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Metabarcoding of Environmental DNA Samples to Explore the Use of Uranium Mine Containment Ponds as a Water Source for Wildlife

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Abstract: Understanding how anthropogenic impacts on the landscape affect wildlife requires a knowledge of community assemblages. Species surveys are the first step in assessing community structure, and recent molecular applications such as metabarcoding and environmental DNA analyses have been proposed as an additional and complementary wildlife survey method. Here, we test eDNA metabarcoding as a survey tool to examine the potential use of uranium mine containment ponds as water sources by wildlife. We tested samples from surface water near mines and from one mine containment pond using two markers, 12S and 16S rRNA gene amplicons, to survey for vertebrate species. We recovered large numbers of sequence reads from taxa expected to be in the area and from less common or hard to observe taxa such as the tiger salamander and gray fox. Detection of these two species is of note because they were not observed in a previous species assessment, and tiger salamander DNA was found in the mine containment pond sample. We also found that sample concentration by centrifugation was a more efficient and more feasible method than filtration in these highly turbid surface waters. Ultimately, the use of eDNA metabarcoding could allow for a better understanding of the area's overall biodiversity and community composition as well as aid current ecotoxicological risk assessment work.

Keywords: biodiversity surveys; vertebrates; wildlife attraction nuisance; ecotoxicology

1. Introduction

Ecotoxicology is the study of contaminant effects on wildlife species, natural communities and entire ecosystems [1,2]. To understand contaminant effects, we must first know which species are in the community, because some contaminants can move through the food chain accumulating in higher trophic levels and effects can be species specific [3]. Thus, biodiversity surveys play an important role in ecotoxicology. An ongoing risk assessment study in the Grand Canyon region is examining which local wildlife species are likely to come into contact with radionuclide and inorganic constituents from uranium mining activity by surveying areas near and within mine sites [4]. Uranium mining has occurred in northern Arizona near the Grand Canyon since the 1940s [5]. Concern over uncertainty in environmental, cultural and social effects of uranium mining activity led to a 20-year withdrawal of mining activity on federal lands [6]; however, several mines with existing permits were unaffected by the withdrawal and remain open or under development. Given the lack of site-specific radionuclide and metal characterization in biota near mining sites in the region, Hinck et al. initiated a study to assess potential contamination as well as the toxicological risk to the local biodiversity [4]. Specific goals of the ecotoxicological work were: (1) to identify contaminants of potential concern and critical contaminant exposure pathways; (2) to conduct biological surveys of plants, invertebrates, amphibians, reptiles, birds, and small mammals to understand the local food web and refine the list of target species

for contaminant analysis; (3) to collect target species to determine baseline chemical contaminant concentrations and radiation levels for an active mine site (Canyon Mine); and (4) to conduct laboratory experiments to describe biological effects of contaminants on target species [4,7]. To fulfill the second goal, wildlife surveys were conducted to assess local biota using a number of methods including: capturing via mist-netting (bats, birds), capturing via traps (small mammals) or cover boards (reptiles); and passive sampling with acoustic monitors (bats, anurans). To supplement these surveys, our study collected environmental DNA (eDNA) samples from a mine containment pond and nearby surface water sources to be analyzed with DNA barcoding methods in an effort to assess how mine containment ponds could act as a potential wildlife attraction nuisance. The containment ponds are artificial ponds with impermeable liners, and they collect surface runoff from rain and snow events within the mine yard and any water pumped from the mine shaft. This water contains a number of elements related to mining activity. Knowing what wildlife could be using this water source will inform researchers on the potential ecological connectivity among species and help in the modeling of exposure pathways and potential biological effects of mining-related elements [4,8]. Our work aims to inform the ecotoxicological study by using eDNA to identify wildlife species in the area that could be attracted to mine containment ponds and subsequently exposed to mining related elements.

Water and soil contain DNA from cellular material shed by organisms into the environment. By amplifying and sequencing this environmentally derived DNA (eDNA), researchers infer the presence of a species through the identification of that species' DNA. Environmental DNA analysis and sequence identification methods are becoming important tools in conservation research and biodiversity surveys, enabling biologists to detect species with less effort in terms of time, labor, and monetary costs and with less invasive methods than established survey techniques [9–12]. Since Ficetola's initial study on invasive American bullfrog detection in European wetlands using eDNA [13], numerous studies have been published using this technique to detect invasive species [14], as well as rare or endangered species [15,16]. Extending beyond basic species detection, eDNA analyses can also address ecological questions such as food web dynamics [17,18], trophic interactions [19,20], and community composition changes [21,22]. These studies use the concept of DNA barcoding to amplify and sequence DNA fragments that are diagnosable to some taxonomic level [23]. In general, barcoding involves extracting DNA from the tissue of a single organism and matching that sequence to a database in order to identify it. Metabarcoding extends this method to the use of bulk samples in which DNA is extracted from either multiple whole organisms (e.g., plankton tow samples and insect trap samples) or environmental (e.g., water, soil and air) samples to identify multiple taxa from a single sample. Although a number of terms have been used to describe this approach of multi-species identification from a single sample, we follow Taberlet and coworkers' term of DNA metabarcoding or eDNA metabarcoding when using an environmental sample [24]. With advances in DNA sequencing technology leading to lower costs per sample and increases in data output, metabarcoding research is rapidly expanding. Nevertheless, as Taberlet et al. point out, applying a metabarcoding approach to eDNA samples poses some limits not found with traditional barcoding studies [24]. For instance traditional barcodes are commonly used genetic markers that are generally 500–800 base pairs long; however, the degraded nature of eDNA makes it less likely that a standard length barcode will amplify, so shorter fragments must be used. Unfortunately, shortened amplicon length can lead to loss in taxonomic resolution. Past studies using eDNA metabarcoding have used short fragments of the 12S rDNA mitochondrial gene to assess fish assemblages [25] and 18S rDNA nuclear gene for zooplankton [21]; however, taxonomic resolution was generally above the species level (family to genus). Furthermore, universal primers used to amplify these markers are generally unable to amplify all targeted taxa with equal efficiency, leading to primer bias favoring amplification of certain taxa over others leading to potential false negatives [26,27].

In this study, we examine the ability of eDNA metabarcoding to detect and survey vertebrate wildlife that use small surface waters in the arid southwestern region of the United States. We compare two markers (12S and 16S) that produce short (<100 base pairs) amplicons and their ability to

identify taxa to the species level. Our eDNA survey efforts will inform the ecotoxicology study by detecting previously un-surveyed taxa and identifying taxa that may be important targets for future ecotoxicology studies. By sampling at sites near and within uranium mines, we show that eDNA can be used to survey the terrestrial species which could be attracted to the mine water sources and subsequently may be exposed to inorganic constituents and radionuclides.

2. Materials and Methods

2.1. Sites and eDNA Sample Collection

Sample sites were chosen based on water availability and site accessibility near uranium mines. At the time of sample collection we only had access to one active mine containment pond (Canyon Mine). The rest of our samples were from man-made cattle tanks or natural pools of water within eight kilometers of active or inactive uranium mines. Pools varied in volume from site to site as well as among years depending on rainfall. Our largest cattle tank, Wild Band tank, was approximately 47 × 59 m and our smallest sampling site was Pine Nut tank in 2016 which was only a 1 × 1 m puddle (Figure 1a,b). Hack tank had completely dried up during our 2016 sampling period, thus no 2016 samples were obtained from this site. The Canyon Mine containment pond is approximately 120 × 90 m, but like the cattle tanks, volume varied over sampling period (Figure 1c). For all sampling points except for Wild Band tank in 2015, Little Robinson tank in 2015, Pine Nut tank in 2016, and Canyon Mine, opposite sides of the pools were sampled resulting in two replicate 45 mL samples per side (four total samples per site) (Table 1). In 2016, additional 100 mL samples at five of the sites were taken. Although originally aiming to filter 100 mL of water from each site through one filter, only 27–100 mL of water were filtered due to the length of time it took to process these turbid samples (Supplemental materials Spreadsheet 1).

2.2. eDNA Sample Processing

All technicians collecting samples wore sterile, nitrile or latex gloves for each set of samples. Water samples were taken using a three meter long sampler constructed from PVC pipe that fit up to four 50 mL tubes (Figure 1d,e). The PVC water samplers were sprayed with a 10% bleach solution and dried in between sampling sites. Sample tubes were dipped in water, filled to 45 mL, capped and immediately chilled on wet ice until they could be placed in a freezer. Samples were frozen and shipped overnight back to the lab. Samples for filtering were filtered using a vacuum flask set up in the field. Water from these samples was poured into new Nalgene™ analytical filter funnels with 47 mm diameter cellulose nitrate (0.45 µm pore diameter) filter membrane with filter funnel adapter (Thermo Scientific, Waltham, MA, USA) and drawn through the filter membrane using the MasterFlex E/S portable sampler with 4.8 ID silicone tubing (Cole-Parmer, Vernon Hills, IL, USA). Due to the turbidity of the water sources, filtration time varied from 20 to 80 min. Samples from Little Robinson and Pine Nut tanks only had between 27 and 50 mL of water filtered because of the turbidity of the samples (Figure 1f), whereas Arizona One, Kanab North and Wild Band tanks had 100 mL filtered. Filters were then folded using forceps and placed into labeled 1.5 mL tubes with 1 mL 100% ethanol. Filters were also shipped back to the lab. All forceps were sterilized by soaking in a 10% bleach solution for 10 to 20 min and then rinsing with purified water before use.

In the lab, frozen samples were thawed and processed via centrifugation. Water samples were centrifuged for 30 min at 5000 RCF at 4 °C. The water was decanted off, and the DNA pellet was suspended in 200 µL GST buffer (IBI Scientific) in 1.5 mL tubes. Samples were then stored at −20 °C until DNA extraction.

For DNA extraction, samples were digested using proteinase K overnight in a shaking 60 °C water bath and subsequently extracted using the gMax Mini Genomic DNA Kit (IBI Scientific, Peosta, IA, USA). Purified DNA from each 45 mL sample was re-suspended in 50 µL 10 mM Tris-HCL. For the filter samples, filters were first cut in half using sterilized scissors and forceps to preserve half the filter

for future use. The halves removed for extraction were allowed to dry and digested overnight with proteinase K. DNA extraction of the filter samples was done using the Qiashredder kit (Qiagen, Hilden, Germany) for cell and tissue lysate homogenization to remove the DNA from the filters, and the DNEasy Blood and Tissue kit for DNA extraction (Qiagen). Purified DNA from each filter sample was re-suspended in 100 µL of 10 mM Tris-HCL. Because the filters were cut in half for extraction, the total volume sampled from each filter was divided in half to represent the total volume of water processed for that filter (Tables 1 and 2).

Figure 1. (**a**) Wild Band tank site during the August 2016 sampling; (**b**) Pine Nut tank site during the August 2016 sampling; (**c**) View of the Canyon Mine containment pond; (**d**) Sampling for eDNA from the Canyon Mine containment pond, using the constructed water sampler; (**e**) Sampling of Little Robinson tank site using the water sampler; (**f**) Filtration of a Pine Nut tank sample from August 2016, note the turbidity.

Table 1. List of sampling sites, number and types of samples, and total volume processed for each site.

Sampling Site	Site Type	Latitude	Longitude	Month-Year Sampled	Number of Centrifuged Samples	Number of Filtered Samples	Total Volume Processed (mL)
Arizona One Tank	Tank	36°29′57.95″ N	112°47′45.32″ W	July-15	4		180
				August-16	4	1	230
Hack Tank	Tank	36°36′52.52″ N	112°50′54.59″ W	March-15	4		180
				July-15	4		180
Kanab North Tank	Tank	36°42′3.53″ N	112°39′39.11″ W	July-15	4		180
				August-16	4	1	230
Little Robinson Tank	Tank	36°30′0.42″ N	112°50′31.07″ W	July-15	2		90
				August-16	4	1	205
Pine Nut Tank	Tank	36°30′26.77″ N	112°43′44.30″ W	July-15	4		180
				August-16	2	2	109
Wild Band Tank	Tank	36°41′29.50″ N	112°49′18.34″ W	July-15	2		90
				August-16	4	1	230
Canyon Mine	Mine	35°52′57.50″ N	112°5′44.52″ W	June-16	1		45

Table 2. Comparison of filtered versus centrifuge (tube) processed samples.

Site	Arizona One Tank		Kanab North Tank		Little Robinson Tank		Pine Nut Tank		Wild Band Tank	
Sample type	**Filter**	**Tube**	**Filter**	**Tube**	**Filter**	**Tube**	**Filter**	**Tube**	**Filter**	**Tube**
Average volume processed (mL)	50	45	50	45	25	45	19	45	50	45
Number of samples	1	4	1	4	1	4	2	2	1	4
Average number of taxa (16S marker)	1	3.5	4	4.5	3	3.25	1	2.5	2	2
Average number of taxa (12S marker)	3	3	0	1.5	0	1.75	0	3	1	2.5

Positive and negative amplification controls were used in library preparations. The positive amplification control consisted of genomic DNA extracted from black carp (*Mylopharyngodon piceus)* tissue. This extraction was tested and verified to amplify with both markers. This positive control enabled us to verify that the polymerase chain reactions worked even when field collected eDNA samples did not appear to amplify. This also allowed us to identify and quantify MiSeq carryover of reads across samples. A negative (no template) control was used to test for contamination during library preparation.

2.3. Library Preparation and Sequencing

For our assays, we used Riaz and coworkers' first 12S-V5 primer pair to amplify an 83 bp amplicon of the vertebrate 12S rRNA gene [28]. For the 16S assay, we modified the mammal 16S primers developed by Taylor to include degenerate sites which increased this assay's ability to amplify other vertebrate taxa [29]. These primers amplified a 93 bp fragment of the 16S rRNA gene.

Samples, positive controls, and negative controls were prepared for paired-end, high-throughput sequencing on the MiSeq platform (Illumina®, San Diego, CA, USA), using a multiple PCR process. In the first step, the target was amplified with assay specific primers (Table 3). A second round of PCR used the previous PCR product as template and primers that were tailed with a 33–34 bp sequencing primer region on the 5′ end (Table 3). For the 12S primer set, each sample was amplified using a 25 µL reaction volume, including 1X AmpliTaq Gold™ 360 Master Mix (Thermo Scientific), 500 nM of each primer, and 2 µL of template DNA. Conditions were: 5 min initial denaturation at 95 °C, followed by 35 cycles of 95 °C for 15 s, 53 °C for 30 s, and 72 °C for 30 s. For the 16S primer set, reactions were performed with a volume of 25 µL, including 1× AmpliTaq Gold™ 360 Master Mix (Thermo Scientific), 800 nM of each primer, and 2 µL of template DNA. Conditions were: 10 min initial denaturation at 95 °C, followed by 35 cycles of 95 °C for 45 s, 55 °C for 45 s, and 72 °C for 45 s. The second reaction was performed on the product of the previous amplification under the same conditions, except using 400 nM of each primer with the 5′ tailed sequence (Table 3). This final reaction was cleaned up using a MinElute PCR purification kit (Qiagen). Product was then sent to the University of Missouri DNA Core for an additional round of PCR and sequencing.

Table 3. Primers used for each assay, oriented 5′ to 3′. For the first PCR round the assay specific (no underline) sequence was used as a primer. For the second PCR round, primers with the assay specific and tail sections (underlined) were used.

Primer Name	Primer Sequence 5′–3′
12S-V5-Tailed-F1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACTGGGATTAGATACCCC
12S-V5-Tailed-R1	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTAGAACAGGCTCCTCTAG
Taylor_16S_DEGEN_F1_Tailed	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTTGGGGYGACYTYGGA
Taylor_16S_DEGEN_R1_Tailed	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGCTGTTATCCCTRGRGTARC

The final PCR step added the paired end indices (IDT, Ultramer Oligos) as well as the P5 and P7 adaptor sequences, which enable the prepared product to bind onto the surface of the Illumina® MiSeq flowcell. The added indices allowed for multiple samples to be pooled together in a single MiSeq run. PCR was carried out in 50 µL reactions including 1× Phusion™ HF buffer, 5 µL (200 nM) of each indexed primer, 2 U of Finnzymes' Phusion™ High-Fidelity DNA polymerase (NEB), and up to 28.5 µL of product from the previous PCR. Conditions consisted of a 3 min initial denaturation at 98 °C, followed by 25 cycles of 98 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s, with a final 7 min extension at 72 °C.

Pooled samples were run on an Illumina® MiSeq with 2 × 300 bp V3 and 2 × 150 bp chemistries. An additional 15% PhiX DNA spike-in control was added to improve library diversity and subsequent sequencing of reads.

2.4. Bioinformatic Analyses

Sequence pairs (2 × 300 bp) from the MiSeq runs were first joined using flash (https://sourceforge.net/projects/flashpage/). Primers at the ends of the successfully joined contigs were removed using cutadapt, and contigs were retained only if both primers were found [30]. The usearch fastq_filter program (http://drive5.com/usearch/manual/cmd_fastq_filter.html) filtered those contigs whose expected number of errors was greater than 0.5 (see https://www.drive5.com/usearch/manual/ for a description of "expected number of errors"). All contigs were clipped from the 5′ end and any contig shorter than 83 base pairs for the 12S assay and 93 base pairs for the 16S assay was rejected. The Qiime command split_libraries_fastq.py was used to format a fasta file of the cleaned, assembled, contigs [31]. The outputs for all samples were concatenated into one file for clustering and chimera removal by the uparse method (http://www.drive5.com/uparse/). Taxonomy was assigned to a representative of each cluster of OTUs (operational taxonomic units) using BLAST and a database of 12S or 16S DNA sequences that was prepared from all available eukaryotic species on GenBank [32].

The search retained each OTU's top two BLAST hit sequences, scientific name, accession number, identity percentage, coverage percentage, and expected (e) value. Only OTUs with > 80% coverage were retained. We used a set of rules for assigning each OTU a taxon. OTUs with both BLAST hits having an identity (percent similarity between query and subject sequence) ≥97% were considered identified to species level. If the two BLAST hits were different species of the same genus but the read was ≥97% to both species, the read was designated to the genus level. If hits to the OTU were >94% but <97%, we ran another BLAST search and examined similarity of reads to other congeners found in the NCBI database. If the OTU showed 94–97% similarity with just members of one genus, the OTU was assigned to that genus. If the OTU showed 94–97% similarity with multiple species in a family, we designated that OTU to family level. For those 90–94%, order was assigned, and we ignored anything below the 90% identity. Taxonomic calling followed these general rules. Finally, all OTUs with singleton reads were removed.

2.5. Carryover Calculations

To remove sequencing carryover or cross contamination of reads among samples we employed a strategy similar to that of other recent studies [33,34]. Using our positive control sample of black carp DNA, we screened for black carp reads in any of our field samples. Since black carp are not present in any of our field sites, we are confident that any black carp reads detected in the field samples are due to cross contamination or sequencing carryover. We used the sample with the highest number of black carp reads and divided that number by the cumulative number of black carp reads in the positive control. We applied this proportion as our threshold. Reads from any OTU in a sample that were lower than this threshold were removed (Supplemental materials Spreadsheet 2).

2.6. Data Accessibility

High-throughput sequence data can be found in NCBI's Sequence Read Archive under project PRJNA414121 (https://www.ncbi.nlm.nih.gov/sra/).

3. Results

3.1. Reads from MiSeq

All non-indexed reads, including the PhiX reads, were removed by the sequencing facility prior to analysis. After removal of non-indexed reads, we recovered 5,460,004 reads for the 12S assay MiSeq run. Approximately 81% of these reads were retained after trimming and quality checks. For the 16S assay, we had 9,307,611 reads, and we retained approximately 63% after trimming and quality checking. The number of reads retained throughout each quality control step can be found in Supplemental materials Spreadsheet 3, which provides the read number per OTU for each sample after trimming, clustering, and chimera removal.

3.2. Taxa Identification per Marker

Twenty-seven OTUs or taxa were identified from the MiSeq runs using both markers. The 12S marker resulted in twenty-one taxa, whereas the 16S marker resolved nineteen taxa (Table 4). Furthermore, the 12S marker resolved twelve of the twenty-one (57%) identified taxa to species level and 16S resolved ten of the nineteen (53%) taxa to species level (Table 4). Of the total twenty-seven taxa, nine were classified as coming from the lab as either our black carp positive control, possible amplicon contamination from the lab (bigheaded carp, common carp, gar, Salmonidae and Colubridae), or potential contamination from lab reagents (chicken, pig, and turkey) [35]. Not including the black carp positive control, reads from the eight non-target lab taxa were considered to come from lab contamination. Reads from each of the eight suspected contaminant, non-target, lab taxa were less than 1% of total reads except for the bigheaded carp being 2.5% in the 12S assay. Summed together, reads from all eight non-target lab taxa made up 1.9% of the 12S assay and 5.2% of the 16S assay (see Table S1). Our negative amplification controls resulted in less than 300 reads. Once carryover calculations were run only five reads belonging to the duck genus *Anas* were retained in our 16S negative control and zero reads were observed in the 12S negative control (Supplemental materials Spreadsheet 1). This suggests that our library preparation methods were clean with minimal amplicon contamination during library preparation. However, we did observe possible amplicon contamination from past studies (carp, gar, colubrid reads) in our field samples, indicating some lab contamination during sample processing. We also observed reads from domesticated animals including turkey, chicken and pig which may be from dNTP or other reagent contamination [35]. Cattle DNA may also come from reagent contamination [35], but, given the high number of reads obtained from cattle, and the known cattle use at our sample sites, we retained cattle as actual field detections. Human DNA reads could also be from sample and processing contamination, but, due to human usage at these sites, they were retained in subsequent analyses as field detections. With the removal of the eight lab contaminant OTUs and retention of human and cattle reads, a total of eighteen taxa were utilized in subsequent analyses as field detections, including mammal, bird, amphibian, and fish taxa (Table 4).

Table 4. Taxon identification and resolution from all samples using both 12S and 16S markers.

Type	Resolution	Scientific Name	Common Name	Marker
Amphibians	Species	***Ambystoma tigrinum***	**Tiger salamander**	12S, 16S
Amphibians	Species	***Anaxyrus punctatus***	**Red-spotted toad**	16S
Amphibians	Genus	***Spea* spp.**	**Spadefoot toads**	12S, 16S
Birds	Genus	***Anas* spp.**	**Duck**	16S
Birds	Family	**Anatidae**	**Duck**	12S
Birds	Family	**Aredeidae**	**Heron**	12S
Birds	Family	**Emberizidae**	**New world sparrows**	16S
Birds	Species	***Falco sparverius***	**American kestrel**	12S
Birds	Order	**Passeriformes**	**Perching birds**	12S
Birds	Species	*Gallus gallus* *	Chicken	12S, 16S
Birds	Species	*Meleagris gallopavo* *	Turkey	12S, 16S
Fish	Species	***Lepomis cyanellus***	**Green sunfish**	12S
Fish	Species	*Cyprinus carpio* *	Common carp	12S
Fish	Genus	*Hypophthalmichthys* spp. *	Bigheaded carps	12S, 16S
Fish	Genus	*Lepisosteus* spp. *	Gar	16S
Fish	Species	*Mylopharyngodon piceus* **	Black carp	12S, 16S
Fish	Family	Salmonidae *	Salmonid fish	12S
Mammals	Species	***Bos taurus***	**Cattle**	12S, 16S
Mammals	Genus	***Canis* spp.**	**Dog**	12S, 16S
Mammals	Species	***Felis catus***	**Cat**	16S

Table 4. *Cont.*

Type	Resolution	Scientific Name	Common Name	Marker
Mammals	Species	***Homo sapiens***	**Human**	12S, 16S
Mammals	Species	***Lepus californicus***	**Black-tailed jackrabbit**	12S, 16S
Mammals	Genus	***Myotis* spp.**	**Myotis bats**	12S, 16S
Mammals	Genus	***Sylvilagus* spp.**	**Cottontail rabbits**	12S, 16S
Mammals	Species	***Urocyon cinereoargenteus***	**Gray fox**	12S
Mammals	Species	*Sus scrofa* *	Pig	12S, 16S
Reptiles	Family	Colubridae *	Colubrid snakes	16S

Bold text, Field Detections; * Taxa identified as lab contamination (from lab or reagents); ** The positive control.

3.3. Taxa Identification across Sites

Read numbers for each taxa were summed up across sampling events (sample site and date), with August 2016 sampling events including data from both centrifuged and filtered samples. For both datasets, the top four taxa included spadefoot toads (*Spea* spp.), humans (*Homo sapiens*), cattle (*Bos taurus*), and tiger salamander (*Ambystoma tigrinum*) with percentages of reads ranging from 2.3% to 47% (Table 5). Most other taxa made up less than 1% of the total read count. However, green sunfish (*Lepomis cyanellus*) made up over 6% of the 12S reads being the fifth most abundant 12S read, following tiger salamander reads at 12%. Results from the one mine containment pond sample only included human reads with the 12S marker, but included both human and tiger salamander DNA with the 16S marker. The proportions of reads for each taxa from the total number of reads were similar even when reads from the contaminant taxa and the positive control black carp sample were included (Table S1).

3.4. Filter versus Centrifuge Processed Samples

The average number of field taxa identified from centrifuged samples was always equal to or greater than the number identified from filtered samples, even though the centrifuged samples generally had a similar volume processed as the filtered samples with the exception of Little Robinson and Pine Nut tanks which had smaller volumes processed (Table 2). This difference could be due to both differences in DNA capture efficiency and DNA extraction efficiency between the two methods.

3.5. Taxa Identification per Site

Figures 2 and 3 show the percentage of reads of each taxon per sampling event (site and date). The different markers amplified taxa with different sensitivities thus site comparisons across markers are not valid. For instance, in Wild Band, the green sunfish is identified with the 12S marker but not observed with the 16S marker. Spadefoot toad DNA is observed at all sites (but not in each year sampled), appearing to be common across the area (Supplemental materials Spreadsheet 1). Tiger salamander DNA was not detected in any of the August 2016 samples.

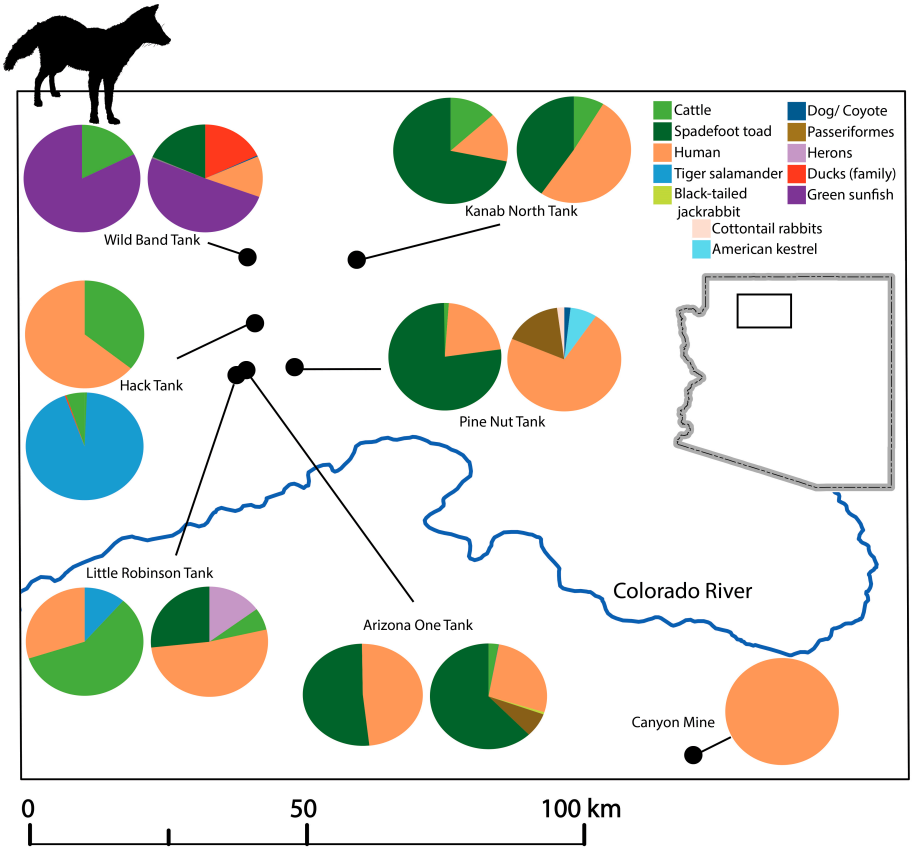

Figure 2. Pie charts show percentage of total reads per site for each taxa for the 12S assay. The left or top pie chart is the earlier date sampled, and the right or bottom chart is the later date sampled for each site. Figure only shows taxa that are 0.3% or greater of the total read counts for that site. See Tables S2 and S3 for all taxa reads per site. (Fox image: Brian Gratwicke—photo; T. Michael Keesey—vectorization; https://creativecommons.org/licenses/by-sa/3.0/).

Table 5. Percentage of reads per field detected taxon for each marker after removal of lab contaminant reads and singletons.

Taxa	Total Number of 12S Reads	Percentage of Total 12S Reads	Taxa	Total Number of 16S Reads	Percentage of Total 16S Reads
Spea spp.	1,417,797	35	*Homo sapiens*	2,227,802	47
Homo sapiens	1,286,429	32	*Bos Taurus*	1,314,121	28
Bos taurus	517,287	13	*Spea* spp.	889,532	19
Ambystoma tigrinum	477,703	12	*Ambystoma tigrinum*	108,565	2.3
Lepomis cyanellus	263,750	6.4	*Canis* spp.	83,389	1.8
Passeriformes	41,122	1.0	Emberizidae	42,153	<1.0
Aredeidae	34,876	<1.0	*Lepus californicus*	20,834	<1.0
Anatidae	26,852	<1.0	*Anas* spp.	15,055	<1.0
Lepus californicus	3596	<1.0	*Myotis* spp.	5351	<1.0
Falco sparverius	1794	<1.0	*Anaxyrus punctatus*	3371	<1.0
Canis spp.	1576	<1.0	*Felis catus*	2884	<1.0
Sylvilagus spp.	1564	<1.0	*Sylvilagus* spp.	191	<1.0
Urocyon cinereoargenteus	310	<1.0			
Myotis spp.	110	<1.0			
Total	4,074,766		Total	4,713,248	

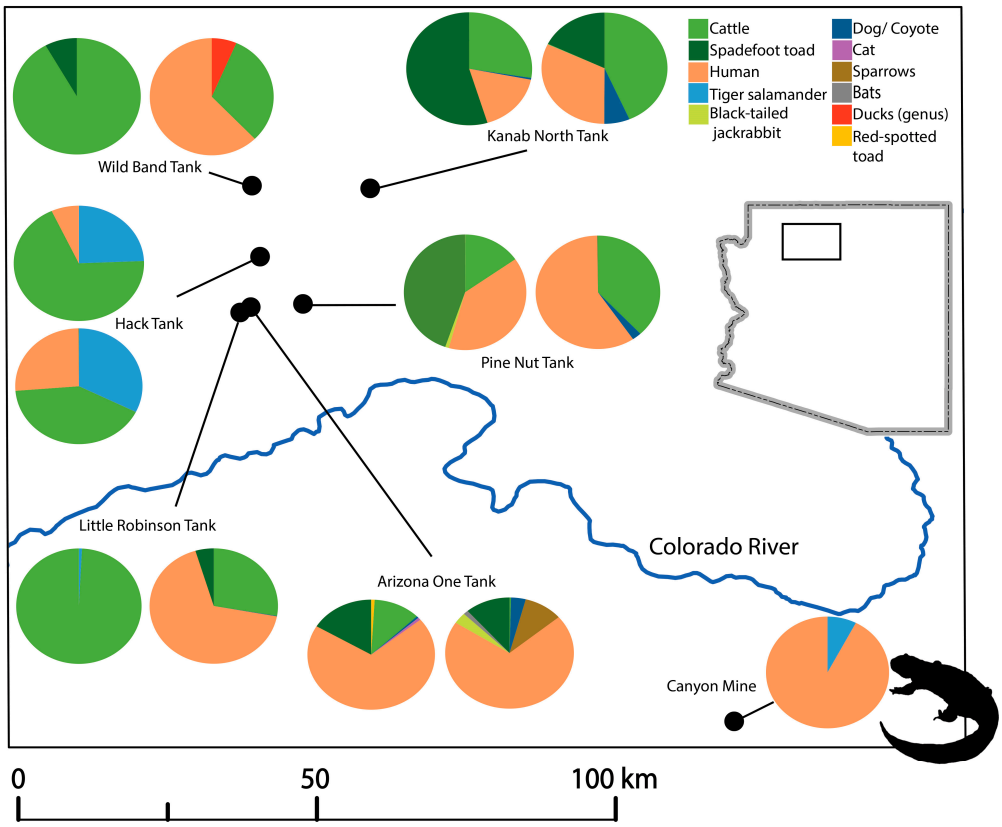

Figure 3. Pie charts show percentage of total reads per site for each taxa for the 16S assay. The left or top pie chart is the earlier date sampled, and the right or bottom chart is the later date sampled for each site. Figure only shows taxa that are 0.3% or greater of the total read counts for that site. See Supplemental Tables S2 and S3 for all taxa reads per site. (Salamander image: Matt Reinbold—picture; Michael Keesey—modification; https://creativecommons.org/licenses/by-sa/3.0/)

4. Discussion

We successfully amplified a range of vertebrate DNA from our eDNA samples taken from surface waters in and near uranium mine sites in northern Arizona, demonstrating the ability of eDNA metabarcoding to detect terrestrial (mammals and birds), semi-aquatic (amphibians) and aquatic (fish) vertebrates using these water sources. Of note, tiger salamander and gray fox DNA were detected from eDNA samples, whereas wildlife surveys in the area and at Canyon Mine failed to detect these species [4,36]. It is not surprising that traditional visual and acoustic surveys did not detect tiger salamanders, given this species' nocturnal activity and non-vocalizing breeding behavior. This species' eDNA was detected at a number of samples sites across the two sampling years suggesting that it is not a rare species, but rather cryptic in our ability to detect it with conventional, diurnal survey methods. This species was also detected in the one mine containment pond (Canyon Mine), and later communication with mine personnel verified the presence of this species at the mine site supporting our results. Tiger salamander DNA was the fourth most abundant OTU for both the 12S and 16S markers; which may indicate a high biomass or repeated occurrence in the sampled area. Given that our sampling covers a large portion of the tiger salamander breeding season and their aquatic egg and larval developmental stages [37], this high abundance of reads is not unexpected. It would be interesting to sample these sites during the non-breeding season as we expect to find few if any tiger salamander detections. In fact our later August 2016 sampling did not detect any tiger salamander DNA at these sites, suggesting that larvae had metamorphosed and already migrated out of breeding ponds. Tiger salamanders exhibit a wide range of life history strategies including paedomoprhic adults, different larval types, and overwintering larvae [38], with strategy dependent upon environmental

factors including permanency of water source [39]. The temporary nature of most of these cattle tanks suggests that the tiger salamanders in this area are unlikely to reside in ponds year round. Spadefoot toads were among the top three most abundant reads for both assays. As with the tiger salamander, our sampling also aligned with spadefoot toad breeding and aquatic larval stages. Both of these explosive breeders return to the terrestrial habitat and estivate during the rest of the year thus we would expect to not detect these species from eDNA samples in the fall or winter. Increased temporal sampling might allow for tracking of seasonal association with surface water in these species. Green sunfish was the fifth most abundant read for the 12S marker and was only detected at the Wild Band tank site. Previous surveys noted the presence of sunfish carcasses on the shore of that cattle tank, supporting our eDNA results. All other identified taxa were terrestrial, and most detections were rare in that they were detected in one out of four samples from a site, and detections were not spread across sites (Supplemental materials Spreadsheet 1). This pattern is likely due to the rare nature of capturing DNA from a species that might visit a water source for a brief period of time to drink (e.g., gray fox, black-tailed jackrabbit) or defecate while flying over (e.g., bats).

Besides demonstrating the ability of eDNA metabarcoding to aid in species surveys, our study also exemplifies some of the challenges of this method. For instance, large biomass organisms such as human, cattle, and breeding amphibians dominated our samples, possibly masking the presence of rarer or lower biomass taxa. It has been demonstrated from environmental samples that quantity of DNA measured as either copy number via quantitative PCR or read number via metabarcoding, is related to the biomass of aquatic organisms [40,41]. Thus, samples contain more DNA from high biomass taxa (i.e., taxa with large or numerous individuals) and less DNA from low biomass taxa (i.e., taxa with smaller or fewer individuals), making the detections of rare species potentially more complicated. In our study non-target taxa such as humans and cattle made up a large proportion of total reads; however, this was not unexpected given the known existence of these taxa in the sampling area. For example, most of the sampled sites were cattle tanks in open range land, with a high biomass of cattle relative to wildlife. Humans also use the area by moving cattle, maintaining stock ponds, and maintenance of the mine containment ponds. One method to decrease the number of reads from field detections of non-target organisms is to use blocking primers to prevent amplification of this non-target DNA [19,42,43]. Developing primers that block amplification of these non-target taxa will improve the detection of rare events such as the usage of these water sources by low biomass taxa.

Another potential cause of certain taxa dominating a multi-template metabarcoding sample is primer bias. This effect results from differences in primer efficiency in multi-template samples, with some templates (taxa) amplifying better with the primers than others, and some templates (taxa) not amplifying at all. For instance, our 12S marker amplified green sunfish, herons, American kestrel, and gray fox DNA, which were not amplified by the 16S marker. However, our 16S marker detected a third amphibian species, the red-spotted toad, which was not identified using the 12S marker. All of these taxa have sequences in the NCBI database at both loci, thus their non-detection is not due to lack of database sequences but likely due to primer bias. Even taxa for which both primers were known to amplify, these taxa were not always amplified from the same sample. For example, our Canyon Mine sample was found to have human and tiger salamander DNA based on the 16S assay; however, only human DNA was detected using the 12S marker, even though the 12S marker amplified tiger salamander DNA in other samples. Even with careful primer design, primer bias can easily disrupt the relationship between biomass and amplicon amplification in complex environmental samples [41]. To reduce the loss of taxon detections because of primer bias, results from our study and others [44] suggest that using multiple primer sets (multiple markers) increases the number of taxa detected. It has been argued that primer and other processing biases will prevent the ability to infer estimates of abundance or relative biomass from metabarcoding eDNA samples [41]. However, others suggest that the use of multiple temporal and spatial sampling along with site occupancy and detection probability may allow for such inferences [34]. Although this debate is beyond the scope of our current study, we do emphasize that abundance or biomass estimates cannot be inferred from our data on terrestrial

taxa as unlike aquatic and semi-aquatic taxa, terrestrial species are not constantly shedding DNA into the water being sampled [45].

Species level resolution is another difficulty in metabarcoding with many studies focusing on an across phyla representation only being able to resolve down to order, family or genus, but not necessarily to species. Studies that focus on specific groups and develop study-specific sequence databases have higher success of obtaining species level confirmation [46,47]. For our study, we focused only on one phylum (Chordata) to distinguish among vertebrate species. We found that our 12S marker had higher species level resolution (65%) compared to our 16S marker (58%). Regardless, both of these markers are less than 100 base pairs which provides few informative sites (base pair differences) for distinguishing among species. For instance we could not distinguish *Canis latrans* from *Canis lupus* with our markers, nor could we distinguish among the three different spadefoot toad species found in this region. These three species only have one to two SNPs that distinguish them in this short fragment; however, with sequencing and amplification error (especially given the high number of reactions each sample went through), only three differences are needed to get below our 97% identity threshold for species resolution. Although shorter fragments are believed to be more readily recovered than larger fragments, recent studies have successfully amplified larger fragments (200–400 bp) from water samples [48], including one study that amplified a 171 base pair length amplicon to identify terrestrial mammals from samples of bathing and drinking water [45]. By increasing length of fragment, we expect to improve our species resolution.

A challenge for any field eDNA study is sample collection and processing. Being able to collect and process samples in the field could be beneficial. A number of studies collect and process DNA in the field by filtering water through a membrane and then later extracting the DNA off of that membrane in the lab. Alternatively, water samples can be collected and then later processed via centrifugation. Immediately after sample centrifugation, the water is decanted off and any extraction method can then be used to extract DNA from the pellet of cellular debris remaining at the bottom of the tube. Eichmiller et al. found that filtering recovered more eDNA than centrifugation [49]. A benefit of the filtration method is that larger volumes (liters versus milliliters) of water can be processed. We found, however, that for our samples which come from very turbid water, filtration was not a practical method. For example, at Pine Nut tank over an hour was required to filter 50 mL of water. We used a smaller pore size filter (0.45 µm) than Eichmiller et al. who used a 1.5 µm size, which likely increased the time for our samples to filter [49]. We also found that our centrifuged samples generally identified the same number or higher of taxa as our filtered samples, even when processing a similar volume (45 mL for centrifuged versus 50 mL for filtered) of water; however, because different extraction methods were used on filter and centrifuge samples, these results may also be due to different efficiencies among the extraction methods. Further refinement of methods to handle turbid samples in the field is needed. Although we were able to freeze and ship our samples within 24 h of sampling to process in the lab via centrifugation, this may not be feasible for other field studies, and shipping problems can lead to delays in sample arrival. Adding Longmire's solution to filter and water samples has been found to stabilize DNA at room temperature and may be an alternative to immediate processing and shipment of samples [50].

Finally, contamination is a major concern for eDNA work in both the field and the lab. In the lab, PCR results in high concentrations of amplicons that can easily contaminate research space and equipment. We were able to identify the source of a number of our non-field, non-target taxa. We suspect possible amplicon contamination from previous studies resulted in the observation of bigheaded carp, common carp, gar, and salmonid fish. The colubrid snake DNA was verified to come from a tissue extraction of *Lampropeltis calligaster* that was used in previous runs to test primer efficiency and resulting amplicon contamination likely occurred. No other DNA from tissue extractions used in primer development were identified in our runs. We cannot account for the chicken, turkey or pig sequences contained in our runs; however, other studies have reported reads from these species as well as from cattle and suggest that these reads may have come from animal feed found in aquaria from

which samples were taken [25], fecal matter from livestock swept down river [51] or contaminated PCR reagents, particularly dNTPs [35]. We believe that most of the chicken, turkey and pig sequence could have come from our PCR reagents, but further investigation is required. As noted in the results these suspected contaminant taxa were a low percentage of the total read numbers for each assay, even lower than what has been reported by other similar metabarcoding studies [45,47]. Furthermore, our negative amplification controls resulted in few reads, indicating that our methods and lab environment were clean during library preparation. We suspect that contamination from lab sources may have occurred during sample processing. To combat contamination, our lab utilizes several procedures to ensure a clean workspace including use of separate spaces for sample extraction, PCR and library preparation; and sterilization of equipment and tools with bleach and UV light. As in other studies, despite our efforts we still observed contamination and amplification of non-targets [45,47]. Future work should include field blank samples that are processed along with regular field samples and extraction blanks to assess the sample processing and extraction steps as potential avenues for contamination.

Overall, our study illustrates the utility of using eDNA sampling in biodiversity and wildlife surveys. We identified one seemingly abundant semi-aquatic organism, the tiger salamander that Hinck et al. did not recover in their surveys [14,36]. Our results show the ability to also detect terrestrial animals using eDNA water samples; however, the detection of some of these terrestrial species was rare, with some taxa only identified in one of the four 45 mL water samples collected per site. Increasing sample volume or taking more samples as well as using blocking primers to avoid non-target taxa that are in the sampling area will increase the chance of detecting more terrestrial vertebrates. Another goal of our surveys was to identify taxa that might be useful in future ecotoxicological research. Amphibians such as the tiger salamander and the spadefoot toads, might be good candidates, given their dependence on these water sources for half of their life cycle. In summary, the application of eDNA metabarcoding to assess species diversity and presence in an ecosystem is rapidly expanding from complementing traditional species surveys for single species or multi-species assemblages to examining community and landscape wide changes in species composition, expanding the utility of this tool to a variety of fields.

Supplementary Materials: The following are available online at www.mdpi.com/1424-2818/9/4/54/s1, Table S1: Percentage of reads per field detected taxon for each marker including contaminant reads, but with singletons removed, Table S2: Percentage of total reads per sampling event for each taxa for the 12S assay, Table S3: Percentage of total reads per sampling event for each taxa for the 16S assay; Spreadsheet S1; Spreadsheet S2; Spreadsheet S3.

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Author Contributions: Jo Ellen Hinck, Catherine A. Richter, Nathan Thompson and Katy E. Klymus conceived and designed the experiments; Nathan Thompson and Katy E. Klymus performed the experiments; Katy E. Klymus and Catherine A. Richter analyzed the data; and Katy E. Klymus wrote the paper.

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