are common to the area. While GenBank did indicate the presence of the falcated teal, fox squirrel, and white-tailed deer at this site, we believe that these are the American widgeon, Arizona gray squirrel, and mule deer, all of which are commonly found species in the Prescott National Forest (Jaworski & Unit, 2011). White Tank Spring is a natural spring located along a hiking trail among a Ponderosa pine stand in the Prescott National Forest; therefore, the presence of human DNA at this spring is unsurprising. Furthermore, White Tank Spring's location is ideal black bear habitat (Varas-Nelson, 2010), meaning that the presence of American black bear DNA at the location is consistent with our knowledge of their habitat.

At Site 3, cattle (*Bos taurus),* human (*Homo sapiens*), American bullfrog (*Lithobates catesbeiana*), and western groundsnake were detected. Again, as an open range area, the presence of cattle and human DNA is unsurprising. During data collection, the researchers heard the call of the American bullfrog, and our detection of its DNA is consistent with our

observational data. The American bullfrog is an invasive species in Arizona (Snow & Witmer, 2010), and our detection represents a next step in better monitoring presence and removing the species. The western groundsnake is more common at lower elevations in the Sonoran Desert, but its range extends into the Prescott National Forest (Goldberg, 2001).

Site 4, one of our most rural sites, detected a variety of vertebrates that were also found at Site 3, in addition to the wild turkey, which is a common bird found in the Prescott National Forest (Wakeling et al., 1998).

Site 5 is located on the edge of the Woodchute Wilderness, and the detection of a variety of species in this arid location is consistent with observational data. As a stock tank located in a high elevation Ponderosa pine stand, Site 5 detection of elk is consistent with our knowledge of their habitat and range in the central Arizona highlands (Jaworski & Unit, 2011). Site 6 was located within five miles of Site 5, but had fewer detection results, which may be due to its proximity to a local camping area. Because of this, detection of human and mallard (*Anas platyrhynchos)* is consistent with the location of the stock tank near human establishments.

Site 7 and Site 8 both had limited results, indicating that there may have been contamination in the sample that day. The only vertebrate detected during that day of sampling was human, but no invertebrates were detected, indicating that there was an issue with sampling protocol at these sites, which is a limitation in this study.

The presence of different species in the natural springs and stock tanks may indicate the disparity in results between these two types of water sources. The water in smaller stock tanks and springs was more stagnant, which could explain the lack of detection of a more diverse collection of vertebrates. Additionally, size of the tank or spring may have an impact on both the types of species that are visiting the tank and the temporal longevity of the eDNA. Continued

research will be necessary to validate the effect of water source size, location, and type on eDNA metabarcoding results.

Project limitations

While metabarcoding techniques may be less expensive in the laboratory than qPCR, this research was limited by the amount of resources available to process samples. To maximize the productivity of sample processing, 90 samples from separate projects were gathered before the data was processed. Because of this, samples collected in the summer and early fall of 2019 were not processed until November 2019. Contamination was also an issue in effective processing. As shown in Table 2, representing round 1 of sampling (Sites 1 and 2), two of the samples for Site 1 were contaminated, and were thus not included in these results. However, as the data analysis process became more streamlined, and the forensics biology laboratory assistant became more practiced in conducting DNA extraction, contamination was more effectively avoided or accounted for. For example, contamination was found in three samples in Round 2 of analysis, but the laboratory assistant was able to recomplete PCR for these samples.

Water collected from some of the more stagnant stock tanks were turbid, requiring recompletion of the PCR process. Although DNA is present in these samples, excess particles collected can prevent inhibition during DNA extraction. Because of this, samples had to be diluted and PCR had to be re-started. Many of the samples collected in Round 2 had to be diluted multiple times to effectively extract DNA, which adversely affected the timeline for completing DNA sequencing.

The recompletion of PCR of these three samples indicates the laboratory assistant's knowledge of the processing technique, but the time necessary to recomplete this analysis also extended the time until data was available for bioinformatics analysis. While this was a

limitation in this particular project, the process of completing DNA sequencing is continuously improving, and contamination and dilution issues that were recognized and rectified in fall of 2019 can be better mitigated in future research.

eDNA metabarcoding limitations

A limitation in eDNA metabarcoding is the inability to assess species density, as results from metabarcoding only display which species are present, and not species density. Further limitations include the collection of "zombie" DNA, or DNA from a dead organism (Thomsen & Willerslev, 2015). While results from eDNA biomonitoring display species presence, it still lacks robustness in further describing populations and communities.

As discussed in the results section, the availability of genetic information in GenBank may make it difficult to distinguish between species; therefore, a knowledge of species presence in the area is necessary to make important distinctions. For example, results from GenBank showed that the white-tailed deer was detected at Site 2; however, it is more likely that the detected species in the Prescott National Forest would be a mule deer (Jaworski & Unit, 2011). Errors such as these can be expected to decrease as the application of eDNA metabarcoding continues to grow. GenBank is consistently updated, and inclusion of DNA representations of specific species will continue to make results of sampling more precise.

Metabarcoding processes are continuing to expand (Klymus et al., 2017). As this expansion continues, we may expect to see greater numbers of detection results. All eDNA collected in a test tube for sampling cannot be processed (Vences et al, 2016). Therefore, it is possible that species are present, but do not appear in the results. For example, the research team saw mule deer visiting Sites 5 and 6 during sample collection, but this species was not detected by the sequencer.

Although the 16S rRNA metabarcoding technique may be useful for vertebrate biomonitoring, it may not be as effective in assessing invertebrates. A variety of different metabarcoding methodologies using different strands of rRNA have been used in research to study the presence of species (Andruszkiewicz, et al. 2017). The 16S methodology was designed specifically for vertebrate detection (Vences et al., 2016). While this metabarcoding technique has identified various invertebrate species, the percentage match is often lower. Furthermore, the initial research done on the Verde River did not result in outcomes that showed the presence of crayfish (*Orconectes virilis*); however, non-native crayfish (*Orconectes virilis*) are common in the Verde River system, and were visually observed by the research team throughout water sample collection. Therefore, the 16S rRNA metabarcoding technique may not be an effective tool in invertebrate biomonitoring.

RECOMMENDATIONS

Current eDNA metabarcoding applications are comprehensive and support a diverse assortment of needs for enterprises ranging from environmental conservation to energy industries (Figure 10). However, there is a need for further research to continue to vet applications of eDNA as a biomonitoring tool, and better assess how viable eDNA is as a survey tool in each realm.

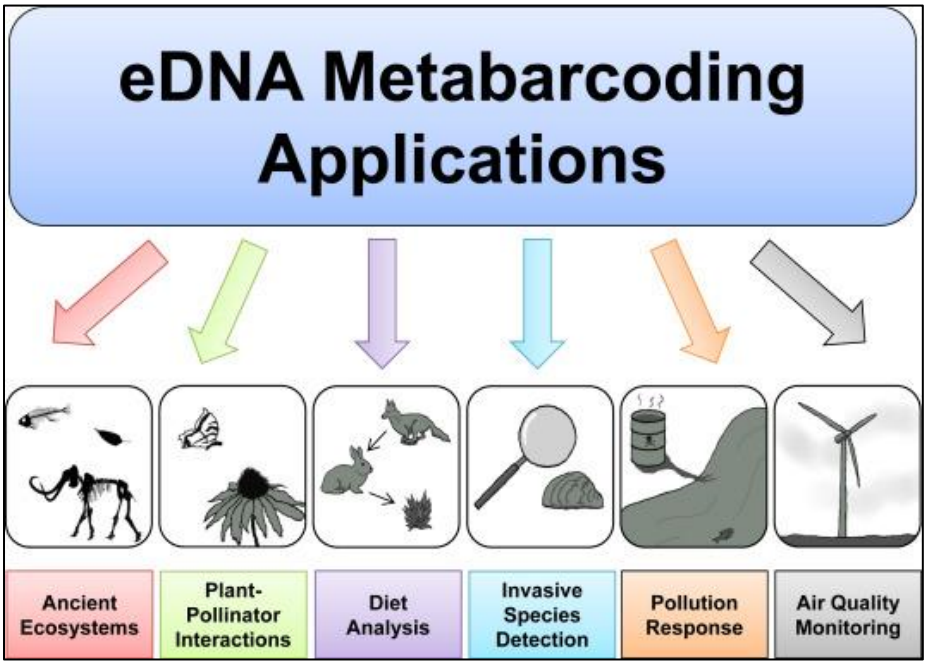

Figure 10. eDNA Metabarcoding Applications (Ruppert et al., 2019).

While the purpose of this paper was to provide a representation of which species are detected at each site, the results of this project provide insight into the presence of an invasive species, the American bullfrog; applications of more refined techniques beyond the use of 16S rRNA extraction can provide more tailored results based on the needs of specific projects. For example, blocking primers can be used in DNA analysis to block specific nucleotides and only present the results of desired species (Klymus et al., 2017). In doing this, invasive or rare species can be targeted for monitoring in data collection.

The results of this research have aided in further demonstrating the usefulness of eDNA metabarcoding in vertebrate species detection. Specifically, by addressing natural springs and human-made stock tanks, this professional paper showed that eDNA biomonitoring is a useful tool in assessing species presence at water sources that are often more stagnant or smaller. The GenBank database is continuing to expand as eDNA biomonitoring research opportunities increase, and the increased availability of species information will result in more accurate species identification, thus resulting in more robust and applicable data.

Furthermore, this research demonstrated the effectiveness of conducting sampling procedures in the field, as opposed to in the laboratory setting. In Round 1 of sampling, we transferred all water samples from Whirl-Pak® bags in the field to Nalgene cellulose nitrate filters in the laboratory, and transferred filters to test tubes in the Biological Safety Cabinet Class II. By transferring filters in the field, we were able to eliminate waste and time necessary to conduct sample analysis. The use of the filters in the field also allowed for decreased potential for contamination or spillage during transportation back to the ERAU Forensic Biology laboratory. In Round 1 of sampling, water samples were transferred back to the laboratory in Whirl-Pak® bags in a large cooler. There was increased opportunity for these bags to break during transit, and the quantity of water that needed to be transported back to the laboratory resulted in fewer opportunities for sampling due to space availability in the cooler and relative weight. Filters in test tubes were much smaller and lighter to transport, meaning that the research team could gather more samples during each day of field work.

Future research

As a continuation of eDNA metabarcoding research on the Verde River in central Arizona, this project demonstrated the efficacy of eDNA biomonitoring in natural springs and

human-made stock tanks. The research team aims to continue to assess different bodies of water in order to further improve the use of eDNA metabarcoding as a viable biomonitoring tool.

Future research should continue to focus on the temporal longevity of eDNA. We would like to address the use of trail cameras in future research due to limited research on the temporal range of eDNA. Past research has addressed the range of eDNA half-lives with varying results, ranging from four days to one month (Dejean et al., 2011). While this research proposal initially included the use of trail cameras to assess when species were visiting springs and stock tanks in order to compare results and establish eDNA temporal longevity, vandalism of the cameras at East Copper Spring resulted in our inability to use them for our research. Upon receiving further funding to purchase additional trail cameras, the research team hopes to re-install them at future sampling spots. Trail cameras that were initially installed in tree stands at East Copper Springs included tags that included Dr. Catherine Benson's contact information and a statement that the cameras were being used for student research. Despite these measures, the trail cameras were still vandalized, and we plan on placing them at sample sites that are more difficult to access for future use. Although the trail cameras that were placed at our sample site were vandalized in this research, they would be an efficient way of monitoring temporal validity of eDNA for each species (Leempoel et al., 2019).

There are further opportunities available to continue to study eDNA. While this research focused on eDNA collection in water, eDNA has also been found in snow, soil, air, and seawater (Le Port et al., 2018). Additionally, further research collaborations can be established to continue to apply eDNA as a biomonitoring tool. For example, Northern Arizona University has conducted ancient mitochondrial DNA analyses in archaeological research (Speller et al., 2010). Future collaboration between the unique resources available at the laboratories at each

university, located less than two hours from one another, could result in more diversified and applicable results of species presence, past and present, in the Southwest United States.

One aspect of eDNA biomonitoring that this research exposed was the application of 16S rRNA assessments in vertebrate and invertebrate biomonitoring. While the 16S technique detected invertebrate species, the research team believes that these results may not be completely accurate. Therefore, future research could focus on the development of an eDNA metabarcoding assay that incorporates both invertebrate and vertebrate species, instead of requiring that different tools be implemented based on types of species that a research team is attempting to detect.

CONCLUSIONS

Overall, this project provided results that are indicative of the viability of eDNA metabarcoding as a tool in vertebrate species detection in springs and stock tanks. From this, future projects can be developed to better understand biodiversity in specific ecosystems. The application of eDNA metabarcoding as a biomonitoring tool is a global project, and scientists throughout the world are making significant contributions to the growth of peer-reviewed research on the subject, including the detection of common mammal visitors to Japanese ponds, the creation of a monitoring plan for the United Kingdom's endangered great crested newt (*Triturus cristatus*), and the identification of newly discovered mammals in the Amazon Rainforest (Ushio et al., 2018; Harper et al., 2018; Kocher et al., 2017). There is still room for improvement in the metabarcoding process, as shown in the results and limitations of this specific study. As the scientific approach to collecting and analyzing eDNA data becomes more streamlined, opportunities for easier sample collection and inexpensive data sequencing will continue to evolve. The use of eDNA as a biomonitoring tool in springs and stock tanks presents logical results of detection of species that are known to inhabit the Prescott National Forest, and represents another step forward in the eDNA metabarcoding research endeavor.

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